



## Sheep lymph-nodes as a biological indicator of environmental exposure to fluoro-edenite



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### ARTICLE INFO

#### Article history:

Received 16 November 2015

Received in revised form

27 January 2016

Accepted 29 January 2016

Available online 6 February 2016

#### Keywords:

Fluoro-edenite

Sicily

Environmental monitoring

Pleural mesothelioma

Biancavilla

### ABSTRACT

A significantly increased incidence of pleural mesothelioma in Biancavilla (Sicily, Italy) has been attributed to exposure to fluoro-edenite (FE), a fibrous amphibole extracted from a local stone quarry.

The lymph-nodes draining the pulmonary lobes of sheep grazing around the town were examined, to gain insights into fibre diffusion.

The pasture areas of six sheep flocks lying about 3 km from Biancavilla were located using the global positioning system. The cranial tracheobronchial and one middle mediastinal lymph-node as well as four lung tissue samples were collected from 10 animals from each flock and from 10 control sheep for light and scanning electron microscopy (SEM) examination.

The lymph-nodes from exposed sheep were enlarged and exhibited signs of anthracosis. Histologically, especially at the paracortical level, they showed lymph-follicle hyperplasia with large reactive cores and several macrophages (conioophages) containing grey-brownish particulate interspersed with elements with a fibril structure, forming aggregates of varying dimensions (conioophage nodules). Similar findings were detected in some peribronchiolar areas of the lung parenchyma. SEM examination showed that FE fibres measured 8–41  $\mu\text{m}$  in length and 0.4–1.39  $\mu\text{m}$  in diameter in both lymph-nodes and lung tissue.

Monitoring of FE fibres in sheep lymph-nodes using appropriate techniques can help set up environmental pollution surveillance.

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### 1. Introduction

Malignant mesothelioma epidemics due to non-occupational exposure to asbestiform fibres have been described in several areas of the world, including Greece, Turkey, Cyprus, Corsica, New Caledonia, Afghanistan, Russia, and Montana (Constantopoulos, 2008; Luce et al., 2004; Srebro and Roggli, 1994; Voisin et al., 1994; Whitehouse, 2004; Tossavainen et al., 2000).

In Italy, a 10 year study of mortality from malignant pleural mesothelioma (1988–1997) highlighted an unexpected cluster in

Biancavilla, Sicily (Paoletti et al., 2000; Comba et al., 2003) that was confirmed by later studies (Bruno et al., 2014).

The municipality of Biancavilla lies 515 m above sea level on the SW slope of the Etna volcano. It extends for about 70 km<sup>2</sup> from Mt. Etna to the river Simeto; the town occupies a twentieth of its territory.

The high mortality rate was attributed to exposure to a fibrous amphibole, identified as fluoro-edenite (FE) (Gianfagna and Oberti, 2001), which is chemically similar to tremolite, except that its OH groups are replaced by fluorine. FE fibres were found in the inert material, such as sand and rubble, extracted from a stone quarry excavated inside Mt. Calvario, which stands on the immediate outskirts to the SE of the town. This material has been employed locally in construction work for about 50 years (Comba et al., 2003; Paoletti et al., 2000). The quarry was sealed in 1998. Data

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from in vivo and in vitro studies (Soffritti et al., 2004; Loreto et al., 2008; Martinez et al., 2006; Musumeci et al., 2011; Ballan et al., 2014; Szychlinska et al., 2014; Musumeci et al., 2015) have contributed to the International Agency for Research on Cancer (IARC; Lyon, France) classification of FE as carcinogenic (Grosse et al., 2014). The findings of some environmental monitoring projects (Famoso et al., 2012; Bruni et al., 2014), suggest that the population has been exposed to high levels of amphibole fibres until mitigation measures, adopted in 2001, gradually reduced them to about 0.1–0.4ff/l (Bruni et al., 2014).

Two recent studies of the health impact of FE exposure on Biancavilla residents have shown an excess of mortality and hospitalizations due to chronic respiratory disease and of hospitalizations due to all respiratory diseases, particularly pneumoconiosis (Conti et al., 2014; Miozzi et al., 2016), while an excess of non-malignant pleural lesions has been described by Rapisarda et al. (2015a) in subjects occupationally exposed to FE. Increased fibulin-3 levels have also been detected in workers exposed to FE (Rapisarda et al. 2015b).

In 2004, FE fibres were detected in the pulmonary parenchyma of eight sheep living in the Biancavilla area (DeNardo et al., 2004). In 2005, Rapisarda et al. (2005) suggested a bioindicator role for sheep as a sentinel species.

This study was devised to establish whether FE fibres are found in the lymph-nodes draining the lung lobes of sheep habitually grazing in the area of the Mt. Calvario quarry and, especially, to measure the concentration of uncoated and coated fibres, i.e. ferrous bodies (FB), in order to establish whether pulmonary lymph-nodes can be used as a measure of atmospheric pollution around Biancavilla.

## 2. Materials and methods

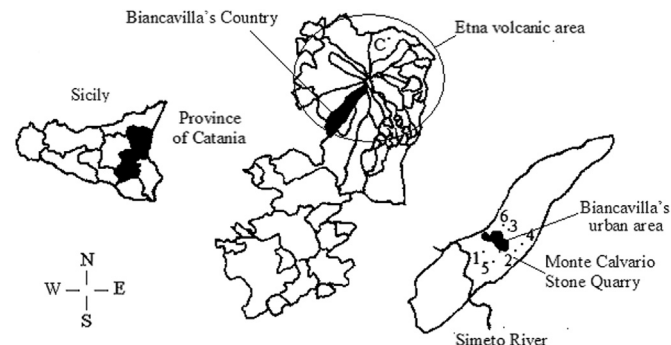
### 2.1. Selection of the flocks

The pasture areas of six flocks of sheep grazing about 3 km from the town were identified using the global positioning system. Their exact position is indicated in Fig. 1 (areas 1–6).

The shepherds of areas 2, 5, and 6 stated that their sheep always grazed in the same zone, whereas those of areas 1, 3 and 4 said that their animals spent the transhumance period (November–March) in a temperate area about 40 km from the quarry. The mean number of sheep per flock was  $190.5 \pm 45.4$ .

### 2.2. Sacrifice and collection of lymph nodes

Ten sheep randomly selected from each flock and 10 control



**Fig. 1.** From left to right: map of Sicily; Province of Catania; SW slope of Mt. Etna (C: pasture area of the control group); and territory of the municipality of Biancavilla with indicating the town, the stone quarry, and the pasture areas of the six flocks of sheep (1–6).

sheep from a flock whose pasture area was about 30 km from the quarry were sacrificed in a slaughterhouse in September–October 2014. A veterinary surgeon conducted ante- and post-mortem examinations to ensure that all sheep were in good health conditions. The age range of exposed and control animals was 4.0–6.5 years.

The cranial tracheobronchial and one middle mediastinal lymph-node, which drain the right apical lobe and the principal and accessory lung lobes, respectively, were collected from each sheep. Lung tissue from each of the four lobes was also collected.

All samples were fixed in 10% formalin for light microscopy and in paraformaldehyde 4% in 0.1 M phosphate buffer (PB) for scanning electron microscopy. Special care was taken to remove all tissue adhering to the lymph-nodes before processing.

A randomly selected half of each cranial tracheobronchial and mediastinal node was used to measure fibre concentration; the other half was used for histological examination.

### 2.3. Quantification of fluoro-edenite fibre concentration

Lymph-nodes were digested in sodium hypochlorite solution (15–16% free chlorine; Subalpina, Torino, Italy) according to Morgan and Holmes (1980); the residues were filtrated and collected on 0.2  $\mu\text{m}$  pore size Nucleopore filters. FB and naked fibres were quantified by light (LM) and scanning electron microscopy (SEM), using a Zeiss Axiophot microscope (Oberkochen, Germany) and a Philips XL-20 electron microscope (Philips, Monza, Italy) equipped with an energy dispersion spectrometry x-ray analysis (EDAX) apparatus. Tissue specimens were analysed by SEM as described by Tuomi et al. (1989). Fibres  $> 1 \mu\text{m}$  in length and  $> 0.3 \mu\text{m}$  in diameter were identified, measured, and counted at 5000X magnification.

### 2.4. Histological examination

Specimens for LM examination were embedded in paraffin wax. Sections 5  $\mu\text{m}$  in thickness were stained routinely with haematoxylin and eosin and Perls' Prussian blue. Specimens for SEM observations were rinsed in 0.1 M PB, dehydrated in graded alcohol, and critical-point dried. Specimens were mounted on aluminium stubs and coated with a layer of gold in a Polaron E 5400 sputter-coater. FB and FE fibres were sought in tissue sections using LM and SEM.

### 2.5. Statistical analysis

All calculations and statistical analyses were generated using SPSS for Windows, version 20 (SPSS, Inc., Chicago, IL, USA). Results were expressed as mean  $\pm$  standard deviation (SD). After variance analysis (ANOVA), data were subjected to Bonferroni's *t*-test. Significance was set at  $p \leq 0.05$ .

## 3. Results

By macroscopic examination, lymph-nodes appeared slightly enlarged but exhibited a normal consistency, colour and shape; the section surface showed clear signs of anthracosis, with the typical black colour.

LM examination evidenced no FB either in digested nodes or in histological sections.

SEM analysis of digested lymph-nodes evidenced some naked fibres; EDAX showed that most had the crystallo-chemical features of FE described by Gianfagna and Oberti (2001). Fibres ranged from 8 to 41  $\mu\text{m}$  in length and from 0.4 to 1.39  $\mu\text{m}$  in diameter. Their dimensions were similar to those described by Paoletti and

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