Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO2 production workers

Markers of lipid oxidative damage in the exhaled breath condensate of nano TiO_2 production workers

Daniela Pelclova[1], Vladimir Zdimal[2], Petr Kacer[3], Nadezda Zikova[2], Martin Komarc[4,5], Zdenka Fenclova[1] Stepanka Vlckova[1], Jaroslav Schwarz[2], Otakar Makeš[2], Kamila Syslova[3], Tomas Navratil[6], Francesco Turci[7], Ingrid Corazzari[7], Sergey Zakharov[1], Dhimiter Bello[8]

1 *Charles University in Prague and General University Hospital in Prague, First Faculty of Medicine, Department of Occupational Medicine, Prague, Czech Republic*
2 *Institute of Chemical Process Fundamentals of the CAS, v.v.i., Prague, Czech Republic*
3 *Institute of Chemical Technology Prague, Czech Republic*
4 *Charles University in Prague, Faculty of Physical Education and Sport, Department of Methodology, Prague, Czech Republic*
5 *Charles University in Prague and General University Hospital in Prague, First Faculty of Medicine, Institute of Informatics, Prague, Czech Republic*
6 *J. Heyrovský Institute of Physical Chemistry of the CAS, v.v.i., Prague, Czech Republic*
7 *Department of Chemistry, "G. Scansetti" Interdepartmental Centre, and NIS Interdepartmental Centre, University of Torino, Torino, Italy*
8 *UMass Lowell, Department of Public Health, College of Health Sciences, Lowell, MA, USA*

Corresponding author: Daniela Pelclova, daniela@.pelclova.cz

Keywords

Nanoparticles; TiO_2; Exhaled breath condensate; Aldehydes; Oxidative stress; Occupational exposure; Monitoring.

ABSTRACT

Nanoscale titanium dioxide (nanoTiO_2) is a commercially important nanomaterial. Animal studies have documented lung injury and inflammation, oxidative stress, cytotoxicity and genotoxicity. Yet human health data are scarce and quantitative risk assessments and biomonitoring of exposure are lacking. NanoTiO_2 is classified by IARC as a group 2B, possible human carcinogen.

In our earlier studies we documented an increase in markers of inflammation, as well as DNA and protein oxidative damage, in exhaled breath condensate (EBC) of workers exposed nanoTiO_2. This study focuses on biomarkers of lipid oxidation.

Several established lipid oxidative markers (malondialdehyde, 4-hydroxy-trans-hexenal, 4-hydroxy-trans-nonenal, 8-isoProstaglandin F2α and aldehydes C_6-C_{12}) were studied in EBC and urine of 34 workers and 45 comparable controls. The median particle number concentration in the production line ranged from $1.98x10^4$ to $2.32x10^4$ particles/cm^3 with ~80% of the particles <100 nm in diameter. Mass concentration varied between 0.40-0.65 mg/m^3. All 11 markers of lipid oxidation were elevated in production workers relative to the controls ($p<0.001$). A significant dose-dependent association was found between exposure to TiO_2 and markers of lipid oxidation in the EBC. These markers were not elevated in the urine samples.

Lipid oxidation in the EBC of workers exposed to (nano)TiO_2 complements our earlier findings on DNA and protein damage. These results are consistent with the oxidative stress hypothesis and suggest lung injury at the molecular level. Further studies should focus on clinical markers of potential disease progression. EBC has reemerged as a sensitive technique for non-invasive monitoring of workers exposed to engineered nanoparticles.

Introduction

Titanium dioxide (TiO_2, CAS No. 13436-67-7) is a white, non-combustible, odorless and poorly soluble powder, that is widely used as a white pigment because of its brightness and very high refractive index. Furthermore, TiO_2 is permitted for use as an additive (E171) in food. NanoTiO_2 is used in numerous applications, including coatings, antibacterial sprays, composite nanofillers, copy toners, printing inks, and cosmetics, as well as in the pharmaceutical industry (Weir et al. 2012, Warheit et al. 2013, Khatri et al. 2013a, Pirela et al. 2015). TiO_2 is a high-volume material, with nano-sized TiO_2 material being increasingly used in many applications. The 2010 estimated production was anywhere from 10,000 to 88,000 metric tons (Lazareva and Keller 2014). TiO_2 is capable of generating free radicals in experimental studies (Toyokuni et al. 2008,).

The physicochemical properties of engineered nanoparticles differ significantly from those of coarse particles of the same composition due to their much larger specific surface area and surface activity *in vitro* (Hsieh et al. 2013), their much higher deposition rate in the respiratory system of animals, longer retention in the lungs, and reduced clearance by macrophages in comparison with the larger particles (Kreyling et al. 2013, Silva et al. 2013). Experimental studies have reported various biological effects of TiO_2 nanoparticles in the respiratory system, including generation of oxidative stress, pro-inflammatory effects and possible development of fibrosis and/or cancer (Shi et al. 2013; NIOSH 2011).

There is increasing experimental evidence that persistent oxidative damage due to nanoTiO_2 occurs to lipids in cellular membranes, proteins, and nucleic acids (Møller et al. 2010, Chang et al. 2013). The *in vitro* well documented relation of oxidative damage biomolecules, such as aldehydes to oxidative stress and upregulation of cell proliferation and cancer is well established (Toyukuni et al. 2008). Hsieh et al. (2013) and Pal et al. (2014) provide a detailed physico-chemical characterization of a large number of commercially important nanoTiO_2 samples, as well as evidence of nanoTiO_2 induced oxidative damage in human serum, measured via a clinically relevant assay (ferric reducing ability of serum or FRAS) and in induced macrophage like THP-1 cells (monitoring the ratio of oxidized vs. reduced glutathione). It is important to note here that such oxidative damage happened in the absence of UV light.

NanoTiO_2 is classified by IARC as a group 2B, possible human carcinogen (IARC 2010) and obviously, a primary area of concern is their potential adverse impact on workers, since they are likely exposed at much higher concentrations than the general public (Liao et al. 2014, Liou et al. 2015).

Oxidative products include several aldehydes, such as malondialdehyde (MDA), 4-hydroxy-trans-hexenal (HHE), and 4-hydroxy-trans-nonenal (HNE), which may be measured in biological fluids or exhaled breath condensate (EBC) (Syslova et al. 2009, Gong et al.

2013). The volatile oxidation products (such as aldehydes) are exhaled as vapors, whereas the non-volatile compounds are released from the airway lining fluid in the form of aerosolized particles. Their formation in the respiratory tract has been attributed either to turbulent airflow or by a process of bubble bursting during opening of the bronchioles following exhalation (Horvath et al. 2005).

Linear aldehydes, such as C_6, C_7, and C_9, are the end metabolites of the lipid peroxidation process of ω-3 and ω-6 fatty acids, which are principal components of phospholipids that form a substantial portion of cell membranes (Huang et al. 2014). Concentration of C_6, C_8, and C_9 are significantly higher in the exhaled air of lung cancer patients than in smokers and healthy controls (Jareno et al. 2012). It has been hypothesized that they might be used as a non-invasive probe for lung malignancies (Fuchs et al. 2010). Antioxidant supplementation, anti-diabetic treatments, weight loss, and a decrease in daily caloric intake have been shown to decrease their formation (Roberts et al. 2007).

Another commonly used marker of lipid oxidation is 8-isoProstaglandin $F_{2\alpha}$ (8-isoprostane), an isoprostane isomer, formed from the free radical oxidation of arachidonic acid from phospholipid membranes. 8-isoprostane causes vasoconstriction of blood vessels and bronchi, lowers blood flow in the kidneys, and participates in the pathology of several diseases, such as lung diseases, atherosclerosis, and diabetes (Syslova et al. 2014, Czerska et al. 2016).

Based on aforementioned experimental data and mechanistic understanding of nanoTiO_2 toxicity, it was hypothesized that inhalation of nanoTiO_2 will sustain high levels of oxidative damage in the lungs of workers, which could be measured noninvasively in their EBC condensate. This hypothesis is further supported by a large study of workers exposed to different types of engineered nanoparticles in 14 nano manufacturing plants, which documented a suppression of antioxidant enzymes (Liou et al. 2012, Liao et al. 2014). Furthermore, markers of oxidative stress were found in copier operators, who are exposed exclusively to nanoparticles, which also contain small amounts of nanoTiO_2 from toners and paper (Khatri et al. 2013b, Martin et al. 2015), and in iron oxide pigment producing workers with a high proportion of nanoparticles in inhaled aerosol (Pelclova et al. 2016b).

This study is the fourth in a series of papers documenting inflammation, protein, and nucleic acids damage in the EBC of the same cohort of nanoTiO_2 manufacturing workers (Pelclova et al. 2015, Pelclova et al. 2016a, Pelclova et al. 2016c). Its objective was to expand the spectrum of investigation to markers of lipid oxidation both in EBC and urine and to identify the most robust oxidative stress markers for routine biomonitoring of exposed workers. This article provides new data on the markers of lipid oxidation following exposure to nanoTiO_2 in long-term exposed manufacturing workers using non-invasive methodologies and discusses relationships of lipid oxidation markers to lung disease development in these workers.

MATERIALS AND METHODS

Process and facility description

TiO_2 is produced in the factory from the ilmenite ore ($FeTiO_3$) via the sulfate process. The finely ground ilmenite ore undergoes a series of chemical reactions, starting with the dissolution in sulfuric acid to form soluble titanium sulfate. The solution undergoes a series of enrichment and purifications steps, during which iron is separated, whereas titanium sulfate is precipitated as titanium dioxide hydrate. Upon calcination in large high temperature rotary kilns (800-900 ^{0}C) (enclosed units), titanium dioxide hydrate is converted into crystalline TiO_2 as a mixture of anatase/rutile crystals. With further heating, phase transformations occur, which converts the anatase form to rutile. Micronisation, i.e. milling the raw form of the pigment, reduces the particles size. Additional proprietary chemical coating processes modify surface characteristics of TiO_2.

The facility operated seven days a week, in three shifts. It was located in a one-floor building, with the calcination furnace and micronisation unit located on the ground floor. The research part of the plant was situated in another separate but adjacent building.

Worker activities

The majority of workers were employed as production workers and worked in three shifts. They worked in four main areas in the facility; three areas corresponded with the main industrial processes: 1) calcination furnace; 2) micronisation unit; 3) post-processing units that included surface coating, filtration processes, and the transport corridors. According to their time-sheets, these workers spent about 31-46% of their 8-h shifts in close proximity to one of the three production units. All workers were exposed to TiO_2 aerosol, which contains both rutile and anatase. The remainder of their 8-hr shift, they spent it in the fourth area, i.e. operating/control room, checking the production lines remotely. The operating room was located close to the production unit and was separated by a closed door. A more detailed description of the workers has been already published (Pelclova et al. 2016a). A summary description of working activities is shown in **Table 1.**

Additionally, four employees worked in the research and development department of the factory, testing new TiO_2 production technologies, which included milling materials. Similarly, they spent most of the time in a control room and on average 38% of the time in this workshop.

Subjects

All workers were men. To meet the inclusion criteria, workers had to be working with TiO_2 for at least 6 months and should not have had a history of tuberculosis, lung cancer,

myocarditis, congenital heart disease, or a recent fever and/or inflammation. All workers were administered a standardized questionnaire in an interview led by a trained interviewer. They answered questions about their personal and occupational history, medication, supplements/vitamins and other treatments, lifestyle (including physical activity) and dietary habits, smoking, and alcohol intake. Furthermore, each subject received a physical examination, including body mass index (BMI), and blood pressure measurements.

The facility was visited twice, one year apart. In the first cross-sectional study in 2012, there were 20 workers from the TiO_2 production plant. Their EBC and urine samples (described later) were collected for each subject both before and after the 8-h shifts in the first half of the working week. In the second campaign performed in 2013, 14 male production workers could be examined (several were not available), and only post-shift.

The mean age of the production workers was 33.5 (95% confidence interval of the mean - CI 29.7-37.3) years. They were employed on average 9.7 (CI 7.0-12.5) years, 50% were current smokers, and 93.3 % were daily alcohol users. Another four employees worked in the research wing of the factory; their mean age was 35.0 (CI 23.0-47.0) years, they were exposed for 3.8 (CI 2.2- 5.3) years, one was smoker (25%), and all were daily alcohol users. In 2013, the mean age of workers was 33.7 (CI 27.9-39.5) years; mean employment was 8.9 (CI 5.5-12.4) years. Thirty-six percent of them were smokers and 100.0% were daily alcohol users.

Among the production workers, eight participated in both phases of the study (57%). Six (43%) subjects were newly recruited in 2013, but they also worked in the TiO_2 production for an average of 9.0 (CI 1- 21) years. All workers were provided with a dust mask to be used in the production areas of the factory.

There were a total of 45 male controls, with a mean age of 34.2 (CI 31.5-36.9) years; 40.0% were smokers and 100.0% were daily alcohol users. They were not occupationally exposed to TiO_2, dusts, or other hazardous substances, and were examined in parallel in 2012 and 2013. Their job title was mostly ‘safety inspector’ or ‘office worker’, and to control for potential differences in their daily physical activity, they were asked if they completed at least 30 min walking/day or a similar physical activity.

The study was carried out according to the Helsinki Declaration. The Ethics Committee of the Charles University approved the study. All participants signed an informed consent form before the beginning of the study.

Bulk TiO_2 material

Samples of TiO_2 collected at the calcination furnace and micronisation production unit were analyzed for crystallinity and crystallite size by X-ray diffraction (XRD) with monochromatic Cu K radiation ($K\alpha_1$ = 1.5406 Å, $K\alpha_2$ = 1.5444 Å) on a Philips 3830 instrument X-ray diffractometer with Bragg-Brentano Θ-2Θ geometry. The pattern profiles

were assigned by comparing the experimental data with standard diffractograms from the International Centre for Diffraction Data (ICDD) database.

Primary particle size and morphology of TiO_2 was examined by scanning electron microscopy (SEM) in a Stereoscan 410 Leica (Germany) at an accelerating voltage of 20 kV. TiO_2 particles were suspended in water and the suspension was dropped on a stub covered with a conductive carbon tape, dried in an oven at 40°C, and then covered with a gold film (thickness <50 nm) to increase sample conductivity.

Exposure assessment

The exposure assessment consisted of the following elements: generation of exposure maps of particle number concentration (20 nm-1 μm, #/cm^3), real-time monitoring of size distribution and number concentration, and integrated sampling on filters.

Exposure concentration maps were first generated for the facility to localize the main sources of aerosol particles. Two portable monitors (particle number concentration, P-TRAK; mass concentrations DustTRAK DRX; both TSI Inc., Minneapolis, USA) were used; P-TRAK measures particles in submicrometer range, while DustTRAK measures particle larger than 0.5 μm. At least 5 min of the measurements were taken at each location over three separate events, and the data were averaged.

Extensive area exposure assessment using real-time monitoring was conducted during 2012 and 2013. In 2012, a scanning mobility particle sizer (SMPS), model 3936 L (TSI Inc., Minneapolis, USA), and an aerodynamic particle sizer (APS), model 3321 (TSI Inc., Minneapolis, USA), were used for continuous monitoring of the particle size distribution (10 nm-20 μm range) during the 8-h shifts with a 5-min sampling frequency. Random checks were performed to compare total particle number concentrations determined by SMPS with the P-TRAK values (the differences between averaged values never exceeded 20%).

The PM_{10} mass concentrations determined by the DustTRAK DRX were similar to the PM_{10} integrated from the APS data when assuming a particle density corresponding to TiO_2 (4 g/cm^3). Two low volume samplers Leckel LVS (Enviro Technology Services, UK) sampled parallel to the SMPS and APS spectrometers for the whole shift and provided average mass concentrations of size fractions particulates smaller than 10 μm (PM_{10}) and 1 μm (PM_1) at each location.

EBC and urine sample collection

Collection of biological samples (EBC and spot urine) in the workers in 2012 was performed both pre-shift and post-shift, whereas in 2013 they were only collected post-shift. The simplified collection protocol in 2013 was a result of multiple factors, especially the findings in the first study where the difference in the pre-shift and post-shift was not

significant in the majority of cases, as the pre-shift levels were already elevated. Another reason was the cost consideration. The controls gave samples only once (half of them in the morning and half in the afternoon).

The EBC samples were collected using the Jaeger Ecoscreen Turbo DECCS, Jaeger, Germany, equipped with a filter. All subjects breathed tidally for 15 minutes through a mouthpiece connected to the condenser (-20 °C) while wearing a nose-clip (Horvath et al. 2005).

Analysis of markers of oxidative stress in the EBC and urine

Oxidation products of C_6-C_{12} hydrocarbons (aldehydes), MDA, HHE, HNE, and 8-isoprostane in the EBC were performed as was previously described (Syslova et al. 2008, Syslova et al. 2010). Briefly, samples were purified and concentrated by solid-phase extraction (SPE) followed by liquid chromatography - electrospray ionization - tandem spectrometry (LC-ESI-MS/MS) analysis using deuterium labeled internal standards. To allow exclusion of EBC samples contaminated by saliva, the concentration of α-amylase was monitored (Horvath et al. 2005) and the conductivity was controlled to avoid differences in the concentration of EBC samples (Effros et al. 2003). All EBC and urine samples were immediately frozen and stored at -80 °C.

Titanium analysis in EBC and urine

Quantitative analyses of titanium in EBC were conducted by inductively coupled plasma mass spectrometry (ICP-MS) on an Agilent 7900 ICP-MS Ultra HMI (UHMI), equipped with MassHunter software and autosampler ASX-520, as previously described (Pelclova et al. 2016a). The method limit of detection (LOD) was 1.2 µg/L and the LOQ was 4.0±0.2 µg/L. Samples below the limit of detection were substituted with LOD/$\sqrt{2}$.

Lung deposition model

The estimated total particle deposition in the human lungs was based on the Multiple Particle Path Dosimetry Model software (MPPD v.2.1) (Anjilvel and Asgharian 1995). This information is important for equivalent dose calculations for *in vitro* and *in vivo* nanotoxicology. The model parameters and results are summarized in **Supplementary Table S1**.

Environmental air pollution data

Information on environmental air pollution was collected from municipal monitoring stations (distance less than 2 km) for the days and locations of EBC samples collection in both

exposed and control subjects. Local monitoring included following parameters: SO_2, NO_2 and CO in 2012 and SO_2, NO_x, O_3, and PM_{10} in 2013.

Statistics

Summary descriptive statistics were computed for all variables, which were subsequently tested for normality using a Kolmogorov-Smirnov test. When comparing workers and controls the independent-groups t-test (normally distributed variables), Mann-Whitney U test (non-normally distributed variables), or Chi-square test (frequency counts) was used. The paired t-test was used to compare workers pre-shift vs. post-shift values while the differences between values in 2012 vs. 2013 were compared using the independent-group t-test.

Subgroups of research workers and production workers in 2012 were compared by nonparametric tests (Wilcoxon signed-rank test for within-group comparisons and Mann-Whitney U test for between-group comparisons). A Spearman correlation coefficient was used to investigate correlations between various variables. Multiple regression analysis was employed to explore relationships between biomarkers of oxidative stress in EBC and several determinants for samples in 2012 and in 2013. Statistical significance was set at $p < 0.05$. All analyses were conducted using SPSS version 22.0 (SPSS, Inc., Chicago, IL).

RESULTS

Subjects

Characteristics of the subgroups of the production workers, research workers, and controls are shown in **Table 1.** No difference was found between the age, prevalence of smoking, or prevalence of alcohol consumption in the groups of workers and controls, nor between the duration of employment in the subgroups of workers studied (all $p>0.05$). The workers and controls differed slightly in BMI only in 2013, which was higher in workers than in controls ($p<0.05$).

Exposure assessment

Airborne TiO2 concentrations

Real time exposure data (particle number and mass concentration) for each production unit determined by SMPS and APS spectrometers are summarized in **Table 2 and Table 3**.

In the workshops, on average, 70-82% of airborne particles were less than 100 nm in diameter. In the research part of the facility, an average of 62% of the particles was less than 100 nm in diameter. The median particle size distributions (representing the one-work shift-

long monitoring) at each of four selected locations determined by SMPS and APS spectrometers in 2012 are shown in **Figure 1**. As can be seen**,** the highest number concentrations measured by SMPS were found in the vicinity of the calcination furnace (peak at 30-40 nm in diameter). The size distribution of particles at the micronisation unit of the plant was flat and broad, with a maximum concentration of the particles in the range of 30-40 nm, as well. A second aerosol peak was present in the 700-800 nm as measured by APS. The number concentration in the control room was one order of magnitude lower than in production units (**Figure 1)**. The highest concentrations were found in the calcination furnace. In the micronisation unit, concentration of particles of the same size was about two times lower than in the calcination, whereas along the transport corridors it was about fourfold lower, which suggests that TiO_2 was transported across the hall. On the other hand, the micronisation process probably emitted particles with diameters under 2 µm and around 6 µm, as the concentration of these particle sizes was higher at the site than in other measured locations. It is likely that aerosol particles from the ambient air of the production plant leaked into the control room from the open windows (the connecting door to the workshops were kept closed), and the majority of these particles were found in the accumulation mode centered on 100 nm in particle diameter. The median size of these particles was 93 nm (interquartile range 54-153 nm).

The overlap in particle size distributions measured by SMPS and APS total concentrations was taken into consideration in the calculation of total mass and total number concentration. This correction lowered the crude data by about 10-25%, depending on the distribution shape.

Lung Dosimetry

The summary statistics of the particle size distribution and the multiple path particle deposition model estimates for human airways during different jobs in the TiO_2 manufacturing facility is shown in **Supplementary Table S1.** Because size distributions and number concentrations varied between different job titles/locations, deposition patterns in different parts of the respiratory tract also changed. For example, the fraction of particles deposited in the head and upper airways varied from 9.6% for calcination (predominantly nanoscale particles) to 21% in the control room where exposures were to mostly larger particles. However, deposited fractions in the alveolar and thoracic regions were similar for different locations - approximately 14-15% for alveolar and 5.9-6.9% for thoracic fractions, respectively (**Supplementary Table S1**). The highest estimated deposited doses were in the upper airways and the alveolar regions of the lungs.

Physicochemical characterization of settled TiO_2 dust and bulk material

The X-ray diffraction analysis of diffraction peaks of two dust samples collected in the workshop and comparison with reference standards from the ICDD database confirmed that both samples were exclusively made of crystalline TiO_2. Only traces of iron (Fe), sulphur (S) and silicon (Si) were found using MicroX-ray fluorescence (Pelclova et al. 2016a). The sample from the calcination furnace was an anatase-rutile mixture (with an approximate anatase: rutile ratio of 2:1), and the sample from the micronisation unit was composed of almost exclusively rutile, as can be seen in **Supplementary Figure S1.** SEM analysis of the settled dust on the floor showed that the settled dust was composed of micrometric and heterogeneous agglomerates/ aggregates of primary particles, which were detected by SMPS and APS. The size of the settled dust showed the aggregation, as displayed in the **Supplementary Figure S1.**

Analysis of titanium in EBC and urine

As was already documented in our previous paper on markers of oxidation of nucleic acids and proteins (Pelclova et al. 2016a), the concentration of titanium in the EBC samples of the subgroups of production workers gave very similar results in both years (means of 22.09 µg/L, 19.38 µg/L, 22.16 µg/L in calcination, micronisation and other processes, respectively). The levels in the research workers (2.00 µg/L) and controls (1.12 µg/L) were significantly lower ($p<0.001$).

Titanium in urine samples of all workers and controls was under the limit of detection (1.2 µg/L).

Analysis of markers of oxidative stress in the EBC and urine

Both pre-shift (in 2012) and post-shift levels (in 2012 and 2013) of all 11 markers of oxidative stress in the EBC of all 34 workers were significantly elevated compared to the controls ($p<0.001$), as shown in **Supplementary Figures S2** and **S3.** The levels of MDA, HHE, HNE, 8-isoprostane, and C_6-C_{12} aldehydes are presented. Most markers did not change significantly during the shift.

When the 30 production workers were divided into subgroups according to their jobs and level of exposure to nanoparticles, it was found that all EBC markers of oxidative damage to lipids were significantly higher ($p<0.001$) in all subgroups of production workers than in the controls. Additionally, all markers, except C_{11} for micronisation and other production, were elevated compared to the research employees ($p<0.05$). The results are shown in **Figure 2**.

In 2012, most pre-shift biomarkers and titanium correlated with their levels in the post-shift samples (except C_{12}). The majority of EBC markers also correlated with other EBC markers, as can be seen in **Supplementary Tables S2** and **S3**.

In the workers, no positive correlation of any EBC marker was found with their non-occupational characteristics, such as age, lifestyle factors (smoking and cigarette pack-years, and physical activity), and diseases, including rhinitis and chronic bronchitis. Furthermore, BMI was not an important factor in the multiple regression analysis, similarly to smoking that was associated with only C_6 in the first year.

Multiple regression analysis confirmed a significant association between occupational exposure and the concentration of all markers of lipid oxidation in the EBC, as shown in **Table 4 and in Supplementary Tables S4 and S5.**

Among the broad spectrum of lipid oxidative damage markers used, HNE, 8-isoprostane, C_7, and C_{10} appeared to be the most robust markers associated with nanoTiO_2 exposure, as they were not associated with any other covariates.

Environmental air pollution data

In 2012 and 2013, all measured air pollution concentration levels were classified as low or mild (data not shown).

No reproducible significant correlation was found between the markers studied and environmental concentrations of SO_2, NO_x, and PM_{10} (all $p>0.05$).

In multiple regression analysis, no environmental pollution parameter was associated more strongly with EBC markers than occupational exposure to TiO_2. SO_2 was associated mostly negatively, as shown in **Table 4.**

DISCUSSION

The results of both studies in 2012 and 2013, which found elevated levels of oxidative products of all 11 markers of lipid oxidation, together with our earlier findings of inflammation and oxidative DNA and protein damage in EBC in the same cohort of workers, support oxidative stress, described *in vitro* and in experimental studies using nanoTiO_2.

Specifically, in an inhalation study by Noël et al. (2013) in rats exposed to nanoTiO_2 at the concentration of 20 mg/m^3 for 6 hours, the oxidative damage measured by 8-isoprostane concentrations in bronchoalveolar lavage fluid was 8–9 fold compared to control rats. Also TiO_2 nanoparticles inhaled for 2 weeks for 6 h/day, 5 days /week at the concentration of 1 1 mg/m^3 led in the rats to histopathological changes in nasal mucosa and the lung tissues (Kwon et al. 2012). Repeated nasal instillation of anatase nanoparticles at a dose of 2.5 mg/kg body weight for 90 days increased the production of MDA in the lung tissues of exposed mice (Li et al. 2013). A single instillation of nanoTiO_2 at 4 mg/kg in rats found the destruction alveolar

septa and corresponding lung functions impairment (Lee JF et al. 2014). The repair processes in these experimental studies are not understood well, in part because they have not been studies systematically or consistently. In the study of Kwon et al. (2012), temporary histopathological changes in the nasal mucosa resolved by day 15. In another study using a single instillation exposure, pulmonary inflammatory/cytotoxic indices persisted up to 90 days (Skocaj et al. 2011).

In our previous study, Raman microspectroscopy revealed anatase and/or rutile particles in 40% of pre-shift EBC samples and 70% of post-shift samples from the workers (Pelclova et al. 2015). In agreement with this observation, elevated markers of lipid oxidation and titanium in the EBC were seen in the workers already pre-shift. This finding is consistent with the persistence of TiO_2 in the lungs, resulting in sustained oxidative stress that is reflected in elevated EBC markers in the pre-shift samples and chronic effect.

Slower clearance and longer retention and biopersistence of engineered nanoparticles in the lungs relative to their larger particles is well documented in animals (Kreyling et al. 2013, Shi et al. 2013). Particles sized 30-300 nm may deposit primarily in the alveolar region of the lungs (Löndahl et al. 2014) and contribute to acute and chronic pulmonary diseases (Möller et al. 2008). Our deposition modeling of the exposure data predicts ~15% deposition in the alveolar region and ~6% in the thoracic region. Chronic exposures over several years may add up to significant lung burden and chronic oxidative stress, which is documented in sustained protein damage, DNA damage, and lipid oxidation. However, it should be born in mind that most of these markers are part of dynamic metabolic cycles, which continuously remove, degrade, recycle, and/or replenish damaged biomolecules. Damaged proteins are removed (e.g. albumin in the liver), degraded, and the intact amino acids recycled and incorporated in the synthesis of new (albumin) protein molecules. Damaged lipids are further degraded, even though they may engage in subsequent damage to other biomolecules (lipids, proteins, etc.) via radical chain reaction/s. Under conditions of sustained and elevated chronic exposures to nanoparticles such as titania, such biomolecular markers tend to plateau at some maximum levels. It can be further argued that, at the molecular and cellular level, all these lipid (or protein) damage biomarkers are removed and, in the absence of exposure for sufficient time periods, they would return to background levels. Damaged DNA also gets repaired and DNA damage markers (such as 8-OHdG) would return to background levels. However, DNA mutations may accumulate over time leading to increased risk of cancers in future years. Therefore, cumulative exposure (i.e. nanoTiO_2 mass accumulated over the years of employment, mg*year) would not be a predictive exposure metric for these biomarkers. Cumulative exposure would be a good metric for irreversible effects such as markers of fibrosis for example) or accumulated DNA mutations. In contrast, average exposure levels may be better predictive of these lipid and protein bimolecular markers.

Exploratory analysis along the lines of aforementioned exposure metrics do in fact support the argument made above. Cumulative exposure was poorly correlated with lipid oxidation biomarker levels (data not shown), whereas average TiO_2 group exposures (and iron oxide in a previous study) were much better correlated with these markers (including research workers as shown in **Supplementary Figure S4 and S5,** and office workers who intermittently visited the workshops (Pelclova et al. 2016d). Furthermore, the amount of Ti in EBC correlated with most EBC markers of oxidative stress. Ti in EBC might therefore serve as a marker of exposure, and can complement markers of biological effect in the EBC.

Similarly, in a study of nine workers involved in the production of multi-walled carbon nanotubes (MWCNT), MDA and HHE were significantly elevated in the EBCs of workers (Lee JS et al. 2014), similar to the study of Liou et al. (2016), suggesting these biomarkers may indeed be useful for the monitoring of workers exposed to nanoparticles. The number of epidemiological studies in nanomanufacturing workers is still limited. From the perspective of risk reduction, insurance premiums, and/or early interventions to prevent disease, all studies, including those with normal results, would be highly valuable; however even such negative studies are missing in the literature.

The quantitative relationship between oxidative stress markers and clinical disease has not been clearly established. It has not been clarified yet at what levels these biomarkers depart from simple perturbations of normal physiology, when they represent reversible damage without disease, or irreversible damage with established development of chronic diseases. After exposure to nanoparticles, impairment of lung function parameters was found in workers in the study by Zhang et al. (2014). In our workers, most lung function parameters were not impaired relative to controls (Pelclova et al. 2015). However, inspiratory vital capacity (%VCIN) and the peak expiratory flow (%PEF) were significantly lower than in controls, and the decline in these parameters was seen in 2012 in the workers with longer than average occupational exposure (Pelclova et al. 2016c).

Urine markers of oxidative stress were elevated only in a few individuals, and only in the first year of the study. One straightforward explanation for this observation is that urine analysis may not be a sensitive approach for lipid oxidation markers of nanoTiO_2 exposure. A second, more plausible explanation is that very little of nanoTiO_2 deposited in the lungs translocate to extra pulmonary organs and circulation. As such, the effects of nano TiO_2 exposures may be primarily localized at the deposition site (the lung tissues) and not systemic. This interpretation is consistent with the current understanding that extra pulmonary translocation of poorly soluble nanoparticles (including nanoTiO_2) is low (typically $<0.1\%$), and the vast majority of circulating nanoparticles accumulate in the liver. However, urine has repeatedly been shown to be a sensitive medium for monitoring systemic changes in other exposure scenarios (Syslova et al. 2014).

This study is in full agreement with our earlier work on DNA and protein damage in the same group of TiO_2 workers (Pelclova et al. 2016a), where markers of oxidation of nucleic acids of

proteins, and markers of inflammation (leukotrienes), (Pelclova et al. 2016c) were significantly elevated and related to occupational exposure to nanoTiO_2. Even office workers from the same factory, who visited the production floor intermittently for an average of 14 min/day, had elevated EBC levels of lipid markers of oxidative stress, compared to the unexposed controls ($p<0.05$), but they were significantly lower ($p<0.001$) than that of the production workers (Pelclova et al. 2016d).

Limitations

We acknowledge several limitations to our study. For a start, markers of oxidative stress are not specific to nanoparticles and other agents/co-exposures cannot be completely excluded, in spite of our efforts to account for several potential confounders (smoking, drugs and vitamins, environmental pollution, etc.). In particular, we acknowledge that nanoparticles other than TiO_2 may have been present in some areas of the production plant and that size selective quantitation of nanoTiO_2 in workplace air is highly desirable. Furthermore, we recognize the opportunity for better personal quantitative exposure assessment, especially personal nanoparticle monitoring, which only now are starting to become more commonplace, and other constraints placed on the research team. Personal monitoring almost always results in higher exposure estimates than area measurements. Chemical analysis of the airborne aerosol on filters could not be measured due to the limited resources, and limit of detection issues, and it would have been highly desirable to also monitor for other co-exposures, such as fumes and gases potentially emitted from furnaces. Diesel exhausts in the transport corridors did not play an important role because real time aerosol exposures in these locations were low. We did not collect information on diet (except for the last meal), cooking fuel at home (gas or electricity), transportation method to work (car, public transport, etc.), which may have contributed to nanoparticle exposures, and/or oxidative stress. Lastly, we could not continue to study this cohort over time to investigate in greater detail changes in their biomarker levels and disease progression (if any), or investigate the impact of exposure reduction intervention efforts on these biomarkers. It is now a well-known reality that access to workplaces is an important bottleneck in conducting human nanotoxicology and molecular epidemiology studies for engineered nanoparticles.

CONCLUSIONS

To our knowledge, this is the first series of studies performed *in vivo* in workers with relatively high levels of exposure to aerosols containing a high proportion of nano-sized TiO_2 particles. A significant pre-shift elevation of all 11 lipid oxidation biomarkers in the EBC, indications of a decline in some parameters of lung function, and consistency of these patterns with that of other DNA and protein damage markers in the same cohort of workers, and strong association with TiO_2 exposures, all point out to subacute or chronic biological effects in the

lungs, rather than acute changes. Taken together, this study and the previous ones on the same cohort of chronically exposed workers suggest molecular and cellular damage in the deep airways, consistent with oxidative stress and inflammation.

This study also shows differences in the concentration of titanium and levels of markers of lipid oxidation in EBC according to the TiO_2 aerosol mass concentration and particles number concentrations among the groups of subjects in the study. We found that HNE and 8-isoprostane were the most robust biomolecular markers of lipid oxidation, similar to findings in patients with silicosis and asbestosis (Pelclova et al. 2007, Pelclova et al. 2008). Urine markers of oxidative stress and of Ti exposure were not sensitive in our study. Oxidative stress markers of lipid, protein, and DNA damage in the EBC samples may be used for periodic examinations of workers.

Authors' contributions

DP designed the study, participated in the field study and drafted the manuscript, VZ, NZ, JS, OM carried out the air monitoring, VZ participated in the design and coordination of the study, KS and PK analyzed the biological samples, ZF and SV participated in the field study and collection of data and samples, SV provided the environmental air monitoring data, FT and IZ performed the physic-chemical characterization of the deposited dust samples. MK, TN and SZ performed the statistical analysis and preparation of data. DB calculated the Multiple Particle Path Dosimetry and revised the manuscript critically for important intellectual content. All authors read and approved the final manuscript.

Declaration of interest

The authors report no conflicts of interest. The authors alone are responsible for the content and writing of this article.

This research was supported by the project of the Charles University P25/1LF/2, P28/1LF/6 and EU Project “Material - technical Research Base for the Diagnostics and Treatment of Environmentally-caused and Oncological Disorders and their Risks, in the General University Hospital in Prague” (reg. No. CZ.2.16/3.1.00/24.12) and Czech Science Foundation project P503/12/G147.

References:

1. Anjilvel S, Asgharian BA. 1995. Multiple-path model of particle deposition in the rat lung. Fundam Appl Toxicol 28:41-50.

2. Chang XH, Zhang Y, Tang M, Wang B. 2013. Health effects of exposure to nano-TiO2: a meta-analysis of experimental studies. Nanoscale Res Lett 8:1-10.
3. Czerska M, Zieliński M, Gromadzińska J. 2016. Isoprostanes - A novel major group of oxidative stress markers. Int J Occup Med Environ Health 29:179-190.
4. Effros RM, Biller J, Foss B, Hoagland K, Dunning MB, Castillo D, et al. 2003. A simple method for estimating respiratory solute dilution in exhaled breath condensates. Am J Respir Crit Care Med 168:1500-1505.
5. Fuchs P, Loeseken C, Schubert JK, Miekisch W. 2010. Breath gas aldehydes as biomarkers of lung cancer. Int J Cancer 126:2663-2670.
6. Gong JC, Zhu T, Kipen H, Wang GF, Hu M, Ohman-Strickland P, et al. 2013. Malondialdehyde in exhaled breath condensate and urine as a biomarker of air pollution induced oxidative stress. J Expo Sci Environ Epidemiol 23: 322-327.
7. Horvath I, Hunt J, Barnes PJ, Alving K, Antczak A, Balint B, et al. 2005. Exhaled breath condensate: methodological recommendations and unresolved questions. Eur Resp J 26:523-548.
8. Hsieh SF, Bello D, Schmidt DF, Pal AK, Stella A, Isaacs JA, et al. 2013. Mapping the biological oxidative damage of engineered nanomaterials. Small 9:1853-1865.
9. Huang J, Kumar S, Hanna GB. 2014. Investigation of C3-C10 aldehydes in the exhaled breath of healthy subjects using selected ion flow tube-mass spectrometry (SIFT-MS). J Breath Res 8:037104.
10. IARC: Carbon Black, Titanium Dioxide, and Talc. In: IARC Monographs on the Evaluation of Carcinogenic Risks to Humans, vol. 93. City: World Health Organization; 2010. ftp://ftp.cdc.gov/pub/Documents/OEL/02.%20Kuempel/References/IARC_2010-Vol%2093.pdf
11. Jareno J, Munoz MA, Maldonado JA, Carrillo B, Trigo GR, Civera C et al. 2012. Volatile Organic Compounds (VOC) in exhaled breath in patients with lung cancer (LC). Lung Cancer 77:S31-S32.
12. Khatri M, Bello D, Pal AK, Cohen JM, Woskie S, Gassert T, et al. 2013a. Evaluation of cytotoxic, genotoxic and inflammatory responses of nanoparticles from photocopiers in three human cell lines. Part Fiber Toxicol 10:42.
13. Khatri M, Bello D, Gaines P, Martin J, Pal AK, Gore R, et al. 2013b. Nanoparticles from photocopiers induce oxidative stress and upper respiratory tract inflammation in healthy volunteers. Nanotoxicology 7:1014-1027.
14. Kreyling WG, Semmler-Behnke M, Takenaka S, Möller W. 2013. Differences in the biokinetics of inhaled nano- versus micrometer-sized particles. Acc Chem Res 46:714-722.
15. Kwon S, Yang YS, Yang HS, Lee J, Kang MS, Lee BS, et al. 2012. Nasal and pulmonary toxicity of titanium dioxide nanoparticles in rats. Toxicol Res 28:217-224.
16. Lazareva A, Keller AA. 2014. Estimating Potential Life Cycle Releases of Engineered Nanomaterials from Wastewater Treatment Plants. ACS Sustainable Chem Eng 2:1656–1665.
17. Lee JF, Tung SP, Wang D, Yeh DY, Fong Y, Young YC, et al. 2014. Lipoxygenase pathway mediates increases of airway resistance and lung inflation induced by exposure to nanotitanium dioxide in rats. Oxid Med. Cell Longev 2014:485604.
18. Lee JS, Choi YC, Shin JH, Lee JH, Lee Y, Park SY, et al. 2014. Health surveillance study of workers who manufacture multi-walled carbon nanotubes. Nanotoxicology 14:1-10.

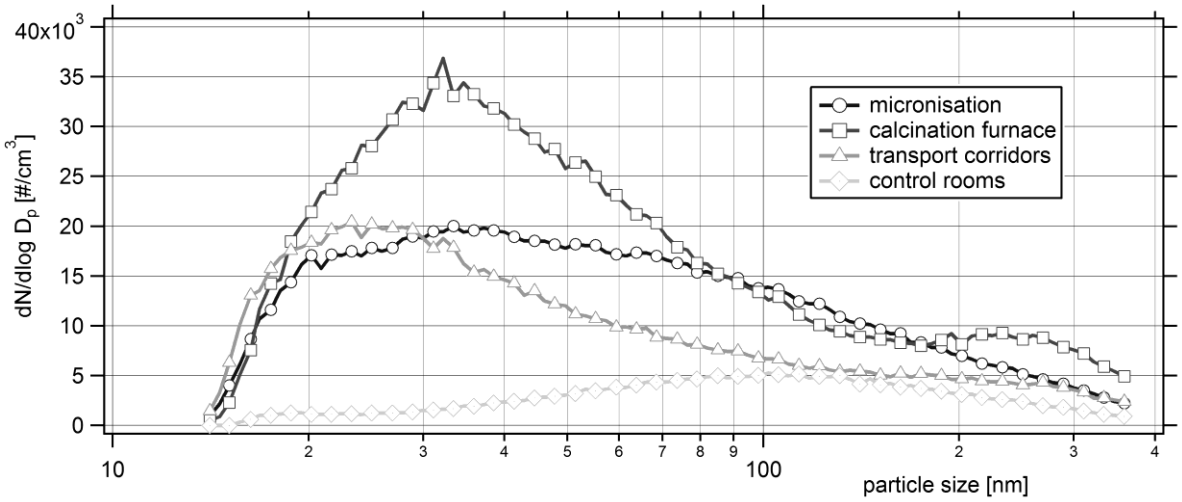
1. Li B, Ze Y, Sun Q, Zhang T, Sang X, Cui Y, et al. 2013. Molecular mechanisms of nanosized titanium dioxide-induced pulmonary injury in mice. PLoS One 8(2):e55563.
2. Liao HY, Chung YT, Lai CH, Wang SL, Chiang HC, Li LA, et al. 2014. Six-month follow-up study of health markers of nanomaterials among workers handling engineered nanomaterials. Nanotoxicology 8 Suppl 1:100-110.
3. Liou SH, Twou TC, Wang SL, Li LA, Chiang HC, Li WF, et al. 2012. Epidemiological study of health hazards among workers handling engineered nanomaterials. J Nanopart Res 14:878-892.
4. Liou SH, Tsai CS, Pelclova D, Schubauer-Berigan MK, Schulte PA. 2015. Assessing the first wave of epidemiological studies of nanomaterial workers. J Nanopart Res17:413.
5. Liou SH, Chen YC, Liao HY, Wang CJ, Chen JS, Lee HL. 2016. Increased levels of oxidative stress biomarkers in metal oxides nanomaterial-handling workers. Biomarkers Mar 24:1-7.
6. Löndahl J, Möller W, Pagels JH, Kreyling WG, Swietlicki E, Schmid O. 2014. Measurement techniques for respiratory tract deposition of airborne nanoparticles: a critical review. J Aerosol Med Pulm Drug Deliv 27:229-254.
7. Martin J, Bello D, Bunker K, Shafer M, Christiani D, Woskie S, et al. 2015. Occupational exposure to nanoparticles at commercial photocopy centers. J Hazard Mater 298:351-360.
8. Møller P, Jacobsen NR, Folkmann JK, Danielsen PH, Mikkelsen L, Hemmingsen JG, et al. 2010. Role of oxidative damage in toxicity of particulates. Free Radical Res 44:1-46.
9. Möller W, Felten K, Sommerer K, Scheuch G, Meyer G, Meyer P, et al. 2008. Deposition, retention, and translocation of ultrafine particles from the central airways and lung periphery. Am J Respir Crit Care Med 177:426-432.
10. NIOSH 2011 Current Intelligence Bulletin 63, Occupational Exposure to Titanium Dioxide, Department of Health and Human Services, Centers for Disease Control and Prevention, National Institute for Occupational Safety and Health, 114 pp. DHHS (NIOSH) Publication No. 2011–160 http://www.cdc.gov/niosh/docs/2011-160/pdfs/2011-160.pdf
11. Noël A, Charbonneau M, Cloutier Y, Tardif R, Truchon G. 2013. Rat pulmonary responses to inhaled nano-TiO_2: effect of primary particle size and agglomeration state. Part Fibre Toxicol 10:48.
12. Pal AK, Hsieh S-F, Khatri M, Isaacs JA, Demokritou P, Gaines P, et al. 2014. Screening for oxidative damage by engineered nanomaterials: a comparative evaluation of FRAS and DCFH. J Nanopart Res 8:9003-9015.
13. Pelclova D, Fenclova Z, Kacer P, Navratil T, Kuzma M, Lebedova J, et al. 2007. 8-isoprostane and leukotrienes in exhaled breath condensate in Czech subjects with silicosis. Ind Health 45:766-774.
14. Pelclova D, Fenclova Z, Kacer P, Kuzma M, Navratil T, Lebedova J. 2008. Increased 8-isoprostane, a marker of oxidative stress in exhaled breath condensate in subjects with asbestos exposure. Ind Health 46:484-489.
15. Pelclova D, Barosova H, Kukutschova J, Zdimal V, Navratil T, Fenclova Z, et al. 2015. Raman microspectroscopy of exhaled breath condensate and urine in workers exposed to fine and nano TiO_2 particles: a cross-sectional study. J Breath Res 9:036008.

16. Pelclova D, Zdimal V, Fenclova Z, Vlckova S, Syslova K, Navratil T, et al. 2016a. Oxidative stress markers are elevated in exhaled breath condensate of workers exposed to nanoparticles during iron oxide pigment production. J Breath Res 10 016004.
17. Pelclova D, Zdimal V, Fenclova Z, Vlckova S, Turci F, Corazzari I, et al. 2016b. Markers of oxidative damage of nucleic acids and proteins among workers exposed to TiO_2 (nano) particles. Occup Environ Med 73:110-118.
18. Pelclova D, Zdimal V, Kacer P, Fenclova Z, Vlckova S, Komarc M, et al. 2016c. Leukotrienes in exhaled breath condensate and fractional exhaled nitric oxide in workers exposed to TiO_2 nanoparticles. J Breath Res 10(3):036004.
19. Pelclova D, Kacer P, Zdimal V, Komarc M, Fenclova Z, Vlckova S, et al. Markers of lipid oxidative damage among office workers exposed intermittently to air pollutants including nanoTiO2 particles. Rev Environ Health, 2016d, in print.
20. Pirela SV, Miousse IR, Lu X, Castranova V, Thomas T, Qian Y, et al. 2015. Effects of laser printer-emitted engineered nanoparticles on cytotoxicity, chemokine expression, reactive oxygen species, DNA methylation, and DNA damage: A comprehensive in vitro analysis in human small airway epithelial cells, macrophages, and lymphoblasts. Environ Health Perspect Jun 16.
21. Roberts LJ, Oates JA, Linton MF, Fazio S, Meador BP, Gross MD, et al. 2007. The relationship between dose of vitamin E and suppression of oxidative stress in humans. Free Radic Biol Med 43:1388-1393.
22. Shi HB, Magaye R, Castranova V, Zhao JS. 2013. Titanium dioxide nanoparticles: a review of current toxicological data. Part Fibre Toxicol 10:15.
23. Silva RM, TeeSy C, Franzi L, Weir A, Westerhoff P, Evans JE, et al. 2013. Biological response to nano-scale titanium dioxide (TiO2): Role of particle dose, shape, and retention. J Toxicol Environ Health 76:953-972.
24. Skocaj M, Filipic M, Petkovic J, Novak S. 2011. Titanium dioxide in our everyday life; is it safe? Radiol Oncol 45:227-247.
25. Syslova K, Kacer P, Kuzma M, Klusackova P, Fenclova Z, Lebedova J, et al. 2008. Determination of 8-iso-prostaglandin F(2alpha) in exhaled breath condensate using combination of immunoseparation and LC-ESI-MS/MS. J Chromatogr B 867:8-14.
26. Syslova K, Kacer P, Kuzma M, Najmanova V, Fenclova Z, Vlckova S, et al. 2009. Rapid and easy method for monitoring oxidative stress markers in body fluids of patients with asbestos or silica-induced lung diseases. J Chromatogr B 877:2477-2486.
27. Syslova K, Kacer P, Kuzma M, Pankracova A, Fenclova Z, Vlckova S, et al. 2010. LC-ESI-MS/MS method for oxidative stress multimarker screening in the exhaled breath condensate of asbestosis/silicosis patients. J Breath Res 4:017104.
28. Syslova K, Bohmova A, Mikoska M, Kuzma M, Pelclova D, Kacer P. 2014. Multimarker screening of oxidative stress in aging. Oxid Med Cell Longev 2014:562860.
29. Toyokuni S. 2008. Molecular mechanisms of oxidative stress-induced carcinogenesis: From epidemiology to oxygenomics. IUBMB Life 60:441-447.
30. Warheit DB. 2013. How to measure hazards/risks following exposures to nanoscale or pigment-grade titanium dioxide particles. Toxicol Lett 220:193-204.

31. Weir A, Westerhoff P, Fabricius L, Hristovski K, von Goetz N. 2012. Titanium dioxide nanoparticles in food and personal care products. Environ Sci Technol 46:2242-2250.
32. Zhang R, Dai Y, Zhang X, Niu Y, Meng T, Li Y, et al. 2014. Reduced pulmonary function and increased pro-inflammatory cytokines in nanoscale carbon black-exposed workers. Part Fibre Toxicol 11:73.

Figure1 Medians of particle number size distributions between 15 and 350 nm as measured by SMPS (A.) and between 0.5 and 10 µm measured by APS (B.).

A.



B.

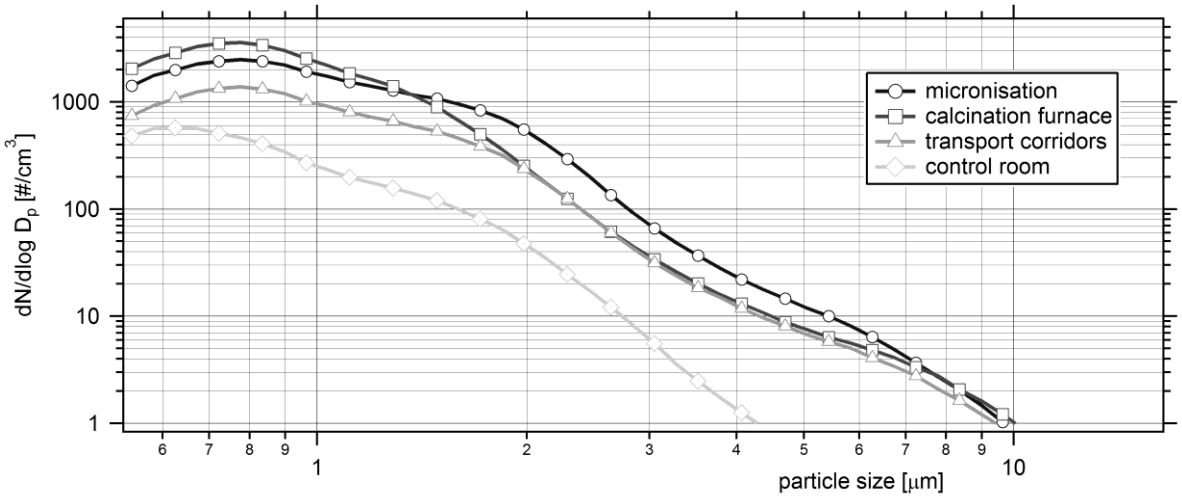
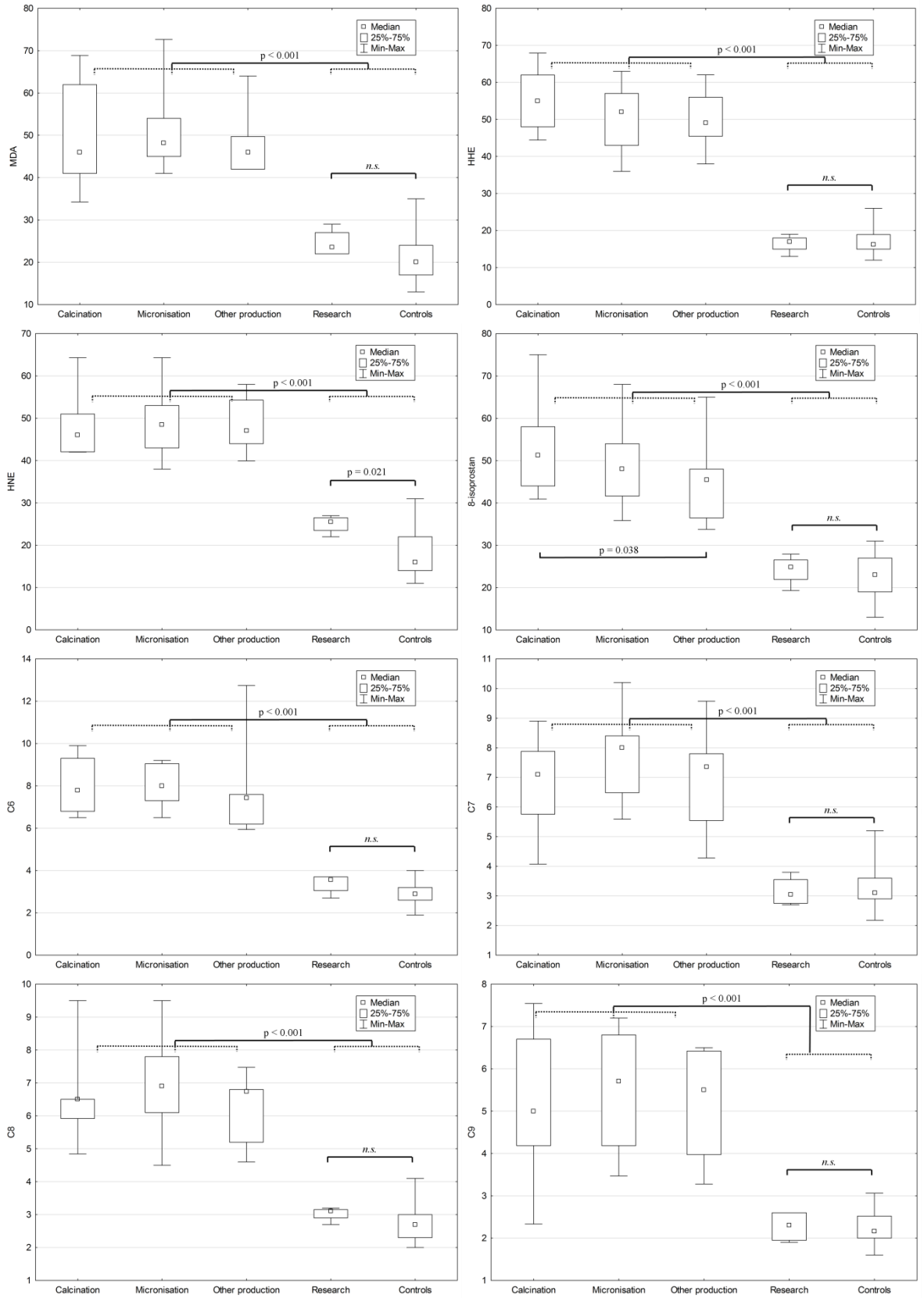


Figure legend: Each median distribution represents an 8-h shift monitored with 5-min time resolution. Axes are on a logarithmic scale. dN/dlogDp = technical term for number concentration normalized by width of the size bin in log scale enabling to compare measurements using instruments based on different physical principles and having different size resolution.

Figure 2 Markers of oxidation of lipids in exhaled breath condensate of the 4 subgroups of workers and controls in both years. § ($p<0.05$), + ($p<0.01$), and * ($p<0.001$)



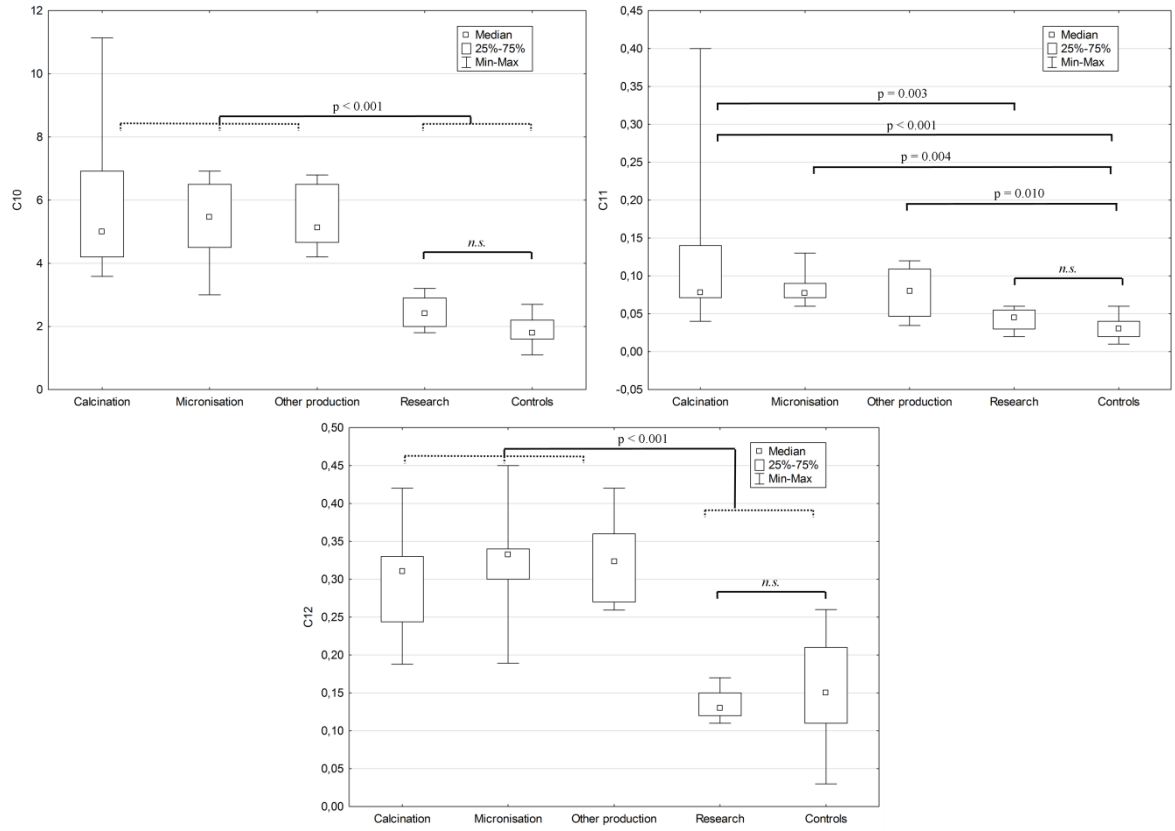

C10
Median
25%-75%
Min-Max
p < 0.001
n.s.
Calcination
Micronisation
Other production
Research
Controls
C11
p = 0.003
p < 0.001
p = 0.004
p = 0.010
n.s.
C12
p < 0.001
n.s.

Table 1 Subjects, their job descriptions and localization of the workplaces in the workers and controls

Characteristics of the subgroups of total 30 production workers, 4 research and development laboratory workers and 45 controls in 2012 and 2013
and the job descriptions and localization of the workplaces.

* calcination group declared less daily alcohol consumption comparing with all other groups,

+ micronisation group had a higher BMI comparing with all other groups, and also declared the highest physical activity.

When all production workers were merged into one group (30 subjects), there was no difference comparing with the control group (45 subjects).

Area	Process	N	Age (yrs) (average) (range)	BMI (average) (range)	Exposure (yrs) (average) (range)	Exposure Duration (hr, % shift)	Alcohol YES %	Physica Activit YES %
Production	Calcination Group	13	33.4 (23–55)	29.3 (23.5–37.4)	9.5 (1.0 – 25.0)	2.5 (31%)	84.6*	46.2
	Micronisation Group	8	37.9 (21-59)	32.2+ (27.4-35.8)	11.7 (0.6-25.0)	3.5 (44%)	100.0	100.0§
	Surface coating, etc. Group	9	29.8 (26-37)	27.1 (20.1-35.5)	8.2 (3.0-14.0)	3.7 (46%)	100.0	44.4
	All 3 Production Workers Groups while staying in the Control Room	30	33.5 (21-59)	29.4 (20.1-37.4)	9.7 (0.6-25.0)	3.0 (37.5%)	**93.3**	**60**

R&D Lab	Milling, etc.	4	35.0 (26.0, 53.0)	25.2 (22.9-28.8)	3.8 (1.5-5.0)	3 -38%	**100.0**	**75.0**
Background	Controls (outside the factory)	45	34.2 (19-59)	26.1 (19.0-35.2)	0	n/a	**100.0**	**51.1**

Abbreviations: R&D =research and development laboratory, BMI = body mass index

Table 2 Summary statistics of airborne TiO_2 exposures as measured by various real-time instruments

(scanning mobility particle sizer SMPS Model 3936 L and Leckel LVS) in major production areas

Area	Process	GM (#/cm^3)	GSD	AM (#/cm^3)	Max (#/cm^3)	$PM_{0.1}$ Mass Conc. µg/m^3	PM_{10} Mass Conc. µg/m^3
Production and Processing	Calcination	$2.35x10^4$	2.29	$2.72x10^4$	$7.21x10^4$	5.12	$3.2x10^3$
	Micronisation	$2.47x10^4$	2.15	$2.77x10^4$	$9.05x10^4$	5.08	$2.8x10^3$
	Surface coating	$1.53x10^4$	2.16	$1.62x10^4$	$3.70x10^4$	2.76	$1.4x10^3$
Control Room	Supervision	$4.68x10^3$	2.15	$4.96x10^3$	$1.07x10^4$	1.36	$0.57x10^3$
R&D Lab	Milling, etc.	$1.33x10^4$	2.35	$1.35x10^4$	$1.91x10^4$	3.07	$2.2x10^2$

Abbreviations: GM= Geometric mean; GSD= Geometric standard deviation; AM=Arithmetic mean, Max= Maximum measured concentration;

PM_{10}=particulate matter <10µm, $PM_{.01}$=particulate matter <0.1µm; $PM_{0.1}$ determined from SMPS, PM_{10} from Leckel LVS,

GM_p=GM of process measurements, R&D Lab=Research and Development Laboratory

Table 3 Summary statistics of airborne TiO_2 exposures as measured by various real-time

instruments (aerodynamic particle sizer APS Model 3321) in major production areas

Area	Process	GM (#/cm³)	GSD	AM (#/cm³)	Max (#/cm³)	PM_{10} Mass Conc. µg/m³
Production and Processing	Calcination	1288	1.42	1429	4045	329
	Micronisation	1264	1.46	1349	4560	588
	Surface coating	740	1.47	775	1564	350
Control Room	Supervision	218	1.43	249	877	42
R&D Lab	Milling, etc.	845	1.37	1083	3198	92

Abbreviations: GM= Geometric mean; GSD= Geometric standard deviation; AM=Arithmetic mean, Max= Maximum

measured concentration; PM_{10}=particulate matter <10 µm; R&D Lab=Research and Development Laboratory