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# Leukotrienes in exhaled breath condensate and fractional exhaled nitric oxide in workers exposed to $TiO_2$ nanoparticles

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## Abstract

Human health data regarding exposure to nanoparticles are extremely scarce and biomonitoring of exposure is lacking in spite of rodent pathological experimental data. Potential markers of the health-effects of engineered nanoparticles were examined in 30 workers exposed to $TiO_2$ aerosol, 22 office employees of the same plant, and 45 unexposed controls. Leukotrienes (LT) B4, C4, E4, and D4 were analysed in the exhaled breath condensate (EBC) and urine via liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS). Fractional exhaled nitric oxide (FeNO) and spirometry was also measured. The median particle number concentration of the aerosol in the production ranged from $1.98 \times 10^4$ to $2.32 \times 10^4$ particles $cm^{-3}$; about 80% of the particles were <100 nm in diameter. Median total mass concentration varied between 0.4 and 0.65 mg $m^{-3}$. All LT levels in workers' EBC were elevated relative to the controls ($p < 0.01$). LTs in the EBC sample were correlated with titanium levels. Urinary LTs were not elevated in the workers and office employees. Office workers had higher LTB4 in EBC ($p < 0.05$), and higher levels of FeNO ($p < 0.01$). FeNO was higher in office employees with allergic diseases and was negatively correlated with smoking ($p < 0.01$). In spirometry significant impairment in the workers was seen only for %VCIN and %PEF (both $p < 0.01$). Multiple regression analysis confirmed a significant association between production of $TiO_2$ and all cysteinyl LTs in EBC ($p < 0.01$) and impaired %VCIN and %PEF (both $p < 0.01$). LTB4 was also associated with smoking ($p < 0.01$). LT levels complemented our earlier findings of DNA, protein, and lipid damage in the EBC of workers with nano$TiO_2$ exposures. Cysteinyl LTs in EBC analysis suggest inflammation and potential fibrotic changes in the lungs; they may be helpful for monitoring the biological effect of (nano)$TiO_2$ on workers. Spirometry was not sensitive enough.

## Abbreviations

| | |
|---|---|
| 5-LO | 5-lipoxygenase |
| 5-HPETE | 5-hydroperoxyeicosatetraenoic acid |
| ALOX5AP | Arachidonate 5-lipoxygenase activating protein |
| 8-isoprostane | 8-isoProstaglandine F2$\alpha$ |
| APS | Aerodynamic particle sizer |
| ATS | American Thoracic Society |
| BAL | Bronchoalveolar lavage |
| DLCO | Transfer factor of the lung for CO |
| DNA | Deoxyribonucleic acid |
| EBC | Exhaled breath condensate |
| ERS | European Respiratory Society |

| | |
|---|---|
| FeNO | Fractional exhaled nitric oxide |
| FEV | Forced expiratory volume |
| FEV1 | Forced expiratory volume at 1s |
| FVC | Forced vital capacity |
| GSTM2 | Microsomal glutathione-S-transferase 2 (muscle) |
| ICP-MS | Inductively coupled plasma mass spectrometry |
| IQR | Interquartile range |
| LC-ESI-MS/MS | Liquid chromatography–electrospray ionization–tandem spectrometry |
| LOD | Limit of detection |
| LOQ | Limit of quantification |
| LSD | Least significant difference |
| LT | Leukotriene |
| NIOSH | National Institute for Occupational Safety and Health |
| PEF | Peak expiratory flow |
| PEL | Permissible exposure limit |
| PM | Particulate matter |
| PSD | Particle number size distribution |
| SMPS | Scanning mobility particle sizer |
| SPE | Solid-phase extraction |
| VCIN | Inspiratory vital capacity |

## 1. Introduction

The increasing worldwide use of nanoparticles in products is resulting in heightened exposure of both workers and consumers.

The paint and coatings industry is known to have significant particulate matter emissions into the working atmosphere. In workers exposed to $TiO_2$ white powder pigment, respiratory diseases have occasionally been described [1–3].

However, exposure levels have not been studied in detail, especially in regards to ultrafine/nano particles (particle diameters below 100 nm). This lack of information becomes alarming when considering the ability of nanoparticles to cross the barriers of the body due to their small size [4, 5]. Responsible development of any technology, including nanotechnology, requires protecting workers, the first people to be exposed to the by-products.

Both experimental and human data show that nanoparticles influence lung physiology; they may have adverse effects due to a larger surface area and higher predicted pulmonary deposition [6]. Information about physicochemical characteristics is essential when judging the pulmonary toxicity of nanoparticles [7]. For example, rats exposed to nano$TiO_2$ particles had a higher infiltration of neutrophils into the lung than those exposed to larger (submicron) particles at the same mass doses [8]. When compared on a mass basis with larger particles nano$TiO_2$ also had an enhanced oxidant capacity and a greater potential to induce pulmonary inflammation in humans [9]. In workers producing nano Fe oxide pigments, markers of oxidation of nucleic acids, proteins, and lipids were elevated in their exhaled breath condensate (EBC) [10].

Several *in vivo* rodent studies show that after inhalation air containing nanoparticles travels from the nose or mouth through the larynx and trachea into bronchi and bronchioles until reaching the alveoli. Some of the particles may be removed by mucociliary clearance in the airways. Other nanoparticles may be able to translocate the mucus layer and reach epithelial cells of the airways and underlying interstitium (with its blood and lymph vasculature). A considerable portion (about 30%) of particles sized between 10–100 nm are deposited in the alveolar region and then pass through the thin epithelial layer of the alveolar wall and cross the lung–blood barrier [11]. Nanoparticles may appear in many compartments of the body within few hours, including the liver, heart, and nervous system. Inhaled nanoparticles of $TiO_2$ have been found on the luminal side of airways and alveoli of rats, in all major lung tissue compartments and cells, and within capillaries [11, 12].

Macrophages in the tissues uptake a portion of the particles and move upward via the mucociliary escalator; if then swallowed, nanoparticles enter the gastrointestinal tract. However, the uptake by macrophages is sporadic and rather unspecific within 24 h after their deposition and witnesses for macrophages play an insufficient role in the key clearance mechanism in the peripheral lungs [12]. If not cleared by the macrophages, nanoparticles can reach the lung interstitium, from where they are transported to the local lymph nodes or reach the blood circulation [11].

Additionally, transport across the olfactory epithelium and to the brain was proven [13]. The particle uptake *in vitro* into cells occurs by diffusion or adhesive interactions [12]. Because the particles within cells are not membrane-bound they have direct access to intracellular proteins, organelles, and deoxyribonucleic acid (DNA), which supposedly greatly enhances their toxic potential.

Nano$TiO_2$-induced pulmonary toxicity and pulmonary emphysema are complicated multifactorial diseases processes.

After a single intratracheal dose of 0.1 mg nano-$TiO_2$/mouse the animals developed pulmonary emphysema, macrophage accumulation, extensive disruption of alveolar septa, and epithelial cell apoptosis. The pathological changes persisted until the second week. The changes were more severe in the higher dose of 0.5 mg/mouse. Additionally, nano$TiO_2$ induced differential expression of hundreds of genes, including activation of pathways involved in cell cycle, apoptosis, chemokines, and complement cascades. These results indicated that nano$TiO_2$ can induce severe pulmonary emphysema by activating the inflammatory pathways [14].

After mice underwent a single exposure (40 $\mu$g/50 $\mu$l) to $TiO_2$ nanomaterials (anatase and rutile) neutrophilia were found in bronchoalveolar lavage (BAL) fluid and

persisted for 7 d. In histology, inflammation of alveolar duct bifurcations was found [15]. Although inflammatory end points may not be the most useful for determining chronic diseases such as fibrosis or carcinogenesis, acute inflammation is still the most sensitive end point for toxicity rankings. In experiments with animal subjects exposed to nanoparticles the full recovery time, in regards to inflammatory changes, lasted up to six months [7].

A few studies measured lung functions and found that exposure to $TiO_2$ nanoparticles was associated with airway hyperresponsiveness, an increase in airway resistance, and a decrease of peak expiratory flow (PEF) [16]. These results are in agreement with another study regarding rats exposed by inhalation to nano$TiO_2$ [17]. Leukotriene (LT) C4 increase in the lung lavage was observed in addition to pulmonary and systemic inflammation and oxidative stress in the lung. Pathologic examination showed emphysematous changes in the lung parenchyma with alveolar wall destruction. Aerosolized nanoparticles of $TiO_2$ induced a significant rise of nitric oxide in blood and BAL fluid. These results suggest that the production of LTs, mediated by oxygen radicals, may play a critical role in the obstructive ventilatory insufficiency induced by nano$TiO_2$ [17]. Accordingly, as previously mentioned, several experimental studies reported histological emphysematous structure [14].

LTs are considered markers of inflammation. They play an active role in the pathogenesis of different respiratory disorders, such as asthma and lung fibrosis. They are primarily produced by leukocytes from arachidonic acid and are known to have very powerful effects over short distances within the body. LTs are not stored in the cells. The release of arachidonic acid from the membranes is mediated by phospholipases. Free arachidonic acid is first modified by the enzyme 5-lipoxygenase (5-LO) to 5-hydroperoxyeicosatetraenoic acid (5-HPETE), with the assistance of the arachidonate 5-lipoxygenase activating protein (ALOX5AP or FLAP), to unstable LTA4. 5-LO acts as a regulator of LT synthesis during a rate-limiting step.

LTA4 can be further metabolized by the enzyme LTC4 synthase to LTC4 after conjugation with glutathione by glutathione-S-transferase 2 (GSTM2, muscle). LTC4 is then transported out of the cells and quickly transformed to LTD4 and then LTE4. As all these molecules (LTC4, LTD4, LTE4) contain cysteine they are known collectively as cysteinyl LTs. After inhalation in an experiment cysteinyl LTs constrict human airways with a thousand times greater potency than histamine [18]. They participate in the pathogenesis of asthma and have been studied in patients with occupational asthma [19–22].

Alternatively, LTA4 can be converted by 4-hydroxylase to LTB4. LTB4 is known for its role in initiating the inflammatory response. Produced by the leucocytes and macrophages residing in the tissue in response to stimuli, such as infection or stress, $LTB_4$ potently promotes adherence to the endothelium and activation of neutrophils and other leukocytes. This way $LTB_4$ attracts and activates leukocytes and its level increases during exacerbation of chronic obstructive pulmonary disease [23] and after smoking [24].

An important feature of the 5-LO pathway is its activation. There is no LT synthesis in the resting state of normal leukocytes without this activation. Stimulation can occur by many different factors, including exposure to nano$TiO_2$ [25]. There appear to be diseases where the 5-LO pathway is constitutively activated and LTs are constantly produced, as in pulmonary fibrosis [26, 27]. LTs promote the proliferation of human bone marrow-derived fibroblasts, which can produce both cysteinyl LTs and LTB4 [28] and participate in tissue remodelling [29, 30].

Besides in the EBC, LTs can be detected in other body fluids, such as plasma and urine, using sensitive analytical methods. LTE4, the end product of biotransformation of cysteinyl LTs, has been non-invasively measured in urine [31]. Limited information is available about other LTs in urine or blood. In patients with asbestos exposure LTB4 and LTE4 levels were elevated in plasma and LTD4 levels were elevated in urine; in patients in silicosis solely LTB4 in plasma was increased. Both groups of patients with pneumoconioses had elevated LTs in the EBC [32].

The objective of this study, the fourth in a series of papers documenting increased LTs in the EBC of the same cohort of nano$TiO_2$ manufacturing workers [33–35], is to expand the spectrum of investigation to markers of inflammation in EBC and urine, fractional exhaled nitric oxide (FeNO) and spirometry, and to identify the most relevant examinations for routine biomonitoring of exposed workers. This article provides new data in chronically exposed manufacturing workers, examined in 2012 and 2013, and office employees from the same plant, examined in 2013. The study used non-invasive methods and explored their utility for biomonitoring of workers.

## 2. Methods

### 2.1. Subjects

#### *2.1.1. Workers*

The study was performed according to the following scheme: the participants were interviewed by trained physicians using a standardized questionnaire concerning personal and occupational history, medical treatments, and lifestyle habits (diet, alcohol intake, smoking, physical activity). The participants then underwent a physical examination, which was followed by the collection of their EBC and urine. Finally, FeNO and spirometry were measured.

**Table 1.** Characteristics of the groups of subjects.

| | Workers 2012 | Workers 2013 | Office 2013 | Controls 2012 | Controls 2013 |
|---|---|---|---|---|---|
| *N* | 16 | 14 | 22 | 20 | 25 |
| Age (years) | 33.31 ± 5.54 | 33.71 ± 5.24 | 44.27 ± 3.86 | 34.80 ± 4.61 | 33.72 ± 3.18 |
| Exposure (years) | 10.41 ± 4.41 | 8.93 ± 3.16 | 15.45 ± 3.62 | — | — |
| Smoking (yes/no) | 9 (62.5%) | 5 (35.7%) | 1 (4.5%) | 9 (45.0%) | 9 (36.0%) |
| Alcohol daily (yes/no) | 14 (87.5%) | 13 (92.9%) | 22 (100.0%) | 20 (100.0%) | 25 (100.0%) |
| Chronic bronchitis (yes/no) | 4 (25%) | 0 (0%) | 0 (0%) | 1 (5.0%) | 5 (25.0%) |
| Chronic rhinitis (yes/no) | 3 (18.8%) | 0 (0%) | 6 (27.3%) | 1 (5.0%) | 1 (4.0%) |
| Asthma (yes/no) | 0 (0%) | 0 (0%) | 2 (9.1%) | 0 (0%) | 0 (0%) |

To meet the inclusion criteria the subjects had to be males and the workers had to be working with $TiO_2$ for at least 6 months. Exclusion criteria for all subjects were: a history of tuberculosis, myocarditis, congenital heart disease, lung cancer, and recent fever and/or inflammation.

A total of 30 male workers were examined during 2012 and 2013. The measurements were performed after 8 h shifts in the first half of the working week. Production workers manufactured the $TiO_2$ pigment. The length of exposure in 2012 was 1–25 years and in 2013 it was 1–21 years (table 1). The workers spent about 40% of their shifts in close vicinity to the particle emitting production units in the calcination, micronisation, surface coating and filtration processes, and in the transport corridors; the remaining time they stayed in the control room separated by a closed door and checked the production lines remotely. The characteristics of the subjects are given in table 1.

#### 2.1.2. *Office employees*

In 2013 22 office employees working in the same building were examined post-shift. Their length of employment in the plant was 1–28 years. They visited the production workshops for a daily average of 0.23 ± 0.15 h (14 ± 9 min).

#### 2.1.3. *Controls*

The control subjects had comparable characteristics to the workers, as can be seen in table 1. These men were not employed in the factory; they worked as healthcare personnel and technical staff and did not handle nanomaterial or dusts/aerosols. The controls only gave samples once, half of them in the morning and half in the afternoon.

### 2.2. Ethics statement

The study was carried out according to the Helsinki Declaration. The Ethical Committee of the 1st Medical Faculty, Charles University in Prague approved the study. All participants were informed about the aim of the study and they signed the informed consent before the beginning of the study.

### 2.3. Lung function testing

Pulmonary function testing was performed by a SpiroPro, Jaeger, Germany. The measurements were carried out according to standard protocols of the American Thoracic Society (ATS) Guidelines [36] and the results were expressed as a % predictive value. The best of three consecutive measurements was chosen. The measurement included forced vital capacity (FVC), inspiratory vital capacity (VCIN), peak expiratory flow (PEF), forced expiratory volume (FEV), and FEV in 1 s (FEV1). FEV1 and FVC were used to measure the FEV1/FVC ratio. The parameters were considered low if they were less than 80% of the predicted values and if the FEV1/FVC ratio was less than 0.70.

### 2.4. Fractional exhaled nitric oxide (FeNO)

Prior to spirometry FeNO was measured by a portable Hypair FeNO, Medisoft, Belgium, analyser according to ATS/ERS recommendations [37]. A FeNO result of more than 19 ppb was considered positive.

### 2.5. Exhaled breath condensate (EBC) and urine collection and analysis

The post-shift EBC samples were collected using an Ecoscreen Turbo DECCS (Jaeger, Germany), equipped with a filter. All subjects breathed tidally through a mouthpiece connected to the condenser (−20 °C) while wearing a nose-clip. A constant collection time of 15 min and/or minimum volume of 120 l of exhaled air were maintained [38]. Immediately after the sample collection the volume was measured and 250 pg of LTE4-d3 was added to 1 ml of EBC as deuterium-labelled internal standard (stable-isotope-dilution assay). All the subjects also gave spot urine samples.

All samples were immediately frozen and stored at −80 °C until analysis. Analyses of LTs LTB4, LTC4, LTD4, and LTE4 were performed as previously described [39, 40]. Briefly, the method consists of a pre-treatment step, solid-phase extraction (SPE) for rapid and effective isolation of biomarkers from the biological matrices (EBC and urine), and

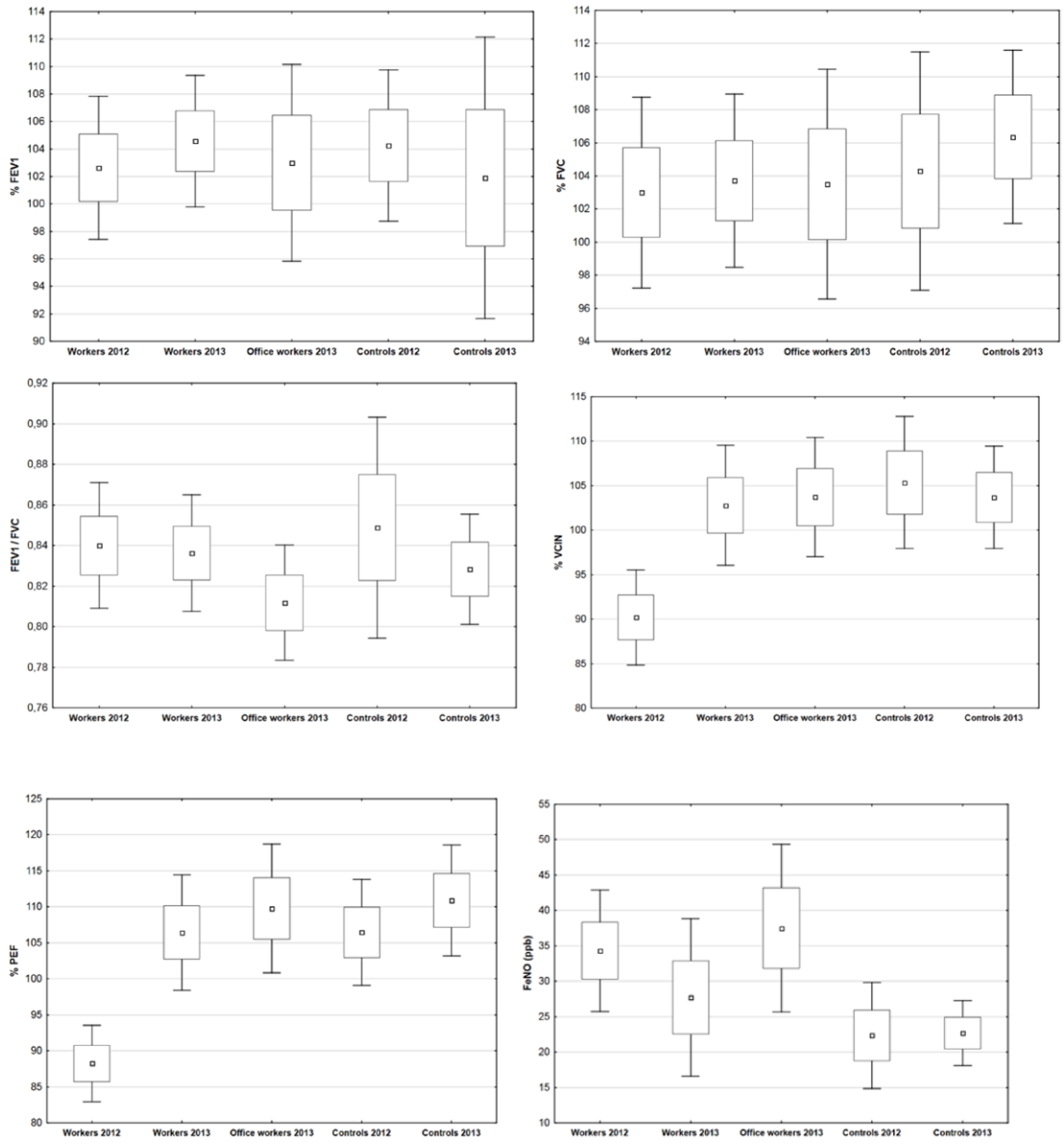


**Figure 1.** Lung functions and fractioned exhaled NO (FeNO) in the groups of workers, office employees, and controls. Mean (point), standard error of the mean (SEM, box), and 95%CI of the mean (whiskers) are presented. % = % predicted.

a detection method using liquid chromatography–electrospray ionization–tandem mass spectrometry (LC-ESI-MS/MS). Contamination of the EBC with saliva eicosanoids and aldehydes was excluded by measuring the concentration of $\alpha$-amylase (UV–VIS absorption spectroscopy). Additionally, the pH of EBC sample was measured [41].

Quantitative analyses of titanium were conducted by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7900 ICP-MS Ultra HMI, equipped with MassHunter software and autosampler ASX-520. Before measurement the liquid samples were evaporated to dryness and mineralized with a mixture of HF and $HNO_3$ (1:3, v/v) in a UniClever microwave decomposition unit (Plazmatronika-Service, Wroclaw, Poland). The method was validated and used for quantitative measurements. The method limit of detection (LOD) was $1.2 \pm 0.2\ \mu g\,l^{-1}$ and the method limit of quantification (LOQ) was $4.0 \pm 0.2\ \mu g\,l^{-1}$, which are three and ten times, respectively, the standard deviation of the mean noise signal of five blank samples. The standard error was determined to be 3.0%. Below the limit of detection titanium values in sample were substituted with LOD/$\sqrt{2}$.

### 2.6. Aerosol measurements

$TiO_2$ pigment (both anatase and rutile form) in the plant is produced from titanium mineral ilmenite by the sulphate process. After the reaction with sulphuric acid titanium hydroxide is precipitated by hydrolysis, filtered, and calcined. During calcination the material is heated to 800–1000 °C and the anatase/rutile crystals are formed. In the finishing operations the crude form of the pigment is milled (micronisation) to produce a controlled distribution of particle size and the surface is treated to improve its functional behaviour. Pilot measurements were carried out for mapping and localisation of the main sources of aerosol particles

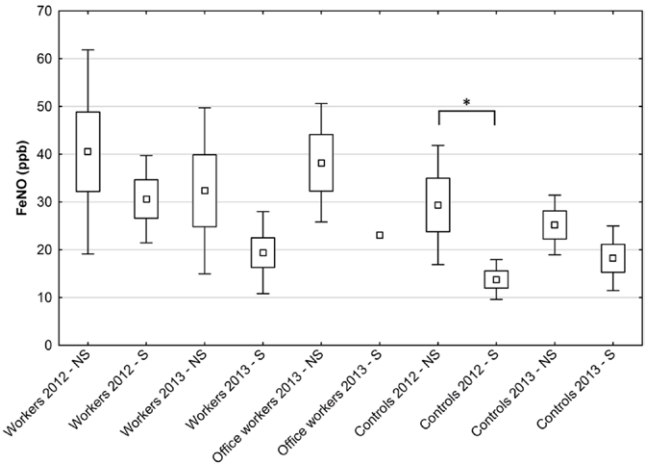


**Figure 2.** Fractional exhaled nitric oxide (FeNO) in the subgroups of non-smokers and smokers in the workers, office employees and controls. Mean (point), standard error of the mean (SEM, box), and 95%CI of the mean (whiskers) are presented. % = % predicted, NS = non-smokers, S = smoker, % = % predicted, $*p < 0.05$.

using a portable particle number concentration monitor P-TRAK, and a portable monitor of particle mass concentrations, DustTRAK DRX (both TSI Inc, USA). The dynamics of aerosol particle number size distributions (PSD) in the workplace were monitored by a scanning mobility particle sizer (SMPS), model 3936L (TSI Inc, USA), as well as an aerodynamic particle sizer (APS), model 3321 (TSI Inc, USA). During the shifts the measurements of highly time- and size-resolved aerosol concentrations were carried out. More detailed descriptions were given in other publications [34, 35].

### 2.7. Statistical evaluation

Basic descriptive statistics (mean, median, CI, SD, skewness, and kurtosis) were computed for all variables, which were subsequently tested for normality using the Kolmogorov–Smirnov test. A $\chi^2$ test was used to compare frequency counts of demographic categorical variables (smoking and alcohol consumption) in the groups of production workers, office employees, and controls. Differences in interval demographic variables were tested using a one-way analysis of variance and independent-groups *t* test, respectively.

One-way analysis of variance with least significant difference (LSD) post hoc tests was used to compare the concentration of titanium, FeNO, and LTs in EBC and urine. The bivariate relationship was assessed using a Spearman correlation coefficient. Multiple regression analysis was used to predict markers of inflammation studied by a set of predictors (exposure in production of $TiO_2$: yes/no, age, smoking: yes/no, alcohol consumption: yes/no). Statistical significance was set at $p < 0.05$. All analyses were conducted using SPSS V.22.0 (SPSS, Inc., Chicago, IL, USA). One-way analysis of variance with LSD post hoc tests were used to compare the concentration of titanium, FeNO, and LTs in EBC and urine, while adjusting for possible confounders (smoking, alcohol, age).

### 2.8. Results

The characteristics of the groups of subjects, such as age, length of exposure, smoking, and respiratory diagnoses in the group of workers producing $TiO_2$ pigment, in the office employees and controls are shown in table 1. The office employees were older than both the workers and controls ($p < 0.05$). Also, their occupational exposure in this factory was longer ($p < 0.05$) and the proportion of smokers in this subgroup was small (one employee only).

The results of the pulmonary function examination (%FEV1, %FVC, FEV1/FVC, %VCIN, %PEF) are shown in figure 1. As can be seen in this figure, the worst finding and the only significant impairment was seen in production workers in 2012 for %VCIN and %PEF (both $p < 0.01$) when compared to the groups of subjects not involved in the $TiO_2$ production. All four workers from this group who had lung function parameters below the lower limit of the normal range were smokers. There was one worker with impaired %VCIN (working in surface coating), two with %PEF (calcination, filtration) and one with both %VCIN and %PEF (calcination). This worker was employed for 25 years in the plant; the mean occupational exposure of the workers with impaired %VCIN was 15.5 years, for the worker with lower %PEF it was 12.7 years. Other parameters, such as %FEV1, %FVC, and FEV1/FVC, did not differ among the groups.

The highest FeNO level was seen in the office employees ($p < 0.01$), as presented in figure 1. This group contained only one smoker (4.5% subjects); in addition eight subjects had allergic diseases. Mean FeNO in these office employees was 48.4 ± 45.9 ppb, while in the rest of the group it was 32.4 ± 9.1 ppb. The only one smoker with allergic rhinitis had 23 ppb. FeNO in $TiO_2$ exposed workers did not differ from the controls.

The distribution of FeNO in the subgroups of non-smokers and smokers is presented in figure 2. There was a trend towards lower FeNO in smokers

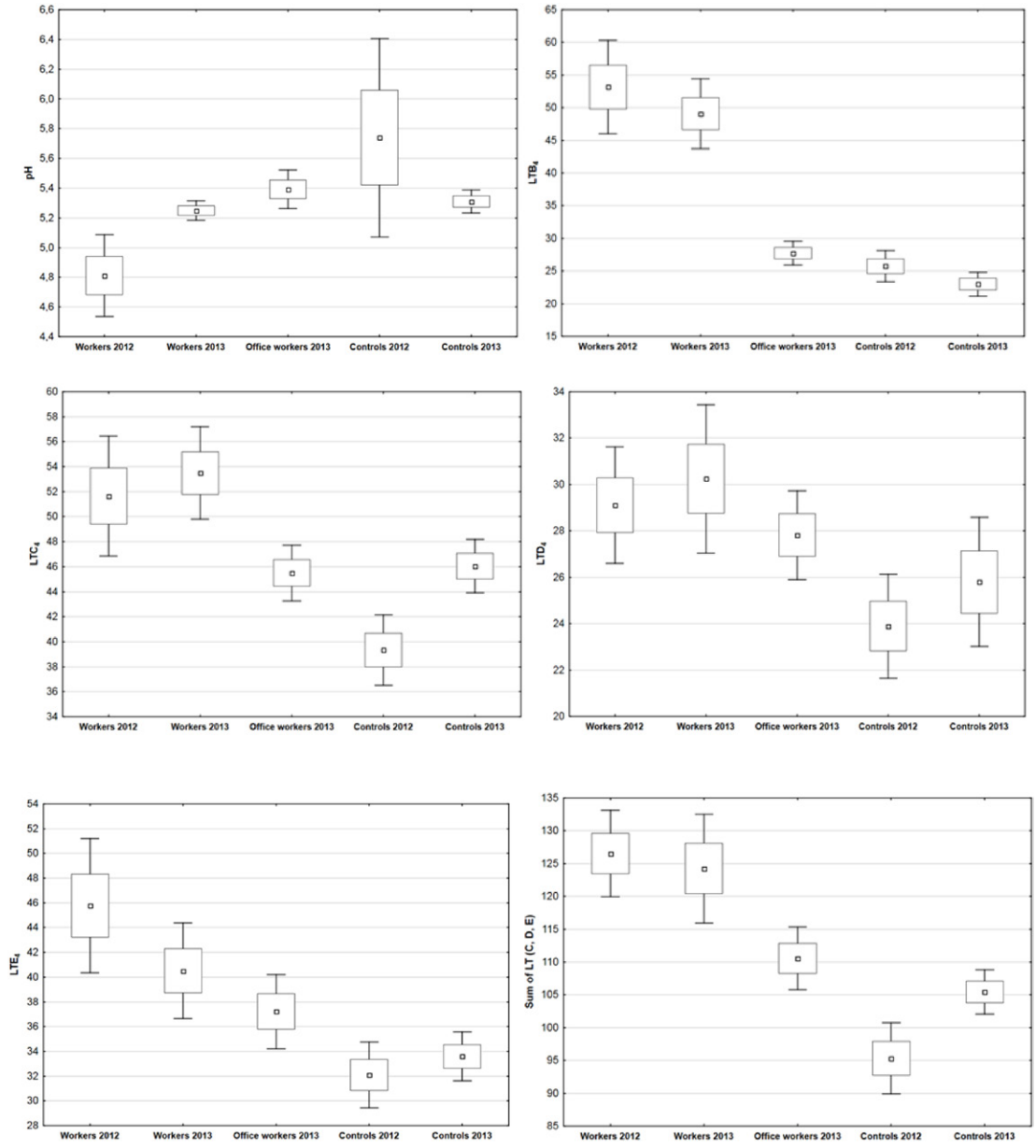


**Figure 3.** pH and leukotrienes (pg ml$^{-1}$) in the exhaled breath condensate in the groups of workers, office employees and controls. Mean (point), standard error of the mean (SEM, box), and 95%CI of the mean (whiskers) are presented. % = % predicted; LT = leukotriene.

in all groups of subjects, however, a significant difference between non-smokers and smokers was found only in the group of the control subjects in 2012 ($p < 0.05$).

The proportion of subjects with FeNO above 19 ppb was higher both in the workers (73.3%) and office employees (90.1%) when compared to control subjects (48.9%), $p = 0.035$ and $p = 0.001$ respectively.

Figure 3 presents the pH and LTs in the exhaled breath condensate in the groups of workers, office employees, and controls. Production workers in 2012 had the lowest EBC pH; pH did not differ from the workers in 2013 ($p = 0.092$), but was lower than both in office employees and controls ($p < 0.01$).

Production workers in both years had significantly elevated LTB4 ($p < 0.001$), LTC4 ($p < 0.01$), LTD4 ($p < 0.05$), LTE4 ($p < 0.001$), and the sum of cysteinyl leukotrienes ($p < 0.001$) in their EBC when compared to the controls. The office employees only had elevated LTB4 ($p < 0.05$) when compared to the controls.

All LTs in EBC correlated with other LTs in EBC, both with LTB4 and with cysteinyl LTs, as can be seen in table 2.

Titanium concentrations did not differ in post-shift EBC samples of the workers in 2012, which were 24.1 ± 1.78 $\mu$g l$^{-1}$ and 20.00 ± 2.21 $\mu$g l$^{-1}$ in 2013, respectively. The levels in the office employees, 0.14 ± 0.08 $\mu$g l$^{-1}$, and in controls, 1.12 ± 0.04 $\mu$g l$^{-1}$, were significantly lower (both $p < 0.001$).

On the other hand, LTs in the urine were not elevated in the workers and office employees when compared to the control subjects, as can be seen in table 3.

Impairment in two pulmonary functions (%VCIN and %PEF) correlated with LTs in EBC: %VCIN with

**Table 2.** Correlations of titanium, selected pulmonary function parameters, FeNO, and leukotrienes in exhaled breath condensate (EBC) in all subjects.

| | Ti | %VCIN | %PEF | pH | FeNO | LTB4 | LTC4 | LTD4 | LTE4 | Cys LT |
|---|---|---|---|---|---|---|---|---|---|---|
| Ti | 1 | | | | | | | | | |
| %VCIN | −0.259[a] | 1 | | | | | | | | |
| %PEF | −0.341[c] | 0.466[c] | 1 | | | | | | | |
| pH | −0.296[b] | 0.086 | 0.073 | 1 | | | | | | |
| FeNO | 0.138 | −0.204[a] | −0.038 | −0.165 | 1 | | | | | |
| LTB4 | 0.758[c] | −0.256[a] | −0.223[a] | −0.214[a] | 0.216[a] | 1 | | | | |
| LTC4 | 0.480[c] | −0.168 | −0.117 | −0.089 | 0.147 | 0.530[c] | 1 | | | |
| LTD4 | 0.296[b] | 0.025 | 0.001 | −0.021 | 0.008 | 0.329[b] | 0.328[b] | 1 | | |
| LTE4 | 0.508[c] | −0.245[a] | −0.217[a] | −0.149 | 0.203[a] | 0.479[c] | 0.324[b] | 0.249[a] | 1 | |
| Cys LT | 0.632[c] | −0.237[a] | −0.186 | −0.148 | 0.254[a] | 0.646[c] | 0.736[c] | 0.612[c] | 0.760[c] | 1 |

[a] ($p < 0.05$).
[b] ($p < 0.01$).
[c] ($p < 0.001$).
*Note.* VCIN = inspiratory vital capacity, PEF = peak expiratory flow, % = percent predicted, FeNO = fractional exhaled nitric oxide, LTB4 = leukotriene B4, LTC4 = leukotriene C4, LTD4 = leukotriene D4, LTE4 = leukotriene E4, Cys LT = cysteinyl leukotrienes.

**Table 3.** Leukotrienes in the urine of the workers, office employees and controls.

| | U LTB4 | U LTC4 | U LTD4 | U LTE4 |
|---|---|---|---|---|
| | ng $g^{-1}$ creat. | ng $g^{-1}$ creat. | ng $g^{-1}$ creat. | ng $g^{-1}$ creat. |
| Workers | 286 ± 68 | 51 ± 14 | 45 ± 8,6 | 50 ± 16 |
| Office | 330 ± 110 | 93 ± 27 | 91 ± 28 | 102 ± 37 |
| Controls | 330 ± 170 | 104 ± 52 | 103 ± 54 | 116 ± 63 |

*Note.* U = urinary, creat. = creatinine, LTB4 = leukotriene B4, LTC4 = leukotriene C4, LTD4 = leukotriene D4, LTE4 = leukotriene E4.

LTB4, LTE4 and cysteinyl LTs, as shown in table 2. LTB4 was negatively correlated with EBC pH.

Similarly, Ti concentration in the EBC correlated with a lowering of %VCIN, %PEF, and with negative EBC pH. It also positively correlated with all EBC LTs, as shown in table 2.

FeNO positively correlated with age, LTB4, LTE4, and cysteinyl LTs in the entire group of subjects. FeNO was correlated with a lowering of %VCIN ($p < 0.05$), but was also negatively correlated with both smoking ($p < 0.001$) and daily alcohol use ($p < 0.05$).

The length of exposure was correlated with LTB4 ($p < 0.05$). There was no correlation of LTs with the respiratory diseases (chronic bronchitis, acute or chronic rhinitis, asthma) or systemic disorders (hypertension, increased cholesterol, cancer, diabetes) in the workers and controls.

No positive correlation was seen between LTs and the age, smoking, or alcohol drinking habits in any groups of subjects.

Multiple regression analysis of nano$TiO_2$ exposure, age, smoking, and daily alcohol consumption confirmed a significant association between occupational exposure to (nano)$TiO_2$ and EBC level of all LTs, FeNO, and impairment of %VCIN and %PEF, as shown in table 4. For all cysteinyl LTs occupational exposure was the only significant parameter, for LTB4 smoking was also associated. FeNO was also negatively associated with smoking and daily alcohol use (both $p < 0.01$).

### 2.9. Workplace area sampling and $TiO_2$ aerosol measurements

The results of the aerosol measurements were given in detail in our previous papers on the same group of production workers [34, 35]. In the workshops the median total mass $TiO_2$ concentrations in 2012 and 2013 were 0.65 and 0.40 mg $m^{-3}$, respectively. The median number of concentrations measured by SMPS and APS were $1.98 \times 10^4$ and $2.32 \times 10^4$ particles $cm^{-3}$, respectively. Importantly, about 80% of those particles were smaller than 100 nm in diameter [35]. The mass concentration did not exceed the permitted exposure level of 10 mg $m^{-3}$ for bulk $TiO_2$.

## 3. Discussion

To our knowledge, this is the first study evaluating LTs in the EBC of workers exposed to engineered nanoparticles. This study found all LTs significantly elevated in the workers producing white $TiO_2$ pigment containing anatase and rutile. Among them, the results were more robust for cysteinyl LTs than for LTB4.

The elevations of LTs and 8-isoprostane have previously been found in the EBC of subjects with asbestos- [42, 43] and silica-induced diseases [32, 39, 42–44]. In the patients with asbestosis the EBC level of cysteinyl LTs correlated with the degree of restrictive lung function impairment (specifically TLC) and transfer factor of the lung for CO (D).

**Table 4.** Multiple regression analysis (95% CI) of $TiO_2$ exposure, age, smoking, alcohol daily, and leukotrienes in the exhaled breath condensate.

| | LTB4 | LTC4 | LTD4 | LTE4 | Cys LT | FeNO | %VCIN | %PEF |
|---|---|---|---|---|---|---|---|---|
| $TiO_2$ exposure (yes/no) | 17.93[c] (13.86, 22.00) | 6.80[c] (3.86, 9.75) | 3.39[b] (1.07, 5.71) | 8.01[c] (4.89, 1.14) | 18.21[c] (12.64, 23.77) | 9.06[a] (1.75, 16.38) | −6,89[a] (−13.01, −0.78) | −8.46[a] (−15.50, −1.42) |
| Age (years) | −0.42[c] (−0.61, −0.23) | −0.13 (−0.27, −0.01) | −0.00 (−0.10, 0.11) | −0.02 (−0.16, 0.13) | −0.14 (−0.40, 0.12) | 0.22 (−0.11, 0.56) | 0.29[a] (0.01, 0.57) | 0.63[c] (0.31, 0.96) |
| Smoking (yes/no) | 8.21[c] (4.17, 12.26) | 2.66 (−0.26, 5.58) | −0.16 (−2.46, 2.15) | −0.87 (−3.97, 2.24) | 1.64 (−3.89, 7.17) | −10.92[b] (−18.18, −3.65) | 0.97 (−5.11, 7.05) | −6.35 (−13.35, 0.65) |
| Alcohol (yes/no) | −9.24 (−22.85, 4.37) | 5.31 (−4.52, 15.14) | 0.88 (−6.87, 8.46) | −5.97 (−16.41, 4.48) | 0.22 (−18.38, 18.83) | −39.35[b] (−63.80, −14.90) | −2.00 (−22.45, 18.45) | 14.66 (−8.89, 38.20) |

[a] ($p < 0.05$).
[b] ($p < 0.01$).
[c] ($p < 0.001$).
*Note*. LTB4 = leukotriene B4, LTC4 = leukotriene C4, LTD4 = leukotriene D4, LTE4 = leukotriene E4, Cys LT = cysteinyl leukotrienes, FeNO = fractional exhaled nitric oxide.

Urinary LTE4 is considered to be the most reliable analytic parameter for monitoring the endogenous synthesis of cysteinyl LTs in the urine. Unlike in the EBC, LTs in urine were not increased in this study, which supports the theory of local effect in the respiratory tract, but not the systemic effect.

These results complete our previous findings. In our pilot study from the same plant in 2012 (20 production and research $TiO_2$ workers) LTs were already elevated before the shift when compared to the controls; all LTs were elevated also post-shift [33]. Accordingly, $TiO_2$ particles were already found in the pre-shift EBC samples of these workers using Raman microspectroscopy [34]. This pre-shift elevation of the inflammation markers studied may point to a subacute or chronic influence.

FeNO in the workers was not increased, probably due to the negative interference with smoking and alcohol intake, however it was also associated with occupational exposure in $TiO_2$ production, as was shown by the multiple regression analysis.

In the low-exposure office employees, who visited the production workshops for average of 14 min daily, the increase of LTs was not found. Only FeNO elevation was seen when compared to controls, which can be partially explained by the lowest proportion of smokers (4.5%) in this group of subjects [45], but also by allergic diseases in the group of office employees.

In our study the impairment of the lung functions was very mild; it concerned only PEF and VCIN in the workers with longer than average occupational exposure. Of course, these findings cannot be overestimated, as both of them depend on the good compliance of the subjects. Due to their correlation with LTs and association with exposure in multifactor analysis, they may represent initial functional impairments of the workers.

Lowering of PEF, FEV1, and FEV1/FVC was observed in a study in highly exposed nanoscale carbon black-exposed workers when compared to the control group, but no subject was diagnosed with chronic obstructive pulmonary disease or asthma [46]. Workers manufacturing multi-walled carbon nanotubes had normal lung function parameters, though they had elevated markers of oxidation of the lipids in the EBC, which shows the lower sensitivity of spirometry [47].

One study focused on FeNO measurement in workers from a large spectrum of nanomaterials handling plants. The multiple regression analysis proved the association of FeNO with a higher risk level of exposure [48]. Another study found a decrease of antioxidant enzymes and higher cardiovascular markers in workers handling nanomaterials [49]. It was also shown that nanoparticles from photocopiers and other engineered nanomaterials induced oxidative stress and upper respiratory tract inflammation in healthy volunteers [50–53].

Until now the human studies are very limited [54]. There is an urgent need for a global assessment of how well workers are being protected from potentially harmful exposures [55]. Nanoparticle products are becoming more and more prevalent in the market place; their potential harm to humans, especially workers, should be the focus of intense research.

The clinical studies are in agreement with the experimental results, however, the significance of the elevation of LTs for the workers is difficult to predict.

Increased LTs were found in patients with pulmonary fibrosis; it concerned both LTB4 and cysteinyl LTs in the lungs in the lung biopsy specimens [19, 26, 56]. In the patients with diseases caused by carcinogenic dusts asbestos [32, 42, 57] and silica [43] who had high LT EBC levels, no correlation with systemic diseases was found [58]. Additionally, LTB4 has been found to be overproduced in ulcerative colitis, Crohn's disease, inflammatory bowel disease, rheumatoid arthritis, atherosclerosis, and cancer [27, 59].

As part of the possible mechanisms within the systemic inflammatory response nanoparticles are known to cause an increase in lipid peroxidation, antioxidant activity, and cellular oxidative stress. LTs are thought to be important contributors to lung pathologies [14, 60]. Induction of LTC4 formation by microsomal glutathione-S-transferase 2 (MGST2) was delineated to be a major mediator of oxidative stress and oxidative DNA damage [61]. This enzyme has also been implicated in several major human pathologies, including metabolic diseases, neurodegeneration, and osteoporosis. Therefore, inhibition of the activation by LTC4 receptor antagonists, already serving as approved asthma drugs, may have broad clinical significance [61].

The link between high LTs and oxidative stress is in agreement with our results in this group of nano-$TiO_2$ workers, who had elevated markers of oxidation of nucleic acids and proteins [35] and LTs in the EBC. Markers of oxidative stress were found also in the workers exposed to nano Fe oxides [10].

All of these findings suggest that lowering exposure may reduce these biological markers of oxidative stress and inflammation. The question of the persistence of the elevation of these markers needs to be answered by further studies. The biological half-times of the $TiO_2$ nanoparticles increased when the dose was increased [62].

The prognosis of the findings in the workers is difficult to estimate, as only a few experimental studies focused on chronic changes. Transient inflammation and the upregulation of chemokines in the bronchoalveolar lavage fluid were observed after a single intratracheal dose for one month; nanoparticles were seen in the cells after six months [63]. Ninety days' exposure of nano-$TiO_2$ in the dose of 2.5–10 mg $kg^{-1}$ led to significant increases in inflammatory cells, production of reactive oxygen species, and peroxidation of lipid, protein and DNA in mouse lung tissue. Nano$TiO_2$ deposition in lung tissue led to severe pulmonary inflammation and apoptosis. Additionally, significant alterations in the expression of genes in the nano$TiO_2$-exposed lung tissues were found; of 521 genes with known

functions, 361 were up-regulated and 160 were down-regulated, which was associated with the immune and inflammatory responses, apoptosis, oxidative stress, the cell cycle, stress responses, and cell proliferation. Up-regulated genes concerned the function of ALOX5AP, activating the LT production [25], which is in accordance with our findings of higher concentrations of LTs in the EBC of the workers.

### 3.1. Limitations

LTs in EBC and FeNO prove the inflammation exists in the respiratory tract; of course they are not specific about the effect of the nanoparticles. The number of workers examined is small; however, the sizes of teams of workers highly exposed to nanoparticles are limited, as are the studies in workers. The office employees' group had the highest FeNO level due to the highest proportion of subjects with allergic diseases. In addition, FeNO was relatively lower in the smokers due to diminished NO production [45].

## 4. Conclusions

This is the first study concerning workers exposed to engineered nanoparticles using LTs in the EBC as a potential marker of the effect of exposure. Significantly elevated LTB4 and cysteinyl LTs were found in the post-shift EBC, but not in the post-shift urine. The results are in agreement with experimental studies and with studies using markers of oxidative stress, i.e. oxidative products of nucleic acids and proteins in the same group of (nano)$TiO_2$ workers [35].

Subjective symptoms were not present in the workers. They did not complain of more respiratory and/or allergy symptoms than the office employees and control subjects.

Pulmonary testing was not sensitive enough, especially for smokers. A mild lowering of VCIN% and PEF% in the production workers was found in the first 2012 study only. This is in agreement with the rare human studies published until now [54]. On the other hand, both cysteinyl LTs and LTB4 were significantly elevated in the workers during both 2012 and 2013. Cysteinyl LTs appear more robust for the examination of the workers, as LTB4 may be influenced by smoking and by the age of the subjects.

FeNO elevation was seen in the office employees only, which relates to the lowest proportion of smokers in this group of subjects. The suppressing influence of smoking (although not significant) was seen in all the groups of subjects. This interfering effect makes FeNO measurement unreliable for the biological monitoring of the workers.

Aerosol levels at this workplace did not exceed the permissible exposure limits (PEL) of 10 mg $m^{-3}$ for bulk $TiO_2$, which suggests that this limit is not appropriate for nano$TiO_2$; a recommended exposure limit of 0.3 mg $m^{-3}$ would better protect the workers [64].

Non-invasive measurement of cysteinyl LTs in the EBC appears to be the method of choice for monitoring exposure to engineered nanoparticles at the workplace, and may be added to other suggested markers measured in the EBC, especially markers of oxidation of nucleic acids and proteins [35] and markers of oxidation of lipids, which were elevated in nano Fe oxide-producing pigments [10].

## Conflict of interest

The authors claim no conflict of interest.

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