



# Assessing the inhaled dose of nanomaterials by nanoparticle tracking analysis (NTA) of exhaled breath condensate (EBC) and its relationship with lung inflammatory biomarkers

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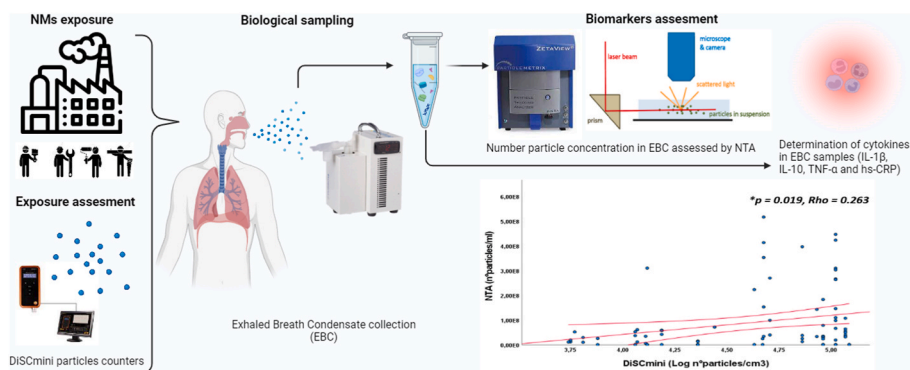
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## HIGHLIGHTS (5 BULLET POINTS)

- The first multi-center occupational study on a cohort of 80 workers exposed to NMs.
- Increasing exposure to NMs revealed increasing particles number in EBC assessed by NTA.
- Nanoparticle Tracking Analysis (NTA) is a possible biomarker of internal dose.
- An increased number of particles in EBC correlates significantly with IL-1 $\beta$  and IL-10.
- This study underlines a lack of occupational studies on non-invasive matrices.

## GRAPHICAL ABSTRACT



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## ABSTRACT

The widespread and increasing use of nanomaterials has resulted in a higher likelihood of exposure by inhalation for nanotechnology workers. However, tracking the internal dose of nanoparticles deposited at the airways level, is still challenging.

To assess the suitability of particle number concentration determination as biomarker of internal dose, we carried out a cross sectional investigation involving 80 workers handling nanomaterials. External exposure was characterized by portable counters of particles DISCminiTM (Testo, DE), allowing to categorize 51 workers as exposed and 29 as non-exposed (NE) to nanoparticles. Each subject filled in a questionnaire reporting working

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practices and health status. Exhaled breath condensate was collected and analysed for the number of particles/ml as well as for inflammatory biomarkers.

A clear-cut relationship between the number of airborne particles in the nano-size range determined by the particle counters and the particle concentration in exhaled breath condensate (EBC) was apparent. Moreover, inflammatory cytokines (IL-1 $\beta$ , IL-10, and TNF- $\alpha$ ) measured in EBC, were significantly higher in the exposed subjects as compared to not exposed. Finally, significant correlations were found between external exposure, the number concentration of particles measured by the nanoparticle tracking analysis (NTA) and inflammatory cytokines. As a whole, the present study, suggests that NTA can be regarded as a reliable tool to assess the inhaled dose of particles and that this dose can effectively elicit inflammatory effects.

### Acronyms/abbreviations

EBC	(Exhaled Breath Condensate)
FFP2	(filtering face piece type 2)
HE	(High-exposed)
CRP	(C-reactive Protein)
IL-	(Interleukins)
LDSA	(lung deposited surface area)
LE	(Low-exposed)
NE	(Non-exposed)
NMs	(Nanomaterials)
NTA	(Nanoparticle Tracking Analysis)
PM	(airborne particulate)
PPE	(personal protective equipment)
TNF	(Tumour Necrosis Factor)

## 1. Introduction

In the last decades, the use of nanotechnologies has experienced significant growth in many industrial sectors, leading to increased nanomaterials (NMs) production and handling with the subsequent likelihood of occupational exposure in Companies and Laboratories (Ghafari et al., 2020; Luo et al., 2022). However, many dry powders used for industrial products (e.g. in paints) can fall under the EU NM definition given by the European Commission (European Commission, 2022; Bergamaschi et al., 2022).

Owing to the low number of human studies, much of our knowledge regarding the potential toxicity of NMs has been evaluated through in vivo and in vitro studies (Gonzalez and Kirsch-Volders, 2016). It has been demonstrated that particles, fine and ultrafine in size, present a large specific surface area, a low coordination of atoms at the surface with other atoms, a high curvature radius, and can have a colloidal nature, all these properties make NMs very reactive (Barbero et al., 2021). Moreover, NMs present ability to penetrate inside the organism and, in his way, interact with biomolecules, potentially inducing local inflammation (Barbero et al., 2017). The main way of penetration of particles into the organism is breathing (Borm et al., 2006; Yah et al., 2012; O'Shaughnessy, 2013) and the quantification of their intake needs to be explored more in depth (Wittmaack, 2007; Ferdous and Nemmar, 2020). Although exposure assessment for nanomaterials has dramatically improved over the last years, relying on innovative approaches, as well as on devices allowing the sampling in the breathing zone of workers and personal monitors translating the aerosol characteristics in relevant metrics, such as the lung deposited surface area (LDSA) (Iavicoli et al., 2018), there is the need to assess both the internal dose and possible effects at the target organ (Bergamaschi et al., 2015). Exposure assessment combined with biomonitoring seems the most useful tool for identifying the causal relationships and the potential risks that workers can be exposed (Bergamaschi et al., 2015). Providing objective demonstration of the absorption of chemicals in the body, exposure

biomarkers can be useful in occupational toxicology for a more accurate risk assessment, reducing misclassification in health studies (Mutti, 2001). Thus, a particle exposure assessment based on the dose deposited in the lungs would be the gold standard for the evaluation of any resulting health effects. Measuring particles in exhaled breath could help to evaluate particle retention in the lungs. By cooling a subject's exhaled breath in a non-invasive way, it is possible to collect a liquid composed mainly of water and a very small amount of airway lining fluids. Exhaled breath condensate (EBC), a non-invasive matrix predominantly composed of water vapour and small droplets from various regions of the respiratory tract, including the bronchial and alveoli regions, is considered a valuable biological to monitoring matrix when traditional matrices, like blood or induced sputum are not feasible (Goldoni et al., 2004, 2006; Hunt, 2007). It is thought that EBC might be a useful biological monitoring matrix where either biological monitoring is currently not possible using traditional biological matrices such as urine or blood (e.g., for dusts or respirable crystalline silica) or where the interpretation of elemental species is difficult in a biological sample (e.g., for hexavalent and trivalent chromium) (Forest et al., 2021; Marie-Desvergne et al., 2022; Forest and Pourchez, 2023).

Innovative techniques and tools, such as nanoparticle tracking analysis (NTA) have been used for the quantification of particles in biological matrices, such as EBC, and can thus support the assessment of internal dose of particles as an exposure biomarker (Guseva Canu et al., 2021).

The aims of the present study are: i) to quantify the number of particles in EBC of workers occupationally exposed to nanomaterials by NTA and ii) to assess the relationship between the number of particles in EBC and the number particles concentration quantified by real time monitoring devices and iii) to explore the relationships between different particle metrics and the pro- and anti-inflammatory biomarkers, which are commonly analysed for characterizing the severity of respiratory diseases (Montuschi, 2007) as well as effect biomarkers for nanomaterial exposure (Ghelli et al., 2022).

## 2. Material and methods

### 2.1. Study protocol

The study protocol was approved by the NanoExplore Consortium and the EU monitor in charge of the NanoExplore project. Moreover, approvals have been obtained from the local ethics regulation organs: the Swiss Ethics in Switzerland (approval 2020-01098); the Bio-ethical Committee of the University of Torino in Italy (approval 336577 August 8, 2020); and the Health and Safety Board of the Catalan Institute of Nanoscience and Nanotechnology in Spain (approval ICN2-22-03-2022). This work was supported by the European Commission LIFE program (Grant LIFE17 ENV/GR/000285).

### 2.2. Study participants & companies involved

A subgroup of workers potentially exposed to nanomaterials belonging to a larger group of subjects recruited for the LIFE NanoExplore project (Grant LIFE17 ENV/GR/000285) was recruited. These workers belong to different companies where paints, adhesives, coatings, construction chemicals are handled and produced. Each company

was identified by a fictitious name based on the type of materials used in their work. Moreover, the presence of the nanomaterials at each company site was investigated by analyzing the filters held in the particle samplers by transmission electron microscopy (TEM) for size and shape and subsequently by energy dispersive X-ray (EDAX) for elemental analysis, as reported more specifically in the study by [Hemmendinger et al. \(2023\)](#); [Guseva Canu et al., 2023](#). In “Company A,” paints, adhesives, and coating materials are produced, and filter analysis identified the following elements Carbon, Oxygen, Titanium, Silicon, and Calcium. These elements also correspond to the main nanomaterials used in manufacturing products such as paints and varnishes. “Company B” produces construction materials of chemical origin. The elements detected at this company site as the main components of the nanoparticles produced in the plant were Aluminium, Silicon, Oxygen, Carbon, Sulfur, Titanium and Calcium. Elements very similar to those found in Company A. Finally, “Company C” is involved in NM research and development, and the material most commonly found in filters was Iron. Whereas people working in companies A and B handled large quantities of materials, the subjects recruited in Company C were mainly involved in research and development, handling small quantities of materials needed for experiments in the research labs.

### 2.3. Exposure monitoring

Exposure monitoring was performed using six particle-size concentration counters DISCmini™ (Testo, DE), placed near different types of workstations (near field measurements). These devices measure the number of airborne particles in the nanometric size range from 10 to 300 nm and the resulting data are expressed as number of particles/cm<sup>3</sup>. Particle size is expressed in nanometers with a time resolution of 1 s. The detection range of the DISCmini™ (Testo, DE) is around 500–1,000,000 particles/cm<sup>3</sup>. Based on the results obtained from the DISCmini™ (Testo, DE) sampling, the LDSA was determined, a metric based on the size-dependent deposition of particles within the lung ([Schmid and Stoeger, 2016](#)).

### 2.4. EBC sampling collection

EBC samples were collected using a Turbo-DECCS™ condenser (Medivac, Parma, Italy) set at –10 °C equipped by a flow meter (VOLTMET 20 Medivac, Parma, Italy), to comply with the American Thoracic Society and the European Respiratory Society Task Force guidelines and normalize the volume of exhaled air collected from different subjects. Workers were required to breathe into the condenser circuit at tidal volume while wearing a nose clip until the air volume of 90 L. This allowed to collect 2–3 ml of EBC. EBC was divided into aliquots of 300 µL and stored at –80 °C until analysis.

The biological sample collection, handling and storage were operated by a dedicated operator in a closed clean room, in different buildings. In this study, EBC sampling was done both at the beginning and at the end of the working week (time A and B), with the aim of identifying possible washout during the weekend or an accumulation over the working week.

### 2.5. NTA-fine tuning methodology

Particle concentration, size distribution and Z potential analysis were performed by the ZetaView® PMX-120 (Particle Metrix GmbH, Germany) nanoparticle tracking analyser, equipped with a light source set to a wavelength of 488 nm. NTA captures the Brownian motion of each particle in a video. The hydrodynamic diameter of the particles is determined based on the Stokes-Einstein relation starting from the obtained diffusion coefficient (size range 30–2000 nm). The particle concentration is determined by counting all objects in the field of view and knowing the measured volume. To optimize the instrumental parameters and the correct sample dilution, a pre-screening on 5 EBC samples

was necessary. The sensitivity, the shutter and the frame rate were finally set at 70, 100 and 30, respectively; 3 × 33 videos of 1 s for each sample were recorded. The dilution of the EBC samples in double-filtered Milli-Q water was set at 1:5, optimal for almost all the analysed samples. Few samples - the most concentrated - were further diluted to carry out a correct analysis. The background noise of the instrument, of the double-filtered Milli-Q water and of the used plastic ware was determined too. A LOD of 5 × 10<sup>6</sup> NPs/mL was calculated.

### 2.6. Inflammation analyses

Cytokines concentrations in EBC, namely IL-1β, IL-10, and TNF-α were determined by Real-Time PCR – linked ELISA (Invitrogen), whereas the C-reactive protein (CRP) was investigated with high-sensitivity ELISA kit (MyBioSource). Real-Time PCR linked ELISA was chosen because cytokine levels in EBC are often highly diluted, resulting in typical concentrations at the pg/mL and a highly sensitive test for their quantification is needed.

### 2.7. Statistical analyses

All environmental and biological data were uploaded and integrated into a database to perform the statistical analysis using SPSS software. Subjects were classified into different groups based on environmental data obtained from DiSCmini™ (Testo, DE), and their profiles were juxtaposed the number of particles (ZetaView® PMX-120 (Particle Metrix GmbH, Germany) nanoparticle tracking analyser) and inflammatory biomarkers in EBC analysing the variance using the non-parametric Kruskal-Wallis’s test.

Additionally, bivariate correlations were established, using "Spearman’s" coefficient depending on the parametric nature of the data, to explore the relationships between DiSCmini™ (Testo, DE) measurements, NTA, and inflammatory markers."

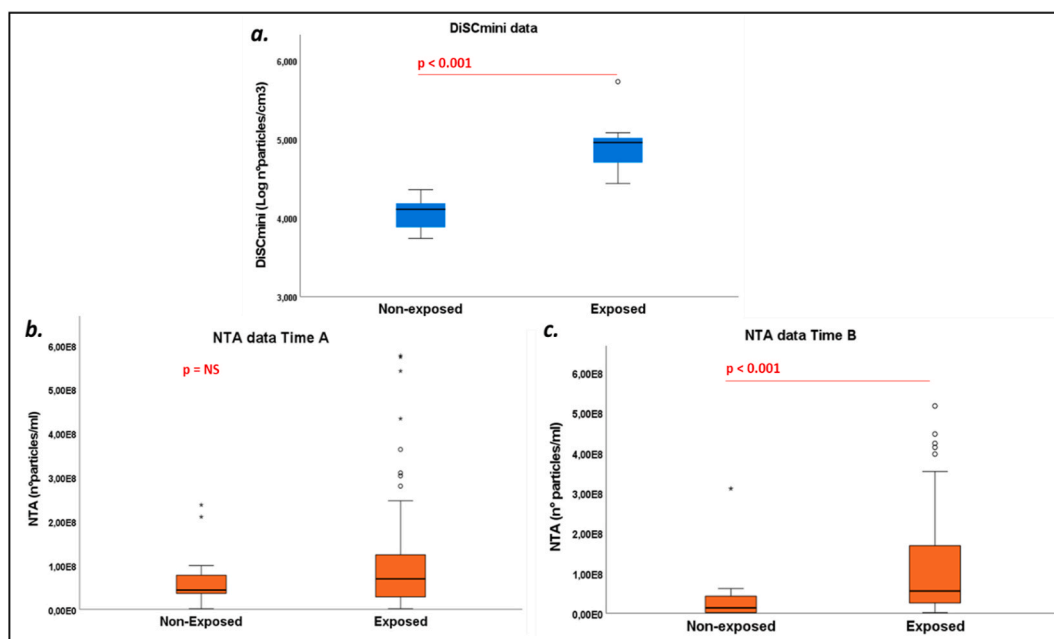
## 3. Results

According to the results of DiSCmini™ (Testo, DE) devices, were identified three subgroups of workers. Subjects exposed to a number concentration (log 10) ranged 3,30–3.88 were classified as non-exposed (NE), workers exposed to NMs ranging between 4.12 and 4.71 were classified as low-exposed (LE), and workers exposed to NMs ranging from 4.92 to 5.74 were classified as high-exposed (HE) group ([Hemmendinger et al., 2023](#)). Thus, from the whole epidemiological sample, were identified 51 NM-exposed workers, of whom 37 were categorized as HE subgroup, 14 LE subgroup and 29 workers with no apparent occupational exposure to NMs were included as controls (NE). [Table 1](#) summarizes the characteristic of the subgroups according to the exposure ranking.

Particle number concentrations recorded by DISCmini were significantly higher in exposed workers as compared to NE ( $p < 0.001$ )

**Table 1**  
Characteristic of the subgroups according to the exposure ranking.

Variables	NE	LE	HE
Subjects n° (%)	29 (36.25%)	14 (17.50%)	37 (46.25%)
Age min-max (mean ± sd)	25–54 (38,6 ± 2,6)	19–60 (36,4 ± 2,0)	22–60 (41,5 ± 1,8)
Male n° (%)	7 (8,75%)	22 (27,50%)	30 (37,50%)
Female n° (%)	9 (11,25%)	6 (7,50%)	6 (7,50%)
Subjects from company A n° (%)	/	14(17,50%)	3 (3,75%)
Subjects from company B n° (%)	/	/	27(33,75%)
Subjects from company C n° (%)	13 (16,25%)		7 (8,75%)
Subjects recruited as controls (NE) n° (%)	16 (20%)	/	/



**Fig. 1.** DiSCmini™ (Testo, DE) data expressed as logarithmic mean of exposed and non-exposed subjects (part a.); Part b illustrates the NTA data expressed as the average particle number concentration of exposed and non-exposed subjects at the beginning of the week (Time A) and at the end (Time B). Blue = DiSCmini data; Orange = NTA data). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

(Fig. 1, a).

The NTA data were consistent with the external exposure data. Particle number quantified by NTA revealed a tendency towards higher values, though not statistically significant, in the exposed subgroup at the beginning of the working week (Fig. 1, b) and a statistically significant difference between NE and the whole group of exposed at the end of the working week (Fig. 1, c).

Both HE and LE subgroups showed significantly higher external exposure values as compared to the NE ( $p < 0.001$ ;  $p = 0.004$ , respectively). Moreover, HE demonstrated a significantly higher concentration than LE ( $p < 0.001$ ). (Fig. 2, a).

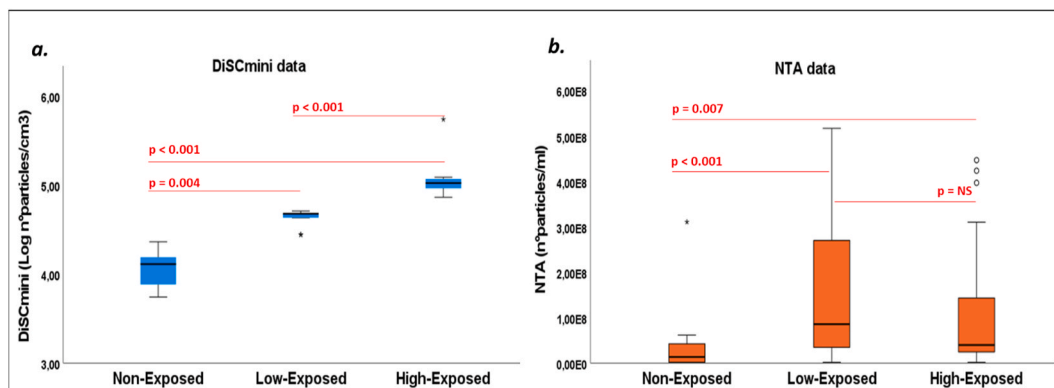
This categorization applied to the NTA data in EBC revealed that subjects belonging to the HE and LE had a significantly higher number of particles than NE ( $p = 0.007$  and  $p < 0.001$ , respectively). However, and unexpectedly, the median value was higher in LE subjects than in HE subjects.

As a whole, a higher concentration of airborne particles at workplace is consistently associated with a greater number of particles in the EBC of exposed subjects as compared to NE subjects.

Considering the epidemiological sample, a positive and significant correlation was apparent between airborne particle number and number of particles measured by NTA in EBC ( $Rho = 0.263$ ;  $p = 0.019$ ), showed a relatively low Rho correlation coefficient, though significant (Fig. 3). The number of particles measured by NTA in EBC was also consistently associated with LDSA ( $Rho = 0.288$ ,  $p = 0.009$ ). The association between the number of particles to which workers are exposed and their presence in exhaled breath appeared weak but statistically significant.

As revealed by the part a. of Fig. 4, the correlation between IL-1 $\beta$  and NTA data, was statistically significant ( $Rho = 0.283$ ;  $p = 0.012$ ). The part b. of Fig. 4 shows the correlation between NTA data and IL-10 levels, indicating a weakly positive relationship ( $Rho = 0.239$ ;  $p = 0.035$ ). Lastly, the part c. of Fig. 4 suggests a positive trend between particle count and TNF- $\alpha$  data but without reaching a significant level.

To further explore any relationships between DiSCmini™ (Testo, DE) and NTA data, it was chosen to aggregate the data according to the different materials produced i.e. aggregating by companies recruited. Statistically significant differences in particle concentration between workers exposed to different occupational settings (A, B, and C) and



**Fig. 2.** DiSCmini™ (Testo, DE) data expressed as Log mean divided into NE, LE, and HE (a.); NTA data expressed as mean particle number concentration of NE, LE, and HE subjects (b.); Blue = DiSCmini data; Orange = NTA data). (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

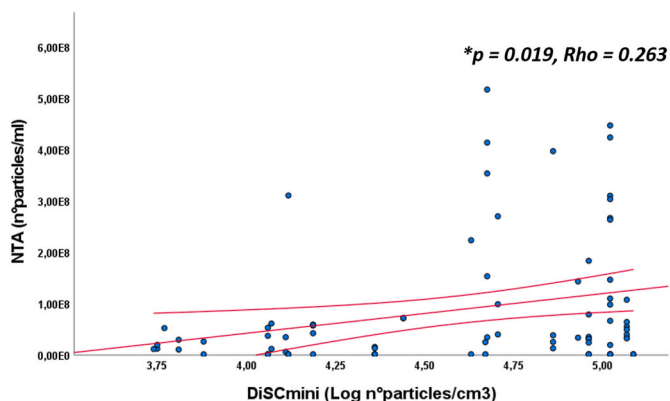


Fig. 3. Correlation between DiSCmini™ (Testo, DE) data and NTA data.

subjects recruited as controls (NE) were observed (Fig. 5, a;  $p < 0.001$ ). When examining the panel b. of Fig. 5, the distributions of NTA across different locations were positively and significantly different in NE subgroup as compared to the exposed workers from company A ( $p < 0.009$ ) and company B ( $p < 0.036$ ) but not significantly different with company A. It is worth mentioning that, whereas the companies are located in different geographical areas, they produced different materials, different amount of dusty materials as well as they undertook

different types of processes, workers belonging to the companies A and B resulting more exposed to particulate matter as compared to those working in company C. These results demonstrate that an increased presence of airborne particles can also be found at the airway level (NTA), even if this finding is not observed in the workers of company C.

#### 4. Discussion

Exposure assessment for nanomaterials has dramatically improved over the last years, now relying on innovative industrial hygiene approaches, new devices for sampling in the breathing zone of workers and new personal monitors translating the aerosol characteristics in relevant metrics. Devices such as DiSCmini™ (Testo, DE), in addition to providing the PNC (particle number concentration) of an aerosol, also provide the LDSA, which is the surface area of particles deposited in the lungs. The latter has been recognized as a more accurate metric for understanding the toxicity of nanoparticles compared to the more commonly used particle mass concentration. LDSA concentrations can be obtained through direct measurements or calculations based on empirical lung deposition models and measurements of the particle size distribution, with the unit of measurement being  $\mu\text{m}^2/\text{cm}^3$ . However, it is important to note that neither LDSA measurements nor size distribution measurements are mandatory or regulated by governmental authorities (Fung et al., 2022). Nevertheless, LDSA has been suggested as a crucial predictor for health outcomes resulting from aerosol exposure, particularly for low- and poorly soluble spherical NPs, as it stands

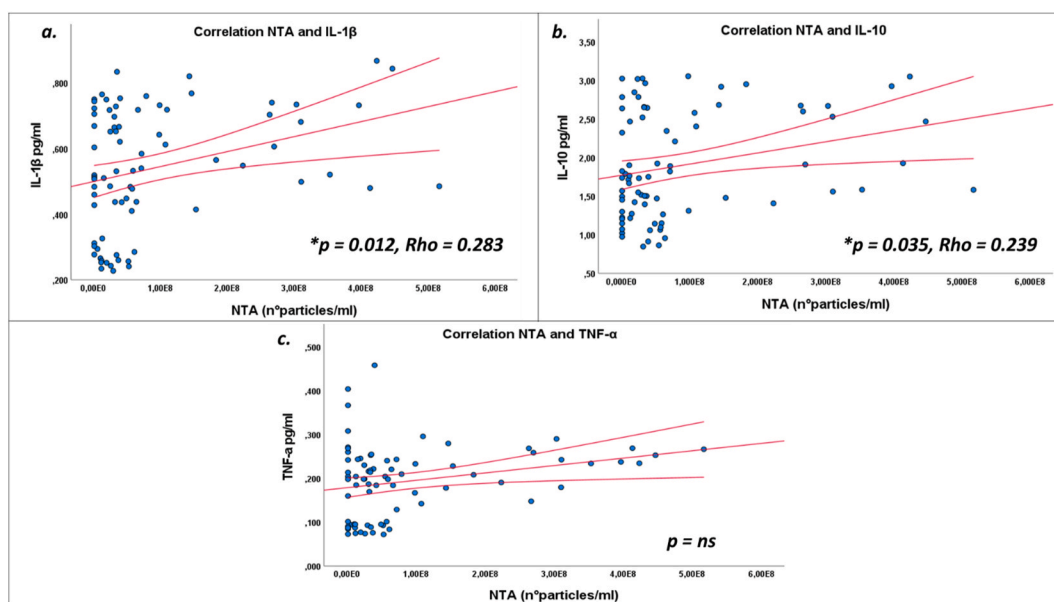


Fig. 4. Correlation graphs between IL-1β (a.), IL-10 (b.), TNF-α (c.) and NTA.

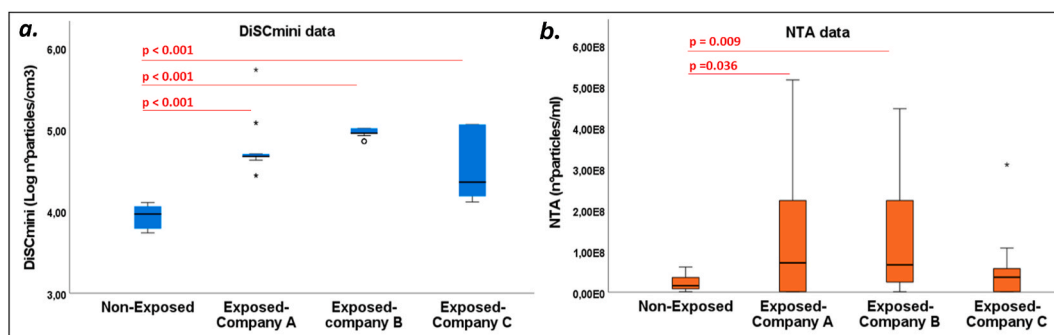


Fig. 5. DiSCmini™ (Testo, DE) data (a.) and NTA data (b.) analysed per company.

out as one of the most effective dose metric for acute pulmonary inflammation (Schmid and Stoeger, 2016).

However, whereas the characterization of external exposure has been improved, there is the need to assess both the internal dose and possible effects at the target organ. Exposure, i.e. the contact between a foreign substance and the body surface to a given chemical, usually results in uptake and leads to an internal dose. For traditional chemicals, the internal dose is usually assessed by both the amount of the substance and/or its metabolites or as a product of interaction with biomolecules in biological fluids. This definition of exposure biomarker cannot simply apply to nanomaterials (Bergamaschi et al., 2017). Available biokinetic data suggest that translocation rates of nanoparticles from the portal-of-entry - the respiratory tract - to secondary organs, is size- and charge-dependent (Choi et al., 2010), but the amount of particles reaching the systemic circulation is actually very low (Kreyling et al., 2002, 2009). As a result, the quantification of particles in biological matrices from lung airways can be regarded as a complementary approach to the definition of exposure and related local effects (Bergamaschi et al., 2017; Marie-Desvergne et al., 2022; Forest et al., 2021; Forest and Pourchez, 2023).

Using light scattering, the NTA technique can detect particles in liquid matrices providing their number-based concentration (Filipe et al., 2010). Therefore, the quantification of breathed particles based on an estimate of the dose deposited in the lungs (Sauvain et al., 2017; McCormick et al., 2021). Several studies are strongly focused on the diagnosis of lung disease (asthma, silicosis, asbestosis, etc.) carried out observing the relationship between exposure to particulate through the respiratory route and increased levels of inflammatory cytokine (Greenberg et al., 2007; Leung et al., 2013; Bhattacharjee et al., 2016).

Various investigations have established a correlation between the number of particles in the air and various health-related indicators, such as particle number concentration and certain biomarkers. Consequently, this metric proves promising for assessing potential health risks associated with particle exposure in both environmental and occupational settings (Chang et al., 2022; Lepistö et al., 2022).

Cytokines play a primary role in the inflammatory process, and their analysis in non-invasive matrices such as EBC is optimal for occupational sampling (Ghelli et al., 2022). Therefore, combining the measurement of particle number in the EBC using NTA, to an analysis of the inflammatory spectrum in the same matrix, could aid in assessing particle retention in the lungs, bridging the gap from exposure to inflammation, and playing a crucial role in primary prevention in occupational settings (Sauvain et al., 2014; Gubala et al., 2018).

In our study, involving a relevant number of workers from three different exposure scenarios, the NTA data were consistent with the external exposure data. Particle number quantified by NTA revealed a tendency towards higher values, though not statistically significant, in the exposed subgroup at the beginning of the working week and a statistically significant difference between NE and the whole group of exposed at the end of the working week. This probably occurred because at the beginning of the week the accumulation process has just begun while, at the end of the working week, this process has progressively occurred, allowing evidence of accumulation during the working week among the workers exposed to particle.

The categorization applied to the NTA data in EBC revealed that subjects belonging to the HE and LE had a significantly higher number of particles than NE. However, and unexpectedly, the median value was higher in LE subjects than in HE subjects. The LE workers are white collars or technical employees working in the same companies. This suggests that workers directly involved in operations with dusty materials, but wearing personal protective equipment (FFP2) are more protected than workers less or not directly involved (LE) who are not used to wear personal protective equipment (PPE), with the likelihood to result more exposed by inhalation.

This study shows that a higher concentration of airborne particles at workplace is consistently associated with a greater number of particles

in the EBC of exposed subjects as compared to NE subjects. In the study by Hemmendinger et al. there were demonstrated strong positive correlations between the airborne particle count, defined by LDSA, and inflammatory cytokines in EBC (Hemmendinger et al., 2023).

As revealed by Fig. 4, the correlation between IL-1 $\beta$  and NTA data was statistically significant, whereas the correlation between NTA data and IL-10 levels was weak ( $Rho = 0.239$ ;  $p = 0.035$ ). Finally, a positive trend between particle count and TNF- $\alpha$  data was observed, though not statistically significant.

Both IL-1 $\beta$  and IL-10 are cytokines, with the former possessing pro-inflammatory properties and the latter acting as an anti-inflammatory agent. Through a negative feedback mechanism, IL-10 helps regulate the synthesis of cytokines, achieving a balance. Additionally, C-reactive protein (CRP), produced by the liver, is employed to identify systemic inflammatory states (Sproston and Ashworth, 2018). Even so, our results reinforce the hypothesis that the number of particles in the EBC is representative of environmental exposure and is associated with and increased level of inflammatory mediators.

It is worth mentioning that, whereas the companies are located in different geographical areas, they produced different materials, different amounts of dusty materials as well as they undertook different types of processes, workers belonging to the companies A and B resulting more exposed to particulate matter as compared to those working in company C. Company C is mainly involved in the research and development of NMs, while the other facilities are involved in the production of paints, adhesives, coating materials, and construction materials. Workers belonging to the companies A and B result more exposed to particulate matter as compared to those working in company C. Company C presents just 7 subjects on 20 categorized as exposed, while the other company workers were all part of the exposed category (Table 1). This discrepancy is undoubtedly influenced by the specific processing activities conducted in these companies and the nature and quantity of materials handled. Although DiSCmini<sup>TM</sup> (Testo, DE) data of company C result significantly higher than the NE subjects (Fig. 5, a), the distribution of NTA in company C subjects exhibits comparable levels to those of NE subjects (Fig. 5, b). The partial discrepancy between DiSCmini<sup>TM</sup> (Testo, DE) data and NTA in company C can be explained by the overall less exposure, the different materials handled, different working procedures and/or more careful use of PPE. This result further highlights the importance of the determination of internal dose biomarker.

As recently observed by Luo and co-workers (Luo et al., 2022), there are still some shortcomings about the use of EBC as suitable matrix for biomonitoring purposes. However, our study shows a significant increase in the number of airborne-derived particles in EBC of the exposed subjects, which is suggestive of a higher deposition of a portion of these particles in their airways. The demonstrated concordance between the environmental and the biological measures, is consistent with other studies which reported an increased number of particles in workers exposed to silica when compared to controls (Sauvain et al., 2017; Hemmendinger et al., 2023).

Furthermore, the burden of deposited particles in the airways is associated with an increased cytokine inflammation.

Therefore, in order to better understand the actual exposure of workers, non-invasive methodologies can be used to improve workers' compliance with biological sampling.

Only a few studies have used EBC to investigate the inflammatory profile in workers. Workers exposed to nanocomposites demonstrated through their EBC samples, an increase of concentrations in biomarkers associated with oxidative stress and inflammation. Several of these biomarkers showed significant changes, although the analysis did not include the quantification of the number of particles present in the samples. Another study revealed an increase in leukotrienes, both at the beginning and end of the work shift, while a follow-up study conducted two years later detected a significant increase in certain cytokines such as IL-4, IL-10, IL-13, and TNF- $\alpha$  after exposure to nanomaterials (Pelclova et al., 2017, 2020). In the EBC samples, in addition to the NTA

analysis, was also measured the inflammatory profile, in particular interleukins 1 $\beta$ , IL-10, and TNF- $\alpha$ .

The exposed subjects exhibited significantly higher levels of all three cytokines when compared to the NE group. Similar trends were also highlighted in the serum in a study conducted by Ursini, which reported an increase in IL-6, IL-8, and TNF- $\alpha$  levels in workers exposed to nanomaterials such as graphene (Hunt, 2002; Ursini et al., 2021). Furthermore, positive correlations were observed between NTA measured in EBC and two of the three inflammatory cytokines: IL-1 $\beta$ , IL-10. Instead, no correlation was found comparing TNF- $\alpha$  and particles measured in EBC with NTA, but only an increasing trend with increasing exposure resulted. A similar answer of TNF- $\alpha$  was found in pathological subjects, in controls after exposure to airborne particulate (PM) (Ghozikali et al., 2022) and in subjects exposed to titanium dioxide. An increase inflammatory levels of IL-1 $\beta$ , IL-6, IL-8, IL-10, and TNF- $\alpha$  in plasma was observed as a result of professional exposure (Zhao et al., 2018). To the best of our knowledge, our study represents the initial attempt to quantify the particles in the air analysed using DiSCmini™ (Testo, DE) and compare them with those found in the exhaled breath of a multicenter cohort of workers exposed to nanomaterials, incorporating inflammatory biomarkers. Our study aims to provide a starting point for the identification of an internal dose marker that can reflect the actual uptake of particles present in the workplace environment.

However, this study presents some limitations, such as the small size of the epidemiological sample which would be useful to expand to acquire greater statistical power and, at the same time, to challenge the above approach against different occupational. Furthermore, an important future perspective will be to discriminate between inorganic and organic particles, to provide an even more accurate measure of particle uptake by the subjects.

## 5. Conclusions

In recent years, the advancement of technology has result in an increasing use of NMs across various industrial and technological sectors. This trend has raised concerns in the scientific community about the toxicological properties of these substances and possible short- and long-term health effects. Consequently, there is a pressing need for a multidisciplinary approach that integrates exposure assessment and quantification of non-invasive biological markers in specific matrices (Schulte et al., 2018; Bergamaschi et al., 2017). Identifying suitable biomarkers reflecting actual exposure to these substances is crucial. This study confirms previous studies and represents a further step in demonstrating the reliability of the analysis of particles in EBC to quantify the number of particles present (using NTA) in subjects recruited from an international multicentre study. The health significance of such internal dose is reinforced by the association with an inflammatory profile analysed in the same matrix. In conclusion, the use of NTA as a tool to investigate the internal dose, integrating the assessment of external exposure, with the inflammatory profile, represents a valid starting point for assessing the fraction of unabsorbed particles that could increase the levels of airways inflammation. Exposure assessment combined with biomonitoring seems the most useful tool for identifying the causal relationships and the potential risks that workers can be exposed (Bergamaschi et al., 2015; Hemmendinger et al., 2023). Further investigations will be needed on a larger sample of workers in diverse company settings.

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## CRediT authorship contribution statement

**Marco Panizzolo:** Writing – original draft, Investigation, Formal analysis, Data curation. **Francesco Barbero:** Writing – original draft, Investigation, Formal analysis, Data curation. **Federica Ghelli:** Writing – review & editing, Visualization, Methodology, Data curation. **Giacomo Garzaro:** Validation, Visualization, Writing – review & editing. **Valeria Bellisario:** Validation, Visualization, Writing – review & editing. **Irina Guseva Canu:** Writing – review & editing, Visualization. **Ivana Fenoglio:** Writing – review & editing, Visualization, Conceptualization. **Enrico Bergamaschi:** Conceptualization, Funding acquisition, Project administration, Writing – review & editing. **Roberto Bono:** Conceptualization, Resources, Supervision, Validation, Writing – review & editing.

## 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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