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Malignant peritoneal mesothelioma in a boar who lived in Calabria (Italy): wild animal as sentinel system of human health

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Abstract

Mesothelioma is a tumor of the serosal membranes described both in human and veterinary medicine. While in humans the relationship between mesothelioma and exposure to asbestos and some other asbestiform minerals is well known, in animals it is still difficult to establish. In this paper a case of malignant peritoneal mesothelioma probably related to asbestos exposure in a wild boar is described. At post-mortem evaluation the peritoneum, diaphragm and serosal surface of liver and kidneys showed isolated to coalescent multiple nodular lesions. Samples from diaphragm, liver and lung were collected to perform microbiological and histological investigations. To assess the presence of asbestos and/or other asbestiform minerals, SEM-EDS investigations were performed on organs and soil samples collected from the area where the wild boar lived.

Microbiological investigations were negative for *Mycobacterium* species. Gross and histological examination were compatible with a biphasic mesothelioma, with nodules composed of epithelioid and sarcomatoid elements with high pleomorphism. Immunohistochemistry revealed only multifocal scattered positivity for WT-1 and D2-40. Asbestos fibres were detected in all samples (organs and soil) by SEM-EDS, demonstrating a potential relationship between the neoplasia and the exposure to naturally occurring asbestos (NOA).

In conclusion, the results of the present study are further confirmation that wild animals, such as the boar, are suitable sentinels to indicate the risk of environmental exposure to asbestos for human populations.

Key words: mesothelioma, asbestos environmental exposure, boar, sentinel animals

Conflict of interest: none

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1. Introduction

Malignant mesothelioma (MM) is a tumor of serosal membranes including pleura, pericardium, peritoneum and tunica vaginalis testis. In humans, MM and its strong correlation with occupational or anthropogenic environmental exposure (at a high dose) to asbestos and other asbestiform minerals has been well documented and confirmed (IARC, 2012) and makes it an epidemiological marker (Pavlisko and Sporn, 2014). It is more difficult to establish effects on human health resulting from chronic low-dose exposures as in the case of a natural environmental background, a very common situation.

The use of asbestos began in ancient times and reached the height during the second half of the 20th century. Actually mining and use of asbestos minerals remain high in a limited number of states in the world (USGS, 2018). Asbestos minerals are present in many environments, i.e. rocks, soils, air and water. Rocks are their primary source and rocks containing asbestos are widespread worldwide. Owing to natural and/or anthropogenic action asbestos is dispersed from these sources and spread in the environment.

The term “asbestos” covers a group of six natural minerals (hydrated silicates), one belonging to the serpentine group and the remaining five to fibrous amphiboles (IARC, 2012).

Owing to the lack of a recognized definition, according to the dimensional definition by World Health Organization (WHO, 1997) and the literature (Belluso et al., 2017), the meaning of

74 the words used in the paper is shown below. The terms "fibre" and "fibrous" are used to
75 address inorganic particles with a length ≥ 5 μm, a width ≤ 3 μm, and a length/width ratio ≥
76 3:1 which present parallel sides when seen in two dimensions, perpendicularly to fibre axis.
77 Asbestiform is an adjective used for non-asbestos-classified fibres with asbestos-fibre-like
78 dimensions and at least one of the asbestos properties such as flexibility or splitting. Fibre
79 bundle is used for a parallel aggregate of mineral fibre.
80 The most widely known fibrous variety of serpentine is chrysotile which is the most
81 commonly used asbestos in industrial production as component of asbestos containing
82 materials (ACM). Chrysotile is abundant as naturally occurring asbestos (NOA), i. e. in rocks
83 . Other asbestiform serpentine varieties are asbestiform antigorite and asbestiform
84 polygonal serpentine, which are difficult to distinguish form chrysotile and often confused
85 with it, depending on the kind of investigation (Belluso *et al*., 2017). Other commercially
86 common asbestos minerals include the amphiboles crocidolite and amosite (also named
87 cummingtonite-grunerite asbestos), tremolite asbestos, actinolite asbestos and
88 anthophyllite asbestos (NIOSH, 2011).
89 Apart from these five asbestos listed above, there are also certain asbestiform amphibole
90 not asbestos classified as, for example, asbestiform fluoredenite (detected in Italy) and
91 asbestiform winchite (detected in USA). Correlation between a high dose exposure to these
92 minerals and MM was recognized through epidemiological investigations in humans affected
93 by MM (McDonald et al., 2004; Paoletti et al., 2000).
94 The presence of these asbestiform minerals was identified only recently, either because
95 they had not been detected in raw materials or because they had been confused with
96 other minerals, such as tremolite asbestos (Meeker et al., 2003).
97
98 Owing to natural and/or anthropogenic action, asbestos are dispersed from these sources
99 and spread in various types of environments. The same origin and the same fate concern

100 other asbestiform minerals not currently classified as “asbestos” that, at the current state of
101 knowledge, are present only in some areas of the world (Cannata et al., 2018).

102 Diffusion of fibrous minerals (whether classified as asbestos or not) in non-occupational
103 environments has been highlighted in the last twenty years by their detection in lungs of
104 animals. In non-experimental animals, MM is generally rare. It has been more frequently
105 described in dogs (Forbes and Matthews, 1991; Glickman et al., 1983;) and cattle (Girard
106 and Cécire, 1995; Klopfer et al., 1983; Zurwieden et al., 1990) but occasionally also in cats
107 (Umphlet and Bertoy, 1988), lambs (Brown and Weaver, 1981), horses (Mair et al., 1992;
108 Ricketts and Peace, 1976), goats (Campopiano et al., 2017) and pigs (Beytut, 2002; Uzal et
109 al., 2016).

110 Animals have been proposed as sentinel system (SSA) for different aerial pollutants
111 including asbestos to complement human epidemiological studies and to demonstrate the
112 interactive effects of these mineral fibres and their role in biological response (Dumortier et
113 al., 2002; Rey et al., 1994). In fact, animals are not affected by occupational exposure, their
114 lungs are easier to obtain for post-mortem examination than human ones and, finally, they
115 can provide an assessment of the kind and amount of the respirable minerals present in the
116 environment where they lived. Therefore, SSA could be indicators of environmental
117 background exposure to inorganic fibres (asbestos and non-asbestos) (Ardizzone et al.,
118 2014; Capella et al., 2017a; Capella et al., 2017b; De Nardo et al., 2004; Dumortier et al.,
119 2002; Fornero et al., 2009; Glickman et al., 1983).

120 In particular, MM in wild animals can be suitable to provide useful indications for
121 understanding the possible correlation between low-dose exposure and carcinogenesis.
122 Regarding the investigated Italian area (Calabria region, south of Italy), in 2017
123 Campopiano et al. identified tremolite asbestos fibres in the pulmonary tissue of sheep,
124 goats, and two boars that lived near disused quarries. In the Calabria region (Monte

Reventino, CZ) several deposits of ophiolite containing mainly tremolite both asbestiform (i.e. asbestos classified) and non-asbestiform (i.e. non asbestos classified) were reported (Zakrzewska, 2008). On the basis of the National Register of mesotheliomas in the same region 6 cases of human tumors related to asbestos exposure were detected in 2012 (INAIL, 2015) confirming the presence of asbestos in the environment and the need to activate a suitable method to evaluate the potential human risk. On the basis of these considerations, the aim of this report is to describe the pathological findings of a case of peritoneal mesothelioma detected in a wild boar (from Calabria) and relate them to the environment where the boar lived.

2. Material and methods

2.1 The boar

A 3-year-old female wild boar of 70 Kg of weight had been killed during the hunting season in Caulonia (Reggio Calabria, Calabria, Italy) and was submitted to post-mortem inspection. The huntsman referred that the boar did not show any sign of sickness before death and it seemed in good nutritional status.

2.2 Microbiological, histological and immunohistochemical investigations

Samples of peritoneal nodules, diaphragm, liver, kidney and lung were collected and frozen to perform microbiological, histopathological and immunohistochemical investigations.

For microbiological investigations tissue samples were frozen and sent to the Istituto Zooprofilattico Sperimentale of Barcellona P.G. (Messina, Italy). Samples were homogenized, decontaminated with 1 volume of 4% NaOH for 30 min at 37°C, neutralized

150 with 0.067 M of phosphate buffered saline (PBS) at pH 7.2 and centrifuged for 15 min at
151 3,000 *g*. Pellets were suspended in PBS, inoculated into Lowenstein-Jensen medium (LJ)
152 (Biolife®, Italy) and LJ medium without glycerol (Biolife®, Italy), then incubated in CO2 for
153 8 weeks at 37 °C, according to the OIE *Manual of Diagnostic Tests and Vaccines for*
154 *Terrestrial Animals* (World Organization for Animal Health, 2009).
155
156 After negative response for tuberculosis, frozen tissues were fixed in 10% buffered formalin,
157 and sent to the Department of Veterinary Sciences, University of Torino for histological
158 evaluation. Tissue samples were embedded in paraffin wax blocks, sectioned at 5 µm
159 thickness, mounted on glass slides and stained with Haematoxylin & Eosin (HE) and
160 Periodic Acid Schiff after diastase treatment.
161
162 Immunohistochemistry was performed on selected sections to characterize the neoplasia in
163 an automated system (Omnis Instrument, Dako, Agilent technologies). The characteristics
164 of the antibodies employed and the corresponding working conditions are detailed in Table
165 1. Reactions were visualized using a polymer-conjugated secondary antibody (Envision
166 Flex, Dako). Sections were counterstained with Mayer's hematoxylin. Positive control was
167 represented by human tissues known to express the different markers and negative controls
168 were carried out by omitting the primary antibodies.
169
170 **2.3 SEM-EDS investigation**
171 To assess the presence of asbestos and asbestiform minerals, and to evaluate a
172 relationship between the suspected mesothelioma and the possible exposure to asbestos,
173 the inorganic residue of lung, liver and diaphragmatic nodules samples were investigated
174 by SEM-EDS at the Department of Earth Sciences, University of Torino following the
175 procedure detailed in Belluso *et al.* (2006). In order to remove the organic fraction, 0.25 mg

of tissue from each sample have been chemically digested in 30 cc of sodium hypochlorite (NaClO). Subsequently, the solution containing the inorganic material has been filtered on a mixed cellulose ester filter with a diameter of 25 mm and porosity of 0.45 μm. The dissolution of NaClO granules has been completed washing the filter with distilled water preheated at 60° C. The filter has been put on a SEM stub and coated with a thin carbon film.

Each membrane has been investigated by SEM (JEOL-IT300LV). Only inorganic particles having aspect ratio >3:1 have been considered and their dimensions have been registered. Their elemental chemical composition has been determined by EDS (Oxford Instrument INCA Energy 2000). The inorganic fibres detected were identified by comparing their EDS spectrum with those collected in the laboratory database. The distinction between chrysotile and asbestiform antigorite, that have the same chemical composition, is possible only by structural determination, but not by SEM-EDS technique (Capella et al., 2017a). As it concerns tremolite and actinolite asbestos, they only differ for a little chemical variation (Hawthorne and Oberti, 2007) that it is not possible to detect on fibres from digested tissue (Goldstein et al., 2012). Therefore, the authors grouped together chrysotile with asbestiform antigorite (i.e. chrysotile/asbestiform antigorite) and tremolite asbestos with actinolite asbestos (i.e. tremolite/actinolite asbestos).

The number of detected inorganic fibres has been normalized to 1 g of dry tissue (ff/gdw) as indicated by international guidelines (De Vuyst *et al*., 1998).

Moreover, SEM-EDS investigations were performed on 5 samples of soil to assess the presence of asbestos in the environment where the wild boar lived. This area is into the woods, far away from footpaths (latitude: 38.371389; longitude: 16.390556). In the same area there are active/abandoned quarries (Italian Institute for Environmental Protection and Research -ISPRA) with some tunnels which can be used as dens by wild animals. There

are also some human settlement (4-5 farms) in the surrounding zone. The collection points were chosen within a circular area of about 3 km^2 around the site where the animal was felled, considered it as its home range (Figure 1). On the basis of these considerations, more or less equidistant points of about 500 meters were identified from the killing point of the animal. For each site, approximately 1 kg of soil to a depth of 30 cm was collected after elimination of the layer of foliage and shrubs typical of the subsoil.

A representative portion of each soil sample was processed following the guidelines of the Italian legislation (Italian Ministerial Decree of September 6, 1994). From the dried fraction with diameter < 2 mm, a portion of soil was ground by using an agate pestle and mortar and 5 mg of obtained powder was suspended into a dispersive solution. A share of this solution was filtered on a polycarbonate filter with a diameter of 25 mm and a porosity of 0.8 μm. The filter was put on a SEM stub and coated with a thin carbon film. The presence of inorganic fibres was evaluated on 0.1 mg of soil by SEM-EDS. Their amount was calculated to obtain the value of mg/kg (ppm) as indicated by the Italian legislation (Italian Ministerial Decree of September 6, 1994; the asbestos pollution being with value equal to or more than 100 mg/kg).

3. Results

3.1 Post-mortem evaluation

At post-mortem evaluation the peritoneum, surface of diaphragm and serosal surface of liver and kidneys showed isolated to coalescent multiple nodular lesions varying from 2 mm to 3 cm in diameter from grey-white to red-yellow color depending on the amount of hemorrhages. The nodules had a smooth and translucent surface (Figure 2). On the serosal surface of the liver some nodules were pedunculated with a cauliflower like surface due to

many complex fissures. No lesions were found in the thoracic cavity. On the basis of the observed lesions tuberculosis/mesothelioma was suspected.

3.2 Microbiological, histological and immunohistochemical investigations

The microbiological exam resulted negative for *Mycobacterium* spp., excluding the hypothesis of Tuberculosis.

The histological features of the nodules were the same in all the samples (diaphragm, kidney and liver serosal surface) and hepatic and renal parenchyma were not affected. Moreover, in liver hemosiderin accumulations were observed probably suggesting previous hemorrhages. Lungs showed no histological alterations.

Nodules were composed by a mixed cell population of epithelioid and sarcomatoid elements with an high pleomorphism. The epitelioid cell population showed oval cells with abundant and light-colored/eosinophylic cytoplasm, round-to-oval nuclei and well-defined cell borders (Figure 3). These cells did not show reactivity with PAS stain indicating the absence of mucin droplets within tumor cells or tubular lamina which were characteristic of adenocarcinoma (Head, 1990). Less commonly binucleate or multinucleate cells were observed. The sarcomatoid pattern was composed by pleomorphic spindled fibroblast-like cells. Areas of necrosis and lymphoplasmacytic infiltrates with rare neutrophils were frequently observed. Both cell populations were mixed in the nodules and tended to dispose in papillary structure, nests or cords.

Most of the immunohistochemical investigations failed to help in the classification of the tumor masses. In fact, only multifocal scattered WT-1 and D2-40 positivity were detected (Figure 4). All the samples showed negativity for Calretinin, cytokeratin 5, HBME-1 and CEA,

but at least for the former three no positivity in internal control cells was observed, thus the reactions were considered inconclusive.

Localization and morphological pattern are compatible with the diagnosis of a biphasic mesothelioma containing both epithelioid and sarcomatoid components at histological examination. Malignancy was supported by the presence of necrosis, increased cellularity, pleomorphism and a diffuse proliferation in the connective stromal tissue supporting mesothelial cells.

3.3 SEM-EDS investigation

The inorganic fibres detected in different organs at SEM-EDS investigation are reported in Table 2. In lung, mineral fibres attributable to phyllosilicates and amphiboles families, have been detected, both asbestos and asbestiform minerals. Within the asbestos minerals, tremolite/actinolite asbestos (Figure 5–a,b), and chrysotile/asbestiform antigorite (Figure 5-c,d) were detected. Regarding asbestiform minerals, horneblende, Na-Ca amphiboles and illite-smectite have been detected.

In the diaphragmatic nodules and in the liver only chrysotile/antigorite asbestiform fibres have been detected.

Inorganic fibres detected in soils samples are reported in Table 3. Chrysotile/asbestiform antigorite is present only in 2/5 samples while fibrous amphibole species (both asbestos and non-asbestos classified) have not been detected.

Two kinds of fibrous species, chrysotile/asbestiform antigorite and fibrous illite/smectite have been detected both in biological and soil samples. In both types of samples, the amount of chrysotile/asbestiform antigorite is predominant compared to the other fibrous minerals.

4. Discussion

277 Primary peritoneal tumors in animals are rare. One of this is represented by peritoneal
278 mesothelioma even if its diagnosis must be made with caution because of the similarity of
279 mesothelial proliferative lesions with chronic granulomatous peritonitis and metastatic
280 tumors in serous membranes (such as carcinoma) (Head, 1990).
281 In the present case Tuberculosis was suspected during post-mortem inspection as the
282 peritoneum was covered by diffuse small nodules. However, histological examination
283 revealed no granulomatous inflammatory process and microbiological investigations
284 excluded the suspect of mycobacterial infection.
285 Gross and histological examination are compatible with a biphasic mesothelioma as the
286 nodules were composed by both epiteliod and sarcomatoid mixed components (Head,
287 1990). Moreover, immunohistochemistry, although focally, was positive for WT1 e D2-40
288 which are specific markers for mesothelioma (Ordoñez, 2013) supporting the hypothesis of
289 a MM in the boar. Indeed, other tested mesothelioma-specific markers, such as calretinin,
290 cytokeratin 5 and HBME-1, were negative both in tumor cells and in internal control cells
291 (such as normal peritumoral mesothelial cells). This finding appears difficult to explain but
292 the authors assume that it can be due to bad antigen preservation in tissue samples. All the
293 samples have been frozen before chemical fixation and the type of freezing-defrosting
294 process (temperature, delay in tissue allocation) can influence the antigen preservation
295 (Pelstring, 1991).
296 In human medicine mesothelioma usually develops in the pleural cavity (Bianchi and
297 Bianchi, 2014). On the contrary, in veterinary medicine a higher number of cases of
298 peritoneal mesothelioma have been reported in literature (Bacci *et al*.; 2006; Beytut, 2002;
299 Brown and Weaver,1981; Forbes and Matthews, 1991; Girard and Cécire, 1995; Umphlet
300 and Bertoy, 1988). This different localization could be explained by the capability of
301 pulmonary macrophages with phagocytosed dust particles to move through alveolar walls
302 towards the pleura. In doing so they may penetrate bronchioles and be transported by the

303 mucociliary staircase or they may penetrate blood vessels and be carried to extrapulmonary
304 sites where they excite a reaction for example in the liver, spleen, kidney and abdominal
305 wall (Holt, 1981). In the boar of the present case an ingestion of asbestos fibres cannot be
306 excluded.
307 The evidence of abundant asbestos fibres in lung, liver and diaphragmatic nodules with
308 SEM-EDS methods seems to support this pathological mechanism and the diagnosis of
309 mesothelioma relating to asbestos exposure.
310 The mineral fibres detected in the biological samples are compatible with the geological
311 characteristics of the area where the wild boar lived. In particular chrysotile/asbestiform
312 antigorite was detected both in biological and in soil samples. The authors cannot exclude
313 that the lack of detection of fibrous amphiboles in soil samples is due to a detection limit of
314 SEM-EDS method.
315 To the best of author knowledge, in humans tremolite asbestos accumulates in lung, while
316 chrysotile is quickly cleared. This difference in pulmonary clearance can explain why
317 tremolite asbestos and amphiboles asbestos in general are considered "2-3 orders of
318 magnitude more carcinogenic than chrysotile" (WHO, 2015). Assuming that pulmonary
319 clearance rate in wild boars is similar to human one, the authors can hypothesize that
320 probably this animal respired more chrysotile/asbestiform antigorite than tremolite/actinolite
321 asbestos, but the most persistent fibres are left the most abundant.
322 Moreover, the amount of fibres detected in the boar lungs is lower than that reported in
323 previous studies.
324 As it concerns the amount of fibres in the lung sample (Table 2), the comparison with
325 literature data shows that amphibole asbestos is much lower than that found in lungs of
326 sheep, goats and two boars from more than 100 km far from the provenance site of the
327 examined boar (Campopiano *et al*., 2017). Similarly, goats from Corsica (Dumortier *et al*.,
328 2002), cows from north-western Italy (Belluso *et al.,* 2017), cats and dogs from California

(Abraham *et al*., 2005) showed lower amount of amphiboles fibres in lung compared to the boar of the present study. Also for chrysotile/asbestos antigorite, the burden in cow lungs from north-western Italy is lower (Belluso *et al*., 2006) than that found in the investigated boar. These animals did not develop MM even if they had a higher fibre burden compared to the boar.

In humans a burden of amphiboles asbestos (fibre with length of > 5 μm) higher than 1 x 10^5 /gdw is considered an indicator of significant asbestos exposure (De Vuyst *et al*., 1998), able to trigger cancerous pathologies. It is interesting to note that the amphiboles asbestos burden in the boar is even lower than the threshold for humans.

This finding, correlated with the mesothelioma diagnosis, seems to support the hypothesis of a cancer risk related to natural environmental exposure (low doses) to asbestos, and the presence of a genetic component of susceptibility to MM (Crovella *et al*, 2016) also in animals.

For these reasons the role of wild boars as SSA has to be considered central in: i) determining the environmental diffusion of asbestos and non-asbestos classified asbestiform minerals; ii) establishing if the natural exposure to asbestos could play a role in the development of human mesotheliomas.

5. Conclusion

In conclusion, the results of this study suggest that an active surveillance on regularly slaughtered domestic/wild animals seems suitable to quantify the risk of exposure to asbestos for human population.

It would be interesting to carry out diagnostic investigations also on MM cases in other wild/domestic animals to confirm the hypothesis that, at least in animals, the low-dose asbestos respiration, presumably continuous, and for a limited period of time (3 years) may induce MM.

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Figures Legends

Figure 1. Geographical area where the boar was killed and soil samples were collected.

Figure 2. Boar, macroscopical findings at post-mortem examination. A: peritoneum, diaphragm and serosal surface of abdominal organs covered by multiple nodules. B: detail of the caudal abdomen characterized by diffuse serosal proliferations.

Figure 3. Boar, histological characterization of the nodular lesions. A: proliferations attached to the diaphragm, Haematoxilin and eosin stain (H-E). B: mixed cell population composed by epithelioid and sarcomatoid elements with disseminated areas of necrosis and lymphoplasmacytic infiltrates. H-E, 100x. C: detail of the tumor masses attached to the serosal surface characterized by pleomorphism and nest or cords organization. H-E, 200x.

Figure 4. Boar, immunohistochemical characterization of the nodular lesions. A: multifocal scattered WT1 positivity. Immunohistochemistry for WT1 detection, haematoxylin counterstaining, 400x. B: multifocal D2-40 positivity. Immunohistochemistry for D2-40 detection, haematoxylin counterstaining, 400x.

Figure 5. SEM images (2000X) and relative EDS spectra of chrysotile/asbestiform antigorite (a,b) and tremolite/actinolite asbestos (c,d) detected in lung samples of the boar.

Table legends

Table 1. Immunohistochemical protocols.

560 **Table 2.** Inorganic fibres detected in different kinds of tissue (ff/ gdt: number of fibres per
561 gram of dry tissue; * fibre bundle of 2 fibres minimum; ** fibre bundle of 6 fibres minimum)
562 **Table 3.** Inorganic fibres detected in soil samples of 5 different areas (* fibre bundle)
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