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# Asbestos fibre burden in gallbladder: A case study

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ABSTRACT

The methods conventionally used to determine the burden of asbestos fibres inhaled/incorporated in lung require chemical digestion of the biological matrix before counting/characterising the inorganic fibrous phases under scanning electron microscopy and energy dispersive spectroscopy (SEM/EDS). Asbestos fibres can also be present in extra-pulmonary organs, and we set out to quantify the fibres in gallbladder. Although the standardised procedure requires approximately $5 \times 10^{-1}$ g of wet tissue, this amount of tissue is not always available. We applied the procedure on about $9 \times 10^{-4}$ g of gallbladder from a patient with known environmental and workplace exposure to asbestos. The patient died of malignant pleural mesothelioma and was also affected by severe bile-tract problems. The traditional procedure of digesting tissue samples in NaClO and filtering the resulting suspension was carried out. The filter was then examined under SEM/EDS using two methods 1. following the standardised procedure to assess the fibre burden in lung by investigating only 2 $mm^2$ of the filter (660 microscopic fields), and 2. analysing all the microscopic fields in one-quarter of the filter (about 82 $mm^2$). In parallel, histological sections (prepared in the usual way for medical diagnosis) were analysed without digestion or manipulation of the sample using variable pressure SEM/EDS. The fibre counts obtained using the two methods were of the same order of magnitude, i.e., $\sim 10^5$ fibres/g of wet tissue. We showed that the counting of fibres in human tissue may be successfully carried out even when a limited amount of tissue is available. We also found that, when exposure to asbestos is considerable, the number of asbestos fibres accumulating in the gallbladder may be significant.

## 1. Introduction

The relationships between asbestos fibres and severe diseases of the respiratory system (e.g., mesothelioma, asbestosis, and pulmonary carcinoma) are now universally accepted (Auerbach et al., 1980; Baumann et al., 2015; Ehrlich et al., 1985; Huang et al., 1988; Muller et al., 2001; Szendröi et al., 1983; Westlake, 1965; Williams et al., 2001). Moreover, some cancers affecting larynx and ovary are recognised by the International Agency for Research on Cancer as being related to asbestos (IARC, 2012). However, the relationships between asbestos and gastrointestinal (GI) cancers are still debated by the scientific community (Constanza Camargo et al., 2011; IARC, 2012; Jacobsen et al., 2013). Consequently, elucidating the role of asbestos in the development of GI cancers is increasingly the object of studies (Brandi et al., 2008, 2013; Boulanger et al., 2015; Di Ciaula, 2017; Di Ciaula and Gennaro, 2016). One approach is to investigate the number, type, and localization of asbestos fibres in the various human organs affected by neoplastic growth. The approach commonly used in previous studies is to digest and analyse wet tissue samples weighing $5 \times 10^{-1}$ g to quantify the number of asbestos fibres (Belluso et al., 2006; Churg, 1982; Churg and Warnock, 1977; De Vuyst et al., 1998; Karjalainen et al., 1996; Tossavainen, 1997). After the sample has been digested, identification and quantification of the fibrous mineral phases can be performed with an optical microscope (De Ridder et al., 2016;

*Abbreviations:* BSE, backscattered electrons; BSED, back-scattered electron detector; GI, gastrointestinal; gwt, gram of wet tissue; IARC, International Agency for Research on Cancer; MFs, microscopic fields; MPM, malignant pleural mesothelioma; NIOSH, National Institute for Occupational Safety and Health; OSHA, Occupational Safety and Health Administration; SEM/EDS, scanning electron microscopy and energy dispersive spectroscopy; VP, variable pressure; WHO, World Health Organization

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Kobayashi et al., 1987; Pairon et al., 1994; Roggli et al., 1986), a scanning electron microscope equipped with an energy dispersive spectrometer (Belluso et al., 2006; Casali et al., 2015; De Ridder et al., 2016; Roggli, 2006; Roggli et al., 1986), or a transmission electron microscope (Albin et al., 1990; De Vuyst et al., 1983; Dodson et al., 1997). A disadvantage of this approach, however, is that digestion destroys the relationship between fibres and biological tissues. Our hospital and university in Alessandria are in a region where asbestos is a severe problem and is responsible for a high percentage of respiratory diseases. Moreover, in recent years, our research group has focussed on the identification of asbestos fibres in bile tract and in gallbladder. We used a variable pressure scanning electron microscope equipped with an energy dispersive spectroscope (VP-SEM/EDS) to analyse bile fluid and thin sections of gallbladder from patients living in asbestos-polluted areas who underwent surgery for gallstones. Also analysed were thin sections of the gallbladder from patients with malignant pleural mesothelioma (MPM) who also had severe gallbladder problems. In all the cases studied, except for a patient too young to have undergone exposure to asbestos, the technique identified asbestos fibres on the basis of their morphology and chemical composition (Grosso et al., 2017, 2015). In our studies, we observed asbestos fibres directly in thin histological sections without digestion of the biological matrix. This meant that the area of the tissue in which the fibres were incorporated could be determined (Grosso et al., 2017). In MPM patients, the analysis of gallbladder sections found a significant number of asbestos fibres. The next step was the quantification of these asbestos fibres. However, because the amount of tissue available for analysis was limited, we applied the traditional procedure on only $9 \times 10^{-4}$ g of tissue included in paraffin. After digestion of the biological matrix, the resulting suspension was filtered. The filter was then examined using SEM/EDS in two laboratories. A detailed study of histological sections of gallbladder from the same patient was also carried out to identify the location of the fibres in the biological medium.

One aim of this study was to assess the scientific reliability and to compare cost/benefit ratios of the two different SEM/EDS methods used for quantification of asbestos fibres. Another aim was to obtain complementary information useful for a deep knowledge of the incorporation mechanism of asbestos fibres in human tissues.

## 2. Materials and methods

Thin sections of gallbladder were obtained from a paraffin-embedded tissue specimen from an 80-year-old woman who died of MPM. The patient was also affected by secondary lymph node localization, necrotic inflammatory changes in the main organs, and acute circulatory problems. For this patient, both environmental and workplace exposures to asbestos were ascertained.

In the current work, we followed the definitions of the National Institute for Occupational Safety and Health (NIOSH, 2011) and Roggli (2014). All particles with length:thickness ratios ≥3:1 were considered “fibres”, and “asbestos fibres” were defined as those with crystal and chemical compositions corresponding to a mineral phase regulated as “asbestos” [i.e., tremolite asbestos, actinolite asbestos, anthophyllite asbestos, cummingtonite-grunerite asbestos (amosite), riebeckite asbestos (crocidolite), and chrysotile] (Hawthorne et al., 2012; IARC, 2012; Italian Legislative Decree 277/1991; Leake et al., 1997; NIOSH, 2011; Rinaudo et al., 2004, 2003; Roggli, 2014; Virta, 2002). Further, the World Health Organization (WHO, 1986) and the US Occupational Safety and Health Administration (OSHA, 1992) have defined “breathable fibres” as crystals with length (l) > 5 µm, diameter (d) < 3 µm, and l/d ≥ 3:1, and crystals with l ≥ 5 µm and l/d ≥ 3:1 respectively. These dimensional criteria refer to fibres biologically active in the alveolar region of the respiratory system, and are therefore fibres that enter the body via breathing. However, it is not clear whether these definitions are valid when fibres are incorporated in the human body through mechanisms other than breathing, e.g., via ingestion.

Mineral phase identification in the current work was based on qualitative SEM/EDS analyses performed on asbestiform crystals collected on a filter after digestion of the biological matrix or in histological sections. Consequently, definite discrimination between tremolite and actinolite was difficult. However, because both were classified as asbestos, in this study their discrimination was not essential. Differentiation between chrysotile and antigorite phases was performed on the basis of their crystal morphology. For all observed fibres, the identification of the mineral phase was performed on the basis of a qualitative EDS spectra database acquired in our laboratories using pure samples of the different asbestos phases that had been carefully characterised with respect to their chemical and mineralogical properties (Grosso et al., 2015; Gualtieri et al., 2013; Rinaudo et al., 2004, 2003).

### 2.1. Without digestion

Fibre observations without digestion of the biological medium were performed directly on 5-µm-thick histological sections cut from cell blocks embedded in paraffin. Three sections of the gallbladder were pasted onto a plastic support to avoid interference with the chemical elements constituting the asbestos minerals, as described in previous works (Croce et al., 2013; Grosso et al., 2017, 2015). These three sections were observed using VP-SEM/EDS to acquire more accurate results concerning the presence of fibres and their mineralogical identification.

Before VP-SEM/EDS analysis, the sections on the plastic support were placed in an oven at 60 °C overnight to eliminate the paraffin film. The sections were then examined by VP-SEM using an ESEM Quanta 200 SEM (FEI Company, Hillsboro, OR, USA) equipped with an EDS (EDAX, Mahwah, NJ, USA) and a back-scattered electron detector (BSED). The back-scattered images (BSE), characterised by white/black contrasts produced by inorganic phases impinging on the biological medium, allowed easy detection of the asbestos fibres, as described by Grosso et al. (2017, 2015). The experimental conditions were a pressure of 90 Pa, a working distance of 10 mm, and an accelerating voltage of 20 kV. The EDS registered spectra were processed with GENESIS software version 3.6. To determine the elemental composition of the observed inorganic phases, EDS microanalyses were carried out both on the inorganic phases and on the organic areas close to them. We thereby acquired several EDS spectra from different points of fibres/bundles of fibres (analyses IN) and from the surrounding area (analyses OUT). The differences between the mean values obtained in the analyses of the inorganic phase (analyses IN) and of the surrounding area (analyses OUT) were established by assessing the qualitative chemical compositions of the observed fibre/bundle of fibres.

### 2.2. After digestion

To perform fibre quantification after digestion of the biological matrix, we analysed $9 \times 10^{-4}$ g of tissue. Five sections of tissue (15 µm thick) were cut from the same paraffin block. The paraffin was eliminated from the sections by washing twice with xylol and twice with ethanol; each washing process continued for 10 min. After drying, the tissue was weighted by using a KERN 770 analytical balance (KERN and Sohn GmbH, Blingen, Germany); the instrumental error was $d = 1 \times 10^{-4}$ g. Chemical digestion was performed at room temperature using 30 ml NaClO (12.5%, Carlo Erba, Cornaredo, Italy) to eliminate the organic fraction. The inorganic material was then recovered by filtering the suspension on a mixed-cellulose-ester filter with a pore size of 0.45 µm and a diameter of 25 mm.

The sample was then made conductive by covering it with a graphite film through a metallization process using an auto carbon coater (JEC-530, JEOL, Tokyo, Japan). Half of the coated sample was analysed using SEM/EDS and the other half was stored for future study.