Faculty of Medicine
School of Public Health
Louvain Centre for Toxicology and Applied Pharmacology

Impact of the environment and lifestyle on the respiratory health of adolescents; application of non-invasive biomarkers of airway damage or inflammation

Erik Van Miert

Thesis submitted in fulfilment of the requirements for
the degree of Doctor (PhD) in Medical Sciences

9 September 2011

For Kato and Anton

Levet Scone

Composition of the jury

PRESIDENT OF THE JURY

Jean-Marie Degryse	Professor Université Catholique de Louvain Faculté de médecine et médecine dentaire Institut de recherche santé et société (IRSS)

MEMBERS OF THE JURY

Alfred Bernard	Directeur de Recherches FNRS Professor Université Catholique de Louvain Faculté de médecine et médecine dentaire Institut de recherche expérimentale et clinique (IREC) Louvain Centre for Toxicology and Applied Pharmacology
Pedro Buc Calderon	Professor Université Catholique de Louvain Faculté de pharmacie et des sciences biomédicales (FASB) Louvain Drug Research Institute (LDRI)
Benoit Nemery de Bellevaux	Professor Katholieke Universiteit Leuven Faculteit Geneeskunde Centrum voor Preventieve Gezondheidszorg Afdeling Pneumologie
Harry Roels	Professor Emeritus Université Catholique de Louvain Faculté de médecine et médecine dentaire
Olivier Vandenplas	Professor Université Catholique de Louvain Faculté de médecine et médecine dentaire Institut de recherche expérimentale et clinique (IREC) Pôle de Pneumologie, ORL et Dermatologie (PNEU)

Acknowledgements

I owe it to a lot of people that I got this far. First of all, I would like to thank the members of the jury who took the time to look into my work during the summer time and who demonstrated a lot of goodwill and flexibility. Special thanks to Professor emeritus Harry Roels, the president of my follow-up commission, and to Professor Jean Degryse, the president of the jury, for managing my rather “unique” case.

The main enabler of this project is undoubtedly my promoter, Professor Bernard, who allowed me the freedom to combine my research work with my job, and whose true researcher attitude, free of dogmatic thinking, truly inspired and challenged me. I would also like to thank the co-workers of Professor Bernard and in particular Dr. Marc Nickmilder who was a major contributor to the epidemiologic study which provided the data enabling me to write 2 of my publication, Dr. Antonia Sardella who did the analyses of nasal lavage samples, and Mr. Xavier Dumont whose technical mastery and kindness made the hours spent in the lab of Professor Bernard highly enjoyable.

I also whish to thank Dr. Francesca Alessandrini (Division of Environmental Dermatology and Allergy, Helmholtz Zentrum/Technische Universität München, ZAUM Center for Allergy and Environment, Neuherberg and Munich, Germany) for involving me in her fascinating research project.

I would like to mention a number of great people I had the privilege of meeting during my professional career and who guided me by example and/or with advice.

- Dr. Piter Terpstra who motivated me to take on this PhD project.
- Dr. Schlage who was initially a member of my follow-up commission.
- Mr. Walter Stinn
- Dr. Kris Meurrens, I would also like to thank Kris for reviewing this document.
- Dr. Patrick Vanscheeuwijck
- Mr. Jan Verbeeck
- Mr. Kristof Schoonjans with whom I designed an optimized exhaled breath condensate collection device. Due to time and resource restraints, I was unable to make it function in a reproducible assay, but I’m confident that it still outclasses most of the currently used exhaled breath collection systems.
- Mrs. Lynda Conroy-Schneider.
- My former colleagues at the Philip Morris Research laboratories who helped me becoming the scientist, but also the person I currently am.
- My colleagues at Solvay S.A. who empower me and give me the opportunity to explore new fields in toxicology and risk assessment.

I would like to thank my parents who gave me the opportunity to do the studies of my preference, who worked hard to provide me everything I needed and who have always respected my choices. Above all, I thank them for showing me the greatness of working relentlessly, yet with a lot of love and modesty, while striving for a challenging objective.

When I told my children that this project, which started well before their birth, was about to reach its final moment they were filled with joy… not because of its scientific merit, but because this project demanded a significant effort not only from me but also from my family. Therefore, I would like to conclude with a big, big thank you to my family and especially to my wife Ann.

Table of contents

1 Summary

In this project we investigated whether lung-specific biomarkers measureable via non-invasive methods allow an evaluation of the respiratory system and an assessment of the impact of factors related to everyday's environment (wood fuel use) or lifestyle (cigarette smoking).

The mechanistic understanding related to changed levels of the Clara cell protein (CC16) in serum and their relationship to changes of the Clara cell population in the lung was further elaborated in 2 *in vivo* studies. In an inhalation experiment with rats, CC16 in serum was found to be a very sensitive marker of permeability changes of the lung epithelial barrier. In an *in vivo* model investigating the interaction of elemental carbon ultrafine particles and allergy, the levels of CC16 in BALF and serum paralleled morphological changes in the respiratory tract. This demonstrated the relevance of the Clara cell population with respect to the inflammatory status of the lung and the usefulness of monitoring CC16 in BALF or serum as a marker of the lung status.

A battery of non-invasive markers, i.e. CC16 in nasal lavage fluid and serum, albumin in nasal lavage fluid, surfactant associated protein D (SPD) in serum, and nitric oxide (NO) in exhaled breath was used in 2 cross-sectional studies on adolescents. We demonstrated the usefulness and sensitivity of two non-invasive approaches, i.e. CC16 in nasal lavage fluid and NO in exhaled breath, to detect early changes in the respiratory system of adolescent smokers well before the appearance of changes in the deeper lung or altered lung function parameters. In a study on non-smoking adolescents, wood fuel use showed to be associated with a decrease of the CC16 to SPD ratio in serum; a change suggesting epithelial changes in the respiratory tract. Additionally, higher frequencies of asthma, hay fever and sensitization against pollen were observed among adolescents with wood fuel use. Using non-invasive approaches enabled us to screen a large study population and to identify significant and objective differences in the status of the respiratory system between exposed and non-exposed adolescents (non-smokers vs. smoker or no wood fuel use vs. wood fuel use). Exploratory studies investigating the applicability of the exhaled breath condensate showed that the methodological requirements to make the assay robust too burdensome to foresee a powerful diagnostic application of this assay in the near future.

Overall, we were able to illustrate that non-invasive biomarkers have the potential to allow the successful screening of large study populations or the repeated measurements of diseased individuals by yielding objective data with high sensitivity. Nevertheless, before these approaches can be routinely used, the methodological performance and robustness of the assays will have to be further improved and the mechanistic investigations which contribute to the understanding of the different parameters influencing the assay outcome will have to be continued.

2 Publications included in the thesis

Study 1

Van Miert E., Dumont, X., and Bernard, A. CC16 as a marker of lung epithelial hyperpermeability in an acute model of rats exposed to mainstream cigarette smoke. Toxicol.Lett. 159(2), 115-123. 15-11-2005.

Study 2

Alessandrini, F., Weichenmeier, I., Van Miert E., Takenaka, S., Karg, E., Blume, C., Mempel, M., Schulz, H., Bernard, A., and Behrendt, H. Effects of ultrafine particles-induced oxidative stress on Clara cells in allergic lung inflammation. Part Fibre.Toxicol. 7, 11. 2010.

Study 3

Van Miert E., Sardella, A., and Bernard, A. Biomarkers of early respiratory effects in smoking adolescents. Eur.Respir.J. 12-5-2011, in print.

Study 4

Van Miert E., Sardella, A., Nickmilder, M.,Bernard A. Respiratory effects associated with wood fuel use: a cross-sectional biomarker study among adolescents. Pediatr Pulmonol. 2011 , in print

3 Introduction

3.1 Non-invasive biomarker assays

The respiratory system is continuously exposed to various biological and xenobiotic agents. In most situations, protective and adaptive processes manage to deal with such challenges, but in few cases these exogenous influences result in disease after prolonged or sometimes even acute exposure. In order to monitor whether the respiratory system is responding to inhaled agents, various approaches have been developed and are still under further development (see Figure 1). Each approach has its strengths and weaknesses and the information provided is often complementary, e.g. imaging data combined with functional data. When following-up a larger population at risk or when a certain situation requires repeated measures, the non-invasiveness of a technique and the ease of sample collection play a major role. Invasive methods such as biopsies or broncho-alveolar lung lavage (BALF) are often time-consuming, not without risk for the individual and require often a certain recovery time before the next sample collection can be performed. On the other hand, technically demanding sample collection processes such as complex imaging techniques, e.g. MRI imaging, typically require a centralised measurement facility and highly trained personnel. In this project we were initially interested to assess and use those non- or less-invasive approaches using blood (serum/plasma), induced sputum, nasal lavage, exhaled breath and exhaled breath condensate. This selection was not based on the assumption that these non-invasive approaches were superior to the others, but it was rather based on their potential to be included in population studies requiring non-invasive and easy sample collection.

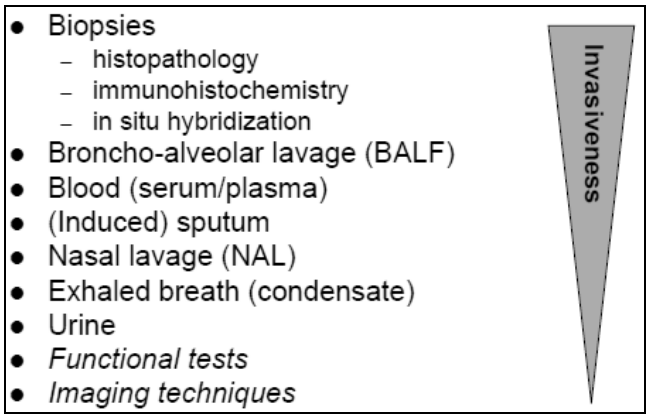

Figure 1 Overview of approaches/matrices used to collect data about health status of the respiratory system

There is already a substantial amount of literature discussing the presence and changes of biomarker levels in non-invasive assays. Table 1 to Table 4 provide an overview of studies in which the abovementioned sample collection methods of interest allowed to differentiate people with and without a respiratory condition. The National Institute of Health (NIH) defined a biomarker as "a characteristic that is objectively measured and evaluated as an indicator of normal biologic processes, pathogenic processes, or pharmacologic responses to a therapeutic intervention" (1). As such, biomarkers can be used as an unbiased indicator of illness onset, as an aid in the classification of a diseased or non-diseased state, and provide the ability to stage disease progression and/or offer insight into its relative severity. An individual's risk of developing a disorder may also be derived from biomarker research; as such a prognostic biomarker could be employed for risk stratification of the general population. In addition to identifying disease, the efficacy of clinical or therapeutic intervention may also be assessed using a biomarker. Because of its objective and quantitative nature, biomarkers can represent a significant added-value to toxicological and epidemiological studies.

Table 1 Overview of biomarker assays in serum which allowed the differentiation of diseased from healthy people (2;3)

Marker	Respiratory disease	Change associated with disease
CC16	chronic bronchitis	↓
	lung cancer	↓
	silicosis	↓
	pulmonary fibrosis	↑
	sarcoidosis	↑
SP-A	acute respiratory distress syndrome (ARDS)	↑
	cardiac lung edema	↑
	pulmonary fibrosis	↑
	asbestosis	↑
	alveolar proteinosis	↑
SP-B	ARDS	↑
	cardiac lung edema	↑
SPD	pulmonary fibrosis	↑
	alveolar proteinosis	↑
	tuberculosis	↑
	sarcoidosis	↑
mucin antigens (KL-6, 17-B1v, 17-Q2)	pneumonitis	↑
	sarcoidosis	↑
	chronic beryllium disease	↑
	tuberculosis	↑
	cystic fibrosis	↑
	ARDS	↑

Table 2 Overview of biomarker assays in (induced) sputum and nasal lavage which allowed the differentiation of diseased from healthy people (2;4-7)

Marker	Respiratory disease	Change associated with disease
Nasal lavage		
IL-8	asthma	↓
(Induced) sputum		
CC16	chronic obstructive pulmonary disease (COPD)	↓
uteroglobin-related protein 1	asthma	↑
uteroglobin-related protein 1	allergic rhinitis	↑
eosinophilia	asthma	↑
albumin	asthma	↑
MUC5AC	cystic fibrosis	↓
MUC5B	cystic fibrosis	↓

Table 3 Overview of biomarker assays in exhaled breath which allowed the differentiation of diseased from healthy people (2;8-12)

Marker	Respiratory disease	Change associated with disease
NO	asthma	↑
	COPD	↑
	allergic rhinitis	↑
	upper resp. tract infection	↑
	bronchiectiasis	↑
	tuberculosis	↑
	lung cancer	↑
	active pulmonary sarcoidosis	↑
	non-asthmatic chronic cough	↓
	pulmonary hypertension	↓
	primary cilia diskynesia	↓
	cystic fibrosis	↓
CO	asthma	↑
	allergic rhinitis	↑
	COPD	↑
	upper respiratory tract infections	↑
	interstitial lung disease	↑
	cystic fibrosis	↑
		↑
hydrocarbons (ethane, pentane, isoprene)	asthma exacerbations	↑
	COPD	↑
	cystic fibrosis	↑
temperature rise (ΔT)	COPD	↓

Table 4 Overview of biomarker assays in exhaled breath condensate which allowed the differentiation of diseased from healthy people

Marker	Respiratory disease	Change associated with disease	Reference
thiobarbituric acid reactive products (TBARs)	asthma	↑	(13)
hydrogen peroxide	asthma	↑	(13) (14) (15)
	COPD	↑	(16)
nitrotyrosine	severe asthma	↓	(9)
nitrosothiols	severe asthma	↑	(8)
	cystic fibrosis	↑	(8)
	COPD	↑	(8)
nitrite (NO_2^-)	severe asthma	↑	(8)
	cystic fibrosis	↑	(8) (17)
	COPD	↑	(8)
8-isoprostane	COPD	↑	(18)
	cryptogenic fibrosing alveolitis	↑	(19)
	fibrosing alveolitis with systemic	↑	(19)
	sclerosis	↑	(19)
	sarcoidosis	↑	(20)
	cystic fibrosis	↑	(20)
LT B_4	asthma	↑	(9;11;21;22)
	chronic bronchitis	↑	(21)
	allergic rhinitis	↑	(21)
	cystic fibrosis + P *aeruginosa*.	↑	(23)
LT E_4	asthma	↑	(9;11)
IL-4	asthma	↓	(24)
INFγ	asthma	↑	(24)
IL-8	cystic fibrosis + P *aeruginosa*.	↑	(23)
pH	lung inflammation	↓	(25)

3.2 The impact of the indoor environment and lifestyle on respiratory health

An individual's respiratory health is the result of a complex interaction of genetic, environmental and behavioural (i.e. voluntary exposures) parameters. The multifactorial and complex nature of the relationships between the environment, lifestyle and respiratory health is well illustrated by the positive association between the individual-level socioeconomic status and the asthma morbidity (26) or by the adjuvant-like activity of outdoor chlorinated pools on the development of atopy (27). Evidence is increasing that indoor air pollution plays an important role in the development of respiratory diseases, especially during childhood (28). A number of risk factors have already been identified, such as volatile organic compounds (29), ultra-fine particles (30), house dust allergens (31), environmental tobacco smoke (32), biomass burning smoke (33), and chlorination products in swimming pools (34).

In this project, we were interested in identifying possible modulators of the respiratory health in adolescents. Children and adolescents are an intensely studied population, because they are generally assumed to have a higher susceptibility to (adverse) influences and because an (in-) adequate adaptation of their still developing respiratory and immune systems to external modulators can have long lasting consequences later in life. A number of recent studies reporting environmental or behavioural modulators of respiratory health of infants, children and adolescents are provided in Table 5. These modulators can be grouped according to lifestyle, outdoor environmental (air) quality and the indoor environmental (air) quality. Considering that a large proportion of these studies are observational studies, causality has not always been demonstrated. Associations have to be addressed cautiously as illustrated by Kovesi et al. who observed a positive association between electrical baseboard heating and exhaled NO (35). Electrical baseboard heating is an easy to install, rudimentary type of electrical heating system, yet without any emissions and as such it should rather be considered as a surrogate marker for the general quality of the in-house environment or as a covariate for a "real modulator".

Table 5 Overview of recent studies suggesting modulators of respiratory health of infants, children or adolescents.

Modulator	Association	Health effect	Reference
Lifestyle			
obesity	+	asthma	(36)
cigarette smoking	+	asthma, wheezing	(37-39)
chlorinated pool attendance	+	atopy, childhood asthma	(40)
	+	adjuvant effect on atopy	(27)
	+	bronchiolitis, asthma, allergic sensitisation	(41)
maternal smoking	-	exhaled NO	(42), (43)
Outdoor environmental (air) quality			
PM2.5	+	Acute airway inflammation decreased lung function	(2)
PM10	+	infant mortality	(44)
swine farm exposure	+	asthma ,wheeze, cough	(45)
rural and farm living	-	asthma atopy	(46)
Indoor environmental (air) quality			
wood smoke particulate air pollution/solid biomass fuel	+	respiratory symptoms lung function (asthmatics only)	(47)
	+	acute lower respiratory tract infection, pneumonia, bronchiolitis	(33)
	+	acute respiratory infection	(48)
improved home heating	-	asthma symptoms, days off school, healthcare utilisation, pharmacist visits	(49)
exposure to moulds	+	asthma	(50)
home dampness/household water damage	+	allergic rhinitis	(51)
	+	asthma, wheezing	(52)
environmental tobacco smoke exposure	+	chronic bronchitis	(53)
	+	IgE sensitisation to indoor inhalation and food allergens	(54)
	+	asthma, wheezing	(52;55)(67)(32)
electrical baseboard heating	+	exhaled NO	(35)
house cleaning with bleach	-	asthma aeroallergen sensitisation	(56)

4 Objective

The overall objective of this project was to investigate the potential of non- or less invasive biomarker methods as tools to study the possible impact of indoor pollution or lifestyle on the respiratory system.

We addressed the following questions:

- How does the Clara cell secretory protein CC16 perform as an endogenous marker for the health status of the respiratory system *in vivo* models of respiratory diseases? What can be learned about the marker's sensitivity and correlations with morphological changes?
- How is the health status of the respiratory system reflected in the composition of nasal lavage fluid, induced sputum, exhaled breath or exhaled breath condensate? Do these assays provide potential lung-specific biomarkers? Do these assays allow screening studies of larger populations or repeated measurements?
- Do non-invasive biomarkers show associations with passive or active cigarette smoke exposure or wood fuel use, suggesting a possible impact of these exposures on the health status of the respiratory system?

5 Overview of the work performed

5.1 Non-invasive assays

5.1.1 Pneumoproteins in serum

In order to increase our understanding of assays in serum assessing the health status of the respiratory system, we studied the changes of the Clara cell protein CC16 in broncho-alveolar lavage (BALF) of rats (Study 1) and mice (Study 2). To investigate possible associations between environmental or behavioural parameters and respiratory health, we used the determination of CC16 and surfactant protein D (SPD) in serum (Studies 3 and 4).

Under normal conditions, the airway epithelium, together with the vascular endothelium, restricts the penetration of exogenous particles and macromolecules from the airway lumen into the interstitium and blood. The integrity of the tight junctions is regarded as the major factor in providing barrier properties to the airway epithelia. Changes in the permeability of the lung epithelial barrier were originally detected by measuring the protein or albumin concentration in the broncho-alveolar lavage fluid (BALF) (57;58) or by measuring the concentration in the blood of radio-labelled tracer compounds such as bovine serum albumin (BSA; MW 69000 Da) or diethylenetriamine pentaacetate (DTPA, MW 492 Da) instilled in the lung (59;60). In case of normal epithelial function and intact tight junctions, only small amounts of intratracheally introduced tracers reach the blood. The disruption of the epithelial barrier results in an increased movement across the airway mucosa (3). This increased permeability is a bi-directional event, i.e. it leads to an increased transport of serum components such as water, solutes and proteins into the air spaces and a concurrent flow of endogenous and exogenous macromolecules from the air spaces into the blood (61). Although the increased permeability of the lung epithelial barrier is most often only transient, the brief period of barrier disruption might present an opportunity for facilitated entry of compounds into the system which then can exert detrimental activities at locations in the body that would normally be inaccessible for them (61). Inhalation of smoke (62) or ozone (61) and intratracheal instillation of LPS (63;64) have been found to increase lung epithelium permeability. Lung hyperpermeability can not only be caused by compounds acting via the air spaces, but also by compounds administered systemically, e.g. an intra-peritoneal injection with 4-ipomeanol (65). Also changes in lung homeostasis can cause altered permeability, e.g. decreases of glutathione (66) or exercise (67).

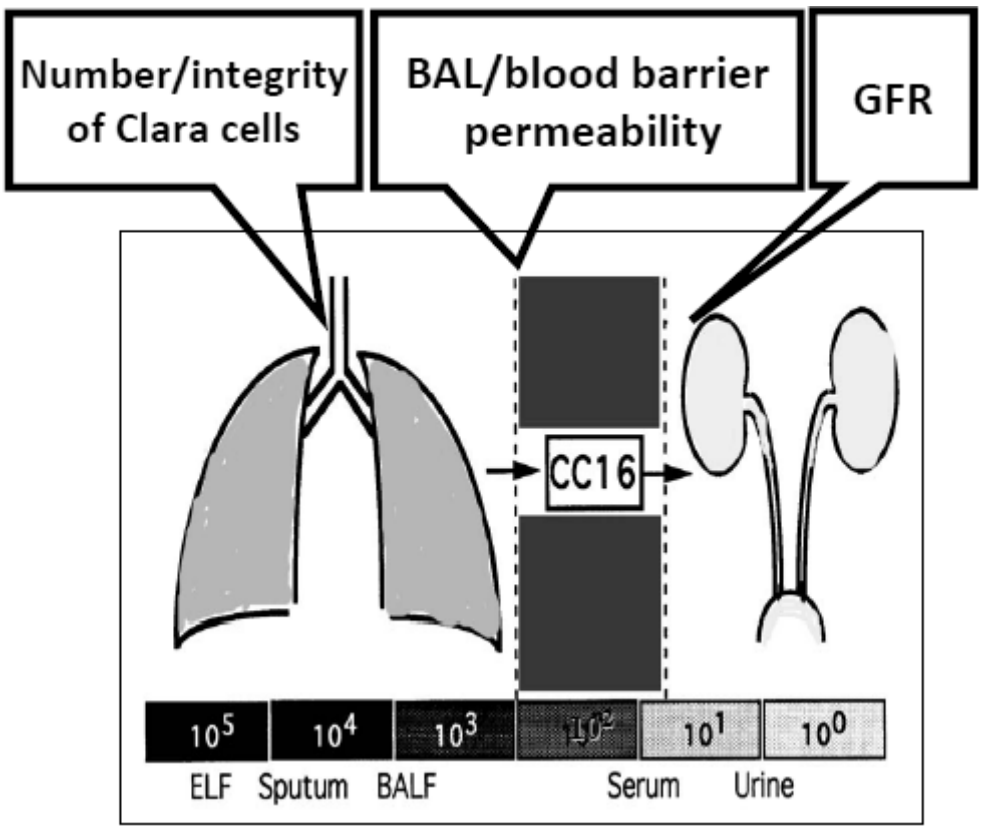

Figure 2 Concept of pneumoproteinemia and the determinants of the serum concentration of a pneumoprotein, illustrated by the case of CC16 (ELF: epithelial lining fluid, BAL(F): broncho-alveolar lavage (fluid), GFR: glomerular filtration rate)

The concept of pneumoproteinemia was already introduced in 1998 and is based on the hypothesis that the determination of the concentration of lung-specific secretory proteins in serum is useful for the evaluation of lung disorders, similarly to the utility of proteinuria in kidney diseases involving the glomeruli (62). Figure 2 illustrates the concept of pneumoproteinemia using the example of the Clara cell protein CC16. The main determinants of the serum concentration of a pneumoprotein are its synthesis in the lung, its transport to the blood and its elimination via the kidneys. The Clara cell protein CC16 is also referred to in literature as CC10, urine proteine-1 and human protein-1. Clara cells are present in the respiratory epithelium along the bronchial tree (3). The functions of Clara cells are mainly oriented towards the protection of the respiratory tract. Clara cells act as stem cells in the repair of bronchial epithelium, have a high xenobiotic transformation capacity and secrete several substances with important biological activities (68). Clara cells are considered to be a privileged target of many pneumotoxicants (65). CC16 (16 kDa) is one of the major proteins secreted by the Clara cells. Cytotoxic or irritating insults causing a decrease of the Clara cell number or activity (CC16 synthesis) result in a reduction of CC16 protein in the lung. Reports state that the majority of CC16 is secreted in the terminal bronchioles, suggesting that changed levels of CC16 in BALF are predominantly caused by effects in the deeper lung (3). In non-smokers, the concentration of CC16 in the epithelial lining fluid has been estimated to be approximately 1000 times higher than in blood. Because of the large gradient, small

changes of the alveolar barrier permeability can generate significant changes of the CC16-concentration in blood. Significant changes of the levels of CC16 in serum have been observed for several pulmonary diseases, see Table 1. When assessing serum concentrations of pneumoproteins, renal clearance should be taken into account as it can significantly alter the serum concentrations. For instance, CC16 concentration is serum is dramatically increased in patients with end-stage kidney failure (3).

The processes described above for CC16 are also relevant for other proteins produced in the respiratory tract, although differences in production (e.g. produced by other cell types) and characteristics (e.g. molecular size) can result in a response to a toxic insult which differs from that of CC16. Surfactant-associated protein D (SPD) is a large (160 kDa), multimeric, water-soluble collagenous glycoprotein produced by pneumocytes type II and Clara cells. The functional properties of SPD are not yet fully elucidated, but SPD does not contribute to the surface-active properties of the surfactant complex. Structural analogy with other proteins suggests that it might play a role in local host defence (3). Contrary to the total phospholipid content, levels of SPD in the broncho-alveolar lavage fluid of smokers have been found to be significantly decreased (69). SPD is also detectable in serum and is prominently increased in patients with pulmonary fibrosis, alveolar proteinosis, tuberculosis and sarcoidosis (3). Moreover, changes in serum SPD levels parallel the changes in health status over a 3 month period in patients with severe COPD (70).

Table 6 shows environmental and lifestyle characteristics which are associated with altered levels of CC16 or the CC16 to SPD ratio in serum. Typically, acute insults to the respiratory system resulted in increased levels of CC16 in serum, whereas decreased serum concentrations of CC16 have been observed in cases of chronic exposure to toxicants such as tobacco smoke and occupational exposures. The ratio of CC16 to SPD in serum integrates the changes of both biomarkers.

Table 6 Overview of changes in serum of CC16 or CC16/SPD ratio observed in studies investigating environmental and behavioral modulators of respiratory health.

Marker	Exposure/treatment	Change	Reference
CC16	wood smoke (acute)	+14 %, $p < 0.005$ (20h after exposure)	(71)
	tobacco smoke (acute)	↑ 2x, $p < 0.001$	(62)
	Ozone (acute)	+10 %, $p < 0.05$	(72)
	infant swimming	- 23 %, $p = 0.01$	(73)
	chlorinated pool attendance	- 30 %, $p < 0.002$	(74)
	cigarette smoking	-30 %, $p = 0.005$	(75)
	cigarette smoking	- 30 %	(3)
	cigarette smoking	-30 %, $p = 0.0014$	(76)
	cigarette smoking	-30 %, $p < 0.0001$	(77)
	NOx	↓	(78)
	aluminium dust	↓ $p = 0.006\%$	(79)
CC16/SPD ratio	infant swimming	- 30 %, $p = 0.003$	(73)

In Study 1, we used the CC16 concentration in serum to demonstrate the kinetics of the cigarette smoke-induced changes of lung permeability. The determination of CC16 in serum allowed us to detect effects at a sensitivity which was clearly higher that that of the conventional approach of measuring albumin in broncho-alveolar lavage (For more details, see Chapter 7.1). CC16 in BALF and serum were also determined in Study 2. The changes of CC16 in BALF paralleled the morphological changes of the lung-epithelial barrier as observed by electron microscopy in an experimental mouse model investigating the interaction of allergy and exposure to elemental carbon ultrafine particulate matter (study 2). Study 2 also confirmed the potential value of CC16 in serum for the detection of changes of the lung permeability. The results of CC16 in BALF and serum illustrated the complex interplay between immune sensitization/challenge, exposure to ultrafine particles and antioxidant treatment. For more details, see Chapter 7.2.

The use of the concept of pneumoproteinemia in 2 cross-sectional studies (Study 3 and 4) is described in Chapter 5.2.

5.1.2 Induced sputum

The aim of sputum induction is to collect an adequate sample of secretions from lower airways in subjects who do not produce sputum spontaneously. Inhalation of isotonic or hypertonic solutions administered by nebulisation has been demonstrated to induce a small amount of airway secretion that can be expectorated and analyzed (80). The analysis of cells and mediators in induced sputum has been widely applied for studying bronchial inflammation in asthma (81). Changes of sputum constituents have also been observed in COPD, cystic fibrosis and allergic rhinitis (see Table 2). Few studies have looked at the impact of exposure to occupational agents in healthy subjects using nasal lavage analysis (80). Vandenplas et al. identified sputum eosinophilia as an early marker of specific bronchial reactivity to occupational agents, which may help to identify subjects who develop an asthmatic reaction only after repeated exposures (82).

We have performed exploratory investigations on a limited set of induced sputum samples from COPD-patients (N=13), kindly provided by Dr. Frans van Overveld (Immunologist, Melsele). The samples were collected according to the ERS recommendations (83). We only used these samples to verify our analytical capabilities for induced sputum samples. No conclusions related to the disease were drawn from these samples as we did not have any information about the samples apart from the COPD diagnosis and the fact that the samples had been treated with dithiothreitol (DTT). We determined the CC16 and uteroglobin-1 concentrations in the samples using our in-house latex immunoassay (84). Uteroglobin 1 (UGRP-1) is a 10-15 kDa homodimeric lung specific protein belonging to the same family as CC16 and it is supposed to share some of its anti-inflammatory properties although its precise functions are still unknown (85). The total protein concentration in the samples was determined using the fluorometric Quant-IT assay (Invitrogen Ltd. Paisley, UK). Figure 3 shows the individual results for CC16 and UGRP-1. Both proteins were detected in all samples. The range of the CC16 concentrations was remarkably wider than that of UGRP-1. The geometric mean of UGRP-1 in the COPD patients in our experiment was about twice as high as that for controls as reported by de Burbure et al. (6).

We also assessed the relationships of both proteins with the total protein concentration; the results are provided in Figure 4. Whereas CC16 displayed a statistically significant correlation with total protein, UGRP-1 did not. Possibly UGRP-1 secretion is dependent on parameters which can differ significantly between COPD-patients and which can eventually lead to significantly different secretions. Significant differences of UGRP-1 secretion are likely to persist irrespective of the total protein concentration which is rather a marker of the induced sputum quality.

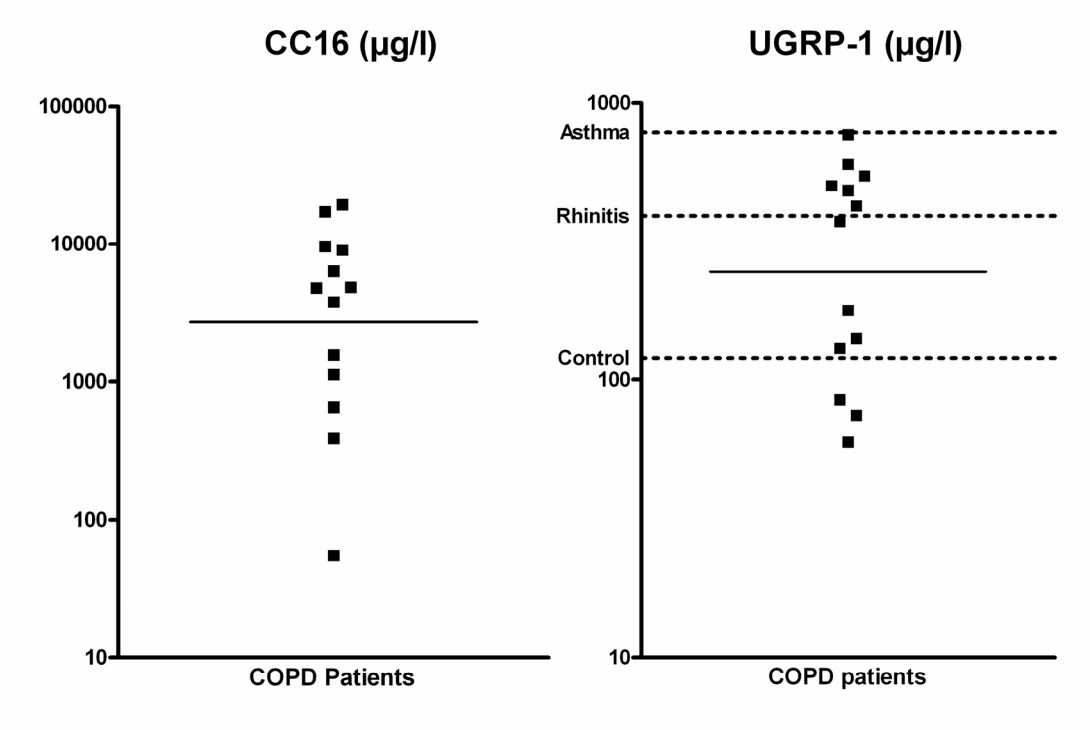

Figure 3 Concentration of CC16 and UGRP-1 in induced sputum samples of 13 COPD patients. The geometric mean is marked by a continuous line. The levels (geometric means) of UGRP-1 as found by De Burbure et al. (6) for controls, allergic rhinitis patients and asthmatics are shown as dotted lines in the chart for UGRP-1.

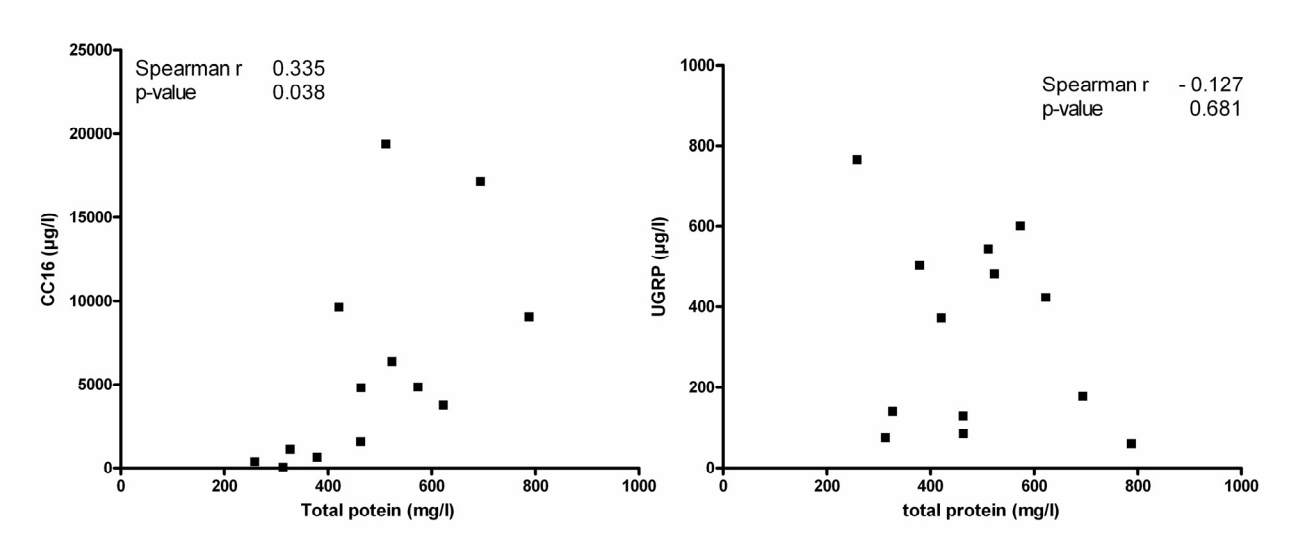

Figure 4 Relationships between CC16 or UGRP-1 and the total protein levels in induced sputum samples of COPD patients. Statistical analysis: non-parametric correlation analysis (Spearman)

Overall we concluded from these exploratory results that we were able to measure CC16 and UGRP-1 in processed samples of COPD patients. Our measurement methods for pneumoproteins could thus be applied on samples which had previously been processed with DTT. Nevertheless, the wide range of CC16 concentrations required further investigations. The difference between the relationship of CC16 and UGRP-1 with the total protein concentration might be indicative of differently regulated secretions.

No further experiments with induced sputum were conducted because we were not able to organise a collaboration to have access to more sputum samples.

5.1.3 Nasal lavage

The collection of nasal lavage fluid (NALF) is a non-invasive approach which recently gained more attention because it was found to mirror changes relevant to the deeper lung (86). Nasal secretions are a complex mixture of water, electrolytes, proteins, lipids and sugars derived from many sources. Serous and mucous glands which secrete the host-defence proteins are plentiful in the nasal mucosa. Plasma proteins may diffuse during periods of vascular permeability or may diffuse from the interstitial tissues into glands and consequently may become incorporated into the glandular secretions (87). Clear clinical, epidemiological and pathophysiological associations between upper- and lower airway inflammation have been observed between rhinitis and asthma suggesting that inflammatory cells may play a key and similar role in both of these syndromes. If one believes that the airway mucosa behaves as a continuum from the nose down to the bronchi, findings from the nasal secretions could be a proxy of those obtained in the less accessible lower airways (4). Barraza-Villareal et al. found a statistically significant decrease of interleukin 8 (- 22 %, $p = 0.002$) in the nasal lavage fluid of asthmatics (2). Ghafouri et al. analysed nasal lavage samples (6 non-smokers and 7 smokers) using two-dimensional gel electrophoresis (2-DE), proteins were identified with peptide mass fingerprinting using matrix-assisted laser desorption/ionization time of flight mass spectrometry. Different levels of CC16, lipocortin-1 and α-antitrypsin were found in the smokers group, respectively -89% ($p = 0.01$), + 70 % ($p = 0.003$) and +250% ($p = 0.02$) relative to the control group.

Nasal lavage fluid was used in Studies 3 and 4 as an information source for the assessment of the possible impact of environmental and behavioural parameters on the respiratory health of adolescents. Protein concentrations in NALF were adjusted for the variable dilution of the recovered epithelial lining fluid, either by calculating the absolute amount of recovered protein or by adjusting the concentration in NALF with the plasma/NALF concentration ratio of urea (87;88). More details about the outcomes of these investigations are provided in Chapter 5.2.

5.1.4 Exhaled breath

The analysis of gaseous constituents in exhaled breath as a way of monitoring inflammation and oxidative stress in the lung is a very appealing approach because it is completely non-invasive; a characteristic which significantly facilitates sample collection. Especially when sensitive online measurement systems are available, this approach has significant advantages. As shown in Table 3, only a limited number of markers have been successfully used in exhaled breath, i.e. nitric oxide (NO), carbon monoxide (CO), ethane or pentane.

A wealth of studies analysing exhaled NO of diseased individuals has been published (see Table 3). Mobile and highly sensitive sample collection and measurement systems are commercially available. The American Thoracic Society and the European Respiratory Society jointly published standardized procedures for the online and offline measurement of exhaled nitric oxide (89). Apart from some non-enzymatous sources, endogenous NO is derived from L-arginine by the enzyme NO synthase (NOS) of which at least three distinct isoforms exist. Two of these enzymes (neuronal and endothelial NOS) are constitutively expressed and are activated by small rises in intracellular calcium concentration, secondary to cell activation. The third enzyme is inducible (iNOS), and has a much higher level of activity. Induction of iNOS can occur by inflammatory cytokines, endotoxin and viral infections (90). NO is produced along the entire length of the human airways, although the cellular source of the NO gas in the lower respiratory tract is not certain yet. The NO in the lower respiratory tract is of mixed origin and may be produced by airway and alveolar epithelial cells. The conducting airways secrete NO into the lumen which mixes with alveolar NO during exhalation, resulting in the observed expiratory concentration. The paranasal sinuses produce a high level of NO. The contribution of endothelial-derived NO is minimal (10).

Exhaled CO and hydrocarbons are believed to be markers for oxidative stress, but considerably fewer studies using these markers have been published. The main source of endogenous CO is the enzyme heme oxygenase which is upregulated during episodes of oxidative stress (10). Ethane and pentane are considered to result from in vivo peroxidation of respectively n-3 polyunsaturated fatty acids and n-6 poly unsaturated fatty acids (91).

Exhaled NO has frequently been used for the detection of effects of various environmental pollutants on the respiratory health of healthy individuals (see Table 7). Typically, pollutants inducing inflammatory processes in the lung are associated with increased levels of NO in exhaled air. Paradoxically, decreased levels of NO have been observed after chronic tobacco smoke exposure, exposure to NO and pure oxygen. Possibly this paradox can be explained by a negative feedback mechanism. A possible limitation

of NO might be that it is most sensitive to changes of the inflammatory status and that it tends to normalize during stable inflammatory situations, e.g. steroid-treated asthma (10).

Table 7 Overview of the effects of environmental pollutants on respiratory health measured as changes of exhaled NO.

Exposure	Effect	Reference
PM2.5	↑	(2)
wood smoking (acute)	+9%, $p < 0.05$ (3h after exposure)	(71)
chlorinated pool attendance	↑, $p < 0.001$	(40)
allergens	↑	(10)
pollen	↑	
air pollution	↑	
ozone	↑	
chlorine dioxide	↑	
formaldehyde	↑	
LPS	↑	
cigarette smoking	- 15 %, $p < 0.01$	(8)
cigarette smoking	- 50 %, $p < 0.01$	(92)
cigarette smoking	↓	(10)
NO	↓	
100% O_2	↓	

The determination of exhaled NO was used in the Studies 3 and 4, more details are provided in Chapter 5.2.

5.1.5 Exhaled breath condensate

Exhaled breath condensate (EBC) is produced by cooling or freezing exhaled air. Its collection method is totally non-invasive. Abnormalities in condensate composition are believed to reflect biochemical changes in the airway lining fluid. Next to water, EBC consists of ions (determining the pH and conductivity), oxidants (H_2O_2), reaction products from oxidative or nitrosative stress (nitrotyrosine, nitrite, nitrosothiols, 8-isoprostane), endogenous eicosanoids (LTB_4, LTC_4, LTD_4, LTE_4) and proteins. Effros et al. estimated that the respiratory fluids represented between 0.01% and 2% of the EBC volume (93). Different levels of non- or semi-volatile molecules have been detected in exhaled breath condensate of diseased people as compared to healthy controls (see also Table 4). Relative to the large number of studies on individuals with a respiratory disease, only few studies have been conducted to screen individuals exposed to air pollutants. A limited number of studies looking at the effects of cigarette smoking has been published (Table 8).

Table 8 Markers determined in exhaled breath condensate which were significantly different in smokers vs. non-smokers

Marker	Exposure/treatment	Change	Reference
Hydrogen peroxide	cigarette smoking	↑5 x, p< 0.01	(94)
nitrate (NO_3^-)	cigarette smoking (acute)	+ 50%, p < 0.02	(95)
nitrosothiols	cigarette smoking	↑4 x, p< 0.01	(8)
8-isoprostane	cigarette smoking	↑2 x, p< 0.05	(18)

After sample collection (several minutes), the small droplets of exhaled breath condensate are collected from the condensation vessel. All constituents in EBC (except water) are present at very low concentrations. Therefore rigorous sample collection and processing procedures are a prerequisite. The European Respiratory Society has published in 2005 a paper providing a number of methodological recommendations and listed also some unresolved questions (96). Despite various efforts, e.g. studies comparing different sample collection systems (97;98), the same issues related to the lack of standardisation and to methodological limitations are still being mentioned in recent reviews, e.g. 2007 (99), 2010 (80) and 2011 (100). Currently, EBC is generally considered as still not being ready for clinical applications. Notwithstanding the technical difficulties, an increasing number of studies using exhaled breath condensate is being conducted and published, see Figure 5.

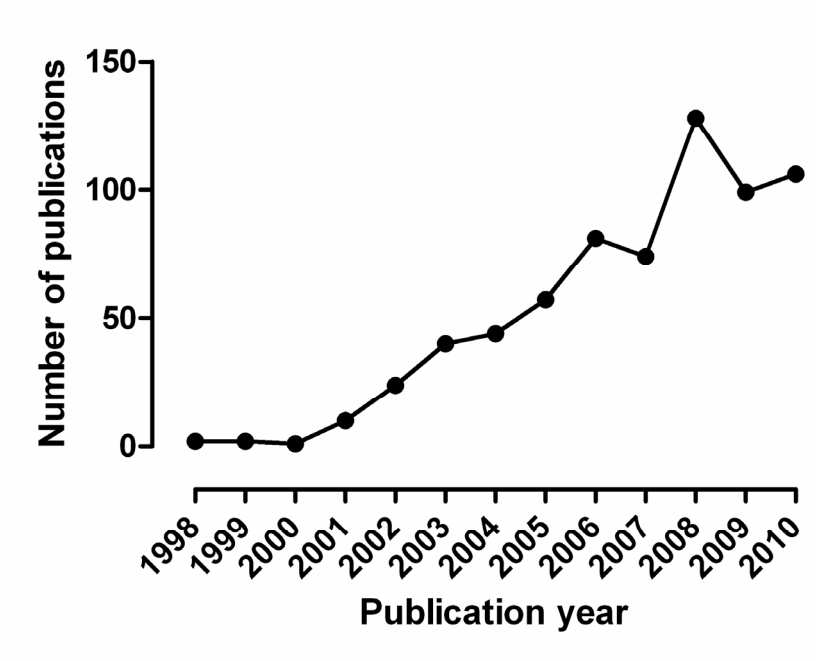

Figure 5 Number of publications with exhaled breath condensate published yearly. Information was obtained from Pubmed (http://www.ncbi.nlm.nih.gov/pubmed) using the search terms *"exhaled breath condensate"[Text Word] AND "xxxx"[Publication Date]*.

5.1.5.1 Exploratory study using the ECoScreen EBC sample collection system

The ECoScreen (Jaeger, Wurzburg, Germany) is one of the most commonly used, commercially available exhaled breath condensate collection devices. We built our initial experience with the ECoScreen system during an add-on experiment to an ongoing study (101). Male (5) and female (9) university students in physical education were asked to repeat a standardized swimming session in a chlorinated indoor pool and an indoor pool sanitized using the copper/silver (Cu/Ag) method. Exhaled breath condensate samples were collected immediately before and at 45 min. and 4h after the swimming session. One week or more separated the swimming sessions. Study participants were asked to breathe tidally for about 10 minutes through the mouthpiece equipped with a two-way valve. Immediately behind the mouthpiece, a cooling device condensed the water together with the aerosolized compounds possibly originating from the lung. The hydrogen peroxide concentration was determined according to the method described by Hyslop et al. (102) and van Beurden et al. (103) which we adapted to the use of a plate reader (96-wells). Van Beurden et al. (103) reported a detection limit of 0.025 µM and a baseline level in EBC of healthy individuals of 0.21 ± 0.03 µM. In our methodology, p-hydroxyphenyl acetic acid (1mM) and horseradish peroxidase (15 U/ml) were added to 250 µl of exhaled breath condensate. P-hydroxyphenyl acetic acid is oxidized by hydrogen peroxide to the stable fluorescent product 2,2'-dihydroxybiphenyl-5,5'-diacetate which can be stored for longer periods. The main advantage of this method is the fact that the reaction with hydrogen peroxide is performed immediately after sample collection, thus limiting possible losses during sample storage. Fluorescence was measured using a

Fluostar Optima (BMG LABTECH, Ortenberg, Germany) with an excitation wavelength of 295 nm and an emission wavelength of 405 nm. Figure 6 shows a typical hydrogen peroxide calibration curve. It can be noted that the performance of the assay in the area around 0.2 µM is reduced. We nevertheless decided to use this method because we prioritized other aspects of the EBC assay development and this method was technically and logistically less demanding. Moreover during the development phase of the EBC assay, we aimed for marked increases relative to baseline, e.g. > 2-fold increases.

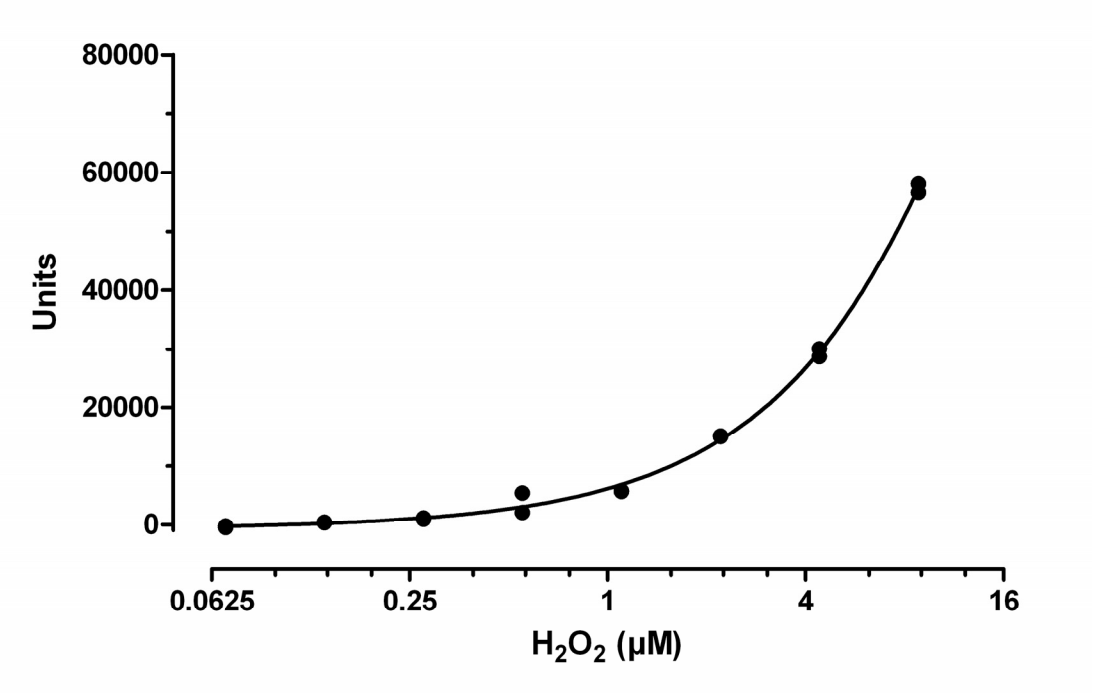

Figure 6 Typical H_2O_2 calibration curve (duplicate samples) obtained using the modified methodology of Hyslop et al. (102) and van Beurden et al. (103).

During the sample collection, we experienced a drawback of the ECoScreen system. The system was built around a single aluminium part on which the exhaled breath condensed. Small droplets which were formed during sample collection had to run down in a plastic cup. When performing a series of sample collections, the same aluminium part needed to be rinsed, cleaned and dried in between, which was a logistical burden but also a potential source of cross-contamination. Additionally, holding the mouthpiece of the ECoScreen in the mouth for 10 min was experienced as uncomfortable because of excessive saliva production.

After sample collection periods of 10 minutes we collected between 470 and 1625 µl of EBC, Figure 7 shows the distribution of EBC-yields. The results of the hydrogen peroxide determinations are shown in Figure 8. Overall, the mean hydrogen peroxide concentration was 0.27 µM with a relative standard deviation (RSD) of 65%. A trend towards increased hydrogen peroxide levels was observed at 4h after swimming. The increase observed after swimming in a chlorinated pool tended to be more pronounced than for the Ag/Cu disinfected pool, although the range of H_2O_2 concentrations at 4h after swimming in a chlorinated pool was significantly larger than that of the corresponding time point for the Ag/Cu pool.

Overall comparison using the non-parametric 1-way Kruskal-Wallis test followed by post-hoc, pair-wise comparisons using the Dunn test did not yield any statistically significant differences between the different time points for the chlorinated pool. The overall analysis for the pool disinfected using the Ag/Cu method showed that the medians differed statistically significantly (P= 0.036), but pair-wise comparisons did not detect a statistically significant difference.

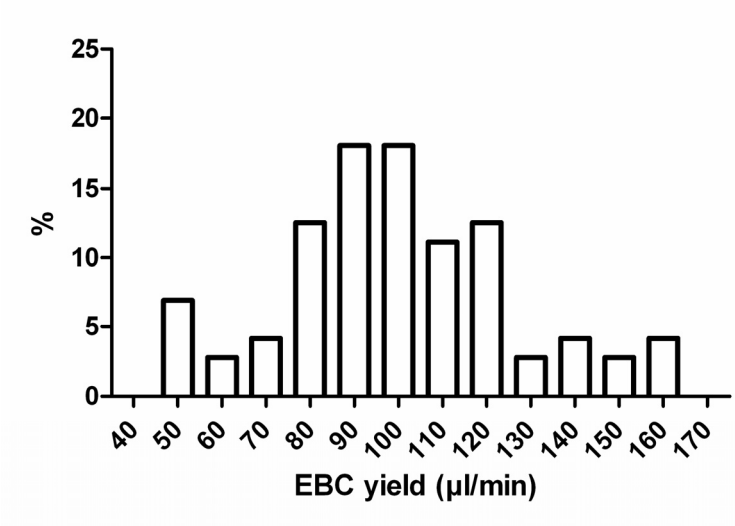

Figure 7 Frequency distribution of EBC yields (µl/min) obtained with ECoScreen during 10 min. sample collection periods, total of 72 EBC sample collections.

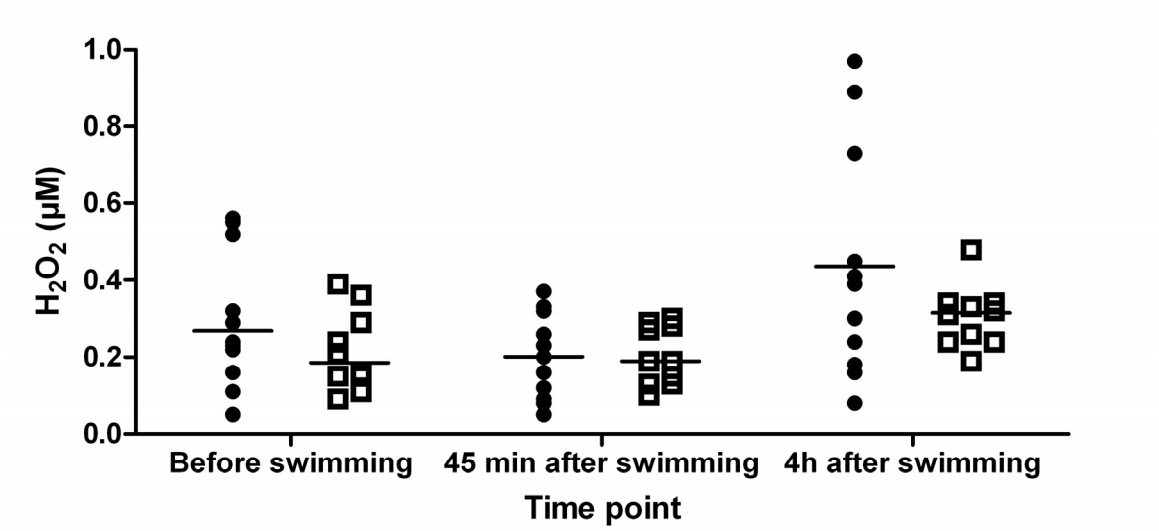

Figure 8 Hydrogen peroxide concentration in exhaled breath condensate of swimmers before and after a standardized swimming protocol. Closed symbols: chlorinated pool, open symbols: pool disinfected with Ag/Cu-method.

5.1.5.2 Creation of custom-made EBC sample collection systems

Based on our experiences during the first study, we chose to develop a custom-made exhaled breath condensation system built around a condensation vessel which was entirely made out of glass (Patent application EP 2 227 142 A2, Application number: 08851238.9). We opted for glass because of its inert nature and the limited protein adsorption. Recent literature has justified our choice for glass (97). The exhaled breath condensate system consisted of a mouthpiece which allowed study participants to deal more easily with saliva, connected to a non-rebreathing valve, a saliva-trap and PTFE tubing towards a cooling unit with the glass condensation vessel (see Figure 9 and Figure 10). The glass condensation vessel could be removed entirely from the cooling unit to allow an efficient transfer of the sample. Multiple condensation vessels were produced, which allowed a more thorough cleaning and drying procedure in-between sample collections.

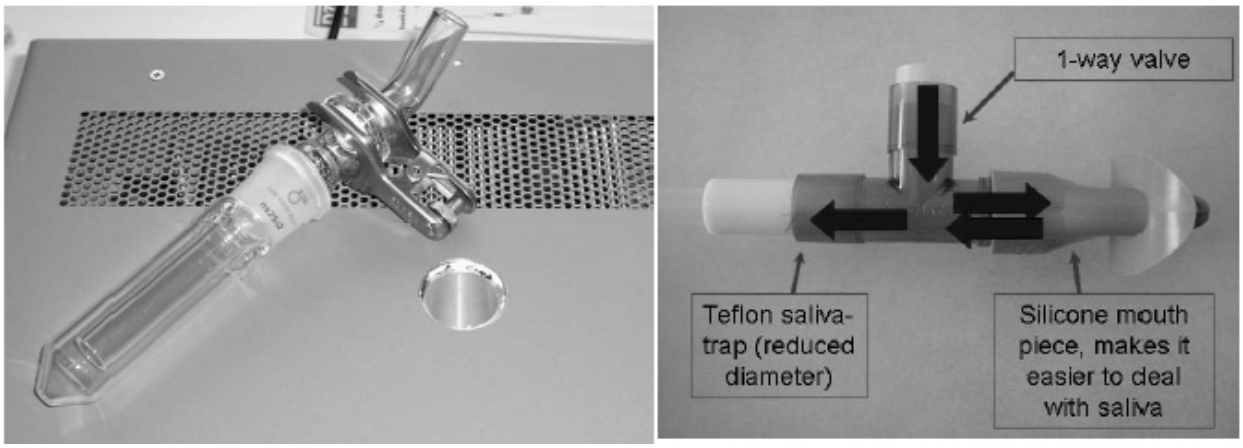

Figure 9 Core elements of custom-made EBC-collection systems for humans, left: glass condensation vessel with refrigeration unit (opening), right: mouthpiece with non-rebreathing valve and saliva trap.

Figure 10 EBC sample collection with custom-made device.

Whereas the ultimate goal was to use the system for the screening of people, we also developed an analogous system for exhaled breath collection from rats. An *in vivo* apparatus would allow us to better optimise the EBC assay under controlled conditions. By using well-known lung toxicants, we expected learn about the dynamic range of changes which could be anticipated for markers in EBC. Additionally, if a working EBC-assay with rats could be established, it could also be used as a surrogate for broncho-alveolar lavage, thus allowing repeated measurements from the same set of animals and consequently limiting the number of animals required to conduct toxicology studies with multiple time points. The exhaled breath condensate collection device for rats was based on the same core principle as the system for humans, i.e. a glass condensation vessel in the shape of a 50 ml centrifugation tube, but it was adapted to be used with a glass flow-past exposure tube. The flow through the system could be set precisely in order to provide sufficient air to the rat while limiting dilution of the EBC sample with excess water originating from non-exhaled air. The system was built to collect samples from 2 rats simultaneously (see Figure 11 and Figure 12).

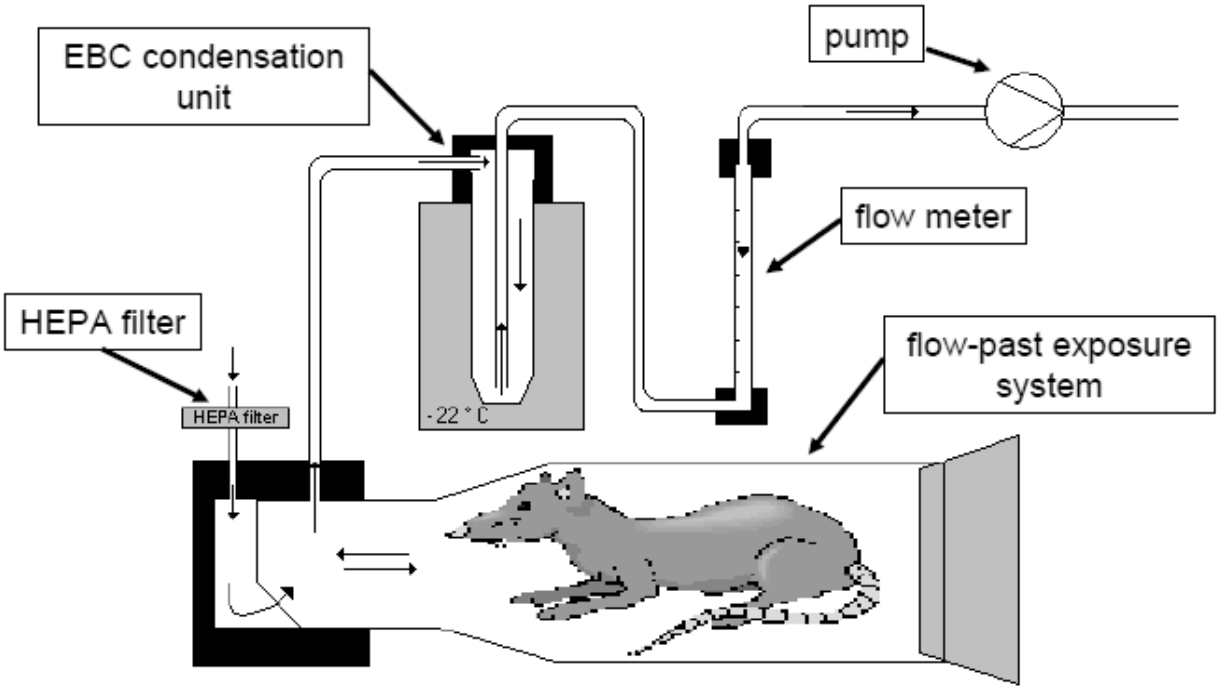

Figure 11 Scheme of custom-made EBC collection system for rats

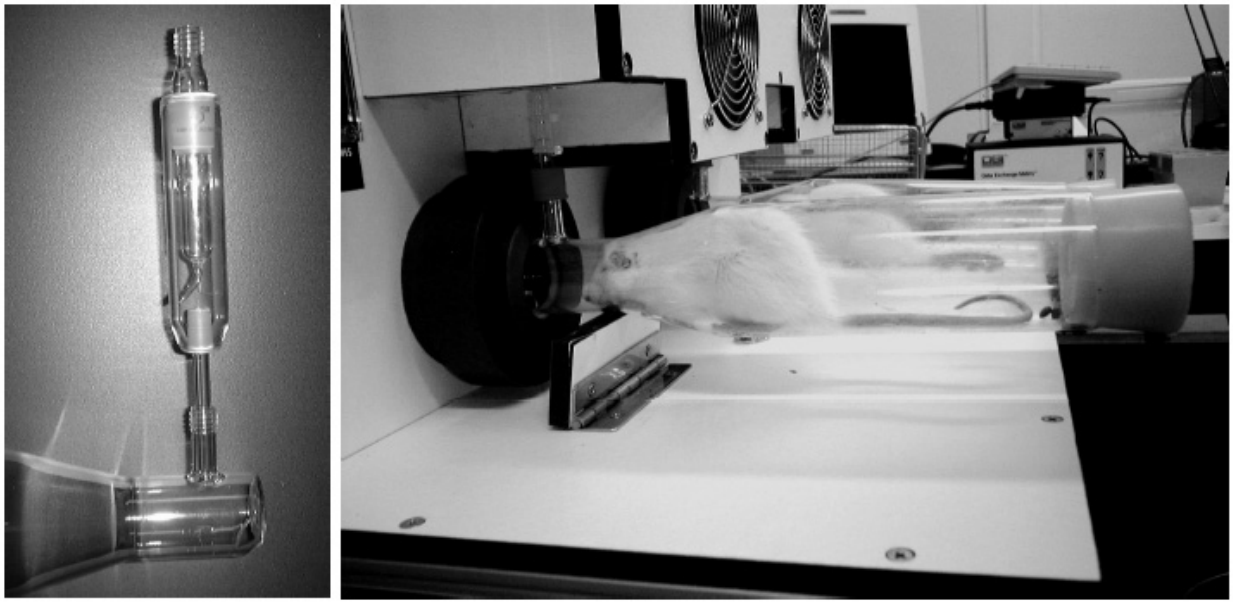

Figure 12 Custom-made EBC collection system for rats, left: glass condensation vessel connected to a glass flow-past exposure tube, right: EBC sample collection from 2 rats simultaneously with custom-made system.

5.1.5.3 EBC experiments with rats

Initial tests showed that there was a clear contribution of the rat to the EBC sample; about 45% of the liquid collected in the system originated from the rat. We considered that lung-specific proteins in EBC would be the most interesting source of information. Therefore, we tried to determine CC16 in EBC from untreated rats using our in-house CC16 latex immunoassay (104), but did not detect its presence. We were also unable to demonstrate the presence of albumin using an ELISA. We tried to detect proteins using SDS-PAGE (PhastSystem™, GE Healthcare Europe GmbH, Freiburg, Germany), followed by Western blotting on a PVDF membrane and silver staining. In an attempt to increase the sensitivity, we lyophilized pooled EBC samples before performing the SDS-PAGE and Western blotting procedure. We also used a more sensitive colloidal gold protein stain (BIO-RAD, Hercules, CA, USA). On a single occasion (1 gel), we observed protein bands (136, 69 and 59 kDa). Because we have not been able to repeat this result, we considered that the most likely explanation for this single result was a contamination of the sample or the buffers used during the electrophoresis or blotting procedure. After these series of negative results, we abandoned our efforts of trying to detect proteins in EBC of rats.

In a second experiment, we wanted to verify whether we were able to detect in EBC of rats treated with paraquat changed levels of hydrogen peroxide as a marker of oxidative stress (105;106). Four male Sprague-Dawley rats received a single IP injection of 30 mg/kg paraquat. EBC samples were collected immediately before and at 2.5h, 5h and 21h after exposure. These sample collection time points were based on the study by Wilhelm et al. who found increased levels in exhaled breath after i.p. injection with paraquat (40 mg/kg) with the maximum increase observed at 5h after treatment (106). The dose of

30 mg/kg instead of 40 mg/kg was selected because it was already half the LD_{50}-value. The sample collection period ranged between 60 and 90 min. The amount of collected EBC ranged between 170 and 290 µl and the yield was 3.1 ± 0.4 µl/min (mean ± SD). Figure 13 shows the hydrogen peroxide concentrations at the different time points. Whereas 2 rats showed an increase of the hydrogen peroxide concentration at 5h (+ 90% and + 50%), the hydrogen peroxide levels for the 2 other rats remained unchanged.

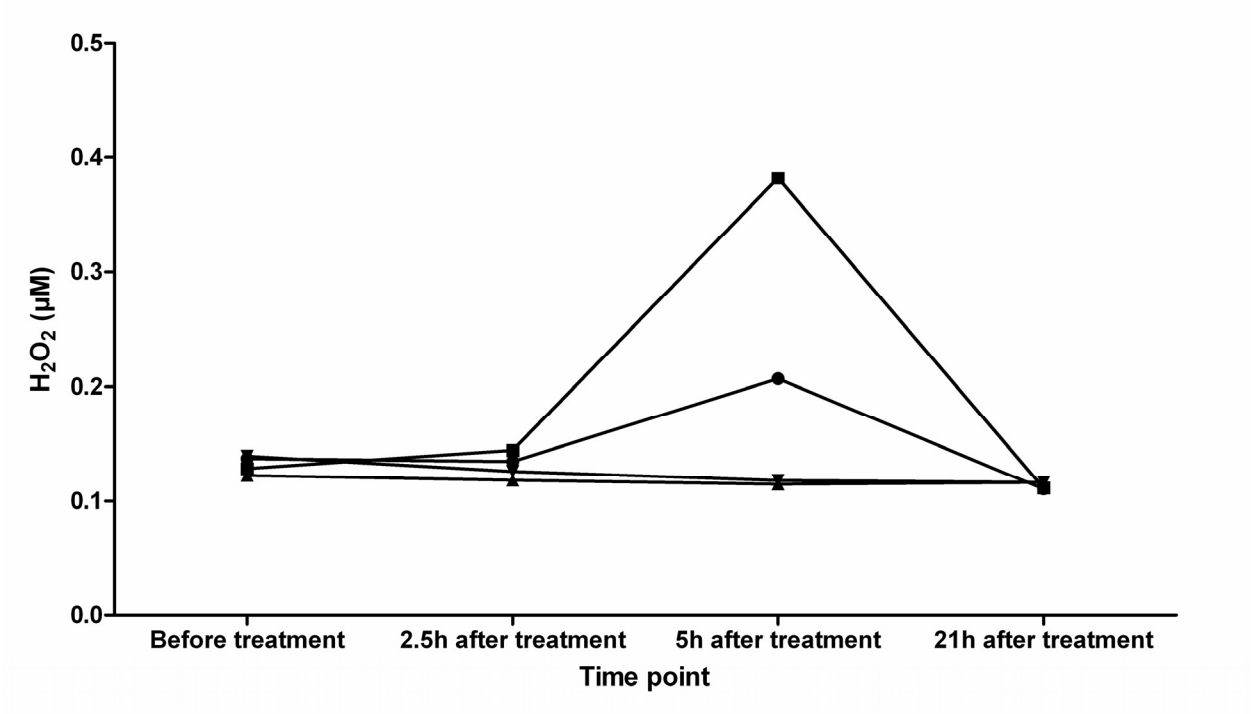

Figure 13 Hydrogen peroxide concentrations in rats treated with 30 mg/kg paraquat (i.p.), results of individual animals shown

Although the results of the experiment with paraquat partially suggested that EBC could be used to detect oxidative stress in the lungs of rats, we decided to give up the analysis of hydrogen peroxide in EBC of rats. We had also learned that any data on hydrogen peroxide would be very difficult to interpret because the rat had a quenching effect on the hydrogen peroxide concentration (data not shown).

In a final attempt to confirm that the rat contributed to the composition of EBC, we decided to screen samples using GC-MS (HP 6890 - HP MSD 5973, Agilent). Six samples of EBC of rats, and 6 corresponding samples of condensed air humidity were spiked with an internal standard solution containing d8-styrene, d8-naphthalene and d6-phenol. Peak identification was done by comparison of spectra of significant peaks of the total ion chromatogram (TIC) with mass spectral libraries (NIST2002, WILEY7). Relative yields (rat vs. air) were derived by semi-quantitatively relating peak areas to those of the internal standard.

As shown in Figure 14, there was clearly a contribution of the rat to the composition of the EBC. The toxicological utility of the compounds (acetone, acetic acid, propionic acid, butyric acid, and p-menth-1-ol) originating from the rat unfortunately appeared rather limited.

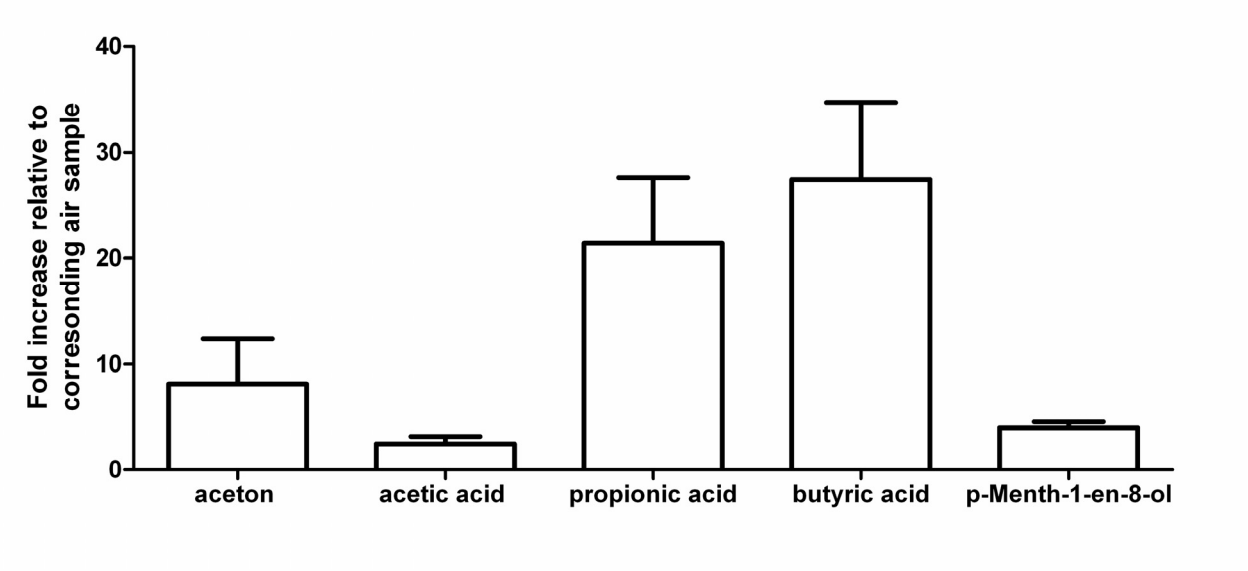

Figure 14 Compounds increased in EBC of rats as compared to condensed air humidity (mean +SEM), N= 6

Although it was not unlikely that LC-MS could well be able to detect larger and potentially more relevant biomolecules (eicosanoids?), we decided to stop our efforts in the development of the EBC assay with rats. Considering that no proteins could be detected, that no reliable determination of hydrogen peroxide was achieved and that it was required to move to advanced analysis techniques, it seemed highly unlikely that the EBC assay with rats would be able to help us in the development and the refinement of the assay with humans.

5.1.5.4 EBC collection from adolescents

In order to investigate the performance of our custom-made system, the collection of exhaled breath condensate was added to the assay battery of a large screening study (107). A total of 104 successful EBC samples were collected from 3rd and 4th grade secondary school students living in the area of Bastogne (girls 64, boys 40). Study participants were asked to breathe tidally through the custom-build EBC collection device during 6 minutes. In conformity with the ERS recommendations (96), the study participants wore a nose-clip. The temperature at the condensation vessel was set at -9 °C.

Planned analyses were the determination of hydrogen peroxide using the modified method of Hyslop et al. (102) and van Beurden et al. (103), and the determination of proteins using the fluorometric Quant-IT assay (Invitrogen Ltd. Paisley, UK). Additionally, 3 markers of oxidative stress, i.e. malondialdehyde, 4-

hydroxynonenal and heptanal would be determined by the research group of Prof. Mutti (Department of Clinical Medicine, Nephrology and Health Sciences, University of Parma) using LC-MC (108). Not every sample could be analysed for all parameters due to the limited sample volume. Additionally, a part of the aliquots for hydrogen peroxide became accidently useless for analysis. In total, 68 samples were analysed for hydrogen peroxide, 40 samples for proteins and 79 samples for malondialdehyde, 4-hydroxynonenal and heptanal. To verify the salivary contamination of the EBC samples, the remaining sample aliquots were used to check for the presence of α-amylase using a sensitive, semi-quantitative latex immunoassay.

The EBC yields are shown in Figure 15. Overall, the yields were lower as compared to the experiment with the ECoScreen, but this was most probably due to the fact that the study population was younger, thus having a lower respiratory minute volume.

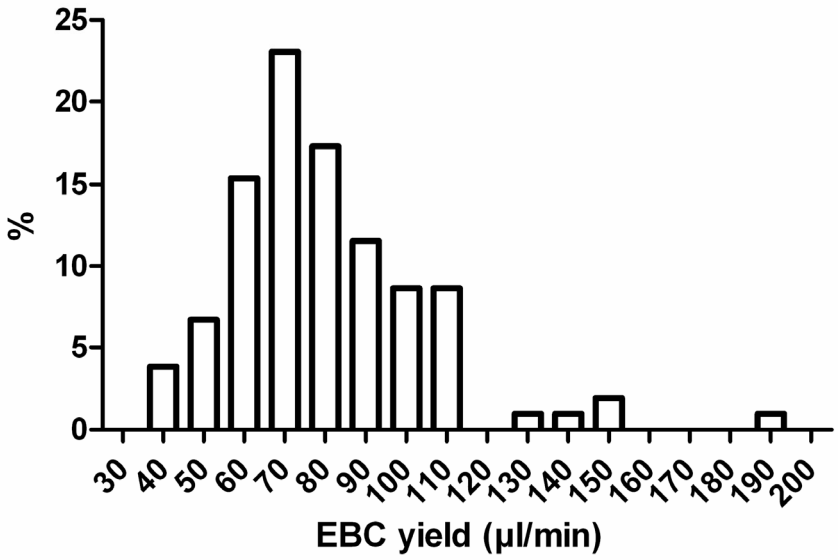

Figure 15 Frequency distribution of EBC yields (µl/min) obtained with a custom-made device during 6 min. sample collection periods, total of 104 EBC sample collections.

The average hydrogen concentration was 1.60 µM with a relative standard deviation (RSD) of 65%, while that of the first study (Chapter 5.1.5.1) was 0.27 µM (RSD 65%). The hydrogen peroxide concentration observed in the current study was also significantly higher than those reported by van Beurden et al. (103;109) and De Benedetto et al. (16) for healthy controls which ranged between 0.12 and 0.35 µM. Since the relative standard deviations were similar for both experiments, one could assume that the quality of the hydrogen peroxide standard was degraded in the second study, thus explaining the shift towards higher measured concentrations. On the other hand, Rosias et al. have reported average hydrogen peroxide concentrations ranging between 1.8 and 2.6 µM in healthy people (98). Clearly, several methodological issues related to determination of hydrogen peroxide in EBC still need to be

addressed. Maybe, a more generalised use of the online hydrogen peroxide measurement method as described by Gerritsen et al. (110) and Gajdocsi et al. (111) might turn out as a significant methodological improvement.

The protein determination was unsuccessful. Only 4 out of the 40 samples analysed gave a result above the lowest point of the calibration curve (25 µg/ml), and a numerical result (median value = 17 µg/ml) could only be obtained for 17 samples by extrapolating below the lowest point of the calibration curve (25 µg/ml). These results are not in line with the study by Effros et al. who measured an average protein concentration of 23 µg/ml in EBC samples of healthy controls (93;112). Malondialdehyde, 4-hydroxynonenal and heptanal were quantifiable in all analysed samples; the median concentrations were 7.83 nM, 2.52 nM and 40.17 nM respectively.

The EBC volumes and the concentrations of hydrogen peroxide, malondialdehyde, 4-hydroxynonenal and heptane for males and females were compared (see Figure 16). No differences were observed except for the EBC volume. Males generated statistically significantly more EBC, most likely due to a higher minute volume. The hydrogen peroxide concentration in EBC was statistically significantly correlated with the malondialdehyde and the 4-hydroxynonenal concentration, but not with the heptanal concentration (Figure 17). Also malondialdehyde and 4-hydroxynonenal (not shown) did not show a statistically significant correlation with heptanal although they were simultaneously measured in the same aliquot. Probably, the determinants of the heptanal concentration differ from those of the other investigated markers of oxidative stress. The correlations between hydrogen peroxide and malondialdehyde, and between hydroxyperoxide and hydroxynonenal showed that the hydrogen peroxide results were not “at random” although hydrogen peroxide concentrations were in the lower part of the measurement range.

Although the custom-made EBC collection device made serial sample collection much easier and an all-glass condensation vessel clearly was an improvement, many technical issues still remained. Study subjects which provided an EBC-sample during a sample collection period of 6 min. clearly had issues with excessive saliva. This problem has not been mentioned in literature although a 10 min. sample collection period is commonly quoted. Additionally, we detected saliva contamination of the EBC samples. We were unable to reliably detect proteins in EBC at the levels reported in literature. It is striking that several authors mention the presence of specific cytokines even though general agreement has not been reached yet for the total protein content of EBC. An issue raised by Sapey et al. holds true for all analytical procedures, i.e. when substances are quantified at concentrations approaching the lower end of the measurement range, the coefficient of variation increases significantly (113). Therefore, quantification limits instead of detection limits should be used.

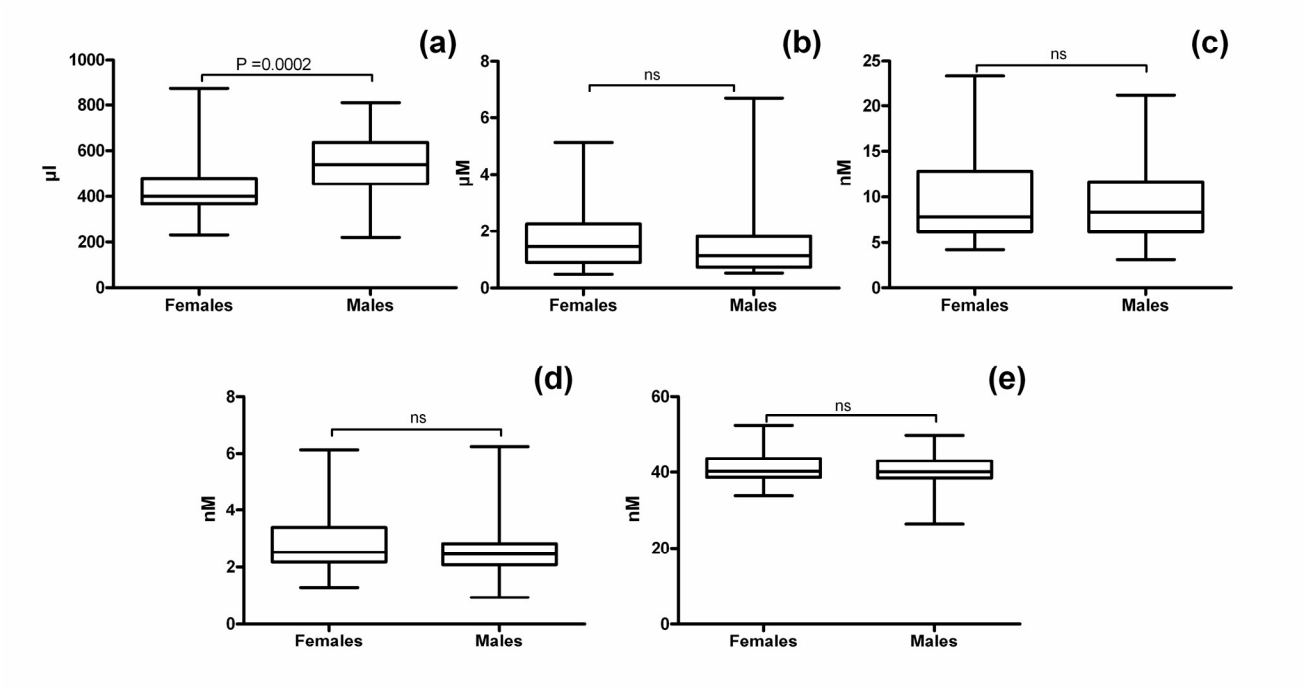

Figure 16 Comparison of the EBC volumes (a) and the concentrations of hydrogen peroxide concentration (b), malondialdehyde (c), 4-hydroxynonenal (d) and heptanal (e) between males and females. Pairwise comparisons were performed using Mann-Whitney U test, ns: not statistically significant.

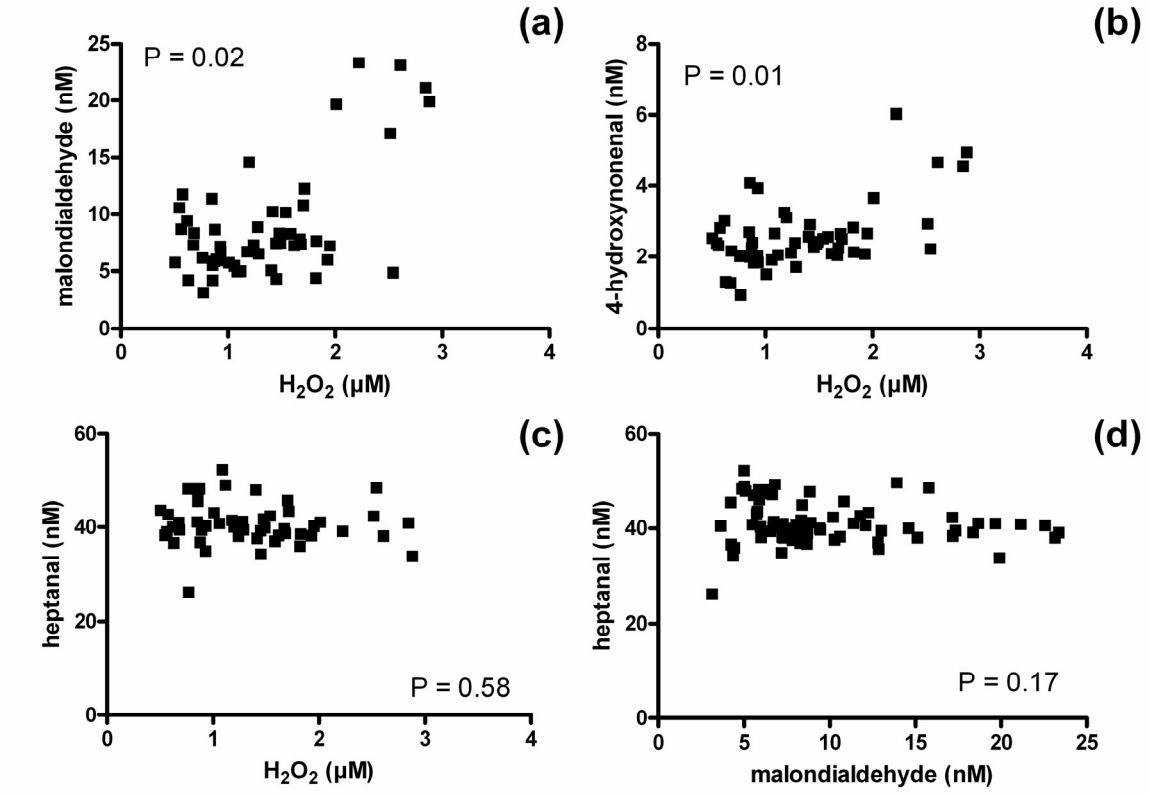

Figure 17 Correlations between hydrogen peroxide and malondialdehyde (a), 4-hydroxynonenal (b) and heptanal (c). Correlation between malondialdehyde and heptanal. Statistical test: non-parametric correlation (Spearman)

5.1.5.5 Conclusions exhaled breath condensate

Overall we identified the following issues with the EBC-assay:

- Sample collection needs to be very well controlled, because respiratory patterns have been found to influence the composition of EBC (114). Moreover the influence of background concentrations in the room air is still unknown.
- Although the impact of salivary contamination might differ for the different markers (99), the issue has not yet been resolved. Several authors have stated that salivary contamination did not impact their study results (103;115), but it is hard to believe that it did not, considering the large amounts of saliva people tend to produce during sample collection.
- The volume of an EBC-sample is most often small, i.e. ± 1ml. The number of aliquots which can be obtained from each sample, and consequently the number of markers or replicates which can be measured are limited.
- In order to adequately measure substances in EBC, advanced analytical techniques which require a high level of expertise are needed. Not every lab is able to set up such analytical capacity.
- Automated on-line measurement procedures of some markers, e.g. hydrogen peroxide or pH, might significantly improve the performance of the assay. Most of such markers are unfortunately rather unspecific, and their diagnostic value is often reduced.

We judged that we required a continued, better controlled EBC sample collection program and a shift towards significantly more sensitive and thus more advanced analytical methods to enable further progress. This escalation of the complexity of the EBC assay did not agree with our initial goal to set up the EBC assay as a tool which could be used for frequent measurements and screening of large populations. We therefore halted our efforts with the EBC assay.

5.2 Associations with respiratory health in adolescents

A cross-sectional survey of more than 800 adolescents performed by our research group resulted in a large database with information on personal and familial characteristics, the in- and outdoor environment, medical characteristics and antecedents, self-reported symptoms and biomarkers. We used this database to answer 2 research questions, i.e.

- Can non-invasive biomarkers of the upper and lower respiratory tract detect sub-clinical changes in the airways of adolescents with an active or passive exposure to cigarette smoke? (Study 3).
- Is wood fuel use associated with respiratory disease, sensitisation to aeroallergens, self-reported symptoms or changes of epithelial biomarkers in serum, exhaled breath or NALF? (Study 4)

We observed that CC16 in nasal lavage fluid was one of the first quantifiable changes in adolescent smokers with very low cumulated cigarette consumption (< 1 pack-year). The decrease in NALF might precede the decrease of CC16 in BALF and serum observed in smokers with much higher cumulated cigarette consumption (75;77). We also confirmed the cigarette smoke-induced decrease of exhaled NO at cumulated cigarette consumption levels which were significantly lower than in earlier studies (92), demonstrating the sensitivity of this non-invasive approach. More details about this study are provided in Chapter 7.3.

We observed lower CC16 concentrations and CC16 to SPD ratios in serum of adolescents with wood fuel use at home. These changes might reflect sub-clinical cell damage in the lower respiratory tract. Wood fuel use was also associated with higher risks of asthma, hay fever, and sensitization against pollen allergens. Although this study did not allow to elucidate the mechanisms underlying these associations, the results pointed towards a possible involvement of wood fuel use in the development of these respiratory effects. More details about this study are provided in Chapter 7.4.

6 Conclusions

The mechanistic understanding related to changed levels of the Clara cell protein (CC16) in serum and their relationship to changes of the Clara cell population in the lung was further elaborated in 2 *in vivo* studies. In an inhalation experiment with rats, CC16 in serum was found to be a very sensitive marker of permeability changes of the lung epithelial barrier. In an *in vivo* model investigating the interaction of elemental carbon ultrafine particles and allergy, the levels of CC16 in BALF and serum paralleled morphological changes in the respiratory tract. This demonstrated the relevance of the Clara cell population with respect to the inflammatory status of the lung and the usefulness of monitoring CC16 in BALF or serum as a marker of the lung status.

We successfully managed to determine lung-specific proteins in induced sputum samples of COPD-patients, but we were unfortunately unable to build further on this promising start. The exhaled breath condensate initially appeared to be a very promising new way to assess the status of the respiratory system, but we failed to set up a robust assay both in rats and in humans. After a long series of experiments, the methodological and analytical requirements to make this assay work appeared too burdensome to foresee a powerful diagnostic application of this assay in the near future.

A battery of non-invasive markers, i.e. CC16 in nasal lavage fluid and serum, albumin in nasal lavage fluid, surfactant associated protein D (SPD) in serum, and nitric oxide (NO) in exhaled breath was used in 2 cross-sectional studies on adolescents. We demonstrated the usefulness and sensitivity of two non-invasive approaches, i.e. CC16 in nasal lavage fluid and NO in exhaled breath, to detect early changes in the respiratory system of adolescent smokers well before the appearance of smoking-related changes in the deeper lung or altered lung function parameters. In a study on non-smoking adolescents, wood fuel use showed to be associated with a decrease of the CC16 to SPD ratio in serum; a change suggesting epithelial changes in the respiratory tract. Additionally, higher frequencies of asthma, hay fever and sensitization against pollen were observed among adolescents with wood fuel use. Using non-invasive approaches enabled us to screen a large study population and to identify significant and objective differences in the status of the respiratory system between exposed and non-exposed adolescents (non-smokers vs. smoker or no wood fuel use vs. wood fuel use).

Study 1 clearly demonstrated the acute effect of cigarette smoke on the epithelial permeability in an in *vivo* model. It was nevertheless a simplification of the situation of a human smoker. Several studies have observed decreased Clara cell numbers and serum CC16 concentration in smokers (3;77). It is unclear whether chronic animal models of cigarette smoke exposure will respond in a similar way, because chronic exposure to ozone has been shown to cause an increased production of CC16 in the lung (116)

and also in Study 2 a compensatory CC16 production was detected 7 days after an allergen challenge of sensitized mice which had been exposed beforehand to elemental carbon ultrafine particles. Study 2 demonstrated the very complex interplay between allergy and particle exposure (oxidative stress). Animal models are valuable tools to screen for sensitizing properties of substances, and to elucidate the very complex mechanisms determining an allergic response, but interspecies differences always remain a factor which needs to be considered when translating results from in vivo models.

Study 3 and 4 were both observational studies, and as such these studies only demonstrated associations, but they could only suggest causal relationships based on mechanistic information or data from other studies. Moreover, the changes of the biomarker levels were in each case limited. Such limited changes should not be a big surprise when screening a population of "healthy" individuals, but the pathological impact of these changes could not be estimated. We only demonstrated that the 2 studied parameters, i.e. cigarette smoking and wood fuel use, were associated with altered levels of biomolecules which are involved in the homeostasis of the respiratory system. This is by itself of course sufficient reason to look further into the issue. As the interplay between the respiratory health and the environment is a complex, multifactorial process, an absence of any confounding effect is very hard to reach. Moreover, a significant part of our data originated from questionnaires which are always susceptible to certain biases. The major limitation of studies 3 and 4 are the absence of a true, objective marker of exposure, e.g. continine or 1-hydroxypyrene. Future studies with a better control of exposure are needed to confirm the outcome of our observational studies. Another interesting field for further investigation is the response of the nasal epithelium against environmental pollutants. Nasal lavage has already been demonstrated to be a useful tool to assess allergy (82) and our study suggests that it might be the same for the early detection of effects caused by environmental pollutants. A better mechanistic knowledge of the nasal epithelium, e.g. the source and regulation of CC16, could facilitate the further development of this assay.

When considering the whole spectrum of non-invasive assays addressed in this project, a general trend was obvious. Similarly to invasive assays such as biopsies, non-invasive assays also come at a "price". Whereas invasive assays are able to provide highly specific and relevant information they have a significant impact on the study subject. With decreasing invasiveness, the impact (risk) for the study subject decreases, but other issues arise. With decreasing invasiveness the methodological requirements increase significantly in order to limit possible influences from confounding factors. Additionally even when a robust methodological approach can be established, the specificity of the non-invasive marker is often an issue, e.g. hydrogen peroxide only confirms a situation of oxidative stress, but provides no information about the underlying cause. Probably because of the multitude of possible confounding factors, it appears much harder to define threshold levels for non-invasive markers. As such,

it looks unlikely that the non-invasive assays assessed in this project will completely replace (more) invasive assays.

Overall, we were able to illustrate that non-invasive biomarkers have the potential to allow the successful screening of large study populations or the repeated measurements of diseased individuals by yielding objective data with high sensitivity. Nevertheless, before these approaches can be routinely used, the methodological performance and robustness of the assays will have to be further improved and the mechanistic investigations which contribute to the understanding of the different parameters influencing the assay outcome will have to be continued.

8 References

(1) Biomarkers and surrogate endpoints: preferred definitions and conceptual framework. Clin Pharmacol Ther 2001 Mar;69(3):89-95.

(2) Barraza-Villarreal A, Sunyer J, Hernandez-Cadena L, Escamilla-Nunez MC, Sienra-Monge JJ, Ramirez-Aguilar M, et al. Air pollution, airway inflammation, and lung function in a cohort study of Mexico City schoolchildren. Environ Health Perspect 2008 Jun;116(6):832-8.

(3) Hermans C, Bernard A. Lung epithelium-specific proteins: characteristics and potential applications as markers. Am J Respir Crit Care Med 1999 Feb;159(2):646-78.

(4) Amorim MM, Araruna A, Caetano LB, Cruz AC, Santoro LL, Fernandes AL. Nasal eosinophilia: an indicator of eosinophilic inflammation in asthma. Clin Exp Allergy 2010 Jun;40(6):867-74.

(5) Braido F, Riccio AM, Guerra L, Gamalero C, Zolezzi A, Tarantini F, et al. Clara cell 16 protein in COPD sputum: a marker of small airways damage? Respiratory Medicine 2007;101.

(6) de Burbure C, Pignatti P, Corradi M, Malerba M, Clippe A, Dumont X, et al. Uteroglobin-related protein 1 and clara cell protein in induced sputum of patients with asthma and rhinitis. Chest 2007 Jan;131(1):172-9.

(7) Henke MO, Renner A, Huber RM, Seeds MC, Rubin BK. MUC5AC and MUC5B Mucins Are Decreased in Cystic Fibrosis Airway Secretions. Am J Respir Cell Mol Biol 2004 Jul;31(1):86-91.

(8) Corradi M, Montuschi P, Donnelly LE, Pesci A, Kharitonov SA, Barnes PJ. Increased nitrosothiols in exhaled breath condensate in inflammatory airway diseases. Am J Respir Crit Care Med 2001 Mar;163(4):854-8.

(9) Hanazawa T, Kharitonov SA, Barnes PJ. Increased nitrotyrosine in exhaled breath condensate of patients with asthma. Am J Respir Crit Care Med 2000 Oct;162(4 Pt 1):1273-6.

(10) Kharitonov SA, Barnes PJ. Exhaled markers of pulmonary disease. Am J Respir Crit Care Med 2001 Jun;163(7):1693-722.

(11) Montuschi P, Barnes PJ. Exhaled leukotrienes and prostaglandins in asthma. J Allergy Clin Immunol 2002 Apr;109(4):615-20.

(12) Paredi P, Caramori G, Cramer D, Ward S, Ciaccia A, Papi A, et al. Slower rise of exhaled breath temperature in chronic obstructive pulmonary disease. Eur Respir J 2003 Mar;21(3):439-43.

(13) Antczak A, Nowak D, Shariati B, Krol M, Piasecka G, Kurmanowska Z. Increased hydrogen peroxide and thiobarbituric acid-reactive products in expired breath condensate of asthmatic patients. Eur Respir J 1997 Jun;10(6):1235-41.

(14) Dohlman AW, Black HR, Royall JA. Expired breath hydrogen peroxide is a marker of acute airway inflammation in pediatric patients with asthma. Am Rev Respir Dis 1993 Oct;148(4 Pt 1):955-60.

(15) Emelyanov A, Fedoseev G, Abulimity A, Rudinski K, Fedoulov A, Karabanov A, et al. Elevated concentrations of exhaled hydrogen peroxide in asthmatic patients. Chest 2001 Oct;120(4):1136-9.

(16) De Benedetto F, Aceto A, Dragani B, Spacone A, Formisano S, Cocco R, et al. Validation of a new technique to assess exhaled hydrogen peroxide: results from normals and COPD patients. Monaldi Arch Chest Dis 2000 Jun;55(3):185-8.

(17) Cunningham S, McColm JR, Ho LP, Greening AP, Marshall TG. Measurement of inflammatory markers in the breath condensate of children with cystic fibrosis. Eur Respir J 2000 May;15(5):955-7.

(18) Montuschi P, Collins JV, Ciabattoni G, Lazzeri N, Corradi M, Kharitonov SA, et al. Exhaled 8-isoprostane as an in vivo biomarker of lung oxidative stress in patients with COPD and healthy smokers. Am J Respir Crit Care Med 2000 Sep;162(3 Pt 1):1175-7.

(19) Montuschi P, Ciabattoni G, Paredi P, Pantelidis P, du Bois RM, Kharitonov SA, et al. 8-Isoprostane as a biomarker of oxidative stress in interstitial lung diseases. Am J Respir Crit Care Med 1998 Nov;158(5 Pt 1):1524-7.

(20) Montuschi P, Kharitonov SA, Ciabattoni G, Corradi M, van Rensen L, Geddes DM, et al. Exhaled 8-isoprostane as a new non-invasive biomarker of oxidative stress in cystic fibrosis. Thorax 2000 Mar;55(3):205-9.

(21) Becher G., Winsel K, Beck E, Neubauer G, Stresemann E. Das Atemkondensat als Methode zur nicht-invasiven Erfassung von Entzündungsmediatoren aus den unteren Atemwegen. Pneumologie 1997;51:456-9.

(22) Montuschi P, Martello S, Felli M, Mondino C, Barnes PJ, Chiarotti M. Liquid chromatography/mass spectrometry analysis of exhaled leukotriene B4 in asthmatic children. Respir Res 2005;6:119.

(23) Bodini A, D'Orazio C, Peroni D, Corradi M, Folesani G, Baraldi E, et al. Biomarkers of neutrophilic inflammation in exhaled air of cystic fibrosis children with bacterial airway infections. Pediatr Pulmonol 2005 Dec;40(6):494-9.

(24) Shahid SK, Kharitonov SA, Wilson NM, Bush A, Barnes PJ. Increased interleukin-4 and decreased interferon-gamma in exhaled breath condensate of children with asthma. Am J Respir Crit Care Med 2002 May 1;165(9):1290-3.

(25) Hunt J. Exhaled breath condensate pH assays. Immunol Allergy Clin North Am 2007 Nov;27(4):597-606.

(26) Bacon SL, Bouchard A, Loucks EB, Lavoie KL. Individual-level socioeconomic status is associated with worse asthma morbidity in patients with asthma. Respir Res 2009;10:125.

(27) Bernard A, Nickmilder M, Voisin C, Sardella A. Impact of chlorinated swimming pool attendance on the respiratory health of adolescents. Pediatrics 2009 Oct;124(4):1110-8.

(28) Pond K, Kim R, Carroquino M-J, Pirard P, Gore F, Cucu A, et al. Workgroup report: developing environmental health indicators for European children: World Health Organization Working Group. Environmental health perspectives 2007;115:1376-82.

(29) Kim H, Bernstein JA. Air pollution and allergic disease. Curr Allergy Asthma Rep 2009 Mar;9(2):128-33.

(30) Kappos AD, Bruckmann P, Eikmann T, Englert N, Heinrich U, Hoppe P, et al. Health effects of particles in ambient air. Int J Hyg Environ Health 2004 Sep;207(4):399-407.

(31) Blanc PD, Eisner MD, Katz PP, Yen IH, Archea C, Earnest G, et al. Impact of the home indoor environment on adult asthma and rhinitis. J Occup Environ Med 2005;47:362-72.

(32) Vork KL, Broadwin RL, Blaisdell RJ. Developing asthma in childhood from exposure to secondhand tobacco smoke: insights from a meta-regression. Environ Health Perspect 2007 Oct;115(10):1394-400.

(33) Fullerton DG, Bruce N, Gordon SB. Indoor air pollution from biomass fuel smoke is a major health concern in the developing world. Trans R Soc Trop Med Hyg 2008 Sep;102(9):843-51.

(34) Bernard A, Carbonnelle S, Michel O, Higuet S, De BC, Buchet JP, et al. Lung hyperpermeability and asthma prevalence in schoolchildren: unexpected associations with the attendance at indoor chlorinated swimming pools. Occup Environ Med 2003 Jun;60(6):385-94.

(35) Kovesi TA, Dales RE. Effects of the indoor environment on the fraction of exhaled nitric oxide in school-aged children. Can Respir J 2009 May;16(3):e18-e23.

(36) Schneider AP, Stein RT, Fritscher CC. The role of breastfeeding, diet and nutritional status in the development of asthma and atopy. J Bras Pneumol 2007 Aug;33(4):454-62.

(37) Genuneit J, Weinmayr G, Radon K, Dressel H, Windstetter D, Rzehak P, et al. Smoking and the incidence of asthma during adolescence: results of a large cohort study in Germany. Thorax 2006 Jul;61(7):572-8.

(38) Strachan DP, Butland BK, Anderson HR. Incidence and prognosis of asthma and wheezing illness from early childhood to age 33 in a national British cohort. BMJ 1996 May 11;312(7040):1195-9.

(39) Sturm JJ, Yeatts K, Loomis D. Effects of tobacco smoke exposure on asthma prevalence and medical care use in North Carolina middle school children. Am J Public Health 2004 Feb;94(2):308-13.

(40) Bernard A, Carbonnelle S, De BC, Michel O, Nickmilder M. Chlorinated pool attendance, atopy, and the risk of asthma during childhood. Environ Health Perspect 2006 Oct;114(10):1567-73.

(41) Voisin C, Sardella A, Marcucci F, Bernard A. Infant swimming in chlorinated pools and the risks of bronchiolitis, asthma and allergy. Eur Respir J 2010 Jul;36(1):41-7.

(42) Gabriele C, Asgarali R, Jaddoe VW, Hofman A, Moll HA, de Jongste JC. Smoke exposure, airway symptoms and exhaled nitric oxide in infants: the Generation R study. Eur Respir J 2008 Aug;32(2):307-13.

(43) Hall GL, Reinmann B, Wildhaber JH, Frey U. Tidal exhaled nitric oxide in healthy, unsedated newborn infants with prenatal tobacco exposure. J Appl Physiol 2002 Jan;92(1):59-66.

(44) Scheers H, Mwalili SM, Faes C, Fierens F, Nemery B, Nawrot TS. Does air pollution trigger infant mortality in Western Europe? A case-crossover study. Environ Health Perspect 2011 Jul;119(7):1017-22.

(45) Merchant JA, Naleway AL, Svendsen ER, Kelly KM, Burmeister LF, Stromquist AM, et al. Asthma and farm exposures in a cohort of rural Iowa children. Environ Health Perspect 2005 Mar;113(3):350-6.

(46) Naleway AL. Asthma and atopy in rural children: is farming protective? Clin Med Res 2004 Feb;2(1):5-12.

(47) Epton MJ, Dawson RD, Brooks WM, Kingham S, Aberkane T, Cavanagh JA, et al. The effect of ambient air pollution on respiratory health of school children: a panel study. Environ Health 2008;7:16.

(48) Po JY, FitzGerald JM, Carlsten C. Respiratory disease associated with solid biomass fuel exposure in rural women and children: systematic review and meta-analysis. Thorax 2011 Mar;66(3):232-9.

(49) Howden-Chapman P, Pierse N, Nicholls S, Gillespie-Bennett J, Viggers H, Cunningham M, et al. Effects of improved home heating on asthma in community dwelling children: randomised controlled trial. BMJ 2008;337:a1411.

(50) Jaakkola JJ, Hwang BF, Jaakkola N. Home dampness and molds, parental atopy, and asthma in childhood: a six-year population-based cohort study. Environ Health Perspect 2005 Mar;113(3):357-61.

(51) Jaakkola JJ, Hwang BF, Jaakkola MS. Home dampness and molds as determinants of allergic rhinitis in childhood: a 6-year, population-based cohort study. Am J Epidemiol 2010 Aug 15;172(4):451-9.

(52) Maier WC, Arrighi HM, Morray B, Llewellyn C, Redding GJ. Indoor risk factors for asthma and wheezing among Seattle school children. Environ Health Perspect 1997 Feb;105(2):208-14.

(53) Evans J, Chen Y. The association between home and vehicle environmental tobacco smoke (ETS) and chronic bronchitis in a Canadian population: the Canadian Community Health Survey, 2005. Inhal Toxicol 2009 Feb;21(3):244-9.

(54) Lannero E, Wickman M, van HM, Bergstrom A, Pershagen G, Nordvall L. Exposure to environmental tobacco smoke and sensitisation in children. Thorax 2008 Feb;63(2):172-6.

(55) Koh MS, Irving LB. The natural history of asthma from childhood to adulthood. Int J Clin Pract 2007 Aug;61(8):1371-4.

(56) Nickmilder M, Carbonnelle S, Bernard A. House cleaning with chlorine bleach and the risks of allergic and respiratory diseases in children. Pediatr Allergy Immunol 2007 Feb;18(1):27-35.

(57) Bronchoalveolar lavage constituents in healthy individuals, idiopathic pulmonary fibrosis, and selected comparison groups. The BAL Cooperative Group Steering Committee. Am Rev Respir Dis 1990 May;141(5 Pt 2):S169-S202.

(58) Nocker RE, van der Zee JS, Weller FR, van Overveld FJ, Jansen HM, Out TA. Segmental allergen challenge induces plasma protein leakage into the airways of asthmatic subjects at 4 hours but not at 5 minutes after challenge. J Lab Clin Med 1999 Jul;134(1):74-82.

(59) Bhalla DK, Mannix RC, Lavan SM, Phalen RF, Kleinman MT, Crocker TT. Tracheal and bronchoalveolar permeability changes in rats inhaling oxidant atmospheres during rest or exercise. J Toxicol Environ Health 1987;22(4):417-37.

(60) Widdicombe JG. Airway liquid: a barrier to drug diffusion? Eur Respir J 1997 Oct;10(10):2194-7.

(61) Bhalla DK. Ozone-induced lung inflammation and mucosal barrier disruption: toxicology, mechanisms, and implications. J Toxicol Environ Health B Crit Rev 1999 Jan;2(1):31-86.

(62) Bernard A, Hermans C, Van HG. Transient increase of serum Clara cell protein (CC16) after exposure to smoke. Occup Environ Med 1997 Jan;54(1):63-5.

(63) Arsalane K, Broeckaert F, Knoops B, Wiedig M, Toubeau G, Bernard A. Clara cell specific protein (CC16) expression after acute lung inflammation induced by intratracheal lipopolysaccharide administration. Am J Respir Crit Care Med 2000 May;161(5):1624-30.

(64) Davreux CJ, Soric I, Nathens AB, Watson RW, McGilvray ID, Suntres ZE, et al. N-acetyl cysteine attenuates acute lung injury in the rat. Shock 1997 Dec;8(6):432-8.

(65) Hermans C, Knoops B, Wiedig M, Arsalane K, Toubeau G, Falmagne P, et al. Clara cell protein as a marker of Clara cell damage and bronchoalveolar blood barrier permeability. Eur Respir J 1999 May;13(5):1014-21.

(66) MacNee W, Rahman I. Is oxidative stress central to the pathogenesis of chronic obstructive pulmonary disease? Trends Mol Med 2001 Feb;7(2):55-62.

(67) Nanson CJ, Burgess JL, Robin M, Bernard AM. Exercise alters serum pneumoprotein concentrations. Respir Physiol 2001 Sep;127(2-3):259-65.

(68) Jeffery PK, Li D. Airway mucosa: secretory cells, mucus and mucin genes. Eur Respir J 1997 Jul;10(7):1655-62.

(69) Honda Y, Takahashi H, Kuroki Y, Akino T, Abe S. Decreased contents of surfactant proteins A and D in BAL fluids of healthy smokers. Chest 1996 Apr;109(4):1006-9.

(70) Sin DD, Leung R, Gan WQ, Man SP. Circulating surfactant protein D as a potential lung-specific biomarker of health outcomes in COPD: a pilot study. BMC Pulm Med 2007;7:13.

(71) Barregard L, Sallsten G, Andersson L, Almstrand AC, Gustafson P, Andersson M, et al. Experimental exposure to wood smoke: effects on airway inflammation and oxidative stress. Occup Environ Med 2008 May;65(5):319-24.

(72) Broeckaert F, Arsalane K, Hermans C, Bergamaschi E, Brustolin A, Mutti A, et al. Serum clara cell protein: a sensitive biomarker of increased lung epithelium permeability caused by ambient ozone. Environ Health Perspect 2000 Jun;108(6):533-7.

(73) Bernard A, Carbonnelle S, Dumont X, Nickmilder M. Infant swimming practice, pulmonary epithelium integrity, and the risk of allergic and respiratory diseases later in childhood. Pediatrics 2007 Jun;119(6):1095-103.

(74) Lagerkvist BJ, Bernard A, Blomberg A, Bergstrom E, Forsberg B, Holmstrom K, et al. Pulmonary epithelial integrity in children: relationship to ambient ozone exposure and swimming pool attendance. Environ Health Perspect 2004 Dec;112(17):1768-71.

(75) Dell'omo, Hermans C, Muzi G, Haufroid V, Bernard A, Carrieri P, et al. Serum Clara cell protein (CC16) in healty young smokers. Biomarkers 2000;5:158-64.

(76) Robin M, Dong P, Hermans C, Bernard A, Bersten AD, Doyle IR. Serum levels of CC16, SP-A and SP-B reflect tobacco-smoke exposure in asymptomatic subjects. Eur Respir J 2002 Nov;20(5):1152-61.

(77) Shijubo N, Itoh Y, Yamaguchi T, Shibuya Y, Morita Y, Hirasawa M, et al. Serum and BAL Clara cell 10 kDa protein (CC10) levels and CC10-positive bronchiolar cells are decreased in smokers. Eur Respir J 1997 May;10(5):1108-14.

(78) Halatek T, Gromadzinska J, Wasowicz W, Rydzynski K. Serum clara-cell protein and beta2-microglobulin as early markers of occupational exposure to nitric oxides. Inhal Toxicol 2005 Feb;17(2):87-97.

(79) Halatek T, Stroszejn-Mrowca, Matczak W, Gruchala J, Trzcinka-Ochocka. Exposure to aluminium dust and respiratory and kidney effects in potroom workers. Inhaled particles 2001.

(80) Quirce S, Lemiere C, de BF, del P, V, Gerth Van WR, Maestrelli P, et al. Noninvasive methods for assessment of airway inflammation in occupational settings. Allergy 2010 Apr;65(4):445-58.

(81) Pavord ID, Sterk PJ, Hargreave FE, Kips JC, Inman MD, Louis R, et al. Clinical applications of assessment of airway inflammation using induced sputum. Eur Respir J Suppl 2002 Sep;37:40s-3s.

(82) Vandenplas O, D'Alpaos V, Heymans J, Jamart J, Thimpont J, Huaux F, et al. Sputum eosinophilia: an early marker of bronchial response to occupational agents. Allergy 2009 May;64(5):754-61.

(83) Djukanovic R, Sterk PJ, Fahy JV, Hargreave FE. Standardised methodology of sputum induction and processing. Eur Respir J Suppl 2002 Sep;37:1s-2s.

(84) Bernard A, Broeckaert F, Hermans C, Knoops B. The Clara Cell Protein, CC16: a Biomarker of Pullmonary Toxicity. Biomarkers 1998;273-83.

(85) Niimi T, Keck-Waggoner CL, Popescu NC, Zhou Y, Levitt RC, Kimura S. UGRP1, a uteroglobin/Clara cell secretory protein-related protein, is a novel lung-enriched downstream target gene for the T/EBP/NKX2.1 homeodomain transcription factor. Mol Endocrinol 2001 Nov;15(11):2021-36.

(86) Irvin CG. The nose: a window into the asthmatic lung? Clin Exp Allergy 2010 Jun;40(6):839-40.

(87) Kaulbach HC, White MV, Igarashi Y, Hahn BK, Kaliner MA. Estimation of nasal epithelial lining fluid using urea as a marker. J Allergy Clin Immunol 1993 Sep;92(3):457-65.

(88) Cavaliere F, Schiavello R, Masieri S, Passali D. Urea to calculate the dilution of nasal secretum collected by lavage. Acta oto-rhino-laryngologica Belgica 1986;40:542-7.

(89) ATS/ERS recommendations for standardized procedures for the online and offline measurement of exhaled lower respiratory nitric oxide and nasal nitric oxide, 2005. Am J Respir Crit Care Med 2005 Apr 15;171(8):912-30.

(90) Kharitonov SA, Barnes PJ. Clinical aspects of exhaled nitric oxide. Eur Respir J 2000 Oct;16(4):781-92.

(91) Risby TH, Jiang L, Stoll S, Ingram D, Spangler E, Heim J, et al. Breath ethane as a marker of reactive oxygen species during manipulation of diet and oxygen tension in rats. J Appl Physiol 1999 Feb;86(2):617-22.

(92) Kharitonov SA, Robbins RA, Yates D, Keatings V, Barnes PJ. Acute and chronic effects of cigarette smoking on exhaled nitric oxide. Am J Respir Crit Care Med 1995 Aug;152(2):609-12.

(93) Effros RM, Hoagland KW, Bosbous M, Castillo D, Foss B, Dunning M, et al. Dilution of respiratory solutes in exhaled condensates. Am J Respir Crit Care Med 2002 Mar 1;165(5):663-9.

(94) Nowak D, Antczak A, Krol M, Pietras T, Shariati B, Bialasiewicz P, et al. Increased content of hydrogen peroxide in the expired breath of cigarette smokers. Eur Respir J 1996 Apr;9(4):652-7.

(95) Balint B, Donnelly LE, Hanazawa T, Kharitonov SA, Barnes PJ. Increased nitric oxide metabolites in exhaled breath condensate after exposure to tobacco smoke. Thorax 2001 Jun;56(6):456-61.

(96) Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Baraldi E, et al. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Respir J 2005 Sep;26(3):523-48.

(97) Rosias PP, Robroeks CM, Niemarkt HJ, Kester AD, Vernooy JH, Suykerbuyk J, et al. Breath condenser coatings affect measurement of biomarkers in exhaled breath condensate. Eur Respir J 2006 Nov;28(5):1036-41.

(98) Rosias PP, Robroeks CM, Kester A, den Hartog GJ, Wodzig WK, Rijkers GT, et al. Biomarker reproducibility in exhaled breath condensate collected with different condensers. Eur Respir J 2008 May;31(5):934-42.

(99) Montuschi P. Analysis of exhaled breath condensate in respiratory medicine: methodological aspects and potential clinical applications. Ther Adv Respir Dis 2007 Oct;1(1):5-23.

(100) Loukides S, Kontogianni K, Hillas G, Horvath I. Exhaled breath condensate in asthma: from bench to bedside. Curr Med Chem 2011;18(10):1432-43.

(101) Carbonnelle S, Bernard A, Doyle IR, Grutters J, Francaux M. Fractional exhaled NO and serum pneumoproteins after swimming in a chlorinated pool. Med Sci Sports Exerc 2008 Aug;40(8):1472-6.

(102) Hyslop P.A., Sklar L.A. A quantitative fluorimeric assay for the determination of oxidant production by polymorphonuclear leukocytes: its use in the simultaneous fluorimetric assay of cellular acitvation processes. Analytical Biochemistry 1984;141:280-6.

(103) van Beurden W.J.C., Harff G.A., Dekhuijzen P.N.R., van den Bosch M.J.A., Creemers J.P.H.M., Smeenk F.W.J.M. An efficient and reproducible method for measuring hydrogen peroxide in exhaled breath condensate. Respiratory Medicine 2002;96:197-203.

(104) Van Miert E., Dumont X, Bernard A. CC16 as a marker of lung epithelial hyperpermeability in an acute model of rats exposed to mainstream cigarette smoke. Toxicol Lett 2005 Nov 15;159(2):115-23.

(105) Etherton JE, Gresham GA. Early bronchiolar damage following paraquat poisoning in mice. J Pathol 1979 May;128(1):21-7.

(106) Wilhelm J, Frydrychova M, Vizek M. Hydrogen peroxide in the breath of rats: the effects of hypoxia and paraquat. Physiol Res 1999;48(6):445-9.

(107) Bernard A, Nickmilder M, Voisin C. Outdoor swimming pools and the risks of asthma and allergies during adolescence. Eur Respir J 2008 Oct;32(4):979-88.

(108) Andreoli R, Manini P, Corradi M, Mutti A, Niessen WM. Determination of patterns of biologically relevant aldehydes in exhaled breath condensate of healthy subjects by liquid chromatography/atmospheric chemical ionization tandem mass spectrometry. Rapid Commun Mass Spectrom 2003;17(7):637-45.

(109) van Beurden WJ, Dekhuijzen PN, Harff GA, Smeenk FW. Variability of exhaled hydrogen peroxide in stable COPD patients and matched healthy controls. Respiration 2002;69(3):211-6.

(110) Gerritsen WB, Zanen P, Bauwens AA, van den Bosch JM, Haas FJ. Validation of a new method to measure hydrogen peroxide in exhaled breath condensate. Respir Med 2005 Sep;99(9):1132-7.

(111) Gajdocsi R, Bikov A, Antus B, Horvath I, Barnes PJ, Kharitonov SA. Assessment of Reproducibility of Exhaled Hydrogen Peroxide Concentration and the Effect of Breathing Pattern in Healthy Subjects. J Aerosol Med Pulm Drug Deliv 2011 Jun 20.

(112) Effros RM, Dunning MB, III, Biller J, Shaker R. The promise and perils of exhaled breath condensates. Am J Physiol Lung Cell Mol Physiol 2004 Dec;287(6):L1073-L1080.

(113) Sapey E, Bayley D, Ahmad A, Stockley R. The validation of assays used to measure biomarkers in exhaled breath condensate. Eur Respir J 2008 Nov;32(5):1408-9.

(114) Schleiss MB, Holz O, Behnke M, Richter K, Magnussen H, Jorres RA. The concentration of hydrogen peroxide in exhaled air depends on expiratory flow rate. Eur Respir J 2000 Dec;16(6):1115-8.

(115) Effros RM, Biller J, Foss B, Hoagland K, Dunning MB, Castillo D, et al. A simple method for estimating respiratory solute dilution in exhaled breath condensates. Am J Respir Crit Care Med 2003 Dec 15;168(12):1500-5.

(116) Dodge DE, Rucker RB, Pinkerton KE, Haselton CJ, Plopper CG. Dose-dependent tolerance to ozone. III. Elevation of intracellular Clara cell 10-kDa protein in central acini of rats exposed for 20 months. Toxicol Appl Pharmacol 1994 Jul;127(1):109-23.