



Malignant pleural mesothelioma: history, controversy and future of a manmade epidemic

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ABSTRACT Asbestos is the term for a family of naturally occurring minerals that have been used on a small scale since ancient times. Industrialisation demanded increased mining and refining in the 20th century, and in 1960, Wagner, Sleggs and Marchand from South Africa linked asbestos to mesothelioma, paving the way to the current knowledge of the aetiology, epidemiology and biology of malignant pleural mesothelioma. Pleural mesothelioma is one of the most lethal cancers, with increasing incidence worldwide. This review will give some snapshots of the history of pleural mesothelioma discovery, and the body of epidemiological and biological research, including some of the controversies and unresolved questions. Translational research is currently unravelling novel circulating biomarkers for earlier diagnosis and novel treatment targets. Current breakthrough discoveries of clinically promising noninvasive biomarkers, such as the 13-protein signature, microRNAs and the *BAP1* mesothelioma/cancer syndrome, are highlighted. The asbestos history is a lesson to not be repeated, but here we also review recent *in vivo* and *in vitro* studies showing that manmade carbon nanofibres could pose a similar danger to human health. This should be taken seriously by regulatory bodies to ensure thorough testing of novel materials before release in the society.



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Malignant pleural mesothelioma is a cancer with increasing death tolls due to the past and present use of asbestos <http://ow.ly/DhA2y>

Background

History of asbestos use and mesothelioma discovery

Asbestos (from the Greek word meaning “inextinguishable”) is the term for a family of naturally occurring minerals that readily separate into thin fibres and are found in many parts of the world. The term *amiantus* (from the Greek “untaintable”, currently used in modern Greek for asbestos) was used by Pliny the Elder (23–79 AD), who described that this material was mined in the mountains of Arcadia, could be spun and woven into material, and was resistant to fire [1]. The Greek geographer Strabo (63/64 BC–ca. 24 AD) described similar use of the so-called “Karystian stone” from the ancient quarry on the island of Evvia in Greece, and Dioscorides (ca. 40–90 AD) described in his *De Materia Medica* the use of this stone for weaving napkins that could be cleaned and whitened by fire [1]. In Finland, asbestos was used in pottery 4500 years ago, and in Norway, bucket-shaped pottery tempered with crushed asbestos was used

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FIGURE 1 Bucket shape pottery from Telemark, also found in several other areas in Norway, from the Bronze Age to the Roman period (400–575 AD). They were used for cooking and storage of food, with estimated asbestos content of 65–80%. Image courtesy of Asbjørn Engevik, University Museum of Bergen, University of Bergen, Bergen, Norway. © Svein Skaare, University Museum of Bergen.

for storing and making food in the Late Roman and Migration periods (*ca.* 350–475 AD) (fig. 1) [2]. Marco Polo (1254–1324 AD) also described asbestos mining in China, so there is clear evidence of asbestos use among various cultures since ancient times [3]. However, mass mining and use did not start until the 20th century, due to the applications of asbestos as insulation against heat, fire and corrosion, and its tensile strength. Asbestos was widely used for insulation of water and combustion pipes, materials used for house construction and shipbuilding, car brakes and gaskets, even toys, jewellery, and cigarette filters; at its peak, 3000 products were registered [4].

The first report of a pleural tumour was in 1767 by Joseph Lieutand; however, mesothelioma was first characterised as an entity by Klemperer and Rabin in 1931 [5]. It took almost a further 30 years to become widely accepted as a separate cancer entity. The definitive epidemiological study linking mesothelioma to asbestos came from South Africa, published in 1960 by J.C. Wagner, C.A. Sleggs and P. Marchand, showing that mesothelioma was very prevalent in people living or working in the crocidolite asbestos mine area [6]. Later, several studies from the USA, Europe, Australia and Japan verified asbestos inhalation as the aetiological cause of mesothelioma [5, 7–11]. The role of asbestos as a separate carcinogen in lung cancer was also described and, even more dramatically, combining cigarette smoking with asbestos increased the risk of lung cancer from 10- to almost 100-fold over that of the unexposed [12]. Asbestos inhalation can also induce: malignant peritoneal mesothelioma, which accounts for approximately 30% of all mesotheliomas; pleural plaques, which are benign and not predictive of mesothelioma; and asbestosis, a pneumoconiosis that may be fatal and was recently shown to increase the risk of cancer of the pharynx, stomach, colon and ovaries, as reviewed in the 2012 *IARC Monograph* [10]. However, this review will focus on malignant pleural mesothelioma, hereafter referred to as “mesothelioma”. Pleural mesothelioma is a relatively chemotherapy- and radiation-resistant cancer that is usually diagnosed in a late stage and has a median survival of 12 months with the current state-of-the-art treatment with pemetrexed and platinum.

Asbestos and nonasbestos fibre types, exposure and mesothelioma epidemiology

Asbestos is classified into two main families, the serpentines and the amphiboles. The serpentines consist of one type, chrysotile, with characteristic short, curly fibres, also called “white asbestos” due to its colour, which accounts for 95% of asbestos in commercial use. The amphiboles, with straight, longer fibres, include crocidolite or “blue asbestos”, amosite, tremolite, actinolite and anthophyllite, and a thorough review of their physical and biological properties has recently been published [10].

The risk of mesothelioma has previously been correlated with fibre type, where shorter fibres (chrysotile) were assumed to be less carcinogenic. This was mainly due to some research findings of high levels of long (amphibole) fibres in the lungs of the deceased patients and the animal experiments by STANTON and WRENCH [13] leading to the “Stanton fibre hypothesis”. Moreover, some researchers claimed that chrysotile could generate mesothelioma only if it was contaminated with an amphibole, the “tremolite contamination hypothesis” [14]. Nevertheless several animal models, including the early work of WAGNER *et al.* [15] from 1974, have pointed to the conclusion that chrysotile is an important carcinogen and risk factor, not only for mesothelioma, but also for lung cancer; the current international perception, according to the World Health Organization (WHO) and the International Agency for Research on Cancer (IARC), is that all types of asbestos are classified as class I carcinogens [10], and that exposure to asbestos is the major cause of both pleural and peritoneal mesothelioma [16]. These findings resulted in the banning of asbestos production and import in several European countries at various time-points after 1970, and in the European Union as late as 2005 [10, 17].

There is a known dose-response pattern of asbestos exposure with mesothelioma and lung cancer but, as stated by the IARC and WHO, no safe lower threshold has been identified. Moreover, BERNSTEIN *et al.* [18] discovered that not only were people working with asbestos at risk but also their families, due to cleaning of contaminated work clothes, and people living close to places where asbestos was mined or processed. In Italy, in Casale Monferrato (Eternit factory), Broni and Bari (Fibronit factory), an epidemic of mesothelioma was registered among inhabitants who were never exposed to the local asbestos factories, where 25% and 33% of cases, respectively, had only one risk factor: living close to an asbestos cement factory. The calculated risk was very high (OR 10.5 and 5.25, respectively) for those living <500 m from the factory and the fibre burden in the lungs of deceased cases was 10-fold that in those from other areas [19].

In most epidemiological surveys, mesothelioma is more common in men (typical male/female ratios were 5/1) and some have inferred that susceptibility is correlated with sex. However, other studies have shown this to be related to exposure and, typically, there is low asbestos exposure in women because the occupations that confer exposure are traditionally men's work. In occupations where women were most exposed, the majority of the victims were women [15].

The extent of import and use of asbestos in a country is closely correlated to mesothelioma incidence. Norway, a small country but with a solid cancer registry and asbestos statistics, exemplifies this point, where the peak of asbestos import was in 1970–1975 and the apparent mesothelioma peak is today, 40 years later (fig. 2). International epidemiological surveys have estimated that the incidence peak in Europe will be reached around 2020 [20].

Asbestos may also cause lung cancer; up to 20 000 asbestos-related lung cancers and 10 000 mesotheliomas are estimated to occur annually across the population of western Europe, Scandinavia, North America, Japan and Australia [21], while registrations are not available in areas that still use asbestos (eastern Europe, South America, Africa, and the rest of Asia, including China). WHO reports that 125 million people encounter asbestos in the workplace. In 2004, asbestos-related lung cancer, mesothelioma and asbestosis from occupational exposures resulted in 107 000 deaths and 1 523 000 disability-adjusted life-years, and we know that these figures are increasing. In addition, several thousands of deaths can be attributed to other asbestos-related diseases, as well as to nonoccupational exposures to asbestos [22]. Currently, the incidence of the disease is still increasing in most countries of the world, and only in countries in which asbestos control measures were taken during the 1970s, such as Sweden and the UK, has this increase levelled off [23]. Thus, the worldwide epidemic is in its beginning [24, 25], and in countries that produce and/or use asbestos, such as China, India, Russia, Zambia, Colombia and Kazakhstan, a sharp rise in incidence could be expected [26–29].

Environmental exposure to carcinogenic fibres that exist on the earth surface is another, more uncontrollable risk factor. An old tradition of whitewashing the houses with soft tremolite in Metsovo,

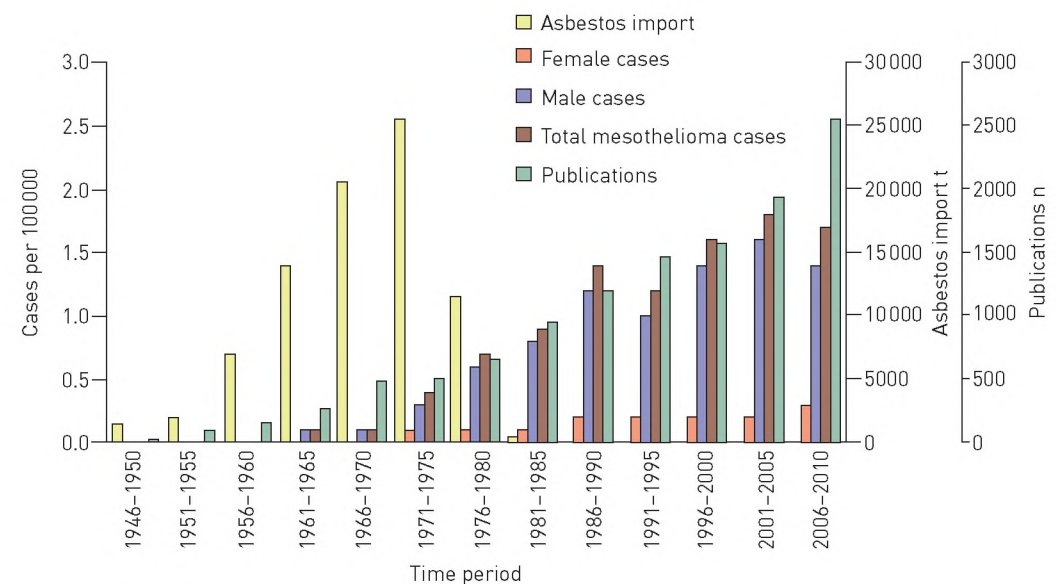


FIGURE 2 Cumulative amounts in 5-year periods from 1946 to 2010 of import of raw asbestos to Norway (data from Statistics Norway (www.ssb.no), 2002), incidence rates of malignant mesothelioma per 100 000 among males and females in Norway (International Classification of Diseases (10th Edition) diagnosis code C45; data from the Norwegian Cancer Registry (www.kreftregisteret.no)) and cumulative number of publications in PubMed with the key word “mesothelioma”.

Greece, was the reason for a cluster of mesotheliomas in young women, as women used to do this work [30]. Another important study by LUO *et al.* [26] documented that the farmers exposed to crocidolite-containing soil in Da-Yao, a province of China, had a death rate of mesothelioma of 365 per million, in contrast to, for example, Norway, with 14 cases per million. Erionite, an asbestos-like mineral from the soil, was revealed as the main factor of mesothelioma in young people in some villages in Cappadocia in Turkey, where more than 50% of inhabitants died from mesothelioma [31]. The study of family trees in these villages showed a strong linkage to certain families, indicating a genetic susceptibility for developing this disease, probably in an autosomal dominant way [32]. Recently, a breakthrough in the study of mesothelioma susceptibility showed that mutations in the gene *BAP1* (BRCA1-associated protein 1) were strongly associated with mesothelioma [33]. This will be discussed later in this review.

Ionising radiation and mesothelioma

Long-term effects of ionising radiation have been aetiologically linked to mesothelioma, although in a much smaller group of individuals than the asbestos exposed. The risk of developing mesothelioma was significantly higher in cases previously exposed to α -particle-emitting agents, such as the radioactive contrast Thorotrast. It is also well-documented that mesothelioma is over-represented in testicular cancer and Hodgkin's lymphoma survivors who have been treated with external radiotherapy [17, 34–36]. However, today, due to knowledge of secondary cancer formation and improved alternatives for these cancers, these treatments are rarely used.

Polio vaccine, simian virus 40 and mesothelioma

One very fascinating, but also very disturbing, hypothesis put forward in 1994 is that inadvertent inoculation of millions of people with an oncogenic virus at the end of the 1950s could be a cause of mesothelioma and other types of cancer [37]. This theory has still not been completely discarded due to the facts reviewed below.

The first inactivated poliovirus vaccine (IPV) and live oral poliovirus vaccine were prepared in primary cell cultures derived from rhesus monkey kidneys. Studies of these vaccines led to the discovery of a new DNA virus called simian virus (SV)40 in 1959 [38], a virus endemic in rhesus monkeys. SV40 did not induce any serious clinical disease in the animals but caused vacuolisation of green monkey cell cultures, and was found to be highly oncogenic in rodents, inducing mesothelioma, ependymoma, osteosarcoma and non-Hodgkin's lymphoma, apparently partly dependent on the route of injection [39]. The formaldehyde treatment that was used to prepare IPV failed to completely inactivate SV40, causing some batches of IPV to contain infectious SV40. As a result, it has been estimated that 30–100 million people in the USA and many more worldwide received potentially contaminated vaccines prepared during the years 1955–1963 [40].

The first known biological effect of SV40 was the development of numerous tumours (polyoma) in hamsters, but also the ability to transform human cell lines. This archetypal virus effect on eukaryotic cells has been crucial in revealing important, basic functions such as replication, transcription and transformation [38]. The strong and consistent relationship between experimental SV40 infection and cancer development in rodents motivated the investigation of its carcinogenic potential in humans. SV40 DNA sequences have repeatedly been reported in cases of human cancers of essentially the same types observed in rodents [37, 41, 42]. In a meta-analysis that included 528 mesothelioma cases and 468 controls from 15 studies, the combined odds ratio for the presence of SV40 DNA sequences in mesothelioma tumour tissue was very high (OR 17, 95% CI 10–28) [43]. Other studies of SV40 sequences in tumour tissue have, however, seriously questioned these results, suggesting that positive findings were caused by contamination or other laboratory artefacts [44, 45]. Even if it is a fact that human infection of SV40 took place on a large scale in the early days of polio vaccination, epidemiological studies have not shown any clear correlation to cancer [46].

The interpretation of the repeated finding of SV40 in human tumours is still controversial. However, recent studies showed that animals infected with SV40 or transfected with SV40 large T-antigen (TAG) were extremely susceptible to asbestos carcinogenesis, and this is currently used as an *in vivo* model of SV40 as a co-carcinogen for mesothelioma development [47, 48]. A molecular epidemiological study has also indicated a co-carcinogen role of SV40 and **studies addressing this question are awaited** [49].

Pathophysiology

Normal pleura physiology and fibre clearance

The pleura is a thin and elastic membrane that covers the entire inner surface of the thoracic cavity. It is almost continuous, so it constitutes an expandable sac with a small amount of lubricating fluid for smooth movement of the thoracic cage, lungs, heart and inner organs. Parietal pleura cover the thoracic wall, mediastinum, heart and diaphragm, and the visceral pleura cover the lungs (fig. 3).

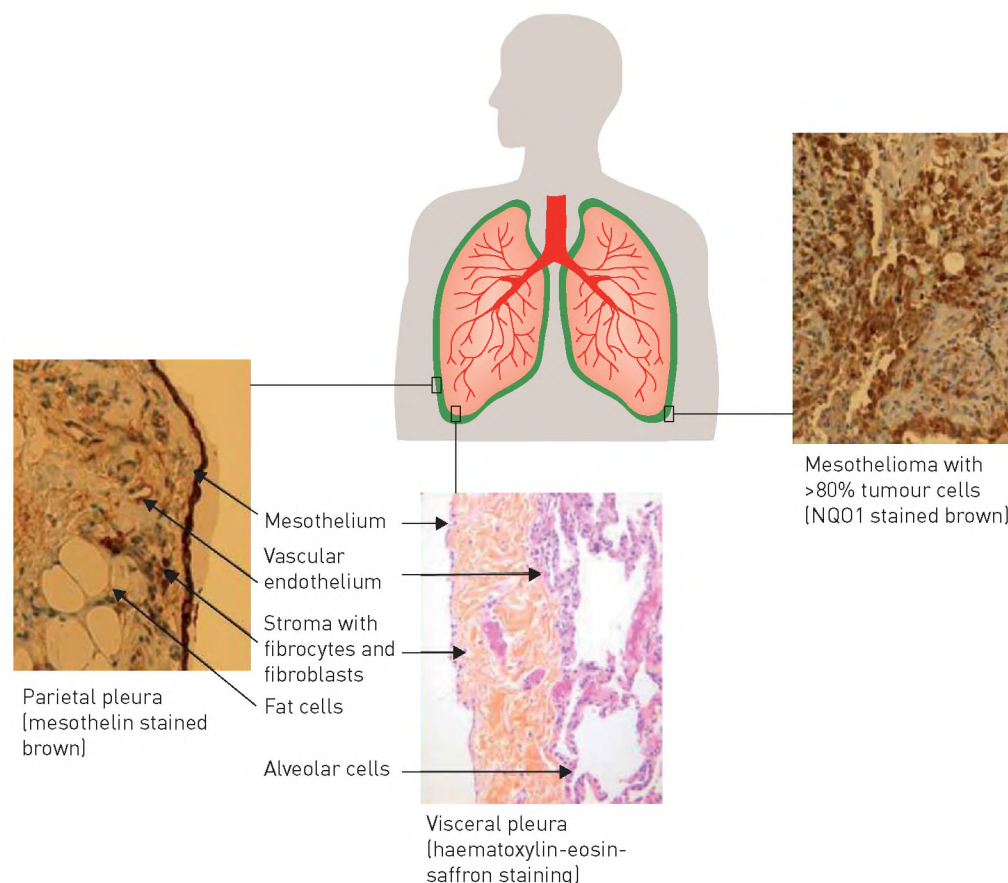


FIGURE 3 Representation of the normal parietal pleura, the visceral pleura and pleural mesothelioma with the most abundant cell types. Reproduced from [50] with permission from the publisher.

Pleura is of mesodermal origin, is present at the seventh week of gestation and comprises five layers: 1) a single layer of mesothelial cells; 2) a thin mesothelial connective tissue layer with a basal lamina; 3) a thin superficial elastic layer; 4) a loose connective tissue layer containing adipose tissue, fibrocytes, fibroblasts, mast cells and other mononuclear cells including telocytes [51], blood vessels, nerves and lymphatics; and 5) a deep fibroelastic layer that adheres tightly to the underlying structures (lung, thoracic muscle, *etc.*). Studies of mesothelial cells have shown them to have a variety of functions, including inflammatory responses and phagocytosis of fibres [52].

Parietal and visceral pleura are similar, but have also significant differences in structure and function (table 1). Interestingly, genome-wide analysis of parietal *versus* visceral pleura from the same noncancer patients reflected this, as described previously in our study (fig. 4) [50].

Why is the pleura, and especially the parietal pleura, the target of asbestos disease? Inhaled fibres enter the visceral pleura and the pleural space to the parietal pleura through the alveoli, or retrograde through the lymphatic vessels [54]. This may be the reason why fibres of different sizes are more abundant in various segments, where the vast majority of chrysotile, the shorter asbestos fibre, is found in parietal pleura [55, 56], while crocidolite and other amphibole fibres are mostly found in the lung parenchyma [56, 57]. Moreover, the chronic inflammatory reaction believed to precede mesothelioma is mostly around the stomata and the lymphoid patches called Kampmeier's foci located in the basal part of the parietal pleura, which is the most common site of mesothelioma.

Origin and site of the mesothelioma cell

Mesothelioma is derived from cells of the pleura, peritoneum or tunica vaginalis, of which pleural location accounts for about 70% of cases [58]. Epithelial mesothelioma, with its cuboidal cells, is the most common subtype, while the sarcomatous type, with spindle-like cells, and the mixed subtype are rarer.

Until recently, it has been taken for granted that the mesothelial cell is the mesothelioma progenitor cell. However, when normal mesothelial cells are damaged, both regeneration from normal mesothelium and

TABLE 1 Structural and functional differences of the parietal and visceral pleura

Biological parameters	Parietal pleura	Visceral pleura
Mesothelial microvilli	Fewer (fewest on the costal surface)	More abundant
Mesothelial cell form	Flat	Cuboidal
Elasticity	Low	High
Permeability	High	Low
Tight junctions	Fewer	More
Intramembranous organisation	Loose	Complex and tight
Innervation	Somatic, intercostal nerves	Vagus, sympathetic trunk
Pain receptors	Yes	No
Vascularisation	Abundant	Poor
Drainage of pleural fluid and particles	Most	Little
Pleural fluid origin	Most	Little
Stomata	Yes, basal part	No
Lymphatic drainage	Abundant (mostly intercostal spaces)	Less, closer to alveoli than the mesothelium
Kampmeier's foci	Yes	No

Information from [53].

development from submesothelial multipotential stem cells are possible mechanisms of repair [59, 60]. Moreover, recent studies showed that adipocytes, circulating multipotential fibrocytes and adult bone marrow-derived stem cells are able to differentiate into both epithelial and mesenchymal cells [61–63]. Interestingly, mesothelial cells also have the ability to change phenotype to become smooth muscle cells and fibroblasts in internal organs and their vasculature, so normal mesothelial cells are multipotential, similar to stem cells [64]. The question that arises is whether the mesothelioma progenitor cell is derived from a submesothelial multipotential cell, from the highly differentiated mesothelial cell or both?

An obvious problem that remains unresolved is why pleural plaques and, most probably, mesothelioma primarily evolve in the parietal pleura [65]. This issue has not been studied in depth, but the aforementioned factors have been hypothesised. However, there is no general consensus on this point. The evidence supporting a parietal origin is that cases with only parietal affection (stage T1a) have a survival of 32.7 months versus only 7 months in T1b, which involves parietal and visceral pleura, indicating that the parietal lesion is an earlier event [65]. Moreover, to our knowledge, cases where only visceral pleura is affected have not been reported [66]. The current concept is that the tumour grows in a locoregional pattern, spreading from the parietal to the visceral pleura and invading the surrounding structures, which induces the clinical picture of pleural fluid, pain and dyspnoea.

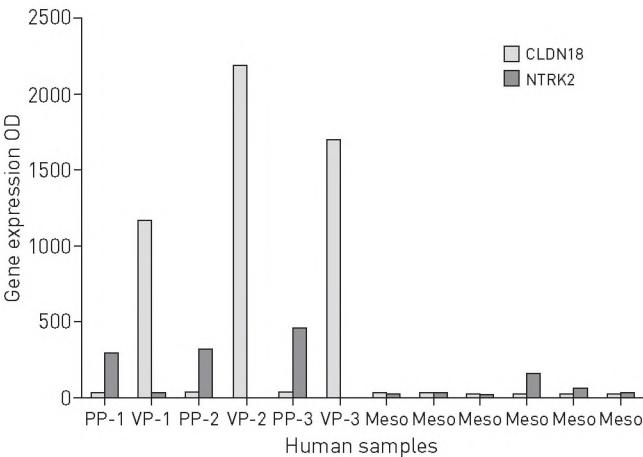


FIGURE 4 Differential gene expression in human samples of the parietal pleura (PP), the visceral pleura (VP) and mesothelioma (meso). PP and PV from the same noncancer patients were analysed with a genome-wide mRNA array (unpublished observations). Tight junctions are much more abundant in the visceral pleura, and several claudins, including the claudin 18 gene (CLDN18), were highly expressed in VP. The type 2 neurotrophic tyrosine kinase receptor gene (NTRK2), which is abundantly expressed in nerve cells, is highly expressed in the PP. Both CLDN18 and NTRK2 had low expression in most of the mesotheliomas analysed. OD: optical density.

Mesothelioma carcinogenesis and molecular profiling

Knowledge of the cytogenetic and molecular aspects of mesothelioma has progressed substantially in recent years, and high-throughput analyses have revolutionised molecular characterisation and our understanding of its underlying biological complexity. However, the carcinogenic mechanism of asbestos is not fully understood. Here, a short overview is provided.

The currently accepted concept is that inhaled asbestos or other carcinogenic fibres end up in the pleura, inducing cytotoxicity, DNA damage, frustrated phagocytosis and chronic inflammation [67, 68]. During the latency period of 20–60 years, several key mechanisms of the mesothelium, such as chromosomal aberrations and epigenetic changes (*e.g.* promoter hypermethylation at tumour suppressor loci), result in functional disturbances measured by gene, microRNA and protein expressions [69–73]. Asbestos fibres are clastogenic and cytotoxic *in vitro*, and induce abnormal segregation at mitosis. Their *in vitro* and *in vivo* transforming activity has been related to fibre dimensions, durability and surface properties [12]. Based on epidemiological data, the long, thin amphiboles (crocidolite and amosite) have been regarded as the most carcinogenic, while the short and curly serpentines (chrysotile) are regarded as less dangerous, but undoubtedly carcinogenic. As noted previously, more recent data point out that chrysotile should probably not be considered less dangerous [74–76].

Cytogenetic studies have shown that mesotheliomas have highly complex and variable chromosomal aberrations [77], and only few features are shared between patients. Loss-of-heterozygosity analyses have demonstrated frequent deletions of specific sites within chromosome arms 1p, 3p, 6q, 9p, 13q, 15q and 22q. Two of these regions are most frequently altered, the tumour suppressors *CDKN2A-ARF* at 9p21, and *NF2* at 22q12. Homozygous deletion appears to be the major mechanism affecting *CDKN2A-ARF*, whereas inactivating mutations coupled with allelic loss occur at the *NF2* locus [78]. Mesothelioma, as opposed to most other cancers, rarely has mutated *TP53*, but loss of p14^{ARF} indirectly inactivates p53 [79]. Restoring p14^{ARF} in a mouse model suppressed tumour growth significantly, showing the importance of this mechanism [80]. Furthermore, retinoblastoma protein and p53 can be inactivated by SV40 TAG, which has been a suggested mechanism of mesothelioma progression [81]. NF-κB (nuclear factor, κ-light chain enhancer of activated B-cells) has been shown to be a survival factor in transformed human mesothelial cells and human mesothelioma cells. It acts as a survival factor in human mesothelial cells exposed to asbestos fibres [82]. Proteasome genes are overexpressed in human mesothelioma. Proteasome inhibition was shown to suppress NF-κB activity in malignant mesothelioma cells, and induce cell cycle blockade and apoptosis *in vitro*, as well as tumour growth inhibition *in vivo* [83]. Recently, *KRAS* and *TP53* missense mutations were shown to induce not only lung carcinomas but also aggressive mesotheliomas in mice [84], showing that only two gene alterations also can induce this tumour. Cell surface heparan sulfate proteoglycans, particularly syndecans, have recently been shown to be expressed in mesotheliomas, interact with growth factors and matrix components, and play a role in epithelial–mesenchymal transition of epithelial mesothelioma to the sarcomatous type [85].

Extracellular signal-regulated kinases (ERKs) are a family of molecules with distinct roles in cell injury, repair, differentiation and carcinogenesis. In several cell line and animal studies, members of this family, especially ERK2, were critical to transformation and homeostasis of human malignant mesothelioma of the epithelial type [86]. The transcription factor activator protein-1, which is a target of asbestos-induced signalling pathways, was also shown to be critical to the transformation of mesothelial cells through ERK-dependent Fos-related antigen (Fra)-1 elevation [87]. Hepatocyte growth factor (HGF)/scatter factor and its receptor tyrosine kinase, c-Met, are highly expressed in most human malignant mesotheliomas and recently, it was shown that HGF-mediated cell proliferation of human mesothelioma cells through a phosphoinositide 3-kinase (PI3K)/MAP kinase 5/Fra-1 pathway [88]. Moreover, c-Met was shown to be a relevant experimental treatment target as well as a negative prognostic factor [89, 90]. Survival pathways, such as PI3K/Akt/mammalian target of rapamycin (mTOR), are involved in cell growth and resistance to apoptosis, and are often activated in mesothelioma. mTOR was shown to mediate survival signals in many mesothelioma tumours and inhibition of mTOR was also proposed as a nontoxic adjunct to therapy directed against mesothelioma [91]. Inflammation has also been incriminated and it was recently shown that inflammation precedes mesothelioma formation. Moreover, asbestos-induced priming and activation of the NLRP3 (nucleotide-binding domain, leucine repeat containing) inflammasome triggered an autocrine feedback loop modulated *via* the interleukin (IL)-1 receptor in mesothelial cells, which is targeted in pleural infection, fibrosis and carcinogenesis [92, 93]. A similar mechanism has been shown for carbon nanotubes (CNTs) (see the section on Manmade CNTs).

High-throughput profiling of mRNA by various array platforms has indicated many interesting features of mesothelioma biology, but the results have not been concordant. This may be due to several factors: use of different array platforms with various numbers of genes; and some researchers using cell lines, others correlating human tissue with cell lines and others examining tumour tissue from the parietal pleura of

lung cancer patients or noncancer patients [50, 94–101]. All these factors may have contributed separately and together to the largely incongruent results.

However, in our genome-wide analysis of six tumour and seven parietal pleura samples, we demonstrated dysregulation of several systems, with overexpression and downregulation of genes reflecting several important biological functions, including DNA replication and repair, and microtubule cytoskeleton organisation and biogenesis [50, 71]. Of the DNA repair entity, genes related to double-strand break repair were over-represented. Several key genes encoding proteins known to be targets, but also to confer chemotherapy resistance (e.g. *NQO1* (NADPH dehydrogenase, quinone 1), *TOP2A* (topoisomerase II α), *TYMS* (thymidylate synthetase), *BIRC5* (survivin) and genes encoding components of the proteasome), and radiotherapy resistance by several DNA repair and damage checkpoint genes (e.g. *BRCA2* (breast cancer 2), *CHEK1* (checkpoint kinase 1), *FANCA* (Fanconi anaemia, complementation group A), *FANCD2* (Fanconi anaemia, complementation group D2), *RAD21* and *RAD50*), were overexpressed in tumours. A novel angiogenic gene, *AGGF1*, was also significantly overexpressed. Several genes encoding detoxifying enzymes, among them the multidrug resistance gene *ABCB1* (ATP-binding cassette subfamily B member 1), were downregulated, as were leukocyte transendothelial migration pathway and signal transduction genes.

Despite the low numbers of cases and controls, the differential gene expression detected was highly significant. Genes known to be overexpressed in mesothelioma were overexpressed here (e.g. the *Ki67*, *syndecan 1*, *survivin* and *vitronectin*). Genes coding for *CD15* and *sialyl transferase*, and negative markers of mesothelioma, such as *FUT4* (fucosyltransferase 4) and *ST6GALNAC3* ((α -N-acetyl-neuraminyl-2, 3- β -galactosyl-1,3)-N-acetylgalactosaminide α -2,6-sialyltransferase 3), were down-regulated [102]. Unexpectedly, genes encoding *calretinin*, *vascular endothelial growth factor (VEGF) receptor* and *mesothelin* proteins overexpressed in mesothelioma were not differentially expressed. However, recent studies showed that these are also expressed in normal mesothelial cells [103–105].

These studies have resulted in the discovery of a novel biomarker, *osteopontin* [106], and the proteasome as a novel treatment target [107, 108], as well as a gene signature for prognostication [109], although it has not gained general acceptance yet [110].

Recently, next-generation sequencing of the whole genome uncovered massive genomic damage in mesothelioma versus normal tissue. This was reflected in a significant aneuploidy, and novel, large-scale, inter- and intra-chromosomal deletions, inversions and translocations. Nearly all candidate point mutations appeared to be previously unknown single-nucleotide polymorphisms. One large deletion in *DPP10* (dipeptidyl peptidase 10) resulted in altered transcription and expression of *DPP10* transcripts correlated with survival in a set of 53 mesotheliomas. Three point mutations were observed in the coding regions of *NKX6-2* (a transcription regulator) and *NFRKB* (a DNA-binding protein involved in modulating NF- κ B), and amplification of genes such as *PCBD2* (pterin-4 α -carbinolamine dehydratase/dimerisation cofactor of hepatocyte nuclear factor 1 α) and *DHFR* (dihydrofolate reductase), which are involved in growth factor signalling and nucleotide synthesis [111].

Mesothelioma susceptibility and the BAP1 cancer syndrome

Mesothelioma develops in a minority of asbestos-exposed individuals. This implies some kind of susceptibility to asbestos fibre carcinogenesis. Previous genome-wide association studies (GWASs) on polymorphisms failed to identify a single gene or even a signature of genes that was reproducible [112]. However, some gene polymorphisms showed increased risks of mesothelioma in a subset of studies, including cases not expressing glutathione S-transferase μ 1 (*GSTM1*-null genotype), as well as two variant alleles of the DNA repair genes *XRCC1* and *XRCC3* [112]. The *GSTM1* protein is important for the detoxification of electrophilic compounds, including carcinogens, drugs, environmental toxins and products of oxidative stress, and its downregulation has been associated with several forms of cancer. More recently, the largest GWAS to date on pleural mesothelioma did not reveal any significant new information, other than indicating that genetic risk factors may play a role in the asbestos-associated mesothelioma [113].

Clustering of mesothelioma cases in some families has been observed by several researchers, and some have argued that this is not only due to shared asbestos exposure [114]. A nuclear protein, *BAP1*, has several proposed functions, including transcriptional regulation, chromatin regulation, and forming part of multiprotein complexes that regulate cellular differentiation, gluconeogenesis, cell cycle checkpoints, transcription and apoptosis [115]. The *BAP1* gene is located on chromosome 3p21, a region that shows loss or deletion in numerous malignant tumours, including 30–60% of mesotheliomas. In the *BAP1* mutation families, there is a dramatically increased incidence of malignant tumours, often developed in an earlier age than observed in the general population [115]. A *BAP1* cancer syndrome has been proposed, including mesothelioma, uveal melanoma, cutaneous melanoma and possibly other malignant tumours [116]. Germ-line *BAP1* mutations have been described in families with extraordinarily high incidence of

mesothelioma [116] and in 25% of sporadic mesotheliomas [117], pointing to *BAP1* as the first gene reported to predispose to mesothelioma, but its role in mineral-fibre carcinogenesis has not been established. Furthermore, there are studies showing that *BAP1* mutations are significantly more common in epithelial than sarcomatous and biphasic mesotheliomas [118, 119]. The discovery of a susceptibility gene will probably be of major importance for defining high-risk *versus* low-risk groups when it comes to screening of asbestos-exposed individuals with novel noninvasive biomarkers.

Biomarkers

Soluble mesothelin-related protein in serum

Mesothelioma has a long latency period: 20–60 years may elapse between asbestos or other oncogenic exposure and the clinical presentation of disease, and the disease is usually diagnosed in a late stage. Noninvasive diagnostic biomarkers aiding early diagnosis and follow-up of patients have not been available.

The first potential mesothelioma tumour biomarker in serum was soluble mesothelin-related protein (SMRP), also called mesothelin [120], and it emerged as a marker for mesothelioma diagnosis [121]. Mesothelin is a family of proteins that are mainly membrane-bound, and expressed in mesothelial cells as well as various cancers, such as pancreatic and ovarian cancer. The SMRP assay can detect three mesothelin variants, where Variant 1 [120] is the predominant form in serum. SMRP is elevated in patients with epithelial mesothelioma, the most common subtype [122]. ROBINSON *et al.* [121] also found SMRP to be elevated 1–5 years before clinical disease in some individuals exposed to asbestos, suggesting that SMRP could be used as a screening test. In addition, elevated SMRP level in serum was recently suggested as an independent negative prognostic factor of mesothelioma [122]. PASS *et al.* [106] not only found a significant elevation of SMRP in mesothelioma *versus* lung cancer, but also between stage I and stage II–IV mesothelioma, and, importantly, it was found to be higher in stage I than in noncancer asbestos-exposed individuals. The biological function of mesothelin is largely unknown but it possibly plays a role in cell–cell adhesion. Interestingly, it may also play a role in peritoneal metastasis of ovarian cancer, as cancer antigen (CA)125 expressed on ovarian cancer cell membranes adhere to mesothelin expressed on normal peritoneal cell membranes [123], facilitating local progression along the mesothelium. Mesothelioma cells also co-express CA125 [124], so the typical growth pattern with local progression and even tumour spread from the parietal to the visceral mesothelium could partly be explained by this mechanism.

SMRP/mesothelin in serum is a noninvasive marker that can be helpful in making the diagnosis of mesothelioma, as the assay in the pivotal study had a sensitivity of 84% for mesothelioma, and 100% specificity in differentiating mesothelioma from other pleural diseases, 95% against other lung cancers and 100% against apparently healthy subjects [16]. However, in another study, the best combination of sensitivity and specificity against controls was 66% and 70.9%, respectively [125]. Moreover, as evaluation of response and recurrence by imaging techniques is difficult in mesothelioma, SMRP level is a sensitive measure of tumour burden as it is increased in more advanced disease [125]. However, this assay failed as an early diagnostic biomarker, as we and others showed no significant difference in preclinical serum levels of cases and controls [126]. Importantly trials are on-going with drugs targeting mesothelin in ovarian cancer, pancreatic cancer and mesothelioma, based on the quite unique expression of mesothelin in these tumours and the lack of expression in vital organs [95]. Thus, mesothelin is currently an important molecule for follow-up of treatment and a putative treatment target for mesothelioma.

13-protein classifier in serum

Proteomic technology has evolved rapidly and a novel assay based on slow off-rate modified aptamers (SOMAmers) are used as capture reagents in order to achieve highly selective protein detection for biomarker identification [127]. The SOMAmers are short, single-stranded deoxynucleotides with an ability to bind discrete molecular targets. The use of SOMAmers as capture reagents carries many advantages over traditional antibody-based immunoassays, including high sensitivity and specificity, dynamic range, accurate quantification, and reproducibility, and has the ability to measure thousands of human proteins in small volumes of biological samples with low limits of detection. Recently, a 13-protein signature was discovered in a cohort of 117 mesothelioma cases and 142 asbestos-exposed control individuals with a diagnostic area under the curve (AUC) of 0.99 in the training, 0.98 in the independent blinded verification and 0.95 in the blinded validation studies. Sensitivity and specificity were 97% and 92% in the training, and 90% and 95% in blinded verification, respectively. Sensitivity also correlated with pathological stage: 77%, 93%, 96% and 96% in stages I–IV, respectively. An alternative decision threshold in the validation study yielding 98% specificity would still detect 60% of mesothelioma cases. When compared to mesothelin in a paired sample set, the 13-protein classifier AUC of 0.99 and 91%/94% sensitivity/specificity were superior to those of mesothelin, with an AUC of 0.82 and a 66%/88% sensitivity/specificity. The candidate biomarker panel consisted of both inflammatory and proliferative proteins that could be associated with

asbestos-induced malignancy [128]. However, there have been no prospective studies on asbestos-exposed cohorts to verify their value for early diagnosis and survival.

Fibulin-3 in plasma

One recently published paper of great interest in the early diagnosis of mesothelioma showed that a previously little-studied protein, fibulin-3, in plasma, could separate asbestos-exposed individuals with mesothelioma from those without mesothelioma, with a sensitivity of 100% and specificity of 94.1%; blinded validation showed AUC 0.87 for plasma specimens from 96 asbestos-exposed persons as compared with 48 patients with mesothelioma. Moreover, fibulin-3 levels in plasma and pleural effusions were significantly different in patients with effusions from mesothelioma *versus* other malignant and benign effusions. Tumour tissue was examined for fibulin-3 by immunohistochemical analysis and 26 out of 26 cases were positive [129]. This is, to date, the strongest diagnostic marker for mesothelioma, but it still needs to be validated in thoracic cancer patients without effusions, as well as in prediagnostic samples. This is very important, as the expression of proteins may vary much in early- *versus* late-stage cancer, and especially in preclinical stages [130].

MicroRNA in plasma

MicroRNA is a novel class of noncoding RNA with several control functions, and high stability in serum and plasma that was found to discriminate cases from controls in several forms of cancer. Currently, there is evidence that increased circulating plasma concentrations of the microRNA miR-625-3p could serve as a potential diagnostic biomarker for patients with malignant pleural mesothelioma [131].

Manmade CNTs

Manmade CNTs and disease: the asbestos of the future?

Among nanostructured materials, CNTs are becoming the best known and studied due to their applications as structural materials in electronics, heating elements, batteries, production of stain-resistant fabric, protection of aerospace materials against lightning strikes, water desalinisation and purification, bone grafting, dental implants, targeted drug delivery, and imaging diagnostics [132–134]. The interest in CNTs is a direct consequence of the synthesis of buckminsterfullerene (or fullerene; molecular formula C_{60}) and its derivatives in 1985. Fullerene is the third allotropic form of carbon after graphite and diamond [134, 135]. It is a 60-atom carbon molecule in a shape of polyhedral cage made of 12 pentagons and 20 hexagons, and can be considered the paradigm of a family of carbon nanostructures characterised by spherical or tubular shapes. The diameter of a nanotube varies from few nanometres up to several micrometres. The CNTs are of essentially two types, namely, single-walled carbon nanotubes (SWCNTs) and multi-walled carbon nanotubes (MWCNTs) [136, 137]. Although CNTs are widely used in several applications, little is known about their potential toxicity in humans. 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 [137].

Current evidence for CNT carcinogenesis in the lung and pleura

Even if the literature on CNT health effects on development of mesothelioma is scarce, several lines of evidence indicate that CNTs behave as biopersistent fibres *in vivo* and have a carcinogenic potential similar to that of asbestos (fig. 5). By inhalation, a significant fraction of CNTs can remain within the lungs for up to several months [138], and in mice, it was shown that MWCNTs migrate and reach the subpleural tissue after a single inhalation of CNTs [139]. Also in mice, PORTER *et al.* [140] showed that MWCNTs of about 4 µm in length are able to reach the pleura and induce pleural inflammation 56 days after a single aspiration, inducing an “asbestos-like pathogenicity”. In a recent publication, rats inhaling sprayed MWCTs weekly for 24 weeks were found to have CNTs that had translocated into the pleural cavity, deposited in the parietal pleura, and induced fibrosis and patchy parietal mesothelial proliferation lesions, very similar to the route and effects of inhaled asbestos [141]. Intraperitoneal injection of MWCTs in heterozygous p53^{+/-} mice that were followed for 1 year showed a dose-dependent development of mesothelioma, in five out of 20, 17 out of 20, and 19 out of 20 mice by increasing the dose, a proof of the principle that MWCTs can produce mesothelioma and at a very high rate [142]. Moreover, chronic exposure to CNTs showed their invasive potential in human pleural mesothelial cells, through an increased expression of matrix metalloproteinase-2 [144], and recently it was demonstrated that respiratory exposure to MWCNTs *in vivo* and *in vitro* induced length-dependent pulmonary fibrosis and epithelial-derived fibroblasts *via* the transforming growth factor-β/Smad pathway [143]. CNTs could also induce metastatic progression of lung carcinoma in mice, mediated by increased local and systemic accumulation of myeloid-derived suppressor cells, as their depletion abrogated pro-tumour activity *in vivo* [145]. One of the theories on asbestos fibre toxicity and carcinogenicity is the “fibre paradigm” [146], where the geometry of fibres contributes their biopersistence [147]. Similarly, it has been shown that the

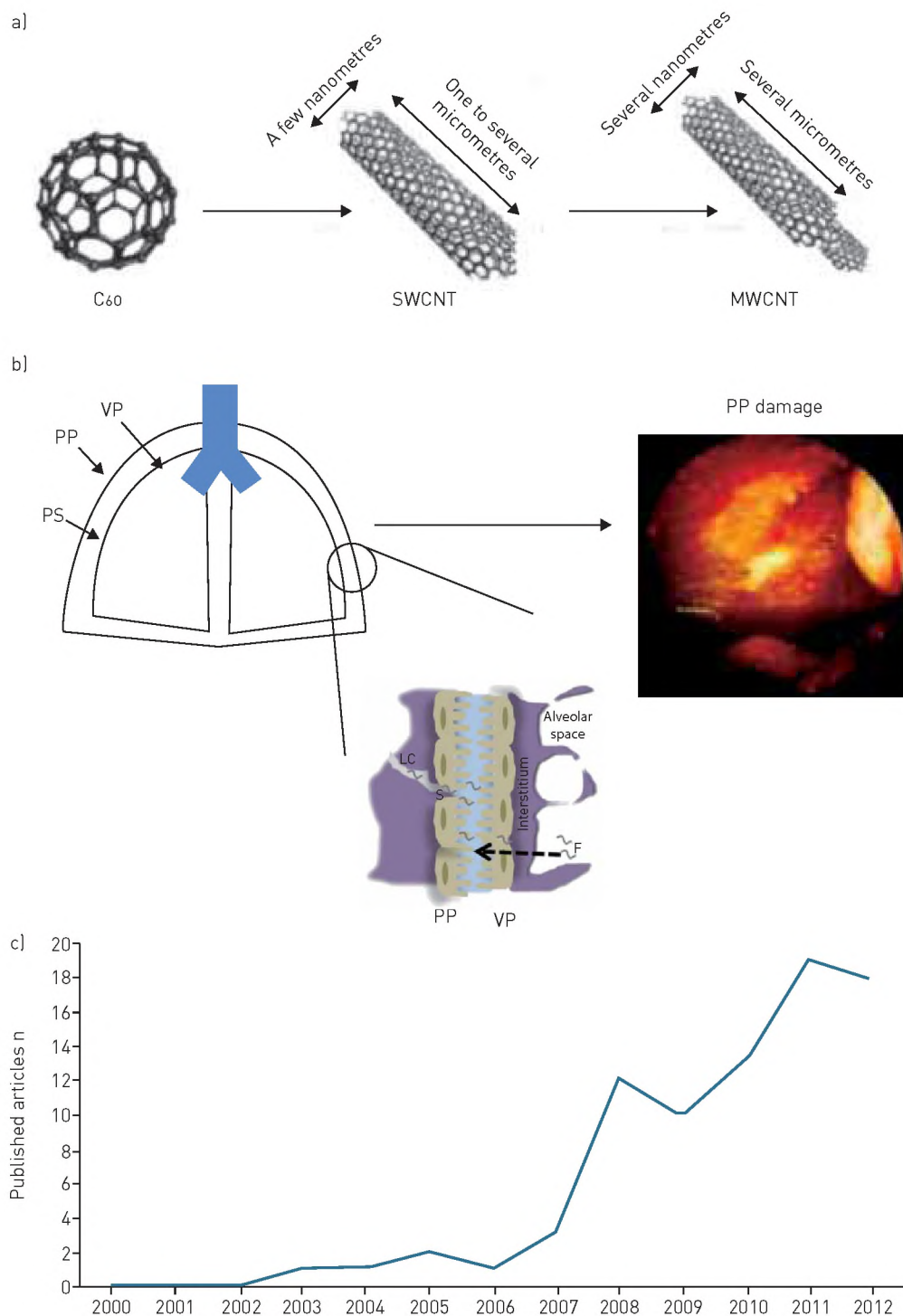


FIGURE 5 Pleural hazard of exposure to carbon nanotubes (CNTs). a) Structures of single-walled CNTs (SWCNTs) and multi-walled CNTs (MWCNTs) derived from buckminsterfullerene (C_{60}). The structure of a SWCNT (<0.4 nm in diameter) can be conceptualised by wrapping a one-atom-thick layer of graphite (or graphene) into a seamless cylinder. MWCNTs consist of multiple layers of graphite rolled on themselves to form a tube shape with an interlayer spacing of 3.4 Å and a diameter ranging from 1 to 50 nm. b) Schematic sequence of steps leading to pleural damage and/or malignant transformation as a consequence of exposure to biopersistent fibres. Long fibres and CNTs are retained at stomatal openings and induce an inflammatory oxidative stress response, which leads to aberrant reactions at the parietal pleura. Accordingly, the primary lesion caused by biopersistent fibres might form at the parietal pleura; this fact is reflected in pleural mesothelioma staging, where the early mesothelioma is confined to the parietal pleura while more advanced mesothelioma involves the visceral layer. LC: lymph channel; F: fibre; VP: visceral pleura; PP: parietal pleura; PS: pleural space; S: space c) Increasing literature on lung and pleura toxicity induced by CNTs in PubMed, searching for “CNT and pleural toxicity + CNT and mesothelioma”.

CNTs also have a length-dependent toxicity and carcinogenicity [147]. Finally, change of gene expression signatures in the mouse lungs following pharyngeal aspiration MWCNTs were associated with human lung cancer risk and progression signatures [148, 149].

Potential mechanisms of CNT oncogenesis

As we have reviewed here, several years of studies on asbestos carcinogenicity have not elucidated its oncogenesis fully. Certainly, knowledge of oncogenesis is rudimentary regarding CNTs, but induction of inflammation and gene toxicity seems to be an important component. Interestingly, a significant acute neutrophilic influx into the lungs was demonstrated in animals just 1 day after CNT inhalation, coexisting with histopathological findings coherent with bronchiolitis/alveolitis [150]. The epithelial damage seems to be mediated by reactive oxygen species (ROS), the production of which has been related to the metallic contamination and impurities of CNTs [151, 152]. Moreover, chronic exposure to CNTs affects the immune response, as it determines the sequestration of surfactant proteins A and D (SP-A and SP-D) and collectins, which, in turn, induce macrophage impairments [153]. Indeed, lowered SP-A and SP-D levels reduce resistance to some infections and induce an emphysema-like alteration of the lungs. Moreover, these changes lead to a progressive accumulation of surfactant phospholipid in the lung tissue and alveolar space, and accumulation of apoptotic alveolar macrophages [154]. Although indirect genotoxic CNT action might be related to inflammation and ROS formation, direct CNT DNA damage in cells has been postulated as being responsible for epithelial and mesothelial malignant transformation. Some *in vitro* and *in vivo* experiments have shown a direct interaction of MWCNTs with DNA [155], suggesting a genotoxic/mutagenic potential, whereas weaker data are available on SWCNTs [156, 157].

Genotoxic damage can occur through direct interaction of retained asbestos fibres and mesothelial cells around the stomata of the parietal pleura as previously described. CNTs have a length-dependent toxicity profile on the mesothelium. Accordingly, long CNTs could be retained around the stomata at the parietal pleura, in a similar fashion to asbestos fibres. It has been reported that macrophages recognise asbestos fibres and CNTs *via* the class A scavenger receptor MARCO (macrophage receptor with collagenous structure) [158], and that the uptake of both types of fibres activates the NLRP3 inflammasome [159]. Recent data demonstrate that asbestos and erionite induce priming and activation of the NLRP3 inflammasome that modulates, through an autocrine feedback loop, the release of IL-1 β , IL-6, IL-8 and VEGF. Production of such critical cytokines and growth factors is probably involved in the initiation of pleural injury and infection, pleural fibrosis, and mesothelioma [160]. Moreover, when exposed to biopersistent fibres, mesothelial cells might avoid apoptosis when stimulated by tumour necrosis factor- α secreted by the activated macrophages. This mechanism has been reported for asbestos and could theoretically be applicable to CNTs [161].

Regulation of health hazards posed by CNTs and future aspects

In the past decade, CNT-related production capacity has increased at least 10-fold and many companies are investing in diverse applications of CNT, from microelectronics to biotechnology, environmental applications and energy storage [162]. Overall, the production capacity for all CNT products is expected to exceed 12 300 tonnes in 2015 and the total production value is expected to reach US \$1.3 billion in 2015 [163]. With such a multitude of applications, a thorough understanding of the associated toxicity is therefore essential. The evaluation of CNT toxic profiles might take in consideration exposure during manufacturing steps as well as their interaction with biological systems. Preliminary data have demonstrated that the main risk to humans is related to chronic occupational inhalation, mainly during those activities involving high CNT release and uncontrolled exposure. There is an urgent need to test these materials extensively and understand their potential role in causing mesothelioma. In 2013, the US National Institute for Occupational Safety and Health (NIOSH) thoroughly revised their reviews of the animal and other toxicological data relevant to assessing the potential nonmalignant adverse respiratory effects of CNT and proposed a recommended exposure limit (REL) of 1 $\mu\text{g}\cdot\text{m}^{-3}$ elemental carbon as a respirable mass (8-h time-weighted average concentration [164]). The NIOSH REL is expected to reduce the risk of pulmonary inflammation and fibrosis. However, given the uncertainty about CNT-associated cancer risk, continued efforts should be made to reduce exposure as much as possible, and a number of strategies for controlling workplace exposures and implementing a medical surveillance programme should be designed. Above all, CNT doses associated with genotoxicity should be determined by *in vitro* and *in vivo* studies. To avoid differences in interlaboratory research protocols contributing to conflicting data in the literature, consortium research programmes are emerging to reduce variability and validate findings [165]. In parallel, a number of workplaces should be monitored, from research laboratories to CNT manufacturing, manipulating and recycling plants. Surveillance programmes should be extended worldwide, manufacturers in North America being focused more on SWCNTs, and Asia and Europe (mostly Japan followed by China) leading the production of MWCNTs [166]. Exposure control challenges should involve both the evaluation of exposure control

techniques, and the management of populations at risk through the identification of work practice recommendations and secondary prevention measures [167, 168]. Due to the probable latency, follow-up analysis should be performed on CNT-exposed people. It should be noted that preliminary reports suggest that functionalisation allowing CNT solubilisation and enhancing their biocompatibility using water-soluble nanoconjugates seems to be emerging as a safe and effective procedure, with no cytotoxicity on macrophages and epithelial cells [169, 170]. In conclusion, these observations have the following relevant implications. 1) Although promising in several fields, including pharmaceuticals and biomedicine, CNTs could exert a toxic and potentially tumorigenic effect. 2) Based on a similar length-dependent pathogenicity, CNTs may pose an asbestos-like mesothelioma hazard. 3) Nanotoxicology requires a multidisciplinary approach to this kind of problem in order to achieve effective risk control.

Conclusion

Malignant pleural mesothelioma is a global, manmade cancer problem with increasing death tolls due to sustained mining and use of asbestos. Preventive measures including a global ban on asbestos should be mandatory. Awareness of the potential danger of new manmade fibres with similar carcinogenic properties, exemplified by CNTs, should be high with thorough and relevant testing before their release into society. Novel diagnostic biomarkers are currently being tried for clinical use but we still do not have markers for early diagnosis, prognosis or prediction of therapy. High-throughput molecular profiling of tumours, blood and pleural fluids is currently revealing novel biomarker candidates for earlier diagnosis and novel targets for improved treatment in the future.

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