ORIGINAL ARTICLE

Markers of oxidative damage of nucleic acids and proteins among workers exposed to TiO_2 (nano) particles

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ABSTRACT

Objective The use of nanotechnology is growing enormously and occupational physicians have an increasing interest in evaluating potential hazards and finding biomarkers of effect in workers exposed to nanoparticles.

Methods A study was carried out with 36 workers exposed to (nano)TiO_2 pigment and 45 controls. Condensate (EBC) titanium and markers of oxidation of nucleic acids (including 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 5-hydroxymethyl uracil (5-OHMeU)) and proteins (such as o-tyrosine (o-Tyr), 3-chlorotyrosine (3-ClTyr) and 3-nitrotyrosine (3-NOTyr)) were analysed from samples of their exhaled breath.

Results In the production workshops, the median *total* mass 2012 and 2013 TiO_2 concentrations were 0.65 and 0.40 mg/m^3, respectively. The median numbers of concentrations measured by the scanning mobility particle sizer (SMPS) and aerodynamic particle sizer (APS) were 1.98×10^4 and 2.32×10^4 particles/cm^3, respectively; and about 80% of those particles were smaller than 100 nm in diameter. In the research workspace, lower aerosol concentrations (0.16 mg/m^3 and 1.32×10^4 particles/cm^3) were found. Titanium in the EBC was significantly higher in production workers ($p<0.001$) than in research workers and unexposed controls. Accordingly, most EBC oxidative stress markers, including in the preshift samples, were higher in production workers than in the two other groups. Multiple regression analysis confirmed an association between the production of TiO_2 and the levels of studied biomarkers.

Conclusions The concentration of titanium in EBC may serve as a direct exposure marker in workers producing TiO_2 pigment; the markers of oxidative stress reflect the local biological effect of (nano)TiO_2 in the respiratory tract of the exposed workers.

What this paper adds

- Little is known about health effects of nanoparticles in workers, that is, those with the highest levels of exposure.
- Methods for monitoring potential biological effects in workers exposed to nanoparticles are missing.
- Local biological effects in the respiratory system—increased levels of oxidative products of nucleic acids and proteins were found in the exhaled breath condensate of the (nano)TiO_2 workers in our study.
- This effect was lower in less exposed workers.
- Markers of exposure and effect in exhaled breath condensate should be further tested for their potential practical use in health surveillance of workers.

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INTRODUCTION

In order to improve product quality, the use of nanotechnology in consumer products continues to grow every year. Since there is uncertainty concerning the health risk for exposed workers, guidelines for medical surveillance are needed. However, methods for monitoring the potential biological effects on workers exposed to nanoparticles are yet to be developed.[1]

Titanium dioxide, TiO_2 (CAS number 13436-67-7), is a white, poorly-soluble powder that, due to its brightness, is used extensively as a pigment. It occurs naturally in three polymorphs—anatase, rutile and brookite—with anatase being the most chemically reactive. There are very limited data about the toxic effects of TiO_2 in humans.[2] More data are available from experimental in vitro and animal studies,[3] where nano-sized TiO_2 induced proinflammatory and cardiovascular effects, and caused pneumotoxicity, neurotoxicity and cancer. The biological effects of TiO_2 particles differ depending on their size.[4]

Nanoparticles can promote the messenger RNA expression of cytokines and increase enzymatic activities during proinflammatory responses in mice and rats. The toxic/genotoxic effects induced by reactive oxygen species (ROS) and reactive nitration species (RNS) can contribute to tumourigenesis in multiple ways, including through the formation of DNA adducts, nucleic acids and proteins' oxidative products.[4 5 6 7] The exact site of the DNA damage is unknown and further research is needed to elucidate the mechanisms for ROS formation and carcinogenesis.[8] Mitochondrial DNA (mtDNA) is even

more vulnerable than nuclear DNA, as it is located closer to the sites of mitochondrial ROS generation, including the so-called displacement loop, which is known as a mutational hotspot. This mutation is associated with hepatocellular carcinoma, and ovarian, breast and colorectal cancer.[9]

Traditionally, TiO_2 has been considered a low-toxicity compound due to its low solubility in water, and it is used as a food (E171) and pharmaceutical additive.[3] TiO_2 is also present in the emissions from laser printers and photocopiers, and nanoparticles emitted from printers and photocopiers may be deleterious to lung cells.[10 11 12]

Genotoxicity information concerning TiO_2 nanoparticles and their effect on humans is currently lacking. The predominant form of free radical-induced oxidative lesions used as a biomarker for oxidative stress and carcinogenesis is 8-hydroxy-2'-deoxyguanosine (8-OHdG or 8-oxodG) in DNA and 8-hydroxyguanosine (8-OHG) in RNA, both of which originate from guanine. Similarly, 5-hydroxymethyl uracil (5-OHMeU) may be formed from thymine in the DNA.[13]

In proteins, modification of aromatic amino acids by ROS/RNS yields several chemically stable markers of oxidative damage. For example, o-Tyr is generated after an attack of hydroxyl radicals on phenylalanine. 3-ClTyr forms in a chemical reaction of tyrosine with hypochlorous acid, which is produced by phagocytes during inflammation.[14] 3-NOTyr is produced from a reaction between tyrosine and peroxynitrite, originating in vivo from superoxide and nitrous oxide or from nitrogen radical by-products of nitric oxide and peroxynitrite transformations.[15]

Exhaled breath condensate (EBC) is a liquid and reflects the composition of the airway lining fluid. It is obtained non-invasively after cooling the exhaled air of a subject.[16] The analysis of EBC allows for tracking the source of markers originally *formed* in the airways and lungs. EBC contains 99.9% water and a minor proportion of water-soluble and insoluble compounds. These non-volatile compounds can range from small inorganic ions, through larger organic molecules (urea, organic acids, amino acids), to proteins and macromolecules. Non-volatile compounds are released from the airway lining fluid in the form of aerosolised particles. Their formation in the respiratory tract has been attributed to either the turbulent air flow or a process of bubble bursting during the opening of the bronchioles following exhalation.[16]

The EBC method of collection and analysis has been used for the detection of biomarkers due to occupational inhalational exposure, including carcinogenic and the fibrogenic minerals silica and asbestos. In the pathogenesis of lung fibroses and cancers caused by these dusts particles, oxidative stress plays an important role.[17] Recently, increased levels of mtDNA mutations were found in the EBC of patients with lung cancer.[18] Therefore, the markers of oxidation of nucleic acids and proteins in EBC could be valuable in expanding our understanding of the effect of nanoparticles.

This article reports the results of a study in TiO_2 workers exposed to aerosol containing nano-sized particles; the first part of this cross-sectional study was carried out in 2012 and the second part in 2013. The aims of the study were to non-invasively measure and evaluate the markers of oxidation of nucleic acids and proteins in the EBC of workers and control subjects.

METHODS

Workplace area sampling and TiO_2 aerosol measurements

In the studied production plant, TiO_2 pigment is manufactured from the titanium mineral ilmenite (iron titanium oxide), by a sulfate process. After reacting of ilmenite with sulfuric acid, titanium hydroxide is precipitated by hydrolysis and filtered. During the process of calcination, the material is heated to 800–1000°C, and anatase/rutile crystals are formed. In the finishing operations, the crude form of the pigment is milled (micronisation process) to produce particles in a controlled size distribution.

The details of the workplace aerosol measurements were described in our earlier report.[19] At the beginning, pilot measurements were carried out to map and localise the main sources of aerosol particles using a portable particle number concentration monitor, P-TRAK, and a portable monitor of particle mass concentrations, DustTRAK DRX (both TSI Inc, Minneapolis, USA). These measurements were then used to design concentration maps for finding the key locations in the workplace. In the next step, a scanning mobility particle sizer (SMPS; model 3936L), and an aerodynamic particle sizer (APS; model 3321; both TSI Inc, Minneapolis, USA), were used (this covered particle diameters ranging from 15 nm to 10 μm). Four representative locations were found for the 8 h shift sampling: the calcination furnace, the micronisation area, the transport corridors and the control room.

Physicochemical characterisation of TiO_2-containing dust from the workplace

X-ray fluorescence spectroscopy (μ-XRF) analysis was carried out using an EDAX Eagle III energy dispersive μ-XRF spectrometer equipped with an Rh X-ray tube and a polycapillary exciting a circular area of 30 μm diameter.

Subjects

Workers

The studies in 2012 and 2013 were performed according to the following scheme: the participants were interviewed by trained interviewers using a standardised questionnaire concerning personal and occupational history, medical treatments and lifestyle habits (diet, alcohol intake, smoking, physical activity). The participants then had a physical examination, including body mass index (BMI), blood pressure and pulse measurements. Finally, their EBC was collected.

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

A total of 36 male workers were examined over 2012 and 2013. In the first year, the measurements were performed both before and after 8 h shifts in the first half of the working week. Production workers were manufacturing the TiO_2 pigment. They spent about 40% of their shifts in the close vicinity of particle emitting production units in the calcination process, in micronisation, in surface coating, in the filtration process and in the transport corridors; the remaining time was spent in the control room, separated by a closed door, where they checked the production lines remotely. Another four workers were working in the research wing of the factory. The characteristics of the subjects are given in table 1, and their exposure data and titanium concentrations in the EBC are shown in table 2.

Based on the results of the 2012 study, in the following year, only workers from the production sector of the plant with higher exposure were examined, and only postshift samples were collected. Among them, 8 (57%) production workers participated in the first and second year. A further 6 (43%) subjects joined the study in 2013. However, their mean length of

Table 1 The characteristics of groups of subjects examined in the years 2012 and 2013

	Production workers 2012+2013	Research workers 2012	Controls 2012+2013	p Value
N	32	4	45	
Age (years), mean (CI)	33.5 (29.7 to 37.3)	35.0 (23.0 to 47.0)	34.2 (31.5 to 36.9)	0.934*
Exposure to TiO_2 (years), mean (CI)	9.7 (7.0 to 12.5)	3.8 (2.2 to 5.3)	–	<0.001†
Current smoker N (%)	15 (50)	1 (75)	18 (40)	0.524‡
Alcohol user (daily) N (%)	28 (93.3)	4 (100)	45 (100)	0.187‡

*One-way ANOVA.
†Independent-sample t test.
‡χ^2 test.
ANOVA, analysis of variance; n, number of subjects; TiO_2, titanium dioxide.

exposure of 9 years (CI 3.6) was not significantly different. All workers in the study received respiratory protective devices to be used during working operations. The length of use of the devices in each shift was not recorded.

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 gave samples only once, half of them in the morning and half in the afternoon.

Ethics statement

The study was carried out according to the Helsinki Declaration. The study was approved by the Ethical Committee of the First Medical Faculty, Charles University. All participants signed an informed consent form before the beginning of the study.

Biological samples

EBC samples were collected using an Ecoscreen Turbo DECCS device, Jaeger. All subjects breathed tidally for 15 min through a mouthpiece connected to the condenser (−20°C) while wearing a nose-clip, which is in accordance with the guidelines of the American Thoracic Society.[16] A minimum constant volume of 120 L exhaled air was maintained. The samples were immediately frozen and stored at −80°C until analysis.

Titanium in EBC

TiO_2 crystallographic measurements were made with a Gemini four circle CCD diffractometer (Gemini, Oxford Diffraction, Ltd), with graphite monochromated Mo Kα radiation (k=0.71073 Å). Quantitative analyses of titanium in the EBC were performed using the ICP-MS technique. An Agilent 7900 ICP-MS Ultra HMI (UHMI) equipped with MassHunter software and an ASX-520 autosampler were used. Before measurement, the liquid samples were evaporated to dryness and mineralised 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 by the artificial addition of known amounts of TiO_2 and used for quantitative measurements. Limit of detection was 1.2 μg/L and the limit of quantitation was 4.0±0.2 μg/L. The SE was 3%.

Analysis of markers of oxidative stress in EBC

Oxidation products of nucleic acids and proteins were analysed after solid-phase extraction by liquid chromatography—electrospray ionisation—mass spectrometry/mass spectrometry (LC-ESI-MS/MS) using deuterium labelled internal standards, as was described in our earlier studies.[20 21] To exclude saliva contamination of the EBC, the concentration of α-amylase was monitored using the following procedure: the hydrolytic activity of α-amylase was determined by measuring the amount of reducing sugars generated from starch. Enzyme reactions were carried out by mixing the EBC with 1% (w/w) starch in a ratio of 1:2 at 37°C. 3,5-Dinitrosalicylic acid was added after 40 min and the reaction was terminated by heating (90°C, 5 min). The concentration of reducing sugar was determined by measuring the absorbance of the reaction product (3-amino-5-nitrosalicylic acid) at 530 nm (Rainbow Reader, SLT, Austria). The analysing personnel were blinded to the samples.

Environmental air pollution monitoring

To exclude the potential effect of environmental air pollution, the concentrations of environmental pollutants were gathered from the National Hydrometeorological monitoring system, as an association between mortality and long-term exposure to particulate matter air pollution has been reported.[22 23]

The monitoring at the station closest to the site of EBC collection (distance less than 2 km) included SO_2, NOx, O_3, $PM_{2.5}$ and PM_{10}. In 2012, NO_2 and CO had also been available. All measured concentration levels were classified as low or mild.

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 χ^2 test was used to compare frequency counts of demographic categorical variables (smoking and alcohol consumption) in the groups of production workers, research workers and controls. Differences in interval demographic variables (age, length of TiO_2 exposure) in these groups were tested using a one-way analysis of variance and independent-groups t test, respectively.

The paired t test was used to compare the concentration of titanium and markers of oxidative stress in EBC (8-OHdG, 8-OHG, 5-OHMeU, o-Tyr, 3-ClTyr, 3-NOTyr) for workers measured preshift and postshift, as well as between 2012 and 2013. The independent-groups t test was used for the following comparisons: Workers 2012 preshift versus Controls 2012, Workers 2012 postshift versus Controls 2012, Workers 2013 postshift versus Controls 2013.

Given the relatively low number of observations in the group of research workers (N=4), the non-parametric tests were used to compare levels of markers of oxidative stress in the EBC within this group of workers in 2012. In particular, a Wilcoxon

Table 2 Job descriptions and localisation of the workplaces in the subgroups of the production workers, research workers and controls; aerosol number, aerosol mass concentration and titanium concentration in 2012 and 2013 exhaled breath condensate (EBC) samples

Job title and location (year)	Job description	Number of workers (year)	Exposure per shift (hours)	Sampling duration in hours (number of samples)	SMPS (10–100 nm) Number concentration (#/cm^3×10^4)	SMPS+APS (10 nm—10 μm) Number Concentration (#/cm^3×10^4)	Total mass concentration (mg/m^3)	Titanium concentration in EBC (μg/L)	Titanium concentration in EBC (μg/L)
A Production workers—CALCINATION (2012+2013)	Controls the calcination process in the production hall	6 (2012) 7 (2013)	2.5 (31%)	6:05 (73 samples) 6:30 (78 samples)	1.97 IQR 1.49–3.89 1.51 IQR 1.20–1.98	2.94 IQR 2.16–4.63 1.93 IQR 1.63–2.41	0.64 IQR 0.46–0.86 0.36 IQR 0.30–0.42	25.67 19.57	22.09
B Production workers—MICRONISATION (2012+2013)	Controls the process of micronisation in the production hall	4 (2012) 4 (2013)	3.5 (44%)	6:25 (77 samples) 6:30 (78 samples)	1.42 IQR 1.19–2.36 2.48 IQR 1.81–3.10	2.00 IQR 1.66–2.95 2.87 IQR 2.15–3.59	0.76 IQR 0.67–0.84 0.43 IQR 0.34–0.55	23.50 19.00	19.38
C Production workers— OTHER JOBS (2012+2013)	Works in surface coating+filtration process and in transport corridors	6 (2012) 3 (2013)	3.7 (46%)	6:15 (75 samples) not measured	1.30 IQR 0.97–1.60 not measured	1.65 IQR 1.27–1.92 not measured	0.41 IQR 0.31–0.52 not measured	23.00 22.33	22.16
A,B,C Production workers—Operating room (2012+2013)	Remotely controls the work in the calcination, micronisation and other production work	16 (2012) 14 (2013)	4.3–5.5 (54–69%)	10:05 (121samples) 10:45 (129 samples)	0.23 IQR 0.16–0.32 0.29 IQR 0.27–0.32	0.45 IQR 0.31–0.63 0.49 IQR 0.47–0.52	0.13 IQR 0.096–0.221 0.050 IQR 0.045–0.056	n/a	
D RESEARCH laboratory personnel (2012)	Tests new production types on a small scale	4 (2012)	3 (37.5%)	2:25 (29 samples)	0.78 IQR 0.64–0.92	1.32 IQR 1.16–1.62	0.16 IQR 0.15–0.22	2.00	
E CONTROLS (2012+2013)	Not exposed (safety inspectors and office employees)	20 (2012) 25 (2013)	NA	NA	NA	NA	NA	1.12	

#/cm^3, particles per cm^3; APS, aerodynamic particle sizer; EBC, exhaled breath condensate; SMPS, scanning mobility particle sizer; NA, not applicable.

signed-rank test was used to evaluate the changes between preshift and postshift levels of the markers in the workers; and Mann-Whitney U test was used to compare preshift and postshift values between production and research workers.

The bivariate relationship was assessed using a Spearman correlation coefficient. Multiple regression analysis was used to predict markers of oxidative stress in the EBC by a set of predictors (TiO_2 exposure: yes/no, age, smoking: yes/no, alcohol consumption: yes/no, BMI, SO_2, NO_2, NOx, $PM_{2.5}$, PM_{10}, O_3 and CO). Statistical significance was set at $p < 0.05$. All analyses were conducted using SPSS V.22.0 (SPSS, Inc, Chicago, Illinois, USA).

RESULTS

Workplace area sampling and TiO_2 aerosol measurements

A description of the aerosol's measurements is presented in detail in our previous paper.[19] In the production 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 1.98×104 and 2.32×10^4 particles/cm^3, respectively; and about 80% of those particles were smaller than 100 nm in diameter. The detailed results from 2012 to 2013 are presented in table 2. Physicochemical characterisation of the dust collected

Online supplementary table S1 reports the punctual (30 μm spot) and average micro-XRF analysis performed on the ilmenite mineral and the dust samples from the calcination furnace and micronisation area. Online supplementary figure S1 represents the X-ray fluorescence maps of Fe, Ti, S and Si for ilmenite, and processed dust from the calcination furnace and micronisation. In these two samples, the occurrence of Ti was homogeneous due to the submicrometric grain of the material. Some iron-rich spots were visible, signalling the occurrence of unprocessed traces of ilmenite.

Subjects

The mean age, prevalence of smoking and alcohol consumption of the subjects is shown in table 1. In these parameters, the control subjects did not differ significantly from the workers.

Biological samples

Titanium in the EBC

The concentration of titanium in the EBC of production workers in 2012 was very stable; the preshift (24.1±1.8 μg/L) and postshift samples (24.1±1.9 μg/L) did not differ significantly. The levels of titanium in all groups of subjects studied are shown in table 2.

Maximal α-amylase activity in all samples did not exceed 0.1% of the saliva activity, proving the absence of saliva contamination in the EBC samples.

Markers of oxidative stress in the EBC

The markers of oxidative damage of nucleic acids and proteins in the EBC were significantly higher in the more exposed production workers (micronisation, calcination and other production jobs) than in the research workers and controls (figure 1), which is in agreement with titanium levels measured in the EBC.

In both years, all mean preshift and postshift markers of oxidative damage were significantly higher (all $p<0.001$) in the workers exposed to TiO_2 than in the controls (see online supplementary figure S2).

The comparison between the markers of oxidative stress in the production workers and less exposed research workers in 2012 is shown in online supplementary figure S3.

In the eight workers who participated in both studies, the following three markers were significantly higher in 2013: 3-NO-Tyr ($p=0.000$), 5-OHMeu ($p=0.001$) and 8-OHdG ($p=0.014$). Another three markers remained stable, and no markers significantly decreased during the 1-year interval. Titanium in the EBC of these eight workers did not significantly differ from the rest of the production workers, and their postshift concentrations were 23.9 μg/L and 21.13 μg/L ($p=0.323$) in 2012 and 2013, respectively.

No correlation was found between the demographic characteristics (age, BMI, smoking, alcohol consumption and physical activity) of the exposed workers, and the concentration of markers of oxidation of nucleic acids and proteins in their EBC samples. Additionally, no diurnal variations in the markers of oxidative stress were noted.

On the other hand, several correlations between titanium levels and several markers of oxidative stress in the preshift and postshift EBC samples existed, as shown in online supplementary table S2.

The multiple regression analysis found a statistically significant association between occupational exposure to TiO_2 and the levels of five markers in the EBC of the workers in 2012 and all six markers in the EBC of the workers in 2013 (table 3). No significant positive associations were observed between the demographic characteristics (age, smoking, drinking habits and BMI) and the level of any of the measured biomarkers. Similarly, no significant association was seen for any marker of the atmospheric pollution measured in both years and the markers of oxidative stress.

DISCUSSION

Analysis of nucleic acid markers of oxidation and proteins in the EBC samples of workers exposed to TiO_2 dust with a high proportion of nanoparticles showed significantly higher levels of markers in the workers than in the controls. Titanium originating from TiO_2 in the EBC can be seen as a marker of exposure to (nano)TiO_2, while the markers of oxidation in the EBC can reflect the biological effect of (nano)TiO_2 in the respiratory tract.

A dose–effect was seen for titanium in the EBC, as the more exposed workers from the production part of this plant had significantly higher levels of titanium than workers from the research wing of the plant. As can be seen in figure 1, the levels of oxidative stress markers are in accordance with these results.

Importantly, the elevations of both titanium and oxidative stress markers, comparing to the controls, were already found in the preshift EBC samples. Therefore, these results probably reflect a subacute or chronic biological effect related to previous shift(s). Additionally, three markers in the EBC increased during the 1-year time interval in the eight workers with repeated examination. The length of persistence of the elevation of titanium and the markers of oxidative stress in the EBC after elimination from the exposure is unknown.

No specific markers are currently available to selectively evaluate the exposure to nanoparticles in the workers. However, our data support the role of oxidative stress, proven by in vitro and in vivo experimental studies testing the effects of nano TiO_2.[3 24]

Multiple experimental studies used identical markers of oxidation of nucleic acids and/or proteins to evaluate the effect of TiO_2 nanoparticles.[25 26] A dose-dependent elevation of markers of oxidation of DNA (8-OHdG) was found in the lung tissues of mice after nasal instillation of anatase nanoparticles for 90 days.[27] The histological samples at the lowest dose used in the study (2.5 mg/kg body weight) exhibited a significant

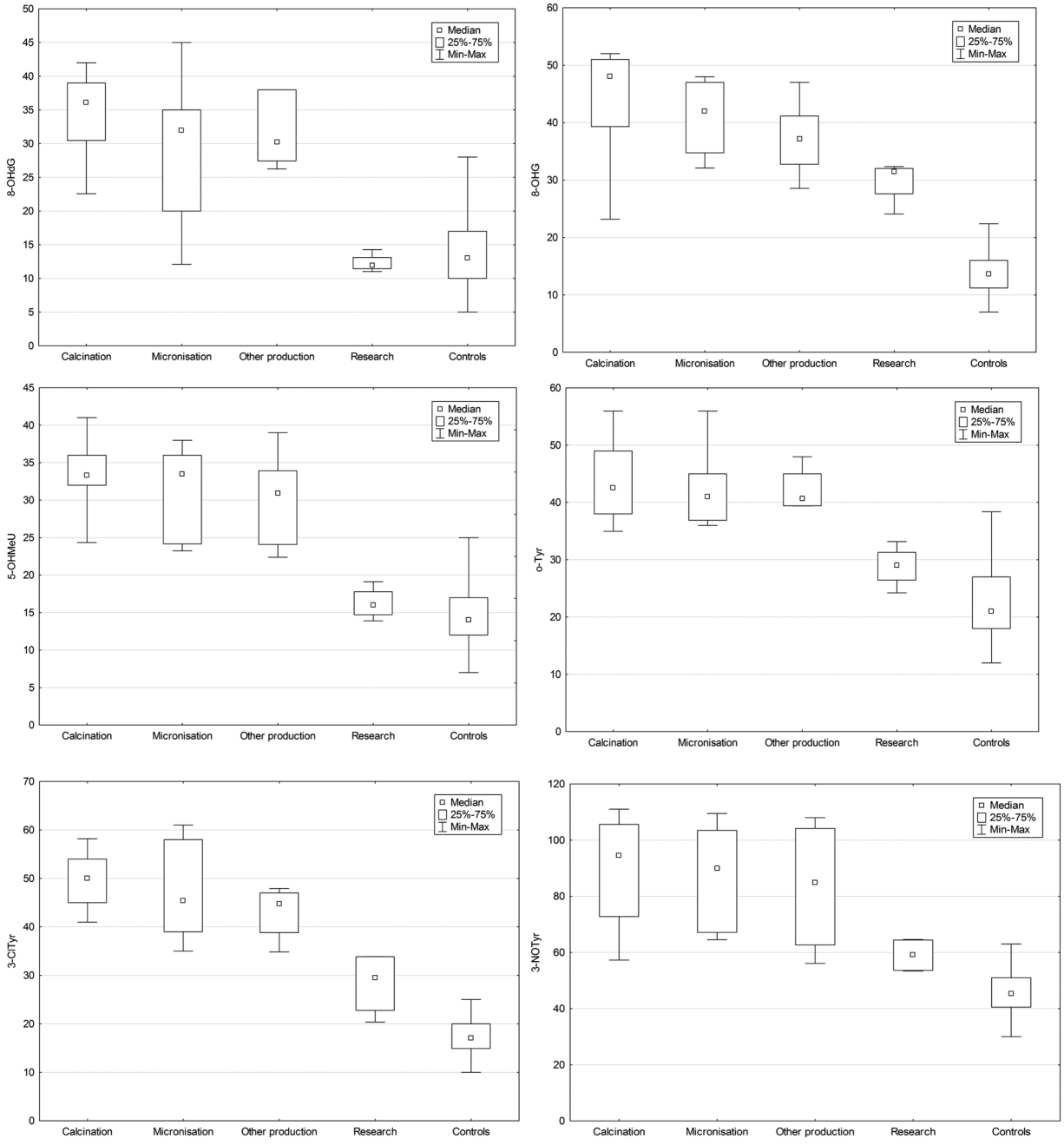

Figure 1 Markers of oxidative stress in exhaled breath condensate of the subgroups of workers and in controls in both years. 8-OHdG, 8-hydroxy-2-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; 5-OHMeU, hydroxymethyl uracil; o-Tyr, o-tyrosine; 3-ClTyr, 3-chlorotyrosine; 3-NOTyr, 3-nitrotyrosine.

shrinkage and chromatin marginalisation of the nucleus and mitochondrial swelling. In this study, the molecular mechanisms were studied in mice and alterations in the expression of 847 genes in the nano TiO_2-exposed lung tissues were found. Among 521 genes with known functions, 361 were upregulated and 160 were downregulated. The function of these genes has been associated with the immune/inflammatory responses, apoptosis, oxidative stress, cell cycle, stress responses, cell proliferation and metabolic processes. Additionally, a further 22 genes involved in oxidative stress were significantly changed in the nano-TiO_2 exposed lung.

Several studies reported the reversibility of pulmonary inflammatory/cytotoxic changes induced by (nano)TiO_2 after a single exposure or exposure lasting 2 weeks at 11.4 mg/m^3. The negative effect persisted only up to 1 week or 1 month after cessation of the exposure.[26 28]

A large follow-up study of nanomaterial-handling workers from 14 manufacturing plants[29] showed that antioxidant enzyme activity was associated with nanomaterial-handling. Recently, increased proinflammatory cytokines have been found in nanoscale carbon-black workers.[30] Another health surveillance study was carried out in workers manufacturing multi-walled carbon nanotubes,[31] where malondialdehyde levels in the EBC of workers were elevated, which was similar to our pilot results.[32] A milder, yet significant, effect was seen in workers exposed to (nano)Fe oxides during the production of Fe oxide pigments (D Pelclova, V Zdimal, P Kacer, *et al*. Oxidative stress markers are elevated in exhaled breath

Table 3 Multiple regression analysis (unstandardised regression coefficients with 95% CI in brackets) of TiO2 occupational exposure, age, smoking, alcohol consumption, BMI, environmental pollution (SO_2, NO_2, NOx, CO_3, PM_{10}) and markers in the EBC

	8-OHdG (pg/mL)	8-OHG (pg/mL)	5-OHMeU (pg/mL)	o-Tyr (pg/mL)	3-ClTyr (pg/mL)	3-NOTyr (pg/mL)
Study 2012						
TiO_2 exposure (Yes/No)	10.06* (0.35 to 19.78)	19.50*** (9.38 to 29.63)	10.17* (2.54 to 17.79)	3.42 (−6.11 to 12.95	24.83*** (13.52 to 36.14)	25.52*** (16.08 to 34.95)
Age (years)	0.04 (−0.18 to 0.26)	0.03 (−0.20 to 0.27	0.04 (−0.13 to 0.22)	−0.09 (−0.31 to 0.13)	0.20 (−0.06 to 0.46)	−0.06 (−0.28 to 0.16)
Smoking (Yes/No)	−0.64 (−5.60 to 4.32)	0.90 (−4.27 to 6.07)	0.95 (−2.94 to 4.85)	1.30 (−3.57 to 6.17)	2.89 (−2.89 to 8.66)	−4.19 (−9.01 to 0.63)
Alcohol daily (Yes/No)†	6.11 (−3.77 to 15.99)	−0.86 (−11.16 to 9.43)	0.04 (−7.71 to 7.79)	2.05 (−7.64 to 11.74)	4.99 (−6.50 to 16.49)	3.91 (−5.68 to 13.50)
BMI(kg/m^2)	0.16 (−0.27 to 0.59)	0.22 (−0.23 to 0.67)	0.09 (−0.25 to 0.43)	0.16 (−0.27 to 0.58)	−0.05 (−0.55 to 0.46)	0.20 (−0.22 to 0.62)
SO_2 ($\mu g/m^3$)	0.30 (−0.40 to 1.00)	0.59 (−0.14 to 1.32)	0.11 (−0.44 to 0.66)	0.59 (−0.10 to 1.28)	0.46 (−0.36 to 1.28)	0.38 (−0.30 to 1.06)
NO_2 ($\mu g/m^3$)‡	−0.05 (−0.17 0.08)	0.04 (−0.09 to 0.17)	0.03 (−0.07 to 0.13)	−0.05 (−0.17 to 0.08)	0.05 (−0.10 to 0.19)	0.10 (−0.03 to 0.22)
NOx	0.02 (−0.09. 0.13)	−0.01 (−0.12 to 0.10)	−0.05 (−0.14 to 0.03)	0.02 (−0.09 to 0.13)	−0.01 (−0.14 to 0.12)	0.04 (−0.06 to 0.15)
CO ($\mu g/m^3$)‡	0.00 (−0.02 to 0.01)	0.00 (−0.02 to 0.01)	0.00 (−0.01 to 0.01)	−0.01 (−0.02 to 0.00)	−0.01 (−0.02 to 0.01)	0.01 (−0.01 to 0.02)
O_3	−0.06 (−0.37 to 0.25)	−0.04 (−0.36 to 0.28)	0.15 (−0.10 to 0.39)	−0.02 (−0.32 to 0.29)	−0.04 (−0.40 to 0.32)	−0.13 (−0.43 to 0.17)
PM_{10}	−0.13 (−0.33 to 0.07)	−0.17 (−0.37 to 0.04)	0.01 (−0.15 to 0.16)	−0.04 (−0.24 to 0.15)	−0.18 (−0.41 to 0.05)	−0.14 (−0.33 to 0.05)
Study 2013						
TiO_2 exposure (Yes/No)	26.73*** (16.99 to 36.47)	27.44 ***(19.03 to 35.85)	18.63*** (11.41 to 25.85)	32.69*** (24.84 to 40.54)	21.26*** (10.67 to 31.86)	47.55*** (30.31 to 64.78)
Age (years)	0.09 (−0.11 to 0.28)	−0.05 (−0.22 to 0.12)	0.05 (−0.10 to 0.19)	0.02 (−0.13 to 0.18)	0.08 (−0.14 to 0.29)	−0.11 (−0.46 to 0.24)
Smoking (Yes/No)	0.98 (−2.69 to 4.66)	0.04 (−3.13 to 3.21)	0.15 (−2.57 to 2.88)	0.10 (−2.87 to 3.06)	−2.15 (−6.15 to 1.84)	0.57 (−5.93 to 7.08)
BMI (kg/m^2)	0.03 (−0.42 to 0.48)	0.31 (−0.08 to 0.69)	0.14 (−0.19 to 0.47)	−0.15 (−0.51 to 0.21)	0.24 (−0.24 to 0.73)	−0.09 (−0.88 to 0.70)
SO_2 ($\mu g/m^3$)	0.05 (−0.11 to 0.20)	−0.03 (−0.16 to 0.10)	−0.05 (−0.16 to 0.07)	−0.11 (−0.23 to 0.01)	−0.08 (−0.25 to 0.08)	0.15 (−0.13 to 0.42)
NOx	0.03 (−0.03 to 0.09)	−0.02 (−0.07 to 0.03)	0.00 (−0.05 to 0.04)	0.01 (−0.04 to 0.06)	−0.01 (−0.08 to 0.05)	−0.03 (−0.13 to 0.08)
O_3	0.10 (−0.01 to 0.21)	−0.05 (−0.15 to 0.04)	−0.03 (−0.11 to 0.05)	0.06 (−0.03 to 0.15)	−0.07 (−0.19 to 0.06)	−0.09 (−0.29 to 0.10)
PM_{10}§	−0.28 (−0.58 to 0.01)	0.10 (−0.15 to 0.36)	0.03 (−0.19 to 0.25)	−0.16 (−0.40 to 0.08)	0.30 (−0.02 to 0.62)	0.14 (−0.38 to 0.66)

*($p<0.05$), *** ($p<0.001$).
†Alcohol consumption was a constant in 2013, therefore it was omitted.
‡CO, NO_2 were available only in 2012.
§$PM_{2.5}$ was excluded from the table due to the high correlation with PM_{10}.
3-ClTyr, 3-chlorotyrosine; 3-NOTyr, 3-nitrotyrosine; 5-OHMeU, 5-hydroxymethyl uracil; 8-OHdG, 8-hydroxy-2-deoxyguanosine; 8-OHG, 8-hydroxyguanosine; (all postshift): BMI, body mass index; EBC, exhaled breath condensate; o-Tyr, o-tyrosine; PM, particulate matter.

condensate of workers exposed to nanoparticles during iron oxide pigment production. *J Breath Res*. Submitted).

The consequence of exposure to nanoparticles in humans has not yet been elucidated. It has been reported that ROS/RNS formation and oxidative stress carcinogenesis is associated with several chronic oxidative conditions, including inflammation, infection and exposure to asbestos or silica.[33 34 35] High concentrations of 8-OHG and 8-OHdG were determined not only in the body fluids or lung tissues in subjects with chronic obstructive pulmonary disease and high air pollution exposure, but also in connection with age-related and/or degenerative diseases, such as type II diabetes, hypertension and several types of cancer.[36] For example, 5-OHMeU has been used as a marker of cardiovascular diseases.[37] A higher concentration of 8-OHG, 8-OHdG, 3-ClTyr and 3-NOTyr was found in patients with Alzheimer's disease, and increased levels of o-Tyr were measured in blood plasma and urine of patients with type II diabetes. Higher plasma or urine levels are related to systemic oxidative stress and increased EBC levels are related to local effects in the respiratory tract.[38]

NIOSH has determined that exposure to ultrafine TiO_2 should be considered a potential occupational carcinogen and recommends an exposure limit of 300 $\mu g/m^3$.[39]

The advantage of our study was the examination of subjects with different levels of exposure to TiO_2 in one industrial plant; and the repetition of the study, which confirmed the first results. Additionally, laboratory proof of the presence of both anatase and rutile in workplace dust was provided.

Measuring titanium in EBC provides this study with unique strengths and offers benefits, as these methods have the potential to provide exposure markers for this industry. This measurement supports the determined oxidative stress effects in EBCs. It is also more reliable than measuring external exposures, as it reflects the internal doses and may point to personal protective equipment failures.

Study limitations

There are several limitations in this study. First is the low number of exposed workers available for the statistical analysis. This is characteristic for production plants using nanotechnologies, as they usually employ few workers. Additionally, there is no motivation for employers to participate in such studies. This is the main reason for the paucity of data concerning workers.[40] Even so, a high statistical significance was found by our study due to the large difference between the level of oxidative stress markers in exposed and control workers.

Another limitation was that direct reading instruments had to be used to measure the workplace aerosol instead of the use of personal samplers. Therefore, to exclude potential external contamination, we thoroughly mapped the workplace, including time integrated area sampling. Chemical analysis of the dust from the filters could not be carried out; however, physicochemical analysis of the sedimented dust from the two workspaces with the highest aerosol concentrations confirmed the presence of rutile and/or anatase,[19] and a minor presence of Fe and other elements. This is in agreement with the particles of TiO_2 found in the preshift and postshift EBC of the workers.[19] The minor proportion of iron in workplace dust does not appear to be the main cause of elevated oxidative stress markers, as in our recent study of workers exposed to (nano) iron oxides, the oxidative stress markers in the postshift EBCs were lower (Pelclova, *et al*, submitted) than in workers producing TiO_2. This hypothesis is in agreement with the lower effect of iron oxides than TiO_2 in the study of Hsieh *et al*.[5]

Another limitation was that the use and effectiveness of personal protective equipment was not the focus of this study. All the workers had devices available; however, the proportion of time these devices were used per shift was not recorded.

The sampling of EBC in the control subjects was carried out only once. Nevertheless, this does not appear important, as no diurnal variations were found in this study. Therefore, EBC marker elevations in the TiO_2 production workers cannot be attributed to diurnal variation.

The potential influence of local environmental air contamination and dating of sample collection was excluded. The multiple regression analysis did not find any association between the examined EBC markers and environmental air pollution.

CONCLUSIONS

The need for data concerning the risk evaluation of nanoparticle exposure for workers has been postulated for more than 10 years; however, until now, very limited data have been obtained from the most exposed subjects—workers.[40] Our study tries to fill this gap. To the best of our knowledge, this is the first study focusing on markers of oxidative stress in workers exposed to relatively high levels of TiO_2 nanoparticles; we proved a significant elevation of markers of oxidation of nucleic acids and proteins in the EBC of these subjects. Our findings also support a dose-dependent biological effect of occupational exposure to TiO_2 nanoparticles, as the results were more elevated in workers with higher exposure. Owing to the low toxic effects of the coarse TiO_2 particles, these effects can be attributed to nanoparticles.

Even if the nucleic acids and protein oxidation markers found in the EBC of workers are non-specific regarding exposure to nanoparticles, their effects were consistent and were proven repeatedly. The preshift biomarkers in the EBC have already been elevated, which supports the possibility of a subacute or chronic effect of TiO_2 nanoparticles. However, both the reversibility and potential of the human body to suppress these biological effects are still unknown.

No guidelines outlining specific testing in workers exposed to nanomaterials are available, and the physical examination and lung function testing may not detect initial biological effects.[19] In addition to the results of the first human studies, the non-invasiveness of EBC collection and analysis make this method potentially useful in the surveillance of workers exposed to nanoparticles.[40] Markers of exposure and effects in EBC should be further evaluated for their practical use in the health surveillance of workers.

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Contributors DP designed the study, participated in the field study and drafted the manuscript. VZ, JS, NZ and OM carried out the air monitoring. VZ participated in the design and coordination of the study. PK and KS analysed the biological samples. ZF, MM and SV participated in the field study. FT and IC performed the physico-chemical characterisation of the deposited dust samples. MK, TN and JB performed the statistical analysis and a graph. SZ was involved in revising the manuscript critically for important intellectual content. All the authors read and approved the final manuscript.

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Markers of oxidative damage of nucleic acids and proteins among workers exposed to TiO 2 (nano) particles

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Notes