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INDOOR RISK FACTORS FOR CHILDHOOD RESPIRATORY DISEASES: DEVELOPMENT AND APPLICATION OF NON-INVASIVE BIOMARKERS

"ANIMO"

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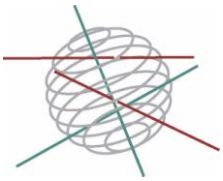

Health and Environment

FINAL REPORT

INDOOR RISK FACTORS FOR CHILDHOOD RESPIRATORY DISEASES: DEVELOPMENT AND APPLICATION OF NON-INVASIVE BIOMARKERS "ANIMO"

SD/HE/05

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SUMMARY

A. Context

The ANIMO project "Indoor risk factors for childhood respiratory diseases: development and application of non-invasive biomarkers" has focused on one of the most common chronic diseases in children in the Western world. Childhood asthma is an heterogenous disease. It may onset early in life but early diagnosis is not yet possible and is mostly based on medical history and clinical manifestations. Its etiology is not well understood but environment and life style may contain risk factors. The goals of ANIMO were to identify environmental risk factors for respiratory complaints in children with special focus on the indoor environment. Another goal was to develop non-invasive biomonitoring protocols and biomarkers which should be easily applicable in children and which may enable to detect adverse effects of respiratory tract status in an early stage allowing preventive measures to be taken before disease outbreak. The goals of the ANIMO project are in line with the priorities of the Belgian environment and health policy plans as laid down in the NEHAP and the themes such as better protection of vulnerable groups, indoor air quality (IAQ), improving policy tools, such as human biomonitoring (HBM),which form the backbone of the current EU-SCALE strategy and EHAP as recently confirmed at the Parma Fifth Ministerial Conference on Environment and Health (March, 2010).

B. Objectives

The specific goals of ANIMO were :

- to identify, standardise and design study protocols for non-invasive biomarkers in environmental health biomonitoring studies addressing children's respiratory health. We evaluated the feasibility of the procedures in young children, the repeatability and major confounders.
- to evaluate the predictive value of these new non-invasive tests for inflammation/epithelium integrity and respiratory complaints in children. We tested the hypotheses that different biomarkers may be needed to characterize different types and stages of airway damage
- to identify environmental risk factors related to changes in the effect biomarkers.

This program has been realised with the collaboration of different cohorts of children. The program was approved by the ethical committees of University Hospital of Antwerp (UZA) and Université Catholic de Louvain (UCL).

C. Conclusions

Biomonitoring and biomarker analysis

The ANIMO project has successfully developed biomonitoring techniques which are applicable in young children due to the non-invasiveness of the sampling method. Protocols have been developed to collect in a standardized way samples of exhaled air, spot urine samples and nasal lavages in school children. After optimization they were first applied in a pilot study of 70 children between 6 and 12 years of age, which was in a second phase expanded to139 children. Exhaled air contains exhaled NO (**eNO**), which is a well-known indicator of deep lung/airway inflammation, especially of eosinophilic airway inflammation. eNO analysis is already used in

clinical practice. 41 of the 70 children succeeded in the test based on ATS[1] recommendations of at least two reproducible measurements, that agree within 10% of each other. Next to a stationary instrument (EcoMedics), we used the mobile NIOX MINO Airway Inflammation Monitor (Aerocrine, Sweden) for eNO analysis because this allowed us to perform measurements at different locations such as schools which is important for biomonitoring studies of the general population. The results of both instruments were well correlated but values obtained with NIOX MINO were slightly higher. Age and height were significant effect modifiers. eNO was higher in boys than in girls in the prospective Walloon cohort study.

Alveolar breath also contains different **volatile organic compounds (VOCs)** that reflect the blood gas content at any given moment. We asked children to donate an exhaled breath sample by blowing into Tedlar bags. This can be done at any place and proved to be easy for children aged 3 years and older. To analyse the volatile organic compounds (VOCs) present in the Tedlar bags, a sampling and thermal desorption gas chromatography – mass spectrometry method were developed that allowed monitoring the metabonome. Two different preconcentration protocols were used focusing on the short C_2-C_5 VOCs and C_5-C_{12} VOCs which are assumed to be linked with oxidative stress. The method showed good repeatability for at least 56 VOCs present in exhaled breath with 89% of the coefficients of variance being less than 30 % (of which 85 % ≤ 20 %). A limited intra-individual variability study in adults allowed to conclude that time of the day and day to day variations in exhaled breath VOC content were negligible (3%) compared to the total variance observed for these VOCs. 35 % of the remaining 97% of variance cannot be ascribed to factors included in this study. It is therefore recommended to study in more detail the different factors that can contribute to fluctuations in exhaled breath VOC content e.g. concentration of VOC in ambient air, diet, health status, genetic polymorphisms, sampling conditions, etc.

Exhaled breath contains also proteins which can be collected after condensation by freezing. **Exhaled breath condensate (EBC)** could be collected in children as young as age 3 years, at any location, by spontaneous breathing during 15 minutes using an RTube (Respiratory Research, Inc). This allowed to collect between 1 and 1.5 mL EBC. We analysed the collected volume, the pH of the condensate, its protein content and potential markers such as leukotriene B4, clara cell protein 16 (CC16) by BIOPLEX (BioRad), Uteroglobin-related protein 1 (UGRP-1) by ELISA, 8-isoprostane (EIA kit, Cayman), cytokines (cytokine antibody arrays, RayBio), and a protein fingerprint. To obtain a protein fingerprint, proteins were first separated by nano-LC and subsequently detected by mass spectrometry MALDI-TOF/TOF 4800 proteomics analyzer, Applied Biosystems and in a later stage by LTQ Orbitrap XL™, Thermo. A study in adults showed high repeatability of EBC volume, pH, protein content with no significant variability in collections within one day or within successive days. Children's age influenced the collected EBC volume, girls had a more acidified condensate than boys. The low protein concentration in the condensate and protein loss during the procedure were major challenges. Concentration of the proteins on beads seemed most successful but further method improvements are required. CC16 and UGRP-1 were not detected in the condensate and most cytokines were below the limit of detection.

[1] American Thoracic Society

The ANIMO project allowed for the first time to identify an extended list of proteins in EBC. The use of the ORBITRAP mass spectrometry allowed to identify, next to a diversity of cytokeratins, also proteins which may be more specifically related to inflammatory and immune processes in the airways. This unique dataset needs further exploration.

The ***nasal lavage (NAL)*** technique was successfully applied in children. The concentration of albumin or other plasma-derived proteins in nasal lavage fluid was used to evaluate acute or chronic disruption of the nasal epithelium associated with inflammation (rhinitis) or exposure to some irritants (e.g. ozone). The CC16 protein which is secreted throughout the airways and predominantly by the bronchiolar clara cell, could be identified in NAL. Concentrations were corrected for urea, which is a reliable dilution marker in nasal lavage.

Spot **urinary samples** were also collected from the children. We have explored whether urinary CC16 might be used as a surrogate indicator of airways integrity after adjustment for the fractional uptake of the protein on the basis of urinary RBP, a reliable marker of the reabsorptive capacity of the proximal tubule.

Diagnostic and predictive value of biomarkers for airway toxicity

The diagnostic and predictive value of the non-invasive biomarkers was investigated in a case/control **pilot study**, with 72 asthmatic children and 67 age matched control children between 6 and 12 years of age, 78 boys and 61 girls. In addition, some of the markers were applied in a large prospective cohort study which has been initiated by the UCL partner and in a follow up study of a Flemish birth cohort.

We could identify markers which were significantly differentially ($p < 0.05$) expressed in children with allergic asthma versus children with non-allergic asthma: the former had higher eNO levels in exhaled breath, the anti-inflammatory CC16 was lower in nasal lavage of allergic asthmatic patients, exhaled breath condensate of allergic asthmatic patients contained higher levels of the oxidative stress marker 8-isoprostane.

When asthmatic children were compared with healthy controls, we showed that exhaled breath pH was lower in asthmatic children. The concentration CC16 in urine showed an inverse association with the risk of asthma or hay fever (asthma, OR 0.14, IC 95% 0.02-0.98; hay fever, OR 0.26, IC 95% 0.10-0.68). In the prospective Walloon study the eNO concentration was significantly higher among children with wheezing, asthma or house dust mite allergy. In the Flemish cohort the eNO concentration was significantly higher in children with doctor-diagnosed asthma and food allergy.

The fingerprints of exhaled gasses and exhaled proteins give new and promising results in the search for non-invasive biomarkers which are specific for asthma status. Based on a database of 821 gasses and 1331 proteolytic peptides, a support vector classifier was used to build predictive models for different health outcomes: asthma, moderately controlled and uncontrolled asthma, or controlled asthma versus healthy controls. A limited number of gasses and peptides was able to discriminate successfully between children with different asthma status. The role of these gasses and proteins in asthma pathogenesis is not completely clear at this moment. We should see this as a first step in the development of novel biomarkers for asthma status in children. This was a pilot study, and the results still need confirmation in other populations. Various proteins that might be relevant in respiratory health outcomes were

identified for the first time in EBC. Additionally, it was shown that exhaled proteins provide unique information from the lower airways, and are not only derived from the mouth.

Environmental risk factors

The newly developed non-invasive biomarkers were applied in 2 different child cohort studies. A new prospective cohort was initiated in the Walloon region. A total of 272 children from 30 schools located mainly in the areas of Brussels and Liège (Belgium) were examined at 5-6 years and 2 years later. 547 children of an existing Flemish cohort were invited for a follow-up at the age of 7 years. Only 65 children and their parents agreed to participate with an health examination in this follow-up study. The new biomarkers were used in combination with classical clinical endpoints (e.g. doctor-diagnosed asthma, respiratory symptoms, lung function, exercise-induced asthma). The parents filled in an extended questionnaire focusing on risk factors for respiratory health outcome and on indoor exposure.

At the age of 6, the prevalence of bronchiolitis, eczema and house dust mite sensitization are high, around respectively 30%, 28% and 25% with increases of the prevalence of house dust mite (HDM) sensitization and eNO between 6 and 8 years of age. The combined database of both cohorts with information from 503 children (7- 8 years of age) showed a prevalence of wheezing, asthma, eczema, food allergy of respectively 13%, 6,6%, 19% , 16%. The study confirmed the protective effects associated with birth at term and day-care attendance. Regarding the risk factors, parental asthma or allergy emerges as a strong predictor in particular for HDM sensitization. Also living near industry was a risk factor for HDM and pollen sensitization at the age of 6, and in the combined cohort living in rural areas before the age of 2 was a protective factor for eNO. The use of air fresheners and environmental tobacco smoke (ETS) were risk factors for asthma at the age of 6. Bleach use for cleaning and ETS were risk factors for wheezing at age in the combined cohort. Another strong predictor identified in our study is the infant swimming practice, which dose-dependently increases the risk of sensitization to HDM or to pollen, and the risk of airways inflammation assessed by the eNO test, while in the combined cohort also the risk for wheezing was enhanced in relation to outdoor swimming pool attendance. This study also stresses the importance of the timing factor to evaluate the respiratory effects of lifestyle or the environment.

D. Contribution of the project in a context of scientific support to a sustainable development policy

The study leads us to conclude that:

Respiratory diseases in young children remain prevalent and need further focus as priority chronic diseases in children. We confirmed that environmental risk factors contribute to the diseases and that timing of exposure may be important. Identification of environmental risk factors such as swimming pool attendance, use of air fresheners, environmental tobacco smoke, which are related to our life style offers opportunities for risk management. Further measures eg to control the indoor air quality environment and sensibilisation may be warranted.

Non-invasive effect biomonitoring is possible in very young children allowing to further disentangle the complex pathologies of airway diseases which may originate early in life. Exhaled NO was confirmed as a non-invasive, repeatable and reproducible diagnostic tool of airway inflammation in children. eNO measurements are currently not reimbursed to the patient

in Belgium. However we conclude that eNO measurement can play an important supplementary role in clinical practice, improving the ability to diagnose asthma.
The volatile organic compounds in exhaled breath and the peptide pattern can be measured in children from 3 years on. First results showed that the obtained fingerprints of gasses and peptides can discriminate between children with different asthma status. This offers new possibilities for understanding the pathology and risk factors of respiratory diseases in children.

The results of the ANIMO project are already published in 6 peer reviewed papers, and several manuscripts are in preparation. The work of ANIMO formed the basis of 2 Ph.D thesis. It was presented at different international congresses. A well attended international workshop (10 December 2010) was organized in Brussels to disseminate the ANIMO results to a wide audience and further links were established with international initiatives such as the COPHES program for harmonization of human biomonitoring in Europe.

E. Keywords

Non-invasive biomarkers, exhaled breath, biomonitoring

1. INTRODUCTION

Children's respiratory health is among the priorities of (inter)national environmental health programs. There is clear evidence that children are more susceptible to some stressors in the environment. Rapid growth, tissue and organ development and vulnerable time-windows of exposure during embryonic or foetal periods make children particularly susceptible. Lower body weight, specific behaviour and time-activity patterns contribute to the differences in exposure between children and adults.

The third Ministerial Conference on Environment and Health in 1999 in London already emphasized the need to develop child-focused environmental protection policies and to establish child specific monitoring tools. On this basis an action plan on children's environmental health in Europe was prepared, the so called "Children's Environment and Health Action Plan for Europe" (CEHAPE). The plan was presented and adopted at the fourth Ministerial Conference on Health and Environment in June 2004 in Budapest. The CEHAPE is a document for policy makers addressing the environmental risk factors that most affect the health of European children. On the basis of the CEHAPE, countries developed national action plans addressing the priority goals (National Environment and Health Action Plan (NEHAP)). Furthermore the Belgian authorities specifically expressed their concerns with respect to the role of indoor pollution.

Respiratory diseases are a major cause of illness in children of developed countries. Furthermore, asthma and allergies are increasing even up to 30% in certain age groups. Environmental factors are thought to affect a child's likelihood to develop these diseases; however the risk factors are largely unknown. Monitoring exposure, effect and susceptibility in children's cohorts, applying mechanistically based biomarkers may help to understand the complex relationship between cause and effect.

Critical needs in children's biomonitoring include exposure and health effect assessment, biological sample collection and ethics. Efforts are made to develop less or non-invasive biomarkers for use in children's environmental research. This research project addresses children's respiratory health by developing non-invasive indicators which should be easily applicable in children and which may enable to detect adverse effects in an early stage allowing preventive measures to be taken before disease outbreak.

Objectives

The project aims to develop and apply non-invasive biomarkers in human biomonitoring programmes especially focusing on children's respiratory health in relation with their environment.

The specific objectives of the ANIMO project are:

- to identify, standardise and design study protocols for non-invasive biomarkers in environmental health biomonitoring studies addressing children's respiratory health.
- to evaluate the predictive value of these new non-invasive tests for inflammation/epithelium integrity and respiratory complaints in children.
- to identify whether environmental risk factors are related to changes in the effect biomarkers.

The performance of the developed non-invasive biomarkers was assessed in a pilot study involving asthmatic and healthy children.
The hypotheses that these new biomarkers are related to respiratory health outcome were tested in an existing and newly established child cohort.
The new cohort of children also assessed the association of the effect biomarkers with indoor environmental risk factors. We tested the hypotheses whether indoor pollutants can promote the development of asthma and make children more sensitive for respiratory diseases.

The project aimed to provide tools to improve the study of respiratory toxicity in children and to provide knowledge to increase understanding and reduce or prevent indoor environmental hazards to children which are a vulnerable group in the population.

2. METHODOLOGY AND RESULTS

2.1. ETHICAL CONCERNS

The project was submitted to the ethical committees of the universities of UCL, UZA and UA. Ethical approval was obtained for the whole project (phase I and phase II) at the two universities. Ethical approval of the pilot study was obtained at the University Hospital of Antwerp (UZA).

2.2. BIOMARKER DEVELOPMENT

Asthma is a heterogeneous disorder with different pathogenic mechanisms, variations in duration and in severity. To characterise the disease and the airway changes that are linked to the onset of the disease, more specific biomarkers are needed. An improved characterization of asthma phenotypes will enhance the insight in risk factors and improve the management of asthma. The hypotheses is that no single biomarker will be sufficient, we may need a panel of biomarkers linked to biological changes which are characteristic for the disease.
The work performed in the first year of the ANIMO project was mainly focused on the development and standardisation of non-invasive biomonitoring methods and biomarker analysis: exhaled breath gases (nitric oxide and other volatile organic compounds), exhaled breath condensate, and nasal lavage fluid. During the second year, these methods were applied in a pilot study involving asthmatic and healthy children in order to evaluate the performance of the non-invasive biomarkers. During the third and the fourth year, biomarker analyses were further optimized. Additional samples were collected and analysed in an enlarged pilot study.

2.2.1 Protocol Development

Four non-invasive sampling methods were used and biomarker analyses were developed and optimized. Descriptions of the tests and the associated protocols have been generated. Details on the standardization and optimization of the non-invasive methods are described below.

2.2.1.1. Exhaled breath gases

Exhaled nitric oxide (eNO)

Nitric oxide (NO) in exhaled air (eNO) is a well-known indicator of deep lung/airway inflammation, especially of eosinophilic airway inflammation. eNO is elevated in asthma but is influenced by different factors such as smoking (McSharry et al, 2005). eNO is being used to monitor asthma. The technique can be carried out in a reliable way in schoolchildren (Buchvald et al, 2005). The collection technique is easy and non-invasive.

A task force of the American Thoracic Society and the European Respiratory Society (ATS/ERS) (Kharitonov et al., 1997) established consensus guidelines (revised in 2001 and 2005 (ATS/ERS 2005)) for the measurement of eNO in adults and children. The single-breath online technique is the "gold standard" technique: the children inhale NO-free gas to total lung capacity and exhale at a constant flow of 50 mL/sec until an NO plateau of more than 2 seconds can be identified during an exhalation of more than 4 seconds. Recently, mobile instruments to measure eNO have become available. This may be an advantage for environmental health monitoring at different locations. Results from a mobile (NIOX MINO) and a static device (EcoMedics) were compared in the pilot study of this research project (see further).

Procedures for use of CLD 88 SP analyser (EcoMedics, Switzerland), NIOX (Aerocrine, Sweden) and mobile NIOX MINO Airway Inflammation Monitor (Aerocrine, Sweden) have been described and tested.

Other volatile organic compounds (VOC) in exhaled breath

It has been shown that alveolar breath contains different volatile compounds that reflect the blood gas content at any given moment (Phillips, 1992). Collection and analysis of exhaled breath gases can therefore be used as a non-invasive tool to monitor the individual exposure to environmental pollutants, but exhaled breath gases can also reflect the individual's respiratory tract status.

GC-MS methodology

GC-MS has been pointed out as the best method to screen VOCs in exhaled air. Exhaled breath of study subjects was collected in Teflon bags. Collection can be performed at any place and is easy for children aged 3 years and older. A Gillian® personal sampler was used to draw the breath content of the sampling bag over a sorbent tube containing 3 cm Carbograph 1TD/ 3 cm Carbopack X. To each sample, the reference compound 2-fluorotoluene was added. For each breath test an equivalent amount of ambient air –

present in the room which the subjects occupied during the breath test – was sampled on a sorbent tube. Although breath consists of a relatively 'clean' sample matrix compared to urine or blood, the high CO_2 content and humidity can turn out to be a serious challenge to GC-MS analysis. Because moisture trapped onto the sorbent tubes was found to interfere with GC-MS output, sorbent tubes were purged with 500 mL Helium (50 mL/min) prior to analysis to expel the moister. Sampled VOCs were recovered from the adsorbent traps by thermal desorption (Markes International Ltd.). Analysis was performed by GC (HP 6890 series) – MS (HP 5973 Mass Selective Detector). The column was an AT-5ms; 60 m x 0.32 mm internal diameter x 0.25 μm film thickness. The GC program was a temperature gradient starting at 35°C (for 1 min) increasing every minute with 5°C until 320°C (for 2 minutes), with a total run time of 60 minutes. The detection was in full scan modus (m/z from 35 to 350). The responses of the different signals from all the samples were combined to one database. Some samples were excluded from the database because the retention time of the reference differed to much from the expected time.

Variability

Repeatability of this method was examined following analysis of 10 breath samples of each of three adult subjects. Coefficients of variance for 56 VOCs were well within acceptable range with 89 % of the coefficients being $\leq$ 30 %. Multiple ANOVA indicated that coefficients of variance were both subject ($p < 0.001$) and component ($p < 0.001$) dependent. Bearing in mind that coefficients of variance of 20 % are normal for standard chemical analysis methods and that we are evaluating a screening method rather than a method optimized to monitor a small selection of compounds, we can conclude that coefficients of variance up to 30 % are acceptable and even better than a lot of other whole organism bioassays.

Part of the variation can be explained by the fluctuations in the signals of the mass detector as was shown by sequential analyses of the (internal) standard cartridge. Thus it is recommended to add an internal standard to each sample to correct for the varying detector signals when calculating and comparing peak surfaces. Circadian rhythm of 4 VOCs in breath was studied using breath of 1 subject. The subject was asked to fill a Teflon bag at different times (9.15 h, 11.15 h, 13.15 h and 15.15 h) for 3 subsequent days. The multiple ANOVA test indicated that variances in VOC abundance due to day ($p = 0.48$) and hour of day ($p = 0.70$) differences were not significant compared to the total variance. As expected there was a marked significance in variance depending on which VOC was studied ($p < 0.001$).

Comparison of preconcentration methods

Low concentrations of VOCs, the high degree of humidity and high CO_2 concentrations in exhaled breath may result in high background values and hamper the analysis. The existing preconcentration methods did not allow to measure easily the short C_2-C_5 VOCs and C_5-C_{12} VOCs, which are assumed to be linked to oxidative stress in the airways (Phillips, 2003). Therefore it was decided to compare two preconcentration methods in the pilot study, each with a different sensitivity and measurable VOC range:

1. Thermal desorption

10L exhaled breath is collected in Teflon® bags. About 7-10 L of the collected air is transferred on a Carbopack X®/Carbograph TD® sorbent cartridge.

2. Entech 7100A Preconcentrator

2L exhaled air is collected in an electropolished stainless steel canister using an Entech Breath Sampler

Statistical analysis

To determine which compounds added to the database were of interest with regard to the classification of the subjects, we used *Support Vector Machines (SVM)*. SVM demonstrate the ability to construct predictive models with large generalization power even in the case of large dimensionality of the data or when the number of observations available for training is low. SVM always seek a globally optimized solution and avoid overfitting. SVM analysis is a learning algorithm that can perform binary classification by nonlinear mapping n-dimensional input space into a high-dimensional feature space. In this high-dimensional feature space, a linear classifier is constructed, and the model is used to discriminate samples belonging to two different groups. Thus, a SVM learns to discriminate between members and the nonmembers of a class (Machado, 2005).

The best subset of compounds was selected using the *attribute selection* option implemented in Weka (Frank, 2004): a collection of machine learning algorithms for data mining tasks. The attribute subset evaluator we used evaluated the worth of a subset of attributes by assessing the individual predictive ability of each attribute along with the redundancy among them. Preferably sets of attributes will be selected showing high correlations with the class and low intercorrelation. Next the selected attributes were evaluated by an SVM attribute evaluator, using recursive feature elimination with a linear support vector machine. Attributes were selected based on the weight of the magnitude as ranking criterion. After every run the least efficient attribute was removed.

All resulting subsets of attributes were analyzed for *classification performance* with use of support vector classifiers based on John Platt's sequential minimal optimization algorithm and the random forest classification algorithm (Platt, 1999).

Tenfold cross-validation was used as test option, both in the attribute selection as for the classification model. This is the standard way of measuring the error rate of a learning scheme on a particular dataset (Witten, 2005).

2.2.1.2. Exhaled breath condensate

Exhaled breath condensate (EBC) is a biological fluid that can be collected by cooling/freezing exhaled air under spontaneous breathing conditions. Collection of EBC is a simple and completely non-invasive method, and as such it is applicable in children aged 3 years and older (Baraldi, 2003). An advantage is that collection can be performed at any place, not only in the lab or in a hospital. Recently, a task force for EBC was established by the American Thoracic Society and European Respiratory Society (ATS/ERS) showing the increasing interest for this approach also for medical applications (Horvath, 2005). EBC is composed of condensed water vapour and aerosol particles from the lower respiratory tract. It is believed that EBC contains molecules that reflect the physiological state of the lung (Hunt, 2003).

Determinants of variability

EBC was collected using a RTube (Respiratory Research, Inc). The aluminum sleeve was stored for at least 30 min in a home freezer (-18°C) before collection. EBC pH was analysed since it has been shown that inflammation causes acidification of the EBC. The protocol to measure pH in EBC was standardized: pH was measured exactly 5 minutes after EBC collection (without deaeration), in a volume of 500 μL EBC. Both the time after collection and the volume in which it is measured, influence this measurement. It is known that pH is influenced by the CO_2, which slowly disappears from the samples. When the latter stays constant, pH measurements are very reproducible. Total protein concentration was measured with a NanoOrange Protein Quantitation kit (Molecular Probes), which is able to determine very low protein concentrations. All EBC samples were checked for saliva contamination by using the Infinity Amylase Liquid Stable Reagent kit (Thermo).

Experiments on repeatability in 20 healthy *adults* sampled at 6 different times showed no significant differences in collected volume, EBC pH or total protein concentration (ANOVA, $p < 0.05$) between samples collected at different days or different sampling times a day. Median volume of EBC collected during 15 minutes of collection was 1.7 mL in adults (interquartile range of 1.40-1.87 mL). Median total protein concentration was 1.02 μg/mL with an IQR of 0.71-1.27 μg/mL. Median pH was 6.17 (IQR: 5.96-6.31). In adults we have shown in this project that gender, age and height of the subjects contributed significantly to the variation in volume and protein content of EBC. They did not affect significantly the pH of EBC (Bloemen et al, 2007).

Children and adolescents (total N = 170; age: 3-23 yrs) were asked to breath tidally through the mouthpiece for 15 minutes while watching a movie. The influence of individual characteristics (gender, age, height, weight) and environmental parameters (environmental temperature and relative humidity) on EBC markers was evaluated by regression analysis. It was concluded that taller and older individuals collected a larger volume of EBC in the same sampling time. Multiple regression showed that especially age has the most influence. Although specific environmental parameters such as environmental temperature and relative humidity had a significant effect on collected EBC volume in a univariate regression, multiple regression did not allow to detect influential parameters with significant effect on EBC volume or pH.

Proteolytic peptide pattern in EBC

Protein profiling of EBC samples was performed on a proteomics technology platform. A protocol was developed to ensure concentration, separation and identification of the proteins present in the EBC samples (published in Proteomics Clinical Applications: Bloemen et al. (2009)). The low protein concentration in the EBC samples is an important problem that had to be handled. Various methods were compared to concentrate proteins, such as TCA precipitation, ultramembrane centrifugation, freeze-drying, speedvac, precipitation with pyrogallol red, and concentration on beads. Protein recovery and reproducibility of the various methods were evaluated, and concentration on POROS R2 beads was selected as the method to use in further research. Additionally, the technique to separate and identify the proteins was optimized. Proteins were first digested into peptides, after which nano liquid chromatography was used to separate these peptides (2D-nanoLC pump; Eksigent). Fractions were collected by using a nano-fraction collector (Probot, Dionex), and measured with a matrix assisted laser desorption ionisation time-of-flight time-of-flight mass spectrometer (MALDI-TOF/TOF MS; 4800 proteomics analyzer, Applied Biosystems). MSMS analysis was performed for protein identification (Figure 1). In a later stage, another mass spectrometer (hybrid Fourier Transform mass spectrometer, LTQ Orbitrap XL™, Thermo) was used to obtain an extended set of protein identifications.

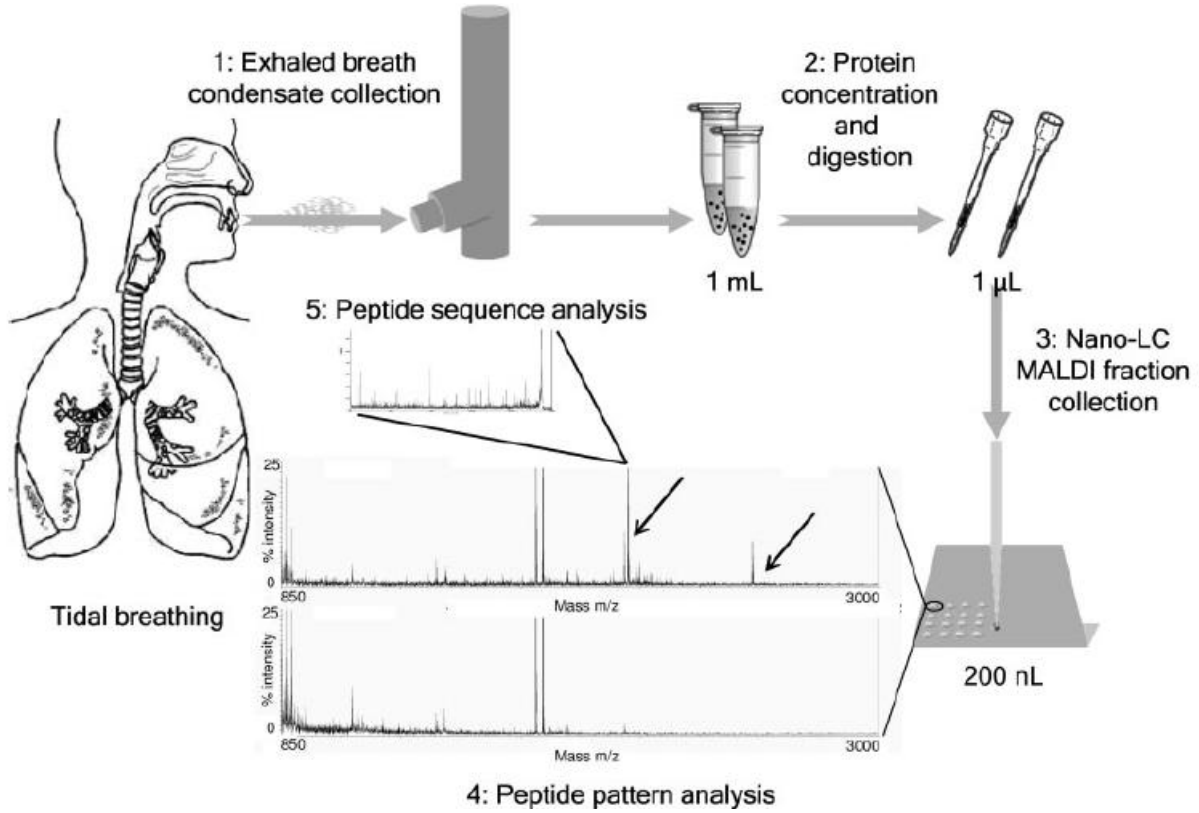


Figure 1. Schematic overview of the workflow for exhaled breath condensate (EBC) analysis. Of the collected EBC (1), 1 mL is used in the proteome analysis. Samples are concentrated on beads and enzymatically cleaved into peptides (2), which are separated using RP nano-LC. Fractions are collected on a MALDI target (3). MS analysis results in elution peptide spectra for each fraction (4), which are used for pattern recognition. MS/MS spectra are used for identification of the protein, by peptide sequence analysis (5).

Refined data processing started with a baseline correction on the MS spectra (DataExplorer version 4.6, Applied Biosystems). Due to the presence of a very regular noise in the first part of all spectra from this study group, a Fourier transformation filter (Kast et al, 2003) was performed on all masses smaller than 1700 Da. Then, peak detection, mono-isotopic peak filtering, and peak deisotoping were performed (DataExplorer). To make it possible to compare these results with spectra from other tests, the area of the transformed peaks was multiplied by a correction factor. Normalization occurred based on an internal standard added in the MALDI matrix (*m/z* 1570.677, fibrinopeptide B; Sigma): the area of each peptide mass was divided by the area of the internal standard of that spectrum. The logarithmic transformation of this ratio was used for further statistical analysis. The same statistical technique as described above for the VOC analysis – Support Vector Machine (SVM) analysis – was used to analyze the protein profiles.

Protein arrays

Protein arrays are a new approach to measure a whole range of known proteins in biological samples. The biggest advantage of this technique is, that when a signal is detected, the identity of the corresponding protein is known. No further identifications are needed as in the LC/MS approach. Already some protein arrays are commercially available. We chose to work with cytokine antibody arrays (RayBio), because these were already successfully used in EBC research (Kullmann et al, 2008). One array contains 60 different cytokines (Figure 2), and the limit of detection was reported to be comparable to current ELISA kits. These protein arrays are a screening technique, that can be used to select potential biomarkers. The signal is evaluated after fluorescence detection in a microarray scanner. Because the technique is semi-quantitative, further validation of the selected biomarkers has to be performed by using quantitative techniques such as ELISA.

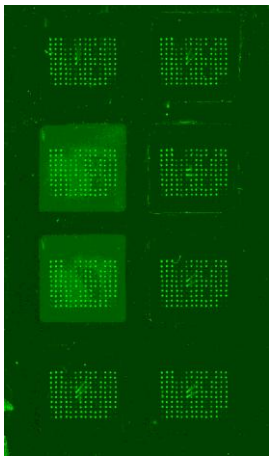


Figure 2. Glass slide containing 8 protein arrays of each 60 cytokines.

Some optimizations were performed on the protein array technique, to improve sample preparation, to reduce the background signal, to select the parameters of the microarray scanner (e.g. laser intensity), and to automate the data analysis. These were all performed on standard mixtures, and on some EBC samples of healthy volunteers. Reproducibility of this technique was good: coefficients of variation were less than 20%. A first screening was performed with 2 pooled samples of healthy controls (1 mL and 3 mL EBC) and 2 pooled samples of asthmatic patients (also 1 mL and 3 mL EBC). These first tests with EBC samples gave promising results: elevated concentrations of IL-1alpha and IL-1beta were found in the asthmatic patient (Figure 3). However, after repeating this test in groups of 10 asthmatic patients and 10 healthy controls, increased variation between the samples was observed, and no clear differences were found anymore between the two groups. We have to conclude that cytokine concentrations in EBC are currently not or difficult to detect using protein arrays, and most measurements are at limits of detection of this assay.

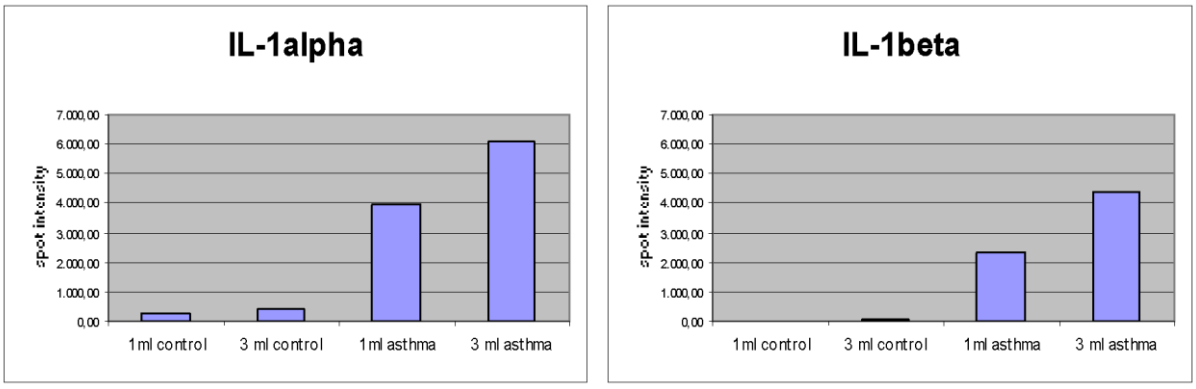


Figure 3. Result from first analysis on protein array.

8-isoprostane

8-isoprostane, a known exhaled marker for oxidative stress, was measured by a commercially available EIA kit (Cayman). However, in the first pilot study, we were unable to detect this molecule in EBC samples. It was suggested that the addition of 0.005% butylated hydroxy toluene (BHT) in ethanol to the EBC samples was needed to stabilize 8-isoprostane in the samples. The optimal volume needed to detect 8-isoprostane in a reliable way in EBC samples, and the effect of storage time at -80°C on the stability were tested as well.

EBC samples were collected once each month in the same individual, during 8 months. One aliquot was frozen immediately, without addition of the stabilizing compound. In a second aliquot, 0.005% BHT was added before freezing. One additional sample was collected immediately before the analysis. 8-isoprostane was measured in these samples for the first time after 3 months, in volumes of 50, 100 and 200 µL. In all samples in which no BHT was added, measurements were at the limit of detection. In the samples were BHT was added, 8-isoprostane was quantifiable. From this first measurement, it was concluded that 8-isoprostane was stable for at least 3 months when 0.005% BHT was added to the samples. Furthermore, it was concluded that a volume of 100 µL of EBC was needed for one measurement. In addition, measurements in samples collected in a RTube coated with 1% BSA and 0.01% Tween-20 resulted in a slightly higher 8-isoprostane concentration. However, also when collected in uncoated RTubes, this compound is reliably quantified.

100 µL of the same samples was measured again after preservation of EBC samples during one year at -80°C. Again, 8-isoprostane was detectable in all samples collected in both coated and uncoated RTubes. It can be concluded that when the BHT buffer is added, the compound remains relatively stable and can be measured up to one year after collection, when appropriately frozen at -80°C. Storage during 6 months or less resulted in the same values. When storage times were longer than 6 months, the concentrations decreased slightly. Based on this comparison, it can however not be

concluded whether this is caused by instability and breakdown of 8-isoprostane, or whether there is another explanation such as the season of sample collection (samples were collected from January until August, once a month).
We can conclude that 8-isoprostane measurement remains stable and reliable when samples are stored together with the BHT buffer up to 6 months at -80°C.

Clara Cell protein 16 (CC16)
BIOPLEX (BioRad) is a new technique including sandwich ELISA and FACS methods. In practice, beads are fluorescent and a fluorochrome (PE = phytoerythrine) is used as a detection method for the ELISA assay. The following reagents were used for our assays:
- monoclonal mouse anti-CC16 antibodies were coupled onto beads
- polyclonal rabbit anti-CC16 antibodies were biotinylated
Several tests were carried out to generate a standard curve. We tried different antigen concentrations, antibodies dilutions and incubation time. No fluorescence could be detected in EBC samples despite checking several possible causes of trouble.

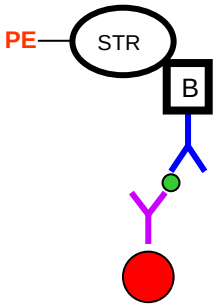


Figure 4. Schematic overview of ELISA compounds.

Uteroglobin-related protein 1 (UGRP-1)
Previously, we showed an increase of UGRP-1 in the sputum of patients with asthma and rhinitis suggesting that UGRP-1 may play a role in these inflammatory diseases (De Burbure, C, 2006). CC16 and UGRP-1 share a sequence homology. We know that UGRP-1 is implicated in lung development and in inflammation. UGRP-1 is a small protein (17kDa) secreted in trachea and bronchi. The observed differences in UGRP-1 and CC16 may be linked to different cell populations being responsible for their secretion, UGRP-1 is mainly secreted in the larger conducting airways whereas CC16 is mainly secreted by the nasal and peripheral airway epithelium.
We have developed a new sandwich ELISA based on a commercial monoclonal antibody and on a polyclonal antibody produced in our laboratory. The method was carefully validated for linearity, accuracy and reproducibility (manuscript in preparation). A typical standard curve is displayed below. This new ELISA was tested on

different types of biological samples, including samples collected in the ANIMO project (see further).

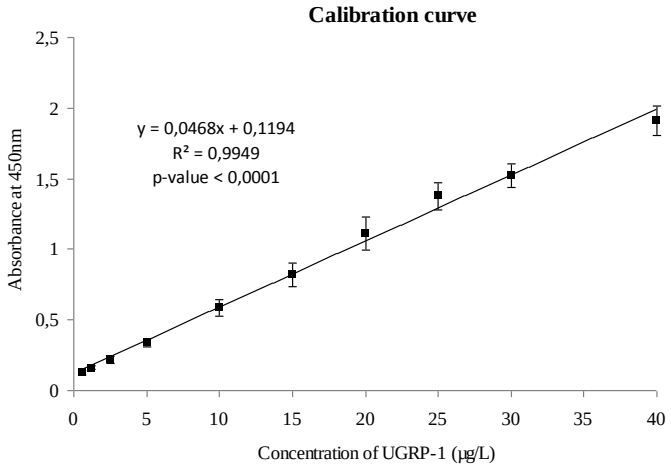


Figure 5. Standard curve for the assay of UGRP-1 by ELISA.

The newly developed ELISA was tested on different types of biological samples, including samples collected in the ANIMO project: urine, EBC collected on coated and uncoated tubes, nasal lavage and saliva samples. The results are shown in the Table I below. It can be seen that UGRP-1 was not detectable in urine, nasal lavage and EBC samples while high concentrations were found in BAL, sputum and amniotic fluid. Concentrations of UGRP-1 in serum are well detectable and in the same range as that of CC16.

Table I. Measurement of native UGRP-1 in biological fluids.

	N	UGRP-1(µg/L)	Median	MIN	MAX
Serum	10	7.2	4.9	1.7	29.1
BAL	16	137.2	93.9	12.3	405.7
Sputum	15	420.3	366.1	81.9	1146
Urine	10	undetectable	-	-	-
Saliva	26	2.7	0.9	0.7	21
EBC	64	undetectable	-	-	-
Nasal lavage	64	undetectable	-	-	-
Amniotic liquid	88	708	472	47	6257

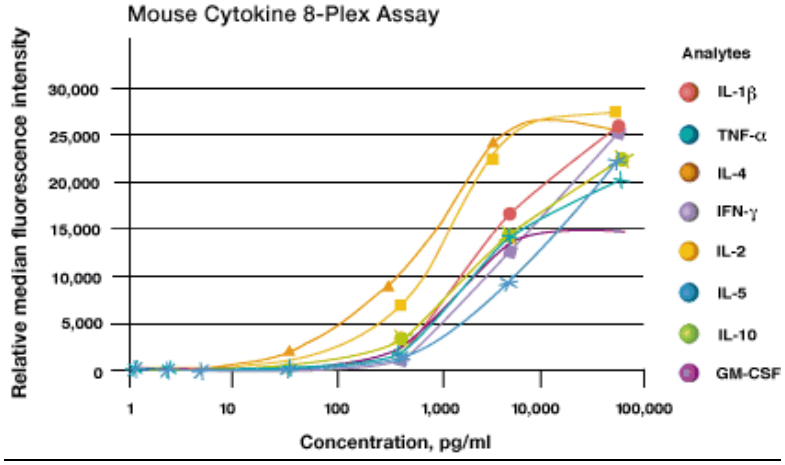
Interleukins

Several cytokines (IL-2-4-6-8-10, IFN-γ, TNF-α) were measured by Multiplex methods (Figure 6):

a) in different EBC dilutions and saliva of children and adults (Table II)

- Pure EBC
- EBC + PBS buffer
- EBC + Biorad buffer
- Pure Lyophilized EBC
- Lyophilized EBC + PBS buffer
- Lyophilized EBC + Biorad buffer
- Pure saliva
- Saliva + PBS buffer
- Saliva + Biorad buffer

b) in BAL and EBC collected in coated and uncoated tubes of children from ANIMO study at 2 different dilutions



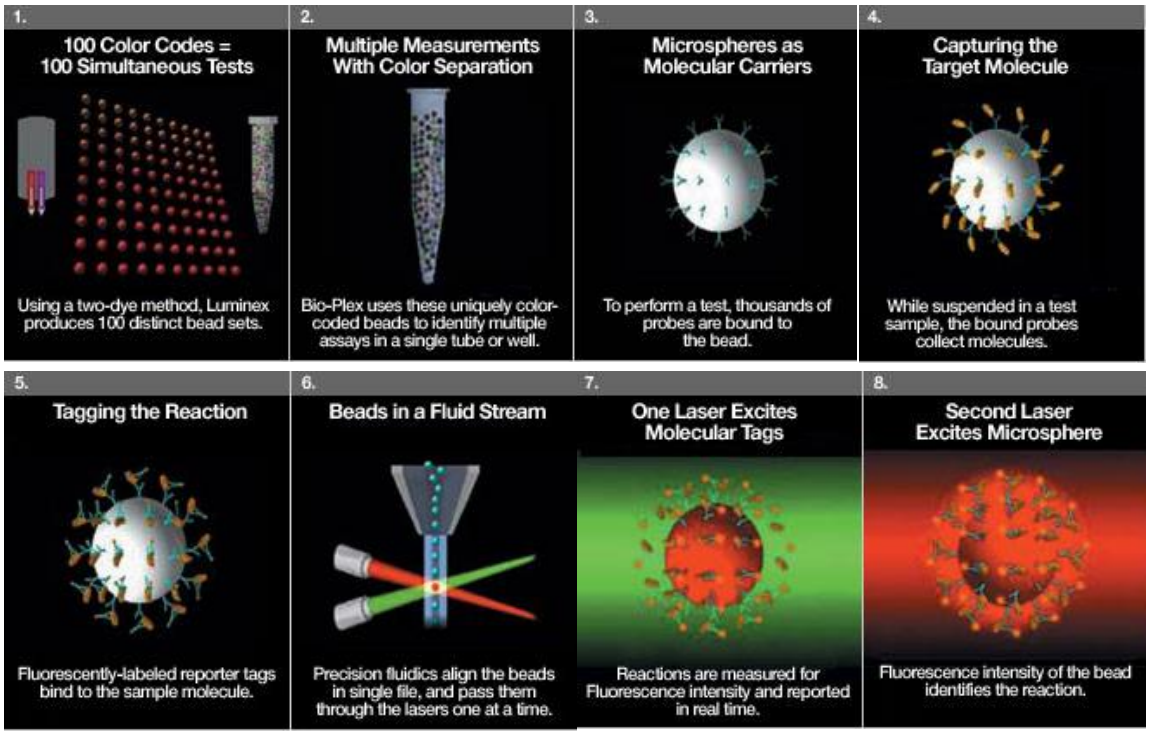


Figure 6. Multiplex method

The observed concentrations were very low and undetectable in most EBC samples collected in children or adults, even in subjects with asthma. We tested also samples of BAL and EBC collected in tubes pre-coated with albumin or not. IL-2 was detected in EBC samples from only 2 asthmatic children (both on uncoated and coated tubes). IL-2,4,6,8, TNF-α, IFN-γ were detected in all BAL samples (results not shown).

Table II. Results of the analyses in different groups.

	Pure EBC	EBC+PBS buffer	EBC+ Biorad buffer	Pure saliva	Saliva+ PBS buffer	Saliva+ Biorad buffer	Pure lyo-EBC	LyoEBC+PBS buffer	LyoEBC+ Biorad buffer
Control - children	IL-10	IL-10, IFN- γ	IL-4,10 IFN- γ	IL-6, 8,10	IL-6, 8	IL-6, 8,10	-	-	-
Asthmatic children	IL-10 IFN- γ	IL-10 IFN- γ	IL-10 IFN- γ	IL-4,6,8 IFN- γ TNF- α	IL-4,6,8 IFN- γ	IL-4,6,8 IFN- γ	-	-	IL-10 IFN- γ
Control -adults	IL-10	-	IL-10	No tested	No tested	No tested	INF-g	INF-g	-
Asthmatic adults	-	-	-	No tested	No tested	No tested	-	-	-

Lyo: lyophilisation

Cytokines measured with the Bioplex methods were undetectable in most EBC samples even in those from subjects with asthma. When some cytokines were found in some samples, we suspect a salivary contamination which was confirmed by the detection of the α-amylase in the EBC samples. In addition, we could not detect CC16 and UGRP-1 in EBC samples despite the use of sensitive immunoassays.

2.2.1.3. Nasal lavage

The technique of nasal lavage (NAL) allows collecting in a completely painless way proteins and other molecules that leak or are secreted at the surface of the nasal epithelium. The concentration of albumin or other plasma-derived proteins can be used to detect an acute or chronic disruption of the nasal epithelium associated with inflammation (rhinitis) or exposure to some irritants (e.g. ozone).

The technique consists in instillating at a constant flow using a peristaltic pump, 2,5 mL of saline (distilled water + NaCl 0.9%) at 37°C in each nostril while holding the head in a downward position. During 20 seconds, the fluid is recovered by returning the head in the upward position. The technique was successfully applied on children. The recovery of proteins such as CC16 and albumin in the nasal lavage sample was checked. The 16 kDa Clara cell protein, which is secreted throughout the airways and predominantly by the bronchiolar Clara cell, could be identified in NAL. CC16 is a very

sensitive marker of increased airways permeability. Because the Clara cell is uniquely sensitive to toxic injury, serum CC16 has been used to evaluate acute or chronic damage to terminal airways, the serum levels of CC16 decreasing proportionally to the amount of protein secreted in the respiratory tract (Bernard, 2005).

Comparisons of the amounts of albumin and CC16 measured in de nasal lavage fluid collected in children from the left and the right nostrils are shown in figure 7 and are in line with previous observations in teenagers. In further analysis in this study, concentrations are corrected for urea, which is a reliable dilution marker in nasal lavage (Sakata, 2000; Kaulbach et al, 1993).

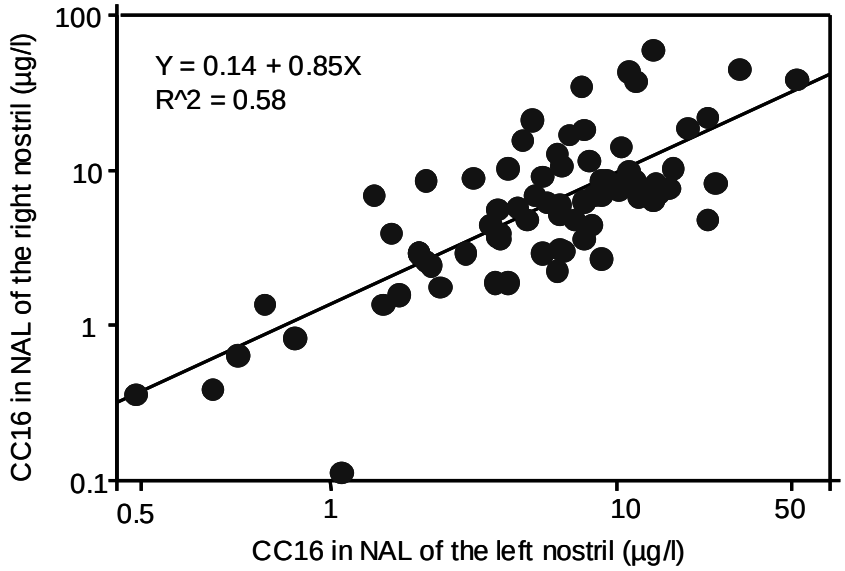


Figure 7. Correlation between the concentration of CC16 in NAL from the right and left nostril (data obtained from 60 children).

2.2.1.4. Urine

Clara cell 16kDa protein (CC16) is produced by the epithelial Clara cells (non-ciliated cells) that are mainly present in the human respiratory bronchioles. The Clara cells have been shown to repair damaged epithelium, detoxify xenobiotics and secrete proteins with important biological activities like CC16. The production of CC16 is most abundant in the respiratory epithelium, but some also takes place in nasal mucosal epithelial cells, the male urogenital tract, the endometrium, the fetal lung, the fetal kidney, the amniotic fluid and the female urogenital tract.

Increased permeability of a damaged respiratory epithelium is thought to increase the concentrations of CC16 in serum. CC16 has been thought to be able to diffuse through the air-blood barrier of the lung epithelium because of its small size. Both anti-inflammatory and immuno-modulatory qualities have been attributed to CC16. Furthermore, CC16 may be able to downregulate oxidative stress and inflammation in the respiratory tract and has been reported to have anti-tumor properties.

The highest concentrations of CC16 are found in pulmonary fluids (ELF, BALF, sputum. Although of great potential for evaluating the extent of inflammation or tissue damage, markers in BAL are not applicable for monitoring population exposed to air pollutants in the environment like children. But CC16 is also present in human amniotic fluid, serum and urine.

CC16 is at the origin of another approach for assessing the integrity of the respiratory epithelium, based on the assay in serum of lung-specific proteins. Serum CC16 can increase for several reasons including increased leakage across the pulmonary epithelium due to increased epithelial permeability, up-regulated CC16 due to anti-inflammatory qualifications, and decreased renal clearance. Both serum and urinary CC16 can be measured using enzyme-linked immunosorbent assay (ELISA) techniques which are now commercially available. So far urinary CC16 has been used mainly as a marker of proximal tubular dysfunction especially in pre-puberty children who have no post-renal secretion of the protein.

Because of their non-invasiveness tests on urine samples are readily accepted and can be applied in very young children. We have thus explored in this study whether urinary CC16 might be used as a surrogate indicator of airways integrity after adjustment for the fractional uptake of the protein on the basis of urinary RBP, a reliable marker of the reabsorptive capacity of the proximal tubule.

Table III describes the factors which are significantly associated to changes of urinary CC16 in 8 years old children with no asthma diagnosis and no airways inflammation ($eNO < 20$ ppb).

Interestingly, the concentration of CC16 in urine expressed per liter, per g of creatinine or as a ratio to RBP was positively association with exposure to tobacco smoke during pregnancy and a cumulated outdoor chlorinated pool higher than 100 hours. Such associations were not found with urinary RBP, which suggests that they may arise from an increased airways permeability. Negative associations were found with BMI, older siblings or mould on bedrooms walls suggesting that these factors might contribute by decreasing the production of the protein (Voisin C et al, article in preparation). Several other interesting observations were made, further suggesting that urinary CC16 might serve as a non-invasive test of airways integrity in children. For instance, the risk of asthma or hay fever showed an inverse association with the concentration CC16 in urine (asthma, OR 0.14, IC 95% 0.02-0.98; hay fever, OR 0.26, IC 95% 0.10-0.68) (Voisin C et al, manuscript in preparation).

Table III. Determinants of urinary CC16 in children

		Regression coefficient	p-value
Log CC16 (μg/g /creatinine)	Smoking during pregnancy	0.260	0.09
	BMI	-0.036	0.04
	Older siblings	-0.172	0.006
	Mould	-0.382	0.01
	Outdoor pool >100hours	0.284	0.01
	Ski Practice	0.310	0.008
Log CC16/RBP	Smoking during pregnancy	0.182	0.04
	Older siblings	-0.063	0.04
	Mould	-0.162	0.02
	Outdoor pool >100hours	0.121	0.06
Log CC16 (μg/L)	Smoking during pregnancy	0.350	0.04
	Outdoor pool >100hours	0.232	0.07
Log RBP (μg/L)	Parental asthma	-0.162	0.19
	ETS	0.126	0.05
	Birth weight	0.00001	0.07

2.2.2. Pilot study

In order to evaluate the performance of the non-invasive biomarkers, a pilot study involving asthmatic and healthy children was organized. Asthmatic children (N = 40) were recruited from the asthma clinic in the University Hospital Antwerp. Ethical approval has been obtained from the ethical committee of the University Hospital Antwerp. Healthy children (N = 30) were recruited from personnel of the University and from a primary school in Antwerp. The children were between 6 and 12 years old. Information sheets for the parents, informed consent documents and a small questionnaire were used. The following selection criteria were set: 5 children per school-year, equal number of boys and girls (asthma: 20/20; controls: 17/13), ratio foreigner/natives: 20/80 (based on selection of names). The examination included NO measurements (both NIOX MINO and Ecomedics device), EBC (RTube; 15 minutes in an uncoated RTube, 10 minutes in a coated RTube), exhaled gases (Tedlar bag), nasal lavage (left and right nostril) and spirometry (only the asthmatic patients). With the NIOX MINO device, one measurement was done, according to the manufacturers recommendations. With the device from EcoMedics, the children were asked to perform 3 repeated measurements. During the EBC collection, that took about 30 minutes, children were allowed to watch a movie.

Recruitment started in November 2007 and ended in April 2008. All tests together took 1 hour in the asthmatic patients (they are used to perform this type of tests), and 1 hour

10 minutes in the control group. Acceptance of the tests was very good (Table IV), and we obtained positive reactions of both children and their parents. Parents were informed on the results of the tests.

Table IV. Number of failed tests in asthmatic patients (N = 40) and control group (N = 30).

Examination	asthma	control
NO (EcoMedics)[a]	18	11
NO (EcoMedics)[b]	9	4
NO (EcoMedics)[c]	3	1
NO (NIOX MINO)	2	4
Exhaled gases (Tedlar bag)	0	0
Spirometry	2	-
Condensate 15 min	0	0
Condensate 10 min	0	0
Nasal lavage right	2	0
Nasal lavage left	2	0

[a] Based on ATS recommendations at least two reproducible measurements, that agree within 10% of each other. 41 children succeeded in this test. 12 children had two measurements that agree within 10-15% of each other, and 9 children had a variability higher than 15%. 8 children failed to perform 2 measurements.

[b] Number of individuals that failed to perform 3 NO measurements, irrespective of reproducibility; 3 trials were performed at maximum. All children were able to perform at least one measurement. 8 children performed only one successful measurement, 5 children only two.

[c] Number of individuals that rejected or failed to perform the maneuver once, irrespective of ATS recommendations concerning reproducible measurements or number of measurements.

Expansion of the pilot study

Based on results from the pilot study and suggestions by the mid-time reviewers, it was decided to collect samples in an additional group of individuals, to increase the statistical power of the obtained results and/or to confirm the results, and to improve the protocols for analysis of non-invasive biomarkers. In addition to the previous individuals, 32 asthmatic patients and 37 healthy individuals were recruited for this purpose in 2010. All children were in the same age range (6-12 years), but more boys than girls were included (asthma: 20/12; controls: 21/16). The examination included exhaled gases (Tedlar bag), exhaled NO (NIOX MINO), exhaled breath condensate (15 minutes in an uncoated RTube), saliva and urine. Also their body weight and height were determined during the examination.

2.2.2.1. Exhaled NO

Comparison of the static and the mobile device

It is known from literature that exhaled NO values obtained with the NIOX might be a little higher compared to those obtained with the NIOX MINO, especially in the higher ranges, but overall the two devices are in good agreement (Alving et al, 2006). We didn't find any comparison of the NIOX MINO and the static device from EcoMedics in the available literature. In our first pilot study (N = 70), those two devices were compared. Only the results obtained by the Ecomedics device that were in accordance with the guidelines of the ATS (2005) were used in further analyses. Table V shows mean values of exhaled NO obtained by both devices. The exhaled NO values obtained by the two devices correlated well with each other ($r = 0.81$, $p < 0.001$; Figure 8). Values were slightly higher in the NIOX MINO compared to the EcoMedics, and when evaluated in a non-parametric test for paired samples – the Wilcoxon sign test – this difference was found to be significant ($p = 0.0005$). Additionally, a Bland-Altman analysis was performed to evaluate the results obtained by the two devices (Figure 9). It can be concluded that the results between the two devices are in good agreement over the whole range. The mean difference between the devices is 2.1 ppb, with a maximal difference of about 10 ppb, which is clinical acceptable. Based on these results, it is recommended not to use results from both devices in the same analysis. In future examinations during this project, at least two measurements will be done, which agree within 10% of each other, irrespective of the device used. However, also for new studies we believe it is recommended to perform two measurements.

Table V. Exhaled NO values obtained by the NIOX MINO and the EcoMedics device.

Subjects	NIOX MINO (ppb)*	EcoMedics (ppb)*
Asthma group	17.50 (12.00 – 35.00)	16.16 (8.53 – 32.07)
Control group	14.10 (10.00 – 18.00)	10.18 (7.70 – 15.65)
All children	16.00 (11.50 – 23.50)	13.36 (7.75 – 20.87)

* Median (interquartile range, IQR).

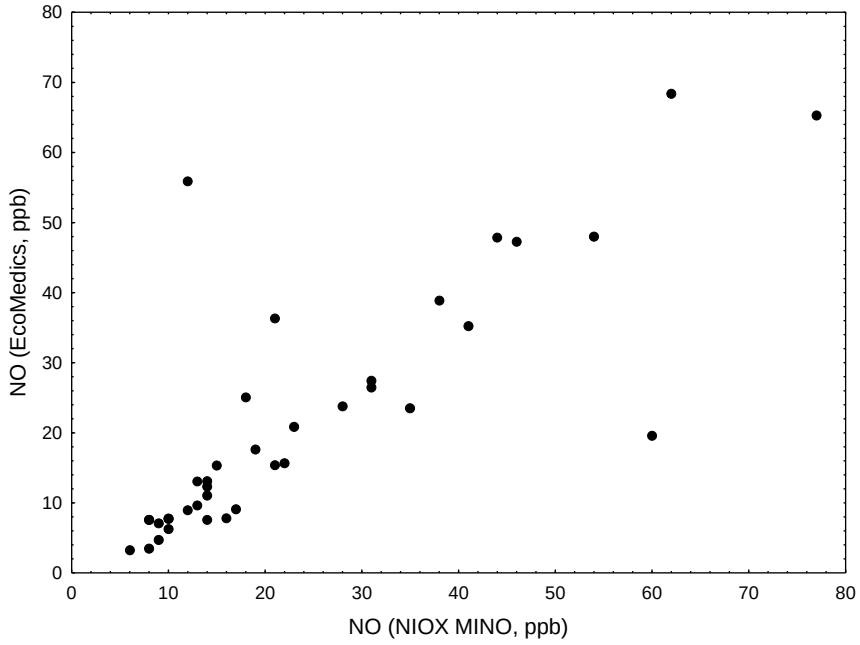


Figure 8. Scatterplot with eNO values obtained with EcoMedics and NIOX MINO devices.

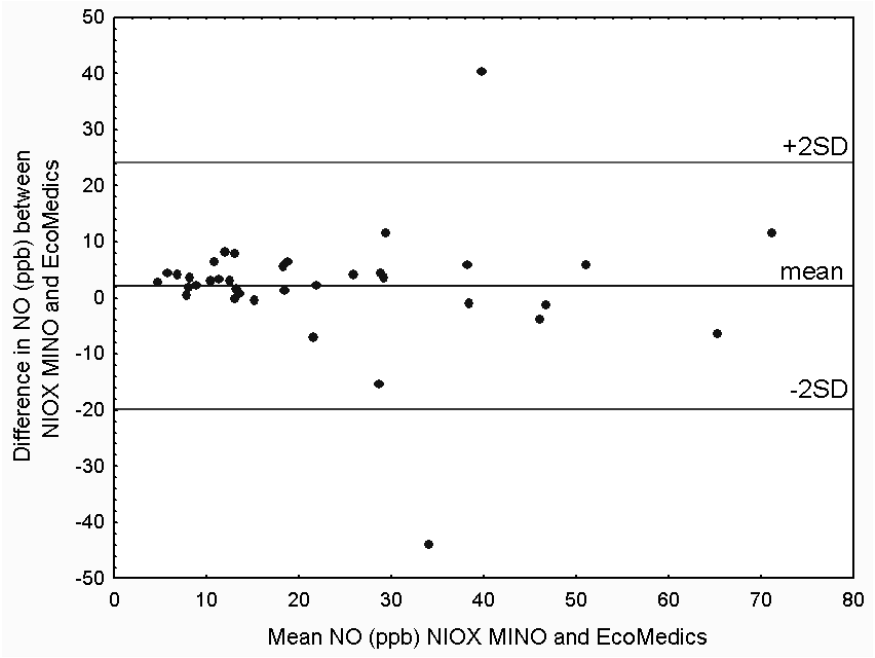


Figure 9. Bland-Altman plot to compare eNO results from NIOX MINO and EcoMedics device. When the 2 outliers are excluded from the analysis, 2*SD is only 10.9, and still all but one point are between mean ± 2*SD.

eNO as non-invasive marker

In the first pilot study (N = 70), higher eNO values in asthmatic patients compared to healthy controls were observed (Mann-Whitney U test: p = 0.011 for the EcoMedics, and p = 0.10 for the NIOX MINO). Together with the results from the expanded pilot study (N = 139), the difference in exhaled NO was borderline not significant between asthmatic patients and healthy controls (NIOX MINO; Mann-Whitney U test: p = 0.057;

figure 10). Exhaled NO was more than 20 ppb In 32 of the 72 asthma patients and in 16 of 67 healthy controls. Higher concentrations than 30 ppb were observed in 24 of the 72 asthma patients and in 6 of 67 healthy controls. The difference in eNO value was more obvious between allergic asthma patients and non-allergic asthma patients (Mann-Whitney U test: p = 0.01, NIOX MINO; figure 11). In the total group, a significant correlation was observed between eNO and age (Spearman correlation: r = 0.17, p = 0.049) and height (Spearman correlation: r = 0.22; p = 0.013).

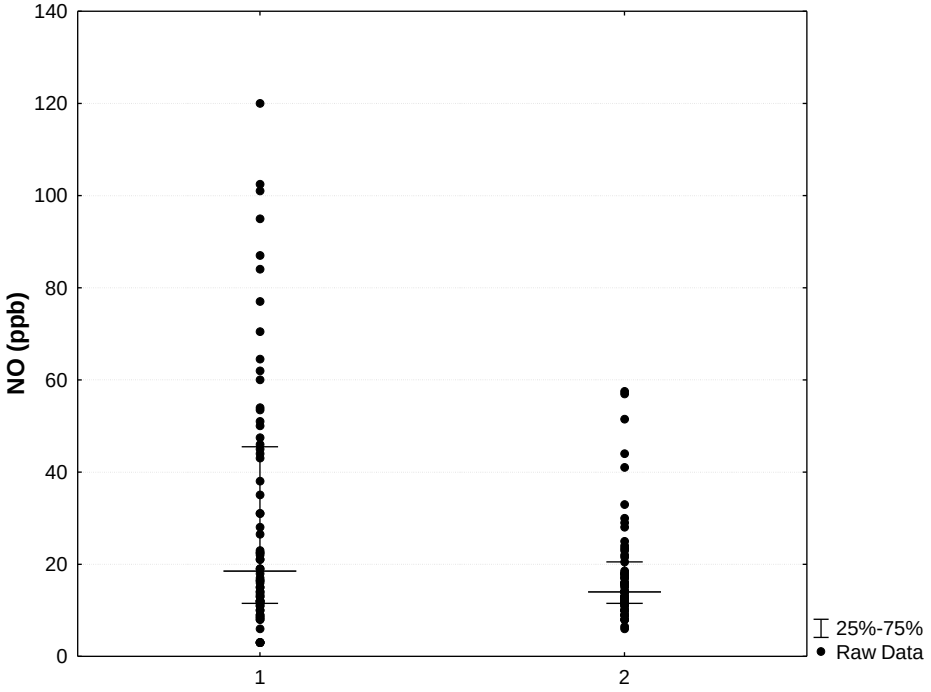


Figure 10. Scatterplot with median value and interquartile range of eNO values (NIOX MINO) from asthmatic (1) and healthy (2) children from the pilot and the expanded pilot study together.

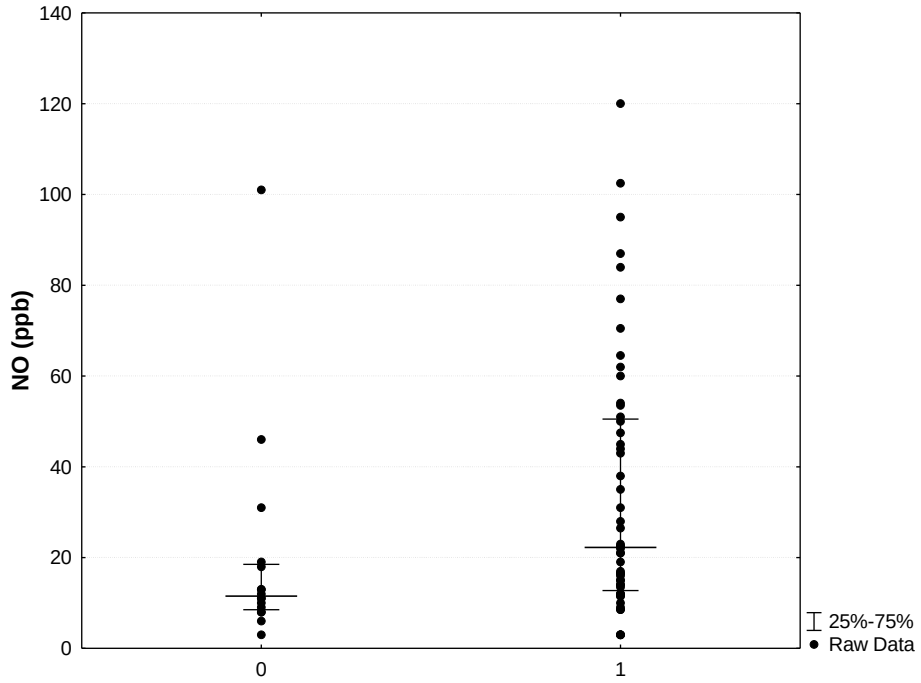


Figure 11. Scatterplot with median value and interquartile range of eNO values (NIOX MINO) from allergic asthmatic (1) and non-allergic asthmatic (0) children from the pilot and the expanded pilot study together.

2.2.2.2. Nasal lavage

Nasal lavage samples were collected in both the right and the left nostril during the first pilot study (N = 70). As previously observed in children and adolescents, significant correlations between the two nostrils were seen in albumin/urea ratio (log) ($r = 0.748$; $p < 0.0001$) and CC16/urea ratio (log) ($r = 0.556$; $p < 0.0001$). Therefore, the average (log) value of the two nostrils was used in further analysis.

Albumin is a carrier protein derived from serum. When epithelial cells from the airways (e.g. in the nose) are damaged, albumin can leak through the epithelium, causing the concentration in the tissues (and as a consequence e.g. in nasal lavage) to increase. In this pilot study, mean ratio albumin/urea ($\pm$SD) for all children was 0.424 ± 0.566. Values in control and asthmatic children were not statistically different (Mann-Whitney U test, $p = 0.49$; figure 12). Also between allergic asthmatic patients and non-allergic asthmatic patients, no significant difference was observed (Mann-Whitney U test: $p = 71$). We found no correlations between albumin/urea ratio (log) and age, gender, weight, height or asthma/control group.

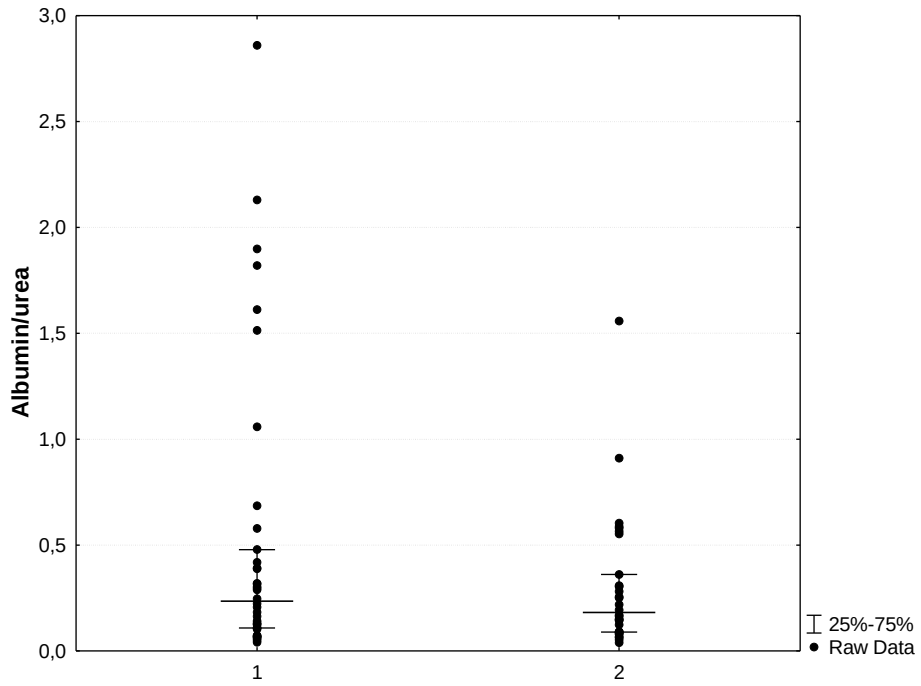


Figure 12. Scatterplot with median value and interquartile range of albumin/urea values from asthmatic (1) and healthy (2) children from the first pilot study.

Clara cell protein 16 (CC16) is an anti-inflammatory protein, predominantly excreted by non-ciliated bronchiolar clara cells. In the first pilot study (N = 70), mean ratio CC16/urea (± SD) for all children was 3.39×10^{-4} ($\pm$ 6.07×10^{-4}). Values in control and asthmatic children were not statistically different (Mann-Whitney U test, p = 0.82; Figure 13). Values were significantly lower in allergic asthmatic patients compared to non-allergic asthmatic patients (Mann-Whitney U test, p = 0.019; figure 14). This result was also reported in the international literature (Johansson, 2005). We found no correlations between CC16/urea ratio (log) and age, gender, weight, height or asthma/control group.

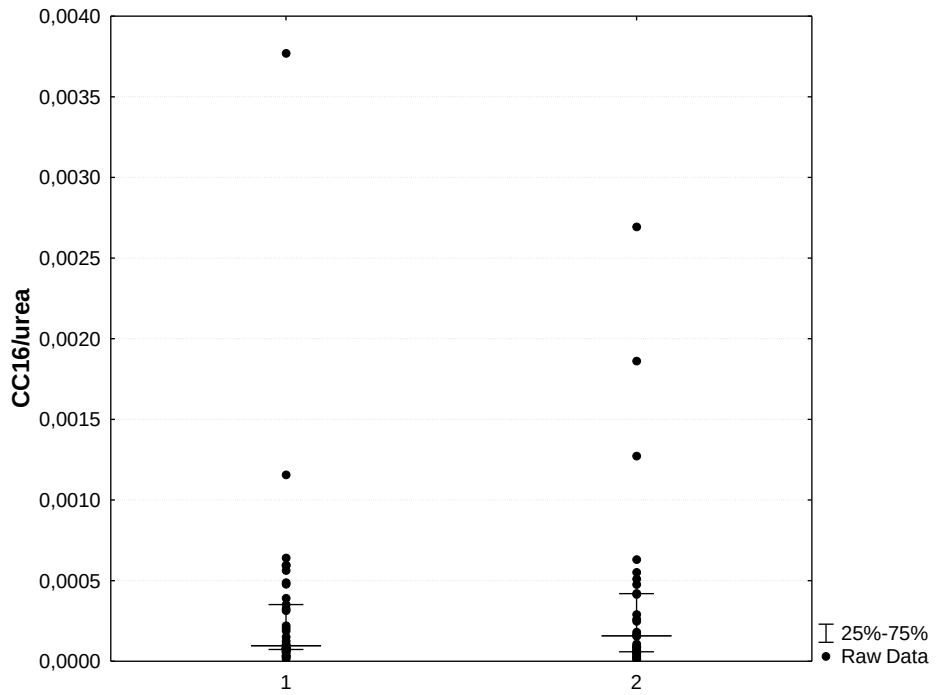


Figure 13. Scatterplot with median value and interquartile range of CC16/urea values from asthmatic (1) and healthy (2) children from the first pilot study.

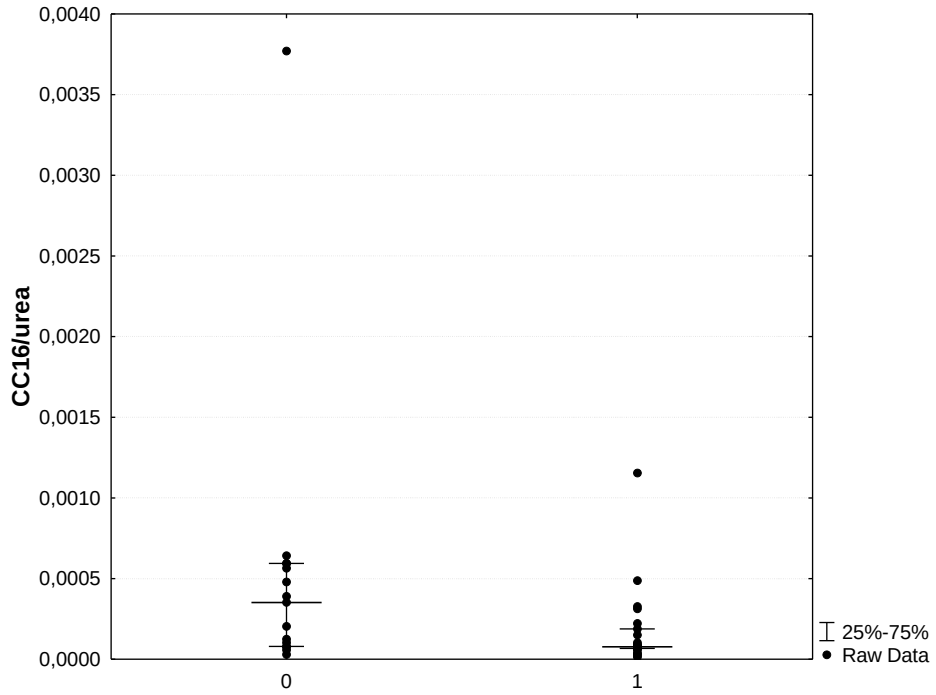


Figure 14. Scatterplot with median value and interquartile range of CC16/urea from allergic asthmatic (1) and non-allergic asthmatic (0) children from the first pilot study.

2.2.2.3. Exhaled breath condensate

EBC was collected twice in the first pilot study (N = 70): the first collection took 15 minutes of tidal breathing through an uncoated RTube. These samples were used for pH measurements and proteome analysis. The second collection occurred during 10 minutes of tidal breathing through a RTube coated with 1% bovine serum albumin (BSA). These samples were used to measure specific molecules in the EBC, such as leukotriene B4 and 8-isoprostane. The coating was applied to reduce loss of molecules on the surface of the collection system. As a result, molecules can be identified using commercially available kits, as initially amounts were below the detection limits.

pH

EBC pH was measured in 500 μL of the first EBC sample (N = 70), collected in the uncoated RTube, exactly 5 minutes after collection, without deaeration. Mean value ($\pm$SD) was 6.17 $\pm$ 0.29. pH was significantly lower in the asthma group (6.07 $\pm$ 0.28) compared to the healthy controls (6.23 $\pm$ 0.29; Mann-Whitney U test: p = 0.047) (Figure 15). The statistical power of this test was 64% (α = 0.05). We calculated that a statistical power of 80% (α = 0.05) can be achieved with 50 individuals in each group. No significant difference was observed between allergic asthmatic patients and non-allergic asthmatic patients (Mann-Whitney U test: p = 0.79). We observed a significant correlation of EBC pH with gender (r = -0.299; p = 0.013): girls had lower pH values compared to boys. In a multiple regression analysis, effects of gender and asthma/healthy control were estimated. The overall effect of these two determinants on EBC pH was significant. In the control group alone, no significant correlation of gender and EBC pH was observed, in the asthmatic patients, this correlation was present (r = -0.37; p = 0.019). Previously, we didn't observe an effect of gender on EBC pH in healthy adults. Unfortunately, our study group was too small to make any conclusions about the effect of the interaction between gender and asthma/control on EBC pH (power = 0.11).

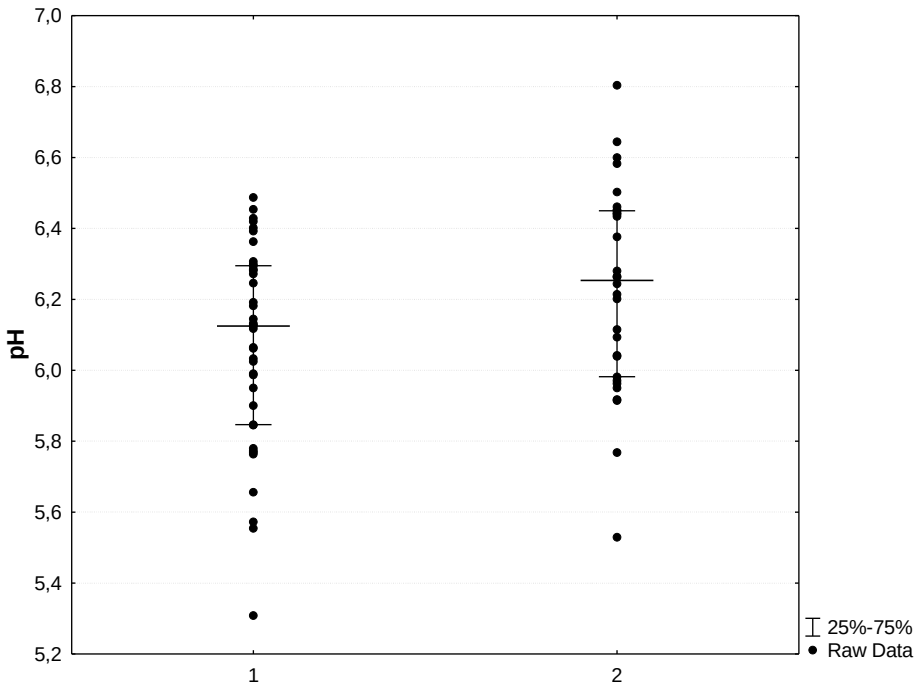


Figure 15. Scatterplot with median value and interquartile range of EBC pH values from asthmatic (1) and healthy (2) children from the first pilot study.

Additionally, pH was measured in the lab after deaeration. In literature, it is stated that this method is more standardized and shows less variation. However, after deaeration during 15 minutes, pH was not stable, and still varied in time. Furthermore, it is not known which gases or molecules, other than CO_2, are removed from the samples. Therefore, it was concluded to measure pH exactly 5 minutes after sampling, without deaeration, in 0.5 ml EBC.

In the expansion of the pilot study, pH could unfortunately not be measured directly after sampling due to technical problems. We were only able to measure EBC pH after deaeration in the lab. We observed the same problems as in the first group concerning variation and standardization. Based on the results after deaeration, no differences were observed between the asthma group and the control group (Mann-Whitney U test: $p = 0.61$ in the new samples only, and $p = 0.53$ taken all samples from both pilot studies together).

EBC Volume

During the first EBC collection (15 minutes), an average of 0.97 mL $\pm$ 0.41 EBC was sampled. In the asthma group, the mean value was 0.84 mL $\pm$ 0.35, and in the control group, it was 1.15 mL $\pm$ 0.42. A positive correlation was found between collected volume EBC and height ($r = 0.26$; $p = 0.037$) and weight ($r = 0.30$; $p = 0.013$) of the individuals. Multiple regression analysis showed significant effects of weight ($p = 0.009$)

and asthma / healthy control ($p = 0.002$) on the collected volume. In our previous study in adults, we found that height (which is well correlated with weight; $r = 0.85$; $p < 0.01$) had a major effect on the collected volume.

8-isoprostane

In the first pilot study, the stabilizing reagent butylated hydroxytoluene (BHT) was not added to the samples, and as a consequence, most measurements were at the limit of detection, and results were not reliable. After further optimization of the assay, it was concluded that the addition of a stabilizing substance such as BHT is needed. In the expansion of the pilot study, this buffer was added immediately after sampling to the aliquot in which 8-isoprostane was measured, which resulted in reliable measurements. Although the 8-isoprostane concentration is reported in the literature to be significantly increased in asthma patients compared to healthy controls, we found no significant difference between the two groups (Mann-Whitney U test: $p = 0.15$; figure 16). More variance was observed in the control group. At this moment, we don't have an explanation for this result, although possibly asthma control status might be an influencing factor. Significant higher values were observed in the allergic asthmatic patients compared to the non-allergic asthmatic patients (Mann-Whitney U test: $p = 0.032$; figure 17). Gender, age, height and weight were not correlated with this molecule (Spearman correlation).

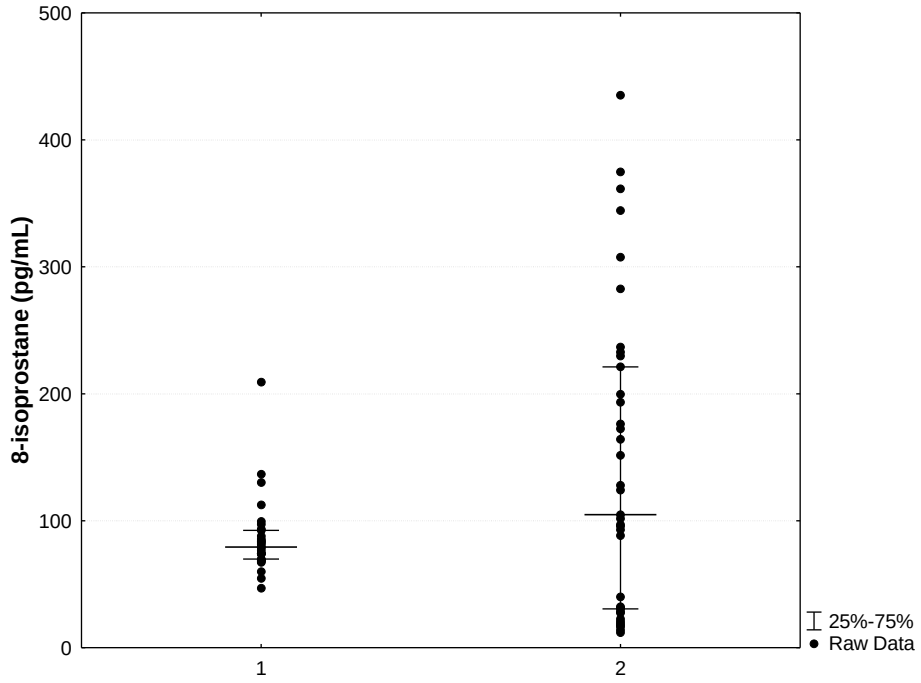


Figure 16. Scatterplot with median value and interquartile range of 8-isoprostane values from asthmatic (1) and healthy (2) children from the expanded pilot study.

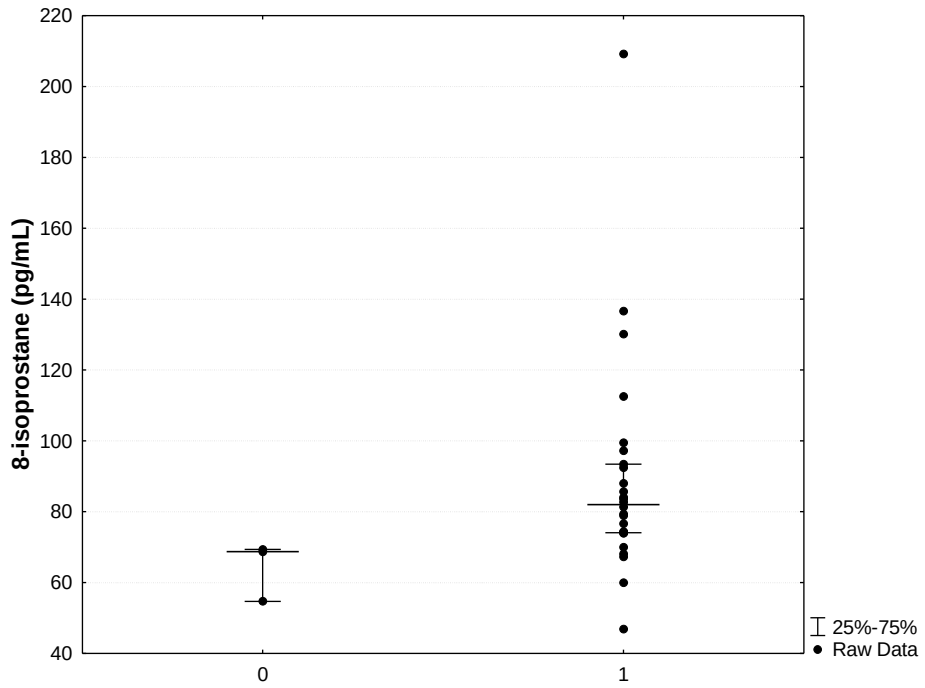


Figure 17. Scatterplot with median value and interquartile range of 8-isoprostane from allergic asthmatic (1) and non-allergic asthmatic (0) children from the expanded pilot study.

Leukotriene B4

Leukotriene B4 (LTB4) is an inflammatory molecule. It is produced from leukocytes in response to inflammatory mediators and is able to induce the adhesion and activation of leukocytes on the endothelium, allowing them to bind to it and cross into the tissue. It is a chemoattractant for neutrophils, and results in airway narrowing. LTB4 was measured in the EBC samples collected in the coated RTube. Measurements were done in duplicate by using the EIA kit from Cayman, and were successful in 100% of the samples. In the pilot study, we found no correlations between EBC LTB4 values and age, gender, height or weight of the children. In the first pilot study, we could demonstrate a correlation between LTB4 and family history of asthma: children from which the parent(s) had asthma, had significant higher LTB4 levels compared to those with parents without asthma irrespective of their individual allergy status (Mann-Whitney U test: $p = 0.038$ and $p = 0.028$, respectively). No significant difference was observed between children whose parents had an allergy and those who did not. Also allergy in the child was not correlated to LTB4 levels in exhaled breath. We did observe slightly higher values in the healthy children compared to the asthmatic children, although not significant. The statistical power of this test was 68% ($\alpha = 0.05$). Based on these results, a statistical power of 0.80 with the same significance would be obtained when 45 individuals are present in each group.

Furthermore, we did observe a positive correlation between LTB4 in EBC and CC16/urea in nasal lavage ($r = 0.3018$, $p = 0.014$).

Together with the results from the expanded pilot study, comparable results were obtained: LTB4 levels in the exhaled breath were slightly higher in the control group compared to the asthma patients (figure 18), but no significant difference between the groups was observed (Mann-Whitney U test: p = 0.087). Values from allergic asthmatic patients and non-allergic asthmatic patients were also not statistically significant (Mann-Whitney U test: p = 0.11). No correlations with individual characteristics were observed.

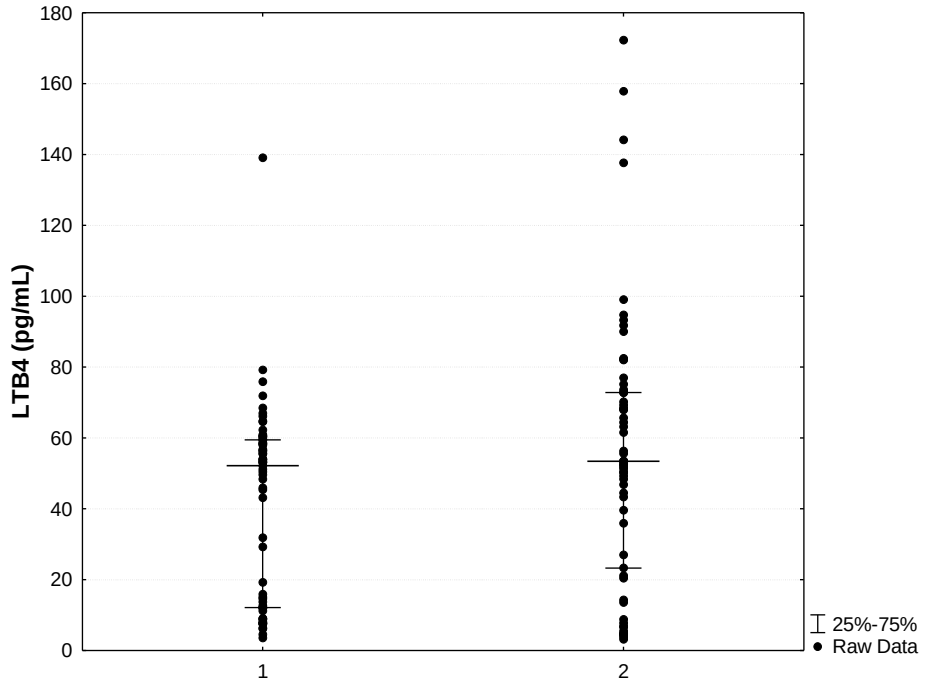


Figure 18. Scatterplot with median value and interquartile range of exhaled LTB4 values from asthmatic (1) and healthy (2) children from the pilot and the expanded pilot study together.

2.2.2.4. Saliva

Because the exhaled breath condensate samples are collected by breathing orally, it is possible that small amounts of molecules originating from the mouth are present in the collected sample. To prevent saliva contamination, volunteers are instructed to rinse their mouth with water before EBC collection, and to swallow or spit out all saliva during collection. In the collection devices, some form of saliva trap or filter is often used to prevent gross contamination by liquid saliva. In the RTube, the collection tube is located above the mouthpiece, which allows only exhaled air to go up. When saliva enters the device, it remains in the mouthpiece itself or in the lower part of the tubing, but not in the collection tube nor in the sample. Of course, molecules present in the mouth might also be aerosolized during breathing, and as such collected in the exhaled breath condensate.

In the expansion of the pilot study, saliva was collected to check for correlations of specific molecules both in the saliva and the EBC derived at the same sampling time, from the same individuals. We measured leukotriene B4 both in both matrices. No correlation was observed between concentrations in saliva and EBC (Spearman correlation: $p = 0.79$; figure 19). Also other studies reported no correlations between measurements in both matrices (Ichikawa, 2007; Baker, 2007; Tsicopoulos, 2000).

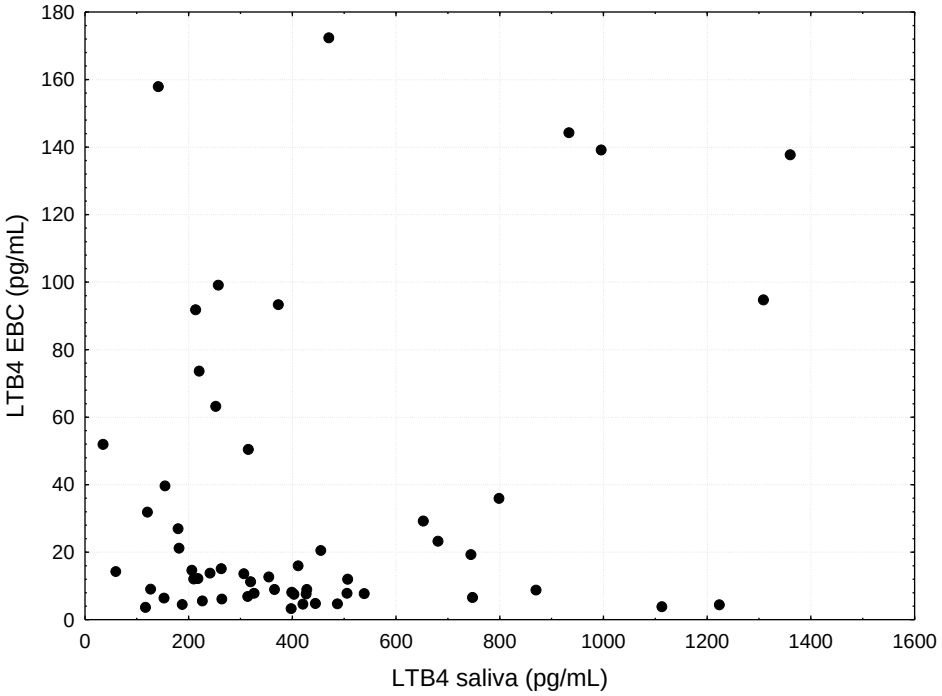


Figure 19. No correlation between LTB4 concentrations in EBC and saliva was observed.

Additionally, EBC samples can be checked for the presence of alpha-amylase activity. Alpha-amylase is the most or one of the more abundant proteins in saliva (Tan, 2007; Carlson, 1993), and is not expected to be present in the lungs or lower airways. Various kits are available for this purpose, however, these kits might not be sufficiently sensitive to identify small contributions of saliva to EBC. We checked every EBC sample for alpha-amylase activity by a commercial kit based on a kinetic, enzymatic reaction (Infinity™ Amylase Liquid Stable Reagent kit, Thermo), and never detected any activity in the EBC samples. Additionally, every sample was checked for peptide masses derived from alpha-amylase (by using mass spectra), but only a few samples were found to be contaminated. The abundance of alpha-amylase in EBC samples is, however, relatively low, especially compared to cytokeratins, but also compared to albumin, hemoglobin, and other proteins identified in EBC. In saliva samples, alpha-amylase is the most or one of the more abundant proteins (Tan, 2007; Carlson, 1993), which is in contrast to our results in EBC.

Based on this information, we can conclude that molecules derived from saliva or the oral cavity can be present in low amounts in EBC. However, both matrices give different information. EBC contains unique molecules, which are derived from lower airways or the lungs and which cannot be detected in saliva.

LTB4 concentrations measured in the saliva were comparable between the asthma patients and the healthy controls (Mann-Whitney U test: $p = 0.5$).

2.2.2.5. Urine

In the expansion of the pilot study, 8-oxo-deoxyguanosine (8OHdG), clara cell protein (CC16), and retinol-binding protein (RBP) were measured in urine samples from the asthma patients and healthy controls.

Urinary 8OHdG is a marker of oxidative DNA damage and oxidative stress, which was measured by ELISA (Gentaur, Belgium). Between the asthma and control group and between the allergic and non-allergic asthma patients, no significant differences were observed (Mann-Whitney U test: $p = 0.21$ and $p = 0.64$, figure 20).

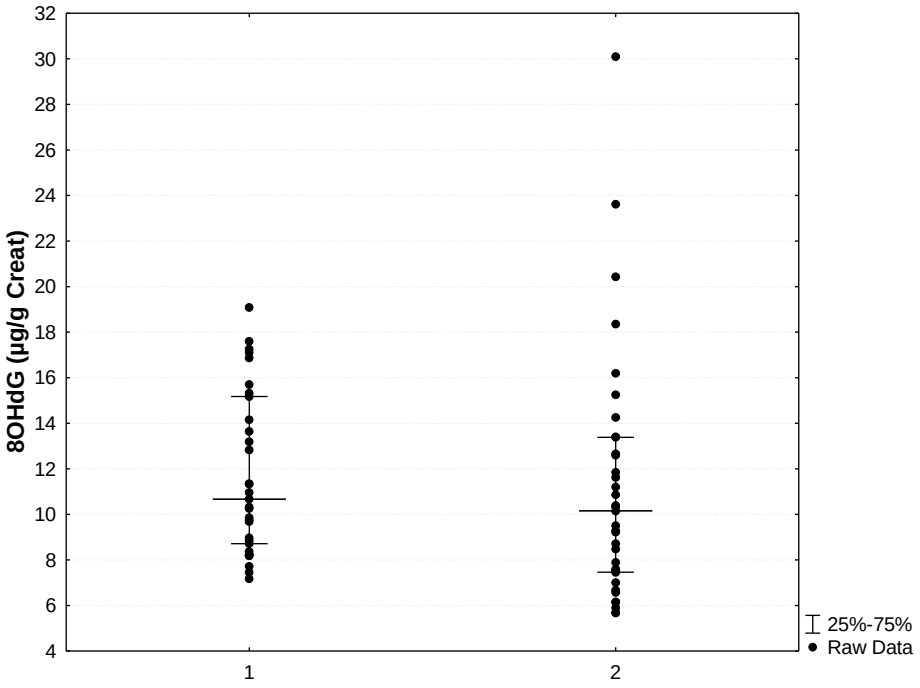


Figure 20. Scatterplot with median value and interquartile range of urinary 8OHdG values from asthmatic (1) and healthy (2) children from the expanded pilot study.

Clara cell protein (CC16) is a lung-specific protein. RBP has been measured to adjust CC16 for variation in proximal tubular function. No significant differences were observed between the asthma patients and healthy controls, nor between the allergic

and non-allergic individuals (Mann-Whitney U test: p = 0.77 and p = 0.27 respectively, figure 21).

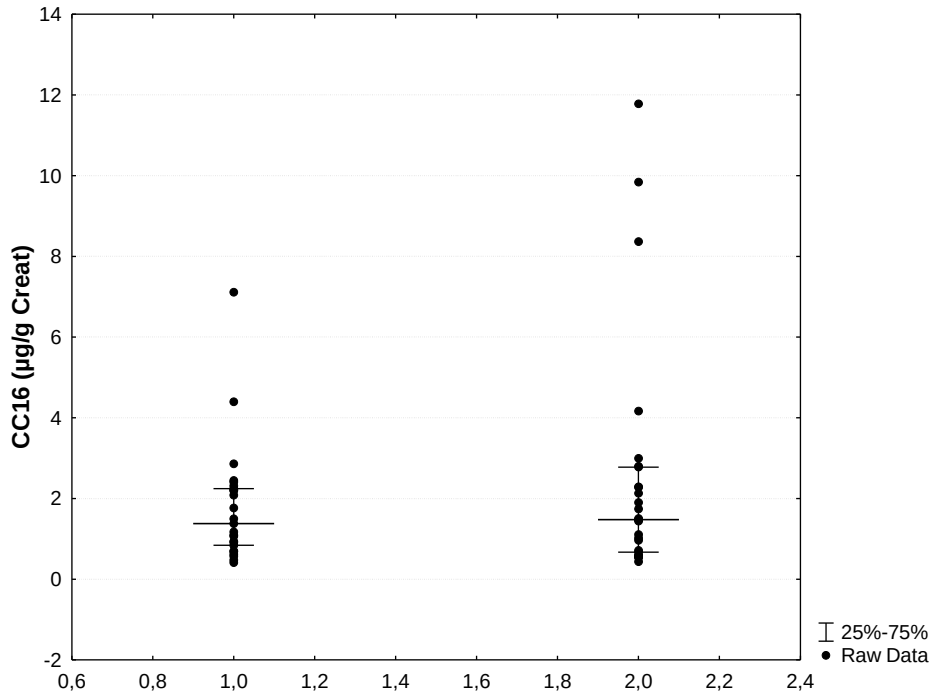


Figure 21. Scatterplot with median value and interquartile range of urinary CC16 values from asthmatic (1) and healthy (2) children from the expanded pilot study.

2.2.2.6. Analysis of exhaled breath fingerprints

Combination of various biomarkers

In both pilot studies, exhaled NO, EBC pH (after deaeration) and EBC LTB4 were measured. These three markers were analysed together in a multiple logistic regression analysis for asthma. eNO and LTB4 were statistically significant in separate analysis, and since they were not correlated with each other, they could be analysed together. In the result from this combined analysis however, only eNO was significantly elevated in the asthma group compared to the healthy individuals.

Proteome analysis

EBC (1 mL) from the collection in the uncoated RTubes was used for proteome analysis. Samples were concentrated on beads and enzymatically digested by trypsin. The resulting peptides were separated by nanoLC. Peptides in all fractions were detected in a MALDI-TOF mass spectrometer. By using this method, a potential biomarker proteolytic peptide pattern was selected. MSMS analyses were performed to identify the various proteins. The whole procedure has been described and published (Bloemen et al, 2009; Bloemen et al, Epub ahead of print, 2010).

Of the 50 samples from the first pilot study that could be used for protein analysis, 22 were from the control group, and 28 from asthmatic patients. In this last group, 5 patients had no doctor-diagnosed asthma (the diagnosis was not completely clear at that time), 13 patients had well controlled asthma, 9 moderately controlled and 1 uncontrolled.

Proteolytic peptide patterns

Data processing was performed as described above (section 2.2.1.2.).

To select a peptide pattern that is able to distinguish between healthy controls and asthmatic patients with as few proteins as possible, we performed support vector machine (SVM) analysis. Peptides that were detected in less than 10% of the samples, were discarded in the analysis. This was done since these peptides did not exert any discriminatory power due to their low occurrence rate, and might introduce noise if implemented into the classification model.

In a first attempt to select a characterizing peptide profile for EBC of asthmatic patients, all patients were compared with the control group. Based on 14 peptides, 98% of the samples were classified correctly. One healthy control was classified in the asthma group.

Next, only data from the moderately and uncontrolled patients (n = 10) were compared to the control group (n = 22). Applying the threshold criterion (presence of the peptides in more than 10% of the samples) resulted in a database consisting of 1331 peptides. The most optimal classification model was based on a support vector classifier using 10 peptides. This model classified all subjects correctly (100%) regarding their asthma status (figure 22, upper graph). Five of these peptides were more abundant in the control group, and 5 were more abundant in the patient group. Three of those peptides were also significantly different in a Student t-test performed between the healthy controls and the 10 moderate and uncontrolled asthmatic patients. Most of the peptides selected because of their discriminating capacity were not abundant enough to allow their identification. Just one peptide was identified as part of cytokeratin 1.

A third model was able to distinguish for 100% correctly between only the controlled asthma patients (n = 13) and the control group (n = 22). In this model, 11 peptides were needed, of which six were more abundant in the asthma group (figure 22, lower graph). One peptide that was more abundant in the control group was also present in the previous model.

A final approach, in which we attempted to discriminate between the five groups (no doctor-diagnosed asthma, controlled asthma, moderate asthma, uncontrolled asthma, and healthy individuals) using one SVM model, resulted in only 73% correct classification based on 41 peptides.

To test the classification model for the moderately and uncontrolled patients versus the healthy individuals described here, some new EBC samples were introduced to the model. 20 EBC samples from healthy children and 5 new EBC samples from uncontrolled asthmatic patients (individuals until now not included in the model) were collected and analyzed using the same start volume EBC and the same protocol. However, it should be noticed that the overall intensity of mass peaks was lower compared to the spectra of the first samples. The ratio 'area under the curve of a standard peptide mixture added to the samples' / 'area under the curve of the internal standard', was in the new samples on average only 80% of this ratio in the first sample series. As a result, not all of the 10 selected peptides were detected in these new samples. Based on maximum 8 peptides, 16 of the 20 control samples were classified correctly in the control group. In the original model, also 80% of the samples could be classified correctly if only those 8 masses were used. For the classification of the uncontrolled asthmatic samples, only 5 peptides were detected and could be used. Two of those were more abundant and 3 were less abundant in the asthma group of the model. It is not possible to classify the samples based on only half of the peptides from the model. When this was tried, only 2 of the 5 samples were classified correctly in the asthma group.

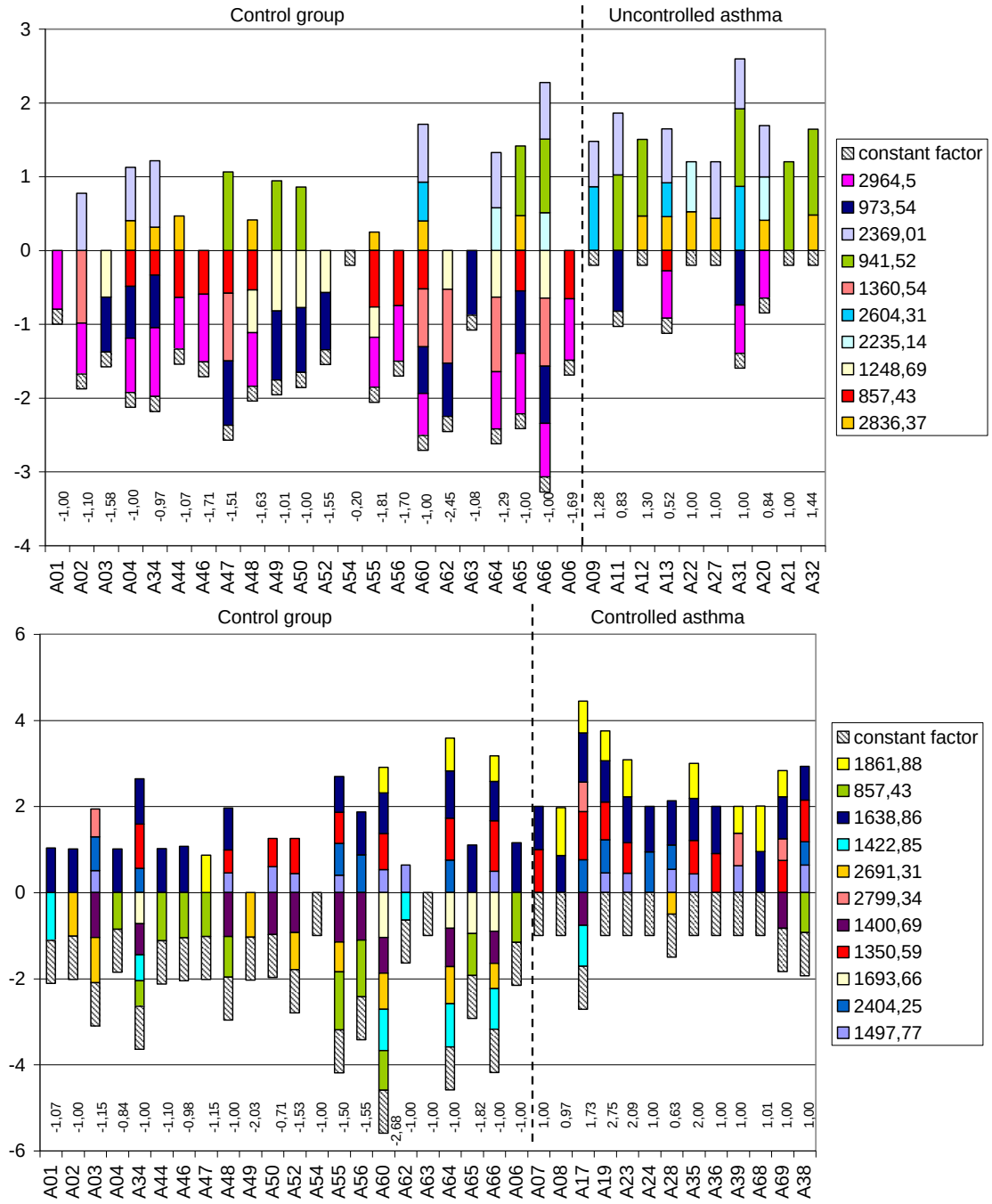


Figure 22. *SVM models able to classify all subjects 100% correctly for asthma status (upper graph: model for moderately and uncontrolled asthma versus healthy controls; lower graph: model for controlled asthma patients versus healthy controls). The X-axis shows the subjects. Left and right of the dotted line depicts subjects belonging to respectively the control and asthma group. The Y-axis represents the normalized and weighted integrated peak area of the selected ln-transformed peptides. The masses of the peptides are shown in the legend. The constant factor from the SVM equation is also shown. The relative abundance of each peptide is shown for every individual. The outcome of the SVM equation, which is the sum of the values from all normalized and weighted ln-transformed peptides and a constant factor, is shown for every sample: in the asthma group, these numbers are positive, while samples in the control group have a negative SVM outcome. The SVM implementing and combining data from all peptides is able to classify all subjects correctly (100%) in the two groups.*

Protein identifications

The most abundant proteins identified in the EBC samples were cytokeratins. Especially the cytokeratins 1, 2, 9, and 10 were found in most samples. Additionally, cytokeratin 5, 6, 8, 14, and 16 were identified in various samples. Although the abundance of the most common peptides belonging to these proteins is mostly higher in the EBC samples from the asthma patients, none of these peptides were significantly different between the asthma and the control group in the Student t-test. However, one peptide belonging to cytokeratin 1 is part of the SVM pattern to distinguish between the asthma and the control groups.

Additionally, this approach for the analysis of EBC samples led to the identification of new proteins in these samples, such as albumin, hemoglobin, lysozyme, actin, dermcidin, and calgranulin A and B. Until now, the more abundant proteins in the EBC samples were identified. Additional measurements on a different mass spectrometer (hybrid Fourier Transform mass spectrometer, LTQ Orbitrap XL™, Thermo) were performed, to allow further identification of exhaled proteins. This resulted clearly in more sensitive measurements: previous identifications were confirmed, and reliable identification of additional, less abundant proteins such as annexin A1, annexin A2, Arginase-1, Cystatin A and B, etc (table VI) was obtained. At this moment, however, not all peptides selected in the profiles, are identified.

Table VI. Identified proteins in EBC and their function

Protein identifications	**Function*** (selection)
Cytokeratin 1 – 2 – 9 – 10	Structural proteins
Albumin	Transport protein
Actin	Cell division, muscle contraction, cell motility, and repair response
Hemoglobin	Produced by e.g. alveolar type II epithelial cells Oxygen transport across air-blood barrier Protects cells against oxidative damage
Lysozyme	Secreted by glands and epithelial cells in the airways Antibacterial compound
Dermcidin	Antibacterial protein, probably derived from sweat glands
Calgranulin B	Role in inflammation
Calgranulin A	Role in inflammation
Arginase-1	Association with asthma, asthma exacerbation and atopy

Protein identifications	**Function*** (selection)
Annexin A1	Anti-inflammatory activity by inhibition of phospholipase A2 (synthesis of inflammatory molecules, e.g. PG, LT)
Annexin A2	Related with S100 proteins. Role in asthma exacerbation

Beta-catenin	Cell adhesion, signal transduction
Caspase-14	Role in apoptosis, especially present in epidermis
Catalase	Protects cells against toxic effects of hydrogen peroxidise; stimulates cell growth (e.g. T cells, B cells, fibroblasts)
Cystatin A	Cysteine protease inhibitor
Cystatin B	Protease inhibitor
Desmocollin-1	Cell adhesion
Desmoglein 1	Component of intercellular desmosome junction; cell-cell adhesion; possibly derived from the tongue
Desmoplakin	Epidermal integrity
E-FABP	Epidermal fatty acid-binding protein
Elongation factor 1α1	Role in translation during biosynthesis of proteins; nuclear export of proteins, role in cell death due to oxidative stress
Filaggrin	Differentiation of epidermis; binds to keratin
Galectin-7	Cell-cell / cell-matrix interactions; associated with apoptosis of bronchial epithelial cells in asthma
GAPDH	Role in glycolysis (antioxidant co-factor); regulates cell death
HSP27	Resistance against stress
Hornerin	S100 protein; may have a role in cornification
Junction Plakoglobin	Cell adhesion
Keratinocyte proline-rich protein	Possible role in differentiation of keratinocytes
NCCRP-1	Role in innate immunity
Peroxiredoxin	Cell proliferation, differentiation; immune response; thiol-specific antioxidant; controls apoptosis
PIP (prolactin-induced protein)	Role in immunoregulation, antimicrobial activity, apoptosis
Serpin B12	Protease inhibitor
Small proline-rich protein 2B	Keratinocyte-protein
Suprabasin	Epidermal differentiation
Zinc-alpha-2-glycoprotein	Stimulates lipid degradation in adipocytes

* Information especially derived from Uniprot database but also from literature search (pubmed, sciencedirect).

Additionally, salivary proteins (e.g. alpha-amylase) were detected in the EBC, although at low abundance. Compared to saliva, in which alpha-amylase is the most abundant protein, EBC contains various more abundant proteins, suggesting that the proteins detected in the EBC are not only derived from the mouth but also from lower airways. Some proteins might be derived from the skin (e.g. cytokeratins, dermcidin): they might be present in the ambient air, and collected in the exhaled air after breathing them in. Most of the proteins identified in the EBC have been described to be present in the airways (e.g. in bronchoalveolar lavage fluid), or were associated with asthma (literature

search). At this moment, we were unable to detect some lung-specific proteins such as surfactant proteins (SP) or clara cell (CC) proteins in the EBC.

Metabonome analysis

VOC patterns

Data processing was performed as described above (section 2.2.1.2.).

To select a VOC pattern that is able to distinguish between healthy controls and asthmatic patients with as few molecules as possible, we performed support vector machine (SVM) analysis. Gasses that were detected in less than 10% of the samples, were discarded in the analysis. This was done since these compounds did not exert any discriminatory power due to their low occurrence rate, and might introduce noise if implemented into the classification model.

The samples from the first pilot study were used to compose this model. The analysis started with 821 gasses (present in more than 3 samples (= 10%). The most optimal classification model for the VOCs classified all subjects correctly (100%) regarding their asthma status (figure 23, table VII). This model was based on the moderately and uncontrolled asthma patients, not on the whole asthma group.

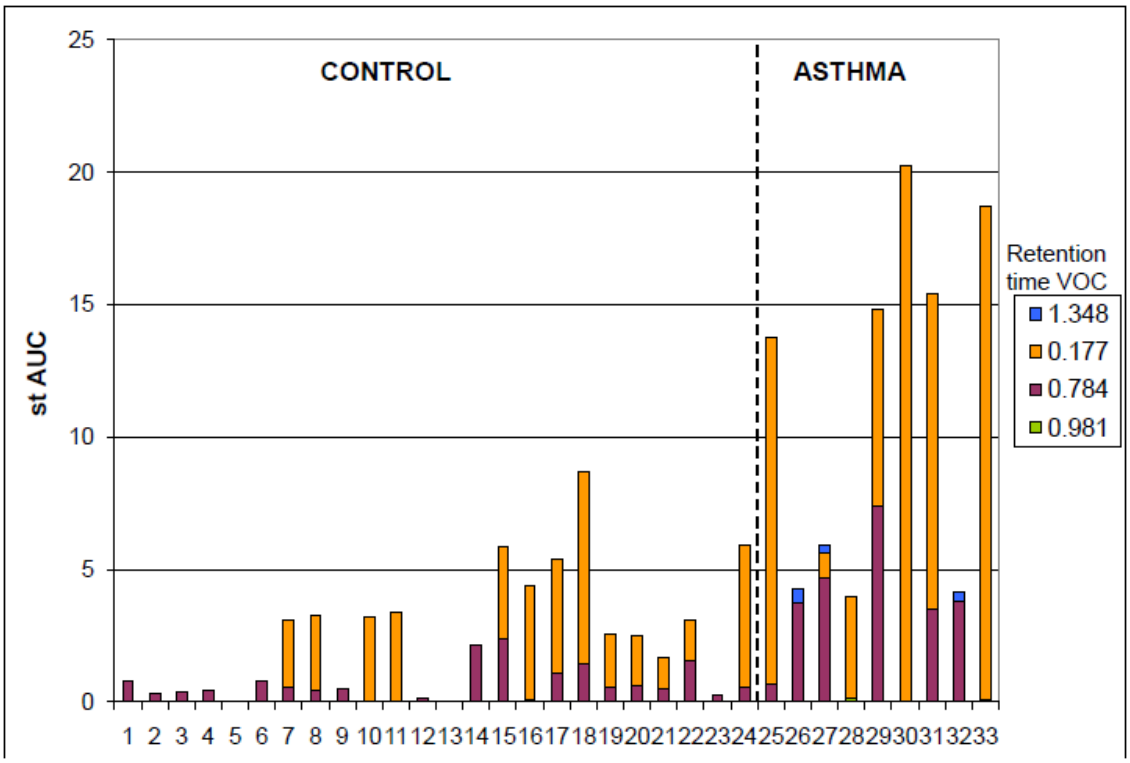


Figure 23. SVM model able to classify all subjects 100% correctly for asthma status (moderately and uncontrolled asthma) versus healthy controls. *The X-axis shows the subjects. Left and right of the dotted line depicts subjects belonging to respectively the control and asthma group. The Y-axis represents the normalized and weighted integrated peak area of the selected ln-transformed gasses. The relative retention times of the gasses are shown in the legend.*

Table VII. Identity of the VOCs in the model (thermodesorption)

Relative Retention Time	VOC
1.348	1-Chlorundecane
0.177	Acetone
0.784	5-Methyldecane or 4-Ethyldecane
0.981	1-Dodecene or 2-Decanon

This model was based on the samples from the first pilot study. In the expansion of the pilot study, new exhaled breath samples from 31 asthma patients and 36 healthy controls were collected and analyzed. The four relative retention times / VOCs mentioned in the model above were searched in this new database, and placed in de Support Vector Machine equation of this model. As a result, 7 of the new asthma patients and 1 healthy control were classified in the asthma group, which gives a sensitivity of 22.6% and a specificity of 97.2% (Table VIII). Please notice that the original model was based on a subgroup of the asthma patients: only those with moderately or uncontrolled asthma were included in the model. In the expansion of the pilot study however, all asthma patients are still included. We have unfortunately no accurate data on the asthma control status of these new individuals, which makes interpretation of the result difficult at this time.

Table VIII. Confirmation of the VOC model

	Classified as asthma in the model	Classified as control in the model
Asthma	7 (correct)	24 (false negative)
No asthma	1 (false positive)	35 (correct)
Total	1	20

Sensitivity: 22.6%; Specificity: 97.2%

The VOCs were analyzed by a second protocol during the first pilot study, in which the exhaled air was brought into canisters that were analyzed by another device with a different preconcentrator. Here, the analysis started with 100 VOCs (present in more than 3 samples (=10%). The most optimal classification model classified all subjects correctly (100%) regarding their asthma status (Figure 24, Table IX).

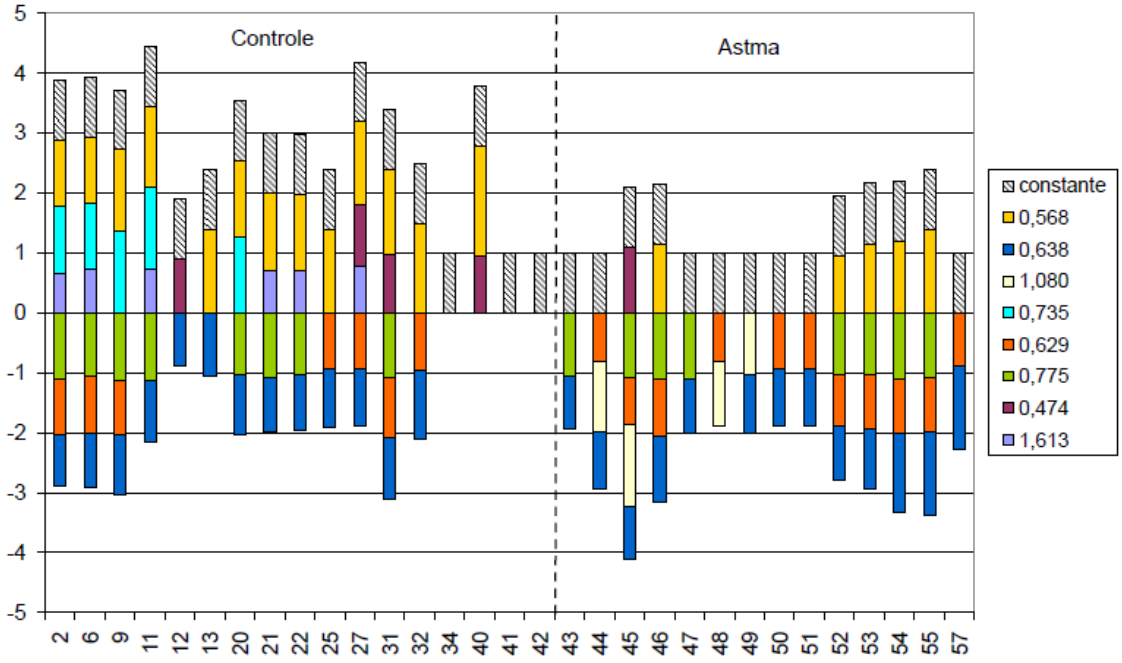


Figure 24. SVM model able to classify all subjects 100% correctly for asthma status (moderately and uncontrolled asthma) versus healthy controls, based on the analysis with the canisters. *The X-axis shows the subjects. Left and right of the dotted line depicts subjects belonging to respectively the control and asthma group. The Y-axis represents the normalized and weighted integrated peak area of the selected ln-transformed gasses. The relative retention times of the gasses are shown in the legend.*

Table IX. Identity of the VOCs in the model (canisters)

Relative Retention Time	VOC
0.568	2-Propenal
1.080	Heptane, 2,2-dimethyl-
0.735	Ethyl acetate
0.775	Cyclopentane, methyl

The identity of the VOCs at the other 4 relative retention times is not yet clear.

Comparison of the two preconcentration methods

In the first pilot study, collected VOC samples were analysed by two different methods. 10 L of exhaled breath was collected in Teflon® bags. For the first, thermal desorption method, about 7 L of the collected air was transferred on a sorbent tube. For the second method, 2 L of exhaled air was stored in an electropolished stainless steel canister until measurement. Both samples were analysed in the lab. By using the first method, on average 220 ± 40 gasses were identified in the samples, compared to an average of 61 ± 32 gasses by the second method. Of course, a smaller volume of exhaled air was used for the second analysis. By using the second analysis method however, more volatile compounds such as ethane and pentane – markers for oxidative stress – should be measurable. In our samples, ethane was detectable in 2 of the 54 samples, while

pentane was detectable in 53 of the 54 samples, after analyses with the second method. By using the first method, these 2 molecules were not detected.

2.2.2.7. Conclusions from the pilot studies

The already well-known non-invasive marker exhaled NO also proved its value in these pilot studies: a significant increase was observed in the asthma group compared to the healthy controls, although there was a large overlap between the groups. Whether eNO is a marker of asthma or rather a marker of atopy is under discussion in the international literature (Scott et al, 2010; Ho et al, 2000). Also in these pilot studies, a significant difference is observed between the allergic asthmatic patients and the non-allergic asthmatic patients.

The markers measured in nasal lavage and urine in this pilot study, were not related to asthma. CC16 in nasal lavage was found to be associated with allergy in asthmatic patients.

From the specific EBC markers measured in the pilot study, pH measured directly after sampling without deaeration, was the only one that was significantly different between the asthma group and healthy controls, although large overlap was observed. 8-isoprostane, which has in literature been shown to be elevated in asthmatic subjects (Caballero Balanza et al, 2010), was in this pilot study only significantly different between non-allergic and allergic asthma patients. The other specific exhaled markers measured during the ANIMO project are, based on the results from the pilot studies, not of interest for the respiratory health outcomes asthma or allergy.

The analytical work performed on the screening of exhaled gasses and exhaled proteins however, gives new and interesting results in the search for non-invasive biomarkers. Models of selected gasses and selected proteolytic peptides were build based on the health outcome asthma. Although the role of these gasses and proteins in asthma pathogenesis is not completely clear at this moment, this is a first step in the development of reliable, non-invasive biomarkers for asthma. This was a pilot study, and these results still have to be confirmed. Various proteins that might be relevant in respiratory health outcomes were identified for the first time in EBC. Additionally, it was shown that exhaled proteins provide unique information from the lower airways, and are not only derived from the mouth.

2.3. CHILD COHORT STUDIES

The newly developed non-invasive biomarkers were applied in 2 different child cohort studies: a new cohort was initiated in the Walloon region, and the children of the existing Flemish cohort were followed-up.

Walloon cohort

Children from 30 schools located mainly in the areas of Brussels and Liège (Belgium) were examined. These children were recruited in the framework of a prospective study on the respiratory impact of indoor air pollution. The children were tested at 5-6 years and 2 years later. The ethics committee of the Faculty of Medicine of the Catholic University of Louvain (Louvain, Belgium) approved the study protocol, which complied with all applicable requirements of the international regulations.

Parents completed a detailed questionnaire inquiring about the health of their child, respiratory symptoms, family antecedents, care during early life style and all lifestyle and environmental factors known or suspected to influence the risk of allergic sensitization. The questionnaire also comprised questions about sport and recreational activities. For swimming practice, parents were asked to specify the type of pool attended by their child, the type of disinfection method used (even though almost all of them use chlorine), the frequency of attendance and the age at which their child started to attend the pool regularly. This information served to calculate the cumulative pool attendance (CPA) at indoor or outdoor chlorinated pools, separately or combined, before the age of 2 years (during infancy) or later in life (during childhood). For the second phase of the study, parents were asked to fill an additional questionnaire to inquire about health status and risk factors during the two last years.

A total of 272 children from schools in Brussels and the Walloons Region were followed since November 2007 until May 2010. They were tested 2 times, first at 5-6 years and the follow took place 2 years later when they were around 7-8 years old. Children were examined in schools from 9h to 13h. Height and weight were measured. Since the examination of children in schools, screening for respiratory allergies was performed noninvasively by measuring specific immunoglobulin E in nasal mucosa using the Rhinostick test (made by F. Marcucci). This test was successfully performed at 6 and 8 years old in 220 children, in whom the following allergens were screened for cat epithelium, *Dermatophagoides Pteronyssinus, Anthoxanthum odoratum, Parietaria officinalis* and a mix of tree allergens containing *Betula odorata, Corylus avellana* and *Alnus incana*. Exhaled NO (eNO) was also measured, this measurements were performed before exercise testing. Single-breath, on-line measurement of eNO was performed in accordance with ATS/ERS recommendations at an exhalation flow of 50 mL/s (Aerocrine NO system; Aerocrine AB; Stockholm, Sweden; and CLD 77 am chemiluminescence analyzer; Eco Physics AG; Duernten, Switzerland). The use of this equipment is well documented.

Flemish cohort

This study included a further follow-up of an existing Flemish child cohort[2] at the age of 7 years. In a subgroup (N = 150) from this cohort, non-invasive measurements were performed earlier in a subgroup, at the age of 3 years. 47 children were asked to collect EBC: during 15 minutes they succeeded to breath about half of this time through the collection tube while watching a movie. The collected volume from 14 of those children was not sufficient to perform proteome analysis. One sample was not analysed due to technical reasons. Protein analysis occurred according to the protocol described above, and was successful in 32 EBC samples. Exhaled breath gasses were collected in 51 children. This sample collection was successful in all 51 children. VOCs in all samples were examined successful according to the protocol described above. At the age of 7 years, the parents were asked for the respiratory health status of their child, as well as housing, cleaning and life style factors in a questionnaire. The questionnaire was send to 105 children. A completed questionnaire was received from 86 children. Five of these children reported to have doctor-diagnosed asthma. These data were used to study the predictive value of the selected non-invasive markers.

In a separate subgroup of this Flemish cohort, non-invasive measurements were performed at the age of 7 years. 547 children were contacted, however the response was very low: only 65 children and their parents agreed to participate to this follow-up study. During the examination, their body weight and height were determined, exhaled NO was measured, urine samples and EBC (during 5 minutes) were collected, and spirometry with free-running test were performed. These last tests, however, were unsuccessful in a lot of children, and as a consequence these data will not be used for analysis. Additionally, an extensive questionnaire was completed.

2.3.1. Relation of the biomarkers with health outcome

2.3.1.1. Diagnostic value of the non-invasive markers

Walloon cohort

The characteristics of the population of children at the age of 6 years and at the age of 8 years are shown in table X. Among them, 118 were boys (53.6%) and more than 51% of the total population had a parental history of asthma or allergy. More than 13% were exposed to maternal smoking during pregnancy and about 80% were breastfed. There were no significant differences at the age of 6 and 8 years regarding main risk factors of respiratory diseases including exposure to tobacco smoke or to pets and living in a rural environment. An increased prevalence for the use of bleach for cleaning was observed in our population between the age of 6 and 8 years. On average, infant swimmers had

[2] Flemish Environment and Health study (2002 - 2006) – www.milieu-en-gezondheid.be.

spent 73 hours in indoor and 203 hours in outdoor chlorinated pools at the age of 6 years and 64 hours in indoor and 129 hours in outdoor chlorinated pools at the age of 8 years.

Table X. Characteristics of children examined at the age of 6 and 8 years (Walloon cohort).

	Age: 6 years		Age: 8 years	
	N	**Prevalence**	**N**	**Prevalence**
Gender-Male	118	53.6%		
Body mass index (mean, SD)	20.8	SD: 3.5	16.7	SD:2.3
Family characteristics				
Parental history of asthma or allergy	114	51.8%		
Older siblings	125.	56.8%		
Infancy				
Maternal smoking during pregnancy	30	13.6%		
Birth at term	171	77.7%		
Breastfeeding	177	80.5%		
Pets exposure before 2 years	95	43.2%		
Day-care attendance	129	58.6%		
Rural environment before 2 years	96	43.6%		
Environment and lifestyle				
Home near a highway	134	60.9%	122	55.6%
Rural environment	95	44.2%	87	42.4%
Exposure to pets	112	50.9%	111	50.9%
Air fresheners use	65	29.5%	53	24.3%
Exposure to mould			13	6.1%
Environmental tobacco smoke	58	26.4%	66	30.4%
House cleaning with bleach	18	8.2%	58	26.7%
Tap water consumption	100	45.5%	128	58.2%
Peanuts consumption	85	38.6%	98	46.8%
Vegetables consumption from garden			45	20.7%
Leisure activities				
Horse riding	13	5.9%	15	6.8%
Infant swimming	116	52.7%		
Indoor chlorinated pool attendance (h)	73.8	SD: 79	204	SD: 193.7
Outdoor chlorinated pool attendance(h)	64.8	SD: 119.9	129	SD: 219.1

The changes in the prevalence of allergic sensitization, respiratory diseases and symptoms between the age of 6 and 8 years are shown in table XI. The only noticeable changes concerned the prevalence of house dust mite (HDM) sensitization and of increased eNO, which rose from 12% to 28% for HDM sensitization and from 20% to 38% for eNO >12ppm or from 14 to 29% for eNO >15ppm.

Table XI. Respiratory diseases and symptoms at the age of 6 and 8 years (N = 272; Walloon cohort).

		Age: 6 years		Age: 8 years
	N	Prevalence	N	Prevalence
Allergic sensitization				
House dust mite sensitization	**27**	**12.9%**	**58**	**28.3%**
Pollen sensitization	36	17.1%	30	14.6%
Cat allergen sensitization	20	9.5%	16	7.8%
Allergic diseases				
Allergic rhinitis	25	11.4%	14	6.4%
Hay fever	16	7.3%	14	6.4%
House dust mite allergy	24	10.9%	19	11.6%
food allergy	37	16.8%	23	10.9%
Pets allergy	14	6.4%	17	7.9%
Eczema	60	27.3%	35	16.5%
Asthma and respiratory symptoms				
Asthma	16	7.3%	15	6.8%
Wheezing	33	15%	28	12.7%
Asthma crisis	10	4.5%	5	2.3%
Chest tightness	7	3.2%	4	1.8%
Shortness of breath	10	4.5%	10	4.5%
Exhaled NO				
eNO > 12 ppb	**44**	**20.0%**	**85**	**38.5%**
eNO > 15 ppb	**32**	**14.5%**	**65**	**29.5%**
eNO (mean (SD))		9 (6)		15.15 (14.9)
Biomarkers in urine and nasal lavage				
Nasal lavage: CC16/Urea (mean (SD))	Left	0.0004 (0.00104)		7.201 (8.791)
	Right	0.0004 (0.0008)		7.122 (10.559)
Urine: Log CC16/Creat (mean (SD))		0.225 (0.626)		0.291 (0.446)

When re-examining the children at the age of 8 years, it was for practical reason necessary to recruit all children of the same classrooms. This resulted in a total population of 444 children, including 214 girls and 230 boys. 272 of these children were already examined before, and their results are also shown separately to compare with the previous result. Table XII presents the characteristics of this population, separately for girls and boys. At the exception of eNO (expectedly higher in boys than in girls), there were no significant differences between girls and boys for the main respiratory outcomes. As observed in the cohort of the first phase, the prevalence of

bronchiolitis, eczema or house dust mite sensitization are still very high, around respectively 30%, 28% and 25%.

Table XII. Characteristics and respiratory health of 8 years old children (Walloon cohort).

	Girls (N = 201)	**Boys (N = 229)**	**p-value**
Belgian (%)	88.2	88.1	0.99
Body weight, kg (mean, SD)	25.2 (4.6)	26.0 (8.8)	0.30
Height, cm (mean, SD)	126.1 (7.2)	125.2 (13.1)	0.45
Wheezing (%)	15.9	19.1	0.37
Asthma (%)	6.1	7.4	0.58
Bronchiolitis (%)	28.6	32.3	0.40
Pneumonia (%)	10.0	12.4	0.43
Exhaled NO (ppb, mean, SD)	13.8 (11.5)	17.2 (20.0)	**0.04**
Allergic rhinitis (%)	9.4	12.2	0.35
Hay fever (%)	7.0	9.6	0.33
House dusts mite allergy (%)	17.3	12.2	0.13
Eczema (%)	26.6	30.4	0.38
Pets allergy (%)	8.4	9.1	0.79
HDM sensitization (RS)	27.2	24.2	0.51
Cat sensitization (RS)	7.2	6.8	0.89
G sensitization (RS)	4.4	4.7	0.89
Mix4 sensitization (RS)	6.7	5.8	0.73
W19 sensitization (RS)	4.4	5.3	0.71

(RS:Rhinostick)

The values of eNO between children with or without respiratory symptoms or diseases are compared in table XIII. The eNO concentration is significantly higher among children with wheezing, asthma or HDM allergy.

Table XIII. Concentrations of eNO of children with and without respiratory problems (8 years old; Walloon cohort, N = 430).

	eNO (mean, ppb)		
Symptoms	**Yes**	**no**	**p-value**
Wheezing	27.1	14.1	**<0.0001**
Asthma	23.0	14.8	**0.04**
Allergic rhinitis	19.2	15.1	0.30
Hay fever	20.3	15.2	0.06
House dusts mite allergy	29.8	14.4	**<0.0001**

Flemish cohort

Tables XIV and XV describe the characteristics and respiratory health of the children from the Flemish cohort followed-up at the age of 7 years. In total, 65 children were examined, of which 42 girls. 69% of the population had a parental history of asthma or allergy. Exposure to maternal smoking during pregnancy was low (4.6%). There were no significant differences between girls and boys for the main respiratory outcomes. Also for exhaled NO, no significant difference was observed between the boys and girls, while in the Walloon cohort, higher values were found in boys.

Table XIV. Characteristics and respiratory health of 7 years old children (Flemish cohort).

	Girls (N = 42)	**Boys (N = 23)**	**p-value***
Belgian (%)	92.8	82.6	0.48
Body weight (kg. mean. SD)	25.6 (4.5)	25.5 (3.9)	0.75
Height (cm. mean. SD)	125.6 (5.8)	128.2 (6.3)	0.15
Exhaled NO (ppb. mean. SD)	13.8 (7.5)	13.7 (11.0)	0.39
EBC pH (mean. SD)	5.7 (0.7)	5.7 (0.7)	0.74
8OHdG urine (μg/g creatinine, mean. SD)	14.1 (6.9)	14.5 (11.4)	0.35
CC16 urine (μg/g creatinine, mean. SD)	1.2 (1.2)	2.1 (5.7)	0.51
Coughing during the night (%)[#]	64.3	73.9	0.48
Coughing during the night while having a	53.5	62.5	0.49
Wheezing (%)[#]	13.6	28.0	0.24
Wheezing related to exercise (%)[#]	0.0	3.8	0.75
Rattling (%)[#]	15.2	11.1	0.75
Shortness of breath (%)[#]	2.1	7.1	0.67
Airway infection before 1 yr of age (%)	16.7	6.9	0.64
Airway infection last 12 months (%)[#]	4.1	6.7	0.54
Airway infection between the 2 periods (%)	20.0	16.1	0.38
Medication for cough (%)[#]	35.3	37.5	0.54
Doctor-diagnosed asthma (%)	5.7	2.9	0.87
Asthma exacerbation (%)[#]	1.9	0.0	0.88
Running nose (%)[#]	60.7	51.4	0.54
Fever (%)[#]	36.2	20.5	0.44
Antibiotics (%)[#]	20.0	9.8	0.50
Food allergy (%)°	3.2	4.7	0.90
Metal allergy (%)°	1.6	0.0	0.59
Insect bite allergy (%)°	12.5	13.3	0.37
Pet allergy (%)°	3.1	2.2	0.80
Medication allergy (%)°	1.5	2.1	0.83
Care products allergy (%)°	7.5	2.1	0.73
Household products allergy (%)°	4.4	2.0	0.81
Itchy rash (%)[#]	15.9	16.0	0.51
Eczema (%)[##]	7.1	7.8	0.68
Eczema confirmed by a doctor (%)	4.2	7.7	0.33

* Mann-Whitney U test; [#] during the last 12 months; [##] during the last 6 months; ° during the last 5 years.

Table XV. Characteristics of 7 years old children (Flemish cohort).

	N	Prevalence
Gender – Male	23	35.4%
BMI (mean, SD)	16.0	SD: 1.8
Family characteristics		
Parental history of asthma or allergy	45	69.2%
Older sibling	28	43.1%
Infancy characteristics		
Maternal smoking during pregnancy	3	4.6%
Birth at term	53	81.5%
Breastfeeding	45	69.2%
Rural environment before 2 years	16	24.6%
Environmental and lifestyle characteristics		
Rural environment	16	24.6%
Exposure to pets	39	60.0%
Air fresheners use	25	38.5%
Exposure to mould	5	7.7%
Environmental tobacco smoke	21	32.0%
House cleaning with bleach	3	4.6%
Tap water consumption	49	75.4%
Consumption of nuts	46	70.7%
Vegetable consumption from own garden	23	35.3%
Leisure activities characteristics		
Infant swimming	39	60.0%
Total indoor chlorinated pool attendance (hours)	81.4	78.0%
Total outdoor chlorinated pool attendance (hours)	104.1	167.6%

The values of eNO between children with or without respiratory symptoms or diseases are compared in table XVI. The eNO concentration is significantly higher in children with doctor-diagnosed asthma or food allergy. No significant difference was observed for wheezing, as in the Walloon cohort.

Table XVI. Concentrations of eNO in children with or without respiratory problems (Flemish cohort).

	eNO (median, ppb)		
Symptoms	**yes**	**no**	**p-value***
Coughing during the night[#]	12.00	9.00	0.08
Wheezing[#]	12.00	12.00	0.55
Rattling[#]	10.50	12.00	0.16
Shortness of breath[#]	10.50	12.00	0.63
Airway infection[#]	7.25	12.25	0.18
Doctor-diagnosed asthma	24.70	11.50	**0.03**
Running nose[#]	12.00	13.00	0.24
Food allergy°	20.00	11.50	**0.02**
Insect bite allergy°	9.00	12.00	0.06
Pet allergy°	14.50	11.50	0.36
Itchy rash[#]	12.00	11.75	0.24
Eczema[#]	14.50	11.75	0.15

* Mann-Whitney U test. Significant differences are shown in bold. [#] during the last 12 months; ° during the last 5 years.

Urinary CC16 concentrations between children with or without respiratory symptoms or diseases are compared in table XVII. The concentration is significantly higher among children with a running nose.

Table XVII. Urinary CC16 in children with or without respiratory problems (Flemish cohort).

	urinary CC16 (median, µg/g creatinine)		
Symptoms	**yes**	**no**	**p-value***
Coughing during the night	0.80	0.41	0.21
Wheezing	0.82	0.69	0.44
Rattling	0.66	0.74	0.50
Shortness of breath	0.00	0.72	0.30
Airway infection last 12 months	0.69	0.73	0.56
Doctor-diagnosed asthma	0.80	0.70	0.81
Running nose	0.81	0.00	**0.007**
Food allergy	1.09	0.69	0.09
Insect bite allergy	1.44	0.59	0.06
Pet allergy	1.46	0.72	0.61
Itchy rash	0.94	0.69	0.60
Eczema	1.02	0.68	0.27

* Mann-Whitney U test. Significant differences are shown in bold.

EBC pH values and urinary 8OHdG concentrations between children with or without respiratory symptoms or diseases are compared in tables XVIII and XIX. No significant results are observed for these markers.

Table XVIII. EBC pH in children with or without respiratory problems (Flemish cohort).

	EBC pH		
Symptoms	**yes**	**no**	**p-value***
Coughing during the night	5.94	5.91	0.41
Wheezing	6.09	5.88	0.52
Rattling	6.15	5.92	0.85
Shortness of breath	6.34	5.9	0.10
Airway infection last 12 months	6.11	5.9	0.83
Doctor-diagnosed asthma	5.98	5.94	0.85
Running nose	5.91	6.09	0.78
Food allergy	5.8	5.97	0.65
Insect bite allergy	6.06	5.89	0.59
Pet allergy	6	5.91	0.68
Itchy rash	5.99	5.94	0.87
Eczema	6.06	5.92	0.89

* Mann-Whitney U test

Table XIX. Urinary 8OHdG in children with or without respiratory problems (Flemish cohort).

	8OHdG (median, μg/g creatinine)		
Symptoms	**yes**	**no**	**p-value***
Coughing during the night	11.25	11.23	0.69
Wheezing	11.14	11.23	0.92
Rattling	10.27	11.24	0.83
Shortness of breath	8.66	11.25	0.27
Airway infection last 12 months	13.17	11.24	0.53
Doctor-diagnosed asthma	10.47	11.25	0.25
Running nose	11.23	11.29	0.97
Food allergy	11.25	11.22	0.84
Insect bite allergy	11.18	11.24	0.94
Pet allergy	12.66	11.15	0.08
Itchy rash	11.23	11.29	0.54
Eczema	10.17	11.38	0.07

* Mann-Whitney U test

2.3.1.2. Predictive value of the non-invasive markers

Exhaled breath condensate

In the pilot study described above (title 2.2.2.), 3 models were developed to discriminate between asthma patients and healthy controls, based on exhaled proteins.

The first model discriminated between uncontrolled or moderately controlled asthma patients and healthy controls, the second model between controlled asthma patients and healthy controls, and a third model between all asthma patients and healthy controls. The proteolytic peptides selected in these three models were searched in the proteolytic peptide profiles from the children from the Flemish child cohort at the age of 3 years. Based on these peptides and the Support Vector Machine equation derived from the pilot study, these children were classified according to the three models. From the questionnaire at the age of 7 years, information was obtained of their current health status, e.g. whether they have doctor-diagnosed asthma, and whether they suffered from wheezing during the last 12 months. Of the 32 children for whom EBC analysis at the age of 3 years is available, we received questionnaires from 21 children. One of those children has asthma, and this one child was also the only one with wheezing symptoms at the age of 7 years (complaints during the last 12 months). Although data from only few children are available, sensitivity (the chance that the model gives a positive result in the children with asthma) and specificity (the chance that the model gives a negative result for healthy children) of the predictive value of the developed models were determined.

Based on the first model (moderately and uncontrolled asthma versus healthy controls), one of the individuals was categorized wrong, although borderline, in the asthma group. The one asthma patients was also categorized borderline wrong in the control group. All 19 other individuals were clearly categorized correct in the control group (table XX).

Table XX. Predictive value of model 1 (moderately and uncontrolled asthma vs healthy controls).

	Classified as asthma in the model	**Classified as control in the model**
Asthma	0 (correct)	1 (false negative)
No asthma	1 (false positive)	19 (correct)
Total	1	20

Sensitivity: 0/(1+0) = 0%; Specificity: 19/(19+1) = 95%

Based on the second model (controlled asthma versus healthy controls), 5 of the individuals were categorized in the asthma group, including the one individual that actually has asthma. One of the other four was classified borderline as an asthma patient (Table XXI).

Table XXI. Predictive value of model 2 (controlled asthma vs healthy controls).

	Classified as asthma in the model	**Classified as control in the model**
Asthma	1 (correct)	0 (false negative)
No asthma	4 (false positive)	16 (correct)
Total	5	16

Sensitivity: 1/(1+0) = 100%; Specificity: 16/(16+4) = 80%

Based on the third model (all asthma patients versus healthy controls), 14 of the 21 children were classified in the asthma group, including the one individual that has asthma (table XXII).

Table XXII. Predictive value of model 3 (all asthma patients vs healthy controls).

	Classified as asthma in the model	**Classified as control in the model**
Asthma	1 (correct)	0 (false negative)
No asthma	13 (false positive)	7 (correct)
Total	14	7

Sensitivity: 1/(1+0) = 100%; Specificity: 7/(13+7) = 35%

Results are identical for the health outcome 'wheezing during the last 12 months', since the asthma patient is the only individual with this complaint.
The individuals that were classified as false positive in the various models, do not suffer from other respiratory complaints such as cough, cold, shortness of breath, airway infection, or fever.

The first and second model seem to give acceptable results in this study. The asthma patient is classified in all three models correct or borderline correct. However, because only 1 individual from whom we have data available at the age of 3 years currently has asthma, we cannot draw any conclusions from this study.

Exhaled VOCs

Also exhaled gasses were collected and analyzed from the 3-year-old children in the Flemish cohort study (another subgroup as those that collected EBC). All data are available to evaluate the predictive value of the VOC model developed in the pilot study. Unfortunately, we were unable to complete this data-analysis within the ANIMO project. We will however complete this work, and when the results will be published, we will acknowledge the Belspo support for the ANIMO project.

2.3.2. Risk factors for respiratory health

Walloon cohort

Table XXIII presents the characteristics of the 8-year old population, separately for girls and boys. At the exception of air fresheners there were no significant differences between girls and boys for the main risk factors.

Table XXIII. Environmental and lifestyle factors likely to influence the respiratory health of children (Walloon cohort).

	Girls (%)	Boys (%)	p-value
Maternal smoking during pregnancy	18.9	15.0	0.73
Siblings	84.5	87.8	0.32
Pets at home	53.2	55.3	0.67
Exposure to ETS	31.0	29.1	0.67
Use of bleach for house cleaning	26.1	24.8	0.75
Air fresheners use	25.6	35.3	**0.03**
Residential swimming pool	18.4	21.8	0.38
Parental asthma	15	14.4	0.86
Parental allergy	40.5	41.2	0.88
Breastfeeding	77.6	76.0	0.69
Swimming during infancy (before two years)	37.5	40.8	0.49
Day care attendance	55.7	51.3	0.36
Swimming pool attendance	70.2	71.2	0.69
Swimming pool attendance during holidays	62.0	58.4	0.45

Table XXIV presents the factors associated with the risks of aeroallergen sensitization and airway inflammation before the age of 6 years and between the age of 6 and 8 years (risk factors for respiratory health effects that appear after the age of 6 years). Multiple regression analyses was used to asses the associations: either logistic for dichotomic variables or linear for continuous variables after log transformation. Before the age of 6 years, day care attendance is associated with a lower risk of sensitization to HDM or pollen, and of elevated eNO (>12 ppb). Birth at term appears also to be protective

towards of the risk of HDM or pollen sensitization. Risk factors of aeroallergen sensitization during childhood include parental asthma or allergy (HDM), older siblings (HDM) and living near an industry (HDM and pollen). Between age 6 and 8, the patterns of risk and protective factors were completely different. No associations were seen with parental asthma or allergy, or day care attendance while associations with older siblings were inconsistent. The only consistent risk factor was infant swimming, which dose-dependently increased the risk of sensitization to HDM and pollen as well as the risk of increased eNO. Infant swimming, an activity practiced by 53.7% of children, was actually the strongest risk factor for increased eNO between the age of 6 and 8 years with an odd ratio of 3.54 for ever swimmers and of 4.45 for children who had swum for a total of more than 10 hours during infancy.

In conclusion, the results of this two-year prospective study among children from kindergarten highlight the potential of non-invasive biomarkers to assess the respiratory health of children and identify protective or risk factors related to the lifestyle or the environment. Our study confirms the protective effects associated with birth at term and day-care attendance towards the risk of developing respiratory allergies and airway inflammation during infancy. Regarding the risk factors, parental asthma or allergy emerges as a strong predictor in particular for HDM sensitization. Another strong predictor identified in our study is the infant swimming practice, which dose-dependently increases the risk of sensitization to HDM or to pollen, and the risk of airways inflammation assessed by the eNO test. This study also stresses the importance of the timing factor to evaluate the respiratory effects of lifestyle or the environment. For instance, the associations of respiratory allergies and airways inflammation with infant swimming were detected only with the outcomes measured between the age of 6 and 8 years. This time lag might be related to the time of outcome development with perhaps a masking effect of inherited or other risk factors (parental allergy, day-care, …).

Table XXIV. Risk factors for aeroallergen sensitization and airway inflammation before age 6 yrs and between 6 and 8 yrs (Walloon cohort).

	House dust mite sensitization		**Pollen sensitization**		**Exhaled NO > = 12**	
	Before 6 years	Between 6 - 8 yrs	Before 6 years	Between 6 -8 yrs	Before 6 years	Between 6 -8 yrs
Day-care attendance	**0.16 (0.04-0.59)**	1.45 (0.57-3.68)	**0.38 (0.16-0.93)**	2.95 (0.55-15.8)	**0.30 (0.12-0.78)**	1.88 (0.82-4.3)
Older siblings	**1.8 (1.0-3.24)**	0.88 (0.37-2.05)	0.86 (0.37-2.01)	**6.7 (1.09-42.3)**	2.18 (0.88-5.41)	**0.56 (0.34-0.93)**
Living near industry	**13.3 (2.39-74.2**)	0.88 (0.37-2.05)	**3.1 (1.06-9.01)**	0.86 (0.05-14.1)	0.73 (0.18-2.9)	0.53 (0.14-2.06)
Parental asthma / allergy	**4.30 (1.12-16.7)**	1.13 (0.47-2.75)	1.59 (0.68-3.71)	2.01 (0.36-11.31)	1.5 (0.6-3.74)	0.66 (0.3-1.44)
Consumption of home vegetables	0.15 (0.02-1.16)	**0.21 (0.04-0.97)**	1.37 (0.4-4.66)	1.17 (0.08-16.5)	0.86 (0.21-3.57)	0.36 (0.08-1.42)
Mould	0.56 (0.04-7.29)	2.19 (0.43-11.2)	1.63 (0.39-6.69)	**78.7 (4.81-12.8)**	-	2.12 (0.93-4.87)
Exposure to pets	0.47 (0.15-1.48)	0.79 (0.36-1.75)	0.89 (0.38-2.11)	**0.11 (0.01-0.87)**	0.51 (0.21-1.25)	**2.3 (0.96-5.24)**
Birth at term	**0.17 (0.04-0.72)**	2.29 (0.78-6.67)	**0.39 (0.16-0.96)**	1.22 (0.25-6.0)	0.39 (0.14-1.06)	0.48 (0.19-1.18)
Air fresheners	2.38 (0.66-8.53)	0.97 (0.39-2.39)	0.71 (0.27-1.87)	1.28 (0.88-1.87)	**3.49 (1.59-7.63)**	1.06 (0.41-2.74)
Infant swimming (before 2 years)						
Ever	0.87 (0.30-2.54)	1.88 (0.86-4.12)	0.81 (0.36-1.83)	2.01 (0.91-4.4)	0.31 (0.11-0.84)	**3.54 (1.26-9.92)**
CPA (hours)						
0	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)	1.0 (1.0-1.0)
1-10	0.57 (0.12-2.78)	1.53 (0.41-4.22)	0.99 (0.33-2.99)	0.38 (0.05-2.75)	**0.14 (0.03-0.72)**	2.69 (0.76-9.53)
> 10	2.02 (0.54-7.59)	**2.6 (1.01-6.67)**	0.68 (0.24-1.9)	**7.68 (1.03-57.2)**	0.38 (0.12-1.18)	**4.45 (1.34-14.8)**
Smoking during pregnancy	1.46 (0.17-12.8)	1.25 (0.44-3.59)	0.72 (0.17-3.06)	0.33 (0.04-2.58)	0.82 (0.16-4.07)	0.51 (0.14-1.83)
Environ. tobacco smoke	0.29 (0.06-1.28)	1.18 (0.44-3.13)	1.09 (0.37-3.18)	1.98 (0.29-13.67)	0.82 (0.28-2.35)	1.41 (0.21-3.25)

Odds ratio's are presented

Table XXV. Risk factors for asthma, wheezing and allergic rhinitis before age 6 yrs and between 6 and 8 yrs (Walloon cohort).

	Asthma		**Wheezing**		**Allergic rhinitis**	
	Before 6 years	Between 6 - 8 yrs	Before 6 years	Between 6 -8 yrs	Before 6 years	Between 6 -8 yrs
Day-care attendance	0.52 (0.16-1.65)	0.08 (0.005-1.22)	0.49 (0.17-1.4)	2.41 (0.45-12.89)	1.75 (0.42-7.34)	0.03 (0.002-3.7)
Older siblings	1.29 (0.38-4.37)	0.43 (0.03-6.03)	1.11 (0.43-2.85)	0.71 (0.17-3.1)	7.56 (2.08-27.5)	-
Parental asthma / allergy	**4.48 (1.16-17.4)**	1.74 (0.11-28.48)	2.26 (0.85-6.01)	0.42 (0.10-1.67)	**18.7 (3.86-91.2)**	0.04 (0.003-5.46)
BMI		**2.44 (1.12-5.33)**	1.07 (0.95-1.21)	**1.73 (1.21-2.46)**	0.95 (0.80-1.12)	1.3 (0.98-1.72)
Exposure to pets	1.75 (0.5-6.15)	6.91 (0.41-115.5)	1.34 (0.52-3.44)	0.37 (0.07-1.86)	**3.65 (1.07-12.43)**	-
Birth at term	**0.12 (0.03-0.57)**	-	**0.38 (0.14-0.99)**	2.51 (0.43-14.69)	0.76 (0.17-3.29)	0.14 (0.001-5.46)
Air fresheners	**4.01 (0.99-16.3)**	-	2.46 (0.9-6.71)	0.72 (0.13-3.87)	1.72 (0.48-6.18)	1.07 (0.05-22.3)
Smoking during pregnancy	1.5 (0.25-8.87)	-	2.34 (0.6-9.11)	0.67 (0.06-7.8)	1.74 (0.33-9.15)	-
Environ. tobacco smoke	**4.06 (1.07-15.4)**	0.33 (0.02-6.53)	0.67 (0.22-2.08)	1.2 (0.26-5.51)	1.06 (0.25-4.55)	0.88 (0.003-11.53)

Odds ratio's are presented

Flemish cohort

Table XXVI presents the characteristics of the 7-year old children (N = 65), separately for girls and boys. There were no significant differences between girls and boys for the main risk factors.

Table XXVI. Environmental and lifestyle factors likely to influence the respiratory health of children (Flemish cohort).

	Girls (%)	Boys (%)	p-value*
Maternal smoking during pregnancy	2.4	8.7	0.84
Siblings	97.6	82.6	0.12
Pets at home	54.8	69.6	0.33
Exposure to ETS	16.6	43.5	0.19
Use of bleach for house cleaning	23.8	39.1	0.65
Air fresheners use	54.8	73.9	0.63
Residential swimming pool	40.5	39.1	0.93
Parental asthma	14.3	13.0	0.44
Parental allergy	61.9	78.3	0.53
Breastfeeding	71.4	65.2	0.90
Swimming during infancy (before two years)	61.9	56.5	0.59
Swimming pool attendance	95.2	95.6	0.98
Swimming pool attendance during holidays	69.0	82.6	0.33

* Mann-Whitney U test

Mann-Whitney U tests were performed to study the effect of the risk factors on the non-invasive markers eNO, EBC pH, urinary CC16 and urinary 8OHdG, although it has to be kept in mind that this is a relatively small group (N = 65). Eating vegetables from the own garden was associated with decreased urinary 8OHdG (median values: 10.17 μg/g creatinine vs 11.91 μg/g creatinine when no self-cultivated vegetables were eaten; p = 0.013). Drinking of tap water was associated with lower values of urinary CC16 (0.68 μg/g creatinine vs 1.44 μg/g creatinine; p = 0.027). Parental allergy was associated with higher EBC pH values (median: 6.05 vs 5.69; p = 0.042) and with higher urinary CC16 values (median: 0.84 vs 0.54 μg/g creatinine; p = 0.025).

Additionally, Mann-Whitney U tests were performed to study the effect of the risk factors on the health outcomes doctor-diagnosed asthma (N = 4), wheezing (N = 13), rattling (N = 10), shortness of breath (N = 3), allergies, etc. Parental asthma was significantly associated with wheezing (p = 0.035), while parental allergy was associated with rattling (p = 0.04). Breastfeeding was found to be a protective factor for asthma (p = 0.048). Total swimming pool attendance (no/yes variable) was a risk factor for shortness of breath (p = 0.018), although only 3 children suffered from this

respiratory complaint during the last 12 months. Expressed in hours, swimming pool attendance gave no significant result.
Due to the small size of this Flemish cohort, these data were further analyzed in a common database, together with the results from the Walloon cohort (N = 504).

Common analysis Walloon and Flemish cohort

The databases of the Walloon and Flemish cohort were combined, and common analysis were performed based on some interesting results from the Walloon cohort. The characteristics of the children from the common database are summarized in table XXVII. Table XXVIII presents the factors associated with the risk of asthma, wheezing, eczema, allergy and airway inflammation. This table cannot directly be compared with tables XXIV and XXV, because for the Walloon cohort, analysis were performed for health effects that appeared between the age 6 and 8 years, while for the common analysis, all individuals with health effects at the age of 8 years were included.

In this common database, birth at term was found to be a protective factor for asthma at the age of 7-8 years. Risk factors for wheezing at age 7-8 years are the use of bleach for cleaning the house, exposure to environmental tobacco smoke, and total outdoor swimming pool attendance (in hours). At the age of 6 years, these environmental factors were not yet indicated as risk factors for wheezing. Family size was associated with higher eNO values in the common database, while living in a rural area before the age of 2 years was associated with lower eNO factors.

Table XXVII. Characteristics of the children (Walloon + Flemish cohort).

Characteristics	Girls	Boys	p-value
N	253	250	
BMI, mean (SD), kg/m²	16.7 (2.42)	16.6 (2.12)	0.89
Belgian, N (%)	225 (90)	219 (89)	0.72
Parents			
Asthma, N (%)	40 (15.8%)	32 (12.9)	0.35
At least one allergy, N (%)	140 (55.3)	114 (45.6)	0.03
Early life			
Birth at term, N (%)	208 (83.2)	198 (80.16)	0.38
Caesarian delivery, N (%)	50 (20.4)	50 (20.9)	0.89
Birth weight, mean (SD), g	3189.01 (628.9)	3345.5 (574.8)	0.005
Breastfeeding, N (%)	194 (77.9)	201 (82.04)	0.25
Older siblings, N (%)	123 (48.6)	128 (51.2)	0.56
Exposure to tobacco smoke			
During pregnancy, N (%)	34 (13.7)	32 (13.06)	0.85
Parental smoking at home, N (%)	75 (30.1)	71 (29.1)	0.80
Cumulative pool attendance over lifetime			
Indoor, median (IQR), hours	145.4 (148.9)	137.6 (136.2)	0.55
Outdoor, median (IQR), hours	88.5 (164.81)	97.7 (172.4)	0.55
Environment and lifestyle			
House cleaning with bleach, N (%)	103. (40.7)	83 (33.2)	0.08
Living close to an industrial zone, N (%)	58 (23.2)	48 (19.3)	0.30
Exposure to pets, N (%)	142 (56.1)	143 (57.2)	0.81
Living in rural area before 2 years, N (%)	93 (36.9)	87 (34.8)	0.62
Air flagrance use, N (%)	77 (30.4)	75 (30)	0.92
Humidity, N (%)	33 (13.15)	38 (15.2)	0.30
Molds, N (%)	17 (6.8)	18 (7.2)	0.84
Open fire place, N (%)	50 (19.8)	72 (28.9)	0.02
Consumption of tap water, N (%)	194 (76.7)	200 (80)	0.37
Respiratory symptoms and diseases			
Wheezing	30 (12.1)	37 (14.9)	0.35
Asthma	16 (6.3)	17 (6.8)	0.82
Bronchitis, N (%)	95 (37.9)	102 (41.46)	0.42
Food allergy, N (%)	37 (14.9)	43 (17.3)	0.48
Pets allergy, N (%)	18 (7.17)	18 (7.26)	0.97
Eczema, N (%)	46 (18.3)	49 (19.8)	0.67
Exhaled NO, mean (SD)	13.9 (10.9)	16.9 (19.4)	0.04

Respiratory diseases were defined as doctor-diagnosed diseases at any time. Wheezing corresponds to episodes of wheezing during the 12 last months.

Table XXVIII. Risk factors for respiratory complaints (Walloon + Flemish cohort).

	Asthma	Wheezing	Eczema	Pets allergy	eNO > 20 ppb
Living in a rural area before age 2 yrs					0.51 (0.29-0.91)
Older siblings				1.96 (0.97-4.02)	
Birth at term	0.43 (0.19-0.95)				
Family size					1.32 (1.03-1.71)
Humidity at home			1.79 (0.99-3.24)		
Exposure to pets		0.59 (0.33-1.06)		2.37 (1.17-4.78)	
Bleach use for cleaning		1.70 (0.96-2.99)			
Total cumulated outdoor chlorinated pool attendance (hours)		1.01 (1.0-1.03)			
Environmental tobacco smoke		2.14 (1.17-3.94)			

Odds ratio's are presented, only for the (borderline) significant results.

3. POLICY SUPPORT

This project focused on the development of non-invasive biomarkers which can be applied in clinical practice for diagnosis and follow-up of respiratory diseases such as asthma in children, as well as in human biomonitoringstudies addressing the effect of exposure on children's respiratory health. Secondly, this project aimed to apply non-invasive markers in the follow-up of children in cohort studies to evaluate their predictive value for inflammation, epithelium integrity and respiratory complaints, and to identify whether environmental risk factors are related to changes in these non-invasive markers.

3.1. RESPIRATORY DISEASES IN CHILDREN SHOULD REMAIN A FOCUS FOR HEALTH POLICIES

The project provided information of respiratory health and atopy status of children from different cohorts in both parts of the country. Questionnaire data and lung function tests allowed us to conclude that respiratory complaints and atopy remain major health concerns which originate early in life. Prevalence rates were high. At the age of 6, the prevalence of bronchiolitis, eczema and house dust mite sensitization are around respectively 30%, 28% and 25% with increases of the prevalence of house dust mite (HDM) sensitization and eNO between 6 and 8 years of age. Data from 503 children at 7and 8 years of age showed prevalences of wheezing, asthma, eczema, and food allergy of respectively 13%, 6.6%, 19%, and 16%. These new observations are in line with previous data and support the concerns expressed at different fora to focus on major health challenges and vulnerable populations. This has been expressed explicitly in the EU strategy "Together for Health" (2008-2013) and is also one of the focal points of the Belgian environmental health strategy.

3.2 BIOMONITORING AND THE NEED FOR NON-INVASIVE BIOMARKERS

The study has demonstrated the need and also the opportunities for new non-invasive biomarkers that can be applied in young children, to monitor exposure, respiratory health effects, and susceptibility to risk factors for atopy and airway diseases. We felt the need for non-invasive biomarkers for clinical diagnosis and for improved risk assessment. Currently, there are no objective measures to evaluate the physiological state of the respiratory tract in young children. The diagnosis of asthma can, until now, only be established reliably in older children while we know that the disease may originate early in life. There is no unique marker. It is also clear that different underlying pathologies may contribute to a heterogeneous spectrum of symptoms. Also the therapy

response among patients differs which illustrates the complexity of the disease. Applying mechanistically based biomarkers in children's cohorts may help to understand the complex relationship between cause and effect. Additionally, these biomarkers will be valuable in early diagnosis and follow-up of the disease and treatment efficacy. It was of utmost importance to develop reliable monitoring protocols that are applicable in very young children. Exhaled breath collection showed feasible in children from 3 years of age.

Also for ethical reasons, the development of non-invasive biomarkers is of importance. The collection of non-invasive markers is easier and more comfortable and child friendly than for example the collection of blood, another matrix in which mechanistically based biomarkers can be measured. In biomonitoringstudies, more children (and parents) will be motivated to participate when only non-invasive measurements are involved. During the ANIMO project, parents were very enthusiast about the methods, and we received various positive reactions.

The ANIMO project was a first step towards the development of non-invasive biomarkers that can be applied in very young children. Various non-invasive matrices were studied, including exhaled breath condensate, exhaled gasses, nasal lavage, and urine. Exhaled NO was also included as the best known non-invasive marker for respiratory complaints at this moment.

The study increased the ability to perform biomonitoring in vulnerable groups in the population such as young children which will definitely improve risk assessment. Human biomonitoring is cited in relation to its potential to meet the EHAP objective - identifying new themes and assessing the effectiveness of policy. The methods which have been developed in the ANIMO project certainly contribute to these needs.

3.3 EXHALED NITRIC OXIDE ALREADY APPLICABLE FOR CLINICAL DIAGNOSIS

The study confirmed that exhaled nitric oxide (eNO) can already be applied in the diagnosis of childhood asthma. Asthma is characterized by episodes of wheezing, shortness of breath, chest tightness and coughing. These symptoms evolve from airflow obstruction, bronchial hyperresponsiveness and airway inflammation. In the search for a biomarker, it is known that the most useful biomarkers are those with the most direct relationship between the marker and the physiopathology of the disease. Airway inflammation is well established as the underlying key feature of asthma. Childhood asthma is currently diagnosed in clinical practice using lung function measurements, including spirometry and provocation test. Spirometry gives an indication of the airflow obstruction, while a provocation test explores the presence of bronchial hyperresponsiveness. Measurement of eNO however offers the possibility to assess the patient's airway inflammation. Therefore we aimed to evaluate the measurement of eNO

as a diagnostic tool in childhood asthma. eNO measurement is non-invasive, repeatable and reproducible and can provide valuable additional information. However, in clinical practice the reimbursement to the patient remains problematic, which leads to the fact that this biomarker is mainly available in major hospitals. Our findings support the use of eNO measurements in the diagnostic process of childhood asthma, since elevated eNO levels indicate the presence of airway inflammation. eNO measurements are currently not reimbursed to the patient in Belgium. However we conclude that eNO measurement can play an important supplementary role in clinical practice, improving the ability to diagnose asthma.

3.4 ENVIRONMENT AND LIFE STYLE RISK ARE MANAGEABLE RISK FACTORS FOR RESPIRATORY HEALTH AND ALLERGY

The study was able to trace risk factors for respiratory health and allergy in young children. Some of them are personal characteristics and unavoidable such as the atopy status of the parents. Other determinants were related to life style and environment. We confirmed protective determinants such as birth at term and day care attendance. This has been observed also in other well conducted international studies and gives support for the robustness of our study results. In addition we identified environmental risk factors such as exposure to environmental tobacco smoke, living near industry, exposure to air fresheners, bleach use for cleaning, mould in the house, humidity at home and swimming pool attendance. Although the data need further exploration and will be further analysed and published, they support the need for further improvement of the air quality and especially the indoor air quality. The findings are a support for the commitments expressed at the Fifth Ministerial Conference on Environment and Health at Parma where the member countries, for the first time, adopted time-bound targets to reduce children's environmental threats and committed to tackling a series of key environmental health challenges including safe indoor environments for children. However, indoor air quality is determined by a number of separate community policy files that go beyond environment and health, such as product emission and construction materials standards as well as climate change. Policy coherence to achieve an overall added value for health is needed.

3.5 THE HEALTH RISK OF SWIMMING POOL ATTENDANCE IN EARLY LIFE STAGES

Special focus of the ANIMO study was on swimming pool attendance at infant age as a potential trigger for later atopy and asthma.

Human exposure to chlorination products has considerably increased during the 20th century especially after the 1950s with the development of public and leisure pools and

other water recreational areas. When attending these aquatic environments, the populations of industrialized countries have been increasingly exposed to powerful chlorine-based oxidants, either through direct contact with chlorinated water or by inhaling them in the form of gases (trichloramine and chlorine gas) or of aerosols (hypochlorite/hypochlorous acid and chloramines). The existence of respiratory and allergic problems in competitive swimmers training in the chlorine-laden atmosphere of swimming pools has also been known for more than two decades. Serious concern about these chemicals arose, however, only recently when it was found that they could affect the lung epithelial barrier of recreational swimmers and increase the risks of atopic diseases such as asthma or hay fever. Furthermore, studies focusing on occupational exposures have demonstrated an increased risk of asthma and respiratory problems among swimming pool workers while ecological studies have brought to light associations between the prevalence of childhood asthma or eczema and the availability of swimming pools or the chlorine level in drinking water. Albeit unexpected, these findings are not really surprising. A variety of experimental studies have shown that hypochlorous acid and chloramines are membrane permeable oxidants capable of rapidly opening the tight junctions of epithelial layers. This led to the suggestion, supported by some epidemiological and experimental observations, that these oxidants closely linked to our Western lifestyle might act as adjuvant in the development of atopic diseases by facilitating the transepithelial penetration of allergens. The ANIMO study confirmed and added to the earlier finding of health risks due to swimming pool attendance: infant swimming practice, increased dose-dependently the risk of sensitization to HDM or to pollen, and the risk of airways inflammation assessed by the eNO test. Infant swimming, an activity practiced by 53.7% of children, was actually the strongest risk factor for increased eNO between the age of 6 and 8 years with an odds ratio of 3.54 for ever swimmers and of 4.45 for children who had swum for a total of more than 10 hours during infancy. This study also stressed the importance of the timing factor to evaluate the respiratory effects of lifestyle or the environment. For instance, the associations of respiratory allergies and airways inflammation with infant swimming were detected only with the outcomes measured between the age of 6 and 8 years.

3.6 FURTHER RESEARCH OPTIONS

The ANIMO project has allowed to open opportunities for gaining further knowledge and has clearly pointed to areas which require further investigations and which are open for future innovation.

Biomonitoring in children at young ages is feasible and useful information is expected if new technologies such as metabonomics and proteomics are integrated in future

biomarker studies. The ANIMO project has allowed us to set the first steps in this complex approach. Promising results have been obtained but confirmation is needed in independent study populations.
We expect that biomarker fingerprints will help to elucidate further diagnosis and follow up of complex diseases such as allergies and asthma. We expect also that risk factors can be reliably determined if the biomarkers are more related to the biology of the disease, than is currently possible. Expanded studies are needed with larger patient groups that are clinically well characterized. This will allow to obtain discriminating profiles for the different groups.

Next to diagnostic markers, there is also a strong need for early warning biomarkers. Early warning biomarkers are sensitive signals which allow to take measures before diseases become manifest. To develop these markers we need a prospective cohort. The ANIMO study has started with this approach , but the study needs to be expanded and should include a larger study population that is followed over a longer time period.

Cohort studies to identify risk factors need to be large enough to draw reliable conclusions. Prospective follow up studies are the most powerful studies for identification and confirmation of risk factors and protective factors for diseases. However, it is important to establish clearly from the beginning of the study a strategy for keeping the study population interested and make sure that they participate also at later time points. A long term strategy has to be set up for extended follow up of children in time. The ANIMO project illustrates again that early life exposures may be among the most vulnerable stages of life for determination of health at an older age. Protecting the child and its environment may have a significant effect on the disease burden of the population. This concept fits into a strategy of preventive health care and healthy aging.

4. DISSEMINATION AND VALORISATION

The development and application of non-invasive biomarkers is still a relatively new and promising area of research, and therefore dissemination of our findings is very important. Here, all dissemination activities (presentations and posters) reporting results from the ANIMO study are listed.

List of presentations

- Oral presentation on 'IV European Asthma congress', 22-25/04/2006 in Spain, by K Bloemen: "Variation in exhaled breath condensate pH, volume and total protein concentration".
- Oral presentation on BELSPO workshop 'health and air pollution' on 05/06/2008 in Leuven by K Bloemen: "ANIMO : Indoor risk factors for childhood respiratory diseases: development and application of non-invasive biomarkers."
- Oral presentation on the 'Third international Symposium on Proteome Analysis' on 19/12/2008 in Antwerp by K Bloemen: "Analysis of the exhaled breath condensate proteome."
- Oral presentation on the symposium 'Indoor air pollution and health problems' on 25/05/2009 in Brussels by K Bloemen: "ANIMO: development and application of non-invasive biomarkers."
- Oral presentation on 'SOT', March 2010, by G Koppen: "Non-invasive biomarker development for airway disease and exposure" (K Bloemen, R Van Den Heuvel, E Govarts, G Koppen, E Witters, K Desager, G Schoeters).

List of posters

- Poster presentation at the 'XXV Congress of the European Academy of Allergology and Clinical Immunology', Vienna (Austria), 10-14/06/2006: "Can profiling of volatile organic compounds in exhaled breath be used to characterize subjects that are/or will be suffering from airway diseases?" (H Dhondt).
- Electronic poster presentation / discussion at the 'Annual European Respiratory Society (ERS) congress', September 2007 in Stockholm: "Proteins in exhaled breath condensate: identification and pattern" (K Bloemen, E Witters, J Hooyberghs, K Desager, G Schoeters).
- Poster presentation at the 'Annual Human Proteome Organization (HUPO) congress' 2008, Amsterdam: "Proteins in exhaled breath condensate: identification and pattern" (K Bloemen, E Witters, J Hooyberghs, K Desager, G Schoeters).

- Poster presentation at the 'Annual European Respiratory Society (ERS) congress', 04-08/10/2008, Berlin: "Determinants of variability in exhaled breath condensate (EBC) in children" (K Bloemen, R Van Den Heuvel, K Desager, G Schoeters).
- Poster presentation at SOT 2009: 'Non-invasive biomarker development for airway disease and exposure' (K Bloemen, R Van Den Heuvel, G Koppen, E Govarts, K Desager, E Witters, G Schoeters).
- Poster presentation at the 'Annual European Respiratory Society (ERS) congress', 12-16/09/2009 in Vienna: "Development of an exhaled breath protein pattern to distinguish asthmatic from healthy children" (K. Bloemen, R. Van Den Heuvel, E. Govarts, G. Koppen, V. Nelen, E. Witters, K. Desager, G. Schoeters).
- Poster presentation at the 'Annual European Respiratory Society (ERS) congress', 12-16/09/2009 in Vienna: "Traffic-related air pollution and exhaled breath condensate (EBC) pH" (K Bloemen, G Koppen, A Colles, V Nelen, K Desager, G Schoeters).
- Electronic poster presentation / discussion at the 'Annual European Respiratory Society (ERS) congress', 12-16/09/2009 in Vienna: "Swimming during infancy increases the risk of bronchiolitis" (C Voisin).
- Electronic poster presentation / discussion at the 'Annual European Respiratory Society (ERS) congress', 12-16/09/2009 in Vienna: "Nasal epithelium damage, chlorinated pool attendance and risk of sensitization to aeroallergens" (A. Sardella).

Workshop

On December 10th 2010, a workshop was organized by the partners of the ANIMO project, together with the European COPHES project: "Workshop on non-invasive human biomonitoring". The program, abstracts and presentations are brought together on the following website: http://cms.ku.dk/sund-sites/ifsv-sites/lisbethe/ animocophes/.

Other

ANIMO is involved in the cluster project with the belspo projects MYC-ATR, PARHEALTH, PM2TEN[3] and SHAPES. ANIMO is also involved in the cluster project AIR-QUALITY[3].

[3] http://www.belspo.be/belspo/ssd/science/pr_health_envir_fr.stm

5. PUBLICATIONS

Peer reviewed publications

A list of all publications is provided here.

- Relation between ambient air and breath volatile organic compounds. Dhondt H, Goelen E, Koppen G, Verschaeve L. WIT Transact. Biomed. Health 2007;11:33-40.
- Non-invasive biomarker sampling and analysis of the exhaled breath proteome. Bloemen K, Hooyberghs J, Desager K, Witters E, Schoeters G. Proteomics Clin Appl. 2009;3(4):498-504.
- Application of non-invasive biomarkers in a birth cohort follow-up in relation to respiratory health outcome. Bloemen K, Koppen G, Colles A, Govarts E, Van Den Heuvel R, Nelen V, Witters E, Desager K, Schoeters G. Biomarkers 2010;15(7):583-593.
- Infant swimming in chlorinated pools and the risks of bronchiolitis, asthma and allergy. Voisin C, Sardella A, Marcucci F, Bernard A. Eur Respir J 2010;36(1):41-47.
- A new approach to study exhaled proteins as potential biomarkers for asthma. Bloemen K, Van Den Heuvel R, Govarts E, Hooyberghs J, Nelen V, Witters E, Desager K, Schoeters G. 2010. *Early online*.
- Respiratory risks associated with chlorinated swimming pools: a complex pattern of exposure and effects. Bernard A, Voisin C, Sardella A. Am J Respir Crit Care Med. 2010. Accepted.
- Risks of allergic sensitization associated with infant swimming. Voisin C, Sardella A, Marcucci F, Bernard A. Article in preparation.
- Measurement of CC16 in urine, environmental insults and risks of respiratory allergies: a biomarker study among school children. Voisin C, Sardella A, Marcucci F, Bernard A. Article in preparation.

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