

## MARKERS OF OXIDATIVE STRESS ARE ELEVATED IN WORKERS EXPOSED TO NANOPARTICLES

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### Abstract

Health-effects of nanoparticles in humans are little understood. Pre-shift and post-shift spirometry, markers in exhaled breath condensate (EBC), and fractional exhaled nitric oxide (FeNO) were measured both in 20 workers exposed to TiO<sub>2</sub> aerosol and 19 controls. Particle number size distributions were monitored by aerosol spectrometers SMPS and APS, covering the overall size range 15 nm-10 µm. Simultaneously, the spatial distributions of total particle number and mass concentrations were determined using a particle number concentration monitor (P-TRAK) and a monitor of particle mass concentrations (DustTRAK DRX).

Malondialdehyde (MDA), 4-hydroxy-trans-nonenal (HNE), 4-hydroxy-trans-hexenal (HHE), 8-isoProstaglandin F<sub>2α</sub> (8-isoprostane), 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), 5-hydroxymethyl uracil (5-OHMeU), o-tyrosine (o-Tyr), 3-chloro-tyrosine (3-Cl-Tyr), nitrotyrosine (NO-Tyr), and leukotrienes (LTs) were analyzed after solid-phase extraction by LC-ESI-MS/MS.

Total aerosol concentrations in the production plant varied greatly in both space and time; number concentrations 1x10<sup>4</sup>-2x10<sup>5</sup> particles/cm<sup>3</sup> and mass concentrations 0.1-30 mg/m<sup>3</sup>. In the workshops, 90% of the particles were smaller than 100 nm in diameter and particle concentrations were 10x higher than in the control room.

All pre-shift and post-shift EBC markers were higher in the workers, excepting pre-shift LT D4. Markers of lipid oxidation were elevated (p<0.001): MDA, HNE, HHE, and 8-isoprostane. Markers of oxidation of nucleic acids and proteins: 8-OHdG, 8-OHG, 5-OHMeU, 3-Cl-Tyr, NO-Tyr, o-Tyr; and LT B4, C4 and E4 were increased (p<0.001). In the workers, EBC pH was lower and FeNO elevated in both samples. This first in-vivo study in workers suggests adverse effects of chronic exposure to TiO<sub>2</sub> aerosol including nano-sized fractions.

**Keywords:** Nanoparticles, exhaled breath condensate, exhaled nitric oxide, oxidative stress, occupational exposure

### 1. INTRODUCTION

The possible adverse health-effects of nanoparticles are as yet little understood [1]. Therefore, this pilot study was performed in workers exposed to TiO<sub>2</sub> aerosol. Experimental studies currently available highlight the proof of biological effects of nanoparticles on the respiratory system with the generation of oxidative stress, pro-inflammatory effects and a possible development of fibrosis, pulmonary emphysema and/or cancer. There is extensive experimental evidence that oxidative damage permanently occurs to lipids of

cellular membranes, proteins, and nucleic acids [2]. In nuclear DNA, 8-hydroxy-2'-deoxyguanosine (8-OHdG) or 8-oxo-7,8-dihydro-2'-deoxyguanosine (8-oxodG) are the predominant forms of free radical-induced oxidative lesions, and has therefore been widely used as a biomarker for oxidative stress and carcinogenesis. Experts' forecast of the European Risk Observatory of the European Agency for Safety and Health at Work (EU-OSHA) puts nanoparticles at the top of the list of emerging risks. No human studies are available so far; therefore, no recommendations are available concerning specific examinations of the workers exposed to dust containing nanoparticles [3]. Non-invasive methods would be the methods of choice. Therefore, the analysis of markers of oxidative stress and inflammation in the exhaled breath condensate (EBC) [4] and the measurement of fractional exhaled nitric oxide (FeNO) [5] as well-tolerated and repeatedly performed methods have been chosen in this study.

## 2. METHODS

The dynamics of aerosol particle number size distributions (PSD) at the workplace was monitored by SMPS and APS aerosol spectrometers sampling synchronously with 5 min time resolution and covering the overall size range from 15 nm to 10  $\mu\text{m}$  in particle diameter with size resolution of 32 channels/decade. Simultaneously, the spatial distributions of total particle number and mass concentrations, respectively, had been determined using a portable particle number concentration monitor (P-TRAK) and a portable monitor of particle mass concentrations (DustTRAK DRX), respectively. During the shifts, measurements of highly time- and size-resolved aerosol concentrations were carried out.

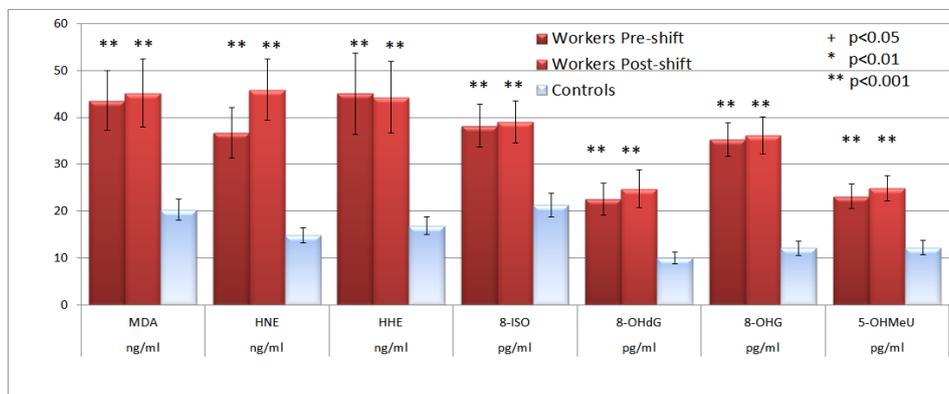
Pre-shift and post-shift spirometry, markers in exhaled breath condensate (EBC) [4], and fractional exhaled nitric oxide (FeNO) [5] were examined in 20 workers (males, mean age  $33.7 \pm 5.2$  years, 11 smokers, 9 nonsmokers) and 19 controls (males, mean age  $34.8 \pm 5.0$  years, 11 smokers, 8 nonsmokers).

Malondialdehyde (MDA), 4-hydroxy-trans-nonenal (HNE), 4-hydroxy-trans-hexenal (HHE), 8-isoProstaglandin  $F_{2\alpha}$  (8-isoprostane), 8-OHdG, 8-OHG, 5-hydroxymethyl uracil (5-OHMeU), o-tyrosine (o-Tyr), 3-chloro-tyrosine (3-Cl-Tyr), that serves as specific molecular marker for production of chlorinating oxidants by the eosinophil peroxidase and myeloperoxidase systems of leukocytes; nitrotyrosine (NO-Tyr), and leukotriene (LT) B<sub>4</sub>, C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub> were analyzed after solid-phase extraction (SPE) by liquid chromatography - electrospray ionization - mass spectrometry/mass spectrometry (LC-ESI-MS/MS) [6, 7], in pre-shift and post-shift EBC samples collected using Ecoscreen, Jaeger. FeNO was measured by Hypair FeNO Medisoft. In the workers the measurements were performed both before and after 8h shifts. The study was approved by the Ethical Committee of the 1<sup>st</sup> Medical Faculty, Charles University. Statistical evaluation using following basic statistical tests was performed: test of normality of distribution, arithmetic mean, standard deviation, skew, median, modus, Student's t-test for equal variances and for equal means, F-test, confidence intervals. Statistical significance was set at  $p \leq 0.05$ .

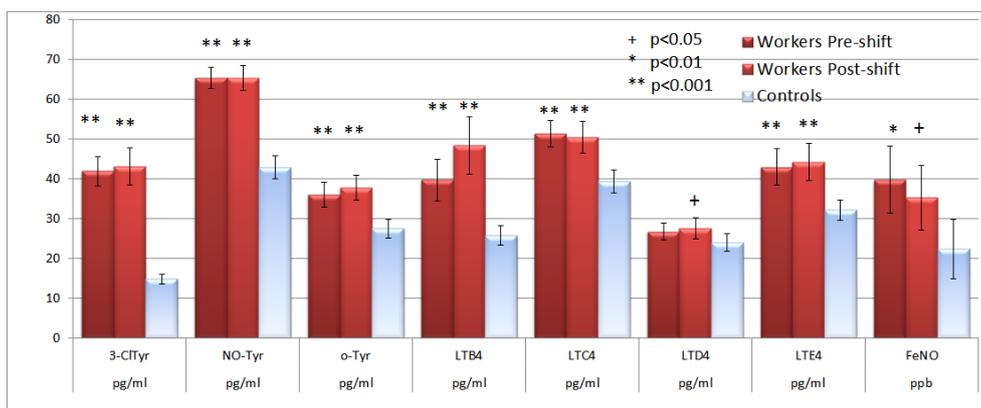
## 3. RESULTS AND DISCUSSION

Measurements in the production plant showed that total aerosol concentrations varied greatly in both space and time; number concentrations were found in the interval from  $1 \times 10^4$  to  $2 \times 10^5$  particles/ $\text{cm}^3$ , mass concentrations varied between 0.1 to 30  $\text{mg}/\text{m}^3$ . According to their time-sheets, the workers under the study spent about 1/3 of their shifts in the vicinity of particle emitting production units, while they were sitting in the control room and checking the production lines remotely in the remaining time. Concerning the control room, most of aerosol particles were transported indoors from the ambient air, with number concentrations between  $3 \times 10^3$  and  $1 \times 10^4$  particles/ $\text{cm}^3$ ; most of these particles being found in the accumulation mode centered around 100 nm in particle diameter. At the other three monitoring positions located close to production units, total particle concentrations were found to be one order of magnitude higher than in the control room. Furthermore, over 90% of those particles were smaller than 100 nm in diameter having the ability to penetrate deeply into the alveolar region of the respiratory tract.

Markers of oxidative stress of lipids and nucleic acids are shown in **Fig. 1**; markers of oxidative/nitrosative stress of proteins, LTs in the EBC and FeNO are presented in **Fig. 2**.



**Fig. 1:** Malondialdehyde (MDA), 4-hydroxy-trans-nonenale (HNE), 4-hydroxy-trans-hexenal (HHE), 8-isoprostaglandin  $F_{2\alpha}$  (8-isoprostane, 8-ISO), 8-hydroxy-2-deoxyguanosine (8-OHdG), 8-hydroxyguanosine (8-OHG), hydroxymethyl uracil (5-OHMeU) in the exhaled breath condensate in 20 workers (pre-shift and post-shift) and 19 controls. The symbols \*\* denote the significance levels of data equivalency gained from Pre-shift and Controls and Post-shift and Controls. The bars denote the significance levels  $\alpha=0.05$ .



**Fig 2:** 3-chloro-tyrosine (3-Cl-Tyr), nitrotyrosine (NO-Tyr), o-tyrosine (o-Tyr), leukotrienes (LTB<sub>4</sub>, C<sub>4</sub>, D<sub>4</sub> and E<sub>4</sub>) in the exhaled breath condensate and fractional exhaled nitric oxide (FeNO) in 20 workers (pre-shift and post-shift) and 19 controls. The symbols \*\*, \*, +, and – denote the significance levels of data equivalency gained from Pre-shift and Controls and Post-shift and Controls. The bars denote the significance levels  $\alpha=0.05$ .

As can be seen in **Fig. 1** and **Fig. 2**, the level of all pre-shift markers studied in the EBC, except pre-shift LT D<sub>4</sub>, were significantly increased in the workers than controls. Post-shift EBC markers in the workers were usually slightly higher, comparing to the pre-shift markers, however no statistical difference was noted, excepting for HNE ( $p<0.05$ ).

Although the breathing volume and EBC volume did not differ significantly between the groups of subjects, EBC pH in the workers was significantly lower ( $p<0.05$ ) both pre-shift (4.83) and post-shift (4.96) than in the controls, where 5.74 was found. Also pre-shift ( $p<0.003$ ) and post-shift ( $p<0.023$ ) FeNO was higher in the workers than in controls.

When the group of workers and controls were divided into the subgroups of smokers and non-smokers, FeNO in both workers and controls was lower; however, the difference was significant only in the controls, as shown in **Tab. 1**.

**Tab. 1:** Fractional exhaled nitric oxide (FeNO, ppb) in the subgroups of non-smokers and smokers in the workers (pre-shift and post-shift) and controls. (N.S. not significant,  $p \geq 0.05$ )

	Controls	Workers Pre-shift	Workers Post-shift
Non-Smokers	29±12	49±17	42±16
Smokers	13.8±4.2	32.0±6.7	29.6±8.4
p	0.025	N.S. (0.063)	N.S. (0.13)

LT B4 was the only marker increased in smoking workers vs. non-smoking workers in both pre-shift ( $44.2 \pm 7.7$  vs.  $34.1 \pm 6.2$  pg/ml,  $p < 0.05$ ) and post-shift samples ( $55.0 \pm 10.0$  vs.  $39.7 \pm 9.0$  pg/ml,  $p < 0.05$ ). LT C4 was elevated in smokers vs. non-smokers in post-shift samples only ( $54.0 \pm 5.7$  vs.  $46.0 \pm 5.2$  pg/ml,  $p < 0.05$ ).

In vitro studies show that nanoparticles generate reactive oxygen species, deplete endogenous antioxidants and produce oxidative damage to lipids, proteins and nucleic acids. Surface area, reactivity and chemical composition play important roles in the oxidative potential of particulates [8, 9]. Due to its apparent simplicity and non-invasiveness [10], the EBC analysis is a promising technique, that brought positive results in subjects exposed to carcinogenic dusts in the past [11]. However, being not yet standardized [12], it is considered only a research method. Potential confounders were eliminated in our study as much as possible by the selection of the control subjects matched by sex, age and smoking status, and pre-shift and post-shift EBC collection. According to the methodological recommendations [10], identical breathing volume (120 liters) was maintained in all subjects; nose breathing was eliminated by nose clips and salivary contamination by amylase analysis. Additionally, the collection of EBC was performed in a clean part of the TiO<sub>2</sub> producing plant, therefore TiO<sub>2</sub> contamination from the working environment had been eliminated in all pre-shift samples. However, the impact of chronic contamination of the airways and lungs of the workers cannot be fully excluded and will be further studied by TiO<sub>2</sub> analysis in the EBC samples. Obviously, the smoking status did not play any important role. The effect of smoking in the workers was seen solely as the LT B4 elevation. It is in agreement with other studies [13]. FeNO, on the other hand, is a standardized method for clinical use [14], measuring NO as a biological mediator implicated in the pathophysiology of lung diseases. The cut point of 25 ppb enables us to diagnose subjects with asthmatic airways inflammation. Recently, inflammation in the lung parenchyma has been associated with increase of FeNO levels [5]. Therefore, both the airways and lungs could have been affected by TiO<sub>2</sub> aerosol. Smoking as a factor, well known to depress FeNO, reduced it in the smokers, both workers and controls; nevertheless this biological marker of inflammation was significantly higher in both pre-shift and post-shift samples of workers, exposed to TiO<sub>2</sub> aerosol.

To our knowledge, this is the first study performed *in vivo* in workers exposed to aerosol containing nano-sized particles showing a positivity of markers of inflammation and of oxidative/nitrosative stress in the lipids, proteins, and nucleic acids. Almost all parameters were elevated in the workers already before the shift and no further increase due to 8h working shift in the working environment was seen. This fact may point to subacute or chronic influence, rather than acute changes.

#### 4. CONCLUSIONS

This pilot study suggests the possibility of biological effects of chronic TiO<sub>2</sub> exposure to aerosol particles containing coarse, fine and nano-sized fractions. The results indicate that oxidative stress and elevated levels of oxidatively altered biomolecules are important intermediate endpoints that may be useful markers in

hazard characterization of particulates. We used non-invasive methods, potentially applicable in the industry. In order to evaluate the effects in similar environments, a broader scale study would be needed.

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