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

"ANIMO"

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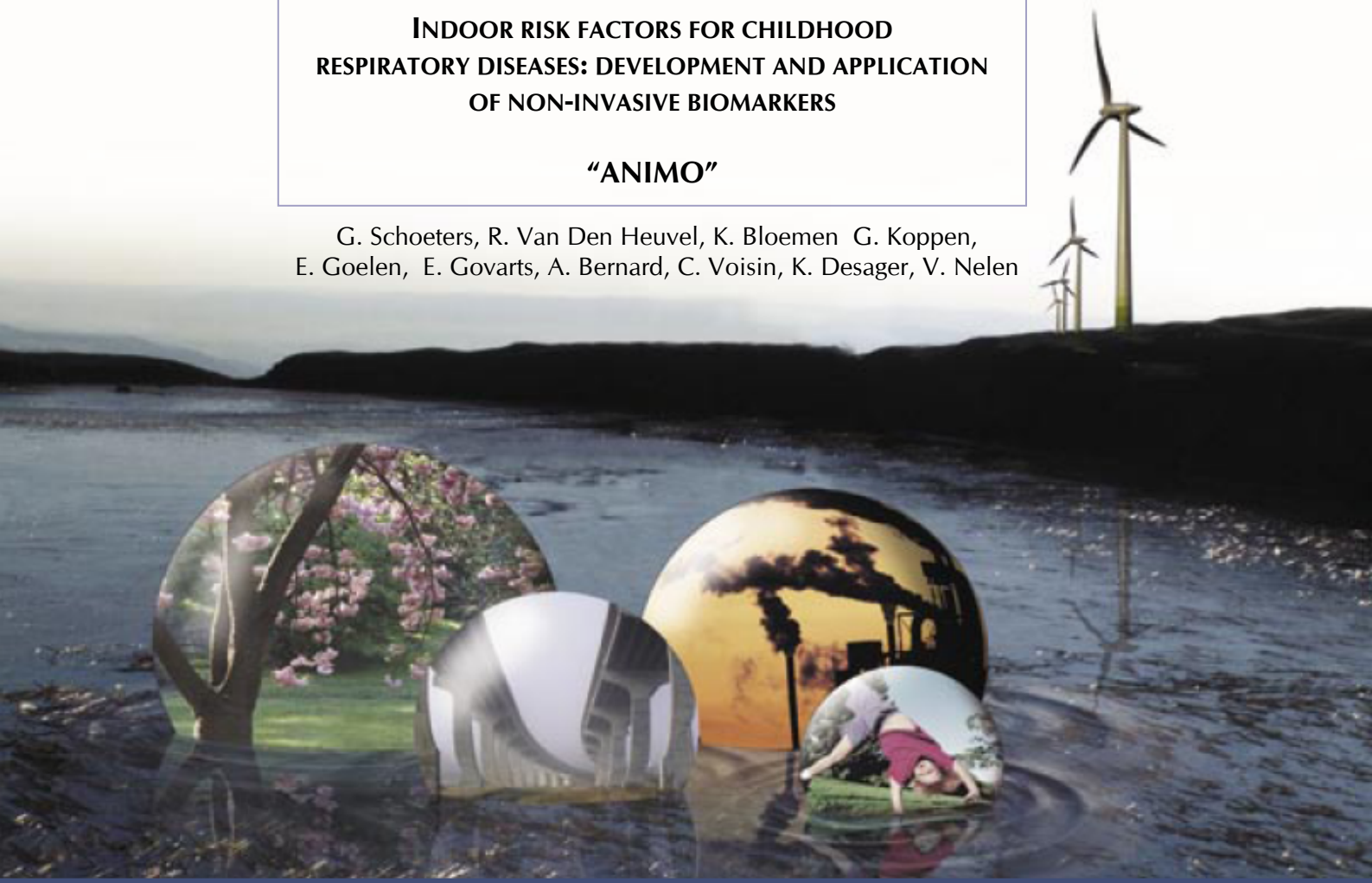

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FINAL REPORT PHASE 1

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

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ABBREVIATIONS

CC16	Clara cell protein 16
EBC	exhaled breath condensate
eNO	exhaled nitric oxide
GC-MS	gas chromatography- mass spectrometry
LC-MS	liquid chromatography- mass spectrometry
NAL	nasal lavage
NO	nitric oxide
RPB	retinol-binding protein
SVM	Support Vector Machines
UGRP-1	Uteroglobin-related protein 1
VOCs	volatile organic compounds

1 SUMMARY

In the last decades, an increase in the burden of respiratory diseases or disorders such as allergies and asthma has been observed in children as they grow up. Environmental factors (outdoor and indoor) may contribute to this increase. There is clear evidence that children are more susceptible to some stressors in the environment.
It is clear that within the EU, human biomonitoring is becoming an important tool for environmental health follow up with special emphasis on children's health. Children's respiratory health is among the priorities of environmental health programs. 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 study addresses children's respiratory health by developing non-invasive markers 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.

Initial activities of the first phase focused on the **development, optimization** and **standardisation** of protocols for four **non-invasive methods** for application in children: exhaled breath gases including NO (nitric oxide) and other volatile organic compounds (VOC), exhaled breath condensate (EBC) and nasal lavage.

NO in exhaled air (eNO) is a well-known indicator of deep lung/airway inflammation. Procedures for use of the mobile NIOX MINO Airway Inflammation Monitor (Aerocrine, Sweden) and the CLD 88 SP analyser (EcoMedics, Switzerland) in children were finalized.

The method to analyse *exhaled breath gases* was optimized. Exhaled breath of study subjects was collected in Tedlar bags. A sampling and thermal desorption gas chromatography – mass spectrometry method was developed that allows monitoring of C_5-C_{12} VOCs in exhaled breath of subjects. A repeatability experiment demonstrated that the method can be considered reliable for at least 56 VOCs present in exhaled breath with 89% of the coefficients of variance being less than 30 % (of which 85 % $\leq$ 20 %).
A limited intra-individual variability study leads us 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.
Attention has been given to the preconcentration step preceding the chemical analyses (GC-MS) of the exhaled VOCs. Two preconcentration methods, the thermal desorption GC-MS method and the Entech 7100A preconcentration GC-MS, were compared, and differ in sensitivity and in the measurable VOC range.

The *nasal lavage* technique was successfully tested on children. Afterwards the procedure was slightly modified to make it even less invasive. After modification, the recovery of proteins such as CC16 and albumin in the nasal lavage sample was checked.

Exhaled breath condensate (EBC) was collected during tidal breathing through an RTube. Determinants of variability of protein content, EBC volume and pH of EBC were studied in

adults. No significant differences between sampling times on the same day or on different days were obtained for pH, volume and total protein concentration, provided that subjects are experienced in collecting EBC. Furthermore, no amylase activity (marker for saliva contamination) was measured in the EBC samples. A protocol to concentrate the proteins in the EBC samples was selected based on protein recovery and reproducibility data. The method using the concentration of the proteins on beads, was retained. After concentration, the proteins were digested and separated by nano-LC and detection was done by using a MALDI-TOF/TOF mass spectrometer.

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. 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. 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). The pilot study should allow to evaluate the feasibility to measure the biomarkers in the selected age group and it should help to identify the determinants of variability for the biomarker measurements.

In the pilot study, the NIOX MINO and EcoMedics device were compared to measure exhaled NO. The obtained values from the two devices correlated well with each other ($r = 0.81$, $p < 0.001$). Values were slightly higher in the NIOX MINO compared to the EcoMedics. Due to the variability between the two devices, it is recommended not to use results from both devices in the same analysis. Furthermore, we would recommend to perform at least two measurements, which agree within 10% of each other, irrespective of the device used.
No significant correlations were found in this study group between exhaled NO and age, height, weight and gender. A significant difference between asthmatic patients and healthy controls was observed ($p = 0.004$ for the EcoMedics, and $p = 0.027$ for the NIOX MINO).

Nasal lavage was collected in both the right and the left nostril. As observed in previous results in adolescents, also in this pilot study, there is a good correlation in albumin/urea ratio (log) ($r = 0.748$; $p < 0.0001$) and CC16/urea ratio (log) ($r = 0.556$; $p < 0.0001$) between the two nostrils. In this pilot study, mean ratio albumin/urea (± SD) was 0.424 (± 0.566). Mean ratio CC16/urea (± SD) was 3.39×10^{-4} ($\pm 6.07 \times 10^{-4}$). No correlations were found between these ratios (log) and age, gender, weight, height or asthma/control group.

Exhaled breath condensate pH was measured in 500 µl of EBC sample, collected in an uncoated RTube, exactly 5 minutes after collection, without deaeration. Mean value (± SD) was 6.17 (± 0.29). pH was significantly lower in the asthma group (6.07 ± 0.28) compared to the healthy controls (6.23 ± 0.29; Mann-Whitney U test: 0.047). LTB4 was measured in the EBC samples collected in the coated RTube. Mean (± SD) LTB4 concentration was 60.05 (± 10.61) pg/ml in the pilot study. We did observe slightly higher values in the healthy children compared to the asthmatic children, although not statistically significant. A positive correlation between LTB4 in EBC and CC16/urea in nasal lavage was observed.
EBC was used for proteome analysis. Samples were concentrated on beads, enzymatically digested, and resulting peptides were separated by nanoLC (liquid chromatography). Peptides in all fractions were detected in a MALDI-TOF mass spectrometer. MSMS analyses were

performed to identify the various proteins. The most abundant proteins in the EBC samples were identified as cytokeratins. Already some additional proteins could be identified. However, most are still under investigation at this moment. To compare the protein pattern between two groups, the area of the peptides in the mass spectra was corrected for the area of the internal standard in that fraction. For further statistical analysis, Support Vector Machine analysis was used. Although the asthma group consists of 4 groups, e.g. no asthma, controlled asthma, moderately controlled asthma and uncontrolled asthma, only the subjects with moderately controlled and uncontrolled asthma were include in the asthma group, and compared with the whole control group. Preliminary analysis of the peptide pattern resulted in a classification model that classified all subjects correctly (100%) regarding their asthma status.

The exhaled gases collected in the Tedlar bags were transferred to thermodesorption tubes and after adding the reference compound (2-fluorotoluene), these were submitted to a GC/MS analysis. The analysis was performed on an a-polar column and the detection was in full scan modus (m/z from 25 to 250). 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. Also here, Support Vector Machines were used for statistical analysis. Again, only subjects with moderately controlled and uncontrolled asthma were included in the asthma group, and compared to the control group. The most optimal classification model classified all subjects correctly (100%) regarding their asthma status.

An existing **child cohort** and a new child cohort will be used for studying environmental risk factors for respiratory diseases in children using non-invasive biomarkers. The optimized non-invasive biomarkers will be applied in
1) a follow-up of the existing child cohort of the Flemish Environment and Health Study at the age of 6 years.
2) a new cohort including schoolchildren exposed to specific risk factors (chlorinated products).
The new biomarkers will be used in combination with classical clinical endpoints (e.g. doctor-diagnosed asthma, respiratory symptoms, lung function, exercise-induced asthma). The cohorts of children will be used to test the hypotheses that the non-invasive effect biomarkers are related to respiratory health outcome.

A questionnaire focusing on respiratory health outcome and indoor exposure was compiled based on the experience from previous studies and on relevant literature data. The questionnaire will asses indoor chemical exposure, other risk factors for asthma and allergy (e.g. family history,…) and respiratory complaints in children. This questionnaire will be used in the follow-up of the existing child cohort and the new cohort. In addition, a statistical analysis plan was developed.

The new cohort was started in September 2007. A total of 425 children in 30 kindergarten schools in Brussels, Liège and Louvain-la-Neuve was recruited. The questionnaire filled by the parents included a total of 60 questions about the health of the child and previous diseases, the respiratory symptoms during the 12 last months, the parental antecedents of asthma and allergic diseases, the general environment, the home environment (pets, ETS, use of cleaners and fresheners…) and sport practices. Examination of the children included the measurement of exhaled nitric oxide, spirometry, EBC collection, urine sample and nasal lavage collection, and a Rhinostick test. Biomarker analyses are currently in progress.

In conclusion, protocols for four non-invasive biomarkers were developed and optimized. Further optimization of exhaled breath fingerprints is needed. The methods were successfully applied in young children. Based on the good methodological results of the pilot study and the successful recruitment of children in the new cohort we are well equipped for the second phase of the ANIMO project. In this new phase the non-invasive biomarkers will be assessed in both the existing Flemish cohort and in the children from the new cohort.

2 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. Our proposal 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.

3 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 our study are:

-to identify, standardise and design study protocols for non-invasive biomarkers in environmental health biomonitoringstudies 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 will be assessed in a pilot study involving asthmatic and healthy children.
The hypotheses that the biomarkers are related to respiratory health outcome will be tested in an existing and newly established child cohort.
The new cohort of children will also assess the association of the effect biomarkers with indoor environmental risk factors. We test the hypotheses whether indoor pollutants can promote the development of asthma and make children more sensitive for respiratory diseases.

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

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).

4 BIOMARKER DEVELOPMENT

The work performed in the first year of the ANIMO project was mainly focused on the development and standardisation of four non-invasive methods. 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.

4.1 PROTOCOL DEVELOPMENT

Four non-invasive methods 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.

4.1.1 Exhaled breath gases

NO (nitric oxide) in exhaled air (eNO) is a well-known indicator of deep lung/airway inflammation. eNO may serve as an early parameter detecting asthma. The collection technique is easy and non-invasive .

A task force of the American Thoracic Society and the European Respiratory Society (ATS/ERS) (Kharitonov, 1997) established consensus guidelines (revised in 2001) 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.

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 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. 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 RTX 502.2 column with a Crossbond phenyl methyl polysiloxane phase (105 m long, 0.32 mm IK and 1.8 µm film thickness).
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 ≤ 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. Intra – individual variability 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, 11.15h, 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 ($p < 0.001$).
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 a Entech Breath Sampler

4.1.2 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).

EBC was collected using a RTube (Respiratory Research, Inc). The aluminium sleeve was stored for at least 30 min in a home freezer (-18°C) before collection. The volume, pH and total protein concentration were analysed in the EBC samples. We have analysed pH of the condensate since it has been shown that inflammation causes acidification of the EBC. pH was measured exactly 5 minutes after collection (without deaeration). Total protein concentration was measured with a NanoOrange Protein Quantitation kit (Molecular Probes). All samples were checked for saliva contamination by using the Infinity Amylase Liquid Stable Reagent kit (Thermo).
Prior experiments on repeatability in 20 healthy adults sampled at 6 different times showed no significant differences (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). 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 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, 2007).
Children and adolescents (total n = 170; age: 3-23 yrs) were asked to breath tidally through the mouthpiece for 15 min while watching a movie. Individual characteristics (gender, age, height, weight) and environmental parameters (environmental temperature and relative humidity) were checked whether or not they had an effect on EBC markers. 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 environmental parameters had a significant effect on collected EBC volume in a univariate regression, multiple regression showed that these parameters had no significant effect on EBC volume or pH.

The protocol to measure pH in EBC was standardized: pH will be measured exactly 5 minutes after EBC collection, 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.

EBC samples were used for protein profiling using a proteomics technology platform. A protocol was developed to ensure concentration, separation and identification of the proteins present in the EBC samples. 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 beads was selected as the method to use in further research.

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. Fractions were collected by using a probot, and measured with a MALDI-TOF/TOF mass spectrometer.

4.1.3 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 to instillate 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 1 and are in line with previous observations in teenagers.

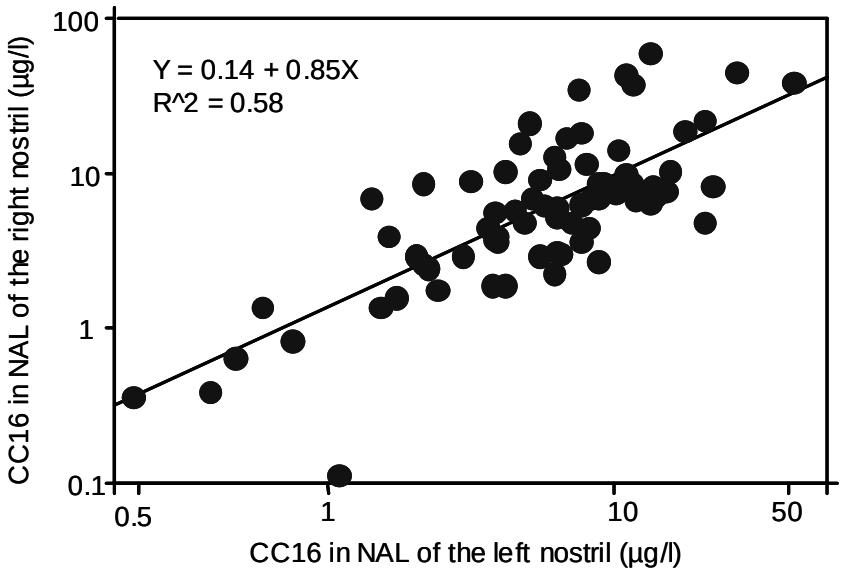

Fig 1: Correlation between the concentration of CC16 in NAL from the right and left nostril (data obtained from 60 children)

4.2 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 are 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, which 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 1), and we obtained positive reactions of both children and their parents. Parents were informed on the results of the tests.

Table 1. Number of failed tests in the asthmatic patients (n=40) and the 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.

4.2.1 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 pilot study, 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. The exhaled NO values obtained by the two devices correlated well with each other ($r = 0.81$, $p<0.001$; Fig. 2). Table 2 shows mean values of exhaled NO obtained by both devices. Results are also shown in a box plot (Fig. 3). Additionally, a Bland-Altman analysis was done to evaluate the results obtained by the two devices (Fig. 4). Values were slightly higher in the NIOX MINO compared to the EcoMedics, but not statistically different (T-test, $p=0.32$). The coefficient of variation between the two devices was 23%, both in the control group and in the asthmatic children. Because of this variability between the two devices, it is recommended not to use results from both devices in the same analysis.

In future examinations, at least two measurements will be done, which agree within 10% of each other, irrespective of the device used.

Table 2. Exhaled NO (Mean ± SD) values obtained by the NIOX MINO and the EcoMedics device.

Subjects	NIOX MINO (ppb)	EcoMedics (ppb)
Asthma group	25.71 ± 20.14	24.09 ± 21.32
Control group	16.26 ± 7.70	11.97 ± 6.80
All children	21.87 ±16.85	18.90 ± 17.69

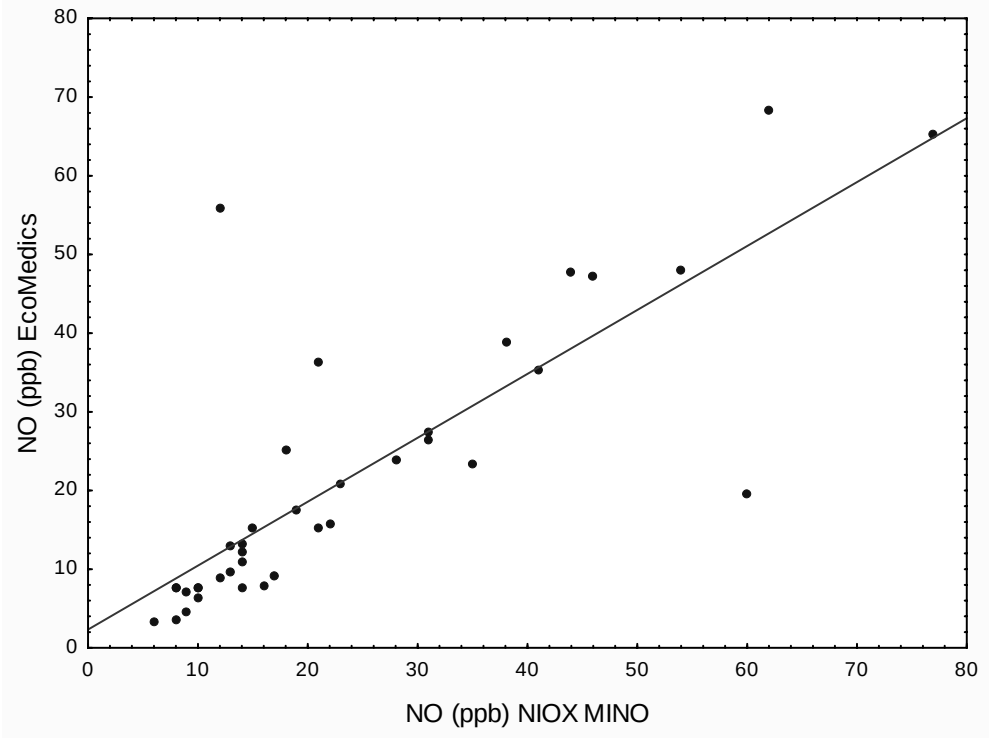

Figure 2.: Scatterplot with exhaled NO values obtained with EcoMedics and NIOX MINO devices.

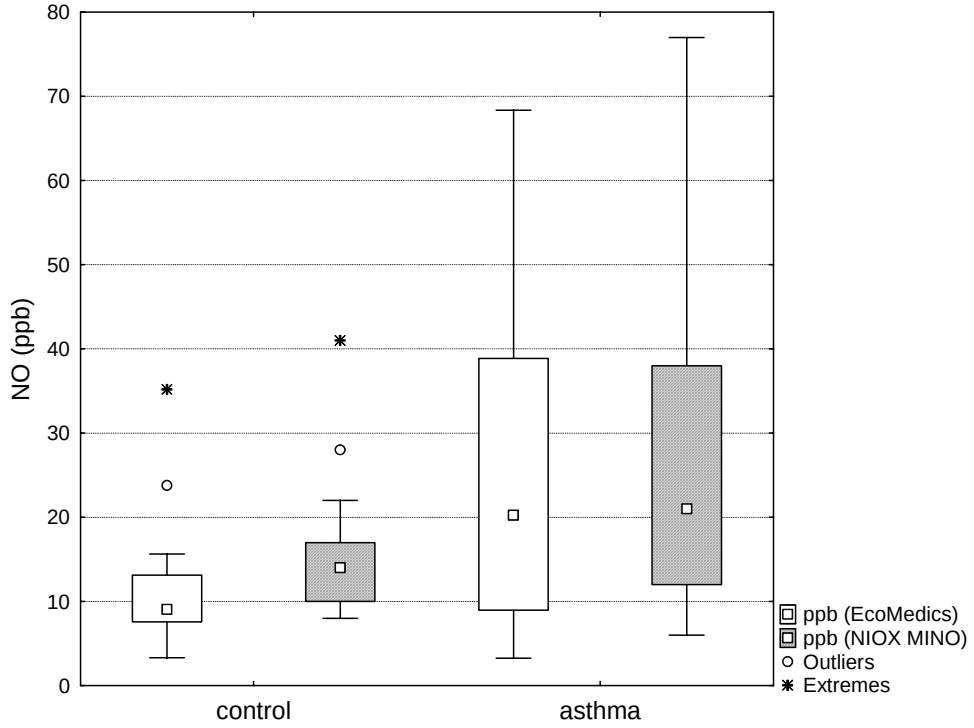

Figure 3. Box plot (Box: median with percentiles; Whisker: non-outlier range) with exhaled NO values from asthmatic and healthy children obtained by EcoMedics and NIOX MINO device.

No significant correlations were found in this study group between exhaled NO and age, height, weight and gender. A significant difference between asthmatic patients and healthy controls was observed ($p = 0.004$ for the EcoMedics, and $p = 0.027$ for the NIOX MINO). After correction for age, height, weight, gender, asthmacontrol, and/or medication, the difference between asthma and control remained significant for both devices.

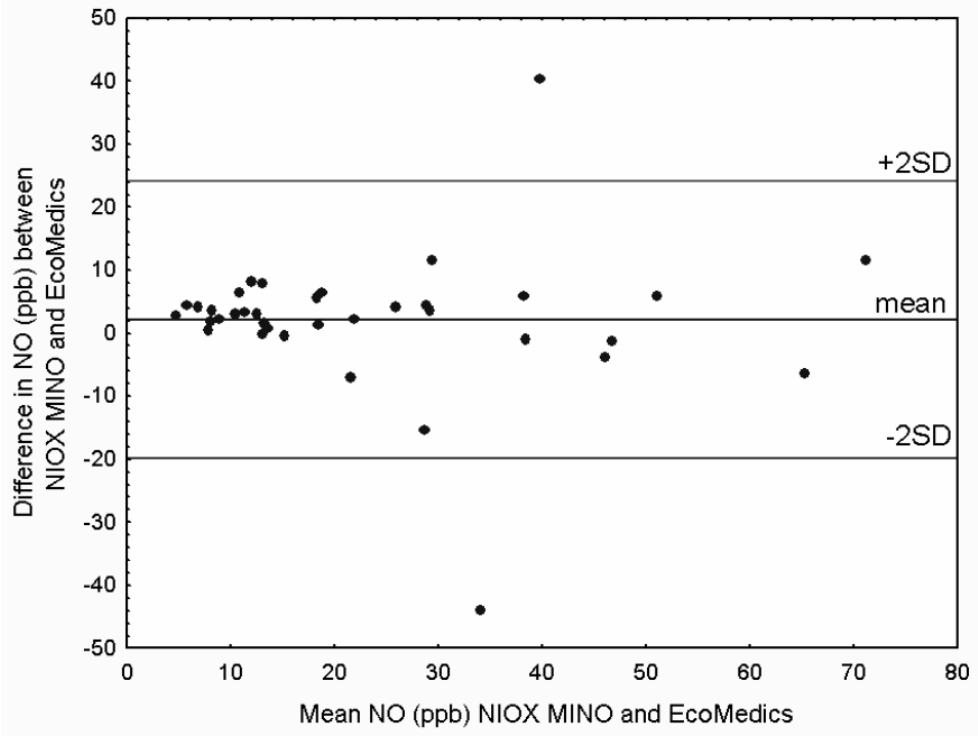

Figure 4: Bland-Altman plot to compare exhaled NO results from NIOX MINO and EcoMedics device.

4.2.2 Nasal lavage

Nasal lavage samples were collected in both the right and the left nostril. 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 (±SD) for all children was 0.424 ± 0.566. Values in control and asthmatic children were not statistically different (T-test, $p=0.11$) (Figure 5). We found no correlations between albumin/urea ratio (log) and age, gender, weight, height or asthma/control group.

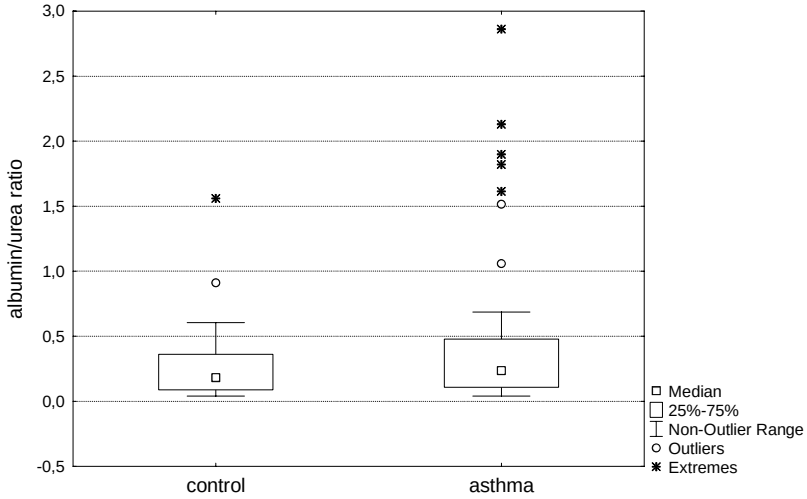

Figure 5: Box plot of albumin/urea ratio in asthmatic patients and healthy control children.

Clara cell protein 16 (CC16) is an anti-inflammatory protein, predominantly excreted by non-ciliated bronchiolar clara cells. In the pilot study, mean ratio CC16/urea (±SD) for all children was 3.39×10^{-4} ($\pm 6.07 \times 10^{-4}$). Values in control and asthmatic children were not statistically different (T-test, $p=0.713$) (Figure 6). We found no correlations between CC16/urea ratio (log) and age, gender, weight, height or asthma/control group.

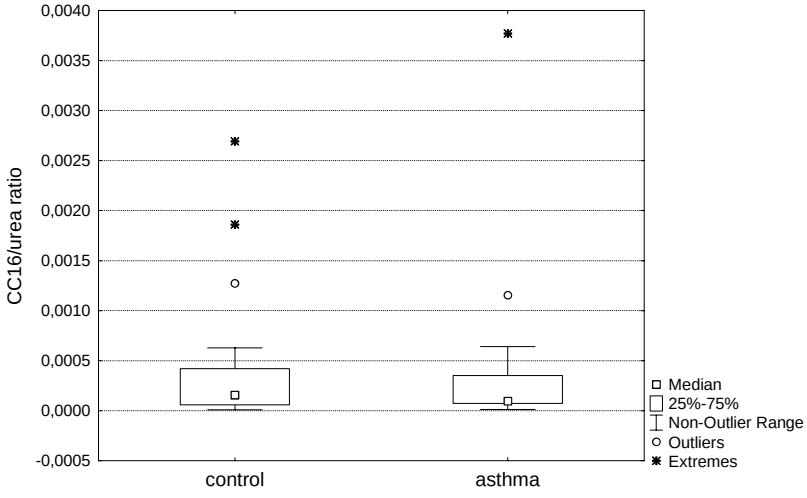

Figure 6: Box plot of CC16/urea ratio in asthmatic patients and healthy control children.

4.2.3 Exhaled breath condensate

EBC was collected twice: 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 tidal breathing through an 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, collected in the uncoated RTube, exactly 5 minutes after collection, without deaeration. Mean value (±SD) was 6.17 ± 0.29. pH was significantly lower in the asthma group (6.07 ± 0.28) compared to the healthy controls (6.23 ± 0.29; Mann-Whitney U test: 0.047) (Figure 7). 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. 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).

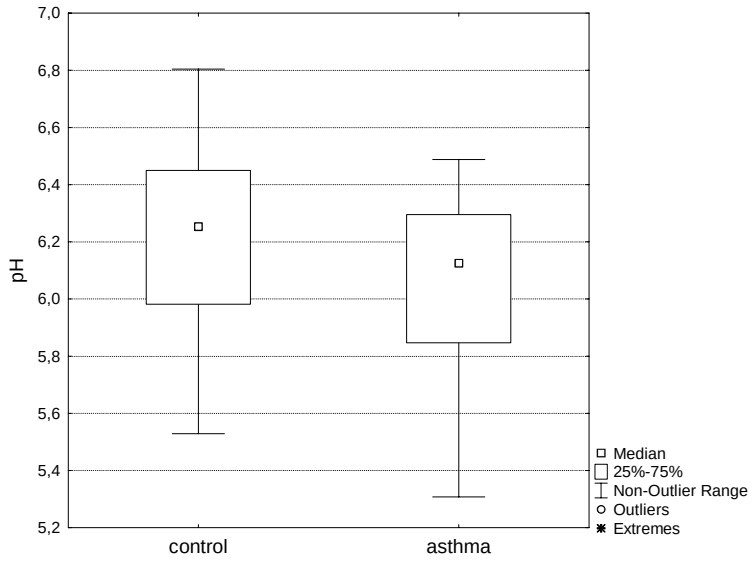

Figure 7. Box plot (Box: median with percentiles; Whisker: non-outlier range) of EBC pH values in asthmatic patients and healthy controls.

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.

EBC Volume

During the first EBC collection (15 minutes), on average 0.97 ml ± 0.41 EBC was sampled. In the asthma group, the mean value was 0.84 ml ± 0.35, and in the control group, it was 1.15 ml ± 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.

Analysis of specific EBC compounds:

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. Mean (± SD) LTB4 concentration was 60.05 (± 10.61) pg/ml in the pilot study. We found no correlations between EBC LTB4 values and age, gender, height or weight of the children. We did find a correlation between LTB4 and family history: 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 (Figure 8). 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 observed a positive correlation between LTB4 in EBC and CC16/urea in nasal lavage ($r= 0.3018$, $p=0.014$).

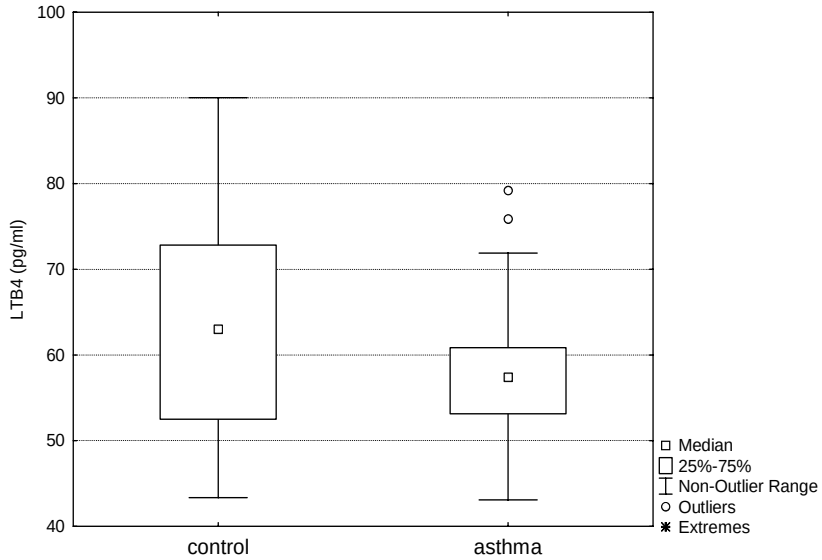

Figure 8. Box plot (Box: median with percentiles; Whisker: non-outlier range) of LTB4 values in asthmatic patients and healthy controls.

8-isoprostane, a marker for oxidative stress, was measured by a commercially available EIA kit (Cayman). Unfortunately, most measurements were around the detection limit, and as a

consequence, results are not reliable. Due to instability of the compound we may need to add a substance to stabilize 8-isoprostane, such as butylated hydroxytoluene.

Additional measurements including inflammation markers and lung-specific proteins (e.g. IL8, CC16 and alpha-amylase) will still be performed on the EBC collected in the coated RTube.

4.2.4 Analysis of exhaled breath fingerprints

Proteome analysis:
EBC (1 ml) from the first collection was used for proteome analysis. Samples were concentrated on beads, enzymatically digested and resulting peptides were separated by nanoLC. Peptides in all fractions were detected in a MALDI-TOF mass spectrometer. MSMS analyses were performed to identify the various proteins. The whole procedure has been described and submitted for publication. By using this method, biomarkers or a biomarker protein pattern might be selected.
The most abundant proteins in the EBC samples were identified as cytokeratins. Already some additional proteins could be identified. However, most are still under investigation at this moment. To compare the protein pattern between two groups, the area of the peptides in the mass spectra, are corrected for the area of the internal standard in that fraction.

Metabonome analysis:
The content of the Tedlar bags was transferred to thermodesorption tubes and after adding the reference compound (2-fluorotoluene) these were submitted to a GC/MS analysis. The analysis was performed on an a-polar column and the detection was in full scan modus (m/z from 25 to 250). 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.

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).

The most optimal classification model for the VOCs classified all subjects correctly (100%) regarding their asthma status. Preliminary analysis of the peptide pattern also resulted in a classification model that classified all subjects correctly (100%).

5 CHILD COHORT STUDIES

The work performed in the first phase of the ANIMO project was mainly focused on the development and standardisation of four non-invasive methods. In addition, a new cohort was initiated and children were recruited and examined for the first time. Non-invasive biomarkers were applied according to the protocols which have been developed. Furthermore, preparations were made for a further follow-up of an existing Flemish child cohort[*].

5.1 APPLICATION OF NON-INVASIVE BIOMARKERS IN EXISTING CHILD COHORT

In Flanders (Belgium), a child cohort was initiated as part of a large scale human biomonitoring study (Flemish Environment and Health study 2002-2006) supported by the Ministry of Health and Environment (Steunpunt Milieu en Gezondheid). Women-child dyads, adolescents and adults residing in 8 different areas of Flanders with different pollution pressure participated in this study. The newborn campaign included the measurement of biomarkers of exposure (Pb, Cd, HCB, pp'-DDE, dioxine-like compounds and PCBs) in cord blood. Clinical parameters (e.g. biometry, apgar score, thyroid hormone levels) for neonates were registered (biomarkers of effect).
A follow-up study (n=170) related to the development of asthma and allergy has been conducted in part of the child cohort at the age of 36 months. These children were living in the Antwerp region or rural Flanders. Clinical parameters, lung function tests, skin prick tests and exhaled NO were examined in the children.

In Phase II of the project, a second follow-up of the children at the age of 5 or 6 years will take place. The non-invasive biomarkers will be applied in a further follow-up of the existing child cohort. The focus will be on the relationship with health outcome.

Related to this follow-up study, a questionnaire focused on respiratory health outcome, risk factors for asthma and allergy (e.g. family history,…) and indoor exposure was compiled. Questions cover different topics including relevant covariables (age, sex, diet, socio demographic information, …), indoor conditions and chemical exposure (eg housing conditions (dampness, mold), cleaning behavior, heating, swimming pool attendance, pet exposure, food consumption,…
The questionnaire was based on the experience from previous national and international studies and on relevant literature data on risk factors for atopy/allergy/respiratory diseases. The following sources were consulted: Alspac study, CDC (NHANES study), EPA, RIOPA study, Flemish Environment and Health Study, ECRHS II study, THADE report, SHER report.
The questionnaire is ready for use in the follow-up of the Flemish child cohort. The questionnaire will be transferred to P2 for use in the second follow-up of the children of the new cohort. This will allow a combined analysis of the data of both cohorts.
A statistical analysis plan was developed for the follow-up study.

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

5.2 APPLICATION OF THE BIOMARKERS IN NEW CHILD COHORT

A new child cohort was initiated in order (1) to conduct a prospective study on young children which will allow to identify indoor pollutants contributing to the development of respiratory and allergic diseases during childhood and (2) to evaluate the predictive value of new non-invasive indicators of airway inflammation and damage with respect to the development of asthma and respiratory allergies

After having obtained the approval of the Ethics Committee of the Catholic University of Louvain and the agreement of school directors, recruitment started in 2007 in 30 kindergarten schools in Brussels, Liège and Louvain-la-Neuve (Figure 11). Schools are located in urban and rural areas.

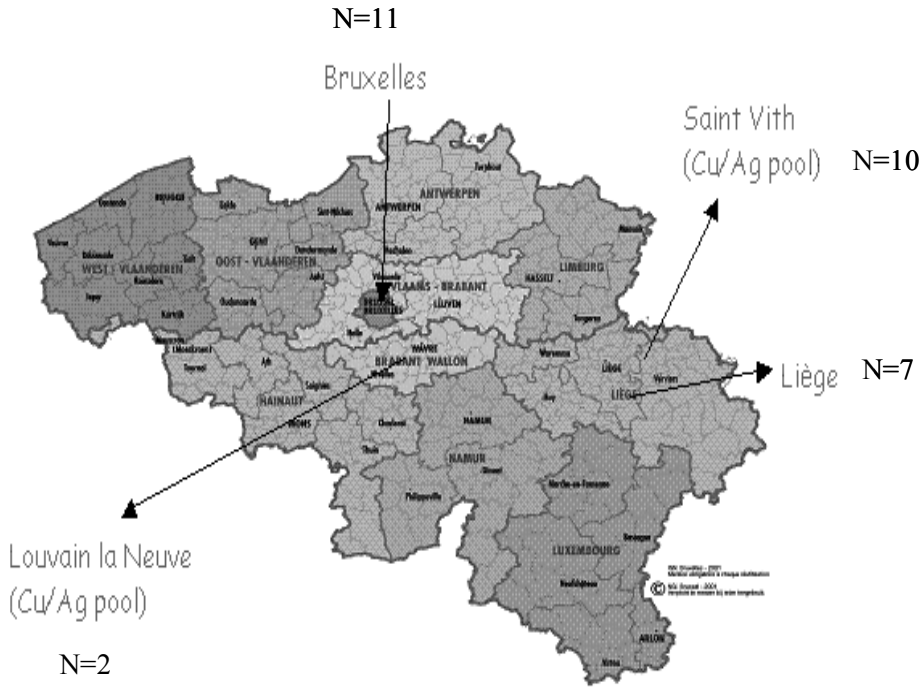

Figure 11 Number of kindergarten schools participating study at Brussels, Louvain-la-Neuve, St Vith and Liège

Informed consent documents together with a questionnaire were distributed to parents of a total of 839 children of the last grade of kindergarten school. The parents of 425 children gave their written agreement for the participation of their child and answered to the questionnaire. School directors were asked to complete a questionnaire inquiring about the school environment and characteristics and the sport activities organized in the schools. The questionnaire filled by the parents included a total of 60 questions about the health of the child and previous diseases, the respiratory symptoms during the 12 last months, the parental antecedents of asthma and allergic diseases, the general environment, the home environment (pets, ETS, use of cleaners and fresheners…) and sport practices. Examination of the children included the following tests and measurements:

a) Measurement of height and body weight
b) Measurement of nitric oxide (NO) in exhaled air with the NIOX analyzer (Aerocrine AB, Sweden). The test was performed according to the guidelines of the American Thoracic Society.

c) Spirometric tests with a Spirostar 2000 (Medriko OY, Finland). FEV1, FVC and PEF measurements were performed according to the standards of the American Thoracic Society.
d) Collection of exhaled breath condensate (EBC) using the TURBO-DECCS (Transportable Exhaled Condensate Collection Systems) for the determination of inflammation markers and lung-specific proteins using ultra-sensitive assays.
e) Collection of an untimed urine sample to measure Clara cell protein (CC16), keratinise and retinol-binding protein (to adjust for variations in the tubular reabsorption of CC16.
f) Collection of a nasal lavage (NAL) sample from the two nostrils separately. The test consists to instillate with a peristaltic pump 1 ml of physiologic water per nostril during 15 second and then to collect the saline with the dissolved protein in it after another 10 seconds. These samples will be use for the assays of albumin, CC16 and urea.
g) Screening of sensitization to the eight most prevalent aeroallergens using the Rhinostick test.

A total of 425 children, all from the third kindergarten schools (mean age, 6 years), had the agreement of their parents to participate to our study. Of these children, 394 were at school the day of the examination and were part of the final cohort for the follow-up. Table 3 and 4 show, separately for boys and girls, some of the final data that are already available concerning the characteristics of the children, their respiratory health and some potential risk factors for respiratory diseases. Except exhaled NO, all these data are based on the information provided by the questionnaire filled by the parents.

Table 3. Characteristics and respiratory health of children (prevalences in %)

	Girls	Boys	P-value*
N	201	229	
Belgian (%)	84.0	84.7	0.86
Body weight, kg (mean, SD)	22.9 (14.2)	25.3 (18.9)	0.16
Height, cm (mean, SD)	111.6 (14.9)	110.3 (20.5)	0.50
Wheezing (%)	19.4	16.1	0.37
Asthma (%)	6.0	8.3	0.35
Bronchiolitis (%)	25.9	35.7	0.03
Pneumonia (%)	10.4	11.7	0.67
Exhaled NO (ppb, mean, SD)	8.9 (4.99)	9.0 (6.22)	0.77
FEV1 (% of predicted value, mean, SD)	104.8 (18.6)	108.1 (18.7)	0.09
Allergic rhinitis (%)	8.6	14.8	0.06
Hay fever (%)	2.49	8.7	0.006
House dusts mite allergy (%)	11.4	9.13	0.43
Eczema (%)	27.0	30.4	0.43
Pets allergy (%)	4.5	7.9	0.15

**t-test and chi-square test*

Table 4. Some environmental and lifestyle factors likely to influence the respiratory health of children (prevalences in %)

	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 freshness 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

**t-test and chi-square test*

There were little differences between boys and girls regarding most risk factors (except for the use of air fresheners). Proportions of children with some classical risk factors of asthma and respiratory allergies (parental asthma and allergy, exposure to ETS or to tobacco smoke during pregnancy) were very similar to those observed in our previous studies (Bernard, 2006; Bernard, 2008). Proportions of children exposed to air fresheners, having access to a residential pool or having swum during infancy were slightly higher than those observed in our previous studies. Boys tended to be more frequently affected by some respiratory allergies (hay fever, allergic rhinitis) and diseases (bronchiolitis). Interestingly, the prevalences of wheezing, doctor-diagnosed asthma and the mean values of exhaled NO were not significantly different between boys and girls, in contrast to what is usually found in older children and adolescents. This suggests that the gender-related differences in these outcomes arise from the exposure to risk factors intervening later during childhood. Of note also, the proportions of children having had a bronchiolitis (more than one fourth) or pneumonia (more than one tenth) were relatively high and there was already a marked difference in the prevalences of hay fever between boys and girls. We will examine whether some of these outcomes, especially those with a high prevalence, can be related to some risk factors linked to the environment, the lifestyle, or the family history of children. In addition to factors listed in Table 4, we will assess the influence of a number of other factors such as socio-economic status (based on parental education), use of pesticides at home, dietary habits, birth weight, breastfeeding or ambient air pollution (e.g. living less than 100 m from a busy road).

In order to assess the validity of the exhaled NO protocol, the concordance of exhaled NO values with the information provided by the parents via the questionnaire was checked. As shown in Table 5, exhaled NO was significantly higher in children with wheezing, asthma, allergic rhinitis, hay fever and house-dust allergy (questionnaire). This indicates that the airways inflammation detected by the biomarker is associated with a poorer respiratory health in terms of wheezing, asthma and respiratory allergies.

Table 5. Concentrations of nitric oxide (NO) in exhaled air of children with respiratory problems and those with a good respiratory health.

	Exhaled nitric oxide (mean, ppb)		
Symptoms/affection	**yes**	**no**	**P-Value***
Wheezing	11.7	8.4	<0.0001
Asthma	13.4	8.5	<0.0001
Allergic rhinitis	11.7	8.6	0.0004
Hay fever	11.2	8.8	0.045
House dusts mite allergy	11.3	8.7	0.0006

** Student t-test*

The analysis of biomarkers in NAL samples (albumin, CC16 and urea) is currently in progress. The samples analyzed so far indicate that the concentrations of CC16 in NAL from the two nostrils are well correlated, as this was already the case in our previous study in adolescents and children. Surprisingly, the concentrations of CC16 in these young children were almost 10 times lower than those observed in adolescents. We will adjust these values for urea concentrations in order to determine to what extent this difference is due to a higher dilution or to a lower production of CC16 during childhood because of physiological or other reasons.

At this stage, we have completed the analyses of the Rhinostick for 320 children. These preliminary results are given in Table 6. Prevalence rates of sensitization to aeroallergen are similar between boys and girls. Overall, about 40% of children in both sexes are sensitized to at least one aeroallergen and 30 % to pollen. Prevalence rates of sensitization to pets (cat) and house dust mite are lower. Table 7 compares the mean eNO level between children who are sensitized to aeroallergen and those who are not. Sensitization to house dust mite and cat allergen is associated with significantly higher levels of eNO but no difference is seen with sensitization to pollen. These findings are expected since it is known that cat and house dust mite allergens penetrated more deeply in the lung than the pollen allergen.

Table 6 . Preliminary results of the rhino-sticks for 313 children.

	Girls	**Boys**	**P-value***
Positive for E1 (cat allergen)	13 (9.3%)	18 (10.4%)	0.74
Positive for D1 (house dust mite)	15 (10.6%)	25 (14.6%)	0.3
Positive for pollen (G, Mix 4, W19)	39 (28.1%)	51 (29.5%)	0.78
Positive for at least 1 aeroallergen	59 (40%)	74 (43.5%)	0.53

* X squared test

Table 7. Association between the allergies detected by rhino-sticks and the results of eNO.

	eNO (mean, ppb)		
	No sensitization	*Sensitization*	P-Value*
Positive to D1 (HDM)	8.4	11.8	<0.0002
Positive to E1 (Cat allergen)	8.6	11.3	<0.0001
Positive to pollen (Mix4, G, W19)	8.8	9.0	0.82

* Student t-test

Concerning the non-invasive bio-markers we are currently analyzing the lung-specific Clara cell protein (CC16) and the retinol-binding protein (to adjust CC16 for variation in proximal tubular function) in the urine-samples). The mean concentration of RBP measured in 300 samples averages 114 µg/l (SD: 90.8).
With respect to EBC, we are currently developing the ultra sensitive Bioplex technique (BioPlex® Bio-Rad Laboratories) to measure CC16 and possibly Uteroglobin-related Protein (UGRP)-1 in the same run as cytokines (IL-8, LTB4).

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6 CONCLUSIONS

Efforts of the first phase of ANIMO addressed 1) method and protocol development, 2) preparation of the cohort studies by writing study protocols, developing questionnaires and obtaining permission of the ethical committees, 3) initiation of the new prospective cohort study in the Brussels and Walloon region.

1) Protocol development
The non-invasive methods (eNO, nasal lavage, exhaled breath condensate and exhaled gases) were successfully adapted and optimized for use in young children. The procedures were written down. The performance of the biomarkers was evaluated in a pilot study consisting of a cohort of 30 control children and 40 children with asthma.
Exhaled NO
Both devices (NIOX MINO and Ecomedics) were well accepted by the children. 57 of the 70 children succeeded to perform three measurements with the EcoMedics device, 64 of the 70 succeeded with the NIOX MINO. Results are in good agreement, although values are a little higher in the NIOX MINO. It is recommended not to use results from both devices in the same analysis. Furthermore, we would recommend to perform maximum 6 measurements, until at least two measurements agree within 10% of each other, irrespective of the device used. Our results correspond with data from literature, and confirm that eNO values are significantly elevated in asthma.
Nasal lavage
Collection of nasal lavage samples was well accepted by the children. CC16 and albumin were successfully measured in the samples.
Exhaled breath condensate
Collection of exhaled breath condensate by using the RTube is well accepted by all children. pH is at this moment the best studied parameter in EBC, and is a marker for airway inflammation. The measurement is easy and results are reliable. The protocol for protein analysis in EBC was optimized (manuscript accepted for publication in *Proteomics Clinical Applications*), and is used now to identify EBC proteins which might be involved in airway disease/inflammation.
Although detection limits of specific compounds by commercially available kits are often a problem in mediator measurements in EBC, leukotriene B4 could be measured in a reliable way. To measure 8-isoprostane however, a substance needs to be added to the samples to stabilize this molecule. New sensitive methods to analyse more compounds are under development.
Exhaled breath gases
All children were able to collect exhaled breath gases into a Tedlar bag. The protocol for metabonome fingerprinting was optimised. Variability has been described and found to be acceptable. The identification of gases needs further exploration.

Variability of the different assays was determined in the pilot study. We found in this group no correlations for exhaled NO, nasal lavage albumin/urea, nasal lavage CC16/urea, and EBC LTB4 with age, height, weight or gender. For EBC pH, a correlation with gender was found but only in the asthma group. Children with a higher weight, collected a higher EBC volume. The pilot study yielded information which is useful for calculating statistical power in the larger cohort studies.

The main purpose of this pilot study was to check the feasibility of the tests in the age group 6-12 years. We can conclude that this is very good. Only some of the youngest children had

some problems with NO measurements. Also protocols to measure the various molecules in the different samples were developed and optimized when needed.

The statistical analysis in the pilot study suggested that some molecules might be able to discriminate between asthmatic and non-asthmatic children. However, this still has to be studied in more detail and confirmed in the subsequent cohort studies.

2) Preparation of cohort studies occurred as planned and permits were obtained.

3) Recruitment of children for the new cohort was successful. A total of 394 young children was recruited and examined and will be followed up during the next two years of the ANIMO project. These children originated from schools located in urban and rural areas. On the basis of data collected so far, there seems to be no major participation bias concerning classical risk factors of respiratory diseases and allergies. Interestingly the mean levels of exhaled and prevalences of wheezing and asthma did not differ significantly between boys and girls, suggesting that the gender-related differences in these outcomes normally found in older children and adolescents is due to risks factors that intervene after the age of 6 years and thus might be identified in the course of this follow up study. The protocols of the non-invasive biomarkers could be applied successfully in almost all children at the exception of the Rhinostick test which a few children (less than 10%) refused to perform. The exhaled NO, NAL and EBC tests did not pose any problem.

Based on the good methodological results of the pilot study and the successful recruitment of children in the new cohort we are well equipped for the second phase of the ANIMO project. In this new phase the non-invasive biomarkers will be assessed in both the existing Flemish cohort and in the children from the new cohort. The age of the children in both cohorts will be comparable (7-8 years). In both cohorts children are from urban and rural areas. We will apply harmonised methods for biomarkers and we will apply the same questionnaire in both studies. This will increase the power of the individual studies separately as we will be able to combine the data for statistical analysis of the risk factors related to indoor air and to evaluate the predictivity of the biomarkers for respiratory toxicity. Each cohort will obtain additional data: the Flemish cohort will allow a further development of exhaled breath fingerprints, the power of the study in the Brussels and Walloon region are the repeated non-invasive biomarker measurements at different ages in the same children.

7 FUTURE PROSPECTS AND PLANNING

As suggested by the experts during the mid term review, in the second phase of the ANIMO project more efforts than initially foreseen will be committed towards further optimization of the biomarkers in exhaled breath. The biomarkers that have been developed in the first phase of ANIMO will be applied in child cohorts. The outcome of the biomarker measurements will be primarily linked with respiratory health outcomes. The reviewers questioned that "the analysis of associations between environmental exposures and asthma or allergy in the Flemish cohort will yield informative results because of the size of the cohort and the expected low incidence of respiratory disease".
In general, for a clinical study, the number of study participants needed is much smaller that for an ecological or epidemiological study. We performed power calculations to predict the sample size needed, based on the results from the pilot study. To evaluate the **predictivity** of the new biomarkers, we could calculate that 2 groups (asthma / healthy control) of 50 individuals are needed to detect a significant difference in EBC pH. 20 individuals in each group were needed for significant differences in exhaled NO, and 45 individuals for significant differences in EBC LTB4. At this moment, we do not know how many of the 159 children from the Flemish cohort have developed asthma. During the follow-up at age 3 years of these children, the Asthma Predictive Index (mAPI) could be determined for 112 of the children. 41 of those had a positive mAPI, which means they have a chance to develop asthma.
Associations between markers of effect with **exposure markers** in cord blood may also be studied in small study populations. Based on the same Flemish birth cohort with less than 200 children, results were published earlier eg. Verhulst et al (2009), Maervoet et al (2007). They reported effects related to prenatal exposure and biomarkers in cord blood. However the statistical outcome will depend on the magnitude of the effect.

As a consequence of the additional emphasis on the new "omics" biomarkers, some tasks that were originally foreseen will not be carried out in the ANIMO project. Statistical analysis of risk factors in the Flemish cohort may be limited. Since we will collect all the samples and since we will further optimize the biomarker analyses, we expect that the new biomarkers may be measured at the end of the project. All valuable information will be collected. But detailed statistical analysis may be postponed and performed in a later stage.

Workpackage 1 : Development, validation and design of study protocols for new biomarkers

Objectives:

- To further optimize the biomarkers in exhaled breath condensate and gases, to further explore the feasibility of the biomarker measurements in children, and to validate the biomarkers derived by the 'omics' approach

Description of the work:
Further efforts will be undertaken to examine the EBC Protein pattern. The Support Vector Machines (SVM) model allowed to identify 16 discriminatory peptide masses to classify all children correctly for asthma status in the pilot study. The results will be futher validated with extra samples that will be collected from asthma patients and from controls. Protein/cytokine arrays will be further introduced in this study to screen EBC samples. It will

be verified whether these techniques are sensitive enough to detect molecules in EBC. An advantage of this technique is that the identity of the proteins present in the samples and on the arrays is immediately known. If appropriate, these arrays will be implemented in the follow-up of the cohort. If the technique is not sensitive enough, the nanoLC-MS approach introduced in Phase 1 will be further optimized and used. This method allows to detect the whole spectrum of proteins present in the samples. Furthermore, a protocol to measure 8-isoprostane in EBC, a marker of oxidative stress, and an ultra sensitive Bioplex technique (BioPlex® Bio-Rad Laboratories) to measure CC16 and UGRP-1 will be optimized

In the pilot study, exhaled gases were collected in two different ways. Exhaled breath of study subjects was collected in Teflon bags collected and in an electropolished stainless steel canister. The samples of the Teflon bags were analysed using thermal desorption. Using SVM statistics allowed to identify 4 molecules which could classify all children correctly for asthma status. Samples collected in canisters will be analysed in the same way. In addition, exhaled breath samples from 3-year-old children will be reanalysed using a similar method.

Workpackage 2: Application of non-invasive biomarkers in existing child cohort

Follow-up study: recruitment, examination and biomarker analyse

Objectives:

- To set-up a follow-up study of the child cohort from the Flemish Environment and Health study
- To assess the new non-invasive biomarkers in the child cohort
- To correlate the health status of the children with the new non-invasive biomarker results

Description of the work:

The non-invasive biomarkers developed in phase I will be applied in a further follow-up of the existing child cohort of the Flemish Environment and Health study. The set-up of the follow-up study in the existing Flemish cohort was prepared in phase I of the project. Mother-child dyads who participated in the first follow-up study (age of 3 years) on the development of asthma and allergy will be asked to join a further follow-up study (n=159).

Children will be asked to donate the following samples following the protocols and study design developed in Phase I of the project:

- exhaled NO: NIOX MINO or EcoMedics (Based on ATS recommendations: at least two reproducible measurements, which agree within 10% of each other);
- exhaled breath condensate using an uncoated RTube for proteome analysis and a second collection using an RTube coated with 1% bovine serum albumin (BSA) to measure specific molecules in the EBC, such as leukotriene B4;
- exhaled breath gases collected in Tedlar bags for metabonome analyses;
- nasal lavage from the two nostrils separately. The test consists to instillate with a peristaltic pump 0.25 ml of physiologic water per nostril at a constant flow and then to collect the saline with the dissolved protein in it after another 20 seconds. These samples will be used for the assays of albumin, CC16 and urea.
- urine sample to measure CC16 (depending on the outcome of the protocol development by P3);
- Rhinostick to measure sensitivity to aeroallergens.

Children will also undergo

- a lung function test (spirometry)

A blood sample will be drawn to determine serum specific IgE and serum pneumoproteins.

The parents will be asked to complete the questionnaire related to indoor exposure, life style factors and respiratory health of the child.
The data will be entered into a database.

Data analyses
Objectives:
- To organize standardized data collection and entry
- To clean the data, manage the database and develop a final data set containing all the questionnaire data, biomarker analyses and health outcome.
- To assess the relationship between the new biomarkers and the health outcomes in the children

Description of the work:
Data analyses of the Flemish child cohort
- To ensure standardized data collection and coding, P1 will develop data-entry screens to correspond to the questionnaire, the various biomarker analyses and health outcomes. The partners will enter all the data they collected into the standardized data-entry screens and forward the entered data to P1.
- The new data set will be merged with the existing database of the Flemish Environment and Health Study.
- Statistical analysis will be performed according to an analysis plan which will be developed before initiating the biomonitoring study.
- The final data set will be used for analyses which will involve descriptive and epidemiological statistical methods.

Analyses of the combined data of the two cohort studies
- The standardized date entry by the different partners will allow merging of the two databases.
- The final data set will be used for analyses which will involve epidemiological statistical methods to study
    - the association between the non-invasive biomarkers of effect with respiratory outcome. This will allow to evaluate the predictive value of the biomarkers

Communication plan
Objective:
- To develop a policy for communication of the results

Description of the work:
To ensure standardized communication to the parents, a communication plan will be developed which will be communicated to the parents in the recruitment phase. Parents will get feed-back on the personnel results of their child as well as on the outcome of the study.

Workpackage 3: Application of the biomarkers in new child cohort

Objectives:
- To complete biomarker analyses of first follow-up study
- To follow-up the new child cohort a second time
- To apply the new non-invasive biomarkers

- To identify indoor pollutants increasing to the risks of allergic diseases in young children

Description of the work:

Biological samples analysis

The first year will be devoted to the completion of biomarkers analyses on samples of EBC and urine collected in the follow-up study during Phase I:

1. NAL and Rhinosticks.
2. EBC samples. A total 397 EBC samples have been collected and stored at minus 20°C. Because of the relatively small volume available (around 300-400 µl), the team will use ultrasensitive immunoassays (BioPlex® Bio-Rad Laboratories) to analyze at least one inflammation marker (e.g. Il-8, LTB-4) and one lung-specific protein (e.g. Clara cell protein, URGP-1) together with a marker of saliva contamination (alpha-amylase).
3. Urine samples. Most children have provided an untimed urine sample on which the team will analyze CC16 and creatinine. The objective is to assess whether as suggested by a recent study (Anderson et al., 2007) urinary CC16 in young children could not serve as a surrogate marker of the serum levels of the protein and hence its production by the airways.

Data analysis and reporting

1. All the results will be introduced in the database which will be checked for accuracy by a third person
2. Preliminary analyses of database will be performed to determine whether the team will proceed with biomarkers analyses separately for each nostril or on whether the team will calculate the mean value for the two nostrils enough anyone result per child. In this evaluation, the team will also look the influence of adjustments for urea for both CC16 and albumin in order to select the best mode for expressing the results.
3. The team will also conduct the preliminary analyses to assess the validity of the results of the different bio-markers and the predictivity of the different biomarkers.
4. The team will explore the possible associations between the different outcome measures and risk factors of asthma and allergies with the special attention paid to dose linked to the indoor air quality. Example, ETS, air-fresheners, presence of moulds, use of cleaning agents and exposure to chlorine when attending indoor or outdoor swimming pools.

Recruitment of the children for the follow-up

From September 2009, the team will initiate the re-examination of the child-cohort who participated in phase 1. Basically, the team will follow the same procedure as in 2007 for contacting school directors, the parents and for examining children in the different schools.

When re-examining the children, the team will take care to contact schools according to the same chronology as in phase I in order to maintain a similar time interval for all children (approximately two years).

The team will repeat exactly the same biological analyses as in phase I. In order to ensure the comparability of results, the team will incorporate in these analyses a small proportion (for example 5%) of the biological samples analyzed during phase 1. This will be done for urine, NAL and for EBC samples.

The database construction and statistical analysis will be performed as described above (first year) in particular for checking the accuracy and validity of the results. The statistical

analyses will be focused on the changes in biomarkers levels that have occurred during the follow-up and on the identification of risk factors that might be associated with these changes. Reporting and publication will also be conducted in a similar way.

Workpackage 4: Project management

Objectives:

- To bring the project to a successful end by appropriate co-ordination and management

Description of the work:

Following steps will be undertaken

P1 will ensure a smooth flow of communication between participants and will be responsible for all contacts with the Federal Science Policy.

P1 will manage the scientific and technological execution of the project to ensure that the objectives will be delivered

P1 will organize consortium meetings and management team meetings at regular basis.

P1 will deliver the requested interim reports and final reports. All partners will contribute towards delivery of the reports.

At the end of phase II, the partners will organize a workshop on non-invasive biomarkers to assess respiratory diseases where the results of the project will be presented.

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9 ANNEXES

ANNEX 1

1. PILOT STUDY DOCUMENTS

1.1 Information sheet

ONTWIKKELING VAN NIEUWE TECHNIEKEN OM ONTSTEKING IN DE LUCHTWEGEN TE METEN.

Beste ouders,

Astma is een chronische ontsteking van de luchtwegen. Steeds meer kinderen hebben deze ziekte. Waarom weten we niet. Erfelijke factoren spelen een rol maar ze kunnen deze stijging niet verklaren. Andere verklaringen zijn waarschijnlijk meer milieu- en levensstijl gebonden; bijvoorbeeld luchtvervuiling, ozon, blootstelling aan gechloreerde gassen, aan sigarettenrook, aan allergenen zoals huisstofmijt en pollen, infecties op jonge leeftijd en voeding.
Een goede behandeling van astma voorkomt ontsteking. Er bestaan nu nieuwe methoden om ontsteking in de luchtwegen te meten op een niet pijnlijke en niet ingrijpende manier. Om deze technieken nog verder te verfijnen zijn wij op zoek naar 30 gezonde kinderen tussen de 6 en de 12 jaar die willen deelnemen aan ons onderzoek. Door deel te nemen helpt u het onderzoek naar een betere behandeling van de ziekte.

Wie en wat onderzoeken we?

WIE? 30 kinderen tussen de 6 en de 12 jaar die geen last hebben van astma.
WAT? In de uitgeademde lucht wordt stikstofmonoxide en eiwitten gemeten.

Wie wordt geselecteerd en waarom?

Alle kinderen tussen 6 en 12 jaar van deze school krijgen deze brief. Uit de toestemmingen die we krijgen kiezen we willekeurig 30 kinderen. Het kan dus zijn dat u wel toestemming geeft maar dat uw kind niet geselecteerd wordt.

Hoe zal het onderzoek praktisch verlopen?

Voor het meten van ontstekingsparameters in uitgeademde lucht is geen ingrijpend onderzoek nodig. Wat verwachten we van uw kind?

wegen en meten
enkele malen uitblazen in een toestel
gedurende 15 min in een toestel ademen.
een zak opblazen zodat wij de uitgeademde lucht kunnen opvangen.
de neus spoelen met een kleine hoeveelheid zoutoplossing, die we na 20 sec terug opvangen.

Deze testen zullen gebeuren op twee verschillende dagen, tijdens de lesuren. Het kind moet dus niet langer op school blijven. Dag 1 zullen de testen ongeveer 45 min in beslag nemen en dag 2 zullen de testen ongeveer 20 min in beslag nemen.

Wat gebeurt er met de resultaten van het onderzoek?

U ontvangt de resultaten van uw kind en de groepsresultaten thuis.

Bent u bereid om mee te werken aan dit onderzoek?
Vul dan het vragenlijstje in en onderteken het toestemmingsformulier.
U mag alles terugbezorgen aan de school.

Alvast bedankt voor uw medewerking!

1.2 Informed consent

TOESTEMMINGSFORMULIER TOT DEELNAME AAN DE STUDIE

GEBRUIK VAN NIEUWE TECHNIEKEN OM ONTSTEKING IN DE LUCHTWEGEN TE METEN.

Onderzoeksstudie van het UZ Antwerpen en het VITO (Vlaams Instituut voor Technologisch onderzoek). Het veldwerk wordt uitgevoerd door de dienst Gezondheid van het Provinciaal Instituut voor Hygiëne te Antwerpen.

Geachte ouders,
Ontsteking in de luchtwegen speelt een belangrijke rol in astma. Een vroege behandeling zorgt ervoor dat astma beter onder controle kan gehouden worden. Methoden om ontsteking te meten zijn meestal ingrijpend. Er bestaan een aantal nieuwe testen die nuttige informatie kunnen geven over de ziekte-evolutie, wat kan bijdragen tot betere opvolging en behandeling.
Voor het meten van ontstekingsparameters in uitgeademde lucht is geen ingrijpend onderzoek nodig. De patiënt moet enkel rustig in en uitademen, daarom zijn deze testen erg geschikt voor kinderen en voor ernstig zieke mensen.
Dit project wil het nut van deze testen in de praktijk nagaan. Daarom onderzoeken we een 40-tal astma-patiënten. Ter controle willen we ook 30 gezonde kinderen testen. Dit wil zeggen kinderen die geen astma of allergie hebben en die niet verkouden zijn op de dag van het onderzoek. Hierbij vragen we of uw kind wil deelnemen aan dit onderzoek.

Verloop van de studie:
De hierboven beschreven testen worden afgenomen in de school tijdens de lesuren. Het onderzoek gebeurt in twee delen. Dag 1 ongeveer 45 min en dag 2 ongeveer 20 min.

Voordelen van de studie:
Deze onderzoeken zijn gemakkelijk uit te voeren en zijn weinig ingrijpend.
U helpt het onderzoek naar betere behandeling van deze ziekte.

Kosten:
Geen enkele test zal worden aangerekend. Alle diensten zijn gratis.

Mogelijke risico's:
Er is geen enkel risico of ongemak verbonden aan het verzamelen van ademlucht. De studie is goedgekeurd door de onafhankelijke ethische commissie van het Universitair Ziekenhuis Antwerpen. De haalbaarheid, het belang,

de veiligheid en de overeenstemming met de internationale aanbevelingen worden door deze commissie geëvalueerd.

Vertrouwelijkheid:
Alle informatie wordt vertrouwelijk behandeld conform de wet op de privacy. De deelnemers worden aan de hand van een code geïdentificeerd. Indien de resultaten van deze studie gepubliceerd worden in een rapport of wetenschappelijk tijdschrift wordt er geen enkele naam vermeld.

Vrijwillige deelname / Intrekking van deelname uit de studie:
U neemt vrijwillig deel aan de studie.
U kan ook beslissen om niet aan het onderzoek deel te nemen en u kan op ieder moment uit het onderzoek stappen, zelfs al heeft u dit formulier getekend. Alles wat u moet doen is de onderzoekers vertellen dat u niet langer wilt deelnemen. Ook kan u vragen dat alle stalen die uw kind gegeven heeft, vernietigd worden en niet langer deel uitmaken van deze studie.

Recht op informatie: U heeft het recht om informatie te vragen over de procedures en het beschreven onderzoeksproject. Alle redelijke vragen voor informatie zullen beantwoord worden door de hoofdonderzoeker naar best vermogen. Als er belangrijke veranderingen zijn in de procedures, de risico's of de voordelen van deze studie dan wordt u op de hoogte gebracht.

Naam en voornaam van de deelnemer:
…….Klas :…….
geboortedatum: …../…../…..

Ik heb de informatieformulier en het toestemmingsformulier gelezen en begrijp de mogelijke risico's en voordelen van deze studie. Ik neem vrijwillig deel aan deze studie. Ik wil *één/geen* (omcirkel één van beiden) kopie van dit formulier.

_____ …./…../…..
Handtekening van één van de ouders
Datum

Verklaring van de hoofdonderzoeker: De hoofdonderzoeker is verantwoordelijk om dit onderzoeksprogramma uit te voeren volgens de voorwaarden die beschreven zijn in dit document.

Hoofdonderzoeker: Prof dr Kristine Desager

1.3 Questionnaire

Als u kind mag meewerken aan het onderzoek gelieve dan onderstaande vragenlijst in te vullen

Wat is de geboortedatum van uw kind: __ /__/ ____

Heeft uw kind ooit last van piepen en fluiten in de borstkas gehad? Ja O Nee O

Gebruikt uw kind puffers op regelmatige basis? Ja O Nee O

Heeft uw kind ooit astma gehad? Ja O Nee O

Zo ja, Werd dit door een arts bevestigd? Ja O Nee O

Heeft uw kind astma, eczeem, hooikoorts of neusallergie? Ja O Nee O

Heeft u, uw partner of een ander kind in het gezin last van eczeem of allergie? Ja O Nee O

datum van vandaag? __/ __ /20__

Naam kind ……Klas: ……

Adres:……
……...

2. NEW COHORT DOCUMENTS

2.1 Information sheet

Woluwe, 10 mars 2008

A l'attention de chaque parent,

Madame, monsieur,

Nous nous permettons de solliciter votre collaboration à notre projet de recherche. Notre équipe de recherche participe à un projet fédéral en collaboration avec le VITO et l'université d'Anvers qui va permettre d'étudier les risques d'affections respiratoires et d'allergies chez l'enfant liés à la qualité de l'air intérieur. Vous trouverez ci-dessous les détails et informations relatives à ce projet mais nous restons bien entendu disponibles pour tout renseignement complémentaire.

ANIMO

Risques d'affections respiratoires chez l'enfant liés à la qualité de l'air intérieur : développement et application de biomarqueurs non-invasifs

1. QUEL EST LE BUT DE CETTE ETUDE ?

Notre environnement est par définition notre lieu de vie, qu'il s'agisse de l'atmosphère qui nous entoure, de l'air de nos espaces intérieurs, l'eau que nous buvons ou encore notre alimentation. Il a un impact incontestable sur nous et sa détérioration peut potentiellement altérer notre santé. L'étude de ses impacts sur notre santé est ainsi devenue une des préoccupations primordiales de notre société. Actuellement, des outils de plus en plus performants sont développés pour mieux comprendre ces relations entre santé et environnement.

L'objectif de l'étude que nous menons actuellement s'inscrit dans ce cadre pour une meilleure compréhension de ces relations chez les enfants et elle a pour but d'identifier les sources de pollution (polluants de l'air, contaminants alimentaires, …) pouvant être impliquées dans l'augmentation moderne d'affections allergiques telles que l'asthme, l'eczéma, le rhume des foins,…. Pour la mener à bien, nous sollicitons votre participation ainsi que celle de votre enfant et nous vous en remercions d'avance.

Cette étude a reçu un avis favorable de la commission d'éthique biomédicale de l'Université catholique de Louvain et est soutenue par le service public fédéral de programmation politique scientifique de Belgique.

2. EN QUOI CONSISTE LA PARTICIPATION DE VOTRE ENFANT?

Si vous êtes d'accord que votre enfant participe à cette étude, on vous demandera de compléter un questionnaire sur son état de santé, ses antécédents familiaux, votre habitation, votre environnement et ses activités sportives.

Votre enfant sera également invité à effectuer quelques tests pendant une partie de matinée scolaire au cours de laquelle :

- Il sera pesé(e) et mesuré(e)
- On lui demandera d'effectuer des tests respiratoires ; c'est-à-dire de souffler dans des appareils. (Spirométrie de base, Niox, EBC)
- Un tampon sera aussi appliqué pour collecter du liquide nasal afin de dépister les allergies
- On recueillera également un échantillon d'urine

Ces tests seront réalisés au sein de l'école de votre enfant au cours d'une matinée scolaire et en présence d'un médecin.
Nous insistons vraiment sur le fait que tous ces tests sont absolument non invasifs et tout à fait indolores.

Même si vous ne désirez pas que votre enfant participe, pourriez-vous nous rendre le questionnaire complété afin que nous puissions nous assurer qu'il n'y aura aucun biais de sélection entre les participants et les non participants (même sans nom ni prénom si vous le préférez)?

3. QUELS SONT LES RISQUES ET INCONVENIENTS EVENTUELS ASSOCIES A L'ETUDE ?

Votre enfant ne prend aucun risque à participer à cette étude. Les tests respiratoires sont des examens indolores qui n'entraînent aucun inconvénient particulier. Seule une sensation de gène pourrait être éventuellement ressentie lors des examens.

4. QUELS SONT LES BENEFICES ASSOCIES A L'ETUDE ?

Grâce à cette étude scientifique, votre enfant bénéficiera gratuitement d'une évaluation de la fonction respiratoire et d'un dépistage des allergies les plus communes (acariens, chat et pollens) à partir du liquide récolté au niveau nasal. Les résultats des mesures seront envoyés à votre domicile.

Comme nous l'avons déjà signalé plus haut, le but de l'étude est d'identifier des facteurs de risque de maladies respiratoires liés aux polluants présents dans l'environnement intérieur et extérieur. Ces observations contribueront à établir un ensemble de recommandations (normes et dispositifs pour des groupes à risques, …) et à accroître l'efficacité d'une politique de prévention contre ces maladies.

5. QU'EN EST-IL DU RESPECT DE LA CONFIDENTIALITE ?

Le secret médical et les exigences légales en matière de vie privée seront respectés (en conformité avec la loi belge du 8 décembre 1992, la loi du 22 août 2002 relative aux droits du patient et la loi du 7 mai 2004 relative aux expérimentations sur la personne humaine).

L'identité de votre enfant ainsi que les données le concernant seront traitées de façon confidentielle. L'ensemble des informations figurant dans le questionnaire, le prélèvement d'urine et les mesures respiratoires seront identifiés par un numéro anonyme. Les informations ainsi codifiées seront traitées par les chercheurs qui analyseront l'ensemble des réponses au questionnaire et les résultats des mesures effectuées. Si vous le désirez, vous pourrez, à tout moment de l'étude, avoir accès ou modifier les données concernant votre enfant.

Nous vous rappelons que vous recevrez de manière confidentielle et personnelle par courrier à votre domicile l'ensemble des résultats des tests réalisés avec votre enfant.

6. COUTS ?

La participation à cette étude ne vous coûtera rien financièrement. Votre enfant sera remercié(e) de sa participation par un petit cadeau.

7. LA PARTICIPATION EST VOLONTAIRE

Votre enfant et vous avez le droit de refuser de participer à cette étude et il pourra la quitter à tout moment.

8. QUELLE UTILISATION SERA FAITE DES RESULTATS DE CETTE ETUDE ?

Dans un but d'information, le rapport final de l'étude sera publié dans une revue scientifique internationale. L'article ne comportera aucune donnée personnelle concernant votre enfant.

9. CONTACTS

N'hésitez pas à nous contacter si vous aviez la moindre question ou inquiétude, nous nous ferons un plaisir d'en discuter avec vous.

Melle Catherine Voisin
(Catherine.Voisin@uclouvain.be) (02/7645343)
Professeur Alfred Bernard
(bernard@uclouvain.be) (02/7645334)

Laboratoire de Toxicologie de l'Université Catholique de Louvain
Avenue Emmanuel Mounier 5302
B-1200 Bruxelles
Fax : 02 764 53 38

2.2 Informed consent

ANIMO

Risques d'affections respiratoires chez l'enfant liés à la qualité de l'air intérieur : développement et application de biomarqueurs non-invasifs

FORMULAIRE DE CONSENTEMENT (A RENDRE COMPLETE ET SIGNE)

Nous, soussignés, ... (nom et prénoms du père et de la mère en majuscules ou des personnes responsables de l'enfant), parents ou responsable(s) de ..... (nom et prénom de l'enfant en majuscules), confirmons que l'équipe de recherche de toxicologie de l'Université Catholique de Louvain a demandé que notre enfant participe à l'étude intitulée «Risques d'affections respiratoires chez l'enfant liés à la qualité de l'air intérieur : développement et application de biomarqueurs non-invasifs ».

Cette étude est soutenue par le service public fédéral de programmation politique scientifique de Belgique. La Commission d'Ethique de l'Université catholique de Louvain a donné un avis favorable à la réalisation de cette étude.

Nous comprenons le but de l'étude à laquelle il est demandé que notre enfant participe. Nous comprenons que si notre enfant participe à cette étude, nous acceptons de remplir le questionnaire. Notre enfant accepte d'avoir des tests respiratoires et de donner un peu d'urine et nous n'y sommes pas opposés. Nous avons été informés que la participation à cette étude ne nous occasionnera aucun frais mais ne nous donne droit à aucune indemnité.

Notre consentement ne dégage pas les chercheurs de leurs responsabilités. Notre enfant garde tous les droits qui lui sont garantis par la loi.

A tout moment, la participation de notre enfant peut être arrêtée, selon son désir ou le nôtre. Nous en informerons le Professeur Bernard. Les données qui concernent notre enfant resteront confidentielles.

Nous pourrons à tout moment demander toute information complémentaire au Professeur Bernard.

◊ **Nous sommes d'accord que notre enfant participe à cette étude dans les conditions précisées ci-dessus : OUI / NON (entourer la réponse qui convient).**

◊ **Signature des parents ou responsable(s) de l'enfant**

Fait à ....., le .....

2.3 *Questionnaire school*

UCL Université catholique de Louvain

Faculté de médecine
Unité de toxicologie industrielle et médecine du travail
Professeur A Bernard

ANIMO

Risques d'affections respiratoires chez l'enfant liés à la qualité de l'air intérieur : développement et application de biomarqueurs non-invasifs

Madame, Monsieur,

Nous vous remercions de votre accueil et de votre collaboration à notre projet. Pourriez-vous compléter ce questionnaire dans son entièreté et nous le faire parvenir soit par fax, soit par mail ?

Nom de votre école :
Nom du directeur :
Adresse :
Tél/fax :
Mail :

Au sujet de l'établissement

1. Quelle est l'année de construction du bâtiment ?
2. Quel est le volume des classes de maternelles ?
3. Combien y a-t-il d'enfants en 3ème maternelle ?
4. Combien d'enfants y a-t-il par classe en maternelles ?
5. Les classes sont-elles ventilées et si oui, à quelle fréquence et de quelle façon?
6. Quels sont la fréquence et le mode de nettoyage des classes ?
7. Utilise-t-on des produits chlorés ? Pour quel usage ? Et à quelle fréquence ?
8. Quels autres produits d'entretien sont-ils utilisés ?
9. Quel est le type des conduites d'eau ?

10. Votre établissement bénéficie-t-il d'un jardin ou d'un parc ?
11. Si oui, quels types d'arbres et de végétation en général y a-t-il ?
12. Y a-t-il des animaux dans les classes ou dans les pièces communes ?
13. Si oui, de quel type d'animaux s'agit-il ?

Au sujet des activités sportives proposées

1. Quelles sont les activités sportives auxquelles les enfants participent ?
2. A partir de quelle année scolaire?
3. A quelle fréquence ?
4. les enfants de maternelle vont-ils à la piscine ?
5. Si oui, à partir de quand ?
6. Dans quelle piscine ?
7. Combien de temps restent-ils dans l'eau ?

Au niveau du mode de vie

1. Les enfants consomment-ils l'eau du robinet ?
2. Les repas sont-ils cuisinés au sein de l'école ou par une société extérieure ?

Contacts

N'hésitez pas à nous contacter si vous aviez la moindre question, nous nous ferons un plaisir d'en discuter avec vous.

Melle Catherine Voisin
(Catherine.Voisin@uclouvain.be) (02/7645343)
Professeur Alfred Bernard
(bernard@uclouvain.be) (02/7645334)

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2.4 Questionnaire child

ANIMO : Environnement et santé des enfants : étude épidémiologique des facteurs de risque

Questionnaire sur la santé de votre enfant et son environnement

Pour chaque question, entourez la mention adéquate ou noircissez le carré.

Son identité

Nom : …… Prénom : ……
Taille : ……cm Poids : ……kg
1. Date de naissance : ……
2. Adresse : rue : …… N° : ……
Commune : …… Code Postal : ……
Téléphone : …… @-mail des parents : ……
3. Sexe : □ Masculin □ Féminin
4. Nationalité : □ belge
□ non belge
Si non belge, précisez :4.1 Pays d'origine : ……
4.2 Date d'arrivée en Belgique : ……

Sa Santé

5. Au cours des 12 derniers mois,
5.1 A-t-il eu une respiration sifflante ? Oui □ Non □
5.2 A-t-il été réveillé(e) par une sensation de poids sur la poitrine ? Oui □ Non □
5.3 A-t-il été réveillé(e) par une sensation de manque d'air ? Oui □ Non □
5.4 A-t-il été réveillé(e) par une crise de toux ? Oui □ Non □
5.5 A-t-il été réveillé(e) par une crise d'asthme ? Oui □ Non □
5.6 A-t-il eu des éternuements, le nez qui coule ou le nez bouché (sans avoir de rhume, de rhino-pharyngite ou grippe) Oui □ Non □
5.7 Quelle(s) maladie(s) a-t-il eue(s) :……
5.8 A-t-il eu des diarrhées ? : Oui □ Non □
5.9 A-t-il eu de la température ? (supérieure à 38.5°) : Oui □ Non □

6. Depuis sa naissance, a-t-il eu les maladies suivantes (diagnostic fait ou confirmé par un médecin) ?
6.1 Asthme : Oui □ Non □
6.2 Bronchite : Oui □ Non □
6.3 Bronchiolite : Oui □ Non □
6.4 Pneumonie ou broncho-pneumonie : Oui □ Non □
6.5 Eczéma : Oui □ Non □
6.6 Verrue : Oui □ Non □

6.7 Mycose (ex : champignon aux pieds) : Oui □ Non □
6.8 Conjonctivite : Oui □ Non □
6.9 Rhume des foins : Oui □ Non □
6.10 Rhinite allergique : Oui □ Non □
6.11 Rhume (plus de 4 fois/an) : Oui □ Non □
6.12 Sinusite (au moins 1fois/an) : Oui □ Non □
6.13 Otite (au moins 1fois/an) : Oui □ Non □
6.14 Infection urinaire : Oui □ Non □
6.15 Diabète : Oui □ Non □
6.17 Hépatite : Oui □ Non □
6.18 Méningite : Oui □ Non □
6.19 Démangeaisons : Oui □ Non □
6.19 Autres affections : ……

7. Si une des maladies suivantes a été diagnostiquée, pouvez-vous préciser à quel âge ?
   7.1 Asthme : ……
   7.2 Eczéma : ……
   7.3 Rhinite allergique : ……
   7.4 Diabète : ……
   7.5 Méningite : ……
   7.6 Hépatite : ……

8. Quels sont les différents vaccins que votre enfant a eus et quand ?
   8.1 ……en ……
   8.2 …… en ……
   8.3 …… en ……
   8.4 …… en ……
   8.5 …… en ……
   8.6 …… en ……

9. A-t-il ou a-t-il eu des caries ? Oui □ Non □
   Si oui, combien d'entre elles ont été soignées (plombages) ? ……

10. Depuis sa naissance, a-t-il eu des allergies ?
    10.1 Alimentaires ? Oui □ Non □
    10.2 Aux œufs ? Oui □ Non □
    10.3 Aux acariens ? Oui □ Non □
    10.4 Aux poils d'animaux ? Oui □ Non □
    10.5 Aux pollens ? Oui □ Non □

11. A-t-il pris ou prend-il des médicaments ?
    11.1 Pour les allergies ? Oui □ Non □
    11.2 Pour l'asthme ? Oui □ Non □
    11.3 Si oui, lesquels ? ……
    11.4 Des antibiotiques ? : Oui □ Non □
    11.5 si oui, quand et lesquels ?: ……

12. Depuis sa naissance, a-t-il été hospitalisé ?
    12.1 Pour infection respiratoire ? Oui □ Non □
    12.2 Pour asthme ? Oui □ Non □

13. Pendant la nuit, combien de fois votre enfant se réveille-t-il ? : ……

14. Sa maman a-t-elle fumé pendant sa grossesse? Oui □ Non □

15. Quel était son poids à la naissance ? ……grammes

16. Est-il né(e) à terme (entre 39 et 41 semaines de grossesse) ? Oui □ Non □
Si non, à combien de semaines gestationnelles est-il né(e) ? ……

17. A-t-il été allaité(e) ? Oui □ Non □
Si oui, pendant combien de mois ? ……

18. Ses biberons étaient-ils préparés avec :
□ De l'eau du robinet ? □ De l'eau du robinet filtrée (Brita) ? □ De l'eau en bouteille ?

19. Pendant sa petite enfance :
19.1 A-t-il mangé des petits pots ? Jamais □ 1 fois/sem □ 2 fois et plus/sem □
19.2 A-t-il eu une tétine ? Oui □ Non □

20. Entre 0 et 2 ans, allait-il à la crèche ? Oui □ Non □
20.1 Si oui, de quel âge à quel âge ? ……
20.2 A quelle fréquence ?
□ 1 jour/sem □ 2 jours/sem □ 3 jours/sem □ 4 jours/sem □ 5 jours/sem

21. Entre 0 et 2 ans, vivait-il ? : □ En ville □ A la campagne

22. Depuis son enfance, boit-il l'eau du robinet ?
□ Oui □ Rarement □ Jamais

23. Votre enfant consomme-t-il les aliments suivants ?
23.1 Pain : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.2 Probiotiques (Actimel, Yakult, …) : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.3 Produits laitiers enrichis au bifidus : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.4 Yaourt, fromage blanc : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.5 Lait frais non pasteurisé : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.6 Produits à base de soja : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.7 Légumes : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.8 Pâté : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.9 Viande : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.10 Fruits frais : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.11 Poisson : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.12 Œufs : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.13 Lait : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.14 Chips : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.15 Gâteaux, biscuits ? : Oui □ Non □

Si oui, à quelle fréquence ? :……
23.16 Produits Beneo ? : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.17 Noix ? : Oui □ Non □
Si oui, à quelle fréquence ? :……
23.23 Cacahuètes ? : Oui □ Non □
Si oui, à quelle fréquence ? :……

Environnement et habitation

24. A proximité de chez vous, y a-t-il ?
    24.1 Une installation industrielle (usine, ….) : Oui □ Non □
    Si oui, laquelle ? ……., à quelle distance ? …… km
    24.2 Un incinérateur, décharge, déchetterie, traitement des déchets : Oui □ Non □
    Si oui, quel est son nom ? ……., à quelle distance ? …… km
    24.3 Un aéroport à moins de 5 km : Oui □ Non □ Si oui, lequel ? …….

25. Votre habitation se trouve-t-elle près d'une route avec un trafic important (ex : chaussée, boulevard, nationale, autoroute) ? Oui □ Non □
    Si oui, à quelle distance ? : □ moins de 10 mètres
    □ moins de 100 mètres
    □ plus de 100 mètres

26. Y a-t-il ou y avait-il un ou plusieurs animaux à la maison ?
    □ Aucun animal
    □ Un ou des animal(aux) à poils : □ chat □ chien
    □ Un ou des animal(aux) à plumes
    □ Autre à préciser : …….
    26.1 Depuis quel âge, vit-il avec lui (eux) ? …….
    26.2 Laissez-vous entrer l'(es) animal(aux) dans sa chambre ? Oui □ Non □

27. De combien de chambres votre habitation est-elle constituée ?
    □ 1 □ 2 □ 3 □ 4 □ 5 □ 6 □ 7 □ 8 ou plus

28. Les chambres sont-elles ventilées ou aérées ?
    1 fois/jours □ 1 fois/2 jours □ 1fois/sem □ 1 fois/mois □ jamais □

29. Utilisez-vous des aérosols ou des parfums d'ambiance ?
    29.1 Si oui, à quelle fréquence ? :
    □ 4fois/mois □ 3 fois /mois □ 2 fois /mois □ 1fois/mois □ jamais

30. Pour le nettoyage du sol sans tapis, utilise-t-on des produits à base d'eau de javel ?
    Oui □ Non □
    30.1 Si oui, à quelle fréquence ? :
    □ 4fois/mois □ 3 fois /mois □ 2 fois /mois □ 1fois/mois □ jamais
    30.2 Si non, quel produit utilise-t-on ? ……

31. Y a-t-il une piscine chez vous ? Oui □ Non □
    31.1 Si oui, est-elle ? □ A l'intérieur □ A l'extérieur
    31.2 Est-elle ? □ gonflable □ construite dans le sol
    31.3 Est-elle désinfectée au chlore ? Oui □ Non □
    31.4 Quand y va-t-il ? □ en été □ toute l'année
    31.5 Depuis quel âge va-t-il dans cette piscine ? ……
    31.6 Combien de mois par an ? ……
    31.7 Combien d'heures par semaine pendant la période où il y va ? …….

Ses activités sportives

32. Quel(s) type(s) d'activité extrascolaire fait-il régulièrement (au moins une heure par semaine) ?
    a) …… b) …… c) ……

33. Fait-il de l'équitation ? Oui □ Non □
    Si oui, depuis quel âge ? : ……

34. Allait-il à la piscine avant l'âge de 2 ans ? : Oui □ Non □
    Si oui, 34.1 Etait-ce des cours de « bébé nageur » ? Oui □ Non □
    De quel âge (mois) à quel âge ? ……
    A quelle fréquence ? □ 1 fois/sem □ 1 fois /15 jours □ 1 fois/mois
    Combien de temps par séance ? □ 10 min □ 20 min □ 30 min □ 1h
    Quel était le nom de la piscine ? ……
    34.2 Etait-ce comme loisir avec ses parents ? Oui □ Non □
    De quel âge à quel âge ? ……
    A quelle fréquence ? □ 1 fois/sem □ 1 fois /15 jours
    □ 1 fois/mois □ 1 fois/3 mois
    Combien de temps par séance ? □ 10 min □ 20 min □ 30 min □ 1h
    Quel était le nom de la piscine ? ……

35. Depuis l'âge de deux ans, va-t-il ou est-il allé à la piscine dans un club, avec ses parents ou avec des amis ? Oui □ Non □
    Si oui, 35.1 De quel âge à quel âge ? ……
    35.2 A quelle fréquence ? □ 1 fois/sem □ 1 fois/15 jours □ 1 fois/mois
    □ 1 fois/3mois □ 1 fois/ans □ jamais
    35.3 Combien de temps par séance ? □ 30 min □ 1 h □ 1 h 30 □ 2 h
    35.4 Quel est le nom de la piscine ? ……

36. Est-il déjà allé dans des lieux de villégiature avec piscine ?
    36.1 Si oui, la piscine était-elle ? □ A l'intérieur □ A l'extérieur
    36.2 Est-elle désinfectée au chlore ? Oui □ Non □
    36.3 Combien de fois a-t-il bénéficié de ce type de vacances ? ……
    36.4 Depuis quel âge ? ……
    36.5 Combien de semaines par an ? ……
    36.6 Combien d'heures par semaine pendant la période où il y va ? ……

Sa famille

37. De combien de personnes son foyer est-il constitué (en le comptant) ? :
    □ 2 □ 3 □ 4 □ 5 □ 6 □ 7 □ 8 ou plus

38. A-t-il des frères et/ou des sœurs ? □ Oui □ Non
    Si oui, 38.1 Combien ?……
    38.2 Combien de sœurs et de frères sont-ils plus âgés que lui ?
    □ Aucun □ 1 □ 2 □ 3 □ 4 ou plus

39. Parmi ses frères et/ou sœurs, certains ont-ils ou ont-ils eu ? :
    39.1 De l'asthme : Oui □ Non □
    39.2 Des allergies : Oui □ Non □

40. A la maison,
    40.1 Sa maman fume-t-elle ? Oui □ Non □

40.2 Son papa fume-t-il ? Oui □ Non □

41. D'autres personnes à la maison fument-elles ? : Oui □ Non □
Si oui, combien ? □ 1 □ 2 □ 3

42. Sa maman souffre-t-elle de ?
42.1 Eczéma : Oui □ Non □
42.2 Asthme : Oui □ Non □
42.3 Rhume des foins : Oui □ Non □
42.4 Allergies : □ Acariens □ Poils d'animaux □ Pollen □ Alimentaires

43. Son papa souffre-t-il de ?
43.1 Eczéma : Oui □ Non □
43.2 Asthme : Oui □ Non □
43.3 Rhume des foins : Oui □ Non □
43.4 Allergies : □ Acariens □ Poils d'animaux □ Pollen □ Alimentaires

44. Concernant les études et les activités de ses parents ?
44.1 Maman : quelle est son activité professionnelle ? ……
quelles études a-t-elle terminées ?
□ L'école primaire □ L'enseignement secondaire
□ Etudes supérieures non universitaires □ Etudes universitaires
44.2 Papa : quelle est son activité professionnelle ? ……..
quelles études a-t-il terminées ?
□ L'école primaire □ L'enseignement secondaire
□ Etudes supérieures non universitaires □ Etudes universitaires

Date et Signature ……

Glissez le questionnaire dans l'enveloppe que vous fermerez et sur laquelle vous écrirez son nom et son prénom ainsi que son école. Un grand merci pour votre collaboration !!!!

ANNEX 2

COMITE DE SUIVI – SSD
Volet "Santé & environnement"

ANIMO		
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