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PERSPECTIVE

Ins and Outs in Environmental and Occupational Safety Studies of Asthma and Engineered Nanomaterials

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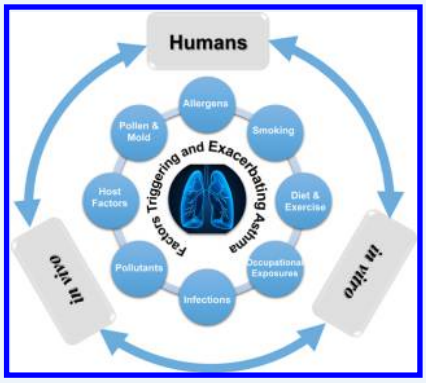
ABSTRACT: **According to the Centers for Disease Control and Prevention, approximately 25 million Americans suffer from asthma. The disease total annual cost is about $56 billion and includes both the direct and indirect costs of medications, hospital stays, missed work, and decreased productivity. Air pollution with xenobiotics, bacterial agents, and industrial nanomaterials, such as carbon nanotubes, contribute to the exacerbation of this condition and are a point of particular attention in environmental toxicology as well as in occupational health and safety research. Mast cell degranulation and activation of Th_2 cells triggered either by allergen-specific immunoglobulin E (IgE) or by alternative mechanisms, such as locally produced neurotransmitters, underlie the pathophysiological process of airway constriction during an asthma attack. Other immune and non-immune cell types, including basophils, eosinophils, Th_1, Th_{17}, Th_9, macrophages, dendritic cells, and smooth muscle cells, are involved in the inflammatory and allergic responses during asthma, which, under chronic conditions, may progress without mast cells, the key trigger of the acute asthma attack. To decipher complex molecular, cellular, and genetic mechanisms, many researchers have attempted to develop *in vitro* and *in vivo* models to study asthma. Herein, we summarize the advantages and disadvantages of various models and their applicability to nanoparticle evaluation in asthma research. We further suggest that a framework for both *in vitro* and *in vivo* methods should be used to study the impact of engineered nanomaterials on asthma etiology, pathophysiology, and treatment.**



Asthma is an inflammatory disorder occurring in the lungs as a result of repeated immediate-type hypersensitivity and late-phase allergic reactions to the inhaled allergen(s). It is characterized by repeated and reversible airway obstruction, chronic bronchial inflammation, hyperreactivity to bronchoconstrictors, and smooth muscle cell hypertrophy of bronchi. Asthma often coexists with chronic obstructive pulmonary disorder (COPD). The combination of these conditions frequently leads to irreversible airway obstruction, morbidity, and even death. According to the Centers for Disease Control and Prevention (CDC), asthma affects ~8% of Americans (~25 million people).[1] Other reports suggest that 15.7% of asthma cases among adults in the United States (~1.9 million cases) are work related.[2] Overall, about 300 million people worldwide suffer from this disorder,[3] and 11% of these cases are associated with workplace conditions, such as exposure to fumes, gases, or dust.[4] The asthma prevalence rate is similar across industrialized countries but is lower in nations with a less developed industrial base. According to the Global Initiative for Asthma (GINA), the prevalence rate has increased by 50% every decade and is accountable for 250,000 deaths annually.[3] Another organization whose research efforts focus on this disorder, the Asthma and Allergy Foundation of America, conveyed that direct (hospital stays and medications) and indirect (missed work hours and decreased worker productivity) annual costs of asthma in the United States total over $56 billion.[5] These data emphasize the impact of the disorder on modern society and justify extensive research in this area.

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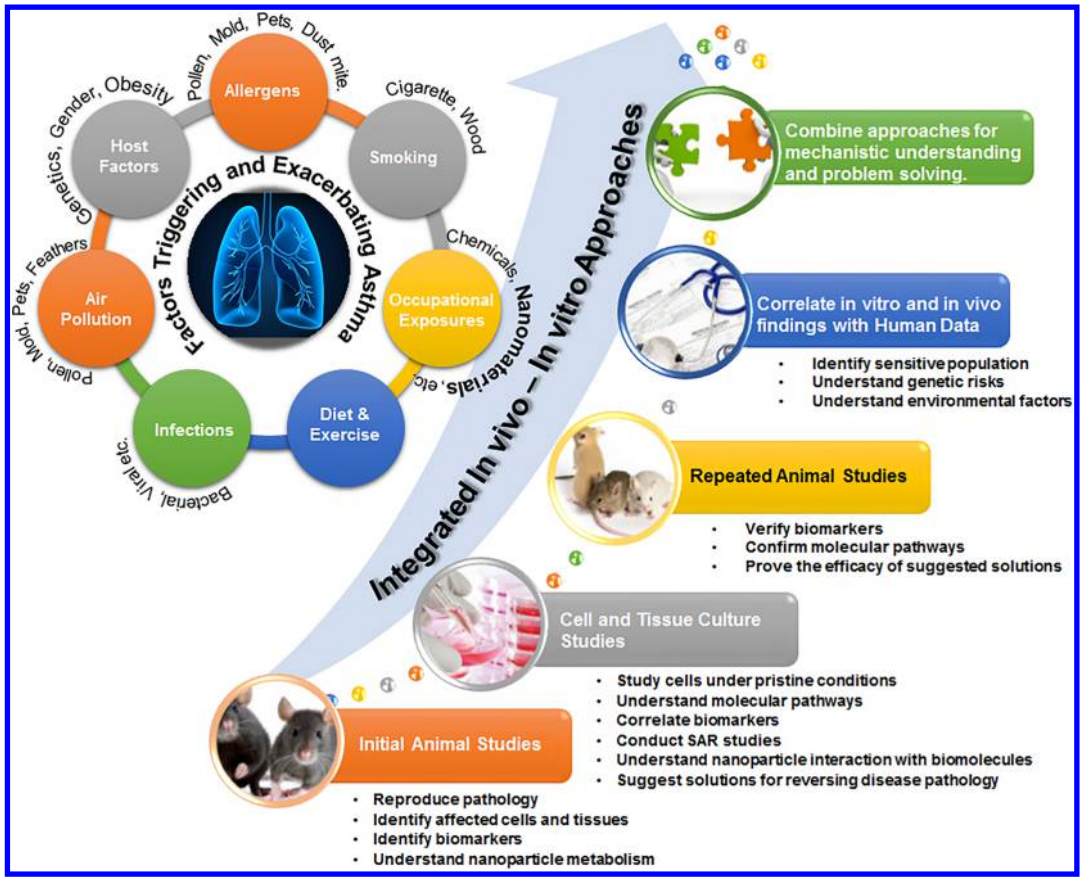

Figure 1. Systemic approaches required for evaluating the asthmatic effects of nanomaterials exposure.

Factors triggering and exacerbating asthma include both immune (*i.e.*, allergen-specific immunoglobulins E, IgE) and non-immune (*e.g.*, cold, certain drugs, exercise) stimuli (Figure 1). Allergen-specific IgE binding to mast cells and basophils and activation of Th_2 cells reacting to allergens trigger the pathophysiologic chain of events culminating in an allergic asthma attack. Mast cell degranulation and release of cytokines by Th_2 cells result in recruitment of progressively increasing numbers of T-cells, eosinophils, and basophils. Although the T-cell population in asthma is dominated by Th_2 cells, other T-cell subtypes are also involved, including Th_1, Th_{17}, and Th_9 cells. The infiltrating airway lymphocytes continue to stimulate the inflammatory response by secreting cytokines and chemokines, which also support the cellular infiltration into the airway. The inflammatory response finally results in hypertrophy of smooth muscles, thickening of basement membranes, hyperplasia of mucous glands, and continuing cellular infiltration. These alterations of the airway, or airway remodeling, may lead to fibrosis and irreversible airway obstruction in some patients. Collectively, cytokines and mediators secreted by lymphocytes trigger hypertrophy and hyperreactivity of bronchial smooth muscle cells, whereas those released by eosinophils, basophils, and mast cells result in the constriction of airway smooth muscles.

In contrast to the engagement of different cell populations, the molecular mechanisms underlying allergic and non-allergic asthma are insufficiently understood and thus represent major gaps in deciphering the pathogenesis of the disease. One such gap is related to the role of environmental factors and conditions. Specifically, it is well-established that air pollution with xenobiotics, bacterial ligands (*e.g.*, lipopolysaccharide), and some industrial nanomaterials (carbon nanotubes, CNTs; carbon black; and some diesel exhaust particles) have a significant influence on the development and progression of asthma. Moreover, the effects of these environmental factors also depend on the age of exposed individuals. One hypothesis, termed the hygiene hypothesis, proposes that more frequent bacterial infections, lower air pollution with xenobiotics and diesel exhaust, and higher exposure to commensal flora in a farm setting lead to regulated maturation of the immune system and promote less frequent occurrence of asthma and other Th_2-driven responses to non-environmental antigens in less industrialized areas. The same hypothesis states that a cleaner bacterial environment accompanied by greater air pollution in industrialized areas are responsible for the higher prevalence of atopic diseases in these regions. However, it is also known that bacterial and viral infections of the respiratory tract predispose a person to the development of asthma and exacerbate pre-existing asthma. The hygiene hypothesis does not explain how such infections promote Th_2 and mast cell responses or clarify how non-immune mediators (*e.g.*, cold, physical and mental stress, chemicals, medications) trigger this disease. Genetic differences may also affect susceptibility to asthma, as well as responsiveness to asthma medications. Therefore, more research is needed to understand both cellular and molecular mechanisms underlying this complex disorder.

The respiratory system represents the main route of exposure to aerosolized nanomaterials, by accident or by occupational factors. Although many studies have explored the respiratory effects of engineered nanomaterials, including CNTs, in different animal models, few publications have documented the effects of respiratory nanomaterials in experimental models

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A variety of *in vitro* and *in vivo* models have been employed to improve our understanding of asthma and the contributions of environmental nanomaterials to its pathogenesis.

of pulmonary disease. Environmental exposure in sensitized individuals is a critical inducer of an airway inflammatory response, which is a hallmark of the asthmatic lung. Although a variety of factors can initiate inflammation *via* different cellular and molecular pathways, their cumulative effects lead to increased bronchial reactivity. In the area of environmental toxicology and occupational health, engineered nanomaterials, and particularly carbon-based particles, have recently attracted significant attention as potential triggers of and contributors to asthma.[6] Due to their high aspect ratio and biopersistance, several studies have emphasized the role of CNTs in asthma (*e.g.*, see refs 7−10). A recent report demonstrated the presence of CNTs in the bronchoalveolar fluid of children with asthma.[11] Another study, comparing gene expression profiles in the blood of individuals exposed to CNTs to those of naïve workers in the same facility, revealed significant alterations in the expression of the genes implicated in diverse cellular processes such as apoptosis, cell-cycle regulation, and proliferation.[12] These findings further fueled the public alertness and triggered more thorough investigations of CNTs' involvement in asthma pathogenesis, including detailed and mechanistic *in vivo* studies.[7−12] A variety of *in vitro* and *in vivo* models have been employed to improve both the understanding of asthma and the contributions of environmental nanomaterials to its pathogenesis. The advantages and limitations of these models are discussed below.

The benefits of *in vivo* studies include the ability to account for particle biodistribution, metabolism, and repeated exposure and to mimic the chronic inhalation conditions that exist in the environment and contribute to lung inflammation and asthma (Table 1). For instance, an *in vivo* study in a model of asthma demonstrated that pulmonary exposure to carbon nanoparticles (CNPs) induces a significant release of pro-inflammatory cytokines, modulates proliferation of splenic T-cells, and alters the pattern of inflammatory cells in the lung.[13] Growing evidence demonstrates that the adverse effects of inhaled CNTs are increased under conditions of pre-existing inflammation such as in allergic asthma. Recent data revealed that exposure to CNTs exacerbates ovalbumin (OVA)-induced allergic airway inflammation in mice by causing airway fibrosis, increasing OVA-induced T-cell proliferation, and amplifying lung Th_2 cytokines and chemokines. Interestingly, induction of allergic responses exaggerated by CNTs involved activation of B-cells and was accompanied by the production of IgE in the absence of pre-exposure to allergen.[14−16] This effect is not unique to CNTs, as the exposure to another carbon-based nanomaterial, graphene oxide (GO), resulted in potentiation of OVA-triggered asthma potentially through the mechanism involving an increase in chitinase production by macrophages.[13] These and other *in vivo* studies in animal models suggest that humans with allergic asthma may potentially be more susceptible to the enhanced immune responses and airway remodeling induced by carbon-based nanomaterials than animals. However, due to genetic diversity between different species and even different strains of animals of the same species, as well as differences in the immune responses elicited

Table 1. Advantages and Limitations of *In Vitro* and *In Vivo* Methods Used To Study the Role of Engineered Nanomaterials in the Development of Asthma

in vitro		*in vivo*	
advantages	limitations	advantages	limitations
able to single out particular cell type	does not account for particle biodistribution and homing	able to account for particle biodistribution	often does not consider the real nanoparticle concentrations in occupational setting
enables deciphering specific molecular pathway(s)	does not account for particle metabolism and degradation	able to account for metabolism and degradation	interspecies immune system differences between rodents and humans make extrapolation of data difficult
less time-consuming	hard to account for mechanisms involving multiple cellular players	enables repeated exposure	difficult to model irregular nature of repeated exposure, especially in the context of other environmental factors
less cost-intensive	cannot account for relevant repeated exposure	enables more close mimicking chronic inhalation	does not account for genetic polymorphisms
high-throughput	low predictive value	enables complex interactions between various cell types	more time-consuming
ability to test human cells and biological fluids relevant to disease		enables modeling of different pulmonary diseases	more cost-intensive
possibility of testing exact doses and concentrations		enables working with transgenic and knockout animals to study specific cell types or molecular pathways	low-throughput
opportunity of long-term cultures and cell lines		enables evaluation of human cells in humanized mouse models	high possibility of lung overload during intratracheal instillation
cell-specific testing of specific inhibitors, antagonists and antibodies		enables testing different routes of administration	difference in biodistribution to secondary organs between inhalation and intratracheal instillation

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by mice and humans, extrapolation of *in vivo* data to the human population is often limited, and it is still unknown whether carbon nanotubes can cause or exacerbate asthma in humans.

Additional technical nuances have to be considered when analyzing and interpreting complex *in vivo* results. Specifically, redistribution of airborne particles from the lungs to other organs and tissues depends on the physicochemical properties of nanomaterials, including composition, size, shape, surface properties, and aspect ratio.[17−19] Routes of nanoparticle entry into the body, their biodistribution, and their metabolism may also contribute significantly to the test results. Administration routes (*e.g.*, inhalation *versus* intratracheal instillation) may affect the outcomes even in the same animal strain and for the same nanomaterial. For example, inhaled iridium nanoparticles were found in the lung and other organs (liver, spleen, brain, and heart) of experimental rats.[20,21] In contrast, these nanomaterials administered *via* intratracheal instillation were not found in secondary organs.[22] The potential explanation for the difference was associated with their partial ingestion after the inhalation; as such, some of the inhaled particles may be ingested and distributed to the liver and spleen *via* gastrointestinal absorption.[21] Thus, the study design is critical for data interpretation.[21] Intratracheal instillation, however, may lead to lung overload, thus causing the exaggerated picture of pulmonary toxicity of nanomaterials administered *via* this route. The importance of the administration route and its relevance to the effects of nanoparticles on humans has recently been emphasized.[23]

Other factors of special relevance to nanoparticle exposure include changes in nanoparticle composition and physicochemical properties following particle interactions with proteins and other biomolecules and the dose. The changes in nanoparticle composition and physicochemical properties are triggered by adsorption of different biomolecules on the nanoparticle surface, biodegradation of nanomaterials *via* enzymatic machinery of inflammatory cells, and direct pro-oxidant effects of nanoparticles. Adsorption of biomolecules on the nanoparticle surface leads to the formation of so-called protein and lipid "coronas" whose composition is dynamic and changes during the redistribution of nanoparticles between different compartments in the body.[24,25] For example, it was demonstrated that different protein and lipid components of the lung surfactant, initially integrated on to the nanoparticle surface after inhalation, were replaced by plasma proteins and lipids after the nanoparticles transferred to the systemic circulation.[26−28] Such "coronas" of nanoparticles may contribute to the particle recognition as pro-asthmatic signals by different types of immune cells.[29−31] The biodegradation of nanoparticles is also essential for their role in the immune responses and pathogenesis of asthma. The major oxidative enzymatic pathways of inflammatory cells, including myeloperoxidase/NADPH oxidase of neutrophils and iNOS/NADPH oxidase of macrophages, were reported to degrade carbon-based nanomaterials catalytically.[32] In addition to destroying nanoparticles and lowering their concentrations in tissues, these oxidative effects may also lead to the appearance of oxidized proteins, lipids, and carbohydrates, thus affecting their immunogenic potential.[33] Although the exact role and significance of these metabolic transformations of nanoparticles in the pathogenesis of asthma remain to be elucidated, the ability of nanomaterials to affect other enzymatic pathways in an experimental mouse asthma model has been already reported. For example, studies of the mechanisms of up-regulated airway responsiveness following pulmonary GO exposure in an animal model of Ovalbumin (OVA) sensitization uncovered an intriguing role of pulmonary macrophage-derived chitinase and chitinase-like molecules.[13] The chitinases are hydrolytic enzymes that break down glycosidic bonds in chitin. These enzymes were found to improve allergic responses in murine models of allergy *via* a mechanism involving the increase in pulmonary macrophages, reduction in alveolar eosinophils, and decrease in serum IgE levels. Interestingly, although the GO induced up-regulation of chitinase expression in the lung, the enzymatic activity of this enzyme was decreased.[13] Based on the molecular modeling studies, the authors suggested that GO binds to the entrance of the catalytic site of chitinase, mimicking interactions with chitinase inhibitors. Thus, by interfering with chitinase, nanomaterials may restrict its role in allergic inflammation and asthma.[13]

The ability of nanoparticles to alter the activity of different enzymatic systems is not limited to chitinase and has also been reported for other enzymes, for example, α-chymotrypsin.[34] The dose of the tested nanomaterials should also be considered. It has been found that dendritic cells' (DCs) exposure to a low or medium dose of fullerenes preceding an antigen (OVA) challenge promoted the ability of DCs to stimulate OVA-specific T-cells. In contrast, a higher dose of fullerenes down-regulated antigen presentation by DCs,[35] whereas carbon nanoparticles did not affect the ability of DCs to stimulate antigen-specific T-cells.[35] These differential effects of carbonaceous nanoparticles on DCs may be associated with their capacity to alter expression of the main proteins involved in antigen processing machinery, such as immunoproteasomes.[35] These results support the notion that nanoparticles may modify the ability of antigen-presenting cells to process and to present antigens (allergens) and, thus, to control Th_1/Th_2 balance in exposed individuals.

Finally, nanoparticles containing transition metals such as biologically common Fe, Cu, Mn, Cr, Ni, and Co as well as those normally not present in the body (Ce) can all affect the redox environment and cause oxidative stress and injury. In the context of asthma and Th_2 inflammation, pro-oxidant conditions are essential factors that influence exacerbations of asthma episodes and worsen its general course and outcomes.[36] These effects may be related to the lowered effectiveness and levels of reduced glutathione (GSH) and GSH-driven enzymes such as GSH-peroxidases.[37] All of the conditions discussed above emphasize the complexity of biological responses to nanoparticles and nanoparticle interaction with various cells, proteins, and other macromolecules. The inherent complexity of *in vivo* responses to nanoparticle exposure limits the value and the adequacy of the *in vitro* studies.

One common criticism of animal studies is their potentially limited relevance to the human population.[23] Among the other limitations of *in vivo* models are their high cost and relatively low-throughput. *In vitro* models help to overcome some of these constraints. They offer higher throughput, are less cost intensive, and enable better insights into the contributions of individual cell types and the isolation of specific molecular pathways. Their major drawbacks include the inability to account for particle distribution and metabolism; inability to mimic repeated exposure to multiple, often fluctuating, environmental factors accurately; and do not represent relevant regulatory interactions between various cells, organs, and tissues. Altogether, these limitations lower the predictive value of *in vitro* models. To overcome the limitations of *in vitro*

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models pertinent to their inability to mimic chronic inflammation, Chortarea *et al.* suggest the use of an air−liquid interface cell exposure (ALICE) system, as reported in this issue of *ACS Nano*.[38] This system is efficient in aerosolization of both spherical and fibrous nanomaterials and permits repeated aerosol exposures of cultured bronchial epithelial cells to CNTs. Cells harvested from healthy individuals and asthmatic patients were cultured at the air−liquid interface and treated *in vitro* with an aerosolized form of CNTs at concentrations corresponding to human occupational lifetime exposures.[38] The results showed that chronic CNT exposure elicited a duration-dependent pro-inflammatory and oxidative stress response as well as a significant alteration of the mucociliary clearance mechanism in both healthy and asthmatic cultures. The latter displayed stronger and more durable long-term effects compared to healthy cells, indicating that individuals with asthma may be more prone to adverse effects from CNT exposure compared to non-asthmatic populations.[17] The results also highlight the importance of occupationally relevant subchronic exposures when *in vitro* models are used in the nanotoxicity hazard assessments. Another recently proposed *in vitro* system reconstitutes organ-level lung functions on a chip.[39] This biomimetic microsystem retains the critical functional alveolar-capillary interface of the human lung. The microdevice reproduces complex integrated organ-level responses to bacteria and inflammatory cytokines introduced into the alveolar space. The lung-mimicking model has been applied to nanotoxicology studies and has demonstrated adequate responses to silica nanoparticles regarding toxic and inflammatory reactions as well as enhanced epithelial and endothelial uptake of nanoparticles and their transport into the underlying microvascular channel.

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For better mechanistic insights into pathophysiological events during asthma development, analysis of *in vitro*−*in vivo* correlation can be performed (Figure 1). The dominant clearance mechanism of inhaled nanoparticles is particle uptake by the lung resident macrophages. As such, oxidative stress, cytokine secretion, and consequent inflammation were commonly reported in studies with inhaled nanomaterials.[40−42] Particle aggregation to larger, micron-sized materials and the presence of other environmental factors, such as endotoxin, were shown to contribute to inflammatory reactions to inhaled nanomaterials. For example, certain airborne particulates are not inflammatory alone but enhance the inflammation caused by endotoxin.[43−46] Therefore, mitigation of the inhalation toxicity of environmental nanoparticles should include control over contributing contaminants such as endotoxin. Oberdorster *et al.* suggested inclusion of several end-points (reactive oxygen species, lactate dehydrogenase leakage, protein oxidation induction, the number of polymorphonuclear neutrophils in bronchoalveolar fluid) in the study design to correlate *in vitro* and *in vivo* toxicology data.[47] Other researchers have suggested that hemolysis correlates with inflammation *in vivo*.[48] *In vitro*−*in vivo* correlation of the hemolysis tests have been demonstrated by several studies (see review by Brown *et al.*[49]). From a mechanistic standpoint, stroma-free hemoglobin can act as a peroxidase, provided a source of oxidizing equivalents is available.[50,51] Under conditions of enhanced inflammation, superoxide radicals generated by macrophages and, to a lesser extent, neutrophils[52] can dismutate to yield H_2O_2 that will feed the peroxidase activity of free hemoglobin. Once formed, this poorly controlled pro-oxidant catalytic center can cause severe oxidative stress and oxidative damage to many cells, including red blood cells, thus perpetuating release of hemoglobin and creating a vicious cycle of pro-oxidant reactions.[53]

Collectively, the available data indicate that current models do not accurately mimic the complex network of cellular, biochemical, and molecular pathways involved in the development of asthma, especially in different environmental conditions. The advantages and disadvantages of each of the mentioned approaches are summarized in Table 1. Recognizing the limitations and non-ideal nature of each of the approaches is important to arrive at accurate conclusions. Future studies would benefit from better understanding of the nanomaterial effects on various types of immune cells beyond alveolar macrophages. More profound insights into nanoparticle effects on mast cells, neutrophils, eosinophils, basophils, and multiple subsets of T-cells (Th_1, Th_2, Th_{17}, Th_9), as well as their communications with and bidirectorial regulation by macrophages and other antigen-presenting cells, is needed. Systematic approaches based on both *in vitro* and *in vivo* studies would fulfill this need (Figure 1).

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Notes

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The authors declare no competing financial interest.

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