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Application of non-invasive biomarkers in a birth cohort follow-up in relation to respiratory health outcome

Karolien Bloemen[1], Gudrun Koppen[1], Eva Govarts[1], Ann Colles[1], Rosette Van Den Heuvel[1], Vera Nelen[2], Erwin Witters[3,4], Kristine Desager[5], and Greet Schoeters[1,6]

[1]Flemish Institute for Technological Research (VITO NV), Environmental Risk and Health, Toxicology, Boeretang, Mol, Belgium, [2]Provincial Institute of Hygiene (PIH), Antwerp, Belgium, [3]Flemish Institute for Technological Research (VITO), Environmental Analysis and Technology, Boeretang, Mol, Belgium, [4]University of Antwerp (UA), Department of Biology, Center for Proteome Analysis and Mass Spectrometry (CEPROMA), and Department of Ecophysiology, Biochemistry and Toxicology (EBT), Antwerp, Belgium, [5]Antwerp University Hospital, Department of Pediatrics, Edegem, Belgium, and [6]University of Antwerp (UA), Department of Biomedical Sciences, Antwerp, Belgium

Abstract

Asthma-related symptoms can manifest in children during the early years, but only some of the children will develop the disease. This feasibility study showed that it is possible to apply non-invasive markers (in urine, exhaled nitric oxide (FENO) and exhaled breath condensate (EBC)) in 3-year-old children, and evaluated the biomarkers in relation to health outcomes and potential modifiers. FENO was correlated with respiratory allergy, and was borderline significantly correlated with wheezing, but not with the asthma predictive index (mAPI). EBC pH and urinary 8-oxo-deoxyguanosine were not significantly correlated with these clinical outcomes. An EBC proteolytic peptide pattern was developed, which could distinguish between mAPI-positive and -negative children. Non-invasive biomarkers may become a promising tool for investigating respiratory health in children but further research is needed.

Keywords: *Exhaled breath condensate; EBC; asthma; asthma predictive index*

Introduction

As the incidence of asthma in children has increased during the past decades (Ronchetti et al. 2001), early diagnosis of this disease and recognition of risk factors are major challenges that will help to develop prevention strategies (Rhodes et al. 2001, Martinez 1999, Castro-Rodriguez et al. 2000). At present, the identification of a child at high risk is not possible with absolute certainty. Current research points to some risk factors including family history of asthma and allergy (especially maternal history), early and severe sensitization to some food antigens (especially hens' eggs) and to aeroallergens, and early viral infection associated with wheeze and adverse environmental exposures (Sly et al. 2008).

The asthma predictive index (API) has been developed to predict which children at preschool age will have the highest risk of persistent symptoms at school age (age 6 years) (Castro-Rodriguez et al. 2000). Recently, this index was adapted into the 'modified Asthma Predictive Index' (mAPI) (Guilbert et al. 2004). This index is based on different criteria such as wheezing, atopic dermatitis and allergic sensitization to aeroallergens in the first 3 years of life, and a positive family history of asthma. However, wheezy children are a heterogeneous group and many patterns of wheezing disorders exist. Although up to 60% of all children under 3 years of age experience recurrent wheeze, the majority have a good prognosis: almost 60% of these children have stopped wheezing by the age of 6 years (Martinez et al. 1995). Most wheezing infants have transient conditions associated with diminished airway calibre and have no increased risk of asthma later in life. In a minority of infants, early wheezing episodes are related to a predisposition to asthma (Sly et al. 2008, Martinez et al. 1995).

Address for Correspondence: Karolien Bloemen, Flemish Institute for Technological Research (VITO NV), Environmental Risk and Health, Toxicology, Boeretang 200, 2400 Mol, Belgium. Tel: +32 14 33 51 07. Fax: +32 14 58 26 57. E-mail: karolien.bloemen@vito.be

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The API index gives a prediction with reasonable accuracy using simple, clinically based parameters. However, there is evidence that the immunopathology of asthma in the airways of young children is present even before symptoms occur (Warner et al. 1998). Measurement of objective parameters is not currently available and is difficult to obtain in very young children. This emphasizes the need for new early biomarkers which are applicable in very young children. Exhaled nitric oxide (FENO) has been shown to be a non-invasive marker of airway inflammation (ATS/ERS 2005). Recently, exhaled breath condensate (EBC), a fluid formed by cooling exhaled air, has been proposed as a simple, safe and non-invasive technique for obtaining samples from the lower respiratory tract to assess airway inflammation, and is of particular interest in paediatrics (Rosias et al. 2004, Romieu et al. 2008). As only tidal breathing is needed, EBC sample collection is already possible in very young children.

This study is a first step in the development and application of non-invasive biomarkers for early diagnosis of asthma in a cohort of 3-year-old children living in Flanders, Belgium. We examined FENO, EBC pH, proteins in EBC and urinary 8-oxo-deoxyguanosine, and studied these in relation to the health outcomes mAPI, wheezing symptoms and results from skin prick tests. This is primarily a feasibility study, to evaluate the applicability of (new) non-invasive markers in 3-year-old children. We investigated their relationship to health outcomes. Additionally, the importance of covariates such as gender, age, lifestyle parameters, food consumption and outdoor exposure parameters (ozone (O_3), particulate matter (PM_{10}) and nitrogen dioxide (NO_2)), were determined.

Methods

Study population

In Flanders (Belgium), a large-scale human biomonitoring surveillance programme was carried out between 2002 and 2006 (FLEHS), by the Flemish Center of Expertise on Environment and Health, commissioned, financed and steered by the Flemish Government. Mother–child pairs, adolescents and adults residing in eight predefined areas in Flanders with different pollution loads, participated in this study. The newborn survey included the measurement of biomarkers of exposure in cord blood (Koppen et al. 2009). Clinical parameters for neonates were registered. Questionnaires on lifestyle, socioeconomic situation and an extensive food questionnaire were completed by the mothers.

A follow-up study related to the development of asthma and allergy was carried out in part of the FLEHS child cohort ($n=134$). The study population resided in the urban area of Antwerp ($n=81$) or in a relatively low-populated region ($n=53$). Data on demographic factors, respiratory symptoms and risk factors for asthma were collected by questionnaires at 6-month intervals. Between the ages of 2.8 and 3.3 years (median 3.1 years), children were enrolled for a follow-up examination, in which skin prick tests (SPT) and FENO measurements ($n=39$) were performed, and EBC ($n=48$) and urine samples ($n=118$) were collected. The last questionnaire was completed in the week before this examination. The mAPI index was determined for each child. Children with signs of infection were excluded from the study. The study protocol was approved by the ethics committees of the University of Antwerp. All parents gave written informed consent.

Nitric oxide measurement

FENO was measured online during spontaneous breathing by the multiple breath test technique (ATS/ERS 2005), because the standardized single breath method is difficult to perform in young children. The child breathed slowly and regularly through a mouthpiece. No active cooperation was required of the child. Nose clips were not used. A rapid response chemiluminescence analyser (CLD88sp; EcoMedics, Duernten, Switzerland) was used for FENO analysis in the range of 0–100 parts per billion (ppb) with a sensitivity of 0.1 ppb. The software (Spiroware; EcoMedics) allowed online quality assurance of the data analysis. Several repeat runs were performed. Exhalations with sudden NO peaks were excluded, as contamination from the nasal cavity could be assumed. Exhalations with a sudden drop in pressure and/or flow were excluded as this reflected leakage. The average of three measurements was taken as the FENO value for the child.

Exhaled breath condensate collection and analysis

EBC samples were collected during 15 min of tidal breathing. The children exhaled about half of this through the device (RTube; Respiratory Research, Inc., Austin, TX, USA), while watching a movie. They were asked not to eat 1 h prior to collection. EBC pH was measured in the samples exactly 5 min after collection without deaeration, and samples were stored within 15 min at -80°C until further analysis. Previously, we examined variability in EBC pH without deaeration of the samples, and concluded that reproducibility of this method was very good when time and volume were standardized (CV 5.2%) (Bloemen et al. 2007). All EBC samples were checked for saliva contamination: α-amylase was measured with the Infinity™ Amylase Liquid Stable Reagent kit (Thermo Fisher Scientific Inc., Waltham, MA, USA). Additionally, the samples included in the proteomics approach were analysed for the presence of α-amylase. None of the samples contained α-amylase. Proteins in EBC were analysed

with liquid chromatography and mass spectrometry (nanoLC-MS) as described previously (Bloemen et al. 2009). In brief, proteins were concentrated on POROS beads (Applied Biosystems, Foster City, CA USA), after which they were enzymatically cleaved into peptides with trypsin. These peptides were separated with LC, and 96 fractions were collected. The peptides in these individual fractions were analysed with matrix-assisted laser desorption/ionization–time of flight–time of flight MS (MALDI-TOF-TOF MS). Comparison between two groups was based on the 96 elution profile (MS) spectra of each sample. Data analysis included standard baseline correction, peak detection, mono-isotopic peak filtering and deisotoping steps. Ratios of the area of the peptides related to the area of the internal standard were used in further statistical analysis. MS-MS spectra were used for identification of proteins of interest, based on homology-based protein identification in a local search engine (MASCOT 2.1; Matrix Science Inc., Boston, MA, USA), using a primary sequence database (Swissprot; Uniprot Release 12.8) for human data.

Urine samples

Spot urine samples were collected at home, maximum 24 h before the examination. Samples were stored at -20°C until analysis. 8-oxo-deoxyguanosine, a marker of oxidative DNA damage and oxidative stress, was measured in the urine (enzyme-linked immunosorbent assay (ELISA); Gentaur, Brussels, Belgium). Results are expressed as g^{-1} creatinine.

Skin prick test

Skin reactivity to cow's milk, egg white, grass and house dust mite (*Dermatophagoides pteronyssinus*; Hallab) was assessed by SPT as described by Pepys (1972). A positive SPT was defined as a weal at least 3 mm in its longest dimension on the place of each skin prick.

mAPI

Symptoms of wheeze were assessed by International Study of Asthma and Allergies in Childhood core questions (Pearce et al. 1993). Based on the longitudinal questionnaire, children were classified into a mAPI-positive and a mAPI-negative group (Guilbert et al. 2004). The mAPI is based on four or more episodes of wheezing in the first 3 years of life, of which one is diagnosed by a physician, and at least one of the major criteria (parental history of asthma, atopic dermatitis and allergic sensitization to at least one aeroallergen) or at least two of the minor criteria (allergic sensitization to milk, egg or peanuts, wheezing unrelated to colds and blood eosinophils above 4%).

Outdoor exposure

Outdoor concentrations for PM_{10}, NO_2 and O_3 from the available Flemish measuring units were interpolated using the RIO model (Residual Interpolation Optimised for ozone) (Janssen et al. 2008). The pollutant concentrations were calculated and averaged for the last 8 days before the child's examination at the age of 3 years.

Statistical analysis

Due to the relative small sample sizes for various parameters, non-parametric tests were used for all analyses. Correlations between the non-invasive biomarkers and SPT, wheezing and mAPI, were evaluated in a Spearman correlation matrix. Mann–Whitney *U* tests were used to evaluate differences between two groups.

The correlation between the measured variables and parameters that might influence the measured variables, such as individual characteristics (body mass index (BMI), gender, born in various seasons), lifestyle factors (living area (urban/rural), medication against fever, antibiotics, vaccination, contact with farm animals, consumption of various foodstuff, and attendance to day-care mother and to day-care centres) and environmental exposure (PM_{10}, NO_2 and O_3 concentration 8 days before the examination), was evaluated in a Spearman correlation matrix. A regression tree analysis was used to predict the values of a continuous non-invasive biomarker variable from one or more continuous and/or categorical predictor variables. The process of computing decision trees can be characterized by four basic steps: tree building, stopping tree building, tree pruning and tree selection (Breiman et al. 1984). The maximized significance criterion was used, which calculates significance values for each split candidate, and uses these rather than the raw values to determine the best split. The process was stopped when there were only five observations in each of the child nodes. Tree pruning was applied to avoid overfitting, and to find the subtree that is most 'predictive' of the outcome and least vulnerable to noise in the data. Statistical analyses were performed using Statistica 8 (Statsoft Inc., Tulsa, OK, USA) and JMP software version 8 (SAS Institute Inc., Cary, NC, USA).

To select a protein pattern in the EBC allowing us to distinguish between two groups of children (mAPI-positive and -negative), the learning algorithm support vector machine (SVM) analysis was used. This technique demonstrates 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 is low. SVM always seek a globally optimized solution and avoid overfitting. This implies a large number of features (i.e. proteolytic peptides) are allowed (Valkenborg et al. 2008, Van Berkel et al. 2008). We used

these SVM to determine which compounds were of interest with regard to the classification of the subjects into two groups, based on as few proteins as possible (Machado et al. 2005). The best subset of compounds was selected using the attribute selection option, implemented in Weka 3.4 (Frank et al. 2004, Witten & Frank 2005), a collection of machine learning algorithms for data mining tasks. Selected attributes were evaluated by a SVM attribute evaluator, and all resulting subsets of attributes were analysed 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). The model with the best classification performance and the lowest number of compounds was selected. Tenfold cross-validation, the standard way of measuring the error rate of a learning scheme on a particular dataset, was used as the test option, both in the attribute selection and for the classification model (Witten & Frank 2005).

Results

Table 1 shows the characteristics of the study population. Out of the 134 children followed up until the age of 36 months, 26 had a positive mAPI index, 18 experienced one or more episodes of wheezing during the last 6 months, and 14 children showed a positive SPT for at least one allergen. The non-invasive measurements including EBC pH, EBC proteolytic peptide pattern, FENO and urinary 8-oxo-deoxyguanosine were performed in subgroups of this study population to evaluate their use as objective measurement in relation to the parameters mentioned above (wheeze, mAPI, SPT).

Feasibility

The success rate of EBC collection was 100%: 48 children were asked and succeeded in donating this sample by breathing through the device for the maximum 15 min. All samples could be used for pH measurement. For 13 out of the 48 children who collected an EBC sample, the collected volume was less than 0.5 ml, which was insufficient for protein analysis. One sample was not analysed due to technical problems. Proteins were analysed in EBC samples of 34 children. FENO measurement was successfully obtained in 39 out of the 81 children after a minimum of two and a maximum of five attempts. Forty-two children were excluded from statistical analysis because of irregular breathing or leakage during measurements.

Health outcome

EBC proteolytic peptide pattern

The EBC samples were grouped based on individuals with a positive or negative score for the mAPI index (9 positive/23 negative). Peptides that were detected in less than 10% of the samples were excluded from the analysis. Peptides which occur in a limited number of samples have less power to contribute to the capacity to discriminate and might introduce noise if implemented into the classification model. By applying this selection criterion, 305 peptides were left for classification based on the mAPI. The SVM classifier was able to classify all individuals 100% correctly for the mAPI index, based on eight peptide masses (Figure 1). One of those peptides could be identified as part of the protein cytokeratin 10. The other seven peptides have not yet been identified.

Table 1. Characteristics of the study population.

Parameter	Total study population($n = 134$)	FENO (subgroup of 39 children)	EBC (subgroup of 48 children)	8-oxodG (subgroup of 118 children)
Individual characteristics				
Sex (girls/boys), *n*	63/71	18/21	25/23	56/62
Body mass index, median (IQR)	15.7 (14.5–16.7)	16.1 (14.6–17.5)	16.0 (14.6–16.7)	15.8 (14.6–16.6)
Health outcome, *n* (%)				
Wheeze	18 (13)	8 (21)	7 (15)	18 (15)
SPT positive	14 (10)	5 (13)	8 (17)	13 (11)
Positive mAPI	26 (19)	10 (26)	14 (29)	24 (20)
Exposure				
Urban/rural living area, *n*	81/53	24/15	48/0	72/46
NO_2 level 8 days prior to examination (ppb)[a], median (IQR)	36.4 (19.9–44.1) *[41.8 (37.4–50.0)/ 17.2 (12.2–22.2)]*	35.4 (19.9–48.5)	39.3 (34.5–43.4)	36.5 (19.8–46.3)
O_3 level 8 days prior to examination (ppb)[a], median (IQR)	48.7 (28.0–59.7) *[45.1 (25.4–56.1)/ 53.2 (36.8–62.5)]*	45.5 (19.5–65.1)	49.2 (40.4–66.9)	48.7 (26.6–62.4)
PM_{10} level 8 days prior to examination ($\mu g\ m^{-3}$)[a], median (IQR)	32.9 (27.1–39.5) *[34.1 (30.0–40.2)/ 29.3 (19.3 –38.3)]*	33.7 (26.7–41.7)	32.0 (28.9–36.4)	33.2 (27.1–39.5)

Characteristics of the children are shown for the total study population as well as for each measurement (exhaled nitric oxide (FENO), exhaled breath condensate (EBC) collection, 8-oxo-deoxyguanosine (8-oxodG) in urine). SPT, skin prick test; mAPI, modified asthma predictive index, IQR, interquartile range. [a]Total group *[urban/rural living area]*.

The individual peptides are not able to discriminate between the groups. The whole pattern has to be used to classify the samples. Based on these eight peptides, a 'SVM factor' can be calculated for each sample. In the mAPI-positive group, the SVM factors for all samples are positive (median 0.99, min. 0.61, max. 1.44), in the mAPI-negative group, the factors are all negative (median -1.103, min. -2.993, max. -0.589).

EBC pH

The median pH of exhaled breath was 6.7 (IQR 5.7–6.9; Table 2). No significant differences were observed in EBC pH based on wheezing, allergy or the mAPI index (Table 2).

Exhaled NO

The median FENO was 3.1 ppb (range 1.3–13.2 ppb; IQR 1.9–4.8 ppb) (Table 2). No significant differences were found in FENO based on mAPI groups, although values were slightly increased in the mAPI-positive group (3.6 ppb) compared with those in the mAPI-negative group (2.9 ppb). FENO values were borderline not significantly increased in the wheezing group ($p = 0.06$), and significantly increased in SPT-positive children, especially for respiratory allergens ($p = 0.04$).

Urinary 8-oxo-deoxyguanosine

The median urinary 8-oxo-deoxyguanosine concentration was 22.5 μg g^{-1} creatinine (IQR 17.1–31.3; Table 2). No significant differences were found in 8-oxo-deoxyguanosine levels if groups positive or negative for mAPI, SPT or wheezing were compared.

Correlations with individual characteristics and environmental parameters

Environmental and lifestyle parameters which might be associated with the non-invasive biomarker measurements were analysed in a Spearman correlation matrix (Table 3).

EBC proteolytic peptide pattern

A significant positive correlation for the SVM factor based on the mAPI was found with 'addition of products to bathwater' ($p = 0.029$; for 18 children products were added to the bathwater), and 'itchy rash' ($p = 0.035$; seven children suffered from itchy rash), but not with environmental exposures. So more children with itchy rash and for whom products were added to the bathwater, can be found in the mAPI-positive group.

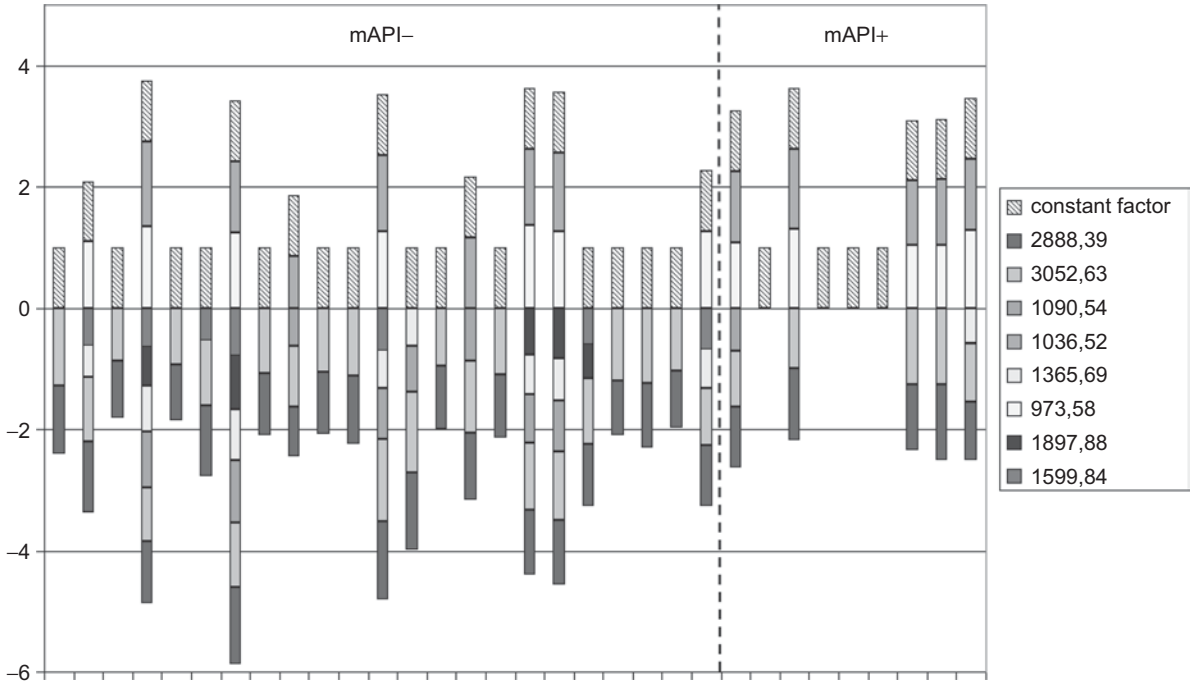

Figure 1. Peptide pattern obtained by support vector machine (SVM) analysis based on the modified asthma predictive index (mAPI) index. The individuals are shown on the *x*-axis. For each individual, the relative contribution of the eight peptides is shown. On the *y*-axis, the factor for each peptide in the SVM equation is shown. The eight peptides together enabled classification of the samples 100% correctly into the mAPI-positive and -negative groups. One of the peptides (mass 3052.627) could be identified as part of the protein cytokeratin 10. The SVM equation, including the eight peptides and the constant factor, results in a positive value for the individuals in the mAPI-positive group, and a negative value for individuals in the mAPI-negative group.

Table 2. Median (IQR) values of the biomarker analysis for each health outcome

		FENO (ppb)	EBC pH	8-oxodG (μg g^{-1} creatinine)
Whole group		3.1 (1.9–4.8)	6.7 (5.7–6.9)	22.5 (17.1–31.3)
mAPI	+	3.6 (1.6–4.3)	6.9 (4.2–7,0)	23.1 (18.1–35.4)
	-	2.9 (2.1–5.1)	6.6 (5.7–6.9)	22.3 (17.0–29.2)
Wheezing	+	4.6 (3.1–8.1)	6.6 (5.3–6.9)	25.6 (20.1–33.7)
	-	2.9 (1.9–4.2)	6.7 (5.7–6.9)	22.3 (17.0–31.3)
SPT	+	7.0 (5.0–7.1)*	6.1 (5.5–6.6)	25.0 (19.7–29.3)
	-	2.9 (1.9–4.0)	6.8 (5.9–6.9)	22.3 (17.0–31.5)

Number of children in each group (n) is shown in Table 1. *Significant difference (p <0.05, Mann–Whitney U test) between the positive and negative group. 8-oxodG, 8-oxo-deoxyguanosine; SPT, skin prick test; mAPI, modified asthma predictive index; IQR, interquartile range.

EBC pH

EBC pH was positively correlated with 'NO_2 concentration averaged over 8 days before the examination' (p = 0.039), and negatively with contact with farm animals (p = 0.046, children in contact with farm animals had a lower EBC pH), although only two of the 48 children came in contact with these animals. In the regression tree, outdoor NO_2 concentration also seems to have significant influence on EBC pH (Figure 2). Explanatory variables involved in constructing the pruned tree are: NO_2 concentration averaged over 8 days before the examination, consumption of yoghurt and meat, and soaping before a bath. The five-folded R^2 of this tree is 0.29, indicating that 30% of the variability in the pH values is accounted for by this tree.

Exhaled NO

Exhaled NO was not significantly correlated with any of the tested individual, lifestyle or environmental factors.

Urinary 8-oxo-deoxyguanosine

A positive correlation between this urinary oxidative stress marker and 'PM_{10} levels 8 days before the examination' (p = 0.0049) as well as 'NO_2 levels 8 days before the examination' (p = 0.046) was observed. Additionally, a significant positive correlation was found with the consumption of fresh milk (p = 0.039), but only seven of those 118 children consumed fresh milk. In the regression tree, outdoor NO_2 concentration has a significant influence on 8-oxo-deoxyguanosine (Figure 3). The five-folded R^2 of this tree is 0.17.

Correlations between markers

No correlation was observed among the various non-invasive markers FENO, EBC pH and urinary 8-oxo-deoxyguanosine, but only 12 children performed both FENO test and EBC collection, which limits conclusions. Also wheezing, positive SPT results and mAPI were not correlated with each other. Wheezing was significantly correlated with coughing (p = 0.009) and airway infections (p = 0.004).

Discussion

Identification of infants and young children who have a risk of developing asthma later in life is very important for the development of a strategy for early intervention aimed at changing the natural course of the disease. Distinguishing transient wheezers from wheezing children who will continue to develop asthmatic symptoms, simply on the basis of their clinical presentation, is currently problematic (Castro-Rodriguez et al. 2000).

This feasibility study, which is a first step in the development of early objective biomarkers, showed that non-invasive markers can be applied in 3-year-old children. All children were able to donate EBC, although the collected volume is sometimes too small to perform extensive analysis such as proteome analysis. Exhaled NO could be measured in about half the children at this age, while others were excluded from analysis due to irregular breathing or leakage during measurements.

We measured the non-invasive biomarkers FENO, EBC pH and urinary 8-oxo-deoxyguanosine, and studied the exhaled proteolytic peptide pattern in 3-year-old children from the follow-up study asthma and allergy from the Flemish birth cohort (FLEHS). The relationship of these markers with health outcome (mAPI, wheezing and allergy) was studied, as well as the influence of individual, lifestyle and environmental variables on these biomarker measurements. FENO correlated with some respiratory health outcomes in this small study population. The other endpoints could not be related to health outcome, most likely because the study was underpowered.

In this study, we applied a proteomics approach on EBC samples derived from 3-year-old children in the search for early biomarkers for asthma development. We were able to select a potential biomarker profile of eight peptides (probably resulting from eight proteins) for objective early diagnosis of asthma, based on the currently used mAPI. The corresponding proteins could not yet be identified due to their low abundance. This study is a first attempt to study exhaled proteins as early biomarkers for asthma. Further adaptations, e.g. the sampling device, specific steps in the protocol, or separation or detection techniques, will lead to a refined

Table 3 Spearman correlation matrix for each of the non-invasive markers.

	FENO			EBC pH			SVM factor			8-oxodG		
	n = 39			*n* = 48			*n* = 32			*n* = 118		
	n	*r*	*p*-Value	*n*	*r*	*p*-Value	*n*	*r*	*p*-Value	*n*	*r*	*p*-Value
Individual characteristics												
Sex		-0.04	0.81		0.10	0.50		-0.06	0.75		-0.07	0.42
BMI		-0.03	0.86		-0.16	0.35		-0.03	0.90		0.19	0.06
Health outcome												
mAPI		-0.08	0.66		0.12	0.43					0.08	0.39
Allergy (positive SPT)		0.34	0.04*		-0.13	0.39		-0.04	0.84		0.05	0.56
Wheezing		0.30	0.06		0.01	0.95		0.03	0.89		0.09	0.35
Environmental exposure												
Urban/rural living area		-0.19	0.25								-0.13	0.16
NO_2 level 8 days before examination		0.02	0.91		0.30	0.04*		-0.13	0.47		0.19	0.05*
O_3 level 8 days before examination		-0.12	0.47		-0.14	0.34		0.05	0.79		-0.002	0.99
PM_{10} level 8 days before examination		-0.31	0.05		0.10	0.52		-0.03	0.88		0.26	0.005*
Lifestyle factors												
Day-care centre[a]	8	-0.02	0.89	12	0.20	0.19	10	0.30	0.09	27	0.03	0.73
Day-care mother[a]	3	-0.19	0.25	4	-0.07	0.63	3	-0.31	0.09	14	0.05	0.58
Times a week in bath[ab]	39	0.19	0.25	48	-0.23	0.11	32	0.28	0.12	108	-0.01	0.92
Addition of products to bathwater[a]	23	0.03	0.85	25	0.01	0.93	18	0.39	0.03*	69	-0.09	0.35
Soaping before bath[a]	24	-0.23	0.17	23	0.21	0.16	14	-0.12	0.50	60	-0.06	0.55
Itchy rash[a]	6	-0.07	0.68	11	-0.08	0.60	7	0.38	0.04*	24	0.003	0.96
Airway infection[a]	10	-0.20	0.22	8	0.17	0.26	4	-0.23	0.21	24	0.003	0.97
Vaccination[a]	2	-0.03	0.85	1	-0.16	0.27	1	0.30	0.09	3	-0.08	0.42
Medication against fever[a]	23	0.22	0.19	29	0.01	0.94	21	-0.15	0.41	67	0.001	0.99
Antibiotics[a]	17	0.01	0.97	10	-0.25	0.10	7	0.14	0.46	37	-0.02	0.83
Ion generator[a]	1	0.19	0.26	2	-0.24	0.11	2	0.01	0.94	3	0.05	0.57
Airconditioning[a]	1	-0.15	0.38	2	-0.11	0.47	2	0.16	0.38	2	-0.04	0.71
Contact with farm animals[a]	5	-0.18	0.28	2	-0.30	0.05*	1	0.06	0.74	18	0.02	0.81
Consumption of yoghurt[ab]	33	-0.25	0.13	8	0.04	0.79	26	0.25	0.17	99	0.04	0.64
Consumption of fresh milk[ab]	2	-0.25	0.13	0			0			7	0.20	0.04*
Consumption of vegetables[ab]	38	0.14	0.41	46	0.06	0.69	31	-0.05	0.80	113	-0.03	0.78
Consumption of meat[ab]	36	0.01	0.97	44	0.14	0.35	31	0.23	0.20	111	-0.08	0.38
Consumption of fruit[ab]	38	-0.20	0.22	46	0.07	0.65	32	-0.03	0.89	113	0.03	0.76
Consumption of fish[ab]	38	0.15	0.38	46	0.006	1.00	32	-0.03	0.89	112	0.15	0.12
Consumption of crustaceans[ab]	9	0.18	0.29	14	0.02	0.91	8	-0.15	0.44	35	0.05	0.63
Consumption of eggs[ab]	36	0.02	0.89	43	-0.07	0.62	31	0.06	0.73	103	0.18	0.05
Consumption of milk[ab]	34	-0.02	0.92	42	-0.10	0.51	28	0.02	0.90	105	-0.04	0.70
Consumption of chips[ab]	35	0.16	0.34	44	-0.18	0.23	30	-0.27	0.14	106	0.14	0.14
Consumption of cake[ab]	36	-0.20	0.25	46	-0.08	0.62	31	0.11	0.57	113	0.05	0.61
Consumption of cheese[ab]	30	-0.05	0.78	42	-0.13	0.37	27	0.02	0.91	97	0.05	0.57
Consumption of nuts[ab]	13	0.17	0.30	24	-0.06	0.70	14	-0.09	0.63	57	0.13	0.15
Consumption of products with nuts[ab]	2	0.21	0.22	5	-0.23	0.14	2	-0.34	0.07	8	-0.07	0.44
Consumption of bread[ab]	38	-0.07	0.69	48	-0.01	0.93	32	0.18	0.33	114	-0.07	0.47
Consumption of probiotica[ab]	8	-0.03	0.85	38	-0.14	0.35	4	0.01	0.98	21	0.03	0.79
Consumption of *Bifidus*[ab]	5	0.05	0.77	36	-0.06	0.71	5	0.19	0.33	17	-0.05	0.61
Born in autumn	12	-0.07	0.66	1	0.16	0.27	0			34	-0.03	0.78
Born in summer	11	-0.07	0.68	13	-0.21	0.16	7	-0.04	0.82	27	0.01	0.94
Born in spring	9	-0.05	0.78	17	-0.03	0.87	12	0.05	0.80	25	0.11	0.23
Born in winter	7	0.22	0.18	17	0.17	0.25	13	-0.01	0.96	32	-0.08	0.39

[a]During the last 6 months before the examination; [b]also frequency (of consumption) was asked and taken into account in the analysis. In the column 'n' it is indicated how many children from the subgroup answered positive to the question. *r*, Spearman correlation coefficient; *significant correlation; FENO, exhaled nitric oxide; EBC, exhaled breath condensate collection; 8-oxodG, 8-oxo-deoxyguanosine; SVM, support vector machine.

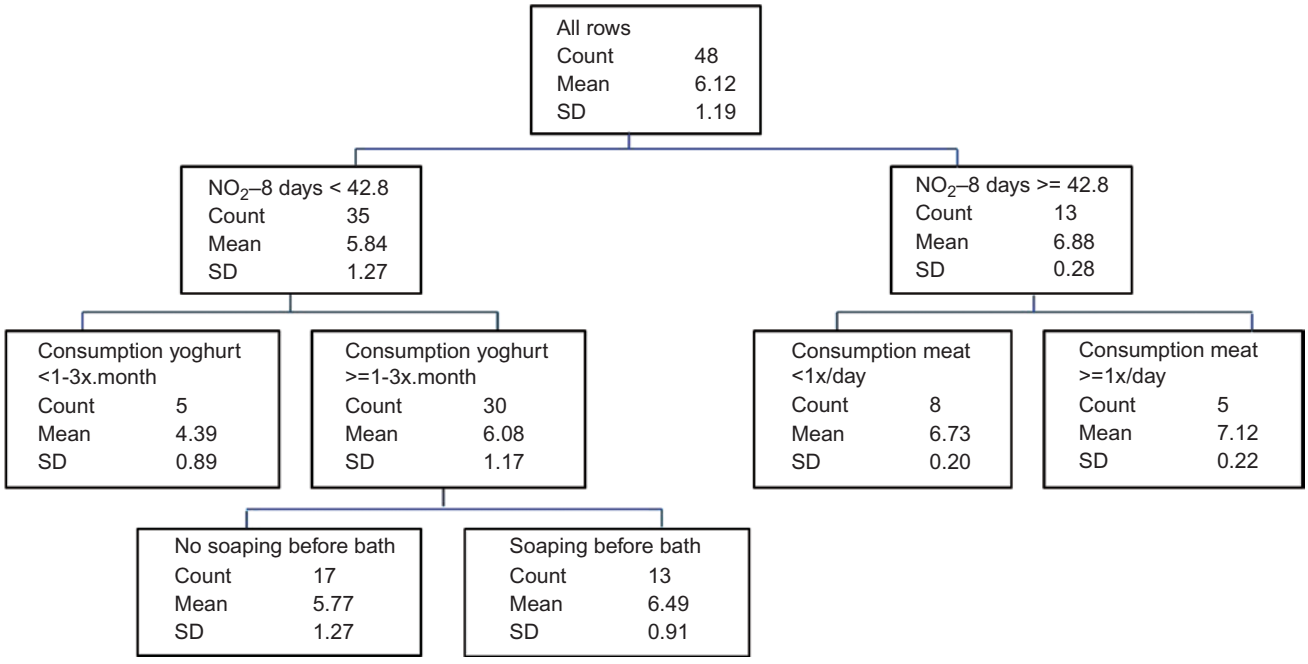

Figure 2. Result of the pruned regression tree for exhaled breath condensate (EBC) pH.

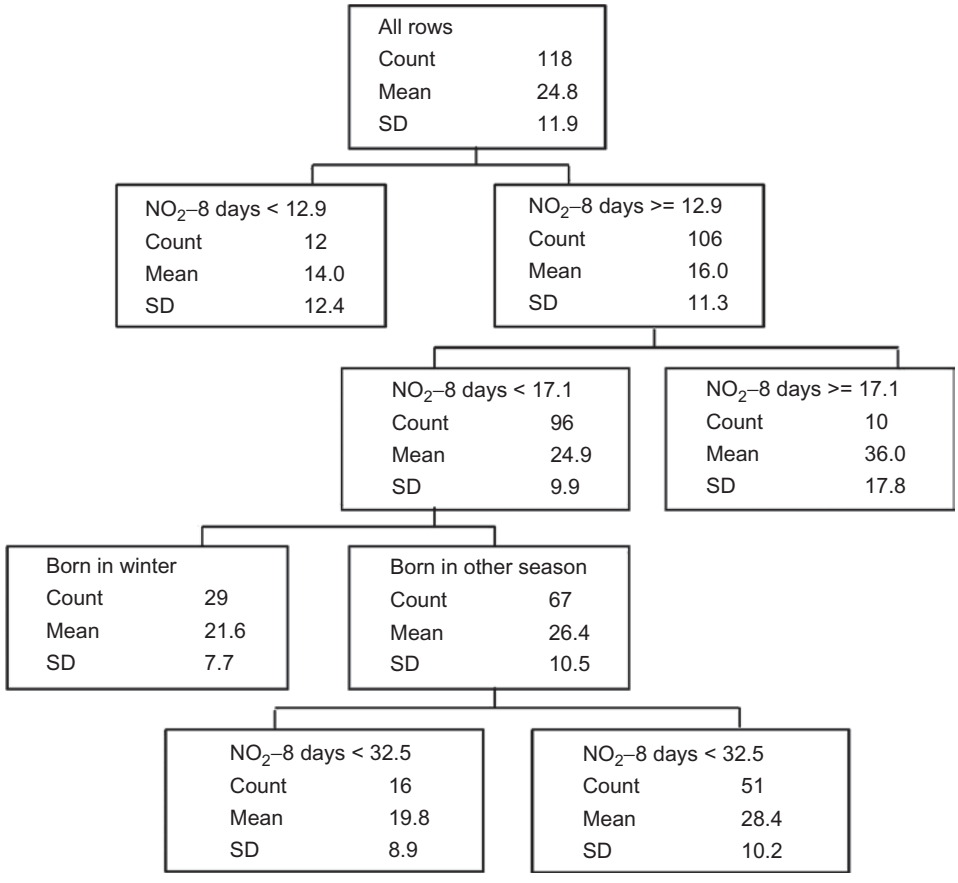

Figure 3. Result of the pruned regression tree for urinary 8-oxo-deoxyguanosine.

protocol to study these samples. This approach can lead to an objective parameter for early diagnosis of those at risk of developing asthma. Additionally, further follow-up of the children is needed when they reach an age on which asthma diagnosis is possible, to confirm the use of this proteolytic peptide pattern as potential early biomarker.

Airway acidification reflects airway inflammation, and occurs during airway diseases such as asthma (Kostikas et al. 2008, Brunetti et al. 2006). EBC pH is currently considered a robust mediator, with good reproducibility (Bloemen et al. 2007, Accordino et al. 2008). In this study group, EBC pH was not associated at this young age with symptoms such as wheezing, which might be transient. Also mAPI, which predicts higher risk for developing asthma at a later age, was not correlated with EBC pH. This might be due to the fact that there is no inflammation present yet. Based on these results, EBC pH does not seem an indicator for early diagnosis of asthma in the small group of young children who have been tested in this study.

The FENO values measured in this study (3 ppb; 2–5 ppb) were comparable with those reported by Buchvald and Bisgaard (2001) in 67 young children of whom 51 were in the age range of 2–5 years: 6 ppb (4–8 ppb) in children with asthma, 5 ppb (3–7 ppb) in children with mild episodic wheezing and 3 ppb (2–4 ppb) in healthy controls. In the present study, we found a significant correlation between FENO and inhalation allergy (positive SPTs for respiratory allergens) in 3-year-old children, which is one of the risk factors for the development of asthma later in life (Sly et al. 2008). No significant correlation with the mAPI and borderline not significant result with wheezing was found, probably due to small sample sizes. This is in line with results from the PIAMA study (Brussee et al. 2005), in which mean FENO values, measured off-line, were higher in 4-year-old children with specific IgE to inhalant allergens compared with those without. This is also in agreement with the results of previous studies in school-aged children (Silvestri et al. 2001, Franklin et al. 2003, Frank et al. 1998). Also no association was found with wheezing phenotypes in the PIAMA study (Brussee et al. 2005).

As oxidative stress plays an important role in various diseases such as asthma, urinary 8-oxo-deoxyguanosine, a non-invasive marker for oxidative stress and DNA damage, was measured (Tsukahara 2007). Although this mediator has – to our knowledge – not been related to human asthma before, it has recently been suggested as a potentially useful biomarker for evaluating the severity of respiratory failure in patients with severe motor and intellectual disabilities (Tanuma et al. 2008). In the present study, no correlations were found between 8-oxo-deoxyguanosine in urine and respiratory health outcome.

Recent research indicates that exposure through skin is also a potential route of sensitization for respiratory allergy (Strid & Strobel 2005). The SVM factor based on the mAPI was correlated with the addition of products to bathwater, as well as with itchy rash. From the nine children in the mAPI-positive group, eight indicated that products were added to the bathwater. From the 34 children, products were added to the bathwater in 18 cases. Seven children suffered from itchy rash during the last 6 months before the examination, of whom four were from the mAPI-positive group. These findings are in line with the recent findings in allergy research (Strid & Strobel 2005).

The present study showed that traffic-related pollutants known to be linked to airway inflammation were correlated with EBC pH, which is a marker of airway inflammation. This confirms the results of earlier studies in which EBC pH was decreased after acute exposure to traffic-related air pollutants in both non-asthmatic ($n = 50$) and asthmatic ($n = 158$) children living in Mexico city (age 6–14 years), and decreased significantly after acute O_3 exposure in asthmatic children (Barraza-Villarreal et al. 2008). A study in adults observed no changes in EBC pH after controlled exposure to O_3 (Corradi et al. 2002). Also other markers in EBC have been studied in relation to air pollution. Romieu et al. (2008) concluded that in children ($n = 107$; mean age 9.5 years), the oxidative stress marker EBC malondialdehyde was related to both traffic-related air pollution exposure, in particular O_3 and $PM_{2.5}$, and changes in lung function and inflammatory markers. They also observed a significant inverse correlation between malondialdehyde levels in EBC and the EBC pH levels (Romieu et al. 2008). There is growing evidence that air pollutants specifically associated with traffic exposure, such as $PM_{2.5}$, NO_2 and O_3, are risk factors for exacerbation but also for the development of asthma and allergic disease (Heinrich & Wichmann 2004, Peden 2005, Koppen et al. Accepted for publication). Prospective studies to evaluate the relationship between traffic-related air pollution and development of asthma at more advanced ages are needed to confirm or refute this association.

No correlation was observed between FENO and exposure to O_3, NO_2 or PM_{10} during the last 8 days before the examination. This was also concluded in controlled ozone-exposure studies in healthy human adult volunteers (Alfaro et al. 2007, Montuschi et al. 2002), in which FENO and NO metabolites remained unchanged. Another recent study in 1613 children aged 9–11 years found a positive correlation between FENO and environmental $PM_{2.5}$ values (24 h average) (Dales et al. 2008).

Urinary 8-oxo-deoxyguanosine has been correlated before with smoking and increased exposures to air pollutants (Sorensen et al. 2003). Our results correspond to recent findings that PM_{10} and $PM_{2.5}$ levels measured

during a 7-day period before urine collection were associated with 8-oxo-deoyguanosine (Svecova et al. 2009).

In conclusion, in this follow-up of part of a Flemish birth cohort study, we demonstrated that it is feasible to perform non-invasive sampling (EBC, FENO and urine) for biomarker measurements to assess respiratory toxicity in 3-year-old children. FENO could be measured in about half of the children and was related to some of the health outcome parameters; EBC could be collected from all the children but was not related to health outcomes. Based on SVM analysis we established a specific peptide pattern which may be useful for discriminating children based on their mAPI index, which is predictive for asthma at later ages. The study was underpowered to establish relations between the biomarkers but we were able to find environmental influential factors on the EBC measurements (NO_2 concentration averaged over 8 days before the examination on EBC pH, addition of products to bathwater and itchy rash on EBC peptide pattern). Studies with more participants are needed to confirm these findings.

Declaration of interest

This study was part of the biomonitoring study carried out by the Flemish Center of Expertise on Environment and Health, which was commissioned, financed and steered by the Flemish Government (Department of Economics, Science and Innovation; Flemish Agency for Care and Health; and Department of Environment, Nature and Energy). Development of EBC biomarkers was supported by the Belgian Science Policy (Contract number SD/HE/05A: ANIMO project). K.B. was supported by a PhD grant from VITO. The authors report no declarations of interest.

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