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ABSTRACT

Nanotechnology applications are fast-growing in many industrial fields. Consequently, health effects of engineered nanomaterials (ENMs) should be investigated. Within the EU-Life project NanoExplore, we developed a harmonized protocol of an international multicenter prospective cohort study of workers in ENM-producing companies. This article describes the development of the protocol, sample size calculation, data collection and management procedures and discusses its relevance with respect to research needs. Within this protocol, workers' ENM exposure will be assessed over four consecutive working days during the initial recruitment campaign and the subsequent follow-up campaigns. Biomonitoring using noninvasive sampling of exhaled breath condensate (EBC), exhaled air, and urine will be collected before and after 4-day exposure monitoring. Both exposure and effect biomarkers, will be quantified along with pulmonary function tests and diagnosed diseases reported using a standardized epidemiological questionnaire available in four languages. Until now, this protocol was implemented at seven companies in Switzerland, Spain and Italy. The protocol is well standardized, though sufficiently flexible to include company-specific conditions and occupational hygiene measures. The recruitment, to date, of 140 participants and collection of all data and samples, enabled us launching the first international cohort of nanotechnology workers. All companies dealing with ENMs could join the NanoExplore Consortium, apply this harmonized protocol and enter in the cohort, concieved as an open cohort. Its protocol meets all requirements of a hypotheses-driven prospective study, which will assess and reassess effects of ENM exposure on workers' health by updating the follow-up of the cohort. New hypothesis could be also considered.

Introduction

Background and rationale

Nanotechnology is an emerging field and fast growing in industry and science (Ellenbecker and Tsai 2015). Engineered nanomaterials (ENMs) are nanosize matter (approximately 1–100 nm) produced and used to develop novel material with exceptional physiochemical properties (European Commission 2011; NIOSH 2018; Kuempel et al., 2021). For these reasons, ENMs are used in many industry sectors including electronics, energy, healthcare, nutrition, and cosmetics (Chen

et al. 2021, Schubauer-Berigan et al. 2020, Ursini et al. 2021, Wu et al. 2021). Although ENMs can be highly beneficial for several industrial fields, there are many unanswered questions about their potential environmental and health effects (Hodson, Geraci, and Schulte, 2019; Riediker et al. 2012).

Most of the current knowledge about ENMs' toxicity comes from experimental *in vitro* and *in vivo* studies. These studies have shown that ENMs can induce harmful effects on cells, tissues, and organs, such as reduction ofimmune cell viability, increase in oxidative stress, inflammations, endothelial

permeability, pro-thrombotic state, airways irritation, and can cause diseases such as cancer (Guseva Canu et al. 2018; Schulte et al. 2009; Mostovenko et al. 2021; Murphy et al. 2016; Müller et al. 2018; Dávila-Grana et al. 2018; Erdely et al. 2009; Li et al. 2007; Shvedova et al. 2005). Indeed, these effects can differ depending on the type of ENMs, but also within the same family of ENMs depending on some specific physicochemical properties (Drew et al. 2017; Stone et al. 2020). Such studies are necessary and important for identifying hazardous effects of nanoparticles, yet they are insufficient in assessing human exposure and health risks, developing recommendations and implementing occupational exposure limits to consistently manage ENMs' risks. First, ENMs may react differently in simplified biological models (e.g. immune cells) compared to complex biological milieu such as specific human tissues, organs and systems (Cedervall et al. 2007; Monopoli et al. 2012). Second, some deleterious effects were observed only for extremely high doses that are not representative of working conditions (Krug 2014; Pauluhn 2011). Although no overt health effects in humans caused by ENMs have been reported yet, ensuring the safety of workers and consumers is mandatory for the responsible development and the long-term sustainability of nanotechnology-enabled industry (European Commission 2014).

Conducting epidemiological studies with a comprehensive exposure and health outcome assessment at short and long terms is, thus, the next necessary step in the anticipation of potential adverse health effects related to exposure to ENMs in humans, particularly for medium and longer-terms (Guseva Canu, Burstyn, and Richardson 2016b).This seems especially urgent, as by the end of 2020, the number of exposed workers has been estimated to be six million worldwide and this number is expected to grow (Ghafari, Moghadasi, and Shekaftik 2020; Roco 2011).

Epidemiological data from human populations specifically exposed to ENMs are currently very limited. A systematic review by Schulte et al. (2019) identified 27 studies in humans exposed to ENMs published over the past 15 years. Eighteen of these 27 studies were cross-sectional, including four studies with no comparison (i.e. non-exposed) group, and a very small sample size (from two to 16 workers), i.e. characteristic of an exploratory, rather than epidemiological study design. Only four research teams conducted repeated measurements of

exposure and outcomes, with variable follow-up duration (i.e. 5 months, 12 months, three and four years), usually by adopting a panel study design. Yet, this design is particularly sensitive to attrition (Lugtig and Smith 2019). In studies of ENM workers, the number of participants remaining at the subsequent follow-up drops dramatically and ranges between less than 30% (Ghosh et al. 2017; Kuijpers et al. 2018) and up to 60% (Afshari 2017; Pelclova et al. 2017) of the initial sample. The highest participation rate (85%) at one and two year follow-up was reported by Pelclova et al, (Pelclova et al. 2020), but the study consisted of only 20 exposed workers. Such settings preclude a causal inference analysis, therefore, the evidence on potential health effects of ENM exposure in humans remains limited (Schulte et al. 2019; Gulumian et al. 2016).

Launching epidemiological studies among nanotechnology workers is challenging (Guseva Canu et al. 2018). Besides methodological limitations pertaining to the epidemiological studies, numerous scientific issues hamper the realization of cohort studies of nanotechnology workers. They include a lack of standardized exposure assessment methods for quantifying ENM airborn concentrations as well as measuring relevant ENM exposure biomarkers; the large variety of existing ENMs and their properties; the lack of knowledge about ENMs toxicological modes of action, dose-response functions and target organs, as well as about specific biomarkers allowing to assess biological and health effects related to ENMs exposures; and the relatively small number of workers exposed to a given (subset of) ENMs for a large period of time (Guseva Canu et al. 2018; Riediker et al. 2012).In this regard, Riediker et al (Riediker et al. 2012) emphasized that, before launching large-scale studies on health monitoring, it is paramount to determine the feasibility of such studies, as well as usefulness and reliability of the results. They strongly suggested establishing well-defined frameworks that will properly identify study populations and select study designs, characterize exposures and define appropriate outcome measures.The usage of biomarkers is encouraged for both assessing and monitoring the exposure to ENMs, and the early biological and health effects of this exposure (Bergamaschi et al. 2015).

According to the World Health Organization (WHO), biomarkers are chemicals, their metabolites, or products of an interaction between a chemical and some

target molecule that is measured in the human body compartments (WHO 2006). Exposure biomarkers reveal the concentration of a parent compound or its metabolites in human biological matrices (Nieuwenhuijsen, Paustenbach, and Duarte-Davidson 2006), such as urinary metal concentration after exposure to metal-containing ENMs. Effect biomarkers are measurable biochemical, physiological, and behavioral effects or other alterations within an organism that, depending on the magnitude, can be associated with an established or possible health impairment or disease (Zare Jeddi et al. 2021). They indicate changes in biological systems resulting from complex pathways of exposure. Since effect biomarkers often reflect subclinical changes before the onset of disease, they are a valuable tool for anticipating the potential adverse effects of ENMs and elucidating dose–effect relationships. However, in practice, the use of biomarkers for environmental and occupational exposure and health surveillance is limited (Viegas et al. 2020). Obstacles to the implementation of biomonitoring include the lack of standards and guidance values needed to interpret the results, as well as organizational and logistical aspects, which may be difficult to manage in some contexts (Crézé et al. 2021; Jones 2020). Biomarkers should ideally be measured in biological matrices collected noninvasively and enabling a routine screening and monitoring of workers (Crézé et al. 2021). In prospective studies, which need repeated measurements of biological matrices, the non-invasiveness of the procedure can increase the participation rate of the workers and of not exposed volunteers, thus allowing a more consistent and representative study sample. Exhaled breath condensate (EBC), exhaled air and urine could therefore be considered the three preferred biological matrices for non-invasive monitoring of ENM workers. Biomarkers measured inEBC can assess acute or long-term changes in lung biopathology, giving insights on oxidative stress and inflammatory response but also on tissue remodeling (e.g. lung fibrosis or COPD) (Pelclova et al. 2018; Graczyk et al. 2015), whereas urinary biomarkers can reflect systemic changes (Smolders et al. 2010; Manno et al. 2010; Bergamaschi et al. 2015; Schulte et al. 2018).

Objectives and research hypotheses

The NanoExplore project aimed at building an integrated network for studying biomarkers for identifying early effects on health related to ENM exposures in nanotechnology workers, in the European Union and Switzerland. In particular, it aims at assessing a representative panel of biomarkers known to reflect particle exposure and various short- and long-term biological endpoints such as inflammation and oxidative stress, immune system dysregulation, host defense impairment and activation of the pro-fibrotic cascade, both at local (pulmonary) and systemic levels.To address these research needs, we propose a harmonized protocol for a prospective epidemiological cohort, encompassing a comprehensive assessment of occupational and environmental exposure to ENMs combined with a feasible biological monitoring. Although we focus on ENMs, the protocol can be adapted to studies investigating exposures to nano-objects and their agglomerates and aggregates and non-engineered ultrafine particles.

As a first preparatory step, we identified and defined target ENMs, manufacturing processes, potentially exposed workers, biomarkers, and biomonitoring procedures relevant for exposure and health assessment by reviewing the published literature and technical reports (Bergamaschi et al. 2019; Domat 2019). The available toxicological findings suggested that the pulmonary exposure to some ENMs can cause cardiovascular and respiratory diseases, particularly the lung fibrosis, but also cancer (Schulte et al. 2018). However, these diseases usually appear after a long latency of several years or decades, while the majority of the current workforce in nanotechnology has not been exposed for a long enough time and/or at high enough level to develop such diseases at this time. Conversely, the early responses to ENM exposure are potentially appropriate outcomes to measure, even though most of them reflect short-term and sometimes reversible changes with uncertain clinical significance. In particular, ENMs are known to elicit the inflammatory responses that could be monitored in blood, serum, induced sputum, urine and EBC of exposed workers. Similarly, ENMs may activate oxidative stress responses that could reduce the concentration of biomarkers of antioxidant defense system at systemic level and increase the level of reactive oxygen species (ROS) (Schulte et al. 2019). We thus included the biomarkers of fibrosis, inflammation and oxidative stress that can be measure at pulmonary and systemic levels.

Second, we conducted an online survey of nano-technology companies to assess the biomonitoring acceptance among managers and workers, as well as the practical aspects affecting its feasibility in the field (Crézé et al. 2021). We also assessed the propensity and barriers to participate in a longitudinal epidemiological study among these specific populations. In this article, we present the harmonized protocol with special emphasis on standardization of procedures, study sample representativeness and recruitment feasibility.

The protocol development integrated several underlying hypotheses. First, we hypothesized that the exposure and effect biomarkers selected for this study are associated with ENM exposure in a dose-response manner and that the fluctuation of ENM exposures would affect within working week the concentrations of the most sensitive biomarkers. Second, we hypothesized that the ENM exposure can also act on a longer-term basis (e.g. by affecting the bio-markers of chronic effect, such as a lung fibrosis or some clinically measured lung function parameters). Furthermore, we hypothesized that ENM exposure reduction recommendations can affect the biomarker profile and disease prevalence at the long term, e.g. between initial and subsequent follow-up campaigns, several years later. This set of hypotheses reflects the current overview of toxicological findings for ENMs, which in large majority suggest some exposure-related changes in early effect biomarkers, although varying depending on the type of ENMs handled. Human studies reporting changes corresponding to the pathological alterations, namely in pulmonary functional parameters, are still limited (Li et al. 2018; Schubauer-Berigan et al. 2018; Zhao et al. 2018; Cao et al. 2020). However, these long-term effects could be better evidenced with a longer follow-up of exposed workers in the context of chronic exposure (Guseva Canu et al. 2018; Mostovenko et al. 2021).

On the basis of the results of pilot studies, we discuss the most challenging aspects to be taken into account in cohort development and future studies.

Material and methods

Setting up the study protocol

There are four main prerequisites for the study protocol: i) to create a well-characterized registry of eligible participants (i.e. ENM worker registry) for prospective epidemiological studies by developing a solid, yet feasible recruitment strategy; ii) to harmonize and standardize all processes in deriving the exposure assessment strategy, in characterizing the population, in verifying pre-analytical requirements for the biomonitoring methods; iii) to select a panel of biomarkers for prospective epidemiological studies by assessing relationships between exposure and health effects at short-, medium- and longer-terms; iv) to evaluate associations between concentrations of airborne particle exposure and biomarkers of exposure and early effects in terms of causal inference after reducing, whenever possible, the extent of exposure (Schulte et al. 2016; Guseva Canu et al. 2013).

Study design

The proposed study design relies on an inter-national multicenter open prospective cohort, with the first follow-up end-point planned at 6 to 9 months since the first recruitment (Figure 1). The precise planning and frequency of the follow-up campaigns will be scheduled in close collaboration with companies, depending on their operational availability and budget.

Study population and sample size

The target population encompasses workers handling ENMs and more generally nano-objects, their aggregates and agglomerates (European Commission 2014) during their occupational tasks. On the basis of the results of our preparatory survey (Crézé et al. 2021), we will focus on companies manufacturing and/or processing metal (Al, Ag, Cu), metal oxide (TiO_2, ZnO, CeO_2, SiO_2 -both crystalline and amorphous-, Fe_xO_y, Al_2O_3, $CaCO_3$), carbon-based ENMs (single- and multi-walled nanotubes, graphene) and nanocellulose. As 50% of responding companies reported to have less than 10 employees handling these ENMs, it is crucial to include a sufficient number of companies and workers to ensure an appropriate gradient of exposure and statistical power for causal inference analysis.

Two complementary approaches were used to determine the minimal sample size needed to attain sufficient statistical power. First, external exposure
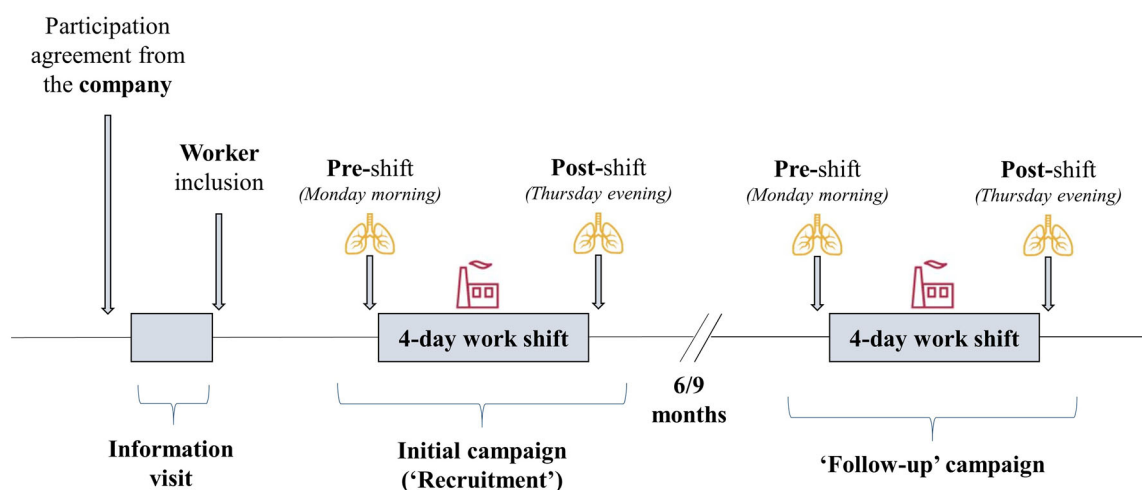
Figure 1. Main steps of the NanoExplore cohort construction and follow-up. An information visit conducted beforehand will serve to collect company-related information, inform and enroll volunteer participants into the cohort. Participants' data on exposure and health outcomes will be collected during the field campaigns. Both the initial ('recruitment') and 6 or 9-month follow-up campaigns will be conducted following similar procedures. Airborne exposure to ENMs (symbol: plant) will be monitored during a 4-day work shift. Biological matrices (i.e. EBC, exhaled air and urine; symbol: lungs) for biomarker quantification will be sampled in pre- and post-shift.

and post- vs. pre-shift variations of the exposure and effect biomarkers were estimated based on the study by Pelclova et al.(Pelclova et al. 2018). The corresponding within-group standard deviation of the individual within-subject variation was approximated based on results of the study by Hopf et al. (Hopf et al. 2019), where the same biomarkers of oxidative stress were quantified in urine and EBC. For all calculations, estimations were performed under the hypothesis of identical standard variation in both exposed and non-exposed workers. All estimations were calculated with a type I error (α) set at 5% and a statistical power ($1 - \beta$) set at 80%.

A second sample size calculation was performed for the exposure biomarkers, i.e. estimation of the difference that we expected to observe in biomarkers concentration between exposed and non-exposed workers. Between-subject internal exposure variations (i.e. particulate number concentration in EBC determined by Nanoparticle Tracking Analysis) were estimated from the study on airport worker populations exposed to ultrafine particles (Marie-Desvergne et al. 2016).

Based on these calculations, we determined that a minimal study sample size of 120 workers (60 exposed, 60 non-exposed) was needed to detect a significant difference of at least 25% between groups in the within-subject variation of biomarkers of effect. Taking into account a lost-to-follow-up

ratio of 20% due to constraints related to the collection of biological samples and employee turnover (Liao et al. 2014), an optimal sample size would include 80 exposed workers and 80 non-exposed workers. For more details of sample size calculation, see Web Appendix A.

It is noteworthy that in many companies the so-called 'non-exposed' workers (whose occupational tasks are not related to ENMs per se, e.g. administrative tasks) may be non-intentionally exposed to ENMs, because of non-effective confinement between production and administration areas (Ellenbecker and Tsai 2015). Therefore, we will split the non-exposed group in two subgroups. A subgroup with negligible or low ENM exposure will consist of administrative office workers from the same companies where workers for the 'Exposed' group will be recruited. A second non-exposed subgroup will consist of workers with confirmed absence of exposure to ENMs in their occupational settings (i.e. workers from companies whose activities are not related with ENMs).

Recruitment strategy

Participants will be recruited in a two-step procedure. First, members of the NanoExplore Consortium will recruit eligible companies in their respective countries to get a representative sample of

companies. For this, Consortium members will make use of professional contacts with companies established during the regular activity of or with occupational physicians, occupational hygienists or researchers during NanoExplore and previously conducted surveys (Schmid, Danuser, and Riediker 2008; Crézé et al. 2021), and with the help of an announcement posted on the NanoExplore website. For this recruitment step, priority will be given to companies with confirmed ENM exposure and with more than five ENM exposed workers).

In a second step, an onsite information visit will be organized in companies that previously gave their agreement to participate in the study (Figure 1). During this visit, a questionnaire will be administered to managers or health and safety specialists to collect standardized information on company activities, processes at risk for ENM exposure, as well as equipment and infrastructure already available for employee protection (further referred to as the company questionnaire and available on Unisanté data repository https://doi.org/10.16909/-dataset/31). Next, eligible workers will be recruited. A member of the NanoExplore Consortium will explain study objectives, procedure and potential risks and benefits related to study participation. Workers willing to participate will then be provided with detailed information about data collection procedures. A written informed consent will be obtained from each participant before her/his inclusion. For practical and logistical reasons, the non-exposed group will consist of workers from NanoExploreConsortium institutions.

Data collection

Individual data on ENM exposure, health outcomes and potentially confounding factors will be collected during the field campaigns, as illustrate in Figure 2.

Main outcomes

In this study, we will assess several types of outcomes. As primary outcomes, we will consider the effect biomarkers reflecting biological changes at pulmonary and systemic levels. Table 1 describes the effect biomarkers and their corresponding analytical methods. We selected both 'traditional'

biomarkers of inflammation, immune dysfunction, and oxidative stress and 'new' biomarkers, namely High-Sensitivity C-Reactive Protein, Surfactant protein-D (SP-D), and Krebs von den Lungen glycoprotein 6 (KL-6), that will be measured in EBC for the first time. While most biomarkers reflect the early or short-term effects of the exposure, the SP-D and KL-6 are regarded as biomarkers resulting from long-term effects (Bergamaschi et al. 2022). The biomarkers will be measured in the initial and follow-up field campaigns in three types of biological matrix: urine, EBC and exhaled air. During each campaign, biological matrix sampling for biomarker analysis will be conducted twice, i.e. before and after a 4-day monitoring of external exposure (further referred to as pre- and post-shift, respectively). Web Appendix B summarizes the standardized biological sampling procedures.

Furthermore, pulmonary function parameters, including the Forced Expiratory Volume in 1 second (FEV1), the Forced Vital Capacity (FVC) and the Forced Expiratory Fluxes at 25% and 75% of FVC (FEF25–75%) will be considered as secondary outcome. These outcomes could reflect the early and/or delayed effects of ENM exposure on the respiratory system. The pulmonary function tests will be performed by an occupational physician of the NanoExplore Consortium (for details, see Web Appendix B).

Finally, some selected diseases diagnosed by participants' physicians and self-declared by the study participants in the epidemiological questionnaire (https://doi.org/10.16909/dataset/31) could be considered as protracted effects of the ENM exposure and analyzed as tertiary health outcomes. These will include cardiovascular, respiratory, allergic, and cancer diseases selected based on the evidence from experimental and human studies on the effects of ultrafine and fine particle exposure.

Exposure assessment

We will follow the Nanoparticle Emission Assessment Technique (NEAT) during the field campaigns. NEAT was established for the identification and measurement of potential inhalation exposure to ENM (Methner, Hodson, and Geraci 2010). Airborne particle number concentration will be measured over the full or a part of work shift for
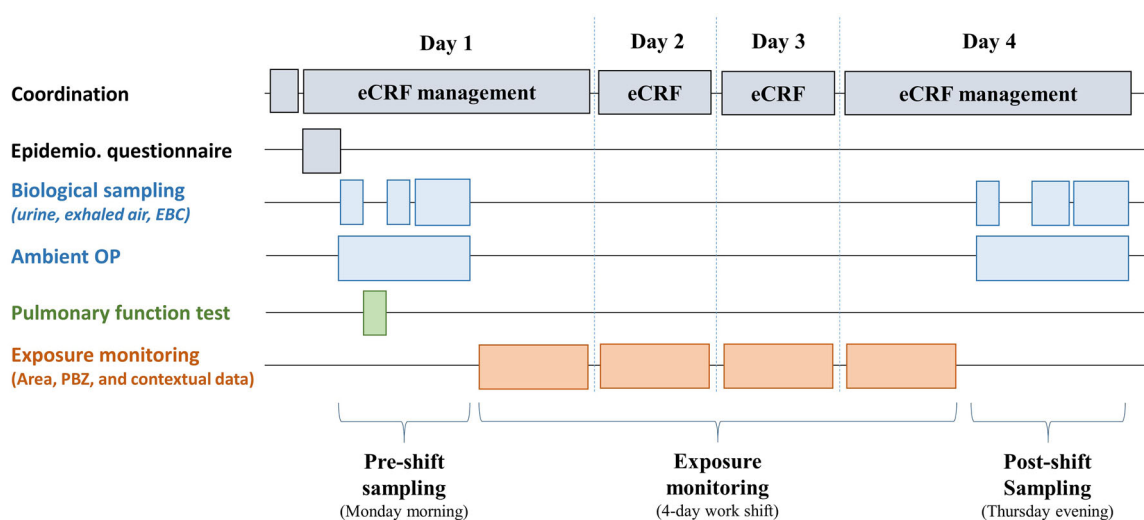
Figure 2. Structure and timeline of the NanoExplore campaign in the field. The field campaign lasts four days. The electronic epidemiological questionnaire is filled once, in the beginning of the campaign. Biological sampling takes place twice, i.e. in pre-shift on the 1st day and post-shift on the last day of the campaign. Pulmonary function test completes the pre-shift sampling. Exposure monitoring is conducted over a 4-day 8-hour work shift. The overall coordination is ensured using workers' individual electronic Case Report Forms (eCRFs). EBC: exhaled air condensate, OP: oxidative potential

four consecutive days, i.e. between pre- and post-shift biological sampling sessions detailed in paragraph 2.5.1. Stationary and personal air monitoring will be conducted using NanoExplore kit of devices whose combined use offers an adequate multi-metric strategy of ENMs exposure assessment (Figure 3), and consists of three built-in measuring devices (Methner, Hodson, and Geraci 2010):

1. The Optical Particle Counting (OPC) module measures mass concentrations for particulate matter (PM) with size lower than 1, 2.5 and 10 μm, respectively (PM1, PM2.5 and PM10 respectively), using real-time light scattering-based diameter measurements.
2. The nanoparticle sensor (Partector 2) measures the electrical diffusion charging behavior of airborne particles – submicronic particles passing through the impactor are electrically charged at the instrument inlet – in order to calculate the particle number concentration and average diameter, and consequently derive the Lung Deposited Surface Area (LDSA) parameter in real-time, with a time-resolution of one second. The mass concentration is thus obtained through a series of approximations on the measured particles (Asbach et al., 2017).
3. The transmission electronic microscopy (TEM) particle sampler consists of a holder of an organic polymer film hosting a metal grid) connected to

a built-in pump (Rotary Vane Pump G 6/02-4 EB from THOMAS), operating at a maximum flow of 2.6 l/min, a maximum pressure of 230 mbar, and a maximum vacuum of 23%.This pump enables collecting airborne particles during ENM-releasing activities. The particles trapped on the filter will be analyzed following standardized laboratory procedures to evaluate particles' chemical profile, average size distribution and shape (see Web Appendix C for details).

The ambient oxidative potential will be measured in order to evaluate the aerosol ability to modify the redox homeostasis in the lungs (Bates et al. 2019). This metric will be used to standardize the oxidative potential measured in workers' exhaled air (OPEA, described in the Table 1) (Goekce et al. 2022).The original approach developed by Unisanté consists of a sampling train made of a Teflon filter inserted in an IOM cassette followed by XAD-2 sorbent tube operating at a pump rate of 2 L/min. OPEA and ambient oxidative potential will be measured just before or just after the urine and EBC sample collection. Upon the end of the sampling period (typically 6–8 hours), both filters and XAD-2 tubes will be stored at 4 °C with the help of a cooler. The oxidative potential analysis will be performed following the procedure described in Sauvain et al (Sauvain et al. 2021). For details on exposure measurement methodology, see Web Appendix D.

Table 1. Effect biomarkers measured in the NanoExplore cohort.

Biomarker	Biological matrix	Biomarker description	Analytical method
8-isoprostane	Urine	Oxidative stress	ELISA
Malondialdehyde (MDA)	Urine	Oxidative stress	Thiobarbituric Acid Reactive Substances assay (Colorimetric)
8-Oxo-2′-deoxyguanosine (8OHdG)	Urine	DNA oxidative damage	ELISA
Total Antioxidant Power (TAP)	Urine	Antioxidant capacity	Cupric ion reducing antioxidant capacity assay (Colorimetric)
Creatinine	Urine	Clinical biomarker for renal excretion, used for volume normalization of spot urine samples	Spectrophotometric method (Kinetic Jaffé procedure)
8-Oxo-2′-deoxyguanosine (8OHdG)	EBC	DNA oxidative damage	ELISA
8-isoprostane	EBC	Oxidative stress	ELISA
Malondialdehyde (MDA)	EBC	Oxidative stress	Thiobarbituric Acid Reactive Substances Assay (Colorimetric)
Tumor necrosis factor (TNF-α)	EBC	Pro-inflammatory multifunctional cytokine; it plays important roles in different cellular events such as cell survival, proliferation, differentiation, and death	Real time PCR- linked ELISA
Interleukin 1 beta (IL-1β)	EBC	Pro-inflammatory cytokine; key mediator of the inflammatory response	Real time PCR- linked ELISA
Interleukin 6 (IL-6)	EBC	Pro-inflammatory cytokine secreted by macrophages in response to specific microbial molecules (PAMPs)	Real time PCR- linked ELISA
Interleukin 10 (IL-10)	EBC	Immuno-suppressive cytokine, which reduces the recruitment of effector T cells and counteracts the effects of TNF-α and IL-1β	Real time PCR- linked ELISA
Leukotriene B4 (LTB4)	EBC	Bronchial tract activation, permeability and local inflammation	ELISA
High-Sensitivity C-Reactive Protein (Hs-CRP)	EBC	Low grade systemic inflammation	ELISA
Surfactant protein-D (SPD)	EBC	Regulation of pulmonary host defense and inflammation; type II alveolar epithelial cells integrity	ELISA
Krebs von den Lungen glycoprotein 6 (KL6)	EBC	Potential biomarkers of interstitial lung disease; activation of pro-fibrotic cascade	ELISA
Nitrotyrosine	EBC	Nitrosative stress	ELISA
Oxidative potential in exhaled air (OPEA)	Exhaled air	Oxidative stress	OPEA analyzer + FOX colorimetric test (6 min including sampling)

Ambient temperature and relative humidity will be continuously monitored using a multifunction measuring instrument TESTO 435 (Strategies 2017). This instrument will be placed near the worker's personal breathing zone to characterize the environmental conditions of the process and the value measured will be taken before the start of the shift or activities with ENMs.

This exposure assessment will be completed with biological exposure monitoring. Exposure biomarkers will be measured in urine and EBC as described in Table 2. While the metal concentration measured in urine is a widespread method of exposure monitoring, the quantification of number concentration of nanoparticles in the EBC samples is still rarely used (Guseva Canu et al. 2021b). This metric can be measured using the nanotracking analysis (NTA), which also determines the hydrodynamic size distribution with a diameter of approximately 40–1000 nm (nm) in liquid suspension (Sauvain et al. 2017).

As effect biomarkers, exposure biomarkers will be measured twice at each field campaign according to the standardized procedures described in Web Appendix B.

It worth to mention that after the each field campaign, companies will receive the coded company-specific results of exposure measurement, and when appropriate, the advices and recommendations of a occupational hygienists from the NanoExplore Consortium to support companies in the implementation of remediation strategies for reducing exposure. The exposure measurements conducted in consecutive field campaigns will allow to evaluate, whether companies follow these recommendations and whether the implement exposure control measures are effective in lowering ENM exposure.

Individual risk factors and co-exposures

In order to reduce the likelihood of biased result interpretation when associating ENM exposure with
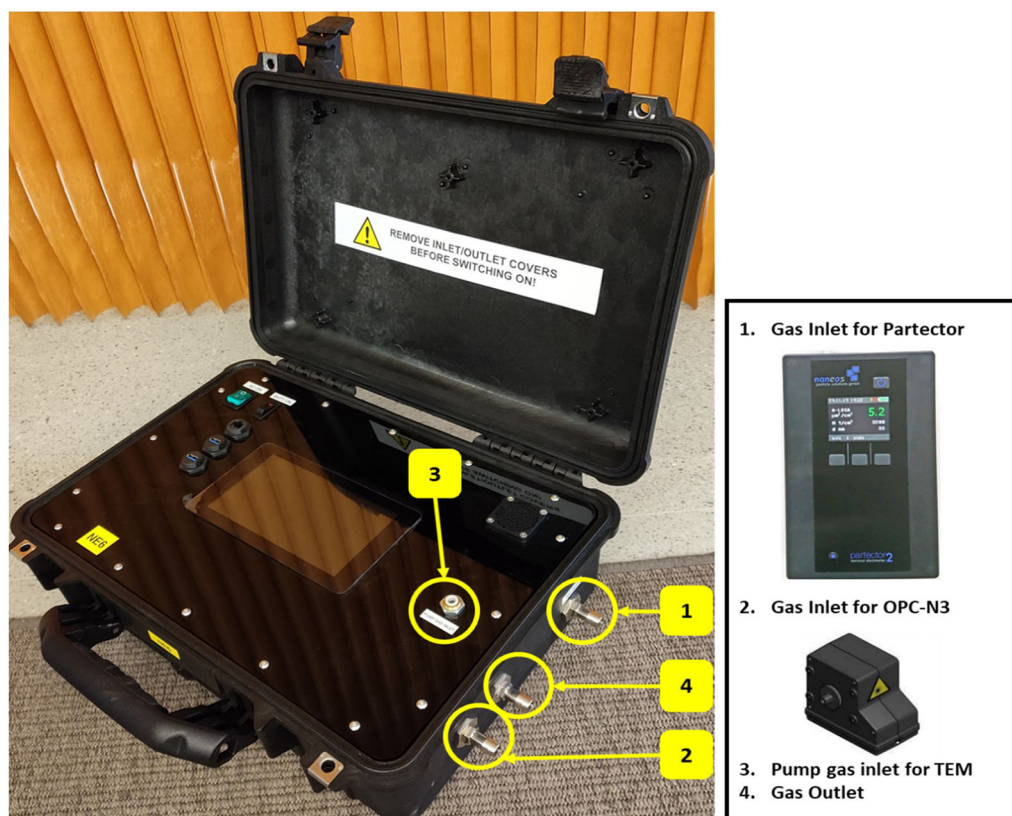
Figure 3. The NanoExplore kit for exposure monitoring. The NanoExplore kit includes three built-in modules: the Optical Particle Counting module (OPC), the nanoparticle sensor (Partector 2), and the transmission electronic microscopy (TEM) particle head sampler.

Table 2. Exposure biomarkers measured in the NanoExplore cohort.

Biomarker	Biological matrix	Analytical method
Metal mass concentration Ag, Cu, Ti, Zn, Ce, Fe, Al, Mo[a]	Urine	ICP-MS
Particle size and number concentration	EBC	Nanoparticle Tracking analysis (NTA)
Metals mass concentration Ag, Cu, Ti, Zn, Ce, Si, Fe, Al, Ca, Mo[a]	EBC	ICP-MS

[a]Measured only in workers exposed to metal containing ENMs

individuals' biomarker concentrations, individual, contextual and potentially confounding factors will be explored using an epidemiological questionnaire. This questionnaire will be administered to all workers enrolled in the study at baseline and will address determinants of occupational, environmental and domestic exposures (both past and present) to fine and ultrafine particles. Moreover, information on the smoking status, life habits related to inflammatory status (e.g. sleep, food and physical activity habits, paraclinical parameters) and health- and allergy-related parameters, with a specific focus on respiratory parameters, onco-hematological diseases, immuno-deficiency syndromes, auto-immune diseases and cardiovascular events (both past and present) will be collected. The baseline epidemiological questionnaireis is available at the Unisanté data repository (https://doi.org/10.16909/dataset/31).

Standardized coordination for field campaigns

The standardized procedure illustrated in Figures 1 and 2 should ensure reproducibility, comparability and precision of data collected during different field campaigns. Procedures for the initial and the follow-up campaigns are nearly identical, except for the epidemiological questionnaire, which is significantly shorter at follow-up and only aims to update the information collected atthe initial campaign.

Once potentially eligible workers are identified during the information visit to the company (see *Recruitment strategy*), they are pre-allocated to the 'Exposed' or the 'Non-exposed' group according to the information about their exposures provided by the company's occupational safety and health specialist. This pre-allocation aims at facilitating participant recruitment, to meet the sample size requirements, while the final classification of participants with respect to ENM exposure will be based

on individual exposure monitoring data. Next, study participant IDs (coded) are created by the study coordinator at Unisanté using the REDCap software (Harris et al. 2009). Links to the questionnaire will be sent to participants by the partner institutions. Recruited participants will then complete the epidemiological questionnaire using either their mobile phone, tablet or personal computer upon a personalized invitation link managed via REDCap. All participants have at least one of these electronic devices available at work and will fill in the questionnaire during their working-time. Throughout biological sampling procedures, individual forms will also be completed in REDCap, thus ensuring standardized collection of information pertaining to biological samplings. The overall collection of all forms and questionnaires pertaining to one study participant constitutes his/her electronic Case Report Form (eCRF). Use of electronic questionnaire and forms was preferred as this prevents data collection from human errors related to entering data from paper questionnaires into the REDCap system. Moreover, REDCap allows the language choice for questionnaire completion between Spanish, Italian, French, German, and English. Electronic data collection and storage will be organized in accordance with the ethical guidelines. Only coded (de-identified) data will be centralized at the study coordinating center (Unisanté)(Tajani and Edtstadler 2018). In order to avoid analytical variability, the collected samples will beanalyzed in the same laboratory at the University of Torino and the coded results will be centralized at Unisanté secured server, curated and analyzed statistically.

A local project coordinator will be available on site to answer questions from workers or company representatives regarding study objectives and procedures during the sampling campaigns. Exposure assessment results will be communicated to companies in the form of a confidential report and explained by an occupational hygienist from the NanoExplore Consortium. Advice regarding exposure reduction measures will also be formulated. Whenever possible, biomarker concentrations will be compared with available reference intervals for European adult population and the meta-estimates of background values in healthy and non-exposed population reported in literature (Graille et al. 2020a; Graille et al. 2020b; Hemmendinger et al. 2020;

Shoman et al. 2020; Toto et al. 2022; Turcu et al. 2022; Guseva Canu et al. 2022).

Code of ethics and participation consent

This study follows the principles of the Declaration of Helsinki. The study protocol was approved by the NanoExplore Consortium (https://www.lifenanoexplore.eu/about/consortium) and the EU monitor in charge of the NanoExplore project. Moreover, approvals have been obtained from the local ethics regulation organs: the Swissethics in Switzerland (approval 2020–01098); the Bio-ethical Committee of the University of Torino in Italy (approval 336577 8.08.2020); and the Health and Safety Board of the Catalan Institute of Nanoscience and Nanotechnology, in Spain (approvall CN2-22-03-2022). All participants have given an individual written informed consent to the inclusion of material pertaining to themselves and acknowledged that they cannot be identified via the paper; and that we have fully de-identified their data.

Statistical analysis

First, the data of the initial campaign will be analyzed as a cross-sectional sample, where non-exposed workers, workers with low exposure and exposed workers will be compared using linear models to ensure comparability in terms of demographic and socio-economic variables.

In a second step, short-term effects from occupational ENM exposures on the within work-week differences in the selected biomarkers will be investigated using multiple linear mixed models. The change in each biomarker concentration over 4-days will be modeled as function of individual ENM exposure measurements accounting for potential confounders or effect modifiers explored using the variables collected by epidemiological questionnaire. Such analyses will be performed on a within-campaign basis, to associate external and internal exposure patterns and biomarker concentrations.The effect of the different types of ENMs identified in the company and epidemiological questionnaires can be assessed and compared in stratified analysis comparing each of the most frequent types of ENMs with the non-exposed participants. Adequate control for multiplicity of tests will be performed using standard

procedures. As a third step focusing on external exposure, the time-dependent minute-by-minute variations in particle number concentration will be investigated with respect to exposure determinants. These time series will be visually inspected for qualitative assessment, then quantitatively described using Bayesian spline models (Pétremand et al. 2021, Pétremand et al. 2022).

Finally, an overall analysis will consist in modeling, again using linear mixed models, the changes in biomarkers occurring along with the implementation of the exposure control measures over the 6/9-month follow-up by companies. These analyses will allow us to identify a set of best-fitting biomarkers to be used in future studies. Differences in exposure patterns (features of the released ENM and concentration) between initial and follow-up field campaigns will be investigated using linear models accounting for company-specific factors, to evaluate the validity of emission reduction strategies, when applicable.

Statistical analyses will be performed using a type I error of 5% ($\alpha = 0.05$; two-sided tests) with the STATA software, version 17.

Discussion

As far as we know, this is the first harmonized protocol for an international multicenter prospective cohort study of ENM workers in the world. It includes harmonization and standardization in ENM exposure assessments and pulmonary functional tests coupled with biological monitoring of early effects from ENM exposures on human health. It has been approved by different research teams, companies and countries. The prospective design of this cohort with regular follow-up campaigns enables nesting intervention studies focused on ENM exposure controls in companies with ENM exposures and monitoring health effects over medium- and long terms.These features are paramount when assessing causality, particularly when the expected diseases are chronic non-communicable diseases with a long latency time (e.g. 2 to 30 years) before their clinical manifestation, such as cancer or cardiovascular diseases (Checkoway et al. 1990; Rose 2005). The originality and strength of this protocol consist of its highly standardized, though adaptive nature, with electronic multilingual procedures,

facilitated by the use of RedCAP software. These procedures and tools for their implementation have been developed based on the previous exploratory studies conducted by the NanoExplore Consortium members (Bencsik, Lestaevel, and Guseva Canu 2018; Bergamaschi et al. 2021; Bergamaschi et al. 2015; Crézé et al. 2021; Graczyk et al. 2015; Guseva Canu et al. 2016a; Guseva Canu et al. 2020; Guseva Canu et al. 2021a; Guseva Canu et al. 2021b; Guseva Canu et al. 2017; Guseva Canu et al. 2016c; Guseva Canu et al. 2018; Hopf et al. 2019; Iavicoli et al. 2019; Sauvain et al. 2022; Bergamaschi et al. 2022), in a highly interdisciplinary framework. In fact, this Consortium includes experts in occupational hygiene, toxicology, biomonitoring, medicine, epidemiology, and biostatistics, air quality monitoring and assessment as well as in communication from six countries. Moreover, most partners are specialized in ENM exposure assessment and/ornano-toxicology, owing to their long-standing activity in EU- and internationally funded research projects and current technical/professional consultancy activity for companies. The protocol was based on the best evidence available as to the health outcomes to be measured and ENM methods of exposure and outcome assessments, in an integrated and practical approach.

This protocol is currently being implemented in a three-country pilot study (Switzerland, Spain and Italy). At the time of manuscript drafting, 140 eligible workers have been included at seven different companies. The same team will conduct the biological sampling and exposure assessments across the facilities, which would improve the consistency of the data. It is noteworthy that the protocol has been tested just at the beginning of the Covid-19 pandemic with complete or partial lockdowns and sanitary restrictions differing between participating countries. During the biological sample collection period, safety measures such as mandatory face mask wearing and disinfection of the equipment can require additional time, financial and human resources. Fear of potential contamination could decrease participation rate among workers. Additionally, exposure to ENMs could differ between the usual situation and pandemic period because of a decreased production activity/volume and an increased use of personal protective equipment. However, the study protocol appeared

sufficiently flexible to include additional safety measures, being applicable even in extremely unusual conditions.

The ongoing pilot study confirmed the importance of a harmonized protocol and standardized procedures which should lead to an effective management of geographical and cultural differences in participating countries and companies. A good level of integration of local study coordinators within the NanoExplore Consortium also appeared paramount for organizational and logistical reasons, especially pertaining to biological sample storage and transportation/shipment (e.g. preserving biological specimens by freezing). Such an integrated approach is crucial for the investigation of ENM effects on human health as it allows standardizing not only the exposure assessment and recruitment process but also the procedures of biomarkers' collection, storage and analyses. The use of EBC as a non-invasive method for the assessment of samples from the airways has become increasingly popular. The analysis of EBC allows for the measurement of mediators of local inflammation synthesized in airways of healthy and ill people as well as for the assessment of systemic inflammation through the determination of mediators coming from the bloodstream. It should be stressed that although promising, biomarkers measured in EBC are not yet validated clinically, in contrast with biomarkers of systemic inflammatory response measured in serum and changes in blood cell counts, considered more robust. Moreover, for some effect biomarkers, such as MDA, 8-isoprostane and 8-OHdG in EBC, the analytical methods still need validation and inter-laboratory comparisons (Hemmendinger et al. 2021). The latter could be facilitated through such an international collaboration. As this study encompasses a broad range of biomarkers, it will give insights on the most sensitive and relevant biomarkers among the selected subset. Their relevance with respect to clinical outcomes can be further assessedusing the Key characteristics framework (Smith et al. 2016; Lind et al. 2021). Non-invasive biological sampling implies minimal risks and burden for participants and investigators. Such procedures are easily accepted and their use is positively associated with participation rates (Crézé et al. 2021). Finally, this protocol meets all requirements of a hypotheses-driven longitudinal study, which

will assess and reassess effects of ENM exposure on workers' health by updating the follow-up of the cohort. A priori defined research questions and hypothesis testing render this study methodologically more robust and therefore more conclusive in comparison with exploratory studies conducted until now (Schulte et al. 2009). Therefore, this study will lead to an increasing knowledge of occupational exposures to ENMs across research fields and countries, and creating awareness of ENM exposures among workers, companies, as well as researchers.

It is worth mentioning that this protocol was developed for the real occupational settings and exposure scenarios. It showed its relevance in a company handling powders of micrometric range in which we found a non-negligible fraction of metal nanoparticles. In fact, several conventional materials (e.g. titanium dioxide or carbon black) considered simply as bulk powders, consist in reality of nanoparticles in aggregated or agglomerated forms (Bergamaschi et al. 2022; Fonseca et al. 2021), which may fall under the EU definition of nanomaterials. Moreover, several studies revealed that the release of ultrafine particles originating from the handling of conventional micrometer-sized materials may be substantial (Viitanen et al. 2017). As a result, workers are often exposed to a heterogeneous mixtures of different particles, which can make the quantitative exposure characterization and risk assessment very complex. Hence, the measurement of particle number concentration, size distribution and LDSA in workers' PBZ represents a pragmatic approach to assess external exposure to nanosized particles considering all potential sources using relatively standardized exposure metrics common for the entire cohort. Several authors found that the surface area concentration of particles may have stronger correlation with the negative health effects than e.g. number or mass concentration and considered LDSA as a relevant metric when it comes to understanding the deposition of particles into the human lungs (Hennig et al. 2018; Patel et al. 2018; Oberdörster, Oberdörster, and Oberdörster 2005; Brown et al. 2001; Lepistö et al. 2022). Nevertheless, none of these metrics is specific with respect to ENM type. That is why, whenever possible, it is recommended to complement them with additional measurements targeting more

specifically certain types of ENMs or some of their physicochemical properties. For instance, for carbon-based ENMs, such as CNTs, the measurement of elemental carbon mass concentration and quantification of CNT agglomerates and/or CNT contained fibers in PBZ could be implemented (Dahm, Bertke, and Schubauer-Berigan 2019), although concerns raised with respect to the standardization of these measures and metrics (Guseva Canu et al. 2020). For a metal-based ENM, the metal mass fraction in airborne particles can be measured, ideally along with assessment of bioavailability and bioaccessibility of this metal (Figueroa-Lara et al. 2019; Guseva Canu et al. 2021a). However, this methodalsolacks consensus (Kastury, Smith, and Juhasz 2017). Although more specific to certain types of ENMs, these additional exposure measures are considered optional in this protocol, unless their cost, in field implementation challenges and lack of standardization can be reduced sufficiently to allow their generalization. It is also worth to stress, that handling only one type of ENM is an unrealistic exposure scenario in industry. A complex exposure assessment protocol with multiple exposure metrics justified in the research setting is hardly acceptable in some industrial settings. Moreover, for a mixed exposure to different ENMs at a workplace, distinguishing the effects of different particles and sizes will be also unrealistic. It would be also misleading from a toxicological point of view (Bergamaschi et al. 2015; Iavicoli et al. 2019; Iavicoli, Leso, and Schulte 2016). In this situation, biomonitoring as the only tool to assess the effects of such mixed exposures (Schulte et al. 2018; Schulte et al. 2016; Schulte et al. 2019; Zare Jeddi et al. 2021).

Importantly, the apparently small minimum sample size of this cohort with 120–160 workers actually corresponds to one of the largest studies of workers exposed to ENMs in the world. For instance, the unique cohort study of ENM workers (the EpiNano cohort) launched in 2012 in France, has included 130 workers so far (Guseva Canu et al. 2016c) while the future US National Institute for Occupational Safety and Health (US-NIOSH) cohort of carbon nanotube and nanofiber workers had 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 ENMs producing plants (Wu et al.

2019). It is noteworthy that in the US-NIOSH study, a personal exposure monitoring has been conducted while in the French cohort, the exposure is assessed only qualitatively since the (semi)quantitative exposure assessment has been discontinued (Renaudie et al. 2018). In the Taiwanese study, the exposure is assessed using control banding despite its high bias potential (Guseva Canu, Burstyn, and Richardson 2016b). The assessment of airborne exposure to ENMs based on aerosol sampling analysis should be considered as the minimal requirement pondering between the study feasibility in different occupational settings and the scientific value of its results. Whenever possible, it should be completed with a more thorough individual exposure assessment.

The relatively small numbers of participants as compared to the estimated nanotechnology workforce consistently reflect the difficulties in endorsement and recruitment of companies for epidemiological studies, and particularly the low response rate of ENM manufacturers. For example, the response rate in the NanoExplore survey was 2.4% (Crézé et al. 2021), in the International Commission on Occupational Health (ICOH) survey 2.6% (Iavicoli et al. 2019), in a Canadian survey 8.4% (Endo, Ostiguy, and Emond 2014), and in the French EpiNano program 16.0% (Guseva Canu et al. 2016c).The two rare examples of high company' participation rates are surveys carried out in Switzerland (58.3%) (Schmid, Danuser, and Riediker 2010) and in the US (80%) (Schubauer-Berigan et al. 2018) in collaboration with the Swiss Ministry of Economy and Industry and the Swiss national work accident insurance, and the US-NIOSH, respectively. The difficulties experienced in research projects regarding company recruitment reflect the lack of awareness and regulation on emerging risks. It is noteworthy that once companies gave their agreement, workers generally have a favorable attitude to such studies and their participation rate can reach 75% (Beard et al. 2018) or even 99% (Guseva Canu et al. 2016c).

A successful implementation of a harmonized protocol in occupational settings concerned with ENM exposures in the ongoing study should demonstrate the feasibility of similar research projects in the future, facilitate further epidemiological studies and health surveillance programs, and inform stakeholders of regulatory aspects targeting occupational

exposure to engineered and incidental nanoparticles. This protocol enables the launching of an international cohort of nanotechnology workers and, more generally, of workers exposed to a broad range of nanomaterials. As an open cohort, it could grow by including additional workers from new nanotechnology companies from various countries, which could join the NanoExplore Consortium and apply this harmonized protocol.

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Disclosure statement

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