Histol Histopathol (2021) 36: 1301-1308

**http://www.hh.um.es**

Histology and Histopathology
*From Cell Biology to Tissue Engineering*

**ORIGINAL ARTICLE** **Open Access**

# ATG7 immunohistochemical expression in malignant pleural mesothelioma. A preliminary report

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**Summary.** Literature evidence has demonstrated a high incidence of asbestos-related malignant pleural mesothelioma (MPM) in a Sicilian town (Biancavilla, Italy), where fluoro-edenite (FE) fibers were discovered some decades ago. As ATG7 immunohistochemical analysis has been ascribed as a prognostic tool of improved survival, we decided to investigate, in MPM patients, exposed and not exposed to FE fibers, the immunohistochemical expression of this autophagy-related protein named ATG7. We analyzed the correlation between ATG7 immunohistochemical level and clinicopathological parameters. Twenty MPM tissue samples, from patients with available clinical and follow-up data, were included in paraffin and processed for immunohistochemistry. The immunohistochemical results confirmed activation of the autophagic process in MPM. Densitometric and morphometric expressions of ATG7 were significantly increased in MPMs when compared to the control tissues. A significant association of a high level of ATG7 with increased survival was demonstrated, with a mean overall survival (OS) of 12.5 months for patients with high expression vs. a mean OS of 4.5 months for patients with low ATG7 expression. In addition, a significant correlation between ATG7 expression and the survival time of MPM patients was observed. This study represents a starting point to hypothesize the prognostic role of ATG7 which could be a reliable prognostic indicator in MPM.

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DOI: 10.14670/HH-18-396

**Key words:** Malignant Pleural Mesothelioma, Autophagy, ATG7, Immunohistochemistry, Asbestiform fibers, *Ex vivo* study

## Introduction

Malignant Pleural Mesothelioma (MPM) represents an aggressive neoplasm in humans and it is strictly related to asbestos and asbestiform fiber exposure (Delgermaa et al., 2011; Filetti et al., 2020a,b). The most relevant prognostic parameters for MPM are represented by the histological subtype, gender, and age at diagnosis (Delgermaa et al., 2011).

In the last decades, a high incidence of MPM has been demonstrated in an Italian town (Biancavilla, Sicily, Italy) where there was a stone quarry contaminated by fluoro-edenite (FE) fibers used to extract building materials (Delgermaa et al., 2011; Ledda et al., 2016a,b; Cavone et al., 2019; Pasetto et al., 2019; Zona et al., 2019; Blanquart et al., 2020; Fazzo et al., 2020). From a pathophysiological point of view, a strict correlation has been shown between fiber exposure and the activation of the apoptotic cascade in cells tissues of the respiratory apparatus (Loreto et al., 2008; Ledda et al., 2020). It has been demonstrated that a "cross-talk" between apoptosis and autophagy also exists (Fimia and Piacentini, 2010; Dyczynski et al., 2018; Follo et al., 2019a) and these processes are not independent of each other; they can be activated by common upstream signals and share molecular switches (Mariño et al., 2014).

Starting from this assumption and our previous work, our research group decided to investigate for the first time, in a small but unique subset of MPM patients, who underwent environmental exposure to FE fibers, the

immunoexpression, and immunolocalization of an autophagy-related protein named ATG7 to depict the activation of the autophagic mechanism by FE. In addition, we compared the ATG7 expression of these cases with MPM cases not induced by FE fibers. We evaluated the correlation between ATG7 immunohistochemical levels and clinicopathological parameters.

Autophagy constitutes the main mechanism for regulating the turnover of components of the cytoplasm and selective removal of damaged organelles, preventing ROS accumulation, inflammation, and the onset of oncogenic mutations (White, 2015; Levy et al., 2017). Autophagy assumes a central role in tissue remodeling during embryogenesis, in cellular differentiation (Levine and Klionsky, 2004), and takes part in the control of cell proliferation (Klionsky, 2005). Thus, it appears quite clear how the dysfunction of this balanced mechanism is implicated in many diseases, including cancer onset and development.

Literature data show that autophagy restrains inflammation through many regulatory interactions with innate immune signaling pathways by removing endogenous inflammasome agonists and acting on immune mediators' secretion. By controlling chronic inflammation, autophagy may contribute to subtract cancer cells' relevant survival signals (Zhou et al., 2011). Despite its tumor-suppressive role in the early stages of tumor onset and development, autophagy has been shown to support cancer survival and the acquisition of particular aggressive tumor features.

To date, more than 37 autophagy-related genes are known to be implicated in different functions of the autophagic process. ATG (autophagy-related) protein constituents represent the core autophagic machinery. The initiation of autophagy requires two separate ubiquitin-like protein (UBL) systems that regulate autophagosome assembly. In these systems, ATG7 plays as an E1-like enzyme facilitating both microtubule-associated protein light chain 3 (LC3)-phosphatidylethanolamine and ATG12 conjugation. Therefore, it represents an essential regulator of autophagosome assembly, assuming a pivotal role in autophagy-related cell homeostasis (Xiong, 2015). Defects in this pathway can lead to tumor formation. For what is stated above, the present paper will analyze its immunoexpression on MPM tissue, which will be framed in the context of the literature to gain insight and postulate the role of the autophagic process in MPM prognosis.

## Materials and methods

### Sample collection

Informed consent, by the ethics committee, was not requested as the present study has a non-interventional retrospective value and the research complied with the Helsinki Declaration.

MPM specimens from twenty patients were retrospectively selected. Of these ten MPM cases were exposed to FE fibers and ten MPM cases were not exposed to FE fibers. For all samples clinical-pathological and follow-up data were available. All patient information was transmitted by the National Registry of Mesothelioma (ReNaM). The samples were collected for diagnostic purposes. Hematoxylin and Eosin-stained sections, for each sample, were analyzed by two surgical pathologists (G.B. and R.C.) who confirmed the MPM diagnosis following the World Health Organization (WHO) criteria. The selected ten patients affected by MPM exposed to FE fibers were residents in the town of Biancavilla or nearby areas where there is environmental contamination of the silicate, while the selected ten patients affected by MPM not exposed to FE fibers were residents in Sicilian towns not located in the Biancavilla area.

The cohort of control cases was composed of eight patients that did not show neoplastic diseases. Control pleural tissues were collected during surgery for pleurisy or pulmonary emphysema.

### Histopathology

After washing in phosphate-buffered saline (PBS; Sigma, Milan, Italy), samples were fixed in 10% buffered formolo, as reported (Loreto et al., 2019). Then, dehydrated in graded ethyl alcohol, passed in xylene, and fixed in paraffin (Loreto et al., 2020a; Broggi et al., 2021). Paraffin-blocks were cut with a microtome and, slides of 4-5 μm in thickness mounted on silane-coated slides (Dako, Glostrup, Denmark). After, sections were stained with Hematoxylin and Eosin and observed using a Zeiss Axioplan light microscope (Carl Zeiss, Oberkochen, Germany) for the morphological evaluation.

### Immunohistochemistry

Sections were processed for the immunohistochemical analysis, as previously described (Leonardi et al., 2007; Angelico et al., 2018). After being dewaxed in xylene, rehydrated with graded ethyl alcohol, slides were incubated for 30 min in 0.3% hydrogen peroxide/methyl alcohol solution to block the endogenous peroxidase activity and finally washed with PBS for the antigen retrieval. Sections were put in a microwave oven (750 W) (5 min x 3) in capped polypropylene slide-holders with citrate buffer (10 mM citric acid, 0.05% Tween 20, pH 6.0; Bio-Optica, Milan, Italy). After dewaxing, sections were incubated at 4°C overnight with anti-ATG7 rabbit monoclonal antibody (Abcam, San Francisco, CA, USA) diluted 1:100 in PBS, as previously described (Lombardo et al., 2019; Loreto et al., 2020b). Immune complexes were then exposed to a biotinylated link antibody for 10 min at room temperature and after were detected with peroxidase-labeled streptavidin (LSAB + System-HRP, K0690; Dako) incubated for 10 min at room temperature. As

reported, the immunoreaction was visualized by 3,3'-diaminobenzidine and 0.02% hydrogen peroxide solution (DAB substrate Chromogen System; Dako) (Loreto et al., 2020c). Sections were counterstained with Mayer's Hematoxylin (Histolab Products AB, Göteborg, Sweden) mounted in GVA (Zymed Laboratories, San Francisco, CA, USA), evaluated and then immortalized with a Zeiss AxioCam MRc5 digital camera (Carl Zeiss).

ATG7 immunoexpression was signed as positive when a brown chromogen was observed at a cytoplasm level (Broggi et al., 2020a). Tissue specimens with known antigenic positivity were used as positive controls. Negative sections have been obtained by slides treated with PBS without the primary antibodies. For each section seven fields were observed randomly, about 600.000 $\mu m^2$, to evaluate the morphometric and densitometric count. The percentage areas (morphometric analysis) stained with anti-ATG7 antibody were expressed as % positive, dark brown pixels of the evaluated fields, while the levels (high/low) of staining intensity of positive areas (densitometric analysis) were expressed as densitometric count ($pixel^2$) of positive, dark brown pixels of the evaluated fields. These parameters were analyzed by image acquisition software (Axio Vision Release 4.8.2 - SP2 Software, Carl Zeiss Microscopy GmbH, Jena, Germany). The results were presented as mean ± standard deviation (SD). Digital micrographs were performed as previously reported.

### *Statistical analysis*

The data were plotted using Prism for Windows v 7.00 (Graphpad Software; CA, USA). Data were tested for normality with the Kolmogorov-Smirnov test. All variables were not normally distributed. Wilcoxon Signed Rank Test was used for comparisons between the means for both percentage area values (morphometric analysis) and $pixel^2$ values (densitometric analysis) of ATG7 immunoexpression of MPM samples vs. controls tissues. Moreover, considering the median of the ATG7 expression values expressed as area % (2.8057), the Hazard Ratio (HR) was calculated using the Mantel-Haenszel test. Cancer-specific survival analysis was performed using the Kaplan-Meier method, and the Mantel-Cox log-rank test was used to compare the survival curves. To evaluate the correlation between clinic-pathological and immunohistochemical data the Spearman correlation was used. In particular, age, gender, pathological subtype, and survival time have been correlated with the ATG7 immunohistochemical level. p-values less than 0.05 ($p<0.05$) were stated as statistically significant.

## Results

Patients with MPM included fourteen men and six women (mean age: 68.2 years ± 10.4; age range: 50–93 years). The histological classification of the cases, according to the WHO criteria, was the following: eleven epithelioid types, two sarcomatoid types, and seven biphasic subtypes (Galateau-Salle et al., 2016). Particularly, two biphasic MPM cases showed a slight predominance of the sarcomatoid component (60% sarcomatoid, 40% epithelioid); one demonstrated a marked predominance of the sarcomatous component with scattered epithelioid elements (80% sarcomatoid, 20% epithelioid), and in contrast one case showed a

**Table 1.** MPM cases' clinic-pathological data and immunohistochemistry (IHC) results of ATG7.

| Case | Age (Years) | Gender | Pathological Subtype | ATG7 IHC (Area %) | ATG7 IHC (Pixel2) | Survival Time (Months) |
|---|---|---|---|---|---|---|
| MPM exposed to FE | | | | | | |
| 1 | 69 | M | Epithelioid | 0.2209 | 2763 | 1.5 |
| 2 | 74 | F | | 1.405 | 17572 | 13 |
| 3 | 85 | M | | 9.3119 | 116461 | 23 |
| 4 | 58 | F | | 2.485 | 23705 | 18 |
| 5 | 55 | M | | 5.355 | 67016 | 37 |
| 6 | 56 | M | | 0.11 | 23705 | 12 |
| 7 | 69 | F | Sarcomatoid | 3.5699 | 44647 | 5 |
| 8 | 50 | M | Biphasic (20% Epithelioid, 80% Sarcomatoid) | 0 | 0 | 16 |
| 9 | 93 | F | Biphasic (40% Epithelioid, 60% Sarcomatoid) | 50.6592 | 633573 | 7.5 |
| 10 | 75 | M | | 29.985 | 375017 | 60 |
| MPM not exposed to FE | | | | | | |
| 11 | 70 | M | Epithelioid | 1.0832 | 13546 | 3 |
| 12 | 74 | F | | 0.4438 | 5550 | 2 |
| 13 | 62 | M | | 5.3319 | 66681 | 12 |
| 14 | 75 | M | | 1.3721 | 17159 | 5 |
| 15 | 59 | M | | 5.8363 | 72983 | 18 |
| 16 | 61 | M | Sarcomatoid | 4.6623 | 58302 | 13 |
| 17 | 70 | M | Biphasic (90% Epithelioid, 10% Sarcomatoid) | 3.8943 | 48698 | 9 |
| 18 | 63 | M | Biphasic (10% Epithelioid, 90% Sarcomatoid) | 1.321 | 16519 | 4 |
| 19 | 73 | F | Biphasic (80% Epithelioid, 20% Sarcomatoid) | 3.1264 | 39080 | 8 |
| 20 | 72 | M | Biphasic (70% Epithelioid, 30% Sarcomatoid) | 0.5923 | 7407 | 3 |

marked predominance of the epithelioid component with scattered sarcomatous elements (80% epithelioid, 20% sarcomatoid); one case demonstrated a prominent epithelioid component with very few sarcomatous elements (90% epithelioid, 10% sarcomatoid), and on the contrary, one showed a high predominance of the sarcomatous component with very few epithelioid elements (10% epithelioid, 90% sarcomatoid); the last biphasic case showed 70% and 30% of epithelioid and sarcomatoid component, respectively. Clinico-pathological data including age, gender, the pathological subtype of MPM, survival time, and ATG7 immunoexpression are summarized in Table 1.

The cohort of control cases was composed of eight men (mean age: 44±25.5 years; age range: 15-76 years). These patients did not live in Biancavilla, and they did not show neoplastic diseases but pleurisy (n=5) and pulmonary emphysema (n=3).

ATG7 expression was confirmed following immunohistochemistry. The Kolmogorov-Smirnov test showed that both the percentage areas stained with ATG7 (area %) and the level of ATG7 staining intensity of positive areas (pixel$^2$) in cases of MPM and controls differ significantly from a normal distribution ($p<0.0001$). As demonstrated in Fig. 1, a significant difference between ATG7 immunostaining in MPM tissue (Fig. 1A) compared to the control tissue was shown (Fig. 1B). In particular, Fig. 1a showed the ATG7 immunostaining software image analysis of the corresponding MPM sample, in which mainly a high immunostained area (red color) was demonstrated.

Densitometric and morphometric expressions of ATG7 were significantly increased in MPMs when compared to the controls ($p<0.0001$) (Fig. 2).

Considering the median overall survival (OS) between high and low ATG7 expression there was an

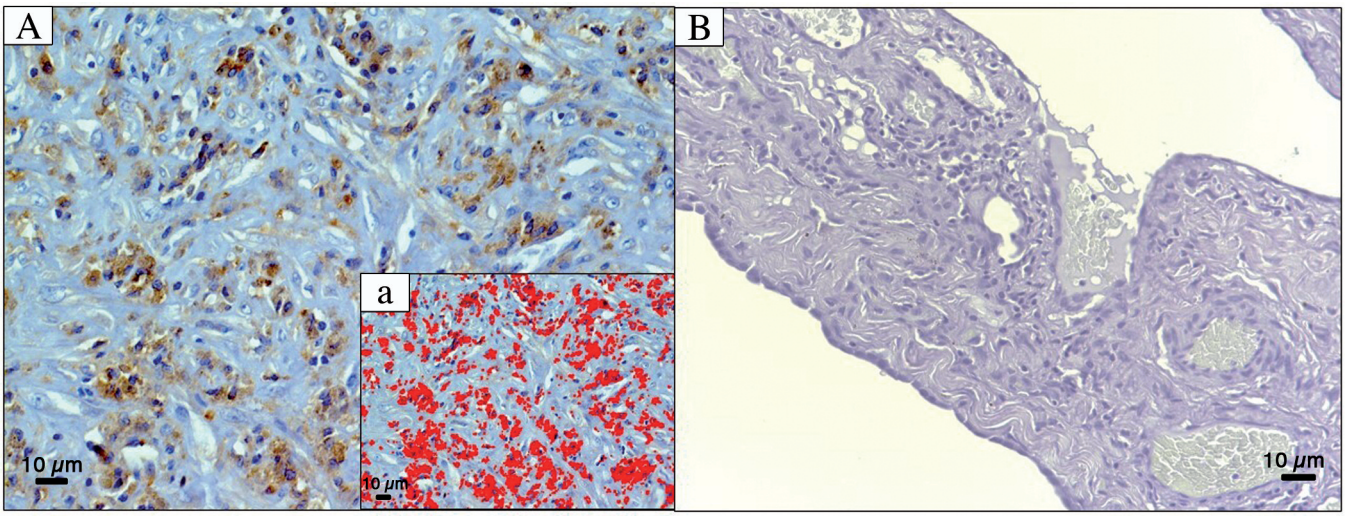


**Fig. 1. A.** High immunohistochemical cytoplasmic expression of ATG7 in a biphasic MPM tissue (immunoperoxidase staining). **a.** ATG7 immunostaining software image analysis of Fig. 1A, in which mainly a high immunostained area (red color) was detected. **B.** Immunohistochemical section of non-neoplastic mesothelial control tissue showing absence of staining with ATG7 (immunoperoxidase staining).

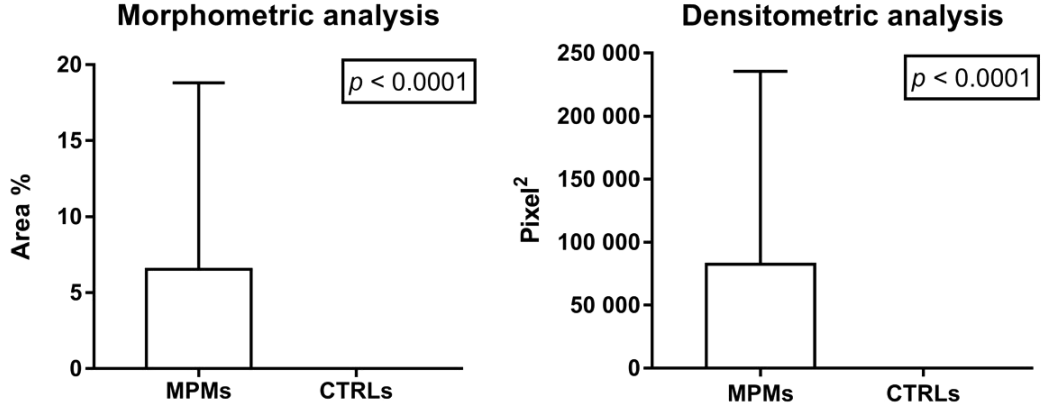


**Fig. 2.** Comparison of the morphometric analysis (area %) and densitometric analysis (pixel$^2$) of the ATG7 immunostaining in the analyzed fields for MPM cases vs. controls. Data are presented as mean ± standard deviation (SD).

association of ATG7 expression and increased OS. The Kaplan-Meier method showed a significant association of ATG7 expression with an increased OS (p=0.0438) and the hazard ratio (HR) was 0.344 with a 95% confidence interval (CI) 0.1219 to 0.9709) (Fig. 3).

Generically, no relationship emerged between ATG7 expression and other clinicopathological variables, but there was a significant correlation between ATG7 expression and the survival time of MPM patients (p=0.0099) (Fig. 4).

## Discussion

The present study has been conducted on an *ex vivo* cohort of patients with MPM. The selected cases of MPM were collected based on exposure and non-exposure to FE fibers. The exposed cases were residents in the Biancavilla area, where high mortality for this neoplasm has been related to FE fibers, while the non-exposed cases were residents in Sicilian towns not located in the Biancavilla area. The immunohistochemical results confirmed tissue activation of the autophagic process, demonstrated by ATG7 overexpression in MPM samples vs. controls. In particular, a strong immunoreactivity against ATG7 was observed in 10 out of 20 MPM cases (50%) while a low reactivity was detected in 10 out of 20 MPM cases (50%). Moreover, a trend of longer OS was found in MPM patients with ATG7 overexpression, with late recurrences and death for the neoplasm. On the contrary, earlier recurrences and the worst prognoses were represented in the 50% of the cases that demonstrated low levels of expression of ATG7. In particular, a statistically significant correlation of ATG7 high expression with increased survival was observed with a mean OS of 12.5 months for patients with overexpression vs. a mean OS of only 4.5 months for patients with low ATG7 immunoexpression. Furthermore, there was a significant correlation between ATG7 expression and the survival time of MPM patients.

Our results are in line with a previous paper that states ATG13 as a prognostic factor for mesothelioma, underlining that autophagy plays an essential role in this type of neoplasm (Follo et al., 2016, 2018, 2019b). The aforementioned studies indicated that higher autophagy initiation status correlates with a better prognosis and suggests that the autophagy process plays a dual role, both pro-survival and tumor-suppressive (Follo et al., 2016, 2018, 2019b). It is possible to assert that autophagy acts as a 'Janus-faced' player in cancer onset and progression. At the beginning of cancer development, autophagy plays an onco-suppressive role in protecting healthy cells and repressing initial steps in carcinogenesis. Later, once the neoplasm is established autophagy mainly supports progression by helping cancer cells to overcome the adverse conditions of the cancer microenvironment (like oxygen and nutrients deprivation) (Gugnoni et al., 2016).

Literature data have correlated a deficit in the autophagic process to the onset of carcinogenesis (Galluzzi et al., 2015; Maes et al., 2013; White, 2015; Lee et al., 2018). This tumor-suppressive role for autophagy is stated by the evidence that allelic loss of Beclin 1 in mice (Beclin1-/- animals are not viable) is associated with the progression of hepatocellular carcinoma and other cancers (Galluzzi et al., 2015; Maes et al., 2013; White, 2015). Hepatocyte-specific deletion of ATG7 promotes liver size, fibrosis, progenitor cell expansion, and hepatocarcinogenesis, which is rescued by concurrent deletion of Yap (Lee et al., 2018), an important transcription activator in MPM (Zhang et al., 2017). More recently, mice harboring tissue-specific deficiency in other key autophagy genes, such as AMBRA1 (an interactor of Beclin 1) (Cianfanelli et al., 2015), ATG5, or ATG7 (Maes et al., 2013; Galluzzi et al., 2015; White, 2015; Lee et al., 2018) showed the

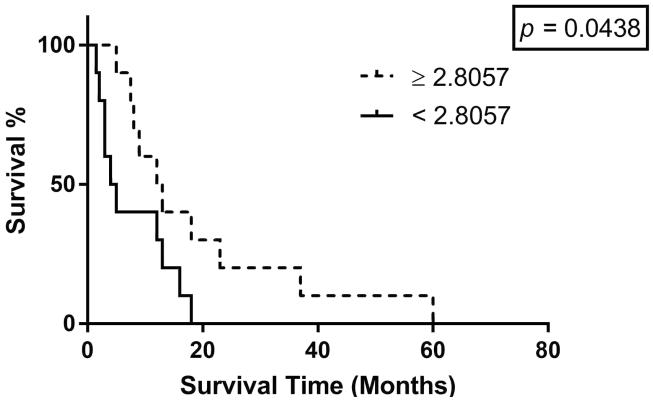


**Fig. 3.** Kaplan-Meier survival curve of ATG7 expression (area %) in MPM patients.

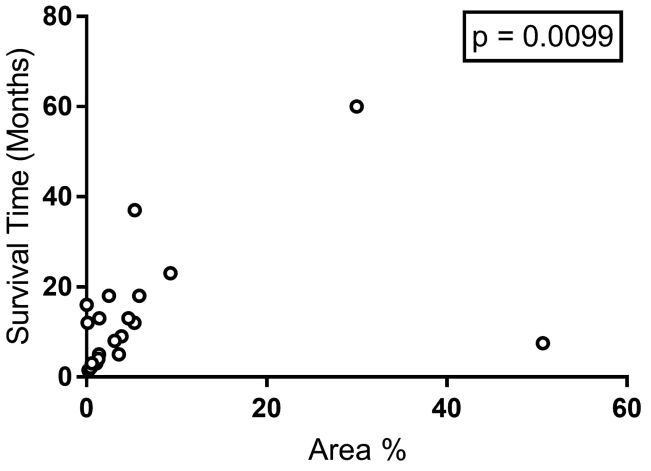


**Fig. 4.** Spearman correlation between survival time (months) of MPM patients and the percentage areas (morphometric analysis) stained with ATG7 expressed as % positive, dark brown pixels of the analyzed fields.

emergence of oncogene-driven pre-malignant lesions and accelerated spontaneous tumors (Maes et al., 2013; Galluzzi et al., 2015; White, 2015) supporting the tumor-suppressing role of autophagy during the initial phases of the carcinogenesis process. In addition, our research group assessed the immunoexpression of three autophagy-related proteins (Beclin-1, p62, and ATG7) in a series of 85 uveal melanomas (UMs), of which 39 were in metastatic stage, and found that the overexpression of Beclin-1 correlated with tumor cell type and better outcome in UM; instead, no significant differential expression of ATG7 and p62 between patients, with or without metastasis, was found (Broggi et al., 2020b).

A large part of the literature data demonstrated that autophagy also represents a mechanism of cell death that can be established even without detectable apoptosis (via autophagic death) or at the same time as apoptosis (Yonekawa and Thorburn, 2013). Besides, autophagy regulatory and executor ATG genes can also interact with other processes, such as apoptosis. It was reported that ATG7 protein, involved in the maturation of the autophagosome, regulates p53 function (Lee et al., 2012). Several common oncogenes (such as those encoding PI3K class I, Pkb, Tor, Akt) inhibit autophagy, while tumor suppressor genes (such as those encoding p53, Pten, Tsc1, Tsc2) induce the autophagic process (Botti et al., 2006).

Then, autophagy constitutes a stress adaptation that escapes cells from apoptosis, whereas it represents an alternative cell-death pathway in other cellular settings. In severe cell damage, both these cell death processes may be activated by common upstream signals and cooperate to escape cell transformation. In this setting, massive autophagy activation leads to cell death through apoptosis (Maiuri et al., 2007). Previous studies conducted by our research group demonstrated the activation of the apoptosis cascade in MPM tissues. This is a critical mechanism that leads tissue to irreparable FE-induced damage and, if not activated enough, predisposes it to a neoplastic evolution (Loreto et al., 2020d).

The finding on tissue samples of protein markers, such as ATG, may provide the physician with important prognostic and predictive data on the therapeutic response (Broggi et al., 2020a).

In the present study, ATG7's high expression represents a promising prognostic tool for patients with MPM; however, the relatively small number of MPM patients represented suggests further validation to establish the definite prognostic value of ATG7 overexpression. Therefore, our future perspective is to demonstrate the role of ATG7 as an independent prognostic marker also when it is corrected for the cancer stage.

In future studies, we aim to integrate the clinical and prognostic implications of ATG7 immunoexpression with other prognostic and predictive parameters. In this view, the tumor immune microenvironment and the complex inter-relationships between tumor cells, mesenchymal stem cells, and cells of the immune system have recently been demonstrated to act a relevant part in MPM tumorigenesis (Hill et al., 2003; Li et al., 2012; Marcq et al., 2016; Cui et al., 2018; Kopecka et al., 2018; Perut, et al., 2018; Serio et al., 2018; Ding and Hong, 2020; Marzullo et al., 2020; Pezzuto et al., 2020). Surely, we will improve this preliminary report, investigating whether nuclear Yap immunoreactivity is inversely correlated to ATG7 immunoexpression in MPM, because YAP is an oncogene candidate that has shown potential to be a therapeutic target of mesothelioma (Zhang et al., 2017).

Finally, due to its ubiquitous presence in the different cited contexts, autophagy has assumed an emerging and relevant modulator of cancer progression that is scientifically intriguing and clinically relevant.

*Acknowledgements.* This research was supported by 2020/2022 PIA.CE.RI., University of Catania, DIPREME project, UPB: 20722142130.

*Informed Consent Statement.* Patient consent was waived due to the non-interventional retrospective nature of the study.

*Conflicts of Interest Statement.* The authors declare no conflict of interest.

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Accepted November 11, 2021