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Particles and health 2021: An international conference addressing issues in science and regulation

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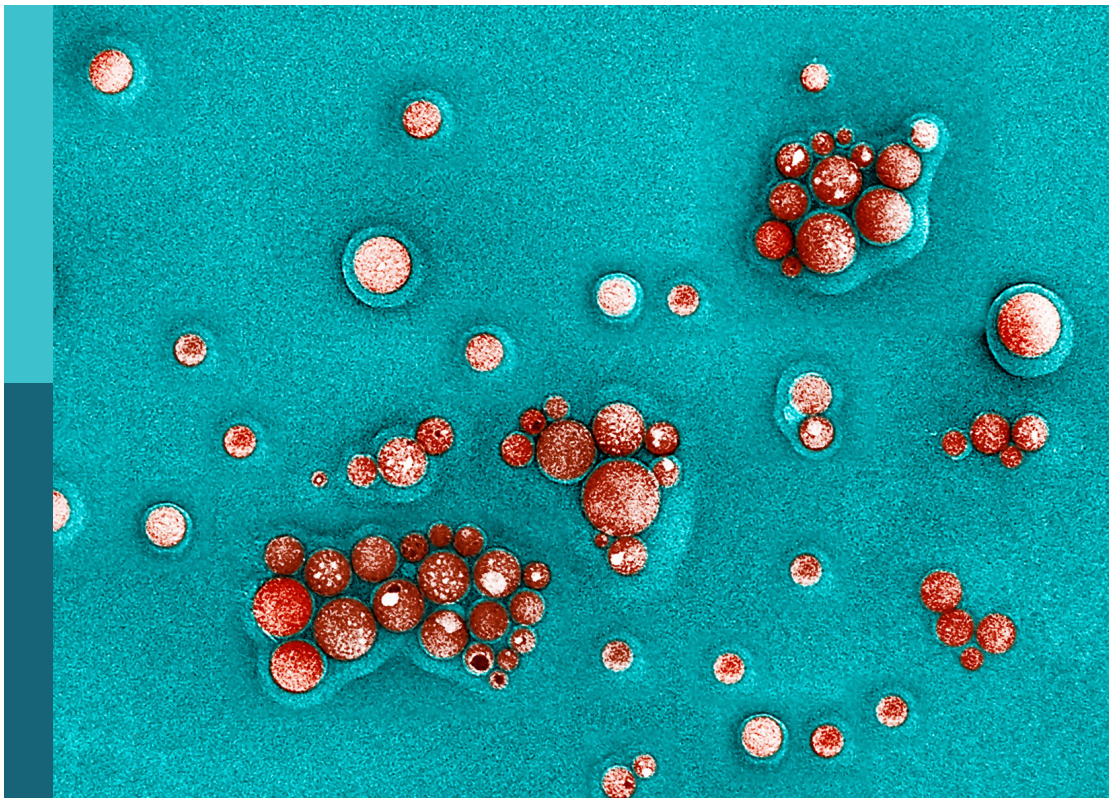

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Particles and health 2021: An international conference addressing issues in science and regulation

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Editorial: Particles and Health 2021: An international conference addressing issues in science and regulation

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Editorial on the Research Topic
Particles and Health 2021: An international conference addressing issues in science and regulation

Regulatory initiatives in the European Union (EU) have led to proposed carcinogenicity classifications for poorly soluble low toxicity particles (PSLTs). Although PSLTs lack a widely accepted definition, commonly cited examples include carbon black, titanium dioxide and iron oxide, among others. In light of the demanding time challenges in proper review and regulation of substances used in commerce, it can be appealing to consider classes of compounds to facilitate regulatory applications. In a similar vein, the German MAK Commission has proposed workplace exposure limits for poorly soluble low toxicity particles. To foster an update on the science regarding PSLTs, and potential regulatory applications, the Particles and Health 2021 conference was designed to update the interdisciplinary science of PSLTs related to toxicology, epidemiology, occupational, and pulmonary medicine and exposure assessment, among others. This special issue focusing on "Particles and Health" includes peer reviewed papers on the topics of human health, the role of rat inhalation studies and inflammatory responses in addressing health risks, followed by papers on non-pulmonary effects of PSLTs and papers related to the regulatory impact of scientific studies related to PSLTs.

Introduction

Regulatory initiatives in the European Union (EU) have led to proposed carcinogenicity classifications for poorly soluble low toxicity particles (PSLTs). Although PSLTs lack a widely accepted definition, commonly cited examples include carbon black, titanium dioxide and iron oxide, among others. In light of the demanding time challenges

in proper review and regulation of substances used in commerce, it can be appealing to consider classes of compounds to facilitate regulatory applications. In a similar vein, the German MAK Commission has also proposed workplace exposure limits for poorly soluble low toxicity particles.

To foster an update on the science regarding PSLTs, and its potential regulatory application, the Particles and Health 2021 conference was designed to update the interdisciplinary science of PSLTs in toxicology, epidemiology, occupational and pulmonary medicine, and exposure assessment, among others (see conference web site www.particlesandhealth.org for more detail). Occupational exposure to PSLTs can occur in many industrial sectors, including mineral mining and milling; welding and asphalt use; and in the manufacture of textiles, glassware, roofing, pulp, and paper products. Nanomaterial manufacturing and use (e.g., gold, copper, titanium dioxide) presents other challenges. PSLT exposures may impact millions of workers globally.

The scientific program committee strove to select disparate perspectives on similar topics to promote discussion for the support of evidence-based scientific underpinning of regulatory standard setting regarding PSLTs. Although regulatory concern regarding PSLTs has primarily focused on lung cancer risk based on rat inhalation overload studies, the conference included presentations on genetic, reproductive and cardiac issues, among others.

The conference goals were to present current scientific information on particles and health regarding risks to human health and the environment while specifically addressing: (1) Uncertainties of defining poorly soluble low toxicity particles (PSLTs); (2) Whether PSLTs should be considered separate entities or broadly defined; and (3) Appropriate regulatory applications of the health related science of PSLTs.

The conference was divided into four major sections held over a two period in which 30 formal presentations were made, including:

1. The role of human studies, including epidemiology, in assessing health risk.
2. The role of animal and *in vitro* studies in assessing inflammation as a key adverse outcome pathway in particle induced effects.
3. The role of nanoparticle toxicology and potential impact of PSLTs on non-pulmonary adverse effects.
4. The critical aspect of appropriate regulatory application of science.

What follows are highlights of the key sections led by the respective moderators of the session.

Human studies

Role of human studies in assessing health risk.

The Health section was introduced by a discussion of the history of The Institute of Occupational Medicine (IOM) from its inceptions in the 1960s when it played a major investigative role in understanding coal workers pneumonoconiosis to today's challenges in addressing the potential impact of nanomaterials (Seaton et al.).

In the regulatory application of scientific studies regarding health implications of the production and use of certain materials, including PSLTs, human studies led by Mundt et al., most notably epidemiological evaluations have particular relevance. In fact, according to CLP regulation (EC) No 1272/2008 (Annex I, Section 1.1.1.4), complementing the European REACH Regulation (EC No 1907/2006), *"Where evidence is available from both humans and animals and there is a conflict between the findings, the quality, and reliability of the evidence from both sources shall be evaluated to resolve the question of classification. Generally, adequate, reliable, and representative data on humans (including epidemiological studies, scientifically valid case studies as specified in this Annex or statistically backed experience) shall have precedence over other data."*

The US Centers for Disease Control has also noted that Epidemiology is the preferred basis of Risk Evaluation. Epidemiology, often described as the basic science of public health, can demonstrate human health risks of PSLTs under the "natural" circumstances of use and exposure. In a presentation regarding the "Role of epidemiology in human risk assessment," Mundt et al. reported on several chronic inhalation studies of PSLTs (e.g., carbon black, TiO2, and talc) in rats that demonstrated risk of lung cancer, but only at "lung particle overload" doses in rats.

Similar findings were not observed in mice, hamsters, and guinea pigs. In contrast, epidemiological studies of talc, carbon black, and titanium dioxide workers have not shown statistically significant associations between PSLT particles and risk of lung cancer (Mundt et al.).

The value of meta-analysis in the evaluation of disparate results in individual studies was addressed by Yong et al. based on a study related to Carbon Black and Lung Cancer Mortality-A Meta-regression Analysis Based on Three Occupational Cohort Studies. Evidence of lung cancer among carbon black production workers was inconsistent: increased lung cancer mortality was indicated in the UK and the German cohorts, while deficit was found in the US cohort (1–3). Lack of exposure- response analyses were identified to be a gap by the IARC working group (4). An updated follow-up study of the US cohort was published in 2015, to address the exposure-response relationship

(3). Based on a sensitivity analysis of cumulative exposure-response estimates the relative risk of lung cancer was 0.99 [95% CI: 0.994–1.005; (5)].

To address whether coal worker mortality studies can offer a perspective in addressing the significance of rat inhalation studies for human risk assessment, McCunney and Yong reviewed the coal worker cohort mortality studies and evaluated whether components of coal warrant its classification as a representative PSLT.

According to the International Agency for Research on Cancer (IARC), coal is a complex mixture of >50 elements and their oxides. Coal dust is composed of numerous substances, including Human Carcinogens, such as crystalline silica, beryllium and cadmium. Moreover, coal mining activities often occur in the context of exposure to machinery that emits diesel exhaust particles—an IARC Type I carcinogen. In contrast to coal, Carbon Black (CAS No. 1333-86-4)—a manufactured product considered a PSLT- is virtually pure elemental carbon (upwards of 98–99%) produced by incomplete combustion of gaseous or liquid hydrocarbons under controlled conditions. Its physical appearance is that of a black, finely divided pellet or powder.

Risk of lung cancer among coal miners has been investigated in cohort mortality studies conducted over nearly 50 years. Over 120,000 coal miners have been part of studies in UK, Germany, Netherlands, USA, Poland, Japan, Czech Republic, and Australia. The weight of epidemiological evidence suggests no increase in risk of lung cancer among coal miners. Slight elevations in SMR cannot lead to a reliable conclusion about an increased risk due to limitations in exposure assessment, and inherent biases in case-control studies, most notably confounding and recall bias.

Despite the lack of scientific appropriateness of using coal as a surrogate for PSLTs in evaluating the significance of rat inhalation studies, the preponderance of epidemiological results of cohort mortality studies of coal-mine workers do not indicate a consistent increase in lung cancer risk.

Since poorly soluble low toxicity particles such as carbon black or titanium dioxide have raised concern about potential malignant or non-malignant adverse effects such as lung cancer or chronic airflow obstruction, Harber summarized the application of causal inference analysis to questions concerning the health effects of such particles. Relationships with malignancies remain uncertain. Inflammation has been postulated as a key intermediary step in the pathogenesis. Observational predictive epidemiologic studies have limited ability to address poorly observable mechanistic steps. Causal inference analysis, such as the use of Directed Acyclic Graphs (DAG), can create a useful analytic framework to allow integration of data from epidemiologic, clinical, and experimental studies to address mechanistic questions. In addition, these methods are useful to clarify potential bona fide and artifactual observed relationships.

As a means of detecting early signs of pulmonary inflammation that may presage malignant and non-malignant lung disease, Professor Chris Fanta summarized the potential utility of measurement of exhaled nitric oxide. Exhaled nitric oxide assessment is a non-invasive, simple, and safe method of measuring airway inflammation that provides a complementary tool to other ways of assessing airways disease, including asthma (6). This parameter is currently used in clinical settings in the diagnosis of asthma, detection of eosinophilic inflammation of the airways, prediction of steroid responsiveness in asthma, monitoring for asthma activity and assessing medication non-adherence (7). Analysis of components of exhaled breath offers a non-invasive assessment of airway and lung pathology. The science of exhaled breath analysis, however, is in its infancy but nonetheless has potential for enhancing the understanding of the lung's inflammatory response to inhaled particles.

Considerable overlap in results may be present, however, between normal and disease and may be Influenced by age, atopy, sinus disease, and cigarette smoking, among others. Dramatic suppression by inhaled corticosteroids, for example, can occur and lead to results in a "gray zone" (20–40 ppb).

Yong et al. describe their evaluation of the effect of cumulative exposure to respirable synthetic amorphous silica (SAS) dust on respiratory morbidity, as assessed by spirometry. Multiple exposure assessments were conducted in a cross sectional analysis of 462 exposed male workers. An averaged cumulative respirable SAS dust concentration of 6.44 mg/m^3 years was estimated. Internal regression models suggested a reduction of 8.11 ml (95% CI: 0.49–15.73) in forced vital capacity (FVC) per 1 mg/m^3 year increase of exposure. No effect on forced expiratory volume in 1 s (FEV1) or the FEV1/FVC ratio was observed in association with exposure to the respirable fraction of SAS. No adverse effects on the occurrence of respiratory diseases were observed. The authors concluded that there was no clear evidence of adverse pulmonary effects from occupational exposure to respirable SAS.

Rat inhalation studies

In a luncheon address, Dr. Paul Brandt-Rauf, the editor of the Journal of Occupational and Environmental Medicine, described some of the vagaries of journal publications. He described the role of the impact factor in raising a journal's reputation and reviewed some of methods employed by editors to raise a journal's impact factor, most notably by publishing major review articles, which customarily receive more citations than individual articles. The afternoon session, moderated by Borm et al. focused on inflammation as a key adverse outcome pathway in particle induced adverse health effects. Dominique Lisone presented a study related to the role of pulmonary macrophages in inflammation and the development of lung cancer. Driscoll then presented paper on particle induced

inflammation and lung cancer based on the outcome of an Edinburgh workshop. Roger Duffin then presented an overview of inflammatory pathways in humans followed by a discussion by Jack Harkema, a pathologist who discussed pulmonary cell proliferation, the missing link in particle induced lung cancer. The session concluded with a group discussion related to the Global Harmonization System (GHS) based target organ toxicity design and interpretation of existing studies.

Nanotechnology and non-pulmonary effects

Nanoparticles can be defined as particulate materials having at least one external dimension smaller than 100 nm. Airborne nanoparticles can occur naturally (silicates, iron oxides); be anthropogenic, arising as by-products of human activity, e.g., fossil fuel combustion; or can be materials purposely engineered to be of nanometer size and possessing unique properties.

The potential of inhaled nanosized particulate to cause adverse pulmonary effects is well-documented; however, their potential to have effects outside the lung is less well-understood. Epidemiology studies have demonstrated associations between inhaled ambient fine particles which includes a nanosized fraction and adverse cardiovascular effects (8, 9). More recently, other non-pulmonary responses have been attributed to inhaled nanoparticles (Scarcello et al.) (10, 11). The session covered several topics including: dose metrics and biokinetics relevant to nanoparticles; the potential for nanoparticle exposure to elicit adverse effects on DNA, the nervous system, the reproductive system and developmental toxicity, the cardiovascular system, and strategies for evaluating the safety of new nanotechnologies.

Drs. Oberdörster, Creutzenberg, and Graham discussed dosimetric and biokinetic considerations of nanoparticles. Important factors differentiating nano from larger micron sized inhaled particles include: the larger number and surface area per unit mass (or volume) of nanoparticles and consequently the potential for greater reactivity; the role of diffusion mechanisms in the deposition of nanosized materials resulting in deposition patterns in the respiratory tract which differ from larger size particles for which inertial mechanism are more important; and the nanosize potentially enabling translocation into cells and along axons and dendrites.

Regarding translocation of nanoparticles, Creutzenberg et al. summarized studies in rats with inhaled and/or intratracheally instilled titanium dioxide, zinc oxide, silica, and carbon black which demonstrated that shortly after exposure deposited nanoparticles were found as aggregates localized in macrophages. Tissue analysis after days or weeks demonstrated retention in lung macrophages, pneumocytes, and translocation to the lung associated lymph nodes. These studies supported the major clearance pathway was *via* the gastrointestinal track with no significant translocation to remote organs.

Dr. Graham described studies conducted studies on human autopsy tissues using high resolution transmission electron microscopy combined with elemental mapping which demonstrated the present of nanosized particles in the olfactory bulbs. The particle composition (e.g., silicon, lead, zinc, nickel) indicated an exogenous origin, hypothesized to be due to inhaled air pollution particles depositing in the nasal passages and translocating to the brain. In support of this hypothesis, studies in rats have demonstrated nanoparticle depositing on the nasal mucosa can translocate to the brain (12). The silicon and heavy metal particles observed in the brain were processed endogenously by ferritin coating and were associated with a localized inflammatory response. The studies on human tissues indicate nanosized particles may translocate to non-respiratory tissues and trigger a localized tissue response. The differences between the animal studies described by Creutzenberg et al. and that observed in humans in extra-pulmonary particle translocation may reflect the sensitivity of the detection methodologies used.

The potential for nanoparticles to elicit effects on DNA and in non-pulmonary tissues was addressed in presentations by Drs. Moller, Schins, Hougaard, and McCunney.

Dr. Moller discussed research on multiwalled carbon nanotubes (MWCNT) reporting MWCNT-7 (a long, straight, thick fiber) elicits inflammation and production of reactive oxygen species when injected into peritoneal cavities of rats; however, no DNA damage was detected. Comparable results were observed with NM-401, another long, thin nanotube, however, intraperitoneal injection of shorter nanotubes did not produce inflammatory effects. *In vitro*, MWCNT-7 nanotubes produce DNA damage, albeit only at extremely high doses. These findings on material dependent differences in inflammatory and DNA damaging effects are consistent with other studies demonstrating differences in bioactivity of various MWCNT (13) and support the concept the composition, in addition to size and shape are key to nanomaterial toxicity. Dr. Moller noted a key gap in our understanding of nanotube genotoxicity is the absence of data indicating whether any the DNA damage detected was irreversible and produced mutation.

Regarding the potential for neurotoxic effects, Dr. Schins reported inhalation of diesel exhaust increases b amyloid protein accumulation in the brains of 5 × FAD mice, a model of Alzheimer's Disease amyloid protein accumulation. These findings suggest inhaled diesel exhaust can have neurological effects distance from the respiratory tract. Additional studies on inhaled engineered nanoparticles (ZrO_2, CeO_2) did not increase brain plaque formation in 5 × FAD mice and were without effect in a mouse model of atherosclerosis. Studies on oral exposure of mice to titanium or silver nanoparticles had no effect on markers of neuroinflammation

with some behavioral changes observed in female mice as well as biochemical changes in cortical tyrosine kinase activity. Overall, the neurotoxicity research summarized supports that inhaled or ingested nanoparticles may have effects distance from the lungs or GI tract with the effects being material dependent.

Dr. Hougaard reviewed the potential developmental and reproductive effects of nanoparticles investigating genotoxic, nervous system, reproductive and immune system changes in offspring of pregnant mice exposure to titanium dioxide, diesel particles, carbon black, and multiwalled carbon nanotubes (MWCNT). The studies supported exposure-associated changes on the central nervous system, immune system, and male fertility. The mechanisms underlying the changes reported by Drs. Schins and Hougaard were not defined and will require further investigation. The association of occupational carbon black exposure with cardiovascular disease was discussed by McCunney et al.. Environmental exposure to PM 2.5 particles has been associated with cardiovascular disease, a relationship considered to be causal in nature, although the mechanisms are unclear (8, 9, 14). Carbon black is >98 carbon and can have polycyclic aromatic hydrocarbons tightly bound to its surface. In the occupational setting, carbon black typically exists as agglomerates of nanosized particles.

McCunney et al. reviewed the three major occupational epidemiology studies on carbon black investigating populations in the United States, Great Britain, and Germany which collectively have includes over 9,300 workers. After accounting for cigarette smoking, none of these studies have demonstrated an increased risk of mortality or cardiovascular disease. A meta-analysis of these studies confirmed an absence of an association between occupational carbon black exposure and heart disease, ischemic heart disease, or acute myocardial infarction. The negative studies on carbon black considered alongside the evidence on air pollution and cardiovascular disease suggest the complex chemical properties of air pollution are a critical factor in the elevated cardiovascular disease risk vs. a specific effect of poorly soluble nanosized particles (15).

To support the development and risk assessment of nanotechnologies, the "Gracious 2020 Project" established a framework to apply grouping and read-across approaches (16). The Gracious framework describes a hypothesis- driven approach for leveraging existing data to streamline safety evaluation in terms of time, cost, and animal usage safety. The framework defines criteria for grouping, selection of appropriate benchmark materials and developing an integrated approach to testing and assessment (IATA). The Gracious framework supports both qualitative and quantitative risk assessments and, when needed, developing an appropriate experimental plan to address data gaps using *in silico*, *in vitro*, and/or *in vivo* testing. A challenge in using this approach may be the paucity of robust inhalation toxicology data on nanomaterials.

Science and regulation

The aim of this final session was to explore how the role of sound and evidence- based scientific underpinning was used in regulatory standard considerations regarding PSLTs and other particles. Although regulatory concern regarding PSLTs has focused on lung cancer risk as a result of rat inhalation overload studies, this conference also addressed all relevant health end points, including respiratory tract and lung inflammatory changes, genetic and reproductive issues, among others, consistent with ECHA and other national and international guidelines. These issues relate to both hazard classification and to risk-based exposure limit setting.

The first-scene setting presentation addressed the regulatory application of science and stakeholder engagement in the setting of EU occupational exposure limits (OELs) by Alick Morris from the EU Health and Safety at Work Unit, DG Employment, Social Affairs and Inclusion, European Commission, Luxembourg. His presentation described how this activity takes place at the EU level when setting limit values (OELs) to protect the health of workers from risks due to occupational exposure to chemicals. Alick Morris described the EU Pillar of Social Rights Action Plan, the EU Occupational Safety and Health strategic framework and how the key steps in setting OELs and Biological Limit Values (BLVs) as well as their benefits. He noted how the social partners (industry, trade unions, and EU Member State governmental representatives) participate in regulatory decision-making through the EU Advisory Committee on Safety and Health (ACSH) which also adopts new lists of priorities for future limit values. He also described some current initiatives driven by the Occupational Safety and Health Directive and the Carcinogens and Mutagens Directive which included recent work on asbestos, lead and diisocyanates. He pointed out how this work was assisted *via* expert committees, formerly though the Scientific Committee on Exposure Limits (SCOEL) but more recently, by the EU European Chemical Agency's (ECHA's) Risk Assessment Committee (RAC) (https://ec.europa.eu/social/main.jsp?catId=148&langId=en).

Schulte et al. from the US National Institute for Safety and Health (NIOSH) described the application of translational science approaches to protect workers exposed to nanomaterials. In his presentation he noted that nanotechnology, like translational science, is a relatively new and transdisciplinary field. Translational science in occupational safety and health (OSH) focuses on the process of taking scientific knowledge for the protection of workers from the laboratory to the workplace and back again. Translational science has been conceptualized as having number of multiple phases of research along a continuum, from scientific discovery (T0) to efficacy (T1), to effectiveness (T2), to dissemination and implementation (D&I) (T3), to outcomes and effectiveness research in populations (T4). The translational research process applied to occupational

exposure to nanomaterials might well-involve similar phases. This includes basic research (T1) in the areas of toxicology, epidemiology, industrial hygiene, medicine, and engineering. He described iterative interrelationship of these four phases in some detail, including potential barriers to the implementation of solutions. Of critical importance, and in accord with the first presentation by Alick Morris, he emphasized that stakeholder participation and engagement was critical to all four phases of the translational continuum.

The next series of presentations dealt with one particulate nanomaterial; synthetic amorphous silica (SAS) to illustrate a number of scientific, ethical, and regulatory issues. In the case of many PSLTs and nanomaterials, including nanostructured SAS, the most important exposure pathway for such materials is inhalation; depending on the possible applications of the substance in foodstuffs, cosmetics, among others that dermal and oral exposure must also be taken into account. Additionally, to reduce the need for animal experiments, *in vitro* test methods are urgently needed to compare different materials, including SAS, and to test their toxicological properties for oral and inhalative exposure.

In the first of these illustrative presentations on SAS, Peter Wick described an oral *in vitro* screening of nanomaterials with an advanced *in vitro* intestine model.

Nanostructured food processing agents, which are added to prevent caking, to improve flowing or change texture of the food, might be ingested. Regulatory authorities, as well as consumers, are concerned about potential adverse effects of nano-sized materials both in food and on public health. Considering the high oral exposure of all these food additives, a better understanding of the uptake, accumulation, and biological effects of food relevant nano-sized materials at the intestinal epithelium is needed. The presentation described 10 distinctly different SAS materials with different surface areas, structures, sizes, and surface charges that had been characterized. Their biological impact was screened in Caco-2 cell line, representative of the most common cell type in the intestine. No acute impairment of viability or barrier integrity was identified. In the second part of this work, an advanced co-culture model was established to better evaluate the impact of food grade materials in an *in vivo* like setting. The exposition of the advanced coculture model with six different SASs selected due to the different production routes, specific surface areas and different silanol content led to no differences in the viability, barrier integrity, microvilli function, and lipid uptake. Nevertheless, the treatment had shown that the mucus production increases after the treatment with SAS materials that present certain aggregate sizes and a high silanol content. A co-effect has been found for the investigation of the iron uptake. Precipitated SAS with a small specific surface area decreased the iron uptake in the advanced co-culture only in iron uptake but not in the also on the gene level. The results show that the use of this advanced *in vitro* model could lead to an improved prediction on potential adverse outcomes of food components on the intestine. Mucus seems to be a very important protective barrier in the interaction of food components with the intestinal epithelium and should be studied in more detail. The advanced co-culture model established in this work can be used for an initial estimate of the interactions of food components with intestinal epithelium and ideally, a further reduction of animal experiments in the future. Overall, this *in vitro* test model for oral intake showed that no adverse effects were observed with SAS. Further information on this alternative screening method for oral exposure is provided by Hempt et al. (17, 18).

Wiemann et al. addressed animal welfare aspects in his presentation: "Can we reduce animal testing- tiered approach using *in vitro* screening?" He presented a well-established macrophage *in vitro* method for comparative studies after inhalation of nanomaterials, which allows a grouping of the substances with regard to their activity in a serum-free test system (19, 20). Serum lowers the bioactivity in this macrophage *in vitro* test system. The influence of serum is described in a separate publication by Wiemann et al. (21). He presented the results of SAS that showed that bioactivity *in vitro* is strongly diminished by protein binding to the particle surface. Of special interest in the investigation was the bioactivity of SAS surface-treated with organosilanes in comparison with non-treated SAS forms using alveolar macrophages as a highly sensitive test system (Wiemann et al.). Five non-treated and nine surface-treated SAS (one hydrophilic, eight hydrophobic, six different coating reagents) were included in the *in vitro* study with alveolar macrophages. Dispersion of the hydrophobic SAS (8/14) required pre-wetting with ethanol and extensive ultrasonic treatment in the presence of 0.05% BSA (Protocol 1). Hydrophilic SAS were suspended by moderate ultrasonic treatment (Protocol 2) and with Protocol 1.

Importantly, the results of these *in vitro* studies correlate very well with the results of subchronic *in vivo* studies (90-day study) with hydrophobic surface-treated SAS (22). In this study, hydrophobic surface-treated SAS showed a weaker inflammatory activity at the end of exposure and faster reversibility of effects in the recovery period compared to pure pyrogenic SAS.

The results above raise the question that if hydrophobic surface-treated SAS materials show reduced bioactivity *in vitro* and in long-term *in vivo* studies, the question arises as to why lethality can be observed in some acute inhalation studies with very high concentrations of hydrophobic surface treated SAS. The answer to this question on lethality was addressed by Jürgen Nolde and Nils Krueger. "The challenge to create particulate aerosols for acute toxicity testing—a systematic approach" was presented by Juergen Nolde. Large differences between lethal and non-lethal concentrations of different forms of the same substance have been documented through a large

number of acute inhalation studies which do not conform to the results from acute oral or dermal studies which did not provide any concerns on potential toxicity for the same substance. Therefore, it is necessary to address the cause of these contrary results in the different behaviors of the particles of the substance in the inhalation equipment, up to the point when the particles are delivered to the nose of the rat. OECD test guidelines for acute inhalation studies require defined maximum particle sizes (MMAD max. 4 μm) and concentrations up to 5,000 mg/m^3 or the maximum technical feasible concentration. This technical feasible concentration, however, and more importantly, its measurement, is not defined. The challenge of creating particulate aerosols for acute toxicity testing using a systematic approach was presented. The aim is to examine optimized aerosol generation and its monitoring, including detailed characterization of the exposure atmospheres in the test equipment (stability, particle concentration, particle size distribution over time) from the point of generation to the point of release out of the system prior to performing OECD animal inhalation studies (Wessely et al.). It was the intention to perform rat studies with the highest technically feasible concentration without significant aerosol altering. Studies of aerosol generation on this scale and detail with several different particular substances, e.g., silica, organic pigments, aluminum oxide, sugar, flour dust, and calcium carbonate have not previously been carried out in any acute animal inhalation studies. A key conclusion was that a detailed evaluation of the aerosol generation can help predict the outcome of rat inhalation studies with particles and therefore, reduce the number of animals required in future acute inhalation tests.

Krueger et al. presented a study on hydrophobic surface treated SAS: "Non-specific particle effects now trigger classification?." The aim of the study was to understand the mechanism of lethality associated with high dose inhalation of hydrophobic surface treated SAS observed in some acute inhalation studies. It was demonstrated that physical obstruction of the upper respiratory tract (nasal cavities) caused effects observed when hydrophobic surface-treated SAS was inhaled (flow-past, nose-only) by six Wistar rats (three males and three females) in an acute toxicity study at a concentration of ∼500 mg/m^3 for 4 h (Krueger et al.). Under the conditions of the test set-up, the concentration applied was found to be the highest that can be delivered to the test animal port without significant alteration of the aerosol size distribution over time. None of the test-material-exposed animals survived the observation period. Histopathology and energy dispersive X-ray (EDX) analysis demonstrated that test material particles agglomerated and formed a gel-like substrate that blocked the upper respiratory airways, which is fatal for the rat as an obligatory nose breather. This observation is in line with the findings reported by Hofmann et al. (23) showing a correlation between lethality and hydrophobicity determined by contact angle measurement.

Summary

This 2 day conference with 30 speakers from Europe and the USA addressed the scientific basis of potential health risks associated with exposure to poorly soluble low toxicity particles (PSLTs), most notably carbon black, titanium dioxide and talc. From this conference, 20 peer reviewed scientific papers were published in this special issue. The role of epidemiology and toxicology in assessing potential human health risks of regulatory impact was emphasized. Clearly, as regulatory challenges persist in establishing appropriate classification schemes and exposure limits for PSLTs, the role of scientific investigations will play a major role.

Author contributions

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of interest

Author RM is Chair of the Scientific Advisory Group (SAG) of the International Carbon Black Association. Author LL is a member of the SAG. Authors KD and PB have served as consultants to the ICBA. Author NK works in the Product Safety Division of Evonik Industries.

Publisher's note

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Science With Purpose: 50 Years of the Institute of Occupational Medicine

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The Institute of Occupational Medicine (IOM) was founded in 1969 by the then UK National Coal Board to complete its nation-wide epidemiological study of lung disease in coal miners, the Pneumoconiosis Field Research. The results quantified risks in the industry and were influential across the world in setting preventive standards. The research, based on epidemiology, was multidisciplinary from the start, and the IOM's broad scientific expertise was applied across many other industries with an increasing focus on environmental measurement and ergonomics. In 1990, as the coal industry declined, IOM became a self-funding research charity with a strong commercial arm. It has expanded its research, often with European collaborators and funding from governments, and has achieved wide recognition. This has most recently been applied during the pandemic in areas of hospital ventilation, personal protection, and viral exposure research, illustrating IOM's ability to respond to new environmental or occupational challenges.

Keywords: multidisciplinary research, occupational health, airborne particles, respiratory protection, ergonomics

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ORIGINS

The nationalization of the UK coal industry in 1946 revealed a huge epidemic of chronic lung disease in miners. With funding from the UK Government, the industry began an epidemiological research programme in selected collieries on 50,000 miners, recording symptoms, lung function, radiology, study of selected lungs *post mortem,* and detailed estimates of exposure to respirable coal dust. The research was intended to address two questions, (i) how much and what kinds of dust caused pneumoconiosis and (ii) what action needed to be taken to prevent disablement of miners? To coordinate and analyse the data and oversee the research, in 1969 the National Coal Board founded the Institute of Occupational Medicine (IOM) in Edinburgh as a research charity. A detailed account of its history was published in 2019 and this provides further detail (1).

From the early years, IOM's staff comprised specialists in occupational medicine, pathology, statistics, physics, chemistry, and ergonomics. Its research was and remains multi-disciplinary. The early results were the first worldwide to show quantitative relationships between dust exposure and risk of pneumoconiosis in coal miners (2, 3). These led to formulation of science-based preventive occupational standards in the UK, USA and elsewhere.

THE FIRST 20 YEARS: 1969–89

The unique contribution of the early research was in quantification of risks in epidemiology. This led not only to understanding of pneumoconiosis but also to quantifying the increased risk of chronic obstructive lung disease and emphysema (COPD) in coal miners (4, 5), related conditions commonly caused by tobacco smoking. This contributed to recognition of COPD for industrial disability compensation in the UK. The research established the IOM's international reputation in occupational medicine and hygiene.

Alongside the coal research, IOM developed a programme of research into asbestos diseases, the other outstanding occupational risk issue of these decades. The emphasis was on understanding experimentally what made asbestos fibers so uniquely dangerous, causing asbestosis, lung cancer and pleural mesothelioma. The answer related to the diameter and length of the fibers and their solubility in lung fluid once inhaled (6, 7). This research enabled more reliable measurement of asbestos in relation to risk, both for epidemiology and in workplace hygiene control. Furthermore, it led to ways of assessing the potential hazard of new non-asbestos mineral fibers being introduced into industry and to a research programme into such fibers (8).

The experience and reputation in epidemiology gained by IOM led to contracts from many different industries including steel in relation to cancer risks, brickworks, and quarries and opencast coal in relation to lung risks. Major international projects included research into risks of shale mining for oil production in the USA (9), assessment of occupational exposures in the mineral wool industries across Europe (10), and studies of risks related to a volcanic eruption in Montserrat (11). In the UK, IOM also carried out unique studies of lung problems in wool workers (12), agricultural use of pesticides (13), dust exposure in the London Underground (14), and exposures in relation to PVC production (15). The coal studies also led to important contributions to understanding the risks of exposure to silica and to the setting of a standard to reduce risks of silicosis (16).

Beside the early research into occupational epidemiology and pathology were two important programmes, occupational hygiene, and ergonomics. The hygiene research addressed the need better to quantify toxic exposures, especially in relation to dust, and how to protect against them. New methods of measuring dust and airborne asbestos fibers developed by IOM exposure scientists have been adopted as standard approaches throughout the world, most notably with the IOM inhalable dust sampler (17), and the Walton-Beckett microscope eyepiece graticule for measuring asbestos and other fibers (18).

Measurement of workplace hazards led naturally to studies of improved methods of protection, especially personal protective equipment (PPE), including programmes on respirators (19), masks and building ventilation that were later to find substantial application in the COVID-19 pandemic.

The IOM was a significant contributor to ergonomics, at a time when this was a relatively young discipline, developing a leading role internationally. The UK Ergonomics Research Society (the oldest such society worldwide, and now the Chartered Institute of Ergonomics and Human Factors) is only 10 years older than ergonomics at the IOM itself. This research had started in the complex activities required in coal mining but spread to developing means of improving the physical and psychological conditions associated with more mundane activities such as keyboard work, bending and lifting, and stress (20–24). Occupational hygiene and ergonomics were both to become fundamental to the IOM's continued existence in more recent years.

By the late 1980s the UK coal industry was in terminal decline and doubt was cast on the IOM's future. However, IOM scientists, many with high international reputations, were publishing over 50 refereed papers annually, and contributing to multiple Government and European committees, and the Institute was counted among the best-known in the world. Sufficient non-Coal Board grant funding was being obtained for some research to continue and a service function based primarily on occupational hygiene and ergonomics together with teaching courses had been developed that made survival appear possible. Costs were cut by reducing staff, all the most senior scientists and the Director taking redundancy and being succeeded by their deputies. Additional short-term funding from the Coal Board to ensure completion of European contracts, and grants from the Colt Foundation and the mineral fiber industries were obtained and in 1989 IOM became a self-funding research charity, supported by an enlarged commercial section to sell its services.

THE INDEPENDENT INSTITUTE 1990–2020

The move to financial independence was a bold one, as few if any research institutes exist without core funding from government or other benefactors. The solution to this was to expand the commercial side of the organization while maximizing grant income for the charitable research. Many of the research areas lent themselves to provision also of consultancy and measurement services, notably with respect to ergonomics and occupational risk and safety. The 1990s were a period when there was increasing recognition of musculoskeletal and psychological problems in workplaces, and these provided opportunities for the IOM. At the same time, new opportunities came from collaborative research in Europe in ergonomics, the application of exposure science in epidemiology, and in the new nanotechnologies.

Ergonomics and Occupational Hygiene

IOM continued its important research in both musculoskeletal disorders and psychological stress, the major causes of work-related absence (20, 25, 26). Other studies embraced protection from heat and noise, safe design of machines and workplaces, and safe handling of materials (21, 23, 24, 27). Starting from the earlier work in mines, IOM developed a research interest in personal protection, and has made significant contributions to the protection of firefighters, attracting grants from the Health and Safety Executive and the Home Office, and contributing to US action in the aftermath of the terrorist attacks known as 9/11. The IOM has won three awards from the Chartered Institute of Ergonomics for its research over the years. In addition, IOM

scientists have much experience in evaluating the effectiveness of respirators and face masks (28–30).

Research continued on the issue of estimating exposures to toxic substances. As a fundamental part of the European research project in the synthetic fiber manufacturing industry, new mathematical modeling approaches in estimating historic workplace exposures were developed; this has subsequently been applied to produce tools to estimate human exposure for European chemical regulation (10). Pioneering work on the measurement of skin exposure and inadvertent ingestion exposure in workplaces has led to IOM becoming one of the main international centers of expertise in these aspects of exposure science (31, 32).

Air Pollution and Nanoparticles

IOM scientists had from 1990 been involved in Government advisory committees on air pollution, stemming from their work in particle inhalation. From this interest arose the concept that the cardiac effects of pollution were caused by nanoparticles derived from burning fossil fuel (33). This coincided with a rising interest in the industrial and scientific use of nanomaterials. A report to Government by the Royal Society and Royal Academy of Engineering drew attention to the need for research in possible hazards in nanotechnologies and European grants subsequently became available for this (34).

With its background in particle toxicology and risk assessment, IOM was well placed to address some of these challenges, particularly those relating to the protection of individuals who might be exposed to these new materials. Its work, with many international collaborators, had defined the physical properties that make particles toxic, including asbestos fibers, which are often of a nanosized diameter. Studies of dust regarded as non-toxic had shown that they might overload defense mechanisms in the lung, leading to an important conceptual advance in toxicology based on the surface area of dust particles rather than the mass (35, 36). These became of critical importance in understanding the toxic properties of nanoparticles. Building on this background, IOM embarked on a strategy to develop nanotechnology risk as both a scientific programme and a business opportunity, seeking to understand and explain risks and how they could be reduced. The proactive approach taken demonstrated that it was possible to raise the profile of a whole area of science, build capacity and credibility, and move the international agenda (37). It enabled IOM to develop in innovative ways, from new ideas about science communication to thinking in a new paradigm where risk research was key to supporting technological innovation.

The expertise of IOM scientists was applied during the COVID-19 pandemic in assisting the NHS in testing the effectiveness of room ventilation in hospital wards and providing advice about the use of respiratory protection. Research was carried out to measure the virus in the air and on surfaces in healthcare settings and developed a mathematical model to predict the effectiveness of different control strategies in reducing the infection risk for staff. IOM is part of the UK PROTECT COVID-19 National Core Study on transmission and environment, assisting with transmission modeling and investigations of risk in specific sectors, such as the food processing industry.

FIFTY YEARS OF MULTIDISCIPLINARY SCIENCE

The past fifty years have provided many challenges, not least to an organization doing medical scientific research initially supported by a dying industry. So far IOM has survived two major coal strikes, two major economic crashes, the loss of its core financial support, Brexit, and now at least four waves of the greatest pandemic since 1918. IOM's history provides many valuable lessons to inform its future success. Of overriding importance has been a commitment to practical and applied science with the purpose of improving the health of workers and the wider population. This ethos, combined with the commitment of its staff, many of whom can look back with pride over decades of work with us, has been the foundation of our success.

Understanding and managing the risks from inhaled particles has been a central theme but the need to adapt both the topics addressed and the methods used has been critical. Evolution of the organizational structure with emphasis on varying types and models of funded work at different times has been necessary to ensure IOM's continued existence (1). Underpinning this has been its enduring commitment to its values of independence, impartiality, integrity, quality and sustainability.

THE CHANGING WORLD OF WORK

IOM's primary concern is in the prevention of occupational and environmental causes of ill health. Since the promotion and maintenance of good health are among the most fundamental and important global challenges, there are many opportunities to apply its scientific and advisory knowledge. During its 50 years, the world of work has changed dramatically. In the West in particular, there is a decline in employment in manufacturing and extractive industries, and an increase in what is thought of as office work. Increasingly people work at home.

The emergence of a 24/7 culture has disrupted traditional patterns of work-life balance and social support mechanisms. The use of artificial intelligence, big data, robotics and the internet is growing rapidly. Millions of people spend their entire working day in front of a computer or other interface device. There are fewer large companies, lots of small to medium enterprises, and many millions of self-employed individuals. People change their jobs and careers often in their working life. There is a huge rise in the "gig" economy, in which people have temporary jobs or are doing separate paid pieces of work, rather than working on a regular contract, and there is much more part-time working.

As western economies age, retirement age also rises, leading to a workforce with increased vulnerabilities, often managing chronic disease or reduced mobilities. Hazards in the modern workplace can also relate to the way that work is organized rather than specific agents, and the consequential harm may be more psychological than physical. Construction is growing rapidly, particularly in developing economies, often with new

techniques and materials. In industrial, scientific, and high-technology manufacturing, people may be exposed to new hazards. In developing economies, millions are still employed in extraction, construction, and manufacturing, often in conditions which have become rare in the West. Exposure to dust, asbestos and agricultural chemicals remains a major issue worldwide.

THE CHANGING ENVIRONMENT

There is an irony in that the IOM arose from the humanitarian desire of a state-owned coal industry to protect the health its workforce, while the product of that workforce, coal for combustion, has subsequently proved to be the basis of the world's greatest threat to civilisation's survival, climate change, and one of its major causes of ill health, air pollution (38). Along with warfare, social injustice, and pestilence these may be seen by many as the fourth metaphorical horseman of the apocalypse.

IOM was early to recognize the commonality of occupational and environmental disease, requiring the same scientific approaches to their investigation and amelioration. In many developing countries infectious diseases remain the most important causes of early death, but the current COVID-19 pandemic caught Europe unprepared. The IOM's expertise in PPE and hospital ventilation (for preventing cross-infection) played an important part in advising governments and in saving lives. However, infection aside, in most countries, adult morbidity and mortality is usually dominated by non-communicable conditions, including heart disease, cancer, diabetes and chronic musculo-skeletal and respiratory diseases. Environmental air pollution, indoor and out, is now recognized as a significant contributor world-wide to death in people with these chronic diseases, including dementia (39).

The United Nations Sustainable Development Goals (SDGs) call on countries to mobilize efforts to end poverty, fight inequalities and tackle climate change, whilst ensuring that no country is left behind. They recognize that ending poverty must go hand-in-hand with strategies that build economic growth and address a range of social needs including education, health, social protection, and job opportunities, whilst tackling climate change and protecting a diverse environment.

VISION FOR THE FUTURE

The world is changing rapidly, perhaps more so now than at any time in IOM's history. In response, IOM continues to adapt and evolve to stay relevant, add value and realize its purpose. IOM's vision is to remain a globally recognized organization that engages and empowers its staff to deliver its mission of contributing to a healthy and sustainable world. Its focus is the application of science to key risk issues of importance, *"to maximize the relevance, benefits and impact of our work"*. This includes development of its existing areas of occupational and workplace health, exposure science, nanotechnology, citizen science, human factors, hospital ventilation, and wellbeing. Within these areas, new topics are emerging such as shiftwork and cancer, dementia in professional sports people, and robotics and automation, where IOM is already beginning to work and achieve prominence. The international dimension of IOM's work is also important, since the issues it addresses are worldwide.

IOM's origin addressed the challenge of answering two questions (i) how much and what kinds of dust caused pneumoconiosis and (ii) what action needed to be taken to prevent disablement of miners. Addressing these questions has opened many opportunities in the workplace and general environment for IOM to build on what has already been achieved. These questions when adapted to new industrial and environmental scenarios and the associated health risks remain pertinent. Future success will depend on IOM's ability to innovate and remain relevant to the changing needs of society.

AUTHOR'S NOTE

All research contracts throughout IOM's history have included a clause ensuring freedom to publish results in the scientific literature.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

AS wrote the first draft of the manuscript. RA, JC, and HC contributed to manuscript revision. All authors read and approved the submitted version. All authors contributed to the content of the manuscript which is an overview of the scientific work of the IOM, in which they all had substantial involvement.

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Carcinogenicity of Poorly Soluble Low Toxicity Particles: Commentary on Epidemiology as a Risk Assessment "Reality Check"

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Inhaled particles that are poorly soluble or insoluble and of low toxicity ("poorly soluble low toxicity" or "PSLT" particles), can accumulate in the lung and at lung overload levels induce lung cancers in rats. The question of whether PSLT particles increase lung cancer risk in humans is complicated by large differences between rats and humans and the relatively large particle doses administered in animal studies even when compared with heavy human occupational exposures. We review the findings of epidemiological studies on occupational exposure to each of three different PSLT particles (carbon black, talc and taconite). The epidemiological evidence indicates that at even very high occupational exposure levels at which non-malignant respiratory diseases including pneumoconiosis and even talcosis are observed, lung cancer risks appear not to be elevated. Although positive human cancer risks might be predicted based on extrapolation from overload doses in rats to relevant exposures in humans, the epidemiological "reality check" based on the three examples indicates that these PSLT particles are unlikely to increase lung cancer risk in humans even at high occupational levels of exposure. Therefore, we propose that careful evaluation of the epidemiological evidence can serve as a "reality check" for human risk assessment and help balance the risk evaluation process.

Keywords: epidemiology, PSLT particles, carcinogenicity, lung cancer, risk assessment, talc, taconite, carbon black

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INTRODUCTION

Several inhaled particulate substances have been implicated in increasing the risk of cancer, and especially lung cancers in humans. For example, certain compounds of arsenic, beryllium, cadmium, chromium and nickel–as well as asbestos and crystalline silica–have been reported in various epidemiological studies to increase lung cancer risk, especially in occupational settings where these substances were produced or used and where workplace exposures were substantial (1). All of these carcinogens are believed to be directly genotoxic and therefore the observed increased risk of lung cancer may be considered biologically plausible.

Despite the relatively clear epidemiological evidence that these genotoxic substances are human lung carcinogens, some epidemiological studies demonstrate that lung cancer risks are not increased among all groups of exposed workers, and may be greatest or even limited to those most highly exposed (2–4). This suggests that for at least some of these substances there are exposure thresholds above which risk is increased, possibly reflecting the impact of one or more protective

mechanisms including DNA and/or cellular repair, clearance, dissolution, chemical reduction or transformation, etc., that reduce or eliminate the carcinogenic responses to the substances. Less obvious, however, is whether inhaled particles of low toxicity that also are poorly soluble (or insoluble) in physiological fluids and that accumulate in the lung also can increase lung cancer risk. These substances are commonly referred to as "poorly soluble low toxicity" or "PSLT" particles. One classic example is coal dust (5, 6), which clearly leads to coal workers' pneumoconiosis (sometimes called "black lung"), or the related industrial chemical, carbon black, that epidemiologically have not been associated with increased risk of lung cancer.

The epidemiological research for these examples demonstrates a wide range of lung cancer risks associated with these known and potential human carcinogens. Additionally, for those substances determined to be lung cancer hazards, epidemiologically based risk evaluation may be capable of identifying evidence of exposure thresholds or the lack of increased risk associated with lower doses or with substances that are unlikely to be carcinogenic at human relevant exposures. This paper will highlight epidemiology in providing a "reality check" regarding the role of occupational exposure to a selection of PSLT particles that have been implicated in animal studies to increase lung cancer risk.

DEFINITION AND SHORT SUMMARY OF PHYSICOCHEMICAL PROPERTIES OF PSLT PARTICLES

According to the European Centre for Ecotoxicology and Toxicology of Chemicals (ECETOC), PSLT particles are those that have dissolution half-lives measured in artificial lung fluids (interstitial fluid, artificial lysosomal fluid, and artificial alveolar fluid) longer than macrophage-mediated clearance times, e.g., titanium dioxide, carbon black, talc, and printer toner (5). These particles are considered to be chemically inert and without any known specific or inherent toxicity (i.e., lack of biochemical reaction(s) between the molecules at their surface or dissolved from their surface and the embedding lung fluid) (5, 6). These properties differentiate PSLT particles from those such as crystalline silica that exhibit significant surface-related cytotoxicity (5). Bio-solubility refers to the solubility of particles in biological systems, including *in vitro* cell systems and *in vivo* biological fluids. The bio-solubility of a substance may differ substantially from its solubility in water and in different biological fluids (5, 7). The bio-solubility of particles determines how easily particles are dissolved in intracellular or extracellular fluids and cleared into the blood or lymphatic circulation, and therefore contributes significantly to clearance rates and the potential for toxicity (7). Bio-solubility is a significant factor in determining whether particles can accumulate in the lung, and the dose at which lung overload may occur (7).

ANIMAL EVIDENCE-LUNG OVERLOAD

Carbon black, titanium dioxide (TiO_2) and "talc not containing asbestiform fibers" all are described by the International Agency for Research on Cancer (IARC) in Monograph Volume 93 as having "inadequate" evidence of carcinogenicity in humans. However, IARC classified the experimental animal evidence for the carcinogenicity of carbon black and TiO_2 as "sufficient" and "limited" for talc (8). In 2010, TiO_2 and carbon black were classified by IARC as Group 2B "possibly carcinogenic to humans" based on inhalation studies in rats and the absence of increased lung cancer in occupational epidemiological investigations and inhaled talc not containing asbestos or asbestiform fibers was classified as Group 3 - "not classifiable as to its carcinogenicity" (8).

The proposed mode of action for the animal evidence for carbon black and TiO_2 was impaired clearance with accumulation of particles in the lung, resulting in inflammation, cell injury, production of reactive oxygen species that eventually lead to mutations and tumors in animal models. This largely was supported by studies in rats demonstrating lung tumors only at high exposure concentrations that increased particle retention, i.e., "lung overload" (9–12). However, studies conducted with mice and hamsters exposed to PSLT particles did not report increased occurrence of lung tumors (5, 8, 13). Results from several PSLT particle inhalation studies indicate the rat lung responds differently from other small animal species, non-human primates and humans [(6, 8, 14–19) as cited by (13)]. The U.S. National Institute for Occupational Safety and Health (NIOSH) considers the key steps leading to lung tumor development in the rat as particle-induced lung inflammation, oxidative stress, lung tissue damage, and epithelial cell proliferation, (i.e., tumor formation is through a secondary genotoxic mechanism) (20). The ECETOC proposed that in particle-overload exposed rats, continuous exposures promote a scenario including enhanced transfer of particles to lymph nodes, accumulation of particles in the lung, increases in lung weight, alveolar macrophage accumulation, pulmonary inflammation, alveolar epithelial hyperplasia (proliferation) and metaplasia, fibrosis and eventually cancer. These are consistent with scientific evidence that supports a threshold model for PSLT particles causing adverse respiratory tract effects in humans (7, 16).

The relevance and applicability of the lung tumor findings in rat studies to humans exposed to PSLT particles have been questioned and are the subject of several comprehensive reviews resulting from scientific conferences, workshops and/or task forces (5–7, 13, 21–23). The development of lung tumors following overload exposures to PSLT particles may be unique to rats and when observed, occurs only under the circumstances of sustained particle overload in the lungs. In contrast, heavy particle exposures resulting lung overload appear not to elicit neoplastic responses in mice or hamsters, or in larger mammals including humans and non-human primates (6).

Adverse outcome pathway (AOP) scenarios for exposure to PSLT particles progressing to the development of lung tumors have not been identified in any species other than the rat. Warheit et al. (6) has identified factors that provide compelling evidence that the rat lung tumor response to PSLT particle overload is not relevant to lung cancer risks in humans. Specifically, the conceptual AOP model for the particle overload response in rats represents a species-specific set of pathological sequelae that is not consistent with the pulmonary effects documented

in PSLT particle-exposed mice/hamsters or in particle-exposed non-human primates or coal miners. In humans and primates, a large proportion of inhaled particles that deposits in the distal lung translocates across alveolar epithelial barriers to interstitial sites. This sequestration of particles in the interstitial (humans and primates) vs. alveolar (rat) compartments most likely has an impact on the types of adverse pulmonary effects observed in humans and rats. In addition, the model for long-term particle retention in the lungs of humans developed by Gregoratto et al. (24) estimates substantially greater translocation of inhaled particles into the interstitial compartment than the International Commission on Radiological Protection (ICRP) Human Respiratory Tract Model (HRTM), which correlates well with the findings of pulmonary responses reported in studies on particle-exposed monkeys and post-mortem evaluations of particle kinetics/responses in the lungs of long-term coal miners. Furthermore, there are differences in the morphology, anatomical locations, and characterization of lung tumor types in PSLT particle-overload exposed rats vs. human lung tumors e.g., those associated with cigarette smoking.

EPIDEMIOLOGY AS A "REALITY CHECK": LUNG CANCER AND OCCUPATIONAL EXPOSURE TO CARBON BLACK, TALC, OR TACONITE

IARC (8) acknowledged that some of the mechanistic steps have been shown to occur in humans exposed to poorly soluble particles (PSP), however, the group concluded that "it is not known to what extent humans are susceptible to particle-induced lung cancers" associated with PSLT particles such as carbon black and talc. While the use of data from animal studies is an important piece of evidence in the classifications of chemicals, epidemiology seems better suited for providing a "reality check" for the potential impact of exposure to PSLT particles. The clear advantage for risk evaluation and quantitative risk analysis is that epidemiological evidence directly reflects relationships between human-relevant exposures (including extreme ones historically occurring in some workplaces) and actual increased risk, if any, of human lung cancer.

Occupational Carbon Black Exposure and Lung Cancer Risk

Carbon black is a powdered form of elemental carbon occurring as near-spherical colloidal particles and coalesced particle aggregates of colloidal size, produced by the controlled combustion or thermal decomposition of hydrocarbons. Carbon black is used in many commercial products such as rubber, particularly in tires, and in plastics (25).

Three large cohort studies of carbon black production workers investigated the association between carbon black exposure and lung cancer mortality risk. Primary results from these lung cancer studies are summarized in **Table 1**. A cohort mortality study of 6,634 US carbon black workers from 18 facilities with high tracing (98.5%) of vital status and over 8,000 time-weighted average measurements summarized using a job-exposure matrix (JEM) analyzed lung cancer mortality by years of employment, years since first exposure, and years since cessation of exposure. The study reported no increase in lung cancer mortality and no relationship with any exposure metric (26).

The German cohort was defined in Wellmann et al. (28), Morfeld et al. (29, 30), and involved 1,522 production workers with a cumulative follow-up from 1976 through 1998. Using national reference rates, an initial evaluation indicated an elevated standardized mortality ratio (SMR) for lung cancer, based on 50 cases (28).

Since the smoking prevalence of this cohort was particularly high, which may have resulted in an overestimation of the SMR for lung cancer, further analyses were performed to clarify the potential role of smoking and the use different (i.e., more local) referent rates. A nested case-control study also was conducted to estimate the potential effects of selection bias and confounding due to smoking, prior exposures, and being a prisoner of war during World War II. The association with carbon black exposure was no longer apparent in analyses of 50 lung cancer cases after taking into account smoking history and using regional reference rates (31). Specifically, the SMRs for lung cancer was reduced to 1.33 (95% CI: 0.98 to 1.77) and no longer statistically significantly elevated when accounting for smoking and based on the referent rates of West Germany. The SMRs for lung cancer further were reduced to 1.27 (95% CI: 0.93 to 1.69) and 1.20 (95% CI: 0.88 to 1.59), using local reference rates from North-Rhine Westphalia and from Cologne, respectively (29).

The UK cohort involved 1,147 male production workers with follow-up from 1951 through 1996. An elevated SMR of 1.61 (95% CI: 1.29–2.00) was noted for lung cancer (27). In their re-analyses, the investigators reported elevated lung cancer risk in only two of the five plants. Furthermore, the investigators focused on the potential risk of lung cancer due to carbon black exposure in the most recent 15 years of employment (32); however, this "lugging" effect (i.e., discounting earlier exposures rather than discounting more recent exposures when "lagging" exposure to account for latency) was not apparent in the German cohort.

Combining the data from these three cohort studies with different exposure levels, a meta-regression reported no exposure-response relationship with lung cancer mortality for each 10 mg/m^3-yr increase in cumulative exposure (beta value $= -0.013$, SE $= 0.065$, p-value $= 0.8469$) (33).

Occupational Talc Exposure and Lung Cancer Risk

Talc is a naturally occurring mineral consisting of hydrated magnesium silicate. It is used in many industrial and consumer applications such as cosmetics, baby powder, pharmaceuticals, ceramics, paper, reinforced plastics, and paint (8).

Several occupational cohort studies of talc miners and millers in regions where the talc deposits do not have more than trace contamination by asbestos have been published and updated over the last four decades. Lung cancer results from each study are summarized in **Table 2** including an overall SMR based on the pooled data. These include separate cohorts of 542 talc workers in the French Pyrenees followed for mortality from

TABLE 1 | Lung cancer results from cohort studies of carbon black workers.

Reference	Cohort size	Lung cancers (*N*)	Period, Location (Reference rate)	Lung cancer SMR* (95% CI)
Dell et al. (26)	6,634 including inception cohort 4,882	184	1940–2011 United States (national)	0.77 (0.67–0.89)
Sorahan et al. (27)	1,147	61	1951–1996 UK (England and Wales)	1.61 (1.29–2.00)
Wellmann et al. (28)	1,522	50	1976–1998 Germany (West Germany)	2.18 (1.61–2.87)
Morfeld et al. (29)**	1,528 including inception cohort 1,271	47	(West Germany) (Northe-Rhine Westphalia) (Cologne)	1.33*** (0.98–1.77) 1.27*** (0.93–1.69) 1.20*** (0.88–1.59)

*SMR, standardized mortality ratio; **more detailed analyses of data from Wellman et al. (29); ***using local reference rates and adjusted for smoking and prior exposure.

1973 to 1995 and 1,070 talc workers from four sites in the Austrian Alps followed for mortality from 1945 to 1996 (36). Talcs from both locations were considered to have low quartz content (36). A cohort study in Norway followed 390 talc miners and millers for mortality from 1952 to 2011 (38, 39). Norwegian talc reportedly contains only trace amounts of quartz, tremolite and anthophyllite (38). A cohort of 427 talc miners and millers in Vermont (US) who began working as early as 1930 were followed for mortality through 2012 (35, 40). The talc was described in the original publication as "free of asbestos and of significant quantities of free silica" (40).

Finally, perhaps the most informative study for purposes of examining risks of lung cancer among heavily talc-exposed workers is the occupational cohort study of 1,749 miners and millers of "a very pure type of talc" in the Val Chisone region in northern Italy (37, 41). These workers were enumerated and have been followed for decades until 77% were deceased (37). Early reports document enormous dust concentrations in both mines and mills, and consequently the risk of non-malignant respiratory disease was profoundly increased, especially silicosis in miners (37, 41). Nevertheless, millers were exposed to high concentrations of pulverized talc (and unlikely silica) and had a non-significant excess of pneumoconiosis (SMR = 2.63; 95% CI: 0.96–5.73, 6 observed cases) (37).

Even without consideration for smoking, a pooled-SMR of lung cancer for the five cohorts with high historical exposures to talc conducted in five different countries was 1.13 (95% CI: 0.97–1.31). Furthermore, there is no increase in pleural cancer/mesothelioma in studies of talc miners and millers in talc with no known or detectable contamination by asbestiform minerals (42). This finding is not surprising due to the lack of a credible level of biological plausibility as well as the lack of substantial exposure to amphibole asbestos associated with these talc mining and milling operations.

Occupational Taconite Exposure and Lung Cancer Risk

Although not commonly identified as a PSLT (5-6), taconite is an iron-rich mineral with uncertain but likely low toxicity extracted as an iron ore that has been mined in the Mesabi Iron Range in Minnesota (US) since 1865. Iron oxide, however, is considered a PSLT. Hematite, a high-grade iron ore, was originally mined in this region, but after reserves were depleted in the 1950's, the mining of the lower-grade taconite began (43). Several publications on the lung cancer mortality of miners and millers exposed to taconite dusts have been generated since the 1980's, in part addressing concerns related to the elongate mineral particle (EMP) characteristics of taconite, and the hypothesis that EMPs might behave as fibers and increase the risk of mesothelioma (44–46). The most recent studies were cohort updates published in 2014-2015 and incorporating substantially larger numbers of workers than in previous reports, with totals exceeding 40,000 (47, 48). Two publications addressed lung cancer incidence among these workers (43, 48). The results of the studies of Minnesota taconite workers and lung cancer risk are presented in **Table 3**.

Higgins et al. (44) compared the lung cancer mortality rate through 1976 of 5,751 taconite workers at one mining company with Minnesota state mortality rates. Cooper et al. (45, 46) similarly compared the lung cancer mortality rate through 1988 of 3,444 taconite workers at two mining and milling operations with Minnesota state mortality rates. Allen et al. (47) expanded the lung cancer mortality evaluation to seven companies which included 31,067 taconite miners for the study period of 1960 to 2010. Lung cancer incidence was evaluated in a taconite worker cohort established by the University of Minnesota and the Iron Range Resources and Rehabilitation Board. The cohort included 40,720 workers who were alive as of 1988 and followed for cancer incidence through 2010 (48). A case-control analysis nested in this cohort was performed, including additional participants and lung cancer occurring after 1960 (43).

The six publications on taconite workers identified (including cohort expansion and updates) have resulted in only one study with a slightly increased but statistically significant overall SMR for lung cancer among taconite miners (47). After an adjustment for smoking, no increase in lung cancer incidence was observed in the largest cohort (48). In a more detailed evaluation of the nested case-control data, no association with

TABLE 2 | Lung cancer results from cohort studies of miners and millers of talc reportedly not contaminated with asbestos [updated from (34)].

Reference	Period, Location	Cohort size	Lung cancers (*N*)	Lung cancer SMR* (95% CI)
Fordyce et al. (35)	1940–2012 Vermont	427	32	1.44 (0.98–2.03)
Wild et al. (36)	1945–1996 France	1,070	21	1.23 (0.76–1.89)
Wild et al. (36)	1973–1995 Austria	542	7	1.06 (0.43–2.19)
Ciocan et al. (37)	1946–2020 Italy	1,749	85	1.02 (0.82–1.27)
Wergeland et al. (38)	1953–2011 Norway	390	21	1.17 (0.73–1.79)
Combined		4,178	166	1.13 (0.97–1.31)**

**SMR, standardized mortality ratio; ** Pooled SMR.*

TABLE 3 | Lung cancer results from studies of taconite workers.

Reference	Period	Size	Lung cancers	Lung cancer SMR, SIR or OR* (95% CI)
Higgins et al. (44)	1952–1976	5,751	15	0.84 (0.47–1.38)
Cooper et al. (45, 46)	1959–1988	3,444	62	0.87 (0.52–0.86)
Allen et al. (47)	1960–2010	31,067	949	1.16 (1.09–1.24)
Allen et al. (48)	1988–2010	40,720	973	1.1 (1.0–1.3)**
Allen et al. (43)	1960–2010	3,381 controls	1,706	Exposure Hematite 0.81 (0.67–0.98) [0.13- <0.45]† 1.0 (0.79–1.25) [0.45- <2.35]† 0.98 (0.77–1.24) [> 2.35]† 0.82 (0.57–1.19)

**SMR, standardized mortality ratio; SIR, standardized incidence ratio; OR, odds ratio. **SIR adjusted for smoking. Unadjusted SIR, 1.3 (1.2–1.4).* † *EMP/cm^3-yrs; EMP, elongate mineral particles.*

taconite exposure indicators was identified (43). Additionally, an absence of increased risk of lung cancer and mesothelioma in the Minnesota community has been noted (49).

DISCUSSION

Overall, the epidemiological evidence (i.e., the most direct, relevant data on health outcomes in humans) for carbon black, talc and taconite as examples of PSLT particles and risk of lung cancer consistently fails to demonstrate elevated risks or compelling exposure–response relationships. This finding is consistent with a meta-regression analysis for titanium dioxide (another PSLT particle not reviewed herein) that reported no elevated risks for mortality due to lung cancer (50). Therefore, the epidemiological evidence for carbon black, talc, taconite and titanium dioxide, though biologically plausible in experimental animal exposure-overload settings, does not support the hypothesis that these examples of PSLT particles increase lung cancer risks at human-relevant exposure levels.

While the "reality check" of epidemiology currently does not support the hypothesis that PSLT particles cause human lung cancer–at least for the three examples presented here–several caveats should be noted.

First, the use of mortality, while a reasonable (but not ideal) surrogate for incidence for some outcomes with high case-fatality rates including lung cancer, does present challenges, especially if mortality due to more rapidly fatal non-malignant conditions occurs (such as silicosis) before cancerous tumors are identified. Also, lung cancer excesses among workers often reflect a higher prevalence of cigarette smoking, which often is not captured and controlled in mortality studies.

Second, that heavy exposure to PSLT particles may not increase the risk of cancer and lung cancer (or mesothelioma) specifically, these exposures can result in increased risk of non-malignant respiratory diseases including the pneumoconioses, one of which is talcosis. Exposure to "dusts" in the workplace–often called "nuisance dusts"–remains common. Exposure to PSLT particles occurs across many industrial sectors such as (1) mineral mining, milling, preparation, (2) welding, asphalt production and application, (3) manufacturing of textiles, glassware, roofing, pulp and paper products, etc., and (4) nanomaterial manufacturing and use of PSLT nanoparticles. Therefore, it is reasonable to conclude that PSLT particle exposures may impact millions of workers globally. Future studies on possible health effects of PSLT exposure should consider and evaluate, when possible, earlier

health endpoints as well as measure disease incidence by exposure levels.

Third, most cohort mortality studies–including those summarized here–reflect exposure, often substantial, occurring several decades prior to the study publication. It is common for historically high exposures to have been reduced as hazards were identified and protections put in place over time. Nevertheless, even if those high exposure scenarios no longer exist, and workers continue to be exposed to the same substances at substantially lower levels, epidemiological studies characterizing exposure groups still can inform risk assessments. As exposure levels continue to decline, there may come a time when no increased risks can be detected, i.e., exposures fall below thresholds of increased risk. This may have been seen in recent decades for crystalline silica, where exposure controls that prevent silicosis also prevent lung cancer that may require higher exposures than can induce silicosis. However, for historical occupational exposure to PSLT particles such as carbon black, talc, and taconite even if orders of magnitude higher than commonly seen in recent decades, these appear not to increase lung cancer risk.

The mechanisms of toxicity of inhaled PSLT particles are increasingly well understood, allowing the establishment of safe exposure levels and identification and use of adequate risk management measures based on non-cancer endpoints. Many toxicological and epidemiological studies provide data that are more relevant to human exposures than rat lung tumors observed under particle overload conditions and appropriate for quantitative risk assessment informing the derivation of exposure limits that are protective against cancer and non-cancer endpoints. For example, an Expert Workshop recently evaluated whether inflammation should be used as the key endpoint in applying Benchmark Dose (BMD); No Observed Adverse Effect Level (NOAEL) or Lowest Observed Adverse Effect Level (LOAEL) approaches with adjustments for lung dose based on physiologic differences in species (e.g., lung deposition, ventilation) (13). The Expert Workshop recommended that occupational or environmental inhalation standards should aim to prevent inflammation as it precedes more serious responses (e.g., fibrosis, cancer, mortality) observed in epidemiological studies and may be detected at lower exposures.

While the current evidence based on cohort mortality studies of workers historically heavily exposed to carbon black, talc and elongate taconite particles does not support an association between exposure to these PSLT particles and the development of respiratory cancers in human populations, there is a need for more sensitive studies on the incidence of objectively measurable health endpoints such as inflammation that are indicative of increased risk of non-malignant respiratory diseases and can inform quantitative risk analyses supporting exposure regulations that protect workers.

CONCLUSION

According to ECETOC (5), promulgating regulatory levels for PSLT particles based on their possible classification as inhalation carcinogens–observed only in rat cancer studies in which exposure overload may have occurred and likely are irrelevant to human health risks–is not epidemiologically supported, and may compromise the value of chemical hazard classification schemes. For human health hazard and risk assessment, it is important to understand not only the type of potential human health effects plausibly caused by PSLT particles in experimental animals, but also whether such exposures are relevant to humans and whether the mechanisms by which PSLT particles may cause cancers in rats occur in or can be extrapolated to humans (19). The Expert Workshop panel stated that, in the absence of supporting data from other species, overload-associated lung tumors in rats do not imply a human hazard, and that lung tumors observed in rats under lung particle overload conditions are not relevant to risks in humans exposed to PSLT particles at human-relevant exposure levels (13). Furthermore, the US EPA Guidelines for Carcinogen Risk Assessment (51) acknowledges, at least in principle, the criticality epidemiological evidence in the risk evaluation process: "Epidemiologic data are extremely valuable in risk assessment because they provide direct evidence on whether a substance is likely to produce cancer in humans, thereby avoiding issues such as: species-to-species inference, extrapolation to exposures relevant to people, effects of concomitant exposures due to lifestyles. Thus, epidemiologic studies typically evaluate agents under more relevant conditions." The epidemiological "reality check" based on the three examples considered aligns with this perspective.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

KM, CBa, AS, and WT substantially contributed to the conception and design of the presentation and manuscript. MY presented the Particles and Health 2021 Conference on carbon black and substantially contributed to the carbon black section. CBo, CBa, and GD substantially contributed to the toxicological sections. All authors contributed to interpreting findings, drafting, revising the manuscript, reading, and approving the final manuscript.

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Coal Miners and Lung Cancer: Can Mortality Studies Offer a Perspective on Rat Inhalation Studies of Poorly Soluble Low Toxicity Particles?

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Inhalation studies involving laboratory rats exposed to poorly soluble particles (PSLTs), such as carbon black and titanium dioxide, among others, have led to the development of lung cancer in conditions characterized as lung overload. Lung overload has been described as a physiological state in which pulmonary clearance is impaired, particles are not effectively removed from the lungs and chronic inflammation develops, ultimately leading to tumor growth. Since lung tumors have not occurred under similar states of lung overload in other laboratory animal species, such as mice, hamsters and guinea pigs, the relevance of the rat as a model for human risk assessment has presented regulatory challenges. It has been suggested that coal workers' pneumoconiosis may reflect a human example of apparent "lung overload" of poorly soluble particles. In turn, studies of risk of lung cancer in coal miners may offer a valuable perspective for understanding the significance of rat inhalation studies of PSLTs on humans. This report addresses whether coal can be considered a PSLT based on its composition in contrast to carbon black and titanium dioxide. We also review cohort mortality studies and case-control studies of coal workers. We conclude that coal differs substantially from carbon black and titanium dioxide in its structure and composition. Carbon black, a manufactured product, is virtually pure carbon (upwards of 98%); TiO_2 is also a manufactured product. Coal contains carcinogens such as crystalline silica, beryllium, cadmium and iron, among others; in addition, coal mining activities tend to occur in the presence of operating machinery in which diesel exhaust particles, a Type I Human carcinogen, may be present in the occupational environment. As a result of its composition and the environment in which coal mining occurs, it is scientifically inappropriate to consider coal a PSLT. Despite coal not being similar to carbon black or TiO_2, through the use of a *weight of evidence* approach-considered the preferred method when evaluating disparate studies to assess risk- studies of coal-mine workers do not indicate a consistent increase in lung cancer risk. Slight elevations in SMR cannot lead to a reliable conclusion about an increased risk due to limitations in exposure assessment and control of inherent biases in case-control studies, most notably confounding and recall bias. In conclusion, the weight of the scientific literature suggests that coal mine dust is not a PSLT, and it does not increase lung cancer risk.

Keywords: coal miners, lung cancer, PSLTs, poorly soluble particles, carbon black, titanium dioxide

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INTRODUCTION

Inhalation studies involving laboratory rats exposed to poorly soluble particles (PSLTs), such as carbon black and titanium dioxide, among others, have led to the development of lung cancer in conditions characterized as lung overload. Lung overload has been described as a physiological state in which pulmonary clearance is impaired, particles are not effectively removed from the lungs and chronic inflammation develops, ultimately leading to tumor growth. Since lung tumors have not occurred under similar states of lung overload in other laboratory animal species, such as mice, hamsters and guinea pigs, the relevance of the rat as a model for human risk assessment has presented regulatory challenges.

Recently, an international panel of scientific and regulatory toxicology, epidemiology and particles scientists discussed the relevance of rat lung tumor data for poorly soluble low toxicity particles (PSLTs) (1). Their consensus views were: *"In summary, the Expert Panel thoughtfully considered the current state of the science for PSLT and reached agreement on several matters relevant to PSLT toxicology, hazard classification and risk assessment*. Specifically, the Expert Panel: (1) outlined an experimental process for determining if a material should be considered as poorly soluble and low toxicity; (2) agreed the rat is a sensitive test species for PSLT inhalation toxicology and supported continued use of the rat for PSLT inhalation toxicology studies; (3) recommended that future studies focus on defining thresholds for inflammation and inflammation be used as a critical endpoint for OEL setting; (4) agreed rat lung cancer occurring only under conditions of lung particle overload, in the absence of corroborating data from other species, should not be interpreted to imply a cancer hazard for humans; and (5) were in consensus that rat lung tumors under lung particle overload are not relevant to health hazard or risk under non-overload exposure conditions."

Inhalation studies of PSLTs, such as carbon black and titanium dioxide, among others, in which lung cancer under conditions of lung overload has occurred in rats, but not mice, hamsters or guinea pigs, has presented regulatory challenges in human risk assessment. It has been suggested by some that coal miners may represent an occupational group in which it may be argued that lung overload has occurred, primarily due to substantial amounts of retained coal dust in miners with coal workers pneumoconiosis, a chronic inflammatory condition that seems primarily due to the iron content in coal and not quartz (2).

It should be noted, however, that biomathematical models have not demonstrated physiological evidence of lung overload in humans (3). The authors studied 131 US coal miners with an average cumulative dust exposure of 107 mg-year/m^3 with 36 years of exposure and a mean coal mine dust concentration of 3 mg/m^3). In the biomathematical analysis, a mean dose of 13.8 grams of coal dust were retained in the miners studied,and found that a three-compartment model with no clearance break-down fit the lung burden best (3).

UK investigators also used statistical and mathematical modeling techniques to analyze data from an autopsy results of 423 UK miners to predict lung and lymph node dust burdens in coalminers with long-term exposure to respirable dust (4). The analysis was based on autopsy data held at the Institute of Occupational Medicine. The mean lung dust burden was 14.4 g (sd = 11.7 g) Like Kuempel et al. described above, Tran and colleagues showed that coalminers did not develop overload even under high exposure scenarios.

In summary, intensive investigations in the US and in the UK showed that coalminers did not develop overload–even under high exposure conditions and are not at risk of lung cancer (5).

Despite these mathematical analyses that indicate that coal miners do not develop "lung overload" as physiologically defined, the term "lung overload" is often used colloquially in regulatory and other settings to describe the extent of dust retention in coal miners in comparison to other occupational groups. In this discussion, we refer to coal miners with pneumoconiosis as representing a clinical state of "overload" as a result of substantial levels of retained dust in the lungs and the corresponding level of chronic inflammation.

As a result, it seems wise for the purpose of this review to raise the question that If coal miners do experience "lung overload" could studies of risk of lung cancer among coal miners provide a perspective on the human significance of rat inhalation studies of poorly soluble low toxicity particles? This question necessitates addressing not only whether coal increases risk of lung cancer but also whether coal is an appropriate surrogate for carbon black and other PSLTs?

The purpose of this report is to (1) address whether coal is a PSLT and an appropriate surrogate for carbon black and titanium dioxide (TiO_2), as examples of PSLTs and (2) whether coal worker mortally studies show an increased risk of lung cancer (6).

METHODS

To address the questions posed about the relevance of coal as a surrogate for PSLTs and to evaluate coal worker mortality studies regarding lung cancer risk, we (1) reviewed the components of coal, carbon black and TiO_2 and (2) identified and reviewed the published coal worker mortality studies.

Addressing Compositional Differences Between Coal, Carbon Black and TiO2

The International Agency for Research on Cancer (IARC) convened a working group in 1997 to address potentail carcinogenic risks in coal miners (7). As in most IARC monographs, considerable attention is devoted to the physico-chemical aspects of the substance under review. IARC, considered an authoritative source, thoroughly reviewed numerous aspects of coal, including its composition. Althought the monogaraph is now over 20 years old, the basic compositional aspects of coal will not be substanatilly different.

Literature Review of Coal Worker Mortality Studies

The same IARC working group described above that reviewed coal in 1997 also reviewed epidemiology mortality studies of coal workers. The results of these studies will be tabulated. In addition,

to identify mortality studies of coal workers published after the 1997 IARC monograph, we conducted an updated literature review *via* PubMed.

RESULTS

Composition of Coal

Is coal an appropriate surrogate to evaluate risk of lung cancer from exposure to poorly soluble particles such as carbon black and titanium dioxide?

Coal is a complex mixture of more than 50 elements and their oxides; the mineral content varies with particle size of the dust and the coal seam (7). The remaining portion consists of a variable mixed dust, introduced into the mine atmosphere through operations other than coal cutting, such as roof bolting or in the distribution of rock dust (a low-silica limestone dust) to prevent explosions. Airborne respirable dust in underground coal mines has been estimated to be 40–95% coal [(8); United States National Institute for Occupational Safety and health, 1995]

In addition to coal itself, coal mining has involved the use of heavy equipment, often run by diesel engines. Operation of diesel equipment underground can lead to the generation of fine dust particulates ($< 1\,\mu m$); the composition of which would be fairly typical of diesel exhaust from industrial machines. Diesel exhaust particles (DEPs) are considered a Type I Human Carcinogen (9). The presence of DEPs in the coal mining environment presents a significant confounder in evaluating the significance of lung cancer results in coal worker mortality studies. Below are a series of tables that describe components of coal.

Table 1 notes the carbon content of various types of coal. As noted, the carbon content can vary from 50% in Peat to 92 to 95% in anthracite (7).

As noted in **Table 2** below, coal also contains Type 1 Human Lung Carcinogens per the International Agency for Research on Cancer (IARC) classification system, including beryllium, cadmium and chromium. The presence of these substances presents significant confounders in evaluating any potentail lung cancer risk in coal miners

Quartz is another component of coal that varies from about 4–8% in British Coal mines (see **Table 3** below). In addition, quartz concentrations in US and German coal mines can vary up to 7 and 5%, respectively [see Tables 7, 8 from IARC's monograph on Coal (7)].

As noted in **Table 4** below, coal contains concentrations of quartz ranging from 3.2 to nearly 7% in British coal mines.

Table 5 below notes the percentage of quartz content by weight and corresponding exposure levels in dust in German coal mines.

In summary, coal contains varying concentrations of carbon as well as carcinogens such as crystalline silica, beryllium and cadmium. In addition, coal mining often necessitates the use of diesel powered machinery that can lead to the generation of diesel exhaust particles, considered by IARC to be a human lung carcinogen.

TABLE 1 | Carbon contents of coal.

Coal type	Rank	Carbon %
Peat		50–65
Lignite	Low	65–70
Sub-bituminous	Low	75–80
Bituminous	Intermediate	80–90
Semi-bituminous	Intermediate	90–92
Anthracite	High	92–95

TABLE 2 | Elements and trace elements in coal.

Constituent	Range (Percentage)	Constituent	Range (ppm)
Aluminum	0.43-3.04	Beryllium	0.2–4
Iron	0.34-5.32	Cadmium	0.1–65
Silicon	0.58-6.09	Chromium	4–54
Titanium	0.02-0.15	Nickel	3–80

TABLE 3 | Compositional data for airborne dusts in a sample of British coal mines prior to 1970.

Coalfield	Quartz %
Scottish	5.8
Cumberland	6.8
Yorkshire	7.8
North Wales	6.9
Warwick	4.2

Abstracted from IARC (7); **Table 6.*

Carbon Black

Carbon black is an intentionally produced substance that is generated by the incomplete combustion of petroleum products, most notably heavy oils. Unlike the multi compositional aspect of coal, carbon black is virtually pure carbon.

According to an update on carbon black in Pattys' Industrial Hygiene and Toxicology text, *"Carbon black is the earliest known synthetic pigment, having been produced by the Chinese more than 1,500 years ago...Carbon black has been commercially produced in the United States for more than 100 years*. Its major use has been as a reinforcing agent in rubber, particularly in tires" (10). "In contrast to diamond and graphite, carbon black is an amorphous carbon composed of particles and fused particle aggregates. Untreated carbon black grades generally contain more than 97% elemental carbon with variable amounts of oxygen, hydrogen, and sulfur. Less than 1% of carbon black particles consist of extractable organic materials. The extractable material, usually in the range of tenths of 1% by weight of the carbon black, consists of a mixture of polycyclic aromatic hydrocarbons (PAHs), lesser amounts of other polynuclear aromatic hydrocarbons (PNAs), and sulfur compounds" (10).

Table 6 below demonstrates the significant compositional differences between carbon black and coal, most notably that carbon black is virtually pure carbon, whereas as noted earlier

TABLE 4 | Quartz percentages in samples of coal dust: USA: 1985–1992.

Occupation	Average quartz content
Roof Bolter	6.97
Miner operator	5.54
Shuttle car operator	4.33
Longwall shearer operator	4.02
Coal Drill operator	3.29

From Tomb et al. (11); abstracted from IARC (7); ***Table 7****.*

TABLE 5 | Mean quartz content in dust in German coal mines.

Particle size	Quartz (% by weight)	Dust concentrations (mg/m^3)
Total dust	4.1 ± 3.3	53.1 ± 29.4
Dust < 7 microns	4.3 ± 3.0	25.3 ± 13.0
Dust < 5 microns	2.9 ± 1.9	9.2 ± 7.9
Dust < 3 microns	2.2 ± 1.6	2.1 ± 1.6

From Leiteritz et al. (12); abstracted from Table 9 (7).

in this report, carbon content in coal varies widely and can be as low as 65% in lignite.

Titanium Dioxide (TiO_2)

Titanium dioxide is a white inorganic compound, which has been used for around 100 years in a vast number of diverse products for its non-toxic, non-reactive and luminous properties, which heighten the whiteness and brightness of many materials.

Titanium dioxide has reflective characteristics and is known for being the whitest and brightest of known pigments, with reflective qualities. This compound has reflective properties and can also both scatter and absorb UV rays. Applications include paints, paper, plastics, pharmaceuticals, sunscreen and cosmetics and skin care, as well as food.

Metallic titanium and TiO_2 forms are insoluble, unreactive, non-metabolized, and virtually devoid of acute toxicity; although chronic overload inhalation exposures to high concentrations of TiO_2 in rat studies can cause lung tumors in particle-exposed rats. With expansion of nanotechnology, the TiO_2 material has been engineered in terms of a variety of shapes and sizes resulting in significant particle size reduction. The reduction of the particle size leads to increased specific surface area, which contributes to increased potential for toxicity of some forms of the engineered TiO_2 nanomaterials.

Toner Mortality Study

Copier toners are fine powders composed primarily of plastics and small quantities of colorants and functional additives. They appear to be fit a description of a poorly soluble low toxicity particle. To evaluate potentail carcinogenic risks from occupational exposure to copier toner, investigators conducted a retrospective mortality study of 33,671 workers potentially exposed to copier toner (28). This study showed statistically significant deficits in all cancers, including lung cancer. No increase in nonmalignant respiratory disease was shown.

TABLE 6 | Components of commercially produced carbon blacks*.

Property	Acetylene black	Furnace black
Carbon (%)	99.8	97.3–99.3
Hydrogen (%)	0.05–0.10	0.45–0.710
Oxygen (%)	0.10–0.15	0.19–1.25
Benzene-extractable organics (%)	0.1	0.01–0.18
Ash (%)	0.00	0.1–1.0
Sulfur (%)	0.02	0.05–1.5

**Abstracted from Table 89.3 of Carbon Black, Pattys Industrial Hygiene and Toxicology, 2012.*

TABLE 7 | Summary of 15 cohort studies of coal miners with lung cancer standardized mortality ratios (SMR) and 95% confidence intervals (CI).

Cohort studies	Countries	*N*	Lung Ca. SMR	95% CI
Liddell (13)	UK	3,169	0.63	n.a.
Costello et al. (14)	US	3,726	0.67	0.4–1.0
Rockette (15)	US	23,232	1.13	1.0–1.3
Armstrong et al. (16)	AUS	213	0.25	0.01–1.4
Atuhaire et al. (17)	UK	3,865	0.78	0.7–0.9
Kuempel et al. (18)	US	8,878	0.77	0.6–1.0
Swaen et al. (19)	NLD	2,941	1.02	0.9–1.2
Starzynski et al. (20)	POL	7,065	1.07	0.9–1.2
Brown et al. (21)	UK	23,630	0.74	0.50–1.06
Miyazaki and Une (22)	JP	5,818	< 15 yr: 1.00	0.41–2.43
			>15 yr: 2.08	1.01–4.27
Morfeld et al. (23)	DE	4,581	0.79	0.64–0.96
Attfield and Kuempel (24)	US	8,899	1.07	0.95–1.19
Miller and MacCalman (25)	UK	17,820	0.99	0.93–1.05
Graber et al. (26)	US	9,033	1.08	1.00–1.18
Tomášková et al. (27)	CZ	6,687 with no CWP	0.83	0.70–0.98
		3,476 with CWP	1.70	1.41–2.04

Coal Worker Mortality Studies

Despite the limitations of using coal as a surrogate for poorly soluble particles like carbon black and TiO_2, let's briefly review the highlights of coal worker mortality studies.

Risk of lung cancer among coal miners has been investigated in cohort mortality studies conducted over nearly 50 years. Over 120,000 coal miners have been evaluated in UK, Germany, Netherlands, USA, Poland, Japan and Australia. Epidemiological studies provide data regarding the risk of lung cancer in workers exposed to coal dust. See **Table 7** below that summarizes the key results of coal worker mortality studies.

As noted in the table, the overwhelming evidence of the cohort mortality studies shows no significant increase in risk of lung cancer among coal miners. Standardized Mortality Ratios (SMRs) are typically <1 and when above 1, the results are usually not statistically significant.

There are two cohort studies (11 and 12) and a recent case-control study (29) that suggest a minor risk elevated risk in lung cancer. The risk estimates from the case-control studies are not in agreement with the results from the cohort studies. The inherent limitation of case-control studies, with lack of clear temporality, sampling bias and information bias is of limited value for a causality evaluation. Nevertheless, these studies will be described below.

In an updated study of US coal miners, Lung cancer SMR was slightly elevated and of marginal statistical significance- as the lower limit of the 95% confidence limits was 1 (SMR = 1.08, 95% CI: 1.00–1.18) (26). It is noteworthy that an earlier follow-up of the same cohort of upwards of 9,000 coal miners through 2,000, showed no association between coal mine dust exposure and lung cancer (24).

This excess in the updated US study has been described as "*unexceptionable*" (5). In this assessment, the authors stated: "*Internal analyses showed an association of lung cancer mortality with coalmine dust exposure but only during the last follow-up interval from 2000 to 2007*" (26).

The US study has a number of limitations that limit drawing broad conclusions about lung cancer risk among coal miners, most notably because the study relies on smoking information collected *only* at the start of follow-up; the models used are unable to adjust for smoking habits after leaving work. The coal miners at the start of the study (1969/1970) smoked cigarettes less than current smokers in the US male population (prevalence of smoking more than 25 cigarettes per day: 12.4% among US coalminers vs. 28.0% in the US male population). This difference has been attributed to prohibition of smoking when miners were working underground. As a result, it is not unreasonable to assume that smoking coal miners increased their intensity of smoking after cessation of coal mining work, and as a result, increased the lung cancer mortality rate of the cohort during the last follow up period when most coalminers had stopped working underground. This issue has also been discussed in the most recent study of UK coal miners, which is the largest study of coal miners to date with better assessment of exposures in a time-dependent manner (25).

The US study is further limited by an incomplete assessment of jobs held, including no start and end date of jobs held before 1969/1971; no information on jobs held after start of follow-up in 1979/1971 and no end date of working as coalminer for 16% of cohort members. Thus, only a crude assessment of exposure to coalmine dust up to the start of follow-up was possible and no time-dependent exposure analysis or lagging or lugging of exposures could be done. Measurements of crystalline silica, an acknowledged Group 1 IARC human carcinogen, were available only after 1982 but had to be allocated to the jobs held before 1969/ 1971. Recall that this updated US publication is a further analysis of US coal miners, in which a mortality study of upwards of 9000 coal miners showed no elevation in lung cancer (24).

Taeger et al., who later performed a case-control study, in a letter to the editor about the Graber study, noted that "emphasizing a 'significant' relationship with lung cancer mortality seems inappropriate in view of borderline results, [as noted above, the lower limit of the 95% CI was 1.0] lacking exposure-response relationship and nonsignificant results of the categorical analysis. Second, SMRs between regions differ considerably, especially those for lung cancer—significantly up to a factor of 3. We would suggest using regional rates for each region and then combining results to avoid bias" (Taeger et al., 2014). Note the importance of using regional rates as a reference group.

A year after the US study, European investigators published a case-control study in which risk of lung cancer among coal miners was addressed (29). Described as the European Synergy Study, the authors evaluated the joint effect of smoking and occupational lung carcinogens in 14 case-control studies comprising 14,251 lung cancer cases and 17,267 controls. Exposure assessment was based on: Employment duration; Time since first employment; and Job titles maintained for at least a year. Exposure concentrations for coal dust or other lung carcinogens-were not available.

This case-control study examined lung cancer risk for coal and ore miners and quarrymen, using the European SYNERGY data base. SYNERGY investigated the joint effect of smoking and occupational lung carcinogens. Numerous studies have been published based on the SYNERGY data base. This study focused on one aspect of SYNERGY: coal miners and lung cancer risk. The quantitative exposure assessment was based on employment duration and time since first employment. For coal miners, employment duration of 1–9 years (OR = 1.46; 95% CI: 1.18–1.80) and ≥20 years (OR = 1.73; 95% CI: 1.14–2.62) implied increased risks, while employment duration of 10–19 years suggested no link with lung cancer (OR = 0.99; 95% CI: 0.67–1.47). This latter pattern is inconsistent with a dose response relationship between coal mining exposure and lung cancer risk. Assessing dose-response is a critical step in evaluating potential causality.

Detailed information on smoking does not compensate for the weakness of the occupational exposure assessment. Exposure to coal mine dust was based on job titles maintained for at least a year. No exposure concentrations- for coal dust or any other known lung carcinogens-were available. As a result, confounding effects from lung carcinogens such as crystalline silica, asbestos, PAH, radon and metals could not be addressed in the analysis. Therefore, the observed association cannot be directly attributed to coal dust. Employment duration was used as proxy of cumulative exposure, which is prone to misclassification as to the degree of exposure. No distinction between long-term but low level exposure and short-term but high level exposure was possible.

While employment duration from 1–9 years and ≥20 years indicated an increased risk for lung cancer, no association for employment duration of 10–19 years was observed. The authors speculated that the finding may reflect healthy worker survivor effect (HWE), although the HWE has not been well studied in case-control studies; thus, this interpretation is not justified. Due to the case-control design, the study is also prone to recall bias with respect to exposure assessment. Recall bias can occur in case-control studies, in which cases are asked to recall events from many years ago. It has been repeatedly shown that people with serious illness tend to inaccurately recall events associated

with historical hazardous exposures and thus present challenges in the interpretation of the results of case-control studies (30–32). Recall bias tends to be most prominent if the disease is highly significant (such as lung cancer), and the patient has a preconception that the exposure is related to the illness.

In summary, the Taeger study did not have measured exposure data on coal mine dust or confounders, such as crystalline silica, and thus does not provide adequate information to reliably address coal mine exposure and risk of lung cancer. Confounding from carcinogens such as crystalline silica, asbestos, PAH, radon and metals could not be addressed. Employment duration was used as proxy of cumulative exposure, which is prone to misclassification as to the degree of exposure. Recall bias-major limitation in case-control studies. The observed association is unlikely to be directly attributed to coal dust. Assessment of risk is most appropriately based on cohort studies as it avoids inherent biases, most notably recall bias, in case-control studies. The weight of the scientific literature suggests that coal mine dust has little or no effect on lung cancer risk (5).

Tomášková et al. (27) compared two cohorts with respect to total mortality and cause-specific mortality, and lung cancer risk to address whether CWP would accelerate the development of lung cancer. The authors defined a cohort of coal miners with acknowledged coal workers' pneumoconiosis (CWP), and another one without CWP for comparison of the outcomes of interest in the Czech Republic through the period 1992–2013.

The cohort of 6,687 former coal miners who did not have CWP through 2013 yielded a significant decreased mortality risk of lung cancer in comparison to the general population (SMR = 0.83, 95% CI: 0.70–0.98), while the coal miners with CWP had a two-fold higher risk (SMR = 1.70, 95% CI: 1.40-2.04). Furthermore, the authors found that the total mortality of the cohort of 6,687 former coal miners without CWP (SMR = 0.86, 95% CI: 0.82–0.91) was significantly decreased than that of the cohort with CWP (SMR = 1.10, 95% CI: 1.02–1.17), compared to the general population. The mean age at death for coal miners with CWP from diseases of the respiratory system was 70.5 (SD: 10.9), while those without CWP died at age 61.1

To address whether CWP accelerates the development of lung cancer, one needs information on the pathway from (1) coal dust exposure, (2) time to development of CWP, and (3) finally time to development of lung cancer. Using time-to-event data is more reasonable to clarify this question. Unfortunately, the authors used SMR instead, which may explain the contradictory findings between the SMR estimates and age at death between the cohorts. In addition, Tomaskova et al. used maximal permissible exposure as a proxy of exposure for the non-CWP cohort, while the exposure level of the CWP cohort remained unknown.

In summary, the study of Tomaskova et al. did not provide sufficient data to evaluate the risk of coal dust exposure on lung cancer risks and to address whether CWP would accelerate the risk of developing lung cancer. Inherent limitations of the study, including the retrospective design, lack of information on exposure of interest and potential confounding factors, such as silica and smoking habits affect drawing definitive conclusions about risk of lung cancer in this cohort. Further commentary on this study is provided by Yong (33).

Rats, Monkeys and Exposure to PSLTs

In light of the different reactions of monkeys and rats to inhaled particles, it is wise to address two year inhalation studies of rats exposed to coal dust. In a study of rats and monkeys exposed to coal dust at 2 mg/m^3 for 7 h/day, 5 days per week for 24 months, rats, but not monkeys, had significant alveolar epithelial hyperplastic, inflammatory, and septal fibrotic responses to the retained particles (34). The response to particles, including alveolar epithelial hyperplasia, inflammation and focal septal fibrosis, was significantly greater in rats than in monkeys. Rats had a significantly greater alveolar epithelial hyperplastic response to particle exposure than monkeys ($p < 0.001$). Rats also had a significantly greater inflammatory response to particles than monkeys ($p = 0.02$). Lung tumors were not demonstrated in either monkeys or rats at exposures of 2 mg/m^3. The authors concluded that "*if human lungs respond to poorly soluble particles in a manner more like monkey lungs than rat lungs, perhaps the pulmonary response of rats particles may not be predictive of the response in human lungs at concentrations representing high occupational exposures*" (34).

DISCUSSION

Is Coal a PSLT and a Suitable Substitute for Carbon Black and TiO_2?

Substantial compositional differences exist between coal, carbon black and TiO_2.

Coal contains significant concentrations of crystalline silica (Type I IARC carcinogen).

Coal mining environment often includes exposure to diesel exhaust particles, another IARC Type I Human carcinogen. As a result, considering coal as a poorly soluble particle is not scientifically justified.

Coal Worker Cohort Mortality Studies

"Using a weight of evidence approach, studies of coal-mine workers, who have been exposed to occupationally relevant levels of dust, do not indicate an increase in lung cancer risk. Classifying all poorly soluble as carcinogenic to humans based on rat inhalation studies in which lung overload leads to chronic inflammation and cancer is not supported by data in humans" [Morfeld et al. (5), p. 12:3, *Particle and Fiber Toxicology*].

CONCLUSION

Using a *weight of evidence* approach-considered the preferred method when evaluating disparate studies to assess risk- studies of coal-mine workers do not indicate a consistent increase in lung cancer risk. Slight elevations in SMR cannot lead to a reliable conclusion about an increased risk due to limitations in exposure assessment and control of inherent biases in case-control studies, most notably control of confounding and recall bias. In conclusion, the weight of the scientific literature suggests that coal mine dust does not increase lung cancer risk. And finally, due to substantial compositional differences between coal

dust, carbon black and titanium dioxide, coal dust cannot be considered representative of a poorly soluble low toxicity particle.

AUTHOR'S NOTE

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AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Extended Investigation of Exposure to Respirable Synthetic Amorphous Silica Dust and Its Potential Impact on Non-malignant Respiratory Morbidity

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Objectives: The present analysis aims to study the health impact of an occupational exposure to respirable synthetic amorphous silica (SAS) dusts, based on the available data from the German study.

Methods: The effect of cumulative exposure to respirable SAS dust on respiratory morbidity were investigated in 462 exposed male workers. Multiple exposure assessments was performed anchored by a most recent measurement series. Internal regression models in addition to Monte Carlo-Multi Model were fitted.

Results: An averaged cumulative respirable SAS dust concentration of 6.44 mg/m^3-years was estimated. Internal regression models suggested a reduction of 8.11 ml (95% confidence interval: 0.49–15.73) in forced vital capacity (FVC) per 1 mg/m^3-year increase of exposure. But no effect on forced expiratory volume in 1 s (FEV_1) and the ratio of the parameters FEV_1/FVC was observed in association with exposure to a respirable fraction of SAS. No adverse effects on the occurrence of respiratory diseases were indicated.

Conclusion: This study provides no clear evidence of adverse health effects from occupational exposure to respirable SAS.

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Keywords: respirable synthetic amorphous silica, lung function, epidemiology, modeling, occupational exposure

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INTRODUCTION

Pyrogenic and precipitated synthetic amorphous silica (SAS) are nanostructured polymorphs of silicon dioxide, according to the International Organization for Standardization (ISO) definition that "material with any external dimension in the nanoscale or having an internal structure or surface structure in the nanoscale." Nanoscale is defined as a size range from ~1 to 100 nm.

Workers can be exposed to dusts of SAS during production and use of the material. Pyrogenic and precipitated SAS havethe internal structure of primary particles in the nanoscale. However, the aggregate is the smallest divisible entity for an amorphous material like SAS.

Note, Synthetic amorphous silica must be distinguished from crystalline silica. Whereas, adverse health effects from exposure to crystalline silica dust have been identified and studied for decades [e.g., (1)] the situation for chronic SAS dust exposure among humans is quite different. The International Agency for Research on Cancer (IARC) classified crystalline silica as a human lung carcinogen but amorphous silica was categorized into group 3, i.e., "not classifiable as to its carcinogenicity to humans" (2). No cancer risk was linked to SAS dust exposure. Therefore, the major interest in exposure to SAS is the potential for non-malignant respiratory effects. However, the documentation and assessment of published results on respiratory diseases in SAS exposed workers have been insufficient for drawing robust conclusions (3). This situation may be due to the fact that the amorphous forms have never drawn attention given their low toxicity potential (no known specific toxicity, amorphous structure, and solubility of SAS). However, according to a review, risk of respiratory diseases like chronic bronchitis from SAS exposure could not be ruled out (4).

Very few epidemiologic studies that investigated the exposure to amorphous silica and health outcomes are existing. Therefore, the documentation and assessment of published results on respiratory diseases in SAS exposed workers have been insufficient for drawing robust conclusions (3).

A cross-sectional study involving five German SAS production sites has been conducted to investigate the long-term exposure to inhalable SAS dust (5) and the effect on lung function parameters, such as forced vital capacity (FVC), forced expiratory volume in 1 second (FEV_1), and maximal expiratory flow at 50% of vital flow capacity (MEF50), respiratory diseases, such as chronic bronchitis, and chronic obstructive pulmonary disease (COPD) and pneumoconiosis (abnormalities in chest radiographs) in 484 exposed male workers from five German SAS production plants (6). Average effects of 80 mg/m^3-years of cumulative inhalable SAS exposure on lung function were estimated (FVC: -48 ml, $p = 0.04$; FEV_1: -28 ml, $p = 0.16$; FEV_1/FVC: $+0.2\%$, $p = 0.39$; MEF50: -12 ml, $p = 0.76$). The reduction of FVC was, however, unexceptional when compared to reference values from the non-smoking healthy general population. Hence, no concern for safety and health was substantiated under the existing working condition.

The size of particulate matter has been considered an important determinant of adverse health effects. A thorough review (7) provided a new perspective that the biological activity of SAS can be related to the particle shape and surface characteristics interfacing with the biological milieu rather than to particle size. In epidemiology, the potential health impact from the occupational exposure to SAS, in particular the respirable fraction, is less investigated.

The present study aims to address the potential impact of the respirable fraction of SAS on non-malignant respiratory morbidity with respect to lung function parameters, and prevalence of respiratory pattern, chronic bronchitis, and chronic obstructive pulmonary disease (COPD), we used the available data from the German study involving 462 SAS production workers (5, 6).

MATERIALS AND METHODS

Study Population

The enumerated study population consists of 522 workers exposed to SAS working at five German plants producing either pyrogenic or precipitated SAS (5). The start exposure period of the five German plants varies from 1959 to 1978. Eligible to participate were all current full-time workers in 1997, who worked for at least 1 month at one of the plants. Due to the small number of female workers (about 5%), only male workers were included in the present analysis. Furthermore, 21 workers without exposure information, missing lung function data, missing prick test, and inconsistent data were excluded (6). In total, 462 exposed male workers were eligible for the present analysis. Among the 462 included workers, there are 158 (34%) workers at Plant 1, 29 (6%) at Plant 2, 165 (36%) at Plant 3, 39 (8%) at Plant 4, and 71 (15%) at Plant 5. All study participants gave their informed consent.

Exposure Assessment

Job-exposure matrices (JEMs) were applied to assess the individual cumulative exposure. Respirable SAS dust concentrations were measured at each of the plants. Within each plant, the same measurement devices were applied, while different teams took the measurement across the plants. The team for Plant 1, 3, and 4 (first team) acted as a trainer for the study teams at Plants 2 and 5. Person-related dust measurements were taken repeatedly and were individually documented. Each participant was measured twice at the first instance and depending on these two measurements a third, fourth, or even fifth measurement was performed. Workers with the same jobs were additionally measured and these measurements were used in later evaluations, although these additional workers were not treated as participants in this study. All measurements were taken in the period from 1997 to 2000 (5). Industrial hygiene and plant experts assessed all jobs across the five plants to create similar exposure groups (SEGs), which means that jobs were rated as one category if the exposure levels in the work environment were comparable (8). The seven different SEGs were categorized as 9 if the categorization was not possible and 0 for the lowest exposure category up to 5 for the highest exposure category. At each plant, changes in production, ventilation, housekeeping, and other factors were reported in detail for each year and SEG since the start of the SAS production. With this information, the experts could assess the exposure levels at each plant by relative scoring (9).

Together with the individual measurement data from the period 1997 to 2000 these relative estimates were anchored to derive the respirable SAS dust concentration estimates for the complete exposure period (10). To do so, a multiple SAS exposure assessment was performed with five calculated statistics to function as anchor values for the individual measurement data.

These five statistics were p25 (first quartile), median, p75 (third quartile), the geometric, and the arithmetic mean. Furthermore, uncertainties in relative scoring were assessed by the experts by estimating low, medium, and high relative SAS dust level changes. Routine data from personnel files of the five plants were used to create the individual working history of each worker. With the anchoring method (p25, median, p75, geometric and arithmetic mean) and the type of backward extrapolation (low, medium, high), an array of 15 exposure scenarios were presented as follows, which led to 15 JEMs.

kum1 mean_high scenario
kum2 mean_medium scenario
kum3 mean_low scenario
kum4 p75_high scenario
kum5 p75_medium scenario
kum6 p75_low scenario
kum7 median_high scenario
kum8 median_medium scenario
kum9 median_low scenario
kum10 p25_high scenario
kum11 p25_medium scenario
kum12 p25_low scenario
kum13 geomean_high scenario
kum14 geomean_medium scenario
kum15 geomean_low scenario

This data was then combined with the job histories to constitute an individual exposure profile and across time to derive 15 basic estimates of cumulative exposure to respirable SAS dust for every worker in the study (11, 12). The procedure is described in detail in earlier publications (5, 6).

In analogy to the exposure assessment of inhalable SAS dust (5), the kum8 scenario (median-medium) was based on the medium backward extrapolation estimate anchored at the median of the respirable SAS dust measurements and was less affected by outliers. The kum8 scenario yield the estimates which proved to present the best agreement with the estimates derived from another JEM procedure based on expert judgments. Therefore, it was chosen to serve as the leading exposure scenario when exploring potential health effects due to respirable SAS dust exposure.

Definition of Health Outcomes

Information regarding demographics, height, occupational history (including prior exposures and co-exposures to hazardous substances), smoking habits, medical history, and current respiratory symptoms were collected with the baseline questionnaires. Interviewers differed between plants and the questionnaires were either interviewer- or self-administered.

The prevalence of respiratory diseases, including chronic bronchitis, chronic obstructive pulmonary disease (COPD), and pneumoconiosis was then determined based on the questionnaires. For chronic bronchitis, the WHO definition of cough and sputum for at least 3 months in at least two consecutive years was applied (13–15). The diagnosis of COPD was based on the GOLD criteria (16, 17). For analysis, two categories, COPD $\geq$ I and COPD $\geq$ II, were defined based on lung function data only. Additionally, obstructive chronic bronchitis was defined as COPD $\geq$ I and COPD $\geq$ II based on lung function data and the presence of chronic bronchitis. Using poster anterior chest radiographs according to the International Labor Office (ILO) classification 1980, pneumoconiosis was defined as profusion category $\geq$1/0 (any type: 1/0 or 1/1) by at least one of the three independent readers (18). Atopy was assessed by a skin prick test using four different allergens (cat, grass, birch, and house dust mite). Furthermore, IgE antibodies for several environmental allergens were applied for atopy assessment.

Spirometry was performed by occupational physicians working at the different plants or a technician of the Institute of Preventive and Occupational Medicine (IPA) (at Plant 2). All measurements were either taken with Masterlab® (Plant 3 and Plant 4), portable pneumotachographs (Flowscreen®, Plant 1 and Plant 5), or Masterscope® (Plant 2) to obtain the forced expiratory volume in 1 s (FEV_1), forced vital capacity (FVC), FEV_1/FVC ratio, and maximal expiratory flow at 50% of vital flow capacity (MEF50). Each of the devices was produced by the former German company Viasys and used the same pneumotachograph. The measurements were performed according to the recommendations of the American Thoracic Society (19), but FEV_1 and MEF50 were based on the best FVC maneuver. For each of the three lung function parameters FEV_1, MEF50, and FVC at least three satisfying forced expiratory maneuvers were done. For Plant 3 and 4, only the best maneuver for FEV_1, MEF50, and FVC was used.

Afterward, values for FVC, FEV_1, FEV_1/FVC ratio, and MEF50 of each worker were expressed as Z-scores according to the reference values according to Quanjer et al. (20) *(European Respiratory Society)*. These Z-scores were then compared to the −1.64 for the lower limit of normality (LLN), i.e., the lower 5% of a normal distribution, to define the respiratory patterns, such as normal (FEV_1/FVC $\geq$ LLN and FVC $\geq$ LLN), obstructive (FEV_1/FVC $<$ LLN), or restrictive pattern (FEV_1/FVC $\geq$ LLN but FVC $<$ LLN) (21).

Statistical Analyses

The continuous variables of the baseline study characteristics and the spirometry parameters were described with mean ± standard deviation (SD), median and interquartile range (IQR), while the categorical variables were summarized in counts and percentages. Multivariable linear regression models were performed to estimate the effect of a unit increase of cumulative respirable SAS dust exposure (1 mg/m^3-year) on the lung function parameters (FEV_1 in ml, FVC in ml, FEV_1/FVC-ratio in %, MEF50 in ml) by the median-medium scenario (kum8). The regression models included a set of covariates to adjust for potential confounding effects. The same covariates used in the assessment of inhalable SAS dust (6) were included in the models of the present study, to ease comparison between the effect estimates of inhalable and respirable SAS dusts. These covariates were planted effect (Plant 2–5 vs. Plant 1 as reference), age in years, height in cm, body mass index in kg/m^2, former and current smoker vs. non-smoker, pack-years of smoking corresponding 20 cigarettes per day during 1 year, atopy assessed by skin prick test and IgE antibodies, use of

TABLE 1 | Study characteristics for 462 SAS-exposed male workers.

Characteristic	Mean (SD)	Median (IQR)	*N* (%)
Age (baseline) (yrs)	41.0 (9.8)	39.5 (33.6–48.3)	
Height (cm)	176.6 (6.9)	176.0 (172.0–181.0)	
Body mass index (kg/m^2)	27.2 (4.1)	26.8 (24.4–29.4)	
Duration of exposure (yrs)	13.2 (8.7)	11.6 (7.2–17.4)	
Year of hire	1983 (9.2)	1985 (1977–1989)	
Year or termination	1998 (1.4)	1998 (1996–1998)	
Smoking			
Non-smoker			109 (23.6)
Former smoker			80 (17.3)
Smoker			273 (59.1)
Pack-Years of smoking	14.8 (15.2)	12.0 (1.5–20.0)	
WHO chronic bronchitis			
Yes			52 (11.3)
No			410 (88.2)
GOLD obstructive chronic bronchitis (stage)			
0			445 (96.3)
I			6 (1.3)
II			11 (2.4)
GOLD spirometric staging for COPD			
0			393 (85.1)
I			45 (9.7)
II+			24 (5.2)
Atopy assessment by prick test			
Positive			163 (35.3)
Negative			299 (64.7)
Atopy assessment by spec. IgE			
Positive			148 (32.0)
Negative			314 (68.0)
Antiobstructive medication			
Yes			14 (3.0)
No			448 (97.0)
Prior exposure to fibrogenic dust			
Yes			77 (16.7)
No			385 (86.3)
Prior exposure to substances causing obstruction			
Yes			126 (27.3)
No			336 (72.7)
Spirometry			
FEV_1 (L)	3.9 (0.8)	3.8 (3.4–4.4)	
>LLN			410 (88.7)
≤LLN			52 (11.3)
FVC (L)	5.1 (0.9)	5.0 (4.5–5.7)	
>LLN			436 (94.4)
≤LLN			26 (5.6)
FEV_1/FVC	0.8 (0.1)	0.8 (0.7–0.8)	
>LLN			394 (85.3)
≤LLN			68 (14.7)
Respiratory outcome			
Normal			367 (79.4)
Obstructive pattern			79 (17.1)
Restrictive pattern			16 (3.5)
Small opacities (radiograph)			
Rounded			
0/0			415 (89.8)
0/1			23 (5.0)
N/A			24 (5.2)
Irregular			
0/0			379 (86.5)
0/1			50 (11.4)
1/0			8 (1.8)
1/1			1 (0.2)
N/A			24 (5.2)
Mixed			
0/0			435 (94.2)
0/1			3 (0.7)
N/A			24 (5.2)

LLN: −1.64 according to GLI 2012.
N/A, chest radiograph not available; SD, standard deviation.

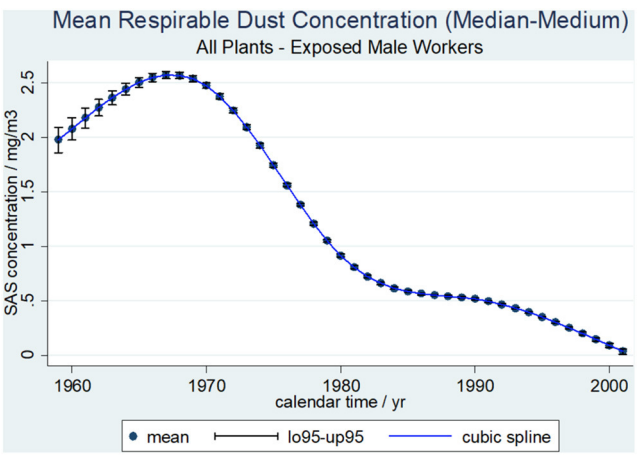

FIGURE 1 | Average respirable SAS dust concentrations (median-medium scenario) and cubic spline function by calendar time with 0.95-confidence limits, 462 SAS exposed male workers.

anti-obstructive medication, and prior exposure to fibrogenic dust and substances causing an obstruction. Adjustment for plants was necessary because not only the teams measuring the lung function but also the measurement devices differed across the plants. To correct for heteroscedasticity, robust variance estimates (sandwich estimator) were used in the regression models (22). Multivariable polytomous logistic regression models yielding odds ratios (ORs) were performed on obstructive and restrictive patterns vs. normal spirometry. Same covariates as in the linear regression models were applied, but the smoking status was changed to current smoker vs. former and non-smoker due to missing former smokers for the restrictive pattern. Cumulative respirable SAS dust exposure was expressed as an

increase of 1 mg/m^3-year. Next to the regression models on the respiratory impairment, multivariable logistic regression models on respiratory diseases (chronic bronchitis, obstructive chronic bronchitis, and COPD) and cumulative respirable SAS dust exposure have been conducted. Cumulative SAS exposure was categorized as ≤2, >2–≤6, and above 6 mg/m^3-year. These categories were based on the distribution, i.e. tertiles (2 mg/m^3 for 33.33% and 6 mg/m^3 for 66.66%) for the median-medium scenario. For smoking status, the categories of the current smoker and former smoker vs. non-smoker as in the linear regression models were used. Values of the lung function parameters measured in this study were expressed as a percentage of the Quanjer reference values and skewness and kurtosis have been calculated by the d'Agostino test for each distribution (20). Average respirable SAS dust concentrations (median-medium scenario) for the whole exposure period from 1960 to 2000 were estimated by spline regression and plotted as cubic spline with corresponding 95% confidence intervals for each exposure year. To investigate a potential dose-response relationship between respiratory impairment, respiratory diseases, and cumulative respiratory SAS dust exposure, restricted cubic splines using previous logistic regression models with the 5th, 50th, and 95th percentile as cubic knots have been calculated and plotted. Correlation between the inhalable and respirable SAS dust fraction of the 462 exposed male workers was calculated to determine the strength of the relationship between the two SAS types. Furthermore, this correlation was evaluated separately in each plant to check for plant-specific differences. Due to uncertainties in the anchoring and backward extrapolation for the exposure assessment, Monte Carlo simulations for the linear and logistic regression models as applied above have been performed with 10,000 repetitions for each of the regression models. The Monte Carlo procedure is described in detail previously (6). Fractional polynomials of degree two were used in order to take into account the possible non-linearity in age trends for the association of cumulative respirable SAS dust exposure and the lung function parameters. Additional age effects on the lung function parameters were assessed by calculating the age coefficients of lung function regression analyses according to reference values of the European Respiratory Society (20). These coefficients were compared to the age coefficients in this study for the whole study group of 462 exposed male workers and a subgroup aged 25 years and above.

All analyses were done with Stata 13 (23). Fractional polynomials were fitted with the "fracpoly" command. Monte Carlo regression analyses were performed with the "simulate" command. The statistical significance level was defined at 5%. Adjustment for multiple testing is not considered.

RESULTS

Characteristics of the Study Population

Characteristics of the study population are summarized in **Table 1**. The mean age at baseline was 41 years, the mean duration of exposure was 13.2 years and 59.1% of this study group were active smokers. A total of 52 cases of chronic bronchitis and 69 cases of COPD were counted among the workers. A total of 17 of the 52 cases with chronic bronchitis were additionally classified as obstructive chronic bronchitis. Cases with chronic bronchitis were identified by questionnaire and COPD cases by spirometry criteria only. Furthermore, there were 79 workers with an obstructive respiratory pattern, 16 workers with a restrictive respiratory pattern, and 367 workers with a normal respiratory pattern.

Exposure Assessment of Respirable SAS Dust

The correlation coefficients between the cumulative respirable SAS and inhalable SAS for the median-medium exposure scenario were examined, respectively, on a linear and logarithmic scale.

Strong correlation, ranging from 0.97 to 0.99 for all plants except for plant 2 (0.28) is indicated, with an overall correlation coefficient of 0.87 ($p < 0.001$). To control for the impact of the outlier measurements, the correlations were examined on the logarithmic scale as well. The correlation coefficients are generally improved, for plant 2 (0.60) particularly. An overall correlation coefficient of 0.92 ($p < 0.001$) was yielded.

Average respirable SAS dust concentrations (median-medium scenario) by calendar time with 0.95-confidence limits for all plants and workers are shown in **Figure 1**. Based on the median-medium scenario the mean SAS concentrations in mg/m^3 were extrapolated from 1956 (the first working year in the study population) onwards. Calendar time points with a respirable SAS dust concentration below 0 mg/m^3 were excluded. Until 1966, the mean SAS dust concentration reached its peak value of above 2.5 mg/m^3 before continuously decreasing to below 0.1 mg/m^3 in 2001. Even in the job category "bagging" with the highest levels of exposure the median dust concentration was below 0.6 mg/m^3 at each of the five plants.

Table 2 summarizes the statistics of the cumulative respirable SAS dust concentrations in mg/m^3-year for the 15 exposure scenarios of all 462 SAS-exposed male workers. Cumulative SAS dust concentrations varied considerably between the 15 exposure scenarios with a mean of 3.45 mg/m^3-year up to 21.97 mg/m^3-year. The exposure scenario used in the regression models in this study (median-medium scenario) had an average respirable SAS dust concentration of 6.44 mg/m^3-year and ranged from 0.2 to 62.7 mg/m^3-year.

Association Between Cumulative Respirable SAS Dust Exposure and Lung Function Parameters

Valid lung function measurements according to the quality criteria of the American Thoracic Society (19) were collected for FVC, FEV_1, and FEV_1/FVC in 462 SAS dust exposed male workers and in 456 SAS dust exposed male workers for MEF50.

External Comparisons

Figure 2 displays the results of external comparisons, based on the predictive values of the respective lung function parameters (FVC, FEV_1, FEV_1/FVC, MEF50) in relation to the reference values of the European Respiratory Society. Given statistics are mean, median, minimum, maximum, and the percentiles

TABLE 2 | Distribution of cumulative respirable SAS dust exposures in mg/m^3-year among the 15 exposure scenarios for 462 SAS-exposed male workers.

Scenario (*N* = 462)	Min	P5	P25	Mean	SD	P50	P75	P95	Max
1 Mean-High	0.1242767	0.8621879	2.11227	18.38822	45.02148	5.086327	13.531	71.5805	333.1182
2 Mean-Medium	0.1242767	0.8621879	2.099591	10.63807	16.15442	4.731598	11.61749	38.02087	105.2348
3 Mean-Low	0.0961178	0.8621879	2.099591	8.291332	9.819764	4.453384	10.38533	31.91607	68.39028
4 P75-High	0.1547988	1.0875	2.795124	21.96905	59.1461	5.754957	14.34165	89.49892	439.2065
5 P75-Medium	0.1547988	1.0875	2.718446	11.92538	20.06919	5.591762	11.5534	39.8041	138.5428
6 P75-Low	0.1093381	1.0875	2.713178	8.938291	10.75261	5.536644	10.23994	29.48778	83.22604
7 Median-High	0.1497006	0.536193	1.373239	10.45706	21.562	3.573217	9.571267	38.40878	152.3228
8 Median-Medium	**0.1497006**	**0.536193**	**1.340482**	**6.436606**	**8.339037**	**3.470625**	**7.77128**	**23.10788**	**62.66677**
9 Median-Low	0.0948276	0.536193	1.340482	5.133449	5.497449	3.312052	6.694469	16.27296	55.3203
10 P75-High	0.0923077	0.3477631	0.817757	7.280382	16.41187	2.354699	6.293403	30.15792	116.9589
11 P75-Medium	0.0923077	0.3477631	0.798005	4.35689	6.141231	2.246712	4.830932	16.65632	39.48246
12 P75-Low	0.0923077	0.3333333	0.798005	3.450836	3.959503	2.044682	4.068177	12.34656	34.85706
13 Geomean-High	0.1205337	0.6104928	1.54758	12.24693	28.64355	3.756728	9.841978	45.68624	209.8146
14 Geomean-Medium	0.1205337	0.6104928	1.526232	7.146067	10.21232	3.672569	8.125386	23.24833	66.39297
15 Geomean-Low	0.0931322	0.5966656	1.479748	5.568611	6.087117	3.501494	6.899977	18.37606	49.44646

N, number of subjects; min, minimum; p5, 5th percentile; p25, 25th percentile; mean, arithmetic mean; sd, standard deviation; p50, median; p75, 75th percentile; p95, 95th percentile; max, maximum.

The exposure estimates based on median-medium scenario (in bold) are used for the regression analyses.

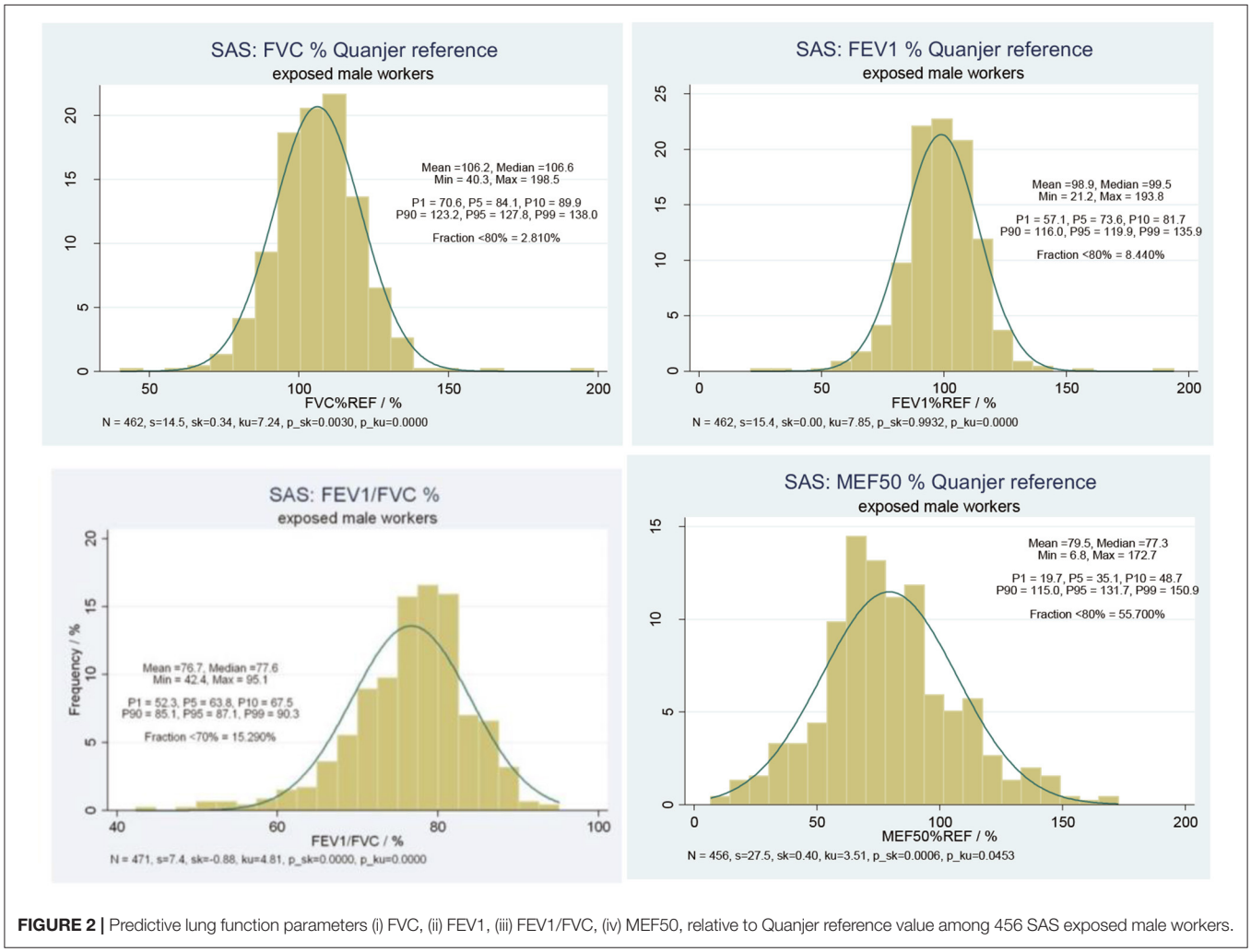

FIGURE 2 | Predictive lung function parameters (i) FVC, (ii) FEV1, (iii) FEV1/FVC, (iv) MEF50, relative to Quanjer reference value among 456 SAS exposed male workers.

TABLE 3 | Multivariable linear regression of FVC and cumulative respirable SAS dust exposure among 462 SAS-exposed male workers by median-medium scenario.

Response FVC (ml)	Linear regression				
	Obs = 462; median-medium scenario*				
	Estimate	SE	Robust 95% CI		*P*-value
Intercept	−4,812.16	1,013.29	−6,803.59	−2,820.74	0.000
Cumulative exposure (1 mg/m^3-year)	−8.11	3.88	−15.73	−0.49	0.037
Plant 2 vs. Plant 1	−96.28	198.76	−486.91	294.35	0.628
Plant 3 vs. Plant 1	−308.25	71.69	−449.15	−167.36	0.000
Plant 4 vs. Plant 1	−294.69	113.57	−517.90	−71.48	0.010
Plant 5 vs. Plant 1	179.52	81.81	18.73	340.31	0.029
Age (years)	−28.10	3.91	−35.79	−20.42	0.000
Height (cm)	64.77	5.18	54.59	74.95	0.000
Body mass index (kg/m²)	−8.81	6.84	−22.26	4.64	0.199
Former smoker vs. non-smoker	214.42	97.30	23.20	405.64	0.028
Current smoker vs. non-smoker	−8.29	92.01	−189.12	172.53	0.928
Pack-Years of smoking	−2.96	2.87	−8.61	2.68	0.302
Atopy assessed by prick test (yes/no)	−24.35	73.40	−168.61	119.91	0.740
Atopy assessed by spec. IgE (yes/no)	56.33	72.18	−85.53	198.20	0.436
Antiobstructive medication (yes/no)	−242.18	253.75	−740.88	256.53	0.340
Prior exposure to fibrogenic dust (yes/no)	−92.10	85.66	−260.44	76.25	0.283
Prior exposure to substances causing obstruction (yes/no)	75.98	72.65	−66.79	218.75	0.296

95% CI, 95% confidence interval.
$^*R^2 = 0.496$.

of interest. Additionally, standard deviation (s), skewness (sk), kurtosis (ku), and the *p*-values (p_sk, p_ku) of the d'Agostino test are reported. Furthermore, the fraction of observations below 80% of reference for FVC, FEV_1, and MEF50 and the fraction of ratios below 70% (16) for the FEV_1/FVC ratio are shown.

The predictive value of FVC yielded a mean value of ∼106% and a median value of about 107%. Only 2.8% of the study population were found with FVC below 80% of the reference value. In parallel, the predictive value of FEV_1 yielded a mean value of about 99% and a median value ∼100%. About 8.4% of the study subjects were found with FEV_1 below 80% of reference values. On average, a reduction of about 23% in comparison to the reference value was found, 14.5% of all measurements were below 70% of reference. The predictive value of MEF50 yielded a mean value of 79.5% and a median value of 77.3%, which made about 55.7% of all study subjects with MEF50 below 80% of the reference value.

TABLE 4 | Overview of effect estimates of cumulative respirable SAS exposure (1 mg/m^3-year) from respective multivariable linear regression of lung function parameters FVC, FEV_1, FEV_1/FVC, and MEF50, among 462 SAS-exposed male workers by median-medium scenario.

Response	Linear Regression				
	Estimate*	SE	Robust 95% CI		*P*-value
FEV_1 (mL)	−5.21	3.43	−11.95	1.52	0.129
FVC (mL)	−8.11	3.88	−15.73	−0.49	0.037
FEV_1 /FVC (%)	0.03	0.05	−0.06	0.12	0.526
MEF50 (mL)	−3.45	7.69	−18.56	11.67	0.654

95% CI, 95% confidence interval.
**Effect estimates are adjusted for plants (plant 1 as reference), age (years), height (cm), body mass index (kg/m²), former smoker vs. non-smoker, current smoker vs. non-smoker, pack-years of smoking, atopy assessed by prick test (yes/no), atopy assessed by spec. IgE (yes/no), antiobstructive medication (yes/no), prior exposure to fibrogenic dust (yes/no), prior exposure to substances causing obstruction (yes/no).*

Internal Comparisons

For internal comparisons, regression models provide the risk estimate per unit increase of cumulative exposure to respirable SAS, after adjustment for potential confounding factors. The effect estimates with respect to FVC are presented in **Table 3**. Cumulative exposure to respirable SAS dust (1 mg/m^3-year) was negatively associated with FVC values with a reduction of 8.11 ml ($p < 0.05$). Apparent heterogeneity across the plants was observed. Compared to plant 1, Plant 3, and Plant 4 presented significantly reduced FVC values ($p < 0.01$). Another plant effect was found in Plant 5, which was positively associated with FVC values ($p < 0.05$). Antiobstructive medication showed a pronounced effect on FVC values in this model, though not reaching statistical significance in this model.

Including the same set of covariates, the risks of cumulative exposure to respirable SAS were estimated with respect to FEV1, FEV1/FVC, and MEF50 as well. The risk estimates from the multivariable linear regression of FEV_1, FVC, FEV_1/FVC, and MEF50 are summarized in **Table 4**.

With respect to FEV_1 (effect estimates in ml), the estimated coefficient of cumulative exposure to respirable SAS was not

TABLE 5 | Multivariable logistic regression of WHO chronic bronchitis and cumulative respirable SAS dust exposure among 462 SAS-exposed male workers by median-medium scenario.

Characteristic	Obs = 462; median-medium scenario				
	OR	SE	Robust 95% CI		*P*-value
Cumulative exposure (mg/m^3-year)					
≤2	1.00				
>2–≤6	2.02	0.83	0.90	4.54	0.090
>6	1.03	0.55	0.36	2.93	0.957
Plants					
1	1.00				
2	0.10	0.14	0.01	1.44	0.091
3	0.64	0.27	0.28	1.46	0.289
4	0.64	0.37	0.20	2.00	0.440
5	1.22	0.52	0.52	2.82	0.648
Age (years)	1.03	0.03	0.98	1.07	0.299
Height (cm)	0.97	0.01	0.95	0.99	0.000
Body mass index (kg/m²)	1.04	0.04	0.97	1.12	0.225
Smoking					
Non-smoker	1.00				
Former smoker	0.74	0.52	0.19	2.92	0.664
Current smoker	2.52	1.50	0.79	8.06	0.119
Pack-Years of smoking	1.03	0.01	1.00	1.05	0.064
Atopy assessed by prick test					
No	1.00				
Yes	0.89	0.32	0.44	1.81	0.746
Atopy assessed by spec. IgE					
No	1.00				
Yes	0.80	0.30	0.38	1.68	0.548
Antiobstructive medication					
No	1.00				
Yes	7.40	4.68	2.14	25.56	0.002
Prior exposure to fibrogenic dust					
No	1.00				
Yes	1.55	0.76	0.60	4.03	0.369
Prior exposure to substances causing obstruction					
No	1.00				
Yes	1.16	0.46	0.53	2.54	0.703

95% CI, 95% confidence interval; OR, odds ratio.

significant ($p = 0.129$). Significant effects were shown regarding the different plants, age, height, smoking, and anti-obstructive medication. Pronounced heterogeneity was observed across the plants. On average, FEV_1 values decreased by 30.55 ml per year of age ($p < 0.01$) and increased by 44.18 ml per cm increase of body height ($p < 0.01$). Smoking status (former smoker, current smoker vs. non-smoker) and pack years were included in the model at the meantime. Higher pack-years were associated with a decrease in FEV_1 values by 8.53 ml for each increment of pack-year ($p < 0.01$), while current smoking status was not significant. Former smokers showed a positive association with increased FEV_1 ($p < 0.05$). A very strong association was indicated from anti-obstructive medication, which was negatively associated with FEV_1 values (−468.5 ml, $p < 0.05$). BMI, atopy, or prior exposure did not show a statistically significant impact.

In contrast to the models of FVC, no significant effect on the FEV_1/FVC ratio was observed. Significant effects were found for plants, age, pack-year of smoking, anti-obstructive medication ($p < 0.01$), and height ($p < 0.05$).

With respect to MEF50, neither the cumulative exposure to respirable SAS dust nor the different plants yielded significant estimates, while age, more pack-years of smoking, and anti-obstructive medication were negatively associated with MEF50 ($p < 0.01$).

TABLE 6 | Multivariable logistic regression of chronic obstructive pulmonary disease (COPD) and cumulative respirable SAS dust exposure among 462 SAS-exposed male workers by median-medium scenario.

Characteristic	Obs = 462; median-medium scenario				
	OR	SE	Robust 95% CI		*P*-value
Cumulative exposure (mg/m^3-year)					
≤2	1.00				
>2–≤6	2.45	0.94	1.16	5.20	0.019
>6	1.42	0.68	0.56	3.62	0.462
Plants					
1	1.00				
2	0.06	0.59	0.01	0.40	0.003
3	0.61	0.23	0.29	1.28	0.189
4	1.06	0.57	0.37	3.03	0.907
5	0.89	0.38	0.38	2.07	0.792
Age (years)	1.02	0.02	0.98	1.06	0.422
Height (cm)	0.98	0.01	0.97	0.99	0.003
Body mass index (kg/m²)	1.00	0.04	0.93	1.08	0.980
Smoking					
Non-smoker	1.00				
Former smoker	0.99	0.51	0.36	2.72	0.982
Current smoker	0.75	0.44	0.24	2.35	0.625
Pack-Years of smoking	1.05	0.02	1.02	1.09	0.002
Atopy assessed by prick test					
No	1.00				
Yes	0.92	0.31	0.48	1.78	0.810
Atopy assessed by spec. IgE					
No	1.00				
Yes	0.87	0.31	0.43	1.74	0.689
Antiobstructive medication					
No	1.00				
Yes	4.04	2.48	1.21	13.43	0.023
Prior exposure to fibrogenic dust					
No	1.00				
Yes	0.91	0.46	0.34	2.45	0.852
Prior exposure to substances causing obstruction					
No	1.00				
Yes	0.85	0.31	0.42	1.72	0.654

95% CI, 95% confidence interval; OR, odds ratio.

Association Between Cumulative Respirable SAS Dust Exposure and Respiratory Pattern, Chronic Bronchitis, and Chronic Obstructive Pulmonary Disease

In **Tables 5**, **6**, the results of the multinomial logistic regression of obstructive ($FEV_1/FVC < LLN$) and restrictive patterns ($FEV_1/FVC > LLN$ and $FVC < LLN$) compared with normal spirometry ($FEV_1/FVC > LLN$ and $FVC \geq LLN$) according to the Global Lung Initiative (GLI) 2012 are shown (24). Cumulative exposure to respirable SAS dust (1 mg/m^3-year) for the median-medium scenario did not reach significance for the obstructive pattern, but for the restrictive pattern (OR = 1.07; 95% CI = 1.01–1.12; $p < 0.05$). While for the restrictive pattern no significant odds ratio was found for the other covariates, pack years of smoking (OR = 1.03; 95% CI = 1.00–1.06; $p < 0.05$) and anti-obstructive medication (OR = 8.17; 95% CI = 2.12–31.53; $p < 0.01$) were significantly associated with the obstructive pattern.

The multivariable logistic regression of chronic bronchitis according to the WHO criteria and cumulative respirable SAS dust exposure based on the leading exposure estimate (median-medium scenario) among the 462 SAS-exposed male workers is shown in **Table 5**. Three categories were classified according to the cumulative exposure: ≤2 mg/m^3-year as the reference category, >2–≤6, and >6 mg/m^3-year. Neither the cumulative exposure categories (mg/m^3-year) nor the different

TABLE 7 | MC Regression models on the respiratory effects of cumulative exposure among SAS exposed male workers.

		Precision weighted wean effects given 10,000 MC simulations		
		Linear regression		
Cumulative exposure) (1 mg/m^3-year)	**Estimate**	**Robust 95% CI**		
FEV_1/ml; $N = 462$	−5.21	−11.93	–	1.50
FVC/ml; $N = 462$	−8.11	−15.71	–	−0.51
FEV_1/FVC/%; $N = 462$	0.03	−0.06	–	0.12
MEF50/ml; $N = 456$	−3.45	−18.52	–	11.63
		Logistic regression		
Cumulative exposure) (1 mg/m^3-year)	**OR**	**Robust 95% CI**		
WHO Chronic bronchitis (no/yes); $N = 462$				
52 cases (11.26%)	1.00	0.96	–	1.04
GOLD spirometric staging for COPD (stage I, II+)				
Stage 1; 45 cases (9.7%):	0.98	0.94	–	1.02
Stage II+; 24 cases (5.2%):	1.01	0.95	–	1.08

TABLE 8 | Effect of age on lung function parameters according to reference values of the European respiratory society (20) and estimated in this study.

	Age coefficients of lung function regression analyses in linear regression		
	Quanjer et al. (20) males	**Exposed males 25+ y $N = 440$**	**Exposed males $N = 462$**
FVC [ml]	−26.01	−28.73	−26.24
FEV_1 [ml]	−29.03	−30.44	−28.75
FEV_1/FVC [%]	−0.13	−0.17	−0.17
MEF50 [ml]	−31.00	−49.73	−45.89

plants showed a significant effect on the development of chronic bronchitis. No exposure-response relationship was observed.

Table 6 presents the multivariable logistic regression of COPDand cumulative respirable SAS dust exposure. Compared to the lowest exposure category (≤2 mg/m^3-year), cumulative exposure to respirable SAS of 2–6 mg/m^3-year seemed to increase the risk of developing COPD significantly (OR = 2.45; 95% CI = 1.16–5.20), while the highest exposure category (>6 mg/m^3-year) was not associated with a significantly increased risk (OR = 1.42; 95% CI = 0.56–3.62). No exposure-response relationship could be concluded.

Table 7 summarizes the results of the Monte Carlo regression models on lung function parameters (FEV_1, FVC, FEV_1/FVC, MEF50), and the prevalence of respiratory diseases (chronic bronchitis, COPD based on spirometric staging only). The risk estimates of cumulative respirable SAS dust exposure on the lung function parameters were not statistically significant for FEV_1, FEv1/FVC, and MEF50, while −8.11 (95% CI: −15.71–−0.51) ml decline of FVC was implied. No increased risk of chronic bronchitis and COPD was indicated.

Effect of Age on Lung Function Parameters

Table 8 shows the effect of age on lung function parameters according to the reference values of the European Respiratory Society published in the study by Quanjer et al. (20) and additionally the estimated effect in this study for the whole study group and restricted to workers aged over 25 years. In addition to the set of covariates used in the former regression models, an interaction between the different plants and the cumulative exposure was included. The effect estimate of age on FEV_1 and FVC was comparable in the three groups, while it was greater on MEF50 in the whole study population (−45.89 ml) and those aged above 25 years (−49.73 ml) compared to the males (−31.00 ml) in the study by Quanjer et al. (20). Furthermore, age had a marginally higher impact on the FEV_1/FVC ratio in the two groups of the present study (−0.17%) than in the Quanjer reference group (−0.13%). Comparing the two groups of the present study, exposed workers aged over 25 seemed to suffer more loss of lung function annually than the whole study population.

DISCUSSION

The present extended analyses aimed to investigate the effect of cumulative exposure to respirable SAS dust on non-malignant respiratory morbidity. The endpoints are (i) lung function parameters (FEV_1, FVC, FEV_1/FVC-ratio, and MEF50); (ii) respiratory pattern (restrictive and obstructive pattern); (iii) respiratory diseases (chronic bronchitis and COPD in 462 exposed male workers of five German plants producing synthetic amorphous silica (SAS). Individual cumulative SAS exposure was calculated by backward extrapolation using 15 different JEMs adapted for uncertainties in historical exposure level estimates. These JEMs were based on anchoring individual working histories; information on changes in production, ventilation, housekeeping, and similar exposure groups (SEGs), and other factors in each plant with the actual SAS measurements in the period of 1997 to 2000.

To our knowledge, apart from the study of Taeger et al. (6) upon which the population of this study was based, only two human studies on occupational SAS exposure and pulmonary function have been performed (25, 26). The cross-sectional study by Wilson et al. consisted of 165 amorphous silica workers. Results showed that pulmonary function and radiographic changes were not significantly associated with

neither duration nor total cumulative SAS exposure. In the study of Choudat et al. (25), 41 workers exposed to amorphous silica were compared with an age-matched control group of 90 non-exposed workers at the same plant. Significant differences between exposed and non-exposed workers were found for lung function parameters of forced expiratory forces. No exposure-response relationship was suggested. Both studies suffered from weak exposure assessments, no distinction between inhalable and respirable dust, and no proper adjustments for covariates. The third study reported the adverse health outcome of exposure to inhalable SAS dust (6). To overcome the uncertainties in exposure assessment, tremendous effort was given to check the robustness of exposure estimates, please refer to Morfeld et al. (5). A reduction in FVC, but no effect on FEV_1 and FEV_1/FVC was observed in association with exposure to an inhalable fraction of SAS.

The present study reported the evaluation of the health effects of exposure to a respirable fraction of SAS, based on the same study population of Taeger et al. (6). In analog, the leading median-medium scenario among 15 JEMs has been used to estimate cumulative SAS exposure for the present study. Results from the multivariable linear regression models did not indicate any adverse effect on FEV_1 and FEV_1/FVC, but a reduction in FVC as a result of exposure to respirable SAS (cf. **Table 3**).

An estimated loss in FVC of −48 ml resulting from a cumulative exposure of 6.44 mg/m^3-years needs a discussion. We compared this amount of loss with the loss expected due to aging and smoking. **Table 7** shows the age-dependency of lung function parameters according to the reference equations published in Quanjer et al. (20) and it presents the age coefficients estimated in this study for the whole study group and restricted to workers older than 25 years. We used the Quanjer equations to calculate the expected loss in FVC over 40 years: 26 ml/year *40 years = 1,040 ml. Thus, the estimated relative additional loss in FVC due to an exposure to respirable over 40 years was 48/1,040, i.e., <5% (had we used the modeling results, this fraction was slightly smaller). Smokers were found to show a decline in FEV1 of about 60 ml/year (27, 28), equivalent to an additional loss of about 33 ml/year which amounts to an overall additional loss of 1,200 ml over 40 years. Thus, the relative additional loss due to smoking (1,320/1,040) is larger than 100%. Note that a smoker's additional decline in FVC is similar to the smoker's additional decline in FEV1 (29). Thus, we conclude that the additional lung function loss in FVC due to SAS dust exposure as estimated from the MC regression models appears to be negligible when compared to the regular loss due to aging and in particular when compared to the effect of smoking on lung function.

The major weakness of this cross-sectional study is a potential underestimation of exposure and effects since diseased workers might terminate the exposure and not be included in this study population. This so-called "Healthy Worker Survivor Effect" noted as a weakness of Taeger et al. (6) is difficult for the researchers to trace back when the diseased workers have left and the causes for the termination (6). The extensive exposure assessment is the key strength of the present study, using multiple exposure scenarios approach with Job Exposure Matrices as in the present study yields a robust estimate of historical exposure levels. Especially, when assessing historical exposures in different plants and for different job categories, it is important to use an approach that deals with uncertainties in exposure measurements and exposure level changes in the past.

In general, the present cross-sectional study reports that historic workplace exposures to respirable SAS were not associated with respiratory morbidity. However, the effect of the respirable fraction seemed to be more relevant than that of the inhalable fraction, given the identical level of exposure. Since a cross-sectional study has its inherent limitation for causal inference because of the ambiguous temporal order of cause and outcome, a prospective follow-up study with extensive exposure assessment of nanoparticles would provide valid evidence for risk assessment.

DATA AVAILABILITY STATEMENT

The datasets presented in this article are not readily available because data protection directives.

ETHICS STATEMENT

The studies involving human participants were reviewed and approved by the German Workers Council. The patients/participants provided their written informed consent to participate in this study.

AUTHOR CONTRIBUTIONS

PM, MY, and RM: study design, study conduction, and drafting and reviewing of the manuscript. All authors contributed to the article and approved the submitted version.

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Causal Inference Analysis for Poorly Soluble Low Toxicity Particles, Lung Function, and Malignancy

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Poorly soluble low toxicity particles such as carbon black and titanium dioxide have raised concern about possible nonmalignant and malignant pulmonary effects. This paper illustrates application of causal inference analysis to assessing these effects. A framework for analysis is created using directed acyclic graphs to define pathways from exposure to potential lung cancer or chronic airflow obstruction outcomes. Directed acyclic graphs define influences of confounders, backdoor pathways, and analytic models. Potential mechanistic pathways such as intermediate pulmonary inflammation are illustrated. An overview of available data for each of the inter-node links is presented. Individual empirical epidemiologic studies have limited ability to confirm mechanisms of potential causal relationships due to the complexity of causal pathways and the extended time course over which disease may develop. Therefore, an explicit conceptual and graphical framework to facilitate synthesizing data from several studies to consider pulmonary inflammation as a common pathway for both chronic airflow obstruction and lung cancer is suggested. These methods are useful to clarify potential bona fide and artifactual observed relationships. They also delineate variables which should be included in analytic models for single study data and biologically relevant variables unlikely to be available from a single study.

Keywords: causal inference analysis, directed acyclic graph, carbon black, chronic obstructive pulmonary disease (COPD), lung cancer, particulate toxicity, causation analysis, pulmonary inflammation

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INTRODUCTION

Poorly soluble low toxicity particles (PSLTs) have received increased interest over the past few years. Concerns about possible malignant or nonmalignant pulmonary disease effects are being addressed. However, available epidemiologic data leave considerable uncertainty about the presence and magnitude of significant effects in humans. In general, studies of chronic pulmonary disorders (e.g., chronic airflow obstruction, CAO) and malignancies such as lung cancer (CA) are particularly challenging because of long latencies and major changes in typical workplace exposures over time. Although more mechanistic data are suggested by animal and *in vitro* studies, the applicability to human health is constrained by species differences and differences in toxokinetics.

Causal Analysis

Causal inference analysis (CIA) provides an explicit framework for identifying and representing interacting factors of different types in complex multi-step causal pathways (1). It is particularly useful for delineating variable sets to be considered as covariates, defining exposure variables more

clearly, and identifying potential biases leading to misleading conclusions. These methods also allow explicit consideration of mediators between exposure and disease outcomes.

Causal considerations have long been implicit in science; research increasingly seeks to provide explanations rather than simple descriptions. A causal relationship between A and B (A→ B) may conceptualized as either deterministic or probabilistic. In a deterministic relationship, A is sufficient to cause B. It is probabilistic if an intervention setting A to a fixed value changes the probabilistic distribution of B. Current assessments of the likelihood that PSLT's produce CA and/or CAO follow the latter inferential probabilistic paradigm.

Significant conceptual advances concerning causation occurred in the early 20th and early 21st. In the early 1900's, Popper conceptualized that an explanation may be considered scientific if it is possible to refute it by empirical observations. Similarly, Fisher, Pearson, and others helped create the basis for modern statistical hypothesis testing and statistical inference techniques.

Observational studies such as those assessing exposures and long-term health in humans must consider multiple factors. Such factors may be empirically measurable for inclusion as variables in statistical calculation models or be unmeasured but of potential interest. Such factors may increase or decrease the apparent relationship between the predictors and outcomes of interest; they may also prevent identification of bona fide causal relationships or lead to the appearance of artifactual relationships. Confounders in regression models are a such empirically measurable factors worthy of consideration. Even unmeasurable factors should be considered for interpreting the significance of calculated models. Occasionally, a measurable instrumental variable may be used to infer an important unmeasured factor.

Focus upon causation evaluation methodologies reignited in the early 21st century to deal with this potential complexity. Causal inference analysis helps identify the set of variables, whether measurable or unmeasured, that should be considered. Directed acyclic graphs (DAGs) are increasingly used to both facilitate ascertaining estimands and to force logical thinking and explicit representation of how they might relate. This paper illustrates how such DAGS may be applied to understanding several potential PSLTs effects.

Using pulmonary inflammatory response as an example, the paper then illustrates how DAGs may be applied to consideration of underlying biologic mechanisms in a single study. It then extends these principles to suggest how approaches analogous to DAGs may facilitate explicitly expressing potential underlying mechanisms even if information comes from separate studies. While mechanistic reasoning is already extensively used, the graphic approach facilitates explicit and logical representation.

Directed Acyclic Graphs (DAGs)

DAGs are based upon underlying quantitative probabilistic models as described by Greenland et al. in 1999 (2). These methods may be applied for both facilitating specific calculations and for forcing explicit consideration of potential relationships (1).

A directed acyclic graph is composed of a series of nodes and links. The graph is built by including the set of all variables that affect the relationship between the predictor and outcome. Thus, for a complex topic such as PSLT effects, the graph may become complex. In this paper, the graphs are built sequentially beginning with very simple relationships and then adding more considerations. By including a full set of variables, the formal graph approach helps determine those variables that would not affect the primary relationship of interest. The DAG may include a series of alternate pathways.

In a DAG, causal relationships must be both directed and unidirectional. The direction of causation is indicated by a single arrow. That is, A→ B and B→ A cannot both be included. Causation generally is probabilistic rather than deterministic. In addition to causal links (represented by arrows), associations not implying causation may also be shown (e.g., by dashed lines without arrows).

The DAG is acyclic, meaning that even with a complex series of nodes and links, a variable may not have a directed path leading back to itself. Thus, A→ B and A→ C→ D→ B may be present but A→ C→ B→ D→ A is not acceptable.

Paper Overview

This paper applies these methods to examine the specific questions: Do PSLTs such as carbon black (CB) cause chronic airflow obstruction (CAO) or lung cancer (CA), and if so, is this mediated by inflammation? The paper is illustrative and does not presuppose that such relationships exist. The approach is described in three sections: Frameworks (Section Framework for DAG Analysis) describes use of directed acyclic graphs (DAGs) to frame hypotheses and identify variables appropriate for analysis. DAGs consist of a series of nodes and links representing segments of potential causal pathways between the exposure and the possible outcomes. They also represent links to variables, both measured and unmeasured, that should be considered to avoid misleading results. Data for some variables identified as relevant may be challenging to acquire in a single study. Available data (Section Available Data) provides examples of data available for the many of the individual causal pathway link segments, although they may not be directly combined in a specific quantitative model. Implications (Section Implications of the Causal Mechanistic Analysis) suggests approaches for understanding disease development mechanisms by considering information from multiple studies. Potential future studies for CAO and CA are suggested. The approach emphasizes how existing data may be applied rather than providing a comprehensive summary of all relevant research data.

These considerations may contribute to understanding of PSLT effects in several ways: 1) Graphical methods such as DAGs make assumptions about causal pathways explicit. 2) It encourages specifically stating the set of variables deemed necessary and sufficient for understanding causal associations. 3) Limitations of data likely to be available from individual studies are described. 4) Examples illustrate how appropriate or inappropriate variable selection or adjustments may impact accuracy of measures of association. 5) Specific suggestions for qualitative inferences from distinct studies are shown.

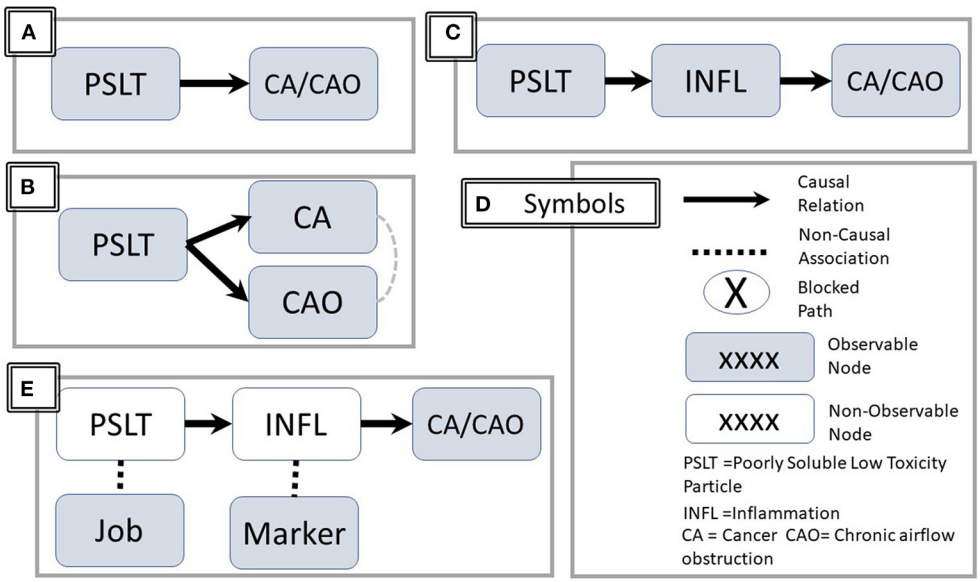

FIGURE 1 | Directed Acyclic Graph (DAG) representations. The figure illustrates three causal sequences: **(A)** PSLT causes CA and CAO (solid line with arrowhead). **(B)** Clarification–the causation of CA and CAO are independent. However, a noncausal association may be observed between CA and CAO (dashed line without arrow). **(C)** PSLT acts through the mediator of inflammation. **(D)** Summary of representational symbols. **(E)** PSLT and INFL are indirectly assessed by associated job and biomarkers respectively. Unfilled nodes are not directly observable. PSLT, poorly soluble low toxicity particles; CA, cancer; CAO, chronic airflow obstruction; INFL, inflammation.

FRAMEWORK FOR DAG ANALYSIS

This section describes applications of DAGs for explicitly representing a framework for causal relationships and lays the foundation for assessing the role of empirical data in Section Available Data in specifying the relevant DAG. This section also illustrates methods to reduce the likelihood of misleading results. It also suggests potential intermediate steps along the path from exposure to disease.

Fundamental Hypothesis

Potential pathways from PSLT exposure to final disease outcomes (CAO and CA) are represented as nodes and links. For simplicity, the nodes (PSLT, CAO, and CA) are shown as binary elements (yes, no). However, these methods may be extended to graded effects (e.g., CAO might be represented with a quantified physiologic airflow obstruction), or the strength of a causal link may be quantified. The figures include a series of DAGs showing progressively richer causal networks. Relationships between nodes are represented by a line with an arrow if the relationship is causal, by dashed lines without an arrow if the association is not causal, and by lighter dashed lines if it may be artifactual.

The traditional epidemiologic mortality study hypothesis asking whether PSLT predicts development of CA and CAO is shown in **Figure 1A**. This illustrates a direct causal or "front door path". This is further clarified in **Figure 1B**, suggesting that PSLT exposure predicts CA and CAO, but they occur independently. In this circumstance, an association of CAO and CA would be observed even if there is no actual causal pathway between these outcomes. In addition, the apparent strength of association between PSLT and CA (e.g., odds ratio) may be attenuated by adjustment for the presence of CAO.

The mechanistic hypothesis that a causal relationship between PSLT and CAO or CA is mediated via pulmonary inflammation is shown in **Figure 1C**. For simplicity, the two health outcomes are combined in a single node. **Figure 1D** summarizes the common symbols.

However, there are several ambiguities in the apparently simple representation of the hypothesis.

- Even if inflammation is a critical intermediary step, inflammation *per se* may not be the final effector for producing airway damage or malignant cellular transformation (i.e., there may be additional intermediary steps).
- The number of potentially observable measures of inflammation, particularly in humans, is limited and may not truly reflect the *in vivo* inflammatory process.
- Inflammation is not a specific entity; rather, there are numerous inflammatory pathways, many of which are interactive and/or cross-regulatory. These are often assessed indirectly by measuring associated biomarkers (**Figure 1E**).
- "PSLT" is not a specific entity, and its definition is ambiguous. While many would agree that carbon black and titanium dioxide fall within this group, inclusion of other materials such as coal dust is less certain.
- Even for a specific agent, potential effects and pathways may depend upon the characteristics of size, particle size, dose, dose rate, charge, and surface properties. These were recently reviewed by Borm and Driscol (3).
- The exposure term (e.g., PSLT) may either refer to the specific agent or to a surrogate of exposure such as job title. Laboratory

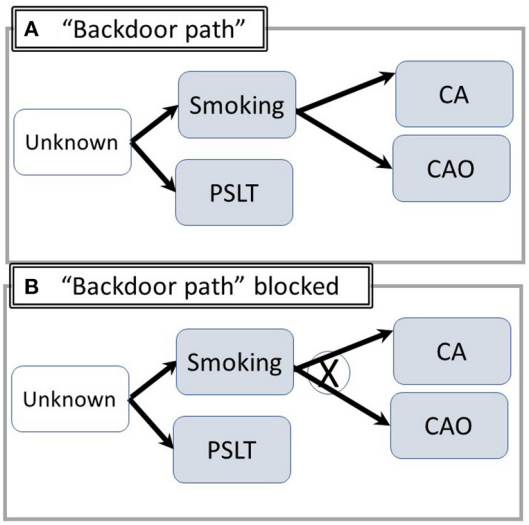

FIGURE 2 | Utilizing DAG's to illustrate "Backdoor Paths" /confounding. **(A)** ("Backdoor Path"): Common unknown/unspecified factors increase the likelihood of both smoking and exposure. An artifactual apparent association between PSLT and CA/CAO may appear. X = pathway blocked by proper adjustment. **(B)** Adjustment for smoking blocks the pathway including PSLT-Unknown-CA (Abbreviations are summarized in **Figure 1**).

studies may use a chemically defined agent with specified dose or even assessed initial or retained dose. Neither exposure nor dose are typically precisely measured or controlled in human studies. Rather, these are assessed with proxy measures such as job title, job duration, etc. as approximate surrogate measures of actual dose (**Figure 1E**). The correlation between the surrogate and the actual dose is likely to be heterogeneous over time and among study sites.

- Time course: Inflammatory events early in the time course may initiate events ultimately leading to CAO/CA, but the mechanistic or exposure events later in the pathway may be different. Implications are discussed below.

Explicit consideration of pathways involving both observable and significant but unknown (non-recorded) factors is essential for analysis and interpretation. Backdoor pathways, representing artifactual associations such as the well-known confounder of tobacco smoking are illustrated in **Figure 2A**. For example, smoking may be associated with exposure if common but unobserved socioeconomic or cultural factors increase the likelihood of working in a heavily exposed job and of smoking. Members of socially disadvantaged groups may be disproportionately likely to have jobs with heavy exposure and come from homes in polluted areas or have grown up with parental smoking. Adjustment for smoking, which produces both CA and CAO (**Figure 2B**) prevents the appearance of an incorrect causal association between PSLT and the outcomes.

Conversely, adjustment for an intermediary step such as inflammation may reduce the likelihood of identifying a true relationship between PSLT and the outcomes. This potential overadjustment is illustrated in **Figure 3A**. Similar considerations apply if there are both a direct and an inflammation mediated pathway (**Figure 3B**) (The figure is simplified by assuming each of the nodes is directly observable). In contrast, adjustment for inflammation may create an artifactual association between PSLT and CA if both PSLT and smoking cause inflammation, but inflammation is not actually the mediator between PSLT and CA (see **Figure 3C**). In this "collider" situation, a false association between PSLT and CA would be observed if the PSLT-CA measure of association (e.g., odds ratio of CA on PSLT) is adjusted for inflammation (Adjustment for smoking would eliminate this effect, but it is preferable to simply not adjust for inflammation). Thus, assessment of which factors to use and when to adjust is facilitated by DAGs.

Constraints Upon Observable Data in Human Studies

In addition to the consequences of long latency and occasional ambiguity in defining "exposure", observational studies in humans require additional framework considerations. Such ambiguities may contribute to inconsistency among studies. These include distinguishing early vs. late steps in the causal chain and practical constraints upon measures of inflammation that may be collected noninvasively.

The importance of time is summarized in **Figure 4**. A study of whether PSLT predicts subsequent CAO or CA must cover a long time span. Even if the worker remains active in the industry until near the date at which diagnosis is made, it is necessary to reconstruct exposures many years in the past. This is particularly important since workplace air concentrations were often orders of magnitude greater in the past. In many instances, retrospective exposure assessment does not occur until near the date of diagnosis and is therefore subject to recall bias and/or missing data. Other workers may have left the industry long before recognition of CAO or CA, making cohort identification challenging.

Many studies utilize estimated cumulative exposure as the predictor of a possible health effect. However, the same cumulative exposure may be accrued with very different dose rates and latencies as shown in **Figure 4**. For example, some may have moderate exposure over many years, while others may have an early brief but very intense exposure. Misinterpretation of causal relationships may result from not considering the time varying nature of longitudinal exposures (4).

Both biologic and operational consequences of the time course are illustrated in **Figure 5**, in which time is divided into early, mid, and late eras. The latter begins when the fully advanced disease is present.

Biologic considerations about PSLT's warrant consideration of the assumption that cumulative exposure is the optimal predictor. This is particularly true for PSLT's for which clearance and deposition may have thresholds, overloading, or significant nonlinearities. For example, clearance processes may be overwhelmed with very high early exposures but not with more moderate exposures. Limited epidemiologic adjustment methods such as overweighting or underweighting exposure by calendar date or excluding either recent or very remote exposures

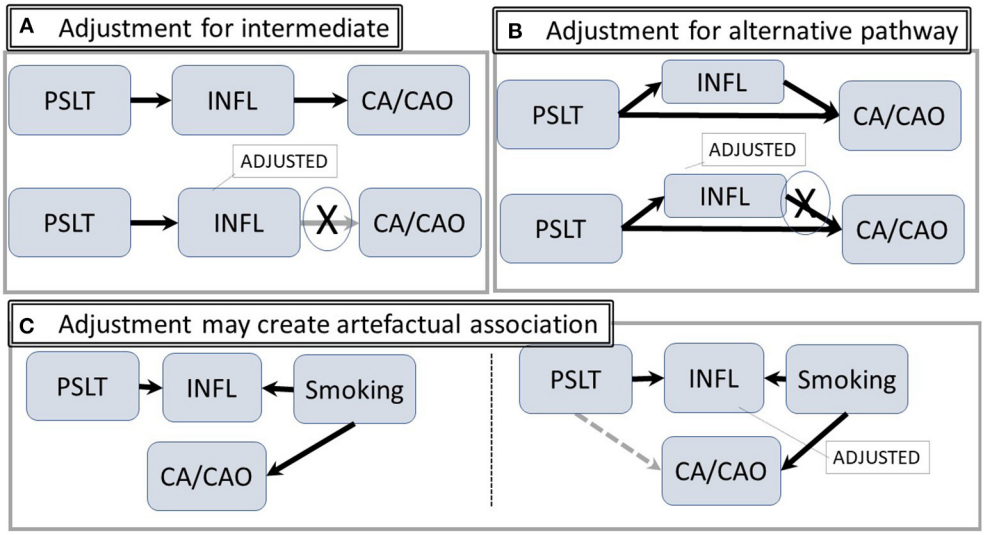

FIGURE 3 | Intermediate and alternative pathways. **(A)** The hypothetical pathway from PSLT to CA/CAO is mediated through inflammation. Statistical adjustment for inflammation may attenuate or block the pathway. **(B)** Inflammation is an alternative pathway from exposure to CA/CAO; adjustment for inflammation will potentially modify the apparent strength of the association. **(C)** There is no path from PSLT to CA/CAO. However, adjusting for inflammation may open an artifactual pathway from PSLT to CA/CAO (shown in gray) (Abbreviations are the same as in **Figure 1**).

FIGURE 4 | Exposure temporal profiles. The figure illustrates that the same cumulative dose may be received with different temporal patterns. **(A)** If exposure controls improve over time, long tenure workers have a relatively high exposure early in their career and then much less later. **(B)** Constant moderate exposure. **(C)** Extremely heavy exposure for short duration, then leaving industry with no subsequent exposure (Abbreviations are the same as in **Figure 1**). Changes in exposures over time are reflected by the height of bars within the PSLT node.

does not fully address biologic considerations such as short-term or long-term clearance, tissue repair, threshold doses, or other mechanistic considerations. Underestimation of remote exposures due to applying more recent data to earlier years (5) may lead to overestimation of the regression coefficient of CAO upon exposure.

The simple DAG of **Figure 1** is augmented for analytic clarity in **Figure 5**. This framework expresses several possible considerations. Examples of several human inflammatory markers are shown in **Table 1**. **Figure 5** represents that inflammation is better represented as a cascade of steps rather than as a single node, and there are multiple inflammatory pathways of which only some are likely to be relevant. Unlike animal and *in vitro* studies, the range of inflammatory biomarkers appropriate for human studies must be limited to noninvasive or minimally invasive procedures. Hence, the observable biomarker for each inflammatory step may be correlated with but not synonymous with the actual mediator (dashed line in the figure); the available biomarkers may therefore be indirect measures rather than direct assessment of the inflammatory steps. They are still useful, but they may only be loosely correlated to the actual inflammatory element.

The figure also suggests that markers measured at various times may reflect various mechanistic aspects differing in their

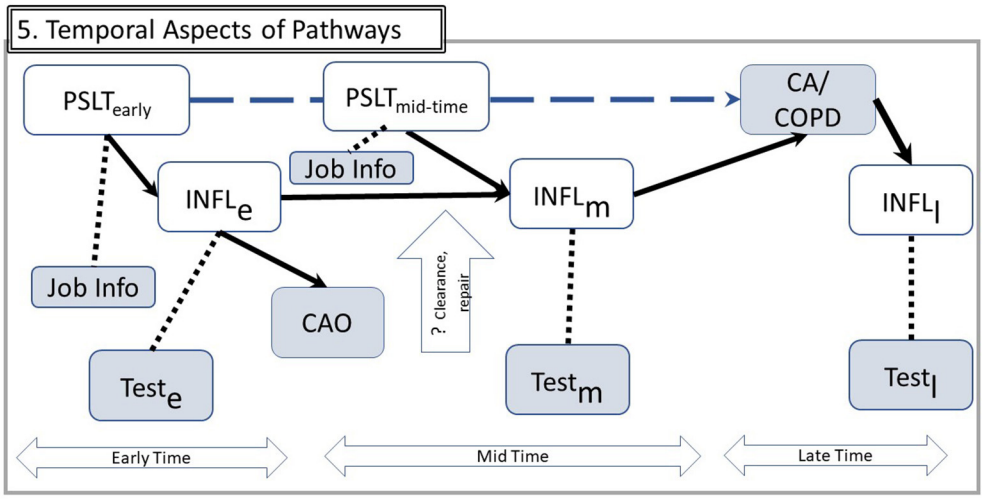

FIGURE 5 | Temporal aspects of pathways. Using example from **Figure 4A**, the implications for observable data over time are illustrated. Time is divided in three eras: Early exposure, Mid for subsequent years of work, and Late after the disease outcome has been diagnosed. Inflammation processes in these three eras may involve different mechanisms, denoted by subscripts e, m, and l. Since INFL is not directly observable, it is assessed by relevant tests in each of these three times. The vertical open arrow indicates that some mechanisms such as particle clearance or repair mechanisms may be specific to one time era (e.g., some particle burden from early exposures may be cleared and therefore reduce transition from early to mid-inflammation). A hypothetical from PSLT to CA/CAO is shown by the dashed upper arrow. Other abbreviations are the same as in **Figure 1**.

TABLE 1 | Markers of pulmonary inflammation (examples).

Invasive:
Animal sacrifice and histology/biomarker measurement
Bronchial biopsy, bronchial lavage
Less invasive human sampling:
Exhaled breath analysis (frozen or otherwise)
Minimally invasive measures:
Exhaled indicators: FeNO: (Exhaled nitric oxide)
(Subject to upper airway contamination)
(Short-term responsive)
(Does this reflect "important" mediator?)
Non-pulmonary biomarkers:
Nonspecific indicators e.g., CRP- C-reactive protein
CC16 – Club Cell 16: Mediator or a protector?
Micro-RNA
Omics:
Genomics
Epigenomics
Proteomics
Metabolomics
Targeted SNP's or hypothesis generating GWAS
Markers of Effect
Lung function
Quantitative CT scan

significance. Measurement of an early step may be more relevant for some outcomes in comparison to others. Measures of inflammation are more frequently assessed proximate to the health effect diagnosis ("mid") than in the early years of exposure. Measurement of inflammatory markers in the several years immediately preceding diagnosis of CA or CAO may overlook the importance of inflammation in the earlier years in initiating the pathogenic sequence.

Reverse causation is another potential concern. For example, rather than PSLT leading to CAO and CA, it is possible that CAO increases the retained dose of PSLT if airway dysfunction reduces clearance of inhaled PSLT and therefore increases the effective retained dose of PSLT. It also is plausible that CA causes inflammation rather than the reverse causal relationship (in the "late" era). This is illustrated by several of the empirical studies discussed below.

Solution to Complexity

The complexity of a causal inference framework, as illustrated by the DAGs, does not imply it is impossibly challenging to understand whether there are bona fide causal relationships between PSLT and the health outcomes.

Much of the complexity is addressable by collecting complete and accurate data on as many variables as feasible in a specific study. This may require long-term studies of a worker group or acquiring data previously obtained (an example is provided in Section Implications of the Causal Mechanistic Analysis). Appropriate adjustments to block backdoor pathways, avoiding adjustments that may introduce artifactual biases, or use of analytical models incorporating do-calculus may help (6). Careful analysis may also identify variables that need not be studied, thereby improving study efficiency (7). In special well-circumscribed circumstances, estimation of effect in one population may be aided by incorporating data from other studies; Pearl applied terms such as transportability and meta-synthesis to these approaches (6, 7). While this paper emphasizes specifying

the DAGs using expert knowledge derived from existing studies, causal structures may sometimes be derived from data themselves with causal distributional notation beyond associational measures (7).

Similarly, information necessary to thoroughly assess the postulated intermediary steps from a single work history—health outcome study. Rather, explicitly delineating the individual node and link segments permits considering available data for each of these segments. For some, analogous human data are available, and for others relevant animal data are applicable. Thus, explicit framework analysis promises to overcome inherent constraints of using a single study and integrating empirical data with conceptual understanding. This approach is illustrated in Part 2 below.

AVAILABLE DATA

The complex causal inference links and nodes shown in **Figure 5** make it unlikely that a single set of human data can traverse all the nodes in the causal pathway. However, data applicable to the each of the links in aggregate may guide consideration of mechanistic hypotheses. The analytic framework described in this paper illustrates how information of several types may be synthesized. It does not provide a specific method for calculating a precise quantitative measure of association (2).

The section includes examples of data types relevant to each of the segments linking particles to inflammation and inflammation to disease. Categories of supportive information include biologic plausibility, disease associations, common exposure associations, common genetic risk factors (e.g., proinflammatory genes), early/pre-lung cancer studies, common mediators, prospective population-based studies, consistency with mediators measured in animal toxicology studies, and internal consistency of postulated mechanisms.

Overall Associations

Several epidemiologic studies have examined the hypotheses that PSLT produces CAO and that PSLT produces CA with studies of carbon black (CB). The CB-CAO relationship was addressed by studies of Harber in North America (8, 9) and Gardiner in Europe and the United Kingdom (5) examining the relationship between estimated cumulative exposure and respiratory outcomes. Both observed that chronic exposure led to a reduction of the forced expiratory volume in one second (FEV1), with the average slope of 0.7 ml and 1.2 m/ per cumulative mg-year/m^3 inhalable dust respectively. And effect upon the forced vital capacity was observed in the North American but not the UK/European study. These were cross-sectional studies in which cumulative exposure was retrospectively estimated for the total duration of work for each subject; North American subjects had an average of 14.1 years of exposure (8, 9).

These studies illustrate the practical impact of retrospective exposure assessments. Cumulative exposure estimates were considerably higher in the Harber than the Gardiner studies since the former used all available data to assign exposures in the earlier calendar periods, whereas the latter applied measurements from the early 1990s to earlier years (5, 8). This methodologic difference is likely to account for differences in regression coefficients for FEV1. Neither considers the temporal pattern or dose rate.

Several epidemiologic mortality studies have addressed the relationship between CB and CA (10–13). In addition, several comprehensive reviews consider this possible association and pointed out the inconclusive nature of the studies (14).

Indicators of Inflammation

As discussed in Section Framework for DAG Analysis, "inflammation" is itself complex since it changes over time and includes many alternate pathways. For human data to be germane to the mechanistic hypothesis under consideration, the measures must be minimally or noninvasive, capable of repeated measurement over time, and reasonably anticipated to correlate well with the actual biologic processes. Potential markers are shown in **Table 1** and their timing is described in **Figure 5**.

Associations Between Diseases

Cross-sectional studies show that several established lung diseases with persistent inflammation are associated with an increased cancer incidence (e.g., tuberculosis, idiopathic pulmonary fibrosis, and CAO) (15–17). These relationships might arise either because inflammation is an underlying cause of both CA and the nonmalignant disease or is a consequence rather than cause of the nonmalignant disease and/or CA. A recent review summarized data supporting a common antecedent for both CAO and CA (18). Many such studies suffer from only looking at "late stage" findings and depend upon fully established clinical diagnoses.

Longitudinal studies overcome this limitation. The US National Health and Nutrition Examination Survey (NHANES) identified 113 incident lung cancers during the 17 year follow-up (19). Moderate or severe CAO at baseline was associated with a significantly elevated risk of subsequent incident CA (OR = 2.6, CI = 1.5–3.8). Analogous data were reported by O'Callaghan (20). Thus, despite limitations, disease association studies provide some support for the proposed mechanism.

Inflammation Concurrent With CA or CAO

Studies have also demonstrated that inflammation is frequently present when CAO or CA is diagnosed. Limited weight should be given to studies showing of inflammation at the time of CA diagnosis such as, the adverse prognostic impact of inflammation at the time of diagnosis (21, 22). These may be important for guiding treatment but have limited import for describing the significant pathway (see **Figure 5**).

Associations Between Particle Exposure and Inflammation

Human and animal data support a role of pulmonary inflammation. These studies include both population-based air pollution studies with long-term exposures as well as shorter-term acute exposures (e.g., air pollution episodes, wildland fires), and laboratory studies in chambers. Animal studies have been particularly fruitful in showing that with adequate dose, PSLT's can lead to inflammation. This is likely to depend upon the dose

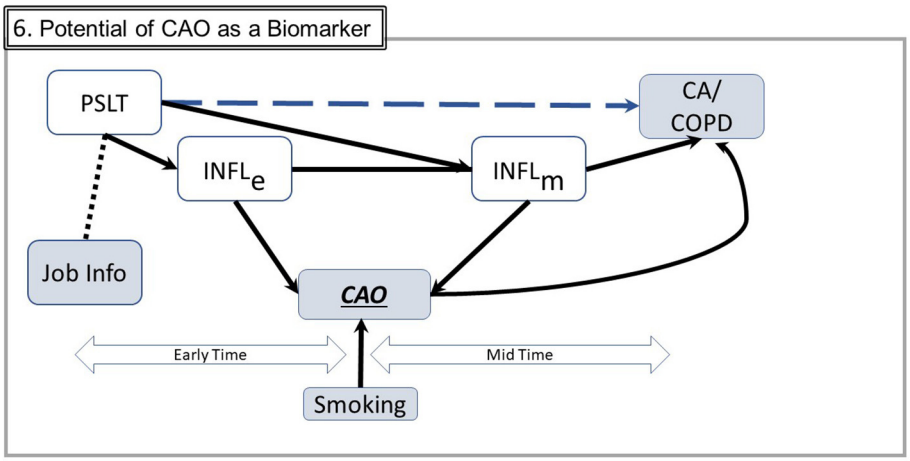

FIGURE 6 | Potential of CAO as a biomarker. This figure includes simplified elements from **Figure 5**. CAO occurs both in the early and the mid times and therefore may be empirically ascertained throughout the long time from exposure to potential illness. In this figure, COPD is chronic obstructive pulmonary disease, a defined disease entity rather than a measurable trait such as CAO. Smoking, commonly the most important confounder, may generally be ascertained and subject to adjustment (Abbreviations are the same as in earlier figures; Several components from prior figures are not shown to facilitate graphical clarity).

time profile and may proceed through several distinct pathways. Human studies are more limited. For example, a study among CB workers and nonexposed controls found increased eosinophils with CB exposure. The differences in peripheral inflammatory markers between CB exposed and unexposed workers were more evident when analyses were stratified by smoking status (23, 24).

Associations Between Inflammation and CA/CAO

Several prospective studies support causal associations between inflammation and CA and CAO. For example, in an 8 year follow-up, elevated C-reactive protein (CRP) was associated with an elevated risk of subsequent lung cancer (hazard ratio = 3.39) (25). Since the increased CRP antedated the CA, reverse causation (CA→ inflammation) is unlikely. An analogous study using high-sensitivity CRP was reported by Muller (26). Association of CRP with lung functional morbidity such as airway hyperresponsiveness or hospitalization has also been reported (27, 28). Genetic studies support this thesis (29, 30).

IMPLICATIONS OF THE CAUSAL MECHANISTIC ANALYSIS

The two preceding sections establish an analytic framework and provide suggestive data applicable to each segment of the pathway. This part suggests their practical implications.

First, causal inferential mechanistic analysis is useful for identifying relevant factors to be considered and suggesting considerations for appropriate and inappropriate adjustments in specific statistical models. Additionally, analogous principles extend to synthesizing data across studies for inferential purposes and informing underlying mechanistic considerations.

Second, translating observed data into practical policy may benefit from mechanistic considerations. Dose and dose rate are particularly germane if the mechanism suggests that clearance may either reduce the effective dose or become saturated at high dose rates. Reliance upon estimated total cumulative dose is not robust to such considerations. In addition, it is useful to consider whether intermediary steps are potentially irreversible or may be counterbalanced by repair mechanisms. Irreversible mutational changes may be contrasted with inflammatory sequences subject to internal homeostatic controls. The analysis presented in this paper does not in itself address these questions specifically for the PSLT's, but it establishes a framework through which other data such as animal toxicology studies may be integrated with observable human data.

Third, these approaches facilitate planning future research studies to strengthen the empirical database. As shown in Section Framework for DAG Analysis, it is unlikely to be practical to measure inflammatory markers throughout a working lifetime and thereafter to link early inflammatory responses to subsequent CAO or CA identified decades later. However, the individual segments (node-link-node) may be studied, albeit in different individuals. For example, the relationship between PSLT and inflammation may be assessed in exposed workers with different temporal dose patterns (see **Figure 3**). Such results may contribute to assessing the significance of current lower-level industrial worker exposures and/or effects upon end-users. Similarly, resolution of inflammatory markers after cessation of exposure may be assessed.

Fourth, the different time courses for detecting CAO and CA might be leveraged to gain insight. Unlike CA, which is typically diagnosed at a specific "late" point in time, CAO develops gradually and is detectable early in its course well before advanced disease such as chronic obstructive pulmonary disease is present (see **Figure 6**). If inflammation is central to the development of

both outcomes, monitoring CAO may provide insight into the temporal/concentration exposure characteristics associated with inflammatory responses. CAO is easily measured repetitively and noninvasively at low cost with spirometry or associated inflammation biomarkers such as exhaled nitric oxide. Assessing temporal course of CAO with different temporal exposure patterns would provide insight relevant to the significance of dose-time exposure patterns. Ongoing industry medical surveillance programs may already have data permitting such analyses. This approach, while currently hypothetical, may be practically implementable.

SUMMARY

Causal inference analysis such as use of DAGs is a very useful tool to help clarify many of the significant questions concerning the health significance of poorly soluble low toxicity particles. It is useful both for representing the set of appropriate variables and guiding analytic models for individual studies. Its principles of identifying relevant variables and their potential effects may be applied as a heuristic to graphically represent relationships explicitly to foster qualitative synthesis of information from disparate studies.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary materials, further inquiries can be directed to the corresponding author.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Systematic review of the scientific evidence of the pulmonary carcinogenicity of talc

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We conducted a systematic review to assess the potential pulmonary carcinogenicity of inhaled talc in humans. Our systematic review methods adhere to Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) guidelines and incorporated aspects from the US Institute of Medicine (IOM) and several United States (US) Environmental Protection Agency (EPA) frameworks for systematic reviews. A comprehensive literature search was conducted. Detailed data abstraction and study quality evaluation, adapting the US Toxic Substances Control Act (TSCA) framework, were central to our analysis. The literature search and selection process identified 23 primary studies that assessed exposure to talc and pulmonary cancer risks in humans (n = 19) and animals (n = 3). Integrating all streams of evidence according to the IOM framework yielded classifications of suggestive evidence of no association between inhaled talc and lung cancer and pleural mesothelioma at human-relevant exposure levels.

KEYWORDS

systematic review, talc, hazard assessment, carcinogenicity, risk assessment, lung cancer, mesothelioma

Introduction

Talc is a hydrous magnesium sheet silicate (Mg3Si4O10(OH)2) with particles that are plate-like in structure. Mined mineral talcs may contain various amounts and forms of accessory minerals. It has been reported that some cosmetic talcs and finished talcum powders may contain trace levels of asbestiform minerals despite the lack of evidence of reliably detectable asbestos at the major sources (1). However, there have been challenges with accurately identifying and quantifying asbestiform minerals in talc (2, 3). As a result, the validity and relevance of these findings remains unclear; however, the epidemiological studies reflect potential risks associated with exposures to talcs including whatever accessory minerals and contaminants that might be present.

In its most recent review of talc, the International Agency for Research on Cancer (IARC) concluded "[t]here is *inadequate evidence* in humans for the carcinogenicity of inhaled talc" (4). They also concluded "[t]here is limited evidence in experimental animals for the carcinogenicity of talc not containing asbestos or asbestiform fibers." This review is now 12 years old, and several studies and reviews on talc exposure and pulmonary cancer risk have been published since the 2010 IARC Monograph (1, 5, 6). The objective of this paper was to apply systematic review methods to critically evaluate and synthesize the scientific evidence addressing the possible relationship(s) between exposure to talc occupationally and from exposure to talc-containing products (primarily talcum powders and cosmetics) and pulmonary cancers, specifically lung cancer and pleural mesothelioma, integrating epidemiology, toxicology, and studies informing potential underlying modes of action.

Materials and methods

This systematic review was conducted in accordance with the Preferred Reporting Items for Systematic Reviews and Meta-Analyses (PRISMA) checklist, using a "hybrid" systematic review framework that incorporates aspects from several recognized systems. We relied most heavily on the U.S. Environmental Protection Agency's (EPA) protocol for systematic reviews conducted under the Toxic Substances Control Act (TSCA) and the Draft Handbook for the Integrated Risk Information System (IRIS) (7). Hazard conclusions were determined using the U.S. Institute of Medicine (IOM) framework (8). An overview of our methods is provided below, and an example of their application can be seen in a recent review on ethylene oxide (9). Our evaluation of reproductive tract cancers, following the same methods as the current review, to be presented in a companion manuscript (10). Additional details of our methodology are provided in the Protocol in the Supplementary material, allowing verification and replication of our review.

We developed *a priori* inclusion and exclusion criteria to identify the most relevant articles for full review consistent with systematic review principles. In brief, selected literature pertained to talc exposure *via* inhalation and addressed potential associations with lung cancers or pleural mesothelioma. We included epidemiological studies, experimental animal studies in mammalian species, and mechanistic studies *in vivo* or in mammalian or bacterial cell lines. We performed literature searches using PubMed and Web of Science and used existing agency reviews as a basis for cross-referencing critical studies. The preliminary search string was as follows: (talc OR "talcum powder") AND ("cancer" OR "carcinogen" OR "mesothelioma"). Additional searches were run using filters for animal/toxicology studies, and for mechanistic/mode of action (MOA) studies, using search terms including, but not limited to the following: micronuclei, sister chromatid exchange, chromosome aberrations, DNA adduct, DNA methylation, inflammation, mechanism, and MOA.

Experimental animal and mechanistic studies were selected based on their overall relevance to chronic health effects (primarily tumor formation and cancers) and adherence to the Population, Exposure, Comparator, and Outcome (PECO) criteria. Epidemiological studies were selected to include groups exposed to talc, including talc miners and millers (the groups historically most highly exposed) as well as groups potentially exposed to cosmetic talcum powders and other products containing talc. Each study was reviewed for relevance, and if the full text met inclusion criteria, study information was extracted into tables and the study was evaluated for reporting and methodological quality.

We followed a modified version of the study quality framework used by U.S. EPA TSCA risk evaluations (11, 12). Specifically, this framework involves reviewing and evaluating studies according to specific quality domains (e.g., outcome assessment and exposure characterization), each of which includes two to seven individual metrics assessing specific study features. All studies were screened and evaluated by two independent reviewers, and any discrepancies discussed and resolved. We employed qualitative and hierarchical or tiered approaches to arrive at the overall study quality score, tailored to each study type and outcome. The tiering system allows for preferentially weighting specific quality domains (e.g., exposure characterization) first, followed by more secondary determinants of study quality. A flow chart for the overall tiering approach used for cohort studies is provided as an example in Figure 1.

For animal toxicology and selected mechanistic studies, we followed the TSCA study quality evaluation framework (12, 13) and assigned relative numerical ranks to each of the outcomes (1, 2, and 3 corresponding to high, medium, and low) for each metric, then averaged the metric scores to arrive at an overall relative rating of high, medium, or low quality. Mechanistic studies were evaluated according to Klimisch scoring (14).

Evidence was synthesized across all epidemiological studies and then integrated with animal study findings and mechanistic considerations to reach conclusions for human pulmonary cancers. Integration of evidence included consideration of consistency, coherence and the presence of exposure-response relationships. Overall conclusions were derived for each cancer: sufficient evidence of a causal relationship; sufficient evidence, suggestive evidence, or inadequate/insufficient evidence of an association; or suggestive evidence of no association. The nomenclature of these classifications is simplified but follows the rationale of the corresponding U.S. IOM classifications for causation (8) (Table 1).

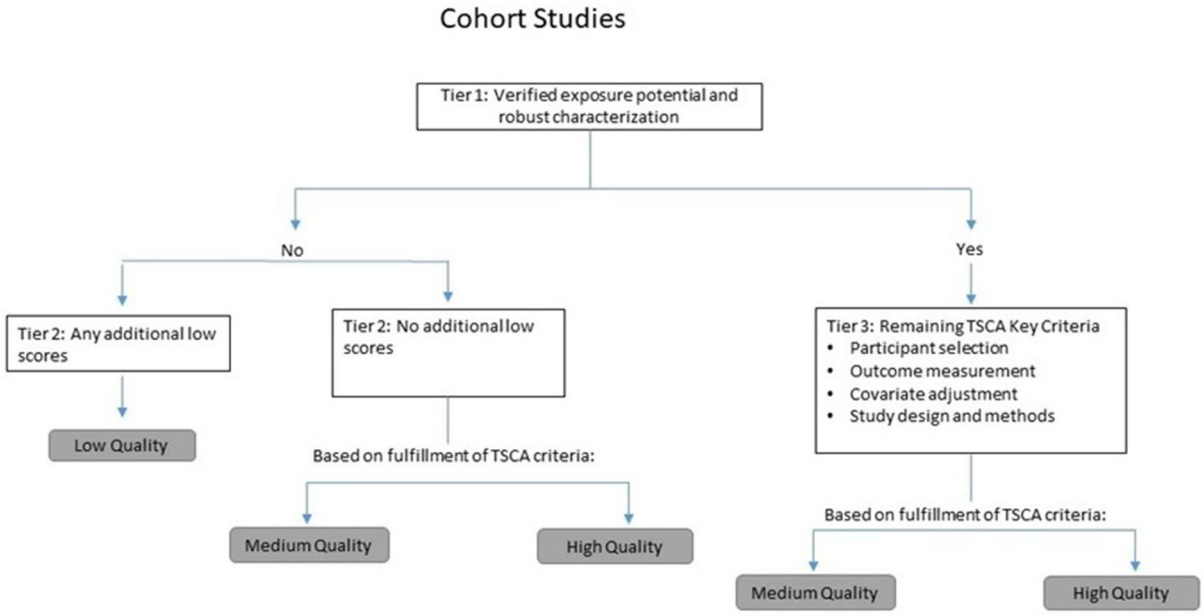

FIGURE 1
Tiering approach used in evaluating cohort studies.

Results

Literature search and selection

The primary literature search for talc exposure and all cancers was performed in PubMed (in April; updated September 2021) and yielded a total of 716 publications. After eliminating duplicates or studies that were subsequently updated, and applying the inclusion and exclusion criteria determined *a priori*, 43 epidemiological and six animal studies remained for detailed review. An additional 10 epidemiological studies were identified through supplemental searches using narrower terms (e.g., "lung cancer," "mesothelioma," "millers," "miners") and considering the tertiary literature (reviews, gray literature). Additional searches in Web of Science identified no additional publications. Of these, 19 epidemiology studies and three animal studies were determined to address pulmonary cancers and included in this systematic review. The results of the literature search and study selection process are summarized in Figure 2.

Pharmacokinetics of talc in the respiratory system

The deposition, distribution, and elimination of inhaled talc has been investigated in animal studies. Generally, aerosolized talc has an alveolar biological half-life of about 7–10 days in animals. In one study, Syrian golden hamsters were administered a single, 2-h, nose-only exposure to commercial baby powder (MMAD of 6.4–6.9 μm) (15, 16). Between 6% and 8% of the inhaled dose was deposited in alveoli. By 132 days after exposure, there were no statistically significant differences in talc burden in the lungs of exposed vs. unexposed hamsters, indicating clearance of the particles.

Experimental animal studies

Our search identified six studies assessing talc exposure and tumor formation in animal models. After excluding studies based on exposure route, non-pulmonary outcome, and administration of talc as part of a mixture, three studies remained for study quality evaluation (17–19). Study details can be found in Supplementary Table 1. Based on the quality evaluation methods (see Materials and methods), the three studies assessing talc toxicology in animals were rated as high quality. A brief overview of the quality evaluation results is presented below, followed by a summary of study findings. Full quality evaluation results are presented in Supplementary Table 2.

Quality evaluation results

Overall, the animal studies all employed designs that properly incorporated a control group for comparison,

TABLE 1 Categorizations for evaluating strength of evidence[a] (8).

Classification	Description
Sufficient evidence of a causal relationship	Evidence is sufficient to conclude that a causal relationship exists between the exposure to a specific agent and a health outcome in humans. The evidence fulfills the criteria for sufficient evidence of an association (below) and satisfies several of the criteria used to assess causality: strength of association, dose-response relationship, consistency of association, temporal relationship, specificity of association, and biological plausibility.
Sufficient evidence of an association	Evidence is sufficient to conclude that there is a positive association. That is, a positive association has been observed between an exposure to a specific agent and a health outcome in human studies in which chance, bias, and confounding could be ruled out with reasonable confidence.
Limited/suggestive evidence of an association	Evidence is suggestive of an association between exposure to a specific agent and a health outcome in humans, but is limited because chance, bias, and confounding could not be ruled out with confidence.
Inadequate/insufficient evidence to determine whether an association does or does not exist	The available studies are of insufficient quality, consistency, or statistical power to permit a conclusion regarding the presence or absence of an association between an exposure to a specific agent and a health outcome in humans.
Limited/suggestive evidence of no Association	There are several adequate studies covering the full range of levels of exposure that humans are known to encounter, that are mutually consistent in not showing a positive association between exposure to a specific agent and a health outcome at any level of exposure. A conclusion of no association is inevitably limited to the conditions, levels of exposure, and length of observation covered by the available studies. In addition, the possibility of a very small elevation in risk at the levels of exposure studied can never be excluded.

Source: IOM (8).
[a]IOM has since updated the classification language, but the same general underlying considerations for reaching each conclusion. The previous classification categories were retained as we believed the previous categories were more easily interpreted than the updated categories.

including randomly allocating test animals into experimental groups to reduce potential bias. All three studies provided explicit descriptions of talc aerosolization with exposure consistently administered across both control and experimental groups (except for some fluctuations in the NTP study). Outcome assessment for these studies typically encompassed a full histological examination or autopsy; clinical and body weight monitoring was also conducted in two of the studies (15, 18, 19). Although these studies overall were considered to be of high quality, there were some important deficiencies that limited their utility, which are discussed below.

Summary of study findings

Wehner et al. (15, 18) exposed groups of 100 Syrian golden hamsters to whole-body inhalation of Johnson's Baby Powder at 8 mg/m^3 for 3, 30, or 150 min/day, for 5 days/week for 30 days, or for 30 or 150 min/day, 5 days/week, for up to 300 days. Two groups of 50 hamsters also were exposed to laboratory air as a control for 30 or 300 days. Calculated cumulative talc exposures were 12, 120, and 600 mg-h/m^3 for 30 days or 1,200 and 6,000 mg-h/m^3 for the 300-day groups. Exposure to talc aerosol produced no statistically significant differences in body weight; survival; or the type, incidence, or degree of histopathological change, relative to unexposed controls. There was one lung carcinoma found in the group exposed to talc for 3 min/day for 30 days, and one lung carcinoma found in the 300-day control group. However, the authors noted that these tumors were metastatic and that there were no primary neoplasms found in the respiratory system.

Wagner et al. (17, 20) exposed Westar rats to Italian 00000 grade talc, superfine chrysotile asbestos, or laboratory air (controls), *via* whole-body inhalation. Talc concentrations of 10.8 mg/m^3 were administered for 7.5 h per day, 5 days per week; 48 animals from each group were exposed for 3 months, 24 animals for 6 months, and 24 animals for 12 months with cumulative doses of approximately 4,100, 8,200, and 16,400 mg/m^3-h for the 3-month, 6-month, and 12-month exposure durations, respectively. Two adenomas (not considered cancerous) were observed in talc-exposed animals, 13 lung tumors were found in chrysotile-exposed animals, and one adenoma occurred in the air-exposed control group. No statistical tests were reported in either publication. In both the talc and chrysotile exposure groups, fibrosis was observed to a similar extent, with minimal to no fibrosis observed in the air-exposed control group.

The National Toxicology Program (19) exposed F344/N rats and B6C3F1 mice to air or micronized Pfizer MP 10–52 talc via whole-body inhalation. Animals were exposed to 0, 6, or 18 mg/m^3 talc for 6 h/day for 5 days/week until death or until the mortality of any exposure group reached 80% (approximately 2 years). There was no evidence of carcinogenic activity of talc in male or female B6C3F1 mice. Generally, lung talc burdens of mice exposed to 18 mg/m^3 were disproportionately greater than those of mice exposed to 6 mg/m^3, suggesting clearance of talc from the lung was impaired in mice exposed to 18 mg/m^3. In male F344/N rats, there was an increased incidence of benign or malignant pheochromocytomas of the adrenal gland, commonly observed in rats (21). In female rats, there was an increased incidence of alveolar/bronchiolar adenomas and carcinomas of the lung and pheochromocytomas of the adrenal gland. There was a single occurrence of malignant

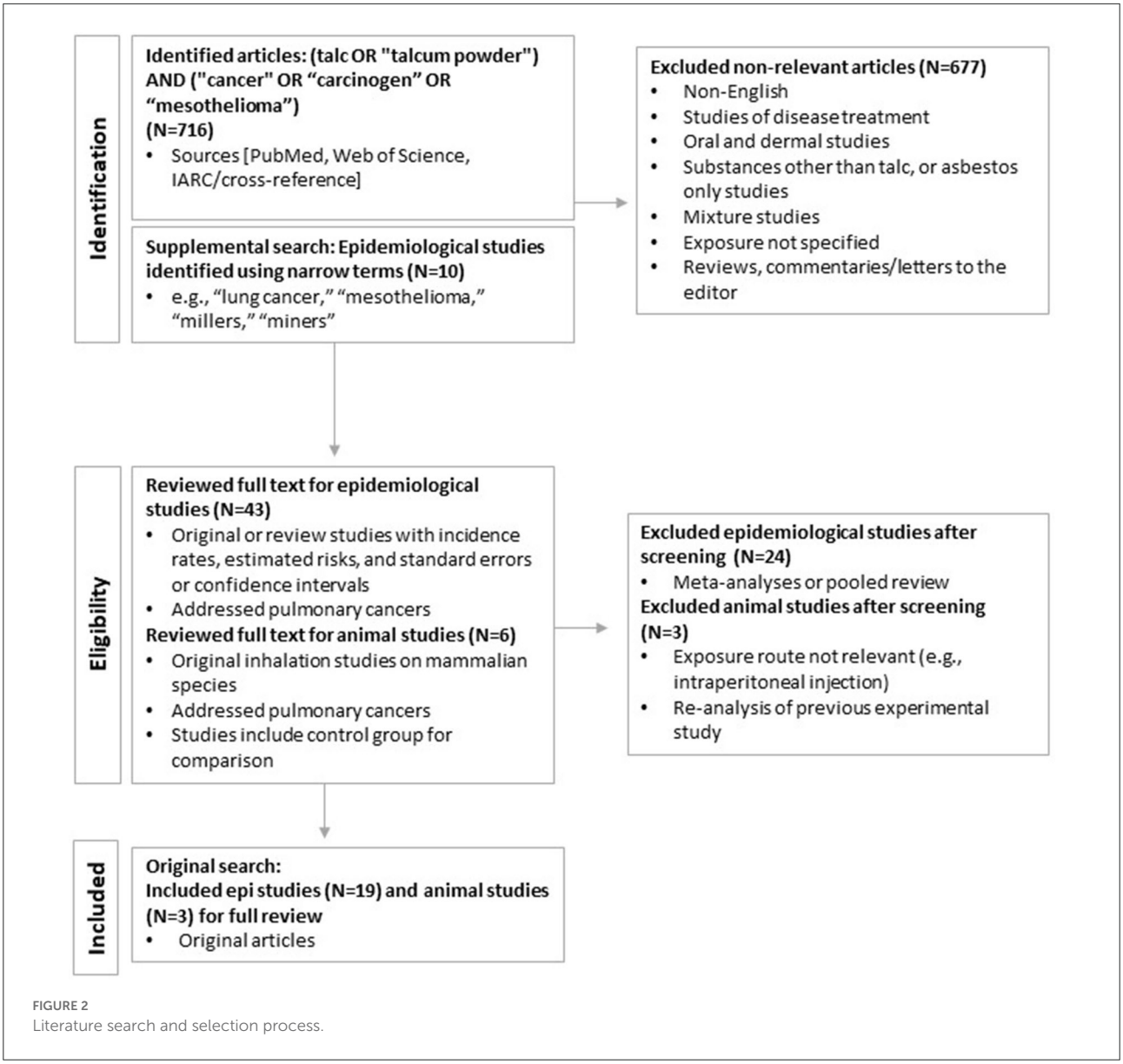

FIGURE 2
Literature search and selection process.

mesothelioma in a high-dose male rat; however, aging F344 rats have been reported to develop spontaneous mesotheliomas (22). In both rats and mice, 2-year inhalation exposure to talc was associated with chronic active inflammation and accumulation of macrophages in the lung (19). The authors reported issues with the consistency of administration of talc aerosol over some weeks in the middle of the study, likely resulting in increased talc exposure. Additionally, the NTP (19) study involved the use of micronized talc, which has a smaller aerodynamic diameter than mined and milled talcs and talc used in talcum powder products. While this does not affect the quality of the study, as discussed below, micronized talc is not used in cosmetic talcum powder products and may limit the study's generalizability for assessing risk associated with the use of cosmetic talcum powder.

Summary and conclusions for animal evidence

Across the animal studies assessing chronic talc toxicity, results largely demonstrate a lack of talc carcinogenicity. Although two of the studies reported no increase in tumor formation among talc-exposed animals (15, 17, 18, 20), NTP (19) reported a higher incidence of lung tumors in female rats in the highest exposure group, relative to controls. However, the lung tumors occurred only in female rats exposed at a dose also inducing significant chronic lung toxicity and high lung talc burden, such that the maximum tolerable dose may have been exceeded (21). Particle overload is common when high doses of poorly insoluble particles are administered – particle clearance

mechanisms of the lung are overwhelmed and carcinogenic processes are initiated (23, 24): see "Mechanistic evidence and Mode of Action." There was no evidence of carcinogenicity in mice exposed to talc. Overall, in available animal studies, there is indeterminate evidence that talc is associated with lung tumors in rodents based on negative findings in several high-quality studies and species, but positive results in a single species and sex (female rats) exposed to high doses of micronized talc that caused particle overload conditions.

Mechanistic and mode of action evidence

Genotoxicity

Talc was negative for mutagenicity and other forms of genotoxicity in all available assays, described in brief below.

In an OECD Guideline 473 *in vitro* mammalian chromosome aberration test (rated as a Klimisch score of two), Endo-Capron et al. (25) reported that talc did not cause significantly increased frequency of sister-chromatid exchange or increased DNA repair synthesis in rat pleural mesothelial cells, relative to positive or negative controls (16).

Talc was negative in a reverse mutation assay *Salmonella typhimurium* strains TA1530 and G46. Talc was also negative in a companion host-mediated mutagenicity assay using male ICR mice injected with *Salmonella typhimurium* and *Saccharomyces cerevisiae*, with and without metabolic activation (16).

In an *in vivo* OECD Guideline 478 Dominant lethal test, male Sprague-Dawley rats were exposed *via* gavage to a single dose or one dose/day for 5 days of 300, 3,000, or 5,000 mg/kg talc. No chromosomal aberrations in the bone marrow or dominant lethal mutations at any dose were observed (16).

Inflammation

As with other particles, one postulated carcinogenic MOA for talc is chronic inflammation resulting from the overwhelming of particle clearance mechanisms (i.e., phagocytosis), long-term tissue irritation and release of inflammatory chemokines and cytokines, and reactive oxygen species (ROS) formation. For pulmonary cancers, it is therefore plausible that talc could cause irritation and a cascade of inflammatory mechanisms.

Beck et al. (26) sonicated respirable granite and talc dust from a talc mine in Vermont (0.8 μm, MMAD of 7.5 μm) in saline and intratracheally instilled it into the lungs of Syrian Gold Hamsters at doses of 0.15, 0.75, or 3.75 mg/100 g (single exposure). Bronchioalveolar lavage (BAL) fluid was collected and assessed at 1 day post exposure (all doses) or 1, 4, 7, and 14 days after talc administration (3.75 mg/100 g). One day after dosing, macrophage numbers were not significantly altered, but there were increases in polymorphonuclear leukocytes (PMNs), lactate dehydrogenase, and peroxidase, indicating cellular injury. Albumin, a marker of pulmonary edema, also was increased. In the time-course evaluation, most findings returned to control levels within 14 days. However, macrophage numbers decreased in days 4–14, and remained decreased, indicative of a chronic effect. Note that this route of administration (intratracheal instillation) produces different lung distribution patterns compared to inhalation, which limits its relevance to inhalation exposures to talc in humans.

Pickrell et al. (27) exposed F344/Crl rats and B6C3F1 mice to unspecified talc *via* whole-body inhalation and compared tumor development in these animals relative to non-exposed rats and mice as controls. Talc was administered to both species for 6 h per day, 5 days per week, for a total of 20 exposure days at talc concentrations of 0, 2, 6, or 18 mg/m^3. Histologic evaluation of lung tissue revealed no exposure-related lesions except for a modest, diffuse increase in free macrophages within alveolar spaces of both rats and mice exposed to the highest concentration of talc. In mice, intra-alveolar macrophages were focally aggregated. The normalized lung talc burdens for both mice and rats were lower at the lowest exposure level than at the two higher exposure levels; however, the difference was statistically significant only for the rats.

Similar findings were reported in rats exposed to non-asbestiform talcum powder for 6 h per day for 4 weeks *via* whole-body inhalation (28). The authors observed increases in macrophage infiltration at 50 and 100 mg/m^3 talc, but not at the lowest concentration of 5 mg/m^3. Markers of oxidative stress (superoxide dismutase 2) were increased at 100 mg/m^3.

Conclusions for mechanistic and MOA evidence

The pharmacokinetic data on the fate of inhaled talc indicate rapid clearance from the lung and body after single doses and no translocation of talc to other organs after single or repeated exposures. The evidence for possible carcinogenic mechanisms of talc is limited; however, a genotoxicity MOA confidently can be ruled out. A few studies provide evidence of some key events in the proposed inflammatory MOA; however, data are limited to non-human relevant exposure pathways and/or cell-based assays.

Markers of inflammation also have been observed in the lungs of rats after talc exposure, but one of the only available mechanistic studies utilized a route of exposure (i.e., intratracheal instillation) that is not comparable to inhalation exposures. Further, some aspects of the physiology and function of the respiratory system of rodents (e.g., a delicate balance of pulmonary surfactants, as well as smaller and fewer macrophages relative to humans) make them highly susceptible to high doses of solid particles relative to humans (29). Overall, the mechanistic evidence is insufficient to support an MOA whereby talc induces pulmonary carcinogenesis.

Epidemiological studies

Nineteen epidemiological studies (17 cohort study publications and two nested case-control studies) satisfied inclusion and exclusion criteria and were selected for full review to assess the possible relationship between occupational talc exposure and pulmonary cancers (specifically lung cancer and mesothelioma). Several of these publications were updates of reports based on the same cohorts. Two nested case-control studies were conducted in tandem with occupational mortality analyses (30, 31).

We also identified several meta-analyses/pooled studies, two large cancer registry-based linkage studies examining cancer risks by occupational group but lacking specific information on individual exposures (32, 33), and several talc pleurodesis studies (34–37). None of these studies met our inclusion criteria and therefore did not undergo full study quality review. No epidemiological studies were identified that assessed consumer use of talc and talcum powder products. Three case series (38–40) drawn from medico-legal consultation practices were identified, but these did not meet the inclusion criteria for epidemiological studies (e.g., because they lack referent or control groups) and thus were not selected for further review.

Therefore, the body of literature eligible for full review consisted of studies evaluating workers occupationally exposed to high levels of talc during mining and milling operations. The 17 occupational cohort study reports evaluated for study quality encompassed talc miners and millers in New York State (41–47), Italy (1, 48–51), Norway (52, 53), France and Austria (30) and Vermont (54–56). Details regarding the cohorts and study methods can be found in Supplementary Tables 3, 4. Briefly, the cohorts ranged in size from about 400 (53) to over 1,700 (1) miners and millers that were followed for mortality for up to seven decades. For each cohort, excluding the Austrian and French cohorts, mortality was updated at least once.

The Italian, Norwegian, French, and Austrian talc mines all produced talcs described as non-asbestiform with various accessory minerals including small amounts of chlorite and quartz (30, 50, 52). Similarly, Vermont talc miners and millers encountered talcs "free of both asbestiform mineral and significant quantities of free silica" (54, 56). Some of the mines in upstate New York were reported to produce talcs with asbestiform or non-asbestiform amphibole minerals including tremolite and anthophyllite (42, 43, 57). Historical occupational talc exposure most often was estimated using duration of employment as a surrogate. Historical air sampling records (i.e., total respirable dust) also were available at some locations. Pulmonary cancer (lung cancer and mesothelioma) mortality generally was ascertained *via* death certificates coded by certified nosologists.

Across all cohort studies, observed numbers of deaths for site-specific cancers among talc miners and millers (either combined or separately) were compared with expected numbers based on national and/or regional reference rates. When available, we focused our analysis on the most recent update that provided the most complete vital status and cause of death ascertainment.

Quality evaluation results

Based on the quality evaluation methods, domains and quality criteria (described above and detailed for all studies in Supplementary Tables 5, 6), most of the studies assessing pulmonary cancer risk among talc miners and millers were rated medium quality. While many studies received high ratings for individual metrics such as study participation and outcome assessment, limitations in other metrics or domains precluded some studies from receiving a high overall rating. Overall ratings of medium quality for 14 cohort studies primarily were driven by potential confounding/variable control (especially for lung cancer and cigarette smoking) and exposure assessment, including characterization of talc exposure (e.g., by job, exposure concentration level and duration). Because the case-control studies were nested within cohorts, we did not expect substantive differences in quality. We pilot tested the case-control quality evaluation for Gamble (31) and determined that its rating was equivalent to the associated cohort study (41) and thus did not separately evaluate the second case-control study.

In general, potential confounders such as age were appropriately considered in the statistical analyses, and employment records were used to extract key demographic and work history information. However, high quality ratings were rare due to inadequate information on prior employment history among the cohort members, which precluded determining the potential for prior occupational exposure to asbestos. For example, Fordyce et al. (56) reported that the death certificate for the single mesothelioma death in the cohort "explicitly mentioned exposure to asbestos"; yet no additional information was available regarding this potential exposure (56). On the other hand, Honda et al. (43) obtained relatively detailed information on prior employment histories through next of kin interviews for the two presumed (but not verifiable) mesothelioma deaths reported in that study.

Epidemiological findings for malignant mesothelioma

None of the 17 cohort studies identified an increased risk of malignant pleural or peritoneal mesothelioma among talc miners and millers. No mesothelioma deaths were observed in most of the cohorts, including the Austrian, French, Italian, and Norwegian talc miners and millers (1, 30, 53). In the Vermont cohort, a single mesothelioma death was reported in the latest update; however, this case was identified through a detailed review of death certificates for the cohort (which was

not performed for the reference group) in a field on the death certificate different from the conventional field for underlying cause of death (56). As noted above, the talcs produced from these mines have been reported not to contain asbestiform minerals and testing has not produced reliably detectable levels of asbestos of any fiber type [e.g., regarding the Italian mines, see (1)].

Kleinfeld and coworkers published two proportionate mortality studies on miners and millers in upstate New York potentially exposed to talc with asbestiform or non-asbestiform amphibole minerals, largely in response to reports of fibrogenic pneumoconiosis (44, 45). One peritoneal mesothelioma was reported in both studies (the same individual) and analyses including this case in the category of "gastric cancers" found no excess risk overall or by specific age categories (44, 45). Methods used to derive cause of death were poorly described, and included a variety of sources including death certificates, employment records, and hospital records, resulting in a low quality rating for that domain, and low overall quality rating (44, 45).

Five additional mortality studies evaluated specific causes of death among New York talc miners and millers (41–43, 46, 47). Lamm et al. (46) reported one mesothelioma (site not specified) in an electrician hired at the plant 15 years prior to his death [possibly the same case reported in (42)]. Brown et al. (41) studied the same plant, but their NIOSH Health Hazard Evaluation Report mentioned no mesothelioma. Honda et al. (43) extended follow-up of this cohort from 1948 through 1989, reporting two deaths from pleural mesothelioma; however, the underlying causes of death on the death certificates officially were coded by the New York State nosologists as "benign neoplasm of the respiratory system" and "malignant neoplasm of bronchus and lung, unspecified," respectively. No specific mention of mesothelioma was recorded on the death certificates.

In summary, no excess of malignant mesothelioma has been reported in any of the available epidemiological studies of talc miners and millers heavily exposed to talc. Although some of the individual cohorts were small, the collective number of workers followed was substantial (6) and the cohorts were followed for many decades. These workers were clearly and highly exposed to the talcs: all except the Norwegian cohort reported statistically significant excess mortality due to pneumonoconiosis, a group of non-malignant respiratory diseases caused by heavy exposure to dusts.

Ierardi and Marsh (58) conducted a power analysis to determine whether the pooled cohorts could detect a true association between talc exposure and mesothelioma risk among talc miners and millers, if there were one. Based on 130,154 total person-years of follow-up across five cohorts, the authors estimated that the pooled cohorts (30, 51, 53, 56) had 59% and 78% power to detect a 2.5-fold or greater and a 3.0-fold or greater increased risk of mesothelioma, respectively. Because the one malignant mesothelioma in the Vermont cohort was identified following a focused search of death certificates for the cohort but not the referent group, its inclusion results in a conservative bias. These findings were confirmed in Ierardi et al. (6), which reported an alternate pooled Standardized Mortality Ratio (SMR) of 0.242 (90% Confidence Interval [CI]: 0.012–1.15) with additional follow up time but no new deaths. When considered together, the moderate confidence in study findings, large study populations, long duration of follow up, and consistency of null findings indicate that talc exposure is not associated with mesothelioma.

Epidemiological findings for lung cancer

Among the most recent mortality updates for the talc miner and miller cohorts (1, 30, 43, 53, 56), Honda et al. (43) was the only study to report a statistically significant excess risk of lung cancer (SMR = 2.32; 95% CI: 1.57–3.29). Lung cancer mortality was slightly elevated, but did not achieve statistical significance, in the French (SMR = 1.23; 95% CI: 0.76–1.89) and Vermont (SMR = 1.44; 95% CI: 0.98–2.03) talc miners and millers but was close to unity for the Austrian (SMR = 1.06; 95% CI: 0.43–2.19), Italian (SMR = 1.02; 95% CI: 0.82–1.27) and Norwegian (SIR = 1.17; 95% CI: 0.73–1.79) talc miners and millers (1, 30, 53, 56).

Most of the available studies that evaluated lung cancers were rated medium quality. The higher quality studies generally did not demonstrate an association, or attributed observed increased risks to potential confounding by cigarette smoking, as risks did not correlate with indicators of talc exposure. Findings for each of the cohort studies that was rated high quality for exposure characterization are discussed more below.

Rubino et al. (50) followed 1,346 talc miners and 438 millers hired between 1921 and 1950 and that worked for at least 1 year in the Val Chisone talc operations. Mortality from lung cancer was slightly lower than expected for the entire cohort. Historical air sampling records were used to estimate cumulative dust exposure for each worker. Internal comparisons by exposure category showed that lung cancer mortality did not increase with increasing exposure for miners or millers. Coggiola et al. (48) updated the cohort and observed 44 lung cancers from 1946 through 1995 (SMR = 1.07; 95% CI: 0.73–1.50 for miners and SMR = 0.69; 95% CI: 0.34–1.23 for millers) (48). When stratified by duration of exposure, no exposure-response pattern was found (1, 48, 51).

Wild et al. (30) reported no increased lung cancer mortality among French (SMR = 1.23; 95% CI: 0.76–1.89) and Austrian (SMR = 1.06; 95% CI: 0.43–2.19) talc mining and processing employees (30). Using detailed employment histories and a job exposure matrix, cumulative talc exposures were estimated, and each cohort member was assigned a concentration of low (2.5 mg/m^3), medium (10 mg/m^3), or high (40 mg/m^3). A nested case-control analysis showed no association of lung cancer with increasing cumulative exposure to talc, after adjusting for smoking, exposure to quartz, and underground work (30). The

authors reported a reduced exposure odds ratio (OR = 0.73; CI not reported) for cohort members in the highest cumulative talc exposure group (≥800 mg/m^3-years).

Honda et al. (43) reported 31 deaths from lung cancer among 809 New York talc miners and millers who had worked at least 1 day between 1948 and 1989 (SMR = 2.32; 95% CI: 1.57–3.29). The increased risk appeared to be limited to workers hired before 1955 (SMR pre-1955: 2.86; 95% CI: 1.90–4.14), as the SMR for workers hired after 1955 was 0.83 (95% CI: 0.17–2.42). The increased risk among those hired before 1955 was strongest among miners (SMR = 3.94; 95% CI: 2.33–6.22, based on 18 cases) and not elevated among millers (SMR = 1.28; 95% CI: 0.51–2.63, based on 7 cases). Lung cancer risk did not increase with increasing cumulative exposure categories after adjustment for age and years since hire (43). Relative to cohort members with the lowest cumulative respirable dust exposure (0 to <95.1 mg/m^3), individuals in the two highest cumulative exposure groups had slightly reduced lung cancer risk (RR for cumulative exposure of 95.1-<987 mg/m^3 = 0.8; 95% CI: 0.3–1.9, and RR for cumulative exposure of 987+ mg/m^3 = 0.5; 95% CI: 0.2–1.3).

Exposure characterization was considered the most critical study quality domain in the quality evaluation of lung cancer studies. Three studies (30, 43, 50) were rated high quality for the exposure characterization domain. In the domain of confounding control, none of the studies had data on individual smoking histories. In order to increase cohort size, the New York cohorts did not limit eligibility based on minimum employment (e.g., 1 year) as is common in cohort mortality studies (42). This might have introduced confounding, as short-term or transient workers tend to have a higher prevalence of smoking and other health risk factors (46). Consequently, half of the talc workers in the mortality study conducted by Dement et al. (42) were employed <1 year (42). Stille and Tabershaw (47) updated the earlier cohort study and reported that all workers combined showed a moderate excess of lung cancer deaths (SMR = 1.57; no CI reported) (47). Smoking histories were not available for many workers, but all lung cancer cases were known to have been cigarette smokers (31).

The impact of prior occupational exposure to respiratory carcinogens also was not considered in most studies. One exception was Stille and Tabershaw (47), which presented stratified analyses for workers with and without known prior employment where exposure to occupational lung carcinogens might have occurred. When stratified by employment history, talc workers with known prior employment also showed excess lung cancer mortality (SMR = 2.14; no CI reported), and talc workers with no known work prior to their talc facility employment showed a slight deficit of lung cancer deaths (SMR = 0.76; no CI reported). An increased risk of death from lung cancer also was restricted to short-term workers in the updated cohort analysis (46). Among 705 white men employed between 1957 and 1977, lung cancer mortality was statistically significantly elevated among workers employed <1 year (SMR = 3.17, based on six lung cancer deaths). The authors attributed the excess lung cancer to previous employment and smoking (46).

In summary, lung cancer mortality was not elevated among most of the cohorts of talc miners and millers exposed to high levels of respirable talcs and accessory minerals. While some cohort mortality studies reported an association between occupational talc exposure and lung cancer mortality, additional epidemiological investigations reported no such association or attributed observed increased risks to potential confounding by cigarette smoking, as risks did not correlate with indicators of talc exposure (43–47). Specifically, individual study quality ratings were high overall and the studies rated as high quality for the exposure characterization domain failed to demonstrate exposure-response patterns between occupational talc exposure and lung cancer mortality (30, 49). Given the moderate confidence in study quality ratings and the lack of a consistent association between occupational talc exposure and lung cancer mortality, the available epidemiological evidence does not demonstrate a causal association between talc exposure and lung cancer mortality.

Evidence integration and hazard characterization

Our conclusions regarding hazard for each cancer type, based on the IOM classification system (8), are described below and in the protocol, and visualized in Table 2. Of three experimental animal studies of inhaled talc, only one mesothelioma was reported in a strain of rats prone to spontaneous mesothelioma. Genotoxicity studies were negative, and the few identified *in vivo* and *in vitro* mechanistic studies did not report strong evidence of the postulated chronic inflammation-mediated MOA in respiratory tissues at human-relevant exposure levels. The body of epidemiological literature evaluating talc exposure and risk of malignant mesothelioma, which consists of highly exposed workers with long follow up periods, fails to demonstrate any increased risk of malignant mesothelioma. Integrating the evidence demonstrating a lack of statistically significant increases in mesothelioma in inhalation rodent bioassays, the null findings in the higher-quality epidemiological literature, and the lack of evidence of a plausible MOA, we conclude that there is suggestive evidence of no association between inhaled talc and mesothelioma at human-relevant exposure levels.

In animal studies, a statistically significant increase in lung tumors was observed after inhalation of talc, although these tumors were limited to females of one species. Lung tumors occurred at very high administered doses that appeared to cause particle overload and using micronized talc (not typical of talc mining or milling exposures, or of cosmetic talcum powder products), limiting the ability to extrapolate these

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TABLE 2 Evidence integration summary judgment: lung cancer and mesothelioma.

Summary of animal, human, and mechanistic evidence					Inference across evidence streams
Evidence from studies of exposed humans					*Suggestive Evidence of no association* • Several medium or high-quality epidemiological studies demonstrate no positive association between talc and LC or mesothelioma. • Three of four studies show no evidence of increased lung tumors • Single positive finding in one species exposed to doses associated with particle overload Other inferences: • Talc is not DNA reactive • Insufficient evidence supporting a MOA
Studies, outcome and confidence	**Key findings**	**Factors that increase certainty**	**Factors that decrease certainty**	**Summary strength of evidence judgment**	
Lung Cancer and Mesothelioma fifteen *medium quality* prospective cohort studies: and one cohort study each in the high and low quality categories	• No elevated risk of LC largely consistent across studies • Few sporadic mesothelioma cases • Include both cosmetic and industrial talc	• Medium quality studies • Highly-exposed millers and miners in several countries • Semi-quantitative exposure characterization in some studies	• Smoking not adequately considered in LC studies • Previous employment not often considered in LC studies	Evidence against	
Evidence from *in vivo* animal studies					
Studies, outcomes, and confidence	**Key findings**	**Factors that increase certainty**	**Factors that decrease certainty**	**Summary strength of evidence judgment**	
Four *high-quality* studies in rats and mice	• Three studies with no lung tumors or mesothelioma • One study with > lung tumors in female rats	• High quality studies	• Particle overload/ exceedance of MTD in positive study • Micronized talc not relevant to human exposure	Indeterminate Micronized talc causes lung tumors in one species and sex of animals at doses >MTD conditions	
Mechanistic Evidence or Supplemental Information					
Biological events or pathways (or other information category)	**Primary evidence evaluated**	**Key findings, interpretation, and limitations**	**Evidence stream summary**		
Genotoxicity, chronic inflammation	• Three GLP/*guideline (K = 1)* genotoxicity studies • Two medium quality ($K = 2$) *in vivo* studies	• Rapid clearance of talc from lungs • Increased macrophages and markers of inflammation *in vitro* and *in vivo* • One study used intratracheal instillation, not relevant to humans	• Available mechanistic evidence is insufficient to support any mode (or modes) of action for talc and lung cancer		

findings to relevant exposures in humans. Two animal studies examining inflammation in the lungs reported increased early inflammatory markers. Specifically, markers of inflammation and injury were observed after intratracheal instillation of large doses of talc, exposure conditions that do not correlate with those of humans. Other studies have reported increases in

macrophages and other inflammatory markers after whole-body exposures, but again only with high talc concentrations.

The body of epidemiological evidence does not demonstrate a clear or consistent increase in mortality from lung cancer among talc miners and millers. A few studies reported excess lung cancer mortality, but lung cancer mortality risk did not increase with increasing talc exposure and the association potentially was confounded by smoking. In contrast to the lack of excess lung cancer deaths among talc miners and millers, a strong and consistent association has been observed between occupational talc exposure and non-malignant respiratory disease (NMRD), including pneumoconiosis (30, 43, 49, 56). A three-fold excess of mortality due to NMRD was reported in the earliest cohort study of New York talc workers, reflecting the high concentrations of dust present in the workplace; prior to 1948, median exposures ranged from 61 to 1,196 mppcf (45). Similarly, in the latest update of the Italian cohort, excess mortality for pneumoconiosis (SMR = 9.55; 7.43–12.1) was observed among talc miners and millers (1). These reported results for exposure and NMRD mortality underscore the magnitude of historical exposures and consequent health risks. Considering the totality of the evidence, we conclude that there is suggestive evidence of no association between inhaled talc and lung cancer at human-relevant exposure levels.

Discussion

Our systematic review of talc and pulmonary cancers generated suggestive evidence of no association for exposure to talc and lung cancer and pleural mesothelioma. The body of epidemiological evidence is reasonably large and robust for lung cancer and mesothelioma and provides the most weight in the evidence integration, complemented by the number of high-quality experimental animal carcinogenicity bioassays, as well as the lack of convincing mechanistic evidence. Although the paucity of mechanistic information remains a limitation, the balance of evidence, especially the volume of epidemiological evidence demonstrating no increased cancer risks associated with even the highest "real world" human-relevant exposures, strengthens the current analysis.

The conclusions we reached in this systematic review are similar to those of IARC. Specifically, our findings for pulmonary cancers are consistent with IARC's classification of inhaled talc not containing asbestos or asbestiform fibers as "not classifiable" as to its carcinogenicity, in that neither identified any clear increase in cancer risk in animals and humans, and no clear MOA for carcinogenesis was identified (4). Although one meta-analysis (5) reported a statistically significant meta-SMR of 1.45 (95% CI: 1.22–1.72) for non-asbestiform talc and lung cancer, this review included several occupational cohorts with mixed exposures to possible lung carcinogens such as silica, asbestos, and radon (miners) resulting in high heterogeneity across reported study results. However, considering several newer studies, combined with a more rigorous systematic review methodology, our evaluation differed from these, indicating suggestive evidence of no association.

Further, the physicochemical properties of non-fibrous talc (e.g., inertness) and recent evaluations of potential MOAs indicate talc poses little to no concern for carcinogenic effects in humans, especially at less than lung overload exposures. It also is worth noting that despite claims that some talcs and therefore some talcum powders contain trace amounts of asbestiform minerals, high occupational exposures leading to large excesses of pneumoconiosis deaths were not associated with increased mortality from lung cancer, and these studies consistently report no excess (in fact, no cases) of malignant pleural mesothelioma.

One strength of our review is that we drew from the strongest aspects of established methodologies of several organizations' systematic review guidance in an attempt to provide a full and transparent evaluation. We recognize, however, that there still may be areas where further refinement in the approach is possible.

In sum, and based on the integration of evidence from animal experiments, mechanistic evaluations, and epidemiological studies all of reasonable methodological quality, it is unlikely that talc and cosmetic talcum powders at human-relevant exposures cause human pulmonary cancers, including lung cancer and mesothelioma.

Data availability statement

The additional study results are presented in the article/Supplementary material; further inquiries can be directed to the corresponding author.

Author contributions

Conception/scoping: KM and HL. Analysis of evidence: HL, DL, OL, RF, AI, WT, JC, AU, and KC. Wrote sections of manuscript: HL, KM, WT, RF, AU, AI, OL, KC, JC, and DL. QA/review: MC, PB, and KM. All authors contributed to manuscript revision, read, and approved the submitted version.

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Conflict of interest

Authors HL, KM, WT, DL, OL, JC, KC, RF, MC, AI, and AU are employed by Stantec ChemRisk, a consulting firm that provides scientific support to the government, corporations, law firms, and various scientific/professional organizations. Authors KM, WT, MC, and AI have been retained as expert witnesses on behalf of defendants in litigation matters in which it has been alleged that products containing talc caused mesothelioma or other cancers. Author PB is Full Professor at the Renaissance School of Medicine at Stony Brook University and Department of Medical and Surgical Sciences, University of Bologna, and Senior Scientific Advisor to ChemRisk; he has no conflicts to declare. The content and the conclusions of the manuscript are exclusively those of the authors.

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Supplementary material

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.989111/full#supplementary-material

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REVIEW
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Applying Existing Particle Paradigms to Inhaled Microplastic Particles

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Ambient particulate pollution originating from plastic contaminates air, including indoor and urban environments. The recent discovery of ambient microplastic (MP) particles of a size capable of depositing in the thoracic region of the airway, if inhaled, has raised concern for public exposure and health impacts following lessons learned from other particle domains. Current microplastic exposure estimates are relatively low compared to total ambient particulate matter, but optimal analytical techniques and therefore data for risk and health impact assessments are lacking. In the absence of such an evidence base, this paper explores paradigms, metrics and dose-response curves developed in other particle domains as a starting point for predicting whether microplastic are of concern. Bio-persistence, presence of reactive sites and soluble toxicants are likely key properties in microplastic toxicity, but these are not measured in environmental studies and hence are challenging to interpret in exposure. Data from a MP inhalation study in rats is available but the study was conducted using conditions that do not replicate the known human health effects of $PM_{2.5}$ or surrogate exposures: compromised, aged animal models are recommended to investigate potential parallels between MPs and $PM_{2.5}$. One of these parallels is provided by tire wear particles (TWP), which form part of current ambient PM and are sometimes regarded as microplastic. A connection to epidemiological studies where PM filters are still available is recommended and consequently analytical advances are required. In summary, established particle domains and existing paradigms provide valuable insight and data that can be used to predict MP toxicity, and direct study design and key properties to consider in this emerging field.

Keywords: microplastic, particle toxicology, inhalation [MeSH], exposure, physicochemical properties, particulate matter

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INTRODUCTION

Microplastic particles (MPs) are comprised of diverse synthetic organic polymeric materials, varying by shape, size, density, weathering state, and chemical and/or microbial load. They originate from a variety of sources (plastic items and synthetic textiles) and activities and contaminate all of Earth's spheres. In air, their concentrations can range from <1 to 1,000 s per cubic meter (m^3). The most frequently observed polymers in air are polyethylene and polyethylene terephthalate, with polypropylene, polystyrene, polyamide, and epoxy resin particles also commonly observed. Most studies find that fragments are the predominant shape, followed by fibers, however, all these variables depend on the sample type, environment, geographical location, and analytical methodology employed (1–4).

Another source of synthetic polymeric aerosols is non-exhaust vehicle emissions. Non-metallic and semi-metallic brake pads may include synthetic polymers in the composite material, and the majority of a (synthetic) tire is comprised of synthetic polymer (e.g., styrene butadiene styrene). Several studies estimate that 3–7% of the particulate air pollution fraction $<2.5\,\mu m$ aerodynamic diameter ($PM_{2.5}$) consists of tire and brake wear [review: (5)] but there is considerable debate over whether these particles should be considered MPs (6). Many authors refer to tire wear particles (TWP) as MP due to its (semi-) synthetic polymer structure, solid state, insolubility, and particle size range (i.e., $<1{,}000\,\mu m$). In the environment, however, pure TWP are rarely found. Instead, hybrid particles consisting of tire and road wear particles are present (7).

Irrespectively, MPs form part of the complex and dynamic particulate matter (PM) exposure profile. Acute and long-term exposure to the $PM_{2.5}$ fraction is associated with increased mortality in cardiovascular and respiratory diseases (8, 9). Both $PM_{2.5}$ and PM_{10} are associated with increased mortality from all causes, cardiovascular disease, respiratory disease, and lung cancer. Associations remain below the current WHO guideline exposure level of 10 $\mu g/m^3$ for $PM_{2.5}$ (8). Since the first study on the link between acute mortality and $PM_{2.5}$ (10), several hundred studies have been published which have led to the estimate that an increase of 10 $\mu g/m^3$ $PM_{2.5}$ increases the risk of (premature) mortality by 1% (9).

Although MPs are claimed to be abundant in the environment, few studies are available that allow for an estimation of their relative contribution to $PM_{2.5}$ or PM_{10} exposure, hence the potential role of MP in premature mortality and non-communicable disease is poorly understood. Irrespective of this, within environmental and occupational particle exposures, there are sub-fractions that are likely to be more harmful than others, such as transition metals in PM_{10} (11–13), which contributes very little to mass. Here, the concentration of transition metals can be seen as the biologically effective dose (BED) [review: (14, 15)]. It is therefore important to determine whether inhaled MPs share a common Mode of Action (MoA) with other inhaled particles or are of disproportionate concern and why. This paper explores the application of existing particle paradigms to MP in the lung environment and, more specifically, investigates the hypothesis that its action may be already part of the ambient particulate air pollution.

PRINCIPLES IN PARTICLE TOXICOLOGY

Over the past 50 years, scientific principles of particle toxicology have been developed in important particle domains reflecting occupational and, later, environmental exposures. Research in coal mine dust (16, 17), respirable crystalline silica (18), asbestos and man-made mineral fibers (19, 20), ambient PM, poorly soluble low toxicity particles such as TiO_2 (21, 22), and engineered nanomaterials (23, 24) has helped define the physicochemical properties and dosimetric factors that influence the pathways leading to particle-triggered adverse outcomes (summarized in **Table 1**). The most important properties and factors are also interconnected. For example, the crucial "retained dose" in the lung following inhalation is equivalent to deposition (dependent on dimension and shape) minus what has been eliminated by defense (macrophage clearance) and/or dissolution (durability). Whilst all properties are important, some are more important in specific particle domains, such as shape/dimension and bio durability in the fiber domain and surface area in the engineered nanomaterials domain.

In the concept of BED, surface, composition, and size/dimension are integrated, where intrinsic reactivity is a multiplier of surface area, depending on the material the particle is made of. In addition, lessons from asbestos and manmade vitreous fibers (MMVF) inform us that, for fibers, length is important and so "shape" needs to be included as a factor. Furthermore, studies on PM_{10} welding fumes, etc., have shown that many particles are complex, containing soluble components

TABLE 1 | Parameters and factors that play a major role in the biological response upon particle inhalation.

Parameter	Description	Relevance for
Durability	Biopersistence, dependent on defense as well as on particle properties (dissolution).	Fibers, PSLT
Density	The density of material, along with size and shape, determines aerodynamic behavior and deposition in the airway.	All
Size/Shape	Size distribution (diameter, length) and shape influence aerodynamic diameter along with density, and macrophage clearance.	AIL, but fibers specifically
Surface area	The quantitative surface available for interaction with the environment.	PSLT, nanomaterials
Chemical composition	Bulk composition is not equal to surface chemistry. In addition, toxic components may be released upon dissolution.	
Surface charge	The presence and composition of functional groups.	Positively charged particles (nylon flock, TWP)
Surface reactivity	Reactive groups and radicals on the surface	Crystalline silica
Dose	Cumulative dose for chronic effects; can be based on particle or fiber mass, number, or surface area. Bulk composition is not equal to surface chemistry.	All
Deposition	Dependent on dimension/shape and density, but also on airway morphology (hot spots).	All
Defense	Mucociliary clearance, macrophage clearance, inflammatory cells. If macrophage clearance is saturated, overload occurs; dose increases exponentially with time.	All
Retained dose	Dose retained in the lung after clearance and dissolution (determined by dose, defense and durability).	All

PSLT, poorly soluble low toxicity particles; TWP, tire wear particles.

that can have considerable toxic potential. Finally, the duration the particle is likely to persist and avoid clearance determines the length of time that the BED is applied to the biological system (see "Durability," **Table 1**); thus, a bio persistence factor is required (14). Taking these factors together, the paradigm that could predict the toxicity of an unknown particle consists of three main attributes for the biologically effective dose:

1. Surface attribute = surface area (SA) × specific surface reactivity (i.e., reactivity per unit SA) × surface availability (not all surface area is available for biological interaction)
2. Dimension attribute = length – diameter (mainly length if greater than a critical length), and when combined with density, influences aerodynamic behavior, deposition and thus exposure
3. Composition attribute = volume × specific volumetric reactivity (i.e., the toxic material per unit volume) × availability (= release rate i.e., amount per unit time)

It is logical to assume that the effects of inhaled MPs in the airway are driven by the same set of particle properties. The challenge is to find those properties that are crucial to describe and regulate its effects along the concept of BED.

MICROPLASTIC INHALATION EXPOSURE

Of the published studies on airborne MP which employed an analytical technique capable of distinguishing between plastics to calculate concentrations, just 10 were found for urban and indoor environments. These environments are considered most relevant to population exposure due to population density and the proportion of time spent indoors, and hence were used. The key details of these studies and characteristics of observed MP are summarized in **Supplementary Table 1**. The study by Soltani et al. (25), whilst on indoor deposition rather than air, calculates an inhalation exposure concentration estimate and is thus included. Most studies have focused on outdoor air, with 30% analyzing indoor air and 20% analyzing both outdoor and indoor samples.

Polyethylene and polyester/polyethylene terephthalate were equally found to be the most common polymers in studies. One of these studies only targets polycarbonate and polyethylene terephthalate (26) and therefore may not be representative of all polymers present. The study which focused on an emission source (synthetic nails/nail salon) found the acrylic nail material to be most common in both indoor and outdoor air (27). Eighty per cent of the studies found fragments and irregular shapes to be most common, not fibers as are often predicted.

Observed concentrations generally increased with increased instrument limits of detection, as expected. Higher concentrations were on the order of 100 s (2–4) to 1,000 s (1, 2) of MPs per cubic meter of air and most studies found the majority of particles to be distributed in the smaller size classes. Few studies explicitly estimate exposure. Chen et al. (28) estimate nail salon employees to be exposed to 260 MPs per day. However, if inhaled, these particles are likely to deposit in the upper airways and be swallowed due to the particle sizes being >25 μm. Just two studies used a method with a limit of detection below 10 μm (1, 4), which is relevant to human exposure in the central and lower airways and subsequent adverse health outcomes. In impactor samples (>2.5 μm and 2.5–1.0 μm aerodynamic diameter) collected near Bremen City, Germany, Kernchen et al. (4) found 67% of observed MP were below 10 μm in size, giving an exposure concentration of 60/m^3. If normal adult minute ventilation is between 5 and 8 liters per minute, this results in an inhalation exposure estimate of 432–691 MPs per day. In a PM_{10} sample collected from an urban roadside site in London, UK, Levermore et al. (1) found 52% of observed MP were between 5 and 10 μm in size, resulting in an inhalation exposure estimate of 9,367–14,988 MPs per day. Whilst both studies adopt similar analytical methods and observe comparative proportions of <10 μm size classes and polyethylene, they are collected in different environments, which could explain the order of magnitude difference in concentration. Using high-density polyethylene (0.97 g/cm^3) as a worst-case representative polymer from these studies, the volume of a 10 μm sphere, and the highest particle number exposure estimates for each study, worst-case mass concentration inhalation exposure estimates are 0.67–14.54 μg/day. It is important to highlight that those studies which do not analyze an aerodynamic fraction, collected by an aerodynamic size selective sampler, observe a very broad and coarse size distribution. Those studies which analyze a sample collected with an aerodynamic size selective sampler (1, 4) observe a narrower size distribution, with a modal size around 10 μm (1).

Some data is emerging to suggest MP is inhaled into the lung, through the analysis of lung tissue digestates (27, 29). Most of the MP observed in these studies are relatively large compared to what is expected (i.e., a median size of 10 μm or below). Whilst there is a small chance that these particles were inhaled from the environment, one would expect to see a greater abundance of smaller MP for the anatomical regions studied and hence more data is needed to draw conclusions from these observations. Additionally, *in situ* analyses, such as via spectral imaging, will strengthen this, such as the method applied by Chen et al. (28) which detected a cellulose fiber in a lung tumor tissue section.

In general, exposure to ambient MPs is largely unknown and has possibly been either underestimated or overestimated due to analytical challenges and sampling methodologies that are often incompatible with routine ambient PM sampling. The assessment of MPs in PM filters is a first step toward assessment of the respirable fraction in ambient air. There is still a great body of work to do to understand the size distribution of airborne MP, which requires physicists, chemists, engineers, and environmental scientists. Furthermore, whilst properties such as size, shape/dimension and bulk composition are often measured and reported, other metrics intrinsic to a structure-activity paradigm, such as the concentration of soluble impurities, presence of reactive sites or surface area are not. Laboratory-based experiments can begin to rank the toxicological importance of the various properties of MP, however, without knowledge on how these relate to environmental exposures, one cannot conclude the level of risk.

TABLE 2 | Typical exposures, health outcomes, and current exposure standards for different particle domains.

Particle group	Main health effects	Exposure characteristics	Material characteristics
Coal mine dust	Lung function decrease, bronchitis, CWP, PMF, emphysema	Occupational, underground and surface (coal) mining 2–40 mg/m^3 for up to 20 years	Mixtures of minerals (crystalline silica) and organic components. Current OEL: 4 mg/m^3
Asbestos mineral fibers	Lung function decrease, lung cancer, mesothelioma	Occupational and consumers, insulation and production 1–1,000 fibers/cc	Fiber shaped minerals. Standards around 1 fiber /cc in most countries, long (>15 μm) and thin (<3 μm) of greatest concern.
Poorly soluble low toxicity particles (PSLT)	Lung function decrease, fibrosis, cancer	Occupational exposure (nuisance dusts). OEL values between 4 and 10 mg/m^3	Diverse group of insoluble materials including polymers, CB, TiO_2, talc, toner pigments.
$PM_{2.5}$/PM_{10}	Increased acute mortality and morbidity in patients with COPD or cardiovascular problems, long term cause of diabetes/lung cancer	Environmental exposure. WHO exposure standard: up to 100 μg/m^3 (24 h)	Complex mix of many components and adsorbed compounds varying per time and space. Includes ultrafine particles. Current standard: 20 μg/m^3 (24 h). No standard for UF particles.
Nanomaterials	No general health effects indicated	Occupational and consumer exposure (particle numbers and surface area instead of mass)	Endless variability in size, surface chemistry, and sub-molecular properties, with at least one dimension measuring <100 nm. Standards available for subtypes (TiO_2, CNT)
Microplastic particles	No general hazard identified	Omnipresent at low levels of exposure. Mainly non-respirable (>50 μm) due to analytical limitations	Synthetic and semi-synthetic materials, usually fibrous and fragments. No standard available

CWP, coal worker's pneumoconiosis; PMF, Progressive massive fibrosis; COPD, chronic obstructive pulmonary disease; CNT, carbon nanotubes; OEL, occupational exposure limit; MAK, German commission for occupational exposure limits; UF, ultrafine.

PARTICLE BIO-PERSISTENCE AND ADVERSE OUTCOMES

An array of lung cells generate inflammatory mediators and intracellular ROS upon exposure to diesel exhaust particles and PM (30) and also to MP model particles (31, 32). *In vivo*, acute inflammation is immediately followed by resolution and tissue repair, mediated through specialized pro-resolving mediators (SPMs) and type 2 cytokines and cells, including M2 macrophages and Th2 lymphocytes. For biopersistent particles and fibers in the lung, dose-dependent inflammation can progress to a type 2 inflammation, which eventually promotes interstitial fibrosis, granuloma formation, and tumorigenesis (reviewed in: 24). Recent studies also reveal the involvement of regulatory T-cells in the subsequent pathogenesis caused by inhaled particulates and therefore a long-term immune response is considered to be part of the long-term outcome (33). The persistence of MPs is thus considered a highly relevant property to consider in MP toxicology. Synthetic fibers (polypropylene, polyethylene, and polycarbonate) showed no dissolution, no significant changes to surface area and very slight weight gain following a 180 day *in vitro* leaching test in physiological fluid (Gamble's Solution), suggesting they may persist *in vivo* (34). However, respirable para-aramid fibers are considered a low risk since they have been observed to shorten in the lung and undergo rapid clearance in *in vivo* experimental studies. It has been hypothesized that lung fluid coats the fibers, catalyzing enzymatic attack and enabling biodegradation of inhaled p-aramid fibers in the lungs (35). Degradation has also been observed for inhaled polypropylene fibers in rats, which increased with exposure concentration and time (36). Whether this is apparent for other synthetic fibers is largely unknown and experiments are needed to assess their solubility, considering pH and enzymes. What is noteworthy, is that cellulose fibers were found to be more persistent than p-aramid fibers *in vivo* (37), and thus manmade cellulose fibers should be included in synthetic fiber hazard assessments. The inhalation of elevated levels of respirable plastic dust in occupational settings has been linked to interstitial lung diseases (38). Whether this pathogenicity is due to biopersistence and particle overload or the positive charges on the nitrogen atoms of nylon flock (39) remains untested.

Given the substantial variation within and between particle domains and MPs in their size, shape, aspect ratio, rigidity, and other physicochemical properties, it is rational to assume that their interaction with the immune system in the lung will differ within and from one another. The physicochemical properties of a particle govern the composition of the protein corona it acquires in biological fluids, which in turn affects bioavailability and fate *in vivo*. Since these properties vary by polymer, it is difficult to predict the behavior of a material *in vivo* (40). However, there are common disease phenotypes among different particles, fibers, and nanomaterials (**Table 2**). Therefore, whilst the molecular initiating events may differ, it is likely that some particulates share a similar adverse outcome pathway, such as inflammation > chronic inflammation and aberrant repair > fibrosis (33).

INHALATION STUDIES WITH MICROPLASTIC PARTICLES OR SURROGATES

Despite being a recognized component of PM for longer, whether ambient tire wear proportionally contributes to the effects of $PM_{2.5}$ is still being determined. Available *in vivo* toxicity data, obtained using TWP collected at a road simulator laboratory, suggest that inhaled TWP exert only mild respiratory toxicity (41). Female Sprague Dawley (SD) rats ($n = 10$/treatment group) were exposed to TWP at 10, 40, or 100 $\mu g/m^3$ via nose-only inhalation for 6 h/day for 28 days and toxicity was assessed following OECD guidelines (TG 412). No TWP-related effects were observed on survival, clinical observations, body or organ weights, gross pathology, food consumption, immune system endpoints, serum chemistry, or biochemical markers of inflammation or cytotoxicity (41). Unfortunately, $PM_{2.5}$ was not used as a reference, although this was part of the study hypothesis, and only diesel exhaust particles and mineral particles such as TiO_2 and SiO_2 were used in a separate study-arm investigating bronchoalveolar lavage (BAL) after intratracheal instillation (41). Based on the NOAEL level from the TWP study (55 $\mu g/m^3$), and exposure estimates for TWP, this group of investigators propose that a margin of exposure of 400–700 is present between the current $PM_{2.5}$ standard and TWP exposure (42, 43).

Important to note is that health effects due to ambient PM exposure are observed mainly in sensitive subgroups within human populations and in animal models mimicking such populations (44). Nearly all reported *in vivo* studies on toxicological responses to ambient PM and/or standards produced no or low responses in normal laboratory animals compared to effects seen in humans. Therefore, spontaneously hypertensive (SH) rats and APO $E^{-/-}$ deficient mice have been used to model acute respiratory and cardiovascular responses to ambient PM (see **Supplementary Table 2**), whereas OECD protocols prescribe the use of normal rats or mice.

A selection of animal inhalation studies with ambient PM or surrogates have been included in **Supplementary Table 2** to illustrate that responses in compromised animals mimic cardiovascular effects such as blood pressure, heart rate variability and systemic inflammation observed in humans in epidemiological studies. However, the concentrations needed to induce systemic effects with ambient air particles are usually higher in (compromised) rats than in man (**Supplementary Table 2**). As a benchmark, intratracheal instillation of 1,000 μg, but not 200 and 500 μg, $PM_{2.5}$ suspension in aged SHR rats led to pulmonary and systemic events (45), whereas instillation of 100 μg of $PM_{2.5}$ suspension in humans led to significant cellular responses in the BAL fluid (11). This underscores the fact that no current animal models reflect human sensitivity to inhaled fine particles.

Therefore, it is no surprise that studies with normal animals, according to OECD protocols, have shown little effects for higher concentrations of polymer, tire and road wear particles (see **Supplementary Table 2**) and pharmaceutical acrylic ester polymers (data not shown). However, rats exposed to polystyrene nanoparticle mass concentrations similar to those of TWP (20, 50 and 100 $\mu g/m^3$) for 6 h per day, 5 days/week for 2 weeks showed a statistically significant decrease in hematological parameters, including white blood cell and lymphocyte counts, and a statistically significant increase in percent and number of eosinophils, but only in female rats (32). Effects on lung lavage were less pronounced, due to high variation, but a potential inflammatory and fibrotic response was observed from the lowest concentration of PS nanoparticles at 22 $\mu g/m^3$ (32). The retained dose per rat (5–34 μg/day) (32) is similar to the alveolar burden in ultra-fine (50 nm) carbon particle studies (45, 46), estimated at 10.6 μg or 5.5×10^{11} ultra-fine carbon particles. It is evident that MP inhalation studies (**Supplementary Table 2**) used longer exposure times than used for $PM_{2.5}$ and focused on sub-chronic effects in the lung; however, the exposure concentrations used are not dissimilar from the PM studies in compromised animals. Thus, a retained dose-based read across is plausible for the inflammatory outcomes in the lung. Effects in compromised animals remains to be studied.

EXPOSURE TO PARTICLE-CHEMICAL MIXTURES

In both $PM_{2.5}$ and PM_{10} epidemiological studies, black carbon has been identified as a separate descriptor of health effects (47, 48) and reduced exposure is recommended. Whilst not directly toxic, toxicological studies suggest that black carbon may operate as a universal carrier of toxic species, providing a mechanism of entry to the human body and transport to target tissues. Low concentrations of components absorbed on the particle surface may actually be the driver of the adverse response. In this context it has been shown that metals and quinones on the particles surface are crucial for initiating an inflammatory response (11) in humans and DNA damage (49) in rats exposed to PM. In addition, bacterial endotoxin (LPS), which is a well-known initiator of inflammatory cascade, is commonly adsorbed on PM, although most prominent in PM_{10} and less in $PM_{2.5}$ (50).

Such a carrier mechanism can be easily assumed for substances absorbed on or contained in MPs, following inhalation. Several different synthetic polymers have been shown to carry different toxic metals (51, 52), including redox-active Cu-ions. The absorption of metals on MPs has also been shown to affect the kinetics and toxicity of both metals and particles in *Daphna magnia* (53) and Zebrafish embryos (54), but little data using human target cells are available. Bradney et al. (55) discussed the potential mechanisms and evidence by which trace elements (including metals) can achieve a different ADME when carried by MP but had no specific outcome for inhaled MPs. A recent review by Hahladakis et al. (56) provides an overview of all potential chemical additives present in plastics. While this overview focused on migration, release and fate of additives, no attention is given as to the additive effect of such components to particle toxicity. From other particle domains, including PM, we know such can be a very important trigger of the inflammatory response such as through redox mechanisms or surface reactivity (49). It also needs to be recognized that in some cases the role of contaminants has been overestimated. Well-designed inhalation

studies showed that the role of polycyclic aromatic hydrocarbons carried by diesel exhaust particles was much less important for toxicity than initially assumed (57, 58).

The absorption of external contaminants on airborne MPs is an area which requires research and modeling. Thermodynamic modeling has shown that at least in the aquatic environment, MP contributes a negligible amount of chemicals to biota, relative to the environment and uptake via natural pray, mostly due to their low concentrations and therefore low likelihood of an organism encountering one relative to pray (59, 60). Studies in ambient PM have shown that particles aggregate and agglomerate during formation and transport and particle composition is determined by condensation and colliding and merging of primary and secondary particles even during the day (61). Based on these observations and the occurrence of many particle types that can be considered as MPs (e.g., tire wear, fibrous fragments) studies are needed to evaluate whether ambient MPs may act as a vector or form particle complexes, which increase exposure and may enhance particle activity, such as for ambient PM. This should be considered for total suspended MP, since inhaled MP $> 10\,\mu m$ in aerodynamic diameter may still deposit in the upper airways.

CONSIDERING MICROPLASTIC AS PART OF $PM_{2.5}$

Another approach to explore potential MP-driven health effects is to study the MP content in $PM_{2.5}$ or PM_{10} filters of exposure studies that have been used in epidemiological studies. Levermore et al. (1) showed that both virgin and environmental MPs ($>2\,\mu m$) can be detected via Raman spectral images. Application of this technique to available filter samples could give an estimate on the percentage of MP in those samples for comparison with available estimates (42, 43). The health impact of that presence could be calculated via the proportion of PM_{10} or $PM_{2.5}$ and associated health risks (top-down approach) or via the rat toxicity data of TWP (42) or model polymers [e.g., (32)], which can be considered as bottom-up. The top-down approach would be based on many samples from all over the globe that are linked to both acute and chronic mortality due to PM. The assumption that the effect is based on the mass or number proportion is simply based on assuming that the best metric of health effects of PM is mass/m^3.

As an example, if 0.1% of the mass on PM filters is microplastic, the proportionate mortality rate at 10 $\mu g/m^3$ increase would be 1%/1,000 = 0.001%, instead of 1% as observed for $PM_{2.5}$ (9). Even though this number is small, the recent analysis by Schwartz et al. (62) on individuals continually exposed to low PM levels found this led to 14,000 premature deaths per year per 1 $\mu g/m^3$ in the USA. Extrapolating this to MP (and 0.1 % of mass) would still lead to significant numbers at the current average PM exposure. Of course, this is with limitations, since it assumes proportional linearity in the toxicity of different PM sources, but it provides a start.

In summary, MPs likely have similar hazards to other particle domains, such as ambient PM, PSLTs and engineered nanomaterials. However, in the absence of inhalation exposure assessments, the level of risk is uncertain. Either way, plastic is a source of PM_{10} and $PM_{2.5}$, which are regulated on a (total) mass basis only. Thus, a better understanding of MP sources and quantification of emissions will determine whether and where interventions are needed to ultimately reduce $PM_{10/2.5}$ exposure and future disease burdens. The concept of BED provides a conceptual basis for read-across. The concept may provide a framework for filling in knowledge gaps for MP such as (gravimetric) exposure, particle dimensions and surface activity in lung response.

AUTHOR CONTRIBUTIONS

SW and PB contributed equally to the conception and design of the article, the acquisition, evaluation and interpretation of the data and the drafting and editing of the manuscript. Both authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.868822/full#supplementary-material

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Inflammation as a Key Outcome Pathway in Particle Induced Effects in the Lung

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Inflammation is considered a key event in the pathology of many chronic diseases, including pulmonary and systemic particle induced effects. In addition, inflammation is now considered as the key response in standard setting for poorly-soluble low toxicity (PSLT) particles and also the critical endpoint to screen for in OECD based sub-chronic animal inhalation testing protocols. During Particles & Health 2021, an afternoon session was dedicated to the subject and a brief summary of the most important messages are summarized in this paper. In the first part of this session, two speakers (Prof. Lison and Dr Duffin) provided state of the art insight into different aspects and sequels to (persistent) inflammation as a protective or adverse response. Most recent insights on the role of different macrophage cell types were presented as well as perspectives and data provided by inflammatory pathways in humans, such as in asthma and COPD. A brief review of the expert workshop on PSLT particles focusing on the regulatory impact of using persistent inflammation as a key outcome was provided by Kevin Driscoll. The second part of the session focused on the outcomes that are associated with inflammation in animal studies, with an emphasis by Drs. Harkema (Michigan State) and Weber (Anapath) on cell proliferation and other pathologies that need to be considered when comparing human and animal responses, such as outcomes from 14- or 28 day inhalation studies used for specific target organ toxicity classification.

Keywords: inflammation, cancer, particles, genomic instability, macrophages, neutrophils, hazard assessment

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NOVEL INSIGHTS INTO INFLAMMATION AND CANCER

Prof. Lison (Université catholique de Louvain, Brussels) gave a key note presentation entitled "Macrophages, inflammation and cancer" and presented the paradigm of cancer development proposed by Mantovani et al. (1), which connects inflammation, oncogene activation and genomic instability. The connection between inflammation and cancer includes two pathways: an extrinsic pathway, driven by inflammatory conditions that increase cancer risk (such as in silicosis) and an intrinsic pathway, driven by mutations in cancer cells (such as oncogene activation) which cause inflammation and neoplasia (**Figure 1**). The intrinsic pathway was uncovered when investigating

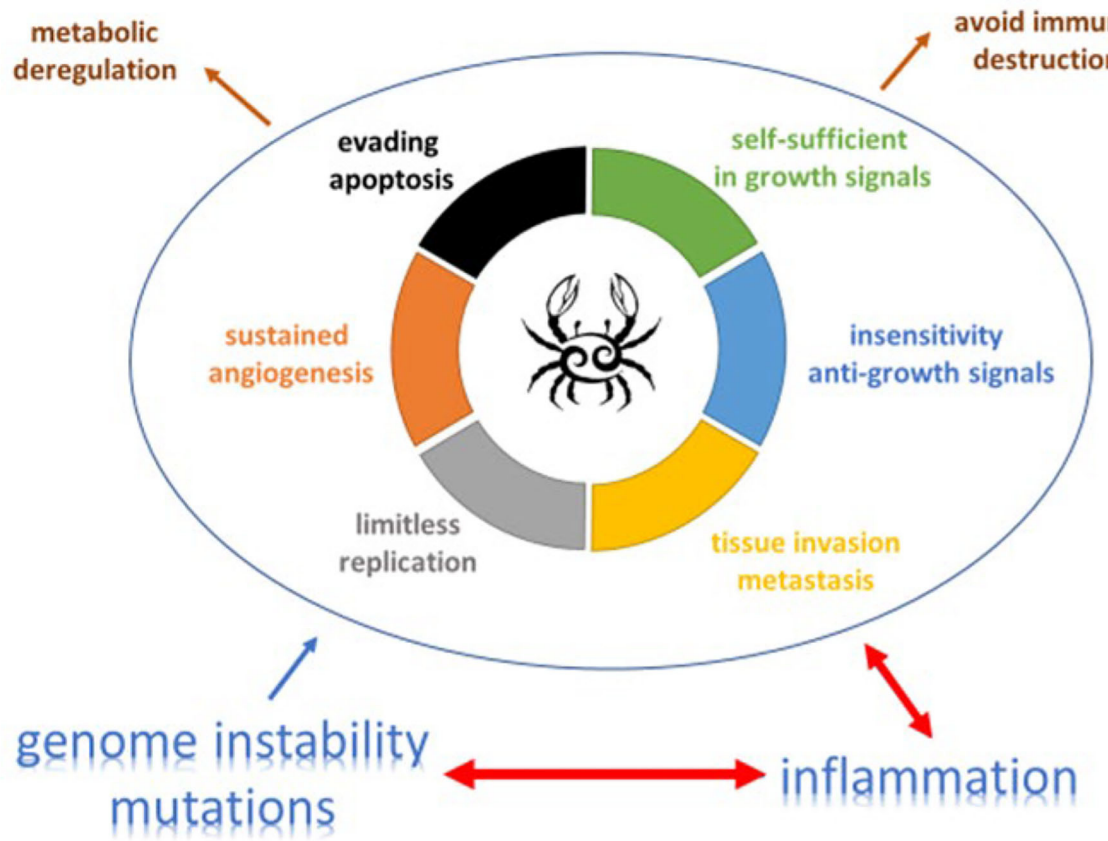

FIGURE 1 | The complex relation and balance between genomic instability, inflammation and cancer promotion and control [adapted from (19) and (20)]. An increasing body of research suggests that two additional hallmarks of cancer are involved in the pathogenesis of some and perhaps all cancers. One involves the capability to modify, or reprogram, cellular metabolism in order to most effectively support neoplastic proliferation. The second allows cancer cells to evade immunological destruction, in particular by T and B lymphocytes, macrophages, and natural killer cells. Additionally, two consequential characteristics (lower half of figure) of neoplasia facilitate acquisition of both core and emerging hallmarks. Genomic instability endows cancer cells with genetic alterations that drive tumor progression. Inflammation by innate immune cells designed to fight infections and heal wounds can instead result in their inadvertent support of multiple hallmark capabilities, thereby manifesting the now widely appreciated tumor-promoting consequences of inflammatory responses.

why inflammatory cells and mediators are present in the microenvironment of most, if not all, tumors. Oncogenes can, for instance, encode tyrosine kinases that are persistently activated in a ligand-independent manner as a result of mutation or chromosomal rearrangement NF-κB is a key transcription factor that coordinates innate immunity and inflammation, but also emerged as an important endogenous tumor promoter (2). The expression of cytokines such as TNF-alpha, TGF-b, IL-6 and other inflammatory mediators is also regulated by NF- κB. NF-κB is crucial both in the context of neoplasia and in the context of inflammatory cell function. It acts downstream of the sensing of microorganisms or tissue damage via the Toll-like receptor (TLR)–MyD88 signaling pathways.

However, one has to realize that inflammation is a dynamic process involving many different effector cells, and these cell populations interact. Macrophages are key in orchestrating the lung inflammatory response to inhaled particles. Recent research has revealed a significant phenotypic heterogeneity in the population of macrophages in lung, and this information has been, so far, insufficiently integrated in particle toxicology. In the lung, as in most other organs, the macrophage phenotype is determined by different parameters, including their ontogeny and tissue localization (niche). Tissue-resident alveolar macrophages (AM) migrated from the embryonic yolk sac and liver to the lung, and have self-renewing potential through local proliferation. Blood monocyte-derived macrophages migrate to the lung in response to insults (viral infection, particle deposition, smoking, etc.,) and can differentiate into interstitial macrophages (IM) or AM. This distinction is important because monocyte-derived macrophages appear to be more reactive than

Abbreviations: AM, Alveolar Macrophages; BrdU, 5-bromo-2'-deoxyuridine (incorporation in DNA); ECHA, European Chemical Agency; IARC, International Agency on Research of Cancer; Il-6, Interleukin 6; MyD88, Myeloid differentiation primary response 88 protein; NETs, Neutrophil Extracellular Traps; NIOSH, National Institute of Occupational Health & Safety; NFkB, Nuclear factor kappa B; NOAEL, No observed Adverse Effect Level; PCNA, Proliferating cell nuclear antigen; PSLT, Poorly Soluble Low Toxicity particles; OECD, Organization for Economic Co-operation and Developmen; RAC, Risk assessment Committee of ECHA; SAS, Synthetic amorphous Silica's; TLR, Toll-like receptor; TGF-B, Transforming Growth Factor-Beta; TNF, Tumor necrosis Factor.

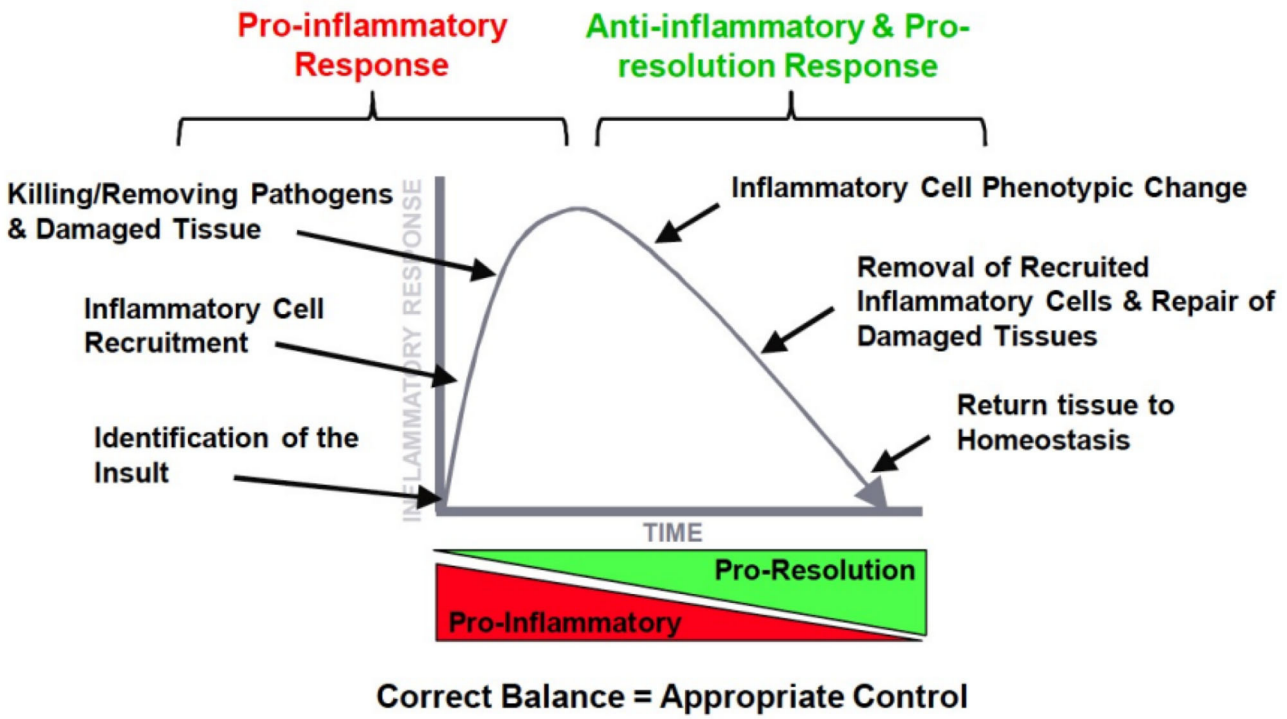

FIGURE 2 | The ideal orchestration of the inflammatory response with an initial pro-inflammatory response and resolution of this response by inflammatory mediators.

resident AM (3). Following successive insults to which the lung is submitted during life, the population of lung macrophages is thus progressively enriched in monocyte-derived cells, which contributes to increasing sensitivity of the lung to subsequent insults (4). It has also been recently discovered that macrophages associated with blood vessels or nerves in the lung tissue express a specific phenotype. For instance, blood vessel-associated IM appear to exert anti-inflammatory and anti-fibrotic functions (5). This phenotypic heterogeneity has important implications for the selection of appropriate *in vivo* models to evaluate inflammatory response to inhaled particles. For instance, rats that are housed in a clean environment have a high proportion of resident AM, which is totally different from those that are pre-exposed to pathogens such as in real-life conditions. Regarding *in vitro* lung macrophage models used in particle toxicology, the relevance of primary cells (generally obtained from clean animals) and cell lines should be carefully considered in terms of the *in vivo* responses that are being modeled.

Most studies of the mechanisms of cancer-related inflammation have focused on the early stages of pathology, but inflammatory mediators and cells are also involved in the migration, invasion and metastasis of malignant cells. Chemokines and cytokines coordinate autocrine and paracrine interactions between malignant cells and infiltrating leukocytes. These interactions increase the migration, invasion and survival of malignant cells. They also affect the growth of the primary tumor and the ability of tumor cells to colonize the metastatic niche (6).

Then of course there is the inhibitory side of inflammation to tumor growth. There is strong evidence from genetic studies using mouse models that cells of the adaptive immune system carry out surveillance and can eliminate nascent tumors, a process called 3mmune-editing (7). Innate immune responses, which manifest as inflammation, are crucial for the initiation of adaptive immune responses. Therefore, the seemingly divergent effects of inflammation and 3mmune-editing are paradoxical. Yet, a recent study in mice (8) shows that the TLR adaptor MyD88 (which is involved in innate immune responses) has a key role in promoting tumor development and that inflammation-induced carcinogenesis and 3mmune-editing can occur in the same tumor model.

RESOLUTION OF INFLAMMATION AS KEY TO PERSISTENT EFFECTS

Dr. Duffin went into more detail on the time course of events, mediators, and cells during the different phases of the inflammatory response. In **Figure 2**, it is illustrated how ideally a pro-inflammatory response turns into a second phase after the insult has been eliminated, that is the resolution of the inflammatory profile by an anti-inflammatory network. The ultimate goal is resolution with a return to tissue homeostasis, but, of course, this is different when returning from a neutrophilic inflammation which is designed to kill, or from a macrophage

influx which is meant to clear invaders from the lung. Inflammation is essential for normal host defense, but a timely switch from inflammation to resolution is essential to limit host damage. The therapeutic potential for manipulating this process is huge.

Neutrophil apoptosis is a key process in inflammation resolution and can lead to different outcomes such as necrosis, NETosis and necroptosis (9). Especially the formation of NETs (Neutrophil Extracellular Traps) is relatively unknown as they are similar to a conserved anti-microbial defense mechanism (10), releasing DNA complexed to neutrophil proteins. The formation and release of NETs are implicated in tissue damage and inflammation in a number of inflammatory and autoimmune conditions (11) and are highly dynamic processes.

Regarding its meaning for PSLT particles, Dr. Duffin concludes that inflammation is a great marker of particle exposure, but inflammation isn't always a straightforward response. Just as explained by Prof. Lison, inflammation is highly dynamic, highly orchestrated, and at times complex, i.e., acute vs. chronic, innate vs. adaptive response. Timing seems to be key, and we don't seem to know what the difference in impact is between an inflammatory response in an already diseased system and as a primary defense compared to that in a healthy uncompromised organism.

INFLAMMATION AND OVERLOAD ARE KEY IN PARTICLE CLASSIFICATION

Dr. Driscoll summarized the premises and outcomes of the PSLT workshop in Edinburgh which was held in April 2019 and its outcomes published in 2020 (12). The background is that rats exposed chronically to high concentrations of respirable PSLT materials develop lung cancer (e.g., titanium dioxide, carbon black). However, lung cancer only occurs at doses which overload lung macrophage particle clearance. Moreover:

- Lung cancer is preceded by marked inflammation and epithelial proliferation.
- Lung cancer after PSLT particulate exposure has not been demonstrated in mice and hamsters.
- PSLT materials are not directly genotoxic.

Therefore, lung cancer in the rat is believed to be a consequence of persistent inflammation and epithelial cell proliferation. Until now epidemiological studies in workers with carbon black, talc, or titanium dioxide have not demonstrated associations between PSLT particle exposure and lung cancer in humans. It is interesting, however, to note differences in how regulatory bodies have dealt with observations in a different manner:

- IARC has classified titanium dioxide and carbon black as possibly carcinogenic to humans based on the rat lung cancer data (and absence of increased lung cancer in epidemiology studies). Talc is not classifiable as a human carcinogen (inhalation route).
- The Committee for Risk Assessment (RAC) from the European Chemical Agency (ECHA) classified titanium dioxide as suspected of causing lung cancer through the inhalation route based on a rat data with titanium dioxide and supporting evidence from rat studies with other PSLTs, i.e., carbon black. Carbon black and talc are currently under review based on submissions of France and Netherlands, and expected to follow the same pathway (Class 2 carcinogen).
- NIOSH differentiated the cancer hazard of titanium dioxide based on particle size and the exposure level in rats causing cancer. Ultrafine size ($<0.1\,\mu m$ diameter) titanium dioxide is considered a potential human carcinogen, while the larger size ($>0.1\,\mu m$ diameter) particles are considered unclassifiable as to human carcinogenicity.

The expert panel that convened in 2019 (12) defined a process for characterizing a material as a PSLT, provided guidance on inhalation study design, and reached consensus on OEL setting: the prevention of lung inflammation should be the driving principle in PSLT particle risk assessment and exposure limit setting. More importantly, consensus was based on the relevance of PSLT particulate exposure and rat lung cancer to humans: (i) the rat lung tumors occurring only under lung overload conditions is not relevant to human lung cancer hazard in the context of non-overload conditions, and (ii) PSLT particles should not be considered as human lung carcinogens based on rat data alone and no additional supporting data (from other species, mechanisms).

Dr. Driscoll's key messages included the fact that we need clarity on what materials are PSLTs and which are not, including the importance of particle size in the definition. Of particular note was his observation regarding coal dust, which has been referenced by regulatory agencies as evidence that PSLT particles can overload humans lungs and potentially cause cancer. The issue that Dr. Driscoll highlights is that coal dust particulates are not a PSLT and, so, comparison to titanium dioxide or carbon black does not have a scientific basis. In current regulatory context, substances are classified and missing pieces of evidence are usually taken from other dossiers, such as in TiO_2. In addition, the current classification in Europe is based solely on lung cancer in rats under conditions of marked overload. Therefore, the PSLT particle hazard classifications need to be revisited to determine if they remain appropriate.

Pulmonary Cell Proliferation: The Missing Link?

Dr. Harkema's talk focused not on inflammation, but on one of the sequels of inflammation leading to lung remodeling, namely epithelial cell proliferation in toxicant-induced lung injury, repair, adaptation, and cancer. He started off with a seminal paper by Evans et al. (13) and more recently by Aspal and Zemans (14) documenting the signaling pathways involved in this differentiation process. After a brief review of general mechanisms in carcinogenicity, dr Harkema showed how cell proliferation should be considered as a key process in particle induced carcinogenesis (15).

Using data from earlier carbon black inhalation studies he illustrated the development of particle-induced proliferative and inflammatory lesions in rodent lungs. Whereas, initially only inflammation is visible, proliferation is evident after 3 months of exposure, while hyperplasia and fibrosis started to occur at 6–12 months. It takes a lifetime exposure (up to 24 months) in rats to develop lung tumors. However, sustained pulmonary epithelial hyperplasia, a key and necessary factor in carcinogenesis, occurs within weeks to months of particle exposure. Therefore, he went into more detail on the potential use of detecting sustained epithelial cell proliferation in short-term bioassays for predicting particle-induced lung cancer.

The standard process for evaluating substances for carcinogenic activity is to perform a dose range finding study, usually 90 days, in the test species, followed by a 2-year bioassay. Now, short-term (28, 90 days) screening assays in rats and other rodents focus on mode of action and human relevance. However, what is ultimately needed is a determination as to whether the inhaled agent or particle poses a cancer risk to humans and not an investigation to determine if a chemical poses a cancer hazard in rats or mice. He proposed a protocol for a short-term inhalation study with evaluations at 1, 4, and 13 weeks with (1). Postexposure evaluation(s) for sustained proliferation, (2). Histopathologic evaluation of alveolar epithelial hyperplasia and immunohistochemistry for DNA synthesis (e.g., BrdU, PCNA), 3) Morphometric analyses for quantitative pathology (labeling index using digital image analysis), 4) Detecting cell proliferation then developing adverse outcome pathway to evaluate human relevance, 5) Relatively new to the field: incorporate transcriptomic analyses (RT-PCR; bulk tissue RNA sequencing), and 6) Additional analyses such as single-cell RNA sequencing and organoid assays as an *in vitro* target system (16).

Pathology to Be Reconsidered?

Dr. Weber (Anapath) discussed a number of important issues in relation to current papers on particle toxicology. Study design weaknesses such as small numbers of animals per treatment group, lack of awareness regarding context, and variables that influence test models are not given due consideration. Such papers present bias and also have regulatory consequences. As an example, he discussed a study by Reuzel et al. (17) in which lung fibrosis was noted in rats, but only at a high concentration of synthetic amorphous silicas (SAS). This paper has been crucial in the regulation of amorphous silicas, but, upon independent review of the slides and original materials, a Pathology Working Group comprised of well-recognized pathologists in the field of inhalation pathology did not find fibrosis at all in a single group or animal. In addition, no irreversible chronic persistent inflammation was observed. No substance induced fibrosis, i.e., all SAS types showed comparable effects, although minimal variations in the degree of inflammatory changes were observed and could be related to physicochemical differences (surface area, particle number). The NOAEL of SAS was between 17 and 46 mg/m^3 and fibrogenesis was a reversible effect that was observed in all SAS exposure groups. It is recommended that material from future and current studies is kept for independent evaluation since:

- 30 Year-old slides can be de-cover-slipped, re-stained (with standard hematoxylin and eosin staining) and then cover-slipped again, whereby the de-cover slipping may potentially have damaged the original tissue samples and fibrosis might be no longer evaluable;
- Single incidences of minimal focal fibrosis can be observed and fibrosis can be a reversible process. Weber et al. (18) noted such lesions without relation to the concentration and a slight increase in fibrogenesis in the high dose males (2/10). There was also an increase in inflammation indicators, comparable with the other effects noted by Reuzel et al. (17).

The message Dr. Weber wants to bring across is that it seems that pathology is only used as circumstantial evidence and not as the real gold-standard endpoint. In addition, the quality of investigations and resulting interpretation need to be defined more carefully in order to avoid misclassification of effects. The discussion with RAC suggests that also the process of re-evaluation of existing information is not optimally integrated in the classification process. Weber proposed that all studies that might cause a large-scale impact on established chemicals should undergo independent, professional peer review and expert pathological assessment. This could be costly, but should be performed at the discretion of the product owner considering the economical of its outcome.

CONCLUSION

Particle induced chronic lung inflammation is seen as the hallmark related to pulmonary effects such as lung fibrosis and lung cancer. Occupational exposure limits for particles are often based on preventing this intermediate, chronic inflammation. The evidence for this Mode of Action is mainly based on rat studies and in that species a one-to-one relationship between inflammation and cancer is assumed. Most studies of the mechanisms of cancer-related inflammation have focused on the early stages of pathology, but inflammatory mediators and cells are also involved in the migration, invasion and metastasis of malignant cells. In addition it is demonstrated that the resolution of inflammation by an anti-inflammatory network and return to tissue homeostasis is an important step in mitigating potential further tissue damage. Structural cell proliferation as the intermediate, local sequel of inflammation should be considered as a key element of *in-vivo* bioassays. Finally, the gold standard for cancer hazard remains pathological evaluation of lung tissue after long term exposures and follow-up and not the short term bio-assays (28 and 90 days) that are often used for particle materials and tend to focus on inflammatory response. Although there is a wealth of evidence of the link between particle-induced inflammation and cancer in the rat, many unknowns in humans are still present and current data (in humans and other species) suggest no such relation. The need remains for a more

sophisticated understanding of inflammation in humans during particle inhalation exposures and its role in overall risk of lung cancer.

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Review of Lung Particle Overload, Rat Lung Cancer, and the Conclusions of the Edinburgh Expert Panel—It's Time to Revisit Cancer Hazard Classifications for Titanium Dioxide and Carbon Black

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Chronic inhalation of titanium dioxide or carbon black by rats at concentrations which overload lung particle clearance can result in lung cancer. Based on this rat lung response, IARC, NIOSH, and ECHA classified titanium dioxide, and IARC classified carbon black, as potential human carcinogens. These classifications have been questioned based on an extensive data base demonstrating: the rat lung cancer occurred only under conditions of extreme lung particle overload; the lung cancer response in rats has not been seen in other animal species; and studies in titanium dioxide and carbon black exposed human populations have not shown an increased incidence of cancer. In 2019 an international panel of science and regulatory experts was convened to document the state of the science on lung particle overload and rat lung cancer after exposure to poorly soluble low toxicity particles. Regarding hazard identification, the expert panel concluded, in the absence of supporting data from other species, lung particle overload-associated rat lung cancer does not imply a cancer hazard for humans. Regarding high to low dose extrapolation, the expert panel concluded rat lung tumors occurring only under conditions of lung particle overload are not relevant to humans exposed under non-overloading conditions. The conclusions of the Edinburgh Expert Panel directly conflict with IARC, ECHA and NIOSH's extrapolation of lung particle overload associated rat lung cancer to hazard for humans. The hazard classifications for titanium dioxide and carbon black inhalation should be assessed considering the state-of-the-science on lung particle overload and rat lung cancer.

Keywords: lung overload, titanium dioxide, carbon black, rat lung cancer, hazard classification

INTRODUCTION

Lung Particle Overload

The term "lung particle overload" refers to impairment of particle clearance from the deep lung after inhalation of high concentrations of poorly solubility, low toxicity materials exemplified by titanium dioxide and carbon black. The impairment of particle clearance under these circumstances was proposed by Morrow (1) to be due to the physical loading of macrophages with the consequent loss of cell mobility. Subsequent research has supported impaired macrophage mobility as a contributing factor to lung particle overload and implicated other mechanisms including translocation of particles to the interstitium and lung lymphatics (2, 3). Critical to the definition of "lung particle overload" is it applicability only to materials of low inherent toxicity which differentiates lung particle overload from particle clearance impairment caused by inherently toxic materials which can directly damage macrophages or other lung cells, and in this way reduce particle clearance. Impairment of clearance by inherently toxic materials reflects a different adverse outcome pathway (AOP) from that described by the term "lung particle overload."

Rat Lung Cancer and Lung Particle Overload

Over three decades ago, Lee et al. (4) reported chronic exposure of rats to high concentrations of titanium dioxide resulted in lung cancer. This study involved exposure to 10, 50 and 250 mg/m^3 of respirable particles with lung cancer observed only for the 250 mg/m^3 exposed rats. Following the Lee et al. (4) publication, additional chronic inhalation studies have reported lung cancer in rats exposed to titanium dioxide and other poorly soluble materials considered to be of low inherent toxicity (summarized in **Table 1**).

There now exists an extensive toxicology data base in rats and other species on the lung response to poorly soluble, low toxicity particles which has implications for the human relevance of the rat lung cancer response. The following are key findings:

TABLE 1 | Chronic inhalation studies in rats producing lung cancer.

Material	Concentration (mg/m^3)	Lung Burden (mg)	Lung Cancer	References
Carbon Black	11.6	43.8	Yes	(5)
Carbon Black	2.5	21.0	Yes	(6)
	6.5	38.5	Yes	
Talc	6	9.7*	No	(7)
	18	26.7*	Yes	
Titanium Dioxide	10	39.2	Yes	(5)
Titanium Dioxide	10	25.5	No	(4)
	50	124.0	No	
	250	665	Yes	

**Lung burden normalized to air control lung weight (g).*

- Rat lung cancer after inhalation of poorly soluble low toxicity particles occurs only under exposure conditions which overload macrophage-mediated particle clearance i.e., cause lung particle overload (3, 8–10).
- A consequence of overloading clearance is a build-up of particulate material in the lung disproportionate to exposures which do not overload lung particle clearance (1, 11).
- In addition to lung cancer, lung particle overload in rats is associated with pulmonary inflammation; lung epithelial cell hyperplasia and metaplasia; and pulmonary fibrosis. These non-neoplastic responses precede development of lung cancer and occur at exposure levels not causing cancer (4–7, 12).
- Lung cancer has not been observed in other animal species (i.e., mice and hamsters) after chronic inhalation exposure to the materials in **Table 1** under conditions of lung particle overload (5–7, 13, 14).
- Epidemiology studies have not demonstrated a significant increase in lung cancer after exposure to the materials in **Table 1** (15–18).

Hazard Classification of Titanium Dioxide and Carbon Black

Several organizations have characterized the health hazards associated with titanium dioxide and carbon black inhalation, including IARC, ECHA, and NIOSH. The outcomes of these evaluations are summarized below.

IARC

IARC classified titanium dioxide and carbon black as "possibly carcinogenic to humans" based on lung cancer occurring in rats (16). The IARC review reported there was no convincing evidence of cancer in humans exposed to these materials. In the same IARC monograph, talc was determined to be not classifiable as to its carcinogenicity based on limited data in animals (e.g., a single chronic inhalation study in rats which showed lung cancer under lung clearance overload and a chronic inhalation study in mice which was determined to be negative for cancer) and, inadequate evidence in humans for talc not containing asbestos or asbestiform fibers.

ECHA

ECHA's Committee for Risk Assessment (RAC) recommended titanium dioxide be classified as suspected of causing lung cancer through the inhalation route (19). This assessment was based on a chronic titanium dioxide inhalation study in rats which, in the words of the study investigators (5): "*induced lung tumours in rats under conditions of marked particle loading in the lung.*" RAC concluded human data do not support an association between occupational exposure to titanium dioxide and risk of lung cancer. ECHA subsequently adopted the RAC recommendation on titanium dioxide hazard. Observations from the RAC documentation on titanium dioxide include:

- RAC did not use the Lee et al. (4) study to support its classification, concluding "*these exposure conditions represent excessive exposure which invalidates the results of the Lee et al. (4) study on their own for classification purposes.*" RAC

noted such a marked condition of overload should not be a determining factor on classification of titanium dioxide.
- RAC's classification of titanium dioxide relied on *"selected carcinogenicity data for poorly soluble low toxicity particles as supporting evidence*." RAC did not provide a definition of poorly soluble low toxicity particles, although carbon black was discussed in this context.
- In selecting relevant studies for classification RAC chose not to follow OECD Guidance Document 116 (20) which recommended safety evaluation not be based on experimental exposure levels of particles resulting in an elimination half-time of ∼1 year due to lung overload. RAC's rational for not following OECD guidance was OECD did not provide a justification for the 1 year half-time. Of note, the titanium dioxide clearance half-time in the rat study RAC used for classification was reported to be 500 days (2).
- RAC discussed coal dust as an example of human exposure to a poorly soluble low toxicity material which supports the potential human relevance of rat lung overload associated cancer (19).

NIOSH

NIOSH differentiated their cancer hazard classification based on the particle size of titanium dioxide (21). Ultrafine titanium dioxide (<100 nm diameter) was classified as a potential occupational carcinogen based on the Heinrich et al. (5) inhalation study in rats. In contrast, NIOSH concluded for fine size titanium dioxide (>100 nm diameter) there were insufficient data to classify as to carcinogenicity. NIOSH recommended separate exposure limits (RELS) for ultrafine (0.3 mg/m^3) and fine size titanium dioxide (2.4 mg/m^3). The RELS were based on an extrapolation of the rat inhalation data to humans using particle surface area as the dose metric which several studies have suggested is a more relevant dose metric for the cancer response in the rat studies (22, 23). Other key observations from the NIOSH discussion of the titanium dioxide inhalation hazard include:

- NIOSH disregarded the Lee et al. (4) study, stating: "*because this dose is considered to be significantly higher than currently accepted inhalation toxicology practice (24), NIOSH concluded that the response at such a high dose should not be used in making its hazard identification*".
- NIOSH analyzed the rat lung cancer and exposure dose relationships for poorly soluble low toxicity materials and concluded the cancer risk of titanium dioxide inhalation is most closely related to the surface area dose of the particulate.
- NIOSH concluded the adverse effects of inhaling titanium dioxide may not be material-specific but due to a generic effect of poorly soluble low toxicity materials. While not providing a definition of poorly soluble low toxicity, NIOSH listed materials in this group as including titanium dioxide, $BaSO_4$, carbon black, toner, and coal dust.
- NIOSH discussed coal dust as an example of poorly soluble low toxicity particulate exposure in humans. NIOSH cited data on lung burden in coal miners as supporting the human relevance of the titanium dioxide lung burdens and lung cancer findings occurring in rats under lung clearance overload.

Edinburgh Expert Workshop on the Hazards and Risks of Poorly Soluble Low Toxicity Particles

In, a panel of scientists and regulators with extensive expertise on particle inhalation toxicology and risk assessment was convened to document the state-of-the science on the hazards and risks of inhaled of poorly soluble, low toxicity materials. This workshop also included observers from government and industry representing important stakeholders on the topics being considered. Details on the experts, the observers, the charges to the panel and the outcomes can be found in Driscoll and Borm (25). For convenience, the expert panel members and observers are summarized in **Tables 2A,B**.

The Edinburgh Expert Panel reached agreement on the state-of-the science for several topics relevant to the application of inhalation toxicology data for hazard identification and risk assessment of titanium dioxide, carbon black and other materials characterized as poorly soluble and low toxicity. Key areas of consensus included:

- In the absence of supporting data from other species, particle overload-associated lung cancer in rats should not be extrapolated to human lung cancer hazard.
- Lung cancer in rats occurring only under conditions of lung particle overload does not imply a cancer hazard for humans under non-overloading exposures.
- Materials with unknown toxicological profiles should NOT be grouped with poorly soluble low toxicity materials without data demonstrating comparable low solubility and low toxicity.
- Increased particle retention resulting from large lung burdens of low toxicity materials is distinct from increased particle retention due to the inherent cytotoxicity of particles (e.g., quartz).

DISCUSSION

The Edinburgh Expert Panel's conclusions on the state of the science regarding extrapolating hazards of poorly soluble low toxicity materials have implications for the cancer hazard classifications developed previously for titanium dioxide (by IARC, ECHA, NIOSH) and for carbon black (by IARC).

Cancer Hazard Classification Based on Inhalation Data From Rats

In their reviews of the titanium dioxide data base IARC, ECHA and NIOSH made several general observations which can be summarized as follows: lung cancer in rats was associated with the overload of lung particle clearance; lung cancer was not observed in other animal species exposed chronically by inhalation; and there was no convincing evidence of lung cancer in humans. In the end, IARC, ECHA, and NIOSH rendered classifications of titanium dioxide based solely on studies in rats in which lung cancer occurred under conditions of lung particle

TABLE 2A | Edinburg Workshop Expert Panelists.

Armelle Baeza-Squiban, Ph.D.	Professor, Functional and Adaptive Biology, Paris Diderot, University
Flemming Cassee, Ph.D.	National Institute for Public Health and the Environment, Netherlands
Rodger Duffin, Ph.D., MRC Path, FRSB	Reader in Respiratory Medicine, University of Edinburgh
Tom Gebel, Prof. Dr.	German Federal Institute for Occupational Safety and Health
Helmut Greim, M.D.	Technical University Munich
Uwe Heinrich, Dr. rer. nat.	Toxicology and Aerosol Research, Medizinische Hochschule, Hannover
Wolfgang G. Kreyling, Dr. rer. Nat.	German Research Center for Environmental Health
Robert Landsiedel, Dr. rer. nat. habil.	BASF SE. Experimental Toxicology and Ecology
Len Levy, Ph.D.	Cranfield University
Dominique Lison, M.D. Ph.D.	Louvain Centre for Toxicology and Applied Pharmacology (LTAP)
Fred J. Miller., Ph.D.	Inhalation Toxicology Division, US EPA; Fred Miller and Associates
Günter Oberdörster, Prof	Dept of Environ. Medicine, University of Rochester School of Medicine and Dentistry
Lang Tran, Ph.D.	Institute of Occupational Medicine, Edinburgh, UK
David B Warheit, Ph.D.	Warheit Scientific LLC
Mei Yong, Dr. rer. Medic.	Inst. for Occup. Epidemiology and Risk Assessment, Evonik Technology and Infrastructure

TABLE 2B | Edinburgh Workshop Observers.

Damjana Drobne	University of Ljubljana, Biotechnical Faculty (SL) *Ad hoc* CARACAL sub-group on ATPs to CLP classification of TiO_2 and mixtures.
Craig Boreiko	Consultant to Antimony Association
Fiona Murphy	Herriot Watt University- Edinburgh; Member of the EU GRACIOUS Consortium
Annie Jarabek	U.S. EPA, National Center for Environmental Assessment (NCEA)
Terry Gordon	New York University School of Medicine, ACGIH TLV Committee
Klaus Kamps	Unifrax; Chair of Eurometaux REACH working group
Roger Battersby	EBRC Consulting
Frank Luetzenkirchen	Quarzwerke GmbH, Frechen, Germany (DE); IMA-Europe: Chairman IMA Technical Board
Robert McCunney	Harvard Medical School; Consultant to International Carbon Black Association
David Lockley	Product Defense and Toxicology Manager, Venator Corp; Chair of Scientific Committee and CLH TF, TDMA
Sue Hubbard	Consultant Regulatory Toxicologist Sah Co., Ltd. (UK); Member of Iron Platform
Andrew Smith	Health and Safety Executive (UK); Chemicals Regulation Division, Team leader: REACH-CLP-PIC; Member of ECHA's Risk Assessment Committee
Tim Bowmer	European Chemicals Agency (ECHA); Chairman of the Committee for Risk Assessment
Ari Karjalainen	European Chemicals Agency (ECHA), Unit C1—Hazard I
Yufanyi Ngiewih	Orion Engineered Carbons GmbH; ICBA Scientific Advisory Board

overload. IARC based its concern classification of titanium dioxide solely on studies in rats in which cancer only occurred under lung particle overload (4, 5). Similarly, in its evaluation of carbon black, IARC based the classification solely on studies demonstrating rat lung cancer under conditions of lung particle overload. NIOSH and ECHA disregarded the Lee et al. (4) study assessing the exposures as to excessive and based their cancer classification on a single study (5). Considering NIOSH and ECHA's basis for rejection of the Lee et al. study (4), questions can be raised as to why these groups accepted of the Heinrich et al. (5) given the rat lung cancer occurred under conditions described by the study investigators as "severe dust overloading," with a titanium dioxide lung clearance halftime of 500 days, and no reversibility of lung clearance (2, 5). The basis of the IARC, ECHA and NIOSH classification conflict with the more recent assessment of the state-of-the science by the Edinburgh Expert Panel regarding the extrapolation of rat lung cancer outcomes, observed on under lung particle overload and with no supporting data from other species.

Coal Dust Is Not a Suitable Reference for Titanium Dioxide

In their classification of titanium dioxide, both ECHA and NIOSH reference coal-dust exposed workers to support the human relevance of the lung burdens in rats causing lung cancer. First, it should be noted that a preponderance of the epidemiology data does not support an association between coal dust exposure and lung cancer or lung clearance impairment (26, 27). Regarding poorly soluble low toxicity dusts, neither NIOSH nor RAC provide a definition, however, NIOSH lists coal along with titanium dioxide, $BaSO_4$, carbon black, toner as a group they consider to be poorly soluble and low toxicity. A scientific issue regarding use of coal dust lung burden data to support the human relevance of the rat cancer after titanium dioxide, is coal is quite different toxicologically. Briefly, coal dust can contain significant amounts of quartz; trace metals such as boron, cadmium, copper, nickel, iron, and zinc; as well as in organic minerals (27). Quartz is a well-established lung toxin, directly toxic to macrophages and other lung cells (27).

Regarding trace metals, studies on coal have demonstrated the iron present generates reactive oxygen species which contributes to coal dust toxicity to lung macrophages and epithelial cells (28–30). Moreover, in studies directly comparing the effects of coal dust and titanium dioxide on human macrophages, coal dust but not titanium dioxide, was shown to activate macrophage release of the potent proinflammatory cytokines tumor necrosis factor α and interleukin 6 which can contribute to lung disease (31). As concluded by the Edinburgh Expert Panel, before grouping materials for safety considerations, there needs to be data demonstrating similarity in solubility and toxicity profiles. Existing data on coal dust demonstrates it is clearly different from titanium dioxide in its inherent toxicity. On a scientific basis such differences arguably preclude the use of coal dust exposure and lung burdens as a surrogate for titanium dioxide, carbon black and other comparable poorly soluble low toxicity dusts.

Does Lung Particle Overload Occur in Humans?

The expert panel agreed that lung particle overload has been demonstrated in all laboratory animal species evaluated. As such, there was agreement lung particle overload could occur in humans, however, there was not agreement on whether this has been proven (25). In this respect, it is noteworthy that even in coal miners with extremely high lung burdens of coal dust, which is inherently more toxic than titanium dioxide or carbon black, significant prolongation of lung particle clearance has not been demonstrated (26, 32, 33). This raises the question: if lung particle overload with its various sequelae can occur in humans, what magnitude of lung exposure to truly low solubility, low toxicity materials (i.e., titanium dioxide and carbon black) would be required? It can be anticipated that the magnitude and duration of such hypothetical exposures would have no relevance to occupational exposures reported for titanium dioxide and carbon black (26, 33).

Summary and Recommendations

The finding that chronic inhalation of titanium dioxide or carbon black results in lung cancer in rats but not in other species and that the rat lung cancer occurs only under conditions of extreme lung particle overload has raised questions on the relevance of overload-associated rat lung cancer to human hazard (8, 11, 13, 34, 35). Despite significant questions on the predictiveness of the rat lung cancer response, IARC, NIOSH and ECHA identified titanium dioxide as a cancer hazard for humans based solely on rat lung cancer. IARC made a similar classification of carbon black, again based solely on lung cancer in rats occurring under lung particle overload.

In 2019, a panel of scientists and regulators expert in inhalation toxicology and risk assessment was convened at the University of Edinburgh to document the state-of-the-science on rat lung cancer and lung particle overload. Regarding hazard identification, the expert panel concluded that in the absence of supporting data from other species, lung particle overload-associated rat lung cancer does not imply a cancer hazard for humans. In the context of high to low dose extrapolation, the expert panel concluded rat lung tumors occurring only under conditions of lung particle overload are not relevant to humans under non-overloading exposures to poorly soluble low toxicity materials. Hazard identification represents an important activity to ensure public health; however, such identification needs to take full account of the state-of-the-science and be updated as scientific understanding advances. In this respect, the conclusions of the Edinburgh expert panel call for a reassessment of the cancer hazard classifications on titanium dioxide and carbon black taking into full account the current scientific understanding of lung particle overload, rat lung cancer and species differences in lung cancer response to poorly soluble, low toxicity materials.

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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Toxicokinetics of Nanoparticles Deposited in Lungs Using Occupational Exposure Scenarios

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Various synthetic powders with primary particle sizes at the nanoscale and a high commercial impact have been studied using Wistar rats. The test materials were metal oxides, i.e., TiO_2, ZnO and amorphous silica, and carbon black (technical soot). Dosing schemes were in the regular ranges typically used in subacute rat studies to simulate occupational exposure scenarios (mg range). Nanoscaled particle agglomerates have the potential to disintegrate and translocate as individual nanoparticles to remote locations following deposition in the lungs. The toxicokinetic fate of metal oxides post-inhalation in lungs/organs was investigated (i) by chemical analysis of the retained particulate/dissolved matter and (ii) by visualization of particles in various remote organs using transmission electron microscopy (TEM). The three titanium dioxides (NM-103, NM-104, NM-105; JRC coding) showed a very slow dissolution in lung fluids. In contrast, the coated ZnO (NM-111) dissolved quickly and was eliminated from the body within approximately 1 day. The precipitated amorphous silica (NM-200) showed a partial dissolution. Chemical analysis in lungs (particulate and soluble TiO_2) and in remote organs (liver and brain) showed a small solubility effect under physiological conditions. The translocation to remote organs was negligible. This confirms that for poorly soluble TiO_2 particles there was no considerable translocation to the liver and brain. The chemical analysis of zinc demonstrated a very rapid dissolution of ZnO particles after deposition in the lungs. Statistically significant increases in Zn levels in the lungs were detectable only on day 1 post-exposure (NM-111). Overall, no relevant amounts of increased NM-111 in the ionic or particulate matter were detected in any body compartment. Amorphous silica (NM-200) particles were found in the cytoplasm of intraalveolar macrophages in the lung and the cytoplasm of macrophages in the lung associated lymph node. Interestingly, these particles were found in a few animals of all treatment groups (1, 2.5, and 5 mg/m^3 NM-200) even after 91 days post-exposure. In all other organs of the NM-200 treated animals such as the nasal epithelium, trachea, larynx, liver, spleen, kidney, and mesenteric lymph node no particles were found at any time point investigated. Carbon black was tagged internally ("intrinsically") with a γ tracer (^{7}beryllium; half-time: 53.3 days). Due to limited amounts, the test item (0.3 mg per rat lung) was intratracheally instilled into the lungs. This dose avoided a particle overload effect, meaning that the toxicokinetic fate of carbon black could be followed under the approximated physiological conditions of lung clearance. Analysis of the γ labeled carbon black confirmed conclusively that there was

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no evidence for the translocation of carbon black beyond the lung into the blood or other body compartments. Very small amounts were only detected in lung-associated lymph nodes (LALN). On day 20 post-treatment, upon necropsy, both carbon black samples were practically exclusively found in lungs (75.1% and 91.0%, respectively) and in very small amounts in the lung-associated lymph nodes (LALN), i.e., ∼0.5%. In the other organs/tissues, the test item was not significantly detectable. Separation of leukocytes and cell-free supernatant of a bronchoalveolar lavagate by centrifugation revealed that carbon black was completely located in the cell sediment, indicating total engulfment by alveolar macrophages. In conclusion, in occupational settings the nanomaterials titanium dioxide, zinc oxide, amorphous silica, and carbon black acted as microscaled agglomerates, not as individual nanoparticles. They displayed no potential to translocate beyond the lung into the blood compartment. Besides lungs, very small particulate amounts were detected only in LALN. This finding is consistent with the behavior of microscaled poorly soluble particles. Overall, there was no evidence of translocation of the nanomaterials following pulmonary exposures.

Keywords: nanoparticles, agglomerates, toxicokinetics, translocation, metal oxides, carbon black

INTRODUCTION

The toxicokinetics of inhaled nanoparticles is predominantly determined by **deposition** characteristics (efficiency, aerodynamic particle size), **dissolution** behavior (in biological media), and **translocation** potential (driven by size-depending migration pathways). In this context, individual nanoparticles behave differently to agglomerated ones with regard to their fate following deposition in the lungs. Various papers have reported biokinetic inhalation studies using extremely small aerosol concentrations ($\leq$1 $\mu g/m^3$) and highly sensitive analytical methods (1, 2). Occupational concentrations of poorly soluble particles are in the range of ∼1 mg/m^3 while environmental levels may reach up to 50 $\mu g/m^3$ (3). In the case of metal oxides, such aerosols consisting of individual nanoparticles can be experimentally established with spark generators and analytics can be refined using the isotope technique. Risk assessment of occupational exposure scenarios in rodent studies uses aerosol concentrations in the range of 1–60 mg/m^3. Dry dispersion of the test powders results in microscaled mean particle sizes even though the test item may have primary particle sizes in the nano range. The reason for this is that at the given aerosol concentrations agglomerate formation is necessarily the dominating implication (4–6). In this perspective article, the toxicokinetic outcome of studies conducted for risk assessment in working areas is discussed. Examples of various studies on **nano-TiO_2, nano-ZnO,** and **nano-SiO_2** as well as **carbon black** were investigated.

MATERIALS AND METHODS

Metal Oxides

Exposure Conditions

The three test items were taken from the repository of the Joint Research Centre, Ispra, Italy, and aerosolized using a dry dispersion technique. The dispersion was achieved by a feeding system and a high-velocity pressurized air dispersion nozzle developed by Fraunhofer ITEM (7). Concentrations were recorded using an aerosol photometer (scattering light signal). Wistar rats were used as a test model and were exposed for 6 h/day, 5 days/week. The aerosols were generated by a flow-past nose-only inhalation exposure system.

Titanium Dioxides

A 28-day nose-only inhalation toxicity study in rats was performed with three TiO_2 varieties: NM-103 (primary particle diameter: 20 nm/rutile/silicone-coated/hydrophobic), NM-104 (PPD: 20 nm/rutile/glycerol-coated/hydrophilic), NM-105 (PPD: 22 nm/anatase-rutile 80%-20%/untreated/hydrophilic). The aerosol concentrations selected were 3, 12, and 48 mg/m^3 for each test item and simulated conditions at an occupational exposure scenario converted to a typical dosing scheme of a subacute rat study. In addition to the regular study design endpoints (OECD 412), investigations on the disintegration of agglomerates in lungs and the identification of the respiratory cell types responsible for uptake (by TEM analysis) were included. Chemical analysis of the retained masses in the lungs was performed on days 1, 28, and 90 post-exposure.

Zinc Oxide

A 90-day nose-only inhalation toxicity study in rats was performed with a coated nanoscaled zinc oxide sample (NM-111; coated with triethoxycapryl silane). The aerosol concentrations selected were 0.3, 1.5, and 4.5 mg/m^3. In addition to the regular study design endpoints (OECD 412), TEM investigations to detect ZnO particles in lungs were performed (magnification up to 40,000× to allow detection of primary nanoparticles). A chemical analysis of the retained masses in the lungs was performed on days 1 and 30 post-exposure (total of Zn^{2+} and ZnO particles).

Amorphous Silica

A 90-day nose-only inhalation toxicity study in rats was performed with an amorphous silica sample (NM-200; synthetic amorphous silica, precipitated). The aerosol concentrations selected were 1, 2.5, and 5 mg/m^3. TEM investigations were performed to detect SiO_2 particles in lungs (magnification up to 40,000×); two samples of the lung and one sample of trachea, larynx, nasal epithelium, lung associated lymph node, liver, spleen, kidney, and mesenteric lymph node were investigated of all animals of the treatment groups and of all time points (1, 30, and 90 days post exposure); control group rats served to acquire the normal biological background of electron dense structures. To achieve better visibility of possible particles in comparison to the biological background, ultrathin sections were not contrasted using uranyl acetate and lead citrate. Chemical analysis of the retained masses in the lungs was performed on days 1, 30, and 90 post-exposure.

Carbon Black

Test Items

Two carbon black samples i.) Monarch® 1,000 (oxidized carbon black grade) from Cabot Corp., USA. and ii.) Printex® 90 (untreated carbon black grade)] from Orion Engineered Carbons GmbH, Germany were investigated. Carbon black is a black, finely divided powder, consisting of aggregates (in the size range between 100 and 1,000 nm) of aciniform morphology (i.e., aggregates that have been strongly fused in a random configuration that resembled grape-like clusters. The carbon black aggregates rapidly form larger agglomerates held together by van der Waals forces.

Production of ^{7}Be-tagged carbon black: Technical soot (carbon black) is synthesized by a thermal process initially forming nanoscaled primary particles. Due to aging the material finally builds aggregates in the size range of 100–1,000 nm and the latter stick together to form microscaled agglomerates.^{7}Be-tagged carbon black can be produced by direct irradiation of carbon black (8, 9). Using the proton irradiation technique the ^{7}Be radioisotope is produced directly in the crystal lattice of carbon without evidently altering the material structure. Nuclear reaction: natC(p,x)^{7}Be, mainly *via* ^{12}C(p,3p3n)^{7}Be channels; proton energy range 24–38 MeV; ZAG Zyklotron, Karlsruhe, Germany; ^{7}Be is a γ tracer with a half-time = 53 days. As only ∼20 mg of carbon black can be activated per run the test item was sufficiently available for an intratracheal instillation study only; however, this approach is a good surrogate. In this study two carbon black samples [Monarch® 1,000 (oxidized carbon black grade) and Printex® 90 (untreated carbon black grade)] were labeled with ^{7}Be.

Purification of the ^{7}Be-tagged carbon black: The test item was liberated from soluble or loosely attached moieties using a triplicate of solvents (EtOH/H_2O 1/1 v/v−0.01 N HCl—Artificial lysosomal fluid—ALF) to consecutively wash and filter the carbon black samples (10). An aqueous suspension of ∼0.3 mg of the ^{7}Be-carbon black sample was taken in a syringe and the carbon black was separated by pressing through a filter (nuclear pore size: 0.4 μm).

TABLE 1 | Retention half-times of test items in lungs.

Retention half-times in lungs (days)	NM-103	NM-104	NM-105
Low dose	59	85	46
Mid dose	162	267	204
High dose	373	315	485

For comparison: Half-time untreated rat is ∼60 days.

Administration of the ^{7}Be-Tagged Carbon Black Samples

Test items were intratracheally instilled into the lungs. A single dose of ∼0.3 mg carbon black suspended in 0.3 ml of sterile isotonic saline was administered. This dose avoided a particle overload effect, thus, the toxicokinetic fate of the test item could be followed under approximated physiological conditions of lung clearance (note that bolus effects could occur following intratracheal instillation).

RESULTS

Metal Oxides

Titanium Dioxides

The chemical analysis of test items retained in the lungs matched the values predicted by the Multipath Particle Dosimetry (MPPD) model v. 3.04 (11). On day 3 post-exposure, in the low dose groups 0.4, 0.4, and 0.5 mg/lung, in the mid dose groups 1.6, 1.7, and 1.8 mg/lung and in the high dose groups 7.0, 3.8, and 5.9 mg/lung were determined for NM-103, NM-104, and NM-105, respectively. The retained masses showed a high similarity in the groups of the same dose. From the calculated retention half-times (kinetic data of the 3 time-points on days 3, 45, and 90 post-exposure) a non-overload, a slight overload, and a moderate overload could be deduced for the low, mid, and high dose groups (**Table 1**).

During the recovery period, almost no lung clearance was observed in the high dose groups. In contrast, in the mid and low dose groups, a partial and a physiological lung clearance were found, respectively. This reflects the different grades of clearance retardation due to the various lung loads.

The soluble moiety of the test items in the lungs - as measured by separating the particulate and dissolved parts by filtration (0.2 μm pore diameter) - reached up to 5.5% of the total mass in the low dose groups; however, it was not more than 2.2 and 0.9% in the mid and high dose groups, respectively. These results suggest that the solubility of the test item is limited by a given maximum under the conditions of the lung ambiance.

The translocation potential from the lungs was very small. TEM analysis revealed that intraalveolar macrophages are the most prominent compartment of particle detection. In the ranking, the compartment pneumocytes type I cells (low and mid doses) and free particles (high doses) finished second.

Zinc Oxide

One day after the end of exposure, in the high dose group of NM-111 the absolute Zn content (35 μg/lung) was slightly increased

(statistically significant) to 180% in the lung as compared to the clean air control group. In all other organs and also at day 30 post-exposure the Zn levels were very close to the control values.

Amorphous Silica

Lung burdens of 91, 172, and 307 μg/lung were analyzed on day 1 after the end of exposure in the low, mid, and high dose groups, respectively. Data at 1 and 3 months post exposure revealed that, in addition to the ∼60 day half-time of physiological clearance, a dissolution effect is the reason for the calculated actual half-times of 30, 32, and 28 days respectively in the low, mid and high dose groups. The chemical analysis of silicon confirmed an evident dissolution of NM-200 under lung ambient conditions. Statistically significant increases in silicon levels in the lungs were detectable on days 1, 30, and 90 post-exposure (NM-200). TEM analysis confirmed the presence of SiO_2 particles in lungs/LALN up to day 90 post-exposure. In these spot checks, in the NM-200 high dose group SiO_2 particles (confirmation by EDX) were detected within the cytoplasm of intraalveolar macrophages (TEM analysis). However, those particles were not detectable in remote organs (nasal epithelium, trachea, larynx, liver, spleen, kidney, and mesenteric lymph node).

Carbon Black

Feces/Urine

In feces and urine, up to day 3 post-treatment ^{7}Be-Monarch® 1,000 was detectable and reached mean values of 17.6 and 6.7%, respectively (**Figure 1**). For ^{7}Be-Printex® 90 the corresponding data were lower and reached 8.2 and 0.4% (**Figure 2**). The percentages measured in feces may have been swallowed directly after administration or after alveolar macrophage clearance. As no significant amounts of the two grades were detected in blood, the levels measured in urine can be traced back to cross-contamination during the feces/urine collection in the metabolic cages.

Blood Kinetics

In blood samples, the two ^{7}Be-carbon black grades were not detectable at significant levels (up to samples collected on days 1–3). Consequently, a maximum concentration (c_{max}) of the test items in the blood (where the organs could have been determined most precisely) could not be identified.

Organs/Tissues

All organs and tissues were analyzed on day 20 post-treatment for radioactivity. ^{7}Be-Monarch® 1000 was detected only in the lungs as the deposition site (75.1%) and, at a low level (0.6%), in the lung-associated lymph nodes (LALN) (**Figure 1**). Correspondingly, ^{7}Be-Printex® 90 values were 91.0 and 0.4% in the lungs and LALN, respectively. In all other organs (including male reproductive organs) no significant amounts of the test items were detected (**Figure 2**).

^{7}Be-Printex® 90 Analysis in Bronchoalveolar Lavage Fluid

Exhaustive lung lavages were performed to harvest a large moiety of the lung leukocyte pool. Subsequent measurements of the radioactivity revealed that ∼50% of the total ^{7}Be-Printex® 90 retained in lungs had been gained by the lavage process. Centrifugation of the bronchoalveolar lavagate showed that the distribution in cell sediment/cell-free supernatant was 100/0%. Cytoslides of the lung leukocytes showed that the carbon black was fully engulfed by alveolar macrophages.

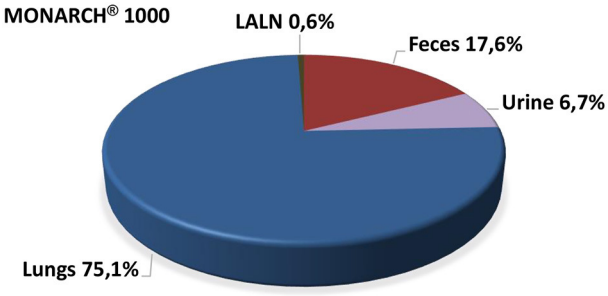

FIGURE 1 | Percentage of body distribution of MONARCH® 1000 post instillation (the entire radioactivity detected in the rat body was set as 100%).

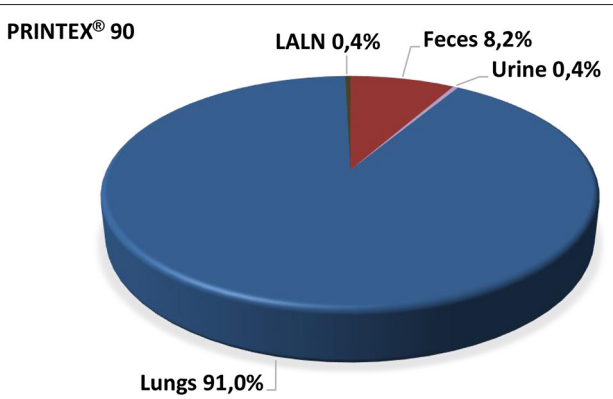

FIGURE 2 | Percentage of body distribution of PRINTEX® 90 post instillation (the entire radioactivity detected in the rat body was set as 100%).

DISCUSSION

Metal Oxides

The three titanium dioxides (NM-103, NM-104, NM-105) are examples of very slowly soluble nanomaterial in lung fluids. In contrast, the coated ZnO (NM-111) shows quick dissolution and is eliminated from the body within ∼1 day. The precipitated amorphous silica (NM-200) shows a partial dissolution. The variation of dissolution behavior among different nanoforms has been discussed for grouping approaches by Keller et al. (12).

Titanium Dioxides

The chemical analysis in lungs (particulate and soluble TiO_2) and in remote organs (liver and brain) showed a small solubility effect under physiological conditions. The translocation to remote organs was negligible. This confirms for the poorly soluble TiO_2 particles to have no considerable translocation to the liver and brain.

Zinc Oxide

The chemical analysis of zinc demonstrated a very rapid dissolution of ZnO particles after deposition in the lungs. Statistically significant increases of Zn levels in the lungs were detectable only on day 1 post-exposure (NM-111); increases of zinc were no longer observed on days 14 or 30 post-exposure. ZnO particles in tissues were not detectable by TEM. The deposited mass of NM-111 in the 90-day exposure period was ∼2,000 μg/lung (calculated by MPPD model), the analytical results thus demonstrate a practically complete dissolution of the retained test item. Overall, no relevant amounts of increased NM-111 in the ionic or particulate matter were detected in any body compartment.

Amorphous Silica

Within the NM-200 treated animals particles were found that consisted of silicon using EDAX-analysis. These particles were found in the cytoplasm of intraalveolar macrophages in the lung and the cytoplasm of macrophages in the lung associated lymph node. Interestingly, these particles were found in a few animals of all treatment groups (1, 2.5, and 5 mg/m^3 NM-200) even after 91 days post exposure. In all other organs of the NM-200 treated animals, such as the nasal epithelium, trachea, larynx, liver, spleen, kidney, and mesenteric lymph node, no particles were found at any time point investigated. Furthermore, no particles were detected in the investigated organs of the clean air treated animals.

Carbon Black

The use of a γ tracer tightly bound into the graphite lattice of carbon black enabled proper analysis of the test items in a biological system. The structure of technical soots (carbon black) is characterized by a strong tendency of the aggregates (size range 100–1,000 nm) to form bigger agglomerates at the micrometer scale. The γ tracer analysis confirmed conclusively that there was no evidence for translocation of carbon black beyond the lung into the blood or other body compartments (very small amounts only detected in lung-associated lymph nodes (LALN). After deposition in lungs, carbon black test items act as insoluble agglomerates in μm size. In occupational inhalation exposure scenarios particles fulfilling the nano definition form agglomerates. Their deposition and translocation behavior is the same as observed for microscaled particles. Particles as such do not translocate to remote organs; in most cases, dissolved moieties of the particulate mass deposited in the lungs are the active agent in remote organs.

AUTHOR CONTRIBUTIONS

OC: study design, study director of all studies, and experimental conduct of the carbon black studies. GP: aerosol generation. DS: TEM investigation. HK: chemical analyses. All authors contributed to the article and approved the submitted version.

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Occupational Exposure to Poorly Soluble Low Toxicity Particles and Cardiac Disease: A Look at Carbon Black and Titanium Dioxide

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Environmental particulate exposure and the potential risk to people with various types of cardiac diseases, most notably cardiovascular disease, have aroused scientific and regulatory interest worldwide. Epidemiological studies have shown associations between exposure to airborne environmental particulate matter (PM) and mortality from cardiovascular disease (CVD). The associations reported, however, are complex and may not involve a direct role for PM, since air pollutants are diverse and highly correlated. This study examines the potential role of occupational exposure to two types of particles, namely, manufactured carbon black (CB) and titanium dioxide (TiO_2), on the risk of cardiovascular disease. To address the risk of cardiovascular disease from exposure to carbon black and titanium dioxide, as reflective of poorly soluble low toxicity particles, we reviewed the published cohort mortality studies of occupational exposure to carbon black and titanium dioxide. Mortality studies of carbon black have been conducted in the United States, Germany, and the United Kingdom. Five mortality studies related to workers involved in the manufacture of titanium dioxide in the United States and Europe have also been conducted. In addition, a meta-analysis of the three-carbon black mortality studies was performed. In the random-effects meta-analysis, full cohort meta-SMRs were 1.01 (95% confidence interval (CI): 0.79–1.29) for heart disease; 1.02 (95% CI: 0.80–1.30) for ischemic heart disease; and 1.08 (95% CI: 0.74–1.59) for acute myocardial infarction (AMI) mortality. A small but imprecise increased AMI mortality risk was suggested for cumulative exposure by a meta-HR = 1.10 per 100 mg/m^3-years (95% CI: 0.92–1.31) but not for lugged exposures, that is, for recent exposures. Results of five cohort mortality studies of titanium dioxide workers in the United States and Europe showed no excess in all heart disease or cardiovascular disease. In the most recent study in the United States, an internal analysis, that is, within the cohort itself, with no lag time, showed that the exposure group 15–35 mg/m^3-years yielded a significantly increased risk for heart disease; however, there was no evidence of increasing risk with increasing exposure for any of the exposure categories. In contrast to environmental studies, the results of cohort mortality studies do not demonstrate that airborne occupational

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exposure to carbon black and titanium dioxide particulates increases cardiovascular disease mortality. The lack of a relationship between carbon black and titanium dioxide and CVD mortality suggests that the associations reported in air pollution studies may not be driven by the particulate component.

Keywords: PSLTs, soluble particles, cardiac disease, carbon black, titanium dioxide

INTRODUCTION

Cardiac disease, most notably cardiovascular disease (CVD), is one of the major causes of death worldwide. According to recent Centers for Disease Control data, CVD accounts for about 659,000 deaths per year in the United States (2019 figures). Epidemiological studies of environmental exposure to airborne particulates have reported associations with a variety of cardiovascular effects, including myocardial infarction, ischemic heart disease, and the major types of CVD (1–3). These effects were first reported among North American and European populations; an analysis of four Chinese studies generated similar results (4). Major risk factors for CVD include smoking, hypertension, high cholesterol, diabetes, family history, and obesity (5).

In light of the potential for environmental particulates to increase the risk of CVD, the American Heart Association published a position paper on particulate matter and heart disease and noted the following: "*It is the opinion of the writing group that the overall evidence is consistent with the causal relationship between* $PM_{2.5}$ *exposure and cardiovascular morbidity and mortality*" (6). The European Society of Cardiology has drawn similar conclusions: "*There is abundant evidence that air pollution contributes to the risk of cardiovascular disease*" (7). The European Society further noted that "*Research should explore optimal methods of air pollution reduction and document the effects of air pollution on the incidence of cardiovascular disease and related mortality to motivate policymakers to intensify legislative efforts on air pollution production*" (7).

In light of these policy statements by professional groups of cardiology specialists in the United States and Europe as well as the results of *environmental* epidemiological studies, this study explores potential relationships between *occupational* exposure to carbon black (CB) and titanium dioxide (TiO_2) regarding the risk of cardiac disease. The purpose of this study was to review the standardized mortality ratios (SMRs) related to cardiovascular disease from the published cohort mortality studies of occupational exposure to carbon black and titanium dioxide, two substances that are considered representative of a category of substances that are considered poorly soluble low-toxicity particles (PSLTs).

Background: Carbon Black and Titanium Dioxide

Carbon black is essentially pure carbon at 98%−99%. The material may also contain traces of polycyclic aromatic hydrocarbons that are *ad*sorbed and tightly bound on the surface of the particle. Carbon black's structure consists of fine particles that form tightly bound aggregates that then form agglomerates (refer to **Figure 1**).

There are 185 carbon black plants globally, with 17 in the European Union, 10 in Eastern Europe, 121 in Asia (81 of which are in China), 21 in North America, 7 in South America, and the remainder in the Mideast or Africa. Approximately 90% of all carbon black produced is used in the manufacturing of tyres and other rubber products, acting primarily as a reinforcing agent (8). The remaining 10% is used primarily in non-rubber industry production, such as black pigment in inks, coatings, and plastics, and to impart electrical conductivity to rubber and plastics.

More than 95% of carbon black worldwide is produced by the oil furnace process. Average primary particle diameters of furnace blacks range from 17 to 70 nm and those of thermal blacks from 150 to 500 nm (refer to **Table 1**). Since primary particles fuse to form aggregates, aggregates are the discrete, dispersible units that constitute the fundamental carbon core of carbon black particles. Clusters of aggregates may form and are known as agglomerates. Carbon blacks are usually highly agglomerated, with 10–1,000 particle aggregates per agglomerate.

Figure 2 shows size distribution data for primary particles and aggregates of carbon black; neither primary particles nor aggregates, however, are generally available under typical settings of its manufacture or use. Complex agglomerates are the predominant carbon black structure outside the environment of the enclosed reaction chamber in which carbon black is manufactured. Agglomerate sizes range from the hundreds of nanometers to the hundreds of microns, which is the typical form of industrial produced and used carbon black. Primary particles, with sizes in the nanometer range, however, can be present in carbon black, but the commercial form of carbon black rarely includes primary particles because of their rapid transformation into aggregates and agglomerates. As a result, carbon black does not meet the EU definition of a nanomaterial, that is, "*a natural, incidental or manufactured material containing particles, in an unbound state or as an aggregate or as an agglomerate and where, for 50% or more of the particles in the number size distribution, one or more external dimensions is in the size range 1 nm−100 nm.*"

Titanium dioxide, a white organic compound has been used for many years in a vast number of diverse products to heighten the whiteness and brightness of materials. It has reflective characteristics and is known for being whitest and brightest of known pigments Metallic titanium and titanium dioxide forms

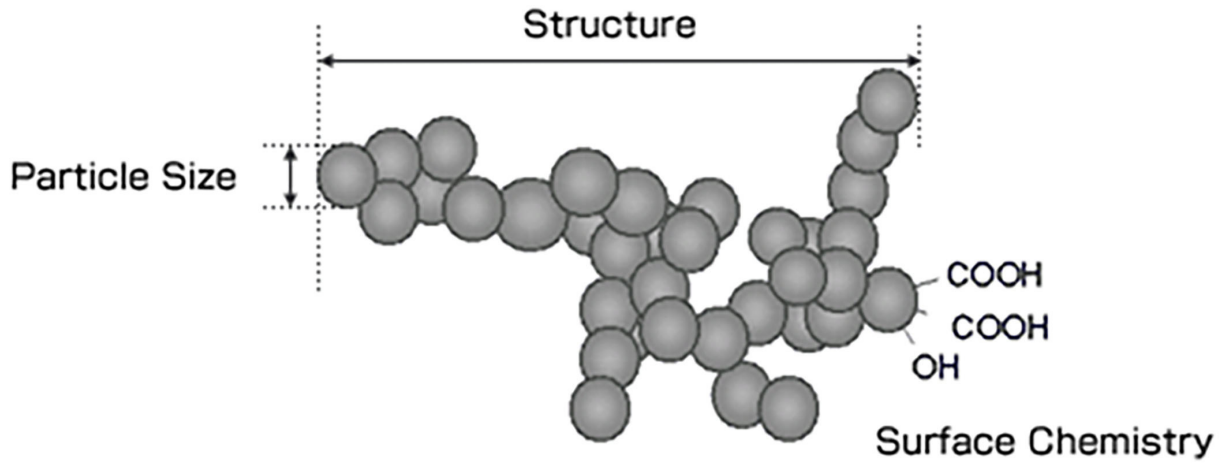

FIGURE 1 | Structure of carbon black.

TABLE 1 | Typical ranges of properties for the five principal types of commercially produced carbon blacks*.

Type of carbon black	Acetylene black	Furnace black	Gas black/channel black	Lampblack	Thermal black
Average aggregate diameter	Not reported	~80–500 nm	Not reported	Not reported	300–810 nm
Average primary particle diameter (nm)	45–50	17–70	13–29	50–100	150–500
Surface area (m^2/g)	60–70	20–300	90–320	20–95	6–15
Carbon (%)	99.8	97.3–99.3	Not reported	Not reported	99.4

*Table abstracted from Table 89.3 of McCunney et al. "Carbon Black," in Pattys Industrial Hygiene and Toxicology (2022, in press). Data are based on original material from M.-J. Wang, C. A. Gray, S. A. Reznek, K. Mahmud, and Y. Kutsovsky, Carbon black, in Kirk-Othmer Encyclopedia of Chemical Technology, 2003, and G. Locati, A. Fantuzzi, G. Consonni, I. Li Gotti, and G. Bonomi, Identification of polycyclic aromatic hydrocarbons in carbon black with reference to carcinogenic risk in tire production. Am. Ind. Hyg. Assoc. J. **40**(7), 644–652 (1979).

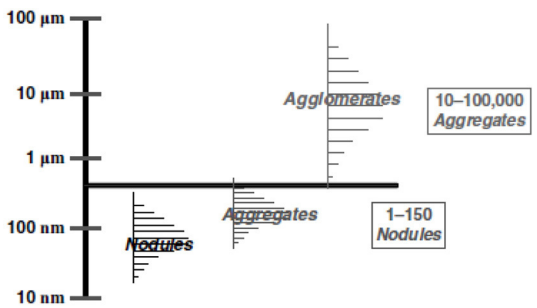

FIGURE 2 | Size distributions of carbon black structural entities*. Abstracted from McCunney et al. Pattys (in press) original figure in Pattys courtesy of Cabot Corporation.

are insoluble and unreactive, devoid of acute toxicity although chronic overload inhalation exposure at high concentrations of TiO_2 in rat studies can increase risk of lung tumors (9). With the expansion of nanotechnology, the TiO_2 material has been engineered into a variety of shapes and sizes, which has led to significant particle size reduction; this reduction of the particle size can lead to increased surface area that may increase toxicity.

METHODS

For this analysis, we reviewed the published occupational cohort mortality studies on carbon black and titanium dioxide in which death rates associated with various types of cardiac disease, including cardiovascular disease, have been evaluated. Cohort mortality studies have been conducted on occupational exposure to carbon black in the United States, Germany, and the United Kingdom (10–13). In addition, a meta-analysis of the three cohort mortality studies of carbon black workers has also been performed (12). Titanium dioxide cohort mortality studies that included assessments of cardiac disease risk have been conducted in the United States and Europe (13–15 and 2010, and 16). A meta-analysis of the cohort mortality studies of titanium dioxide reported no significant risk of all-cause or lung cancer mortality but did not address the risk of cardiac disease (14).

RESULTS

Carbon Black: United States Cohort Mortality Study

The largest mortality study of carbon black workers conducted to date was performed in the United States and included 6,634 workers (10). Some of the workers began employment

TABLE 2 | Cardiac mortality in US cohort.

	Full Cohort *N* = 6,634				Inception Cohort. *N* = 3,890			
	Obs	**Exp**	**SMR**	**95% CI**	**Obs**	**Exp**	**SMR**	**95% CI**
Diseases of the heartICD-9 410-429	616	790	0.78	0.72-0.84	332	394	0.84	0.75-0.94
Ischemic Heart DiseaseICD-9 410-414	272	309	0.82	0.75-0.90	272	309	0.88	0.78-0.99

TABLE 3 | Cardiac mortality in German cohort: reference population: West Germany (from 17).

	Full Cohort. *N* = 1,535			Inception Cohort. *N* = 1,276		
	Obs	**SMR**	**95% CI**	**Obs**	**SMR**	**95% CI**
Diseases of the heart ICD−9: 410–429	103	1.29	1.05–1.57	60	1.39	1.06–1.79
Ischemic Heart Disease ICD−9: 410–414	75	1.30	1.02–1.63	43	1.36	0.98–1.83
Other Heart Disease ICD−9 415–429	28	1.28	0.85–1.85	17	1.47	0.86–2.35

TABLE 4 | Cardiac mortality in German cohort: reference population: North Rhine–Westphalia.

	Full Cohort. *N* = 1535			Inception Cohort. *N* = 1,276		
	Obs	**SMR**	**95% CI**	**Obs**	**SMR**	**95% CI**
Diseases of the heart ICD−9: 410–429	103	1.17	0.96–1.42	60	1.28	0.98–1.65
Ischemic Heart Disease ICD−9: 410–414	75	1.19	0.94–1.49	43	1.27	0.92–1.71
Other Heart Disease ICD−9 415–429	28	1.13	0.75–1.63	17	1.31	0.76–2.10

in the industry as far back as the 1930s. In this study, cumulative inhalable carbon black was assessed by individual lifetime exposure for all cohort members, with the metric being mg/m^3-years. The study had a 98.5% ascertainment of vital status.

In the US cohort mortality study, 616 deaths occurred due to heart diseases (ICD-9 410–429), whereas 790 were expected. These results led to an SMR of 0.78 (95% confidence interval (CI): 0.72–0.84) in the full cohort (10). An evaluation of an inception cohort, that is a group of workers who were followed from the *inception*, i.e., the beginning, of their employment in the carbon black industry, was also performed. Among this group, 332 deaths from ischemic heart disease and the major type of cardiovascular disease occurred, whereas 394 were expected. The SMR was 0.84 (95% CI: 0.75–0.94; refer to **Table 2**).

Ischemic heart disease in the full cohort accounted for 511 deaths, whereas 622 were expected. The SMR was 0.82 (95% CI: 0.75–0.90). An evaluation of an inception cohort yielded 272 deaths, whereas 309 were expected. The SMR was 0.88 (95% CI: 0.78–0.99). In summary, a cohort mortality study of the largest cohort of carbon black workers showed no increase in the risk of death from diseases of the heart or ischemic heart disease (refer to **Table 2**).

Carbon Black: United Kingdom Cohort Mortality Study

A cohort mortality study was conducted among five plants in the United Kingdom that included 1,147 workers employed over the period of 1951–1996 (13). In this analysis, the SMR of diseases of the circulatory system (ICD 8: 390–458) was 1.0 based on 157 deaths (95% CI: 0.85–1.17). This cohort was later updated to assess the risk of lung cancer (15) and further updated in a meta-analysis of all three carbon black cohorts in which additional deaths from heart disease were tabulated and analyzed (12). In this analysis, all of the ICD codes used in the respective mortality studies for "all heart disease" were harmonized and divided into acute myocardial infarctions and the category "all heart disease."

TABLE 5 | Meta–analysis of US, UK, and German cohort mortality studies.

Cardiac Disease	SMR	95% Confidence Intervals
Heart disease	1.01	0.79–1.29
Ischemic heart disease	1.02	0.80–1.30
Acute myocardial infarction	1.08	0.74–1.59

Carbon Black: German Cohort Mortality Study

A cohort mortality study of 332 deaths at the largest carbon black plant in Europe showed a SMR of 1.26 based on 103 observed deaths of heart disease [ICD-9: 410–429; 95% CI: 1.03–1.53; (11)]. German national rates were used as the reference population. Workers who smoked more than 24 cigarettes per day had the highest relative risk of heart disease (7.50; 95% CI: 1.53–36.68).

TABLE 6 | US cohort mortality study of DuPont TiO_2 production workers (17).

1576 participants	Number of deaths	SMR	90% CI	Reference rates
All heart diseases	107	0.93	0.80–1.09	US
Ischemic heart diseases	76	0.93	0.78–1.13	US
All heart diseases	102	1.15	0.98–1.40	DuPont
Ischemic heart diseases	73	1.09	0.90–1.35	DuPont

TABLE 7 | Internal comparisons for all heart disease (18).

Fryzek et al., 14				
Cum. Expo.	**Number of deaths**	**HR**	**95% CI**	**Adjustments**
Low	22	1		Date of hire, age, sex, and geographic are
Medium	57	1.1	0.7–1.8	
High	44	0.8	0.5–1.4	

TABLE 8 | European cohort study on TiO_2.

Boffetta et al., 16	*N* = 15,017				
Ischemic heart disease	**Sex**	**Number of deaths**	**SMR**	**95% CI**	**Reference rates**
ICD:410–414	Men	629	0.88	0.81–0.95	national
	women	5	0.63	0.20–1.41	national

An inception cohort was also evaluated. Ischemic heart disease was significantly elevated: SMR 1.30 based on 75 deaths (95% CI: 1.02–1.63). Other heart disease ICD codes 415–429 indicated non-statistically significant excesses of SMR 1.28 in the full cohort and 1.47 in the inception cohort. These latter results were based on the reference population of West Germany.

The findings were slightly different in an analysis that used the German State (North Rhine-Westphalia), where the carbon black factory was located, as the reference population. North Rhine-Westphalia was used as a more appropriate reference group because of the high rate of smoking in this area. SMRs for heart disease, ischemic heart disease, and other heart diseases were all non-statistically significantly elevated (refer to **Tables 3**, **4**; 17).

In summary, cohort mortality studies of more than 9,000 carbon black workers in the United States, United Kingdom, and Germany indicated the following:

- No excess in cardiac-related mortality was detected in the UK and US cohorts.
- In the German cohort, the cardiac excess detected was of borderline significance when German national death rates were used as the reference population but not when state rates, most notably, those of North Rhine-Westphalia were used as the reference population (16).

In follow-up to these three studies, a meta-analysis was conducted to determine whether there was an elevated risk of cardiac disease in carbon black workers as reported in the three cohort mortality studies described earlier (12). To conduct the meta-analysis, it was necessary to combine standardized mortality ratios and Cox proportional hazard results from the US, UK, and German carbon black production workers. Fixed and random-effects analyses were performed. The analysis addressed mortality for heart disease, ischemic heart disease, and acute myocardial infarction. The results for the full cohort random-effects SMRs were 1.01 (95% CI: 0.79–1.29) for heart disease, 1.02 (95% CI: 0.8–1.30) for ischemic heart disease, and 1.08 (95% CI: 0.74–1.59) for acute myocardial infarction [refer to **Table 5**; (12)].

The authors addressed the highest SMR found for acute myocardial infarction (AMI) [see Tables 11, 12 in (12)]. Random-effects hazard ratios for AMI in an internal analysis indicated a relative risk of 1.10 (95% CI: 0.92–1.31) for cumulative exposure based on 432 deaths among the three cohorts. An internal analysis of an inception cohort that included 138 observed cases showed a relative risk of acute myocardial infarction based on a cumulative of 1.05 (95% CI: 0.97–1.13).

Titanium Dioxide (TiO_2) Cohort Mortality Studies

There have been five published reports of cohort mortality studies conducted among workers involved in the manufacturing of titanium dioxide in the United States and Europe (17–21).

In the first cohort mortality study of titanium dioxide workers in the United States, 1,576 workers exposed to TiO_2 were observed from 1956 to 1985 at two DuPont TiO_2 production plants in the United States (17). Diseases of the circulatory system accounted for 107 deaths, whereas 115.5 were expected. Ischemic heart disease accounted for 76 deaths, whereas 81.5 were expected (refer to **Table 6**). The standardized mortality ratios (SMR) showed no excess in all heart disease

TABLE 9 | Results from extended follow–up of the DuPont cohort.

Ellis et al., (20)				
	Number of deaths	**SMR**	**95% CI**	**Reference rates**
All heart diseases	306	0.92	0.82–1.03	US
	305	1.04	0.93–1.16	DuPont

TABLE 10 | US TiO_2 workers at DuPont plants.

Ellis et al., (21)				
All heart diseases	519	0.82	0.75–0.89	US

or ischemic heart disease. The results were based on both US and DuPont employee reference rates. As noted in **Table 6**, the results showed no statistically significant elevation in the categories "all heart diseases" or "ischemic heart diseases," while larger but non-significant SMRs resulted from a comparison with the mortality rates of DuPont employees. Note that the figures for the number of deaths when the respective US and DuPont reference rates were used were taken directly from the published report. Chen and Fayerweather did not describe the basis of the discrepancy. Nonetheless, the differences are negligible and do not affect the overall conclusion of the study.

US Study on TiO_2

A retrospective cohort mortality study was conducted among 4,241 US TiO_2 workers who were employed for at least 6 months, on or after 1 January 1960, at four TiO_2 plants in the United States (18). Among 171 deaths,[1] in comparison to US disease rates, the SMR was 0.9 (95% CI: 0.7–1.0). These results indicate that there was no increase in mortality from all heart diseases. In an external analysis, there was no dose-response relationship shown between three categories of exposure (low, medium, and high) in relation to the category "all heart disease." No significant association was observed with increasing cumulative exposure (p-value of 0.10; refer to **Table 7**).

European Cohort Mortality Study of TiO_2 Workers

A mortality follow-up study of 15,017 workers (14,331 men) employed in 11 factories producing TiO_2 in 6 European countries [Finland, France, Germany, Italy, Norway, and the United Kingdom; (19)]. The start of follow-up varied by plant and ranged from 1950 to 1972, and the end of follow-up ranged from 1997 to 2001. During the follow-up, 2,652 cohort members died.

Country-specific SMRs were calculated using national mortality rates and pooled across countries using a Poisson model. Therefore, the expected numbers of deaths are not the integral numbers.

The risk of ischemic heart disease mortality was statistically *decreased* significantly in men (refer to **Table 8**).

Authors of another study in the United States defined a much-expanded cohort of DuPont TiO_2 production workers including the oldest and largest facility studied by (17), with a longer follow-up period, and better exposure data. The first of these investigations studied the mortality of a cohort of 5,054 individuals (90% male, 81% white) employed at three DuPont TiO_2 production facilities (20) evaluated cause-specific standardized mortality ratios (SMRs) and stratified the results by the plant for workers employed for at least 6 months between 1935 and 2005 (20). No excess mortality in all heart diseases was observed. The SMR was larger but not significant, in comparison with the mortality rates of DuPont employees (refer to **Table 9**).

Following this study, a cohort of 3,607 workers employed in the same three DuPont titanium dioxide production facilities was followed from 1935 to 2006. This study examined exposure-response between mortality and cumulative exposure to TiO_2 and $TiCl_4$. The more restrictive entry criteria were applied in an effort to reduce the uncertainty in the exposure estimates.The cohort included 833 deaths. Like the earlier report, no excess in all heart disease was observed (15; refer to **Table 10**).

In an internal analysis (**Table 11**) with no lag time, only the exposure group 15–35 mg/m^3-year yielded a significantly increased risk for heart disease, while the relative risks of other 4 exposure categories were not significantly increased. When exposure was lagged 10 years, results were similar with the relative risk estimates similar or slightly higher for each exposure level.

The authors argued that these results were driven by the sub-cohort at Edgemoor plant (EM). Lagging exposure of 10 years would have the least effect on cumulative exposure for the EM cohort since it is the oldest. There was no evidence of an increasing risk of cardiac mortality with increasing exposure to titanium dioxide.

DISCUSSION

The meta-analysis of carbon-black workers included all three carbon-black cohort mortality studies published to date in the United States, the United Kingdom, and Germany. Thus, it has the greatest potential to identify cardiovascular disease risks among these carbon black workers. The meta-analytic procedures that were used to combine specific results of the three-carbon black cohort mortality studies led to enhanced statistical power to identify even small associations. In the analysis, SMR and Cox proportional hazard results focused on ischemic heart disease and acute myocardial infarction as the key components of cardiovascular disease. The availability of reasonably detailed employment histories and exposure assessments in the three cohorts allowed quantitative evaluation of the risk of cardiovascular mortality through the use of standardized individual carbon black exposure estimates. These meta SMRs were unexceptional and showed

[1]*Note that the ICD codes were not specified in the article but are presumed to be ICD-9-410-414.*

TABLE 11 | Internal analyses (21).

Ellis et al. (21)	Lag = 0 year		Lag = 10 years		
	HR	95% CI	HR	95% CI	Adjustments
< 5 mg/m^2 years	1		1		Age, gender, ethnicity, plant first employed, and calendar time
5–15	1.30	0.89–1.89	1.47	1.02–2.11	
15–35	1.61	1.13–2.31	1.65	1.16–2.34	
35–80	1.32	0.90–1.94	1.36	0.92–2.00	
80+	1.27	0.84–1.90	1.51	1.00–2.25	

no association with the duration of exposure to carbon black. These results in a weight of the evidence assessment indicate that carbon black exposure does not increase cardiac disease mortality.

A weight of the evidence assessment of titanium dioxide cohort mortality studies of workers in the United States and Europe also found no significant increase in the risk of cardiovascular disease. We conclude that occupational exposures to carbon black and titanium dioxide indicate no increase in the risk of death from cardiovascular disease.

The discordance of our results of occupational exposure to these particulates in contrast to environmental exposure to ambient pollution that includes particulates and other substances may reflect differences in particle size or that environmental exposures include many other substances, in addition to particulates.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: MY was employed by MY EpiConsulting. DW was employed by Consulting. RJM, MY and DW are consultants to the International Carbon Black Association.

The remaining author declares that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

The reviewer KM declared a past co-authorship/collaboration with the author MY to the handling editor.

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Genotoxicity of Particles From Grinded Plastic Items in Caco-2 and HepG2 Cells

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Large plastic litters degrade in the environment to micro- and nanoplastics, which may then enter the food chain and lead to human exposure by ingestion. The present study explored ways to obtain nanoplastic particles from real-life food containers. The first set of experiments gave rise to polypropylene nanoplastic suspensions with a hydrodynamic particle size range between 100 and 600 nm, whereas the same grinding process of polyethylene terephthalate (PET) produced suspensions of particles with a primary size between 100 and 300 nm. The exposure did not cause cytotoxicity measured by the lactate dehydrogenase (LDH) and water soluble tetrazolium 1 (WST-1) assays in Caco-2 and HepG2 cells. Nanoplastics of transparent PET food containers produced a modest concentration-dependent increase in DNA strand breaks, measured by the alkaline comet assay [net induction of 0.28 lesions/10^6 bp at the highest concentration (95% CI: 0.04; 0.51 lesions/10^6 base pair)]. The exposure to nanoplastics from transparent polypropylene food containers was also positively associated with DNA strand breaks [i.e., net induction of 0.10 lesions/10^6 base pair (95% CI: −0.04; 0.23 lesions/10^6 base pair)] at the highest concentration. Nanoplastics from grinding of black colored PET food containers demonstrated no effect on HepG2 and Caco-2 cells in terms of cytotoxicity, reactive oxygen species production or changes in cell cycle distribution. The net induction of DNA strand breaks was 0.43 lesions/10^6 bp (95% CI: 0.09; 0.78 lesions/10^6 bp) at the highest concentration of nanoplastics from black PET food containers. Collectively, the results indicate that exposure to nanoplastics from real-life consumer products can cause genotoxicity in cell cultures.

Keywords: nanoparticles, microplastic, oxidative stress, DNA damage, comet assay

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INTRODUCTION

The impact of plastic litter on the ecological system has been a matter of concern for decades, although potential hazards to humans until recently appears only to have evoked modest attention. One reason could be that particle toxicologists have focused on health effects related to air pollution and particles that are smaller than 100 nm in diameter (i.e., so-called nanomaterials). The toxicology of nanomaterials has been highly useful for the understanding of ultrafine air pollution particles (1). The diameter of microplastics are between 1,000 nm and 5 μm in diameter, whereas nanoplastics are smaller than 1,000 nm.

Studies on stool samples have documented that humans are exposed to microplastic via the gastrointestinal tract (2). Infants appear to have higher exposure to microplastics than adults do

(3). Consumption of microplastic via diet is an important source of particles in adults (4). House dust has been shown to be an important source of microplastics for children (5). It has also been shown that synthetic polymers and fibers are important airborne microplastics in dwellings (6). Microplastics have been detected in human lung (7) and placenta tissue (8).

One detrimental outcome of particle exposure is genotoxicity, which may be a direct consequence of interaction between particles and DNA or an indirect effect related to oxidative stress and inflammation (9). The single cell gel electrophoresis (comet) assay has been used in many cell culture studies, animal experiments and human exposure studies on both air pollution particles and nanomaterials (10). Similarly, the comet assay has been used extensively in ecotoxicology, using various sentinel species (11, 12). Interestingly, the research on genotoxic effects of plastic particles is much more developed in ecotoxicology as compared to human toxicology. **Table 1** summarizes results from a survey of plastic particles in various studies on DNA damage measured by the comet assay in marine and terrestrial animals. Collectively, the studies indicate that the majority of studies have used standard particles such as polystyrene (23 studies) and polyethylene (6 studies), whereas only few studies have investigated the effect of particles from "real-life" plastic items. The latter group encompasses a study on microplastics from items collected from grinded cutlery, litter on sandy beaches and tumble dryer lint. These studies have shown genotoxicity in animal species after exposure to microplastic particles (13–15). To the best of our knowledge, there are no studies on genotoxic effects of real-life plastic debris particles in human cells (38). However, recent studies have used the comet assay to assess DNA strand breaks and oxidatively damaged DNA on human cells after exposure to either polyethylene or polystyrene (39–41).

The aim of this study was to assess genotoxicity of plastic debris particles from real-life consumer products, namely food containers that were purchased in a local supermarket. There is no standardized procedure for degrading real-life plastics or minimal requirements for sufficient characterization of the particle suspension. The article describes results from experiments on ways to obtain particle suspensions from real-life plastic items. We have characterized the particle size in suspensions, whereas a complete chemical analysis of additives and other chemical compounds has not been done. There are approximately 7,000 additives used for the production of real-life plastic items, although it depends on type of polymer and expected use (42). We selected food containers made of polypropylene (PP) and polyethylene terephthalate (PET) to avoid types of plastics with potentially hazardous additives. The food containers were grinded with different blenders to plastic suspensions that was used to expose cells from the digestive system (i.e., Caco-2 cells) and the liver (i.e., HepG2 cells).

METHODS

Preparation of Suspensions of Nanoplastic

The objective of the generation of nanoplastics was to set up a relatively fast method for grinding and subsequent isolation of small size particles in a harmless medium to human cells. The first part of the experiments used PP and PET plastic products (called "pilot study" in the article). The second part of the experiments focused on the production of PET plastic particles, where more effort was put into method development of the production part (called "main study" in the article).

For the pilot study, transparent plastic food containers of either PP or PET from a Danish supermarket were used. We used food containers because they were easy to obtain and relatively thin (i.e., They were easy to break and subsequently grind). Food containers are found as litter and might degrade to nanoplastics in the environment, whereas it seems unlikely that food containers release large amounts of nanoplastics and contaminate the food. PP and PET were chosen because they seemed to be the most common types of plastics for food containers, commonly found in environmental samples, and we considered them easier to grind than softer types of plastics such as polyvinyl chloride. They were washed with ethanol and left to dry. Afterwards the containers were cut into smaller pieces with a scissor. The small pieces in sterile isotonic water were grinded using an ultra-turrax T-45 blender for approximately 10 min on ice. This produced a slurry of large particles and hazy suspension of smaller particles that was extracted with a pipette. Afterwards the suspensions were filtered with a 0.45 μm sterile filter. The filtered suspensions were left to air dry on a heating block at 60–70°C in a LAF bench for approximately 5 days until the water had evaporated. **Figure 1** shows images of steps in preparation of nanoplastics from transparent PP and PET food containers.

For the second part of the study, we chose to use only PET material and selected raw meat containers from a Danish supermarket. **Figure 2** shows images of steps in preparation of nanoplastics from black PET food containers. The containers contained a minimum of 80% recycled PET material. For the production, 100 g of pre-cut PET was suspended in 800 ml of 96% ethanol for 10 min at room temperature and subsequently grinded using an immersion blender (BOSCH MSM66020). The suspension was left to sediment for 5 min and 400 ml was extracted using a plastic syringe. The suspension was further filtered using a Whatman filter and finally run through a 0.45 μm sterile filter. The filtered solution was placed in a pre-weighted glass beaker and left to evaporate on a heat block at approximately 65°C. Two control beakers containing ethanol were also placed on the heat blocks and treated similarly to the PET suspension. The procedure was repeated three times adding three layers of particles and the beakers were weighed after each addition. The average weight change of the control beakers was estimated and subtracted from the weight of the beaker containing PET. Using a cell scraper, the PET particles were detached from the bottom of the beaker and placed into an Eppendorf tube and sterile water for injection (Gibco® Water for Injection for Cell Culture) was added to the PET to create a concentration of 10 mg/ml. The suspension was further filtered through a 0.45 μm sterile filter and sonicated in a water bath for 1 h, resulting in a stock suspension in water for injection that was diluted in cell media before exposure.

In the present study, we used the highest concentrations possible from the nanoplastic slurries in the cell culture

TABLE 1 | DNA damage measured by the comet assay in marine or terrestrial species after exposure to plastic particles[a].

Sample	Species	Number of exposure groups (time)	Effect	References
Tumble dryer lint (fibers)	Mussels (*Mutilus galloprovinciallis*); hemocytes	3 (7 days)	Increased at highest (25%) and middle (17%) exposure level compared to control (2%)	(13)
Cutlery (PS, 65–125 μm, grinded)	Earthworm (*Eisenia fetida*); coelomocytes	3 (28 days)	Increase (concentration-dependent: 10, 15, 25 vs. 2% in controls)	(14)
Microplastic from sandy beaches (Hawaii) [consisting of 27% PE, 72% PP and 1% PS], which were grinded and filtered (600 μm, D50 = 305 μm)[b]	Japanese medaka larvae	3 (14 days)	Increased in lowest (8%) and middle (5%) exposure groups compared to controls (2%)	(15)
Microplastic from sandy beaches (Eastern Islands), which were grinded and filtered [consisting of 94% PE, 6% PP and 1% PS] (316 μm)[b]	Japanese medaka larvae	3 (14 days)	Unaltered (22%) compared to control (22%)[c]	(15)
Microplastic mixture [40% LDPE, 25% HDPE, 25% PP, 10% PS] (< 600 μm; D50 = 409 μm)[b]	Japanese medaka larvae	1 (14 days)	Unaltered (4 vs. 2% in controls)	(15)
PE (<100 μm)	Mussels (*Mutilus galloprovinciallis*); hemocytes	1 (7 days)	Increase (30 vs. 10% in control)	(16)
PE (300 μm) [in cadmium-contaminated soil]	Earthworm (*Eisenia fetida*); sperm	4 (28 days)	Increased (4.5, 4.0, 6.5, and 8.5 vs. 2% in controls)	(17)
PE (with TiO_2, 10–90 μm)	Neotropical teleost (*Prochilodus lineatus*); erythrocytes, gill and hepatic cells	1 (24 and 96 h)	Increased in erythrocytes (180 vs. 80 a.u.) at 96 h. Increased in liver at 24 h (80 vs. 20 a.u.) and 96 h (125 vs. 50 a.u.). No effect in gill cells	(18)
LDPE (11–13 μm)	Clamps (*Scrobicularia plana*); hemocytes	1 (14 days)	No effect (results reported as tail length and tail moment)	(19)
LDPE (11–13 μm)	Clamps (*Scrobicularia plana*); hemocytes	1 (14 days)	No effect (results reported as tail length and tail moment)	(20)
LDPE (20–25 μm)	Mussels (*Mutilus galloprovinciallis*); hemocytes	1 (7, 14, or 28 days)	No effect (22%, 37% in exposed and unexposed at 7 and 14 days; 30% vs. 25% at day 28 in exposed and controls, respectively)	(21)
PS (<100 μm)	Mussels (*Mutilus galloprovinciallis*); hemocytes	1 (7 days)	Increase (22 vs. 10% in control)	(16)
PS (110 nm)	Mussels (*Mutilus galloprovinciallis*); hemocytes	5 (4 days)	Increased at three highest concentrations (maximally 40% increased as compared to controls)	(22)
PS (0.5 μm)	Mussels (*Mutilus galloprovinciallis*); hemocytes	1 (7 and 26 days)	Increase (40 vs. 25% in control) after 26 days. No effect after 7 days	(23)
PS (4.5 μm)	Mussels (*Mutilus galloprovinciallis*); hemocytes	1 (7 and 26 days)	Increase (40 vs.25% in control) after 26 days. No effect after 7 days	(23)
PS (55 nm)	Zebrafish (*Danio rerio*); blood cells	1 (1, 3, or 5 days)[d]	Increase (15 vs. 7% in controls)	(24)
PS (100 nm)	Zebrafish (*Danio rerio*); blood cells	1 (1, 3, or 5 days)[d]	Increase (12 vs. 7% in controls)	(24)

(Continued)

TABLE 1 | Continued

Sample	Species	Number of exposure groups (time)	Effect	References
PS (5–12 μm)	Zebrafish (*Danio rerio*); gill cells or liver)	1 (21 days)	Increase in gill cells (20 vs. 2%) and liver (21 vs. 1%)[e]	(25)
PS (23 nm)	Grass carp (*Ctenopharyngodon idella*); blood	1 (3 days)	Increase (3 vs. 1% in controls)	(26)
PS (23 nm)	Grass carp (*Ctenopharyngodon idella*); blood	3 (20 days)	Increase (18, 28, and 38% vs. 3% in controls)	(27)
PS (100 nm)	Earthworm (*Eisenia fetida*); coelomocytes	2 (14 days)	Increase (10 and 20%) at both exposure levels compared to controls (7%)	(28)
PS (1.3 μm)	Earthworm (*Eisenia fetida*); coelomocytes	2 (14 days)	Increase (16 and 22%) at both exposure levels compared to controls (7%)	(28)
PS (100 nm)	Earthworm (*Eisenia fetida*); coelomocytes	1 (21 days)	Increase (8 vs. 6% in controls)	(29)
PS (1 μm)	Earthworm (*Eisenia fetida*); coelomocytes	1 (21 days)	Increase (7 vs. 6% in controls)	(29)
PS (10 μm)	Earthworm (*Eisenia fetida*); coelomocytes	1 (21 days)	Increase (12 vs.6% in controls)	(29)
PS (100 μm)	Earthworm (*Eisenia fetida*); coelomocytes	1 (21 days)	Increase (11 vs. 6% in controls)	(29)
PS (65–125 μm)	Earthworm (*Eisenia fetida*); coelomocytes	3 (28 days)	Increase (concentration-dependent: 12, 20, 33 vs. 2% in controls)	(14)
PS (0.5 μm)	Clamp (*Tegillorca granosa*); hemocytes	1 (14 days)	Increase (reported as degree of DNA damage)	(30)
PS (30 μm)	Clamp (*Tegillorca granosa*); hemocytes	1 (14 days)	No effect (reported as degree of DNA damage)	(31)
PS (30 μm)	Clamp (*Tegillorca granosa*); hemocytes	1 (14 days)	No effect (reported as degree of DNA damage)	(32)
PS (20 μm)	Clamps (*Scrobicularia plana*); hemocytes	1 (14 days + 7 days depuration)	Increase (17 vs. 14% in controls)	(33)
PS (220 nm)	Gill and intestinal epithelial cell lines from rainbow trout	1 (48 h)	Unaltered in gill (3 vs. 1%) and intestinal epithelial cells (1 vs. 1% in controls)[f]	(34)
PS (8 μm)	Zebrafish (heart)	1 (21 days)	Increased (18 vs. 0.2% in controls)	(35)

[a] *The literature survey encompasses only pristine particles or debris from plastic litter. Studies were identified by search on PubMed using microplastic, nanoplastic and comet assay as terms. Additional studies were obtained from reference lists of the identified articles. The genotoxicity results are reported as percentage of the fluorescence in the comet tail (%) or arbitrary units (a.u.) as primary comet assay descriptors. Abbreviations are median diameter size (D50), low-density polyethylene (LDPE), polyethylene (PE), polypropylene (PP) and polystyrene (PS).*

[b] *The authors have also published an assessment of extractable organics from microplast samples (36, 37).*

[c] *Exposure to microplastics produced genotoxicity, measured by the Fpg-modified comet assay.*

[d] *Results are only reported from one time point, although which is not specified.*

[e] *Essentially the same results in Perch fluviatilis (gill cells: 22 vs. 2%, liver 24 vs. 1%).*

[f] *The study included the Fpg-modified comet assay (unaltered level of genotoxicity after particle exposure).*

experiments. As such, the higher concentration in the second trial is due to refinement of the plastic grinding and isolation procedure, which made it possible to obtain higher concentrations. It is not possible to compare the concentrations in the present study to the actual exposure in the gastrointestinal tract, because the magnitude of nanoplastic ingestion in humans is uncertain. In general, it is uncertain if the biological response to the same concentration of particles *in vitro* and human tissue is comparable as cultured cells are maintained in a non-physiological environment in the culture flasks and there is a selection of robust cells during the isolation from the original tissue (43).

Hydrodynamic Particle Size

The hydrodynamic particle size of the final plastic suspensions was analyzed by Nanoparticle Tracking Analysis (NTA)

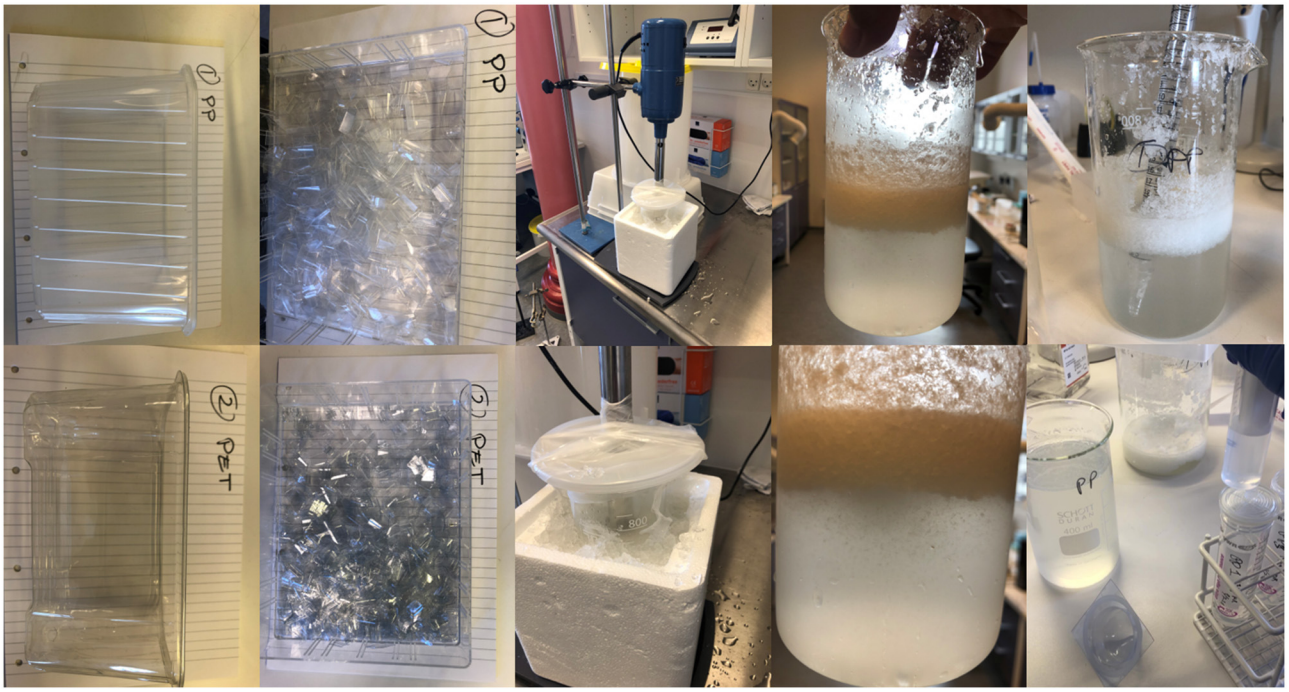

FIGURE 1 | Preparation of polypropylene (PP) and polyethylene terephthalate (PET) suspensions for the pilot study. The images demonstrate from left to right the original food containers, pieces of containers, grinding process, primary slurry of PP and PET plastics particles.

(NanoSight LM20). We have previously demonstrated that similar estimates of the hydrodynamic particle size is obtained with NTA and dynamic light scattering on suspensions of latex beads (100 nm), nanosized carbon black (Printex 90), and nanosized and fine titanium dioxide (44). We used isotonic saline for the hydrodynamic particle size measurements because there is variation in background levels of particles in full cell culture medium (45, 46), which we suspect originates from batch variation in FBS. In the present study, the background level of particles (and their sizes) were high in minimal essential medium (MEM, Sigma, Cat no. M2279) as compared to isotonic saline (results not shown). Previous studies on particles from combustion of fossil diesel, biodiesel, candle lights as well as urban dust (standard reference material 1649b and food-grade titanium (E171) have not revealed a systematic difference in particle sizes of suspensions in isotonic saline and cell culture medium (45, 47–51).

The analysis was performed in filtered water for injection as the cell medium with serum contained a high background in the NanoSight measurements. Five 1-min videos were recorded and analyzed by the NanoSight optical tracking system 3.0. The analysis determines the size distribution and the particle number concentration, from which the mass concentration was calculated by using a density of 1.38 and 0.9 g/cm^3 for PET and PP, respectively. The PET suspension (5 μg/ml) was tested for presence of endotoxins with the Limulus Amebocyte Lysate (LAL) Pyrogent kit (Lonza E194-06), using a standard curve with *Escherichia coli* endotoxin O55:B5 (Lonza Material number: 7360, Batch no: 0000378664) (0.03, 0.06, 0.125, 0.25, and 0.5 EU/ml). No presence of endotoxins was detected in the particle suspension. Endotoxin-spiked samples (0.25, 0.125, 0.0625 EU/ml) showed unequivocal clot formation at 0.25 EU/ml, which was higher than the threshold of clot formation in the standard curve (i.e., 0.125 EU/ml) suggesting a slight inhibition of the biological response of endotoxin by nanoplastics.

Cell Culture

Human hepatocellular carcinoma (HepG2) and colorectal adenocarcinoma epithelial (Caco-2) cell lines were obtained from the American Type Culture Collection (Manassas, VA, USA). HepG2 and Caco-2 cells are widely used in toxicology, including studies on the comet assay (52, 53). Both cell types can internalize particles such as polystyrene nanoplastics (41, 54, 55). In addition, we use these cells because of the high biosafety level (i.e., virus-transformed cells have typically biosafety classification that warrants special laboratory facilities to culture the cells). The cells were cultured in Eagle's Minimum Essential Medium supplemented with 10% fetal bovine serum (FBS), 1% L-glutamine and essential amino acids, and 1% penicillin/streptomycin (all from Gibco). Incubations were carried out at 37°C in an atmosphere of 5% CO_2. All experiments were performed on cells within passage 4–25.

Cytotoxicity (WST-1 and LDH Assays)

We have used water soluble tetrazolium 1 (WST-1) and lactate dehydrogenase (LDH) assays because they detect different

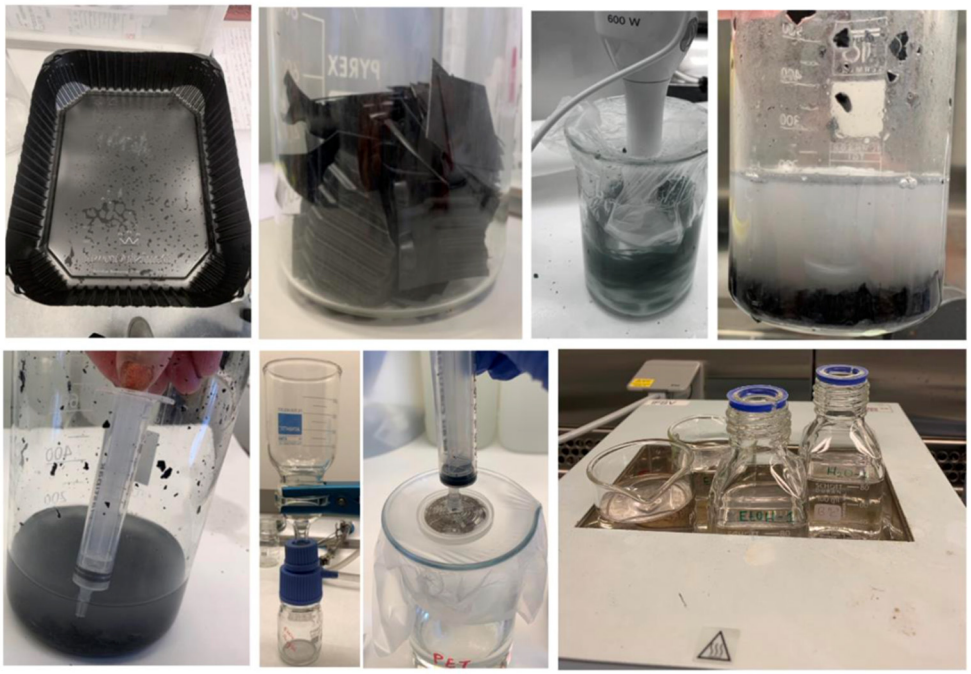

FIGURE 2 | Preparation of PET particles for the main study. Top from left to right: the PET food container was first sterilized using 96% ethanol. The plastic was then cut into 5 cm long strips. The pieces were mixed with ethanol for 10 min at room temperature. The plastic-ethanol suspension was left to sediment for 5 min and 400 ml of the suspension extracted using a plastic syringe and filtered through a paper filter using a vacuum pump. The filtrate was further filtered through a 0.45 disk filter. The final suspension was left to evaporate at 65°C in a heating block.

mechanisms of action of cytotoxicity (i.e., cell membrane damage and metabolic activity). The cells were exposed to nanoplastics for 24 h to increase sensitivity of the cytotoxicity assays and avoid false positive comet assay results due to dying cells, which may not be detected after 3 h exposure. Three technical replicates in each exposure group of HepG2 and Caco-2 cells were seeded into a 96-well-plate (50,000/well), and the next day the cells were exposed to particle suspensions. Medium without particles was used as concurrent negative control. Cells exposed to cell medium with 1% Triton X-100 (Sigma-Aldrich, USA) served as positive control. After 24 h of exposure, the cell medium with particles was removed (and saved for the LDH assay), and the WST-1 reagent (Roche Diagnostics GmbH, Mannheim, Germany) in fresh cell medium (10% in 100 µl medium) was added to each well. The plate was incubated for 2 h before the absorbance was measured with a spectrophotometer (Multiskan Ascent, USA) at a wavelength of 450 nm with 630 nm as reference wavelength.

The LDH assay was performed in the same experimental setup as the WST-1 assay. The LDH activity measurement was performed with the Cytotoxicity Detection Kit (Lactate Dehydrogenase Activity, Roche Diagnostics GmbH, Germany) according to the manufacturer's protocol. The LDH working solution was added to the supernatant from the cell culture and left to incubate for 30 min before analysis with a spectrophotometer. The absorbance was measured at a wavelength of 500 nm with 630 nm as reference wavelength.

Cell Cycle Distribution

HepG2 or Caco-2 cells were seeded in a 6-well plate overnight (500,000 cells per well). The next day the cells were exposed to particles from grinded black colored PET food containers for 24 h at 37°C and 5% CO_2. Serum free cell medium (SFM) was used as control for impeded progression of the cell cycle (i.e., positive control). Following the exposure, medium was removed, and the cells were washed with 1 ml of phosphate buffered saline (PBS) with 2% bovine serum albumin (BSA) and harvested using 350 µl of 0.05% trypsin-EDTA and 650 µl of cell medium. The cells were subsequently centrifuged for 5 min at 500 g, the supernatant was removed, and the cell pellet was resuspended in the small amount of remaining supernatant. The resuspended cells were fixed using 1.3 ml of cold methanol and incubated for 24 h at −20°C for the HepG2 cells and 1.3 ml of cold ethanol incubated for 2 h at −20°C for the Caco-2 cells. Following the incubation, the cells were centrifuged for 5 min at 500 g, and the supernatant was discarded. The cell pellet was resuspended and the cells were washed with 1 ml of PBS with 2% BSA and centrifuged for 5 min at 500 g, and the supernatant removed. The cell pellet was resuspended in 500 µl of FXCycle^{TM} (Thermo Fisher Scientific) staining solution containing 50 µg/ml propidium iodide and 100 µg/ml RNAse A in PBS and incubated for 30 min in 37°C and 5% CO_2.

The stained cells were analyzed using a BD Accuri C6 flow cytometer with excitation at 488 nm. Forward scatter (FSC) and side scatter (SSC) were used to record information on cell size

and granularity (complexity) of the cells, respectively. For each sample, 5×10^4 cells were counted in a gate excluding cell debris and dead cells. A second gating was used to only include single cells in a plot of FSC-H vs. FSC-A. The cell count vs. fluorescence (propidium iodide staining) were used to assign cell cycle phases accordingly.

Intracellular ROS Production

HepG2 or Caco-2 cells were seeded overnight in a black bottomed 96-well plate at a density of 50,000 cells per well. The next day the cells were incubated for 15 min with 10 μM of 2'7'-dichloroflourescin diacetate (CAS no. 4091-99-0, Sigma-Aldrich, Cat no. D6883) in Hank's balanced salt solution (HBSS, Gibco) at 37°C and 5% CO_2. After the incubation with the probe, the cells were washed with HBSS to remove extracellular probe and subsequently exposed to 200 μl of the microplastic suspension. H_2O_2 (CAS No. 7722-84, Sigma-Aldrich, Lot. 18A164128, 1 mM for HepG2 cells and 500 μM for Caco-2 cells) was used as positive control. The H_2O_2 concentration differed because HepG2 cells appeared to be less sensitive than Caco-2 cells in preliminary experiments. Exposures were carried out in technical triplicates on 3 different days. The cells were incubated for 3 h at 37°C and 5% CO_2. After the incubation, the cells were washed twice with HBSS and finally resuspended in 100 μl of HBSS. Fluorescence was measured at 490 nm emission and 520 nm excitation.

DNA Damage Analysis by the Comet Assay

The comet assay is widely used in particle toxicology to detect DNA damage by exposure to a range of nanoparticles as well as complex mixtures such as combustion or air pollution. The standard comet assay—like the alkaline elution and alkaline unwinding assays—detects DNA strand breaks or lesions that are converted to breaks by the alkaline condition (56). It is well-known that procedures affects the characteristics of comets (e.g., the comet tail length is proportional to the electrophoresis time) (57). Thus, comet images or primary comet descriptors (e.g., percent tail DNA) are not equivalent to the actual number of DNA lesions. By using calibration with ionizing radiation, it is possible to obtain information on the actual number of lesions in DNA from primary comet descriptors (58). Using this conversion of primary comet descriptors to actual numbers of lesions in DNA, the comet assay has been validated against chromatographic assays for detection of oxidatively damaged DNA (59–61). The European Comet Assay Validation Group conducted a number of ring-trials with the aim of assessing the inter-laboratory variation procedures and results by the comet assay (62–68). Lately, the hCOMET project are conducting ring-trials to test potassium bromate as a positive assay control in cryopreserved samples (69, 70). The comet assay has been thoroughly validated in studies that led up to the adoption of the technique for OECD guideline test no 489 (71–73). Lastly, it has been shown that the comet assay has reasonable sensitivity (79%) and specificity (76%) in multi-organ tests on rodents (74, 75). In addition, recent pooled analysis from human biomonitoring studies have demonstrated that high levels of DNA strand breaks in blood cells predicts risk of death (76, 77).

HepG2 or Caco-2 cells were seeded overnight in a 24-well plate at a density of 250,000 cells per well. On the day of experiment, the cell culture medium was removed and medium with nanoplastics or H_2O_2 (100 μM, positive control) was added. The cells were subsequently exposed at 37°C and 5% CO_2 for 3 h (nanoplastics) or 15 min (H_2O_2). The incubation period for H_2O_2 is short because it is a fast-acting oxidant and DNA strand breaks are repaired relatively fast after H_2O_2 exposure. Levels of DNA strand breaks were determined by the alkaline comet assay as previously described and reported according to the Minimum Information for Reporting on the Comet Assay (MIRCA) recommendations (78, 79). The exposure medium was removed and the cells were washed with PBS. Subsequently, 150 μl of trypsin was added and the cells were incubated 15 min, until the reaction was terminated by addition of 350 μl medium. Suspensions of cells (75 μl) were mixed with 600 μl of 0.75% agarose gel and 120 μl of this suspension was applied onto Gelbond films (Cambrex, Medinova Scientific A/S, Hellerup, Denmark). The gel-embedded cells were lysed overnight (2.5 M NaCl, 100 mM Na_2EDTA, 10 mM Trizma base, pH = 10). The samples were afterwards placed in electrophoresis buffer (1 mM Na_2EDTA, 300 mM NaOH, pH > 13.1) for 40 min, and the electrophoresis was subsequently run for 25 min at 300 mA and 20 V (0.83 V/cm; cathode to anode). The samples were placed in neutralization buffer (0.4 M Trizma base) for 15 min, followed by overnight treatment in 96% ethanol to preserve the embedded samples. The nuclei were stained with YOYO-1 dye (CAS No. 143413-85-8; 491/509 Thermo Fisher Scientific, Waltham, MA, USA) and scored manually under an Olympus CX40 fluorescence microscope at 40x magnification. The samples were blinded when scoring the comets, and the level of DNA damage was determined by using a five-class scoring system (arbitrary score range 0–400). For each sample, 100 randomly chosen nucleoids per slide were visually scored. The comet score was transformed to lesions per 10^6 base pairs (bp) by the use of a calibration curve from the European Comet Assay Validation Group where one arbitrary unit (0–100 a.u. scale) corresponds to 0.0273 lesions/10^6 base pairs as described previously (63).

Statistics

The results were analyzed by linear mixed effects model, and subsequently linear regression or ANOVA in separate strata of HepG2 and Caco-2 cells. The mixed effect linear regression model contained the cell type as categorical variable and concentration as continuous variable. Cell culture medium without nanoplastics was used as vehicle control. Statistical analyses were carried out in Stata 15 (StataCorp LCC, College Station, TX, USA). The results are reported as mean and standard error of the mean (SEM) of 2–3 independent experiments. Net inductions of DNA strand breaks and 95% confidence interval (95% CI) are reported to give an impression of the effect size and experimental variation.

RESULTS

Hydrodynamic Particle Size of Nanoplastic Suspensions

Particle size distributions of nanoplastic suspensions of PP and PET food containers are shown in **Figure 3**. The mechanical degradation of PP plastic food containers resulted in suspensions

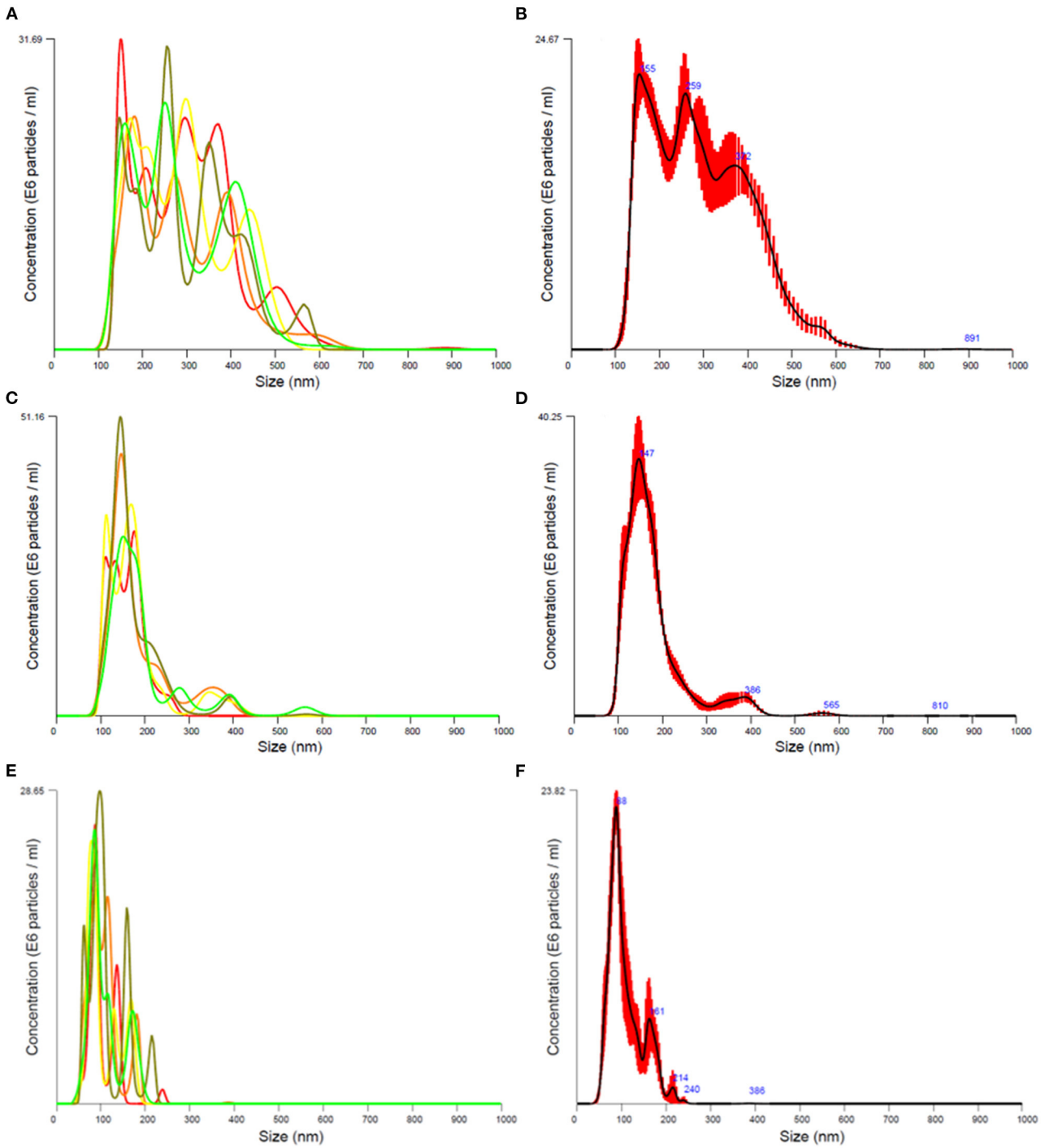

FIGURE 3 | Particle size distribution of final exposure suspensions from transparent polypropylene **(A,B)** and polyethylene terephthalate plastics **(C,D)** in the pilot study, and suspensions from black polyethylene terephthalate plastics in the main **(E,F)** study. The suspensions were analyzed in filtered water for injection. The mean particle size distribution **(B,D,F)** has been obtained from five consecutive size distribution measurements **(A,C,E)**.

with a broad particle sizes distribution in two larger fractions, 80–250 nm and 200–600 nm. The suspension from transparent PET plastic food containers had a primary size distribution peak at 80–250 nm, and a lower peak of particles with particle sizes between 300 and 400 nm. The suspension from black PET containers contained mainly particles with diameters less than 240 nm. The particle number concentration was approximately 20 times lower than expected (for the main study) after particles were passed

TABLE 2 | Hydrodynamic particle size and concentration data from the Nanosight experiment[a].

Characteristic	PET (main study)	PET (pilot)	PP (pilot)
Mean diameter (nm)	107 (10)	252 (100)	158 (55)
Particle number concentration (number/ml)	52×10^{11} (1.5×10^{11})	2.3×10^{10} (9.1×10^{8})	2.2×10^{10} (8.7×10^{8})
Stock suspension (mg/ml)	0.471 (0.14)	0.175	0.063
Final suspension (μg/ml)	0.6–7.1	0.001–0.063	0.003–0.175

[a] *Results are reported as mean and (standard deviation).*

through a sterile filter (0.45 μm) in the end (**Table 2**). The mass concentration has been estimated (and reported in the article) on basis of the particle number concentration, mean particle size and density of the particles.

Effects of Transparent PP and PET Particle Exposure in HepG2 and Caco-2 Cells

Results from the LDH and WST-1 experiments did not indicate cytotoxicity after 24 h exposure to PP in HepG2 and Caco-2 cells (**Figure 4**). The exposure to PET reduced the metabolic activity (WST-1 assay) in HepG2 cells (slope $= -0.39 \pm 0.12$, $P < 0.01$, linear regression) and increased LDH leakage in Caco-2 cells (slope $= 0.27 \pm 0.10$, $P < 0.05$, linear regression). Nevertheless, the cytotoxicity response was not consistent across cell types as mixed effect linear models were not statistically significant for PET (WST-1 assay: slope $= -0.24 \pm 0.12$, $P = 0.053$; LDH assay: slope $= 0.11 \pm 0.06$, $P = 0.09$).

Effects of PP and PET nanoplastic exposure on DNA strand breaks are shown in **Figure 5**. The exposure to PET was associated with a concentration-dependent increase in DNA strand breaks, which was not different in the two cell types (slope $= 0.28 \pm 0.11$, $P < 0.05$; single-factor of "cell type": $P = 0.71$). Based on this model, the highest concentration of PET generated 0.28 lesions/10^6 bp (95% CI: 0.04; 0.51 lesions/10^6 bp). However, the linear regression analyses and ANOVA in separate strata of HepG2 and Caco-2 cells did not indicate statistically significant effects. The exposure to PP was also positively associated with DNA strand breaks in mixed effects linear model, although this was not statistically significant (slope $= 0.10 \pm 0.06$, $P = 0.15$). The induction of DNA strand breaks at the highest concentration was 0.10 lesions/10^6 bp (95% CI: −0.04; 0.23 lesions/10^6 bp). The positive control (H_2O_2) increased the level of DNA strand breaks in both HepG2 and Caco-2 cells in a concentration-dependent manner ($P < 0.001$, linear regression).

Effect of Particles From Black Colored PET Food Container in HepG2 and Caco-2 Cells

The exposure to particles from black colored PET food containers did not affect the level of cytotoxicity in cells (**Figure 6**) or distribution of cells in G0/G1, S or G2/M phases of the cell cycle (**Figure 7**). Cultures of HepG2 and Caco-2 cells in the positive control (SFM) has a slightly shifted cell cycle with more cells in G0/G1 phase (8.0%, 95% CI: 0.5%, 15%) and less cells in S phase (decline: 4.7%, 95% CI: 8.7%, 0.8%) and M phase (decline: 3.2%, not statistically significant). The exposure to PET nanoplastics did not affect the ROS production level in HepG2 and Caco-2 cells after 3 h exposure (**Table 3**), whereas the positive control (H_2O_2) was associated with a 2.2-fold increase in ROS production ($P < 0.05$, linear mixed effects model). The exposure to PET nanoplastics was associated with increased levels of DNA strand breaks in HepG2 and Caco-2 cells ($P < 0.05$, linear mixed effect model) and the concentration-response relationship was the same in the two cell types (**Figure 8**). The induction of DNA strand breaks at the highest concentration was 0.43 lesions/10^6 bp (95% CI: 0.09; 0.78 lesions/10^6 bp). The positive control (100 μM H_2O_2) generated very high levels of DNA strand breaks (2.49 ± 0.06 lesions/10^6 bp). These comet assay experiments also included cryopreserved negative (0 μM H_2O_2) and positive (50 μM H_2O_2) controls of monocytic THP-1 cells. These assay controls did not indicate a difference between individual experiments (i.e., mean and standard deviation 0.08 ± 0.03 and 1.76 ± 0.18 lesions/10^6 bp).

DISCUSSION

Relative few studies have assessed the hazards of real-life plastic debris particles on mammalian cells. A pioneering study, published in 2008, grinded a polymer fabric of polyethylene and PET in a freezer/mill and incorporated the material into standard rodent chow (80). Rats were exposed for 13 weeks and no toxicological effects relevant to the treatment were observed (80). The particle size of the microplastics was not determined in the original study, although a later study has estimated the particle size to 1–50 μm (average size 15–35 μm), based on the grinding procedure (81). A more recent study milled PP microplastics (25–200 μm in diameter) to particles with a size of 20 μm (50% of the particles were 5–10 μm) and showed modest effects in term of cytokine release and ROS production in human cell lines (82). Another study on real-life plastic used exposure to visible/ultraviolet light as environmental weathering process and showed that a disposable coffee cup lid was gradually degraded over a 56-day period to particles with an average size of 224 nm (83). We experimented with relatively low-tech procedures to grind plastic items and obtain suspensions in the size range of nanoplastics (i.e., less than 1,000 nm in average diameter). The size range in PP suspensions was 100–600 nm, whereas that of PET was 100–400 nm (pilot study) and <240 nm (main study). It has been shown that 4 mm PET plastics could be milled and sieved to suspensions of particles with sizes smaller than 200 μm and peak number distribution at approximately 150 nm (84). At least based on these examples, it appears applicable to obtain suspensions of nanoplastics, whereas it might be challenging to obtain nanoparticles (i.e., smaller than 100 nm) from plastic items.

There are notable differences between the studies using the comet assay in ecotoxicological studies and cells from humans

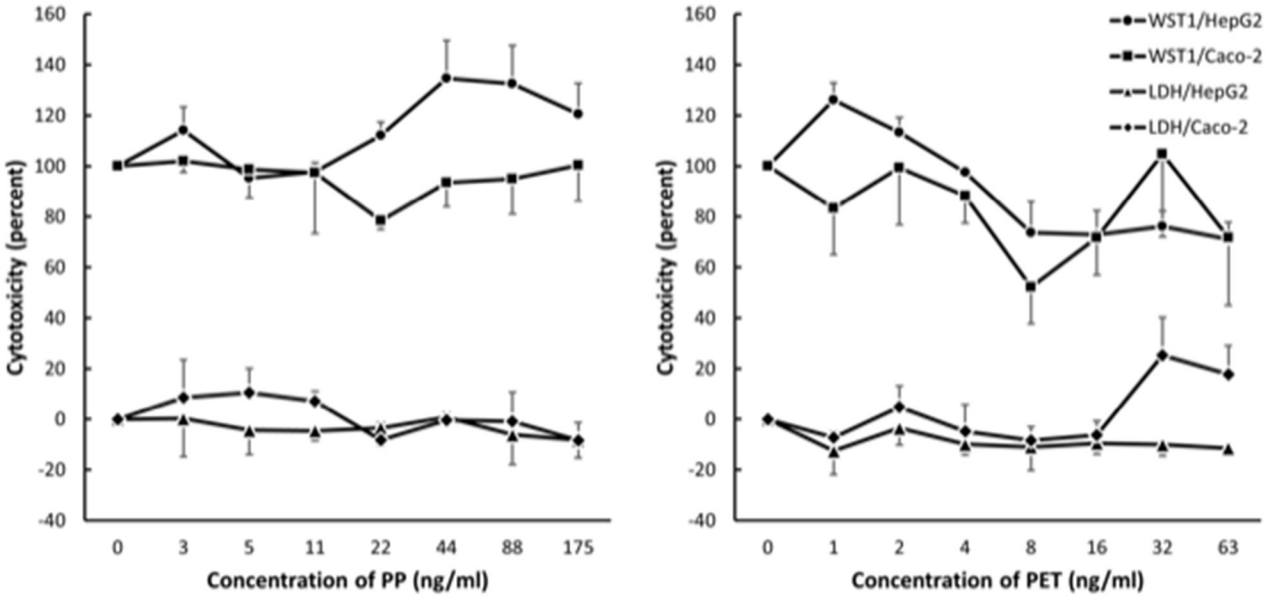

FIGURE 4 | Cytotoxicity in Caco-2 and HepG2 cells after 24 h exposure to polypropylene (PP) and polyethylene terephthalate (PET). The results are reported as fold-difference compared to the positive control (LDH assay) and unexposed (WST-1 assay). Symbol and error bars are means and SEM from three independent experiments.

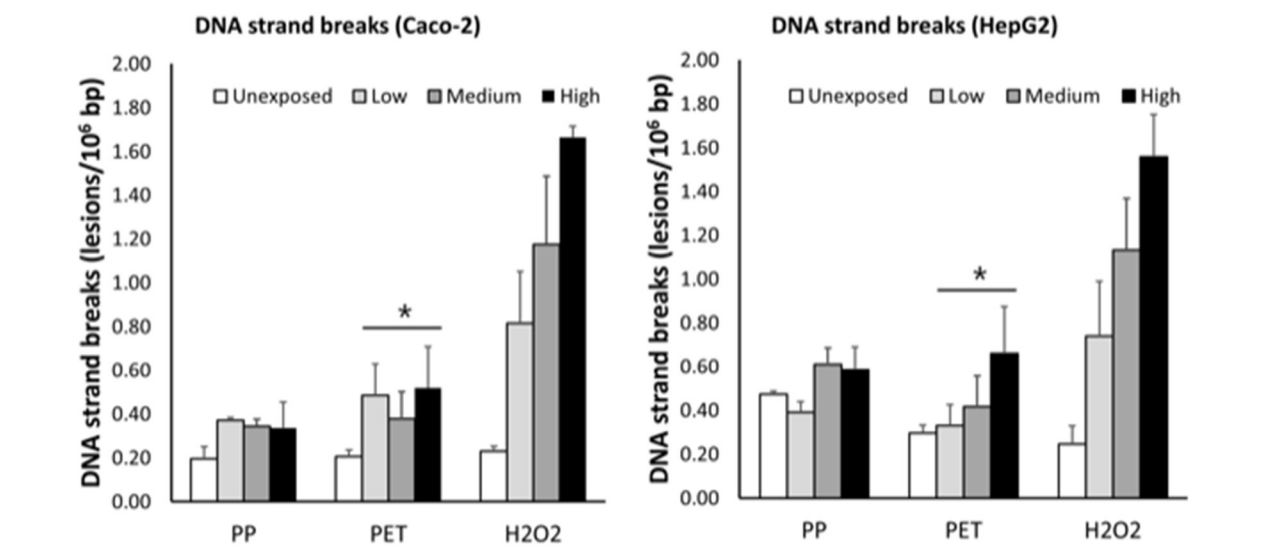

FIGURE 5 | Levels of DNA strand breaks in Caco-2 and HepG2 cells after 3 h exposure to grinded particles of polypropylene (PP) and polyethylene terephthalate (PET) food containers. The high concentration is 63 ng/ml, 175 ng/ml, and 100 μM of PET, PP, and H_2O_2, respectively. The medium and low concentrations correspond to sequential two-fold dilutions. Each bar is the mean and SEM of three independent experiments, except H_2O_2 in Caco-2 cells ($n = 2$). *$P < 0.05$, linear mixed effect model.

(**Table 1**). The exposure time is much longer in non-human species (i.e., typically weeks in ecotoxicology as compared to hours-days in human toxicology). It is also interesting that the ecotoxicological studies indicate that even large polystyrene particles with diameters larger than 1 μm are genotoxic in the comet assay (14, 23, 25, 28, 29, 31, 33). On the contrary, studies on human cells have indicated very little effect on levels of DNA strand breaks measured by the comet assay. Domenech et al. did not observe genotoxicity in Caco-2/HT29 intestinal cells, with or without co-culture with Raji-B cells

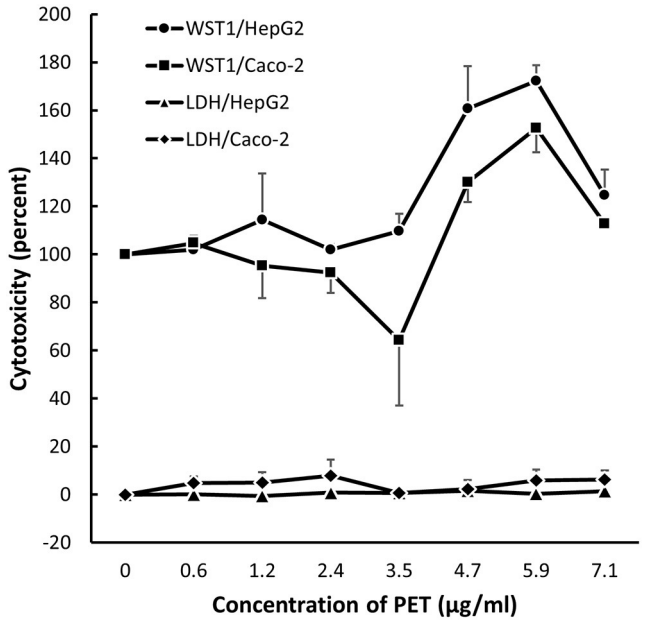

FIGURE 6 | Metabolic activity (WST-1 assay) and cell membrane leakage (LDH assay) after 24 h exposure to nanoplastics from black PET food containers (main study). The results are mean and SEM from three independent experiments.

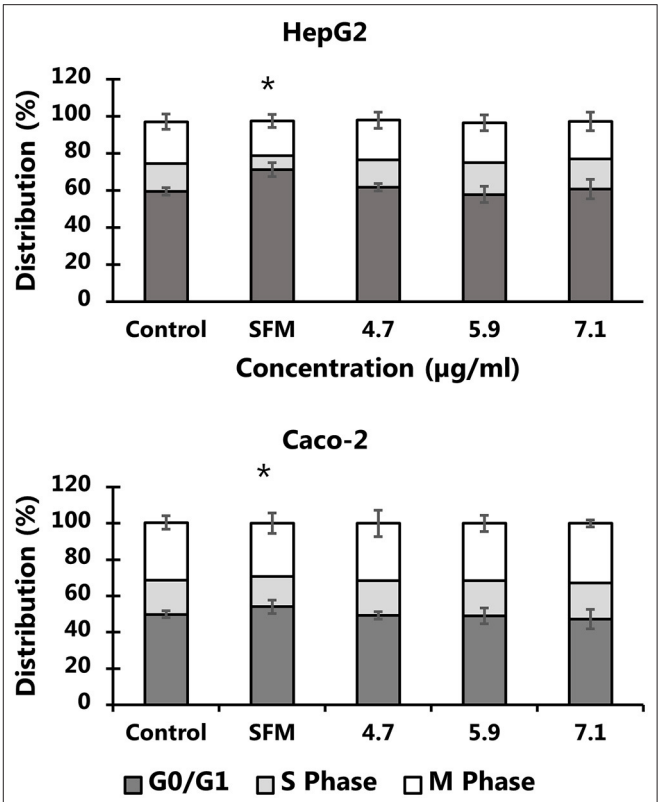

FIGURE 7 | Cell cycle distribution HepG2 and Caco-2 cells after 24 h exposure to PET nanoplastics from black food containers. The results are means of 2-3 independent experiments (mean and standard deviation). The exposure to nanoplastics is not associated with changes in the cell cycle distribution ($P > 0.05$), whereas culture of cells in serum free medium (SFM) shifted the cell cycle to G0/G1 phase from DNA synthesis phase (S). *$P < 0.05$, linear mixed effects model.

after exposure to nanosized polystyrene particles for 24 h (40). Likewise, Busch et al. reported unaltered levels of DNA strand breaks in co-cultures of intestinal cells after exposure to non-modified polystyrene and polyvinylchloride particles (85).

The statistical analysis of the comet assay results indicates a net increase between 0.10 and 0.27 lesions/10^6 bp of PP and PET, respectively (pilot study). The concentrations and net induction of DNA strand breaks was slightly higher in the main study on recycled PET nanoplastics (i.e., 0.43 lesions/10^6 bp). We have previously obtained statistically non-significant increases of approximately 0.23 DNA strand break/10^6 bp in HepG2 and A549 cells after exposure to liposomes for 3 h (86). Studies on combustion-derived particles from our laboratory have typically demonstrated net increases of DNA strand breaks in the range of 0.2–0.8 lesions/10^6 bp in cells after exposure to 100 µg/ml for 3–4 h (87–89). However, it should also be noted that we have used dispersion protocols with high-energy sonication that favors stable particle suspensions, whereas the sedimentation rate of particles might be lower. Using carbon-based nanomaterials, we have observed that approximately 10% of the administered dose deposits on cells at the bottom of the cell culture wells (90, 91). Longer incubation time increases the deposition, but it does not necessarily increase the level of DNA damage after particle exposure, which may be due to concurrent repair of DNA lesions. For instance, we have shown that 24-h exposure to carbon nanotubes increased the DNA repair activity in A549 cells (92).

It should be noted that the cell cycle distribution was unaltered after 24 h exposure, suggesting that the types of DNA lesions do not stall the replication. This suggests that the nanoplastics have produced relatively simple lesions such as single strand breaks and alkali-labile sites, whereas complex

TABLE 3 | Intracellular reactive oxygen species (ROS) production (relative to control) in HepG2 and Caco-2 cells after exposure for 3 h to nanoplastic from recycled PET food containers.

Concentration (µg/ml)	HepG2	Caco-2
0 (control)	1	1
0.1	1.02 ± 0.11	1.17 ± 0.11
0.2	1.22 ± 0.19	1.15 ± 0.09
0.4	1.14 ± 0.11	0.96 ± 0.16
0.9	0.80 ± 0.10	1.31 ± 0.22
1.8	0.94 ± 0.09	1.33 ± 0.21
3.6	1.43 ± 0.37	1.02 ± 0.26
7.1	1.04 ± 0.17	0.84 ± 0.19
Slope (± SEM)*	0.11 ± 0.20 (P=0.61)	−0.26 ± 0.19 (P=0.19)

**Linear mixed effects model indicated no statistical significance of the nanoplastic exposure (Slope = −0.08 ± 0.14, P = 0.58). The positive control (H_2O_2) was associated with a 2.2-fold increase in ROS production (95% CI: 1.1, 5.3-fold) in linear mixed effects model (P < 0.05). Results are mean and SEM of three independent experiments.*

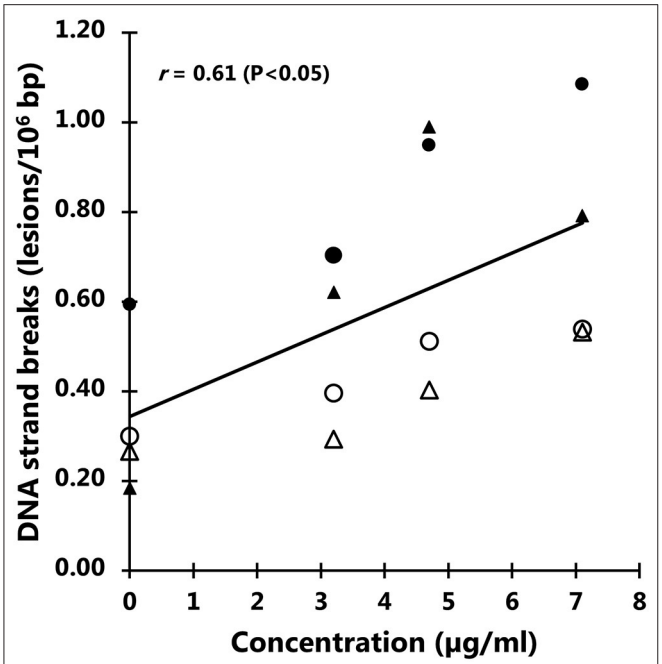

FIGURE 8 | Levels of DNA strand breaks in Caco-2 and HepG2 cells after 3 h exposure to particles from black polyethylene terephthalate (PET) food containers. The correlation coefficient refers to the concentration response relationship in linear mixed effect model. Symbols are individual experiments. The positive control (100 µM H_2O_2) is 2.49 lesions/10^6 bp (standard error of the mean = 0.06 lesions/10^6 bp).

lesions (e.g., DNA cross-links or double strand breaks) have not been predominant. However, the unaltered ROS production in both HepG2 and Caco-2 cells suggests that the genotoxic mechanism of action is not oxidative stress. It is possibly a direct physical interaction between nanoplastic particles with DNA or replication machinery or leakage of chemicals from suspended nanoplastics are causing DNA damage. Nevertheless, we considered that food contact materials are included in stricter regulation due to safety concern than other types of plastics.

The present study has certain important limitations. The chemical composition of the plastic items has not been assessed. It can be speculated that hazardous chemicals have not been added to the plastic items that we have used because they are used for food products. As such it could be argued that the present study may underestimate the genotoxic effect of microplastics that are found in nature as they come from all sorts of plastic items. The physical characteristics (e.g., shapes, agglomeration, and sizes) of the nanoplastic suspensions have not been determined. These characteristics may have an influence on the biological activity of particles, although it—to the best of our knowledge—is uncertain if such characteristics systematically affects the genotoxic potential determined for instance by the comet assay. In addition, it should be noted that differences in physical characteristics has been a relevant issue in studies on well-defined nanomaterials. Research on real-life nanoplastics is somewhat similar to studies on air pollution particles; both are complex mixtures of particles and fibers with many different shapes, sizes, chemical constituents and agglomerates. Another uncertainty is the mass concentration, which was extrapolated from particle number concentrations. There is a risk of the exposure being in the low end of the concentration-response range, although it could also be argued that the concentrations in the present study are closer to realistic exposures than most other studies on particles that uses up to 10-fold or higher concentrations.

In conclusion, the present study shows that real-life plastic materials relatively easy can be degraded to nanoplastics by mechanical processes. Suspensions of these nanoplastics from PP and PET food containers generates genotoxicity in terms of DNA strand breaks measured by the comet assay, without cytotoxicity, ROS production or altered cell cycle distribution.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MH, IK, EM, and JS performed the experiments. MR and PM drafted the manuscript, which was approved by all authors. All authors contributed to the article and approved the submitted version.

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Neurotoxicity of Engineered Nanomaterials: Testing Considerations

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As with toxicology in general, major challenges have emerged in its subfield neurotoxicology regarding the testing of engineered nanomaterials (ENM). This is on the one hand due to their complex physicochemical properties, like size, specific surface area, chemical composition as well as agglomeration and dissolution behavior in biological environments. On the other hand, toxicological risk assessment has faced an increasing demand for the development and implementation of non-animal alternative approaches. Regarding the investigation and interpretation of the potential adverse effects of ENM on the brain, toxicokinetic data are relatively scarce and thus hampers dose selection for *in vitro* neurotoxicity testing. Moreover, recent *in vivo* studies indicate that ENM can induce neurotoxic and behavioral effects in an indirect manner, depending on their physicochemical properties and route of exposure. Such indirect effects on the brain may proceed through the activation and spill-over of inflammatory mediators by ENM in the respiratory tract and other peripheral organs as well *via* ENM induced disturbance of the gut microbiome and intestinal mucus barrier. These ENM specific aspects should be incorporated into the ongoing developments of advanced *in vitro* neurotoxicity testing methods and strategies.

Keywords: neurotoxicity, nanomaterials, *in vitro*, toxicokinetic, engineered nanomaterials

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INTRODUCTION

The steady development and production of engineered nanomaterials (ENM) and their expanding range of uses in various fields requires an appropriate assessment of their potential health effects in humans. Initial concern about the harmful effects of ENM emerged from: (i) early inhalation toxicology studies with specific types of ultrafine particles, showing increased local pulmonary toxicity compared to larger particles of the same chemical composition, and (ii) studies that revealed evidence of increased uptake and translocation of nanoscale particles to tissues and organs beyond the initial deposition site in the respiratory tract (1, 2). The importance of toxicology research on ENM has been substantiated by studies that support a role of ambient ultrafine particles (UFP) in air pollution-associated diseases (3). Similarly, increased attention has been given to the potential neurotoxicity of ENM (4–6). A pioneering inhalation study by Oberdoerster and co-workers (7), demonstrated that insoluble carbon particles in the nano size range can rapidly translocate to the brain upon deposition in the nasal mucosa in rats. This observation formed a major trigger for further research to determine potential adverse health effects of inhaled UFP and ENM on the brain.

Adverse effects on the brain have been shown nowadays in various studies. Investigations with diesel engine exhaust (DEE), collected diesel exhaust particles (DEP) or ambient particulate matter (PM) (6, 8) as well as on-site-exposure studies with concentrated ambient PM (CAPs) have provided important support for the growing number of epidemiological studies that link air pollution to neurological diseases including dementia (9, 10). Neurotoxicological studies with ENM in rodents have been performed with the most widely produced and used materials like Ag, SiO_2, TiO_2, CeO_2, ZnO and carbon black. However, also less common materials have been studied like gold, quantum dots or iridium nanoparticles (11–13).

In the context of regulatory testing, neurotoxicity of ENM can be defined as a direct or indirect adverse effect caused by such particulate materials on the structure or functioning of the nervous system (14, 15). The scope of this mini-review is not to provide an in-depth overview of all studies that have been performed with various types of ENM and to provide a state of the art on their identified underlying mechanisms of neurotoxicity. For this we refer to various reviews by others [e.g. (5, 16, 17)]. In this paper, we highlight some of the unique physicochemical properties and associated effects of ENM that should be considered during testing and interpretation of their potential neurotoxicity.

In general, as with other effects and associated disease outcomes (e.g., in the respiratory or cardiovascular system), oxidative stress and inflammation are also considered key processes of potential neurotoxic and neurological consequences following ENM exposure (5, 6). Oxidative stress in ENM exposed cells, can cause activation of redox-sensitive signaling cascades involved in activation of pro-inflammatory cytokines and chemokines, proliferation, apoptosis and DNA damage induction (18), representing mediators or processes that all have been implicated in neurological and neurodegenerative diseases (5, 19). When taken together, current available neurotoxicological studies with ENM suggest substantial differences in hazards, strongly depending on their physicochemical properties, including the chemical composition. However, as we will discuss later, such an interpretation must take into account specific differences in dosimetry, (i.e., dose and route of administration) as well as the species selected for investigation (e.g., rat vs. mouse) used in the various studies, in perspective to realistic human exposures.

NEUROTOXICITY-RELEVANT EXPOSURE ROUTES OF ENM

Originally, nanotoxicology research has strongly focused on the evaluation of the potential adverse health effects that involve the inhalation route of exposure and pulmonary health risks. This obviously relates to the principal relevance of occupational exposures during manufacturing and handling of ENM. The emerging consensus that short- and long-term exposure to ambient PM is a risk factor for cardiovascular disease (20) has been a main drive to initiate studies that explored cardiovascular effects and underlying mechanisms of action for ambient UFP as well as for various types of ENM *via* inhalation exposure (20–22). Regarding this exposure route, for neurological evaluations, the importance of translocation of nasally deposited particles to the brain is now widely recognized. Herein, it is proposed that ENM can reach the olfactory bulbs of the brain following deposition on the nasal olfactory epithelium and subsequent translocation along the olfactory nerve (7). However, various additional routes of translocation into the brain should be acknowledged. In the upper respiratory tract, this may for instance also include translocation *via* the trigeminal nerve, while alveolar deposited ENM may enter the brain upon their translocation into the circulation (6). In contrast to the neuronal routes, this latter pathway also requires a subsequent passage of the blood-brain barrier (BBB).

The relative importance of translocation from the upper *vs.* lower respiratory tract has been elegantly explored in inhalation studies using radiolabelled iridium nanoparticles in rats and revealed the significance for both deposition sites (23). However, it should be emphasized that the significance of these various translocation routes may largely differ between various types of ENM depending on their composition and physicochemical properties and the associated impact of differently absorbed proteins and lipids (corona) on their translocation kinetics (6). Obviously, regarding the deposition and subsequent rate of translocation of nano-sized particles from the respiratory tract system, the distinct anatomical and physiological differences between rodents and humans must be taken into account as well (19).

While inhalation is the primary exposure route of interest for most bulk-manufactured ENM in occupational settings, other routes should also be considered regarding potential neurotoxicity. Systemic availability *via* dermal exposure, either occupationally or, for example, through its presence in cosmetics, is unlikely to be a major concern for neurological disease risks. In terms of dosimetry and accumulation into the brain, dermal uptake has been generally considered as negligible (2) although this may differ for instance in relation to impaired skin conditions during occupational exposure (24). Exposure to ENM *via* the oral route can be considered much more relevant and has therefore also become a subject of increasing interest in neurotoxicology research. Concerns about the adverse health effects of ingested ENM have increased with their growing number of applications in nanomedicine and particularly in the food sector, where they are used, for example, as food additives or incorporated in food packaging (25, 26). Accumulation of ENM in the brain following oral uptake is envisaged by their successive translocation from the intestine into the systemic circulation and BBB passage. This therefore also represents a potential translocation route for the fractions of inhaled UFP and ENM that are swallowed following mucociliary clearance (1, 27). Translocation from the intestine has indeed been demonstrated for specific ENM of poor solubility in rodents. However, quantitative findings by and large indicate that the amount of accumulation into the brain following oral exposure may be minimal to absent as well [e.g., (28–30)]. A schematic outline of the various exposure and translocation routes that may

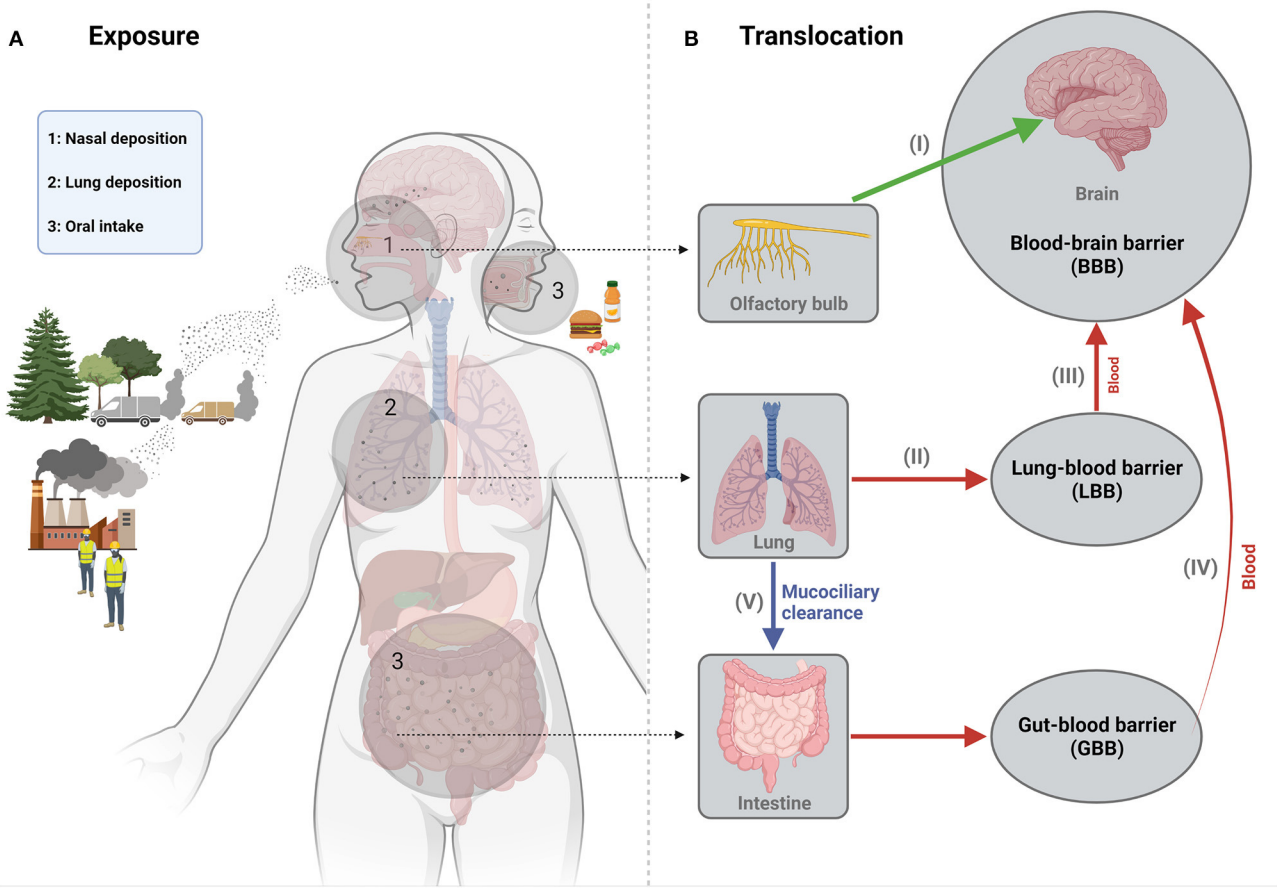

FIGURE 1 | Schematic representation of the main exposure **(A)** and translocation **(B)** routes of engineered nanomaterials in relation to their potential neurotoxicity. Concerns about the neurotoxicity of nanoparticles via inhalation exposure exist in particular for UFP originating from combustion processes at transport-dominated locations. Inhalation represents the most important occupational exposure route for ENM, during their manufacturing or further processing. The most relevant pathway of translocation to the brain for such inhaled particles involves their uptake and retrograde transport along the olfactory nerve upon deposition in the nasal cavity (I). UFP and ENM deposited in the lower respiratory tract can also translocate from this region into the bloodstream upon crossing the "lung-blood barrier (LBB)" (II) and a subset of these particles may thus enter the brain parenchyma across the blood-brain barrier (III). In non-occupational settings, the oral exposure route is particularly relevant for ENM, which may be present in food as additives or contaminants. Uptake across the intestinal mucosal barrier into the bloodstream (IV) of these ENM may thus also result in translocation into the brain. This pathway should also be considered for the fraction of inhaled small particles that is swallowed upon lung mucociliary clearance (V). Depending on exposure levels and their physicochemical properties, accumulation of ENM in the brain may thus directly affect brain structures and cells. However, neurotoxicity and neurological disturbances may also proceed in an indirect manner, for instance, driven by inflammatory effects of inhaled or ingested ENM at the organ of entrance or by ENM induced gut microbiome dyshomeostasis. Figure created with Biorender.com.

result in accumulation of UFP and ENM in the central nervous system is shown in **Figure 1**.

NEUROTOXICITY EVALUATION OF ENM

Neurotoxicity of ENM has been explored in rodent studies as well as in a rapidly growing number of *in vitro* studies [e.g., see reviews (5, 16, 17)]. While neurotoxicology research involves the investigation of effects of toxicants on both the central and peripheral nervous system (14, 15), current ENM research has strongly focused on the brain. Taken together, current available literature indicates that various ENM may have neurotoxic potential. However, studies cannot always be interpreted unambiguously, not least because of the unique physicochemical properties and behavior of the investigated nanomaterial in biological environments. Since the importance of research into the potential toxicity of ENM was recognized, large-scale research initiatives have been launched to dedicate research to support and improve their risk assessment. For instance, the extensive OECD Testing Programme of Manufactured Nanomaterials was launched, among others, to explore to what extent existing strategies and methods for the safety testing of chemicals are also directly applicable to ENM (31). Over the years, a vast array of studies, have been devoted to the investigation of the toxicity of various ENM, including large-scale interdisciplinary research projects. In this context, several assay artifacts could be identified when testing ENM that could lead to false negative or positive data. As a result, specific assay modifications were developed and further testing recommendations were introduced

to minimize potential misclassification [e.g., (32)]. It is crucial that such modifications also be deployed in current available *in vitro* neurotoxicity assays as well as in early stage development of novel test strategies in this research field (33, 34).

In **Table 1**, we have summarized intrinsic characteristics and properties of ENM in biological systems that may affect neurotoxicity assay outcomes as well as approaches that can be used to avoid such assay artifacts and potential misclassification. Above all, the reliability of *in vitro* testing approaches for neurotoxicity relies heavily on the use of appropriate ENM dispersion protocols which have been developed over many years for *in vitro* testing in general [e.g., (35, 36)]. Neurotoxicity testing should also incorporate the necessary control experiments to ensure that assay readouts are not disturbed due to interference of ENM with assay components such as, for instance, by adsorption of dyes, inactivation of reagents or scattering or quenching of (fluorescence) light. Approaches for such control experiments have been proposed and described by various investigators [e.g., (36–38)] and can be used as a basis when developing novel neurotoxicity assays. A particularly great progress in improving *in vitro* assays for ENM has been made following the recognition of the role of the protein/lipid corona in the toxicity of ENMs. In addition to the aforementioned establishment of the importance of the corona in the toxicokinetics of ENMs, its demonstrated influence on cellular uptake and toxicity has been used for further assay adaptations. Specific *in vitro* protocols nowadays incorporate a pre-coating step of the pristine ENM with e.g., (lung) surfactants or serum, or include digestion simulation protocols with gastrointestinal model fluids, to better reflect "bio-nano" interactions that are considered to take place *in vivo* depending on the route of exposure (39, 40).

Interpretation of neurotoxicity findings with ENM from *in vitro* studies will also benefit from improved dosimetry considerations. The strength of outcomes of *in vitro* studies increases with the selection of relevant test concentration ranges that are guided by outcomes from toxicokinetic investigations in rodents (25, 41), and support human health risks estimations under realistic exposure scenarios. Indeed, concentrations of ENM applied in neurotoxicity tests are often in the same order of magnitude as those used for *in vitro* investigations of effects at entrance organs (i.e., lung, intestine), while available toxicokinetic studies indicate large differences in gradients of locally achieved doses. Unfortunately, in-depth quantitative toxicokinetic studies and physiologically based pharmacokinetic (PBPK) modeling studies with ENM in rodents are currently still scarce [e.g., (30, 46–48)]. Moreover, such investigations are typically limited to one or few specific (model) compounds that may not be representative for other types of ENM, in view of each material's unique physicochemical properties. Common quantitative analytical methods used in toxicokinetic studies include AAS and ICP-MS. However, these methods cannot distinguish particulate from non-particulate (e.g., dissolved) compounds. Approaches that do allow for appropriate morphological and size-resolved analyses like electron microscopy are merely qualitative and thus may have limited relevance in toxicokinetic studies. However, these methods can provide important information to complement *in vitro* data. For instance, if a toxicokinetic investigation with a metal-based ENM would reveal exclusive accumulation of dissolved metal in the brain, *in vitro* neurotoxicity studies with the pristine particulate material would be irrelevant and redundant.

In vitro neurotoxicity studies can contribute to hazard identification and are nowadays increasingly used and further developed with high-content and high-throughput adaptations to further reduce animal studies (5, 49). Yet, rodent studies currently still remain a major component of neurotoxicity risk assessment. They can simultaneously tackle multiple aspects of neurotoxicity *via* evaluation of neurophysiological, neurochemical, neuroanatomical and behavioral endpoints (8). *In vivo* neurotoxicity of ENM can, for instance, be investigated along the design recommendations of the OECD Test Guideline TG 424 ("Neurotoxicity Study in Rodents") or embedded in subacute/subchronic oral toxicity studies (e.g., TG 407, 408) or inhalation toxicity studies (e.g., TG 412, 413) by inclusion of specific biochemical and molecular markers of neurotoxicity or neurobehavior tests. Apart from the selection of dose range and exposure duration, also the method of administration of ENM is important for the interpretation of the *in vivo* neurotoxicity findings. For instance, instead of inhalation exposures, studies have used intranasal administration with ENM at very high doses that do not reflect realistic deposition kinetics during inhalation exposure (6, 23). Such intranasal application in terms of dosimetry can exaggerate the neurological impact of ENM *via* the olfactory route, e.g., as a result of local damage and tissue impairment. Conversely, pharyngeal aspiration and intratracheal instillation studies bypass the nasal compartment. While these respective approaches can be very useful for specific mechanistic evaluations including toxicokinetic studies, they are obviously of lower relevance for quantitative risk assessment compared to inhalation studies.

Along the same lines, oral exposure studies with ENM also can involve various methods of administration that may have a large impact on toxicokinetics and toxicodynamics and thus study outcome interpretation. The most commonly applied method of oral administration is gavage. However, like the methods of intranasal instillation, intratracheal instillation and pharyngeal aspiration for the inhalation route, this represents a bolus dose delivery of ENM. Application of ENM in drinking water or in food thus represent more realistic administration methods. Marked differences in accumulations of (elemental) silver in brain were for instance observed following single oral gavage versus application in food pellets (42). However, also studies that address neurotoxicity upon administration in drinking water or food as "vehicles" require additional interpretation. Application in drinking water may affect dosing as well as the physicochemical properties of ENM as a result of agglomeration, sedimentation or dissolution. Introduction in food unavoidably results in complex ENM food matrix interactions that can strongly impact on various physicochemical properties as well including surface-reactivity. Drinking water vs. food applications will also result in different stomach and intestine passage times as well as digestive processes. Accordingly, the selection of the method and regime of application may have major potential

TABLE 1 | ENM neurotoxicity testing considerations.

	ENM characteristics and properties	Considerations and recommendations	References
In vitro assays	Effects of agglomeration status and dissolution rate on ENM toxicity	Use of standardized dispersion protocols; characterization of the physicochemical properties of ENM in test environment (e.g., cell culture medium)	(35, 36)
	Interferences with *in vitro* assays (e.g., adsorption and/or inactivation of assay reagents, disturbance of assay readouts by quenching, auto-fluorescence)	Inclusion of non-particulate assay controls; testing of adsorption quenching using ENM spiking at different concentrations	(36–38)
	Formation and alteration of "corona" upon ENM entrance and distribution in biological systems; associated alterations in toxicokinetic and toxicodynamic properties	Testing of pristine ENM vs. ENM (pre)treated with "corona" mimicking compounds and further ENM-surface modifying environments, e.g., (model) lung surfactant, serum proteins, artificial digestion fluids (stomach, intestine)	(39, 40)
	Dosimetry aspects	Use input from toxicokinetic/PB-PK modeling studies for *in vitro* dosing justification and outcome interpretation	(25, 41)
In vivo assays	Effects of exposure route (inhalation, ingestion) and selected application method on physicochemical properties of ENM	Critical evaluation of the limitations and potential flaws by non-physiological (bolus) administration of ENM, i.e.,: - Inhalation vs. intranasal instillation, intratracheal instillation, pharyngeal aspiration - Application in drinking water or food vs. oral gavage	(42, 43)
	Toxic effects of ENM on entrance organs, e.g., induction of lung inflammation, disturbance or gut homeostasis	Evaluation of inflammation, oxidative stress and barrier integrity effects for organ of entrance (respiratory tract, gastrointestinal tract); analyses of ENM effects on microbiome	(5, 6, 27, 44, 45)

impact on neurotoxic effects in animal studies. Various *in vitro* methods have been developed in recent years to simulate how digestion processes can affect the physicochemical properties of ENM (43).

INDIRECT EFFECTS OF ENM ON NEUROTOXICITY

A final important aspect in neurotoxicity risk assessment of ENM is the consideration of direct vs. indirect effects. On the one hand, it is recognized that *in vivo* neurotoxicity studies may detect indirect effects that are not neurotoxicity-specific. For instance, adverse behavioral responses in exposed rodents may be the consequence of the animals' responses to impairments in other organs and thus a mere reflection of general sickness (8). Inclusion of neurotoxicity-independent indicators of systemic toxicity in the study design, for instance by histology and clinical biochemistry, thus benefits insight on underlying mechanisms of identified neurobehavioural responses in the rodent models and improve judgement of their relevance for neurotoxicity risk in humans. In this context, it is pointed out to be particularly careful when interpreting single-dose animal studies performed at very high dose levels (8). On the other hand, studies may also identify neurotoxicity effects that, albeit indirect, may have a strong mechanistic basis and relevance for humans. The importance of indirect effects of inhaled particles, including ENM, in mutagenesis in the respiratory tract is nowadays well recognized, and considered to be driven by oxidative and proliferative mediators released from recruited inflammatory phagocytes (50, 51). Sustained pulmonary inflammation by ENM has also been proposed as a mechanism for systemic responses e.g., in the cardiovascular system (52). In turn, it is nowadays also increasingly recognized that systemic peripheral inflammation can contribute to neurotoxicity and neurological disease, e.g., *via* activation of microglia by inflammatory cytokines and other mediators or as a result of infiltration of the brain by peripheral immune cells [reviewed by (44)]. Interestingly, these indirect effects can be amplified in conditions of BBB impairments, and effects on the integrity of this barrier and associated neurotoxic responses have indeed been demonstrated for inhaled ENM (53, 54).

Finally, indirect mechanisms of neurotoxicity should also be recognized for ENM in relation to oral exposure. It has emerged that oral exposure to ENM can lead to changes in the intestinal microbiome (45, 55). Intestinal dyshomeostasis resulting from alterations in microbiota composition and their products (e.g., short-chain fatty acids and associated bidirectional gut-brain-axis signaling process that influence mucosal immune responses and the integrity of the intestinal barrier (56, 57). As such, one can even hypothesize that potential effects of inhaled ENM on the brain could involve microbiome-gut-brain axis effects following their mucociliary clearance (27).

DISCUSSION

With regard to the potential neurotoxic effects of ENM, inhalation and ingestion represent the two most relevant exposure routes. Depending on the route and exposure levels as well as on their specific physicochemical characteristics, ENM may reach the brain *via* various pathways (**Figure 1**). While available toxicokinetic/PBPK studies generally indicate that translocation to the brain will be low or even absent, this cannot necessarily be extrapolated to all types of ENM. Further research is needed to guide *in vitro* dosimetry of molecular mechanistic and animal-alternative neurotoxicity testing methods as well as to substantiate assessment of translocation and potential

accumulation of ENM for the human brain. In contrast to current *in vitro* methods, *in vivo* studies can also identify neurotoxic effects that results from indirect mechanisms of action of ENM, for instance, mediated by pulmonary and systemic inflammatory mediators or altered gut-brain axis signaling (**Figure 1**).

To avoid misclassification of ENM regarding their potential neurotoxicity, dosimetric and mechanistic aspects discussed in this paper and summarized on **Table 1**, should be critically considered in the current and future development of alternative testing methods and strategies. In the regulatory context, the assessment of neurotoxicity has traditionally been strongly focused on the evaluation of *in vivo* data, whereas *in vitro* assays have been seen merely as complementary (8, 49). The respective advantages and disadvantages of *in vivo* and *in vitro* tests have been discussed and weighed over the years with respect to their values in human neurotoxicity risk assessment [see, for instance: (58–60)]. The importance of animal testing reduction and replacement methods in toxicology is widely recognized today, both in basic and applied research. The extent to which currently available and newly developed *in vitro* neurotoxicity tests are reliable for the assessment of ENM increases with the recognition and active investigation of the potential occurrence of assay artifacts with these complex particulate substances. At the same time, there is a need for further research into the role of ENM-induced peripheral inflammation, gut dyshomeostasis and other possible indirect mechanisms in the causation of neurological and neurodegenerative diseases.

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All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Physical Obstruction of Nasal Cavities With Subsequent Asphyxia, Causes Lethality of Rats in an Acute Inhalation Study With Hydrophobic HMDZ Surface-Treated Synthetic Amorphous Silica (SAS)

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The aim of the present study was to understand the mechanism of lethality associated with high dose inhalation of a low-density hydrophobic surface-treated SAS observed in some acute inhalation studies. It was demonstrated that physical obstruction of the upper respiratory tract (nasal cavities) caused the effects observed. Hydrophobic surface-treated SAS was inhaled (flow-past, nose-only) by six Wistar rats (three males and three females) in an acute toxicity study at a concentration of ∼500 mg/m^3 for an intended 4-hr exposure. Under the conditions of the test set-up, the concentration applied was found to be the highest that can be delivered to the test animal port without significant alteration of the aerosol size distribution over time. None of the test-material-exposed animals survived the planned observation time of 4 h; three animals died between 2¾ h after starting exposure and cessation of exposure at 3¼ h, two died after transfer to their cages and the remaining animal was sacrificed due to its poor condition and welfare considerations. Histology accomplished by energy dispersive X-ray (EDX) analysis demonstrated that test material particles agglomerated and formed a gel-like substrate that ultimately blocked the upper respiratory airways, which proved fatal for the rat as an obligatory nose breather. This observation is in line with the findings reported by Hofmann et al. showing a correlation between lethality and hydrophobicity determined by contact angle measurement. The aerosol characterizations associated with this study are provided in detail by Wessely et al.

Keywords: synthetic amorphous silica, inhalation, rat, contact angle, physical obstruction, suffocation, asphyxia

INTRODUCTION

Particles with low systemic toxicity such as titanium dioxide (TiO_2) or different forms of synthetic amorphous silica (e.g., SAS, SiO_2) are currently under regulatory scrutiny. The classification of certain forms of titanium dioxide (TiO_2) as suspected carcinogens by inhalation was published on February 2, 2020 amending the EC regulation no. 1272/2008 (CLP Regulation). TiO_2 as E 171 was removed from the EU list of approved food additives [Com Reg (EU) 2022/63] based on a scientific opinion by the European Food Safety Authority (EFSA). The origin of this regulatory pressure is partly due to the particles falling under the nanomaterial definition but also to guidance values for classification and labeling that have never been validated for the local respiratory effects caused by particles. Strictly following the CLP guidance would result in classifications for these particles based on the animal toxicity studies; however, it is important to consider whether the substance form reflects exposures for humans. The particles of these and other substances have in common a low bulk density (according to TRGS 527) and no systemic toxicity but, are either already classified, or in the process of being classified as 'a hazardous or toxic substances' under CLP regulation in the EU with severe consequences for production processes and applications, whereas TRGS 527 is suggesting an occupational exposure limit (OEL) as an alternative.

Low-density particles are extremely light and fluffy. This not only means that on a mass basis there are many more particles compared to a standard dust aerosol, but also that the quite large fluffy aggregates and agglomerates would result in easier blockage of the nasal cavities in the rat compared to standard dust particles. Therefore, high concentrations of low-density particles, such as hydrophobic SAS, can cause lethality in acute inhalation tests at concentrations <1,000 mg/m^3 [ECETOC JACC Report No. 51, (1)]. In OECD guidance 403 and 436 studies (2, 3), observed lethality below 1,000 mg/m^3 within 4 h can lead to skull and crossbones labeling (toxic by inhalation) with respect to the CLP classification and labeling criteria. Recently, the European Risk Assessment Committee (RAC) proposed the Acute Tox Cat 2 (with a hazard statement: "Fatal if inhaled") classification for HMDZ (hexamethyldisilazane), a surface-treated SAS, and one form of hydrophobic SAS, based on the lethality in test animals observed within 4 h at 540 mg/m^3 in an acute inhalation study with read-across to hydrophobic DDS (dimethyldichlorosilane) surface-treated SAS showing a calculated LC50 of 450 mg/m^3 (4). No mortalities were observed with this substance in the low-concentration group (210 mg/m^3), seven of 10 died in the mid-concentration group (540 mg/m^3) and all the rats died (100% mortality) in the high-concentration group (2,100 mg/m^3). The calculated LC50 of 450 mg/m^3 is generally an extremely high concentration for particles, particularly for SAS when taking into account that the occupational exposure limit (OEL) for SAS in the German TRGS 900 is 4 mg/m^3 inhalable which is more than 100 times lower. Furthermore, amorphous silica is not associated with any intrinsic toxicity. SAS forms are used in food, feed and cosmetic applications. Lindner et al. (5) were able to show in their study that nanostructured biogenic amorphous silica (BAS) occurs in food products such as common horsetail and oat husk and that electron microscopical examination showed no morphological differences from synthetic amorphous silica. *In vitro* studies performed by Wiemann et al. (6) showed that surface treatment with hydrophobic coating reagents (organosilanes) strongly reduces the bioactivity of SAS. Against this background, the question arises as to what could cause HMDZ surface-treated SAS lethality in acute inhalation studies. Is it systemic toxicity of the substance or is it more likely that, based on the high concentrations applied in acute inhalation studies, we are dealing with a high-dose phenomenon associated with physical obstruction of the upper respiratory tract? Physical obstruction at high particle concentrations is considered in OECD guidance 39 (7); paragraph [69] explicitly states that "*At very high concentrations, dry powder aerosols... tend to form conglomerates in the proximal nose causing physical obstruction of the animals' airways (e.g., dust loading) and impaired respiration which may be misdiagnosed as a toxic effect.*"

Regarding the objective of clarifying the cause of lethality associated with physical obstruction of the upper respiratory tract, it needs to be clearly stated that the studies carried out under the OECD guidelines cannot answer this question. In cases of lethality, OECD 403 and 436 studies for acute inhalation require a dead animal count and only a cursory macroscopical examination of the outer surfaces of the organs in the abdominal and thoracal cavity. Thorough pathological and histopathological examinations of the entire respiratory tract, especially the upper respiratory tract (nasal cavities) is not required. These OECD guidelines are fully sufficient to identify general systemic effects but specific particle- related questions on fatal local activity cannot be answered, e.g., the physical obstruction of the upper respiratory tract leading to suffocation as cause of mortality. Rat respiratory tract anatomy differs to that of humans: there is mainly monopodial branching of airways, smaller ventilatory unit volume, smaller alveolar size and a lower average number of cells per alveolus (8). Unlike to humans, the rat is an obligatory nose breather; while fixed in a tube for 4 h during acute inhalation studies, the rat can neither protect its nose nor can it even carry out normal cleaning behavior. Taking all these points into account, a new GLP acute inhalation study with HMDZ surface-treated SAS was performed to allow possible areas of obstruction to be identified. Additional parameters were considered in the design of this new study (**Table 1**). A thorough histopathological examination of the entire respiratory tract, including different levels of the nasal cavities, larynx, trachea and lung was included and EDX analysis was performed to determine the chemical identity of agglomerated material. To characterize hydrophobicity of the tested substance, contact angle measurements were made. Aerosol generation considerations are the subject of a separate publication (9). To assure the validity of this new acute inhalation toxicity study with HMDZ surface-treated hydrophobic SAS, it was necessary to select the highest technically feasible concentrations without significant aerosol alteration.

Considering the rat nose, the mass median aerodynamic diameter (MMAD) of the aerosol had to fulfill the

TABLE 1 | Extended experimental design of the acute inhalation test.

1	To take four-chamber aerosol samples during a 4 h study (gravimetrical filter analysis) at an outlet port of the nose-only platform; the aerosol concentrations 50, 500, 1,000, and 2,000 mg/m^3 required by OECD TG 436 will be adjusted to use a target concentration of 500 mg/m^3 as a starting point; the exact value may increase during the 4 h exposure (saturation effect of the exposure unit). *Due to dead/moribund animals, the exposure was stopped after 3 h and surviving animals were transferred to cages. Because of the shortened exposure period, only three gravimetrical filters were taken. 494.0, 519.8, and 537.9 mg/m^3; mean: 517.2 ± 18.0 mg/m^3 (N = 3). An on-line recording of the aerosol concentration was performed in parallel using an aerosol photometer.*
2	To determine the particle size distribution at least twice during a 4 h study; cascade impactor; aerosol generation must be established and controlled over 4 h to avoid too strong agglomerate formation at the outlet ports of the nose-only platform. *Only one value determined because of the shortened exposure period: MMAD = 1.31 μm*
3	To record body weights during the day 1 and 14 post-exposure observation periods.
4	To record gross pathological changes of those rats that died or were killed in a moribund condition prior to the end of the day 1 and 14 post-exposure observation periods.
5	To record gross pathological changes of all animals at terminal sacrifice.
6	To characterize hydrophobicity of the tested substance contact angle measurements were conducted.
7	To histopathologically examine the entire respiratory tract of the animals at days 1 and 14 post-exposure including different levels of the nasal cavities, larynx, trachea and lung. Histopathology was performed in combination with EDX analysis to determine chemical identity of agglomerated material. *Due to dead/moribund animals, the exposure was stopped after 3 h and histopathological as well as EDX examinations were performed for day 1 only.*
8	All observations helping to explain the potential cause of mortality will be documented. This includes photographs, especially of the external nose area (nostrils and whiskers) during and after exposure, to make sure that important details, such as a clot of particulate material at the tip of the nose, are documented (the rat is an obligatory nose breather).

≤4 μm criterion. The test concentration in this study was 500 mg/m^3.based on the investigations from Wessely et al. (9).

MATERIALS AND METHODS

Extended Study Design

Table 1 lists the parameters that were used in addition to those indicated in OECD TG 436 as minimum obligatory requirements for a regulatory test of acute toxicity following inhalation.

Test Item

HMDZ surface-treated SAS (CAS number: 68909-20-6; CAS name: *Silanamine, 1,1,1- trimethyl-N-(trimethylsilyl)-, hydrolysis products with silica*), lot #150060338, was provided by Evonik Operations GmbH, Germany. The test material exhibits the following physicochemical properties: BET: 230–290 m^2/g (medium surface area), purity 99.8% (based on ignited material), delivered as fluffy, white powder with a skeletal density of 2.3 g/cm^3 at 20°C by helium pycnometry (3P Instruments) and a tamped density of about 0.06 g/cm^3.

Test Item Characterization and Optimization

Figure 1 shows the contact angle of the test material as measured according to DIN 55660-2 (11) and Hofmann et al. (12). The measurement was performed using a contact angle instrument, the DSA100 Drop Shape Analyzer and ADVANCE software (KRÜSS Scientific, Germany).

Aerosol Generation

The rats were exposed to an aerosol generated by a flow-past, nose-only inhalation exposure system which has been used for previous inhalation studies at Fraunhofer ITEM (13). In this system, the animal's snout is placed in the anterior end of the tube, which is connected to the exposure cylinder by means of a push fit. The aerosols enter continuously the animal's nasal region through a small tube. The exposure cylinder is operated at slightly positive pressure with respect to the surrounding air. This ensures that a continuous air flow is passing through the animal's breathing zone. In this system, the aerosol is supplied to each rat individually and exhaled air is immediately removed and drained out of the test system. Therefore, oxygen supply is always sufficient and measurement of the oxygen concentration is unnecessary.

The airflow or aerosol flow to each rat port was ∼1 L/min, which is assumed to be laminar. The total flow rate through the test unit with 16 ports was ∼40 L/min; the total volume of the inhalation system (excluding the mixing box) ensures that the intended concentration of the test item was reached shortly after start of exposure (50% concentration value after ∼4 min). The test item was aerosolized using a dry dispersion system operated with pressurized air. The aerosol generator (TOPAS Co.) was provided by Technical University Dresden, Germany and had already been used in the preparation period at TU Dresden to characterize and optimize the physicochemical properties of the aerosol and to investigate aerosol altering, re-agglomeration and precipitation generated mass loss all over the test unit. The feasible limit concentration for non-altering aerosol concentration was determined using this equipment. Because the impact of equipment, hoses, hose material, hose length/diameter and other design parameters heavily influences aerosol behavior, the animal test set-up was designed to duplicate as closely as possible the TU Dresden (9) set-up to create valid aerosol generation. The system was tested without animals to demonstrate the test system compliance (9). This technical prework guaranteed that there were no aging effects on particle agglomerates in the 4 h exposure period of the animal experiment. An aerosol photometer signal was used to control the dispersion system feed rate to maintain a constant aerosol concentration in the inhalation unit. Actual test item concentrations were measured in the breathing zone of the animals.

Monitoring and Controlling the Exposure Atmospheres

Air flow, temperature and relative humidity were measured continuously and recorded as 10-min means. The limits were set

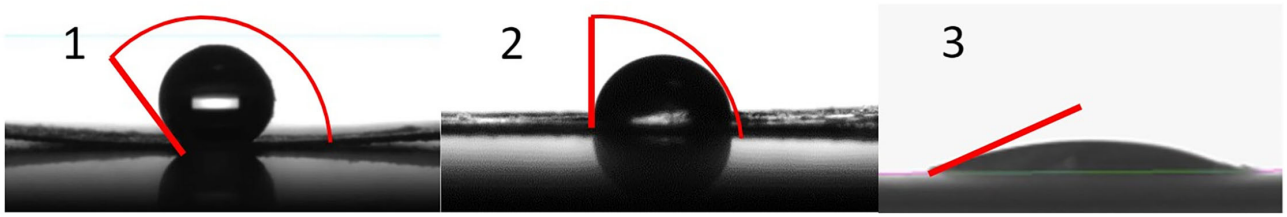


FIGURE 1 | Contact angle for different synthetic amorphous silica products, (1) HMDZ surface- treated SAS, (2) untreated pyrogenic silica, (3) untreated silica gel (10).

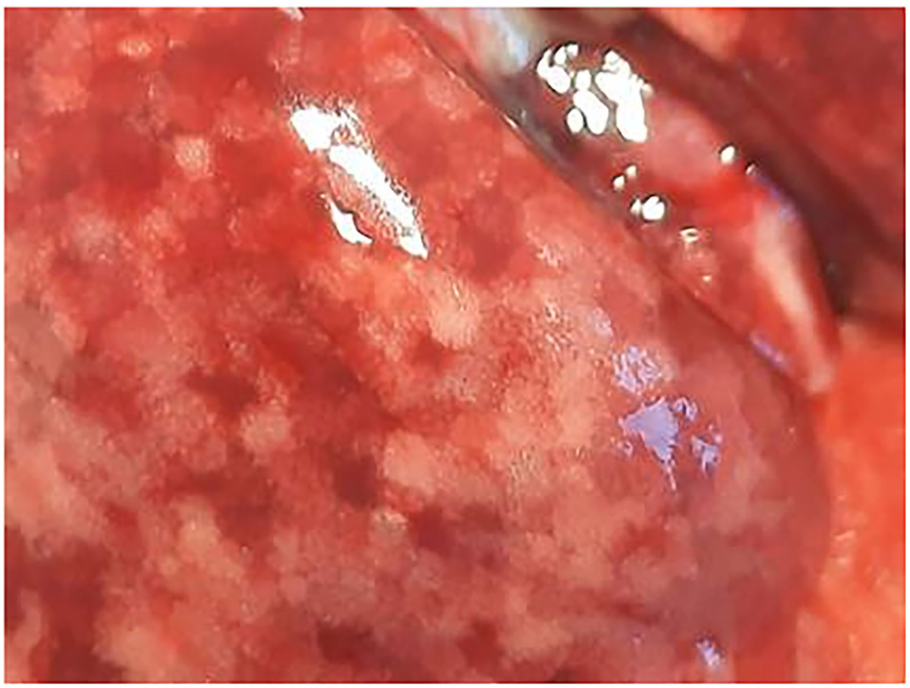


FIGURE 2 | Male rat. Deceased 1 h. after inhalation (in cage). Marbled discoloration of lung surface due to congestion. Visible focal petechia. Whitish areas are representative of air-filled alveolar structures (indicative of acute emphysema).

at 22 + 2°C for temperature and 55 + 15% for relative humidity. Animal room lighting was on a 12 h light/dark cycle controlled by an automatic timing device.

An aerosol photometer developed at Fraunhofer ITEM was used to continuously monitor the aerosol concentration. To adjust the photometer, the aerosol concentration was determined gravimetrically using filter samples (four times per 4 h inhalation).

The required MMAD range in acute toxicity studies is 1–4 µm. Due to the low test-item density, the aerosol showed rapid aging and a strong tendency to form large agglomerates out of this range. Thus, the experimental design given by the OECD TG needed adjustments (**Table 1**). The MMAD of the aerosol phase was determined by measuring the dry aerosol (optical particle sizers; two MMAD determinations were done within the 4 h exposure period).

The exposure set-up was documented by taking photographs. This included the formation and deposition of particle agglomerates in the interior conducting pipes of the exposure unit as well as external deposits in the animals' nasal areas (nostrils and whiskers).

Animal Allocation and Treatment

Three young adult Wistar Crl:WI (Han) rats per sex (Charles River Deutschland, Sulzfeld, Germany), ∼7 weeks of age at delivery, were allocated to this study. The animals were allowed to adjust and become acclimatized to the Fraunhofer ITEM environment for ∼2 weeks. Animals were group-housed (separated by sex) in Makrolon® (polycarbonate) type IV cages. Cages and absorbing softwood bedding material (Lignocel BK8-15) were changed once weekly. Tap water from the Hannover city water supplier was offered fresh weekly in a Makrolon® bottle fitted with a stainless-steel nipple top with a hole ∼0.5 mm in diameter. As diet, a commercial chow in pellet form was used, identified as ssniff V1534, purchased from ssniff-Spezialdiaeten GmbH (Soest, Germany). The diet was offered fresh weekly.

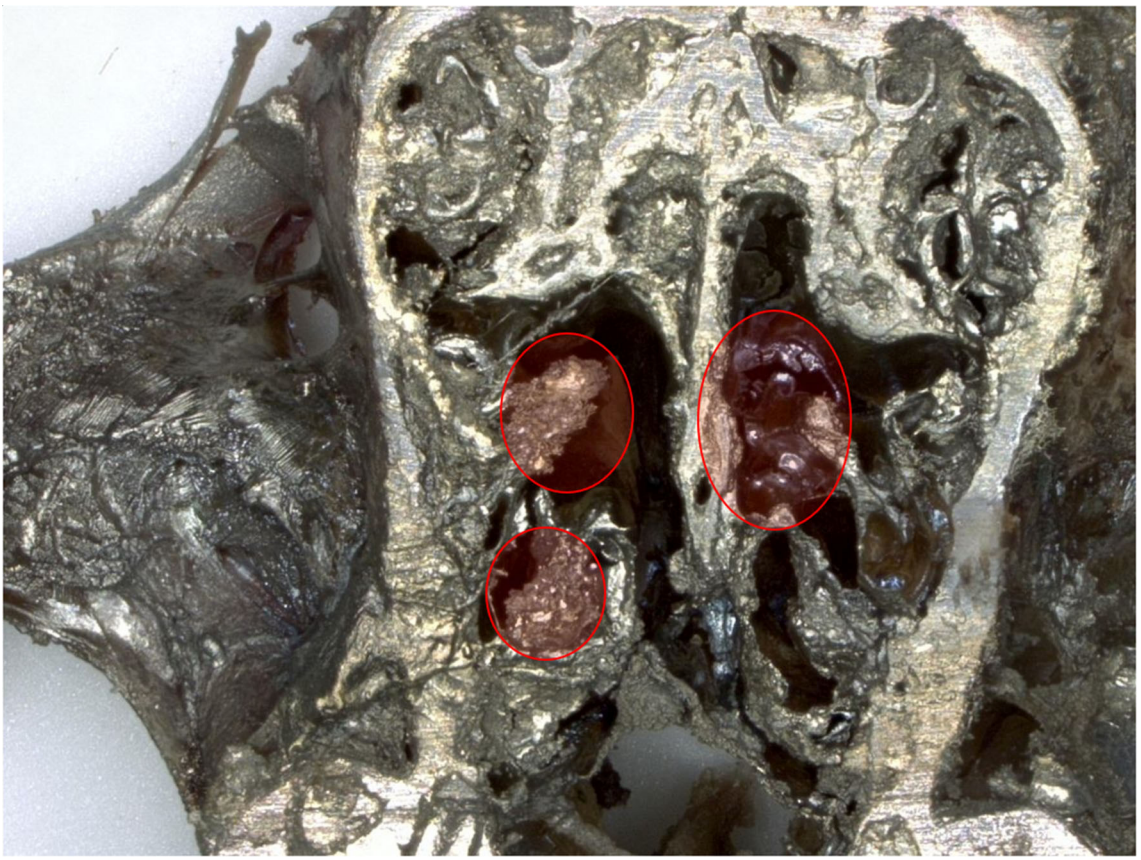


FIGURE 3 | Male rat. Deceased after 2¾ h after start of inhalation. Nasal cavity level 4 (frozen, dried, sputtered sample): partial blockage by deposited test item. Digital microscopy, lens x30.

The temperature and the relative humidity of the animal room were monitored electronically and recorded on a continuous basis. The limits were set at 22 + 2°C for temperature and 55 + 15% for relative humidity. A 12 h light/dark cycle was used controlled by an automatic timing device. The air exchange rate was at least 10 times per hour. Clinical observations were made during inhalation and, when inhalation was stopped ahead of schedule (∼3 h after the start of the experiment), the three surviving animals were observed in their cages. Two of these animals died spontaneously after transfer to the cage and one animal was euthanized.

Body weights were measured during the acclimatization period (once; randomization weighing).

The rats were exposed to an aerosol generated by a flow-past, nose-only inhalation exposure system at Fraunhofer ITEM, Hannover, Germany. For 2 weeks before exposure, rats were trained to the exposure tubes avoiding undue stress on the animals. Animal restraining tubes are constructed in such a way that hyperthermic effects on rats cannot occur. In this system, the aerosol is supplied to each animal individually and exhaled air is drawn off immediately. The rats are placed around the exposure cylinder in tapered acrylic glass tubes with adjustable backstops. Historical measurements have confirmed that there are no differences in concentrations among the different outlets.

Necropsy and Histological Processing

Necropsies were performed at Fraunhofer Institute for Toxicology and Experimental Medicine (ITEM), Hannover, Germany. The lung and the lower half of the trachea were weighed and used for histopathology. The lungs were inflated under a pressure of about 20 cm water column with formalin and fixed by immersion. An extensive set of organs and tissues (nasal cavities, larynx/laryngopharynx, trachea, lungs, heart, thoracic aorta, tongue, esophagus, salivary glands (mandibular, sublingual, parotic), gastrointestinal tract including Peyer's patches, liver, pancreas, thymus, spleen, lymph nodes (bronchial, mandibular, mediastinal, mesenteric), kidneys, urinary bladder, testes/epididymides, prostate, seminal vesicles, ovaries/uterus with cervix and vagina, thyroids with parathyroids, adrenals, pituitary gland, brain, spinal cord, sciatic nerve, eyes with optic nerves and Harderian glands, skin, mammary glands, skeletal muscle, sternum and femur with bone marrow) was sampled and subjected to macroscopic evaluation. The tissues were fixed in 10% neutral-buffered-formalin, the eyes with optic nerves and Harderian glands were fixed in Davidson's fixative and the nasal cavities were deep frozen with liquid nitrogen to avoid loss of SAS in the cavities from rinsing out with liquid fixing solution. All samples were transferred to AnaPath Services GmbH, Liestal, Switzerland, where histological processing, EDX analysis and

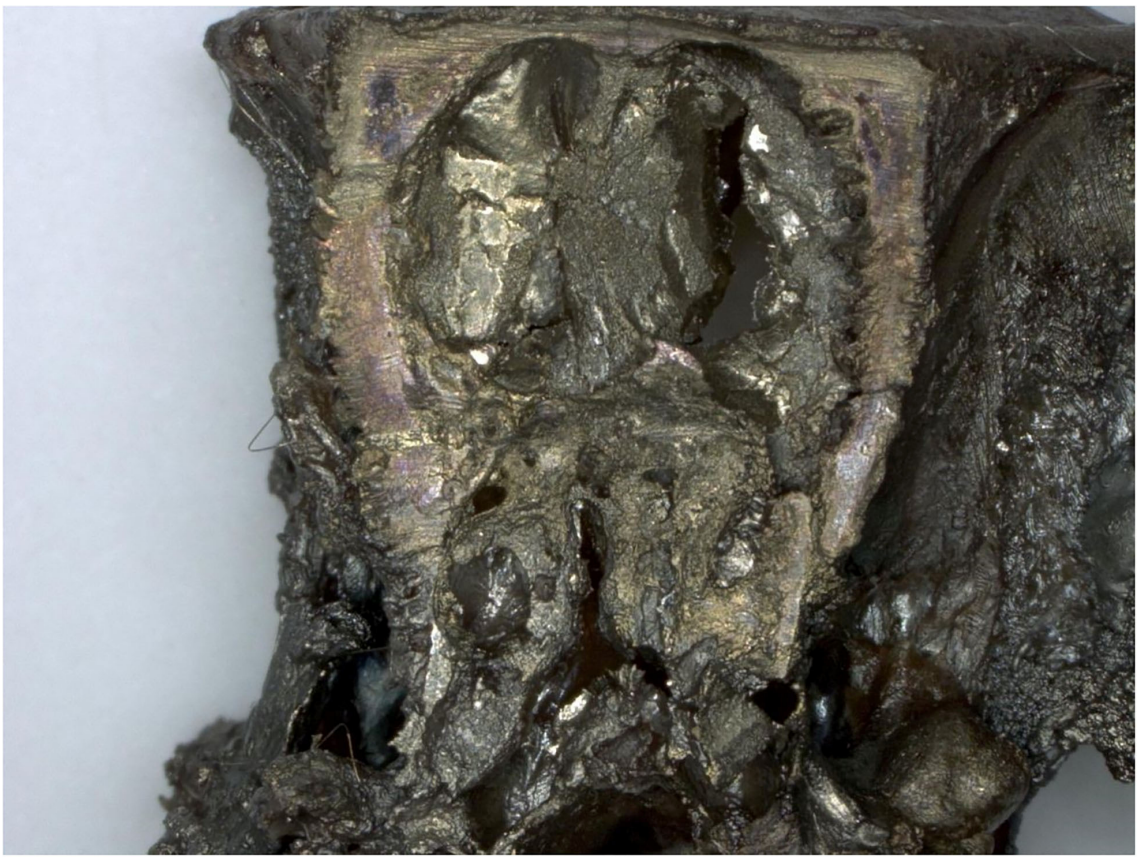


FIGURE 4 | Female rat. Deceased 1 h. after transfer to cage. Nasal cavity level 4 (frozen, dried, sputtered sample): complete blockage by deposited test item. Digital microscopy, lens x30.

histopathological evaluation (Oberbuchsiten, Switzerland) were performed.

The lungs were trimmed according to Ruehl-Fehlert et al. (14), Kittel et al. (15) and Morawietz et al. (16). The left lobe was split at the bifurcation with one part frozen and cryosectioned for EDX analysis. The larynxes were frozen, trimmed at three levels according to the previously cited RITA recommendations and cryosectioned. At each level, one section was stained with HE and one was used for EDX analysis. The tracheas were frozen, trimmed longitudinally and cryosectioned, with one section used for HE-staining and one for EDX analysis. The nasal cavities were transferred frozen to AnaPath Services GmbH. For the two deceased males and the deceased female that died during the inhalation procedure, the nasal cavities were sawn with a diamond blade according to trimming scheme of Young (17) and Kittel et al. (15) and dried for EDX analysis. For the remaining animals, the nasal cavities were submerged and fixed in 100% ethanol and embedded in methyl methacrylate resin before being sawn with a diamond blade according to the same trimming scheme for EDX analysis.

All other tissues were trimmed, dehydrated, embedded in paraffin wax, sectioned at an approximate thickness of 4–5 µm and stained with hematoxylin and eosin (HE) according to AnaPath Services GmbH SOPs. The microscopic tissue sections were quality-controlled under the light microscope before being transferred to the study pathologist.

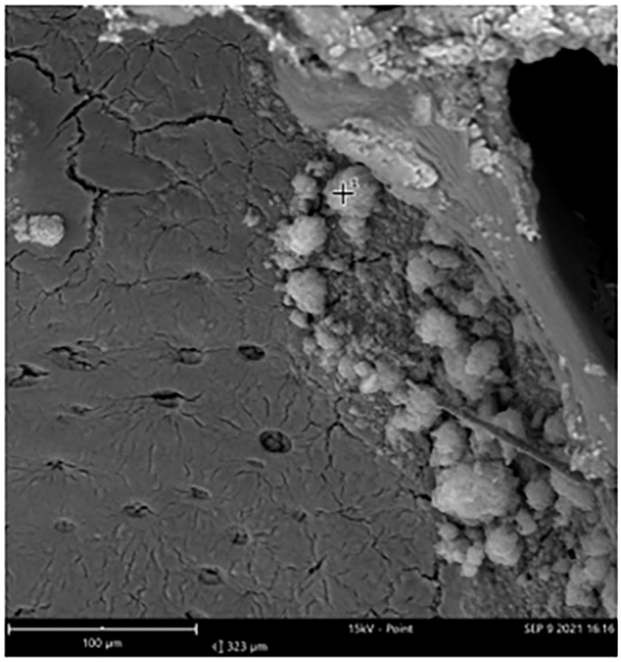
Histopathological Evaluation

Histological changes were described, wherever possible, according to distribution, severity and morphologic character. Severity scores were assigned on a scale of 1–5.

Grade 1, Minimal: This corresponds to a histopathologic change ranging from inconspicuous to barely noticeable but so minor, small, or infrequent as to warrant no more than the least assignable grade. For multifocal or diffusely distributed lesions, this grade was used for processes where $< \sim$10% of the tissue in an average high- power field was involved.

Grade 2, Slight: This corresponds to a histopathologic change that is a noticeable but not a prominent feature of the tissue. For multifocal or diffusely distributed lesions, this grade was used for processes where between ∼10 and 25% of the tissue in an average high-power field was involved.

Grade 3, Moderate: This corresponds to a histopathologic change that is a prominent but not a dominant feature of the tissue. For multifocal or diffusely distributed lesions, this grade was used for processes where between ∼25 and 50% of the tissue in an average high-power field was involved.



Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	65.33	50.80
14	Si	Silicon	24.29	33.15
6	C	Carbon	7.67	4.48
47	Ag	Silver	2.02	10.60
11	Na	Sodium	0.28	0.32
16	S	Sulfur	0.12	0.19
20	Ca	Calcium	0.12	0.23
12	Mg	Magnesium	0.07	0.09
17	Cl	Chlorine	0.06	0.11
15	P	Phosphorus	0.04	0.05
13	Al	Aluminum	0.00	0.00

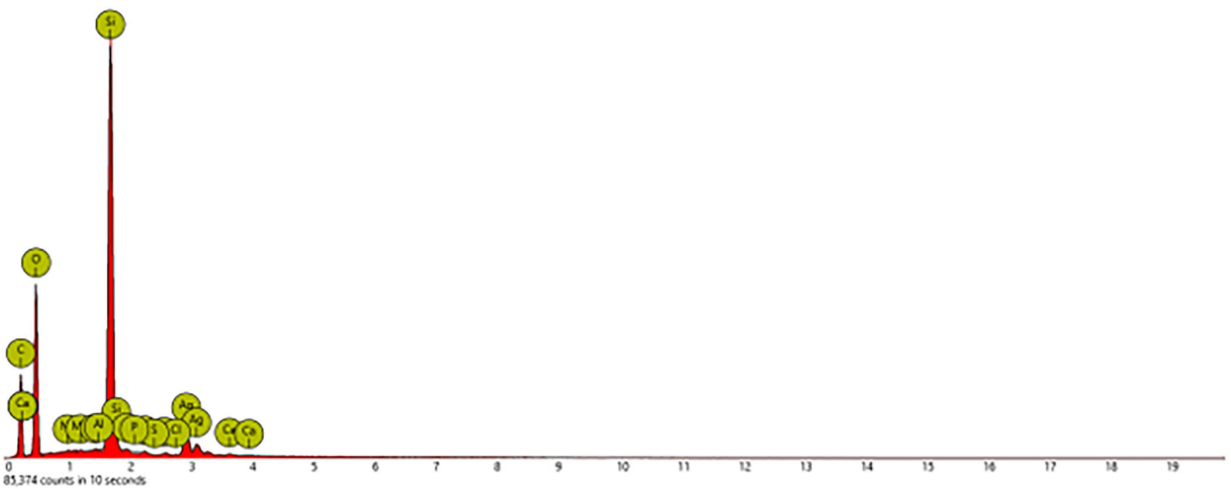


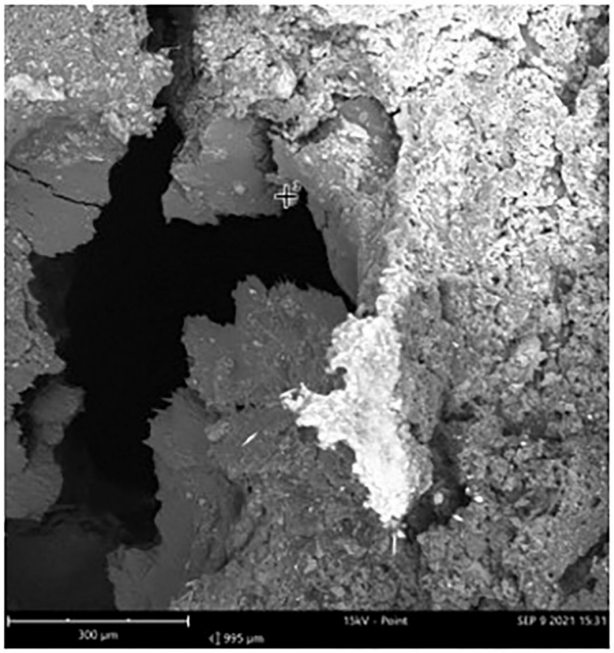
FIGURE 5 | Male rat. Deceased after 2¾ h after start of inhalation. Nasal cavity, frozen and dried, level 3, area 3, EDX point analysis showing SEM image, EDX spectrum, and atomic and weight concentration table of detected Si. Note: agglomeration of particles.

Grade 4, Marked: This corresponds to a histopathologic change that is a dominant but not an overwhelming feature of the tissue. For multifocal or diffusely distributed lesions, this grade was used for processes where between ∼50 and 95% of the tissue in an average high-power field was involved.

Grade 5, Severe: This corresponds to a histopathologic change that is an overwhelming feature of the tissue. For multifocal or diffusely distributed lesions, this grade was used for processes where more than ∼95% of the tissue in an average high-power field was involved.

EDX Analysis

Sections from frozen tissues were placed on silicon-free plastic slides (EMS plastic slides, Electron Microscopy Sciences), silver coated at 10 µm thickness and inserted into the Phenom^{TM} Pharos (Thermo^{TM} Scientific) SEM for scanning electron microscope imaging and energy dispersive X-ray (EDX) point analysis. The tissues were systematically searched and particles/spots with suspicious contrast or morphology were point analyzed. The analyses were conducted using the following specifications: mode 15 kV, intensity point, detector BSD Full and vacuum 60 Pa. The frozen nasal cavities were dried, sawn with a



Element Number	Element Symbol	Element Name	Atomic Conc.	Weight Conc.
8	O	Oxygen	59.63	47.66
14	Si	Silicon	33.94	47.62
6	C	Carbon	5.73	3.44
11	Na	Sodium	0.24	0.28
20	Ca	Calcium	0.20	0.40
12	Mg	Magnesium	0.13	0.16
47	Ag	Silver	0.06	0.35
15	P	Phosphorus	0.06	0.09
13	Al	Aluminum	0.00	0.00
16	S	Sulfur	0.00	0.00
17	Cl	Chlorine	0.00	0.00

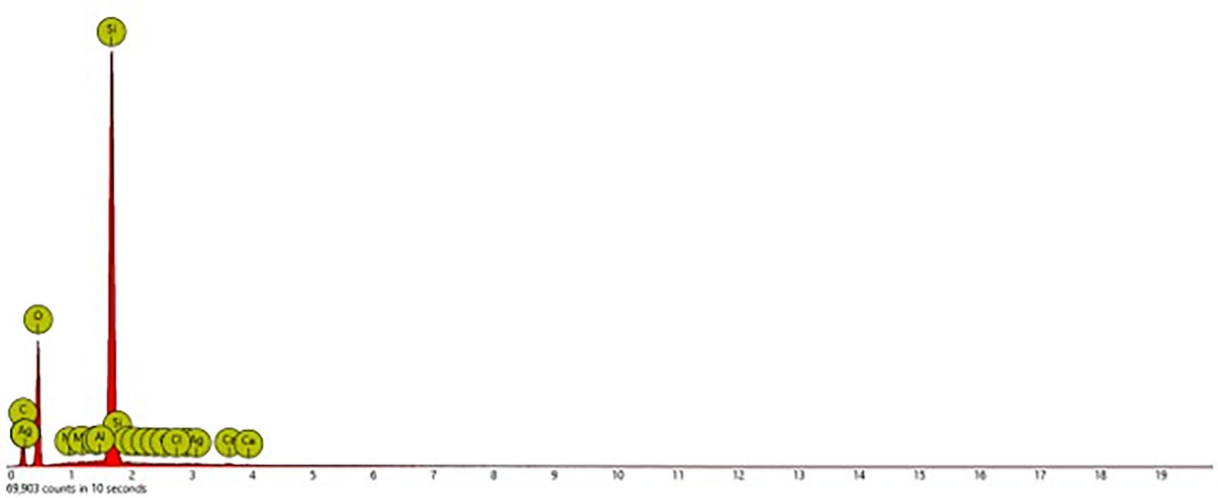


FIGURE 6 | Male rat. Deceased after 3¼ h after start of inhalation, Nasal cavity, frozen and dried, level 4, area 2, EDX point analysis showing SEM image, EDX spectrum, and atomic and weight concentration table of detected Si.

diamond blade, resin embedded and sawn again, then coated and analyzed using an identical procedure.

RESULTS

Contact Angle Measurement of Test Item (Prüfbericht TU Dresden 2021)

The contact angle of 147.21 ± 5.95° revealed hydrophobic properties of the test item (**Figure 1**).

Aerosol Characterization

Due to dead/moribund animals, the exposure had to be stopped after 3 h and surviving animals transferred to cages. Because of the shortened exposure period, only three gravimetrical filters were taken: 494.0, 519.8 and 537.9 mg/m^3; mean 517.2 + 18.0 mg/m^3 ($N = 3$).

Aerosol concentration was recorded on-line in parallel using an aerosol photometer (light scattering).

As is normal, the particle size distribution was measured at one port of the test system using a cascade impactor indicating at the time of measurement a MMAD of 1.31 µm. However, the pretest comparison, without rats, using laser diffraction and cascade compactor measurements for the test unit and test conditions over 4 h clearly demonstrated that the cascade impactor results always led to a much finer particle size distribution due to the induced shear forces on the agglomerated

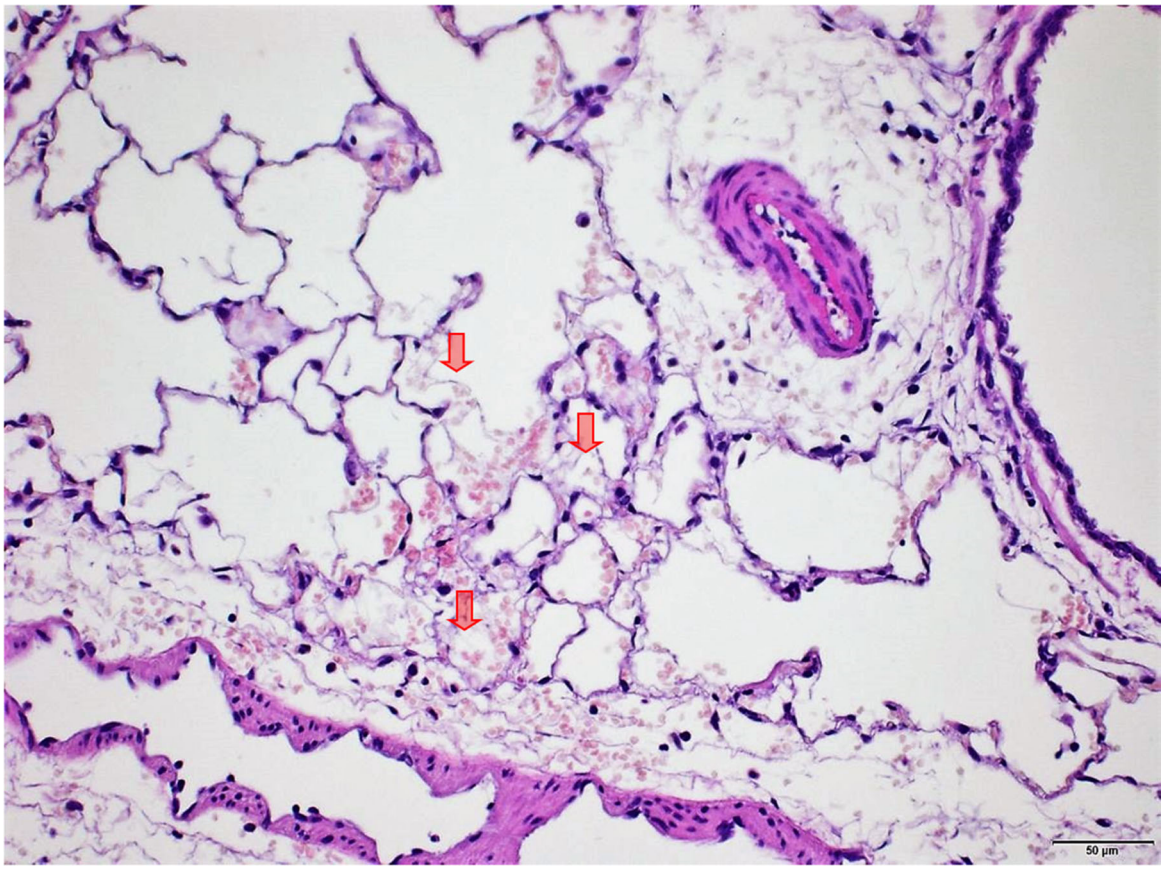


FIGURE 7 | Male rat. Deceased 2¾ h after inhalation start. Lung, right caudal lobe. Note fibrin in alveoli (arrows) associated with tiny hemorrhagic foci. HE, original magnification x20.

aerosols in the cascade impactor. Therefore, the MMAD results of the cascade impactor provided the wrong impression of fine particles while in reality the particles size though ongoing agglomeration was actually increasing over the time, more rapidly at the beginning of the test but dynamically, all over the time. Details are provided by Wessely et al. (9). Cascade impactor measurements subpress the aerosol altering through re-dispersion, as part of the measurement systematic and thus cannot used as quality criteria for fluffy powders with low tapped density.

In-Life Observations Including Inhalation and Post-Inhalation Period

The first male was found dead after 2¾ h of exposure, 5 min after showing a reduced respiratory rate, followed by one male and one female 3 h after the start of exposure, prior to death, the rats showed signs of anemia and a reduced respiratory rate. Because of these mortalities, the exposure was stopped after 3¼ h for animal welfare reasons and the remaining male and two females were transferred to their cages. One hour later, one further male and female with respiratory distress were found dead. The last animal was sacrificed for concurrent moribund conditions and respiratory distress.

Gross Lesions

The animals underwent necropsy within 10 min after death. No external deposits of test material were observed in the animals' external nasal areas (nostrils and whiskers). The lungs of all animals were discolored dark red or spotted and showed a spongy consistency (**Figure 2**). These findings were consistent with congestion, edema, acute emphysema and petechiae. In the trachea of the deceased animals there was a foamy content.

Histopathology and EDX Evaluation Nasal Cavities

In nasal cavities processed from frozen material and sputtered for subsequent EDX analysis, the evaluation under digital microscopy revealed deposition of foreign material in all nasal cavity at levels 3 and 4. Only a minimal deposition was noted in nasal cavity at level 3 (**Figure 5**). In contrast there was partial to almost complete blockage by foreign material deposition in nasal cavity level 4 (**Figures 3**, **4**). By SEM-EDX evaluation, the test

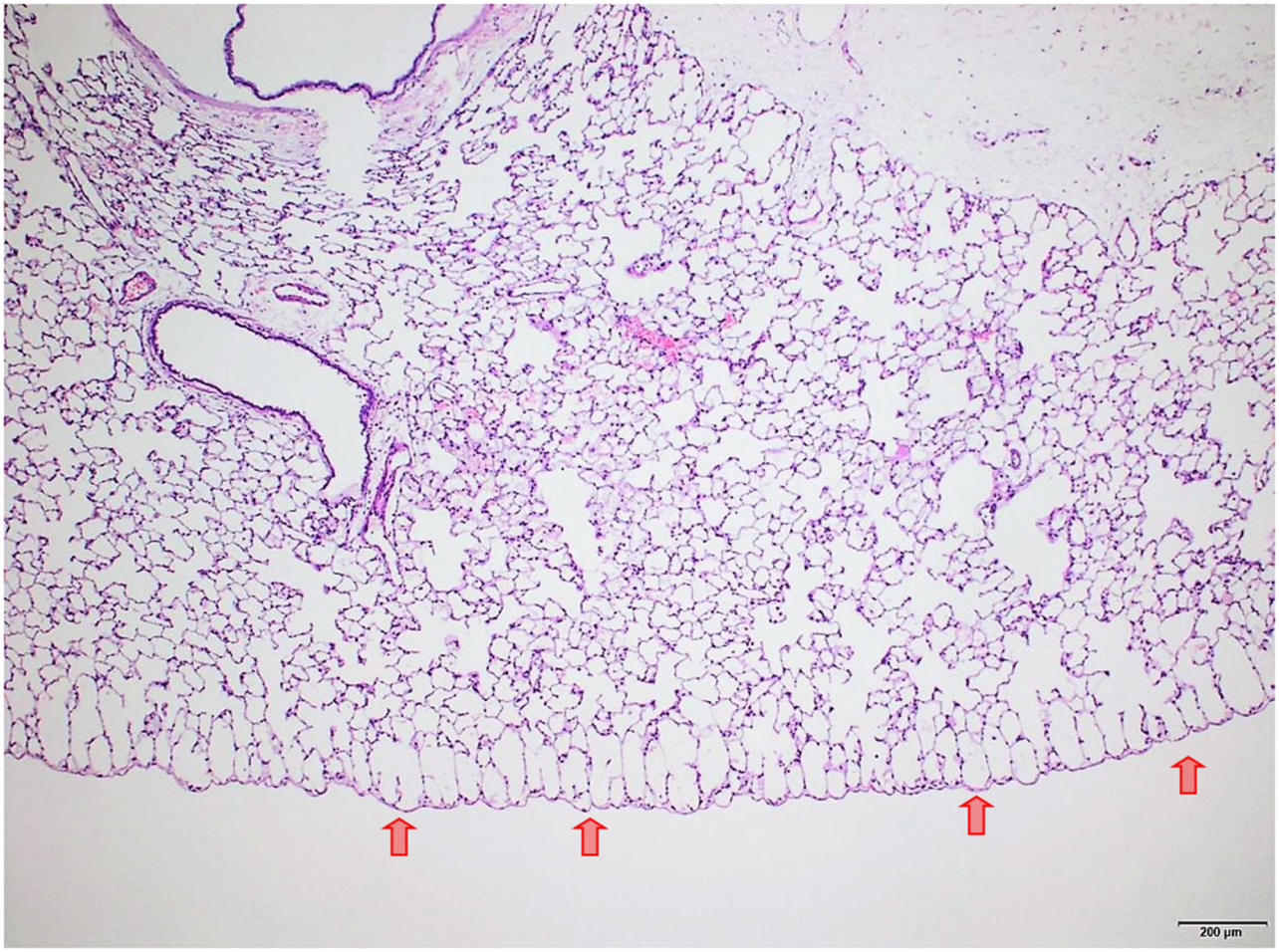


FIGURE 8 | Male rat. Deceased 3¼ h after inhalation start. Lung, left lobe. Focal subpleural acute emphysema (arrows). HE, original magnification x20.

item was found accumulated as larger SAS particles with a more gel-like appearance (**Figures 5**, **6**).

Larynx, Trachea With Bronchial Bifurcation and Carina

Histologically, there were no findings in larynx, trachea, bronchial bifurcation or carina. By EDX, minor amounts of Si were detected in the larynx and trachea of only one animal, the small speck detected in the trachea was deemed to be from background contamination.

Lungs

Several findings were noted in lungs that are related to respiratory failure, including focal to multifocal hemorrhages (**Figure 7**) representing petechiae, alveolar fibrin (**Figure 7**), focal to multifocal acute emphysema (**Figures 8**, **9**) and, rarely, focal alveolar wall necrosis (**Figure 9**). Subsequent mixed-cell infiltrate and macrophages were noted in several samples (**Table 2**). No Si was detected in lungs by EDX.

Other Organs

In four animals (two per sex), there were focal to multifocal hemorrhages in the thymus. No findings in the remaining organs could be attributed to treatment or as the cause of morbidity/death.

DISCUSSION

The European Risk Assessment Committee (RAC) proposed the classification for hydrophobic HMDZ surface-treated SAS as Acute Tox Cat 2 (fatal if inhaled) based on the lethality to test animals observed in an acute inhalation study performed in Wistar rats with a calculated 4 h LC50 of 450 mg/m^3 (18). Re-evaluating these data, it was assumed that no conclusion could be drawn as to the cause of lethality in the seven of 10 animals that died at 540 mg/m^3 during the study based on macroscopical examination of the outer surfaces of the organs in the abdominal and thoracal cavity only. Although clinical effects of respiratory distress and macroscopical findings, such as lumps of white particles and slime in the nose and focal hemorrhages on the

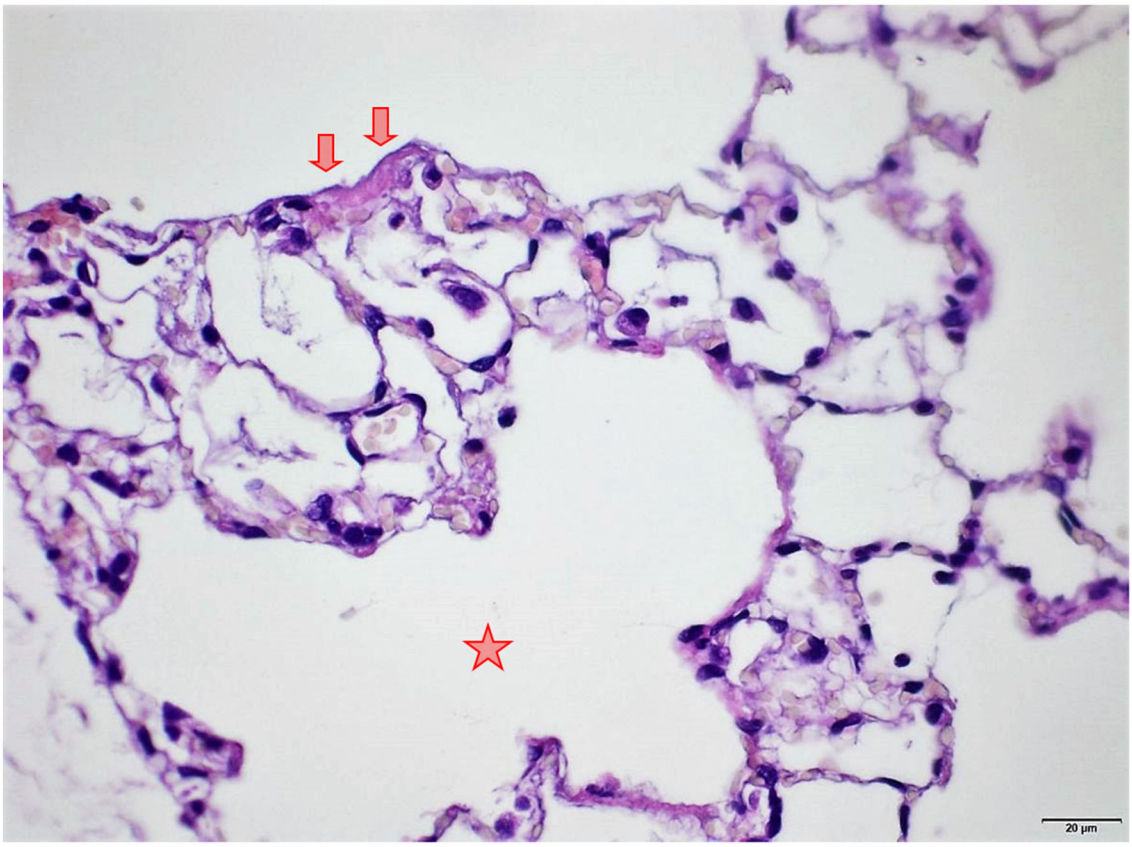


FIGURE 9 | Female rat. Deceased 1 h. after transfer to cage. Lung, right middle lobe. Focal acute emphysema (asterisk) and focal alveolar wall necrosis (arrow). HE, original magnification x20.

TABLE 2 | Findings in lungs separated per lung lobe.

Lung Lobe	Left		Right Cranial		Right Medial		Right Caudal		Accessory	
No. of samples/sex	**(18) M**	**(18) F**	**(18) M**	**(18) F**	**(18) M**	**(18) F**	**(18) M**	**(18) F**	**(18) M**	**(18) F**
					No. affected/mean severity					
Alveolar hemorrhage	2/1.5	2/1.0	1/1.0	0	1/1.0	1/1.0	2/1.5	2/1.0	0	1/1.0
Fibrin, alveolar	2/1.0	2/1.0	2/1.0	0	2/1.0	1/1.0	2/1.5	3/1.0	1/1.0	0
Alveolar macrophages	2/1.0	2/1.0	1/1.0	0	2/1.0	0	1/1.0	1/1.0	1/1.0	0
Infiltrate, mixed cell	1/1.0	0	1/1.0	0	1/1.0	1/1.0	0	1/1.0	1/1.0	1/1.0
Emphysema, acute	2/1.0	1/1.0	1/1.0	0	3/1.3	3/1.0	1/1.0	3/1.0	0	2/1.5
Alveolar wall necrosis	0	0	0	0	0	1/1.0	1/1.0	1/1.0	0	0

lung surface representing petechiae, are described as indicators of suffocation, no definite proof of physical obstruction is given in these existing studies. Other unpublished studies, which were conducted in the same decade showed no mortality or mortality at other concentrations but the reports contained even less information. While conformity with guidelines was cited in all these studies, the scientific protocol is considered inadequate to answer the cause of mortality observed in these studies. Because the investigations required to address the exact cause of lethality in connection with particles was not considered in these previous existing studies, a new acute inhalation study with an extended design was conducted. As a first step, a dry run was performed without animals for technical optimization. This consisted of monitoring and detailed characterization of the aerosol in the test equipment (i.e., MMAD stability, particle concentration, particle size distribution over time, aging effect) at various sites of the exposure tower, focusing on the point of aerosol generation and the main outlet port. Overall, it was shown

that in a concentration range of 500–600 mg/m^3 it was feasible to generate an atmosphere that fulfilled the OECD 436 requirement of MMAD stability over 4 h [within 1–4 μm range (9)].

The TU Dresden investigation and the dry run with the Fraunhofer equipment set-up which was later used, without modification, for the animal study clearly demonstrated that the test aerosol atmosphere at a concentration of 500 to 600 mg/m^3 is sufficiently stable and remains within the required MMAD window at the point of exposure. The starting concentration in this study was chosen as 500 mg/m^3 hydrophobic HMDZ surface-treated SAS for 4 h based on the results provided by TU Dresden regarding still acceptable aerosol alteration in the test unit in line with the OECD 436 requirements. The aerosol was supplied to the rats by a flow-past, nose-only inhalation exposure system at Fraunhofer ITEM, Hannover, Germany. This ensured the highest standards in having the same test atmosphere in each animal's breathing zone. Three young adult Wistar Crl:WI (Han) rats per sex were allocated to this study. Five animals died spontaneously during the study, and one animal was euthanized for animal welfare reasons. All these animals showed respiratory distress, such as preterminal gasping and a reduced respiratory rate prior to death. A few animals appeared to be anemic.

The working hypothesis was that a single inhalation of this high concentration may cause a physical obstruction of the rat upper respiratory tract and that mechanical blockage of the respiratory tract associated with suffocation as cause of mortality would be made evident by histopathological examination, especially when the upper respiratory tract is included. The animals underwent an immediate necropsy (within 10 min. after death to avoid autolysis) and an extended list of organs and tissues was sampled according to an optimized protocol that ensures preservation of the organs, including deposits. The fixed tissues underwent histological and EDX evaluation. Tissues taken from nasal cavities, parts of the lungs, trachea and larynx were initially frozen to avoid loss of SAS in the cavities from rinsing out with liquid fixing solution.

In nasal cavities processed from frozen material and sputtered for subsequent EDX analysis, the evaluation under digital microscopy revealed deposition of foreign material in all nasal cavity at levels 3 and 4. Only minimal deposition was noted in nasal cavity level 3. However, there was almost complete blockage by foreign material deposition in nasal cavity level 4. By SEM-EDX evaluation, the material was confirmed as the test item by the presence of Si. Rats are obligatory nose breathers (19) and sudden death by deposition of material in the nasal cavities, e.g., reflux (20, 21) has been reported. EDX could only be performed on frozen tissues from the three sampled rats, i.e., resin embedded nasal cavities from the other three animals revealed no results. Note that the EDX beam can only penetrate ∼2 μm (of solid carbon), therefore, only Si within this depth could have been detected. However, the Si in nasal cavity samples was found at deeper levels; the frozen, dried, and sawed (Si-free diamond blade) nasal cavities showed remarkably large areas of accumulated Si in all three animals at nasal cavity levels 3 and/or 4. Aside from normal tissue elements Ag from the sputtering and Cl from the plastic slide section were also detected. The visible test item morphology detected in the frozen samples was observed to have changed into larger particulate structures with more gel-like appearance.

During necropsy, the lungs of all animals were described as dark red with a sponge-like consistency or as dark red and spotted. The reason for these changes was macroscopically visible as congestion, edema, acute emphysema and petechiae. Histologically, there were focal to multifocal hemorrhages, alveolar fibrin, focal to multifocal acute emphysema, and, occasionally focal alveolar wall necrosis associated in some samples with subsequent mixed-cell infiltrate and macrophages. All these findings are associated with respiratory failure, i.e., mechanical asphyxia (22). Test item (Si) was not detected in the lungs by EDX. The lesions in lungs are typical of suffocation, i.e., "*Pulmonary edema with or without hemorrhage, atelectasis and interstitial emphysema are common macroscopic and microscopic lesions in animal and human victims.*". In obstructive asphyxia, there is immediate dyspnea with convulsions, followed by bradycardia and apnea, an isoelectric EEG, agonal respirations, and cardiac arrest usually within 4 to 6 min (23).

In four animals (two animals per sex), there were focal or multifocal hemorrhages representing petechiae. Petechiae are not definitive proof but rather, a feature supporting asphyxia (22, 24).

Foamy content in the trachea of the animals was described during necropsy and is considered to be a consequence of asphyxia associated with preterminal gasping and edema. A spot with Si was only found in one animal and the larynx of another animal was found to contain Si, however, no deposit was noted histologically. This is due to the loss of the test item from water solubility during processing. Histologically, there were no findings in the larynx, trachea, bronchial bifurcation or carina. Settlement of the test item in the larynx of one animal may be considered supportive of asphyxia caused by obstruction of the nasal cavities as demonstrated in this study.

However, it is not possible to determine exactly at which concentration the physical obstruction of the nasal cavity for the respective hydrophobic particulate substance occurs, e.g., some hydrophobic organic pigments show 4 h LC50-value above 1,000 mg/m^3 (12). In this study, mortality is discussed as a consequence of the limited wettability of the test material. The contact angle is a possible way to express the wettability and to predict the test material behavior. A contact angle of 147.21 ± 5.95° represents a very reduced wettability. Therefore, a re-agglomeration of particles through a physical effect is highly probable. It is known from investigations of the contact angle of different organic pigments that the higher the contact angle, the higher the mortality in acute rat studies; whereas, hydrophilic particles with a lower contact angle are unlikely to cause death by asphyxiation due to their low re-agglomeration potential (12). When characterizing the test material in the present study, the contact angle was also measured. The results show that it is extremely difficult to apply a defined water droplet on the surface of the test material because the droplet can move easily due to the lack of interaction with the test material, resulting in a contact angle above 140°. It can be concluded that in the present case no inhalation test equipment design would lead to different results, i.e., the study outcome depends on the physicochemical properties of the test item.

Hofmann et al. (12) showed that contact angle measurements resulting in angles demonstrating hydrophobicity are considered indicative of animal study outcome, with high hydrophobic particle concentration leading to lethality in rats. Hydrophobicity is another physicochemical property of HMDZ surface treated SAS which, in combination with the low density of the large light and fluffy agglomerates, leads more readily to upper respiratory tract blockage. The mechanism causing lethality of hydrophobic particles by mechanical obstruction of nasal cavities was convincingly demonstrated in this study, Determining exact respective 4 h LC50 values would require numerous animal testing for each hydrophobic material. However, the scientific value of 4 h LC 50 inhalation studies to determine the concentration leading to physical obstruction and suffocation is highly questionable. This also applies to repeated testing in subacute or subchronic animal inhalation studies to detect nasal cavity obstructions for regulatory purposes, This is also totally unjustifiable from an animal welfare point of view.

CONCLUSION

The observation of lethality with low density surface treated hydrophobic SAS in acute high dose inhalation studies with rat led to wrong interpretations when toxicological assumptions were derived from this. We have clearly shown that the lethal effect in rat was caused by mechanical obstruction of the upper respiratory tract based on physicochemical properties of the inhaled particles. Based on differences in respiration and respiratory tract (upper and lower) anatomy, results from rats cannot be transferred to humans. In contrast to rats, humans are capable of breathing through the nose and mouth unlike the rat which is an obligatory nose breather. The scientific value of acute or repeated dose animal studies for all REACH registrations of hydrophobic particles to determine the exact concentration causing airway obstruction in the upper respiratory tract is not justifiable when considering animal welfare and it is also unnecessary from a regulatory point of view as OECD guidance 39 paragraph 51 (OECD, 2009) expressly points out that physical obstruction should not be misdiagnosed as a toxic effect. Physical obstruction of the nasal cavities in the obligatory nose-breathing rat occurs at an SAS concentration of 450 mg/m^3 (18), suggesting that the numeric cut-off criteria for acute inhalation toxicity study for CLP classifications, are too high for hydrophobic low density powder particles and are not reflective of what could happen in humans.

The assumption that an exposure to concentrations of 450 mg/m^3 and even greater in the rat experiments can allow statements on the toxicological effect of the substance seems very inappropriate A hazard classification for hydrophobic surface treated SAS particles, based on physical obstruction of the rat upper respiratory tract is thus unwarranted.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/supplementary material, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work, and approved it for publication.

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REVIEW
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A Systematic Review on the Hazard Assessment of Amorphous Silica Based on the Literature From 2013 to 2018

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Background: Nanomaterials are suspected of causing health problems, as published studies on nanotoxicology indicate. On the other hand, some of these materials, such as nanostructured pyrogenic and precipitated synthetic amorphous silica (SAS) and silica gel, have been used for decades without safety concerns in industrial, commercial, and consumer applications. However, in addition to many *in vivo* and *in vitro* studies that have failed to demonstrate the intrinsic toxicity of SAS, articles periodically emerge, in which biological effects of concern have been described. Even though most of these studies do not meet high-quality standards and do not always use equivalent test materials or standardized test systems, the results often trigger substance re-evaluation. To put the results into perspective, an extensive literature study was carried out and an example of amorphous silica will be used to try to unravel the reliability from the unreliable results.

Methods: A systematic search of studies on nanotoxicological effects has been performed covering the years 2013 to 2018. The identified studies have been evaluated for their quality regarding material and method details, and the data have been curated and put into a data collection. This review deals only with investigations on amorphous silica.

Results: Of 18,162 publications 1,217 have been selected with direct reference to experiments with synthetically produced amorphous silica materials. The assessment of these studies based on defined criteria leads to a further reduction to 316 studies, which have been included in this systematic review. Screening for quality with well-defined quantitative criteria following the GUIDE nano concept reveals only 27.3% has acceptable quality. Overall, the *in vitro* and *in vivo* data showed low or no toxicity of amorphous silica. The data shown do not support the hypothesis of dependency of biological effects on the primary particle size of the tested materials.

Conclusion: This review demonstrates the relatively low quality of most studies published on nanotoxicological issues in the case of amorphous silica. Moreover, mechanistic studies are often passed off or considered toxicological studies. In general, standardized methods or the Organization for Economic Cooperation and Development (OECD) guidelines are rarely used for toxicological experiments. As a result, the

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significance of the published data is usually weak and must be reevaluated carefully before using them for regulatory purposes.

Keywords: nanotoxicology, synthetic amorphous silica, hazard assessment, study quality, nanostructured particles, nanomaterials, database

INTRODUCTION

The development of innovative materials, such as nanomaterials, is strongly upward and will continue to rise in the future. Even if not all materials find their way to the market, an enormous number of new nanomaterials are being researched in the world's laboratories. This is the main reason why the number of published studies on new materials is also steadily increasing. Around 5,000 published articles per year on "nanotoxicology" (1) make it almost impossible to consider all results when it comes to risk analysis for the use of nanomaterials in our daily life. Moreover, many studies are still being conducted on nanostructured materials that have been on the market for decades, such as synthetic amorphous silica (SAS) and nanoforms of titanium dioxide. The reason for this development is still the working hypothesis right from the beginning: the smaller the nanomaterials, the higher their potency to induce adverse effects. The basis for this working hypothesis lies in the following assumptions that have been described elsewhere (2): specific physicochemical properties, such as smallness lead to better transport in biological systems; a much larger specific surface area compared to larger particles induces higher reactivity and specific material modifications, e.g., one-, two-, or three-dimensional materials add some specific aspects of toxicology. The question remains to what extent these smallest particles can fulfill this paradigm and trigger size-dependent toxicological effects (3), and whether this also applies to materials that have been on the market for more than eight decades, such as synthetic amorphous silica (4, 5), or it is still simply dose-dependent (6).

Unfortunately, the situation in hazard and risk assessment of nanomaterials is not as clear as expected when considering the huge amount of publications during the last two decades of research based on many funding programs at the national or international level (7) resulting in a multitude of studies and publications (compare[1]). As mentioned above, actually, there exist more than 50,000 articles on the biological effects of nanomaterials (8). One could imagine that this might lead to higher safety at workplaces or for the consumer or the environment. Unfortunately, this is not the case, as we have the obscure situation that many publications on the topic "nanotoxicology" do not deliver relevant toxicological data (9–11), and there is still no consensus on the toxicity of nanomaterials (12).

Difficulties in assessing toxicological studies on nanomaterials have been described in detail 10 years ago by Card et al. (13), and they found that 75% of published studies have deficiencies in their study design and are not fully reliable for risk assessment or regulatory purposes. At the same time in 2010, we described first a criteria catalog for assessing the minimum study quality for our informative website of the DaNa-project[2] (download of the criteria catalog[3]) and Card and Magnuson (14) published their concept for quality scoring of toxicological studies for nanomaterials. This resulted in the demand for reliable and comparable results based on the principle of "stable stool," in this case with four legs: (i) validation, (ii) traceability, (iii) quality-management system, and (iv) measurement uncertainty (15). Without adherence to these basic principles, it is difficult to classify studies as reliable for regulatory-accepted risk assessment. As the quality of published toxicological data has not increased over the last 10 years (1), the question may be allowed: what is the reason for this lack of reliability? Many scientists and working groups have been looking for what could be the cause that statements of toxicological studies are, in many cases, not very reliable and describe various pitfalls and flaws in nanotoxicology. In general, there exist many interferences of nanomaterials with the used assay systems for human toxicological (16, 17) as well as ecotoxicological studies (18). Moreover, specific interactions in testing by flow cytometry (19) using the comet assay (20, 21), investigating immunomodulatory effects (22), or simply using common cytotoxicity assays (23) are often not respected. Another very important point is the possible contamination of nanomaterials with bacterial endotoxins (24), which induce false-positive inflammatory effects (25, 26). Additionally, investigated materials, such as SAS, may interfere with the determination of endotoxin contamination in the common LAL test and cause wrong results (27).

These and many more mistakes can be committed in experiments with nanomaterials when investigating their toxicological potential (28). The above-mentioned pitfalls and contradictions specifically in hazard assessment of nanomaterials have been the starting point for a project, which, in the end, collected more than 25,000 citations. Nearly 9,000 studies have been evaluated further, and this resulted in more than 6,500 datasets on various nanomaterials. This data collection is the basis for an extensive literature study on amorphous silica that is shown here.

METHODS

Search Strategy

Several online libraries, such as PubMed®, Google Scholar, and Isi Web of Knowledge, were searched from 2013 to 2018, with a search profile to find all publications on the toxicology of nanomaterials (for the specific search profile, refer to **Supplementary Material**). The keywords were directly

[1]https://www.nanosafetycluster.eu/outputs/nsc-compendium/

[2]www.nanoobjects.info

[3]https://nanopartikel.info/en/knowledge/literature-criteria-checklist/

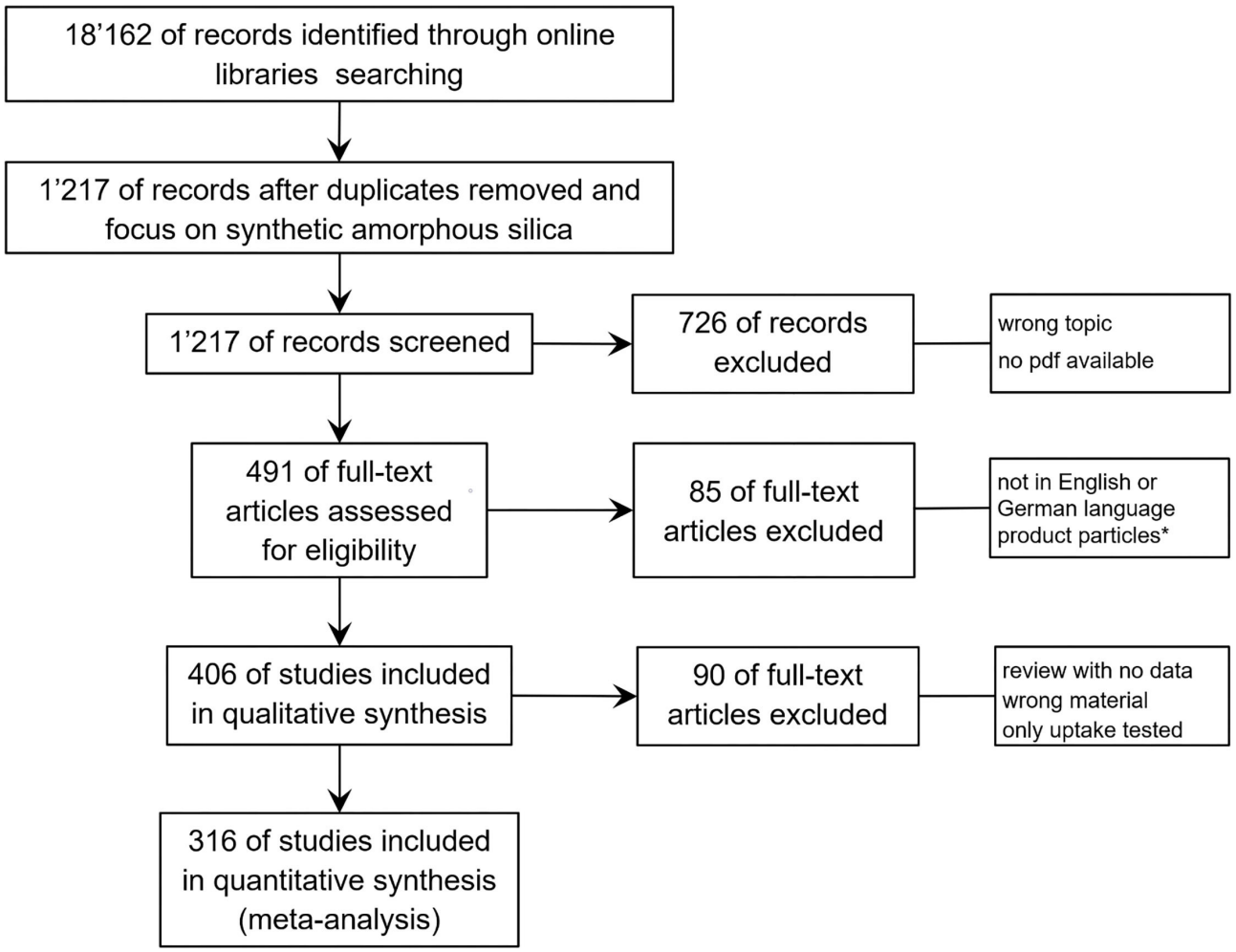

FIGURE 1 | PRISMA flow diagram demonstrating the process of systematic selection of relevant publications for synthetic amorphous silica (SAS) safety studies. Starting with 18,162 citations for the period 2013–2018 on nanotoxicology, 1,217 have been selected with the keyword "synthetic amorphous silica." Evaluating these studies in more detail, the final database contains 316 publications that have been assessed for all study details. *Studies with particle mixtures from commercially available products tested, such as paints, have been excluded.

related to this topic, such as: "nanotox,*" "nanotube,*" and "toxic;*" "nanoparticle* and toxic*;" "nanomat* and toxic*;" and some more combined with the year of appearance. The asterisk represents a wildcard within the search terms. With these search terms, between 3,000 and 5,000 publications have been found per year (1).

Selection of Studies on Amorphous Silica

The references that we found have been screened in the first round roughly for their toxicological content. Many publications use buzzwords, such as "toxic" or "toxicity" in the Introduction or Discussion section, without performing any toxicological experiment in the core test. These publications have been excluded by a quick pass through the literature. For this systematic review, all remaining studies are searched for "synthetic amorphous silica (SAS)" or "silica, excluding crystalline silica" again, and the resulting publications have been included in the evaluation of their eligibility. Further exclusion criteria in the next steps were as follows: (i) research not involving animals or cells or tissues and the material "amorphous silica" was only mentioned but not investigated; (ii) the study was not published in the English or the German language, and only particles from commercially available products, such as paints, have been analyzed; (iii) for the type of publication (review, conference abstract, editorial, etc.), the wrong material was used (crystalline silica, mixtures of silica with other oxides or polymers, etc.) or only uptake into tissues or cells were tested. The PRISMA flow diagram of the literature search and selection process is depicted in **Figure 1**.

Quality Assessment of Studies

In the data collection, all publications from 2013 onward are re-evaluated regarding the quality of study details. Various suggestions exist on how the quality of studies could be assessed

(14, 29–31). Detailed and quantitative evaluation is made possible by the criteria catalog of the European GUIDE nanoproject (29). This tabular record of study data was expanded for automatic use, and this was completed for all publications. For detailed information on which criteria are important and have to be given in the published studies, please refer to Table 5 (*in vivo* studies), Table 6 (*in vitro* studies), and Table 9 (material characterization) in the cited reference (29). Evaluation factors for characterization of the materials used (S-factor) and the toxicological study (*in vitro*, *in vivo*, or ecotox = K-factor) are offset against each other and resulted in four different values for quality (Q-score): "0" means no reliable study, "0.5" means low reliability, "0.8" means good reliable study, and "1" means very reliable study.

Data Extraction and Analysis

Besides the bibliographic information of each study, relevant data from the publications were extracted and put in the data collection in four different groups. The first section regards the material properties given by the authors, including source, size of primary particles, specific surface area, and all additional information available. The second section contains information about the investigated biological model, such as species, strain, source of animals or cell type, and source for cell and tissue cultures. All details of housing, medium, exposure pathway, treatment design and duration, repeats, etc., are collected for the second section. The third section reflects the used doses or concentrations for testing and additional information about repeated-dose experiments, recovery and observation time, overload scenarios, or more details about the amount of material used for experiments. Finally, the fourth section is related to the biological endpoints investigated and which methods were used. The table contains for each investigated endpoint the no-observed-adverse-effect-level (NOAEL) for *in vivo* studies or the no-observed-effect-concentration (NOEC or EC_0) for *in vitro* studies. Not all studies allow for direct determination of the NOAEL/EC_0 from the data because the concentration range investigated was not chosen properly. Therefore, in such a case, either the lowest-observed-adverse-effect-level (LOAEL) or the lowest-observed-effect-concentration (LOEC) is given if even the lowest dose used in the experiment still induced an effect, or the upper-no-observed-effect-level (UNOEL) or upper-no-observed-effect-concentration (UNOEC) if even the highest dose used did not induce an effect *in vivo* or *in vitro*, respectively.

Possible Subjective Perception

This study on synthetic amorphous silica has been carried out by the author of this publication in person. The detailed pre-selection process of more than 1,000 publications on synthetic amorphous silica safety and evaluation detail of 316 studies took several months. The extraction of data and assessment of literature quality have been conducted in all conscience, but of course, there are personal limits to attention, and the author is aware of his subjectivity. Thus, it may have happened that several publications have not been considered for a certain issue or the results have been misinterpreted and differ from the opinion of the authors of the studies. As a result, the pictures drawn and the data presented are, to some extent, a personal view of the actual situation of the published data on amorphous silica nanotoxicological issues between 2013 and 2018.

RESULTS

Study Characteristics

Searching the online libraries for nanotoxicological studies for the period 2013 to 2018 resulted in 18,162 references, which have been further selected following the PRISMA flow diagram shown in **Figure 1**. The number of publications was reduced by focusing on the material (SAS, synthetic amorphous silica), and exclusion of all studies showing no toxicological data or in which one of the other exclusion criteria were applied (**Figure 1**). This resulted in 316 publications providing 973 data sets on different SAS modifications studied in different cell types or animal species and presenting data for NOAEL or EC0 for more than 85 biological endpoints (key events or KEs). Most of the KEs are described only once or <5 times, and for 32 KEs more than 20 datasets exist.

At this point, it is worth noting that the above-mentioned SAS modifications refer to the large number of chemical processes used for the synthesis of SAS in all the studies. The processes do not fulfill, in most cases, the criteria of market production. The most common manufacturing processes are pyrogenic (fumed) silica, precipitated silica or silica gel for powders, and colloidal silica for dispersions. Depending on the process, the final material reaches different states of aggregation (32). Pyrogenic (fumed) silica, precipitated silica, and silica gel are nanostructured materials. The particle size is characterized by different levels of structures, namely, internal structures, aggregates, and agglomerates. Internal structures (also referred to as primary particles or constituent particles) could be visually recognized by TEM because of their curvature inside the aggregate skeleton, but they cannot be isolated. There are no phase boundaries inside an aggregate (32). The typical size of internal structures ranges from ∼3 to 50 nm (d50, number-based, TEM). Aggregates represent the smallest dispersible unit in synthetic amorphous silica and are formed during the production process by the fusion of primary structures by covalent bonds. Aggregates (33) usually have a particle size above 100 nm, although some fractions below 100 nm may occur. Agglomerates are formed out of aggregates by weaker forces, e.g., the Van der Waals force. Synthetic amorphous silica is usually placed on the market in the form of agglomerates. The typical size of agglomerates is >1 μm.

Several studies have been performed with isolated individual particles, which can be achieved by surface treatment or different dispersion methods. For evaluation of size-dependent effects, the authors' information on primary particle size was used without considering aggregation or agglomeration. Primary particle size is usually analyzed visually in the studies by transmission electron microscopy. This information is important for the comparison of the shown data with real market products, such as E 551 (food-grade SAS).

For all publications in the data collection, a rigorous quality check was conducted using the quality criteria checklist established by the EU project GUIDEnano. Only 3.4% of the studies reached the highest classification of "1" (very

TABLE 1 | Modifications of amorphous silica used in the 316 selected studies.

Type of material	Amorphous silica modification					
	Technical food-grade (SAS)	Technical non-food-grade (SAS)	Technical—unspecified	Self-made (unknown process)	Self-made (Stöber process)	Core-shell particles (shell made from amorphous silica)
# of datasets	14	140	448	212	123	36

TABLE 2 | Biological models (animals or cell and tissue cultures) used in the 316 selected studies.

Species	Animal models									Cell models (170 different)	
	Cnidaria	Sea urchin	Nematode	Fish	Fly	Chicken	Mouse	Rat	Rabbit	Human	Animal
# of datasets	1	1	3	25	6	1	125	79	3	447	282

reliable) and 24% reached the level "0.8" for good reliability. Conversely, however, this also means that more than 70% of the studies do not provide reliable data. A comparison to quality classification regarding the literature published before 2013 (9, 10) resulted in no increase in the reliability of data regarding the toxicological content of the more recent studies evaluated in this systematic review.

General Results

Although the overall number of datasets is high, the range of variation in the different material modifications (**Table 1**) and the tested biological models (**Table 2**) is very high as well, which considerably worsens the comparability of the results. Several studies used core-shell materials for which the shell was made from amorphous silica most often following the water-glass method. These silica-coated materials (core-shell particles, **Table 1**, last column) were taken into consideration as well, as they demonstrated the "detoxification" effect of different materials using the silica shell. Eleven different materials, such as gold, zinc oxide, and iron oxide, coated with amorphous silica were used in 21 studies mostly to reduce the toxicity of the core material (34–42). Also of significance is the fact that only 14 datasets resulted from experiments with food-grade silica, which is the most relevant modification for human toxicity testing, and four of these datasets were questionable because no source or product number was given; whereas 140 datasets were produced with technically specified SAS made for a huge variety of products, such as paints, surface coatings, rubber, and plastics, and most of the studies used unspecified SAS or self-made silica particles. In many cases, material properties were not analyzed sufficiently, such as surface charge and specific surface area, and were mentioned by the authors in only 52 and 27% of the studies, respectively.

In addition, the range of variation in the experiments is further increased by the fact that very different concentration units for the treatment of cells or animals were given in the experiments (**Table 3**). Moreover, some of the units used did not make any sense if in combination with nanostructured materials or nanoparticles, as it is difficult or impossible to repeat experiments with the given concentration of nM or μM for particulate materials.

TABLE 3 | Dose and concentration units applied in the experiments of the 316 studies on amorphous silica toxicity.

Concentration units (*in vitro* studies)	Dose units (*in vivo* studies)
#/cell	μg or mg/animal
#/ml	μg or mg/kg
μg/cm^2	mg/m^3
μg/ml	μg/area skin
μg or mg/plate or well	μg/ear
μl	μg/eye
nM or μM	#/animal
ppm	μg/embryo

The data collection additionally recorded typical material characteristics, such as the size of the primary particles or the shape of the materials. To discriminate between size-dependent effects, the data were classified into six different size groups regarding primary particle size given in the studies (**Table 4**). As mentioned above, during the synthesis process, frequent aggregation and agglomeration took place, which was described in 332 datasets (roughly 30% of all the datasets). Here, the particle sizes are between 100 nm and often far above 1 μm. Moreover, some materials had a very special shape (rods, nanowires, spindles, rattles, or irregular aspects were used in 28 datasets), but most of the experiments were carried out with aggregates or agglomerates from spherical primary particles (**Table 4**).

Most of the studies presented data on specific biological endpoints or key events. These played a role in different pathways of toxicity (43) or adverse outcome pathways (44). **Table 5** depicts the most important pathways of toxicity (PoT) that have been addressed in the studies and the number of datasets existing for each of the PoT. Induction of a PoT does not imply that the material is highly toxic, as no concentration or dose-relationship is included in this table, and mostly only high concentrations have induced the respective key event.

TABLE 4 | Distribution of the datasets over the size classes and different material forms.

	Distribution of datasets over six size groups					
Size group (nm)	**0–10**	**11–20**	**21–50**	**51–100**	**101–500**	**>500**
# of datasets	70	250	269	209	108	67
	Occurrence of different primary particle shapes[#]					
Shape	**Spherical***	**Rods**	**Nanowires**	**Spindles**	**Irregular**	**Rattles**
# of datasets	908	8	1	2	14	3

[#]*For the 37 missing datasets, no information on shape was available [no transmission electron microscopy (TEM) pictures are shown or no information is given by the authors of the studies].*

**Synthetic amorphous silica (SAS) produced for the market belongs to the spherical form.*

TABLE 5 | Involved pathways of toxicity in the studies on amorphous silica as mentioned by the authors.

PoT	**Cell viability**	**Apoptosis**	**Membrano-lysis**	**Oxidative stress**	**Stress kinases**	**Immune response**	**Inflammation**	**Gene expression**	**DNA damage**	**Tissue protection**	**No-effect study**
# of datasets	91	27	3	88	11	8	128	11	17	8	456
Quality* rel./not rel.	22/69	7/20	0/3	22/66	5/6	3/5	42/86	3/8	9/8	3/5	139/317

* *Quality as evaluated; rel., reliable (Q-score 0.8 or 1); not rel., not reliable (Q-score 0 or 0.5).*

In vivo Results

Most of the *in vivo* studies on amorphous silica were carried out with mice (45) or rats (37), and other organisms (**Table 2**) played only a minor role. The main exposure pathways chosen by the authors of the studies were intraperitoneal (IP) or intravenous (IV) injection, intratracheal instillation or aspiration, inhalation, and ingestion. For a better comparison of the results only those studies have been selected for the following detailed discussion which have applied "μg/kg bodyweight" in injection or instillation experiments and "mg/m^3" for inhalation studies.

IP Injection

Only seven studies used this exposure pathway, four of them injected amorphous silica, and three studies used other materials with a silica coating. The main effects described were oxidative stress and inflammatory responses, but as all the studies got a quality score of "0," they were not further considered.

IV Injection

Direct exposure by IV injection of amorphous silica particles was carried out in 23 studies. Half of the datasets showed no effect even at very high doses. There was only one study that discussed DNA damage based on observed p53 activation, but this effect was described at 50 mg/kg only in rats and could not be confirmed by comet assay (insignificant) as discussed by the authors (46). For this exposure pathway, the most often observed effect was liver injury (18 of 63 datasets), which is hardly surprising after the injection of doses in nearly all cases above 10–250 mg/kg. Only one study with a low-reliability score ("0") showed impairment of survival of mice at concentrations of 17.5 mg/kg and above with 10-nm amorphous silica particles (47). Taken together, all the results of IV injection of amorphous silica particles in rats and mice only induced slight effects at very high doses.

Intratracheal Instillation

If administered by instillation or aspiration, the dose rates were very high (48). Moreover, in most of the studies, relatively high doses were applied. Within the five repeated exposure studies in mice (3 to 14 repeats), 1 to 25 mg/kg bw have been instilled which results in overload of the lungs except in only one study. In the studies carried out with rats, 75–125 mg/kg bw were instilled in single and repeated-dose experiments, which all ended up with overload doses. Because of these facts, high doses and high dose rates, and inflammatory effects, such as immune cell migration and cytokine production, were observed in most studies, which is the normal tissue response under these conditions. Nevertheless, two-thirds of the studies were not reliable based on the evaluated quality criteria, and many of them went beyond overload concentrations for rats ($\geq$2.5 mg per lung) and mice ($\geq$0.5 mg per lung) (45, 49–62).

Inhalation

The number of inhalation studies was generally low, and in this period, only six studies on amorphous silica or silica-coated materials were found. Only two studies had a low-reliability score and were not considered here. The other four studies, three on rats and one on mice showed no effects on the lungs of the treated animals (63–66) except for the study of Leppanen et al. (66) who used 10–40 nm rod-shaped silica-coated titanium dioxide particles at overload dose (exposure treatment: 30 mg/m^3, 4 days a week, 4 weeks, calculated deposited dose 575 μg/lung in mice). One study with high reliability investigated five different surface modifications of SAS following the OECD guideline 412/403

(65). For the unchanged pristine material (15 nm amorphous silica) a NOAEL of 2.5 mg/m^3 has been observed, whereas all coated silica materials (acrylate, PEG, phosphate, NH_2) had no effect even at the highest used dose and the NOAEL was stated to be higher than 50 mg/m^3. The only effect described for the coated materials was the acrylate modified amorphous silica induced some increase in weight of the spleen and accumulation of nanoparticles and thrombocytes at a NOAEL of 0.5 mg/m^3. However, this effect was fully reversible after the treatment phase.

Ingestion

The data collection contains 14 studies that exposed animals *via* the gastrointestinal tract resulting in 35 datasets. Completely unexpectedly, none of the studies was performed with food-grade silica, and only five followed the OECD guidelines.

Test Guideline 408: Repeated Dose 90-day Oral Toxicity Study in Rodents (67, 68).
Test Guideline 414: Prenatal Developmental Toxicity Study (69).
Test Guideline 416: Two-Generation Reproduction Toxicity Study (70).
Test Guideline 420: Acute Oral Toxicity: Fixed Dose Procedure (67).
Test Guideline 474: Mammalian Erythrocyte Micronucleus Test (71).

Only four of the 14 studies fulfilled the criteria for quality scoring of good studies (67, 69, 72, 73), and a fifth study used a well-characterized material from the JRC repository but without any own detailed analysis especially for contaminants (70). The four studies were conducted with rats except for one with very small particles (between 12 and 26 nm). All of these were "no-effect studies," although some exposed the animals over 90 days to relatively high doses (up to 1,500 mg/kg bw/day). In the high-exposure scenario chosen by Liang et al. (72), only some histopathological observations on the liver and lungs were conducted, but the control animals had slightly higher liver degeneration compared to the treated groups. Moreover, in the same study, two different sizes of silica particles were tested, one with 26-nm primary particle size and the bigger one with $>1\,\mu$m; both have the same weak effect on the lungs, but there were no effects on survival and body weight, and there were no hematological changes.

The ingestion pathway is of special interest as amorphous silica is a registered food ingredient. For this reason, the studies with low-quality scores were also included for a detailed analysis. The low-quality studies did not change the overall picture of the low toxicity of amorphous silica. One study was scored "0" for quality because of missing material characterization showing DNA damage in peripheral blood lymphocytes after a single dose of 3, 7, 10, and 13 mg/kg bw silica (74), but none of the other studies could find a similar effect. In another study on mice with only one dose tested (750 mg/kg bw/day for 14 days), the authors observed some effects on cytokines and oxidative responses with colloidal silica particles of two different sizes (20 and 90 nm) and two different coatings (citrate and L-arginine) (75). In the case of citrate-coated SAS, Il-12p70 was reduced to some extent and arginine-coated particles induced intracellular reactive oxygen species (ROS) production slightly. All shown effects were very weak or statistically insignificant. The authors stressed the point that their experiments demonstrated immunosuppression, which was not confirmed by the results as the materials were not characterized very carefully, and no contaminants or endotoxins were analyzed.

In vitro Results

The number of *in vitro* studies was much larger than that of *in vivo* studies. A total of 729 datasets represented a good basis for reliable statements. Nevertheless, the overall number of studies with comparable experimental study designs was still low; most studies were not conducted using standardized protocols. Most of the experiments were carried out under submersed conditions. More complex exposure methods, such as the air-liquid interface method, to compare inhalation conditions were also established, and as of 2018, 15 datasets for amorphous silica were available for this exposure method (76). Moreover, the incubation conditions also played a role. Cells in their normal environment "see" a mixture of biological molecules, including proteins and peptides, thus the data presented here were chosen from experiments carried out with full medium-containing serum. A total of 180 datasets showed results with coated or functionalized particles. The coatings/functionalization can be mainly divided into 4 classes: fluorescence dye molecules, polymers, carboxylation, and amination. Although the match in the design of the experiments was relatively low, often, different assays were used and quite different concentration units were given (**Table 3**), and there remained sufficiently large group numbers for some of the endpoints investigated to illustrate the results graphically. There were several important experimental variables, such as dose metric and treatment period. For this reason, certain limitations were placed on the comparative analysis. To increase the comparability, only datasets that performed 24-h incubation were chosen for the following evaluation. For the applied dose, only data from studies that presented concentration as μg/ml or if the concentration could be recalculated to this unit by the information provided by the authors were included. Regarding the concentration of nanomaterials, a certain degree of uncertainty remains, since, in a large number of publications, the exact amount of nanomaterials applied cannot be re-calculated because of a lack of information on the number of cells or the volume of the medium above the cells.

Biological endpoints (key events) with the highest number of datasets were chosen for further presentation in detail. The four key events were cytotoxicity, most often measured by MTT assay, represented by 403 datasets; membrane integrity, analyzed by LDH measurement or trypan blue staining, with 231 datasets; detection of ROS, usually tested by dichloro-dihydrofluorescein diacetate (DCFH-DA) assay and represented by 167 datasets; cytokine production, most often tested by ELISA and represented by 116 datasets. To assess the influence of quality evaluation on the appearance of the data, the next figures show the data points for (A) all available datasets and in the other figure those for (B) the high-quality studies only. Moreover, the data are distributed in six size groups (compare **Table 4**) to be able to recognize a

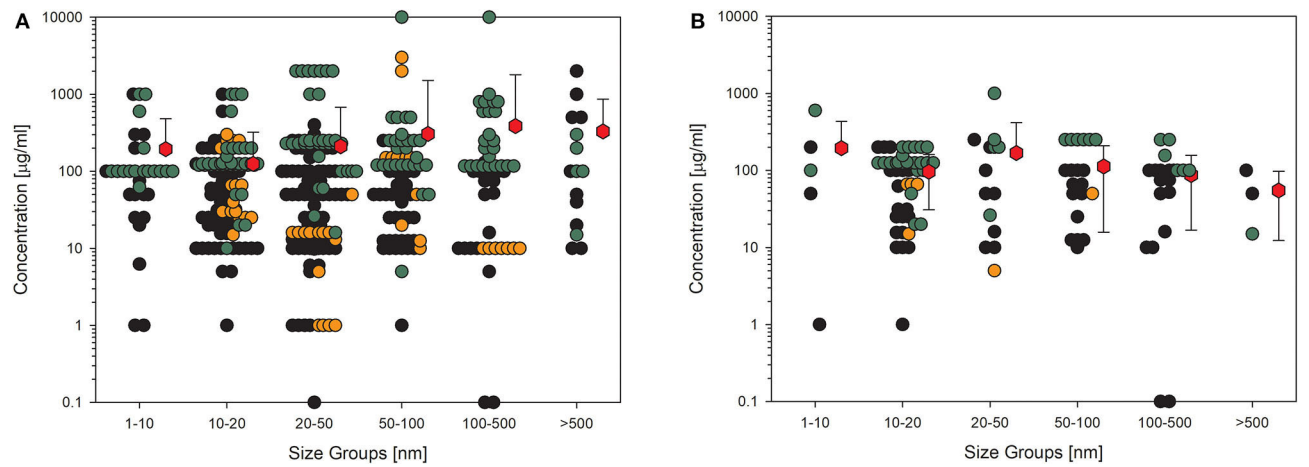

FIGURE 2 | Results of the evaluation of *in vitro* studies on induction of cell death in cells treated with amorphous silica. Data have been collected in six size groups for primary particle size as indicated on the abscissa. **(A)** All available datasets; **(B)** datasets from studies with high-quality scores of 0.8 or 1. Shown are the concentrations given in the publications for the no-observed-effect concentration (EC_0): black circles (●) or the lowest-observed-effect concentration (LOEC; if no EC_0 was available): orange circles (●) the upper-no-observed-effect concentration (UNOEC; if the highest tested concentration could not induce the investigated effect): green circles (●). The red hexagons (⬢) indicate the mean of all data for one size group ± SD. Data points taken from the references are given in the **Supplementary Material**.

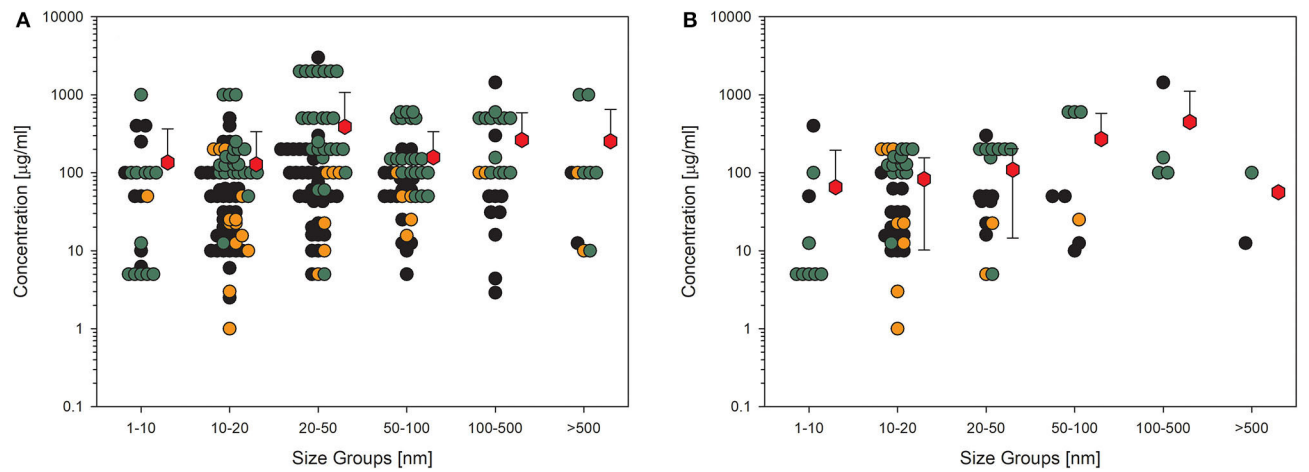

FIGURE 3 | Results of the evaluation of *in vitro* studies on disruption of the membrane integrity of cells treated with amorphous silica. Data have been collected in six size groups for primary particle size as indicated on the abscissa. **(A)** All available datasets; **(B)** datasets from studies with high-quality scores of 0.8 or 1. Details are as those shown in **Figure 2**. Data points taken from the references are given in the **Supplementary Material**.

possible size-dependent effect regarding the primary particle size of the investigated material. The following figures show data points in three different colors. This is to represent the three different categories of concentrations, the NOEC or EC_0 is shown with black dots, the LOEC is shown in orange dots, and the green dots represent the values for the UNOEC. This also explains the appearance of the orange dots often at the lower concentration range and the green dots frequently at the higher concentration range. **Figure 2** shows the first example of the possible cytotoxic effect of amorphous silica on cells.

The data shown in **Figure 2** impressively show how high the variance is in the different studies. The values within one size group scatter over more than five orders of magnitude, which demonstrates the missing use of standardized protocols. The same has been shown for ecotoxicological studies in which the values are distributed over up to six orders of magnitude (77). Reducing the data points to only the high-quality studies (**Figure 2B**) reduces the distribution for all size groups but has nearly no influence on the size of the mean values, which is, in all cases, larger than 100 µg/ml except for the largest size group and high-quality studies. The mean is relatively the same for all the six size groups, indicating that there appears to be no size dependence for this endpoint. Also striking are the recognizable outliers at very low concentrations, which can be explained as

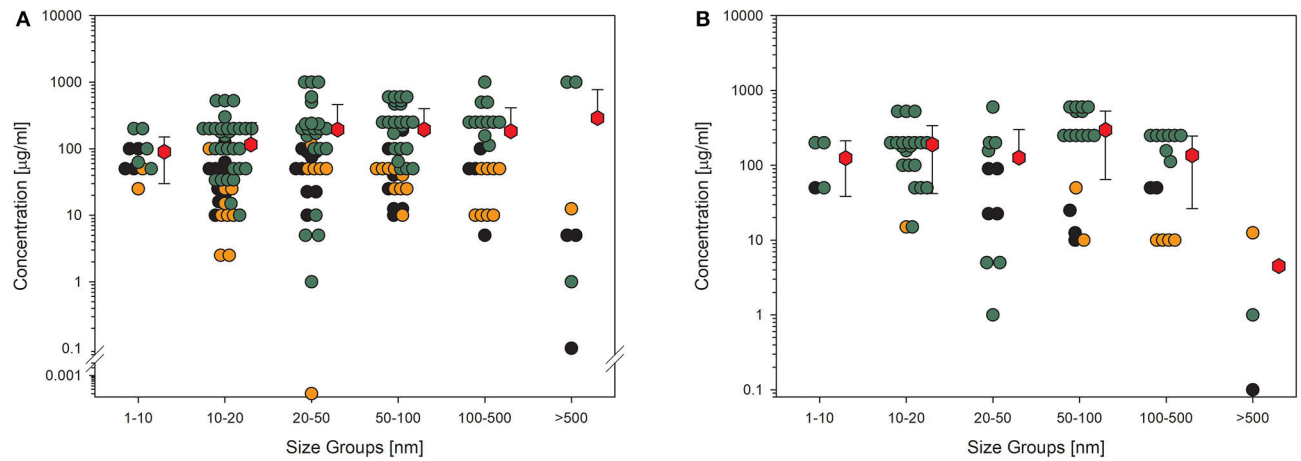

FIGURE 4 | Results of the evaluation of *in vitro* studies on induction of ROS production in cells treated with amorphous silica. Data have been collected in six size groups for primary particle size as indicated on the abscissa. **(A)** All available datasets; **(B)** datasets from studies with high-quality scores of 0.8 or 1. Details are as those shown in **Figure 2**. Data points taken from the references are given in the **Supplementary Material**.

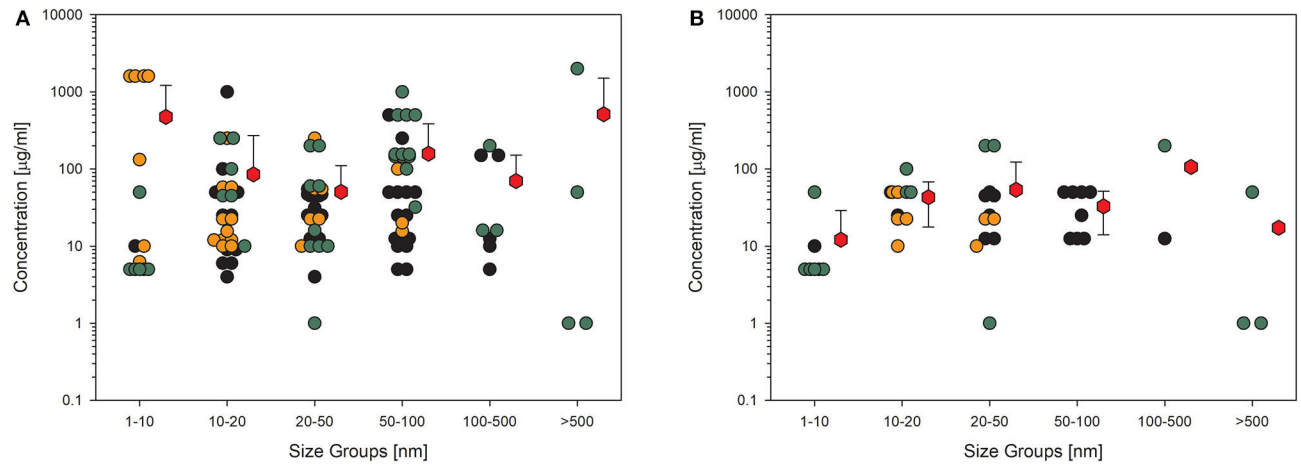

FIGURE 5 | Results of the evaluation of *in vitro* studies on cytokine production in cells treated with amorphous silica. Data have been collected in six size groups for primary particle size as indicated on the abscissa. **(A)** All available datasets; **(B)** datasets from studies with high-quality scores of 0.8 or 1. Details are as those shown in **Figure 2**. Data points taken from the references are given in the **Supplementary Material**.

follows: the EC_0 of 0.1 µg/ml in the 20–50 nm group is due to amorphous silica coated with amino groups, which significantly increases toxicity. The two data points in the 100–500 nm group are due to a self-made material with the Stöber-method (78), and the reduction in viability at concentrations lower than 10 µg/ml is very small.

A similar picture results from the data on membrane integrity of the treated cells (**Figures 3A,B**). The values here also lie in the same range, although the overall number of data points is lower. This is more important if only the high-quality studies are considered (**Figure 3B**), which reduces the number of data points dramatically, and for some size groups, no standard deviation can be calculated because of the low number of values. Nevertheless, nearly all the mean values are above 100 µg/ml.

A common endpoint related to nanomaterial exposure is the formation of ROS and oxidative stress (79). This is possibly the reason why many studies included this endpoint in their experiments (**Figures 4A,B**). The entire dataset for this KE paints an identical picture compared to the acute toxicity testing. Mean values are above 100 µg/ml, except for the largest particle group and high-quality studies, and for this case, too few values are available (**Figure 4B**). Again, some outliers are obvious, with one concentration being extremely low in the group of 20- to 50-nm-sized particles (**Figure 4A**). This study reached only a low-quality evaluation and was carried out with 35 nm amorphous silica particles bought from a company (80). Contaminants were not analyzed, and the dilution steps for this extremely low concentration applied were not explained in the

study description. This is the only study demonstrating an effect at such low concentrations of nanoparticles. Compared to the huge amount of green data points, which means that the concentrations are not able to induce the formation of various oxygen species, this outcome may be questionable.

The last example shows data for another biological endpoint, which is representative of classical cell response, production, and release of cytokines. Although this KE is very important for the assessment of further adverse outcome pathways, such as fibrosis, it is not as much investigated as the other shown endpoints. This implies higher uncertainty in the statistical significance even if the mean values are all above 50 μg/ml (**Figure 5A**). In this case, it is also noticeable when the studies with low quality are omitted (**Figure 5B**), then, the number of remaining data points is so low that a reliable statement is no longer possible. Then, the number of remaining data points is so low that a reliable statement is no longer possible.

For toxicological studies, it is often the question of which biological model should be used. So far, the gold standard seems to be animal testing. However, several studies have shown the limitations of animal studies, which sometimes have higher uncertainty than cell culture experiments (81). Moreover, the same group around Thomas Hartung has shown in an internationally acclaimed study that the readout of big data from existing toxicological data is outperforming animal testing (82). Furthermore, cell culture systems increase in complexity, reflecting more and more the situation in the whole organ and thereby replacing animal studies (83). Going through the collection of data on nanomaterial toxicology used for this study, the number of *in vitro* models is tremendously high. The authors of the studies often look for similarities in tissues of the exposure pathway of interest, e.g., lung epithelial or gastrointestinal tract cells. Moreover, to compare the results between animals and cell cultures, the respective cells should come from the same species, and to compare the results to humans, the cell culture

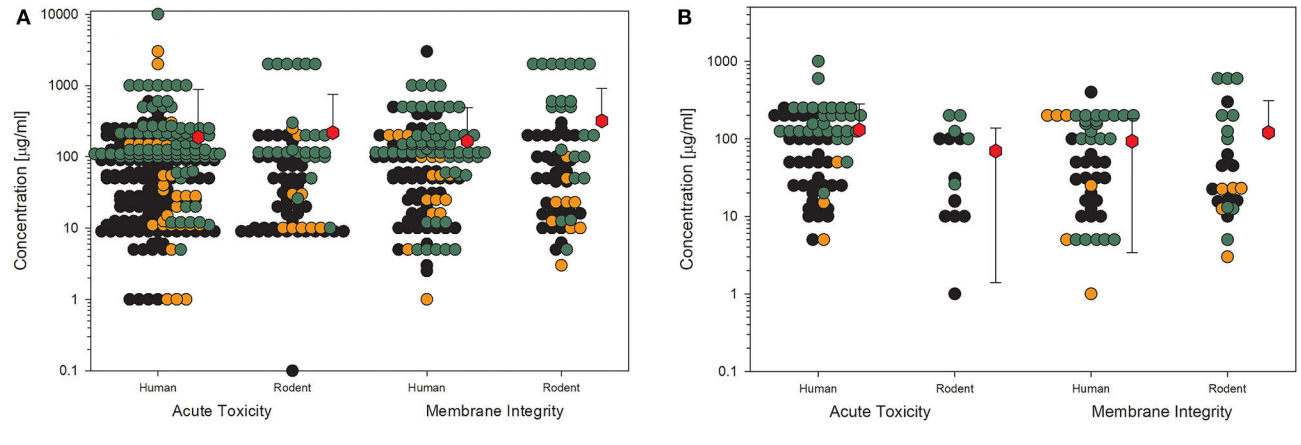

FIGURE 6 | Comparison of human and rodent cell lines in terms of their sensitivity to amorphous silica treatment. Datasets on the key events "acute toxicity" and "membrane integrity" are shown. For this comparison, only data for particles with primary sizes below 100 nm were included. Mean values are calculated from each dot cloud (red hexagons). **(A)** All available datasets; **(B)** datasets from studies with high-quality scores of 0.8 or 1. Details are as those shown in **Figure 2**.

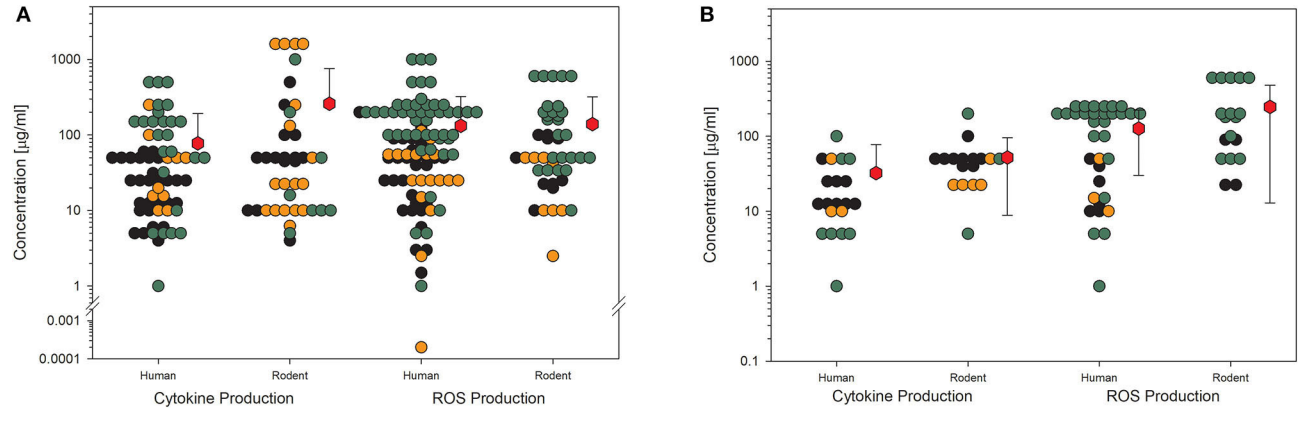

FIGURE 7 | Comparison of human and rodent cell lines in terms of their sensitivity to amorphous silica treatment. Datasets on the key events "cytokine production" and "ROS production" are shown. For this comparison, only data for particles with primary sizes below 100 nm were included. Mean values are calculated from each dot cloud (red hexagons). **(A)** All available datasets; **(B)** datasets from studies with high-quality scores of 0.8 or 1. Details are as those shown in **Figure 2**.

models should reflect the tissues of both species, animals, and humans (2). Finally, the comparison between cellular responses to nanomaterials of animal cells and human cells might be of interest to demonstrate if there is a difference in sensitivity or not. The same four KEs, which have been shown above, have been analyzed with the existing data on amorphous silica studies for possible differences in the biological response in rodent cell cultures compared to human cell cultures (**Figures 6**, **7**). The data from the literature between 2013 and 2018 justifies the statement that there is no difference in sensitivity to amorphous silica between human and rodent cell lines. All four shown KEs do not show any differences between both types independent of the quality of the studies. The mean values are nearly identical not only for the different species but also for all the four KEs. The lowest mean value can be seen for cytokine production in human cells for high-quality studies with 32 μg/ml (**Figure 7B**). All the other mean values are above 50 μg/ml, mostly far above 100 μg/ml.

DISCUSSION

This systematic review focused on synthetic amorphous silica (SAS), a material that has been produced for more than 80 years, has been investigated in hundreds of studies, and is used in cosmetics, pharmaceuticals, as a food additive, and special modifications of many other products. Important is the fact that all publications selected from the available libraries have been evaluated regarding the mentioned "primary particle sizes" of the used amorphous silica materials. Dry powder materials produced for the market exist as aggregates and agglomerates with sizes ranging from several hundreds of nm to above 1 μm. Aggregates are individual indivisible units without well-defined physical boundaries between the primary particles (32). Primary particles usually do not exist in the products on the market but are often produced specifically for the studies described in this review. To better isolate the particles, they are often sonicated with different forms of energy to get the particles in suspension. Moreover, suspensions of primary particles are often stabilized by additives or applied surface charges, and these variables make studies even more diverse. Thus, the comparability of the published data is severely limited and must be interpreted very cautiously. Nevertheless, this overview of the effects of amorphous silica and SAS *in vivo* and *in vitro* attempts to provide a good insight into current nanotoxicological studies as has been shown for other materials as well (9, 10, 84).

A key point of this literature review is to assess the quality of studies regarding their nanotoxicological potential. It has been assumed that over time, with most national- and international-funded projects, the quality of toxicological publications will increase. During the last two decades, unfortunately, this has not been the case. The evaluation of studies from the first decade of the century showed that only 30% of the studies provided reliable data (9, 10, 13), and this low reliability was also evident in the evaluation of studies from the subsequent decade, as shown in this and other evaluations (29). In order not to be too critical, it should also be mentioned that a significantly higher level of quality has been achieved in ecotoxicological studies with algae (47%) and fish (63%) (29).

Selective assessment of the four most often investigated biological endpoints *in vitro* (85) and most important exposure scenarios in animal studies leave no doubt that amorphous silica and, especially SAS, are non-toxic. It is noticeable that there appear from time-to-time publications with special conditions and materials that observe serious effects, but these are usually not representative of the materials on the market. For example, all animal studies on gastrointestinal exposure were conducted with non-food amorphous silica in this period, but in these publications, it is then discussed that "SAS" as a food additive could have negative consequences on consumers, which is a wrong conclusion. Avoiding this problem is only possible if material characterization will be intensified and the relationship to real-life scenarios will not be forgotten in the study design of toxicological experiments.

For various nanomaterials, it could be observed that the strength of a biological effect depends on the size of primary particles or, better, on the increased specific surface area of smaller particles compared to their bigger counterparts. Twenty years ago, Oberdörster (86) published important data on the comparison of ultrafine and fine titanium dioxide demonstrating that effects after lung exposure are quantitatively size-dependent on the particles applied. Following the first principle in nanotoxicology, the transport principle (2), this might be due to better transport of smaller particles, as this could be clearly shown in studies on the human placenta (87) and the lungs (88, 89) or a combined effect of transport and faster solubility because of the larger surface area shown for another material than the one investigated in this study, silver nanoparticles (90). On the other hand, there are also publications demonstrating total independence from particle size (91, 92) or a direct relationship to the applied mass of the nanomaterial (93). This review and another publication in parallel demonstrated *in vitro* experiments with no size-dependent effects for amorphous silica (this study) or titanium dioxide (8). Moreover, most of the animal studies evaluated in this systematic review do not show a size-dependent effect either (data not shown), but the statistical significance of this statement is weak because of the low number of comparable studies and the low potential to induce biological effects of the amorphous silica material.

Certain parameters exist in the publications that have a severe influence on the interpretation of the results and the repeatability of the experiments. The most common flaw is still the missing information about the exact cell number or the exact experimental volume of each sample for *in vitro* experiments. The simple information "the cells have been treated with 20 μg nanomaterial per milliliter" is not enough to recalculate the exact amount of nanomaterial the cells have "seen" in this experiment. During the experiments, a certain Petri dish with a specific number of cells may be filled with 0.5, 1, or 2 ml of a nanomaterial suspension. This results in an overall amount of 10 μg in the first case, 20 μg in the second, and 40 μg in the third case. The difference in dose for the cells is 1:4! The same is true for the dose metric $\mu g/cm^2$ if the cell number and surface area of the Petri dish are not given in parallel. There is a perpetual discussion among scientists about dose-metrics in nanotoxicology, and the most appropriate units have been suggested to be mass per volume (more traditional), mass

per surface area or surface area per cell, and particle number per volume or cell (94–99). It does not matter which unit is chosen for an experiment; the most important criterion remains to be the traceability or repeatability of studies. As described above, the specification of mass per volume is not sufficient to unambiguously repeat an experiment, it is also necessary to know how much total volume was used in the treated sample and, of course, the treated cell number per sample. The same applies to the unit mass per surface. Here, the surface area of cells and the surface area of vessels must also be specified, since no repeatability is possible without this information. The unit particle surface per cell or cell number, on the other hand, is less suitable for normal laboratory use, since the falsification of the dose would be enormous here because of the different agglomeration and aggregation status of the nanoparticles. The same applies to particle number per cell number since here, the question also arises as to which particles are counted. Are they isolated primary particles or agglomerates/aggregates, and how are they isolated? For good traceability, therefore, mass per volume or surface area data are suitable for routine operation, but with the additional information outlined above, without which such data make no sense.

Another aspect is the so-called "landslide effect". It has been demonstrated by Wittmaack (100, 101) that a concentration of 27 μg/ml of TiO_2 applied to a cell culture on a 96-well-plate with a total amount of 8.4 or 25.2 μg/cm^2 induces a total coverage of cells by the nanomaterial because of rapid sedimentation of particle agglomerates. This example makes clear that first, the dose unit μg/ml is susceptible to misinterpretations; second, concentrations above 30–50 μg/ml, referring to 20 or more μg/cm^2, do not make sense for materials with a density higher than 1. The material will build up a layer of more than 50–100 nm in thickness, hindering nutrient and oxygen diffusion to the cell surface and leading to higher susceptibility to disturbing factors. Comparing the data in **Figures 2–5**, the majority of data points are produced with concentrations above this threshold, and, surprisingly, no higher toxicity was found. This might be another evidence of the low toxicity of amorphous silica.

The points presented above show the weaknesses of many studies, which may be the reason for the possible misinterpretation of their results. Detailed explanations should shortly demonstrate where reviewers of manuscripts must look closer in the future to increase the quality of published studies because "non-repeatability" reduces the reliability of experiments dramatically. Finally, in addition to the qualitative deficiencies that the studies exhibit, this review shows that there is low or no toxicity of synthetic amorphous silica and even of the self-made materials not produced for the market.

CONCLUSION AND RECOMMENDATIONS

Conclusion

In vivo Data

- Systematic investigations on size dependency are missing. The overall number of studies that are comparable in the model, application route, dose, and test design is very limited.
- Data gaps exist in the published literature, especially for *in vivo* studies.
- To observe a trend for size dependency, the number of data points is too small to come to a significant conclusion.
- The only two studies out of six that performed inhalation experiments following OECD guidelines (63, 65) did not observe effects at 5 or 10 mg/m^3, thus, the NOAEL can be higher.
- All effects observed *in vivo* after instillation or injection are induced at very high doses, mostly above 10 mg/kg.
- Oral application did not result in any effects. Not even after 90 days application of 2,000 mg/kg bw/day provoked any adverse response in rats.

In vitro Data

- As the number of data points increases, the quality of studies does not increase over time, as is hoped in various funding programs. Funded projects do not use the majority of the established protocols from former projects; thus, the quality of data is not better in the period 2013–2018 compared to the results for the period between 2000 and 2013 (data not shown).
- Size dependency is not apparent; all particles of all size groups have the same potency or, better, the same inertness.
- Outliers may be explained by sensitive models, wrong study design, or specific surface reactivity of self-made materials. The number of outliers is very few.
- No difference in sensitivity can be observed between human and animal cell lines.
- The mean concentrations of amorphous silica inducing biological effects in cells are around or above 100 μg/ml, which is far beyond reasonable concentrations.
- The systematic review of the literature on amorphous silica supports the hypothesis of the very low toxicity of amorphous silica to humans and animals. Especially considering the majority of "self-made" materials, which are not produced for the market under the restrictions of the law, it can be expected that relevant SAS produced for the market is much less harmful, and this leads to the overall conclusion that there is no reason for concern regarding the hazard of silica particles in the form of nanoparticles, larger particles, or agglomerates/aggregates.

Recommendations

For future studies with animals, the study design and applied OECD guidelines should be carefully considered, as, without the use of harmonized protocols, toxicological studies on nanomaterials make no sense. The emphasis here is on the term "toxicological" because mechanistic studies should still be possible but should also be considered as such. To assess the hazardous effects of a given substance or material, dose-response relationships must be established for well-known biological endpoints. To classify a nanomaterial in a given scenario as the IARC classification for cancerogenicity, the key event is well-defined, and the experimental design is well-described (compare OECD Testing Guidelines). The study scenario is different from mechanistic studies. The objective of mechanistic studies is to look for the mode

of action of a given substance or a material that often includes unknown endpoints and non-standardized protocols, which are newly developed or adopted. Exactly, this step of using non-standardized experimental protocols is a substantial difference from toxicological studies that should use SOPs, harmonized protocols, or OECD testing guidelines with well-defined biological endpoints in mind.

It has been often criticized that spurious data of high-dose experiments in single, not comparable studies are of little value (102).

It is of utmost importance to use only nanomaterials for toxicological studies that have been extensively characterized. To increase the quality of toxicological studies, the following prerequisites should be fulfilled for the underlying experiments:

1. a rigorous and adequate physicochemical characterization of the test materials is needed (compare mandatory and desirable properties at the DaNa4.0 website[4]);
2. adequate particle controls and appropriate positive controls for a specific biological endpoint should be included;
3. possible contaminants, such as endotoxins, should be analyzed;
4. interferences of the tested material with the assay should be excluded;
5. high dose experiments designed to produce toxicological effects, which are publishable (and sensational), should be avoided but
6. dose-response studies should cover the entire range, from no-effect concentration (EC_0/NOAEL) to a concentration inducing biological response;
7. dosimetry should be meaningful and traceable;
8. sedimentation rate and cellular uptake should be considered;
9. improved sophisticated *in vitro* models (e.g., ALI) should be used to reflect more realistic conditions.

Evaluation of articles with specific regard to these points has sorted out 70–90% of the respective publications (9, 10). During the Quality Nano-project meeting in Heraklion, Greece in 2015, the first literature study on the content and quality of published studies between 2000 and 2013 was presented (9). The disappointing result of more than 75% of toxicological studies not meeting the quality standards was intensely discussed, and official representatives of the European Commission were hopeful that this will change with new projects. This has not become true, although several national and European projects established new SOPs and some published collections on the web (e.g., DaNa4.0[5], EU-project PATROLS[6]). However, these SOPs will not be used in future projects as is obvious when comparing the outcomes of manifold published studies from more recent years. This systematic review of amorphous silica studies that were published during the period 2013 to 2018 shows clearly that only 3.4% of the studies reach the highest quality score of 1 and further 24% the level of 0.8. Thus, the recent literature fails to meet the expectations not only of the EU officers to show higher quality than earlier publications. For a future increase in the quality of toxicology-oriented studies, new funding programs at the national and international levels should insist that SOPs or OECD guidelines should be used in projects as a matter of principle besides own-developed protocols.

[4]https://nanopartikel.info/wp-content/uploads/2020/11/DaNa_criteria_checklist_2016tox_en.pdf

[5]https://nanopartikel.info/en/knowledge/operating-instructions/

[6]https://www.patrols-h2020.eu/publications/sops/index.php

AUTHOR CONTRIBUTIONS

The author confirms being the sole contributor of this work and has approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.902893/full#supplementary-material

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Comparison of Biogenic Amorphous Silicas Found in Common Horsetail and Oat Husk With Synthetic Amorphous Silicas

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The present study summarizes the current literature on the presence and the structure of biogenic amorphous silica (BAS) in nature. Based on this review, it is shown that BAS is ubiquitous in nature and exhibits a structure that cannot be differentiated from the structure of synthetic amorphous silica (SAS). The structural similarity of BAS and SAS is further supported by our investigations—in particular, specific surface area (BET) and electron microscope techniques—on oat husk and common horsetail. Many food products containing BAS are considered to be beneficial to health. In the context of the use of SAS in specific applications (e.g., food, feed, and cosmetics), this is of particular interest for discussions of the safety of these uses.

Keywords: synthetic amorphous silica, biogenic silica, nanostructure, toxicity, common horsetail, oat husk, transmission electron microscopy (TEM), scanning electron microscopy (SEM)

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INTRODUCTION

Synthetic amorphous silica (SAS) can be produced by either a vapor-phase hydrolysis process, yielding pyrogenic (fumed) silica, or by a wet process, yielding hydrated silica (precipitated silica, silica gel, or colloidal silica). During the manufacturing process of these SAS materials, the primary particles are strongly (covalently) bonded or fused to form aggregates (1). Physical attraction forces (van der Waals interaction and hydrogen bonding) lead to the formation of agglomerates in the micron-size range. Typically, isolated nanoparticles do not occur. In contrast, colloidal SAS dispersions may contain isolated primary particles in the nano-size range, which can be considered nano-objects. SAS powder is placed on the market as micron-sized aggregates and agglomerates with an internal structure in the nanoscale (ISO definition of nanostructured material).

Synthetic amorphous silicas can be regarded as nanomaterials (NM)[1] and have been produced and marketed for many years. Fumed silica production began in 1944, and even in 1950, they had been described as "milli micron particles"; "milli micron" at this time was the equivalent of today's term "nano." Interestingly, the current popular term "nano materials" (NMs) had been coined just a few years prior to the beginning of the new millennium. Since the great interest in NMs in the

[1] Definition according to ISO/TS 80004-1.

TABLE 1 | Quantitative analysis of both horsetail and oat husk from samples "as received".

		Horsetail	Oat husk
Loss on drying (105 °C over night	% Mass	8.4	6.8
Redidue on ignition 540 °C over night	% Mass	17.7	3.1
Na	µg/g	780	79
Mg	µg/g	53,00	330
K	µg/g	2.9	0.43
Ca	µg/g	1.7	0.04
P	µg/g	2,900	260
Al	µg/g	2,100	<10
Fe	µg/g	1,600	11
Ti	µg/g	140	<5
SiO_2	% mass	6.8	2.2

early 2000s, the word "nano" can be found in a wide variety of diverse scientific and technical fields. Following the establishment of the technical committee for nanotechnologies (ISO/TC 229) in 2005, international standardization evolved and finally, an NM is now defined by ISO/TS 80004-1 as "material with any external dimension in the nanoscale (1–100 nm) or having an internal structure or surface structure in the nanoscale." This generic term includes nano-object and NM.

The new nomenclature of NMs led to the impression that these were "novel materials," whereas it has been overlooked by the general public that some of these NMs have been deliberately produced and/or used either for many decades (e.g., SAS and carbon black), centuries (e.g., gold ruby glass), or even millennia (e.g., clay). In addition, there are many NMs naturally occurring in the environment originating from natural sources (e.g., volcanos and minerals *via* wear and abrasion) or from biological processes. We should also note the unintended environmental and occupational production of NMs, e.g., *via* combustion products (automobile engines and wildfires), welding, and other man-made or natural activities.

Biological processes, both in fauna and flora, not only synthesize organic NMs, such as liposomes but also inorganic NMs, such as iron oxides (e.g., magnetite in pigeons) or amorphous silica (e.g., in many kinds of grasses, such as rice, oat, wheat, or sugar cane; diatoms/algae, and sponges).

Helpfully, in the "ISO/TR 18401 Nanotechnologies—Plain language explanation of selected terms from the ISO/IEC 80004 series," the nanostructured amorphous silica found in rice husk is named as an example of a naturally-occurring NM.

Synthetically manufactured NMs are strictly regulated in a number of industries. In this respect, extensive physicochemical, ecotoxicological, toxicological, safety, and epidemiological data have been collected for SAS, and no environmental or health risks have been identified provided SAS is produced and used under current occupational hygiene standards and recommendations for its safe use in various applications. It is therefore of interest to consider if these synthetic NMs can be differentiated from the ubiquitous, naturally occurring biogenic amorphous silica (BAS)[2] NMs. In the present study, therefore, SASs (e.g., food additive E 551) are compared with their natural counterparts: BAS. It will be shown that the NMs of BAS are naturally present, especially in plants, and they can exhibit a similar nanostructure and surface area compared with SAS types used for food/cosmetics. In fact, due to their usual daily uptake, humans are evolutionarily adapted to the intake of BAS, whether it is in a particulate nanoform or dissolved. This is particularly relevant as the average human has about 1.5 g of silicon dioxide (SiO_2) in their body (2). It is a vital chemical for all cells, and especially important for connecting tissues, such as the skin, bones, cartilage, tendons, and ligaments. It has been noted that the amount of silica in the body decreases with age (3–5).

Biogenic amorphous silica thus plays a vital role in the living environment and shows nanostructures that are very comparable with manufactured SAS. For oat husk (*Avena sativa)* and common horsetail (*Equisetum arvense*), their structures are even below 20 nm. This was shown for the latter from as early as 1991 (6). This present article reviews existing studies on the structure of BAS along with our study results. For the above-mentioned products, we have been able, for the first time, to compare the specific surface areas of SAS and BAS by Brunauer, Emmet, Teller (BET) and show that the results for BAS were as high as 150 m^2/g.

MATERIALS AND METHODS

For our studies, we used samples of the common horsetail (*E. arvense*) supplied from Blanks GmbH & Co. KG ("Schachtelhalmkraut, geschnitten"), often used as a herbal tea. Oat husk (*A. sativa*) was obtained from Bio Bäckerei Spiegelhauer ("Haferspelzen, fein vermahlen"); usually this is often used for sprinkling proofing baskets in bread making.

Sample Preparation for Both Common Horsetail and Oat Husk

To obtain an initial indication of the composition, a quantitative analysis of the content of SiO_2 and other inorganic components in the horsetail and in the oat husks raw material was performed.

The samples were prepared according to the following procedure for the detailed investigation of the structure of the silicon dioxide present in the materials.

Schematic sample preparation steps for both raw BAS materials and naming of samples for later analysis.

- Starting material: raw material as received samples as received
- Digestion starts with about 30% hydrochloric acid at room temperature
- Multiple decanting/adding water = dilution (each cycle about 2–3 days) until HCl-concentration < 0.05%
- Centrifugation at 20,000 rpm
- Drying at 105°C for 16 h
- Calcination (in small portions) at 540°C for 4 h—calcined without washing

[2] Alternatively, the abbreviation bASi is also used in soil sciences.

FIGURE 1 | Light microscope picture of parts from common horsetail (*Equisetum arvense*).

- Thoroughly washing (employing ultrasonication and centrifuge)
- Drying at 105°C for 16 h—final sample

For comparison, samples of the raw materials were dried at 105°C (just dried; those have been calcined at 540°C for 4 h have been prepared (calcined raw materials), and washed with water (calcined raw materials/washed).

The common horsetail, as well as the oat husk, were digested with hydrochloric acid at room temperature for several days. Using successive dilution with deionized (DI) water, prolonged sedimentation, and decantation, the resulting greenish (common horsetail) to brown (oat husk) samples were then dried at 105°C overnight and subsequently calcined at 540°C for 4 h. This yielded a white powder which was thoroughly washed with DI water, centrifuged at 20,000 rpm, and finally dried at 105°C overnight.

In parallel, comparative samples were prepared for all steps, e.g., without digestion by hydrochloric acid, to ensure that any observed structures are not confounded by the sample preparation process.

BET and Electron Microscopy

The Multi-Point BET Was Measured With an AUTOSORB® iQ Station

The scanning electron microscopy (SEM) imaging was done with a Jeol JSM-7600F/Oxford-EDX Aztec and the energy dispersive X-ray (EDX) analysis with Thermo Fisher Scientific Ultradry EDX. The transmission electron microscopy (TEM) analysis was performed with a Jeol 2010F. The quantitative analysis was done with an ICP-OES iCAP 6500 Duo (Thermo Fisher Scientific GmbH). Species preparation took place with a lithium-metaborate melt.

RESULTS

Quantitative Analysis of the Samples as Received

To determine the amounts of Na, Mg, K, Ca, P, Al, Fe, Ti, and SiO_2 with ICP-OES, the samples were calcined at 540°C overnight and a lithium metaborate melt was used in the preparation of the measurements. The SiO_2 content was calculated from the data for elemental Si. Values are given about the original biological sample as received (**Table 1**).

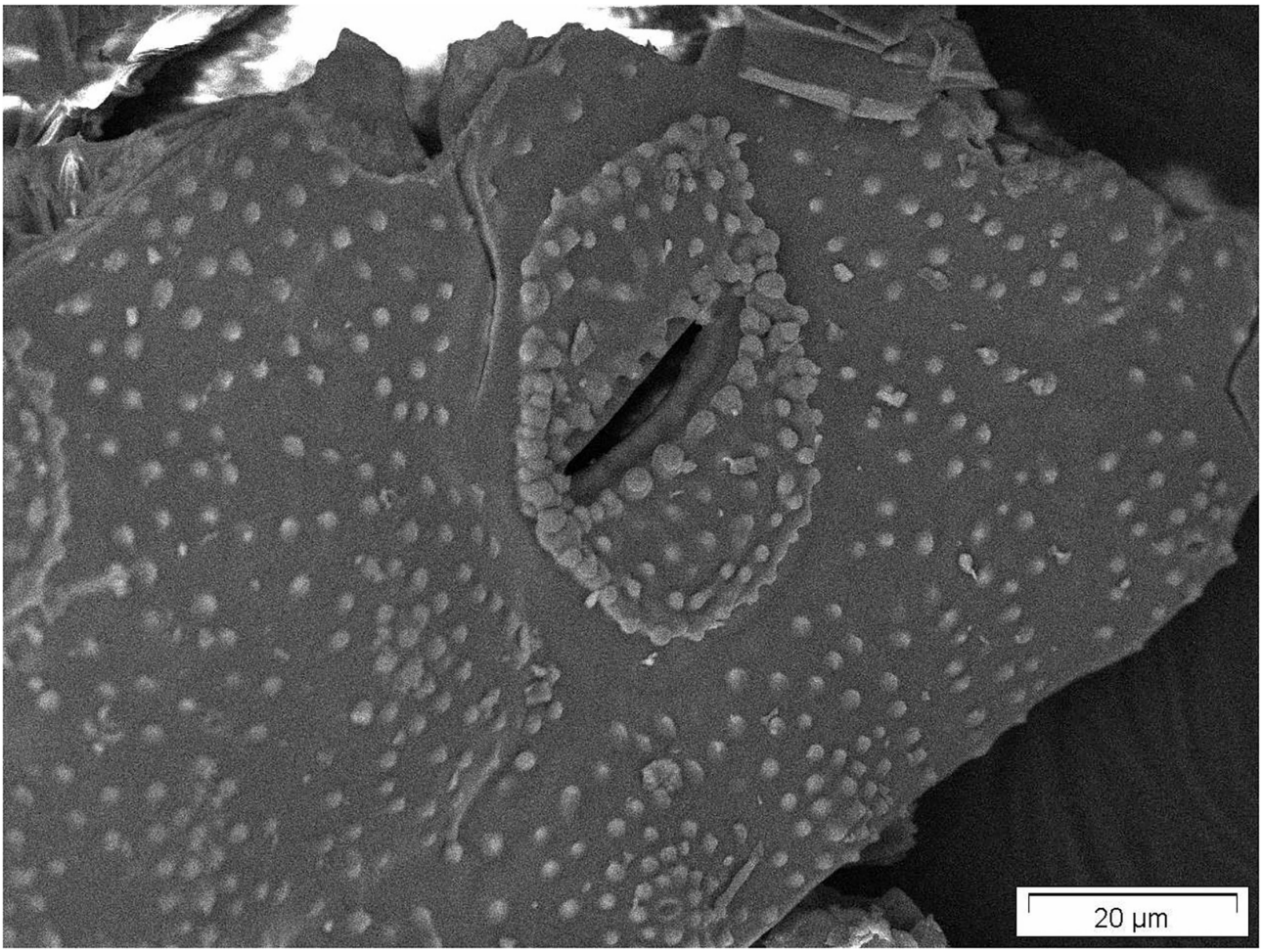

FIGURE 2 | Stomata of just dried common horsetail showing nicely subcuticular incorporated silica particles.

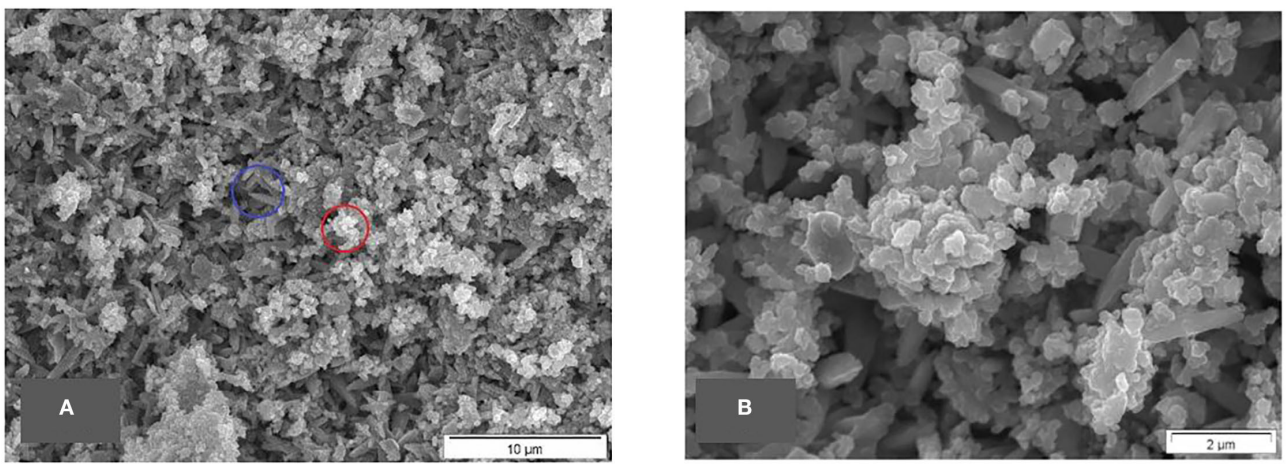

FIGURE 3 | (A,B) SEM micrographs of calcined raw materials/washed common horsetail, typical silica structure highlighted by a red circle, structures from other material highlighted by a blue circle.

Results With Common Horsetail

Light Microscopy

The light microscope picture of untreated common horsetail (*E. arvense*) is given in **Figure 1**.

SEM Microscopy

Horsetail SEM pictures, which have just been dried, reveal the full biological structures *(just dried)* and show, e.g., stomata with clearly shown subcuticular incorporated silica particles (**Figure 2**).

Following calcination and washing and with greater magnification, the structure of inorganic residues was examined. These pictures (**Figures 3A,B**) show that besides the structures which are typical for amorphous silica, there is also some crystalline matter.

At the end of the full sample preparation protocol as described above (final sample), biological structures still can be seen in the lower magnifications but, on greater magnification, the typical structure of amorphous silica can be identified. A series of SEM micrographs at different magnifications is depicted in **Figures 4A–D**.

BET Surface Area

The surface area of the final sample was evaluated according to the BET method, the value of 146 m^2/g (multipoint) for this horsetail-derived BAS is typical for nanostructured amorphous silica. This measurement corresponds very well with the SEM and TEM pictures, which also reveal a nanostructured biogenic amorphous silica (BAS).

SEM-EDX Analysis of the Calcined Raw Material

The SEM-EDX-analysis of the calcined raw material horsetail revealed, besides silicon and oxygen, the presence of Mg, P, S, Cl, K, and a large amount of Ca. This is consistent with the elemental analysis of the raw material described above.

The SEM-EDX-analysis of the samples, which had been prepared according to the above-described digestion method (i.e., final sample), only shows the presence of silicon and oxygen. Thus, this method of SiO_2 extraction was shown to be highly effective as well as being rather mild.

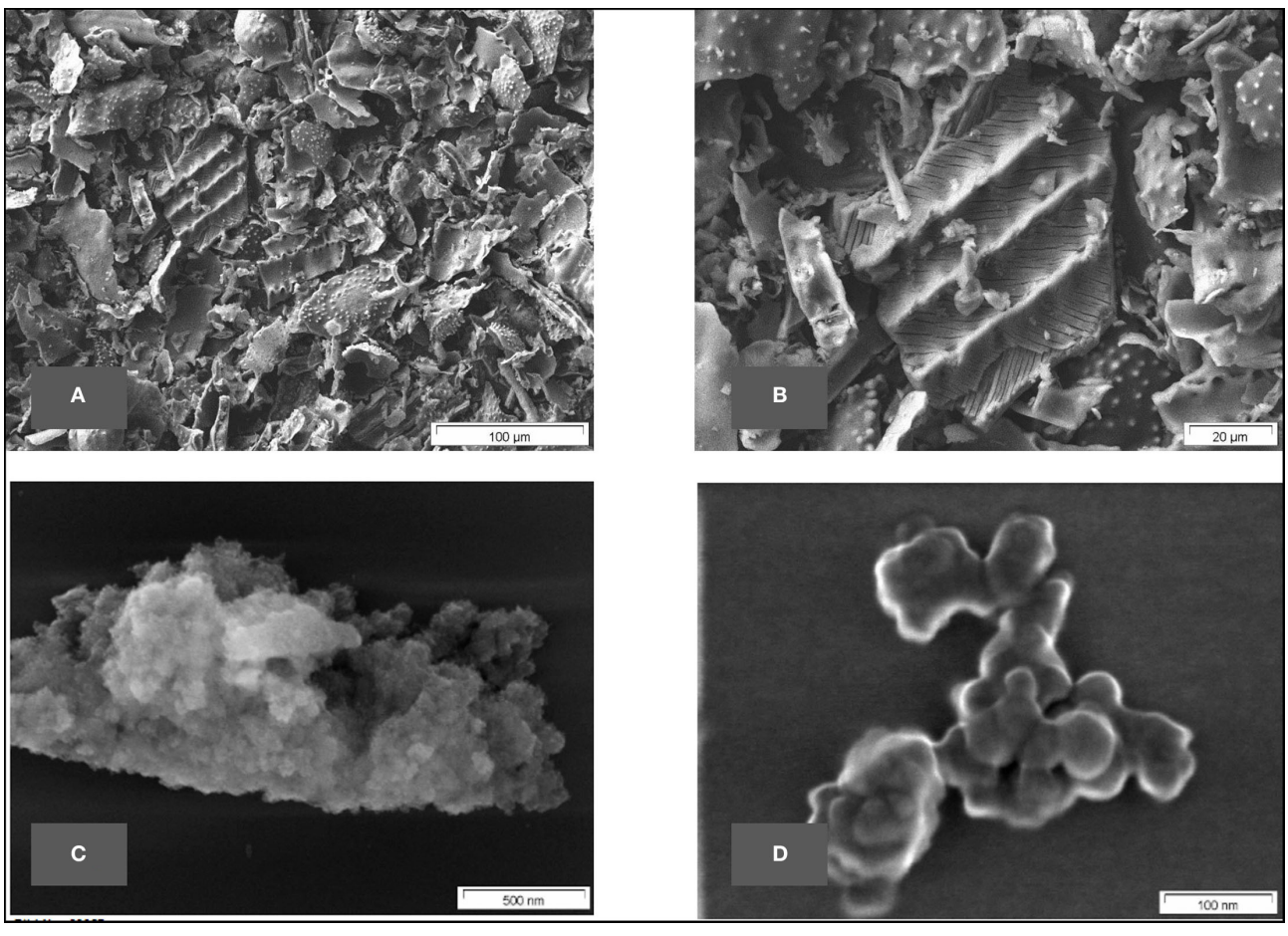

FIGURE 4 | (A–D) SEM micrographs of final sample of common horsetail.

SEM-EDX—Quantitative Analysis—of the Final Sample of Horsetail

The *final sample* shows in the EDX spectrum the presence of Si and O only; thus, confirming the identity as pure silica. **Figure 5** shows an SEM micrograph indicating the areas from which the spectra are taken.

TEM Microscopy

To visualize the smallest structures within the BAS derived from the horsetail sample, TEM micrographs have been performed as well as the SEM micrographs. The images were prepared only on the *final sample*. The TEM micrographs reveal nanostructured amorphous silica (BAS) consisting of fused primary structures in the range of a few nanometers. **Figures 6A–D** shows the TEM micrographs at different magnifications.

Results With Oat Husk

Following the same initial procedure, as was used for the common horsetail, a quantitative analysis of the oat husk raw material was performed in the first stage to get an overview of the composition of the inorganic matter (as shown in **Table 1**).

For the electron micrographs, the sample preparation was focused on obtaining a pure sample. Again, this was performed similarly to the method described for the sample preparation of the horsetail samples. During this sample preparation, no additional quantitative analysis was performed.

However, the relatively coarse oat husk particles did not appear to readily break down prior to the calcination step, i.e., sedimentation and decantation were very effective without the obvious loss of material. Thus, several samples had been quantitatively analyzed for the loss by calcination with an average value of ∼96.1%, i.e., ∼4% of the final sample = silica could be recovered (as shown in **Table 1**). This accords very well with values in the literature where it is reported to be about 5% of silica based on dry oat husk (7, 8).

Optical Microscopy of Oat Husks

Light microscope pictures of the untreated oat husks are given in **Figures 7A,B**.

Scanning Electron Microscopy

Figures 8–10 show SEM micrographs of oat husks at different stages of sample preparation at low magnification.

Close examination of the sample of oat husk, which had been only just calcined (540°C) and washed with DI water, revealed easily identifiable nanoscale structures (**Figure 11**).

When treated with hydrochloric acid, thus transferring the cations into their corresponding readily soluble chloride salts, and after calcinating and thorough washing, the silica nanostructures can be very clearly seen in **Figures 12A,B**. As previously noted, there are a variety of differently-sized primary structures. The interpretation of the measured BET surface area (multipoint) of ∼114 m^2/g is, therefore, an average value of a range of very small and far larger amorphous silica structures.

In the samples prepared without HCl treatment, nanostructures can already be seen but still seem to be covered by some non-silica material and are difficult to identify clearly. In the fully-treated samples, however, the purified silica reveals unambiguously its nanostructure (e.g., **Figures 6**, **12**).

TEM Microscopy

As with the horsetail, TEM images were prepared in addition to SEM pictures to investigate the structure of oat husk-derived BAS in more detail at higher magnifications. **Figures 13A–D** show

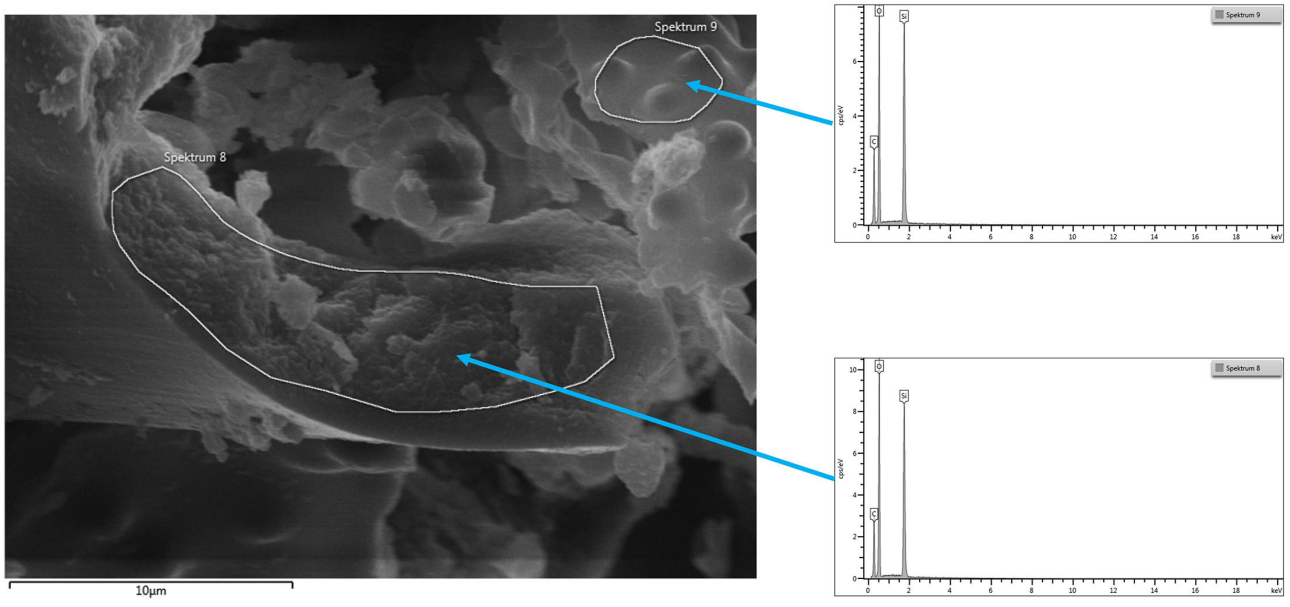

FIGURE 5 | SEM micrograph common horsetail final sample with areas taken for EDX.

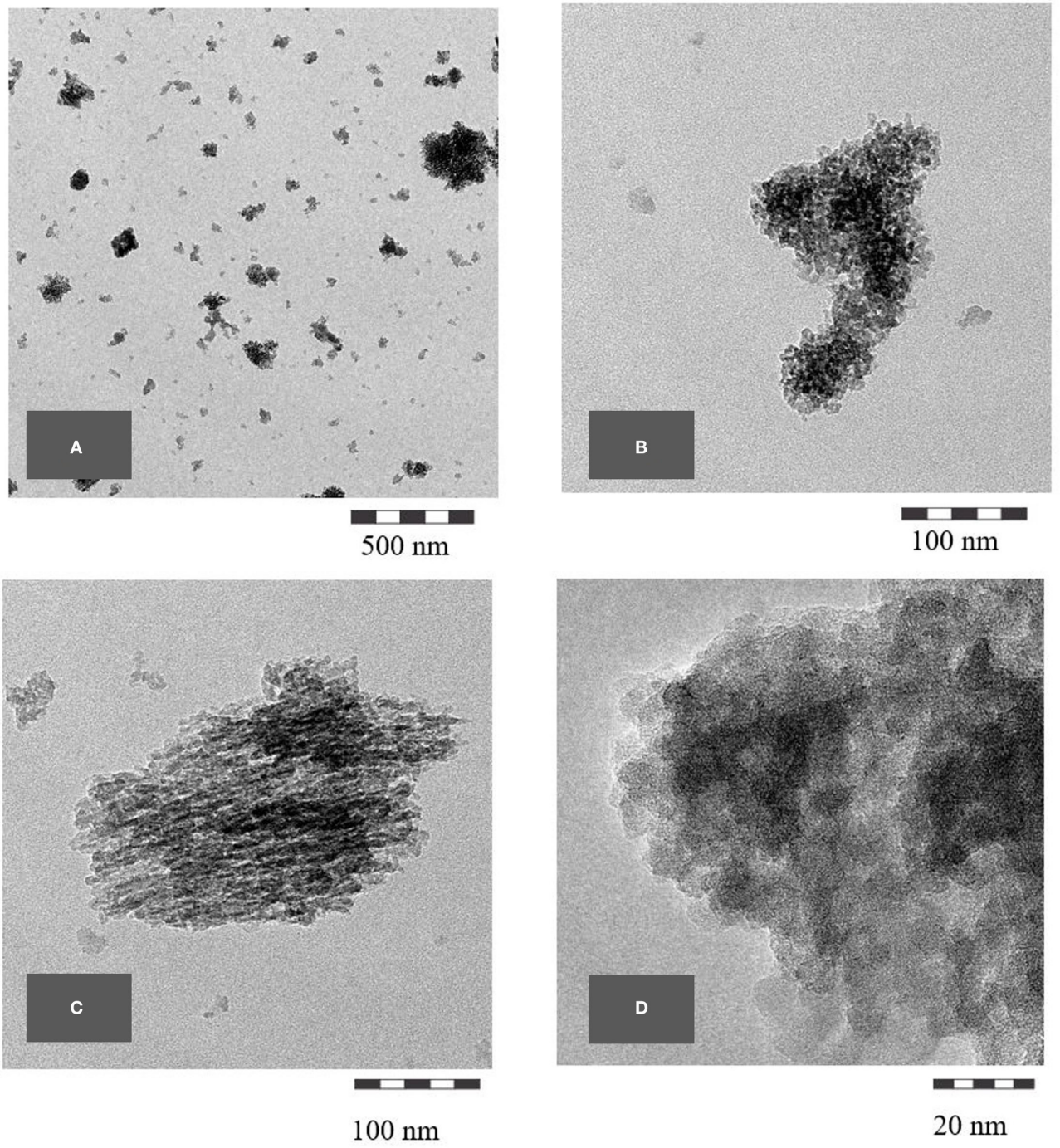

FIGURE 6 | (A–D) TEM micrographs of common horsetail final sample.

very clearly that the nanostructure of these samples is very similar to the structures of SAS.

Several ATEM-EDX spectra of the final sample revealed that besides silicon and oxygen, only very minor impurities are present, i.e., about 0.06–0.25% (atom) K, up to 0.23% (atom) Al, and up to 0.06% (atom) Ca.

Comparison of BAS to Commercial SAS Products Using TEM and BET

In terms of the chemical composition of SiO_2 and its amorphous structure, as well as their nanostructured appearance with similar size ranges in the corresponding primary structures, both BAS and SAS are highly comparable.

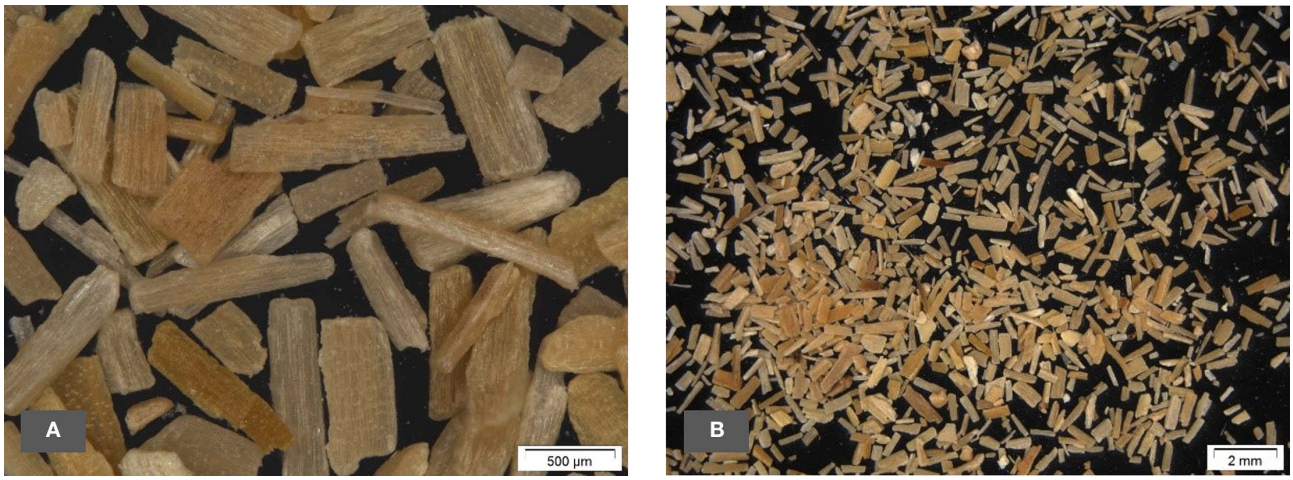

FIGURE 7 | (A,B) Light microscope pictures of oat husks.

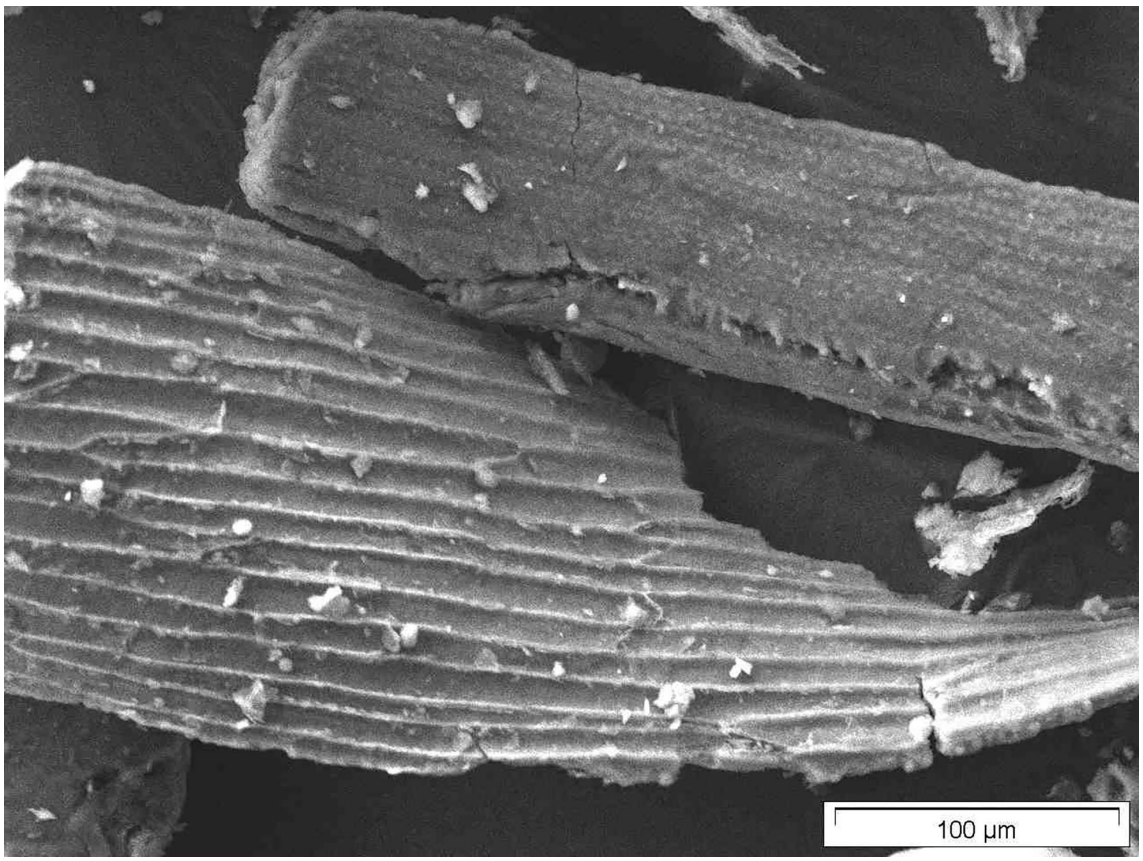

FIGURE 8 | SEM micrograph of out husk (just dried).

TEM pictures, as shown below, demonstrate the similarity between BAS and SAS. Thus, it is not possible to easily distinguish between BAS and SAS based on the results obtained by this described analytical methodology. The specific surface area (BET) also shows that the examined BAS substances are NMs with values $>100\ m^2/g$.

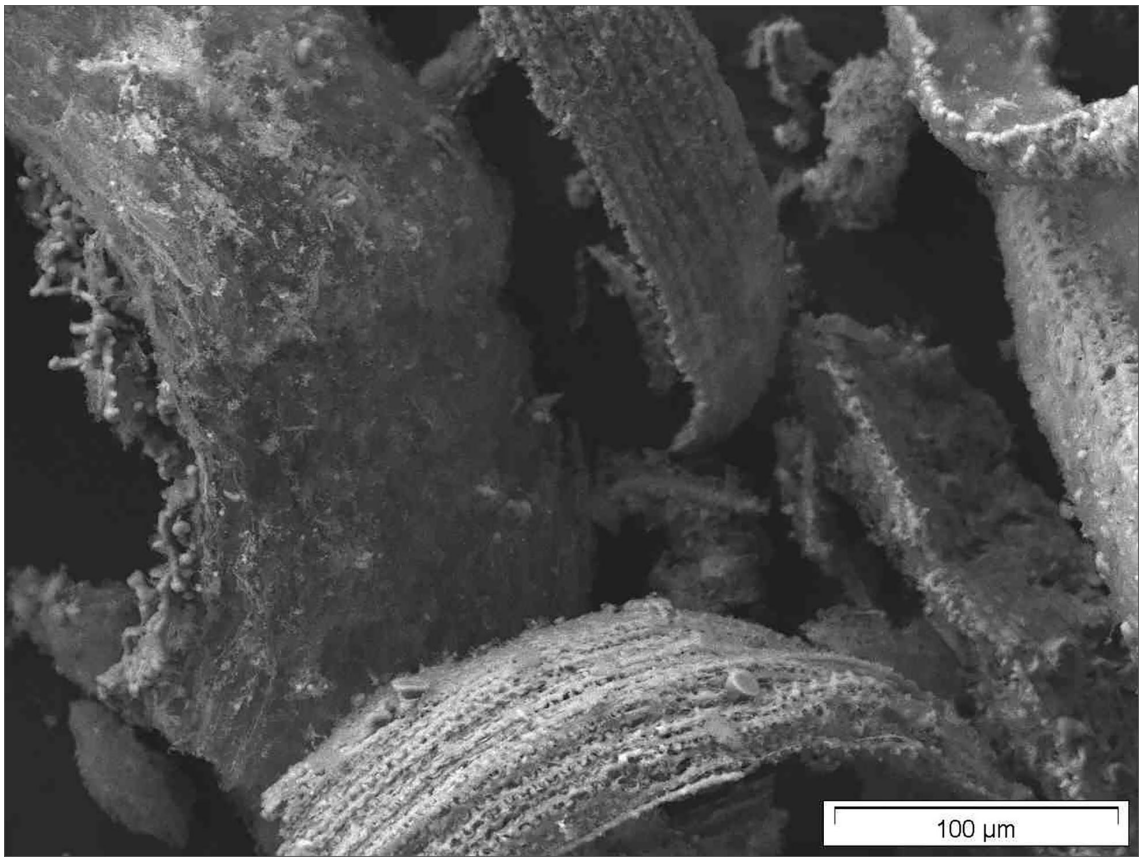

FIGURE 9 | SEM micrograph of calcined oat husk (calcined raw materials).

TEM of Commercial SAS Grades

Figures 14, **15** and **Supplementary Figure 1** show TEM micrographs of different commercially available pyrogenic and precipitated SAS grades. The following commercially available SAS grades have been used.

Precipitated SAS: SIPERNAT® 160 BET 170 m^2/g

Precipitated SAS: SIPERNAT® 22 BET 180 m^2/g

Fumed SAS: AEROSIL® 200 BET 200 m^2/g

[Characteristic BET-surface data taken from (9, 10)]

The images show clearly a very similar nanostructure compared with the BAS grades described above.

DISCUSSION

Background and Literature Review

As early as 100 years ago, silica was found to occur naturally in the human body (3). Based on this observation, it can be assumed that this form of silica has been routinely taken up by the daily diet (11). The European Food Safety Agency (EFSA) notes the Si content in their Scientific Opinion of the Panel on Food Additives and Nutrient Sources added to Food from 2009 (12) for several cereals, e.g., oat 3,910–4,310 mg/kg, barley 2,610–2,720 mg/kg, wheat flour 2,610–2,720 mg/kg, and for polished rice 55–57 mg/kg dry weight.

Martin (13) found that the human body contains about 1.5 g of SiO_2, presumably both in the particulate and dissolved forms (this is in accordance with Merck, 1921). Robberecht (14) provided a comprehensive overview of silicon in food (meat, see food, cereals, milk, vegetables/fruit, and drinks), its content, and bioavailability. The fate and biological responses to SAS in food have been recently described by Yu (15) and an advanced intestinal *in vitro* model has been addressed by Hempt (16). Due to the uptake of silica from different sources of biogenic and artificially generated origin, it is almost impossible to differentiate between the two sources. Theoretically, all sources should have different ratios of Si/O-isotopes; thus, a distinct separation might be possible but to date, this has only been done within a rather limited narrow field of observations (17).

It needs to be noted that in the literature, one needs to be cautious as the word "silica" has been applied to both crystalline (quartz) and amorphous species (1, 18) and sometimes without further clarification. In this article, we have been careful to discuss only amorphous particulate silica.

As noted earlier, amorphous silica can be found in the skeletons of algae (diatoms) (19–26), even in deep-sea sponges and molluscs (27).

Interestingly, there is a DFG Research Unit 2038 "Nanomee" (http://www.nanomee.de/) in Germany that is tasked "to obtain a

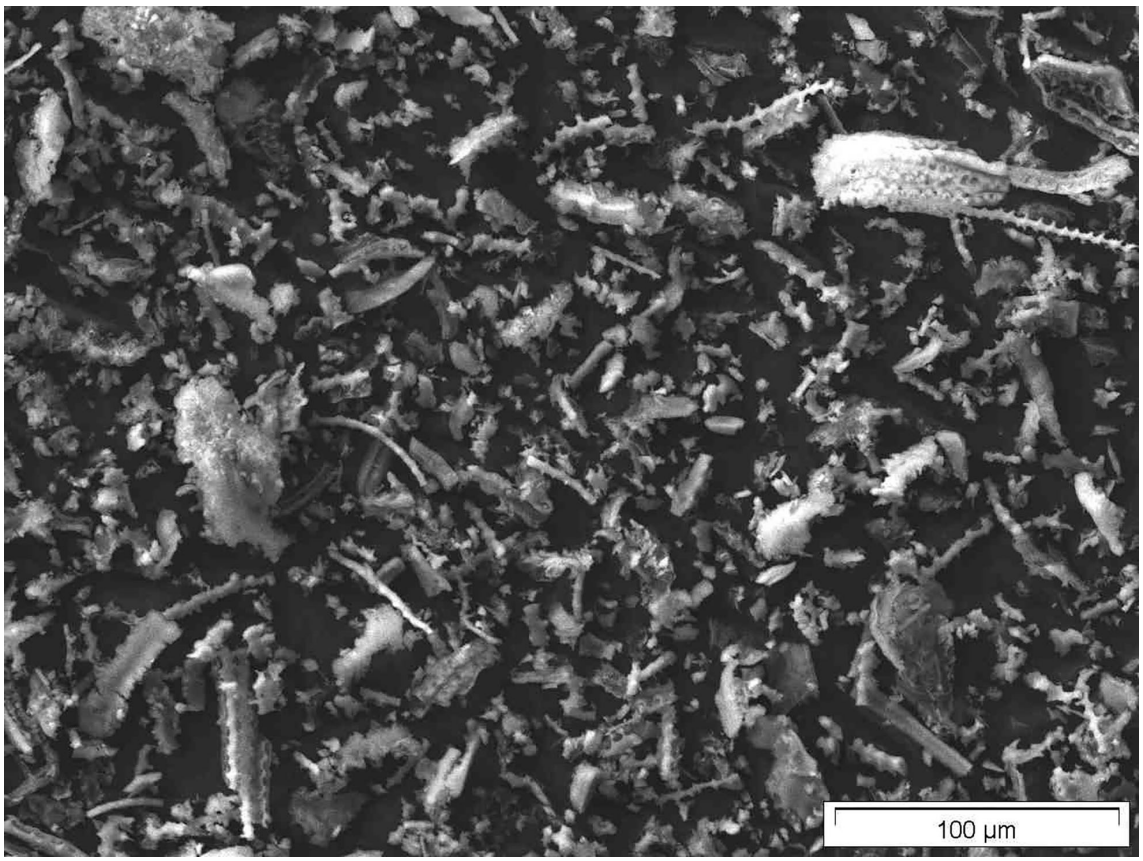

FIGURE 10 | SEM micrograph of final sample of oat husk.

detailed understanding of the biomolecule-controlled nano- and microscale processes that enable diatoms to biosynthesize their species-specifically patterned SiO_2-based cell walls." (28–31).

Plants require BAS—*via* biomineralization—for their growth and strength in so-called biogenic silica structures (32–40).

So-called phytogenic silicas are characteristic of different plant species and of the different functions/locations where they occur.

Amorphous silica is essential for plant growth in terms of not just contributing their physical strength to assist plant structure, but also for their health. Surprisingly, the most abundant and ubiquitously-found silica source in nature, quartz, however, is not available to plants due to its very low water solubility. Therefore, plants have to rely on the presence and adequate concentration of amorphous silica in the soil. The grass types—among them wheat, rye, oat, rice, and others—grow smaller and are more susceptible to diseases when the accessible silica has been substantially reduced by extensive farming. BAS has been found to enhance the resistance of plants to biotic stress, e.g., diseases, such as powdery mildew and pests, such as stem boring insects (41, 42).

Additionally, it has been found that amorphous silica is important for the water storage capacity of the soil and its ability to release immobilized phosphorous, thus making it available to plants (43–50).

The common horsetail is considered to contain the highest percentage of amorphous silica of all living plants, up to 25% (34)—with especially high values in the stabilizing joints. It is remarkable to note that its evolutionary ancestors, from ∼300 million years ago, reached up to well above 20 m in height, with almost the same appearance as seen now. This has been only possible due to the biogenic silica structures stabilizing such constructions.

The first TEM images of the BAS in common horsetail were obtained by Perry and Fraser in 1991 (6) and display distinctive structures of different plant areas with certain "tasks" (e.g., leaves and joints). In 2007 (32), TEM images were obtained showing even more clearly the similarity of BAS to SAS in the case of horsetail. Indeed, the TEM images obtained in this study revealed that BAS looks rather like mirror images of SAS.

As for the BAS in grasses, and those in all grains, amorphous silica NMs in rice husk (from *Oryza sativa*) have been the most extensively studied and the subject of many publications (51–53). In the oat plant, the BAS is also mainly concentrated in the oat husk, too. Moreover, oatmeal is considered a

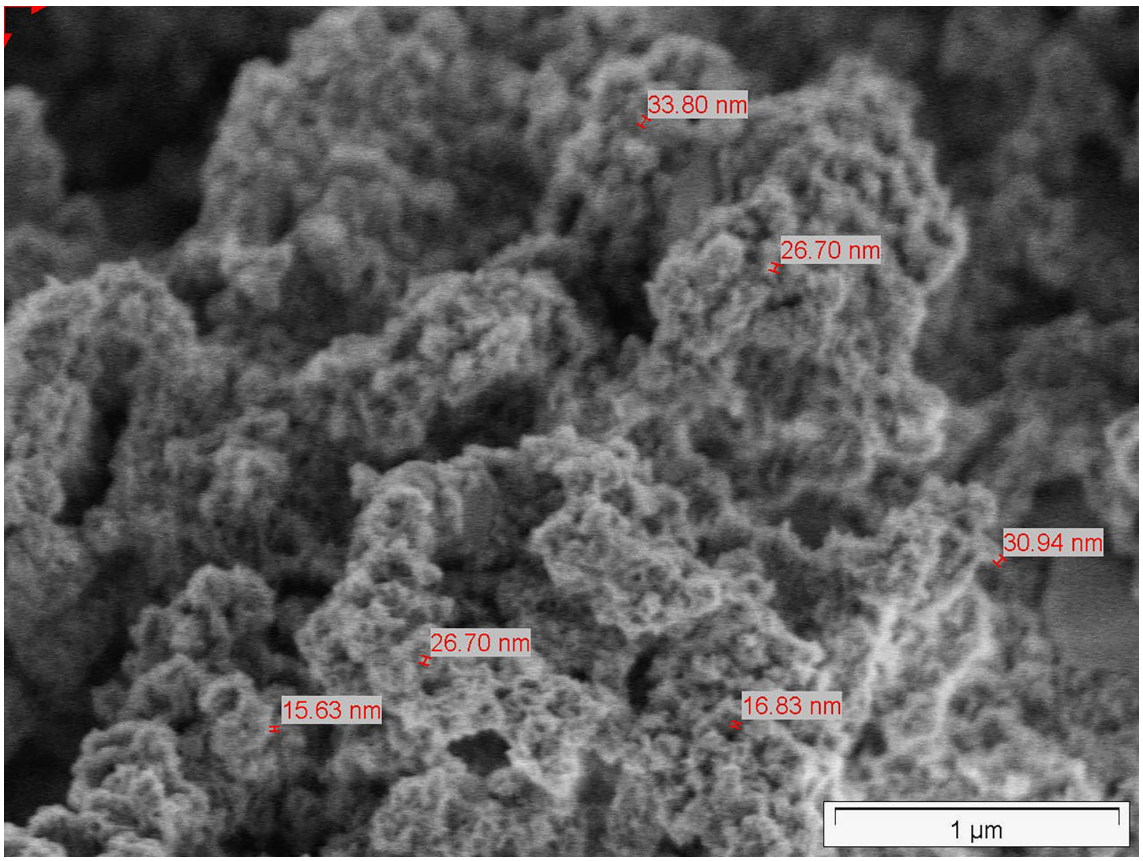

FIGURE 11 | SEM micrographs of calcined oat husk w/o digestion (calcined raw materials/washed) exhibiting nanostructures. Even not as clear as in the final samples they already be identified.

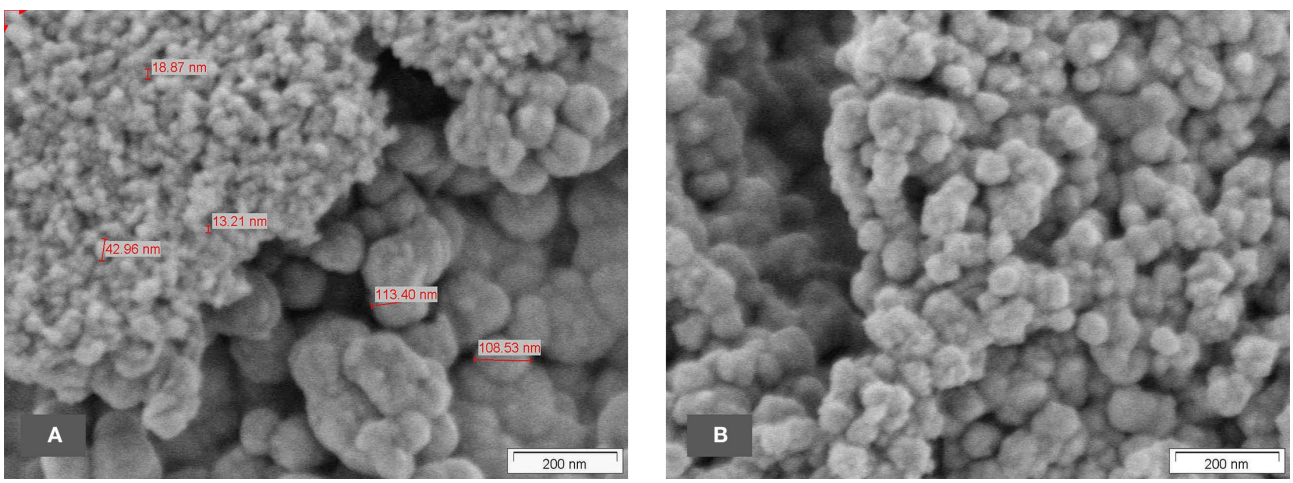

FIGURE 12 | (A,B) SEM micrographs of the final sample of oat husk.

viable daily source of silica in modern breakfast. Therefore, for this reason, the oat husk was chosen in this current investigation as the second natural organic source for the determination of BAS. Interestingly, using SEM imaging, it is possible to identify nanostructured silica even in the oat husk ash directly.

The ubiquitous occurrence of BAS and its having a very similar nanostructure to SAS is an interesting aspect that needs to be

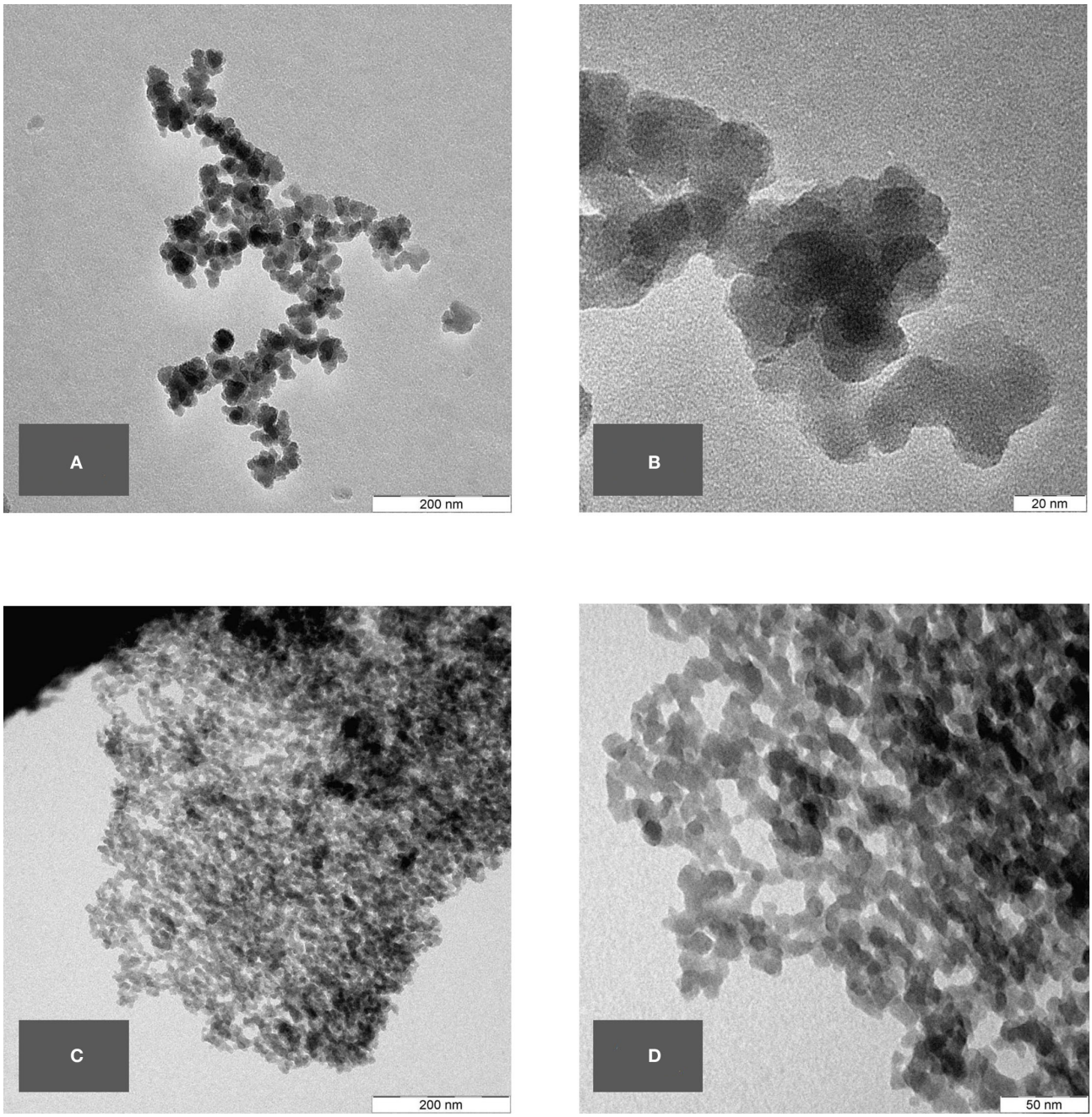

FIGURE 13 | (A–D) TEM micrographs of final sample from oat husk at different magnifications.

considered and should be taken into account in any discussions about the safety of NMs, especially in the context of the oral uptake of SAS in food, feed, and cosmetic applications.

Taken all together, commercial SAS forms are not entirely new NMs with unknown properties, but are well-studied materials that have been in use for decades without changes in their basic physicochemical structure.

Interpretation of the Presented New Studies With Common Horsetail and Oat Husk

In this current investigative series performed on oat husk and common horsetail, we have examined the nanostructure of BAS by SEM and TEM analysis and compared them with the nanostructure of commercially available SAS materials.

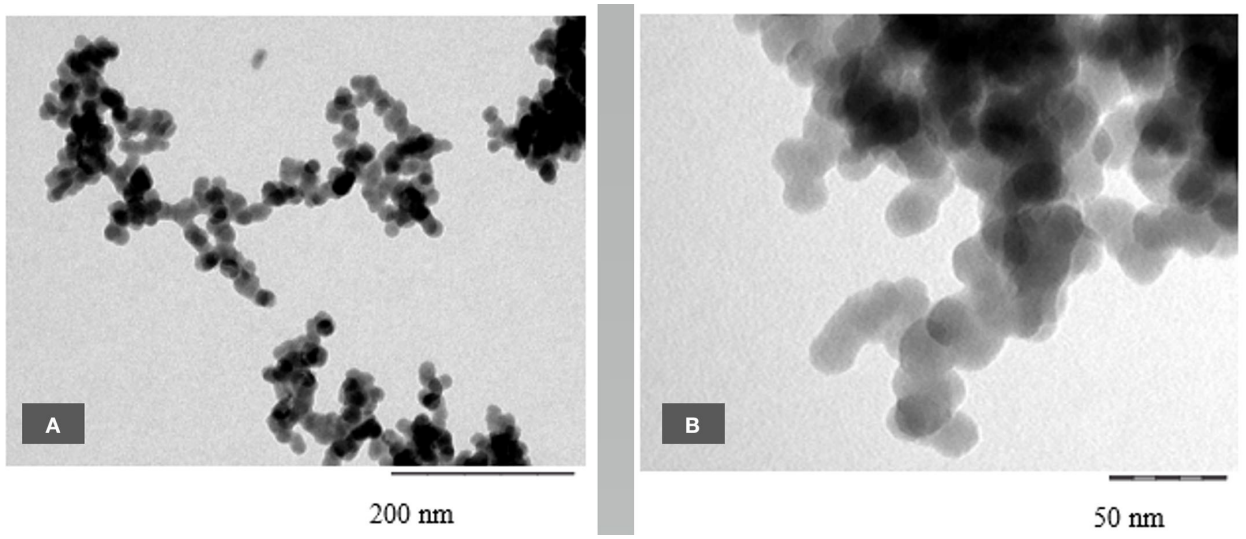

FIGURE 14 | (A,B) TEM micrographs of SIPERNAT® 160 at different magnifications.

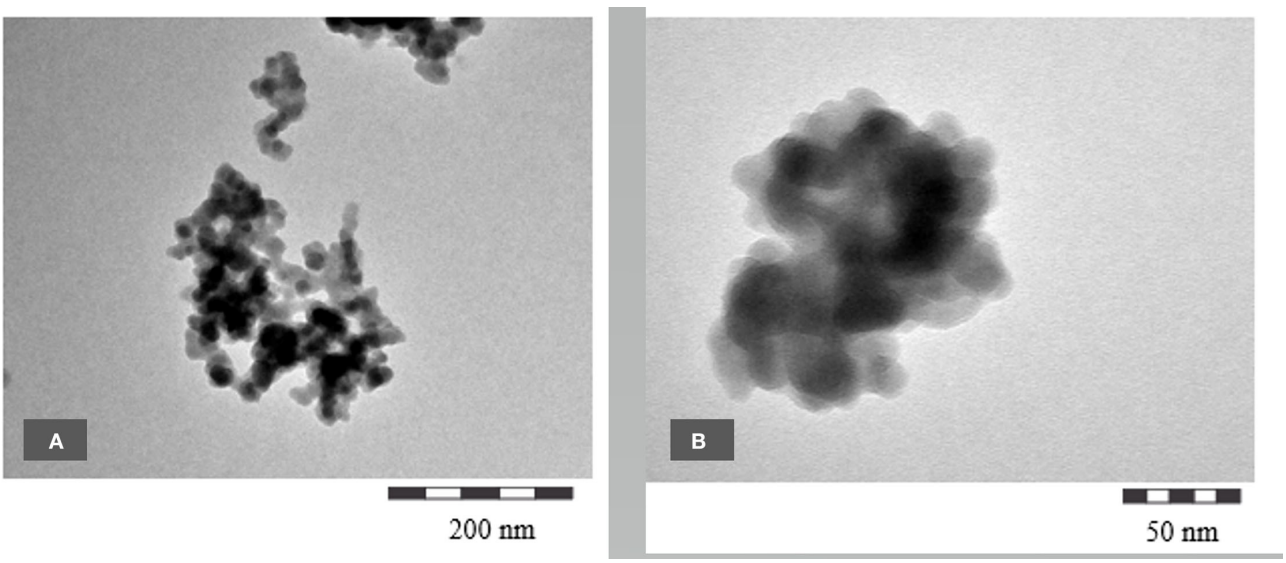

FIGURE 15 | (A,B) TEM micrographs of SIPERNAT® 22 at different magnifications.

Furthermore, we have also performed BET analysis in both types of natural and SAS products.

Scanning electron microscopy is an established method to evaluate the structure of materials down to the nanoscale. In our SEM overview images, it could be shown that the structural analogy between SAS and BAS can be found throughout the whole of the biological sample and not only in isolated locations. Using TEM images, which are suitable to show the structure at even greater magnifications, a direct comparison between BAS and SAS was possible and demonstrated that BAS is structurally very similar to SAS. Thus, the naturally-occurring BAS may even be considered a bio-analog to SAS. This is also confirmed by a comparison of the surface area of both these two amorphous silica types.

Based on our findings and the identical chemical composition, there seems no reason to differentiate between naturally-occurring BAS, which is present in many food sources and is perceived beneficial to health, and SAS.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.909196/full#supplementary-material

Supplementary Figure 1 | (A,B) TEM micrographs of AEROSIL® 200 at different magnifications.

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REVIEW
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Applying Translational Science Approaches to Protect Workers Exposed to Nanomaterials

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Like nanotechnology, translational science is a relatively new and transdisciplinary field. Translational science in occupational safety and health (OSH) focuses on the process of taking scientific knowledge for the protection of workers from the lab to the field (i.e., the worksite/workplace) and back again. Translational science has been conceptualized as having multiple phases of research along a continuum, beyond scientific discovery (T_0), to efficacy (T_1), to effectiveness (T_2), to dissemination and implementation (D&I) (T_3), to outcomes and effectiveness research in populations (T_4). The translational research process applied to occupational exposure to nanomaterials might involve similar phases. This builds on basic and efficacy research (T_0 and T_1) in the areas of toxicology, epidemiology, industrial hygiene, medicine and engineering. In T_2, research and evidence syntheses and guidance and recommendations to protect workers may be developed and assessed for effectiveness. In T_3, emphasis is needed on D&I research to explore the multilevel barriers and facilitators to nanotechnology risk control information/research adoption, use, and sustainment in workplaces. D&I research for nanomaterial exposures should focus on assessing sources of information and evidence to be disseminated /implemented in complex and dynamic workplaces, how policy-makers and employers use this information in diverse contexts to protect workers, how stakeholders inform these critical processes, and what barriers impede and facilitate multilevel decision-making for the protection of nanotechnology workers. The T_4 phase focuses on how effective efforts to prevent occupational exposure to nanomaterials along the research continuum contribute to large-scale impact in terms of worker safety, health and wellbeing (T_4). Stakeholder input and engagement is critical to all stages of the translational research process. This paper will provide: (1) an illustration of the translational research continuum for occupational exposure to nanomaterials; and (2) a discussion of opportunities for applying D&I science to increase the effectiveness, uptake, integration, sustainability, and impact of interventions to protect the health and wellbeing of workers in the nanotechnology field.

Keywords: dissemination, implementation, nanotechnology, research, impact

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INTRODUCTION

Nanotechnology and the development of nanomaterials have yielded a large number of commercial products and exposures to workers (1–3). The diffusion of nanotechnology into commerce in the early 2000's raised concern about potential hazards to workers (4–6). The small particle size, novel characteristics of nanoparticles and hypotheses about interaction with biological molecules and structures prompted public health authorities to consider whether workers could be at risk of adverse effects from exposure to engineered nanomaterials (ENM). To prevent such effects, if they were to occur, occupational public health authorities promoted and conducted research and issued guidance for employers and workers on risk management (6–8). The overarching questions are identifying the extent to which guidance was used, and whether the guidance made a difference in the health and safety of workers. Unfortunately, these and related questions have not been the focus of much research. In fact, such research has rarely, with notable exceptions, been considered or implemented in the occupational health field, in general, let alone in the area of exposure of workers to ENM (9–11). To address this shortfall there has been an effort in recent years to promote research on the "downstream" part of a continuum beyond basic research to impact (**Figure 1**) (11). This research, which includes dissemination and implementation (D&I) research, can fall under the umbrella of "translational science." Traditionally, translational science has focused largely on barriers to intervention development at the efficacy and effectiveness stages (T_1 and T_2). Once an evidence base is established, D&I science has focused on barriers to the adoption, use, and sustainment (T_3 and T_4) of evidence-based interventions (12). **Table 1** illustrates key characteristics of dissemination and implementation research and hypothetical examples for the occupational safety and health field. **Figure 1** depicts the translational science cycle, conceptualized as crossing all translational or "T" phases of the research continuum, from scientific discovery (T_0) to efficacy (T_1), effectiveness (T_2), dissemination and implementation (T_3), and the outcomes and effectiveness and impact of research in populations (T_4). OSH interventions (such as those represented in **Table 2**) may be situated anywhere along the research continuum, and, in reality, do not necessarily pass through all the T stages. In the context of OSH research (and beyond), there is a dearth of research conducted in the T_3 and T_4 stages of the research continuum (14).

While closely connected, the primary focus of translational science is *not* to do translation or its synonymous activity, research-to-practice (R2P) [an approach to collaborations with partners on the adoption, use, and adaptation of knowledge, interventions and technologies (15)]. Rather, it is the study of

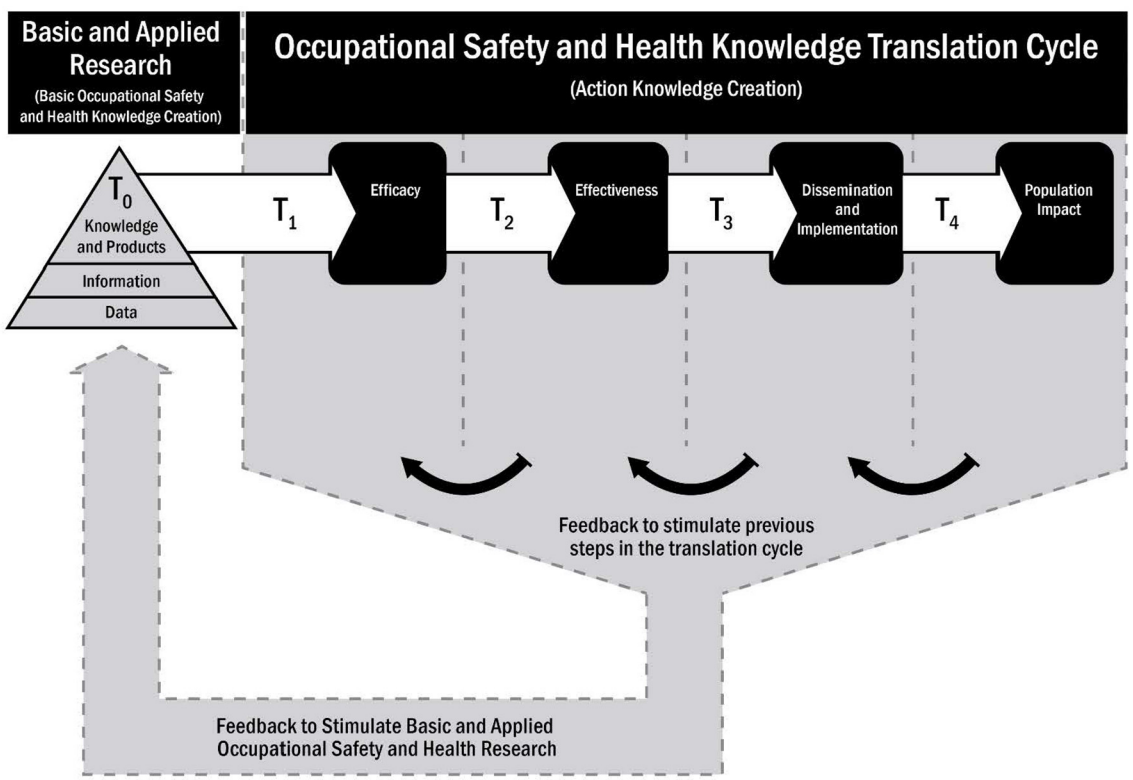

FIGURE 1 | Overview of translational science for occupational safety and health [adapted from Schulte et al. (11)].

TABLE 1 | Ideas for advancing dissemination and implementation science in occupational safety and health.

Key characteristics to consider for dissemination and implementation (D&I) research for occupational safety and health (OSH):

- Understand how effective OSH interventions work, particularly multi-level or multi-component interventions, to inform how those interventions can optimally be delivered when implemented in various workplace settings.
- Understand the relevance of OSH interventions, where applicable, to meet the needs of underserved populations and/or low-resource settings.
- Incorporate theories, models, and/or frameworks appropriate for D&I to inform study hypotheses, measures, and outcomes.
- Consider extant literature on barriers to and facilitators of the dissemination and implementation of practices to improve worker's OSH.
- Consider and characterize the multi-level context and environment in which the proposed research will be conducted.
- Consider the use of qualitative and/or mixed methods approaches.
- Focus on issues of resources expended, program costs, cost-effectiveness, or other economic outcomes related to dissemination and/or implementation.
- Incorporate stakeholder-relevant outcomes (i.e., outcomes relevant to workers, employers, insurers, and/or policymakers).

Hypothetical examples of relevant research topics may include but are not limited to:

- Studies of the implementation of multiple evidence-based practices within businesses and sectors to meet the needs of employers and workers.
- Studies of the business adaptation of evidence-based OSH practices in the context of implementation.
- Longitudinal and follow-up studies on the factors that contribute to the sustainability of evidence-based interventions in business sectors that lead to the reduction in work-related morbidity and mortality.
- Studies of the relationship of context and local capacity of business and sector settings to adoption, implementation, and sustainability of evidence-based practices.
- Studies of influences on the creation, packaging, transmission, and reception of evidence for effective OSH interventions.
- Studies of strategies to impact organizational structure, safety climate, safety culture, and processes to enable D&I of OSH information and effective OSH interventions.
- Studies that focus on the testing of relevant D&I theories, models, and frameworks.
- Studies of policies and other contextual factors that influence the success of dissemination or implementation efforts.

Adapted from NIH (13).

how these activities and processes (i.e., uptake, implementation, sustained use) work, what their multilevel barriers and facilitators are, and what intermediate and ultimate impacts they have on diverse outcomes. It should be noted that the processes shown in **Figure 1** are not linear but represent a dynamic and iterative interplay across stages and involve many factors that interact and provide feedback to previous and consecutive stages. The challenge is to study the main causal factors while accounting for and acknowledging the complex context within which they exist.

Ultimately, there is a need to investigate many casual pathways that influence worker safety and health outcomes. Sorensen et al. (16) developed a conceptual model "based on the premise that addressing multiple pathways in an integrated manner with a focus on the conditions of work will contribute to greater impairment in worker and enterprise outcomes than addressing each pathway separately" (16). This integrated approached can guide research and intervention designs and may be a framework for T_1 and T_2 efforts.

TABLE 2 | Examples of interventions to address worker exposure to ENMs.

Intervention	Example
Program	OECD Testing Programmers of Manufactured Nanomaterials
Practices	Occupational Exposure Limits
Principle	Consider ENM hazardous until proven otherwise
Procedures	Nanomaterial Exposure Assessment Technique
Products	Direct reading instrumentation
Policies	ISO/TC 229 Nanotechnologies; WHO guidance on protecting workers from potential risks of manufactured nanomaterials.

In this paper we examine how research and guidance on ENMs are situated within the translational science continuum, and identify gaps related to, and opportunities for, increasing research efforts particularly at the later stages (T_3 and T_4) of that continuum. To that end, the paper will provide: (1) an illustration of the translational research continuum for occupational exposure to ENMs; and (2) a discussion of opportunities for expanding efforts in D&I science to increase the effectiveness, integration and impact of interventions to protect the health and wellbeing of workers in the nanotechnology field.

For illustrative purposes, we present three examples of OSH interventions— broadly defined as programs, practices, policies, recommendations, and guidelines (17)—that can be considered relevant for occupational exposure to ENMs and could give rise to translational research questions: (1) National Institute for Occupational Safety and Health (NIOSH) recommended exposure limits for titanium dioxide (TiO_2) and carbon nanotubes and nanofibers, (CNTs/CNFs) (18, 19); (2) World Health Organization (WHO) guidelines for protecting workers (20); and (3) The International Risk Governance Council (IRGC) guidance on nanotechnology risk governance (21, 22). These examples were selected because they are widely known by investigators and practitioners in the field of nanomaterial workers' health and illustrate how translational science can be applied to different situations.

What Is the Evidence and What Are Interventions for ENM?

Critical in translational science is understanding how evidence is defined. When is it decided that the science or assembled information is sound enough to transmit to end users? (23). Too much waiting for interventions to meet specific evidence standards may contribute to translational lag while also leading to the generation of interventions that cannot be replicated in real-world settings such as worksites (24, 25). Applying a translational science lens to the development and implementation of interventions related to ENMs might change our definition of evidence and what activities can be undertaken depending on the stage on the translational science continuum. While the evidence for basic science is well-established and is based on more traditional scientific experimentation randomized control trials (RCT), evidence at later stages of the continuum may vary

and can be based on the synthesis of information and peer review of guidance or a critical review of the literature based on results from diverse study designs (e.g., cross-sectional, case control, or longitudinal studies). When establishing evidence for later stage translational work, traditional RCTs can become problematic. For example, the use of RCTs in occupational settings, arguably, could be seen as unethical if an intervention judged to be efficacious and safety or health-enhancing is withheld from some segment of the workforce exposed to a potential hazard (26, 27).

It is also important to identify what an intervention is with regard to ENMs. The interventions to protect workers from adverse effects may vary substantially. Generally, in translational science, interventions can be described in broad terms as one of the "7 P's": programs, practices, principles, procedures, products, pills and policies (28). In the ENM arena most of these, except pills, would be considered as interventions to address occupational hazards (**Table 2**). **Table 2** was developed to help the nanotechnology community understand that "intervention" can have a wide range of meanings. These interventions can fit at all stages of **Figure 1**. The target audiences for translational science are the organizations and decision-makers who develop, disseminate, or implement interventions. These audiences include government authorities, decision-makers, employers, trade associations, and unions. Ultimately the interventions will be implemented to protect workers but the pathway generally will be through employers.

TRANSLATIONAL SCIENCE

It is useful to envision the continuum of translational science from beyond basic research through the impact of that research on populations. Translational science has been conceptualized as having multiple phases of research beyond scientific discovery (T_0), to efficacy research (T_1), to effectiveness research (T_2), to dissemination and implementation research (D&I) (T_3), to outcomes and effectiveness research in populations (T_4) (12, 29–35). From an occupational safety and health perspective, for translational research findings to make a difference in the safety and health of workers it is necessary to determine the best ways to direct basic research findings and guidance information to employers, workers and authorities promotes the uptake of that information, and determines whether it made a positive impact.

The T_0 stage is focused on scientific discovery driven by basic science. While there are important feedback loops between the T_0 and later T_1 through T_4 stages, this paper will focus on T_1 to T_4 that is, how well the products of basic research can be utilized to affect and protect ENM workers and, specifically, how to study the stages leading to such protection. To better view the stages between T_1 and T_4 this paper reviews various examples of the continuum applied to hazard information and interventions related to ENMs. In the next sections and in **Tables 3–5** we present three examples.

Translational science is a field that merits support because it is a crucial that stakeholders receive and use recommendations and interventions as a means to responsible development of a technology, corporate responsibility, and worker and population wellbeing (37–42). The successful commercialization of nanotechnology depends on societal acceptance of it. Societal acceptance will be influenced by, among other things, whether workers are protected from nanotechnology hazards. When it is apparent that workers are at high risk of adverse health effects and not protected, the societal response toward

TABLE 3 | Utilization of translational science pertaining to NIOSH recommended exposure limits for TiO_2 and CNT/CNF.

Example 1: Recommended exposure limits (CNT/CNF & TiO_2)		
T_0	Basic science	Ultrafine and fiber toxicity Specific studies of ENMs Quantitative risk assessment
T_1	Efficacy research	Sensitivity analysis of risk assessment Historical basis for OELs
T_2	Effectiveness research	Citation analysis/downloads Cross-sectional study (CNT)
T_3	Dissemination & implementation research	No examples Hypothetical questions
T_4	Population impact	Use of intermediate indicators [e.g., (36)] Longitudinal studies

NIOSH (18, 19).

TABLE 4 | WHO Guidance for protecting workers.

Example 2: WHO guidance for protecting workers		
T_0	Basic science	Historical and contemporary toxicity data Stakeholder requests
T_1	Efficacy research	PECO analysis International expert assessment
T_2	Effectiveness research	Historical evidence Cross-sectional and prospective studies
T_3	Dissemination & implementation research	Implementation plan No D&I research
T_4	Population impact	No population data Use of intermediaries

WHO (20).

TABLE 5 | International Risk Governance Council guidance.

Example 3: International Risk Governance Council guidance		
T_0	Basic science	Historic and contemporary toxicity data Explosiveness data Stakeholder requests
T_1	Efficacy research	Scoping review on deficit in guidance
T_2	Effectiveness research	Synthesis of evidence for risk governance Applicability of guidelines
T_3	Dissemination & implementation research	No studies Possible research: extent employers adopted guidance/best means of dissemination and implementation strategies
T_4	Population Impact	No studies

IRGC (22).

acceptance of the technology is likely to be negative (37). Therefore, it is incumbent on employers and authorities to support the development of translational science as a means to support responsible development of nanotechnology. Supporting translational science will result in broad utilization of guidance and increase involvement of stakeholders in that process (37, 43–47). For example, Brownson (47), building on work of Curry (48) and Anderson et al. (49), described a push-pull model for strategic public health science that could be adopted to strategic transitional science for occupational safety and health: "This model posits that for science to effect practice there must be a combination of push (a basis in science and technology) and the pull (a market demand from practitioners), and the capacity (delivery ability of public health systems)." The adaption would be that practitioners would be considered as employers, workers, unions, trade associations, and other decision-makers that affect worker protection.

Example 1: NIOSH Recommended Exposure Limits for TiO_2 and CNT

T_0: Basic Science - Discovery Data

Table 3 illustrates the translational science continuum for this example. Titanium dioxide (TiO_2) and CNT/CNF are ENMs that were studied early on in the commercialization of nanotechnology [see (18, 19) for review] and found to have adverse health effects in laboratory animals. Clearly, there are many types of TiO_2, and CNT/CNF and the toxicity information only pertained to specific chemical and physical types. The type of toxicological assessments that is most relevant to occupational safety and health are rodent inhalation studies. TiO_2 and other poorly soluble particles of low toxicity (PSLT) of fine and ultrafine sizes were found to show consistent dose-response relationships for adverse pulmonary inflammation and lung tumors when dose was expressed as particle surface area. Specifically, the chronic animal inhalation study of TiO_2 (50) demonstrated the development of lung tumors (bronchioloalveolar adenomas) in response to exposure to a relatively large dose of 250 mg/m^3.

Subsequently, Henrich et al. (51) showed a statistically significant lung cancer excess in rats exposed to ultrafine TiO_2 at a concentration of 10 mg/m^3.

Studies in rats and mice have shown that CNTs and CNFs can pose a respiratory hazard due to pulmonary inflammation and rapidly developing, persistent fibrosis (19, 52). Occupational exposure to CNTs and CNFs has been associated with biomarkers of fibrosis, inflammation, oxidative stress, and cardiovascular responses in workers (53).

T_1: Efficacy Research

The translational science T_1 phase refers to the development of an intervention and studies of its efficacy, which is a term to describe whether an intervention can work in an optimal and highly controlled laboratory setting. For controlling exposures, occupational exposure limits (OELs) [for example, NIOSH Recommended Exposure Limits (RELs)] are well-established interventions (7, 18, 19). The focus of this example are the NIOSH RELs for TiO_2 and CNTs/CNFs. The RELs are generally based on quantitative risk assessments of appropriate toxicity data sets. In the realm of ENMs T_1 can be thought of as whether a risk assessment is robust. For TiO_2, animal data were analyzed in a dose-responsive quantitative risk assessment and two categories of TiO_2 were assessed: fine (>100 nm) and ultrafine (≤100 nm), and respective RELs of 2.4 and 0.3 mg/m^3, as a time-weighted average concentration for up to 10 h per day during a 40 h work week were determined. These RELs corresponded to lifetime risk estimates associated with <1/1,000 excess risk for lung cancer. To assess the efficacy of this risk determination a model averaging approach was utilized. This approach uses all the information from many exposure-response models and weights them by the Akaike information criteria for model fit and constructs an average dose-response model (54, 55). CNT animal studies indicated the CNT exposure may result in localized and systematic inflammation, cytotoxicity, pulmonary, interstitial fibrosis, mutagenesis, and the potential for lung cancer (19). NIOSH performed a quantitative risk assessment using dose-response data of adverse lung effects in rats following subchronic inhalation of CNTs and CNFs, and also evaluated rodent studies of lung effects by other routes of exposure. The NIOSH REL of 1 μg/m^3 for CNTs/CNFs (as a respirable mass 8-h TWA concentration) was set at the limit of quantification (LOQ) of the analytical method for element carbon (NIOSH method 5040).

In addition to the RELs for TiO_2 and CNT/CNFs, NIOSH provided guidance for hazard and risk management in the NIOSH Current Intelligence Bulletins for TiO_2 and CNT/CNF that could be considered for expository purposes as part of the intervention (18, 19). The T_1 efficacy research for this intervention included peer, stakeholder, and public reviews. In general, these reviews supported and validated the recommendations in the Current Intelligence Bulletins.

T_2: Effectiveness Research

Studies conducted under the T_2 stage of the translational continuum are focused on assessing the effectiveness of an intervention. This refers to whether the intervention "works" in the real world outside the laboratory where the conditions are less controlled. Can it protect workers and prevent disease from exposure to TiO_2 and CNT/CNF? And can results from these studies be generalized to other ENM workers/settings?

While not specifically designed as a T_2 study, a cross-sectional study showed that in 12 facilities handling CNT/CNFs that approximately 93% of averaged samples collected at the respirable fraction for EC mass were below the NIOSH REL of 1 μ/m^3 (56). This indicates that the REL could be met in practice. No other studies were identified to assess the effectiveness of the NIOSH RELs.

T_3: Dissemination and Implementation Research

Dissemination and implementation (D&I) science is defined as the study of methods and strategies for bridging the gap between public health research and practice (13). D&I scientists use a number of empirically tested theories, models and frameworks (TMFs) to plan, evaluate, or understand barriers and facilitators to D&I processes (57–62). Some commonly used TMFs among D&I researchers (63) include the Consolidated Framework for Implementation Research (CFIR) (57), the RE-AIM (Reach, Effectiveness, Adoption,

Implementation, Maintenance) framework and its contextually expanded version the Practical Robust Implementation and Sustainability model (PRISM) (61, 64, 65), the EPIS (Exploration, Planning, Implementation, Sustainment) framework (66, 67), the Diffusion of Innovation theory (68), and the Theoretical Domains Framework (TDF) (69). However, more than 150 D&I TMFs have been identified (70).

In regard to nanotechnology and worker's health dissemination is the active and targeted distribution of information and intervention materials to a specific business audience. Critical to this is the involvement/engagement with stakeholders early on in the research process so the interventions and strategies for distribution are feasible, relevant, and equitable. Dissemination research is defined as the scientific study of these phenomena for the purpose of understanding how best to spread knowledge required to adopt or deploy an intervention. Implementation of the REL is the critical intermediate step in the continuum from research to prevention. Implementation can be defined as the adoption and integration of evidence-based health intervention (in this case RELs) into business practices.

Implementation research is the study of this process (12). Important translational research questions include what prompts employers to consider the uptake of, and implementation into routine practice and use of such guidance, and what are the key barriers and facilitators to the use of those interventions across diverse workplace settings with different resources (14). D&I science inquiry addresses what works, for who, how, why, in what settings, and how it is sustained over time (34).

Implementation strategies that might be relevant for study include engaging trusted intermediaries and developing a business case.

In the T_3 D&I research stage, there are many rigorous study designs that have been used (28, 71). "These include both experimental (e.g., randomized controlled trial, cluster-randomized controlled trial, pragmatic trial, stepped wedge trial, dynamic wait-listed control trial) and quasi- experimental (e.g., nonequivalent groups, pre-/post-, regression discontinuity, interrupted time series), non-experimental or observational (including designs from epidemiology) designs as well as qualitative (e.g., focus groups, semi-structured interviews), mixed-methods (i.e., the collection and integration of qualitative and quantitative data), and system science (e.g., system dynamics, agent-based modeling) approaches" (71). Except for a few qualitative studies, rarely, have any of these study designs been applied to ENM interventions (36).

It appears that there are no published examples of T_3 D&I studies related to ENM safety and health so we provide a hypothetical example of questions that could be investigated. Numerous law firms, trade associations, and labor groups served to amplify the REL information published by NIOSH. Translational research in the T_3 stage could address what were the best strategies to get the REL information (i.e., intervention) to employers, what are the factors that hinder/facilitate adoption of REL information by employers, how REL information should be packaged and what additional information and resources are needed to facilitate broad dissemination, uptake, and initial implementation by organizations and what are key strategies and resources needed for the sustained use of REL information over time across diverse workplaces. It is critical at this stage and all stages of the research continuum, to consider questions around occupational health equity. These include: how diverse stakeholders including employers, employees labor groups, and professional organizations, will be engaged in the research process and the delivery and implementation of the intervention (i.e., REL information); and will the intervention be feasible for lower resource workplaces employing individuals with high vulnerability?

Measuring success in the T_3 stage can take various forms. A possible indicator of success might be to assess the extent to which an intervention, in this case NIOSH guidance, was adopted by companies as the basis for risk management efforts for TiO_2 exposure. To make this determination it would be necessary to survey employers on whether they based risk management decisions on NIOSH guidance. However, for such surveys it is difficult to get appropriate participation possibly because the information requested may be viewed as "business confidential". It may be that tracking the uptake and use of specific guidance may be quite difficult and it might be better to attempt to track uptake of authoritative guidance in general.

Moreover, tracking use of guidance assumes a linear process that a certain authoritative report will influence an employer to take action when clearly employer decision-making is influenced by a large number of factors.

T_4: Population Impact

Cross-sectional data shown under the T_2 effectiveness stage indirectly demonstrates the population impact of REL information within the population of ENM workers and employers. Although due to the limitations of the design it is challenging to establish a causal pathway between the intervention (REL information) and the observed outcomes of REL usage and exposure control. More data are needed on potential impact of the REL intervention from workplaces where exposure could occur. Also needed are data on the prevalence of control use. There are few studies of the use of controls for ENM interventions in general. Iavicoli et al. (36) found that controls were used substantially but their results were based on a small response rate. This may in part be due to concerns about confidentiality of business information. Ultimately, there is need for studies of the extent of disease in workers over time. Ideally, these would be longitudinal studies or two point in time surveillance assessments (72) and would include diverse process and impact outcomes.

Example 2: WHO Guidance: Protecting From Potential Risks of Manufactured Nanomaterials

T_0: Basic Science - Discovery Data

Table 4 illustrates the translational science continuum for this example. In addition to the type of toxicity data discussed earlier for TiO_2 and CNT/CNFs, discovery data may be driven from stakeholders' concerns. For ENMs it was noted in 2007 that "Non-governmental organizations (NGOs) had been active in calling for worker protection

in emerging nanotechnology industries (https://www.who.int/occupational_health/background_review_1.6.12.pdf; line 10)." In 2010 a European Trade Union Confederation recommended application of the precautionary principle because of potential hazards of ENMs. The focus of this example is the WHO guidance on protecting workers from potential risks of manufactured nanomaterials (20). Assessment of the health impacts of new technology is one of the activities of the Global Plan of Action on Worker's Health adopted in 2007. WHO was concerned that the increased production and use of ENM, meant that workers would be at the "…front line of exposures to these materials placing them at an increased of potential adverse health effects (20)." These guidelines were meant to prevent such effects.

T_1: Efficacy Research

The determination of efficacy of control guidance (i.e., the intervention) was based on years of practice controlling ultrafine particles (6–8). This was supported by evidence-based literature. Specifically, for efficacy of the guidance information created by the WHO workgroup, they used the PECO (Population/Situation Exposure-Comparison-Outcome) approach and the GRADE (Grading of Recommendations Assessment, Development and Evaluation) framework for the judgement of the quality of the evidence (20). The target group for the guidance was in phase 1: policy makers from low- and medium-income countries and in phase 2, an implementation guide for employers and workers. The policy was designed to fill critical knowledge gaps such as: (1) Can an algorithm be developed to classify engineered nanoparticles by degree of potential hazards? (2) Which characteristics of particles and which measurement techniques should be used for the assessment of exposure to engineered nanoparticles? (3) What is the exposure to engineered nanoparticles in the workplace? (4) What are the limits of engineered controls and personal protective equipment (PPE) with regard to engineered nanoparticles? (5) What occupational health surveillance should be recommended for workers potentially exposed to engineered nanoparticles? (6) Should exposure registries be established for various groups of workers potentially exposed to engineered nanoparticles? (7) Should engineered nanoparticles be treated as "new" substances and evaluated for safety hazards? The guidance was developed by an international group of experts with the assistance of the WHO Global Network of Collaborating Centres who conducted systematic review studies. The guidelines were peer reviewed by external reviewers.

T_2: Effectiveness Research

The effectiveness of the guidance can be assessed against past experience and is similar to the efficacy determination. As noted, one way to assess the effectiveness is through citation analysis. Translation research approaches could be used to determine the extent to which exposure data have been collected in locations where the guidance has been implemented. What was found? No studies on this aspect were identified.

T_3: Dissemination and Implementation Research

The foundation of the D&I stage is the causal evidence that underlies the intervention and the organizational change envisioned. In the WHO guidelines the intervention is recommendations for the safe use of nanomaterials in the workplace.

The WHO Guideline Development Group (GDG) prepared an implementation plan, which included the following activities:

1. Translating to other languages;
2. Integrating the recommendations of the WHO guidelines into training courses on nanosafety (e.g., UNITAR);
3. Providing and disseminating information:
   - web communication;
   - conference presentations;
4. Providing information on the E.U. and U.S. regulations for nanomaterials in the workplace;
5. Developing Safety Data Sheets for nanomaterials in collaboration with the WHO/ILO International Chemical Safety Cards program and UN GHS: nanoscale titanium dioxide (TiO_2) ICSC#1782 and nanoscale zinc oxide (ZnO);
6. Developing a corporate governance document in collaboration with OECD;
7. Developing a list of practical tools for guideline implementation;
8. Assessing the use of nanomaterials in countries;
9. Providing real-world examples on the use of hazard information and proposed OELs in countries and in workplaces.

The GDG started working on the first five activities but did not complete this plan due to competing demands and priorities. Various D&I research studies could examine aspects of the adoption, implementation and sustainment of this plan, however, no studies of the barriers or facilitators in the plan were conducted. This is what would be needed for T_3 stage.

T_4: Population Impact

There are no data to describe the population impact of the WHO report on intermediate indicators such as the use of recommended risk management practices or on ultimate indicators of morbidity or mortality.

Example 3: The International Risk Governance Council (IRGC) White Paper on Nanotechnology Risk

Table 5 illustrates the translational science continuum for this example. The International Risk Governance Council commissioned Renn and Roco to develop a white paper [distilled into a journal article (21)] on a risk governance framework for emerging nanotechnology.

T_0: Basic Science - Discovery Data

The many facets of emerging nanotechnology coupled with many unknowns and extensive societal concerns in the early 2000's indicated that a comprehensive governance guidance was needed to protect citizens in general and workers in particular who might be at risk. The basic concern was that ENMs may potentially have toxic effects on people exposed to them. The purpose of the IRGC white paper on nanotechnology risk governance was

to present decision makers with a systematic and integrated approach to analyzing and managing anticipated risks from exposure to ENMs (22). While the white paper was expected to have interest to governments, industry, academia, and NGOs, the prime focus was on governments' responsibility for developing and implementing policies.

There was also concern based on the history of dust explosions that nanoparticles could also be explosive. The white paper stated that, "There seems to be no lower particle size limits below which dust explosions could not occur. It may be possible that the increased surface area of nanoparticles could also include the likelihood that they become self-charged and ignite (22)."

T_1: Efficacy Research

T_1 research also involves assessing the potential of the IRGC to recommend risk governance guidance (intervention) (22). An initial scoping workshop involving a broad range of stakeholders and experts from academia, government, industry, insurance, law, and NGOs, was aimed at identifying large governance deficits for nanotechnology. Surveys of experts like those at the scoping workshop were also conducted. These surveys and workshops indicated that industry was highly aware of the potential impact on levels of innovation that could be caused by inadequate risk governance of human health and the environment, and the perception of the public. The major focus identified in the survey responses was research on Environment, Health and Safety and, in particular, the development of guidelines for worker health and safety and the establishment of an international methodology and nomenclature (22).

T_2: Effectiveness Research

While many people and groups participated in the development of the IRGC risk guidance this is not necessarily evidence that it is effective. It does support, to some extent, its application potential and acceptance. Translational research could include: synthesis of evidence for risk governance and risk management of fine dust and powders, in particular, as well as efforts to explore applicability of governance guidelines for specific situations. In general, evidence gathering on effectiveness and value of governance could also be accomplished by multi-stakeholder evaluation (73).

T_3: Dissemination and Implementation Research

T_3 D&I studies would focus on research on the dissemination and implementation of the IRGC risk guidance. There were no examples in the published literature of T_3 studies for the IRGC risk guidance. In the T_3 stage, assessing the barriers, facilitators, and best practices for the dissemination and implementation of the IRGC risk guidance intervention could be undertaken. For dissemination, one might investigate the extent to which employers and other decision-makers received the governance guidance, what were the best strategies for dissemination, and was there variation in who did and did not receive the guidance (i.e., equity perspective). For implementation, research could focus on how employers adopted, used, and sustained practices based on the IRGC risk guidance.

T_4: Population Impact

Like the other examples, the ultimate impact of an intervention is prevention of or decrease in morbidity and mortality associated with the ENM hazard potential. However, the ultimate impact of prevention may take a long time to obtain and the pathway between guidance and prevention may be indirect and involve diverse factors that are difficult to identify. Translational research may need to focus on intermediate outcomes, chiefly utilization of risk assessment and management practices included in the guidance. There were no published examples of T_4 studies for the IRGC risk guidance.

CONCLUSION

If occupational safety and health research on ENMs is to lead to protection of workers exposed to ENMs, there is a need to assure that the full continuum from basic research to population impact is considered. Historically, there has not been sufficient focus on the stages that appear "downstream" from basic research. The types of evidence needed and factors and processes associated with these later stages of the continuum can substantially differ from those relevant in earlier stages. Furthermore, these later stages (i.e., understanding what will determine eventual dissemination, implementation, and broad impact of an intervention) can have an important impact on how earlier stages of research (i.e., basic science and efficacy) are done. This paper provides a framework for considering the application of translational science to protect workers exposed to ENMs. Three examples were provided to illustrate this application. The key issues in applying translational science to ENM exposures is the need to invest in such science and the ability to get the results of such research to decision-makers and employers. Translational science may also include explorations of the information uptake and use by employers and decision-makers and identification of barriers and facilitators to these functions.

Clearly, the uptake and use of translational science pertaining to ENMs occurs against a backdrop of societal rules, laws and norms with regard to worker protection. Nonetheless, the outputs of translational science can provide useful information that can lead to the protection of workers. This paper is a call for utilizing translational science on a regular basis. If that is to occur there is need for funding of a workforce with translational science skills. With limited funding for ENM research in general it may be necessary to shift funding for better balance along all phases of the research to impact continuum.

AUTHOR CONTRIBUTIONS

PS wrote the first draft. RG and BR made major revisions. TC, LH, and VM provided useful comments and example materials. All authors contributed to the article and approved the submitted version.

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Experimental Study on the Transport and Alteration Behavior of Aerosols From Low Density Powders for Acute Inhalation Toxicology Studies

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Low density powders have a bulk density of less than 100 kg/m^3 and are produced technically by flame pyrolysis of silicon tetrachloride (pyrogenic powders such as pyrogenic silica) or wet-chemically by sol-gel processes (e.g. silica-gel) or precipitation reactions using sodium silicate solution and a mineral acid. The transport and alteration behavior of aerosols from low density powders was investigated in a device for toxicological inhalation studies. The test conditions corresponded to those for acute toxicology studies according to OECD Guideline 436. The use of cascade impactors, required by guideline, has not proven successful for low density powders as the fragile agglomerate structures are destroyed during the measurement. As an alternative and non-invasive measurement method, laser diffraction spectroscopy has proved very successful in the present investigations. In particular, aerosols from pyrogenic powders of low density showed a distinctive tendency to re-agglomerate, especially at concentrations >500 mg/m^{3}mm^3. Investigation results indicate that aerosol particle size must be monitored over the entire acute inhalation test period for acute inhalation studies to be performed reliably.

Keywords: acute inhalation toxicology, pyrogenic powder aerosols, aerosol transport, particle size, MMAD, aerosol measurement, laser diffraction

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INTRODUCTION

For particulate systems with low bulk or tapped density, it is difficult to achieve a specific stable target aerosol concentration to be used in acute inhalation toxicity testing without altering and deposition. Thus, the OECD Guideline 39 states that ""…. *elaborate pre-tests without animals may be needed to achieve a specific temporarily stable atmosphere concentration and particle size distribution* [(1), p. e2–e4]. The particle behavior without such a pretest is unpredictable, in particular the tendency for aerosol altering, i.e., agglomeration and precipitation in the test chamber, which can result in misleading or inconsistent results in the animal studies according to OECD Guidelines 403 and 436. Previous animal studies mention difficulties with the atmosphere generation and different maximal technically achievable concentrations for particular low-density systems, in some cases, even with the same substance/grade when different atmosphere generation technologies were applied. Furthermore, OECD Guideline 39 states in paragraph 51: *It can be difficult or*

impossible to generate a respirable (MMAD of 1-4 μm) liquid or solid aerosol at this concentration without encountering experimental shortcomings. As aerosol concentration increases, particle size also increases due to the aggregation of solid particles or coalescing of liquid particles. The usual consequences are (1) a decrease in the respirable particle size fraction (and thus reduced toxicity), (2) increased fluctuation and variability in inhalation chamber concentrations accompanied by increased spatial inhomogeneities, (3) overloading of equipment used to characterize test atmospheres, and (4) a divergence of nominal and actual concentrations. At very high concentrations, dry powder aerosols and chemically reactive liquid aerosols (e.g., polymers) tend to form conglomerates in the proximal nose causing physical obstruction of the animals' airways (e.g., dust loading) and impaired respiration which may be misdiagnosed as a toxic effect [(1), p. 37].

To provide a standardization of this pretest suggested by OECD Guideline 39, a characterization method was investigated that included aerosol generation, particle size measurement at the point of generation, point of delivery, mass flow correlation and influence of standard particle size measurement equipment in relation to the specific physical chemical parameters of several test materials with low toxicity and low density. Aerosol behavior can have, as already mentioned in the respective guideline, a significant impact on the results of acute inhalation tests and lead to severe misinterpretation.

For acute inhalation studies according to the OECD Guideline 436, aerosols of powdered substances must be generated and available at the point of delivery over a period of 4 h, with a mass medium aerodynamic diameter (MMAD) of 1–4 μm and a geometric standard deviation σ_g of 1.5–3 (2).

According to the OECD concentration specifications and CLP regulations, (3) the required test concentrations are 5,000, 1,000, 500 mg/m^3, or up to 50 mg/m^{3}m in the tiered test program for the classification and categorization of particulates. In addition to mass concentration, the volumetric flow rate and particle size distribution (PSD) of aerosols at the point of delivery are specified. The mass particle size distribution is thereby provided as the distribution of the aerodynamic diameter x_{ae}, i.e., the particle density is defined at 1,000 kg/m^3. The median value of this PSD is the above mentioned MMAD. During the test, the particle concentration and particle size distribution are measured and documented at prescribed frequencies. Recommended measurement methods are gravimetric concentration determination using an absolute filter and for the particle size distribution (PSD), aerodynamic measurement methods such as cascade impactors or aerodynamic particle sizers.

In the past, conflicting test results have indicated that greater attention must be paid to the particle size distribution and stability of the aerosol atmosphere at the point of delivery. Even if the aerosol generator runs within the required aerosol size specification, changes in PSD and concentration often occur during the aerosol flow through piping and distribution systems to the point of delivery. This is caused by effects such as agglomeration, wall buildup and associated segregation/precipitation phenomena. These effects are strongly influenced by particle concentration, particle properties (size, effective density, shape, surface properties, surface charge), and the conditions in the test apparatus.

Particularly critical products in this respect are aerosols from so-called low-density powders, which have a bulk density of less than 100 kg/m^3. In this publication, the PSDs of a characteristic selection of powders were investigated at the inlet and after passing through a complete inhalation apparatus as a function of the operating time. The investigations were carried out at two concentrations in each case, a "maximum" particle concentration in the range of 4,500–7,000 mg/m^3 and a "technically feasible" concentration for most powders of about 500 mg/m^3.

Due to the special particle properties of the aerosols, the PSD could not be measured with a cascade impactor. The agglomerates formed in the airstream are very sensitive to shear forces and thus the shear forces induced by the cascade impactor change the particle size. Laser diffraction spectroscopy was selected and used as an alternative measurement method. Consequently, it was necessary to convert the aerosol specification based on the aerodynamic diameter into a geometric diameter distribution of the aerosols for each powder investigated. Unlike the aerodynamic diameter, the geometric diameter can be directly related to the geometric dimensions of the respiratory tracts of the test animals. The low dynamic and thus shear forces applied to any particles in the rat nose is based on the low flow velocity in the nasal cavities which was examined and modeled by Shang et al. 2015 (4). In addition, the geometric diameter of the low-density powders studied in this publication is up to four times larger than the aerodynamic diameter.

The results of the animal test are subject of a seconds publication, Krüger et al. Physical Obstruction of Nasal Cavities with Subsequent Asphyxia, causes lethality of Rats in an Acute Inhalation Study with Hydrophobic HMDZ Surface- Treated Synthetic Amorphous Silica (SAS) (under preparation).

In conclusion, essential corrective actions are recommended to ensure guideline-compliant aerosol atmosphere generation for acute inhalation studies.

Abbreviations: CLP, Classification, Labeling and Packaging; HMDS, Hexamethyldisilazan; OECD, Organization for Economic Co-operation and Development; PSD, Particle size distribution.

FUNDAMENTALS

Aerosol Characteristics and Specifications According to OECD Guideline 436

Aerosol Characteristics

The relevant properties of aerosols can be described with the number or mass concentration of the particles and with the volume-related or number-related particle size distribution in general. Technical applications of aerosols generally involve the generation of the desired aerosol from powder, transport to the point of application and the application itself. At the point of delivery, the aerosol should be present for a constant time at the desired concentration and particle size distribution.

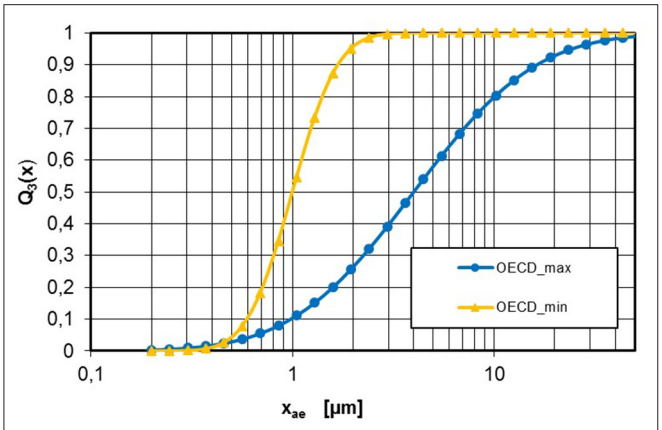

FIGURE 1 | Range of aerodynamic PSD according to OECD Guideline 436: $OECD_{min}$ → $x_{ae,50}$ = MMAD = 1 µm, σ_g = 1.5; $OECD_{max}$ → $x_{ae,50}$ = MMAD = 4 µm, σ_g = 3.

Both the aerosol concentration and particle size distribution (PSD) of low density powders undergo dynamic changes after the generation.

For laminar flow conditions, Marshall investigated these effects with a two-dimensional soft-sphere discrete-element simulation as a function of particle size and particle interaction forces. Among other things, it could be shown that for the same particle concentration, the effects of wall bonding and agglomeration strongly depend on the particle-particle interaction forces (5).

In addition, phenomena such as classifying effects, sedimentation, wall bonding or the debonding and redispersion of deposits back into the aerosol atmosphere must also be considered.

The challenge of the temporal and local change of powder aerosols is therefore relevant for all applications and, as necessary, must be investigated metrologically.

Acute Inhalation Toxicity Test Specifications According to OECD Guideline 436

In this publication, the transport behavior of powder aerosols is investigated as it applies to inhalation toxicology studies according to OECD Guideline 436 (2). Acute inhalation studies are part of the mandatory data set for powdery substances according to REACH Annex VIII (6). In acute inhalation studies according to OECD Guideline 436, rats are exposed for 4 h to an aerosol concentration in a range of 50–5,000 mg/m^3 (graded at 50, 500, 1,000, and 5,000 mg/m^3). The particle size distribution of the aerosol particles at the point of delivery (exposure port of the inhalation chamber) is specified with an aerodynamic diameter of $x_{ae,50}$ = MMAD = 1–4 µm with a permissible geometric standard deviation of σ_g = 1.5–3. The MMAD describes the 50% quantile of the mass-wise cumulative distribution $Q_3(x)$ of the aerodynamic diameter x_{ae}. In the case of a logarithmic normal distribution, this results in limit curves for the aerodynamic diameter according to OECD Guideline 436 (**Figure 1**) (2).

Results from animal studies with this kind of inhalation testing devices and set-up showed unexpected results are sometimes obtained especially for low density powders. These include negative results with known toxic powders as well as positive results with expectedly safe powders. Although the aerodynamic PSD is determined during the cascade impactor tests, a correlation of these contradictory results with the particle size distribution at the point of delivery, the exposure port of the inhalation chamber, is suggested. Agglomerates could block the upper airways and, in certain cases, lead to suffocation of the test animal.

Need for and Qualification of Alternative Measurement Techniques for PSD Determination

Dry sand has a bulk density of about 1,200 kg/m^3. In contrast, low density powders, such as pyrogenic, silica gel or precipitated amorphous SiO_2O, often have a bulk density of less than 100 kg/m^3. These powder aerosols have the following special features:

- low effective particle density and agglomeration tendency
  - low density due to high porosity
  - geometric diameter > aerodynamic diameter
- high agglomeration tendency
  - large specific surfaces
  - rough, form-fitting particle surfaces
- shear-sensitive, fragile particle structures
- In the case of surface treatment, the surfaces are not wettable and the large bulk volume is maintained even in the presence of liquids.

Guideline-compliant low-density powder aerosol agglomerates in the airflow in the micrometer range (single- to double-digit figures); they are very fragile and cannot be detected non-destructively with the prescribed aerodynamic methods (e.g., cascade impactor). The measurement procedures recommended in OECD Guideline 436 for determining aerodynamic PSD, such as cascade impactors or aerodynamic particle sizers (APS), can destroy fragile agglomerates due to internal mechanical stress (acceleration forces, shear forces in nozzles and hose lines) and thus significantly alter the aerosol state in the measurement result. Consequently, the cascade impactor measures a PSD shifted to finer particles at the point of delivery, which in fact does not exist.

Furthermore, at the required concentrations, APSs require aerosol dilution by a factor of 1,000, which is associated with additional mechanical stress on the particles in the injector system due to the addition air needed for the dilution. The measuring methods suggested cannot detect the larger agglomerates due to the upper limit of the measurement range. Corresponding test results for the use of cascade impactors are presented in the test results section.

Due to the concentration range and the expected maximum agglomerate sizes >30–50 µm at the point of delivery, a commercial laser diffraction spectrometer of the type Helos-KR (Sympatec GmbH) was used as an alternative measurement

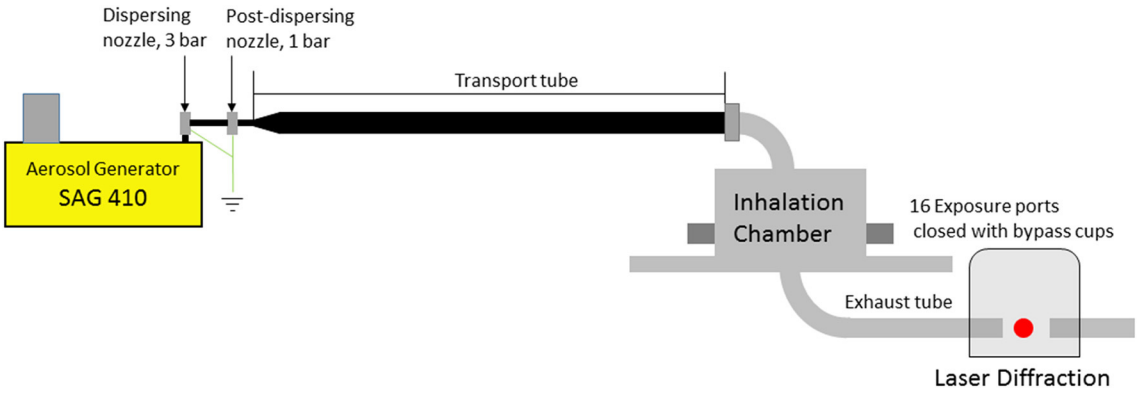

FIGURE 2 | Test set-up for simulating inhalation experiments: aerosol generator and inhalation system, consisting of transport tube and inhalation chamber; laser diffraction particle sensor.

system. Particle concentrations from about 300 to 5,000 mg/m^3 can be analyzed with the used measuring device without dilution. The open optical system of the instrument allows the PSD to be measured in free flow without contact and almost without mechanical stress. Dilution is unnecessary. The actual state of the aerosol almost without distortion can only be recorded in this way.

The particle sizes determined with a laser diffraction spectrometer represent physically the diameter of laser diffraction equivalent spheres. The particle size can be interpreted as geometric diameter, x. To allow comparison with the specification of OECD Guideline 436, the aerodynamic PSD "OECD$_{max}$" in **Figure 1** was converted to corresponding geometric PSD using Equation 1 for all powders.

Previous studies have shown that it is possible to convert aerodynamic diameters to laser diffraction measurement data using effective agglomerate densities (7). The relationship between the geometric diameter x and the aerodynamic diameter x_{ae} is described by Equation 1:

$$x = \sqrt{\frac{\rho_{1000}}{\rho_{eff}}} x_{ae} \quad with \ \rho_{eff} = \rho_s(1-\varepsilon_p)(1-\varepsilon_{Agg}) \qquad (1)$$

ρ_{1000} = 1,000 kg/m^3 and represents the reference density of the aerodynamic diameter (water). The effective particle density ρ_{eff} depends on the porosity of the particles ε_P and, in the case of agglomerates, additionally on ε_{Agg}. For non-porous particles, ρ_{eff} takes the value of the solid's (skeleton) density ρ_s. For pyrogenic powder systems (e.g., pyrogenic amorphous silica, alumina), the tamped density of the powder in case of strong dispersion can be used for ρ_{eff} (3). For the other powdered substances, values based on the effective particle density were applied. Using that assumption, it is possible to convert the OECD MMAD specification for each powder to the measured geometric diameters. The measurement results are presented as geometric diameter x, which can be firmly correlated with the MMAD of max. 4 µm required in OECD GD 39 and OECD TG 436 using the method explained above.

Experimental Set-Up and Test Materials

Test Set-Up and Procedure

Based on experimental investigations on different synthetic amorphous silica and calcium carbonate powder aerosols, the changes in the aerosol atmosphere during aerosol transport through a nose-only-exposure system (inhalation chamber) for rodents are presented.

The chosen experimental set-up is based on equipment used at Fraunhofer ITEM Hannover for inhalation studies according to OECD 436 and other OECD Guidelines (8, 9). The main components are the inhalation chamber, the aerosol generator and a transport tube to the inhalation chamber. The experimental set-up is shown in **Figure 2**. **Table 1** quantifies all essential details of the experimental set-up.

The particle size distribution of the aerosols was determined directly at the aerosol generator (input), at the exhaust tube and, in some cases, directly at four interconnected exposure ports of the inhalation chamber. Measuring the PSD at the exposure ports required sampling at four ports and sample transport through four 40-cm-long tubes with a diameter of 10 mm to achieve the minimum sample quantity for a measurement. This necessary procedure leads to additional changes in the aerosol condition. For this reason, the measurements were preferably performed directly at the outlet of the inhalation chamber. The aerosol condition at the ports and at the outlet are almost identical. To be able to estimate the particle losses in the system, the concentration at the above-mentioned locations was determined gravimetrically using a filter system.

Preliminary investigations have shown that the PSD of the aerosol can change significantly after passing through the test apparatus with increasing operating time. One reason for this is increasing particle deposits in the apparatus, which affect the agglomeration equilibrium of the aerosol (5). For this reason, the measurements were performed for different operating times, e.g., at the beginning, after 5, 10, 20 min, etc. The dispersion conditions at the aerosol generator and the volume flows through the system were constant (**Table 1**). The materials and the aerosol concentration varied. As mentioned

TABLE 1 | Parameters of the test apparatus.

Parameter	Value
Aerosol generator SAG 410	
Dispersing pressure and feed rates	3 bar, feed rate 0 - 100%
Post-dispersing-nozzle pressure	1 bar
Air flow rate main nozzle and diluting nozzle	42 L/min
nozzle driving air without secondary air from the environment	∼20°C, 7 to −8% rel. humidity
Relative humidity measured at exposure unit	25 to −40% rel. humidity
Transport tube	Length 1,500 mm, diameter 40 mm
Inhalation chamber	
Height/diameter of chamber segment	110 mm, 160 mm
Height / diameter of inner cylinder	60 mm, 125 mm
Chamber inlet diameter	33 mm
Exposure port nozzle diameter	6 mm
Exposure port outlet diameter	26 mm
Main exhaust diameter	22 mm
Number of exposure ports	16 (closed with 16 bypass cups)
Air flow rate per port	1 L/min
Exhaust flow rate	42 L/min
Aerosol measurement	
PSD with laser diffraction (Helos)	
- Exhaust	No sampling, complete exhaust flow
- Ports	4 ports, 4 L/min 10 mm tubes to instrument
- Duration for a measurement	30 s, 1 min (low opt. conc.)
- Measuring range	0.9 to −175 µm
Purpose	PSD monitoring versus exposure time
Gravimetric mass concentration	
- Filter diameter	47 mm
- Sampling flow rate exhaust	40 L/ min
- Sampling flow rate at 4 ports	4 L/min
- Min. sampled mass	5 mg
- Purpose	Mass conc. in the generator and behind the inhalation chamber

above, two aerosol concentrations were investigated in each case, the high concentrations range from 4,500 to 7,300 mg/m^3, within the range of maximum test concentrations. A range around 500 mg/m^3 was selected for the seconds concentration. Preliminary investigations had shown that concentrations in this latter range represent those that are technically feasible without significantly altering the aerosol for most powders.

To investigate the effect of mechanical stress in a cascade impactor on PSD, 3 different powder aerosols were measured simultaneously using the laser diffraction spectrometer and cascade impactors. The impactor measurements were performed simultaneously with an ITEM impactor of the type Marple NS 298 at the point of delivery and with an impactor of the TU Dresden at the exhaust tube of the inhalation apparatus. This impactor was built for the particle size range 0.35–15 µm at the Martin Luther University Halle-Wittenberg, Department of Chemical Engineering, in Merseburg in 1985. The laser diffraction measurements were performed immediately before the impactor measurements.

To compare the measured particle size distributions at the outlet of the inhalation system with the OECD Guideline 436 requirements, the limiting curve "$OECD_{max}$" (see **Figure 1**) was converted into a geometric particle size distribution x based on Equation 1. For pyrogenic powders, as mentioned above, the tapped density was used as the effective density, because the porosity of the flakes is nearly identical to that of the dispersed particles (7). For silica gel and calcium carbonate powders, this approach is not possible because the effective densities of the dispersed aggregates will be significantly different from those of the resulting agglomerates. Further investigation is needed to clarify these relationships. Nevertheless, in order to give a conservative estimate, the $OECD_{max}$ curves are calculated based on the effective density of the aggregates. In the case of a very strong agglomeration, these curves will be too fine, i.e., more stringent than the OECD Guideline 436 MMAD specification.

Substance and Particle Characterization

The investigations were carried out on four powdered materials as shown in **Table 2**. The four investigated materials can be divided into two groups, pyrogenic amorphous silica powder and wet-chemically precipitated calcium carbonate and amorphous silica gel produced by a sol-gel process. The final particle size of the precipitated calcium carbonate and the amorphous silica gel is typically archived by milling or intensive milling, depending on the targeted PSD for the products.

In terms of particle structure, the powdered materials differ as shown below:

1. wet chemical powders (silica gel and calcium carbonate): stable, compact aggregates with a tendency to lower agglomeration;
2. pyrogenic powders (untreated and treated pyrogenic silica): fractal, aggregate-like particles of low effective density and high tendency to form large agglomerates; the agglomerates have very low shear stability (fragile).

The particle sizes given in **Table 2** represent the state of the aerosol at the outlet of the SAG 410/U aerosol generator in which the powder particles are accelerated to about 50 m/s in an injection nozzle. These PSDs represent the aerosol state at the inlet of the transport tube of the inhalation apparatus. For pyrogenic powders such as fumed silica, the PSD represents an equilibrium state depending on the dispersion energy (10).

RESULTS

PSD—Cascade Impactor vs. Laser Diffraction

In the first section, cascade impactor measurements and laser diffraction analysis results using three powders as examples will be compared and discussed. For this purpose, it was necessary to convert results of the impactor measurements into geometric diameters using Equation 1.

Figure 3 shows good agreement between the impactor results and the laser diffraction analysis using the example of the calcium carbonate system, which has good dispersibility and no tendency to agglomerate. The effective density of the particles can be

TABLE 2 | Products investigated, PSD at the aerosol generator, bulk and tamped density of powders.

Substance	SiO_2	SiO_2	SiO_2	$CaCO_3$
Substance type	Pyrogenic	Silica gel	Pyrogenic	Precipitated
Surface treatment	HDMS-treated	Untreated	Untreated	Untreated
Product	AEROSIL® R 812	SYLOID® 244 FP	HDK® N 20	SOCAL® UP-G
$x_1x_{0;3}$ [µm]	5.15	1.10	5.15	0.89
$x_1x_{6;3}$ [µm]	6.25	1.31	6.29	1.20
$x_5x_{0;3}$ [µm]	11.6	2.31	13.6	3.31
$x_8x_{4;3}$ [µm]	20.0	3.62	31.6	7.15
$x_9x_{0;3}$ [µm]	23.3	4.05	40.1	8.41
Bulk density [kg/m^3]	40.8	61.0	32.5	226
Tamped density [kg/m^3]	64.0	83.7	46.7	447
Eff. particle density [kg/m^3]	64.0	242	46.7	1,000
x/x_{ae}	4	2	4.6	1

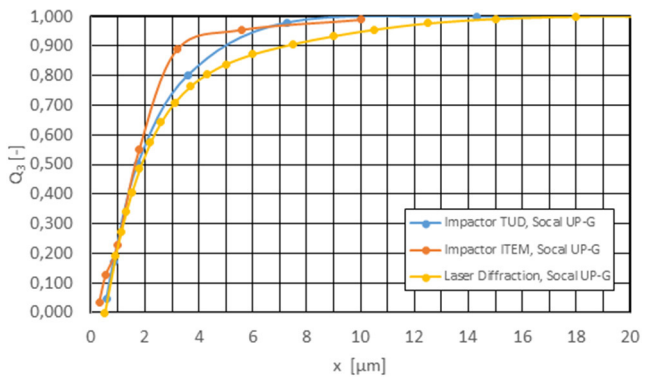

FIGURE 3 | Comparison cascade impactor—laser diffraction for calcium carbonate after 3 h 30 min test duration; $c_m = 500$ mg/m^3, $\rho_{eff} = 1{,}000$ kg/m^3.

estimated from the tap density of 447 kg/m^3 at an assumed bulk porosity, the substance not tending to agglomerate, of 0.5 with about 1,000 kg/m^3. This value was confirmed by our own impactor measurements.

In the case of aerosols which tend to form fragile agglomerates, these will be expected to be at least partially destroyed in the impactor due to shear stresses. Low-density powders, on the other hand, can have adhesion problems on the impactor plates due to the low particle density because a large particle volume must be deposited for a weighable mass. Measurements with silica gel and surface-treated pyrogenic silica therefore show finer measurement results, as expected (**Figure 4**).

In the case of silica gel, the cascade impactor provides values in the range of the known aggregate size distribution (**Figure 4**, left). The laser diffraction measurement also detects some coarser agglomerate structures due to the shear-free measurement. This effect occurs very clearly with pyrogenic powders, here the surface-treated pyrogenic silica, and provides unrealistic values as fragile agglomerate structures are largely destroyed in the aerosol stream insight the cascade impactor (**Figure 4**, right). In this case, the measurement results of the cascade impactors are complete unrealistic. For this reason, all measurements for aerosol characterization were subsequently carried out using laser diffraction spectroscopy.

Particle Losses and One Worst-Case Example

The particle size distributions shown in **Figure 5** are laser diffraction measurements represented as a volume distribution of the geometric diameter. All measurements were made on the set-up as shown in **Figure 2**.

The worst-case example of a pyrogenic powder is used to illustrate the problems that can occur when high concentrations of powder aerosols are applied. **Figure 5** shows the deposits in the inhalation system after exposure to an untreated pyrogenic silica aerosol - mass concentration at the aerosol generator 4,700 mg/m^3 - over a period of 120 min. The deposits are clearly identifiable as agglomerates both in the inhalation chamber and at the exposure port.

By using a laser diffraction meter at the inhalation chamber outlet, it was possible to detect even very large agglomerates. As shown in **Figure 6**, the aerosol already changes so much in the first minute of system operation that after 20 min the volume fraction of particles larger than 100 µm is already 50%. Under these conditions, an inhalation test would not be at all successful.

The particle deposits in the entire apparatus reveal the fact that the mass concentration both at the inhalation port and at the inhalation chamber outlet is significantly below the supplied concentration (**Figure 7**).

The mass losses in the inhalation test system depend on the powder material, the concentration and the test conditions. Mass losses in the range of 20–50% were determined for the investigated materials. These losses can be estimated in a preliminary test and readjusted at the aerosol generator. However, especially in the case of powders with a low bulk density and necessarily high aerosol concentration, there is a risk that the system can become heavily clogged within a short time and that the deposits can penetrate as far as the inhalation port.

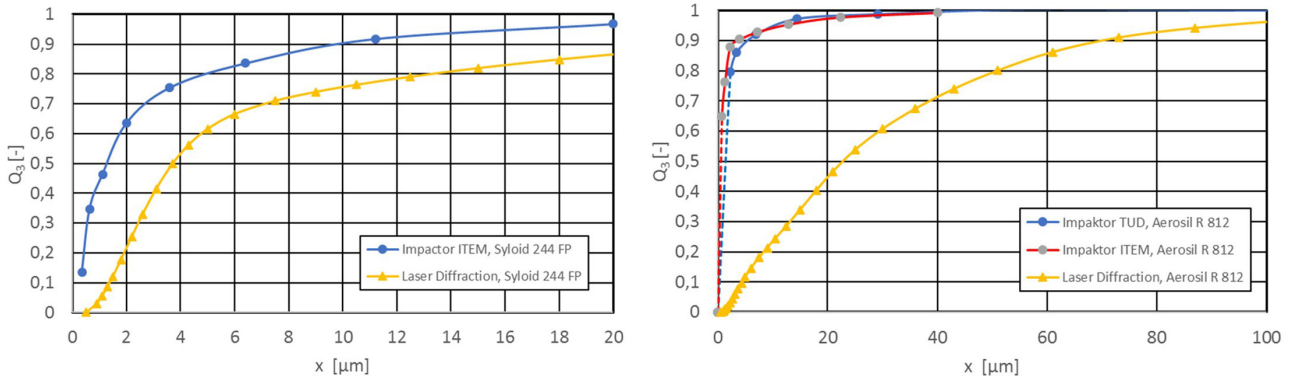

FIGURE 4 | PSD - Comparison of cascade impactor and laser diffraction after an operating time of 3 h 50 min: left: silica gel, 500 mg/m^3, ρ_{eff} = 242 kg/m^3; right: surface-treated pyrogenic silica, c_m = 500 mg/m^3, ρ_{eff} = 64 kg/m^3 (tamped density).

FIGURE 5 | Untreated pyrogenic silica, particle deposits in the inhalation chamber after an operating time of 120 min with c_m = 4,700 mg/m^3, left: exposure port, right: inner cylinder and chamber.

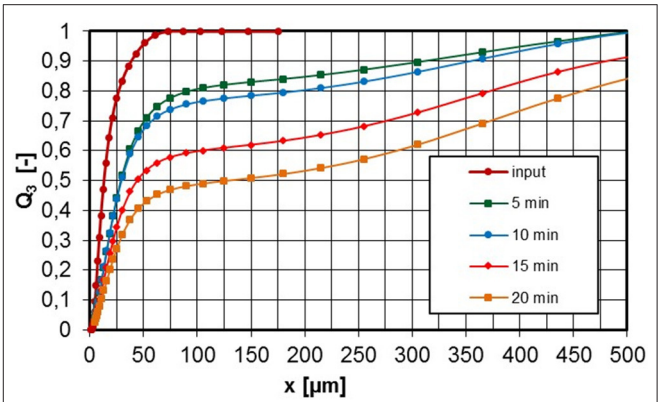

FIGURE 6 | PSD at the inlet and exhaust tube of the inhalation chamber depending on the operating time, untreated pyrogenic silica, c_m = 4,700 mg/m^3.

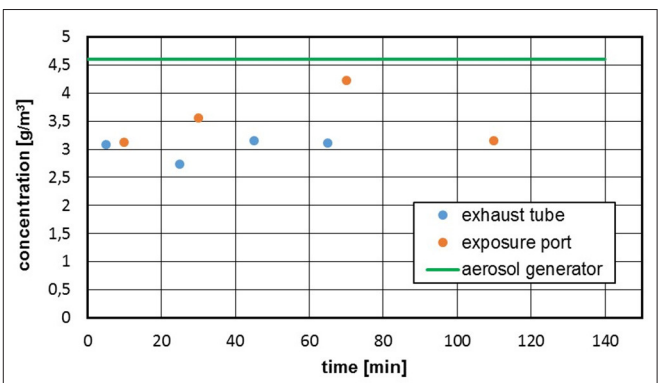

FIGURE 7 | Untreated pyrogenic silica, c_m = 4,700 mg/m^3, gravimetric mass concentration after different run times; green line: at the aerosol generator.

Comparison of Aerosol Condition at Different Concentrations

Experimental results are presented below for all investigated materials, documenting the geometric particle size distributions at the outlet of the inhalation system as a function of the operating time for two mass concentrations in each case. The concentrations given correspond to the values at the aerosol generator.

Figure 8 shows measured values for aerosols from untreated pyrogenic silica at concentrations of 4,700 and 500 mg/m^3. As already described, the measurements were taken at the aerosol generator inlet and at the exhaust tube after the aerosol had passed through the entire inhalation system for different operating times of the apparatus. In addition, the maximum permissible PSD, $OECD_{max}$, according to OECD Guideline 436 is entered according to **Figure 1**. The conversion into an aerodynamic diameter is again carried out with Equation 1 based on the effective particle density ρ_{eff}. If the measured PSD lies to the left of the $OECD_{max}$ curve, the PSD meets the requirements for an inhalation experiment.

The left diagram in **Figure 8** shows that for an aerosol concentration at the apparatus inlet of 4,700 mg/m^3 there is already a volume-related agglomerate fraction of 20% >100 µm after 5-min operating time. According to the $OECD_{max}$, the permissible fraction >100 µm would be about 5%. After 20-min operating time, this proportion increases further to >50%

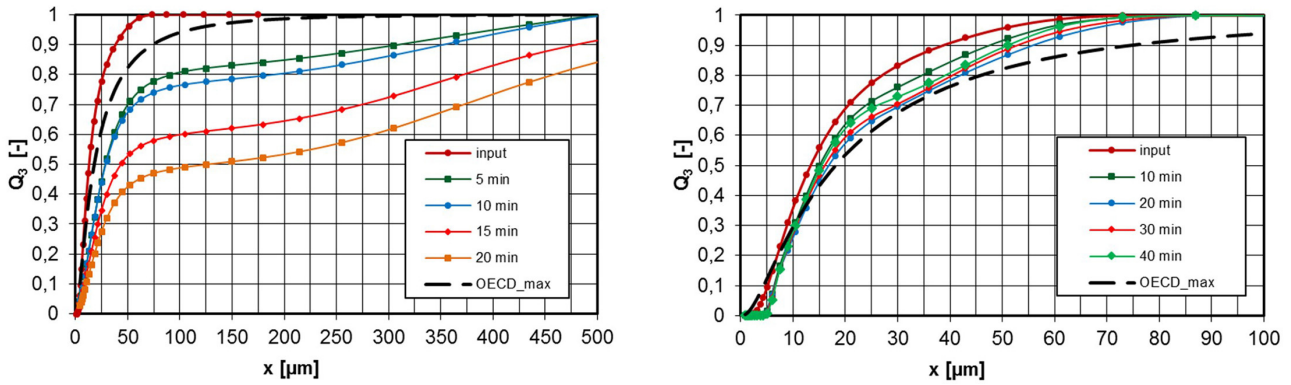

FIGURE 8 | PSD at the inlet and exhaust tube of the inhalation chamber for different operating times, untreated pyrogenic silica, ρ_{eff} = 46,7 kg/m^3 (tamped density), left: c_m = 4,700 mg/m^3, right: 500 mg/m^3.

and more than 10% of the particle volume is >500 µm. Inhalation tests under these conditions are invalid because different problems can occur, e.g., missing inhalable fraction or blockage of the upper respiratory tract (nasal cavities). If the aerosol concentration is reduced to 500 mg/m^3, the agglomerate formation is significantly reduced and the $OECD_{max}$ specification was met over the investigated period of 40 min.

A seconds pyrogenic powder, HMDS surface-treated pyrogenic silica was investigated in the same way. At a concentration of 5,100 mg/m^3, the PSD is already outside the $OECD_{max}$ specification after 5 min (**Figure 9**, left). As expected, the lower concentration of 500 mg/m^3 showed a smaller change in the PSD due to agglomeration. Therefore, the specification was met over a period of 40 min.

Due to the higher primary particle density and the less fractal and coarser particle structure, as shown in **Figure 10** for calcium carbonate, wet-chemically produced powders exhibit a lower tendency to agglomerate in the aerosol phase than pyrogenic produced powders. Even at a concentration of 6,300 mg/m^3, the calcium carbonate aerosol was within specification for 30 min. At 500 mg/m^3 a very stable aerosol atmosphere could be detected.

Figure 11 shows measured values of the silica gel powder, which is also produced by wet-chemical means (sol-gel process). The graph on the left shows the PSD for two aerosol concentrations in comparison with the $OECD_{max}$ specification. The tendency to agglomerate depends on the concentration but is still acceptable for the high concentration in the period investigated. In the right graph of **Figure 11**, the measurement results are plotted as a volume density distribution. It can be clearly seen that part of the particle population (input) changes into an agglomerate population. The proportion of the aggregate population decreases with increasing agglomeration.

SUMMARY

In summary, it can be stated that the agglomeration of powder aerosols can significantly influence the results of acute inhalation tests. Strong agglomeration can lead to the absence of inhalable particles due to excessive particle sizes which could cause blockage of the inhalation ports. In either case, the test results would be unsatisfactory. However, even if the PSD of the aerosol is within specification according to OECD Guideline 436, agglomeration in the air stream can have a significant effect on the deposition site of the particles in the respiratory tract of the test animals, thus changing the effect of the aerosol and influencing the test result. Shang et al. (4) stated that particles of 3 µm geometric size are deposited to 100% in a rate nose model in the vestibule (the upper nose region) furthermore the velocity of the air is beside nose tip rather low and in the laminar region not inducing significant shear forces to the particles.

For the reasons mentioned above, it seems necessary to monitor the particle size of the aerosols from low density powders over the entire acute inhalation test period to reliably perform acute inhalation studies. For low-density powders (bulk density ≤100 kg/m^3) used in this study, the use of cascade impactors has proven unsuccessful because the agglomerate structures are destroyed during the measurement. As an alternative and non-invasive measurement method, laser diffraction spectroscopy was very successful in the present investigations. With laser diffraction spectroscopy, concentrations from about 300 mg/m^3 could be investigated. A significant advantage of this measurement method is that it can record the agglomeration tendency of a powder aerosol in real time and without shear.

From a metrological point of view, there is a need to develop an effective process sensor system that can detect and classify even the largest particle agglomerates as non-invasively as possible.

The particle measurement technology used should not destroy the fragile agglomerates that may form and must be capable to detect and ensure that the full size-range (measurement range) of low density powder aerosols is measured.

The maximum concentration that can be applied over 4 h depends to a large extent on the type of powder and should in any case be determined by preliminary tests. The operating

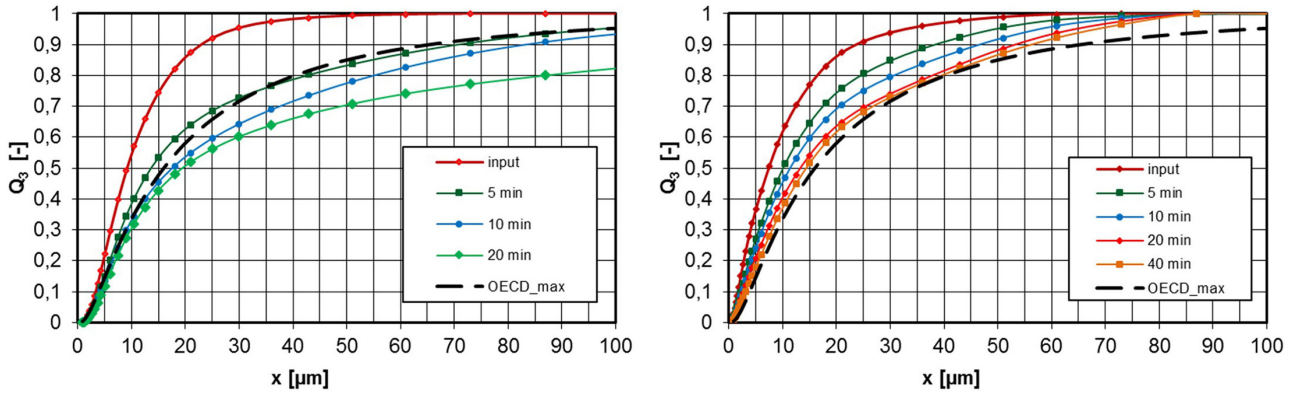

FIGURE 9 | PSD at the inlet and exhaust tube of the inhalation chamber for different operating times, HMDS surface-treated pyrogenic silica, ρ_{eff} = 64 kg/m^3 (tamped density), left: c_m = 5,100 mg/m^3, right: 500 mg/m^3.

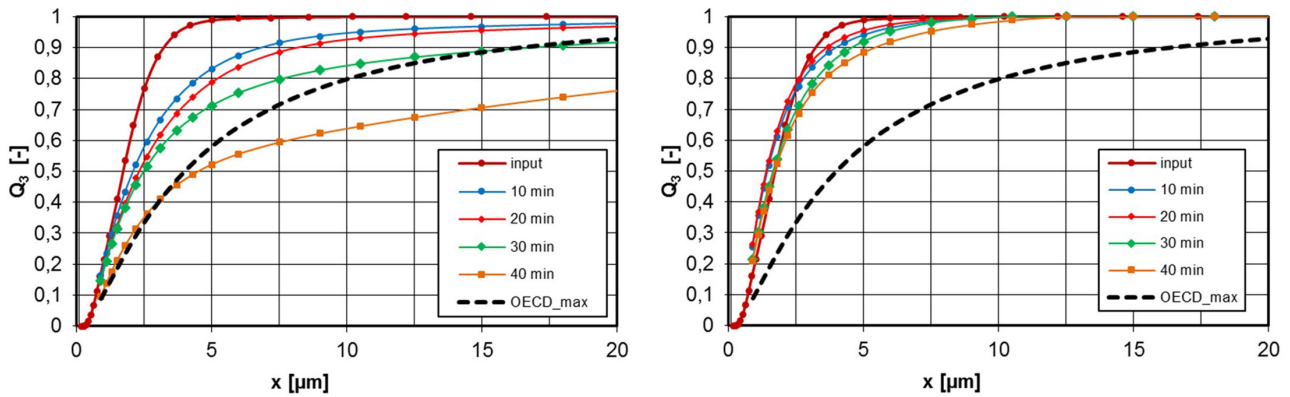

FIGURE 10 | PSD at the inlet and exhaust tube of the inhalation chamber for different operating times, calcium carbonate, ρ_{eff} = 1,000 kg/m^3, left: c_m = 6,300 mg/m^3, right: 500 mg/m^3.

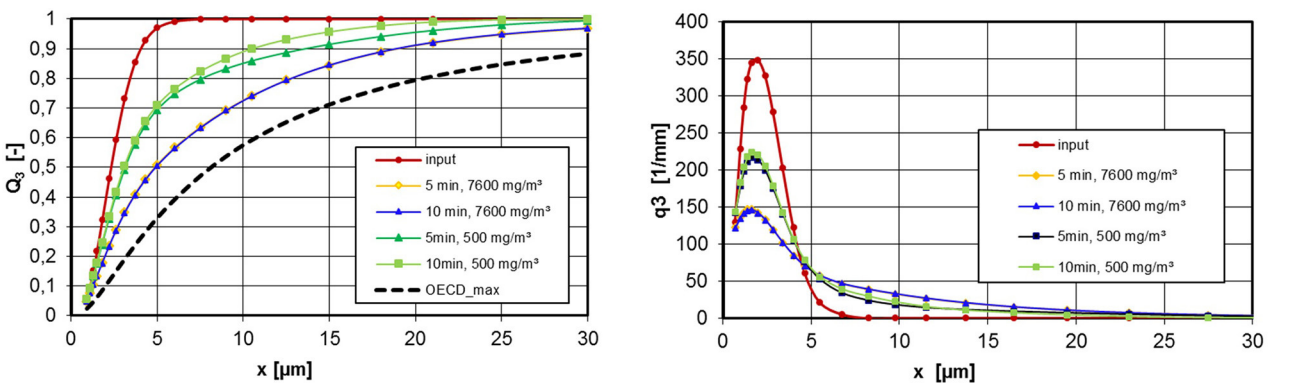

FIGURE 11 | PSD at the inlet and exhaust tube of the inhalation chamber for different operating times, silica gel, ρ_{eff} = 242 kg/m^3, left: c_m = 7,600 mg/m^3 and 500 mg/m^3, right: volume density distributions.

conditions of the system, the concentration and the total test duration must all be considered. For the powder systems investigated in this study, it has been shown that concentrations up to 500 mg/m^3 was technically feasible.

DATA AVAILABILITY STATEMENT

The raw data supporting the conclusions of this article will be made available by the authors, without undue reservation.

AUTHOR CONTRIBUTIONS

All authors listed have made a substantial, direct, and intellectual contribution to the work and approved it for publication.

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Conflict of Interest: The authors declare that the research was conducted in the absence of any commercial or financial relationships that could be construed as a potential conflict of interest.

Symbols.

Symbol	Description	Unit
c_m	mass concentration	[mg/m^3]
MMAD	mass median aerodynamic diameter	[μm]
q_3	volume density distribution	[1/mm]
Q_3	cumulative volume distribution	[-]
x	geometric particle diameter	[μm]
x_5x_0	median diameter	[μm]
x_{ae}	aerodynamic particle diameter	[μm]
$x_{p;3}$	particle size of the percentile p of Q_3Q	[μm]
ε_{agg}	agglomerate volume porosity	[-]
ε_p	particle volume porosity	[-]
ρ_{1000}	density = 1000	[kg/m^3]
ρ_{eff}	effective particle density	[kg/m^3]
ρ_s	true density of solid	[kg/m^3]
σ_g	geometric standard deviation	[-]
σ_{ln}	logarithmic standard deviation $\sigma_{ln=}$ ln(σ_g)	[-]

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Surface Treatment With Hydrophobic Coating Reagents (Organosilanes) Strongly Reduces the Bioactivity of Synthetic Amorphous Silica *in vitro*

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Synthetic amorphous silica (SAS) is industrially relevant material whose bioactivity *in vitro* is strongly diminished, for example, by protein binding to the particle surface. Here, we investigated the *in vitro* bioactivity of fourteen SAS (pyrogenic, precipitated, or colloidal), nine of which were surface-treated with organosilanes, using alveolar macrophages as a highly sensitive test system. Dispersion of the hydrophobic SAS required pre-wetting with ethanol and extensive ultrasonic treatment in the presence of 0.05% BSA (Protocol 1). Hydrophilic SAS was suspended by moderate ultrasonic treatment (Protocol 2) and also by Protocol 1. The suspensions were administered to NR8383 alveolar macrophages under serum-free conditions for 16 h, and the release of LDH, GLU, H_2O_2, and TNFα was measured in cell culture supernatants. While seven surface-treated hydrophobic SAS exhibited virtually no bioactivity, two materials (AEROSIL® R 504 and AEROSIL® R 816) had minimal effects on NR8383 cells. In contrast, non-treated SAS elicited considerable increases in LDH, GLU, and TNFα, while the release of H_2O_2 was low except for CAB-O-SIL® S17D Fumed Silica. Dispersing hydrophilic SAS with Protocol 1 gradually reduced the bioactivity but did not abolish it. The results show that hydrophobic coating reagents, which bind covalently to the SAS surface, abrogate the bioactivity of SAS even under serum-free *in vitro* conditions. The results may have implications for the hazard assessment of hydrophobic surface-treated SAS in the lung.

Keywords: synthetic amorphous silica, surface treatment, hydrophobicity, organosilanes, siloxanes, alveolar macrophage

INTRODUCTION

A large variety (precipitated, pyrogenic, silica gel or colloidal forms) of synthetic amorphous silica (SAS) is produced and used for many industrial applications. SAS is incorporated in consumer products, cosmetics, feed, pharmaceuticals, or food (1–4) and serves as thickeners, fillers, flow enhancing agents, or stabilizers (1, 3, 5–7). However, several of these applications are incompatible with the hydrophilic SiO_2 surface. Chemical modifications are in use to render the SAS particle surface hydrophobicity, thus allowing their incorporation, for example, into polymers, such as silicone rubber, non-water-based paint and coating formulations, toner products, adhesives and sealants, cable compounds, and resin systems. A versatile and widespread industrial process to

achieve the hydrophobicity of SAS is the surface treatment with organosilanes as shown in **Figure 1**. A multitude of different hydrophobic moieties is available, enabling inventors to design and adapt the properties of SAS (such as polarity) to specific material requirements. Organosilanes are chemically bound through SiO_2 (**Figure 1**) or form other types of bonds as outlined in **Table 1**. Silanol groups are suspected to be mainly responsible for the biological activity of SAS (9). A chemical modification or capping of these groups may, therefore, lead to a markedly reduced bioactivity, which should be demonstrable *in vitro*. However, the effects of SAS surface treated with organosilanes have not yet been systematically investigated with sensitive *in vitro* test systems.

The choice of an *in vitro* testing system should reflect the relevant route of particle uptake. In case of pyrogenic ("fumed"), precipitated, or dried colloidal surface-treated SAS, which are distributed as dry powder nanostructured materials, unintentional inhalation needs to be considered as a possible way of particle uptake into the body (10, 11) and the appropriate risk assessment should focus on possible effects of SAS in the lung. SAS, especially without surface-functionalization, is known to induce transient and gradually more pronounced lung inflammation in rodents (10, 12–17). In line with this, also *in vitro* studies carried out with various cell types revealed the effects of SAS (1, 16, 18–22). However, it turned out that the cell culture and incubation conditions strongly influence the outcome of *in vitro* tests. While the effect of SiO_2 is mostly low in the presence of serum proteins, serum-free testing conditions augmented the bioactivity of SAS on cells *in vitro* several-fold (23–28).

Considering silanol groups as reactive sites, several *in vitro* and *in vivo* studies provided evidence that blocking and/or inactivating these reactive groups lowered the bioactivity of crystalline silica such as quartz. As early as 1961, Schlipköter and Brockhaus proposed polyvinylpyridine-N-oxide (PVNO) as a substance mitigating the inflammatory and profibrotic effects of quartz in the lung (29), an effect which also reduced the toxicity of crystalline silica *in vitro* (30, 31). Also, other substances such as Lewis acids were successful in this respect. Comparing the density and steric properties of silanol groups of crystalline and amorphous silica revealed that the hemolytic properties of quartz increase with the number of geminal, but not single silanol groups (32). Only recently, however, a oligomeric, amino-

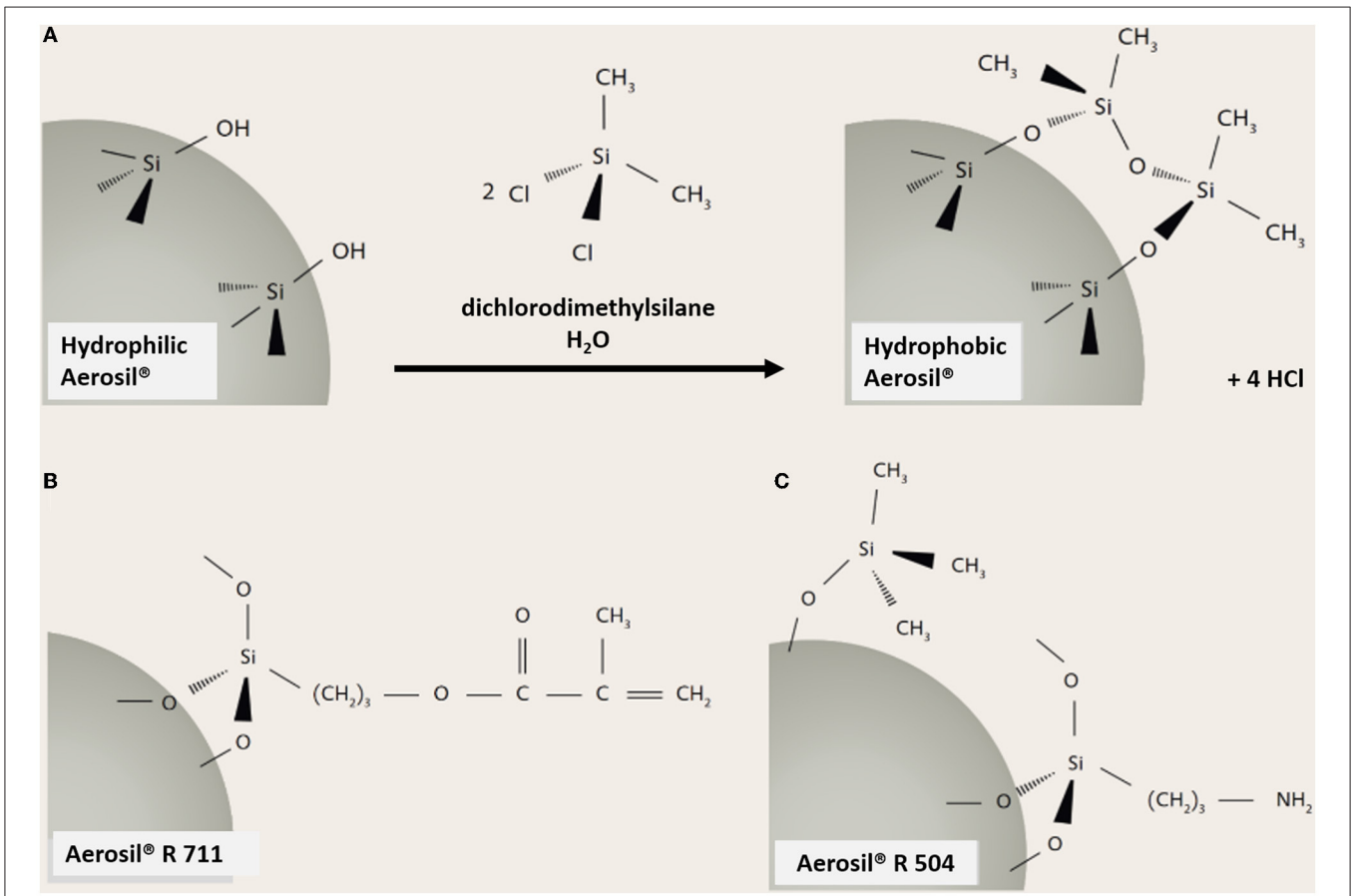

FIGURE 1 | Reaction of organosilanes with silanol groups of SAS and typical reaction products. **(A)** Reaction of dichlorodimethylsilane (DDS) with a hydrophilic Aerosil® generates a hydrophobic AEROSIL®; **(B)** methacrylate functionalities at the surface of AEROSIL® R 711; **(C)** partly aminated surface of AEROSIL® R 504. All parts of this figure are taken from "Evonik Industries. AEROSIL®—Fumed Silica, Technical Overview" (8).

TABLE 1 | List of materials, coating agents, surface modifications, and properties of SAS.

Substance name	Source	Type	Treating agent, abbreviation (CAS No.)	Chemical name (CAS No.)	Primary particle size (TEM)	Aggregate size (TEM)	Skeletal density (g/ml)	BET (m^2/g)	Further properties/purity
LUDOX® SM	GRACE	CS	None	112926-00-8 (ex 7631-86-9)	7 nm	No aggregates	2.2	320–400	30% in H2O; negatively particle charge stabilized with Na+
LUDOX® TM-50	GRACE	CS	None	112926-00-8 (ex 7631-86-9)	22 nm	No aggregates	2.2	110–150	50% in H2O; negatively particle charge stabilized with Na+
CAB-O-SIL® S17D	CABOT	FS	None	112945-52-5 (ex 7631-86-9)	9 nm[1]	D50: 66 nm, D10: 31 nm, D90: 123 nm	2.3	390–430	Non-porous
CAB-O-SIL (E)L-90	CABOT	FS	None	112945-52-5 (ex 7631-86-9)	16 nm[1]	D50: 143 nm, D10: 60 nm, D90: 278 nm	2.3	83–97	
AEROSIL® 50	EVONIK	FS	None	112945-52-5 (ex 7631-86-9)	25 nm[1]	Feret min, D50: 263 nm	2.3	35–65	SiO2 content (based on ignited material): ≥99.8%
AEROSIL® R816	EVONIK	FS	Trimethoxyhexadecyl-silan (199876-45-4)	Silane; hexadecyltrimethoxy-, hydrolysis products with silica (199876-45-4)	8 nm[1]	Feret min, D50: 121 nm	1.9–2.5	170–210	SiO2 content (based on ignited material): ≥99.8%, Carbon content 0.9–1.8%
CAB-O-SIL® TGC413TRD	CABOT	dCS	Hexamethyldisilazane HDMZ (999-97-3)	Silanamine; 1,1,1-trimethyl-N-(trimethylsilyl)-, hydrolysis products with silica (68909-20-6)	50 nm	No aggregates	2.2–2.3	45–70	Non-porous, colloidal silica [(trimethylsilyl)oxy]-modified
CAB-O-SIL®TS610	CABOT	FS	Dichlorodimethylsilane DMS (75-78-5)	Silane; dichlorodimethyl-, reaction products with silica (Silica-[(dimethylsilyl)oxy]-modified) (68611-44-9)	12 nm[1]	D50: 106 nm, D10: 49 nm, D90: 203 nm	2.2	105–145	Non-porous, silane, dichlorodimethyl-, reaction products with silica
CAB-O-SIL® TS720	CABOT	FS	Polydimethylsiloxane, PDMS (63148-62-9)	Silicones and siloxanes, dimethyl-, reaction products with silica ((67762-90-7)	12 nm[1]	D50: 122 nm, D10: 52 nm, D90: 215 nm	1.9	105–135	Non-porous, siloxanes and silicones, di-Me, reaction products with silica
HDK® H15	WACKER	FS	Dichlorodimethylsilane DMS (75-78-5)	Silane; dichlorodimethyl-, reaction products with silica (Silica-[(dimethylsilyl)oxy]-modified) (68611-44-9)	15 mn[1]	Number-based D50: 248 nm, D10: 43 nm, D90: 554 nm.	2.2	150[2]	SiO2 content (based on ignited material): ≥99.8%
HDK® H2000	WACKER	FS	Hexamethyldisilazane, HDMZ (999-97-3)	Silanamine; 1,1,1-trimethyl-N-(trimethylsilyl)-, hydrolysis products with silica (68909-20-6)	12 nm[1]	Number:-based D50: 63 nm, D10: 24 nm, D90: 160 nm	2.2	200[2]	SiO2 content (based on ignited material): ≥99.8%
AEROSIL® R504	EVONIK	FS	3-Aminopropyl-triethoxysilane, AMEO (919-30-2) plus Hexamethyldisilazane, HDMZ (999-97-3)	Silanamine; 1,1,1-trimethyl-N-(trimethylsilyl)-, hydrolysis products with silica and 3-(triethoxysilyl)-1-propanamine (199876-44-3)	n.m.	n.m.	2.2	125-175	SiO2 content (based on ignited material): ≥99.8%, Carbon content: ca. 2.0–4.5%; Trimethylsilyl and Aminopropylsilyl groups

(Continued)

TABLE 1 | Continued

AEROSIL® R711	EVONIK	FS	Trimethoxysilyl propylmethacrylate, MEMO (2530-85-0)	2-Propenoic acid; 2-methyl-, 3-(trimethoxysilyl)propylester, reaction products with silica (100402-78-6)	8 nm[1]	Feret min, D50: 123 nm	1.9–2.5	125–175	SiO2 content (based on ignited material): ≥99.8%, Carbon content: ca. 4.5–6.5%; Methacrylsilyl groups
SIPERNAT® D17	EVONIK	PS	Polydimethylsiloxane PDMS (63148-62-9)	Silicones and siloxanes, dimethyl-, reaction products with silica (67762-90-7)	15 nm[1]	Feret min, D50: 89 nm	2.0	100	Dimethylsilyl groups, Carbon content: ca. 1.7%

FS, fumed (pyrogenic) silica; CS, colloidal silica; dCS, dried colloidal silica; PS, precipitated silica. [1] *Constituent particle size (internal structures which cannot be isolated, D50, number-based by TEM).* [2] *Prior to coating.*

modified siloxane was found to inhibit the *in vitro* and *in vivo* effects of quartz (33).

The effects of organosilane-treated SAS have mainly been studied *in vivo*. Older studies on the acute, subacute, and chronic inhalation exposure of rats mostly to "reaction products of dichlorodimethyl silane (DDS) with silica" are summarized by Becker et al. (34) (see Table 4 of that reference). Overall, these studies showed minor or absent effects in the lower concentration range. Even in cynomolgus monkeys, a concentration of 10 mg/m^3 administered for 1 year elicited no adverse effects (see Ref. 68 in Becker et al. 2013). Similarly, Lewinson et al. (1994) found no toxicity in rats of DDS-treated hydrophobic amorphous silica [DDS reacts with silanol groups and converts them to methylsilyl groups (-Si-CH_3)] (35). The concentrations of >30 mg/m^3 in a 90-day study of organosilane-treated pyrogenic SAS were reported to evoke diffuse or reversible fibrogenesis-like symptoms, but in these cases, the effects were most likely related to high-concentration phenomena (17, 34).

Unlike inhalation experiments, *in vitro* studies offer the possibility to study larger numbers of surface-treated SAS, compare effects of different coating reagents and/or particle sizes, and investigate the cellular modes of action of organosilane-treated SAS. To this end, we studied the cytotoxicity and pro-inflammatory effects of nine surface-treated vs. five untreated SAS with alveolar macrophages. These cells are responsible for clearing inhaled particles from the lung parenchyma where they form a first line of defense against inhaled microorganisms and respirable dusts. Here, we use a well-established *in vitro* test based on the rat alveolar macrophage cell line NR8383 to determine the *in vitro* toxicity of SAS (36). This assay has been validated against 18 short-term inhalation studies and was carried out under protein-free conditions, as this allows to analyze the effects of surface modification without the formation of a protein corona (36). Cytotoxic, activating, and pro-inflammatory effects as well as oxidative stress are analyzed from the cell culture supernatant of cells which are exposed to particles under submersed conditions. However, the dispersion of highly hydrophobic powder materials requires a special strategy. While powders of non-treated (rather hydrophilic) SAS, which contain aggregates and/or agglomerates, can be dispersed with moderate ultrasonic dispersion (USD) energy (3, 37), highly hydrophobic surface-treated SAS demands a different protocol in which particles are pre-wetted with ethanol before they can be immersed in aqueous media, where they are then subjected to extensive USD in the presence of a minimal amount of bovine serum albumin.

Using these dispersion strategies, the particle size distribution and effects of organosilane-treated SAS, as shown in **Figure 1**, on NR8383 alveolar macrophages will be described and compared to those of more hydrophilic SAS dispersed with both protocols. The results show that hydrophobic surface treatment with organosilanes can abrogate or at least largely diminish the bioactivity of SAS under *in vitro* conditions.

MATERIALS AND METHODS

Particle Properties

Powder materials (12/14) and colloidal SAS delivered as suspension (LUDOX® SM, LUDOX® TM 50, all industrial grades) were provided by members of the consortium SASforREACH GbR as listed in **Table 1**, together with the details of surface treatment, chemical modification, primary particle, and aggregate sizes, as well as specific surface area according to the Brunauer–Emmett–Teller (BET) method. Micron-sized corundum and quartz DQ12 particles were included in the study as negative and positive particle controls, respectively, as previously described (36, 38).

Preparation of Particle Suspensions

Dispersion of Hydrophobic SAS: Protocol 1

Highly hydrophobic SAS was dispersed according to the NanoGenoTox Protocol (39) for which a minor adaptation was necessary. To achieve a complete wetting of the powders, 15.36 mg of the powder materials was wetted with 60 µl of ethanol (instead of 30 µl). The samples were then mixed with 6 ml of H_2O containing 0.05% of bovine serum albumin fraction V (BSA), vortexed, and subjected to ultrasonic treatment for 16 min on ice, using a Branson 450D Sonifier, equipped with a 1 cm sonotrode (applied energy density 3,140 J/ml). By this, stable stock suspensions of surface-treated SAS were created (2.56 mg/ml) which were used for all *in vitro* tests. Of note, the final concentrations of ethanol and BSA in all cell assays amounted to 0.04 and 0.002%, respectively, due to further dilution.

Dispersion of Hydrophilic SAS: Protocol 2

To prepare particle stock suspensions for cell culture studies from powder materials, 25.6 mg was transferred into 10-ml sterile pyrogen-free H_20 (B. Braun Melsungen AG, Melsungen, Germany), vortexed, and ultrasonicated on ice for 15 × 12 s, using a Branson 450D Sonifier, equipped with a 5 mm sonotrode; total ultrasonic energy delivered this way amounted to 270 J/ml (37). For colloidal SAS (LUDOX® SM, LUDOX® TM 50), the concentration was adjusted to 2.56 mg/ml, secondary to a gravimetrical measurement of the dry mass of the original suspensions using a Mettler Toledo AT20 microbalance. All suspensions showed no or only minimal settled material and were used throughout the study.

In an additional set of experiments, hydrophilic SAS was dispersed as described for the hydrophobic SAS and with the same final amounts of BSA and ethanol.

Measurements of Particle Size Distribution by Particle Tracking Analysis

In addition to the particle sizes provided in **Table 1**, we determined the particle size distribution in the cell culture medium, that is, under assay conditions by particle tracking analyses (PTA). A NanoSight LM10 instrument equipped with a violet laser (405 nm), an Andor CCD camera, and particle tracking software (NTA 3.0, Malvern Instruments GmbH, Herrenberg, Germany) was used. To measure particle and/or aggregate/agglomerate sizes of SAS suspensions under cell culture conditions, the aqueous particle suspensions were incubated under cell culture conditions (37°C, 5% CO_2) for 90 min in KRPG, or for 16 h in F-12K medium, respectively. The suspensions were serially diluted with the respective medium (H_2O, KRPG, or F-12K medium) to optimize the apparent particle concentration to PTA requirements (∼5 × 10^8 particles/ml). The results and respective dilution factors are presented in **Table 2**. Since the technique is limited by the light-scattering properties of particles, (colloidal) SAS particles smaller than 50 nm were not detected (**Supplementary Figures S1, S2**).

Sterility Testing

To test for any fungal or bacterial contaminations, 100 µl of the final aqueous suspension as prepared for *in vitro* testing was plated onto caso agar and malt extract agar (both from Applichem GmbH, Darmstadt, Germany) and incubated at 37°C for 3 days. Neither bacterial nor fungal contaminants were detected.

Cell Culture and *in vitro* Testing

NR8383 cells (ATCC, USA; ATCC® Number: CRL-2192TM) were cultivated in F-12K medium supplemented with 15% fetal calf serum (FCS), 100 µg/ml streptomycin, 100 U/ml penicillin, and 2 mM L-glutamine (all from PAN Biotech, Aidenbach, Germany) as described.

Assays were carried out as described (36). Cells were seeded into 96-well plates (3 × 10^5 cells/well) and kept at 37°C and 5% CO_2. Each well contained 200 µl F-12K cell culture medium in which the concentration of FCS was reduced to 5%. After 24 h, the medium was replaced by serum-free suspensions of the test materials, which were diluted to 90, 45, 22.5, and 11.25 µg/ml either with KRPG buffer (129 mM NaCl, 4.86 mM KCl, 1.22 mM $CaCl_2$, 15.8 mM NaH_2PO_4, 5–10 mM glucose; pH 7.3–7.4) or with serum-free F-12K medium.

To measure the release of H_2O_2, the particles were administered in KRPG buffer. Released H_2O_2 was measured after 90 min using the Amplex Red® assay. The optical density of resorufin was measured photometrically at 570 nm (reference value: 620 nm) with a plate reader (Tecan Infinite F200Pro, Tecan GmbH, Germany). Positive controls were run with zymosan (180 µg/ml). All values were corrected for background absorbance using cell-free particle controls and converted into absolute concentrations of H_2O_2 using the molar extinction coefficient of resorufin (54,000 L × mol^{-1} × cm^{-1}).

To determine the release of LDH, GLU, and TNFα from the cells, the test materials were administered in serum-free F-12K medium and supernatants were retrieved after 16 h. LDH activity was measured using the Roche Cytotoxicity Kit (Sigma-Aldrich, Taufkirchen, Germany). To measure GLU activity, the supernatant (50 µl) was mixed with 100 µl 0.2 M sodium acetate buffer (pH 5) containing 13.3 mM p-nitrophenyl-D-glucuronide and 0.1% Triton X-100. The color reaction was stopped with 100 µl 0.2 M NaOH terminated; the optical density was measured at 405 nm. LDH and GLU measurements were background corrected and normalized to the positive control values (set to 100%) obtained by lysing the cells with 0.1% Triton X-100 in F-12K.

The concentration of tumor necrosis factor α (TNFα) was determined with a specific enzyme-linked immunosorbent assay (ELISA) for rat TNFα (Quantikine ELISA Kit, Bio-Techne GmbH, Wiesbaden-Nordenstadt, Germany). The TNFα-forming capacity of NR8383 cells was controlled by adding lipopolysaccharide (0.1 µg/ml, Sigma-Aldrich, Taufkirchen, Germany).

In all assays, cell controls were carried out by adding particle-free vehicle, that is, F-12K medium only, or F12-K medium containing BSA and ethanol in concentrations as stated above.

Light Microscopy

To supplement the size data from PTA analysis, to verify particle uptake by cells, and to describe cell morphology, phase contrast micrographs were taken using a Zeiss Axiovert 40-C Microscope. Gravitationally settled particles were micro-graphed under cell culture conditions in the absence of cells with a Nikon BioStation equipped with a 20× phase contrast optics.

Statistical Evaluation

in vitro data were generated in triplicates, and three independent repetitions were carried out. To test for significant differences, values from each concentration were compared to non-treated controls using two-way analysis of variance (ANOVA) with Dunnett's multiple comparison test. A value of $P \leq 0.05$ was considered significant (*). All data were expressed as mean ± standard deviation (SD). All calculations were carried out with GraphPad Prism software.

TABLE 2 | Hydrodynamic diameter of SAS particles in H_2O, KRPG buffer, and F-12K medium.

Particle Name	Protocol	Fluid	Hydrodynamic diameter [nm]				
			Mean ± SEM	Mode ± SEM	d10 ± SEM	d50 ± SEM	d90 ± SEM
CAB-O-SIL® S17D	2	H_2O	136.3 ± 2.2	125.6 ± 5.8	87.1 ± 0.6	123.8 ± 3.3	182 ± 3.9
	1	H_2O	136 ± 1.5	117 ± 1	86 ± 0.4	122 ± 1	183 ± 2.4
	2	KRPG	152.3 ± 1.6	135.1 ± 9.6	93.3 ± 1.2	141 ± 1.7	213 ± 9.6
	2	F-12K	224.8 ± 21.5	163.8 ± 22.8	111.7 ± 33.9	213.4 ± 18.5	345.4 ± 24.9
	1	F-12K	149 ± 2.5	134 ± 1	107 ± 2.7	137 ± 3.6	192 ± 3.9
CAB-O-SIL® (E)L-90	2	H_2O	243.4 ± 1	229.5 ± 17.4	154.8 ± 0.9	226.7 ± 4.6	334.7 ± 2
	1	H_2O	202 ± 1	169 ± 8.1	132 ± 1.2	188 ± 1.5	284 ± 1.7
	2	KRPG	274.8 ± 14.9	194.7 ± 20.4	172.1 ± 10.7	253.5 ± 16.8	389.7 ± 20.4
	2	F-12K	301.3 ± 15.4	254.6 ± 41.4	182.9 ± 9	289.1 ± 15.5	417.4 ± 21.5
	1	F-12K	260 ± 13	212 ± 35.9	164 ± 8.3	250 ± 11.3	356 ± 17.3
AEROSIL® 50	2	H_2O	233.7 ± 4	195.3 ± 12.1	148.9 ± 2.9	210.6 ± 2.3	334.2 ± 8.9
	1	H_2O	221 ± 0.5	183 ± 9.4	139 ± 3.3	205 ± 1.6	305 ± 4.6
	2	KRPG	308.8 ± 6.1	266.9 ± 44.9	195.1 ± 3.8	296.2 ± 16.5	438.2 ± 19
	2	F-12K	297.8 ± 5.2	278.2 ± 6	185.3 ± 5.1	283.6 ± 4.1	409.6 ± 2.7
	1	F-12K	295 ± 9.7	282 ± 11.9	183 ± 6	279 ± 5.2	413 ± 22.5
AEROSIL® R 816	2	H_2O	206.9 ± 7.1	166.2 ± 12.6	133.4 ± 2.3	191.9 ± 5.8	281.6 ± 13.4
	1	H_2O	149 ± 2.1	125 ± 5	98 ± 1.4	134 ± 1.4	204 ± 6.3
	2	KRPG	388.9 ± 24.3	297.5 ± 40.9	227.2 ± 15.1	369.1 ± 16.8	559.4 ± 34.2
	2	F-12K	372.9 ± 9.9	334.1 ± 77.1	190.6 ± 6.8	372 ± 10.1	528.9 ± 9
	1	F-12K	241 ± 2.9	152 ± 5.4	134 ± 3.1	202 ± 1.2	396 ± 10.9
CAB-O-SIL® TGC413TRD	1	H_2O	103.4 ± 0.5	94.3 ± 1.7	68.4 ± 1.5	93.7 ± 1.2	133 ± 1.9
	1	KRPG	88.9 ± 0.4	75.9 ± 2	58.5 ± 1	78.7 ± 0.7	116.3 ± 0.7
	1	F-12K	114.7 ± 1.5	101.9 ± 0.3	67.7 ± 1.5	102 ± 1.2	156.3 ± 2.7
CAB-O-SIL® TS610	1	H_2O	368.9 ± 1.6	394.1 ± 13.2	211.1 ± 5.1	369.5 ± 2.4	500.7 ± 18.7
	1	KRPG	170.7 ± 0.9	149.5 ± 4.8	112.9 ± 2	156.2 ± 0.9	226.4 ± 2.2
	1	F-12K	182.9 ± 2.7	130.6 ± 6.1	115.9 ± 2.4	166.6 ± 3.2	250.5 ± 3.8
CAB-O-SIL® TS720	1	H_2O	380.6 ± 12.2	349.3 ± 48.1	233.7 ± 13.9	383.8 ± 11.5	501.5 ± 12.8
	1	KRPG	174.9 ± 1.9	142.8 ± 15.5	115.2 ± 0.2	160.7 ± 3.2	238.2 ± 3.9
	1	F-12K	185.7 ± 1.6	145.3 ± 12	111.6 ± 0.8	162.3 ± 2.2	260 ± 5.7
HDK® H15	1	H_2O	224.5 ± 5.7	187.6 ± 24.1	136 ± 3	203.3 ± 4.4	334.8 ± 16.2
	1	KRPG	184.1 ± 1.8	141.2 ± 3.2	125.8 ± 1.2	167.8 ± 2.4	249.6 ± 2.3
	1	F-12K	203 ± 1.7	203.3 ± 9.6	129.5 ± 0.4	191 ± 2.5	284.4 ± 4.6
HDK® H2000	1	H_2O	252.4 ± 4.5	148.2 ± 13.2	115.6 ± 1.1	226.2 ± 3.4	419.3 ± 9.2
	1	KRPG	323.5 ± 13.7	249.3 ± 62.9	147.9 ± 10.3	301.7 ± 17.1	523.4 ± 9.7
	1	F-12K	441.3 ± 9.6	392.2 ± 61.9	204.7 ± 11.5	425.4 ± 6.8	682.4 ± 38.2
AEROSIL® R 504	1	H_2O	262.9 ± 21.7	170.8 ± 33.8	131.8 ± 15.2	238.7 ± 19.7	407.3 ± 25.2
	1	KRPG	249.2 ± 31	130.6 ± 26.3	121.1 ± 23.6	215.4 ± 34.6	428.2 ± 47.3
	1	F-12K	300.1 ± 2.7	258.4 ± 29	145.4 ± 3.2	279.9 ± 9.7	474.5 ± 16.8
AEROSIL® R 711	1	H_2O	377.7 ± 10.1	379.7 ± 41.4	216.7 ± 10	374.1 ± 15.5	532.6 ± 12.4
	1	KRPG	179.7 ± 0.8	131.6 ± 5.2	110.1 ± 1	159.6 ± 0.9	263.5 ± 3.6
	1	F-12K	181.5 ± 3.1	137.8 ± 7.3	116.1 ± 0.7	163.7 ± 3.1	262.8 ± 15.5
SIPERNAT® D 17	1	H_2O	266.8 ± 4.6	183.3 ± 12.7	154.8 ± 1	241.2 ± 2.6	408.7 ± 12.8
	1	KRPG	214.3 ± 1.4	154.5 ± 6.1	136 ± 1.2	186.9 ± 2.5	319.4 ± 9.9
	1	F-12K	198.8 ± 4	160.2 ± 8.3	122.7 ± 1	172 ± 2.7	295.6 ± 16.2

Particles were dispersed in H_2O following Protocol 1 or 2 as indicated, diluted in either H_2O, KRPG, or F-12K medium to 90 μg/ml and incubated at 37°C for 90 ± 20 min (H_2O, KRPG) or 16 ± 1 h (F-12K medium) to mimic culture conditions. HDs are means ± SD from three technical replicates. No background correction was carried out because H_2O, KRPG, or serum-free F-12K medium contained no PTA-detectable particles. Values for d10, d50, and d90 describe the cumulative particle size distribution at 10, 50, and 90% of the maximum value.

RESULTS

Size Determination of SAS in Cell Culture Experiments

in vitro effects of particles on phagocytic cells depend on particle size and gravitational settling. To obtain insight into the so-called particokinetics (40) of surface-treated and unmodified SAS, we measured the hydrodynamic diameter (HD) of suspended particles under cell culture conditions. Particle tracking analysis (PTA) was used as it allows to detect light-scattering nanoparticles at relatively low concentration.

The HD data, from size distribution plots (**Supplementary Figures S1, S2**), are summarized in **Table 2**, where the (ultra)fine fraction of SAS particles primarily dispersed in H_2O is compared to the HD found in KRPG buffer after 90 min and in F-12K medium after 16 h, respectively. The non-treated SAS CAB-O-SIL® S17D Fumed Silica (CAB-O-SIL® S17D), CAB-O-SIL® (E)L-90, and AEROSIL® 50, as well as the slightly hydrophilic surface-treated AEROSIL® R 816, when dispersed with Protocol 2, tended to slightly agglomerate. This was reflected by an increase in HD (mode value) of 42.4% for AEROSIL® 50 and 101.0% for AEROSIL® R 816 (**Table 2** and **Supplementary Table S1**). Hydrophobic surface-treated *SAS*, which had to be dispersed with Protocol 1, behaved less uniform, since we observed increases in HD of up to 164.6% (HDK® H2000) and decreases down to −63.7% (AEROSIL® R 711).

In line with these measurements, most SAS did not further agglomerate under cell culture conditions. Nevertheless, phase contrast microscopy revealed low numbers of aggregates/agglomerates (e.g., CAB-O-SIL® TS 720, CAB-O-SIL™ TGC413TRD treated silica) or a thin layer of loose precipitates (CAB-O-SIL® (E)L-90, AEROSIL® R 816, HDK® 2000, and AEROSIL® R 504) all of which were visible under cell-free conditions after 16 h of incubation (**Figure 2** and **Supplementary Figures S4, S5**). Overall, gravitational settling of SAS tested in this study was very limited.

In vitro Effects of SAS on Alveolar Macrophages

To study the biological activity of untreated and surface-treated SAS *in vitro*, we used the well-established alveolar macrophage test, which analyzes the releases of lactate dehydrogenase (LDH), glucuronidase (GLU), tumor necrosis factor α (TNFα), and H_2O_2 from NR8383 cells into the cell culture supernatants. Vehicle-treated cells were used as negative controls. Micron-sized corundum and quartz DQ12 (0–180 μg/ml) were used as negative and positive benchmark controls.

To compare *in vitro* effects of surface-treated and untreated SAS, we administered serially diluted particle suspensions (11.25–90 μg/ml) to the cells. Particle uptake was confirmed by light microscopy at least for the gravitationally settled

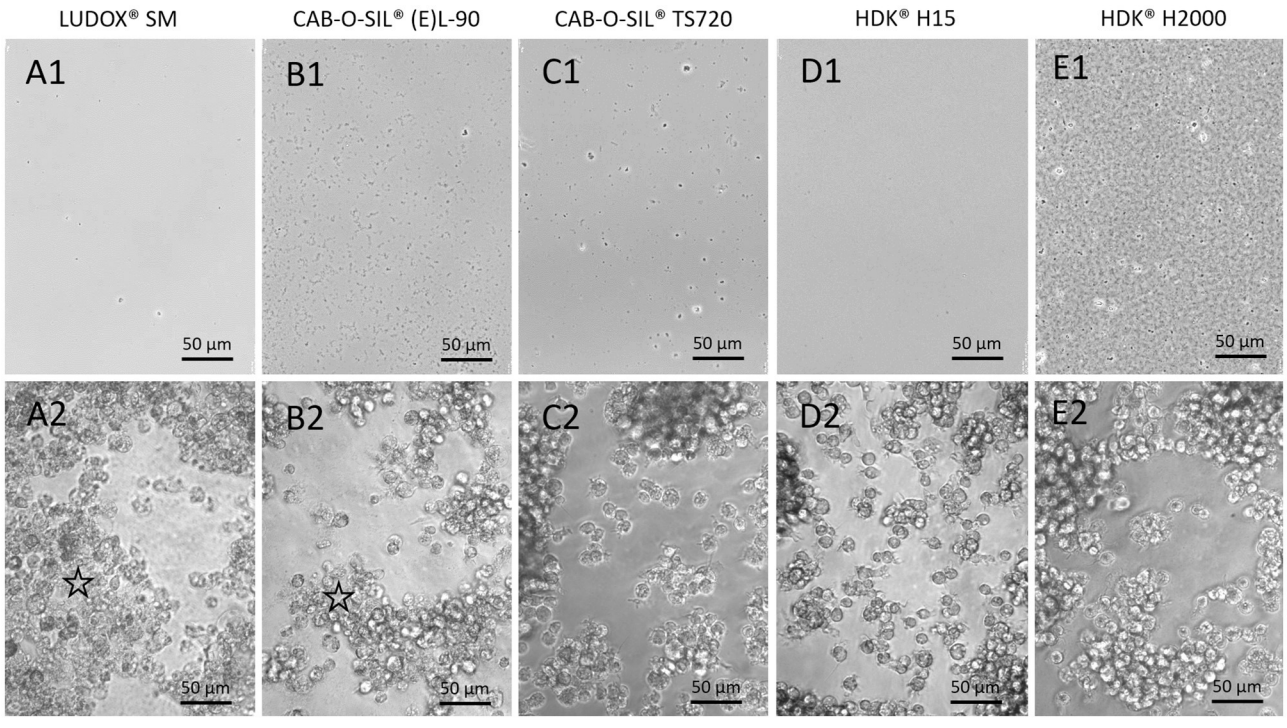

FIGURE 2 | Gravitational settling and uptake of selected SAS. Phase contrast images from cell culture experiments with 90 μg/ml of indicated SAS in the absence (A1–E1, cell-free controls) and presence of NR8383 cells (A2–E2). **(A,B)** Untreated SAS dispersed with Protocol 2, **(C–E)** surface-treated SAS dispersed with Protocol 1. Note that particle settling is obvious at different degrees in cell-free controls (A1–E1). In the presence of cells, precipitates are no longer visible indicating particle uptake. Areas with granular, deteriorated cells (asterisks) are seen in the presence of untreated SAS only. See text for further explanation.

aggregates/agglomerates which are visible with phase contrast optics (**Figures 2B,E** and **Supplementary Figures S3–S5**).

Controls

While corundum elicited no adverse responses in all tests, quartz DQ12 led to cytotoxicity, activation, and pro-inflammatory response indicated by increased release of LDH, GLU, and TNFα, respectively (**Table 3**). The TNFα responsiveness of the cells was controlled with lipopolysaccharide (LPS, 0.5 μg/ml) which increased TNFα concentration to 1,027 ± 164 pg/ml. The induced formation of H_2O_2 was typically low upon both corundum and quartz DQ12 (<1.5 μM), but increased to 23.4 ± 2.0 μM upon zymosan, due to its well-known induction of the NADPH oxidase reaction in macrophages. Overall, the effects of cell and particle controls were within the borders of our historical records, indicating the responsiveness of NR8383 macrophages and the validity of the assay.

Untreated SAS

In general, the majority of untreated SAS, when dispersed with Protocol 2 (**Table 3**), induced a largely similar pattern of responses characterized by dose-dependent cytotoxic effects (LDH) and releases of GLU. Low-observed effect concentrations (LOECs) were generally ≤22.5 μg/ml for LDH and 22.5–45 μg/ml for GLU. Unexpectedly, CAB-O-SIL® S17D elicited an inverse dose response in both assays, starting with a full-blown response upon 11.25 μg/ml. The induction of TNFα was mostly biphasic with a maximum between 22.5 and 45 μg/ml, and LOECs ranged from 11.25 (CAB-O-SIL® S17D) to 22.5 μg/ml (other untreated materials). A significant dose-dependent release of H_2O_2 was found for CAB-O-SIL® S17D at concentrations ≥22.5 μg/ml. All other untreated or hydrophilic SAS elicited no change in the extracellular H_2O_2 concentration.

Untreated SAS was also dispersed according to Protocol 1 to compare the releases of LDH and GLU to those induced by surface-treated SAS. The effects of CAB-O-SIL® S17D, CAB-O-SIL® (E)L-90, AEROSIL® 50, and LUDOX® TM 50 were strongly mitigated, while the effects of both LUDOX® variants were less affected or remained even identical (**Table 3**). Of note, ethanol wetting together with pronounced ultrasonic treatment in the presence of low amount of BSA (Protocol 1) shifted the dose–response curves rightwards (**Supplementary Figures S6, S7**), thus increasing EC_{50} values up to 2.8-fold with colloidal SAS being least affected (**Table 4**). Importantly, the application of Protocol 1 to hydrophilic SAS did not reduce the bioactivity down to control level as observed for highly hydrophobic SAS. Overall, untreated SAS exhibited a high bioactivity in the alveolar macrophage assay.

Surface-Treated SAS

The majority of hydrophobic SAS, when dispersed with Protocol 1, elicited no changes in LDH, GLU, and TNFα (**Table 5**). AEROSIL® R 504, whose surface treatment combines HDMZ with AMEO, induced a small increase in LDH and GLU, which was significant for LDH upon 90 μg/ml only. Another exception was the slightly hydrophilic AEROSIL® R 816, whose effects resembled the non-treated SAS (c.f. **Table 3**). There were also no results pointing to an induction of H_2O_2 formation by surface-treated hydrophobic SAS. However, some hydrophobic variants decreased the small resorufin signal below cell controls (**Table 5**). This phenomenon was interpreted as an assay interference (possibly caused by adsorption of colored reactants to hydrophobic surfaces). Taken together, surface-treated hydrophobic SAS showed no or a very limited biological activity. The direct comparison of untreated and surface-treated SAS is shown in **Figure 3**.

DISCUSSION

In this study, we showed that surface-treated highly hydrophobic SAS elicits neither cytotoxic nor pro-inflammatory effects in alveolar macrophages *in vitro*. While untreated SAS was highly bioactive under the serum-free testing conditions, thus inducing the release of LDH, GLU, TNFα, and, partially, also of H_2O_2 from NR8383 alveolar macrophages, the organosilane-treated SAS elicited no such effects up to 45 μg/ml, and only two materials showed minor effects at the highest dose (90 μg/ml). This is a striking finding because the effects of many poorly soluble nanomaterials on cells or in the lung correlate with the specific BET surface areas (41) which are comparatively large (BET: 58–200 m^2/g) for surface-treated SAS of this study. With respect to SAS, it should be added, however, that the bioactivity seen in *in vitro* and *in vivo* studies is not merely a function of the BET surface, especially if pyrogenic, precipitated, gel and colloidal SAS are compared (20, 37, 42). Moreover, this is most likely influenced by surface characteristics (e.g., steric properties of silanol groups) as well as the occurrence of micro- and mesopores.

Our previous studies with the same cell culture model have shown that NR8383 cells are highly sensitive to SAS (28, 36, 37). Accordingly, the EC_{50} values for the release of LDH, which indicate membrane damage and cytotoxicity, were in the range of 7–30 μg/ml for most non-treated SAS in the absence of protein (37). Considering that particles inside the lung will come into contact with the lung surfactant and proteins of the lung lining fluid, the omission of protein under testing conditions may be regarded a somewhat artificial situation. However, as an established routine method, it allows to disclose biological effects of different particle surfaces (43).

To use the test system for highly hydrophobic surface-treated SAS, particles had to be wetted with ethanol and subjected to very high USD energy in the presence of a low, stabilizing concentration of BSA. One might object that the missing bioactivity of surface-treated SAS, when compared to untreated SAS, mainly results from a lowered particle size which might have caused lower gravitational settling and, thus, reduced particle uptake. However, HD values in the cell culture medium were similar in both groups (mode values were 163–278.2 and 101.9–392 nm for untreated and surface-treated SAS, respectively) suggesting a very similar particokinetics of both types of SAS. Also the concentration ranges in the cell culture medium of both types of particles were highly similar, as estimated from the PTA measurements (**Supplementary Figures S1, S2**, and **S8**). Overall, the PTA data suggest that there were no major differences in size

TABLE 3 | Effects of non-treated SAS dispersed with Protocol 1 and 2 on NR8383 cells.

Concentration	[μg/ml]	Protocol	LDH [% pos. Control] Mean ± SD	GLU % pos. Control Mean ± SD	H_2O_2 [μmol/L] Mean ± SD	TNFα [pg/ml] Mean ± SD
Corundum	0	2	10.3 ± 2.4	1.6 ± 0.2	0.8 ± 0.1	37.6 ± 23.6
	22.5	2	7.8 ± 1.4	1.3 ± 0.1	0.9 ± 0.1	28.5 ± 11.2
	45	2	9.3 ± 1.2	2.2 ± 1.6	1.0 ± 0.0	32.5 ± 13.6
	90	2	11.1 ± 1.1	2.6 ± 2.2	1.0 ± 0.1	33.5 ± 15.8
	180	2	12.8 ± 1.3	2.5 ± 1.8	1.2 ± 0.3	36.8 ± 21.4
Quartz DQ12	0	2	10.3 ± 2.4	1.6 ± 0.2	0.8 ± 0.1	37.6 ± 23.6
	22.5	2	9.1 ± 0.9	2.4 ± 1.6	1.0 ± 0.2	31.9 ± 17.2
	45	2	11.7 ± 1.9	1.2 ± 0.6	1.0 ± 0.2	45.4 ± 23.3
	90	2	27.2 ± 4.0***	3.6 ± 0.7	1.1 ± 0.2	90.7 ± 41.4
	180	2	64.2 ± 3.4***	12.5 ± 1.2***	1.3 ± 0.7	233.0 ± 93.1***
LUDOX® SM	0	1	15.1 ± 4.3	1.6 ± 0.4	–	–
	11.25	1	31.9 ± 11.4*	2.8 ± 0.6	–	–
	22.5	1	46.0 ± 13.0***	4.6 ± 1.0	–	–
	45	1	79.0 ± 22.0***	9.5 ± 2.4	–	–
	90	1	98.8 ± 17.3***	15.2 ± 1.2	–	–
	0	2	10.3 ± 2.4	1.6 ± 0.2	0.8 ± 0.1	37.6 ± 23.6
	11.25	2	14.4 ± 4.2	2.0 ± 0.4	0.8 ± 0.1	47.2 ± 14.3
	22.5	2	40.2 ± 7.7***	2.7 ± 4.1	0.8 ± 0.2	165.5 ± 47.8*
	45	2	77.6 ± 9.2***	11.9 ± 2.4***	0.8 ± 0.4	479.1 ± 250.8***
	90	2	89.3 ± 5.1***	14.1 ± 2.1***	0.8 ± 0.7	541.4 ± 111.1***
LUDOX® TM-50	0	1	15.1 ± 4.3	1.6 ± 0.4	–	–
	11.25	1	15.6 ± 3.8	1.4 ± 0.3	–	–
	22.5	1	19.9 ± 7.5	1.7 ± 0.7	–	–
	45	1	50.5 ± 13.3***	4.2 ± 0.9**	–	–
	90	1	86.3 ± 6.6***	9.5 ± 1.7***	–	–
	0	2	10.3 ± 2.4	1.6 ± 0.2	0.8 ± 0.1	37.6 ± 23.6
	11.25	2	14.8 ± 6.2	1.6 ± 0.7	0.8 ± 0.1	110.8 ± 26.0
	22.5	2	42.8 ± 7.9***	4.2 ± 0.9*	0.9 ± 0.3	299.4 ± 4.5***
	45	2	75.7 ± 8.8***	12.1 ± 1.9***	1.1 ± 0.7	449.5 ± 99.2***
	90	2	84.8 ± 12.7***	17.4 ± 1.9***	1.0 ± 0.8	202.8 ± 120.2**
CAB-O-SIL® S17D	0	1	15.1 ± 4.3	1.6 ± 0.4	–	–
	11.25	1	15.6 ± 5.4	1.7 ± 0.5	–	–
	22.5	1	53.0 ± 15.2***	6.6 ± 1.5***	–	–
	45	1	92.7 ± 14.2***	15.2 ± 1.3***	–	–
	90	1	95.5 ± 15.8***	15.5 ± 1.2***	–	–
	0	2	9.5 ± 1.8	1.3 ± 0.4	0.8 ± 0.2	49.3 ± 19.5
	11.25	2	82.8 ± 3.3***	17.4 ± 1.0***	1.5 ± 0.2	537.2 ± 210.2***
	22.5	2	81.3 ± 7.3***	15.0 ± 0.2***	2.2 ± 0.5***	412.9 ± 15.7***
	45	2	63.9 ± 8.0***	11.8 ± 0.4***	2.8 ± 0.9***	242.8 ± 7.5***
	90	2	33.2 ± 3.3***	7.2 ± 0.2***	2.8 ± 1.3***	92.5 ± 25.4
CAB-O-SIL® (E)L-90	0	1	15.1 ± 4.3	1.6 ± 0.4	–	–
	11.25	1	14.1 ± 4.2	1.5 ± 0.3	–	–
	22.5	1	18.5 ± 5.6	2.0 ± 0.7	–	–
	45	1	81.9 ± 22.8***	12.6 ± 3.6***	–	–
	90	1	101.7 ± 15.8***	23.6 ± 0.8***	–	–
	0	2	9.5 ± 1.8	1.3 ± 0.4	0.8 ± 0.2	49.3 ± 19.5
	11.25	2	24.6 ± 10.7***	3.9 ± 1.7*	1.0 ± 0.1	128.2 ± 38.6
	22.5	2	75.7 ± 4.7***	15.3 ± 1.8***	0.9 ± 0.4	252.5 ± 22.4***

(Continued)

TABLE 3 | Continued

Concentration	[μg/ml]	Protocol	LDH [% pos. Control] Mean ± SD	GLU % pos. Control Mean ± SD	H_2O_2 [μmol/L] Mean ± SD	TNFα [pg/ml] Mean ± SD
	45	2	80.5 ± 5.1***	18.6 ± 0.9***	1.0 ± 0.7	216.7 ± 19.4***
	90	2	80.6 ± 6.3***	18.1 ± 0.4***	1.0 ± 0.9	175.5 ± 23.3*
AEROSIL® 50	0	1	13.2 ± 4.6	1.4 ± 0.2	–	–
	11.25	1	12.0 ± 4.1	0.9 ± 0.5	–	–
	22.5	1	12.5 ± 3.7	1.9 ± 0.1	–	–
	45	1	23.2 ± 8.2	1.9 ± 0.5	–	–
	90	1	90.0 ± 9.7***	3.5 ± 1.4	–	–
	0	2	9.5 ± 1.8	1.3 ± 0.4	0.8 ± 0.2	49.3 ± 19.5
	11.25	2	11.1 ± 4.1	1.8 ± 0.4	0.9 ± 0.2	58.9 ± 17.0
	22.5	2	41.8 ± 13.4***	7.0 ± 2.4***	1.0 ± 0.3	188.0 ± 32.1**
	45	2	87.4 ± 5.7***	18.3 ± 1.9***	1.3 ± 0.8	289.8 ± 42.9***
	90	2	87.5 ± 0.6***	19.0 ± 1.6***	1.4 ± 1.1	226.9 ± 40.7***

*Mean values and standard deviations from three independent experiments. LDH, lactate dehydrogenase; GLU, glucuronidase; ROS, reactive oxygen species (H_2O_2); TNF, tumor necrosis factor α (TNFα). Values significantly different from cell control are marked by asterisks (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Two-way analyses of variance (ANOVA) and Dunnett's test were used to compare means from the control and treated groups. n.m., values not measured.*

TABLE 4 | EC_{50} values of LDH and GLU release obtained with Protocol 1 or 2.

		EC50 LDH release [μg/ml]			EC50 GLU release [μg/ml]		
		Protocol 1	Protocol 2	Fold change	Protocol 1	Protocol 2	Fold change
LUDOX® SM	EC_{50}	26.3	26.1	1.0	37.5	34.2	1.1
	R^2	0.9	1.0		0.9	0.9	
LUDOX® TM-50	EC_{50}	44.3	24.4	1.8	49.9	36.4	1.4
	R^2	0.9	1.0		0.9	1.0	
CAB-O-SIL® S17D*	EC_{50}	23.2	–	–	25.0	–	–
	R^2	0.9	–		1.0	–	
CAB-O-SIL® (E)L-90	EC_{50}	37.1	14.2	2.6	44.7	15.9	2.8
	R^2	0.9	1.0		1.0	1.0	
AEROSIL® 50	EC_{50}	58.0	24.1	2.4	53.6	26.3	2.0
	R^2	1.0	1.0		0.6	1.0	

**No curve could be fitted to the data from Protocol 2. All EC_{50} values were calculated for a 95% confidence interval.*

and number of the small (<500 nm) particles. SAS particles can hardly be viewed inside cells by conventional light microscopy, and the measurement of silicon inside cells requires advanced methods (28) and was not carried out in this investigation. Therefore, indirect methods may be used to estimate the SAS particle concentration inside cells: Particle sedimentation models (44) have shown that SAS with a primary particles sizes of 15–50 nm resulted in settled fractions of 15.8–55% under cell culture conditions (42). The direct measurement with high-resolution ICP-MS showed that 20 or 60%, for example, of pyrogenic AEROSIL® 380 or precipitated SIPERNAT® 160 were associated with NR8383 cells (28). While these data show that the uptake of the small SAS particle fraction is most likely incomplete, settled agglomerates of both untreated (e.g., CAB-O-SIL® (E)L-90) and surface-treated highly hydrophobic particles (e.g., HDK® H2000) were found to be completely cleared from the bottom of the cell culture vessels. This observation shows that organosilane coating does not prevent the ingestion of surface-treated SAS by NR8383macrophages *per se* and suggests that an uptake of the diffusible fraction of hydrophobic SAS is highly likely as well. Taken together, the missing effect of surface-treated highly hydrophobic SAS on LDH, GLU, and TNFα is unlikely caused by a lowered particle uptake. Instead, surface properties need to be considered.

Two aspects of the surface treatment of SAS with organosilanes shall be discussed. The first aspect is the covalent chemical modification itself, which certainly lowers the number of accessible silanol groups. These functional groups have been identified as reactive sites especially in crystalline silica (41) where they can be neutralized with polyvinylpyridine-N-oxide (PVPNO) *in vivo* (29, 30, 33). While the capping of silanol groups may convincingly explain the reduction of acute bioactivity of surface-treated SAS, dissolution rate and long-term stability of surface-modified SAS may be involved as well, especially dissolution rates depend not only on the type of coating but also on curvity and other factors (45, 46) of the core material, such that coated material may display an altered dissolution rate in biological fluids (46). Although the solubility of SAS is lowest at physiological pH and also at pH 4.5 which is typically found inside phagolysosomes (45, 46), the persistence and stability of

TABLE 5 | Effects of surface-treated SAS on NR8383 cells.

Concentration	[μg/ml]	Protocol	LDH [% pos. Control] Mean ± SD	GLU % pos. Control Mean ± SD	H_2O_2 [μmol/L] Mean ± SD	TNFα [pg/ml] Mean ± SD
AEROSIL® R 816	0	1,2#	13.2 ± 4.6	1.4 ± 0.3	0.8 ± 0.2	49.3 ± 19.5
	11.25	1,2#	13.2 ± 5.4	0.9 ± 0.4	1.0 ± 0.2	38.2 ± 12.1
	22.5	1,2#	14.2 ± 4.6	1.3 ± 0.4	1.0 ± 0.2	53.0 ± 8.4
	45	1,2#	17.6 ± 3.7	2.7 ± 0.7	1.0 ± 0.4	164.7 ± 36.6*
	90	1,2#	41.8 ± 14.8***	15.9 ± 2.5***	1.2 ± 0.8	201.2 ± 16.6**
CAB-O-SIL® TGC413TRD	0	1	11.7 ± 2.0	0.9 ± 0.9	0.8 ± 0.1	47.5 ± 38.0
	11.25	1	9.4 ± 2.8	1.4 ± 0.4	0.5 ± 0.0	58.2 ± 54.8
	22.5	1	9.9 ± 1.2	1.2 ± 0.2	0.3 ± 0.1	56.0 ± 50.2
	45	1	11.9 ± 1.5	1.4 ± 0.5	0.0 ± 0.4	61.8 ± 53.3
	90	1	13.1 ± 0.8	1.7 ± 0.7	n.m.	61.3 ± 53.1
CAB-O-SIL® TS610	0	1	11.7 ± 2.0	0.9 ± 0.9	0.8 ± 0.1	47.5 ± 38.0
	11.25	1	9.6 ± 0.5	1.1 ± 0.2	0.5 ± 0.1	60.3 ± 63.3
	22.5	1	7.0 ± 5.4	1.3 ± 0.2	0.4 ± 0.2	56.3 ± 57.4
	45	1	11.4 ± 1.8	0.7 ± 0.1	0.1 ± 0.2	60.7 ± 53.9
	90	1	11.8 ± 0.7	1.1 ± 0.8	n.m.	61.6 ± 56.7
CAB-O-SIL® TS720	0	1	14.0 ± 2.3	1.6 ± 0.6	0.9 ± 0.1	52.2 ± 25.5
	11.25	1	10.0 ± 1.1	1.2 ± 0.1	0.8 ± 0.0	49.2 ± 22.5
	22.5	1	10.5 ± 0.7	1.7 ± 0.2	0.7 ± 0.1	44.3 ± 23.3
	45	1	12.7 ± 0.9	1.5 ± 0.3	0.4 ± 0.3	47.1 ± 25.4
	90	1	15.8 ± 1.0	1.5 ± 0.8	0.2 ± 0.3	47.4 ± 25.7
HDK® H15	0	1	11.7 ± 2.0	0.9 ± 0.9	0.8 ± 0.1	47.5 ± 38.0
	11.25	1	9.0 ± 1.6	1.2 ± 0.2	0.6 ± 0.1	56.6 ± 45.4
	22.5	1	10.0 ± 1.5	1.0 ± 0.0	0.2 ± 0.1	51.3 ± 32.6
	45	1	10.8 ± 1.6	1.2 ± 0.4	n.m.	57.7 ± 47.5
	90	1	11.6 ± 1.3	1.6 ± 0.5	n.m.	58.3 ± 47.8
HDK® H2000	0	1	14.0 ± 2.3	1.6 ± 0.6	0.9 ± 0.1	52.2 ± 25.5
	11.25	1	10.4 ± 0.2	0.8 ± 0.2	0.9 ± 0.0	43.1 ± 27.8
	22.5	1	10.8 ± 0.7	1.0 ± 0.2	0.7 ± 0.2	43.6 ± 28.8
	45	1	12.8 ± 1.2	1.4 ± 0.1	0.7 ± 0.5	44.8 ± 29.6
	90	1	17.4 ± 1.1	2.0 ± 0.5	0.8 ± 0.6	39.5 ± 31.6
AEROSIL® R 504	0	1	11.7 ± 2.0	0.9 ± 0.9	0.8 ± 0.1	47.5 ± 38.0
	11.25	1	9.3 ± 1.6	0.9 ± 0.5	0.6 ± 0.1	51.1 ± 47.7
	22.5	1	9.7 ± 1.1	0.9 ± 0.2	0.3 ± 0.1	58.8 ± 49.1
	45	1	10.4 ± 2.9	1.3 ± 0.3	0.1 ± 0.1	56.8 ± 43.8
	90	1	26.7 ± 6.0***	2.8 ± 1.0	0.2 ± 0.2	65.6 ± 52.5
AEROSIL® R 711	0	1	14.0 ± 2.3	1.6 ± 0.6	0.9 ± 0.1	52.2 ± 25.5
	11.25	1	11.6 ± 0.6	1.4 ± 0.3	0.6 ± 0.1	57.2 ± 23.4
	22.5	1	13.7 ± 2.7	1.5 ± 0.4	0.4 ± 0.2	55.7 ± 26.7
	45	1	17.6 ± 4.2	1.8 ± 0.6	0.1 ± 0.5	58.5 ± 30.1
	90	1	19.7 ± 3.9	1.5 ± 0.2	n.m.	56.3 ± 33.9
SIPERNAT® D 17	0	1	14.0 ± 2.3	1.6 ± 0.6	0.9 ± 0.1	52.2 ± 25.5
	11.25	1	11.4 ± 0.5	1.4 ± 0.2	0.7 ± 0.2	50.2 ± 30.4
	22.5	1	12.2 ± 1.0	1.4 ± 0.3	0.5 ± 0.2	51.4 ± 35.4
	45	1	14.4 ± 2.4	1.6 ± 0.2	0.3 ± 0.4	54.6 ± 39.0
	90	1	20.0 ± 1.8	2.5 ± 0.7	0.1 ± 0.5	53.2 ± 36.1

*Mean values and standard deviations from (n = 3) experiments. LDH, lactate dehydrogenase; GLU, glucuronidase; ROS, reactive oxygen species (H_2O_2); TNF α, tumor necrosis factor α. Values significantly different from cell control are marked by asterisks (*P ≤ 0.05, **P ≤ 0.01, ***P ≤ 0.001). Slightly negative values (n.v.) resulting from color interference and background subtraction were omitted. #Values for H_2O_2 and TNFα were obtained with Protocol 2.*

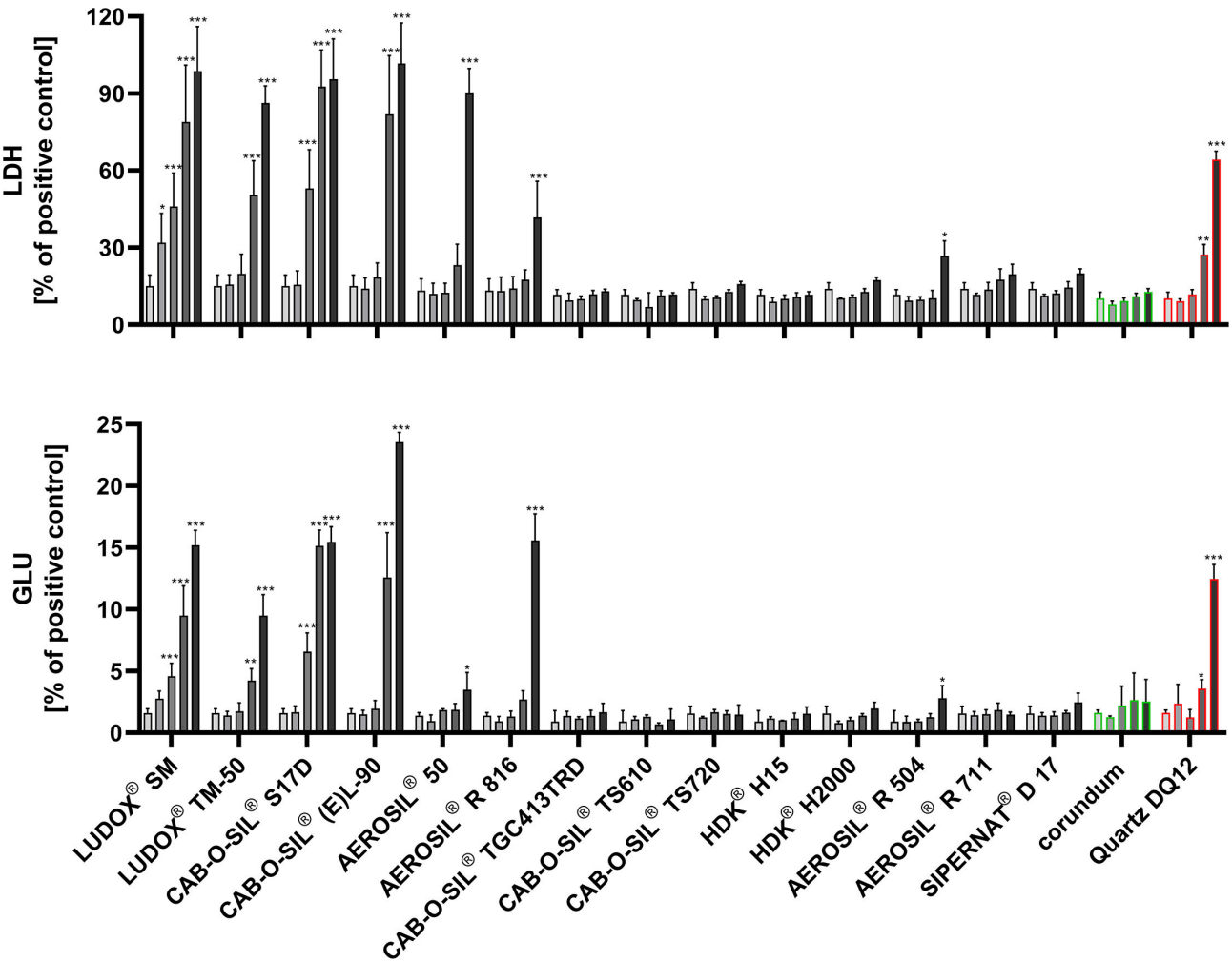

FIGURE 3 | Comparison of the *in vitro* response of NR8383 alveolar macrophages to untreated and surface-treated SAS. All SASs were dispersed with Protocol 1 (columns from left to right: 0, 11.25, 22.5, 45, and 90 μg/ml). (Upper panel) lactate dehydrogenase activity (LDH); (lower panel) glucuronidase activity (GLU); LDH and GLU values are shown relative to the triton X-100 positive control. Untreated SAS: LUDOX® SM, LUDOX® TM 50, CAB-O-SIL® S17D, CAB-O-SIL® (E)L-90, AEROSIL® 50. Surface-treated SAS: AEROSIL® R 816, CAB-O-SIL® TGC413TRD, CAB-O-SIL® TS610, CAB-O-SIL® TS720, HDK® H15, HDK® H2000, AEROSIL® R 504, AEROSIL® R 711, SIPERNAT® D 17. Corundum and quartz DQ12 particles (0, 22.5, 45, 90, and 180 μg/ml) were used as negative and positive controls. Values significantly different from cell control are marked by asterisks (*$P \leq 0.05$, **$P \leq 0.01$, ***$P \leq 0.001$).

the different covalently bound organic residues (see **Table 1**) in biological systems is an open question. A former 90 days inhalation study on rats reported that the organosilane-treated AEROSIL® R 974, which is highly similar to the CAB-O-SIL® TS610 used here, persisted longer in the lung than non-treated SAS (47). At the same time, AEROSIL® 200, AEROSIL® R 974 failed to attract neutrophilic granulocytes directly after exposure indicating absence of inflammatory reaction. Because an erroneously reported pro-fibrotic effect of AEROSIL® R 974 was refuted later (17), the appropriate interpretation of the study of Reuzel et al. (47) is that organosilane treatment can in fact dampen the transient pro-inflammatory effects of untreated SAS in the lung. However, it should be kept in mind that the *in vitro* test as carried out here describes the acute situation only and awaits further confirmation as to whether organosilane coating remains stable under physiological conditions.

In an intratracheal instillation study, a 15-nm-sized colloidal silica and its phosphonated variant were compared for inflammatory effects and local phospholipid distribution after intratracheal instillation of 0.36 mg per rat lung (43). Also, this surface treatment abolished the pro-inflammatory effect of the colloidal silica. In the accompanying *in vitro* study, cytotoxic and pro-inflammatory effects on NR8383 cells were clearly diminished, though not fully suppressed. In this context, it is important to note that phosphonated silica particles were hydrophilic, suggesting that hydrophobicity adds to the diminution of SAS effects.

The cellular mode of actions of SAS has been extensively studied by Karkossa et al. (48). Using two Pluronic F108 dispersed hydrophobic AEROSIL variants coated with organosilane (TMS2 and TMS3), they showed that the hydrophobic surface treatment abolished any effect on both the proteome or metabolome of RLE-6TN cells at a concentration of 10 $\mu g/cm^2$. This result is perfectly in line with our findings and underlines the biocompatibility of organosilane-treated SAS.

The second aspect of the hydrophobic surface treatment concerns the small protein concentration used to disperse hydrophobic particles in cell culture media. It is known that serum proteins, or more precisely the protein corona formed thereof, lower the bioactivity of SAS *in vitro* (24, 25, 27). However, we found that this effect is not uniform for all SAS (28). While the addition of a serum concentration typically used in cell cultures (10%, v/v) clearly inhibited the bioactivity of a SIPERNAT® 50 (a precipitated SAS) without influencing uptake and subcellular distribution of particles, the latter aspects were different for AEROSIL® 50 (a pyrogenic SAS), thus complicating the interpretation of *in vitro* findings. Of note, low concentrations of BSA (<1% w/v) hardly reduced the bioactivity of hydrophilic SAS (data not shown) such that the effects of 0.05% BSA (Protocol 1) are deemed unlikely to effectively inactivate hydrophilic SAS. Being aware that protein adsorption may be higher or different for hydrophobic SAS, we conclude that the uniform reduction of bioactivity found here for surface-treated SAS primarily relies on the capping/chemical modification of silanol groups with only a small, if any, supplementation due to limited BSA binding.

To further unravel the possible effects of Protocol 1 on SAS effects, we also subjected the hydrophilic SAS to this protocol. Generally, there was a rightward shift of the dose–response curves for LDH and GLU quantitatively reflected by increased EC_{50} values (**Supplementary Figures S6, S7** and **Table 4**). We hypothesize that this mitigation of bioactivity may have been caused by a reduction of aggregate/agglomerate size which likely lowers particle settling and, therefore, reduces the availability of particles at the bottom of the culture dish. In fact, CAB-O-SIL® S17D and CAB-O-SIL® EL-90 showed a reduced HD of aggregates/agglomerates in F-12K medium. It is furthermore in line with this hypothesis that shifts of the EC_{50} values upon Protocol 1 were hardly measurable for LUDOX® TM-50 and LUDOX® SM, both of which are colloidal SAS whose single monodisperse particles were not degraded by increased USD energy. We cannot exclude that the combined ethanol/BSA pre-treatment of Protocol 1 contributes to the mitigation of the bioactivity of hydrophilic SAS. However, the missing effect of Protocol 1 on the bioactivity of LUDOX® SM argues against this assumption. A major difference between Protocol 1 and 2 concerned CAB-O-SIL® S17D: while Protocol 1 led to monophasic dose–response curves of CAB-O-SIL® S17D (**Figure 3**), biphasic dose–response curves were obtained upon protocol 2 for the release of LDH, GLU, and TNFα (**Table 3**). At least for TNFα, similar biphasic responses to SAS have been found earlier and have been interpreted as a rapid destruction of the cells and/or antigens due to elevated particle concentrations (37). Although this may apply also for enzymes such as LDH and glucuronidase, further analysis is needed to understand the biphasic effects of CAB-O-SIL® S17D. Overall, changes in particle size may account for the EC_{50} shift of untreated SAS upon Protocol 1.

In any case, it should be kept in mind that—regardless of the dispersion protocol used—non-treated but also the slightly hydrophilic surface-treated SAS AEROSIL® R 816 was far more bioactive than surface-treated highly hydrophobic SAS. Unlike untreated SAS, all surface-treated hydrophobic SASs were classified as passive materials (**Supplementary Table S1**), following the previously established evaluation criteria which consider the BET surface and the number of positive test results in the alveolar macrophage assay (34). While organosilane treatment can also dampen the bioactivity of crystalline silica (33, 49), we propose that the mechanism by which the *in vitro* bioactivity of SAS is ruled out not only involves the inactivation of silanol groups but also benefits from hydrophobic molecules at the particle surface.

DATA AVAILABILITY STATEMENT

The original contributions presented in the study are included in the article/**Supplementary Material**, further inquiries can be directed to the corresponding author/s.

AUTHOR CONTRIBUTIONS

MW and TS took a leading role in project administration and prepared the manuscript. AV was involved in toxicity testing, particle size measurements, and preparation of the manuscript. JN served as scientific advisor and prepared the manuscript. NK was involved in project planning, funding acquisition, and preparation of the manuscript. All authors contributed to the article and approved the submitted version.

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SUPPLEMENTARY MATERIAL

The Supplementary Material for this article can be found online at: https://www.frontiersin.org/articles/10.3389/fpubh.2022.902799/full#supplementary-material

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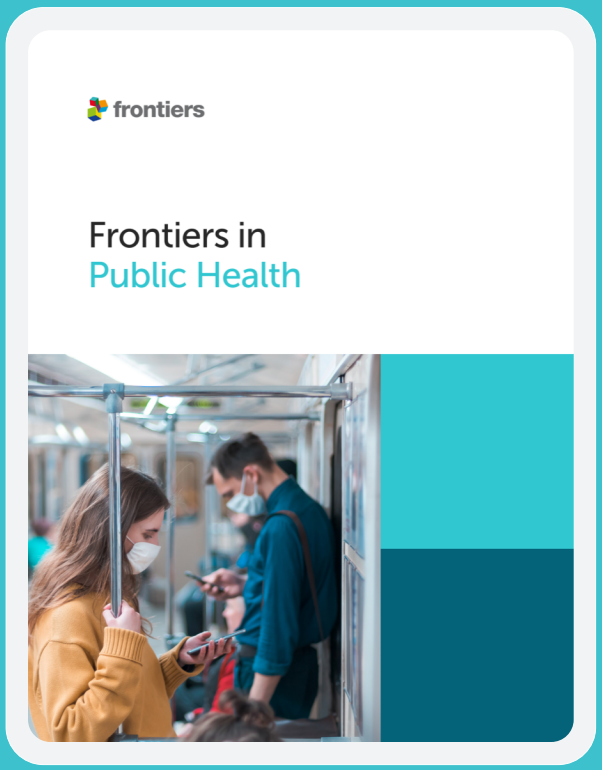

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