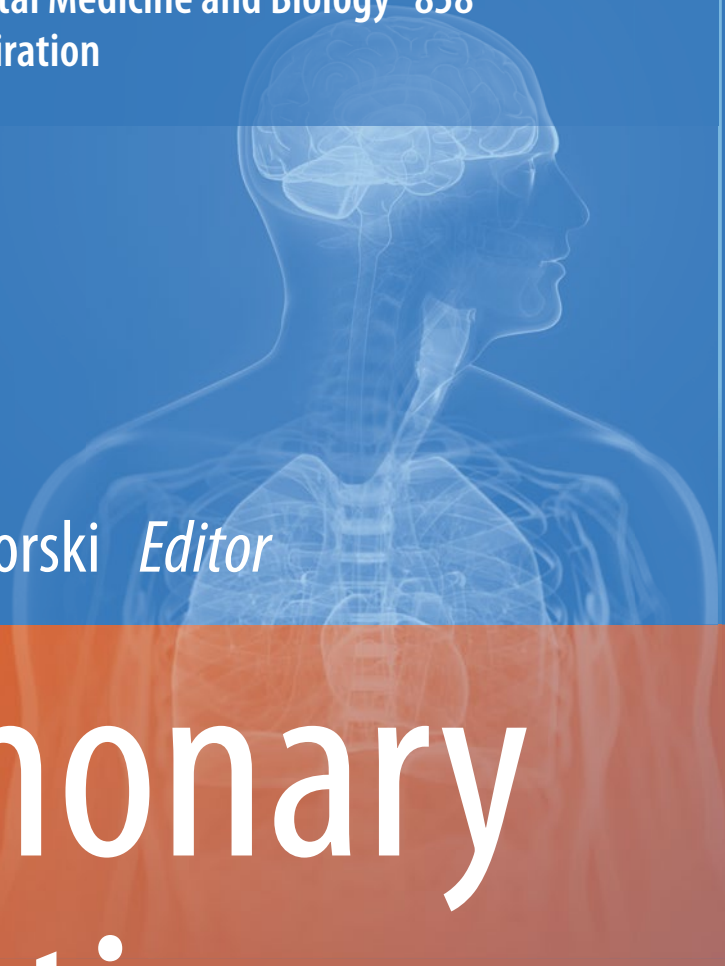
Advances in Experimental Medicine and Biology 858
Neuroscience and Respiration



Mieczyslaw Pokorski *Editor*

# Pulmonary Function

Springer

# Advances in Experimental Medicine and Biology

## Neuroscience and Respiration

## Volume 858

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Editor

# Pulmonary Function

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# Preface

The book series *Neuroscience and Respiration* presents contributions by expert researchers and clinicians in the field of pulmonary disorders. The chapters provide timely overviews of contentious issues or recent advances in the diagnosis, classification, and treatment of the entire range of pulmonary disorders, both acute and chronic. The texts are thought as a merger of basic and clinical research dealing with respiratory medicine, neural and chemical regulation of respiration, and the interactive relationship between respiration and other neurobiological systems such as cardiovascular function or the mind-to-body connection. The authors focus on the leading-edge therapeutic concepts, methodologies, and innovative treatments. Pharmacotherapy is always in the focus of respiratory research. The action and pharmacology of existing drugs and the development and evaluation of new agents are the heady area of research. Practical data-driven options to manage patients will be considered. New research is presented regarding older drugs, performed from a modern perspective or from a different pharmacotherapeutic angle. The introduction of new drugs and treatment approaches in both adults and children also is discussed.

Lung ventilation is ultimately driven by the brain. However, neuropsychological aspects of respiratory disorders are still mostly a matter of conjecture. After decades of misunderstanding and neglect, emotions have been rediscovered as a powerful modifier or even the probable cause of various somatic disorders. Today, the link between stress and respiratory health is undeniable. Scientists accept a powerful psychological connection that can directly affect our quality of life and health span. Psychological approaches, by decreasing stress, can play a major role in the development and therapy of respiratory diseases.

Neuromolecular aspects relating to gene polymorphism and epigenesis, involving both heritable changes in the nucleotide sequence and functionally relevant changes to the genome that do not involve a change in the nucleotide sequence, leading to respiratory disorders will also be tackled. Clinical advances stemming from molecular and biochemical research are but possible if the research findings are translated into diagnostic tools, therapeutic procedures, and education, effectively reaching physicians and patients. All these cannot be achieved without a multidisciplinary, collaborative, bench-to-bedside approach involving both researchers and clinicians.

The societal and economic burden of respiratory ailments has been on the rise worldwide, leading to disabilities and shortening of life span. COPD alone causes more than three million deaths globally each year. Concerted efforts are required to improve this situation, and part of those efforts are gaining insights into the underlying mechanisms of disease and staying abreast with the latest developments in diagnosis and treatment regimens. It is hoped that the books published in this series will assume a leading role in the field of respiratory medicine and research and will become a source of reference and inspiration for future research ideas.

I would like to express my deep gratitude to Mr. Martijn Roelandse and Ms. Tanja Koppejan from Springer's Life Sciences Department for their genuine interest in making this scientific endeavor come through and in the expert management of the production of this novel book series.

Opole, Poland Mieczyslaw Pokorski

# Volume 13: Pulmonary Function

The book discusses new concepts and findings in the field of pulmonary function. This function is notably associated with spirometry and gas exchange at the lungs. The technique of spirometry, its clinical meaning, and reference values have all been refined over decades of its use. Although spirometry remains ancillary in diagnosis-making, it seems hardly replaceable in monitoring of lung disease progression and treatment efficacy. Pulmonary function goes far beyond spirometry. It encompasses interactions with the cardiovascular system, sleep disordered breathing, etiological factors like occupational bio aerosol exposure or cigarette smoke related issues. Pulmonary function may crumple in any respiratory ailment, the case in point being all too often respiratory tract infections. Chapters contribute to the latest thinking on molecular mechanisms underpinning pulmonary function, on patient care and attempt to keep up-todate with current clinical and research progress. The book will be of interest to both clinicians and biomedical researchers.

# Contents

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# Intermittent Hypoxia Impairs Endothelial Function in Early Preatherosclerosis

I. Tuleta, C.N. França, D. Wenzel, B. Fleischmann, G. Nickenig, N. Werner, and D. Skowasch

**Abstract**

Intermittent hypoxia seems to be a major pathomechanism of obstructive sleep apnea-associated progression of atherosclerosis. The goal of the present study was to assess the influence of hypoxia on endothelial function depending on the initial stage of vasculopathy. We used 16 ApoE−/− mice were exposed to a 6-week-intermittent hypoxia either immediately (early preatherosclerosis) or after 5 weeks of high-cholesterol diet (advanced preatherosclerosis). Another 16 ApoE−/− mice under normoxia served as corresponding controls. Endothelial function was measured by an organ bath technique. Blood plasma CD31+/annexin V+ endothelial microparticles as well as sca1/flk1+ endothelial progenitor cells in blood and bone marrow were analyzed by flow cytometry. The findings were that intermittent hypoxia impaired endothelial function (56.6 ± 6.2 % of maximal phenylephrine-induced vasoconstriction *vs.* 35.2 ± 4.1 % in control) and integrity (increased percentage of endothelial microparticles: 0.28 ± 0.05 % *vs.* 0.15 ± 0.02 % in control) in early preatherosclerosis. Peripheral repair capacity expressed as the number of endothelial progenitor cells in blood was attenuated under hypoxia (2.0 ± 0.5 % *vs.* 5.3 ± 1.9 % in control), despite the elevated number of these cells in the bone marrow (2.0 ± 0.4 % *vs.* 1.1 ± 0.2 % in control). In contrast, endothelial function, as well as microparticle and endothelial progenitor cell levels were similar under hypoxia *vs.* control in advanced preatherosclerosis. We conclude that hypoxia aggravates endothelial dysfunction and destruction in early preatherosclerosis.

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**Keywords**
Breathing disorder • Compensatory mechanisms • Endothelial damage • Endothelium-dependent vasorelaxation • Endothelial precursor cells • Hypoxia-reoxygenation

## 1 Introduction

Obstructive sleep apnea (OSA) is an independent risk factor for the development of atherosclerosis (Marshall et al. 2008). However, the exact pathomechanisms of OSA-induced cardiovascular complications are still not enough explored. The key event in the process of atherosclerosis is the disturbance of endothelial layer (Yeboah et al. 2007). Intermittent hypoxia, as a main component of OSA involved in vascular pathology, has been shown to impair endothelial function in animal models of OSA and in OSA patients (Hernández-Guerra et al. 2013; Namtvedt et al. 2013; Faulx et al. 2004). Hypoxia-associated endothelial dysfunction is accompanied by endothelial destruction indicated by the augmented release of endothelial microparticles in blood (Ayers et al. 2009). However, some experiments failed to confirm the increase in endothelial microparticle levels under hypoxia (Akinnusi and El Solh 2009). Also the data referring to the possible activation of endothelial repair mechanisms involving endothelial progenitor cells (EPCs) are not consistent presenting enhanced (Kizawa et al. 2009), unchanged (Jun et al. 2010), or even lower (Jelic et al. 2009) levels of these cells in blood in face of hypoxic stimulus. Additionally, little is known about the activation of EPCs in the central sites of their production and preservation in bone marrow under hypoxic conditions in frame of OSA.

One explanation of the above outlined discrepant results concerning endothelial changes and induction of repair pathways under hypoxia seem to be differences in the intensity of hypoxia across various studies, as it is known that the more pronounced the hypoxia is, the faster the vasculopathy proceeds (Seif et al. 2013). Our hypothesis is that also the initial stage of vessel pathology determines the extent of vessel disease progression and induction of related pathomechanisms underlying hypoxia. Therefore, the goal of the present work was the assessment of potential hypoxia-associated alternations of both endothelial function and the levels of EPCs in bone marrow and blood, depending on the initial degree of atherosclerotic artery disease.

## 2 Methods

### 2.1 Animal Model of Sleep Apnea

All animal protocols were approved by the local Ethics Committees and the studies were performed according to the "Guide for the Care and Use of Laboratory Animals" (National Research Council 1996). Ten- to twelve-week-old male (n = 12) and female (n = 20) apolipoprotein E-deficient (ApoE −/− C57BL/6 J genetic background) mice were purchased from Charles River Laboratories (Calco, Italy). After the acclimatization time of 2 days, animals were randomly assessed to one of the four groups (n = 3 male and n = 5 female each). The first two groups were exposed to a 6-week-intermittent hypoxia (33 cycles of oxygen concentration fluctuations between 21 and 5 %, 8 h per day) either immediately (early preatherosclerosis group) or following 5 weeks of a high-cholesterol diet (advanced preatherosclerosis group). The remaining animals were kept under normoxia and served as corresponding controls. Throughout the duration of the experiment, all animals were fed with a cholesterol-rich diet (21 % fat, 19.5 % casein, 1.25 % cholesterol; Ssniff, Soest, Germany). For *ex-vivo* tests, mice were sacrificed by intraperitoneal injection of xylazine (Rompun) (Bayer HealthCare; Leverkusen, Germany) and ketamine

(Ketalar) (Pfizer; Berlin, Germany) (1:2). Blood was collected by puncture of abdominal aorta, bone marrow was isolated from leg bones and thoracic aorta was excised.

### 2.2 Organ Bath Assay with Isolated Aortic Rings

The thoracic aorta was immediately isolated and cut into 3-mm long segments (4 for each animal) which were mounted in organ-bath chambers filled with a Tyrode buffer (millimolar composition: NaCl 118.0, KCl 4.7, $MgCl_2$*6 $H_2O$ 1.2, NaEDTA 0.03, $KH_2PO_4$ 1.2, $CaCl_2$ 2.5, $NaHCO_3$ 25, and D(+)glucose 5.5), bubbled with oxygen and maintained at 37 °C, pH = 7.4 (Tuleta et al. 2014). The force of isometric aortic ring contractions was recorded by a transducer connected to an amplifier-recorder. A basal tension of 10 mN was stepwise applied to each ring. Then, aortic segments were stimulated with potassium chloride (KCl: 20 and 40 mmol/l) which was afterwards washed out until resting tension was again obtained. Thereafter, the aortic rings were precontracted with phenylephrine in increasing concentrations (1 nmol/l – 10 μmol/l) until a stable plateau was reached. Relaxation responses were determined by application of endothelium-dependent dilator carbachol and endothelium-independent vasorelaxant nitroglycerin in increasing concentrations (10 nmol/l – 100 μmol/l; 1 nmol/l – 10 μmol/l, respectively).

### 2.3 Fluorescence-Activated Cell Sorter (FACS) Analysis of CD31+/Annexin V+ Endothelial Microparticles and sca-1+/flk-1+ Endothelial Progenitor Cells

For quantitative analysis of endothelial microparticles, blood plasma was centrifuged at 13,000 g for 2 min to obtain platelet-poor plasma. Then, platelet-poor plasma (20 μl) was incubated with CD31-apc (4 μl) for 45 min and fluorescein isothiocyanate-conjugated annexin V (annexin V-FITC) for 1 h. IgG $2_a$-fluorescein isothiocyanate (Pharmingen; San Diego, CA) served as a negative control. The measurements were performed by means of FACS Calibur instrument (Becton Dickinson; Heidelberg, Germany). The analysis of data was done using CellQuest software (Becton Dickinson; Heidelberg, Germany), as previously described (Tuleta et al. 2014).

Following red cell lysis and Fc blockade, the experiments with sca-1+/flk-1+ cells in blood and bone marrow were conducted analogically to those with endothelial microparticles. In detail, sca-1-apc and flk-1-PE (Becton Dickinson; Heidelberg, Germany) were used for detection of corresponding markers. In each experiment isotype identical antibodies and unstained samples served as negative controls.

### 2.4 Statistical Analysis

The data are expressed as mean ± SE. The differences between the means of two groups or multiple groups were compared with a two-sample *t*-test or ANOVA test, respectively. The SPSS for Windows software ver. 10.0 was used for statistical analysis. A probability value of $p < 0.05$ was considered statistically significant.

## 3 Results

The main finding of the present study concerned the influence of intermittent hypoxia on endothelial function at different stages of preatherosclerosis. In detail, endothelial function was impaired under hypoxia *vs.* control in early preatherosclerosis (56.6 ± 6.2 % and 35.2 ± 4.1 % of maximal phenylephrine-induced vasoconstriction, respectively, $p < 0.05$, Fig. 1a). In contrast, hypoxia did not significantly worsen the endothelial function compared to control in advanced preatherosclerosis (59.6 ± 9.7 % and 52.4 ± 11.6 % of maximal phenylephrine-induced vasoconstriction, respectively, $p > 0.05$, Fig. 1b).

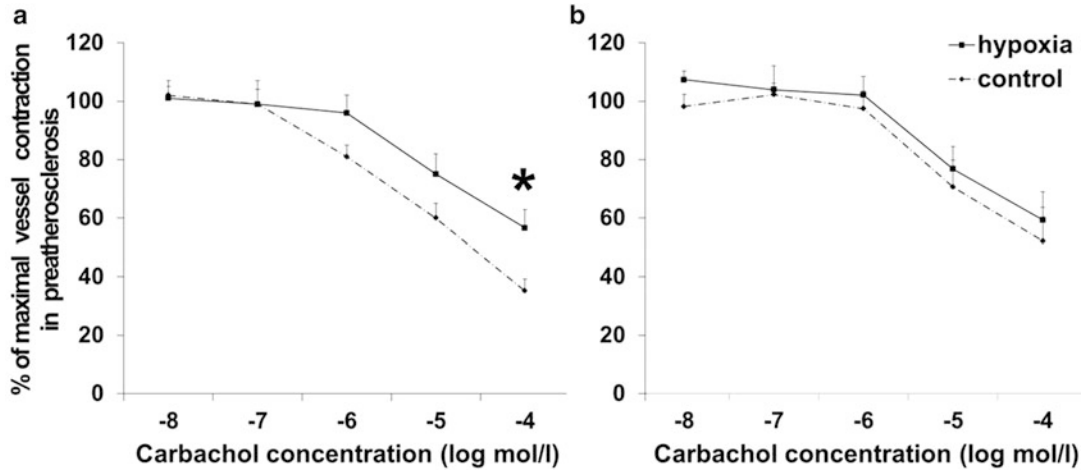


**Fig. 1** (**a**) **Impairment of hypoxia-induced endothelium-dependent vasorelaxation** compared to control in early preatherosclerosis, *p < 0.05; (**b**) No significant influence of intermittent hypoxia on endothelium-associated vasorelaxation in advanced preatherosclerosis

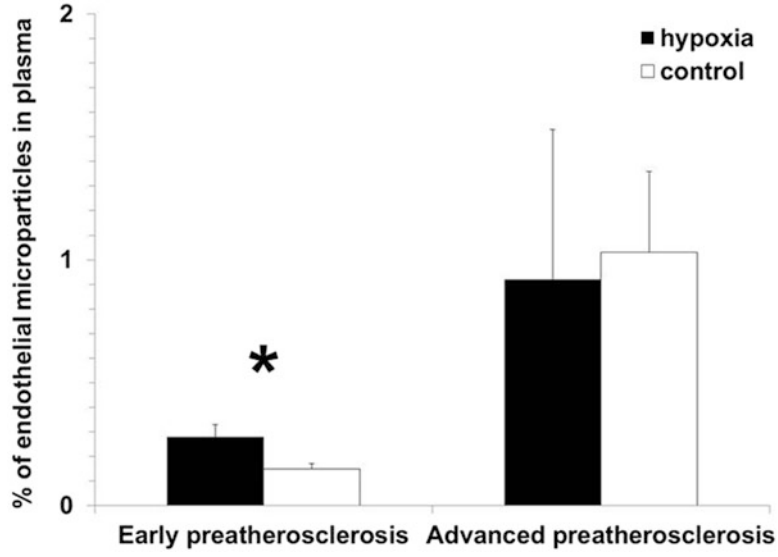


**Fig. 2 Higher levels of endothelial microparticles in blood plasma under intermittent hypoxia** compared to control in early preatherosclerosis. No relevant differences were noted in the percentage of endothelial microparticles between hypoxia and control group in advanced preatherosclerosis, *p < 0.05

Consequently, levels of endothelial microparticles which mirror the degree of endothelial destruction were higher under hypoxia *vs.* control only at early stages of vascular disease (0.28 ± 0.05 % and 0.15 ± 0.02 %, respectively, $p < 0.05$, Fig. 2), without showing any relevant differences between these groups in more advanced vasculopathy (0.92 ± 0.61 % and 1.03 ± 0.33 %, respectively, $p > 0.05$).

The analysis of compensatory pathomechanisms involving the presence of endothelial progenitor cells in bone marrow and blood showed significant changes in the number of these cells under hypoxia *vs.* control in early preatherosclerosis. Specifically, the percentage of EPCs increased in bone marrow (2.0 ± 0.4 % and 1.1 ± 0.2 %, respectively, $p < 0.05$, Fig. 3) and decreased in blood (2.0 ± 0.5 % and 5.3 ± 1.9 %, respectively, $p < 0.05$, Fig. 4) under hypoxia

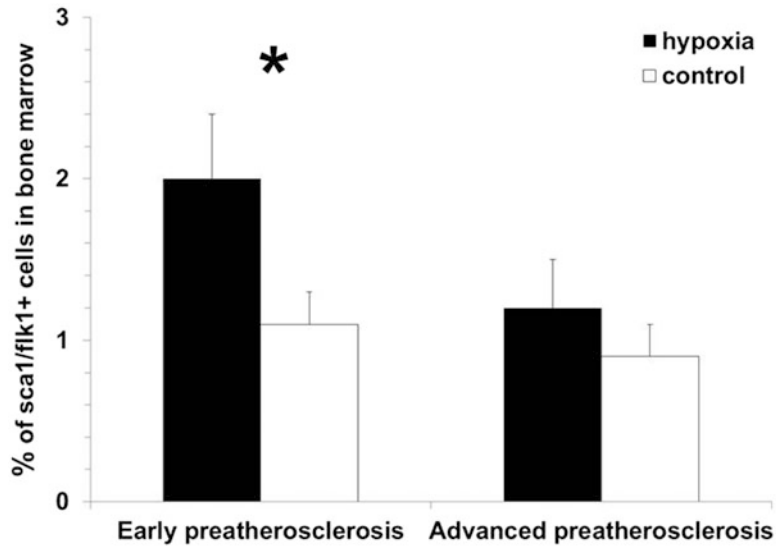


**Fig. 3 Elevated numbers of endothelial progenitor cells in bone marrow** under intermittent hypoxia *vs*. control in early preatherosclerosis in contrast to similar levels of these cells in both groups in advanced preatherosclerosis, *p < 0.05

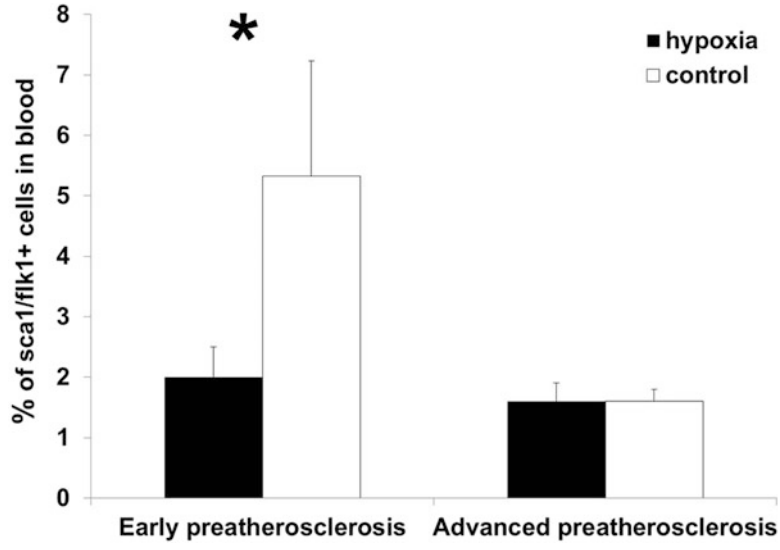


**Fig. 4 Reduced levels of endothelial progenitor cells in blood** under hypoxia compared to control in early preatherosclerosis. No significant differences in percentage of these cells between both groups in advanced preatherosclerosis, *p < 0.05

compared to control. Such alternations were not observed during a further time course of endothelial dysfunction (bone marrow: 1.2 ± 0.3 % and 0.9 ± 0.2 %, respectively, $p > 0.05$; blood: 1.6 ± 0.3 % and 1.6 ± 0.2 %, respectively, $p > 0.05$).

## 4 Discussion

This study demonstrates the influence of intermittent hypoxia on endothelial dysfunction and endothelial repair capacity depending on the degree of advancement of thoracic artery preatherosclerosis. In particular, we showed that intermittent hypoxia decreased endothelial function at early stage of vascular disease. Similar results have been obtained in animal models and human trials (Dematteis et al. 2008; Phillips et al. 2004; Ip et al. 2004). However, dependence of the hypoxia-induced endothelial dysfunction on the initial extent of vasculopathy has not been specifically considered. In the present work, we showed that intermittent hypoxia impairs

endothelial function mainly at the very beginning of vascular disease. During a further time course of vascular disorder the negative effects of hypoxia on endothelium are limited. It seems that additional deleterious stimulus such as hypoxia play a rather subordinated role in the worsening of endothelial function once the process of endothelial damage has reached an advanced stage. In line with the above data, we identified higher levels of endothelial microparticles in blood reflecting vascular injury under hypoxia *vs*. control in initial phases of vasculopathy. As endothelial dysfunction progressed, the overall percentage of microparticles increased, but hypoxia did not significantly augment the levels of endothelial microparticles compared to control. This finding may at least in part explain some previous apparently contradictory data demonstrating enhanced or unchanged endothelial microparticle numbers under hypoxia in comparison to control (Ayers et al. 2009; Akinnusi and El Solh 2009), possibly resulting from investigating of microparticles under hypoxic conditions in different stages of vascular pathology.

The next issue of the present study was the analysis of endothelial repair capacity mediated by endothelial progenitor cells. The number of endothelial progenitor cells increased in bone marrow under hypoxia in early preatherosclerosis. In contrast, the percentage of these cells was lower in blood in the hypoxia *vs*. control group. These findings point to the hypoxia-induced activation of central compensatory mechanisms. However, the peripheral repair capacity in blood seems to be attenuated under hypoxia. The last result may be explained due to the reduced matrix metalloproteinase-9-dependent release of endothelial progenitor cells from bone marrow to the blood, which has been shown by our group in a previous work (Tuleta et al. 2014). Interestingly, the levels of blood EPCs decreased under normoxia as endothelial function worsened suggesting progressive depletion of repair mechanisms. Hypoxic conditions had no relevant influence on the number of these cells in bone marrow or blood in the stage of advanced endothelial dysfunction. Thus, our data showing different responses of EPCs to the hypoxic stimulus depending on the severity of vascular disease may contribute to the explanation of the fact that several studies concerning circulating endothelial progenitor cell levels provide inconsistent results (Jelic et al. 2008; Kizawa et al. 2009). This is probably due to the investigation of populations with different extent of preatherosclerotic vessel changes.

In conclusion, intermittent hypoxia influences endothelial function and endothelial repair capacity mainly in early stages of preatherosclerosis.

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**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# The Mechanisms of Compensatory Responses of the Respiratory System to Simulated Central Hypervolemia in Normal Subjects

M.O. Segizbaeva, Zh.A. Donina, V.G. Aleksandrov, and N.P. Aleksandrova

**Abstract**

The compensatory responses of the respiratory system to simulated central hypervolemia (CHV) were investigated in 14 normal subjects. The central hypervolemia was caused by a short-time passive head-down tilt (HDT, −30°, 30 min). The results show that CHV increased the mechanical respiratory load and the airway resistance, slowed the inspiratory flow, increased the duration of the inspiratory phase, reduced the respiratory rate, but not changed the minute ventilation. CHV induced a significant rise in inspiratory swings of alveolar pressure (184 %), based on the inspiratory occlusion pressure measurement. These changes indicate a compensatory increase in the inspiratory muscle contraction force. A stable level of minute ventilation during CHV was an effect of increased EMG activity of parasternal muscles more than twice ($P < 0.01$). A contribution of the diaphragm and scalene muscles to ventilation during spontaneous breathing in HDT was reduced. An increase of genioglossus contractile activity during HDT contributed to the stabilization of airway patency. These results suggest that a coordinated modulation of inspiratory muscles activity allows preserving a constant level of minute ventilation during a short-time intrathoracic blood volume expansion. The mechanisms of respiratory load compensation seem to be mediated by afferent information from the lung and respiratory muscle receptors and from the segmentary reflexes and intrinsic properties of the muscle fibers.

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**Keywords**
Airway resistance • Diaphragm • Electromyogram • Genioglossus • Head-down-tilt • Parasternal • Sternocleidomastoid

## 1 Introduction

When decreasing the effects of gravity in humans, such as by anti-orthostatic posture changes or immersion into the water, venous return is increased by some 25 % (Norsk 2005). A redistribution of blood from peripheral portions of the body to the intrathoracic circulation leads to central blood volume expansion – central hypervolemia (CHV), which is accompanied by changes in pulmonary hemodynamics and may have an effect on the respiratory system (Bettinelli et al. 2002; West 2002). An increase in central venous pressure, a decrease of functional residual capacity and lung compliance at a postural change from supine to head-down tilt (HDT) of 30° has been shown in anesthetized cats (Aleksandrova et al. 2007; Donina et al. 2013). Increased blood supply to the lungs, which occurs under these conditions, reduces their elastic properties, causing narrowing of the airways and increasing the airway resistance (Estenne et al. 1992; Prisk 2000). The thoracic blood volume expansion changes the lung and diaphragm position and the chest configuration, which may lead to a decrease in lung volume and changes in breathing pattern (Prisk 2000; Prisk et al 2002; Estenne et al. 1992). The compensatory responses that keep the required level of ventilation and gas exchange under these conditions have not been sufficiently studied. Little is known about the inspiratory muscle function during CHV.

The purpose of this investigation was to examine the respiratory responses and compensatory capabilities of the respiratory system in normal subjects submitted to acute cardiopulmonary blood volume expansion by head-down tilt of 30°.

## 2 Methods

### 2.1 Subjects

The study was approved by a local Ethics Committee and conducted in accordance with the ethical standards of the Helsinki Declaration for Human Experimentation. Fourteen healthy volunteers (F/M-4/10) participated in the study. All subjects were familiarized with the experimental procedures and gave informed consent. Their mean age was 22.4 ± 0.9 (19–25) years. Anthropometric data for men were as follows: height 178.1 ± 6.8 (167–186) cm, weight 77.6 ± 6.5 (65.2–84.5) kg, and vital capacity (VC) 4.3 ± 0.5 (3.6–5.1) l, and for women: height 163.2 ± 2.4 (160–171) cm, weight 57.1 ± 4.3 (51.9–65.9) kg, VC 3.3 ± 0.3 (2.9–3.6) l. All subjects had no pulmonary, cardiovascular, or neuromuscular disorders and had the ventilatory function within normal limits.

### 2.2 Ventilatory Parameters

Inspiratory flow ($V_I$) was measured with a pneumotachograph (Fleish No. 3), connected to the inspiratory port of a low-resistance valve (Hans Rudolph 2700, Shawnee, Kansas). The inspiratory flow signal was electronically integrated to obtain tidal volume ($V_T$) and was displayed on the multichannel recorder (Biograph, St. Petersburg, Russia). Inspiratory and expiratory time ($T_I$, $T_E$), total breath cycle ($T_T$), and breathing frequency (f) were measured from this tracing. Minute ventilation ($V_E$) was calculated as a product of $V_T$ and f. Volume calibration was performed before each test using a 1 L syringe.

## 2.3 Mouth Pressure and Airway Resistance

An inspiratory mouth pressure ($P_{mI}$) was measured using a pressure tap at the mouthpiece connected to a differential pressure transducer (PDP 1000 MD, St. Petersburg, Russia). The occluding valve was actuated during quiet breathing in control (standing position) and at 1, 10, 20, and 30 min of HDT in order to obtain the alveolar pressure values ($P_{occl}$). The interrupter technique assumes that immediately after airflow interruption, mouth pressure equilibrates with alveolar pressure (Oswald-Mammosser et al. 2009). Airway resistance ($R_{aw}$) measurements were performed at the spontaneous breathing frequency of the subject. $R_{aw}$ was calculated by the following formula: $R_{aw} = P_{occl}/V_I$; where $P_{occl}$, corresponding to alveolar pressure, is estimated during the 100 ms occlusion and $V_I$, corresponding to peak inspiratory flow, is taken at the mouth immediately before the occlusion.

## 2.4 Maximal Inspiratory Pressure and Peak Flow Measurements

Maximal inspiratory pressure (MIP), peak inspiratory flow (PIF), and peak expiratory flow (PEF) were measured in each subject before the start of experiment in standing position (control) and after 30 min of spontaneous breathing in the HDT position to evaluate the reserve capabilities of the respiratory system. The MIP was measured with a portable device (PowerBreath KH1, Southam, Warwickshire, UK) in accordance with the ATS/ERS Statement (ATS/ERS Statement 2002) and was recorded at the mouth during a quasi-state short maximal inspiration against occluded airways (Müller's maneuver). The maneuver was performed at residual volume (RV) (Troosters et al. 2005). Each participant was asked to perform a few maximal inspiratory efforts to adopt for the correct performance of this test during experiment. Maximal inspiratory efforts were maintained for 3–4 s separated by at least 1 min intervals. The subjects had a nose clip in place during the maneuver. The subjects were verbally encouraged by the operators to achieve a maximal effort. For each body position, the subject performed a minimum of three maneuvers until two maximal pressure values were obtained which did not differ by more than 5 %; the higher of the two was chosen for analysis. For the sake of convenience, the MIP was expressed in positive values. The peak inspiratory flow was measured with the same portable device and the peak expiratory flow with a peak flow meter (MicroPeak, Micromedical, Rhymney, Wales, UK).

## 2.5 EMG Recordings

The electromyograms of the diaphragm (EDI), parasternal (EPS), scalene (ESC), and genioglossus (EGG) were obtained with surface electrocardiographic electrodes (ARBO, TYCO Healthcare Deutschland GmbH, Neustadt/Donau, Germany). The skin was cleaned with alcohol. The surface EDI was recorded with electrodes applied to the skin over the seventh and eighth intercostal spaces close to the upper rib edge, while the EPS was recorded with electrodes placed in the second right intercostal space close to the sternum. The ESC was obtained from electrodes placed in the posterior triangle of the neck (right side) at the level of the cricoid cartilage. The place was located during sniff maneuvers through palpation of the neck in the lower third of a line drawn between the middle of the mastoid process and the sternal notch. Within each electrode pair, the inter-electrode distance was $<2$ cm. The EGG was obtained from two electrodes placed longitudinally on the underside of the chin at 5 and 10 mm from the inferior margin of the mandible after having checked that minimal inspiratory electrical activity was present during spontaneous breathing. All the EMGs were amplified and continuously recorded on a six-channel recorder (Biograph, St. Petersburg, Russia) and were displayed simultaneously. Data were stored on PC for future analysis. To quantify the EMG, the signals were filtered (10 Hz–1,000 kHz) and integrated on a

moving-time-average basis with a time constant of 150 ms. The peak amplitude of integrated EMG was measured for each inspiratory muscle during quite breathing and during Müller's maneuver throughout the study. The amplitude was measured in arbitrary units and then expressed as a percentage of the mean values reached during spontaneous breathing and maximum Müller's maneuver in the standing position.

### 2.6 Study Design

A head-down tilt of 30° that increases the central venous pressure due to fluid shifts within the entire body was used as a model for simulating central hypervolemia. After completing the MIP, PIF, and PEF measurements, the subject breathed quietly for several minutes to establish a stable pattern of breathing in the standing position. When the baseline parameters were collected, the subject was placed in the supine position using a tilting table and allowed resting in this position for 5 min. Then, the subject was exposed to 30-min tilt. After taking the measurements above outlined again, the subject was moved to the supine position for 5 min and then to the standing position. Ventilation, inspiratory swings of mouth pressure, and the EMGs were continuously monitored throughout the study.

### 2.7 Data Analysis

Baseline (control) respiratory variables and their values during the HDT were expressed as absolute values. Data were presented as means ± SE. Control measurements were performed during spontaneous breathing in the standing position. Differences between respiratory variables and the peak integrated EMGs during quite breathing and Müller's maneuver in the HDT were compared with those of the standing position with a *t*-test. $P < 0.05$ was defined as the criterion of significant differences.

## 3 Results

### 3.1 Respiratory Pattern, Ventilation, and Time-Volume Parameters

The mean data for time-volume variables and minute ventilation in the upright posture and during the 30-min HDT are shown in Table 1. When compared with the control level, blood volume expansion significantly decreased the mean inspiratory flow (30 %). A longer $T_I$ and $T_T$ ($P < 0.05$), but no $T_E$ resulted in a reduction in respiratory rate (15 %). Minute ventilation tended to decrease, but this change was insignificant. Central hypervolemia did not evoke significant changes in tidal volume.

### 3.2 Airway Resistance and Reserve Capacity of the Respiratory System

A significant increase in inspiratory occlusion pressure, which equals alveolar pressure, was observed immediately after the head-down-tilt (Fig. 1). All subjects demonstrated an approximately two-fold increase in $P_{occl}$ and $R_{aw}$ (Table 1). These changes were reversed on return to the standing position. The MIP ranged from 52 to 113 cm $H_2O$ in the standing position. These values are approximately in the normal range as found by others (Sachs et al. 2009; Hautmann et al. 2000). The HDT lowered the MIP by 17.4 % ($P < 0.05$). After 30 min of head-down-tilting, PIF and PEF decreased significantly by 16.3 % and 20.0 %, respectively, compared with the standing position.

### 3.3 Electromyographic Responses

During quiet breathing while standing, phasic inspiratory activity was observed in the D and PS muscles of the subjects. In HDT, there are marked differences in the patterns of respiratory muscle activity during quiet breathing, compared

**Table 1** Volume-time respiratory parameters, inspiratory occlusion pressure, airway resistance, peak respiratory flows, and maximal inspiratory pressure in control and during CHV

| Variables | Control (Standing position) | HDT (−30°) (30 min) |
|---|---|---|
| $V_I$, l/s | 0.53 ± 0.07 | 0.37 ± 0.05* |
| | (0.39–0.64) | (0.26–0.47) |
| $V_T$, l | 0.57 ± 0.10 | 0.56 ± 0.16 |
| | (0.46–1.10) | (0.37–1.20) |
| $V_E$, l/min | 9.47 ± 0.71 | 8.20 ± 0.80 |
| | (6.11–13.83) | (5.08–13.10) |
| $T_T$, s | 3.60 ± 0.41 | 4.01 ± 0.66* |
| | (2.42–4.38) | (3.03–5.01) |
| $T_I$, s | 1.34 ± 0.12 | 1.78 ± 0.63* |
| | (1.11–1.66) | (1.40–2.92) |
| $T_E$, s | 2.30 ± 0.39 | 2.39 ± 1.10 |
| | (1.19–4.50) | (1.72–3.48) |
| $f_b$, breaths/min | 17.51 ± 1.80 | 14.88 ± 2.19* |
| | (10.94–23.02) | (10.42–16.99) |
| $P_{occl}$, cm $H_2O$ | 2.50 ± 0.30 | 4.60 ± 0.40* |
| | (1.80–2.90) | (4.10–5.30) |
| $R_{aw}$, cm $H_2O/l{\cdot}s^{-1}$ | 2.70 ± 0.30 | 4.90 ± 0.40* |
| | (2.15–2.80) | (3.30–8.55) |
| PEF, l/min | 559 ± 62 | 448 ± 41* |
| | (370–800) | (320–590) |
| PIF, l/s | 5.50 ± 0.60 | 4.60 ± 0.50* |
| | (3.80–6.80) | (3.60–5.60) |
| MIP, cm $H_2O$ | 86 ± 11 | 71 ± 9* |
| | (52–113) | (38–105) |

Values are means ± SE
$V_I$ mean inspiratory flow, $V_T$ tidal volume, $V_E$ minute ventilation, $T_T$ total breath cycle, $T_I$ inspiratory cycle, $T_E$ expiratory cycle, $f_b$ breathing frequency, $P_{occl}$ occlusion inspiratory pressure, $R_{aw}$ airway resistance, *PEF* peak expiratory flow, *PIF* peak inspiratory flow, *MIP* maximal inspiratory pressure (range)
*P < 0.05 compared with control


**Fig. 1 Typical inspiratory occlusion pressure swings**: *Panel A* – control (standing position) and *Panel B* – after 30 min of head-down-tilting

with baseline, which may be related to CHV. Phasic PS activity increased more than twice (P < 0.01), which may contribute to the inspiratory rib cage expansion in this condition (Fig. 2A). Peak amplitude of the integrated EMG of the diaphragm decreased immediately the onset of HDT; the decrease was maintained (~40 %) throughout the 30-min tilt (P < 0.01). In contrast, after shifting to supine and upright position, a reverse pattern of EMG-responses was observed; peak amplitude of D and PS EMG returned to the control levels (Fig. 2A). Furthermore, minimal electrical activity was present in the SC and GG muscles during quiet breathing in the upright position. This activity was phasic, occurring during inspiration

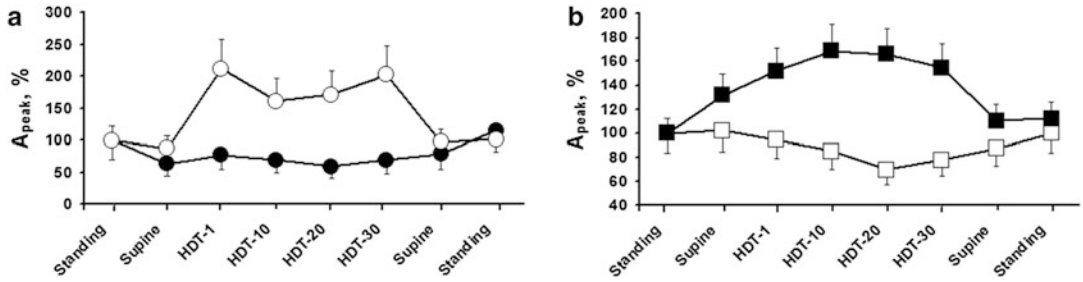


**Fig. 2 Changes in peak amplitude ($A_{peak}$) of integrated EMG** activity of the diaphragm (*solid circles*) and parasternal (*open circles*) (*Panel A*), genioglossus (*solid squares*) and scalene (*open squares*) (*Panel B*) during spontaneous breathing in the supine position and the head-down-tilt after 1, 10, 20, and 30 min and then on return to the previous positions. $A_{peak}$ was expressed as a percentage of control (standing position)

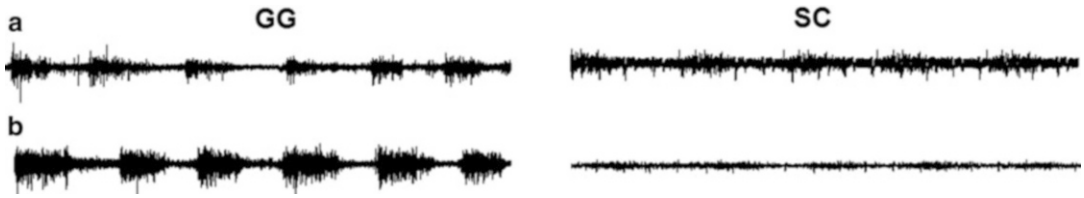


**Fig. 3 Representative EMG recordings** of the scalene (*SC*) and genioglossus (*GG*) muscles during spontaneous breathing in the standing (**a**) and HDT (**b**) positions

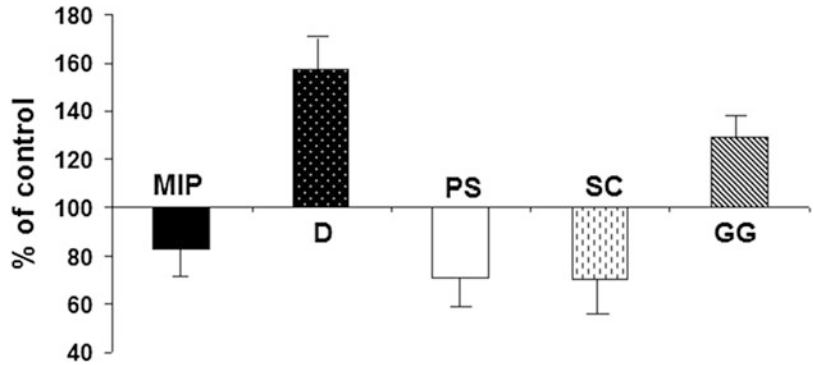


**Fig. 4 Changes in maximal inspiratory pressure (MIP)**, peak amplitude ($A_{peak}$) of integrated EMG activity of diaphragm (*D*), parasternal (*PS*), genioglossus (*GG*) and scalene (*SC*) during Muller's maneuver 30 min after assuming the head-down tilt. Each *column* represents the relative value as a percentage of control (standing position)

(Fig. 3). The transient EMG responses to shifting from the upright to HDT position consisted of a rapid increase in peak amplitude of GG EMG (~65 %), whereas SC activity decreased during spontaneous breathing (~25 %) (Figs. 2B and 3).

All subjects developed phasic inspiratory activity in the D, PS, SC and GG muscles during the voluntary maximal inspiration against closed airways (Müller's maneuver). Integrated EMG of each muscle was expressed as a percentage of its value in the standing position, taken as 100 %. We found differences in the inspiratory muscle activation during Müller's maneuver during the 30-min HDT compared with quite breathing. The peak magnitude of D EMG increased by 57 % during tilting ($P < 0.05$) (Fig. 4). As illustrated in Fig. 4, lower than control values for integrated EMG of PS and SC, by 29 % and 30 %, respectively, were achieved during Müller's maneuver in HDT ($P < 0.05$). The

amplitude of GG EMG was the greatest in the HDT positions during Müller's maneuver (130 % of control) ($P < 0.05$).

## 4 Discussion

During the head-down-tilt or head-out water immersion, intrathoracic blood volume increases due to central translocation of circulating blood and the central hypervolemia develops (Lin 1984; Norsk 2005). It is known that central hypervolemia induces changes in the cardiovascular system and in pulmonary mechanics. However, little is known about the compensatory responses of the respiratory system and about the inspiratory muscle function during the expansion of intrathoracic blood volume. Our present findings show that central hypervolemia increased airway resistance, but the compensatory responses provided the maintenance of the minute ventilation at the stable level. These responses were expressed in an increase of contractile activity and redistribution of the degree of participation of different groups of inspiratory muscles in the respiratory act. We found differences in the pattern of respiratory muscle use during quiet breathing in normal subjects submitted to acute cardiopulmonary blood volume expansion. The maintenance of adequate pulmonary ventilation was provided by a two-fold increase in the activity of the inspiratory muscles of the chest. This group of inspiratory muscles compensates for increased resistive load, ensuring the growth of alveolar pressure for adequate tidal volume during CHV. Electrical activity of the diaphragm was reduced compared with the usual conditions for human hemodynamics, so we can assume that the diaphragm's contribution to the compensation of respiratory effects of hypervolemia was reduced. The redistribution in the inspiratory muscle activity during CHV may be associated with the principle of energy optimization of respiratory movements underlying the patterning of breath (Otis et al. 1950; Segizbaeva 2010). In HDT an effective diaphragmatic contraction is energetically less profitable, since the implementation of the inspiratory efforts are needed to offset the abdominal content, putting pressure on the diaphragm. It is likely that in such a condition an increase in inspiratory muscle contraction of the chest would be more favorable energetically. Accordingly, this group of muscles provides the required level of inspiratory oscillations of alveolar pressures during quite breathing in HDT. A decrease in EMG activity of the SC muscle in the HDT position is explicable by the muscle's initial position (muscle length) and biomechanical conditions of respiratory movements. Possibly, a decrease in the length of the muscle's fibers, which occurs due to mechanical changes during antiorthostatic body position, does not allow increasing its activity. The genioglossus is a major dilator muscle of upper airways. GG does not participate in the generation of inspiratory pressure, but contributes to the stabilization of airway patency. Maximal activation of GG was obtained in the HDT position in all subjects. Upper airway dilator muscles are activated in phase with the respiratory cycle generated by the central nervous system. Studies have shown that non-physiological upper airway mechanoreceptive stimuli (e.g., rapidly imposed pulses of negative pressure) also activate these muscles. Such reflexes may become activated during conditions that alter airway resistance in order to stabilize airway patency (Akahoshi et al. 2001).

The mechanisms responsible for CHV-induced compensatory responses of the respiratory system seem complex. CHV reduced the inspiratory flow; therefore, stimulation of receptors sensitive to a dynamic component of lung tension was weaker. Decreased inhibitory afferentation from pulmonary stretch receptors could render higher EMG activity and power of inspiratory muscle contractions. The involvement of vagal afferents in the intensification of inspiratory efforts induced by CHV has been supported by studies on animal models. The CHV-induced esophageal pressure response is strongly suppressed by transection of the vagal nerves (Aleksandrova et al. 2007; Donina et al. 2013). The human upper respiratory tract has a rich sensory supply and the upper airway

receptors may play a significant role in the formation of adaptive reactions to increased resistive load (Winning et al. 1985). Furthermore, afferent information from intercostal muscle proprioceptors provides both the additional activation of the related spinal alpha-motoneurones (Corda et al. 1965) and the immediate information transmission to the bulbar respiratory structures, with the resultant changes in central inspiratory activity (Shannon et al. 1985). It is possible that the intrinsic properties of inspiratory muscles may also be essential for the respiratory load compensation during CHV, because the force of muscle contraction depends on the velocity of its shortening and the initial muscle length (Sharp 1980).

Analysis of changes in MIP, PIF, and PEF indicates that the intrathoracic blood volume expansion decreased the reserve capacity of the respiratory system and weakened the load compensatory responses. The HDT significantly decreased the indices outlined above compared with their control levels. Changes in muscle mechanics might influence MIP when moving from the standing position to HDT. Gravity pulls the abdominal content caudally, increasing the vertical diameter of the thorax in the standing position (Castile et al. 1982). In the HDT, the abdominal content pushes the diaphragm up into the thoracic cavity, raising the diaphragm length and decreasing functional residual capacity (FRC) relative to the standing condition. It is interesting that the maximal inspiratory efforts during HDT evoked the opposite EMG activity pattern; the contribution of inspiratory thoracic muscles decreased and diaphragm's EMG activity increased compared with spontaneous breathing.

In conclusion, the present study showed that in normal humans exposed to intrathoracic blood volume expansion there is an increase in respiratory loading and the development of compensatory responses. These responses are expressed by an increase in contractile activity and redistribution of the participation of different groups of the respiratory muscles. The mechanisms of respiratory load compensation seem to be underlain by the afferent information from the lung and respiratory muscle receptors, the segmentary reflexes, and the intrinsic properties of muscle fibers. Respiratory effects of central hypervolemia are compensated during spontaneous breathing, but the maximal reserve capacity of the respiratory system decreases during intrathoracic blood volume expansion.

**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# Cellular and Soluble Inflammatory Markers in Induced Sputum of Composting Plant Workers

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**Abstract**

Inflammatory processes, including respiratory symptoms, can be induced among workers in composting plants exposed to bioaerosols containing microorganisms and their compounds. We evaluated inflammatory processes in the lower respiratory tract *via* cellular and soluble mediator profiles in induced sputum (IS). IS samples of 140 current (35 % smokers) and 49 former compost workers (29 % smokers) as well as 29 white-collar workers (17 % smokers) were collected and analyzed for the cell count and composition, and for soluble biomarkers. Significant differences between current and former compost workers and white-collar workers were detected for total cell count ($p = 0.0004$), neutrophils ($p = 0.0045$), sCD14 ($p = 0.008$), and 8-isoprostane ($p < 0.0001$). IS of non-smoking former compost workers showed lower concentrations of IL-8, total protein, immunoreactive MMP-9 and sCD14, compared with non-smoking current compost workers. 10.1 % of the study population was suffering from chronic bronchitis with significant differences ($p = 0.018$) between former compost workers (24.5 %), current workers (5 %), and white-collar workers (10.3 %). Significantly lower IL-8 ($p = 0.0002$), neutrophils ($p = 0.001$), and MMP-9 ($p = 0.0023$) values were measured in healthy subjects compared with subjects with chronic bronchitis. In conclusion, changes in lower airways were detected by analysis of biomarkers in IS of current exposed and, to a lesser extent, in IS of former compost workers. These effects are especially pronounced in subjects with chronic bronchitis.

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**Keywords**
Composting plants • Induced sputum • Non-invasive methods • Inflammatory markers

## 1 Introduction

Bioaerosols contain variety of different airborne biological agents and are associated with a wide range of potential health problems (Douwes et al. 2003; Eduard et al. 2012). Exposure to bioaerosol components (e.g. fungi, bacteria, mycotoxins, allergens, and endotoxins) in the working environment has emerged as a dominant health concern in some occupational settings such as wastewater treatment and composting facilities (Chang et al. 2014). Composting is a natural biological process to biodegrade organic waste such as food and green waste, paper, manure and crop residues, which is mainly driven by a complex microbial community (van Kampen et al. 2014; Chang et al. 2014). Inhaled bioaerosol components can attach to epithelial cells in terminal airways and cause harmful effects. There is increasing knowledge that the respiratory symptoms induced by a complex mixture of several so-called pathogen-associated molecular patterns (PAMPs) (e.g. cell wall components like endotoxin and ß-(1–3) glucans) are mainly based on non-allergic inflammation (Schlosser et al. 2012). Cross-sectional and cohort studies (Bünger et al. 2007; van Kampen et al. 2012) showed that workers exposed to organic dust from composting plants had a higher prevalence of inflammatory response of the upper airways and eyes, the so-called mucous membrane irritation syndrome (MMIS). In addition, cases of hypersensitivity pneumonitis (HP), organic dust toxic syndrome (ODTS), and allergic bronchopulmonary aspergillosis (ABPA) were reported (Allmers et al. 2000; Bünger et al. 2000).

The impact of current or former bioaerosol exposure during working in composting plants on the inflammatory response in the lower airways is not well known so far. Non-invasive methods like the measurement of fractional exhaled nitric oxide (FeNO) and the collection and analysis of exhaled breath condensate (EBC) (Hoffmeyer et al. 2009) and induced sputum (IS) are useful methods in identification of adverse respiratory effects in exposed workers (Hoffmeyer et al. 2009; Quirce et al. 2010; Raulf-Heimsoth et al. 2011).

The objective of the present study was to evaluate the inflammatory processes in the lower respiratory tract *via* cellular and soluble mediator profiles in IS taking into account confounders like smoking and clinical symptoms. The study was conducted in current compost workers in comparison to former compost workers and to white-collar workers.

## 2 Methods

The study design and the protocol were created in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Ruhr-University in Bochum, Germany. All study participants gave written informed consent to the study protocol.

### 2.1 Study Group

As part of a cross-sectional study, 140 current and 49 former compost workers from 31 composting plants in North Western Germany and 29 white-collar workers were examined. The study protocol and the exposure circumstances of the study group were recently published (van Kampen et al. 2014). Smoking status was based on the self-assessed information by interview and study participants were classified as current, former, and never-smokers. For the classification of the study group according to their clinical symptoms, like cough, obstruction, or chronic bronchitis, data of a detailed questionnaire were used and verified by lung function parameters forced expiratory volume in 1 s ($FEV_1$) and forced vital capacity (FVC). The atopy status of the workers was determined serologically using the immunoglobulin E (IgE) measurement in response to a variety of environmental

allergens (sx1 Phadiatop, ThermoFisher Scientific/Phadia, Uppsala, Sweden). A positive atopic status was assumed in case of sx1 values ≥0.35 kU/L.

### 2.2 Collection and Analysis of Induced Sputum

Induced sputum (IS) of each subject was collected after inhalation of isotonic saline (0.9 %) aerosol, generated by an ultrasonic nebulizer for 10 min during a midweek working shift, as described earlier (Raulf-Heimsoth et al. 2011). The subjects were motivated to cough actively, clear their throat, and expectorate sputum. The volume of the IS was determined and an equal volume of 0.1 % sputolysin (dithiothreitol) was added. The samples were mixed gently by vortex mixer and incubated for 30 min at 37 °C to ensure a complete homogenization. After centrifugation the cell-free supernatants were aliquoted, stored at −80 °C under argon protection until further analysis of soluble markers. The cell pellets were resuspended and the total cell number was determined. For differential cell counts of sputum cells, slides were prepared by cytospin (Cytospin 2, Shandon Corp., Pittsburgh, PA) and stained with May-Grünwald-Giemsa. Three independent observers counted 200 cells on each slide by light microscopy. Their results were expressed as a percentage of the total cell numbers and absolute numbers of the cell population (without correction of squamous cells). The inflammatory mediators were determined in the thawed cell-free supernatants of the IS samples. All samples underwent only a single freeze-thaw cycle. The following soluble markers were measured in the IS samples: interleukin-8 (IL-8), total protein content, soluble (s) CD14, matrix metalloproteinase (MMP)-9 and 8-iso-$PGF_{2\alpha}$ (8-isoprostane). IL-8 was measured with the OptEIATM ELISAs (BD Biosciences Pharmingen, Heidelberg, Germany) in a standard range of 3–200 pg/ml. Determinations of sCD14 and MMP-9 were performed with the DuoSetTM ELISA Development system (R&D Systems, Wiesbaden Germany) in a standard range of 62.5–4,000 pg/ml for sCD14 and 31.2–2,000 pg/ml for MMP-9. Total protein content was determined according to the method of Bradford with bovine serum albumin as standard solution (range 10–100 µg/ml) (Bradford 1976). 8-iso-$PGF_{2\alpha}$ was quantified with a specific sandwich immunoassay kit (Assay Designs, Ann Arbor, MI) with a limit of quantification of 6.1 pg/ml.

### 2.3 Statistical Analysis

Data were expressed as median with interquartile range. Values distribution was assessed using the D'Agostino & Pearson omnibus normality test. Values below the limit of quantification (LOQ) were set at 2/3 of the LOQ. Comparisons of unpaired data were performed with Mann-Whitney U test or Kruskal-Wallis test and the distribution of habits, like smoking, between different groups was compared with the Chi-square test. Spearman rank correlation test was used to determine correlations between different biomarkers. A-two-sided significance level of 0.05 was chosen for all tests. Data were evaluated with GraphPad Prism ver. 5.01 for Windows (GraphPad Software, San Diego, CA).

## 3 Results

Sputum induction and analysis was possible in all 218 subjects, 140 current and 49 former compost as well as 29 white-collar workers, the last group without any exposure to compost-plant specific bioaerosols. All subjects tolerated the procedure well, without adverse reactions. Table 1 presents the characteristics of the study

**Table 1** Study group included in the sputum analysis (n = 218)

| | Compost | | White-collar workers (controls) |
|---|---|---|---|
| | Current workers | Former workers | |
| n | 140 | 49 | 29 |
| Gender, male (n%) | 135 (97 %) | 44 (92 %) | 28 (97 %) |
| Age (year; mean ± SD) | 45 ± 9.1 | 51 ± 10.3 | 57 ± 6.5 |
| sx1-pos (atopics) (n%) | 48 (34.5 %) | 18 (36.7 %) | 5 (17.2 %) |
| Current smokers (n%) | 48 (35 %) | 14 (29 %) | 5 (17 %) |

**Table 2** Comparison of current and former compost workers with the white-collar workers (controls)

| | Compost | | | | | | White-collar workers | | | |
|---|---|---|---|---|---|---|---|---|---|---|
| | Current workers (I) | | | Former workers (II) | | | Controls (III) | | | |
| Parameter | n | Median | Interquartile range | n | Median | Interquartile range | n | Median | Interquartile range | |
| Total protein (µg/ml) | 140 | 270 | (121–564) | 49 | 207 | (96–620) | 29 | 355 | (224–642) | n.s. |
| IL-8 (pg/ml) | 139 | 3,266 | (1,034–9,991) | 49 | 2,044 | (1,031–6,930) | 29 | 1,889 | (1,012–5,279) | n.s. |
| MMP-9 (ng/ml) | 122 | 270 | (109–673) | 48 | 138 | (42–468) | 27 | 295 | (137–394) | n.s. |
| sCD14 (pg/ml) | 122 | 6,122 | (1,265–10,859) | 45 | 2,378 | (212–9,949) | 24 | 8,546 | (6,965–14,546) | $p = 0.0008$ |
| | | | | | | | | | | I *vs.* III |
| | | | | | | | | | | $p < 0.05$ |
| | | | | | | | | | | II *vs.* III |
| | | | | | | | | | | $p < 0.001$ |
| 8-Isoprostane (pg/ml) | 128 | 3,346 | (1,730–5,976) | 44 | 2,297 | (1,086–5,953) | 18 | 14,699 | (7,575–26,032) | $p < 0.0001$ |
| | | | | | | | | | | I *vs.* III |
| | | | | | | | | | | $p < 0.001$ |
| | | | | | | | | | | II *vs.* III |
| | | | | | | | | | | $p < 0.001$ |

*n.s.* not significant

group, which was predominantly male. Age and the atopy status (determined by serum specific IgE to ubiquitous aero-allergens, sx1) were not significantly different between the three groups. Current smoking was reported by 35 % of the current compost workers, by 29 % of the former workers and by 17 % of the white-collar workers; these differences were not significant.

Table 2 summarizes the results of soluble biomarkers measured in IS samples. No significant differences were detected for total protein, IL-8, and immunoreactive MMP-9 concentrations in IS of the current and former compost workers as well as of the white-collar workers. In contrast, differences between the following soluble IS biomarkers were statistically significant: sCD14 ($p = 0.0008$) and 8-isoprostane ($p < 0.0001$), especially when comparing with the white-collar worker group. The concentrations of all these biomarkers were lower in IS of the former compost workers compared with the current compost workers, without reaching the statistically significant level. The total cell count showed lower values in the former compost workers than in the current ones (median: $6 \times 10^5$ *vs.* $11 \times 10^5$, $p = 0.001$) and in white collar-workers ($p < 0.01$) (data not shown). The percentage of neutrophils was significantly higher in the current workers compared with the white-collar workers ($p < 0.01$) (Fig. 1). Additionally, there was a high correlation between the IL-8 concentration and the number of neutrophils in IS ($r_s = 0.669$; $p < 0.0001$) (Fig. 2).

Classification of the subjects according to their smoking habits into current smokers and non-smokers (including also ex-smokers) (Table 3) demonstrated that IS concentrations of several soluble biomarkers are highly influenced by smoking. Non/ex-smokers in the group of former workers showed lower biomarker concentrations compared with the non/ex-smoker group of current workers. Especially the comparison of MMP-9 and sCD14 concentrations in IS samples of these two groups reached the statistically significant level (MMP-9: $p < 0.001$; sCD14: $p < 0.05$). Smokers in the group of current workers and former workers showed the highest IL-8 concentrations (Fig. 3). Therefore, for further biomarker analysis smoking habits were taken into account.

An additional approach was to clarify the association between clinical symptoms (like

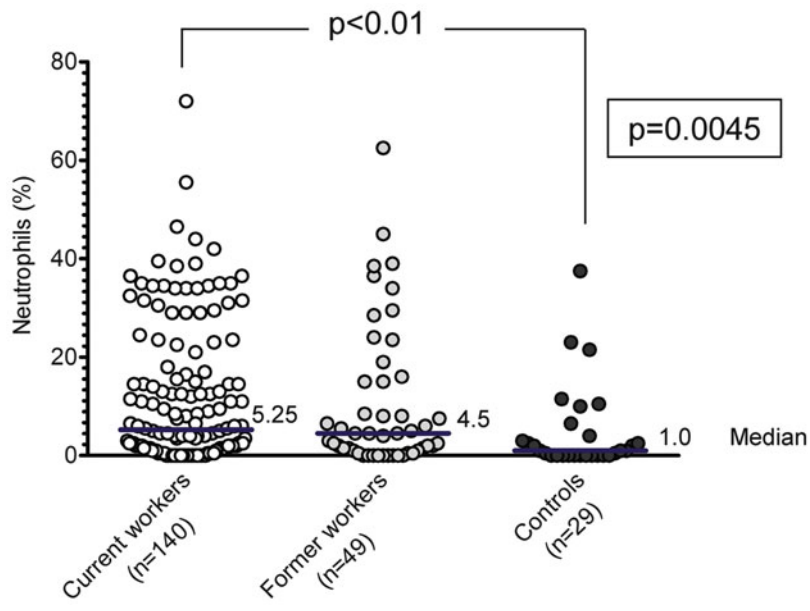


**Fig. 1 Percentage of neutrophils in induced sputum** of current compost workers, former compost workers, and white-collar workers (controls). Using Kruskal-Wallis test with Gaussian approximation the p-value between all three groups is 0.0045 and a significant difference exists between the percentage of IS neutrophils in current workers compared with white-collar workers

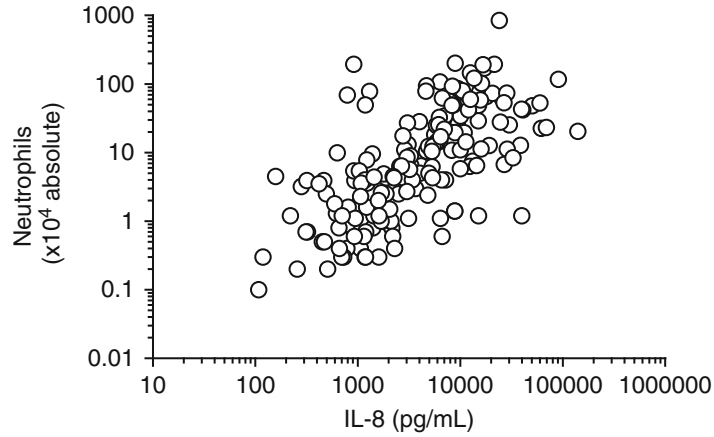


**Fig. 2 Correlation between IL-8-concentrations and neutrophils in induced sputum**. Spearman's $r = 0.669$, $p < 0.0001$

cough or chronic bronchitis) and inflammatory markers. According to this, the study group was divided into three groups: (I) healthy subjects ($n = 111$), (II) subjects with cough and/or obstruction ($n = 84$), and (III) subjects with chronic bronchitis ($n = 22$). As presented in Table 4, 10.1 % of the study population was suffering from chronic bronchitis with significant differences ($p = 0.018$) between former compost workers (24.5 %), current workers (5 %), and white-collar workers (10.3 %). 55 % of the subjects with chronic bronchitis were current smokers in contrast to 28 % in the healthy subject group. Twelve out of the 22 subjects (54.5 %) with chronic bronchitis are former workers. Dividing each of the three groups into non/ex-smokers and current smokers, cell differential analysis of the IS showed that the percentage of neutrophils in each group was higher in the current smokers than that in the non/ex-smokers, and the percentage of neutrophils increased with an augmentation of clinical symptoms (healthy subjects < subjects with cough/obstruction < subjects with chronic bronchitis) (Fig. 4a). A significantly higher ($p < 0.05$) percentage of neutrophils was measured in IS of

**Table 3** Comparison of current and former compost workers with the white-collar workers (controls)

| | Compost | | | | White-collar workers | | |
|---|---|---|---|---|---|---|---|
| | Current workers (I) | | Former workers (II) | | Controls (III) | | |
| | A | B | C | D | E | F | |
| Parameter | Non/Ex-Smoker (n = 91) | Smoker (n = 48) | Non/Ex-Smoker (n = 35) | Smoker (n = 14) | Non/Ex-Smoker (n = 24) | Smoker (n = 5) | p-value |
| Total protein (μg/ml) | 231 | 349 | 155 | 634 | 377 | 260 | $p < 0.0001$ |
| | (102–521) | (142–653) | (69–249) | (505–925) | (222–561) | (232–844) | C *vs.* D $p < 0.0001$ |
| | | | | | | | C *vs.* E $p < 0.05$ |
| IL-8 (pg/ml) | 2,746 | 6,959 | 1,217 | 8,267 | 1,893 | 1,574 | $p < 0.0001$ |
| | (879–7,701) | (1,475–13,286) | (696–3,002) | (5,048–38,914) | (934–5,185) | (980–24,626) | C *vs.* D $p < 0.001$ |
| MMP-9 (ng/ml) | 298 | 265 | 80.6 | 421 | 295 | 231 | $p = 0.0029$ |
| | (120–724) | (106–620) | (18.6–202) | (263–1,088) | (212–396) | (100–363) | A *vs.* C $p < 0.001$ |
| | | | | | | | C *vs.* D $p < 0.001$ |
| sCD14 (pg/ml) | 5,676 | 6,501 | 725 | 10,852 | 8,165 | 13,606 | $p < 0.0001$ |
| | (1,181–10,427) | (1,607–10,980) | (974–6,005) | (6,240–16,969) | (6,683–13,361) | (7,684–17,562) | A *vs.* C $p < 0.05$ |
| | | | | | | | C *vs.* D $p < 0.001$ |
| | | | | | | | C *vs.* E $p < 0.001$ |
| 8-Isoprostane (pg/ml) | 3,578 | 3,028 | 1,894 | 2,423 | 15,733 | 11,501 | $p < 0.0001$ |
| | (1,741–6,458) | (1,632–5,264) | (1,068–5,975) | (1,450–5,950) | (7,673–15,733) | (7,028–15,975) | A *vs.* E $p < 0.001$ |
| | | | | | | | C *vs.* E $p < 0.001$ |

Comparisons were done between A and B, C and D, E and F, A and C, A and E, C and E, B and D, and D and F

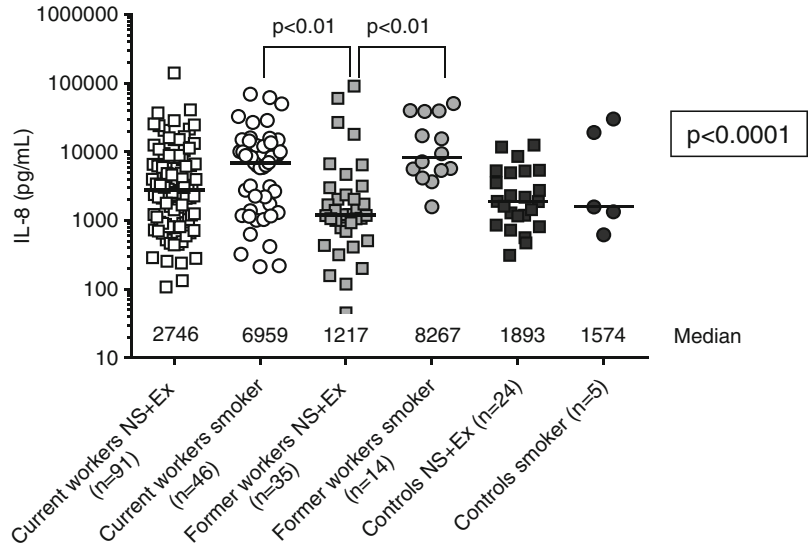


**Fig. 3 Influence of smoking habits on the IL-8-concentration in induced sputum** of current compost workers, former compost workers, and white-collar workers (controls). There were significant differences ($p < 0.001$) between non/ex-smoking former workers and smoking former workers ($p < 0.001$)

**Table 4** Study group classified according to the intensity of respiratory symptoms

| | Total | | Compost | | | | White-collar workers | |
|---|---|---|---|---|---|---|---|---|
| | | | Current workers (I) | | Former workers (II) | | Controls (III) | |
| | n | Smoker (%) | n | Smoker (%) | n | Smoker (%) | n | Smoker (%) |
| Healthy | 111 | 28 | 78 | 35 | 21 | 14 | 12 | 7 |
| Cough and/or obstruction | 84 | 27.4 | 54 | 33 | 16 | 25 | 14 | 14 |
| Chronic bronchitis | 22 | 55 | 7 | 43 | 12 | 58 | 3 | 67 |

smoking subjects suffering from chronic bronchitis compared with the healthy non/ex-smokers. A similar pattern was detected for the IL-8 sputum concentrations: higher IL-8 concentration in the smokers in each group and increasing of IL-8 concentration with an augmentation of respiratory symptoms (Fig. 4b). In addition, sputum immunoreactive MMP-9 concentrations (Fig. 4c) increased also significantly with an augmentation of symptoms, but in contrast to the other biomarkers mentioned above, the MMP-9 sputum concentration was not influenced by smoking habits (Fig. 5).

## 4 Discussion

In addition to a recently published cross-sectional study of compost workers (van Kampen et al. 2012), the major result presented here within the study group is the detection of inflammatory effects in the lower respiratory tract (assessed by the analysis of the cellular and soluble biomarkers of IS samples) in the currently exposed and, to a lesser extent, in the former compost workers. These effects were particularly pronounced in the subjects with chronic bronchitis.

Working in a compost plant is associated with exposure to bioaerosols, which are important air pollutants that are recognized to play an important putative role in lung inflammatory process leading to COPD and exacerbation of COPD. These bioaerosols are complex and diverse mixtures of PAMPs, which are able to activate immune inflammatory pathways postulated to be important in the development of airway disease (Eduard et al. 2009; Harting et al. 2012; Kline et al. 2004; Sarir et al. 2008). As published for our study group (van Kampen et al. 2014) the highest values of cultivable microorganisms in

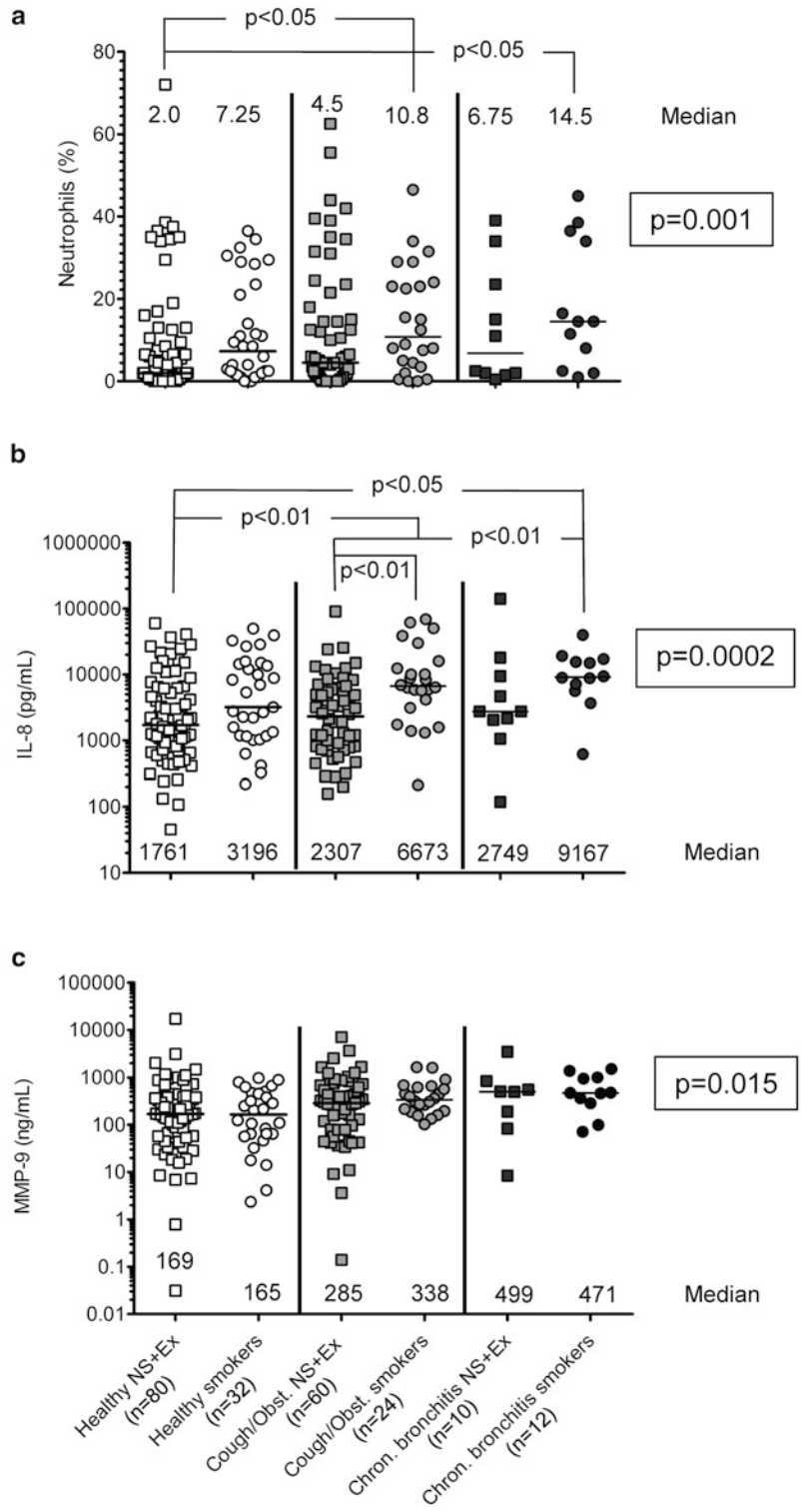


**Fig. 4 Comparison of the percentage of neutrophils (a), IL-8 concentrations (b), and immunoreactive MMP-9 concentrations (c)** in induced sputum samples of healthy subjects, subjects with cough/obstruction, and chronic bronchitis (each group was differentiated into non/ex-smokers and current smokers)

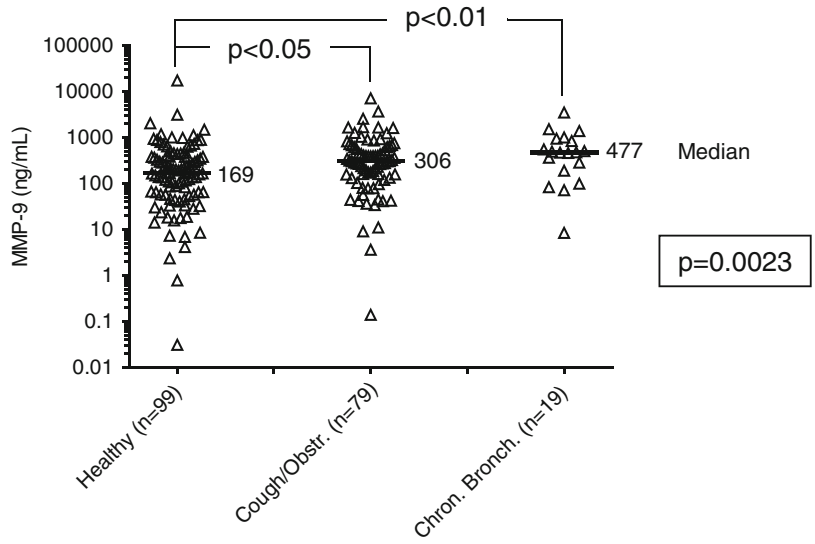


**Fig. 5 Comparison of immunoreactive MMP-9 concentrations in induced sputum** in healthy subjects, subjects suffering from cough/obstruction and suffering from chronic bronchitis. Significant differences were detected between all groups. The highest MMP-9 concentrations were measured in the group with chronic bronchitis

composting plants were demonstrated during shredding, processing, and in sorting cabins and can be substantially reduced by personal or technical means of protection. To avoid misclassification concerning exposure levels and changing working tasks, we analysed the data of the 218 subjects firstly with respect of current working in compost plants in comparison to former working and to white-collar workers without occupational bioaerosol exposure, and secondly with respect to clinical respiratory symptoms. Since cigarette smoking is a well-known inducer of lung inflammatory processes, smoking habits were taken into account for data analysis.

In the present study, a biologically plausible correlation was found between the increased sputum levels of the neutrophil chemoattractant IL-8, a key mediator of neutrophil-mediated acute inflammation, and the neutrophil response. It is well known that sputum IL-8 concentrations and neutrophil counts are related to the intensity of chronic airway obstruction (Bartoli et al. 2009). Matrix metalloproteinase (MMP)-9 is known to be involved in structural changes of the bronchial epithelium, like degradation of extracellular matrix, in response to a prolonged period of epithelial repair. MMP-9 is constitutively expressed by neutrophils, but inflammatory stimuli can induce MMP-9 expression by other airway cells (Chang et al. 2014; Chakrabarti and Patel 2005; Devereux et al. 2014). MMP-9 immunoreactivity has been demonstrated to be associated with the severity of classic asthma, and MMP-9-deficient animals exhibit reduced airway inflammation (Ma et al. 2014). CD14 is the initial principal receptor together with Toll-like receptors mediating LPS-induced inflammation *in vivo,* and its soluble form (sCD14) can be found in human airway fluids (Sahlander et al. 2012).

All soluble biomarkers measured in this study (total protein, IL-8, MMP-9, and sCD14), with the exception of 8-isoprostane, were significantly affected by current smoking. The influence of current smoking was particularly pronounced among former workers without current exposure to bioaerosols. Comparison of the biomarker concentrations of non/ex-smokers within current and former compost workers clearly showed that the cessation of occupational exposure to bioaerosols reduced the concentrations for IL-8, total protein (without reaching the significance level), and significantly so for MMP-9 and sCD14. These findings suggest a remission of a 'subchronic' inflammation in workers exposed to bioaerosols once exposure is terminated. Similar effects were described by Sikkeland et al. (2012) in a group of workers formerly exposed to organic dust containing moderate up to high endotoxin concentrations 1 year after cessation

of exposure. They measured sputum markers of airway inflammation and innate immune function and demonstrated that, for instance, the sputum neutrophil proportion and numbers, IL-8, IL-1ß, and eNO were significantly decreased 1 year after cessation of exposure. The authors concluded that changes induced by bioaerosol exposure were partly reversible among workers who were no longer exposed, in this case, to endotoxin.

Sahlander et al. (2012) described in their study that pig farmers, with a high daily exposure to PAMP, had lower levels of soluble sCD14 in sputum than unexposed healthy subjects. However, the authors failed to take the smoking habits into account. In the present study we also showed a significantly lower sCD14 concentration in the current workers compared with white-collar workers. Similar to the pig farmers who regularly inhale high amounts of LPS, also compost workers have this working environment. Hence, reduced levels of sCD14 may stem from LPS-binding to sCD14 which, as a result, may become undetectable with the ELISA method used.

Taking the severity of respiratory symptoms of the study group into account, participants with chronic bronchitis had elevated sputum levels of cellular and soluble biomarkers of inflammation. With the exception of immunoreactive MMP-9 concentrations, all other biomarkers were affected by smoking. In a previous study with workers exposed to vapors and aerosols of bitumen (Raulf-Heimsoth et al. 2011), we had observed a similar effect that sputum IL-8 concentrations were significantly increased by smoking and bitumen exposure, whereas sputum MMP-9 concentrations were only significantly affected by bitumen exposure but not by cigarette smoking.

In conclusion, inflammatory effects in the lower respiratory tract could be detected by analysis of the IS biomarkers for currently exposed and, to a lesser extent, in former compost workers. The effects were particularly pronounced in subjects with chronic bronchitis. Our study showed that implementation of sputum induction and analysis is useful to assess the inflammatory processes in the airways of workers exposed to bioaerosols. The assessment of airway inflammation using sputum or other sources, like exhaled breath condensate, should, as a rule, consider smoking habits for a meaningful evaluation and interpretation of the effects and to detect the risk factors.

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**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# The Influence of the Reference Values on the Interpretation of Lung Function in Children: Comparison of Global Lung Initiative 2012 and Polish 1998 Reference Values

Joanna Peradzyńska, Katarzyna Krenke, Anna Szylling, Rafał Krenke, and Marek Kulus

**Abstract**

Interpretation of spirometry strongly depends on the applied predicted values. New Global Lung Initiative (GLI) reference values have recently been published but their influence on spirometry interpretation in children has not been widely evaluated. The aim of the study was to compare the interpretation of spirometry using GLI-2012 *vs.* Polish-1998 reference values. Spirometry results of 315 Caucasian children aged 4–18 were analyzed. Airway obstruction was defined as $FEV_1/FVC < LLN$ (lower limit of normal: 5th percentile, −1,64 standard deviation), while restrictive ventilatory pattern as $FVC < LLN$ and $FEV_1/FVC > LLN$. The findings were that $FEV_1$ and FVC expressed as GLI-2012 or Polish-1998 z-scores differed significantly ($p < 0.05$). The mean $FEV_1$ z-score was $-0.68 \pm 1.25$ *vs.* $-0.13 \pm 1.70$ and the mean FVC was $-0.34 \pm 1.08$ *vs.* $0.30 \pm 1.15$ for GLI-2012 and Polish-1998, respectively. There was no difference for $FEV_1/FVC$ z-scores. Obstructive and restrictive ventilatory patterns were diagnosed in 20.3 % and 7.6 % children using GLI-2012 values compared with 17.5 % and 3.8 % when using Polish-1998 reference values, respectively. In conclusion, the use of GLI-2012 reference values in the population of Polish children increases the number of detected lung function abnormalities compared with Polish-1998 reference values.

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**Keywords**
Children • Global lung function • Initiative lung function • Predicted values • Reference values • Spirometry

## 1 Background

Spirometry is a relatively simple diagnostic procedure measuring lung function. As it provides data on lung volumes and airflow, it is widely used in the diagnosis and monitoring of lung diseases. The reliability of spirometry depends on a variety of factors, such as patient-technician cooperation, patient's effort, quality of the measuring system, and technician skills and experience. The interpretation of spirometry is based on the comparison of the measured variables with reference values. Thus, the use of proper reference values plays a critical role in the diagnosis of pulmonary function impairment. Application of inappropriate reference values may lead to overestimation or underestimation of pulmonary function. That may, obviously, result in misdiagnosis and may hamper treatment.

Many different equations have been proposed to calculate predicted spirometric values. In children, the most commonly used formulas include those published by Hankinson et al. (1999), Wang et al. (1993), Knudson et al. (1983), Zapletal et al. (1977), and Polgar and Promadhat (1971). For the population of Polish children, distinct spirometric reference equations have been proposed by the authors from the Institute of Tuberculosis and Lung Diseases – Rabka Branch (Willim et al. 1998). These equations were published in 1998 and since then have been widely used in Poland.

The ERS task force, known as the Global Lung Function Initiative (GLI) published a new multiethnic reference values for spirometry in 2012. The unique features of these reference values include their applicability for both children and adults (age range between 3 and 95 years) and worldwide coverage (Quanjer et al. 2012).

The influence of the GLI-2012 reference values on spirometry interpretation has been addressed in several studies (Pereira et al. 2014; Quanjer and Weiner 2014; Stanojevic et al. 2014; Quanjer et al. 2013; Ben Saad et al. 2013). However, children were included only in two of those publications (Quanjer and Weiner 2014; Stanojevic et al. 2014). To our knowledge, there have been no previous reports evaluating the relationship between the use of the GLI-2012 reference values and lung function interpretation in Polish children. Hence, we undertook a study to compare spirometry results interpreted with the use of GLI-2012 and Polish-1998 reference values in a population of in Polish children.

## 2 Methods

### 2.1 Study Design

The study was approved by the Ethics Committee of the Medical University of Warsaw in Poland. This prospective study included 315 consecutive Caucasian children, who underwent spirometry in the Department of Pediatric Pneumology and Allergy, Medical University of Warsaw between October 2013 and February 2014. The patients were referred for spirometry either due to new signs and symptoms (wheezes, dyspnea, and chronic cough) or to assess the presence and severity of lung function impairment due to previously diagnosed diseases, mainly asthma.

There were 129 girls and 186 boys aged 4–18 (mean age 12.3 $\pm$ 3.3 years). On the day of the test, all children were measured with the use of a stadiometer and the exact age was calculated. Spirometry was performed by an experienced technician with a certified commercial spirometer (Lungtest 1000, MES, Cracow, Poland). Three technically correct and repeatable flow-volume curves were recorded and the best curve

**Table 1** Regression equations recommended by Willim et al. (Polish-1998)

| | | Boys | | | Girls | | |
|---|---|---|---|---|---|---|---|
| Variable | Regression model | a | b | SD | a | B | SD |
| $FEV_1$ | $V_n = \exp(a + b \cdot x)$ | −2.06 | 0.02 | 0.11 | −2.01 | 0.02 | 0.13 |
| FVC | $V_n = \exp(a + b \cdot x)$ | −2.20 | 0.02 | 0.13 | −2.10 | 0.02 | 0.13 |
| $FEV_1$/FVC | $V_n = a + b \cdot x$ | 108.49 | −0.15 | 7.03 | 102.86 | −0.10 | 7.19 |

$V_n$ – predicted value, a, b – coefficients, x – height in cm, SD – standard deviation

was selected for analysis. The variables included: forced expiratory volume in 1 s ($FEV_1$), forced vital capacity (FVC), and the $FEV_1$/FVC ratio. The results were compared with two different reference values: reference values for the Polish Children and Adolescents published by Institute of Tuberculosis and Lung Diseases in Rabka in 1998 (regression equations are provided in Table 1) and GLI-2012. GLI calculations were made using GLI recommended software (Quanjer and Weiner 2014; Willim et al. 1998).

Airway obstruction was defined as $FEV_1$/FVC < LLN (lower limit of normal), while a restrictive ventilatory pattern was diagnosed when FVC was below the LLN and $FEV_1$/FVC > LLN. The LLN was the 5th percentile (−1.64 standard deviation from the predicted value) for all reference equations. The results were provided as z-score, defined as the number of standard deviations from the mean. The number and percentage of abnormal results found with the use of two different predicted values (Polish-1998 and GLI-2012) were compared.

## 2.2 Data Elaboration

Data were analyzed as follows: (1) entire group analysis, (2) male and female subgroup analysis, and (3) age-defined subgroup analysis. First, the values expressed as z-scores of predicted value calculated with the use of Polish-1998 *vs.* GLI-2012 reference equations were compared. Then, the number and percentage of abnormal results defined by either source of reference values were compared in the entire study group. Subsequently, similar analyses were performed separately in the subgroups of girls and boys. Finally, the results were compared in three age-groups defined as 4–10 years (Group 1), 10–14 years (Group 2), and 14–18 years (Group 3).

To assess the agreement between spirometry expressed as z-score obtained with GLI-2012 *vs.* Polish-1998 reference equations, the results were denoted as 0 and 1 (normal and abnormal results, respectively) and then the analysis for the inter-rater agreement was performed.

Data were presented as means ± SD. Differences between groups were tested using an unpaired *t*-test. $Chi^2$ test was used to assess the proportion of abnormal results using two different reference values. The agreement between two observations was assessed using Scott's pi for bivariate analysis. All p values were two-tailed and $p < 0.05$ was considered statistically significant. Analysis was performed using the Statistica software, ver. 10.0 (StatSoft, Tulsa, OK).

# 3 Results

There were significant differences between the predicted values of $FEV_1$ and FVC calculated with the GLI-2012 and Polish-1998 formulas. The mean predicted $FEV_1$ was 2.75 ± 0.90 L *vs.* 2.51 ± 0.84 L, while the mean predicted FVC was 3.17 ± 1.11 L *vs.* 2.91 ± 1.06 L, according to the GLI-2012 and Polish-1998 equations, respectively ($p < 0.05$). The comparison of $FEV_1$ and FVC which were expressed as z-score of GLI-2012 *vs.* Polish-1998 predicted values is presented in Fig. 1. Significant differences were demonstrated between either $FEV_1$ z-score values or FVC z-score values ($p < 0.05$). The mean $FEV_1$ z-score was (−0.68) ± 1.25 *vs.* (−0.13) ± 1.70, while the mean FVC z-score was (−0.34) ± 1.08 *vs.* 0.30 ± 1.15 when

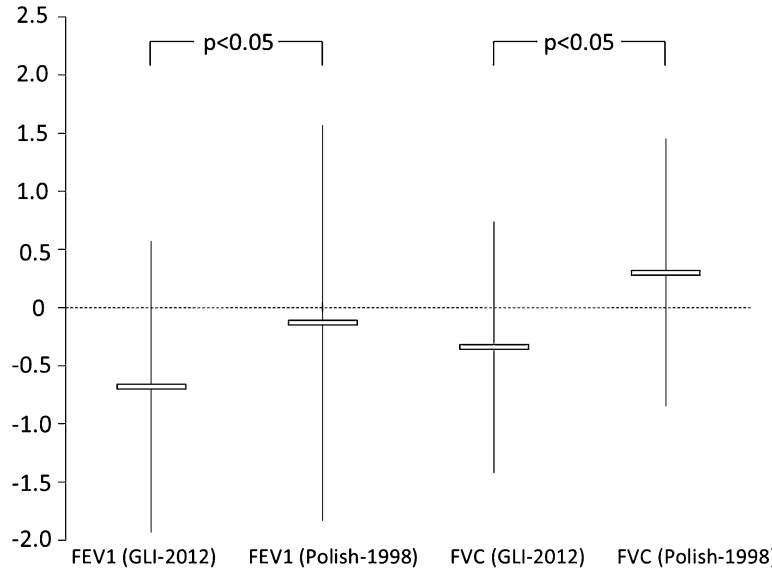


**Fig. 1** Differences between $FEV_1$ and FVC expressed as GLI-2012 and Polish-1998 z-scores. *Horizontal rectangles* show mean values, while *vertical lines* represent SD

GLI-2012 and Polish-1998 reference values were used, respectively. There was no significant difference for $FEV_1$/FVC z-scores.

There was a significant difference in the proportion of abnormal spirometry results when GLI-2012 *vs.* Polish-1998 reference equations were used to calculate the FVC predicted values. A restrictive ventilatory pattern was diagnosed in 7.6 % of patients assessed according to the GLI-2012 predicted values compared with 3.8 % when the Polish-1998 equation was used ($p = 0.04$). A similar trend was noted in the proportion of patients with airway obstruction; albeit the difference here was inappreciable; the respective percentages amounted to 20.3 % *vs.* 17.5 %.

Comparison of spirometry variables for males and females gave virtually the same results as presented above. Significant differences in terms of predicted $FEV_1$ and FVC calculated with the GLI-2012 and Polish-1998 equations were demonstrated in both gender groups (Tables 2 and 3). In consequence, $FEV_1$ and FVC expressed as z-scores of GLI-2012 and Polish-1998 predicted values were significantly different. As reported for the entire group, $FEV_1$/FVC ratio expressed as z-score in the GLI-2012 and Polish-1998 was similar in males and females (Tables 2 and 3). An analysis performed

**Table 2** Predicted spirometry values and measured spirometry variables expressed as GLI-2012 *vs.* Polish-1998 z–scores in boys

| Variable | GLI-2012 | Polish-1998 | p |
|---|---|---|---|
| $FEV_1$ z-score | −0.79 (1.22) | −0.24 (1.79) | 0.007 |
| $FEV_1$ predicted (L) | 2.81 (1.05) | 2.53 (0.95) | 0.021 |
| FVC z-score | −0.40 (1.09) | 0.28 (1.18) | 0.001 |
| FVC predicted (L) | 3.28 (1.25) | 3.02 (1.22) | 0.003 |
| $FEV_1$/FVC z-score | −0.60 (1.30) | −0.63 (1.32) | 0.860 |
| $FEV_1$/FVC predicted (%) | 86.52 (1.32) | 86.20 (2.85) | 0.170 |

Data are means (SD)
*GLI* Global Lung Initiative reference values

in three different age subgroups showed inconsistent results. In older children (≥10 years), $FEV_1$ and FVC z-scores expressed as z-score of GLI-2012 and Polish-1998 predicted values were significantly different (Table 4). In younger children, the predicted $FEV_1$ and FVC values did not differ. Hence, there were also no differences in $FEV_1$ and FVC expressed as the GLI-2012 and Polish-1998 z-scores. In none of the three age subgroups, differences between $FEV_1$/FVC ratio presented as z-scores of GLI-2012 and Polish-1998 were demonstrated (Table 4).

Agreement analysis of the results interpreted with the use of GLI-2012 and Polish-1998 predicted values revealed a good agreement for

**Table 3** Predicted spirometry values and measured spirometry variables expressed as GLI-2012 *vs*. Polish-1998 z–scores in girls

| Variable | GLI-2012 | Polish-1998 | p |
|---|---|---|---|
| $FEV_1$ z-score | −0.53 (1.28) | −0.04 (1.54) | 0.001 |
| $FEV_1$ predicted (L) | 2.66 (0.75) | 2.42 (0.63) | 0.006 |
| FVC z-score | −0.24 (1.06) | 0.35 (1.18) | 0.002 |
| FVC predicted (L) | 3.00 (0.85) | 2.76 (0.77) | 0.018 |
| $FEV_1$/FVC z-score | −0.44 (1.31) | −0.38 (1.30) | 0.700 |
| $FEV_1$/FVC predicted (%) | 89.37 (0.74) | 88.12 (1.51) | 0.001 |

Data are means (SD)
*GLI* Global Lung Initiative reference values

**Table 4** Differences between measured spirometry variables expressed as GLI-2012 *vs*. Polish-1998 z-scores in three age subgroups

| | GLI-2012 | Polish-1998 | p |
|---|---|---|---|
| | Age 4–10 (n = 16) | | |
| $FEV_1$ z-score | −0.61 (1.15) | −0.36 (1.60) | 0.610 |
| FVC z-score | −0.31 (1.30) | 0.16 (1.50) | 0.350 |
| $FEV_1$/FVC z-score | −0.38 (1.34) | −0.60 (1.36) | 0.650 |
| | Age 10–14 (n = 134) | | |
| $FEV_1$ z-score | −0.76 (1.23) | −0.39 (1.59) | 0.032 |
| FVC z-score | −0.36 (1.11) | 0.27 (1.17) | 0.001 |
| $FEV_1$/FVC z-score | −0.60 (1.35) | −0.81 (1.26) | 0.180 |
| | Age 14–18 (n = 165) | | |
| $FEV_1$ z-score | −0.62 (1.28) | 0.10 (1.76) | 0.001 |
| FVC z-score | −0.32 (1.04) | 0.35 (1.11) | 0.001 |
| $FEV_1$/FVC z-score | −0.52 (1.27) | −0.28 (1.32) | 0.100 |

Data are means (SD)
*GLI* Global Lung Initiative reference values

$FEV_1$ and $FEV_1$/FVC, and a good one for FVC (kappa 0.83; 0.9; 0.7, respectively).

## 4 Discussion

The present study shows significant differences in the assessment of spirometry variables expressed as z-score when GLI-2012 reference values were used instead of Polish-1998 values. The implementation of the GLI-2012 reference values for the interpretation of spirometry results in Polish children yields a higher number of results qualified as abnormal as compared with the commonly used Polish-1998 reference equations. Although a statistical difference for spirometry variables expressed as z-score was found for both FVC and $FEV_1$ (but not $FEV_1$/FVC), we believe that the discrepancy in the assessment of FVC may be more important from the clinical and epidemiological perspective. The percentage of patients with a potential restrictive ventilatory pattern FVC < LLN and $FEV_1$/FVC > LLN was significantly higher when the GLI-2012 reference values were applied. The proportion of patients with airflow limitation (defined as $FEV_1$/FVC < LLN) was also somewhat higher, but the difference was relatively small and did not reach statistical significance. It should be stressed that although the difference in the percentage of children with FVC < LLN defined by GLI-2012 and Polish-1998 prediction formulas was only 3.8 %, this corresponds to a 100 % increase in the percentage of abnormal results (3.8 *vs*. 7.6 %). In the absolute numbers, replacement of Polish-1998 reference equations by the GLI-2012 formula yielded 12 additional children (12 *vs*. 24) with a probable lung restriction. In terms of the number of patients with an obstructive ventilatory pattern the difference was less significant (55 *vs*. 64). All the above calculations are based on the assumption that the lower limit of normal is −1.64 SD (5th percentile). This is the recommended and commonly used cut-off point for abnormal results. However, some experts, including the authors of Polish reference values for children and adolescents suggest the 3rd and 97th percentile (±1.96 -z scores) as the lower and upper limit of normal (Willim et al. 1998). If we follow this suggestion, the difference between the prevalence of abnormal results identified by using GLI-2012 and Polish-1998 reference equations would be even more pronounced.

There are several factors that may explain the reported differences. Firstly, the GLI-2012 equations include more variables (age, height, sex, and race) than the Polish-1998 equations which include only height and sex. Secondly, there might be significant socioeconomic and cultural differences influencing the lifestyle of the tested subjects and, in consequence, their general health. Thirdly, the technical differences and quality of spirometry may also impact the results.

The GLI-2012 equations were based on the spirometry results of an enormous number of patients (over 70,000). This number was approximately 100-fold higher as compared with that reported by Polish authors of Polish-1998 reference values. Since the GLI-2012 patients were recruited in many centers in various world regions, lung function tests were performed and recorded by large number of technicians. As children's age and height are often expressed as the numbers containing fractional component they are commonly rounded off. Thus, a potential bias related to the quality of the recorded maneuvers but also to patients' age and height cannot be excluded.

To our knowledge, our report is only a second study, after that by Quanjer and Weiner (2014), which evaluated the effect of GLI-2012 implementation on spirometry interpretation in relation to children's sex and age. We found an almost uniform pattern in all studied subgroups. In both girls and boys and in all children older than 10 years the results of FVC and $FEV_1$ expressed as z-score of GLI-2012 formulas were significantly lower as compared with the Polish-1998 z-scores. This was directly related to significantly higher FVC and $FEV_1$ predicted values calculated with the use of GLI-2012 equations. In none of the analyzed subgroups, $FEV_1$/FVC expressed as GLI-2012 z-score was significantly different than that of the Polish-1998. The only subgroup where the differences in FVC and $FEV_1$ expressed as z-score were insignificant was Group 1, i.e. the youngest children aged between 4 and 10 years (Table 4). However, it must be stressed that also in this group FVC and $FEV_1$ expressed as z-score showed a clear tendency to be lower when the GLI-2012 prediction equations replaced the Polish-1998 equations. As this group was notably smaller (only 16 subjects) than the two other groups, we can speculate that if the number of subjects in this group had been higher, then the difference would probably have reached statistical significance. Interestingly, although we found significant differences in terms of both spirometry values expressed as z-scores and in the number of results assessed as abnormal, the agreement test showed nearly very good and good agreement for $FEV_1$, $FEV_1$/FVC, and FVC, respectively.

The results of earlier studies which evaluated the influence of replacement of previously used reference equations with the GLI-2012 reference values are inconsistent. This refers to numerous previously used formulas, including those proposed by Polgar and Promadhat (1971), Knudson et al. (1983), Zapletal et al. (1977), Hankinson et al. (1999), Wang et al. (1993) and other local or national equations (Pereira et al. 2014; Quanjer and Weiner 2014; Stanojevic et al. 2014; Quanjer et al. 2013; Ben Saad et al. 2013). Some of these studies demonstrated a higher percentage of abnormal spirometric results when the GLI-2012 prediction equations were used, while some other reported the opposite effect.

The results of the largest study to-date have been published by Quanjer et al. (2013). The authors evaluated the diagnostic and interpretative consequences of adopting the GLI-2012 spirometric prediction equations as compared with the European Community for Steel (ECCS) and with the National Health and Nutrition Examination Survey (NHANES) III predicted values. The study included data of 17,572 subjects aged 18–85 provided by two lung function laboratories in Australia and one in Poland. The authors found that FVC and $FEV_1$ predicted values calculated with the GLI-2012 equations were similar to those obtained with the NHANES III equations, but significantly larger than those calculated with the ECCS equations. In consequence, the differences in the LLN led to an important increase in the prevalence of a low FVC compared with the ECCS equations, and a significant decrease compared with the NHANES prediction equations (Quanjer et al. 2013). Adopting the GLI-2012 equations had only small effects on the prevalence of airway obstruction. In this context, the results of our study are similar to the findings reported by Quanjer et al. (2013) which referred to the consequences of adopting the GLI-2012 reference equations instead of those proposed by ECCS.

The results of a Tunisian study by Ben Saad et al. (2013) are largely different from our

findings. The study compared the GLI-2012 prediction equations with local Tunisian predicted values in adults aged 18–60. The authors found a significantly lower proportion of patients with restrictive spirometry pattern when the GLI-2012 reference values were used as compared with that identified by the use of Tunisian predicted values (8.4 % *vs*. 19.0 % respectively). The percentage of patients with airway obstruction was similar, irrespective of predicted equations used (4.2 % for GLI-2012 *vs*. 6.7 % for Tunisian). It should be noted that the proportions of patients with a restrictive and obstructive ventilatory pattern in this study were clearly different than those demonstrated in our present study, which is likely related to differences in the age of the groups studied.

There is much less data on the consequences of adopting the GLI-2012 reference values in children as compared with adults. The results of a large study by Quanjer and Weiner (2014) which included 4,781 children, aged 6–18, have been published. The study evaluated the sequel of transition from different prediction equations used in children (Polgar and Promadhat 1971; Knudson et al. 1983; Zapletal et al. 1977; Hankinson et al. 1999; Wang et al. 1993) to the GLI-2012 equations. There was a good agreement within the ethnic groups between $FEV_1$, FVC, and $FEV_1$/FVC predicted values calculated with GLI-2012 and those of the Hankinson et al. (1999) and Wang et al. (1993) equations. However, a near normal $FEV_1$ and above normal FVC, contributed to a lower $FEV_1$/FVC, particularly in African Americans. For the remaining predicted equations above outlined, the authors found disparate results. There was a significantly lower prevalence of FVC and $FEV_1$ below LLN when the Knudson et al. (1983) equations were applied in both sex subgroups. In this context, the results reported by Quanjer and Weiner (2014) are similar to those found in our present study. Application of the Zapletal et al. (1977) reference values yielded a significantly higher prevalence of FVC below LLN in both girls and boys, but a higher prevalence of low $FEV_1$ was reported only in boys. Quanjer and Weiner (2014) concluded that transition from the Hankinson et al. (1999) and Wang et al. (1993) equations to GLI-2012 leads to grossly similar prevalence rates of abnormally low values for $FEV_1$, FVC, and $FEV_1$/FVC, unlike the transition from the equations proposed by Polgar and Promadhat (1971), Knudson et al. (1983), and Zapletal et al. (1977).

Stanojevic et al. (2014) have assessed the influence of switching from the Knudson et al. (1983), Hankinson et al. (1999), and Wang et al. (1993) prediction equations to the GLI-2012 equations upon interpretation of annual spirometry measurements in 7,530 patients with cystic fibrosis (CF) from the UK. The study included both children and adults. Unfortunately, the authors did not provide the number of children in this study. Nonetheless, the results were similar to those found in our present study. The Stanojevic et al. (2014) study has documented that the Knudson et al. (1983), Hankinson et al. (1999), and Wang et al. (1993) equations overestimated the percentage of predicted values in pediatric patients, and, in consequence, a greater proportion of patients had lung function values within the normal range. It should be emphasized that the percentage of abnormal results in the Stanojevic et al. (2014) study was significantly higher (56–64 %) than in our present as well as in other studies. Those results were probably related to the characteristics (patients with CF) of the study group. In individual patients, the influence of switching equations varied greatly with age. The overall conclusion of the Stanojevic et al. (2014) study was that a unified approach to interpreting spirometric measurements would enable to better compare spirometry results during longitudinal patient's observation in different centers.

The present study has limitations. The number of patients included is relatively small as compared with other publications. On the other hand, the prospective character of the study, small sample size, and the fact that all spirometry measurements were performed in one lung function laboratory helped ensure the highest data quality. Thus, it should be underlined that our data are not merely the patients' spirometry records saved in the memory card during

everyday practice which could be biased by inaccuracy of anthropometric and demographic variables. The limited applicability of the prediction equations (only Polish lung function laboratories) used as a comparator to the GLI-2012 equations might be considered as another drawback of our study. However, as Polish-1998 prediction equations are used in virtually all Polish lung function laboratories, the study may have a major influence on spirometry interpretation in the children population in a large European country. Although a higher prevalence of decreased FVC was the major result of our study, we cannot assume with certainty that the children with this result had a restrictive ventilatory defect. This would require the measurement of total lung capacity (TLC). That is why we could only use the term potential restrictive ventilatory pattern. However, we made an attempt to identify this group as reliably as possible. To this end, we used an additional criterion to define restrictive ventilatory pattern ($FEV_1$/FVC $\geq$ LLN) to exclude children with the so called 'mixed pattern'. This criterion was also applied by Quanjer et al. (2013).

Summarizing, the study demonstrates that adopting the GLI-2012 reference values in the population of Polish children increases the number of detected lung function abnormalities as compared with the widely used Polish-1998 reference values. As this finding is concordant with the results of other recently published studies, we agree with the opinion expressed by various authors that more evidence is needed that the GLI-2012 reference equations can equally be used in all populations, regardless of their differences in the socioeconomic and nutritional status, ethnicity, culture, and life style. If the GLI-2012 equations are to become a worldwide 'gold standard', manufacturers of spirometric software should be encouraged to use them as default reference equations in their equipment.

**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# Variability of Transcutaneous Oxygen and Carbon Dioxide Pressure Measurements Associated with Sensor Location

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**Abstract**

Transcutaneous measurement of oxygen and carbon dioxide pressure ($PtcO_2$ and $PtcCO_2$) is useful in gas exchange monitoring. However, the relationship between $PtcO_2$, pulse oximetry ($SaO_2$) and arterial blood gases (ABG) is unclear. The aim of the present study was to compare $PtcO_2$ and $PtcCO_2$ with $SaO_2$ and ABG, to evaluate the effect of sensor location on the results and stability of $PtcO_2$ and $PtcCO_2$, and to assess the impact of body composition on $PtcO_2$ and $PtcCO_2$. $PtcO_2$ and $PtcCO_2$ were measured in 20 healthy volunteers at three locations: right second intercostal space, lateral surface of the abdomen, and the inner surface of the left arm. The results were recorded 10, 15, and 20 min after sensor fixation and compared with $SaO_2$ and ABG measured 20 min after electrode placement on the chest. Body composition was evaluated by bioimpedance. The findings were that $PtcO_2$ was stable on the chest; but on the arm and abdomen it increased and reached maximum at 20 min. Transcutaneous $PCO_2$ stabilized at 10 min in all the three locations. No significant correlations between $PtcO_2$ and $SaO_2$ or $PaO_2$ were found. Transcutaneous $PCO_2$ correlated with $PaCO_2$. Both $PtcO_2$ and $PtcCO_2$ were not influenced by body composition. We conclude that the value of $PtcO_2$ in monitoring of blood oxygenation was not unequivocally confirmed; $PtcCO_2$ reliably reflects $PaCO_2$, irrespective of sensor location. Body composition does not affect $PtcO_2$ and $PtcCO_2$.

**Keywords**

Blood gas monitoring • Body mass index • Electrode location • Healthy subjects • Transcutaneous carbon dioxide tension • Transcutaneous oxygen tension

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## 1 Introduction

Monitoring of gas exchange requires multiple arterial blood sampling. Pulse oximetry which is widely used in the clinical setting has certain limitations, mainly related to the physicochemical properties of hemoglobin. The oxyhemoglobin dissociation curve clearly shows that a drop of $SaO_2$ from 100 to 90 %, i.e., by only 10 % corresponds with a decrease in the partial pressure of oxygen ($PaO_2$) by as much as 40 mmHg. Moreover, pulse oximetry reflects alterations associated with $O_2$, but not with $CO_2$. This merits search for alternative methods which would be more reliable than pulse oximetry and less invasive than repeated blood sampling. Transcutaneous oxygen ($PtcO_2$) and carbon dioxide ($PtcCO_2$) monitoring, introduced in the 1970s, is based on the principle that dissolved gases diffuse through tissues. Due to this property, gas pressure may be measured by a sensor placed on the skin. The measurement requires local heating of the skin to 44 °C to obtain congestion, and subsequently increase in arterial blood supply to the skin capillary bed directly under the sensor, which results in accelerated gas diffusion (Fuke et al. 2009; Huch et al. 1976). In clinical practice, this method is mainly used to assess gas exchange in infants, children, and adults with acute and chronic respiratory failure (Rudiger et al. 2007; Janssens et al. 1998). It may also be applied in monitoring mechanically ventilated patients undergoing major surgery, invasive diagnostic or therapeutic procedures, wound care, and limb ischemia management (Restrepo et al. 2012; Griffin et al. 2003; Evans et al. 1998).

As the essence of transdermal gas monitoring is to provide information about the function of the respiratory and circulatory system without the need for multiple blood sampling, it is also a point of interest for clinical research. The present study is a part of a larger project aimed at the evaluation of various pathophysiological aspects of thoracentesis and pleural fluid withdrawal. This imposed the search for an optimal site for continuous transcutaneous $PO_2$ and $PCO_2$ measurement, which would guarantee stable and reliable measurements and would be convenient for the patient. So far, no single fixed site has been recommended, the sensor may be placed in various locations including the earlobe, the intercostal spaces, the abdomen, the inner surface of a thigh, and the arm or forearm (Restrepo et al. 2012). The sites of sensor fixation must have good perfusion, should be well-cleaned, stripped of skin lesions (e.g., scars) and deprived of hair. In routine clinical practice $PtcO_2$ and $PtcCO_2$ are usually measured in patients in supine position, and so the most common sensor locations are the anterior surface of the chest or the earlobe (Fruchter et al. 2011; Senn et al. 2005; Janssens et al. 2001). Transcutaneous $PO_2$ and $PCO_2$ may be influenced by many different factors. These include local skin perfusion, presence of right-to-left shunting, local edema, and patient positioning (Restrepo et al. 2012; Tobias 2009). It seems that adipose tissue may also affect the results of $PtcO_2$ and $PtcCO_2$ measurements. However, some authors documented that transcutaneous gas tensions do not depend on body mass index (BMI) (Soto et al. 2014; Maniscalco et al. 2008; Janssens et al. 2005).

The aim of the present study was the following: (1) to compare $PtcO_2$ and $PtcCO_2$ with arterial blood oxygenation saturation measured by pulse oximetry ($SaO_2$) and with arterial blood gases, (2) to evaluate the effect of sensor location on the results and stability of $PtcO_2$ and $PtcCO_2$ measurements, and (3) to assess the influence of body composition on $PtcO_2$ and $PtcCO_2$.

## 2 Methods

The study was approved by the institutional Research Review Board (KB/42/2014). Twenty healthy volunteers, recruited from the medical staff of the Department of Internal Medicine, Pneumology and Allergology, Medical University of Warsaw, Poland, were enrolled. All the participants signed informed consent.

Transcutaneous $PO_2$ and $PCO_2$ were measured with the use of Monitor TCM Combi

M (TCM4; Radiometer Medical AsP, Brønshøj, Denmark). The working temperature of the transcutaneous electrode was set at 44 °C. The measurements were performed in the sitting position at three preselected sensor locations: (1) second right intercostal space in the midclavicular line, (2) lateral surface of abdomen at the level of the umbilicus in the mid-axillar line, and (3) lower third of the inner surface of the left arm. The duration of measurement at each electrode location was at least 20 min. Before sensor fixation, the skin and electrode were thoroughly cleaned, one drop of contact gel was applied, and calibration was performed according to the manufacturer's recommendations. A new adhesive ring was applied before every sensor location change. The values of $PtcO_2$ and $PtcCO_2$ were recorded at 10, 15, and 20 min from the sensor fixation. Stabilization of measurement was assumed when $PtcCO_2/PtcO_2$ value alterations did not exceed ±2 mmHg within 1 min. The arterial oxygen saturation ($SaO_2$) with a finger clip pulse oximeter (Palm SAT 2500; Nonin Medical, Plymouth, MN) was simultaneously monitored.

Arterial blood sampling was performed only once, 20 min after sensor placement on the chest. Blood was drawn with a 25 gauge needle from the left radial artery with PICO 50 Arterial Blood Sampler and gas content was analyzed with an ABL 90 FLEX blood gas analyzer (Radiometer Medical AsP, Brønshøj, Denmark). Body composition was evaluated by bioimpedance (Tanita T5896; Tanita Corporation of America, Arlington Heights, IL). Fat and muscle mass, also water content were expressed as kilograms and percent of total body weight.

All results were presented as median and 25–75 interquartile range (IQR) and were analyzed with non-parametric tests (ANOVA Friedman, Wilcoxon signed-rank test). Correlations were analyzed with Spearman's rank correlation test. A p-value of less than 0.05 was considered statistically significant. Statistical elaboration was performed with Statistica 10 (StatSoft inc. USA).

## 3 Results

The study group consisted of 11 women (55 %) and 9 men (45 %), median age 39.5 years (IQR 25–42), with a BMI of 24.6 kg/m$^2$ (IQR 22.4–26.7). The characteristics of the study group is shown in Table 1. Sensor placement with adhesive rings was stable in all the three measurement sites. The tolerance of the electrode was good, no signs of skin irritation or erythema were noted at the end of monitoring.

### 3.1 Variability of $PtcO_2$ with Time

The results of transcutaneous $PO_2$ measurements in three different sites and at three time points (after 10, 15, and 20 min) are shown in Table 2.

The median $PtcO_2$ tended to increase with time in all the measurement sites and reached the highest value at 20 min. A significant increase in $PtcO_2$ values at 10, 15, and 20 min was found in the arm and abdominal locations (Table 2; $p < 0.05$). Conversely, only a tendency for increase was found when the electrode was placed on the chest. At each time point, the highest $PtcO_2$ values were noted when the sensor was located in the lateral region of the abdomen, while the lowest were measured by the electrode placed at the chest. Significant differences between the measurements taken in three different locations were found at 15 and 20 min

**Table 1** Characteristics of the study group (n = 20)

| Variable | Median (IQR) |
|---|---|
| Age (years) | 39.5 (25.0–42.0) |
| Weight (kg) | 73.4 (65.3–82.0) |
| Height (cm) | 171.0 (166.7–180.0) |
| BMI (kg/m$^2$) | 24.5 (22.3–26.7) |
| Fat mass (%) | 25.4 (19.1–30.3) |
| Fat mass (kg) | 19.9 (13.8–23.9) |
| Muscle mass (%) | 71.1 (66.4–77.2) |
| Muscle mass (kg) | 50.2 (45.6–59.3) |
| Total body water (%) | 54.6 (50.9–59.2) |
| Total body water (kg) | 38.7 (35.3–45.4) |

*BMI* body mass index, *IQR* interquartile range

**Table 2** Transcutaneous $PO_2$ at the selected time points in the three measurement locations

| Time (min) | $PtcO_2$ on arm | $PtcO_2$ on chest | $PtcO_2$ on abdomen |
|---|---|---|---|
| 10 | 71.5 (65.5–79.5) | 70.5 (52.5–74.5) | 74.5 (57.0–82.0) |
| 15 | 75.0 (66.0–80.5) | 72.0 (51.0–78.0) | 78.0 (60.0–85.0) |
| 20 | 76.5 (71.5–82.0) | 74.0 (55.0–77.0) | 80.5 (63.0–85.5) |
| p | <0.05 | NS | <0.05 |

Data are median (IQR)
*$PtcO_2$* transcutaneous oxygen tension, *IQR* interquartile range, *NS* not significant

**Table 3** Transcutaneous $PCO_2$ at the selected time points in the three measurement locations

| Time (min) | $PtcCO_2$ on arm | $PtcCO_2$ on chest | $PtcCO_2$ on abdomen |
|---|---|---|---|
| 10 | 38.0 (37.0–39.0) | 39.0 (38.0–41.5) | 38.5 (36.5–40.5) |
| 15 | 38.5 (37.0–40.0) | 39.5 (37.0–42.0) | 38.5 (37.0–40.5) |
| 20 | 38.0 (36.5–39.0) | 38.5 (37.0–41.0) | 39.5 (37.0–40.5) |
| p | NS | NS | NS |

Data are median (IQR)
*$PtcCO_2$* transcutaneous carbon dioxide tension, *IQR* interquartile range, *NS* not significant

($p < 0.05$ for both). These differences were due to a significantly higher $PtcO_2$ on the arm and abdomen vs $PtcO_2$ on the chest.

## 3.2 Variability of $PtcCO_2$ with Time

The summary of $PtcCO_2$ measurements is demonstrated in Table 3.

In all the sensor locations, the values of $PtcCO_2$ were stable at 10, 15, and 20 min (no significant differences between $PtcCO_2$ at the respective time points). Although the numerical values of $PtcCO_2$ measured in the three different locations at the first time point (10 min) are very similar, there was a significant statistical difference between the value obtained from the sensor on the arm and on the chest ($p < 0.05$). Comparison of $PtcCO_2$ at 15 and 20 min across the three measurement sites showed no significant differences.

## 3.3 Transcutaneous $PO_2$ and $PCO_2$ in Relation to $SaO_2$ and Arterial Blood Gases

The value of $SaO_2$ measured by pulse oximetry was stable during the entire measurement period; the median $SaO_2$ was 97.9 %, (IQR 97.4–98.6). The median $PaO_2$ and $PaCO_2$ was 96.3 mmHg (IQR 91.3–109.0) and 38.3 mmHg (IQR 35.9–40.2), respectively (Table 4). Transcutaneous $PO_2$ did not correlate with $SaO_2$ at any of the measurement locations ($r = 0.11$, NS).

**Table 4** Arterial blood gases in the study group

| Variable | Median (IQR) |
|---|---|
| $PaO_2$ (mmHg) | 96.3 (91.3–109.0) |
| $PaCO_2$ (mmHg) | 38.3 (35.9–40.2) |
| pH | 7.44 (7.40–7.50) |
| $HCO_3^-$ (mmol/l) | 25.7 (25.0–26.8) |
| $SaO_2$ (%) | 97.9 (97.4–98.6) |
| ctHb (g/dl) | 14.2 (13.3–15.0) |

*$PaO_2$* partial pressure of oxygen, *$PaCO_2$* partial pressure of carbon dioxide, *$HCO_3^-$* bicarbonates, *$SaO_2$* blood oxygenation, *ctHb* hemoglobin concentration, *IQR* interquartile range

The median $PtcO_2$ was significantly lower than $PaO_2$ (74.7 mmHg *vs.* 96.3 mmHg, respectively, $p < 0.05$). No correlations between the values of $PtcO_2$ and $PaO_2$ were found ($r = 0.09$, NS). The median $PtcCO_2$ and $PaCO_2$ were comparable in all the three time points and at all the measurement locations. The strongest correlation was demonstrated on the arm and on the chest at

20 min ($r = 0.66$ and $r = 0.66$, respectively; $p < 0.05$).

### 3.4 Transcutaneous $PO_2$ and $PCO_2$ in Relation to Body Composition

The median BMI in our study group was 24.5 kg/m$^2$. Five subjects were overweight (BMI 25–30 kg/m$^2$) and three were obese (BMI >30 kg/m$^2$). There were no significant correlations between $PtcO_2$ or $PtcCO_2$ and age, BMI, and nutritional parameters assessed by bioimpedance. The highest correlation coefficients between $PtcO_2$ and muscle mass, fat mass, and total body water were 0.32, 0.21, and 0.22, respectively (NS). The respective correlation coefficients for $PtcCO_2$ were 0.37, −0.34, and 0.35 (NS).

## 4 Discussion

The present study demonstrates that transcutaneous blood gas monitoring needs elaboration and improvement. This particularly refers to $PtcO_2$. We demonstrated that contrary to $PtcCO_2$, $PtcO_2$ changes significantly between the 10th and 20th minute from the onset of measurement. Moreover, its value depends significantly on electrode location. The optimal stability of measurements carried out between the 10th and 20th minute from the starting point was observed with the sensor located on the chest (right second intercostal space). Our results failed to confirm the influence of body composition on the values of $PtcO_2$ and $PtcCO_2$.

Although a number of publications have addressed transcutaneous $PO_2$ and $PCO_2$ measurement, there might be some important differences between the previous reports and the present study. As in clinical setting the transcutaneous measurement of $PO_2$ and $PCO_2$ is mainly used for monitoring gas exchange in patients with acute and chronic respiratory failure or in patients requiring ventilatory support during surgery (Restrepo et al. 2012), many previous studies included severely ill patients or patients under general anesthesia in whom the measurements were performed in the supine position (Soto et al. 2014; Nishiyama et al. 2006). Our study involved healthy volunteers and the measurements were performed in the sitting position. To our knowledge there have been no previous reports on the transcutaneous $PO_2$ and $PCO_2$ measurement while sitting. However, it must be admitted that some publications involving healthy subjects do not mention the position in which the measurements is performed (Weaver 2007; Wimberley et al. 1985). As the present study was undertaken to find optimal sensor location enabling $PtcO_2$ and $PtcCO_2$ monitoring during therapeutic thoracentesis, which is almost always performed while sitting, sitting position was the obvious choice.

There are also other significant differences between the previous and present investigations. These include the evaluation of the effect of body composition (fat mass, muscle mass, and total water) on $PtcO_2$ and $PtcCO_2$. Since we were not able to find such evaluation in earlier studies, we believe our study adds on some new data to the existing literature on the transcutaneous $PO_2$ and $PCO_2$ measurement.

Even though the transcutaneous measurement of $PO_2$ and $PCO_2$ has important advantages, it must be emphasized that this method differs considerably from the measurement of partial gas pressures in the arterial blood (Hasibeder et al. 1991). Transcutaneous $PO_2$ is always lower than $PaO_2$. The most important factor to be responsible for this difference is the tissue use of oxygen. The present study confirmed the presence of the lower values of $PtcO_2$ as compared with $PaO_2$. The difference between the median $PtcO_2$ measured on the abdomen, where we obtained the highest $PtcO_2$ values, and the median $PaO_2$ was 15.8 mmHg. This means that $PtcO_2$ was approximately 16.4 % lower than the respective $PaO_2$. In a report by Weaver (2007) the difference between $PtcO_2$ and $PaO_2$ was <10 %, but that study involved healthy subjects in hyperbaric conditions. In reports on transcutaneous gas measurement in severely ill patients,

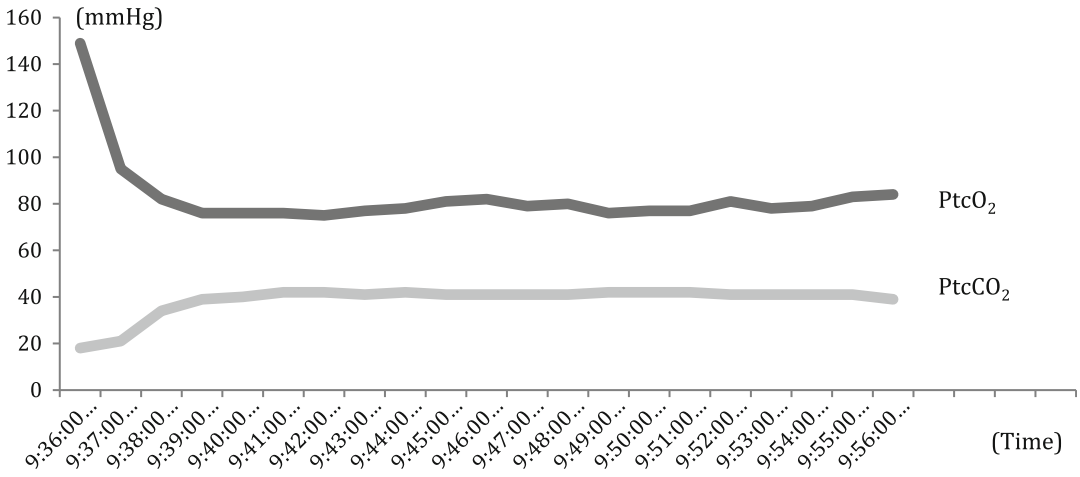


**Fig. 1** An example of 20 min continuous $PtcO_2$ and $PtcCO_2$ recording (the sensor fixed on the arm)

the $PtcO_2/PaO_2$ difference was even more pronounced (Hasibeder et al. 1991). However, the comparative analysis of results from healthy subjects and patients with various respiratory and circulatory disorders may be misleading and requires caution, as the measurements in the latter can be influenced by hypoperfusion and alterations in tissue metabolism. Despite differences in the values of $PtcO_2$ and $PaO_2$, a relationship between these two parameters has been documented (Weaver 2007; Kesten et al. 1991). Some authors have shown that the strongest correlation between $PtcO_2$ and $PaO_2$ was found for $PtcO_2$ values measured on the chest (Nishiyama et al. 2006; Hasibeder et al. 1991). The present results failed to confirm this observation. Furthermore, we did not find any correlation between $PtcO_2$ and $PaO_2$.

Contrary to $PtcO_2$, $PtcCO_2$ is usually higher than $PaCO_2$, due mainly to carbon dioxide production during tissue metabolic processes (Kesten et al. 1991). In the present study, we did not show significant differences between $PtcCO_2$ and $PaCO_2$ in all the three measurement sites at the selected time points and the two parameters correlated with each other.

An important point in the assessment of the reliability of $PtcO_2$ and $PtcCO_2$ measurement is the accuracy and reproducibility of the results. It is well known that a reliable $PtcO_2$ and $PtcCO_2$ measurement requires dynamic equilibrium at the surface of the sensor. Therefore, there must be some delay between the start of the measurement and achieving the accurate $PtcO_2$ and $PtcCO_2$ values. The time necessary to achieve plateau of $PtcO_2$ and $PtcCO_2$ measurement is significantly different. Previous studies have shown the stabilization time of 10–17 min and 3–7 min for $PtcO_2$ and for $PtcCO_2$, respectively (Thomsen 2011). In our patients, $PtcO_2$ gradually increased and reached the highest value at 20 min. This was the case in all the sensor locations. The median $PtcO_2$ measured after 20 min was 1.5–2.5 mmHg higher than that measured after 15 min. Thus, we believe that the achievement of the accurate and stable $PtcO_2$ values may be delayed even 20 min. However, an analysis of the last 3 min of measurement showed that $PtcO_2$ values reached a plateau, which indicates that further $PtcO_2$ increase should not be expected (Fig. 1). We also demonstrated that the median difference between $PtcO_2$ measured in different sensor location may vary from 4.0 to 6.5 mmHg. Since the highest $PtcO_2$ was registered from the sensor placed on the abdomen, we would recommend this sensor position when monitoring $PtcO_2$ and $PtcCO_2$ while sitting.

The pattern of $PtcCO_2$ changes was different than that of $PtcO_2$. Stable $PtcCO_2$ values in all three sensor locations were found already from the 10th minute on, with no further increase as demonstrated for $PtcO_2$. This might be easily

explained by the differences in physical properties of oxygen and carbon dioxide (Kesten et al. 1991). The maximal median difference between $PtcCO_2$ measured by sensor placed in the three different locations was only 1.5 mmHg. These data show that $PtcCO_2$ is a parameter, which is significantly more stable and less influenced by sensor location than $PtcO_2$.

As the transcutaneous gas measurement depends on a number of factors, including body temperature, local capillary blood flow, and tissue metabolism (Restrepo et al. 2012), we hypothesized that body composition may also affect $PtcO_2$ and $PtcCO_2$. However, to our knowledge, the impact of body composition on the results of transcutaneous blood gas measurements has not been evaluated in earlier studies. In fact, we did not find any publications related to this specific issue. Thus, one of the important points of our study was the evaluation of the relationship between body composition measured by bioimpedance and transcutaneous $PO_2$ and $PCO_2$ measurement. Surprisingly, we did not find any correlation between $PtcO_2$ and $PtcCO_2$ and BMI or body composition. Basing on the results of our study, we may speculate that local conditions at the site of sensor location, including the thickness and conductivity of the skin are more important for $PtcO_2$ and $PtcCO_2$ measurement than whole body composition (Kesten et al. 1991). This is supported by the findings of other authors who demonstrated that even in morbidly obese patients $PtcCO_2$ monitoring is a reliable method and its results correlate with arterial blood gases (Soto et al. 2014; Maniscalco et al. 2008).

We are aware of some limitations of our study. The study group, which comprised only 20 subjects, was relatively small. However, it must be emphasized that most of the studies in adults that have addressed the transcutaneous measurement of $PtcO_2$ and $PtcCO_2$ included a comparable number of patients. Larger groups have been reported in papers presenting the results of transcutaneous $PO_2$ and $PCO_2$ monitoring in children and neonates (Tingay et al. 2013; Bernet-Buettiker et al. 2005; Palmisano and Seberinghaus 1990). In our study, the arterial blood samples were obtained only once during transcutaneous gas monitoring. This was because we wanted to minimalize the number of invasive procedures in healthy volunteers. Comparison of $PtcO_2$ and $PtcCO_2$ in all the measurement sites with arterial blood gases would require repeated arterial blood sampling (thrice) within 1 h. Obviously, such an invasive procedure in a healthy subject would raise objections from the ethical point of view. Thus, we made an assumption that arterial blood gases are relatively stable in a healthy subject at rest. We believe that additional blood sampling would not add new or different data to our study. Finally, there were only three obese and five overweight subjects in our study group, while the remaining participants presented normal body weight. This may, to some extent, explain the lack of correlations between body composition and $PtcO_2$ and $PtcCO_2$.

## 5 Conclusions

The value of $PtcO_2$ in non-invasive monitoring of blood oxygenation was not unequivocally confirmed. Our results failed to confirm the correlation between $PtcO_2$ and $SaO_2$ or $PaO_2$. It seems that the chest is the optimal site for $PtcO_2$ monitoring as it was the only location which provided stable measurements in all the selected time points. Transcutaneous $PCO_2$ reliably reflects the $PaCO_2$, irrespective of sensor location. Body composition does not affect the $PtcO_2$ and $PtcCO_2$.

**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# Crosstalk Between Co-cultured A549 Cells and THP1 Cells Exposed to Cigarette Smoke

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**Abstract**

Cigarette smoke (CS) is considered as a major etiological factor in the pathogenesis of chronic obstructive pulmonary disease. In this study we used A549 cells and THP-1 cells grown for 24 h in monoculture or in co-culture in CS-conditioned media and changes in their proliferation, viability, acetylated histone H3 levels and expression of extracellular antigens CD14, HLA-DR, CD11a, and CD11b were assessed. CS was highly toxic to A549 cells but not to THP1 cells. In A549 cells, oxidative stress reached the highest values after 1 h of CS exposure and then decreased. In THP1 cells oxidative stress was lower and increased progressively with time. CS decreased proliferation of A549 and THP1 cells by about 80 % and 21 %, respectively. CS did not alter acetylated histone H3 levels in A549 cells, while in THP1 cells the levels were reduced by about 35 %. CS significantly increased expression of CD14, HLA-DR, CD11a, and CD11b in THP1 cells. In co-culture, naïve or CS-pretreated THP1 cells significantly protected A549 cells against CS toxicity but had higher death rates. These results show that epithelial cells are more fragile to CS than monocytes and that CS-activated monocytes may protect epithelial cells against CS-induced cytotoxicity.

**Keywords**

A549 cells • Cell culture • COPD • Inflammation • THP1 cells

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## 1 Introduction

Cigarette smoke (CS) is considered a major etiological factor in the pathogenesis of chronic obstructive pulmonary disease (COPD), which is characterized by a progressive development of airflow limitation. In COPD lowered lung function is associated with local and systemic

inflammation, resistant to anti-inflammatory drugs including steroids. Activated leukocytes, especially macrophages but also T-cells, B-cells, and neutrophils release cytokines, chemokines and proteases, affect physiology of non-immune cells of respiratory tract and produce increased proliferation of lining epithelial cells, airway remodeling and peribronchial fibrosis leading to emphysema (GOLD 2013). Moreover, a number of compounds found in CS can directly damage lung tissue and induce oxidative imbalance, adding noxious exogenous chemical stimuli to complex endogenous inflammation. The molecular mechanism responsible for COPD is unknown and several animal and cellular models were described to mimic human disease and to study different aspects of the disease (Adamson et al. 2011). Cell lines are commonly used in *in vitro* studies to model COPD. It was shown that human bronchial epithelial cell lines NCI-H292, 16HBE14σ, and BEAS-2B are affected when grown in CS-conditioned media and several biochemical and functional alterations have been described (Heijink et al. 2010). In the present study we used human alveolar epithelial cell line A549, which is the most exploited alveolar epithelial cell line. In our experimental model, naïve or CS-treated A549 cells were grown in monoculture or in co-culture with intact or CS-exposed THP-1 cells, a human monocyte cell line which is able to produce inflammatory mediators, to examine how both cell types respond to CS and how naïve and CS pretreated cells, which share common culture medium, interact in a co-culture system that does not allow physical contact between both cell types.

## 2 Methods

### 2.1 Cell Culture

Two types of cells were used: A549 (ATCC® CCL185™) cells growing in ATCC-formulated F12K medium supplemented with 10 % fetal bovine serum (FBS) and THP1 (ATCC® TIB202™) cells growing in ATCC-formulated RPMI 1640 medium, supplemented with 2-mercaptoethanol to a final concentration of 0.05 mM and with FBS to a final concentration of 10 %. Cells were maintained in 37 °C in an incubator in a humidified atmosphere containing 5 % $CO_2$. For particular experiments, cells were plated out onto 6, 24, or 96 well plates and grown in control or smoke conditioned media for 24 h.

#### 2.1.1 Cell Co-cultures

Naïve A549 cells or A549 cells grown for 24 h in smoke-conditioned media were co-cultured in 6-well cell culture plates with naïve or smoke-pretreated (24 h) THP1 cells. THP1 cells were added to the co-culture inserts (Translucent PET membrane, RoTrac; Greiner Bio-one, Courtaboeuf, France) which were placed in upper parts of culture dishes. The pores of membranes in co-culture inserts were 0.4 μm large and did not allow THP1 cells to pass and to contact physically with A549 cells. Cells were kept in co-culture in CS-free medium for 24 h.

### 2.2 Preparations of CS-Conditioned Media and Cells Treatment

The smoke of four full-strength Red Marlboro cigarettes (Phillip Morris, Cracow, Poland) containing 8.0 mg of tar, 0.6 mg of nicotine and 9.0 mg of carbon monoxide per cigarette was passed through 100 ml of culture media using low pressure vacuum pump. Cigarette filters were removed before the procedure. The pump pressure was set to give a combustion time for each cigarette of about 1 min. To ensure similar level of smoke saturation between different batches of smoke-conditioned media, the nitrate/nitrite levels were measured in media using Griess reagent. Freshly prepared stem cell were diluted with standard media to obtain 30 μM nitrate/nitrite content in each batch. Stem cell media were subsequently sterilized using 0.22-μm filters and were used immediately to cell culture. A549 or THP1 cells were plated at low density in 6 or 24-well plates, and 24 h after seeding cultures were switched to smoke conditioned media and were further incubated

maximally for 24 h. In some experiments cell treatment was shorter to acquire time-effect data. Samples of culture media were collected during incubation to estimate the activity of lactate dehydrogenase (LDH). In co-culture experiments naïve or CS-pretreated (24 h) A549 cells were grown without physical contact with naïve or CS-pretreated (24 h) THP1 cells which were added to co-culture inserts allowing to diffuse soluble molecules to common culture medium (smoke free 1:1 mixture of F12K and RPMI 1640). Cells were kept in co-culture for 24 h.

## 2.3 Cell Growth, Proliferation, and Viability

Cell growth and proliferation was quantified in flow cytometry (Epics XL flow cytometer, Coulter Electronics, High Wycombe, UK) using propidium iodide DNA staining and cell cycle analysis (Brown et al. 1996). Histograms of propidium iodide fluorescence distributions were quantified using MultiCycle software and cells were quantified by their relative distribution in the damaged-subdiploid ('early' G0/G1 cells), diploid (G0/G1 zone)-pre-DNA synthesis/resting, S-phase-DNA synthesis, and G2/M-post-DNA-synthesis/mitosis phases (Fig. 1). Each histogram was derived from analysis of 5,000 cells and six samples were analyzed in each group. Cytotoxicity was expressed as a fraction of damaged – 'early' G0/G1 cells while proliferating cells were quantified as S + G2/M cells.

Cell viability was quantified after 24 h of cell growth in CS-conditioned media using mitochondrial-dependent MTT (3-[4,5-dimethylthiazolyl-2] 2,5-diphenyltetrazolium-bromide) reduction to purple formazan, with colorimetric detection (Niks and Otto 1990). Changes in absorbance in viable cells were measured at 570 nm, with 630 nm as a reference wavelength. Cell viability was estimated as a percentage of the control. Time-dependent toxicity was assessed using lactate dehydrogenase (LDH) release assay (LDH cytotoxicity kit, ScienCell,

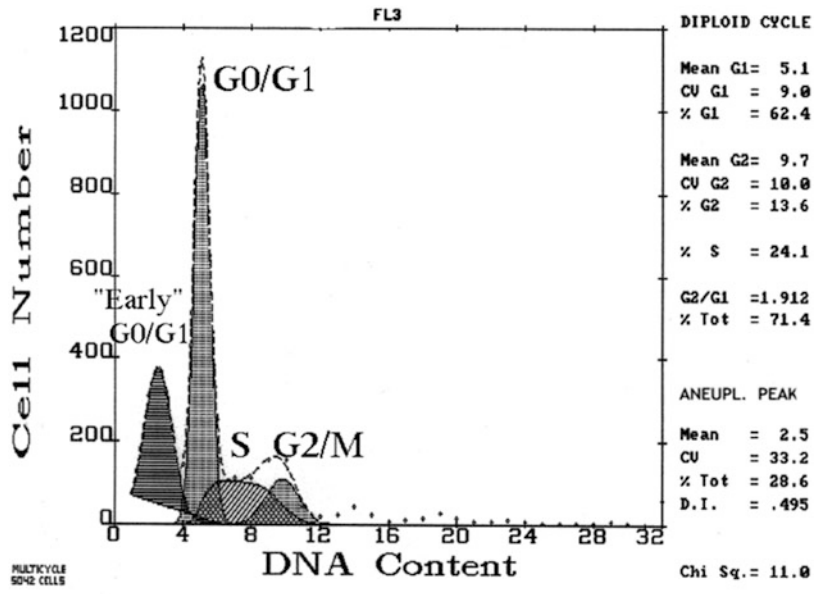


**Fig. 1** MultiCycle software-transformed histogram of propidium iodide-DNA fluorescence of THP1 cells grown for 24 h in co-culture with A549 cells. Original histogram was obtained using epics XL flow cytometer. Gating was set for the control probe (THP1 cells) and applied to all experimental samples. Cell distribution was quantified using MultiCycle software as subdiploid ('early' G0/G1 cells), diploid (G0/G1 peak)-pre-DNA synthesis/resting, S-phase-DNA synthesis, and G2/M-post-DNA-synthesis/mitosis phases

Carlsbad, CA). LDH release to the culture medium was compared to total enzyme activity in sonicated cells.

### 2.4 Oxidative Stress

Reactive oxygen intermediates were quantified using dichlorodihydrofluorescein diacetate (H2DCFDA; Sigma-Aldrich, St. Louis, MO) (Ubezio and Civoli 1994). Cells were loaded with 5 μM H2DCFDA for 30 min, washed, resuspended in phosphate-buffered saline, and assayed by flow cytometry. Green dichlorofluorescein (DCF) fluorescence was captured on F11 channel of flow cytometer (Epics XL, Coulter Electronics, High Wycombe, UK) and registered as histograms of fluorescence distribution.

### 2.5 Expression of Acetylated Histone 3 and Extracellular Activation Markers

Acetylated histone H3 levels were measured in formaldehyde (1 %)-fixed and ethanol (70 %)-refixed cells with acetylated histone H3 (AcH3)-specific monoclonal fluorescent antibodies (Acetyl-Histone H3 (Lys9) Antibody; Rabbit mAb Alexa Fluor 488 Conjugate; Cell Signalling Technology Inc., Danvers, MA), corresponding isotype control antibody, and flow cytometry (Coulter Electronics, High Wycombe, UK) detection (Ronzoni et al. 2005).

The percentages of $CD14^+$, $HLA\text{-}DR^+$, $CD11a^+$, and $CD11b^+$ THP1 cells were determined using specific monoclonal fluorescent antibodies (Beckman-Coulter, Warsaw, Poland), corresponding isotype controls, and flow cytometry detection. Cells were diluted to $10^5$ cells per ample and 10 μl of a commercial antibody solution was added to cell suspension and allowed to bind for 30 min at room temperature in darkness. The cells were washed with phosphate buffered saline, fixed with CellFIX™ (Becton Dickinson, Oxford, UK) and run on an Epics XL flow cytometer (Coulter Electronics, High Wycombe, UK). Three thousand total events were collected per sample.

### 2.6 Statistical Analysis

Results were expressed as means ± SD of 6–10 assays. Statistical analysis was performed with a statistics package-Statistica 6.0 software (Statsoft, Cracow, Poland) using one-way or two-way ANOVA followed by Bonferroni *post-hoc* tests for selected pairs of data. A p value of less than 0.05 was considered statistically significant.

## 3 Results

Cytotoxicity of CS applied for 24 h to the cells was tested initially by the MTT test (Table 1). In A549 cells grown for 24 h in CS-conditioned medium very significant toxicity was detected. MTT values in this group were lower by about 78 % ($p < 0.01$), while no toxicity was noticed in THP1 cells. Since cytotoxicity data in the MTT test reflect not only cell damage but also alterations in cell proliferation, CS cytotoxicity to A549 cells was further characterized in time-dependent experiments. Increased LDH levels were found in the culture medium of A549 cells exposed to CS already after 1 h of cell treatment ($p < 0.05$). Then, LDH levels increased with incubation time to reach 36 % ($p < 0.01$) of total enzyme activity (sonicated cells) after 24 h of cell treatment.

We also examined oxidative stress in cells grown in CS-conditioned media. In A549 cells, the stress was the highest after 1 h. At that time registered values of DCF fluorescence were more than 8 times higher ($p < 0.01$) than baseline reference values. In the 6th hour, oxidative stress was still very high (increased by more than 5 times; $p < 0.01$) and then lower values were observed, but after 12 h of cell growth in CS-conditioned media the stress was still

**Table 1** Cigarette smoke (CS) cytotoxicity (MTT test), proliferation (PI-DNA assay), oxidative stress (DCF fluorescence), expression of acetylated histone H3 (flow cytometry) and expression of extracellular markers of immune activation (flow cytometry) in control alveolar epithelial cells (A549) and in human monocyte cell line (THP1) as well as in cells grown in smoke-conditioned medium for 24 h

| | A549 cells | | THP1 cells | |
|---|---|---|---|---|
| | Control | CS | Control | CS |
| MTT (% of control) | 100 ± 16 | 22 ± 6** | 100 ± 17 | 118 ± 24 |
| LDH (% of total activity) | | | | |
| 1 h | – | 11 ± 5* | – | – |
| 6 h | – | 18 ± 7** | – | – |
| 12 h | – | 27 ± 9** | – | – |
| 24 h | – | 36 ± 11** | – | – |
| Oxidative stress (relative units) | | | | |
| 1 h | 100 ± 11 | 844 ± 55** | 100 ± 16 | 153 ± 66** |
| 6 h | 100 ± 13 | 527 ± 66** | 100 ± 17 | 187 ± 34** |
| 12 h | 100 ± 17 | 161 ± 41* | 100 ± 21 | 221 ± 42** |
| 24 h | 100 ± 19 | 62 ± 33* | 100 ± 18 | 266 ± 48** |
| Cytotoxicity (% of 'early' G0/G1 cells) | 7 ± 3 | 47 ± 11** | 6 ± 3 | 11 ± 6* |
| Proliferation (% of S-G2/M cells) | 43 ± 7 | 9 ± 3** | 27 ± 5 | 19 ± 4* |
| Expression of acetylated histone H3 (relative units) | 100 ± 14 | 85 ± 17 | 100 ± 19 | 65 ± 17** |
| $CD14^+$ (% of cells) | – | – | 5 | 94 ± 11** |
| $HLA\text{-}DR^+$ (% of cells) | – | – | 5 | 47 ± 9** |
| $CD11a^+$ (% of cells) | – | – | 5 | 69 ± 10** |
| $CD11b^+$ (% of cells) | – | – | 5 | 46 ± 8** |

To visualize time-dependent cell membrane damage and alterations in oxidative stress during 24 h of cell growth in CS-conditioned medium Lactate dehydrogenase (LDH) liberation to the culture medium and oxidative stress were assessed in the 1st, 6th, 12th, and 24th hour of experiment. All other parameters were quantified after 24 h of cell growth in CS-conditioned medium

*$p < 0.05$; **$p < 0.01$ for comparisons with the corresponding control cells

significant (increased by 61 %; $p < 0.05$). After 24 h, the DCF fluorescence was lower than control values but at that time DCF fluorescence histograms became broad and bimodal (results not shown) due to significant toxicity. In THP1 cells, oxidative stress increased progressively with time attaining the highest values (about 2.5 times higher than baseline) after 24 h of cell treatment with CS.

Table 1 shows flow cytometry data of PI-DNA fluorescence reflecting both cytotoxicity of CS and changes in proliferation rates of A549 and THP1 cells grown for 24 h in CS-conditioned media. Damaged cell numbers were assessed as 'early' G0/G1 cells, while cell proliferation was quantified as fractions of S + G2/M cells (Fig. 1). About 47 % of A549 cells were damaged ($p < 0.01$) by CS, while in THP1 cells the fraction of damaged cells was about 11 % ($p < 0.05$). CS decreased cell proliferation particularly in A549 cells, where an almost 81 % ($p < 0.01$) decrease in cell growth dynamics was observed comparing to control cells. In THP1 cells, proliferation was also reduced, by about 21 % ($p < 0.05$).

Next we determined the expression of acetylated histone H3 (AcH3) in both cell types. CS did not alter AcH3 levels in A549 cells, while in THP1 cells AcH3 level was reduced by about 35 % ($p < 0.01$). It should be stressed however, that expression of AcH3 was assessed only in viable cells. Incubation of THP1 cells in a smoke-conditioned medium resulted in increased expression of antigens typical for monocyte activation. At baseline, 5 % of cells in each sample were set as antigen positive. CS treatment significantly increased CD14-positive cell numbers (to 95 % of cells; $p < 0.01$), HLA-DR

**Table 2** Cytotoxicity and alterations in cell proliferation in naïve or CS-treated A549 or THP1 cells grown in co-cultures

| | A549 cells | | THP1 cells | |
|---|---|---|---|---|
| | Cytotoxicity (% of 'early' G0/G1 cells) | Proliferation (% of S + G2/M cells) | Cytotoxicity (% of 'early' G0/G1 cells) | Proliferation (% of S + G2/M cells |
| Control | 5 ± 2 | 42 ± 6 | 7 ± 3 | 22 ± 5 |
| CS | 48 ± 7** | 2 ± 1** | 14 ± 3** | 29 ± 6* |
| A549 + THP1 | 8 ± 3 | 30 ± 6* | 22 ± 4** | 34 ± 7** |
| $A549^{CS}$ + THP1 | 18 ± 4**$^{\#\#}$ | 10 ± 3**$^{\#\#}$ | 27 ± 6**$^{\#\#}$ | 27 ± 8 |
| A549 + $THP1^{CS}$ | 5 ± 2 | 26 ± 5** | 26 ± 6$^{\#\#}$ | 49 ± 8$^{++}$** |
| $A549^{CS}$ + $THP1^{CS}$ | 24 ± 6$^{\#\#}$ | 9 ± 3$^{\#\#}$ | 53 ± 9$^{\#\#\wedge\wedge}$ | 19 ± 4$^{\#\#\wedge\wedge}$ |

Naïve or CS-pretreated (24 h) A549 cells were grown in co-cultures without physical contact with naïve or CS-pretreated (24 h) THP1 cells which were grown in co-culture inserts allowing to diffuse soluble molecules to common culture medium (1:1 mixture of F12K and RPMI 1640 media). Cells were kept in co-culture in cigarette smoke-free medium for 24 h

*$p < 0.05$; **$p < 0.01$ for comparisons with the corresponding control cells

$^{\#\#}p < 0.01$ for comparisons with the CS-treated cells

$^{++}p < 0.01$ for comparisons with A549 + THP1 group

^^$p < 0.01$ for comparisons with $A549^{CS}$ + THP1 group

was expressed in 47 % ($p < 0.01$) of cells, while corresponding values in CD11a and CD11b were 69 % ($p < 0.01$) and 46 % ($p < 0.01$), respectively.

In co-culture experiments we estimated CS cytotoxicity using propidium iodide DNA staining and alterations in cell proliferation (Table 2). Co-incubation of CS-pretreated A549 cells with naïve THP1 cells or THP1 cells pretreated with CS resulted in significantly lower ($p < 0.01$) toxicity and increased ($p < 0.01$) but not normalized cell proliferation. Naïve THP1 cells and CS-pretreated THP1 cells exerted similar cytoprotection to CS-pretreated A549 cells. CS was toxic ($p < 0.01$) to THP1 cells grown in co-culture medium and increased ($p < 0.05$) THP1 cell proliferation. In THP1 cells, CS toxicity was about 2 times higher when CS–pretreated cells were incubated in co-culture with A549 cells ($p < 0.01$) and almost 4 times higher ($p < 0.01$) when CS–pretreated THP1 cells were grown in co-culture with CS-pretreated A549 cells. Both naïve A549 cells and CS-pretreated A549 cells exerted similar cytotoxic effect, but only to naïve THP1 and not to CS-pretreated THP1 cells coincubated with naïve or smoke-pretreated A549 cells, where striking ($p < 0.01$) differences in THP1 cells growth and cytotoxicity were observed.

## 4 Discussion

This study demonstrated that the alveolar epithelial cell line and monocyte cell line significantly differed in their response to CS. When naïve or CS-pretreated cells were grown in a co-culture system physically separating monocytes from epithelial cells, changes in cell viability and growth rates were observed. A substantial fraction of epithelial cells died in the first few hours of culture in CS-conditioned medium, while monocytes not only survived but also became activated and to some extent protected epithelial cells against CS-induced cytotoxicity.

Alveolar epithelial cell line A549 is considered as relatively resistant to chemical-induced cytotoxicity, but it has been shown that CS induces apoptosis and alters immunity of A549 cells (Sohn et al. 2009). It should be stressed that CS extract contain several toxic compounds as nicotine, acrolein, formaldehyde, hydrogen cyanide, polycyclic aromatic hydrocarbons, and nitrosamines (Moylan et al. 2013) and A549 cells have low activity of cytochrome P450 enzymes, which are responsible for metabolism of several xenobiotics (Yatzeck et al. 2008). Published CS toxicity data in A549 cells are highly variable

depending on experimental systems and smoke exposure, but it has been reported that CS extract dose-dependently decreases glutathione concentration, increases 4-hydroxy-2-nonenal levels, and induces necrosis in A549 cells (Kode et al. 2006). In our experimental model, cell exposure to CS was rather extensive and nitrate/nitrite levels in smoke-conditioned media were relatively high comparing to other models (Naik et al. 2014). Nonetheless, our smoke-conditioned medium was not toxic to the monocyte cell line, which apparently is more resistant to noxious compounds of CS. It is possible that in A549 cells CS cytotoxicity starts with chemically induced oxidative stress and is followed by cell membrane damage as evidenced by changes in DCF fluorescence assay and LDH release. A major role in CS cytotoxicity in A549 cells may be played by acrolein and hydrogen peroxide (Aoshiba and Nagai 2003). Monocytes are more resistant to oxidative stress, but activation of THP1 cells by CS may be mediated by nicotine, which is able to activate immune cells (Zhou et al. 2013). It has been shown that antioxidants prevent CS toxicity in A549 cells (Banerjee et al. 2008) and decrease protein damage, inflammation, apoptosis, and lung injury in smoke-exposed animals (Rahman 2012), supporting the major role of oxidative stress in CS-induced cytotoxicity.

Another parameter, which may be related to altered inflammatory signaling and steroid resistance in COPD is acetylated histone H3 (Sundar et al. 2013). Histones are responsible for transcriptional regulation of inflammatory signaling and it has been shown that acetylation of core histones may increase expression of inflammatory genes in inflammatory lung diseases (Marwick and Chung 2010). In our model, decreased levels of acetylated histone H3 were detected in monocytes exposed to CS but not in epithelial cells, where only slight but not significant decrease was observed. Our data show that histone acetylation may depend on the cell type. It should be stressed that we quantified acetylated histone H3 in viable cells, without strenuous cell homogenization and histone extraction, which may affect labile histone acetylation status. The quantity of acetylated histones may be relevant to inflammation in COPD and experimental data indicate that in COPD histones are hypercetylated due mostly to decreased activity of histone deacetylases (Yao and Rahman 2012). Recently published data seem to support our observations, since it has been shown that a potential major carcinogen of CS-acrolein inhibits acetylations of N-terminal tails of cytosolic histones H3 and H4 (Chen et al. 2013). It seems that the role of epigenetic signaling in COPD may be different in immune and non-immune cells and should be further evaluated.

CS has been shown to either activate or inhibit activation of cells in culture (Adamson et al. 2011). We have shown that smoke increases expression of CD14, HLA-DR, CD11a, and CD11b antigens on THP1 cells. In COPD, there is increased expression of adhesion molecules and increased differentiation of inflammatory cells (GOLD 2013). Clinical studies also evidenced specific distribution of adhesion molecules in the airways and parenchyma that was consistent with the inflammatory response (González et al. 1996). It has been shown that CS activates human monocytes and macrophages to release chemokines and increases proinflammatory potential of cytokines and tumor necrosis factor-α (Walters et al. 2005). Another study has shown that after 24 h of CS exposure more than 300 genes in THP1 cells are activated, while a similar number of genes is repressed including inducible antioxidants, chaperone proteins, and the ubiquitin/proteosome proteins (Wright et al. 2012). It seems that increased adherence of monocytes may help to protect injured or damaged cells but also to induce an allergic response in the airways.

There are only few studies on airway cell co-cultures exposed to CS. In a co-culture model using A549 cells and fetal lung fibroblasts, low CS concentrations have induced epithelial-mesenchymal transition, observed but in co-cultured A549 cells and not in cell monoculture (Wan et al. 2009). When human alveolar epithelial type II (AT-II) cells were co-cultured with human pulmonary microvascular endothelial

cells, AT-II cells differentiated into AT-I like cells (Hermanns et al. 2009). In another study, lung microvascular endothelial cells (MVECL) grown in co-culture with AT-I cells have been treated with CS extract. Endothelial cells have demonstrated about 50 % reduction in hydrogen peroxide production comparing to monocultures (Downs et al. 2011). Also in our model, co-incubation of CS-pretreated A549 cells with naïve THP1 or CS-pretreated THP1 cells decreased CS cytotoxicity to A549 cells and partly restored A549 cell proliferation. Considering CS cytotoxicity in THP1 cells exposed to CS in monoculture or in co-culture, there was no toxicity when cells were exposed to CS in a dedicated medium and only small toxicity when THP1 cells were grown in co-culture medium mixture (1:1). It should be stressed that CS cytotoxicity becomes more relevant in co-culture with naïve A549 cells and remarkably high when smoke pretreated THP1 cells were co-cultured with smoke pretreated A549 cells.

In conclusion, we demonstrated that during co-culture of A549 cells and THP1 cells there is a bi-directional crosstalk between both cell types cells *via* medium-soluble mediators and that THP1 cells may to some extent protect A549 cells against CS toxicity.

**Conflicts of Interest** The authors had no conflicts of interest to declare in relation to this article.

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# Evaluation of Airway Inflammation in Compost Workers Exposed to Bioaerosols Using Exhaled Breath Condensate and Fractional Exhaled Nitric Oxide

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**Abstract**

Occupational bioaerosol exposures are capable to cause respiratory diseases. We studied the relationship between exposure to bioaerosols and biomarkers' concentration in exhaled breath condensate (EBC) and fractional exhaled nitric oxide (FeNO) in 119 bioaerosol-exposed compost workers taking into account atopy and smoking habits. Atopy was classified according to specific IgE concentrations to common inhalant allergens (sx1). Bioaerosol exposure was estimated according to job title, duration of employment, results of ambient monitoring at the workplaces, and shift time worked under protection of filtered air supply. Concentrations of 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$), prostaglandin $E_2$ ($PGE_2$), leukotriene $B_4$ ($LTB_4$), and acid-base balance (pH) in EBC and FeNO were assessed in 59 never-smoking (NS) and 60 smoking (S) compost workers. We found that atopic subjects were equally distributed among NS and S (n = 16 each). Levels of 8-iso-$PGF_{2\alpha}$ were significantly higher in workers considered highly exposed to bioaerosols than in low exposed workers (86.6 (66.1; 128.8) pg/mL *vs*. 74.4 (56.3; 96.7) pg/mL, p = 0.047). No associations could be observed between exposures and biomarkers concerning compost workers in total, but there were some in atopic workers (duration of employment and FeNO: r = 0.376, p = 0.041; filtered air supply and FeNO: r = −0.335, p = 0.071). Smokers had significantly lower pH values compared to NS (non-atopic, p = 0.041; atopic p = 0.050). In conclusion, EBC and FeNO

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might be useful tools for monitoring of inflammation due to bioaerosol exposures, especially in atopic subjects. Besides smoking also atopy should be considered when investigating airway inflammation.

**Keywords**
Biomarkers • Eicosanoids • Exhaled breath condensate • Nitric oxide • Occupational exposure • Respiratory distress

## 1 Introduction

When handling compost, microorganisms which are essential for the biological decomposition of organic material become airborne (bioaerosols, organic dust) and can be inhaled. Adverse effects on the respiratory tract have previously been reported due to irritative-toxic or allergic features of bioaerosols (Bünger et al. 2000, 2007; Domingo and Nadal 2009; Schlosser et al. 2009). Different tasks at composting plants are associated with different exposure intensities. Concerning molds and bacteria, highest values of cultivable microorganisms were demonstrated during shredding, processing, and manual waste sorting (van Kampen et al. 2014). Exposures can be reduced by personal or technical means of protection. In this respect, a significant reduction of organic dust components results from filtered air supply in wheel loader cabins (WLCs) (Schlosser et al. 2012).

There is evidence that the major pathological reaction caused by organic dust is inflammation based on an allergic and non-allergic reaction (Rylander 2004). Fractional exhaled nitric oxide (FeNO) reflecting the activity of NO synthases (NOS) is primarily considered a surrogate marker of eosinophilic inflammation. Prostaglandin $E_2$ ($PGE_2$) synthesized via the cyclooxygenase pathway and leukotriene $B_4$ ($LTB_4$) *via* the lipoxygenase pathway have been associated with the inflammatory burden, whereas 8-iso-prostaglandin $F_{2\alpha}$ (8-iso-$PGF_{2\alpha}$) is produced mainly by a non-enzymatic pathway catalysed by free radicals thereby reflecting oxidative stress (Pelclová et al. 2008). These biomarkers and the acid-base balance (pH) could be assessed in exhaled breath condensate (EBC) (Hoffmeyer et al. 2009). Collection of EBC is non-invasive and does not interfere with an underlying disease (Horvath et al. 2005). Levels of FeNO, pH, and biomarkers in EBC were reported to be influenced by smoking habits (Kharitonov et al. 1995; Koczulla et al. 2010; Koutsokera et al. 2008). The usefulness of respiratory biomarkers in exhaled breath, induced sputum and EBC was recently reviewed in medical surveillance and identification of adverse respiratory effects in exposed workers (Chérot-Kornobis et al. 2012; Quirce et al. 2010; Corradi et al. 2010).

In the present study, we investigated the effects of exposure to bioaerosols on FeNO and effect markers in non-invasively collected EBC of compost workers taking atopy and smoking habits into account. Former smokers are a highly heterogeneous group and for that reason we focused on never-smokers and current smokers in our analyses.

## 2 Methods

The study design and the protocol were created in accordance with the Declaration of Helsinki and approved by the Ethics Committee of the Ruhr-University Bochum. All study participants gave written informed consent to the study protocol.

### 2.1 Study Participants and Exposure Assessment

In 2009, 190 currently exposed compost workers were examined at 31 composting plants

in North-western Germany. The study protocol was recently published (van Kampen et al. 2012). Smoking habits were assessed as current, former, and never-smokers by interview. Former smokers are a highly heterogeneous group with respect to smoking history and resulting adverse health effects (Malerba and Montuschi 2012). Therefore, we focused on never-smokers (NS) and current smokers (S) in our analyses. One assigned non-smoker was reclassified as current smoker based on CO vol% (fractional carbon monoxide in exhaled breath) in exhaled breath and the nicotine metabolite cotinine in urine (>100 μg/L). Cigarettes smoked per day were investigated and pack-years calculated. Atopy was classified according to specific IgE concentrations to common inhalant allergens (sx1, Phadiatop, ThermoFisher, Uppsala, Sweden). A positive atopic status was assumed in case of sx1 $\geq$ 0.35 kU/L.

Information regarding occupational exposure to organic dust in the past was derived from duration of employment and job tasks, e.g., wheel loader cabin (WLC), sorting cabin, delivery place, piles, shredding, processing, or office work. According to results of ambient monitoring at workplaces (van Kampen et al. 2014) and time spent at respective places, exposure was estimated and categorized in rather low and high. We considered workers serving most of their current daily work-time (>5.5 h) in WLCs or performing office work indoors being rather low exposed to organic dust. Moreover, time daily worked under protection of filtered air supply in WLC was assessed and taken into account.

## 2.2 Collection of EBC and Determination of pH and Biomarkers

EBC was collected according to general methodological recommendations (Horváth et al. 2005) at a midweek working shift. We used the commercially available temperature-controlled device Turbo DECCS (Medivac S.r.l., Parma, Italy) at a maintained temperature of −5 °C. The collection time was exactly 10 min and subjects used a nose clip during tidal breathing through a mouthpiece. Before sampling the subjects were asked to rinse their mouths with water and were instructed to swallow excess of saliva after coming off the mouthpiece.

EBC samples were kept in a container for less than 24 h at 4–6 °C. After transfer from workplaces to our laboratory, samples were deaerated by argon gas, aliquoted in separate tubes according to the intended analysis to avoid multiple frosting-defrosting cycles and frozen at −70 °C (Hoffmeyer et al. 2007). The pH was measured using a pH-meter with a glass-electrode (Mettler Toledo, Giessen, Germany) and specific enzyme immunoassay kits were used to detect 8-iso-$PGF_{2\alpha}$, $LTB_4$ or $PGE_2$ (Assay Designs, Ann Arbor, USA), as previously described (Hoffmeyer et al. 2007, 2012). Briefly, in each assay, the lowest standard was set as the limit of quantification (LOQ) of the assay. The LOQ of 8-iso-$PGF_{2\alpha}$, $LTB_4$, $PGE_2$ was 6.1 pg/mL, 11.7 pg/mL, and 39.1 pg/mL, respectively.

## 2.3 FeNO Measurement

Fractional exhaled nitric oxide was measured according to the European Respiratory Society (ERS) and American Thoracic Society (ATS) guidelines (2005) using a electrochemical analyzer with a measurement range of 5–300 ppb (NIOX Mino; Aerocrine, Sweden).

## 2.4 Statistical Analysis

Biomarkers in EBC with values below the limit of quantitation (LOQ) were set 2/3 of LOQ. $LTB_4$ was transformed into binary categories using the LOQ as cut-off, because just slightly more than 15 % of the values were over the detection limit. Value distribution was assessed using the D'Agostino & Pearson omnibus normality test. Comparison of data was performed with paired *t*-test and Wilcoxon matched-pairs signed rank test (paired data) or unpaired *t*-test and Mann Whitney test (unpaired data), where

appropriate. A two-sided significance level of 0.05 was chosen for all tests. Data are expressed as median with interquartile range. Spearman's rank correlation test or Pearson's test were used to determine correlations between markers of exposure and effect. The data were analyzed and visualized using GraphPad Prism version 5.01 for Windows (GraphPad Software, USA).

## 3 Results

### 3.1 Study and Occupational Exposure Characteristics

After validation of smoking habits, 59 never, 71 former, and 60 current smokers (addressed as smokers) could be identified. Further analyses were based on stratification according to smoking habits and atopic status. Atopic subjects were equally distributed among never-smokers (NS) and smokers (S) ($n = 16$ each). After stratification for atopy, no differences in IgE levels to sx1 could be revealed in NS and S. In reverse, after stratification for smoking habits, CO vol% was similar in non-atopic and atopic subjects corresponding to reported cigarettes/day (16.5 *vs*. 14.2, $p = 0.312$). Study characteristics are presented in Table 1. In summary, age in groups was significantly different from each other ($p = 0.008$) with smoking atopic subjects being younger than non-smoking ones ($p = 0.014$). Age was positively correlated to duration of employment ($r = 0.551$, $p < 0.0001$) and to pack-years in smokers ($r = 0.544$, $p < 0.0001$). In smokers, actual exposure to tobacco smoke (CO vol%) was associated to overall exposure (pack-years; $r = 0.337$, $p = 0.0009$). Age of workers or duration of employment was not significantly correlated to time worked under protection of filtered air supply ($p = 0.300$ and $p = 0.287$, respectively). Stratification for smoking or atopy did not change the results significantly. No obvious differences were observed for the indicated subgroups with respect to exposure levels ($p = 0.343$).

### 3.2 Biomarkers in Exhaled Breath and Exhaled Breath Condensate

Due to the narrow time schedule or technical reasons, FeNO measurement and EBC sampling could not be applied in 14 and 5 workers, respectively. FeNO measurements revealed a median value of 14 (9; 21) ppb. EBC-pH could be determined in each sample with a median of 6.71 (6.25; 7.04). Based on the EBC volume collected, there were 110, 93, and 105 samples available for measurements of 8-iso-$PGF_{2\alpha}$, $PGE_2$, and $LTB_4$,

**Table 1** Personal, work and exposure characteristics of the study group stratified according to smoking history and atopy

| | Non-smoking | | Smoking | | |
|---|---|---|---|---|---|
| | Non-atopic (n = 43) | Atopic (n = 16) | Non-atopic (n = 44) | Atopic (n = 16) | Statistics |
| Age (years) | 44.9 (38.9; 48.8)[a] | 44.8 (36.9; 55.0) | 47.3 (41.1; 54.0) | 35.6 (25.4; 44.6) | 0.008 |
| BMI ($kg/m^2$) | 26.0 (24.2; 29.5) | 27.4 (23.4; 29.8)[a] | 26.1 (24.4; 30.4) | 25.2 (23.2; 29.2) | 0.803 |
| CO (vol%) | 0.32 (0.16; 0.48)[a] | 0.48 (0.32; 0.64) | 2.96 (2.12; 4.16)[a] | 3.52 (2.52; 4.40) | <0.0001 |
| Pack-years | 0 | 0 | 21.0 (9.5; 30.0)[a] | 9.0 (4.6; 25.3) | <0.0001 |
| sx1 (kU/L) | 0.03 (0.02; 0.05)[a] | 4.67 (0.88; 13.21)[a] | 0.03 (0.02; 0.08)[a] | 1.33 (0.74; 12.04)[a] | <0.0001 |
| Occupational exposure | | | | | |
| Duration (years) | 12.3 (5.4; 17.1) | 15.8 (6.4; 23.3) | 10.8 (6.2; 18.3)[a] | 4.4 (1.8; 15.6) | 0.048 |
| Level (low/high) | 14/29 | 8/8 | 18/26 | 9/7 | 0.343 |
| Filtered air supply (h) | 2.0 (0; 4.0)[a] | 1.5 (0; 6.0) | 2.0 (0; 6.0)[a] | 1.0 (0.3; 4.8) | 0.820 |

[a]Data not normally distributed. *BMI* body mass index, *CO* carbon monoxide, *sx1* specific IgE concentrations to common inhalant allergens. Atopic status defined as sx1 $\geq$ 0.35 kU/L

respectively. In all samples the respective biomarkers were detectable, but partly below the LOQ. In detail, values below the LOQ were 1/110, 55/93 and 89/105 for 8-iso-$PGF_{2\alpha}$, $PGE_2$, and $LTB_4$, respectively. Concentration of 8-iso-$PGF_{2\alpha}$ and $PGE_2$ in EBC was 82.1 (61.9; 118.0) pg/mL and 26.1 (26.1; 54.8) pg/mL, respectively. $LTB_4$ was transformed into binary categories using the LOQ as cut off. FeNO concentrations were not correlated to pH or biomarker concentrations in EBC when referring to all subjects or subgroups (data not shown). The only significant correlation between pH and an EBC biomarker concentration was observed in case of $PGE_2$ in the subgroup of smokers ($p = 0.007$). Within EBC-biomarkers, 8-iso-$PGF_{2\alpha}$ was positively correlated to $PGE_2$ ($p = 0.020$). Concentrations of 8-iso-$PGF_{2\alpha}$ and $PGE_2$ were significantly higher in samples with $LTB_4 >$ LOQ compared to <LOQ (108.9 (77.8; 262.6) pg/mL *vs.* 76.3 (59.5; 99.2) pg/mL, $p = 0.012$ and 78.8 (65.2; 125.0) pg/mL *vs.* 26.1 (26.1; 50.1) pg/mL, $p < 0.0001$).

## 3.3 Intensity of Occupational Exposure and Markers of Effect

A rather high current bioaerosol exposure could be suspected in 70/119 compost workers. No differences with respect to the exposure intensity (high *vs.* low) could be observed for FeNO (14 (9; 19) ppb *vs.* 14 (10; 22) ppb, $p = 0.806$), pH (6.74 (6.30; 7.00) *vs.* 6.66 (5.93; 7.16), $p = 0.459$), $PGE_2$ (26.1 (26.1; 54.8) pg/mL *vs.* 26.1 (26.1; 62.4) pg/mL, $p = 0.780$) or $LTB_4$ (> LOQ/< LOQ; 12/53 *vs.* 4/36, $p = 0.278$). Levels of 8-iso-$PGF_{2\alpha}$ were significantly increased in workers considered highly exposed to organic dust (86.6 (66.1; 128.8) pg/mL *vs.* 74.4 (56.3; 96.7) pg/mL, $p = 0.047$). Results of FeNO, pH measurements, and 8-iso-$PGF_{2\alpha}$ concentrations in EBC stratified according to smoking habits and atopy are depicted in Fig. 1. No further differences could be revealed in case of $PGE_2$ or $LTB_4$ after stratification of results according to smoking habits and atopy (data not shown). Smoking demonstrated a significant negative impact on FeNO levels both in low ($p = 0.0001$) and high ($p < 0.0001$) exposed workers. A trend for higher FeNO levels in atopic subjects was observed in low exposed workers ($p = 0.152$). EBC-pH was significantly lower in smoking compared to non-smoking subjects which was more apparent in high exposed subjects (6.46 (6.02; 6.95) *vs.* 6.83 (6.48; 7.05), $p = 0.009$) compared to low exposed subjects (6.58 (5.84; 7.01) *vs.* 6.89 (6.45; 7.27), $p = 0.049$). EBC-pH was not different when referring to atopic status and exposure intensity. No association between pH and cumulative smoking dose (pack-years) could be revealed ($p = 0.848$) in smokers. Concerning 8-iso-$PGF_{2\alpha}$, differences between high and low exposed workers could be mainly attributed to differences in the subgroups of non-smokers ($p = 0.087$) and the subgroup of non-atopics ($p = 0.072$).

## 3.4 Correlation Between Markers of Effect and Exposure

Correlations between current (CO vol%) or cumulative cigarette exposure (pack-years) and current (clean air supply) or cumulative (years of employment) occupational exposure and effect markers referring to all subjects under investigation ($n = 119$) are shown in Table 2. $LTB_4$ was not used for correlation analyses because of the high amount of values below the LOQ (84.8 %). Both current and cumulative cigarette exposure was negatively correlated to FeNO ($p < 0.0001$, each) and pH (0.005 and 0.010, respectively). No associations could be revealed between biomarkers in exhaled breath and EBC and occupational exposure.

After stratification according to atopic status, an association could be suspected between FeNO and time daily worked under clean air supply in atopic workers ($r = -0.335$, $p = 0.071$; Fig. 2a). Moreover, there was a statistically significant correlation between FeNO and duration of employment in this subgroup ($r = 0.376$, $p = 0.041$; Fig. 2b). No associations were observed in any subgroup between means of exposure and biomarkers assessed in EBC (data not shown).

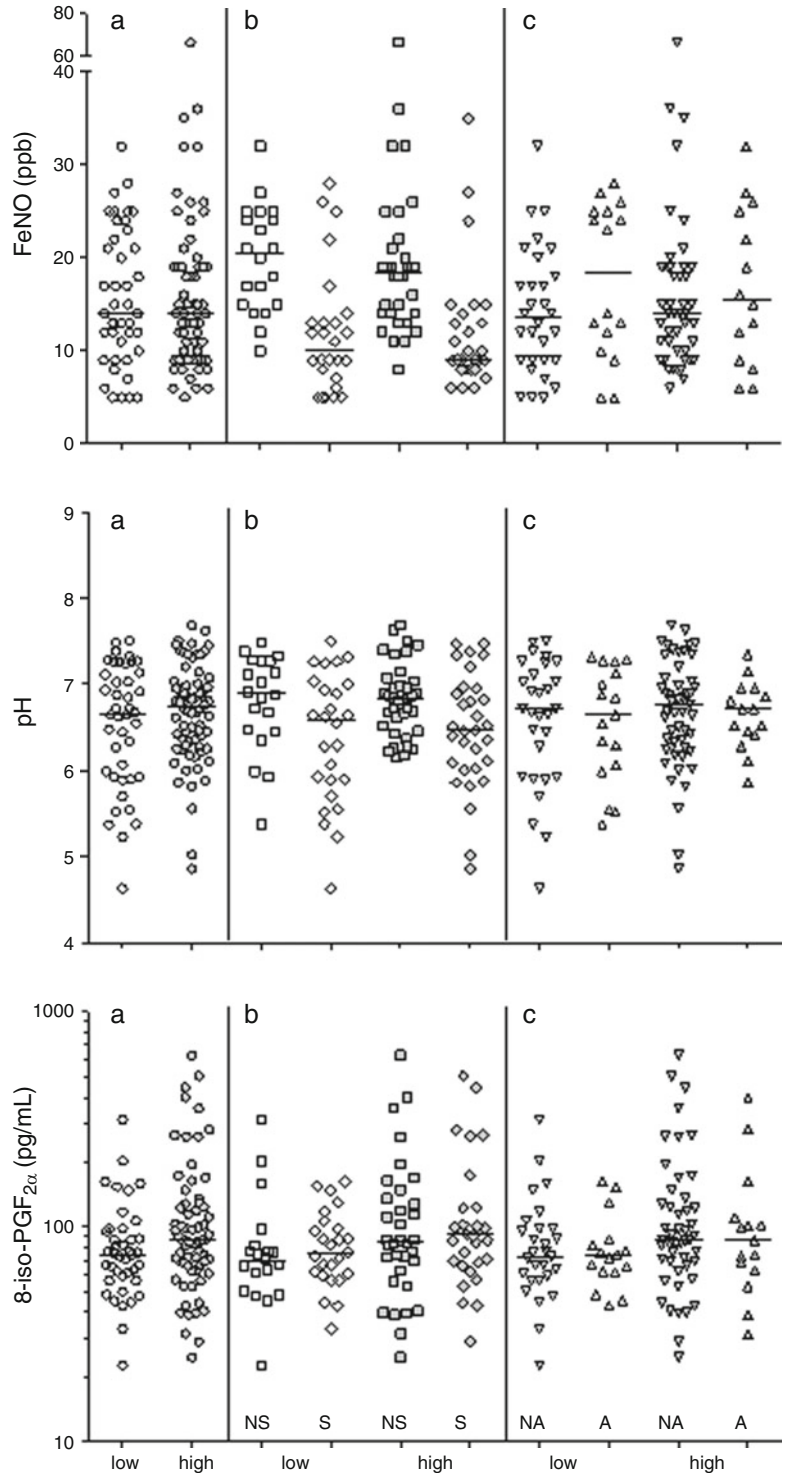


**Fig. 1 FeNO, pH, and 8-iso-$PGF_{2\alpha}$ concentrations in EBC** referring to all subjects (*a*) and stratified according to smoking habits (*b*), and atopy (*c*). *L* low exposure, *H* high exposure, *NS* never smokers, *S* smokers, *NA* non-atopic, *A* atopic

**Table 2** Correlations of exposure characteristics and biomarkers of inflammation

| | | FeNO (ppb) | pH | 8-iso-$PGF_{2\alpha}$ (pg/mL) | $PGE_2$ (pg/mL) |
|---|---|---|---|---|---|
| CO (vol%) | r | −0.516 | −0.264 | −0.002 | −0.034 |
| | *p* | *<0.0001* | *0.005* | *0.980* | *0.746* |
| Pack-years | r | −0.510 | −0.242 | 0.030 | −0.025 |
| | *p* | *<0.0001* | *0.010* | *0.760* | *0.813* |
| Clean air supply (h) | r | −0.148 | 0.051 | 0.040 | 0.016 |
| | *p* | *0.132* | *0.588* | *0.676* | *0.878* |
| Employment (years) | r | 0.017 | 0.059 | −0.095 | −0.083 |
| | *p* | *0.862* | *0.533* | *0.324* | *0.428* |

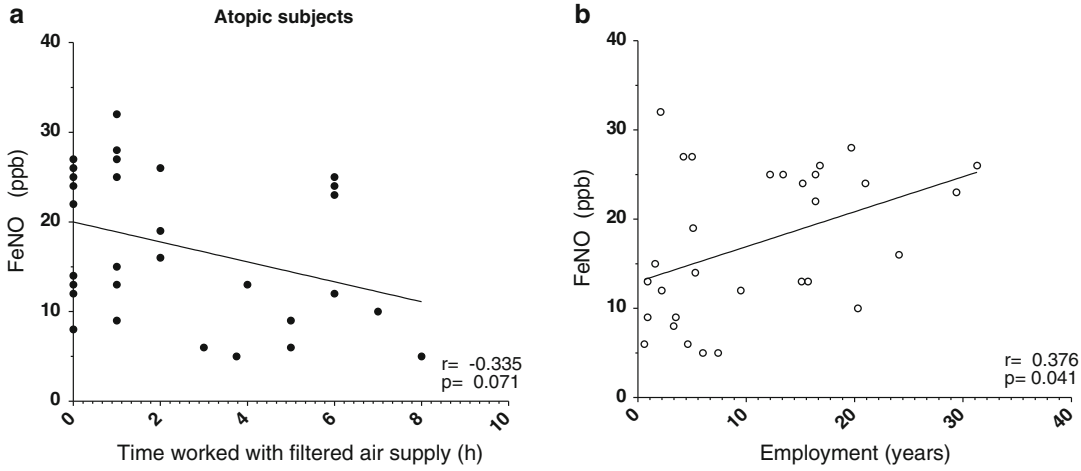


**Fig. 2** **Correlations between FeNO in atopic subjects** and (**a**) the time daily worked with filtered air supply and (**b**) the duration of employment

## 4 Discussion

In this analysis within a recently published cross-sectional study of compost workers (van Kampen et al. 2012) we found that besides smoking habits also the atopic status (defined as specific IgE concentrations to common inhalant allergens (sx1) $\geq$ 0.35 kU/L in this current study) has to be considered when assessing inflammatory markers in exhaled breath (FeNO) and EBC with respect to organic dust exposures. We observed that differences in 8-iso-$PGF_{2\alpha}$ levels between high and low exposed workers could be mainly attributed to differences in the subgroups of non-smokers and non-atopics. In addition, associations between means of exposure and FeNO could be revealed only when referring to the atopic status.

Highest values of cultivable microorganisms in composting plants were demonstrated during shredding, processing, and in sorting cabins (van Kampen et al. 2014). In our study, the number of workers in each participating composting plant was rather small and workers fulfilled different job tasks. Thus, we estimated cumulative occupational exposures by duration of employment rather than by creating a detailed job exposure matrix. However, exposure can be substantially reduced by personal or technical means of protection. In this respect, a significant reduction of organic dust components results from filtered air supply in wheel loader cabins (WLCs) (Schlosser et al. 2012). Thus, for our analysis we considered workers serving most of their current daily worktime (>5.5 h) in WLCs or performing office work indoors being rather low exposed to organic dust. In addition, time worked under

filtered air protection was used as continuous dimension to evaluate effects of organic dust exposure on biomarkers of effect.

Smoking contributes to the known high inter-individual variability shown for inflammatory biomarkers and complexity is even enhanced when referring to former smokers (Malerba and Montuschi 2012). Former smokers are a highly heterogeneous group with respect to duration and intensity of previous nicotine consumption, interim withdrawals, and time since final smoking cessation. Therefore, we focused on never-smokers and current smokers in our analyses. Smoking was assessed by means of current (CO vol%) and cumulative (pack-years) exposure. In our study atopic subjects were defined using measurement of sensitization to common allergens (sx1). Work-related complaints indicative of allergic asthma were only reported by some subjects. In fact, they suffer from eye and nose irritation (van Kampen et al. 2012). Overall, there was a low prevalence of workers under study demonstrating chronic bronchitis (4.2 %) or allergic asthma (6.7 %). This might be explained by individuals leaving the workplace due to health problems (healthy-worker effect). Subjects with allergic asthma may be particularly prone to adverse effects of organic dust.

The usefulness of respiratory biomarkers in exhaled breath, induced sputum and EBC in medical surveillance and identification of respiratory adverse effects in exposed workers was recently reviewed (Chérot-Kornobis et al. 2012; Quirce et al. 2010; Corradi et al. 2010). Measurement of FeNO is well standardized, widely applied and our results were obtained with an established device (Niox Mino, Aerocrine, Sweden).

Referring to EBC, a decrease in aerosolization of the airway lining fluid was suggested as a result of cigarette smoke induced epithelial injury (Morrison et al. 1999). Therefore, we accounted for the EBC volume and also calculated the absolute amounts of $PGE_2$, $LTB_4$, and 8-iso-$PGF_{2\alpha}$ without revealing different results (data not shown). Measurement of pH was performed after deaeration with argon in accordance to published guidelines (Horváth et al. 2005). The array of effect markers was chosen to account for different aspects of inflammation. We observed that biomarkers in EBC were correlated to each other, whereas pH and FeNO conferred independent information as has been previously reported. Do et al. (2008) suggested that airway acidity and oxidative stress in terms of 8-isoprostane level reflect distinct components of airway reactions. Also, measurement of hydrogen peroxide, another marker of oxidative stress, and FeNO in asthmatics provided complementary data and FeNO was found to be independent from EBC-pH (Dressel et al. 2010; Chérot-Kornobis et al. 2012).

Increased levels of 8-iso-$PGF_{2\alpha}$ in EBC of asthmatic patients were reported to correlate with disease severity (Klusáčková et al. 2008). We showed that compost workers considered to be highly exposed to organic dust demonstrated higher 8-iso-$PGF_{2\alpha}$ levels compared to rather low exposed ones. This result is in line with a previous study addressing organic dust exposure in grain workers (Do et al. 2008). Workers demonstrated increased 8-iso-$PGF_{2\alpha}$ levels positively correlated with the intensity of exposure to grain dust and endotoxin. In our study, subjects were further stratified and differences could be mainly attributed to non-atopics and never-smokers. In other words, smoking or atopy may have obscured effects resulting from occupational exposure. This is in accordance with results previously reported for the dominant impact of smoking on welding fume associated 8-iso-$PGF_{2\alpha}$ increases (Hoffmeyer et al. 2012).

Our study revealed an association between increase of EBC acidification (lower pH) and current intensity (CO vol%) as well as cumulative effects of smoking (pack-years), but not with occupational means. An inverse correlation between pack-years and EBC-pH was also shown in grain workers (Do et al. 2008). In addition, an association between the duration of work and decreased pH levels was found among the grain workers. The lack of correlation with duration of employment in our study might be explained by the more intensive organic dust exposure in composting plants. In a cross-week study of two sawmills demonstrating different

exposure characteristics, acidification of EBC was only observed in the sawmill demonstrating higher mould concentration (median 8,620 CFU/$m^3$) even though endotoxin levels in both sawmills were above 100 EU/$m^3$ (Ljubičić Ćalušić et al. 2013). The median exposure levels to moulds determined in composting plants of this study were previously reported with 6,900 CFU/$m^3$ (WLC) and up to $1.6 \times 10^6$ CFU/$m^3$ (processing). We did not include non-invasive diagnostic tools for the white collar workers serving as reference group in our study on compost workers (van Kampen et al. 2012). However, we observed EBC-pH levels of non-smoking workers in an order of magnitude lower than previously reported for healthy, non-smoking subjects (EBC-pH 7.47) using the same methodological approach (Hoffmeyer et al. 2015). Thus, in compost workers a low categorized exposure level might be sufficient to induce a relevant acidification of EBC that did not further increase in a dose-response manner. In this line, concerning smoking we could demonstrate significantly lower values in smokers than never-smokers but there was no correlation with pack years in smokers.

We observed that airway inflammation in atopic workers reflected by FeNO level increases with duration of employment in composting plants. FeNO is produced by the action of different NOS and activation of inducible NOS can be triggered by endogenous mediators and by exogenous stimulants such as allergens and endotoxins (Munakata 2012). Elevated FeNO levels have been reported in working populations exposed to low and especially high molecular weight substances either in occupational field settings or after specific allergen challenge. In healthy, young subjects, an increase of FeNO could be induced after short-term exposure to organic dust in a swine confinement facility (Sundblad et al. 2002). In a study among farmers and workers from agricultural processing industries, Smit et al. (2009) observed that wheezing was significantly associated with FeNO. However, an exposure-response relationship between cumulative endotoxin exposure and FeNO was only revealed in non-atopic, non-smoking subjects. The authors speculated that exposure-response relationships could be missed among chronically high exposed atopic subjects with already elevated FeNO values that might not further increase. In fact, an inverse association could be observed for FeNO in atopic workers with respect to time daily worked under clean air supply in the present study. Protection with half-mask was shown to inhibit the increase of FeNO after acute exposure to swine dust (Sundblad et al. 2002). In addition, preventive measures at the workplace could be verified by decrease of FeNO in serial measurement among farmers (Dressel et al. 2007). In summary our results on FeNO suggest that inflammation (based on a specific immunologic mechanism) was enhanced by long-term effects but still could be modulated by reduction of the current intensity of dust exposure by filtered air supply.

Our results were namely observed in respiratory healthy subjects at a subclinical level and potential confounding factors like smoking habits or atopic status have been taken into account. A limitation of the presented study is the number of participants and missing reference group. We assumed that differences in means of occupational exposures would enable us to unravel dose-effect relations. Our study incorporated information on additional environmental exposures (smoking habits) and a modulating genetic background (atopic status) but interpretation is hampered by the fact that subgroups under study had different distributions for age and duration of employment in composting plants.

In conclusion, results from this study support the usefulness of FeNO and collection of EBC in the evaluation of airway inflammation in workers exposed to bioaerosols. Recording of smoking habits and atopic status are recommended in view of a meaningful interpretation of measured effects and to detect subgroups at special risk.

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**Competing Interest** All the authors declare that they have no competing interests that might be perceived to influence the results and discussion reported in the present manuscript.

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# Plasma Fibrinolysis Parameters in Smokers and Non-smokers of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study

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**Abstract**

Cardiovascular diseases (CVD) are an important cause of morbidity and mortality worldwide. Parameters of coagulation and fibrinolysis are risk factors of CVD and might be affected by cigarette smoking. Aim of our study was to analyze the effect of cigarette smoking on parameters of fibrinolysis in active smokers (AS) and life-time non-smokers (NS) of the Ludwigshafen Risk and Cardiovascular Health (LURIC) Study as well as the use of these parameters for risk prediction. We determined plasminogen activator inhibitor-1 (PAI-1), tissue plasminogen activator antigen (t-PA), protein C activity, and D-dimers in 3,316 LURIC patients. Smoking status was assessed by a questionnaire and measurement of plasma cotinine concentration. Cox regression was used to assess the effect of parameters on mortality. We found that of the 3,316 LURIC patients 777 were AS and 1,178 NS. Within the observation period of 10 years (median) 221 AS and 302 NS died. In male AS *vs*. NS, PAI-1 (19.0 (10.0–35.0) *vs*. 15.0 (9.0–29.0) U/ml; $p = 0.026$) and t-PA antigen (12.7 (9.6–16.3) *vs*. 11.6 (8.9–14.6) μg/l; $p = 0.020$) were slightly increased, while t-PA activity was slightly decreased (0.63 (0.30–1.05) *vs*. 0.68 (0.42–1.10) U/l; $p = 0.005$). In female AS *vs*. NS, t-PA antigen

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(10.5 (8.3–13.9) *vs.* 11.5 (8.8–15.0) μg/l; p = 0.025) and protein C (108.0 ± 24.1 % *vs.* 118.0 ± 25.7 %; p = 0.004) were decreased. All parameters except for protein C were predictive for mortality in AS. Fully adjusted hazard ratios (95 % CI) were 1.14 (1.04–1.25), 1.19 (1.06–1.34), and 1.29 (1.11–1.49) per 1SD increase for D-dimer, t-PA, and PAI-1, respectively. Including fibrinolysis parameters in risk prediction models for mortality improved the area-under-the-curve (AUC) significantly compared with the conventional risk factors. In conclusion, we found alterations in the fibrinolytic system in smokers, which were more pronounced in male AS. PAI-1, t-PA and D-dimers were significant predictors of mortality in AS in LURIC and should be included into the assessment of cardiovascular risk particularly in patients at risk.

**Keywords**
Fibrinolysis • Hemostasis • Mortality • Smoking • Thrombosis

## 1 Introduction

Hemostasis is a process that regulates the intravascular balance of antithrombotic and prothrombotic factors as well as profibrinolytic and antifibrinolytic factors. Cigarette smoke (CS) disturbs this balance in multiple ways (Barua and Ambrose 2013) by affecting the function of endothelial cells, platelets, and the coagulation cascade. Several studies could demonstrate that CS or its isolated components were associated with decreased NO availability by altering the expression and activity of the endothelial NO synthase enzyme (Barua et al. 2001, 2003). Nitric oxide, besides being an important vasodilator, is also involved in the regulation of inflammatory processes, leukocyte adhesion, platelet activation, and thrombosis (Loscalzo 2001). Endothelial cells also secrete both fibrinolytic tissue plasminogen activator (t-PA) and antifibrinolytic plasminogen activator inhibitor-1 (PAI-1). t-PA activates plasminogen leading to the initiation of fibrinolysis and is itself inhibited by PAI-1. It has been shown that CS affects this secretion thereby contributing to the generation of a hypercoagulable state (Benowitz 2003; Barua et al. 2002; Newby et al. 2001; Zidovetzki et al. 1999). However, our understanding of the underlying pathological mechanisms is still far from being complete. Therefore, the aim of this study was to analyze parameters of fibrinolysis in active smokers (AS) and lifetime non-smokers (NS) in a cohort with moderate-to-high risk for coronary heart disease and to investigate the effect of these parameters on the long-term clinical outcome.

## 2 Methods

### 2.1 Study Population

The study was approved by the 'Landesärztekammer' Ethics Committee of the Rheinland-Pfalz state in Germany. All patients signed informed written consent at study entry. The LUdwigshafen RIsk and Cardiovascular Health (LURIC) study is an ongoing prospective study of 3,316 patients of German ancestry who had an indication for coronary angiography and were recruited between June 1997 and May 2001 at the Ludwigshafen Cardiac Center (Winkelmann et al. 2001). All patients were clinically stable (except for acute coronary syndromes). Information on vital status was obtained from local registries. Death certificates were obtained in 97 % of dead participants. Of the persons studied, 523 deaths (26.8 %) occurred during a median follow-up of 10 years. Smoking status was assessed based on a questionnaire and verified by measurement of serum cotinine concentration.

### 2.2 Laboratory Procedures

Fasting blood samples were taken by venipuncture in the early morning prior to angiography. Aliquots were frozen at −80 °C. Cholesterol and triglycerides were measured with enzymatic reagents from Wako (Neuss, Germany) on an Olympus AU640 analyzer (Center Valley, PA). Fibrinolysis factors were analysed at the hemostaseology laboratory of the Ludwigshafen Heart Center at the same day. PAI-1 and t-PA were measured using the corresponding TintElize™ assays (Biopool; Atlanta, GA), D-dimers were measured using the STA Liatest D-DI assay (Roche Diagnostics, Indianapolis, IN), activated protein C was measured using the COMATIC® assay (Chromogenix Instrumentation Laboratory; Milano, Italy), and protein S activity was determined using the Asserachrom® assay (Stago Diagnostica/Roche, Germany). Endogenous thrombin potential (ETP) was determined from frozen aliquots of baseline samples using Innovance ETP on a BCS coagulation analyzer (Siemens Healthcare Diagnostics Inc.; Eschborn, Germany). Circulating serum oxLDL levels were assayed with a competitive ELISA utilizing a specific murine monoclonal antibody (Mercodia, Uppsala, Sweden; detection limit <0.3 U/l). hsCRP was determined by immunonephelometry on a Behring Nephelometer II (N High Sensitivity CRP; Dade-Behring, BN II, Marburg, Germany). Galectin-3 concentration was measured in plasma samples on an Architect analyzer (Abbott Diagnostics, Abbott Park, IL).

### 2.3 Statistical Elaboration

All continuous variables were checked for normality and variables showing a skewed distribution were logarithmically transformed to get normal distribution. Continuous variables were compared between groups by Student's t-test. Associations between categorical variables were examined by chi-square testing. To examine the relationship of fibrinolysis factors with mortality, we calculated hazard ratios and 95 % confidence intervals (95 % CI) using the Cox proportional hazards model. Multivariable adjustment was carried out as indicated. The proportional hazard assumption was checked by examination of scaled Schoenfeld residuals. IBM SPSS Statistics v. 21.0 (IBM Corporation) and R statistical software v. 3.1.1 (http://www.r-project.org) was used for all analyses. ROC curves were compared using the method from Delong as implemented in the R package 'pROC' (Robin et al. 2011).

## 3 Results

Off the 3,316 LURIC patients 777 were active smokers (AS) and 1,178 were lifetime non-smokers (NS). There was a predominance of men in the AS group, and this group had a lower mean age than NS (Table 1). AS showed lower LDL-C and HDL-C but a higher concentration of oxidized LDL and triglycerides as compared with NS. Systemic inflammation as measured by the concentration of hsCRP was higher in AS but there was no difference in the concentration of galectin-3, a marker of fibrosis. AS had a higher mean estimated glomerular filtration rate (eGFR), but there was a higher percentage of patients suffering from coronary artery disease and hypertension as well as a higher proportion of patients treated with lipid lowering drugs (mainly statins) in the AS group as compared with lifetime non-smokers.

AS showed a higher thrombogenic potential demonstrated by a lower international normalized ratio (INR) and a higher endogenous thrombin potential (Table 1) as well as higher concentrations of fibrinogen and soluble fibrin (only men) (Table 2). Regarding the fibrinolytic system we found no difference in the concentration of D-dimers between AS and NS. While there were significant increases in PAI-1 activity and t-PA antigen as well as a decrease in t-PA activity for male AS, there was a small decrease in t-PA antigen for female AS. Regarding active protein C, only female AS showed a significant decrease.

**Table 1** Selected anthropometric data of patients at study onset

| | Never smokers | Active smokers | P |
|---|---|---|---|
| N | 1,178 | 777 | |
| Age (years) | 65.3 ± 10.1 | 56.2 ± 10.3 | <0.001 |
| Male sex (%) | 45.4 | 77.9 | <0.001 |
| BMI | 27.4 ± 4.2 | 27.0 ± 4.2 | 0.833 |
| LDL-C (mg/dl) | 119.1 ± 36.4 | 117.5 ± 32.1 | 0.012 |
| oxidized LDL-C (U/l) | 73.5 ± 26.7 | 78.4 ± 25.4 | <0.001 |
| HDL-C (mg/dl) | 41.2 ± 11.1 | 36.2 ± 10.2 | 0.002 |
| Triglycerides (mg/dl) | 136 (102–192) | 154 (112–218) | <0.001 |
| Galectin-3 (ng/ml) | 15.8 ± 6.3 | 15.5 ± 7.3 | 0.496 |
| hsC-reactive protein (mg/l) | 2.72 (1.17–7.04) | 4.93 (1.84–10.30) | <0.001 |
| eGFR (ml/min/1.73 $m^2$) | 78.7 ± 19.1 | 88.2 ± 20.1 | <0.001 |
| INR | 1.05 (1.00–1.10) | 1.03 (0.98–1.08) | <0.001 |
| ETP (%) | 94.4 ± 28.5 | 99.1 ± 24.5 | <0.001 |
| Coronary artery disease (%) | 68.1 | 80.1 | <0.001 |
| Diabetes mellitus (%) | 38.3 | 36.0 | 0.314 |
| Hypertension (%) | 76.6 | 63.3 | <0.001 |
| Lipid lowering therapy (%) | 42.4 | 52.8 | <0.001 |

Data are means ± SD or median and 25–75th percentile
*BMI* body mass index, *ETP* endogenous thrombin potential, *HDL-C* high density lipoprotein cholesterol, *INR* international normalized ratio, *LDL-C* low density lipoprotein cholesterol

**Table 2** Parameters of the fibrinolytic and coagulation systems in non-smokers and active smokers

| | Men | | | Women | | |
|---|---|---|---|---|---|---|
| | Never smokers (n = 535) | Active smokers (n = 605) | P | Never smokers (n = 643) | Active smokers (n = 172) | P |
| D-dimers (mg/l) | 0.33 (0.22–0.57) | 0.35 (0.22–0.65) | 0.323 | 0.38 (0.23–0.65) | 0.39 (0.22–0.65) | 0.362 |
| PAI-1 (U/ml) | 15.0 (9.0–29.0) | 19.0 (10.0–35.0) | 0.026 | 20.0 (10.0–36.0) | 18.0 (10.0–33.0) | 0.182 |
| t-PA antigen (μg/l) | 11.6 (8.9–14.6) | 12.7 (9.65–16.3) | 0.002 | 11.5 (8.80–15.0) | 10.5 (8.30–13.9) | 0.025 |
| t-PA (U/l) | 0.68 (0.42–1.10) | 0.63 (0.30–1.05) | 0.005 | 0.61 (0.32–1.01) | 0.63 (0.38–0.95) | 0.473 |
| Protein C (%) | 105.0 ± 22.4 | 106.0 ± 25.7 | 0.623 | 118.0 ± 25.7 | 108.0 ± 24.1 | 0.004 |
| Protein S (%) | 119.0 ± 34.0 | 124.0 ± 36.3 | 0.100 | 111.0 ± 31.2 | 107.0 ± 30.5 | 0.365 |
| Factor V (U/dl) | 109.0 ± 20.1 | 115.0 ± 22.0 | 0.001 | 116.0 ± 22.0 | 115.0 ± 23.4 | 0.816 |
| Factor VIII (U/dl) | 160 (122–206) | 154 (116–198) | 0.014 | 184 (144–226) | 174 (116–216) | 0.002 |
| Fibrinogen (mg/dl) | 381 ± 107 | 420 ± 114 | <0.001 | 387 ± 93 | 405 ± 113 | 0.053 |
| sol. fibrin (U/ml) | 60.0 (35.0–87.1) | 64.6 (42.5–97.9) | 0.017 | 53.3 (33.8–82.1) | 59.4 (37.9–79.5) | 0.339 |
| TFPI (μg/l) | 1.21 ± 0.34 | 1.31 ± 0.37 | <0.001 | 1.25 ± 0.39 | 1.27 ± 0.38 | 0.482 |

Data are means ± SD or median and 25–75th percentile
*PAI-1* plasminogen activator inhibitor 1, *TFPI* tissue factor pathway inhibitor, *t-PA* tissue plasminogen activator antigen

**Table 3** Cox regression analysis of all-cause mortality per 1SD increase

| | Never-smokers | | | Active smokers | | |
|---|---|---|---|---|---|---|
| | n | HR (95 % CI) | P | n | HR (95 % CI) | P |
| *Model 1* | | | | | | |
| D-dimers | 1,164 | 1.09 (1.01–1.16) | 0.022 | 770 | 1.13 (1.04–1.24) | 0.006 |
| t-PA antigen | 1,175 | 1.21 (1.12–1.31) | <0.001 | 777 | 1.16 (1.04–1.30) | 0.007 |
| PAI-1 | 1,175 | 1.08 (0.99–1.17) | 0.082 | 777 | 1.23 (1.09–1.40) | 0.001 |
| Protein C | 383 | 0.95 (0.76–1.18) | 0.629 | 294 | 1.09 (0.87–1.37) | 0.453 |
| *Model 2* | | | | | | |
| D-dimers | 1,162 | 1.07 (1.00–1.16) | 0.068 | 766 | 1.14 (1.04–1.25) | 0.006 |
| t-PA antigen | 1,175 | 1.15 (1.05–1.25) | 0.003 | 771 | 1.19 (1.06–1.34) | 0.004 |
| PAI-1 | 1,175 | 1.03 (0.90–1.17) | 0.668 | 771 | 1.29 (1.11–1.49) | 0.001 |
| Protein C | 382 | 0.91 (0.71–1.16) | 0.434 | 292 | 1.15 (0.89–1.48) | 0.275 |

*Model 1,* adjusted for age and gender; *Model 2,* adjusted for age, sex, LDL-C, HDL-C, logTG, BMI, diabetes, hypertension, and logCRP

**Table 4** Area-under-the-Receiver-Operator-Characteristics-curve for different risk prediction models in active smokers

| Model | AUC (95 % CI) | P for comparison with basic Model | P for comparison with Model 2 | P for comparison with Model 3 |
|---|---|---|---|---|
| (1) Basic* | 0.707 (0.666–0.747) | | – | – |
| (2) Basic + fibrinolysis | 0.716 (0.675–0.757) | 0.041 | – | – |
| (3) Basic + coagulation | 0.737 (0.698–0.776) | <0.001 | 0.018 | – |
| (4) Basic + fibrinolysis + coagulation | 0.741 (0.702–0.781) | <0.001 | 0.001 | 0.068 |

*Age, gender, BMI, LDL-C, HDL-C, logTG, logCRP, diabetes, and hypertension

We next examined whether fibrinolytic parameters were associated with mortality in AS and NS by Cox regression analysis. In NS the only parameter that showed a statistically significant association with mortality after adjustment for other cardiovascular risk factors was t-PA antigen with a HR (95 % CI) of 1.15 (1.05–1.25) per 1SD increase (Table 3). In the group of active smokers, D-dimers, t-PA antigen, and PAI-1 activity were associated with an increased risk of death with HR of 1.14 (1.04–1.25), 1.19 (1.06–1.34), and 1.29 (1.11–1.49), respectively.

We also tested the death predictive value of fibrinolysis and coagulation parameters in the AS group by calculating the area under the receiver operator curve (AUC) (Table 4). The basic model including age, gender, BMI, LDL-C, HDL-C, logTG, logCRP, diabetes, and hypertension had the AUC of 0.707 (0.666–0.747). Adding either factors of fibrinolysis or coagulation led to a significant improvement of the model with the AUC of 0.716 (0.675–0.757) and 0.737 (0.698–0.776) for fibrinolysis or coagulation factors, respectively. Adding both kinds of parameters to the basic model further increased the AUC to 0.741 (0.702–0.781), although the increase was statistically insignificant as compared with the model with coagulation factors alone.

## 4 Discussion

In the LURIC study participants we found changes in some parameters of the fibrinolytic system in active smokers compared with lifetime non-smokers. While in non-smokers only the

concentration of t-PA antigen was associated with the risk to die, in active smokers t-PA, PAI-1, and D-dimers were all significantly associated with mortality. Adding markers of fibrinolysis to basic risk models significantly improved the risk prediction as shown by an increased AUC.

Recently we reported significant changes in individual parameters of the coagulation system in active smokers of the LURIC study compared with lifetime non-smokers (Delgado et al. 2015). To expand those findings, we now analyzed markers of fibrinolysis in the same cohort and found a small but statistically significant increase of t-PA as well as a decrease of protein C activity in AS. The term "smokers' paradox" has been coined to describe the reduced mortality after ST segment elevation myocardial infarction (STEMI) in smokers. This effect has been attributed to the "open artery hypothesis" that suggests that early reperfusion of the occluded coronary artery is beneficial because it limits the size of infarction, reduces the degree of left ventricular dysfunction, and improves survival (Braunwald 1989). Smokers show a higher 'thrombolysis in myocardial infarction' (TIMI) flow grade 3, indicating enhanced thrombolysis leading to early reperfusion (Angeja et al. 2002; Kirtane et al. 2005). We and others have shown increased fibrinogen in smokers (Delgado et al. 2015; Dotevall et al. 1994) which points to the possibility that their thrombus may be enriched in fibrin making them more susceptible to fibrinolytic therapy (Kirtane et al. 2005).

The principal regulator of fibrinolysis is t-PA which catalyzes the conversion of plasminogen to plasmin. Most of the circulating t-PA is bound to its inhibitor PAI-1, a member of the serine protease (serpin) family, and this complex can be measured as t-PA antigen. In a small study by Barua et al. (2010) thrombus from otherwise healthy smokers did not show an enhanced lysis when exposed to t-PA and there was no difference in the concentration of fibrinogen, PAI-1, or t-PA between smokers and non-smokers. It has been shown that thrombi formed post-smoking were composed of significantly thinner fibrin fibres and had a denser, more tightly knit cross-linked architecture, as compared with control samples, making them more resistant to thrombolysis (Barua et al. 2010; Scott et al. 2004). In contrast to those results we did observe increases in fibrinogen and t-PA antigen in our smokers. t-PA activity was significantly diminished only for male AS. An increase in t-PA was also reported in neonates that had been exposed to tobacco smoke *in utero* (Mitsiakos et al. 2009). Increased t-PA would suggest an enhanced ability for fibrinolysis, but a rapid release of t-PA from the vascular endothelium is essential for an effective fibrinolysis. Studies could show that the t-PA release from the vascular bed was impaired in smokers (Lang et al. 2008; Newby et al. 1999; Takashima et al. 2007) suggesting an impaired capacity of the endothelium to release t-PA acutely, despite high baseline concentrations of t-PA.

PAI-1 is an acute-phase protein and its concentration in plasma is tightly regulated (Dellas and Loskutoff 2005). Some studies have reported increased concentrations in smokers (Simpson et al. 1997), others have found decreased plasma levels of PAI-1 in chronic smokers compared with non-smokers within 30 min of both groups smoking two cigarettes (Ozdemir et al. 1992), while still others have reported downregulation of PAI-1 mRNA and protein synthesis in heart tissue of mice exposed to tobacco smoke for several weeks (Halappanavar et al. 2009). In the present our study we observed a slight increase of PAI-1 plasma concentration only in male AS.

Protein C activation takes place mostly in the endothelium of small vessels and requires the catalytic action of the endothelial transmembrane complex of thrombomodulin and thrombin (Esmon and Owen 1981). Fernandez et al. (2002) have found that the level of circulating activated protein C was 23.3 % lower in smokers as compared with non-smokers, with no difference in protein S concentration or activity. The authors speculate that cigarette smoking might cause an acquired activated protein C deficiency contributing to the generation of a prethrombotic state in smokers. We also detect a decrease in active protein C in our study, but only in female AS.

D-dimers are one of the cross-linked degradation products generated by the cleavage of fibrin and fibrinogen by plasmin and elevated concentrations reflect ongoing fibrinolysis. Increased D-dimer concentrations have been reported in current smokers (Wannamethee et al. 2005) and studies of smoking cessation showed a significant decrease in D-dimers over time in ex-smokers (Caponnetto et al. 2011; Wannamethee et al. 2005). We did not detect a difference between AS and NS regarding this marker for our study participants.

Among the investigated markers of fibrinolysis only t-PA antigen showed an association with all-cause mortality in NS after adjustment for other cardiovascular risk factors. In contrast to that three out of four markers (D-dimers, t-PA, and PAI-1) were associated with increased mortality in the groups of AS. The risk increase inferred by elevated t-PA was comparable in both groups. The association of D-dimers and t-PA with coronary heart disease (CHD) and CHD death has been described by numerous studies and a recent meta-analysis of findings from general population studies reported adjusted RRs of 1.13 (1.06–1.21) and 1.23 (1.16–1.32) per 1SD higher baseline levels of t-PA antigen and D-dimer, respectively (Willeit et al. 2013), which are in a similar range like the HRs calculated for our present study. The highest risk increase for AS was inferred by elevated PAI-1. PAI-1 appears to be the primary and fastest acting inhibitor of plasminogen activation *in vivo*. It can also bind to the extracellular matrix, mainly by binding to vitronectin, which suggests the possible role in the regulation of cell adhesion by interfering with the binding properties of vitronectin and αvβ3 integrin, or the uPA receptor. Furthermore, the ternary PAI-1-uPA-uPAR complex is cleared from the cell surface through internalization by scavenger receptors leading to the possibility of PAI-1 being involved in initiating or modulating intracellular signalling cascades (Kietzmann and Andreasen 2008). PAI-1 has been associated with mortality in a number of different diseases, e.g., sepsis (Lorente et al. 2014), lung injury (Prabhakaran et al. 2003), or pneumococcal meningitis (Brouwer et al. 2014).

Having shown the association of different parameters of fibrinolysis with mortality we next examined whether the inclusion of these parameters in models predicting all-cause mortality in AS would improve risk prediction. Adding markers of fibrinolysis improved the AUC significantly; although to a lesser extent as compared with coagulation markers.

The present study has got some limitations. All participants were of European ancestry and were recruited at a tertiary referral center. Therefore our findings may not be representative for a random population sample or applicable to other ethnicities. Furthermore, we only investigated active smokers and lifetime non-smokers, and excluded former smokers from the analysis. Direct measurements of fibrinolysis were unfortunately not available. The major strengths of the LURIC cohort are, however, the precise clinical and metabolic characterization of the participants, including the detailed characterization of the coagulation cascade, and the cross-sectional and prospective design. We conclude that beside other risk factors for cardiovascular disease (e. g., lipids or life-style), the parameters of the coagulation and fibrinolytic systems should additionally be determined for risk prediction in active smokers.

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**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# Vertigo with a Vestibular Dysfunction in Children During Respiratory Tract Infections

E.A. Dzięciołowska-Baran and A. Gawlikowska-Sroka

**Abstract**

Sudden balance disorders with violent vegetative symptoms (nausea and vomiting) pose a diagnostic and therapeutic problem. In children vertigo/dizziness with symptoms of vestibular dysfunction is rare, but as vascular etiology is unlikely in children such symptoms arouse concern. This article presents two cases of this type of vertigo. The patients were two boys (6 and 9 years old). They came down with similar symptoms: sudden dizziness, disabled walking, nausea and vomiting, spontaneous nystagmus, and a positive Romberg test. The onset of the balance disorder was preceded by respiratory infection: common cold with symptoms of inflammation of the mucous membrane in the nose and throat. Laboratory tests revealed increased levels of C-reactive protein only in the older boy. Neuroinfection and a displacement process were ruled out. Videonystagmography revealed vestibular dysfunction and vestibular neuronitis on the left side.

**Keywords**

Dizziness • Vestibular neuronitis • Paediatric patients • Otolaryngology • Viral and bacterial infections • Cold

## 1 Introduction

### 1.1 Functions of the Balance System

The balance system is one of the most complex systems in the human body. It is formed by at least three sensory organs: the vestibular system,

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the visual system, and the proprioceptors, which work together to maintain proper orientation of the body and enable postural reflexes. The balance system also signals the brain about current body position, direction, and speed of motion. It responds when the body position is changed with respect to gravity (to prevent falls) and controls eye movement to ensure we perceive the correct image of the surrounding space.

### 1.2 Treatment of Balance Disorders

Balance disorders, popularly known as dizziness, are common complaints reported by patients visiting family doctors (Yardley et al. 1998). Doctors then, depending on their findings, refer patients to neurologists if central vertigo, i.e. other than vestibular, is suspected, or refer them to be consulted by an otolaryngologist if peripheral vertigo is suspected. Each case of vertigo, due to its specific nature, is difficult to handle, both by patients and doctors. Patients feel insecure and often terrified that the world is spinning around them, they are unable to move without assistance, are bothered by nausea and vomiting, and are mistaken for drunks by other people. Doctors find this medical condition problematic because of its multifactorial aetiology. Most patients suffering from vertigo are adults. The incidence of vertigo increases with age and slowly emerges as a separate medical condition. In fact, there is a group of symptoms associated with balance disorders in elderly people (Belal and Glorig 1986). In pediatric patients balance disorders are rare. They can be difficult to diagnose because children are not always able to describe symptoms. On the other hand, their sudden onset and violent course may induce fear in patients, but first and foremost, they can cause real panic in their caregivers (Uneri and Turkdogan 2003). It should be pointed out that in each case vertigo and dizziness are symptoms, not diagnoses (Casselbrant and Mandel 2005). A detailed interview, physical examination, and laboratory tests in patients from every age group help to identify the factors responsible for these ailments.

### 1.3 Classification of Vertigo in Children

Disorders associated with vertigo in children can be classified into three broad categories: (1) acute nonrecurring spontaneous vertigo; (2) recurrent vertigo; (3) nonvertiginous dizziness, disequilibrium, and ataxia (Casselbrant and Mandel 2005). The first category includes rare pediatric diseases: acute labyrinthitis, vestibular neuronitis, labyrinthine concussion, and perilymphatic fistula. The second category, with recurrent episodes, includes Meniere's disease, migraine, benign paroxysmal vertigo (BPV), seizure disorders, and periodic ataxia. The last category covers motion sickness, central nervous system lesions, bilateral vestibular loss, otitis media (Eustachian tube dysfunction with or without middle ear effusion), drug-induced dizziness, and non-neurotologic disorders such as psychiatric problems, ocular disorders, and balance dysfunction in the course of hearing loss.

## 2 Methods

This article presents two cases of sudden vertigo in children. Both patients were boys and were hospitalized at the Regional Hospital in Szczecin. The research was performed in conformity with the Declaration of Helsinki for Human Experimentation. We received the permission to present the results from the children's parents.

### 2.1 Case Report – Patient 1

A 5.5-year-old boy, while in kindergarten, suddenly experienced severe balance disorders followed by nausea and vomiting. The symptoms were violent and disturbing because the boy was unable to stand, and an ambulance was called for. The patient, despite being terrified and unwell, reported feeling a spinning motion, like being on a merry-go-round. He had left-beating nystagmus, and his Romberg test was positive. The patient was taken to the hospital Otolaryngology Unit for Adults and Children, and stayed there for 5 days. The results of laboratory tests carried

out on admission were unremarkable. Computed tomography done on the day of admission did not show any changes in the central nervous system, apart from apneumatic lesions in single ethmoids on the right side. The electronystagmogram (ENG) revealed a 52 % deficit in excitability of the left labyrinth, and no spontaneous nystagmus was found. Hearing, tested by audiometry, was normal, and only a drop to 35 dB for 6,000 Hz was found in the right ear. Bilateral tympanogram was of type A, with a normal acoustic stapedial reflex. An interview with the parents revealed that before the onset of vertigo the boy was well, but several children in the kindergarten had symptoms of a viral infection, manifested by cold of various severity – rhinitis, weakness, and diarrhoea with nausea and vomiting, and enteroviral infection was suspected in two cases.

The boy had been a patient of the outpatient Otolaryngology Clinic for over a year. At age 4 he had myringotomy with adenotomy due to bilateral exudative otitis with conductive hearing loss. A few months after surgery his parents again observed a slight hearing impairment, and the boy also had subacute otitis media with rhinitis, which subsided in quite a short time after nasal obstruction was eliminated. In tympanometry, a reduction of pressure in the middle ear was recorded at that time, especially on the left side, with a type B tympanogram. The symptoms of hearing loss resolved after treatment with a nasal steroid drug. Metoclopramid and rehydrating drips were given during the present hospital stay. The child was discharged in good health, with very discrete balance disorders. A quiet lifestyle was recommended for about 4 weeks, along with a check-up visit to a laryngologist in the outpatient clinic. A check-up 3 weeks later found the patient in good health, with no balance disorders. The results of laboratory and audiologic tests were normal.

## 2.2 Case Report – Patient 2

A 9-year-old boy was admitted to the pediatric hospital unit due to sudden vertigo accompanied by vomiting and severe balance disorders. An interview with the mother revealed that the child was currently being treated with antibiotics (amoxicillin with clavulanic acid) due to upper respiratory tract infection. Previously, the boy was physically active, had no history of laryngological problems, and generally did not fall ill too often. Even during infection, his general status was good, and he only complained about sore throat and had fever for a short time. Suddenly, on the second day of infection the boy's health deteriorated and this made parents seek medical help at the hospital. On admission, the boy was lethargic, reposed, sleepy, and could not keep his balance, leaning to the left. His Romberg test was positive, and right-beating nystagmus was diagnosed. The boy had nausea and moderate vomiting.

The patient was hospitalized for 4 days. Lumbar puncture was performed to rule out neuroinfection. No abnormalities were found in the cerebrospinal fluid, and a head MRI ruled out lesions in the central nervous system. Laboratory tests revealed increased monocyte count in blood smear, without leukocytosis, and slightly increased C-reactive protein (CRP; 7.7 mg/l). The viral tests were the following: Epstein Barr virus antibody to nuclear antigen, EBV NA-IgG (−), to viral capsid antigens, EBV VCA IgG – 2.11(+) and IgM 0.05 (−), and cytomegalovirus, CMV IgG −50.5 (+) and IgM (−). The Lyme disease was ruled out. A urine test was normal, and pathogenic flora were not identified in throat or nose smears. The boy was seen by an ophthalmologist who found periodic horizontal right-beating nystagmus; by a neurologist who found a discrete horizontal right-beating nystagmus, shaking in the Romberg test, unsteady walk, without coordination problems and abnormalities in physical examination, and by a laryngologist who found discrete spontaneous right-beating nystagmus, leaning to the left when walking, and no abnormalities in physical examination. The ENG performed 2 days later, i.e. 1 day before discharge, showed a 40 % deficit in the left labyrinth without spontaneous nystagmus. The boy was treated with a second generation cephalosporin and rehydrating drips. He was

discharged home in good health, and visited the laryngology outpatient clinic 2 weeks later for a check-up, where no abnormalities were found. The boy felt well and returned to football training.

## 3 Results and Discussion

### 3.1 Comparison of Cases

Both boys fell ill in the spring, 2 weeks apart from each other. The disease onset was almost identical and characterized by sudden, severe balance disorders with vertigo and vegetative symptoms. In both cases, the health problem caused serious concern in parents, but in the case of the 9-year-old boy the family reacted in a calmer manner because the boy clearly had infection symptoms and had already been treated. In this case a decision was made to hospitalize the patient at the pediatric unit, and treatment with antibiotics was continued. The other younger boy presented with no evident symptoms of acute infection. In both children the involvement of the central nervous system was ruled out at an early stage by imaging examinations and cerebrospinal fluid test. The viral tests were carried out in the older boy. In the younger boy admitted to the laryngology unit a prompt test of labyrinth function was done, and treatment was only symptomatic, although finally the boy was diagnosed with sinusitis ethmoidalis. Both patients were diagnosed with vestibular neuronitis and recovered in a short time.

It is interesting to see a different approach of pediatricians to the strategy adopted by otolaryngologists, but this probably stemmed from the nature of the case. In the first patient, initially suspected to have vestibular dysfunction, further procedures were limited to the confirmation of the diagnosis, without establishing the etiology of this dysfunction. Doctors assumed that the child had subclinical symptoms of upper respiratory tract infection, and did not search for a causative factor (viral or bacterial). In the second case the diagnosis of vestibular neuronitis was confirmed relatively late by testing the labyrinth functions, but before establishing the diagnosis doctors made attempts to determine etiology. This was important for the choice of treatment.

### 3.2 Factors Responsible for Vertigo in Children

The incidence of vertigo in children is much lower than in adults. Unfortunately, balance disorders in pediatric patients are difficult to diagnose. This applies especially to the symptoms that have no spectacular clinical picture, as is the case in diseases with vestibular shock. The difficulties probably result from the fact that most pediatric patients are unable to describe their condition (Casselbrant and Mandel 2005). At the same time, young age allows for ruling out other causative factors usually associated with co-morbidities in adults.

Vertigo most commonly occurs in children with migraine equivalents. Unlike in adults, migraine in children is not always manifested by headache, but disequilibrium and recurrent vertigo instead. Precise diagnosis in such cases is also easier because of recurrent vertigo. Benign paroxysmal vertigo of childhood (BPVC) is also most likely migraine-related, and is often classified as a single disease or treated as a migraine subtype (Casselbrant and Mandel 2005). In many reports, BPVC is the most common cause of vertigo in children (Baloh 2003; Uneri and Turkdogan 2003). Manifestations of acute vestibular dysfunction in young children include paroxysmal torticollis and paroxysmal tortipelvis. They are considered migraine equivalents because of the strong association with migraine later in life (Eviatar 2005).

Balance disorders in children may be caused by head injuries, relatively common in this age group. Various theories explaining this process have been proposed, including direct injury (vestibular concussion) and pressure injury transmitted *via* skull bones or the cochlear aqueduct, leading to fracture of the membranous labyrinth or damage to hair cells, hair bundles, or other specialized structures in the ampulla or macula. In such cases dizziness is accompanied mainly by hearing disorders (sudden

hearing impairment or loss, tinnitus), and sometimes bloody drainage from the ear. Other causes of posttraumatic vertigo include injury of the central nervous system (especially of the brainstem, or cerebellar contusion) or temporal bone fracture (Gagnon et al. 2004).

Another disease frequent in young children and manifested by balance disorders is otitis media with effusion (OME) (Rine 2009; Riina et al. 2005). OME is associated with abnormal middle ear ventilation, leading to temporary vestibular dysfunctions. The pathomechanism has not been fully identified, but serous labyrinthitis may be one option (Golz et al. 1998).

Acute labyrinthitis should also be considered when analyzing the infective factors of vestibular dysfunction. Causative factors responsible for acute labyrinthitis have not been identified, but there is a theory that bacterial toxins or other biochemicals contained in the middle ear fluid are probably absorbed by the round or oval window (Casselbrant and Mandel 2005). This usually happens in chronic otitis media with concomitant abnormalities of the temporal bone (congenital or acquired, e.g. posttraumatic). Another important issue is inflammations of specific etiology. Currently, with the incidence of tuberculosis on the rise, it should be considered that it also may affect the middle ear. In otoscopic examination of children, the tympanic membrane affected by tuberculosis looks intact, but thinned and has dilated blood vessels. Also syphilis, in any of its manifestations, may be responsible for osteolytic and degenerative changes to the labyrinth.

Many medications are also known to induce vestibular dysfunctions. These include ototoxic antibiotics, such as aminoglycosides, especially gentamycin (causing oscillopsia). Phenytoin, used in the treatment of epilepsy, may cause vertigo and nystagmus due to intoxication. The vestibular organ can also be irritated by chemotherapeutic drugs, loop diuretics, quinine, thalidomide, and very popular nonsteroidal anti-inflammatory drugs.

## 3.3 Vestibular Neuronitis

Vestibular neuronitis or neuritis is a rare disease in children under 10 years of age (Casselbrant and Mandel 2005). Nonetheless, it is usually listed as the third most common cause of vestibular disorders in pediatric patients (Brandt 2003). The clinical manifestation of the disease is characteristic enough to enable diagnosis. The onset of vertigo and balance disorders is sudden and very severe, and is accompanied by nystagmus, nausea, and vomiting. In many cases the problem occurs in children who have previously suffered from upper respiratory tract infections (Bujak and Kasacka 2007; Eviatar 2005).

Vestibular dysfunction as a distinct clinical entity was proposed by Ruttin in 1909 (Ruttin 1909) and vestibular neuronitis was clinically defined by Nylen in 1924 (Nylen 1924). Later on, the condition was investigated in more detail by Hallpike (1943) and Dix and Hallpike (1952) who carried out observations of patients and found that the inflammatory process affects the vestibular ganglion and may be caused by a focal infection. Many clinicians believe that vestibular neuronitis is strongly associated with viral infections caused by herpes simplex virus (HSV), enteroviruses, or parainfluenza virus (Pyykko and Zou 2008; Ergul et al. 2006; Simonsen et al. 1996). This is also supported by the fact that symptoms are epidemic, e.g. more than one family member suffers from the disease (Baloh 2003). Other reported viral diseases clinically manifested by vestibular dysfunctions defined as vestibular neuritis include mumps, rubella, and those caused by cytomegalovirus and Epstein Barr virus. Vestibular research resulted in the elaboration of evoked myogenic potential (VEMP) test which is a clinical method for assessing balance disorders (Murofushi and Kaga 2009).

Despite the characteristic combination of symptoms in vestibular neuritis, the disease is not always correctly and promptly diagnosed because of its low incidence in children. Normally, imaging or cerebrospinal fluid tests are not recommended in non-feverish patients. However, due to a dramatic onset and need to reassure the parents, physicians focus on ruling out neuroinfection and displacement processes in the brain. A perfect solution would be to test vestibular functions in the child, but this is unfortunately possible only at laryngology units.

Another problem is the lack of effective communication with pediatric patients. Therefore, an interview with parents and information from school or kindergarten personnel are highly valuable. They may reveal that the child had asymptomatic or subclinical infection, or had contact with ill children. Significantly more children than adults have a history of infection preceding the onset of vestibular neuronitis (Tahara et al. 1993).

It seems unnecessary to use diagnostic methods targeted at impaired cerebral circulation, which often causes similar disorders in elderly people. Also, the controversial assessment of the cervical spine in children has no diagnostic value. Close cooperation between different specialists, i.e. laryngologists, paediatricians, neurologists, and ophthalmologists is the most important factor.

The treatment of the disease is symptomatic, except for patients with symptoms of acute infection. The prognosis is good in most cases, and symptoms subside within a week or two. Some patients may have prolonged sensitivity of the vestibular system to strain caused, e.g. by dancing, intense physical exercise, or travel by car (Hotson and Baloh 1998). Recurrence of vestibular neuronitis in children is very rare.

## 4 Conclusions

Respiratory tract infections, even if apparently mild, may be associated with vestibular dysfunctions. Etiological factors responsible for the disease still need to be investigated in detail, but viruses and bacteria are indicated most frequently. The essential elements of the diagnostic procedure include an interview, followed by a cooperative effort of various medical specialists, who should carry out a thorough physical examination. In most cases the treatment of the disease is symptomatic.

**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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# The Prevalence of Oral Inflammation Among Denture Wearing Patients with Chronic Obstructive Pulmonary Disease

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**Abstract**

Oral inflammation is an important contributor to the etiology of chronic obstructive pulmonary disease, which can impact patient's health status. Previous studies indicate that people with poor oral health are at higher risk for nosocomial pneumonia. Denture wearing is one promoting factor in the development of mucosal infections. Colonization of the denture plaque by Gram-negative bacteria, *Candida spp*., or other respiratory pathogens, occurring locally, may be aspirated to the lungs. The studies showed that chronic obstructive pulmonary disease (COPD) patients treated with combinations of medicines with corticosteroids more frequently suffer from *Candida*-associated denture stomatitis. Treatment of oral candidiasis in patients with COPD constitutes a therapeutic problem. Therefore, it is essential to pay attention to the condition of oral mucosal membrane and denture hygiene habits. The guidelines for care and maintenance of dentures for COPD patients are presented in this paper. The majority of patients required improvement of their prosthetic and oral hygiene. Standard oral hygiene procedures in relation to dentures, conducted for prophylaxis of stomatitis complicated by mucosal infection among immunocompromised patients, are essential to maintain healthy oral tissues. The elimination of traumatic denture action in dental office, compliance with oral and denture hygiene, proper use and storage of prosthetic appliances in a dry environment outside the oral cavity can reduce susceptibility to infection. Proper attention to hygiene, including

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brushing and rinsing the mouth, may also help prevent denture stomatitis in these patients.

**Keywords**
Denture plaque • Denture stomatitis • COPD • Oral hygiene • Oral inflammation

## 1 Introduction

Chronic obstructive pulmonary disease (COPD) is characterized by irreversible, limited flow of air through the respiratory tract, related to a chronic inflammatory process in lung vessels, destruction of lung parenchyma, and progressive morphological changes in pulmonary alveoli (Murray and Lopez 1997). COPD mainly develops in active and passive smokers or persons exposed to air pollution. The course of the disease depends on patients' general health, age, and the existence of comorbidities. COPD is currently one of the leading health issues in the world and is projected to be the third most common cause of death by 2020, one which substantially diminishes the quality of life (Zhou et al. 2011). Patients with frequent exacerbations require hospitalization and increasing financial expenditure for medical services. Infections constitute the main cause of exacerbations in the course of COPD, whereas a significant part of nosocomial pneumonias is initiated through the aspiration to the lower respiratory tract of opportunistic bacteria colonizing oral cavity and nasopharynx. Microorganisms mainly causing pneumonia in the course of COPD belong to the commensal bacterial flora colonizing the epithelium of the nasopharynx: *Haemophilus influenzae, Moraxella catarrhalis,* and *Streptococcus pneumoniae*. In patients with severe COPD exacerbations, following types of bacteria are isolated from saliva: *Pseudomonas aeruginosa, Escherichia coli,* and *Proteus mirabilis* (Sethi 2010). Chronic local and systemic inflammation related to the colonization of bacteria in the respiratory tract impairs the mucociliary clearance, leading to the destruction of pulmonary alveoli and obturation of the respiratory tract. The etiology of COPD exacerbations depends on the severity of the disease, their frequency of appearance, and the applied antibiotics. Patients with COPD belong to the group of persons with acquired immunodeficiency, who are at risk of *Candida* infections and bacteremia. Chronic local oral cavity inflammation and systemic inflammation impair the mechanisms of humoral and cell-mediated immunity, which promotes the colonization of upper and lower respiratory tract pathogens. Hospitalized patients with advanced COPD are at the highest risk of developing pneumonia and bronchitis (Murphy 2006; Didilescu et al. 2005; Scannapieco et al. 2003).

Over the past decade there has been an increased interest in the link between respiratory tract diseases and infections within oral cavity and nosopharynx (Paju and Scannapieco 2007; Scannapieco 2006; Mojon 2002). Microorganisms exist in the oral cavity not in the form of single cells, but as organized structures forming an ecological niche called biofilm. The ability to form biofilm decides about the pathogenicity of the microorganism, and in this way directly influences the soft tissues of the upper respiratory tract. Their products have been shown to stimulate mucin secretion and may result in the release of antigens, including endotoxins, lipoproteins, peptidoglycans, and other molecules enhancing the effectiveness of anti-inflammatory activities in the respiratory tract and the whole body. Previous research indicates that insufficient oral cavity hygiene contributes to the development of pneumonia. A significant part of nosocomial pneumonias is initiated through the aspiration into lower respiratory tract of bacteria colonizing oral cavity (Scannapieco 2006).

Removable prosthetic restorations create convenient conditions for the growth of bacterial and fungal microflora in the oral cavity. Broad denture plaque impedes the saliva flow and its antiseptic action, limits the ingress of oxygen to the mucous membrane epithelial cells, causes a pH decrease and an increase in temperature, as well as contributes to the build-up of food debris. Elderly patients use dentures for many years, without appearing for control visits to reline them or replace with new ones. Poor stability and retention of prosthesis related to this fact becomes a traumatogenic factor for the oral cavity mucous membrane. Among COPD patients, predominate elderly persons using removable prosthetic restorations made of acrylic materials. These materials are characterized by porosity and surface roughness, which promote the adherence of microorganisms, food debris, and the formation of biofilm of the denture plaque. Strong acrylic surface adherence is shown by *Candida albicans*, owing to the presence of phospholipases and other hydrolytic enzymes. Poor hygiene of oral cavity and prosthetic restorations promotes the build-up of bacterial-fungal plaque, which interacts directly with the mucous membrane of the prosthetic area (Sumi et al. 2002). In certain patients, denture plaque contributes to the development of prosthetic stomatitis, a chronic local inflammation of the oral cavity mucous membrane. Denture stomatitis mostly concerns removable dentures, and affects from 15 % up to 70 % of denture users. Among the risk factors for the occurrence of stomatitis are: *Candida spp* fungal infections, mechanical damage, poor oral hygiene, and round-the-clock use of dentures (Ramage et al. 2004).

COPD patients are encumbered with a high risk of bacterial and fungal infections, particularly *Candida spp*. Our previous studies indicate that multiple pathogenic species related to pneumonia have been isolated from the removable denture plaque of COPD patients. Sixteen bacterial strains responsible for exacerbations of the disease have been identified, including: *S. aureus, P. aeruginosa, E. coli, K. pneumoniae,* and *Serratia spp*. COPD patients show poorer denture hygiene and prosthetic stomatitis complicated by fungal infection, being more frequent that could be explained by the use of chronic inhalation glucocorticosteroids and home oxygen therapy in this group of patients (Przybyłowska et al. 2015).

The elimination of denture plaque, dental plaque and the reduction of bacteria and fungi adherence to the porous surface of acrylic material ought to constitute the basic measure in reducing general inflammations in the oral cavity (Abe et al. 2006; Gornitsky et al. 2002).

## 2 Guidelines for Oral and Denture Hygiene for COPD Patients

Proper daily hygiene of removable dentures is necessary for the prophylaxis and reduction of bacterial plaque adherence, food debris build-up, and the formation of tartar. Moreover, it is relevant for the maintenance of the health of gums and teeth, as well as for the elimination of mouth odor. It is advisable to clean dentures mechanically, chemically, or by combining these two methods of removing denture plaque. Removable dentures are recommended to be cleaned after each meal using mechanical methods, with a special denture brush, with the use of soap and warm running water, for 5 min (Rathee et al. 2009). For persons with hindered manual ability, it is recommended to perform hygiene procedures over a sink lined with a towel, or filled with water, to minimalize the risk of denture damage. When cleaning the prosthetic restorations, patients should also clean the remaining teeth and the tongue. Toothpastes or pastes with added abrasives should be avoided while cleaning dentures, as these may scratch the acrylic material, of which the denture is made. Ultrasound denture cleaners with various types of antiseptic liquids or detergents ought to be used in nursing homes and hospitals. Alternatively, cleaning enzymes in the tablet form, which dissolve and chemically remove the plaque biofilm, can be applied (Adachi et al. 2007). It is unacceptable to place dentures in solutions containing sodium hypochlorite for

more than 10 min, as this could damage the acrylic material. After careful cleaning, dentures ought to be stored in a dry environment to limit the ingrowth of bacteria and fungi in the porous structure (Jackson et al. 2014). For stomatitis prophylaxis, it is advisable to observe a several hours' night break in using dentures.

COPD users of removable dentures often report a burning sensation and the feeling of oral cavity dryness when inhale drugs containing glucocorticosteroids (Sjorgren et al. 2008). Moreover, broad denture plaque hinders the oxygen ingress to the mucous membrane, disturbs the balance between certain types of microorganisms, and causes more frequent inflammatory changes (prosthetic stomatitis) than in other groups of patients. In the etiology of stomatitis, a particular role belongs to *Candida spp* fungal infection. Therapeutic proceedings in denture stomatitis complicated by a fungal infection are based mainly on a combination of prosthetic and pharmacological therapy. In case of suspected fungal infection, swabs from denture plate, alternatively from the mucous membrane of the palate, cheeks or tongue are collected, which are then cultivated on Sabouraud agar under aerobic conditions. The replica method is an alternative where fungal infections cannot be confirmed due to negative mucous membrane swab test, and patients subjectively report discomfort in the oral cavity. An agar model cast from denture plate is incubated under aerobic conditions, and the cultivated *Candida albicans* colonies are located exactly in the place of abutting mucous membrane. Immunocompromised and elderly persons ought to carefully follow the guidelines for oral cavity hygiene. It is recommended to brush teeth after each meal, alternatively each morning and evening, for 3 consecutive minutes using a soft brush and fluoride toothpaste, as well as to use dental floss to clean the areas between teeth (Ramage et al. 2004). Patients with limited manual ability are recommended to use electronic or sonic toothbrushes. Patients on nebulized inhalation drugs ought to brush teeth and irrigate the mouth immediately after taking them (Godara et al. 2011). Patients at advanced disease stage and elderly persons with gums or mucous membrane inflammation are recommended to use 0.12 % chlorhexidine mouth rinse (DeRiso et al. 1996). Scannapieco et al. (2003) proposed the use of 0.20 % chlorhexidine gel twice a day in hospitalized patients, which may decrease the incidence pneumonia in intensive care patients.

In case of prosthetic restorations or retention systems supported by remaining teeth or implants, such overdentures with ball attachment, bars, and telescopic crowns, it is also recommended their surface be cleaned with dental toothpastes, special dental flosses, single-tuft toothbrushes, and oral irrigators. Elements fixed in removable overdentures ought to be cleansed with toothbrush and soap. Mouth rinses without alcohol are advisable.

## 3 Discussion

Negligence of hygiene of oral cavity and prosthetic restorations is a relevant factor predisposing to the occurrence of mucous membrane infections at the base of denture attachment. The elimination of denture and dental plaque, as well as the reduction of bacteria and fungi adhesion to the porous surface of acrylic material ought to constitute the basic manner of reducing general inflammations. Research indicates that the presence of respiratory tract pathogens in dental plaque and in dentures modulates the immunoresponse of the host, influencing the course of the basic disease (Sethi 2010; Przybyłowska et al. 2014). Adachi et al. (2007) have shown that a combination of professional mechanical and chemical oral cavity cleaning substantially reduces the number of pathogens in dental plaque of elderly persons. The adherence to oral hygiene guidelines is of notable importance, particularly in patients on inhaled steroids or other immunomodulating drugs, in order to prevent the passage of bacteria and fungi into the lower respiratory tract.

The research conducted by Liu et al. (2012) indicates that poor oral hygiene and ample dental plaque build-up increase the frequency of COPD exacerbations. Rosenblum (2010) has shown in

his metaanalysis that mechanical cleaning procedures in oral cavity may prevent death due to aspiration pneumonia, in one out of ten elderly nursing home residents. Regular seeing dentists by COPD patients wearing removable dentures allows for prophylaxis and early detection and treatment of oral cavity inflammatory states. Prophylactic dental check-ups and elimination of denture plaque during regular visits, and the adherence to every day hygiene clearly decrease the risk of COPD exacerbations and thus also enable a better control of this chronic disease.

**Conflicts of Interest** The authors declare no conflicts of interest in relation to this article.

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