Carbon nanotubes and pleural damage: Perspectives of nanosafety in the light of asbestos experience

Giulia M. Stella[a)]
Department of Hematological, Pneumological and Cardiovascular Sciences, Section of Pneumology, University of Pavia and Fondazione IRCCS Policlinico San Matteo, 27100 Pavia, Italy

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Carbon nanotubes are molecular-scale one-dimensional manufactured materials which display several potential applications in engineering and materials science. Burgeoning evidence demonstrates that carbon nanotubes and asbestos share comparable physical properties. Therefore carbon nanotubes might display toxic effects and the extent of the toxicity is more specifically directed to lung and pleura. These effects are related to properties of carbon nanotubes, such as their structure, length, aspects ratio, surface area, degree of aggregation, extent of oxidation, bound functional group, method of manufacturing, concentration and dose. At the present there is no global agreement about the risk of carbon nanotubes on human health and in particular on their transformation capacity. Safety concerns regarding carbon nanotubes can be ameliorated. In this context, it is important to put the known hazards of carbon nanotubes into perspective. Here is presented an overview about toxicity issues in the application of carbon nanotubes to biological systems, taking into consideration the already known asbestos-induced mechanisms of biological damages. © *2011 American Vacuum Society.* [DOI: 10.1116/1.3582324]

I. BACKGROUND

Malignant pleural mesothelioma (MPM) is an aggressive tumor with an ominous prognosis. Still lacking of effective therapeutical regimens, MPM represents a critical medical problem, with increasing incidence as a result of widespread exposure to asbestos. However, this disease has a very long latency period and might not become evident until 20–30 yr after exposure. Asbestos has been proven to induce *in vivo* chronic inflammation and carcinogenesis of pleural mesothelium. Several studies have indeed demonstrated that asbestos fibers longer than 8–20 μm and thinner than 0.25 μm are more frequently involved in pleural transformation due to their geometric properties and their pathogenic effect.[1,2] Asbestos fibers are able to initiate a number of signaling and survival pathways in mesothelial cells with overexpression of the same molecular transducers, which are known to be deregulated in pleural carcinogenesis and resistance to chemotherapy. These oncogenic pathways might be activated either by direct interaction of asbestos fibers with receptors on cell surface and interaction with integrins or by producing reactive oxygen species catalytically generated on the fiber surface or after incomplete phagocytosis. Inflammation and interaction of asbestos fibers with other cell types (e.g., macrophages) may also play a role in cytokines elaboration and deregulation of proproliferative and antiapoptotic pathways.[3]

On the other hand, carbon nanotubes (CNTs) are molecular-scale one-dimensional manufactured materials that have the potential to be used in several applications in engineering and materials science.[4] Indeed, CNTs and asbestos share comparable physical properties. Growing evidence suggests that carbon nanotubes might display toxic effects and that the extent of toxicity is more specifically directed to the lung and pleura and depends on the properties of the CNTs, such as their structure, length, aspects ratio, surface area, degree of aggregation, extent of oxidation, bound functional group, method of manufacturing, concentration, and dose. Although the origin of CNTs is highly different, a number of studies hypothesize that CNTs may induce pleural inflammation and transformation in a fashion similar to asbestos. Thus, at present, there is no global agreement about the risk of CNTs on human health and, in particular, on their transformation capacity. A better investigation of toxicokinetics and studies on the effects after chronic exposure of CNTs should be clearly prioritized before taking up any venture, mainly in the biological system.

II. BASIC CHARACTERISTICS OF CARBON NANOTUBES

Nanotechnology is the science that studies the creation of materials, tolls, and devices through control of matter at the nanometric scale. Nanomaterials can thus be defined as materials that have structural components smaller that 1 μm in at least one dimension; nanoparticles are particles with at least one dimension smaller than 1 μm and potentially as small as atomic and molecular length scales and thus they should be considered as a distinct state of matter in addition to solid, liquid, gaseous, and plasma state due to their distinct properties (large surface and quantum size effects).[5]

It is quite different to bring together single atoms (on the scale of fraction of nanometers) to create traditional solids compared to assembling single units composed of tens or hundreds of thousands of atoms to create nanostructures in which each fundamental subunit maintains its individuality. In other words, the atoms of a solid lost their identity to cooperate in determining its physical and chemical proper-

a)Electronic mail: g.stella@smatteo.pv.it

ties. The dimensions of a solid do not interfere with its properties at the macroscopic scale, but they become relevant in getting near the nanometer scale where quantistic effects impact the collective behavior of the atoms. Therefore, a nanostructured material is defined by a series of nanometric units, which, consequently, displays a hierarchical organization that defines their mutual interaction and interdependence and leads to the creation of bigger "meta units;" from this perspective, nanostructured materials can be compared to organisms of biological origin.

The importance of carbon in the "macroworld" is well-known and documented. Carbon atoms can organize and form different structures, from the planar structures of graphite to the diamond tetrahedral. Although derived by the same atom, these two allotropic forms display different—and sometimes opposite—structural (hardness and attrition elasticity) and functional (electric conductibility and color) properties. However, both structures do not use the nanotechnology approach to materials synthesis.

The discovery that carbon could form stable, ordered structures other than graphite and diamonds stimulated the search for other allotropes. Really, the interest in carbon nanotubes is a direct consequence of the synthesis of buckminsterfullerene, C_{60}, and its derivatives in 1985. Fullerenes, in fact, represent the third allotropic form of carbon. The form and stability of a molecule of 60 atoms of carbon in a shape of a polyhedral cage made of 12 pentagons and 20 hexagons have been hypothesized—based on considerations on stability and symmetry—also several years before its experimental discovery in 1985. A further key finding was achieved in 1990 when Krätschmer *et al.*[6] demonstrated that C_{60} could be produced in a simple arc-evaporation apparatus readily available in most laboratories. In 1991, Iijima[4] discovered fullerene-related carbon nanotubes using a similar evaporator. So, a nanotube (also known as buckytube) is a member of the fullerene structural family.

C_{60} should be considered the paradigm of a family of nanostructures of carbon characterized by a spherical or a tubular shape: a number of polyhedral structures featuring various shapes and dimensions, simple or concentric, can be obtained by arranging multiple hexagonal, pentagonal, and heptagonal rings of carbon; the diameter of a nanotube is determined to be on the order of few nanometers and can be up to several micrometers in length.

Nanotubes are composed of sp^2 bonds, similar to those observed in graphite and they naturally align themselves into ropes held together by the van der Waals force. CNTs are essentially of two types, namely, single-walled carbon nanotubes (SWCNTs) and multiwalled nanotubes (MWCNTs). SWCNTs were discovered in 1993 (Ref. 7) and most of them have a diameter close to 1 nm, with a tube length that may be many thousands of times larger and up to the order of centimeters.[8] The structure of a SWCNT can be conceptualized by wrapping a one-atom-thick layer of graphite (or graphene) into a seamless cylinder. Graphene sheet wraps can be represented by a pair of vector (n,m), named chiral vector, the relationship between n and m defines three categories of CNTs: (i) arm chair ($n=m$ and chiral angle=30°), (ii) zigzag ($n=0$ or $m=0$ and chiral angle=0°), and (iii) chiral (other values of n and m and chiral angles between 0° and 30°). SWCNTs feature relevant important electric properties and are excellent conductors. They were used in the development of the first intramolecular field-effect transistors and intramolecular logic gate using SWCNTs.[9] MWCNTs consist of multiple layers of graphite rolled in on themselves to form a tube shape with an interlayer spacing of 3.4 Å. The diameter of MWCNTs ranges from 1 to 50 nm, while the inner diameter is several nanometers. Two models are used to describe MWSCNTs: (i) the *Russian doll* model, where the sheets of graphite are arranged in concentric cylinders, and (ii) the *parchment* model, where a single sheet of graphite is rolled in around itself as the scroll of parchment or a rolled up newspaper.[10]

Several techniques are being employed to produce carbon nanostructures: carbon arc discharge, laser ablation, high-pressure carbon monoxide (HiP_{CO}), and chemical vapor deposition (CVD). Among them, the CVD method seems to be the most promising in terms of price/unit ratio. The arc-evaporation method produces the best quality nanotubes and involves applying a current of about 50 A between two graphite electrodes in a helium atmosphere. This results in graphite evaporation, part of which condenses on the walls of the reactor vessel and part of the cathode. Deposit on the cathode usually contains CNTs. In laser-ablation technique, intense laser pulses are used to ablate carbon target. The pulsed laser ablation of graphite in the presence of an inert gas and catalyst yields CNTs in the form of ropes or bundles of 5–20 nm in diameter and tens to hundreds of micrometers long.[11] CVD technique involves the reaction of a carbon-containing gas (e.g., methane, acetylene, ethylene, and ethanol) with a metal catalyst particle (usually cobalt, nickel, and iron) at temperatures above 600 °C. CVD technique is also emerging as a key growth technique to produce vertically aligned CNTs.[12] Although both arc discharge and laser-ablation techniques produce SWCNTs in high yields (more than 70%), they have some disadvantages: (i) tangled CNTs that are synthesized to make purification and applications of CNTs difficult and (ii) these processes rely on the evaporation of carbon atoms at temperatures higher than 3000 °C.

More importantly, all the applications of SWCNTs require pure SWCNTs, but in most cases, SWCNTs obtained through these procedures contain carbonaceous impurities, a sort of carbon soot made of amorphous carbon, fullerenes, nanoparticles, and transition metals introduced during the SWCNT synthesis. It should be highlighted that a threshold between all these structures is somehow arbitrary since the carbon organizes itself in three-dimensional structures that vary from fullerenes to parchments to CNTs without solution of continuity. Moreover, the obtained structures display a high percentage of crystalline defects, so they are often very different from the corresponding idealized models. This assemble of carbon forms can be considered as a composite nanostructured matter of which each components can be separated by the others through defined physical and chemi-

cal techniques. Methods adopted to purify SWCNTs include hydrothermal, gaseous or catalytic oxidation, nitric acid reflux, peroxide reflux, cross-flow filtration and chromatography, and chemical functionalization.

Due to their electronic behavior, CNTs are able to promote electron transfer when used as electrode materials. It has been recently demonstrated that CNTs possess a good electrocatalytic activity toward biomolecules such as dopamine and epinephrine.[13] Recent experiments suggest that CNT surfaces show enhanced electron transfer rates when used as electrodes in electrochemical reaction. Moreover, CNTs act as sensing materials in pressure, flow, thermal, gas, optical, mass, position, stress, strain, chemical, and biological sensors. In the biochemical industry, CNT-incorporated sensors are expected to bring about revolutionary changes. A model is the glucose sensing application. CNTs are also suitable as implantable sensors: implanted sensors can be used for monitoring pulse, temperature, blood glucose, and for diagnosing diseases. For example, nanotubes can be used to track glucose levels in the blood without the need for taking samples by pricking patient's fingers. Various kinds of CNT-containing sensors and biosensors are employed in a number of industrial applications, such as food industry (e.g., control of contamination of foods by bacterial pathogens), agriculture and fishing industry (as pressure sensors to uniform spraying of liquid fertilizers, insecticides, and pesticides, and as *p*H sensors for the growth of cultured fishes), as well as in manufacturing industry and security.

III. TOXICITY AND BIOPATHOLOGICAL PROFILE OF CARBON NANOTUBES

Although CNTs are widely used in several applications, yet, to date, little is known with regard to their potential to cause damage to both human health and environment. Nanotoxicology is indeed identified as the branch of toxicology that addresses the adverse health effects caused by nanoparticles.[14] In 2008, the European Union (EU) funded ENRHES (Engineered Nanoparticles: Review of Health and Environmental Safety, http://www.nmi.jrc.ec.europa.eu/project/ENRHES), a 12-month project that has performed a comprehensive and critical scientific review of the health and environment safety of fullerenes, CNTs, metal, and metal oxide nanomaterials. Based on the findings of the ENRHES reports, toxicity and toxico-kinetics of CNTs have been extensively reviewed by Johnson *et al.*[15] with a main focus on the physicochemical properties of CNTs that lead to their toxicities. The REACH regulation (Registration, Evaluation, Authorization and Restriction of Chemicals) entered into force on 2007 and is the current regulatory framework for chemical risk assessment of EU. Although REACH applies to engineered nanomaterials, the Technical Guidance Documents of the European Chemical Agency (ECHA,[16] http://www.guidance.echa.europa.eu/guidance_en.htm) for preparing risk assessment currently include very little reference to nanoparticulates. In 2007, the EU Scientific Committee on Emerging and Newly Identified Health Risks [SCENIHR (Ref. 17)] conducted an analysis in order to evaluate the applicability of existing risk assessment approaches to nanomaterials: those SCENIHR documents concluded that current methodologies are likely to be able to identify the hazards associated with the use of nanomaterials, but that modifications are required for the guidance on the assessment of risks. This was further detailed in SCENIHR (2009) focusing on the limitation in high quality exposure and dosimetry data both for humans and environment. However, exposure through medical applications was outside the scope of the assessment.

A material is defined as toxic when it displays the property to disturb, in a reversible or irreversible manner, a physiological process. It is important to be aware that all materials theoretically have a certain degree of toxicity, which is dependent on the "dose (D)," defined as the product of quantity absorbed (Q) multiplied for the time (T) of exposure ($D = Q \times T$). In general, with respect to the different effects of toxic substances, a "no-effect" exposure level should be identified. Within the no-effect interval, the body reacts to the injuries received after exposure through compensatory mechanisms. By increasing the dose, a first compensatory phase is initiated. It means that from the no-effect (which does not correspond to "no-dose") level, it could then reach the disease effect level and also the lethal effect through a panel of biologic variations related to absorption (Fig. 1 and Table I). In other words, the quantity corresponds to a threshold value or to an acceptable concentration limit, below which the toxic effects are minor and not prejudicial to health. From this perspective, suitable dose descriptors are identified from hazard studies in order to determine human no-effect levels. The standard method to identify the safety threshold for humans is to reduce the no observed adverse effect level, which corresponds to the dose with no observed effect by *in vivo* animals studies, with a safety correction factor from 10 to 100 based on the available information on toxicity. By these analyses, the acceptable daily intake can be defined and it corresponds to the daily quantity of a substance that if absorbed for the life period could be considered as lacking of effects on human health. More importantly, it should be noted with respect to carcinogens that a threshold dose cannot be easily defined since, at least theoretically, a single molecule could be enough to induce transformation.

A. Occupational exposure assessment to CNTs

In occupational settings, exposure to CNTs could occur at all phases of the material life cycle. Human exposure to CNTs is not usually expected during the synthesis phase of commercial production since this process is performed in a closed reaction chamber; it is more likely in subsequent phases where the reaction chamber is opened to recover the product or during extraction and transport of the produced materials and cleaning of the system. Workers involved in several downstream applications can be potentially at risk. The inclusion of CNTs in composites may lead to exposure when the materials are machined or drilled and during dis-

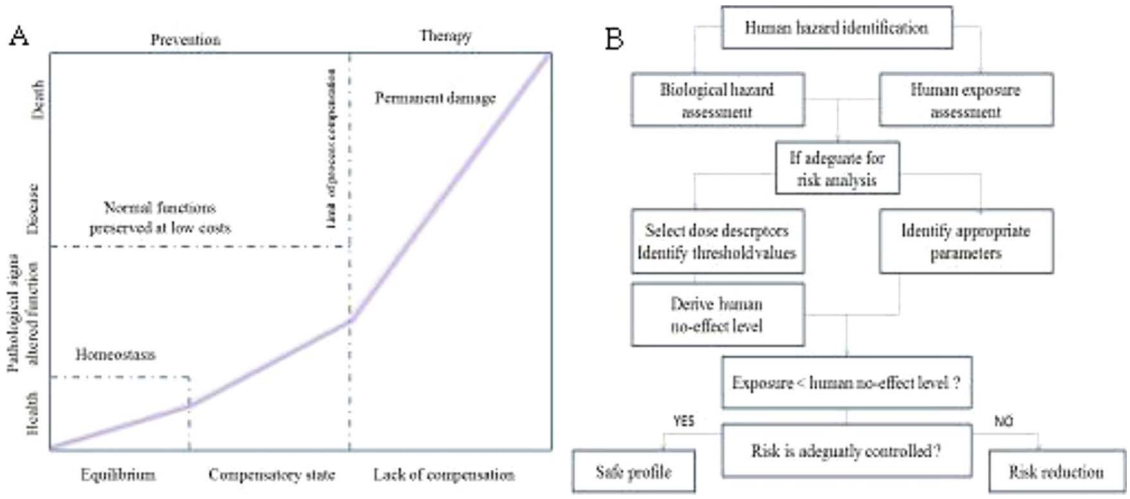

FIG. 1. (Color online) Exposure to toxic substance: biological responses and risk. (A) Process of human body adaptation to a toxic material. (B) Overall process flow of the risk characterization procedure [modified from ECHA 2008 (Ref. 17)].

posal. The use of CNTs in drug delivery system and imaging may also give rise to occupational exposure to those who manufacture and administer them.

The main exposure routes in occupational settings are known to be inhalation and dermal contact. Ingestion could also occur as a consequence of swallowing of the inhaled materials following mucociliary clearance or as a result of hand-to-mouth contact.

1. Inhalation exposure to CNTs

CNT inhalation has been deeply investigated: it has been demonstrated that subsequent to pulmonary exposure, a significant fraction of CNTs remained within the lung for up to several months, thus suggesting that CNTs act as a biopersistent material[18–20] (Fig. 2).

Maynard *et al.*[21] measured SWCNT aerosol concentration and size distribution after generation in laboratory and during the handling of unrefined material. Two techniques for producing SWCNTs were investigated: (i) laser-ablation process (formation of carbon plugs and ablation by laser in an inert gas stream) and (ii) HiP_{CO} process. Estimates of nanotube concentrations of personal air samples (based on the catalytic metal Ni and Fe) ranged from 0.7 $\mu g/m^3$ in the ablation facility to 53 $\mu g/m^3$ in the HiP_{CO} process. Scanning electron microscope analysis on filter samples showed that the particles appeared compact rather than having an open, low density structure more generally associated with unprocessed SWCNTs; some open structures were also observed as well as some large, not respirable, clumps. [The respirable fraction is defined in ISO7708:1995 with a d50 of 4 μm and is considered to represent the fraction of aerosol able to penetrate to the alveolar region of the respiratory tract.]

Han *et al.*[22] monitored the exposure to MWCNTs released in CNTs research laboratory and reported both mass and fiber concentration. Airborne mass concentration was reported as 430 $\mu g/m^3$ during blending prior to implementation of exposure controls. After implementation, maximum measure concentrations were reduced to 40 $\mu g/m^3$. Scanning transmission electron microscope analysis revealed various MWCNT shapes, including individual tube structures, ropes, and clumps. For blending and mixing activities, high fiber

TABLE I. Summary of hazard assessment of CNTs.

	Exposure route	Results	Ref.
Absorption	Inhalation	Deposition in lungs	19
		Limited clearance	20
		Dose-dependent	78
		Traslocation to pleura	48
	Oral	Limited absorption	18
	Dermal	No conclusive data	
Distribution	Intravenous	Accumulation in liver, kidney, lung, spleen	56
	Intraperitoneal	Phagocytosis by Kuppfer cells	43
Metabolism elimination		Biopersistent, clearance by macrophages	19
		Excretion via urine	44
			43
			45

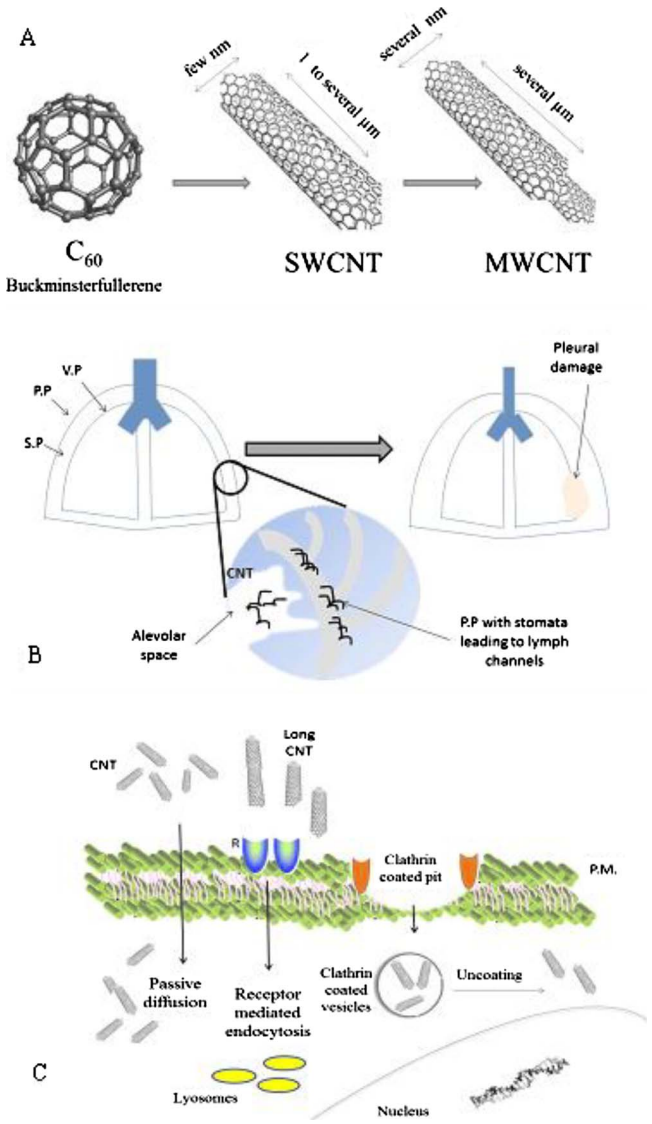

FIG. 2. (Color online) Inhalation exposure to CNTs. (A) Examples of SWCNT and MWCNT derived from buckminsterfullerene (C_{60}). C_{60} could be considered as the paradigmatic structure of the family of tubular or spheric nanotubes. Starting from hexagonal, pentagonal, and heptagonal rings, it is possible to compose several polyhedral structures. For example, it is possible to divide the fullerene into two halves and to create a tubular structure by adding carbon atoms to the broken bonds, thus obtaining hexagonal chains. (B) Hypothesized sequence of events inducing pleural damage after CNT inhalation and retention. (C) Mechanisms of cellular uptake of CNTs. They include passive diffusion, receptor mediated endocytosis, and clathrin or caveolae mediated endocytosis (PP: parietal pleura; VP: visceral pleura; SP: pleural space; and PM: plasma membrane).

concentrations between 173 and 194 fiber/cm^3 were found, based on both personal and area samples. There appeared to be a strong tendency to bundle together in ropes due to the van der Walls force. The maximum length of these fibers was observed to be 1.5 μm.

Bello *et al.*[23] reported no increase in the total particle number concentration at any particle size range as compared to background during CVD growth of CNTs and subsequent handling (removal from the furnace and detachment from the growth substrate). They subsequently measured airborne exposure to nanoparticles and fibers during dry and wet abrasive machining of hybrid advanced composites and found samples containing CNTs at the emission source and at the breathing zone of the workers.[24] Besides, they reported significant exposure to nanoscale particle compared to background during dry cutting of all composites. Airborne concentrations of respirable fibers (5–20 μm in length) were measured to be 1.6 (CNT aluminia and base aluminia) and 4.8 fibers/cm^3 (base carbon) at source during dry cutting and were reduced to 0.2 fiber/cm^3 at the breathing zone. Of them, 71%–89% of the total surface area was dominated by respirable (1–10 μm) aerosol fraction. Interestingly, Yeganeh *et al.*[25] conducted measurements in a commercial nanotechnology facility in the United States that produced fullerenes, CNTs, and other carbonaceous nanoparticles by arc reaction. Airborne particle concentrations were measured during the manufacturing of nanoparticles inside a fume hood, just outside the fume hood, and in the background. The authors demonstrated that the engineered controls at the facility appear to be effective at limiting exposure to the produced nanomaterials.

The National Institute for Occupational Safety and Health (NIOSH) recently published a couple of papers[26,27] describing the development of a "nanoparticle emission assessment technique (NEAT)," which is a structured process to assess potential inhalation exposures in facilities that handle and produced engineered nanomaterials. NEAT utilizes portable direct-regarding instrumentation (condensation particle counter and optical particle counter) to detect releases of airborne nanomaterial, supplemented by filter-based air sampling and subsequent chemical and microscopic analysis for particle identification and chemical speciation. Particle identification is crucial to detect particle source and to differentiate between process-related and incidental nanomaterials. This approach also provides information on the form of the nanomaterials emitted, such as agglomerates, clusters, bundles, or individual fibers and spheric particles. These studies evaluated research laboratories and manufacturers working with MWCNT and CNT. Nanomaterial emission was evident in different tasks, such as opening of the growth chamber, weighing, mixing, and sonication. During opening of growth chambers after the production of CNTs via pulsed laser deposition and chemical vapor deposition, maximum particle concentrations of 42.400 (10–10 000 nm), 0.35 (300–500 nm), and 0.4(500–1000 nm) particles/cm^3 were measured. The same activity in a sealed system with vacuum exhaust reduced the number to 300 particles/cm^3 and 0. Johnson *et al.*[15] described the potential for occupational exposure to CNTs in environmental laboratories. The use of nanomaterials in biological assays usually requires continuous mixing or sonication to deagglomerate. This process results in the release and dispersion of nanomaterials into the air via small water droplets. The highest airborne particle number concentrations were detected during the handling of raw MWCNTs at the 300 nm size, followed by the 500 nm size. Sonication increased airborne raw MWCNT particle number concentration in the 10–1000 nm size. Weighing and

sonication of functionalized MWCNTs resulted in lower numbers of 676 and 726 particles/cm^3, respectively. Nanomaterials containing water droplets have the potential to be deposited on the surfaces within the sonication cabinet and in the laboratory. Takaya *et al.*[28] measured exposure of workers to MWCNTs in a packing facility. Nanoparticles and submicron particles were measured using a scanning mobility particle size and an optical particle counter. The exposure concentration of the workers was 2.9/0.39 (total/respirable) μg/m^3 and the automatization of the process reduced the exposure significantly to 0.29/0.08 (total/respirable) μg/m^3.

A limited number of studies reported a wide range of exposure values. The exposure level mainly depends on the activity (process) and on the effectiveness of exposure control. The determined particle concentration in the air also probably depends on the particle characteristics (agglomerates and dustiness) and on the accurateness of measurements. However, three exposure values to represent low, medium, and high (uncontrolled) occupational exposure were selected for the risk characterization appraisal. The studies from which these values have been derived determined CNT concentrations in terms of mass concentrations (mass/volume), which can be compared to concentrations tested in the available inhalation toxicity studies. A value of 0.7 μg/m^3 from laser-ablation facility is suggested as a low occupational exposure value.[20] A value of 53 μg/m^3 from the HiP_{CO} process has been chosen as a reference concentration for occupational toxicities. Exposure values of 439–1094 μg/m^3 are suggested as high exposure values for conditions without implementing exposure control and for short high peak exposure activities.

2. Dermal exposure to CNTs

Another relevant effect of exposure to CNTs is dermal toxicity, even though limited studies exist. It seems that in mice dermally exposed to MWCNTs, inflammatory cascade activation is demonstrated by the increase in fibroblasts, mast cells, and neutrophil in the dermis; a significant increase in collagen accumulation is also reported at the highest doses tested.[29] The latter is also associated with an enhanced release of inflammatory cytokines IL-10 and IL-60, while no changes are reported in monocyte chemotactic protein-1 (MCP-1), interferon γ (INF-γ), and tumor necrosis factor α (TNF-α) and IL-12.

3. Consumer exposure to CNTs

Due to the several applications of CNTs, one of the main ways of exposure is derived from the abrasion of products. Exposure via medical devices (e.g., internal exposure by targeted drug delivery or contrast agents) is highly investigated.

4. Environmental exposure to CNTs

Exposure to CNTs via environment is mainly derived from incineration of discarded articles or wastes that contain CNTs and by particulate that is generated from wear and tear of products containing CNTs.[30] Data published by Murr and co-workers[31,32] identified MWCNTs and carbonaceous nanoparticles in methane or propane flames generated by kitchen stoves and from fuel-gas combustions. Notably, Wu *et al.*[33] reported that CNTs have been found in lung tissues from subjects exposed to dusts and smoke during the 11 September 2001 collapse of the World Trade Center in New York. Muller and Nowack[34] calculated the predicted environmental concentration for CNTs in the air and they estimated a value between 0.0015 μg/m^3 (defined as "realistic exposure") and 0.0023 μg/m^3 (high exposure). For human environmental exposure to CNTs, a value of 0.0066 has been used as an estimation for a peak/local environment exposure and the values 0.0015–0.0023 μg/m^3 for regional (background) environment exposure.

B. Pharmacokinetic of CNTs and hazard assessment

From the perspectives of characterizing potential adverse effects, a full understanding of pharmacokinetic profile of carbonaceous nanomaterials in the body is needed to define and assess the quantitative risk and to identify a safe (no-effect) systemic dose of CNTs. Pharmacokinetics is defined as the science of quantifying the rate and extent of the absorption, distribution metabolism, and elimination of chemicals and drugs in the body using mathematical modeling approaches. Some descriptive parameters might be defined: (i) volume of distribution (Vd): proportion of drug distributed between plasma and the rest of the body after oral or parenteral administration (Vd=concentration/dose); (ii) clearance (Cl): efficiency of the removal of a compound from the blood. It might be calculated for the whole body or for specific organs (e.g., liver or kidneys); (iii) half-life ($T_{1/2}$): the time it takes for 50% of a process to be completed; (iv) mean residence time: similar to $T_{1/2}$, defines the average time a compound remains in the body; and (v) bioavailability: the fraction of an administered dose of unchanged drug that reaches the systemic circulation. This is calculated from the blood as the ratio of the area under the curve in the blood after a specific route divided by that seen after intravenous (bioavailability: 100%) administration.

As carefully reported by Riviere,[35] most of the pharmacokinetic models investigate substance deposition by blood. However, little is known about nanomaterial biodistribution and kinetics. Some data have been derived from the analysis of proteins as well as of viruses and lipid particles due to their similar size as the CNTs.

Nanopharmacokinetic studies—being quite different from classical approaches for drugs and chemicals—are mainly focused on those physiological functions represented by cellular recognition, opsonization, adhesion, and uptake processes. Some points might be kept into consideration. The first is that for nanomaterials, decay in blood concentrations might be related to the compound movement into tissue from which further excretion does not occur. Indeed, when intravenously injected, most of the nanomaterials tend to accumulate in the liver and to be sequestered at reticuloendothelial system bound to tissue proteins. In those cases, blood $T_{1/2}$ may result paradoxically short. The second is that nano-

materials may also be transported through lymphatic ways and this fact may complicate pharmacokinetic analysis based on blood tests. Another important implication is that all such transported materials have the potential to interact with the immune system resident in regional lymph nodes.

Absorption. As discussed above, one of the main ways of CNT absorption is inhalation. Subsequent to pulmonary exposure, it has been demonstrated that a fraction of CNTs remained within the lungs for up to several months following exposure. Therefore, the behavior of CNTs within the human body is likely to be dependent on the doses to which individuals are exposed and on the properties of CNTs. It is important to underline that after inhalation CNTs are likely to deposit and persist within the lungs, and macrophage-mediated clearance and translocation into the pleural layer have been demonstrated (see below). Long thin fibers may penetrate deeper into airways and aggregate; these CNT agglomerates require removal by phagocytes or, if they overcome epithelial barriers, they could reach other organs through circulation. In particular, it has been shown that 28 days after intratracheal administration, 20% of MWCNTs remained in the lung, whereas elimination of CNTs from the lungs is mainly due to alveolar macrophages. Elgrabli *et al.*[21] reported that following phagocytosis of MWCNTs, the macrophages undergo apoptosis with no inflammatory responses or other physiological and histological pathology. Besides, it has been suggested that MWCNTs may increase lung paracellular permeability, which might thus allow translocation of CNTs into the blood. From these bases, not only exposure times are relevant for the interpretation of exposure tests but also an observation period. Consistent with the long retention times observed and the long half-life of poorly soluble particles, even short-term inhalation studies might require post-exposure periods of at least 3 months to reveal CNT's depositional toxicological profile.[36,37]

Interestingly, after oral administration of 10 μg/mouse, Deng *et al.*[19] demonstrated that majority of MWCNTs were evident in mouth, stomach, as well as in small and large intestines, in the absence of detectable transport into the blood. The MWCNTs remained unchanged and behave as biopersistent materials. In 2009, Folkmann *et al.*[38] suggested an oral absorption of CNTs. Very few data on dermal absorption have been studied on dermal absorption.

Distribution. The distribution of CNTs to various organs after intravenous (IV) exposure shows a predominant localization within the liver, lungs, and spleen.[12,39–41] Yamago *et al.*[42] studied a ^{14}C labeled lipophilic water soluble C_{60} after IV and oral administration to mice and Fisher rats. In both species, oral absorption was minimal, while after IV injection, only 5% of the compound was excreted from the body, all by fecal route. Most radiolabeled C_{60} was retained in the liver after 30 h (primarily in Kuppfer and in perisinusoidal fat cells and not in hepatocytes) and some C_{60} derivatives were also located in the spleen, kidney, and interestingly in the brain. It has been demonstrated that following intraperitoneal administration, SWCNTs accumulate in a number of organs (mainly bone, stomach, and kidney) with their elimination mainly within urine;[43] besides, other works reported that when intravenously injected, MWCNTs were phagocytosed by Kupffer cells in the liver, with no toxicities subsequent as demonstrated by histopathological analysis.[12] It is conceivable that exposure through other routes might follow a similar distribution pattern, but further investigations are needed to confirm this hypothesis. As described above, it is unlikely that CNTs are degraded due to their biopersistent nature[12,13] even if it has been shown that shorter fibers are more easily cleared by macrophages.[13]

Metabolism. It is unlikely that CNTs are degraded due to their biopersistent nature.[19,20] However, shorter fibers are known to be more easily cleared by macrophages.[20]

Elimination. It has been demonstrated in animal models that SWCNTs injected into mice (up to 40 μg/mouse) were rapidly excreted via the kidneys, with a blood half-life of 3 h.[44] Besides, if CNTs are well individualized and sufficiently short ($<$300 nm), they could be eliminated also through the bile ducts.[45] Subsequent to lung exposure, CNTs are likely to be deposited and to persist within the lungs. Macrophage clearance and translocation toward the pleura layer have been demonstrated. Epithelial barriers at the exposure sites are protected from the transfer of CNTs to circulation; however, if protection is overcome, they could reach several organs, among which are liver, spleen, and kidneys. More importantly, the behavior of CNTs within the human body is regulated on the doses to which humans are exposed and on the properties of CNTs. Long thin fibers may penetrate deeper into the airways and then aggregate. Functionalization and surface modification can also impact the solubility and biokinetics of CNTs: in particular, surface modification of CNTs impact their interaction within cells and organelles.

1. Acute CNT toxicity

Several studies have investigated pulmonary effects subsequent to instillation, aspiration, and inhalation. Although intratracheal instillation and pharyngeal aspiration are not physiological routes of exposure for humans, they represent techniques used to investigate pulmonary and systemic toxicities in mouse models. Following single intratracheal instillation[46,47] and pharyngeal aspiration,[48] CNT exposure in general results in an acute, neutrophil-drive inflammation and subsequent fibrotic response, with development of granulomas associated with CNT aggregates. It has been documented that SWCNTs are usually more potent than MWCNTs at comparable doses in inducing inflammation and granulomas: this fact might be related to the usually higher concentration of catalytic metal impurities. Although reported in animals, death via asphyxiation is unlikely in humans due to the difficulties in generating enough CNT aggregates.

In general, intratracheal instillation results in more severe effects if compared to inhalation since higher doses of CNTs might reach lungs when directly instilled. Acute MWCNT inhalation exposure has been investigated,[23] showing a concentration-dependent pulmonary inflammation with evidence of regression over time. MWCNTs induce a less pro-

nounced inflammatory response with a downregulation of inflammatory genes signature, various stress, and fibrotic responses. When mice are exposed to aerosol of SWCNTs for more prolonged periods (subacute inhalation exposure), inflammatory, oxidative, fibrotic, and mutagenic responses are documented with more persistent effects even if only few studies are available for this kind of analysis. In a follow-up study, Mitchell *et al.*[49] demonstrated that inhaled CNTs activate the release of transforming growth factor β (TGF-β) in the lung, which is a mediator of fibrotic process and is postulated to have a direct effect in inducing immunosuppression. Based on these studies, it can be concluded that absorption of CNTs from the lungs seems to be not necessary to induce systemic immunity effects.

2. Subchronic CNT exposure effects

Two guideline works have recently investigated subchronic MWCNT exposure to CNTs. Following 13 weeks of aerosol exposure, a first study[50] reported the absence of any pathological response in major organs such as liver, kidney, and heart based on histopathogical examinations; however, lungs were at higher weights carrying pronounced multifocal granulomatous inflammation, diffuse histiocytic and neutrophilic inflammation, and intra-alveolar lipoproteinosis. The incidence and severity of the effects were concentration-related. A second 90-day inhalation study[51] demonstrated that translocation of MWCNTs into lung associated lymph nodes were detectable only after 13 weeks and sustained elevation in neutrophils in bronchoalveolar lavage fluid occurred at highest exposure concentrations. Granulomatous changes and time-dependent increase of bronchoalveolar hyperplasia increased in intensity from the 8th week of exposure. Overload-associated inflammation was observed at high concentration exposure and was not reversible within the postexposure period of 6 months of observation. No systemic toxicity was detected at any concentration tested. In summary, both studies identified MWCNTs as pneumotoxicant by a physiologically relevant route of exposure and provide hazard criteria basis for establishing risk assessment determinations. Importantly, both studies reported no systemic (extrapulmonary) toxicity based on the histopatological evidence.

3. Biological bases of CNT toxicity

One of the most important mechanisms of CNTs induced toxicity seems to be oxidative stress, which induces inflammation via the activation of oxidative-stress-responsive transcription factors. As for toxicity due to metallic contamination of CNTs, numerous *in vitro* and *in vivo* studies have shown that CNTs and/or associated contaminants or catalytic materials that arise during the production process may induce oxidative stress, prominent pulmonary inflammation, apoptosis in different cell types, and induction of cytotoxic effects on lungs. Despite the differences regarding their wall number, source, metal contamination, and particle dimensions, burgeoning evidence demonstrates that several types of CNTs are able to induce similar pathological effects. With respect to lung toxicity, the molecular mechanisms of epithelial cell damage are mainly mediated by enhanced reactive oxygen species (ROS) production that can be partially blocked by metal chelators, thus suggesting that metal components in CNTs (nickel and iron) are able to contribute to the oxidant response reported.[52] Also, impurities that are contained in CNTs may contribute directly to lung epithelial toxicity. Purified CNTs, on the contrary, were shown to decrease local oxidative stress development,[48] suggesting that similar to fullerenes, ROS can be grafted to the surface of CNTs via radical addition due to their high electron affinity. CNTs could therefore potentially trap the radicals released by macrophages, recruited during inflammatory response. SWCNTs seem to impair phagocytosis more than MWCNTs and C_{60}.[53] It has also been demonstrated that chronic exposure to CNTs results in a sequestration of surfactant proteins A and D (SP-A and SP-D) and collectins, which are known to play a relevant defensive role against infections within the lung.[54] As a consequence, a relevant reduction in immune defense is induced due to both macrophages impairment and direct binding of surfactant proteins. The extent of these effects is likely to be driven by morphology and dimensions of CNTs.

With respect to direct mutagenicity capacity of CNTs, several studies have been performed, but at present, results are inconclusive. Several *in vitro* reports suggest that genotoxic properties may be consequent to two main mechanisms (Table II): (i) direct DNA damage and (ii) inflammation and formation of ROS. More importantly, a dose-dependent DNA damage by a mixture of CNTs has been reported, suggesting that ROS derived by catalysis metals of unpurified CNTs might be involved in DNA damage induction.[55] The paper from Pacurari *et al.*[53] tested raw SWCNTs and suggested that DNA damage was a consequence of a direct interaction of SWCNTs and DNA and to SWCNT-induced ROS production. Yang *et al.*[56] evaluated that highly pure SWCNTs induced more DNA damage but less toxicity related to oxidative stress and concluded that genotoxicity might be mostly due to direct properties of CNTs (e.g., shape), while cytotoxicity can be mainly attributed to oxidative stress.

Enough evidence derived by both *in vitro* and *in vivo* studies sustains that exposure to CNTs is not able to induce DNA damage (point mutations).[57] More importantly, recent researches on nanoparticles have shown that they display more carcinogenic properties if compared to microparticles, suggesting that particle size, distribution, shape, and agglomeration might be kept in consideration when establishing exposure guidelines. Although other mechanisms have been hypothesized, it seems that SWCNTs might act as "nanoneedles"[58] that could penetrate cell membrane without endocytosis and induces changes in cells signaling and regulation [Fig. 2(C)]. A close relationship between electronic properties of CNTs (that are negatively charged) and genotoxicity has been hypothesized. This action seems to be relevant in genotoxic effects of MWCNTs, which are known to interact in cell division process since they are able to induce

TABLE II. Summary of CNT genotoxic studies.

In vitro/in vivo	Genotoxicity assay	Toxicity assay	Physicochemical analysis	Findings	Ref.
Mouse lung epithelial cell lines	Comet assay[a]		Surface area analysis	SWCNTs and C_{60} cause a dose dependent ROS increase, with SWCNTs > C_{60}	63
Type II pneumocytes; MCF-7, RLE cell lines	*Ex vivo* and *in vitro* Mn assay[b]		Surface area and thermal properties analysis	Dose-dependent increase in metals in both *in vitro* and *in vivo* tests after MWCNT administration	34
Wistar rat bronchoalveolar fluid; cultured rat lung epithelial cells	Mn assay		Surface area and elemental analysis, spectroscopy, adsorption microcalorimetry	MWCNTs induce acute pulmonary toxicity	34
Normal mesothelial cells, MPM cells	Comet assay, H2AX phosphrylation[c]	MTT assay, LDH activity, Typan blue staining	Surface area analysis	ROS induce mesothelial transformation Dose-dependent decline in cell viability NF-kB activation; ↑ DNA damage and H2Ax phosphorylation ↓ metabolic activity and cell growth	53
Chinese hamster lung fibroblasts (V79), *Salmonella typhimurium* (YG1024,YG1029)	Comet assay, Mn assay, Ames assay[d]	Trypan blue staining	Surface area analysis, spectroscopy	DNA damage Cellular apotosis	65
MES	Double strands break repair protein; adenine phosphorybosyltransferase assay	Alkaline phosphatase detection		↑ expression of base excision repair proteins ↑ mutation frequency	81

[a]The Comet assay (single-cell gel electrophoresis assay) is a sensitive assay used to evaluate DNA single and double strand breaks.
[b]Mn assay stand for micronuclei assay, which is used to detect the mutagenic chemicals that can induce formation of micronuclei in the cytoplasm of interphase cells by interfering the chromosomes structure and segregation.
[c]H2AX staining is used to detect the H2A histone variant, which is phosphorylated in response to DNA double strand breaks.
[d]The Ames assay is used to evaluate the mutagenic potential of a substance by using several strains of *Salmonella typhimurium*, each of them carries different gene mutations.

micronuclei formation and anaphase bridges among nuclei in binucleated cells.[59] Besides, MWCNTs with structural defects seem to be more effective than pure CNTs in inducing the formation of micronuclei in lung epithelial cells.[35]

Reactive oxygen species are defined either as "primary" or "secondary." Primary ROS (e.g., superoxide O_2^-) are generated through metabolic process or through activation of oxygen, which results in the formation of a reactive nucleophilic molecule of oxygen (superoxide anion); this radical does not react directly with DNA. However, these radicals may interact with other molecules such as redox active transition metals (e.g., iron) or enzymes, thus resulting in the production of secondary ROS (e.g., $^{\bullet}OH$ radical). The latter is the main mediator of DNA damage. The majority of HO radicals generated *in vivo* are derived from the metal catalyzed breakdown of hydrogen peroxide according to the Fenton reaction, according to the following reactions: $2O_2^{\bullet-}+2H^+ \rightarrow H_2O_2+O_2$ (dismutase reaction), followed by $M^n+H_2O_2 \rightarrow M^{(n+1)+}+{}^{\bullet}OH+OH^-$ (Fenton reaction, in which M stands for a transition metal).[59,60] Transition metal ions (such as cadmium, chromium, cobalt, copper, iron, nickel, titanium, and zinc) are released from certain nanoparticles and have the potential to induce conversion of cellular oxygen metabolic products such as H_2O_2 and superoxide anions to hydroxyl radical ($^{\bullet}OH$), which is one of the primary DNA damaging ROS. Iron can also induce the production of H_2O_2 from molecular O_2, which can diffuse through the cellular and nuclear membrane and directly react to Fe bound to DNA, also resulting in the generation of $^{\bullet}OH$. The latter is involved in causing thymine-tyrosine (DNA-histone protein) cross-links in chromatin. Besides, free iron ions can result in $^{\bullet}OH$-induced purine and pyrimidine changes.[61] Thus, potentially, CNTs with iron components could result in an increased source of iron, which contributes to produce a high quantity of ROS.

In vivo data on genotoxic effects are still uncertain at present. Szendi and Varga[62] reported the lack of mutagenic effects in the urinary Ames test after oral exposure to 50 mg/kg MWCNTs and SWCNTs. In contrast, in 2009, Folkman *et al.*[39] observed oxidative damage DNA in liver and lungs of rats exposed to low doses of SWCNTs and hypothesized direct genotoxic ability and an inhibition of repair systems. Other studies investigated DNA damage subsequent to direct lung exposure.[63,64] MWCNTs increased micronucleated cells in rat lungs after intratracheal administration of MWCNTs in response to marked pulmonary inflammatory response. Besides, it has been shown that the occurrence of Kirsten rat sarcoma 2 viral oncogene homolog mutations within pulmonary tissues of mice was greater after SWCNTs inhalation than aspiration, thus suggesting that exposure method could impact results on CNT toxicity studies.[65] The most important target organ of carcinogenicity and transformation properties of CNTs are the lung and pleura (due to inhalation exposure) and these effects are discussed in Sec. IV.

In summary, the genotoxic potential of CNTs is not clearly determined. Genotoxicity can be derived from direct interaction of CNTs with DNA after cellular internalization (primary genotoxicity) and by ROS production (secondary genotoxicity), which follows frustrated phagocytosis of CTN aggregates. Besides, chemicophysical CNT properties, such as electrochemical (negative) charge, size, and shapes, well as surface properties (related to functionalization and structural defects), might contribute to genotoxic effects. Finally, the experimental setup might be kept in consideration in results analysis.

IV. EXPERIMENTAL EVIDENCE AND OPEN ISSUES ON PLEURAL INFLAMMATION AND TRANSFORMATION FROM CNTS

The lungs are the most likely site of exposure to CNTs and—as described above—CNT toxicity is mainly related to their capacity to form aggregates and fiberlike structures that, as a consequence, induce *frustrated* phagocytosis and formation of granulomas. In general, fibers can be cleared by several mechanisms, including mucociliar escalator, removal by macrophages, or through their splitting and chemical modification. According to fiber characteristics of length and diameter (aspect ratio), fibers with high aspect ratio (10–15 nm in diameter and containing two different length distributions of 450 ± 230 and 10451 ± 8422 nm in length) are more toxic to the lung than low-aspect-ratio fibers (10–15 nm in diameter and length of 192 nm).[66] Thus, depending on their size and dimensions, inhaled CNTs may penetrate respiratory tract to distal airways and reach alveolar space.

Fiber diameter is relevant in defining aerodynamic diameter (D_{ae}) and the dependence of pulmonary deposition on D_{ae}.[67] Fiber length has little impact on D_{ae} in the case of thin fibers, except in the case that length is sufficient to cause interception, a mechanism of particle deposition that is confined to fibers following a downstream at a bifurcation when the tip of the fiber makes contact with the wall resulting in local deposition. The penetration of long fibers (>50 μm) beyond the ciliated epithelium is defined on the basis that the D_{ae} of a straight fiber is around three times its actual diameter.[68] This is derived from the alignment to the airflow as the fibers move aerodynamically through the bronchial tree. The retention half-time ($T_{1/2}$) of a compact inert respirable particle or a short fiber is about 60 days; long fibers are more slowly cleared and accumulate in macrophages.[69] The evidence that length is a key factor in the pathogenetic potential of fibers is derived from a number of toxicological experimental studies: in particular, Stanton and coll.[70] carried out a large number of experiments aimed to clarify the role of fiber characteristics in inducing pleural mesothelioma and identified that carcinogenicity is related to "durable" fibers longer than 10 μm.

According to what has been defined as "fibers paradigm,"[77] the geometry of fibers is the most important toxicological feature compared to chemical composition, except in the contribution to fiber biopersistence; in other words, both fiber length and biopersitence interact in determining the clearance of l fiber from the lungs. As described above, CNTs can exist as compact tangles of nanotubes that

are essentially particles or straighter as longer fibers that potentially have a similar propensity to display a length-related toxicity. *Frustrated* phagocytosis of long fibers is likely to be applied to asbestos and long CNTs as well. Therefore, several studies have demonstrated that carbon nanotubes in the form of long fibers display an asbestoslike, length-dependent toxicity and that according to the fibers paradigm, they should be considered as thin, long, biopersistent matter, but unlike other materials, they could exist in forms that do not comply within the paradigm (e.g., in the case of singlet CNTs).

The chest cavity or pleural cavity is the cavity that surrounds lungs, heart, and mediastinum, comprising the ribs and associated muscles and connective tissue. This cavity is covered by a pleural single layer that is constituted of mesothelial cells, which is defined as "parietal" (which corresponds to the chest wall and "visceral" in the tract that is in contact with the lung surface). In physiological settings, these two mesothelial layers are closely opposed and there is a thin space between them containing the pleural fluid and a population of pleural macrophages. Pleural mesothelium has a number of biological functions: the pleural fluid is constantly produced by hydrostatic pressure from the subpleural capillaries and supplemented by glycoproteins secreted by the mesothelial cells. The pleural fluid and its constant outflow keep the lungs tightly coupled to the chest wall, allowing diaphragmatic muscle contraction and relaxation to expand and passively relax the lung during breathing movements. Thus, the pleural space is variable (about 20 μm). The pleural fluid turns over rapidly and continuously exits through stomata that are present in the parietal pleural layer via lymphatic capillaries; the stomata openings on the parietal pleura are between 3 and 10 μm in diameter. The drainage of fluid from the pleural space carries particles in the lymph nodes, mediastinal lymph nodes, and posterior mediastinal lymphoid tissue. The stomata are more densely situated in the most caudal and posterior intercostals space, although they are lightly scattered in more cranial and anterior intercostals regions. Based on this observation, it could be argued that since fibers produce pleural damage while particles do not, fibers must reach the pleura and particles need not. However, a body of literature supports the fact that all particles reach the pleura, pass through the pleural space, and exit through the stomata.

Anatomopathological lesions related to asbestos exposure may affect the pleural layer, the lung parenchyma, the airways, and the lymph nodes (Fig. 3). Pleural lesions are the common ones and vary from by pleural plaques to localized or spread fibrosis to mesothelioma: all of them could be accompanied with pleural effusion. At the early onset, mesothelioma should appear as a solitary intrapleural mass or as spread nodules; however, in the vast majority of cases, it starts as a thickening or a plaque, which includes a part or the total of the corresponding lung surface. Macroscopically invasive pleural tumors can be roughly divided into three main histotypes: epithelial (60%–70% of all MPM diagnosis), sarcomatoi or mesenchimal, and biphasic. Pulmonary

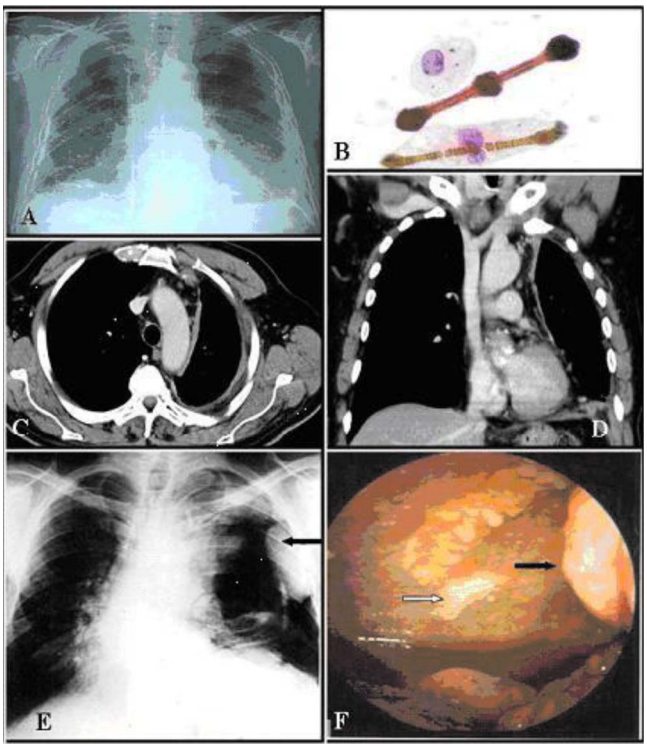

FIG. 3. (Color online) Anatomopathological findings in asbestos related diseases. (A) Chest x ray in the case of asbestosis (benign disease) with plaques on diaphragmatic and mediastinal pleura. Pleural plaques are localized scars (fibrosis) consisting of collagen fiber deposits that form as a result of exposure to asbestos. They are the most common manifestation of exposure to asbestos. Normally, pleural plaque is found in the parietal pleura (on the inside of the diaphragm), but in very rare cases, they can also be found near the ribcage. Asbestosis is a chronic inflammatory and fibrotic medical condition affecting the parenchymal tissue of the lungs caused by the inhalation and retention of asbestos fibers. It specifically refers to interstitial (parenchymal) fibrosis from asbestos, and not pleural fibrosis or plaguing. (B) Asbestos bodies found in sputum. Asbestos bodies are constituted of a central asbestos fiber surrounded by a lining of iron and proteins, which is often segmented. They have a median width of 2.5 μm and a median length of 20–50 μm and generally have a straight shape. [(C) and (D)] Thoracic CT scan—trasversal (C) and coronal (D) sections in MPM, showing the thickening of both parietal and visceral pleural layers. [(E) and (F)] Proliferative epithelioid mesothelioma, chest x rays (E) and the corresponding thoracoscopy view (F); black arrow ($\rightarrow$) indicates malignant areas, while the white one ($\Rightarrow$) denotes an isolated hyaline plate. Hyaline pleural plaques generally occur as discrete elevated gray-white areas, usually involving the parietal layer and without associated effusions or adhesions. They are composed of laminated hyaline collagen; there is no suggestion of vasculature or granulation. They are usually detected in the case of asbestos exposure. Thoracoscopy is a medical procedure involving internal examination, biopsy, and/or resection of disease or masses within the pleural cavity and thoracic cavity. It is the golden standard procedure for MPM diagnosis. (Courtesy of E. Pozzi and P. Cremaschi, Cardio-Thoraco Vascular Dept., Section of Pneumology, Fondazione IRCCS Policlinico San Matteo, Pavia, Italy.)

findings include interstitial fibrosis (asbestosis), ringing atelectasis, peribronchiolar fibrosis, and lung carcinoma. Moreover, the evidence of asbestos exposure should be documented by the finding in tissue sections of the so-called "asbestos bodies." The latter is frequently identified in the lung parenchyma, although they could be present in the airway tract or at lymph nodes as well. Detection of these bodies in bioptic tissue sections, pulmonary secretions, or bronchioal-

veolar lavage fluid is not predictive of the occurrence of an asbestos-induced disease but denotes that the patient has been exposed to a significant fiber concentration.

How asbestos fibers reach the pleural space is still quite obscure. It could be hypothesized that lymphatic flow from the lung parenchyma even if this mechanisms is not fully documented. A possible model could be that they reach pleural space through normal lymph flow centrally to the mediastinum and then into the blood via the thoracic duct, followed by extra vacation in the pleural capillaries during the formation of pleural fluid. The second and more likely route hypothesized requires inflammation in the parenchyma caused by the fibers to reverse both the normal flow of lymph and the normal transpleural pressure, thus resulting in a net flow of fluid and fibers directly into the pleural space from the underlying parenchyma.[71]

It should be concluded that there is enough evidence to support the contention that a fraction of all deposited particles reach the pleura by an obscure pathway and that short fibers and compact particles leave the pleura through the stomata openings. Moreover, most of the particles are transported to lymph nodes and some enter the interstitium at the mouth of the stomata to form what has been defined "black spot." The latter eventually identifies the area around the stomata where mesenchymal cells are activated and proliferate, depending on the toxicity and dose of the particle.[15] Coherent with this model, long fibers that reach the pleural space have potential to physically block the stomata and interfere within the walls of stomata openings and with lymph vessel walls. This is likely to lead to mesothelial and endothelial damage, inducing inflammation and accumulation of pleural macrophages attempting to phagocytose these retained fibers. The macrophages are likely to undergo frustrated phagocitosys in attempting to enclose these long fibers and so release cytokines and oxidants, which amplifies inflammatory cascade and induces fibrosis and genotoxicity. Direct interaction between retained long fibers and mesothelial cells around the stomata could also induce direct genotoxic damage (see below).

Accordingly, the primary lesion caused by long fibers must form at the parietal pleural, the site of retention of long fibers, and the site of biological response. Mesothelioma would therefore originate not at the visceral pleural but at the parietal pleura. This is reflected in the staging of MPM, which identifies the early mesothelioma confined at the parietal pleura while more advanced MPM involves the visceral pleura.[72]

In the case of nonbiopersistent fibers, the degree of their biopersistence, specified by their $T_{1/2}$, impacts the likelihood that they will reach the pleura and the effects that they will have there. In the case of fibers of very low biopersistence such as the crysotile fibers with a $T_{1/2}$ of about 1 day, it seems that they undergo dissolution and breakage in the lung parenchyma in the hours following deposition, such that no long fibers are likely to reach the pleura. For fibers that are moderately biopersistent, long fibers may retain their structure toward the pleura while undergoing dissolution and breakage. If fibers are sufficiently biopersistent, they retain their fibrous structure long enough to reach the pleura and be retained at parietal stomata, initiating frustrated phagocytosis and granuloma formation. Depending on the extent of biopersistence, fibers could eventually dissolve and break within the macrophages as a result of the high *p*H within the phagolysosomes, allowing the granuloma to resolve. With respect to CNTs, it has been demonstrated that in less than 1 day following inhalation of short CNTs, they should be evident in the subpleura extracellular matrix. The issue of the potential mesothelial toxicity of CNTs is based on the attempt to determine whetted similar to asbestos; CNTs show a length-dependent toxicity to mesothelium. Accordingly, long CNTs could be retained at the parietal pleural around the stomata, thus mimicking the asbestos behavior. As discussed above, two different mechanisms should be postulated to determine the mechanisms of the proinflammatory effects of long fibers in the mesothelium, shared by both asbestos and long MWCNTs: (i) failure of long fibers to negotiate the stomata with subsequent retention; (ii) at the point where long fibers accumulate, macrophages attempt phagocytosis stimulating inflammation and mesothelial cell damage, leading to chronic inflammation and granuloma development.

The molecular mechanisms of pleural damages induced by biopersitent fibers have been extensively described in the case of exposure to asbestos. It is conceivable that exposure to nanomaterials and mainly to carbon nanotubes should be a similar potential health ratio due to their similarities toward asbestos. Asbestos species are roughly divided into two mineralogic groups: amphiboles and serpentines. The amphiboles include crocidolite, anthophyllite, and actinolite; among amphiboles, only crocidolite and amosite have widespread commercial use, the not commercial amphiboles are primarily contaminants of other minerals, such as chrysotile. As discussed above, asbestos and CNTs share similar physical and chemical characteristics.[73] Both asbestos and CNTs are not uniformly similar materials and every fiber in a mass displays its own length diameter, crystallinity, and contaminant materials.

Moreover, at present, it is not possible to standardize the production of CNTs due to the nature of synthetic procedures. The problem is also reflected during experimental studies as, in most experiments, fibers are suspended in the solution for easy handling and safety of investigators. However, it should be noted that it is not easy to standardize suspensions of CNTs since they consist of almost pure carbon that makes them extremely hydrophobic. Therefore, various solutions containing proteins[74] or detergents[75] have been used to suspend CNTs and the influence of these components might be kept in consideration in the analysis of the experimental results. Notably, the effect of loading fibers on cells or animals is still an open issue. Usually, in *in vitro* experiments cells are exposed to suspended fibers, whereas in *in vivo* studies animals undergo both aerosolized (which better reflects human exposure mechanisms) or suspended and injected. Moreover, there are significant differences in the respiratory systems between humans and rodents: in par-

ticular, it has been suggested that fiber deposition rate in humans is lower than in rats since rodents are nose breathers and rodent's turbinates are more complex and act as a good filter.[76] A simple translation to humans of the results obtained in these models is not possible, thus it is should be modified. To experimentally evaluate the tumorigenic potential of fibers, they are usually directly injected into somatic cavities of rodents. Through this approach fibers are directly in contact with pleural mesothelium and their transformation capacity could be easily evaluated, although this result does not necessarily correlate with those obtained when fibers are inhaled. This could be otherwise relevant because when injected, fiber metabolism can escape from some biological steps such as fibers impact at respiratory tract, deposition at alveoli, penetration of alveolar epithelial cells, and translocation to pleural layer or lymph or blood stream that are important in mesotelioma onset and that are better reflected during inhalation studies. It should also be noted that clearance from respiratory tract in humans is different in case of smoking habits; cigarette smoke might impair the clearance of short fibers, which consequently persist in the airway system and can bring out their effects.

Although many kinds of cells are involved in response to fibrous materials, the main actors are epithelial/mesothelial cells and macrophages. We can classify the effects of fibers on these cells either into indirect (macrophages) or direct (epithelial/mesothelial).[74] Furthermore, both macrophages and epithelial and mesothelial cells interplay and enhanced each other. In particular, mesothelial injury is unique to biopersistent fibers. Due to their size, particulate materials remain uncovered and could interact with cells other than macrophages. Inhaled nonmaterial might thus translocate to the subpleural tissue and elicit their carcinogenic potential. On the other hand, fibers might be isolated through formation of a granuloma, but it is not known if granuloma is required to induce carcinogenesis.[77] In studies with intraperitoneal injection, long fibers are trapped at lymphatic stomata on the peritoneal surface of diaphragm and induce mesothelial injury and inflammation; the shorter ones are cleared through stomata and not induce pleural damage. Thus, it should be expected that long MWCNTs would be more potent in inducing chronic inflammation and tumorigenity. Activated macrophages release several cytokines and ROS. When exposed to biopersistent matters, indeed mesothelial cells might avoid apoptosis when stimulated by TNF-α secreted by activated macrophages. This mechanism has been demonstrated in the case of asbestos exposure but could be theoretically applicable also for CNTs.

More importantly, Rayman-Rasmussen *et al.*[78] demonstrated the migration of MWCNTs to the subpleura after a single inhalation exposure of mice to high concentrations ($30\ mg/m^3$). It is conceivable that fibrous shape rather than their chemical fullerenic structure of the materials is important in determining the movement of nanotubes through the lungs. To support these hypothesis, Porter *et al.*[49] found that MWCNTs of about 4 μm long reached the pleura and induce pleural inflammation 56 days after a single aspiration of 10–80 μm in mice. For MWCNTs, the authors proposed an "asbestoslike pathogenicity:" indeed, as asbestos inhaled MWCNTs might deposit at the alveolar level and subsequently reach the subpleura. However, the pathological lesions reported were different since asbestos fibers mainly induce pleural inflammation (granulomas) and diffuse fibrosis (plaques), while MWCNT-related lesions were subpleural focal and regional fibrosis together with mononuclear aggregates.

Direct DNA damage by MWCNTs is still controversial; this fact is mainly related to the limed experimental data available on the genotoxicity of CNTs.[74] Notably, mesothelioma does not rapidly develop in mice also after asbestos inhalation, except in p53 deficient models. Asbestos and MWCNTs both induce p53.[79,80] It is unknown whether other types of CNTs would induce the same response.

V. MOLECULAR BASIS OF PLEURAL MESOTHELIAL TRANSFORMATION

Mesothelial cells normally facilitate the free movement of pleural surfaces during respiration by enmeshing lubrificating glycoproteins. These cells rapidly proliferate in response to injury and growth factors.[81] Asbestos is the principal carcinogen known to be involved in the onset of malignant mesothelioma.[82] It should be noted that Simian Virus 40 (SV-40) has also been implicated as a cofactor in causing mesothelioma.[83] It acts by blocking tumor-suppressor genes and is a potent oncogeninc virus in human and rodents cells; SV-40 DNA sequences have been found in lymphomas, brain, and bone tumors and in mesotheliomas as well as in atypical mesothelial proliferation and not invasive pleural lesions.[84,85] Molecular mechanisms of pleural damage induced by biopersistent fibers are shown in detail in Fig. 4.

There are four principal processes by which asbestos affects the pleura: (i) by inducing a direct effect of irritation; the peculiar shape of asbestos fibers and mainly the ratio of their length to their width determine how deeply they penetrate into the lung. Penetrating fibers might enter and irritate the pleural layer and induce disease featured by scarring (plaques) or by a frankly malignant growth (pleural mesothelioma);[86] (ii) asbestos fibers might pierce the mitotic spindle of cells and disrupt mitosis, thus resulting in cell aneuploidy and chromosomal damage;[87] in particular, loss of chromosome 22 is the most common change, but structural rearrangements of 1p, 3p, 9p, and 6q are often found;[88,89] (iii) by inducing generation of iron-related ROS that are able to cause direct DNA damage;[90] and (iv) by inducing phosphorylation of the mitogen-activated protein (MAP) kinases and their extracellular signal-regulated kinases (ERK) 1 and 2. Phosphorylation of these kinases increases the expression of several oncogenes and several growth factors.[91]

The first evidence showing that asbestos fibers might induce aberrant transcriptional responses, cell proliferation, and cell transformation is derived from the studies in which asbestos fibers caused induction of the *c -fos* and *c -jun* proto-oncogene mRNA and of the activator protein-1 (AP-1) transcription factor in pleural mesothelial cells and tracheo-

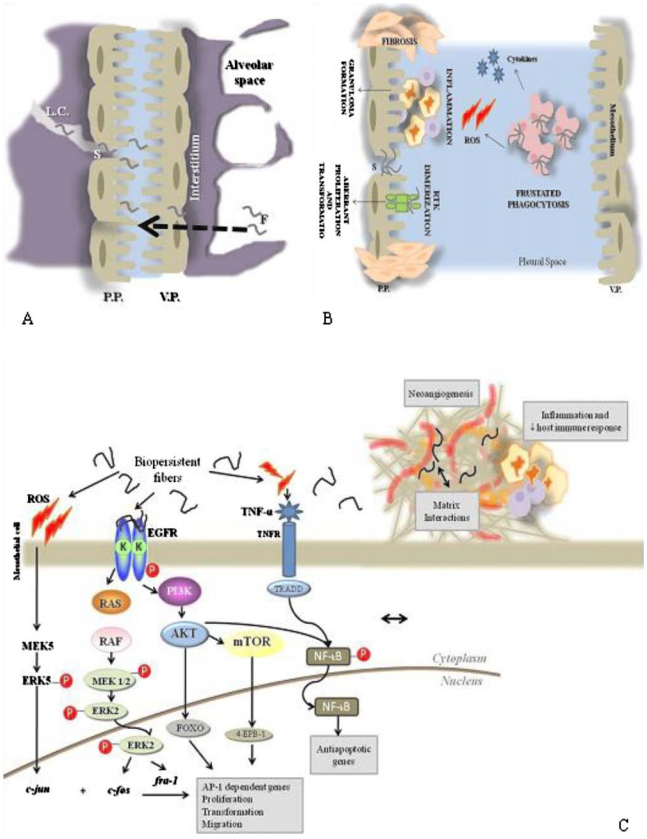

FIG. 4. (Color online) Biomolecular mechanisms of pleural damage induced by biopersistent fibers. (A) The anatomical way covered by an inhaled fiber. When fibers are inhaled, most of them are expelled, but some can become lodged in the lungs and accumulate. Then by going across the lung interstitium, fibers might reach the alveolar space; from alveoli, they could reach the pleural space and eventually the lymphatic vessels. (B) Pathological and biological effects induced by biopersistent fibers in the pleural space: on one hand. the frustrated fibers phagocytosis in macrophages leads to inflammatory response and to granuloma formation; on the other hand, the direct interaction of fibers to transmembrane tyrosine kinase receptors induces receptor activation and enhances a series of oncogenic pathways, which leads to pleura cell proliferation and transformation. (C) Molecular pathways activated during pleural malignant transformation. Biopersistent fibers directly interact both with pleura cells, which acquire neoplastic phenotype, and with the extracellular matrix in which orchestrate the neoangiogenetic process and the impairment of host immunity. ROS produced during frustrated phagocytosis could directly activate the MAPK kinase pathway and *c-jun* gene. Besides, fibers may directly induce dimerization and activation of tyrosine kinase receptors on epithelial and mesothelial cells surface; the latter promotes proliferative and antiapoptitic signals; the tumor necrosis factor alfa (TNF-α) is generated during fiber metabolisms and sustains cell prevention from apoptosis (F: fiber; PP: parietal pleura; VP: visceral pleura; LC: lymphatic capillary; S: stomata; RTK: receptor tyrosine kinase; and ROS: reactive oxygen species).

bronchial epithelial cells.[92] More importantly, induction of these oncogenic mediators has been demonstrated to be dose related and to occur at subcytotoxic concentrations of asbestos fibers being most striking with crocidolite as compared with chrysotile as comparable concentrations.[93]

It is now well established that mesothelioma cells display increased or a finalistic growth properties. They are able to produce and respond to several growth factors. Among them, the epidermal growth factor receptor (EGFR) family of transmembrane proteins might be activated by asbestos fibers, chrysotile and crocidolite. Receptor tyrosine kinases (RTKs) have been demonstrated to have a causative role in many solid cancers, among which is the non-small-cell lung carcinoma (NSCLC).[94] Kinases tend to be altered by heterozygous missense mutations that affect residues involved in their enzymatic activity. This evidence suggests that mutations are activating and operate by increasing the catalytic activity of the mutated protein and also points out that mutated kinase genes act as dominant oncogenes.[95,96] Besides, RTK translocation as well as increased gene copy number have been described in a number of human cancers, such as NSCLC: relevant examples are the transforming ALK-EML4 fusion gene,[97] on one hand, and EGFR (Ref. 98) and MET receptor[99] genes amplification, on the other hand. Notably, EGFR is rarely mutated in MPM, as reported by online available catalogs (COSMIC database, website at www.sanger.uk). Besides, we and others have also reported wild-type sequences in mutational analysis of a panel of kinases and proteins involved in epithelial transformation.[100] These data, although preliminary, are coherent with the already published data reporting no direct mutagenic properties for both asbestos and CNTs (see above).

It has been demonstrated that long (>20 μm) crocidolite asbestos fibers deposited on the cell surface of immortalized human mesothelial cells (MET5A) are physically associated with the EGFR, suggesting that long fibers might induce receptor dimerization and consequent activation.[101] This interaction activates either directly or through adaptor proteins, downstream components of signaling pathways, such as RAS-RAF-MEK, mainly involved in promoting cell proliferation and PIK3CA-mTOR-AKT, which sustains cell motility and invasion; other critical activated pathways include the signal transducer and activator of transcription signal cascade and ERBB-mediated angiogenesis.[102] Activation of the RAS pathway controls expression and transcription activity of the *Fos* family members of the AP-1 transcription factor.[103] The ERK family of serine-threonine kinases regulates several biologic functions, which are able to induce cell proliferation, motility, and neoplastic transformation. Notably increased amounts of phosphorylated ERK 1/2 mediators are observed in small airways epithelium after inhalation of crocidolite asbestos.[104,105] Asbestos also phosphorylates ERK5 (also known as big MAP kinase) in an EGFR-independent manner. Through AP-1, ERK1/2 and ERK5 promote several biological effects including cell proliferation, cell migration, and regulates neoplastic transformation.

Notably, the activation of the PIK3CA downstream mediator mTOR protein is documented in MPM: more importantly, we and others reported that this activation seems not to be a consequence of the occurrence of somatic mutations at the PIK3CA sequence. Aberrant activation of the mTOR signaling actually represents one of the most promising targets in therapeutical approach to MPM.

Asbestos fibers also cause activation of another transcription factor, NF-$_K$B. *In vitro* data report that NF-$_K$B is activated by asbestos in tracheal epithelial cells, mesothelial cells, and lung epithelium after asbestos inhalation.[106,107]

Moreover, asbestos fibers induce transcriptional activation of a number of NF-$_K$B dependent genes, including the proto-oncogene *c -myc*.[30] Activation of NF-$_K$B represents a critical step in upregulating the expression of many genes lined to proliferation, apoptosis, and chemochine/cytokine production. For example, Dostert and co-workers showed that frustrated phagocytosis of asbestos fibers by human monocytes activates the NALP3 inflammasome that produces active IL-1β (Ref. 108) and interleukin that binds to the IL-1 receptor 1. On the other hand, intracellular adapters that include TNF receptor (TNFR) associated factor 6 (TRAF6) are recruited to IL-1 receptor 1 and potentially activate both NF-$_K$B and AP-1 signals.[109] Alveolar macrophages are an early marker of inhalation asbestos fibers. In response to asbestos, they release TNF-α, which cooperates in the activation of the RAS/MAPK/NF-$_K$B pathway in lung epithelial cells.[110] TNF-α promotes both apoptosis and compensatory proliferation in mesothelial cells.

Asbestos fibers might also induce cell senescence, lytic cell death, and apoptosis. The proapoptotic properties of asbestos are related to the generation of ROS and to physical interaction of fibers to plasma membrane and cellular organelles. Several pathways are involved in these biological events: (i) the intrinsic or mitochondria-regulated pathway that is p53 or protein kinase C dependent;[111,112] (ii) extrinsic pathways induced by death-receptor ligands such as TNF- α, TNF-related apoptosis-inducing ligand (TRIAL); and FasL (Ref. 113) and increased activity of the antiapoptotis moleculae Bcl-X_L;[114] and (iii) compensatory proliferation pathways mediated by prolonged activation of AKT and MAPKs.[115–117]

MPM cells are known to produce collagen and matrix metalloproteinases as well as inflammatory cells and cytokines.[118,119] The latter acts as tumor own nutrients and facilitate matrix interactions to provide a supportive environment. Transformed cells can also produce angiogenetic factors, such as vascular endothelial growth factor (VEGF),[120] and it has been demonstrated that VEGF blockade reduces mesothelioma growth in animal models.[121] Increased vascularity in mesothelioma specimens derived from biopsy is associated with a worse prognosis as compared to those in which vasculature is not increased[122] even if few data are available on this issue.

VI. CARBON NANOTUBES: ARE THEY THE NEW ASBESTOS?

Human exposure to nanoparticles from natural and anthropogenic sources has occurred since ancient times. We are always exposed to nanosized entities, such as viruses, nucleic-acid- based structures, terrestrial dusts, and indoor pollutants. Nanotoxicology addresses the adverse health effects of engineered nanoparticles and structures encompassing the toxic effects of atmospheric particles as well as fundamentals of virology and bacteriology.

Carbon nanotubes are a class of fullerenes consisting of graphene arranged into small cylindrical structures. There are essentially two types of CNTs: SWCNTs, with a diameter ranging from 0.4 to 0.3 nm, and MWCNTs made of multiple layers of CNTs with a diameter of 1.4–100 nm up to several microns. Due to their aspect ratio, CNTs are thought to behave as biopersistent fibers *in vivo* and have proposed a carcinogenetic potential in a manner similar to asbestos. The size of CNTs is mainly responsible of their toxic profile. Indeed, pristine CNTs are inherently hydrophobic; therefore, aggregation is expected to be observed *in vivo*.

Since their discovery, the prospect of possible risks for human health effects has been deeply evaluated. With a multitude of opportunities from CNTs use in pharmaceutical and biomedical application, a thorough understanding of associated systemic toxicity is mandatory. The unique properties of CNTs have facilitated their applications in industrialized world: fabrics, filtrations, dental materials, surface disinfectants, diesel and fuel additives, hazardous chemical neutralizers, automotive components, electronics, scientific instruments, drug delivery systems, and pharmaceuticals. Biomedicine represents a relevant field of CNT applications: in medical imaging, as nanoscaffolds used to regenerate central nervous system and possible other organs, as antimicrobial nanopowders and coatings, as membranes utilized in selective transport of molecules, in drug delivery, and in gene transfection. Thus, evaluation of CNT toxicity profile might take exposure during manufacturing steps as well as their interaction to biological systems into consideration. Only through a relative comparison one can understand the dangers of functionalized CNT administration against other treatment options. This would also warrant development of a protocol for the toxicity evaluation of CNTs. Preliminary studies have indicated that the main risk for humans is related to chronic occupational inhalation, mainly during activities involving high CNT release and uncontrolled exposure. The most important target organ is the respiratory tract—as a consequence of inhalation; however, inflammation has also been demonstrated in the skin.

In particular, great interest is addressed on the role of parietal pleura as a target for long fibers hazard following pulmonary deposition and the site of initiation of malignant mesothelioma. There is a need to deeply test these materials and understand their potential role in causing mesothelioma. Therefore, mesothelioma continues to be a global problem due to the ongoing exposure to biopersistent fibers, mainly represented by asbestos. Lung tissue burdens of asbestos have long been used as an index of exposure, but lung diseases (e.g., lung cancer and asbestosis) are not a good indicator of pleural retention since there is no relationship between parenchymal and pleural concentration. This evidence might be kept in consideration in planning studies to evaluate fiber pleural toxicity and to obtain representative samples of early mesothelioma induced by inhalation of CNTs. At present, it is not possible to draw definitive conclusions with regard to the potential risks to long CNT. However, growing evidence suggests that retention at the pleural layer due to the length-restricted clearance through normal stomatal clearance system induces inflammation and pleural pathological reactions. Data from thoracoscopic exams indicate that the parietal pleura is the site of origin of pleural me-

sothelioma. This evidence supports the general hypothesis on the pathogenetic role of length-restricted biopersistent fibers. New techniques, such as laser captures, might allow investigators in identifying and study in detail the areas of parietal pleura where the stomata openings occur in order to evaluate the presence of fibers and their molecular consequences. The risk assessment data mainly are derived from subacute and subchronic toxicity studies. However, since mesothelial transformation is known to occur after a long latency from asbestos exposure, it is clearly evident that these studies might not be conclusive with respect to the evaluation of carcinogenic potential of CNTs. Moreover, toxic effects of different CNTs seem to depend on their form (length, shape) and their physicochemical properties (e.g., metal content, surface chemistry, and functionalization), and the experimental design of the toxicity test (e.g., animal models, way of administration, exposure time, and follow-up analysis) may influence the test result. Exposure measurements have to be improved to provide a better estimate of the real exposure of humans to CNTs under realistic conditions.

Overall, these observations allow relevant implications: (i) although promising in several fields, among which is biomedicine, CNTs could exert a toxic and tumorigenic potential and, in light of asbestos experience, this fact should be considered in industrial and research settings; (ii) nanotoxicology, a relative emerging subset of toxicology, requires a multidisciplinary approach to problems in order to achieve an effective risk control.

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