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

"ANIMO"

G. Schoeters, R. Van Den Heuvel, K. Bloemen G. Koppen,
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FINAL REPORT PHASE 1
SUMMARY

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

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Promotors

Greet Schoeters
Vlaamse Instelling voor Technologisch Onderzoek (VITO)
Environmental Risk & Health
Mol

Alfred Bernard
Université Catholique de Louvain (UCL)
Unité de toxicologie industrielle et de médecine du travail
Bruxelles

Kristine Desager
Universiteit Antwerpen (UA)
Pediatrics and Respiratory Medicine
Antwerp

Authors
G. Schoeters, R. Van Den Heuvel, K. Bloemen
G. Koppen, E. Goelen, E. Govarts - VITO

A. Bernard, C. Voisin - UCL

K. Desager - UA

V. Nelen - Provincial Institute of Hygiene – Antwerp

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BELGIAN SCIENCE POLICY

Rue de la Science 8
Wetenschapsstraat 8
B-1000 Brussels
Belgium
Tel: +32 (0)2 238 34 11 – Fax: +32 (0)2 230 59 12
http://www.belspo.be

Contact person: Emmanuèle Bourgeois
+32 (0)2 238 34 94

G. Schoeters, R. Van Den Heuvel, K. Bloemen G. Koppen, E. Goelen, E. Govarts, A. Bernard, C. Voisin, K. Desager, V. Nelen ***"Indoor risk factors for childhood respiratory diseases: development and application of non-invasive biomarkers "ANIMO"*** Final Report Phase 1 Summary. Brussels : Belgian Science Policy 2009 – 6 p. (Research Programme Science for a Sustainable Development)

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.