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Occupational exposure to nanomaterials and biomarkers in exhaled air and urine: Insights from the NanoExplore international cohort

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ABSTRACT

The current evidence on nanomaterial toxicity is mostly derived from experimental studies making it challenging to translate it into human health risks. We established an international cohort (N = 141 workers) within the EU-LIFE project “NanoExplore” to address possible health effects from occupational exposures to nanomaterials. We used a handheld direct-reading optical particle counter to measure airborne nanoparticle number concentrations (PNC) and lung-deposited surface areas (LDSAs). Airborne particles were characterized by TEM and SEM-EDAX. We assessed oxidative/nitrosative stress with a panel of biomarkers in exhaled breath condensate (EBC) (8-isoprostane, malondialdehyde, nitrotyrosine), inflammation (high-sensitivity C reactive protein (hs-CRP), IL-1 β , TNF- α , IL-10) and KL-6 (considered as biomarker of interstitial lung fibrosis) and urine (total antioxidant power (TAP), 8-isoprostane, and malondialdehyde). Exhaled breath sampled in gas-sampling bags were assessed for oxidative potential. These biomarkers were quantified pre-shift at the beginning of the workweek and post-shift the 4th day. Relationships between airborne nanoparticle concentration and biomarkers were assessed by multiple linear regression with log-transformed exposure and biomarker concentrations adjusted for potential confounders. We found a positive dose–response relationship for three inflammation biomarkers (IL-10, IL-1 β and TNF- α) in EBC with both PNC and LDSA. A negative dose–response relationship was observed between PNC and TAP. This study suggests that occupational exposures to nanoparticles can affect the oxidative balance and the innate immunity in occupationally exposed workers. However, owing to the intrinsic variability of biomarkers, the observed changes along with their health significance should be assessed in a long-term perspective study.

1. Introduction

Nanotechnology is defined as a range of applications manipulating the matter on a nanoscale to generate new materials (Institute, 2020). For the past two decades, nanotechnology has rapidly developed in a

wide range of sectors including medicine (Leucuta, 2010), pharmaceuticals (Jeelani et al., 2020), electronics, and construction (Pacheco-Torgal et al., 2019) driven by the unique physical–chemical properties of nanomaterials (Ellenbecker, 2015). However, the introduction of nanotechnology in various industrial sectors and applications rises the

Abbreviations: 8-OHdG, 8-hydroxy-2'-deoxyguanosine; CNT, carbon nanotubes; DAG, directed acyclic graph; EBC, Exhaled Breath Condensate; hs-CRP, high-sensitivity C reactive protein; IARC, International Agency for Research on Cancer; IL- β , interleukin beta; IL-10, interleukin 10; LDSA, lung-deposited surface area; KL-6, Krebs von den Lungen glycoprotein 6; MDA, malondialdehyde; NMs, nanomaterials; OPEA, Oxidative potential in the exhaled air; PPE, personal protective equipment; PNC, particle number concentration; ROS, reactive oxygen species; TAP, total antioxidant power; TNF- α , Tumor necrosis factor alpha.

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concerns for workers' health. Several occupational hygiene studies have shown that nanomaterials may be generated by various industrial operations and suggested that occupational exposure to nanomaterials is underestimated (Audignon-Durand et al., 2021). For example, exposure to carbon nanotubes (CNT) have been reported for workers in research and development (R&D), pilot-scale manufacturing, and industrial primary and secondary manufacturing activities where CNTs are produced or incorporated into other materials (e.g.e.g., textile, lithium batteries, and composites for aerospace vehicles) (Guseva Canu et al., 2016; Bergamaschi et al., 2021; Guseva Canu et al., 2020). Moreover, recent studies have shown that the release of nanoparticles originating from the handling of conventional micron-sized materials may be substantial. As a result, during material processing, workers are exposed to a heterogeneous mixture of particles with different sizes and physical chemical properties making the quantitative exposure characterization as well as the risk assessment challenging (Viitanen et al., 2017).

In vivo and *in vitro* experimental studies have demonstrated that oxidative stress, genotoxicity, and inflammation are mechanisms of action (MoA) for the observed adverse effects of nanomaterials (Ou et al., 2016; Mendoza and Brown, 2019). The generation of oxidative stress can be mediated by a direct formation of free radicals (i.e., reactive oxygen species (ROS)) (Mendoza and Brown, 2019; Pratsinis et al., 2013), which has been observed for soluble metal-based nanomaterials that generated intracellular ROS as a result of a Fenton-like reaction (Valko et al., 2005). The free radicals may also be formed indirectly via activation of the redox machinery of the cell, particularly in mitochondria (Shvedova et al., 2012). Free radicals then react with several cellular structures, including proteins, lipids, and nucleic acids, thus inducing oxidative damage and depletion of antioxidants (Du et al., 2012; Song et al., 2012). Nanoparticle and nanofiber sizes are comparable to those of viruses and bacteria (Shvedova et al., 2010). Once in the body, they are recognized by the immune system (e.g., macrophages) that triggers the production of different signaling cytokine proteins including interleukin 10 (IL-10), interleukin 1 beta (IL-1 β), or tumor necrosis factor alpha (TNF- α) (Borgognoni et al., 2018). Nanoparticles can cross biological membranes (including the blood-brain barrier) and are thus able to translocate into several organs where they exert persistent pro-oxidant and inflammatory effects (Oberdörster et al., 2005). A persistent chronic inflammation status in the airways would compromise epithelial integrity towards many other sensitizing agents, such as viruses, and promote aberrant tissue remodeling that could potentially lead to pulmonary fibrosis (Byrne and Baugh, 2008).

Inhalation is the main exposure pathway for nanoparticles and nanofibers in workers (Oberdörster et al., 2005; Borm et al., 2006). Consequently, nanomaterial exposure could play a key-role in the occurrence of chronic respiratory diseases including cancer (Schulte et al., 2019; Zhang et al., 2014). On the basis of the available evidence, the International Agency for Research on Cancer (IARC) classified black carbon and the Mitsui-7 CNTs as possibly carcinogenic to humans (Group 2B) (Baan, 2007). Although, titanium dioxide (TiO₂) was also classified as an IARC 2B carcinogen, its consideration under the European Regulation for Classification, Labeling and Packaging of chemicals is still debated due to inconsistent experimental (Charles et al., 2018; Yamano et al., 2022) and epidemiological evidence (Guseva Canu et al., 2020; Guseva Canu et al., 2022).

The epidemiological evidence regarding health effects in humans due to exposure to nanomaterials (summarized in Supplementary material Table S1) is currently limited (Schulte et al., 2019). Epidemiologists face scientific, methodological, political and regulatory challenges in identifying and enrolling sufficient participants in a cohort (Guseva Canu et al., 2018). Exposure assessors need to find new methods as conventional mass dose-based airborne concentration is insufficient for quantitative exposure assessment of nanomaterials. The conventional methods do not account for nanoparticle reactivity due to their large surface and number per mass when compared with large particles. The

emission potential parameters are often evaluated alone and cannot predict workers' individual exposures (Bergamaschi et al., 2015). Moreover, workers handle several types of nanomaterials simultaneously to a varying degree and/or during a short time. This heterogeneity is rarely assessed in published studies (Liou et al., 2015; Lee et al., 2015). Therefore, many human studies on nanomaterials are cross-sectional, based on a small study sample, and exploratory in nature (Schulte et al., 2019).

We established an international cohort (N = 141 workers) within the EU-LIFE "NanoExplore" project to address possible health effects from occupational exposures to nanomaterials and developed a harmonized protocol integrating quantification of airborne nanoparticles and biomarkers in EBC and urine (Guseva Canu et al., 2023). We present here dose-response associations between nanomaterial exposures expressed as either particle number concentration (PNC) or lung deposited surface area (LDSA) and relevant biomarkers.

2. Material and methods

2.1. Study design and participants

This multicenter prospective cohort study is based on a harmonized protocol detailed elsewhere (Guseva Canu et al., 2023). The study involved three NanoExplore project partner-countries: Switzerland, Italy, and Spain. In each country, the participating companies were enrolled based on a confirmed prior knowledge of their activities related to the handling of nanomaterials. Two companies did not conduct nanomaterial-related activities and workers at these companies served as non-exposed workers (controls) in our study. A walk-through visit was conducted for the five companies handling nanomaterials by the company's environmental health and safety professional with an occupational hygienist associated with this study. Workers identified as potentially exposed and non-exposed were invited to participate in the study and workers who provided a written consent for participation were included in the cohort.

2.2. Nanomaterial exposure assessment

Airborne nanoparticles were measured over a work shift (6–8-hour) for two or four consecutive days depending on the facility (Table 1) using a direct reading optical particle counter, the "DiSCminiTM" (Testo, Mönchaltorf, Switzerland). DiSCmini devices were placed in close proximity to the workstations. The DiSCmini measures the particle number concentration (PNC) (#/cm³) within a detection range of 500–1,000,000 #/cm³, the particle size (particles of aerodynamic diameter ranging from 10 to 300 nm) and the lung-deposited surface area (LDSA) ($\mu\text{m}^2/\text{cm}^3$), with a time resolution of 1 s. The LDSA corresponds to the probability of particle deposition in the tracheobronchial and alveolar regions of the lung (Kuuluvainen et al., 2016), and is considered the most relevant exposure metric with respect to nanoparticles' toxicity (Oberdörster et al., 2005; Schmid and Stoeger, 2016).

Participants' work histories, nanomaterials used at work as well as exposure to nanomaterials from other sources were collected with two questionnaires, one completed by the company managers before the visit and another completed by participants at the beginning of the field campaign. We confirmed the presence of the reported nanomaterials handled at each company site by analyzing filters held in the particle head sampler of the NanoExplore kit by transmission electronic microscopy (TEM) for size and shape and further by the energy dispersive X-ray (EDAX) for elemental analysis (Supplementary Material Figure S1).

2.3. Biological sampling and biomarker quantification

Biological samples were collected twice: 1st day of the campaign week pre-shift and post-shift either the second or fourth day. Exhaled breath was collected from participants using a gas-sampling bag (1L,

Table 1
Work activities related to nanomaterials by participating company and country as well as air and biomarker sampling schemes.

Center	Main activity	Country	Nanomaterial activities	Nanomaterial type	Air sampling duration	Biomarker collection day
1	Academic research (no chemical exposure)	Switzerland	-	Indoor environmental background	4 days Monday to Thursday	Monday - Thursday
2	Academic research (no chemical exposure)	Italy	-	Indoor environmental background	4 days Monday to Thursday	Monday - Thursday
3	Production of paints and coatings	Italy	Downstream user, Research and Development, Maintenance of equipment	Calcium carbonate, siliceous sands, cellulose powder, titanium dioxide, talc and other oxides, Black CarbonAL, Fe	2 days Monday -Thursday	Monday - Thursday
4	Production of adhesives and paints	Italy	Downstream user, Research and Development, Maintenance of equipment	Calcium carbonate, siliceous sands, cellulose powder, titanium dioxide, talc and other oxides, Black CarbonAL, Fe	2 days Tuesday -Friday	Tuesday - Friday
5	Construction chemicals	Italy	Downstream user	Cements, siliceous sands, calcium carbonate, natural limes, Black Carbon	2 days Monday -Thursday	Monday - Thursday
6	Construction chemicals	Italy	Downstream user	Cements, siliceous sands, silicon carbonate, calcium carbonate, natural limes	2 days Tuesday -Friday	Tuesday - Friday
7	Research institute in nanoscience and nanotechnology	Spain	Research and development	AgNPs, AuNPs, BaTiO ₃ , FeO, biodegradable polymers, methacrylate and acrylate polymers, Al ₂ O ₃ , SiO ₂ , ZnO, graphene, carbon nanotubes, black carbon	4 days Monday - Tuesday - Thursday - Friday	Monday - Thursday - Friday

TEDLAR, CELScientific corp., Cerritos, USA). The bags were immediately connected to the oxidative potential of exhaled air (OPEA) analyzer (i.e., a colorimetric measurement based on a photonic approach with multi-scattering absorbance enhancement) (Goekce et al., 2022). OPEA results are expressed as a dimensionless ratio between the oxidative potential of the exhaled air (OP_{exh}) and the oxidative potential of the ambient air (OP_{amb}) (Guseva Canu et al., 2022).

EBC samples were collected using a portable collection device (Turbo-DECCS™, Medivac, Parma, Italy) according to recommendations of the American Thoracic Society and the European Respiratory Society Task Force (Horváth et al., 2017). A flow meter (VOLMET™ 20 Medivac, Parma, Italy) was used to normalize the volume of exhaled air collected from different subjects. A volume of 2–3 mL of EBC was collected from each participant (sampling time ~ 15 min). EBC and spot urine samples

were immediately aliquoted and frozen at -20 °C (5 days) before being stored at -80 °C until analysis. All EBC and urine samples were transported and analyzed in the same laboratory. In EBC, we measured 8-isoprostane, malondialdehyde (MDA), nitrotyrosine (considered as biomarkers of oxidative/nitrosative stress), hs-CRP, IL-1β, TNF-α, IL-10 (considered as biomarkers of inflammation), and the Krebs von den Lungen glycoprotein 6 (KL-6), considered as a biomarker of early lung fibrosis. In urine, we measured total antioxidant power (TAP), 8-isoprostane and malondialdehyde. Urinary creatinine concentration was measured according to the kinetic Jaffé procedure to normalize the concentration of urinary biomarkers to urinary volume, expressed as gram of creatinine. Supplementary material Table S2 presents the biomarkers measured in the collected samples along with the analytical method and their limit of detection (LOD).

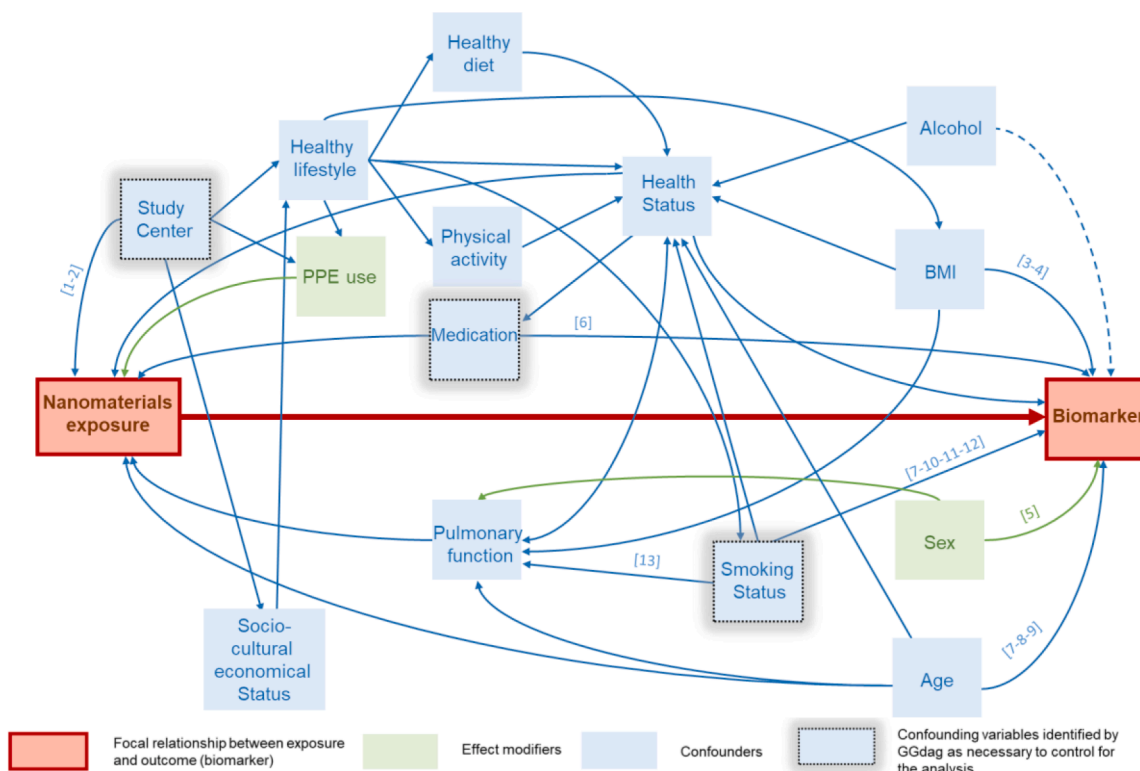


Fig. 1. Directed acyclic graph showing the assumed causal relationship between nanomaterials exposure and an effect biomarker.

2.4. Statistical analysis

We considered each biomarker as independent of the other biomarkers. We created a directed acyclic graph (DAG) (Textor et al., 2016) to identify potential confounders or effect modifiers in the causal network that links nanoparticle exposures and biomarkers based on available literature (Fig. 1). We identified the minimally sufficient set of variables that should be included in the multiple regression using model R Package Dagitty, GGdag (Textor et al., 2016). As shown in Fig. 1, these variables were age, smoking, medication, and study center. Sex and the use of personal protective equipment (PPE) were identified as potential effect modifiers and further assessed in the sensitivity analysis.

Alcohol was linked to biomarkers with a dotted arrow because the scientific evidence is inconsistent. References to the relevant sources are shown in squared brackets and are provided in the Supplementary material File 1.

We used multilevel mixed-effect interval regression models to properly manage the biomarker concentrations below the limit of detection (LOD) or in the interval between the LOD and the limit of quantification (LOQ). We fitted log-transformed exposure dose-metrics as independent variables on the log-transformed biomarkers to test for a first order association between biomarker concentrations with current or short-term exposures to nanomaterials. PNC and LDSA were used as fixed effect independent variables and fitted for each biomarker in separate models. When a statistically significant relationship was detected, the residuals were examined as a function of the exposure variables to detect any non-linearity. In the multiple mixed models, we performed the adjustment suggested by DAG (Fig. 1). Center was added as the first level random effect variable and the participant's ID being the second level random effect variable. The other variables identified as potential confounders were considered as fixed effect variables.

2.5. Sensitivity analysis

We conducted stratified analysis to assess whether exposure to a particular type of materials (collected with the questionnaires and verified with elemental analysis (EDAX)) would have an association with the different biomarkers. We created five binary variables (yes, no) to distinguish exposure to black carbon, TiO₂, SiO₂, CNT, and CaCO₃ nanomaterials. We focused on these types of nanomaterials because of their widespread use and numerous industrial applications on the one hand and the concerns regarding their toxicity and classification on the other hand. The models were performed both with and without adjustments according to DAG. Furthermore, we fitted the previously specified models stratified by sex and PPE use, one at a time, since these variables were identified as potential effect modifiers in the DAG. It is worth mentioning that the study was partially conducted during the COVID-19 pandemic when masks usage became compulsory. Consequently, we created a three-class variable based on questionnaire data to distinguish workers with systematic use of PPEs (PPE available and always used), occasional use of PPEs (PPEs not always available and/or not always used) and none (PPEs unavailable and/or never used).

Data management and statistical analysis were conducted with R version 3.6.2 and STATA version 16 (STATA, College Station, TX, USA) statistical software. A *p*-value < 0.05 was considered as statistically significant. However, when considering the five different types of nanomaterials, for which no a priori hypotheses have been formulated, the *p*-value for statistical significance was set to $0.05/5 = 0.01$ to account for multiplicity of tests by a Bonferroni-type correction. We believe that such correction is not needed when considering independent modelling of the different biomarkers as a function of a single quantitative exposure marker, especially as the selection of confounders was done *a priori* based on the DAG.

3. Results and discussion

3.1. Descriptive results

3.1.1. Study sample

We recruited 141 participants from seven companies: two research facilities with confirmed absence of nanomaterial exposure and five companies with confirmed nanomaterial activity (Table 1). Three participants did not complete the epidemiological questionnaire and did not provide EBC and urine samples, while two did not provide EBC nor urine samples, and one did not provide an EBC sample (Figure S2). The demographic, exposure, lifestyle, and health data are presented in Table 2 and Table S3.

Participants had on average been working for the same company for 5–8 years. Women represented approximately 50% of the participants in the non-exposed group, whereas 20% for the exposed group (Table 2). Women tended to hold administrative jobs more often than jobs in the industrial production.

The exposed group had a higher prevalence of tobacco consumption,

Table 2
Description of the study sample.

Variables	Non-exposed	Exposed
Recruited participants n (%)	43 (30.50%)	98 (69.50%)
Age (in years, mean (sd))	37.7 (10.00)	39.8 (10.30)
Sex		
Women n (%)	22 (51.20%)	21 (21.60%)
Men n (%)	21 (48.80%)	76 (78.40%)
Country of employment		
Italy n (%)	27 (62.80%)	67 (69.10%)
Spain n (%)		30 (30.90%)
Switzerland n (%)	16 (37.20%)	
Employment duration (in years)	5.5 (7.80)	7.1 (7.30)
BMI (mean (sd))	23.4 (3.50)	25.3 (4.00)
PPE use		
None / Not necessary n (%)	43 (100.00)	33 (34.00)
Not systematic n (%)	0 (0.00)	5 (5.20)
Systematic n(%)	0 (0.00)	59 (60.80)
Particle number concentration (mean (sd))	6357.6 (4046)	100,315 (99909.9)
Particle size (in nm, mean (sd))	58.4 (23.50)	27 (5.90)
LDSA (in µm ² /cm ³ , mean (sd))	19.1 (12.10)	112.8 (69.60)
General health status score (mean (sd))	77 (17.60)	67.5 (20.30)
Tobacco consumption (during year; daily > 1 g nicotine) n (%)	4 (9.30)	21 (21.60%)
Alcohol consumption (>1 cons. per week) n (%)	19 (44.20)	52 (54.20)
Medication n (%)	3 (7.00)	18 (18.60)
Vaccination (last three months) n (%)	9 (20.90)	42 (43.80)
Vitamin supplementation n (%)	7 (16.30)	16 (16.50)
Respiratory diseases diagnosed by physician		
Abnormal pulmonary function (FEV ₁) n (%)	2 (5.00)	13 (14.10)
COPD n (%)	1 (2.30)	0 (0.00)
Pneumonia n (%)	4 (9.30)	6 (6.20)
Asthma n (%)	5 (11.60)	7 (7.20)
Sinusitis n (%)	3 (7.00)	8 (8.20)
Bronchiolitis n (%)	1 (2.30)	5 (5.20)
Sleep apnea n (%)	0 (0.00)	2 (2.10)
Cardiac diseases diagnosed by physician		
Infarctus n (%)	0 (0.00)	1 (1.00)
Angina pectoris n (%)	0 (0.00)	1 (1.00)
Arrhythmia n (%)	0 (0.00)	6 (6.20)
High blood pressure n (%)	0 (0.00)	8 (8.20)
Other diseases diagnosed by physician		
High cholesterol n (%)	1 (2.30)	6 (6.20)
Type 1 diabete n (%)	0 (0.00)	1 (1.00)
Type 2 diabete n (%)	1 (2.30)	3 (3.10)
Cancer n (%)	0 (0.00)	1 (1.00)

“Diagnosed by a physician”: diagnosed diseases self-reported by workers. Percentages are given according to the positivity within the same group. BMI = Body mass index, COPD = Chronic obstructive pulmonary disease, PPE = personal protective equipment.

Table 3

Median biomarker concentrations and interquartile range (25th percentile – 75th percentile) by nanomaterial exposure status in exhaled air, EBC and in urine.

Biomarker	Non-exposed	Exposed
Exhaled air		
OPEA	0.66 (0.35 – 1.03)	0.57 (0.33 – 0.92)
EBC		
MDA* (ng/mL)	36.00 (36.00 – 36.00)	36.00 (36.00 – 144.00)
8-isoprostane (pg/mL)	1.50 (1.50 – 1.50)	6.00 (1.50 – 11.39)
NOTyr (ng/mL)	2.03 (0.90 – 2.61)	2.12 (1.86 – 2.40)
hs-CRP (ng/mL)	7.02 (6.68 – 7.36)	6.70 (5.78 – 8.18)
KL-6 (ng/mL)	48.47 (44.64 – 51.98)	52.06 (43.46 – 63.16)
IL-10 (pg/mL)	1.58 (1.48 – 1.68)	1.83 (1.40 – 2.68)
IL-1 β (pg/mL)	0.26 (0.24 – 0.29)	0.64 (0.51 – 0.73)
TNF- α (pg/mL)	0.09 (0.08 – 0.10)	0.24 (0.20 – 0.29)
Urine		
MDA (μ g/g creatinine)	228.03 (76.28 – 332.26)	216.79 (110.40 – 426.07)
8-isoprostane (μ g/g creatinine)	3.78 (2.74 – 5.29)	3.61 (2.61 – 5.02)
TAP (μ g/g creatinine)	1.19 (0.95 – 1.41)	0.86 (0.72 – 1.01)

OPEA is standardized as \log_{10} (oxidative potential in exhaled air/oxidative potential in ambient air). Values of biomarkers in urine are standardized by urinary creatinine concentration. *For MDA, most results were below LOD or below LOQ.

high blood pressure and a lower general health score compared to the non-exposed group.

Mean LDSA concentrations were from 19 to 162 $\mu\text{m}^2/\text{cm}^3$ and increased with PNC concentrations. To date, no LDSA reference values exist due to lack of data. Nevertheless, the concentrations observed for the most exposed participants (162 $\mu\text{m}^2/\text{cm}^3$) were significantly higher than those previously reported in general population studies conducted at polluted urban sites (Kuuluvainen et al., 2016).

The most common nanomaterials reported by the companies were CaCO_3 and SiO_2 followed by TiO_2 and iron oxides. Study participants reported SiO_2 and TiO_2 to be the nanomaterial most often used (Supplementary material Table S3).

3.1.2. Biomarkers

Table 3 summarizes the mean values for OPEA and biomarkers with their corresponding interquartile range (25th percentile – 75th percentile). Mean OPEA values among non-exposed participants were much higher than the general population reference interval for OPEA (Guseva Canu et al., 2022). EBC biomarker concentrations were in general lower among unexposed compared to exposed participants (Table 3) and in accordance with concentrations reported in the literature, except for MDA. Surprisingly, high MDA concentrations in EBC were quantified for non-exposed participants and much higher than reference values established in a recent meta-analysis (Turcu et al., 2022). All participants had slightly higher urinary biomarker concentrations compared with the MDA and 8-isoprostane reference values from the literature (Graille et al., 2020).

3.2. Relationships between particle concentrations, lung deposited surface area and biomarkers

Tables 4 and 5 present the regression coefficients beta (i.e., direction and strength of the relationship) for PNC and LDSA, respectively. 8-isoprostane in EBC was strongly and positively associated with both PNC and LDSA while no statistically significant association was detected between these and OPEA (Tables 4-5). These associations lost their statistical significance after adjustments, however, despite the moderate size of exposure effect ($\beta = 0.26$ for LDSA in Model 2, Table 5). We observed consistent positive relationships between IL and 10, IL-1 β and TNF- α with both PNC and LDSA (Tables 4-5). There was no indication of non-linearity in any of the models when examining the residuals. These relationships remained statistically significant after adjustment for potential confounders. Accounting for these confounders in the model decreased the estimated effect of nanomaterial exposure, particularly for IL-1 β . The estimated β coefficient for LDSA dropped from 0.34 in Model 1 to 0.07 in Model 2 (Table 5, Figure S4). This means that per one unit

increase of LDSA at log10 scale, the log of IL-1 β concentration in EBC increased by 7% when controlled for participants' age, tobacco smoking, medication, and center in Model 2. No association was observed between nanomaterial exposure and MDA, KL-6, hs-CRP or NOTyr in EBC.

Urinary TAP was associated with both PNC and LDSA. When examining the residuals for TAP, there was no non-linearity in any of the models. Contrary to the biomarkers measured in EBC, this dose-response relationship was negative and remained statistically significant with the same effect size after adjustment for potential confounders identified by DAG (Table 4 and 5, Supplementary material File F1).

3.3. Results of sensitivity analysis

The stratified analysis assessing whether the PNC and LDSA relationships with biomarkers differs across types of nanomaterial are summarized in Tables 6 and 7. Exposure to TiO_2 and SiO_2 induced an increase in 8-isoprostane in EBC with the highest estimated β coefficient compared to all the other biomarkers (Table 6). Yet, for CNT exposure this relationship with was of borderline significance after correction for multiple testing. Exposure to CNT but also to black carbon significantly increased KL-6 level in EBC. It is worth noting that without adjustments, some weak associations were also observed between this fibrosis biomarker and silica and CaCO_3 . Finally, a very weak negative association was observed between hs-CRP and TiO_2 that became not statistically significant after adjustment for potential confounders.

The positive associations observed for IL-1 β and TNF- α (Table 4-5) remained significant for exposure to all types of nanomaterials compared to non-exposed group (Table 6). For IL-10, only exposure to black carbon triggered a positive statistically significant effect (Table 6), whereas IL-10 was associated with all nanomaterial exposures (Tables 4-5, expressed as PNC and LDSA). This result (Table 6) can reflect a lack of statistical power in the stratified analysis.

TAP presented a consistent negative association relative to all nanomaterials considered while neither of the urinary lipoperoxidation biomarkers were associated with nanomaterial types (Table 7).

Participant sex seems to have a limited influence on the main findings. The stratified analysis assessing whether the PNC and LDSA relationships with biomarkers differs by sex are presented in the Supplementary material Tables S4 and S5, respectively. In men, most associations identified in the main analysis remained statistically significant. Only the association of IL-1 β with PNC and LDSA in Model 2 turned statistically non-significant. Moreover, a negative association with MDA measured in EBC was observed with both PNC and LDSA (Model 1 in Tables S4 and S5), yet without confirmation in the Model 2. In women, the association between 8 and isoprostane measured in EBC

Table 4
Relationship between PNC ($\mu\text{g}/\text{m}^3$) and biomarkers (\log_{10} -transformed).

Biomarker	Model 1 ^a			Model 2 ^b		
	β	95%-CI	<i>p</i> value	β	95%-CI	<i>p</i> value
Exhaled air						
OPEA	0.06	−0.09	–	0.04	−0.11	–
Exhaled breath condensate						
MDA (ng/mL)	−0.31	−0.59	–	0.27	−0.27	–
8-isoprostane (pg/mL)	0.45	0.31	–	0.20	−0.07	–
NOTyr ⁺ (ng/mL)	0.01	−0.02	–	0.01	−0.03	–
hs-CRP (ng/mL)	0.01	−0.01	–	0.03	0.00	–
KL-6 (ng/mL)	0.02	0.00	–	0.08	−0.01	–
IL-10 (pg/mL)	0.10	0.06	–	0.06	0.01	–
IL-1 β (pg/mL)	0.27	0.24	–	0.05	0.01	–
TNF- α (pg/mL)	0.29	0.26	–	0.08	0.01	–
Urine						
MDA ($\mu\text{g}/\text{g}$ creatinine)	0.03	−0.07	–	0.05	−0.08	–
8-isoprostane ($\mu\text{g}/\text{g}$ creatinine)	−0.01	−0.06	–	−0.02	−0.10	–
TAP ($\mu\text{g}/\text{g}$ creatinine)	−0.10	−0.14	–	−0.10	−0.14	–

^a Univariate (unadjusted) multilevel interval regression with participant ID included as a random effect variable.

^b Adjusted model according to the DAG. CI = 95 % confidence interval.

* The random effect variable Center could not be used for the NOTyr measured in EBC (no convergence when this variable was added in the model). β is calculated as \log_{10} (unit biomarker) per $\log_{10}(\mu\text{g}/\text{m}^3)$.

Table 5
Relationship between LDSA ($\mu\text{m}^2/\text{cm}^3$) and biomarkers (\log_{10} -transformed).

Biomarker	Model 1 ^a			Model 2 ^b		
	β	95%-CI	<i>p</i> value	β	95%-CI	<i>p</i> value
Exhaled air						
OPEA	0.06	−0.12	–	0.04	−0.15	–
Exhaled breath condensate						
MDA (ng/mL)	−0.31	−0.70	–	0.16	−0.53	–
8-isoprostane (pg/mL)	0.57	0.39	–	0.26	−0.11	–
NOTyr ⁺ (ng/mL)	0.01	−0.04	–	0.00	−0.04	–
hs-CRP (ng/mL)	0.01	−0.01	–	0.04	0.00	–
KL-6 (ng/mL)	0.02	−0.01	–	0.20	−0.06	–
IL-10 (pg/mL)	0.14	0.10	–	0.08	0.01	–
IL-1 β (pg/mL)	0.34	0.30	–	0.07	0.01	–
TNF- α (pg/mL)	0.36	0.31	–	0.14	0.04	–
Urine						
MDA ($\mu\text{g}/\text{g}$ creatinine)	0.01	−0.13	–	0.02	−0.14	–
8-isoprostane ($\mu\text{g}/\text{g}$ creatinine)	−0.02	−0.09	–	−0.03	−0.14	–
TAP ($\mu\text{g}/\text{g}$ creatinine)	−0.14	−0.19	–	−0.14	−0.20	–

^a Univariate (unadjusted) multilevel interval regression with participant ID included as a random effect variable.

^b Adjusted model according to the DAG. CI = 95 % confidence interval.

* The random effect variable Center could not be used for the NOTyr measured in EBC (no convergence when this variable was added in the model). β is calculated as \log_{10} (unit biomarker) per $\log_{10}(\mu\text{m}^2/\text{cm}^3)$.

and both dose metrics turned statistically non-significant. IL-10 lost its relationship with PNC but remained associated with LDSA in Model 2. Furthermore, a positive association between hs-CRP and both dose metrics appeared, although it was of borderline statistical significance.

The results of stratified analysis assessing whether the PNC and LDSA relationships with biomarkers differs by the PPE use are presented in the [Supplementary material Tables S6 and S7](#), respectively. For most biomarkers identified in main analysis we found no influence of the PPE use in terms of association with PNC or LDSA. However, the identification of a differential pattern of dose–response association according to PPE seems challenging. This can be due to several factors that we reported in the [Supplementary material File 2](#).

The first model is a univariate (unadjusted) multilevel interval regression with participant ID included as a random effect variable. The second model is adjusted according to the DAG. The *p*-values considered statistically significant after application of the Bonferroni correction for multiple testing (i.e., $p < 0.01$) are shown in bold.

Values of urinary biomarkers are normalized for creatinine concentration. The first model is a univariate (unadjusted) multilevel interval regression with participant ID included as a random effect variable. The

second model is adjusted according to the DAG. The *p*-values considered statistically significant after application of the Bonferroni correction for multiple testing (i.e., $p < 0.01$) are shown in bold.

3.4. Result interpretation from a mechanistic perspective

This study offered new insights into the development of early biological responses in workers after exposures to different types of nanoparticles. Moreover, we assessed whether repeated exposures in the same setting led to subtle lung changes as revealed by KL-6, a biomarker of interstitial lung fibrosis. Taken together, the results suggested an activation of the innate immune response rather than oxidative stress as the main effect of the mixture of nanomaterials investigated.

3.4.1. Innate immune and inflammatory response

After adjusting for relevant confounders identified by DAG, the most notable findings were the significant increases in IL-1 β , TNF- α and IL-10 in EBC with increasing PNC and LDSA. Pro-inflammatory cytokines (IL-1 β and TNF- α) are mainly produced by monocytes and macrophages and their concentration in EBC could represent reliable biomarkers of early

Table 6
Difference between exposed and unexposed groups regarding biomarkers in EBC and different types of nanomaterials.

Biomarker	Nanomaterial type	Univariate model					Adjusted model				
		β	95%-CI			<i>p</i>	β	95%-CI			<i>p</i>
MDA (ng/mL)	TiO ₂	-0.83	-1.36	-	-0.30	<0.01	-0.83	-2.11	-	0.45	0.20
	SiO ₂	-0.61	-1.01	-	-0.20	<0.01	-0.54	-1.50	-	0.42	0.27
	CNT	-0.24	-0.73	-	0.25	0.34	-0.19	-1.21	-	0.82	0.71
	CaCO ₃	-0.60	-1.15	-	-0.04	0.04	-0.56	-1.71	-	0.59	0.34
	Black carbon	-0.57	-1.13	-	0.00	0.05	-0.64	-1.79	-	0.52	0.28
8-isoprostane (pg/mL)	TiO ₂	0.86	0.59	-	1.12	<0.001	0.92	0.39	-	1.46	<0.001
	SiO ₂	0.80	0.56	-	1.04	<0.001	0.82	0.27	-	1.36	<0.01
	CNT	0.40	0.20	-	0.61	<0.001	0.58	0.11	-	1.05	0.02
	CaCO ₃	0.67	0.42	-	0.93	<0.001	0.62	0.02	-	1.23	0.04
	Black carbon	0.69	0.37	-	1.01	<0.001	0.69	0.04	-	1.35	0.04
NOTyr (ng/mL)	TiO ₂	0.04	-0.03	-	0.11	0.25	0.04	-0.04	-	0.11	0.34
	SiO ₂	0.04	-0.03	-	0.10	0.24	0.03	-0.03	-	0.10	0.31
	CNT	0.07	-0.04	-	0.17	0.20	0.07	-0.04	-	0.18	0.20
	CaCO ₃	0.04	-0.06	-	0.13	0.45	0.01	-0.09	-	0.11	0.84
	Black carbon	0.07	-0.03	-	0.16	0.19	0.06	-0.04	-	0.16	0.26
hs-CRP (ng/mL)	TiO ₂	-0.05	-0.08	-	-0.03	<0.001	-0.04	-0.14	-	0.05	0.37
	SiO ₂	0.00	-0.03	-	0.02	0.89	0.00	-0.03	-	0.03	0.97
	CNT	0.01	-0.01	-	0.03	0.41	0.02	-0.01	-	0.04	0.18
	CaCO ₃	0.00	-0.02	-	0.02	0.97	0.00	-0.02	-	0.03	0.81
	Black carbon	-0.02	-0.04	-	0.00	0.09	-0.03	-0.05	-	0.00	0.02
KL6 (ng/mL)	TiO ₂	0.01	-0.01	-	0.04	0.33	0.00	-0.05	-	0.05	0.94
	SiO ₂	0.03	0.00	-	0.06	0.04	0.02	-0.01	-	0.06	0.14
	CNT	0.08	0.04	-	0.12	<0.001	0.08	0.04	-	0.11	<0.001
	CaCO ₃	0.04	0.00	-	0.07	0.03	0.01	-0.02	-	0.05	0.49
	Black carbon	0.06	0.03	-	0.09	<0.001	0.05	0.02	-	0.09	<0.01
IL10 (pg/mL)	TiO ₂	0.05	0.02	-	0.09	<0.01	0.07	-0.09	-	0.22	0.38
	SiO ₂	0.12	0.08	-	0.17	<0.001	0.09	-0.08	-	0.26	0.28
	CNT	0.03	-0.02	-	0.08	0.27	0.04	-0.01	-	0.09	0.10
	CaCO ₃	0.20	0.17	-	0.23	<0.001	0.11	-0.07	-	0.29	0.22
	Black carbon	0.09	0.05	-	0.13	<0.001	0.07	0.03	-	0.12	<0.01
IL-1 β (pg/mL)	TiO ₂	0.35	0.31	-	0.38	<0.001	0.36	0.24	-	0.47	<0.001
	SiO ₂	0.39	0.36	-	0.42	<0.001	0.38	0.29	-	0.48	<0.001
	CNT	0.34	0.30	-	0.38	<0.001	0.34	0.30	-	0.38	<0.001
	CaCO ₃	0.42	0.39	-	0.45	<0.001	0.42	0.39	-	0.46	<0.001
	Black carbon	0.36	0.32	-	0.40	<0.001	0.36	0.32	-	0.40	<0.001
TNF- α (pg/mL)	TiO ₂	0.42	0.38	-	0.46	<0.001	0.42	0.35	-	0.49	<0.001
	SiO ₂	0.45	0.42	-	0.48	<0.001	0.45	0.40	-	0.49	<0.001
	CNT	0.40	0.36	-	0.45	<0.001	0.41	0.36	-	0.45	<0.001
	CaCO ₃	0.48	0.44	-	0.52	<0.001	0.46	0.39	-	0.53	<0.001
	Black carbon	0.44	0.40	-	0.48	<0.001	0.43	0.38	-	0.48	<0.001

Table 7
Difference between exposed and unexposed groups regarding urinary biomarkers and different types of nanomaterials.

Biomarker	Nanomaterial type	Univariate model					Adjusted model				
		β	95%-CI			<i>p-value</i>	B	95%-CI			<i>p value</i>
MDA (ng/mg creatinine)	TiO ₂	0.09	-0.07	-	0.26	0.27	0.09	-0.09	-	0.27	0.33
	Silica	0.06	-0.10	-	0.22	0.44	0.09	-0.07	-	0.26	0.26
	CNT	0.04	-0.19	-	0.27	0.75	0.02	-0.21	-	0.25	0.89
	CaCO ₃	0.01	-0.18	-	0.21	0.90	0.05	-0.17	-	0.26	0.67
	Black carbon	0.08	-0.12	-	0.28	0.44	0.12	-0.10	-	0.33	0.29
8-isoprostane (ng/mg creatinine)	TiO ₂	0.03	-0.05	-	0.12	0.44	0.03	-0.05	-	0.12	0.46
	Silica	0.00	-0.09	-	0.08	0.93	-0.04	-0.19	-	0.11	0.59
	CNT	-0.09	-0.21	-	0.04	0.17	-0.12	-0.31	-	0.07	0.23
	CaCO ₃	0.03	-0.08	-	0.14	0.58	-0.03	-0.22	-	0.16	0.73
	Black carbon	0.00	-0.11	-	0.12	0.95	-0.04	-0.20	-	0.13	0.67
TAP (ng/mg creatinine)	TiO ₂	-0.16	-0.22	-	-0.10	<0.001	-0.17	-0.23	-	-0.10	<0.001
	Silica	-0.15	-0.21	-	-0.10	<0.001	-0.16	-0.22	-	-0.10	<0.001
	CNT	-0.14	-0.22	-	-0.05	<0.01	-0.16	-0.24	-	-0.08	<0.001
	CaCO ₃	-0.16	-0.24	-	-0.09	<0.001	-0.19	-0.25	-	-0.12	<0.001
	Black carbon	-0.14	-0.21	-	-0.07	<0.001	-0.15	-0.22	-	-0.08	<0.001

local inflammatory response. The increase in the release of these pro-inflammatory cytokines has been already observed in several *in vitro* and *in vivo* studies after exposure to nanomaterials (Zhang et al., 2016; Bhattacharya et al., 2017; Låg et al., 2018). On the other hand, IL-10 has a central role following inflammation stimuli by limiting the immune response. The increased levels of IL-10, as observed in this study, concomitant with increased TNF- α and IL-1 β in exposed workers

suggests a feedback loop including pro-inflammatory and anti-inflammatory cytokines, representative of homeostatic mechanisms in the lung of exposed individuals. An increase in pro-inflammatory cytokines has been previously found in the blood of workers either exposed to black carbon (Zhang et al., 2014), carbon nanotube and carbon nanofibers (Fatkhutdinova et al., 2016) and in EBC of workers handling pigment-grade TiO₂ (Bergamaschi et al., 2022). In the latter study, both

TNF- α , IL-1 β and of IL-10 were significantly different between groups ($p = 0.003$, $p < 0.001$, $p < 0.001$, respectively).

Pro-inflammatory cytokines such as TNF- α and IL-1 β , act on the liver and stimulate the production of acute-phase proteins, especially hs-CRP (Devaraj et al., 2005). CRP induces tissue factor secretion, increases ROS and inducible nitric oxide production, and promotes monocyte chemotaxis thus increasing uptake of oxidized low density lipoprotein (Devaraj et al., 2005). hs-CRP plasma levels vary relatively little over time; therefore, a small modification is reliable evidence of an acute inflammation. hs-CRP measured in blood is recognized as major cardiovascular risk marker (Halcox et al., 2014). Given its significance as a biomarker, we quantified hs-CRP in EBC, but found no relationship with PNC nor LDSA and the weak negative relationship found with TiO₂ exposure was no longer significant in the adjusted model. Only one study has investigated the associations between TiO₂ exposure and hs-CRP in blood. This study found no difference in hs-CRP concentrations among workers exposed to TiO₂ nanoparticles and unexposed workers (Zhao et al., 2018). It was found that levels of IL-1 β and TNF- α increased among exposed workers, which supports our findings here.

3.4.2. Oxidative stress response

One of the main mechanisms by which nanoparticles induce adverse health effects is the generation of ROS and oxidative stress (Mendoza and Brown, 2019). Surprisingly, we did not observe significant increases in oxidative stress biomarkers in EBC in this study. Some authors reported high levels of oxidative stress biomarkers in EBC in workers handling CNTs (Lee et al., 2015) and significant exposure-dependent associations between TiO₂ nanoparticles and biomarkers of lipid oxidation in EBC (Pelclova et al., 2017). No associations were found for oxidative stress biomarkers in serum and nanoparticle exposures (Liou et al., 2012). Pro-inflammatory cytokines measured in sputum were not related to CNT exposure whereas oxidative stress biomarkers in sputum were strongly associated (Beard et al., 2018). This is contrary to what we observed in our study and could possibly be due to the difference in nanomaterial exposures, particle versus fibers.

The maintenance of redox homeostasis involves complex mechanisms and excessive oxidative stress may have other effects than lipid peroxidation. The human body is equipped with antioxidant defense mechanisms able to neutralize ROS and scavenge free radicals. Damage to cellular components would only become significant when the body is no longer able to maintain cellular redox homeostasis under chronic challenges. The biomarkers investigated in EBC did not reveal an excess of oxidative stress in the lung district of exposed workers nor an indication of nitration mediated by reactive nitrogen species (determined by the analysis of nitrotyrosine). This information is consistent with a preservation of the redox homeostasis in the lungs of exposed workers.

The only statistically significant finding concerning oxidative stress in this study was lower values of the urinary TAP in exposed workers. TAP reflects cumulative effects of all antioxidants from various endogenous anti-oxidative defense systems thus limiting the noxious effects caused by oxidative stress. The absence of an increase in oxidative stress biomarkers in EBC and the negative dose-response relationship with TAP in urine suggest efficient antioxidant defense mechanisms to maintain the redox balance in exposed workers. Thus, biomarkers reflecting the status of antioxidant defense networks, such as superoxide dismutase (SOD) and glutathione peroxidase (GPX), which have been found increased in the serum of nanomaterial exposed workers should be considered in future studies (Liou et al., 2012).

It is also likely that the extent of exposure did not reach levels inducing oxidative damage in our workers. For example, Pelclova et al. found that significant increases in oxidative stress markers measured in workers were dependent on nano-TiO₂ exposure levels (Pelclova et al., 2017). Compared to this study, NanoExplore participants had low exposure to nanomaterials. This exposure level could lead to more subtle changes in cell signaling activated in fine antioxidant systems and result in non-toxic modulation of redox signaling (Mendoza and Brown, 2019).

Complexing the matter further is that not all nanomaterials cause inflammation via an oxidative stress mechanism (Horie and Tabei, 2021).

3.4.3. Fibrogenic response

Recent clinical studies have suggested that KL-6 determined in blood is a potential biomarker of interstitial lung disease (d'Alessandro et al., 2020). KL-6 is a pulmonary epithelial mucin more prominently expressed on the surface membrane of alveolar type II cells when these cells are injured (Ishizaka et al., 2004). Our study is the second study where this biomarker has been measured in EBC (Bergamaschi et al., 2022). We found slightly elevated ($\beta = 0.08$) KL-6 values in EBC of workers who reported exposure to CNTs. One published study found similar KL-6 results as we report here, but in sputum of workers exposed to CNTs (Fatkhutdinova et al., 2016). Changes in KL-6 values would occur after a long-term exposure and support the idea that exposure to CNTs might play a role in development of idiopathic pulmonary fibrosis (Ursini et al., 2021). This specific pathogenicity of CNTs and particularly multi-wall CNTs could be due to their similarities in structure to asbestos fibers (Fatkhutdinova et al., 2016). Depending on the fiber length and stiffness (Donaldson et al., 2013; Nagai et al., 2011), CNTs may undergo an effective or an incomplete phagocytosis leading to the ROS production and inflammation or physical interference with cells (i.e., needle-like piercing of the cellular membrane) (Shvedova et al., 2012).

In a recent study on workers occupationally exposed to pigment-grade TiO₂ with a mean exposure duration of 14 years, Bergamaschi et al. found higher KL-6 concentrations in EBC of workers than in controls (Bergamaschi et al., 2022). This finding suggested an activation of pro-fibrotic cascade, leading to interstitial lung damage. In the present study, a specific association between KL-6 and TiO₂ was not apparent. However, we observed an increase of KL-6 in EBC of workers handling CNTs or SiO₂, both chemicals known to induce lung fibrosis (d'Alessandro et al., 2020). The lack of association between TiO₂ and KL-6 in the present study may be attributed to the short latency since the beginning of exposure (8 years on average) and an insufficient time span to lead to a clear-cut effect, which is expected from a long-lasting (and heavy) exposure. Furthermore, Bergamaschi et al. used a specific experimental design to track TiO₂ occupational exposures (Fatkhutdinova et al., 2016), while we relied on self-reported information for past exposures and operations records.

3.5. Study strengths and limitations

This study has several important strengths. This is the first extensive study exploring dose-response associations between nanomaterial exposure and early biological responses in humans, while exhaustively checking the possible factors that could influence this relationship (e.g., health status and lifestyle habits). An important attention was also paid to the choice of statistical methods and model selection to control for potential bias, by using causal framework and the DAG (Schubauer-Berigan et al., 2023).

We focused on early respiratory effects in workers exposed to nanomaterials since oxidative stress and inflammation represent the initial steps of an adverse outcome pathway leading to pro-fibrogenic activation pathways and/or overt fibrosis. Non-invasive biological sampling was positively associated with participation rates (Cr ez e et al., 2021). We used a new, inexpensive, and versatile device, OPEA, to assess oxidative potential in exhaled breath from workers and this device appear promising but still requires further developments.

In the present study we included for the first time, novel biomarkers such as KL-6, a fibrogenic biomarker and hs-CRP. These have never been measured in the EBC collected from nanotechnology workers. In contrast to urinary biomarkers, EBC biomarkers are not yet routinely used in occupational medicine and research, and the study is therefore of great importance for their validation and development.

Importantly, this study is one of the largest studies of

nanotechnology workers in the world. Similar cohorts are the EpiNano cohort of nanotechnology workers launched in 2012 in France including 130 workers so far (Guseva Canu et al., 2016) and the US National Institute for Occupational Safety and Health (US-NIOSH) cohort of CNT and nanofiber workers including 108 participants at baseline (Beard et al., 2018). The Taiwanese national panel study currently includes 206 exposed and 108 unexposed workers recruited at 14 different nanomaterial producing plants but have no exposure measurement data (Wu et al., 2019).

The exposure assessment strategy we used presents some limitations. We used a stationary device (DiSCmini) to measure airborne nanoparticle concentrations because as far as we know, no device exists for this kind for measurements that can be worn by the study participants in the breathing zone (Guseva Canu et al., 2023). Personal air samples were collected with a filter to determine type of nanoparticles.

Another limitation is the number of EBC samples below the LOQ. The EBC constituents are often highly diluted, resulting in typical concentrations at the pg/mL (Hemmendinger et al., 2021). In our case, 8-hydroxy-2-deoxyguanosine (8-OHdG), IL-6, circulating surfactant protein D and leukotriene B4 were initially considered for analysis in EBC, but we faced many technical issues (not accurate, repeatable or low reproducible quantification). Concerning MDA, the ELISA kit was not sufficiently sensitive and > 20% of MDA values were not detectable (data not shown). Similarly, 8–26% of 8-isoprostane values were not detectable across the exposure subgroups. Nevertheless, the method sensitivity for both of these analytes was not an issue (LOD = 3 pg/mL), and could be related to the low exposures observed in our study (Hemmendinger et al., 2022).

Although some studies consistently found changes in hematological parameters or serum cytokines as biomarkers of systemic inflammation and oxidative stress (Schulte et al., 2019), the nature of our study - which needs repeated measurements of biological matrices - only allowed the adoption of non-invasive methods of sampling collection. This approach, which can certainly be regarded as a limitation, increased the participation rate of the workers and of not exposed volunteers, thus ensuring a better response-rate.

The generalizability of the study findings should be considered with caution because the study included a relatively modest number of participants from three countries and companies handling various nano- and bulk materials. Thus, our source population can differ from the target population (104). Invited workers were identified during the preparatory company visits and recruited during the field exposure assessment campaign. We therefore expect a limited selection bias in this study due to the absence of health or biomarker information (Schubauer-Berigan et al., 2023). In fact, participants with missing data were equally distributed across exposure categories and their incomplete participation was likely determined by COVID-19 symptoms rather a self-selection based on the health outcomes that might be related to the nanomaterial exposure. Another possible limitation is the possibility of false positive results due to a multiplicity of tests. Within the results presented in tables 6 and 7, a formal Bonferroni correction was applied as no a priori hypotheses had been formulated as to which of the nanomaterials was potentially more toxic. With respect to the single-exposure models presented in tables 4 and 5, no such correction could nor should be applied as the different models apply to different but equally relevant biomarkers of oxidative/nitrosative stress. Nevertheless, it seems safe to interpret cautiously the individual positive results for which the p-value is close to the nominal level of 0.05. The more convincing result is the fact that several of the biomarkers, especially in the EBC matrix, were positively related to the exposure markers rather than the individual biomarker which may (or not) be statistically significant.

The planned follow-up study of the recruited participants will probably clarify the above-mentioned issues.

4. Conclusion

This study showed a significant dose–response relationship between IL and 10, IL-1 β and TNF- α measured in exhaled breath condensate and particle number concentration as well as lung-deposited surface area. These results suggest an activation of the innate immune response rather than oxidative stress as the main effect after exposure to the mixture of nanomaterials investigated. Conversely, no oxidative stress was observed in the exhaled air samples. A significant negative association was observed between nanomaterial exposure and urinary total antioxidant power. We will continue to follow this cohort prospectively. Altogether, this study contributed to improve evidence regarding exposure to nanomaterial and risks to human health.

CRediT authorship contribution statement

Maud Hemmendinger: Writing – original draft, Investigation. **Giulia Squillaciotti:** Investigation, Writing – review & editing. **Thomas Charreau:** Formal analysis, Validation, Data curation. **Giacomo Garzaro:** Investigation, Writing – review & editing. **Federica Ghelli:** Investigation, Writing – review & editing. **Roberto Bono:** Investigation, Writing – review & editing. **Jean-Jacques Sauvain:** Methodology, Writing – review & editing. **Guillaume Suarez:** Methodology, Writing – review & editing. **Nancy B. Hopf:** Methodology, Writing – review & editing. **Pascal Wild:** Formal analysis, Methodology, Writing – review & editing. **Athena Progiou:** Methodology, Writing – review & editing, Project administration. **Carlos Fito:** Methodology, Writing – review & editing. **Enrico Bergamaschi:** Conceptualization, Methodology, Writing – review & editing, Project administration. **Irina Guseva Canu:** Conceptualization, Supervision, Project administration.

Declaration of Competing Interest

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Data availability

The data that has been used is confidential.

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Informed Consent Statement

Written consent to participate were obtained from all participants involved in this study.

Appendix A. Supplementary data

Supplementary data to this article can be found online at <https://doi.org/10.1016/j.envint.2023.108157>.

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