Pleural Mesothelioma and Lung cancer: the role of asbestos exposure and genetic variants in iron metabolism and inflammation genes

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Abstract

Two of the major cancerous diseases associated with asbestos exposure are malignant pleural mesothelioma (MPM) and lung cancer (LC). In addition to asbestos exposure, genetic factors have been suggested as involved in asbestos-related carcinogenesis and lung genotoxicity. While genetic factors involved in the susceptibility to MPM have been reported, to date the impact of individual genetic variations on asbestos-related lung cancer risk is still poorly understood. Since inflammation and disruption of iron homeostasis are hallmarks of asbestos exposure affecting the lung tissue, in this study we aimed at investigating the association between iron-metabolism and inflammasome gene variants and susceptibility to develop LC, by comparing an asbestos exposed population affected by LC with an "asbestos-resistant exposed population". We employed a retrospective approach same as our previous autopsy-based pilot study and we also replicated our previous findings on MPM by repeating the analysis in a novel cohort of autoptic samples. We confirmed the protective role of *HEPH* coding SNP but also of the two non-coding SNP, either in *FTH1* or in *TF,* in a novel cohort of mesothelioma exposed individuals and we found the same protective genetic variants in a cohort of LC exposed individuals, from the same geographic area of MPM subjects, suggesting a common "protection profile". No association was found between *NLRP1* and *NLRP3* polymorphisms with susceptibility to develop MPM and LC. Further research into a specific MPM and LC "genetic signature" would be needed to broaden our knowledge of the genetic landscape causative of MPM and LC.

Keywords: Mesothelioma, Lung Cancer, Formalin-fixed and Paraffin-embedded tissue samples, Inflammasome, Hephaestin, Iron, Polymorphisms, Asbestos exposure.

Introduction

Malignant pleural mesothelioma (MPM) and lung cancer (LC) represent two types of diseases associated to asbestos exposure (Andujar et al., 2016; Gilham et al., 2016; Lemen, 2016). Even though the mechanisms underlying lung and pleural cells injury are still far from being unraveled, the exposure to these fibrous minerals seems not to be the sole cause responsible for the development of such these devastating diseases (D. W. Kamp & Weitzman, 1999; B. T. Mossman, 1990). In the last decade many studies reported that also genetic factors are involved in promoting asbestos-related carcinogenesis and lung genotoxicity.

A number of studies have compared past asbestos exposure and genetic polymorphisms using either candidate genes approaches (Betti et al., 2017; Borelli, Moura, Trevisan, & Crovella, 2015; Christiani, 2000; Crovella et al., 2016; Dianzani et al., 2006; Gemignani et al., 2009; Girardelli et al., 2012; Hirvonen et al., 1996; Landi et al., 2007; London et al., 1995; Murakami et al., 2012; Neri et al., 2008; Schabath et al., 2002; Schneider & Bernges, 2009; Tunesi et al., 2015; Wang, Neuberg, & Christiani, 2004) or whole-genome association studies for both MPM and LC (Cadby et al., 2013; Kettunen et al., 2017; Matullo et al., 2013; Wei et al., 2012). While low- (Cadby et al., 2013; Matullo et al., 2013) and high-risk (Betti et al., 2017; Ohar et al., 2016) genetic factors for MPM have been reported, even defining germline variants in *BAP1* tumor suppressor gene as high-risk factor (Ohar et al., 2016), the impact individual genetic variations have on asbestos-related lung cancer risk is still poorly understood. Candidate gene approach studies have reported that polymorphisms in genes encoding for xenobiotic metabolizing enzymes (e.g. *GSTM1, GSTT1, MPO, CYP1A1* and *CYP2E1*) and manganese superoxide dismutase (*SOD2*) are associated with asbestos-related lung cancer risk (Schabath Wang et al, 2004; Schneider et a., 2009). Although recent genome-wide association studies (GWAS) have pinpointed novel loci for lung cancer risk, few have addressed genome–environment interactions. In particular Wei and colleagues (2012) suggested that immune function regulation-related pathways (Fas pathway)

might be mechanistically involved in asbestos-associated lung cancer risk. Then Liu's and colleagues (2015) proposed MIRLET7BHG (MicroRNA Let-7b Host Gene) as an possible important predictive marker for asbestos exposure-related lung cancer. Finally Kettunen and colleagues (2017) identified novel DNA methylation changes associated with lung tumors and asbestos exposure.

Lung inflammation and disruption of iron homeostasis have been observed both in animal models and patients affected by asbestos-related lung disease (Dostert et al., 2008; Ghio et al., 2008; Ghio, Pavlisko, & Roggli, 2015; Jiang et al., 2012; B. T. Mossman, 1990; B. T. Mossman & Churg, 1998; Brooke T. Mossman et al., 2013) and are considered important mechanisms of pulmonary toxicity induced by asbestos (Ather, Martin, Ckless, & Poynter, 2014; A. E. Aust, Cook, & Dodson, 2011; E. A. Aust, Lund, Chao, Park, & Fang, 2000; Chew & Toyokuni, 2015; Ghio et al., 2008, 2015; Liu et al., 2015; Shannahan et al., 2011).

We have previously studied the association between selected iron-metabolism and inflammation-associated genes with susceptibility to develop MPM in a highly selected asbestos exposed population employing post-mortem paraffin-embedded tissues. Three SNPs, localized in the ferritin heavy chain, transferrin, and hephaestin genes resulted protective against the development of MPM. No associations were found, instead, between polymorphisms in inflammation-associated genes NACHT, LRR, FIIND, CARD domain and PYD domains-containing protein 1 and 3 (*NLRP1* and *NLRP3*), both constituents of a multiprotein oligomer called "inflammasome" and responsible for initiating the inflammatory response (Martinon, Burns, & Tschopp, 2002), and susceptibility to MPM development (Borelli et al., 2015; Crovella et al., 2016).

Since inflammation and iron homeostasis disruption are both hallmarks of lung damage by asbestos exposure, in this study we investigated the possible association between iron-metabolism and inflammasome gene variants and LC developing risk, comparing an asbestos exposed LC affected cohort with an "asbestos-resistant exposed population". We employed a retrospective approach relying

on our autopsy-based pilot study and we additionally replicated our previous findings on MPM assaying a much wider cohort of autoptic samples.

Materials and Methods

Population-based autopsy study

Our samples originate from Monfalcone area, as in a previous study (Crovella et al., 2016). This area is characterized by high incidence of asbestos-related mesothelioma (C. Bianchi, Brollo, Ramani, & Zuch, 1993) and by the frequent presence of pleural plaques observed after the necroscopic examination (Bianchi et al., 1991). Samples in this study have been obtained from autopsies performed between 1980 and 2015 on asbestos-exposed subjects. All necroscopic examinations were conducted at Monfalcone Hospital and were firstly reviewed for signs of asbestos exposure and then for asbestos-related neoplasms (pleural mesothelioma and lung cancer) or for the absence of asbestos-related diseases to select a control population (see below). Asbestos exposure was objectively established for all autopsies by evaluation, during the necroscopic examination, of the presence of pleural plaques and/or Asbestos Bodies (AB) in routine lung sections obtained at necropsy. Pleural plaques were examined and classified in three stages, as previously described (Crovella et al. 2016) and AB were also quantified, using the Smith-Naylor method (Smith e Naylor 1972), with AB lung burden expressed as number of AB per gram dry lung tissue. A documented occupational history of asbestos exposure was collected for the majority of subjects. On the basis of occupational history, we subdivided individuals into 5 categories: maritime, having worked on merchant ships; builder, being involved in construction/refurbishment of houses/industrial warehouse; shipyard, being employed either directly or indirectly in shipbuilding on Monfalcone shipyard; domestic, being a wife of a worker in Monfalcone shipyard; other, not pertaining to aforementioned categories.

Study population: individuals exposed to asbestos who developed mesothelioma or lung cancer.

Autopsies were performed between 2009 and 2015. Inclusion criteria were: subjects who presented objective signs of asbestos exposure (presence of AB and/or pleural plaques during routine lung sections examination) and developed either pleural mesothelioma (Asbestos Exposed with Mesothelioma = AEM, n=52) or lung-cancer (Asbestos Exposed with Lung Cancer = AELC n=57); subjects' characteristics are summarized in table 1 and 2. Diagnosis of pleural mesothelioma or lung cancer was confirmed or obtained during necropsy and evaluated by histological examination. Information on histological type of neoplasia was collected for each patient, both mesothelioma or lung-cancer and the patients were subdivided into categories based on this information. The majority of malignant pleural mesotheliomas cases can be differentiated into three different histological types: epithelioid, possessing mostly cells with epithelial morphology; sarcomatoid, with cells having a spindle morphology; biphasic, with cells belonging to both categories (spindle and epithelioid) (Inai, 2008). The majority of lung cancers can be classified into two large histological categories: small cell lung cancer or non-small cell lung cancer, further subdivided into adenocarcinoma, squamous carcinoma and large cell carcinoma. (Travis et al., 2015). In AELC patients, information regarding smoking status was obtained and used to classify subjects in non-smoker (N) or smokers (S).

Control population: individuals exposed to asbestos without mesothelioma or lung cancer development (AE).

Autopsies were performed between 1980 and 2000. Inclusion criteria were: subjects (n=48) presenting objective signs of asbestos exposure (presence of pleural plaques and/or of AB during routine lung sections examination), who developed neither of the asbestos-related diseases (specifically mesothelioma or lung cancer and also other form of asbestos-induced tumors, such as laryngeal,

gastrointestinal and ovarian cancer (International Agency for Research on Cancer 2012) and died of other causes after 75 years age. The threshold of 75 years of age was chosen on the basis of latency period for asbestos-related diseases, occupational exposure and age at the time of death, hinged on previously reported data from a series of mesothelioma cases in the same geographical area (Bianchi, 2007). The study was approved by the regional ethical committee for Friuli Venezia Giulia.

Postmortem samples

The archives of the Department of Pathological Anatomy of Monfalcone Hospital stored the histological samples from all autopsies. Myocardial tissue was chosen as the starting material for DNA extraction, selected to be free from neoplastic cells and thus without somatic alterations due to tumorigenic transformation (Bisel, Wroblewski, & Ladue, 1953). Mean age ± S.D. of the material at the time of DNA extraction was 14 ± 11,69 years, ranging from 4 to 35 years. Fixation was made in 10% formalin for all the samples; from the same paraffin block, forty to fifty slices were cut with a 5-7 µm thickness and processed for DNA extraction.

DNA extraction from Formalin-fixed Paraffin-Embedded (FFPE) samples

Genomic DNA was extracted using QIAamp DNA FFPE Tissue Kit (Qiagen, Hilden Germany), following manufacturer instructions, and eluted in 30 µL final volume of TE buffer. Extracted DNA was qualitatively and quantitatively evaluated using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis; final concentration for genotyping was 50 ng/ml.

HEPH, TF, FTH1, NLRP1 and NLRP3 polymorphism analysis in AEM, AELC and AE population

We selected genetic variants, previously analyzed in a population from the same area, located in three iron metabolism-related genes, namely hephaestin (*HEPH*), transferrin (*TF*) and ferritin heavy polypeptide 1 (*FTH1*) as in (Crovella et al., 2016), and in two inflammation-related genes, namely nucleotide-binding domain, leucine-rich-containing family, pyrin domain-containing-3 (*NLRP3*) and *NLRP1* as in (Borelli et al., 2015).

Genotyping was performed on an ABI7900HT Fast Real-time PCR (polymerase chain reaction) instrument, using fluorescent TaqMan SNPs genotyping assay and TaqMan Genotyping Master Mix (Applied Biosystems-Life Technologies, Carlsbad, CA) following manufacturer's instructions. Samples have been run in duplicate.

For *HEPH* and TF polymorphisms, rs3747359 and rs2715631, pre-designed SNP genotyping assay were used (C__27476246_20 and C____148065_20, respectively); for *FTH1* rs76059597 a custom TaqMan SNP assay (Life Technologies, Foster City, CA) was employed. For NLRP1 polymorphism, rs12150220 and rs6502867 pre-designed SNP genotyping assay were used (C___1600653_10 and C__29222211_20). For NLRP3 polymorphism, rs35829419 and rs10754558, pre-designed SNP genotyping assays were used (C__25648615_10 and C__26052028_10) Data analysis was performed both manually and automatically using SDS software version 2.1 (Life Technologies, Foster City, CA).

Statistical analysis

Test for Hardy-Weinberg equilibrium (HWE) either on autosomal or sex chromosomes was made with the *HardyWeinberg* R package (Graffelman, 2015). Allelic and genotypic differences between the groups were determined using the Fisher exact test whereas odds ratio (OR) and 95% confidence interval were estimated using Woolf's method. Calculations were done for the different genetic models as indicated in Lewis and Knight (Lewis & Knight, 2012) Statistical calculation was performed using

R-studio (*RStudio: Integrated Development for R. RStudio, Inc.*, 2015) and dedicated packages. Power analysis was performed using GPower (G*Power, version 3.1.9.4, Universitat Kiel, Germany) (Faul, Erdfelder, Buchner, & Lang, 2009). Probability calculation was made using guidelines from Andrade (2015) and Norton et al., (2018) and performed with the following formula

$$P = \frac{Fractional\ O.R.numerator}{(Fractional\ O.R.numerator)+(Fractional\ O.R.denominator)}$$

with P as probability.

Results

Study and Populations characteristics

The study was performed on autopsy samples derived from individuals exposed to asbestos who developed either pleural mesothelioma (AEM, *n*=52) or lung cancer (AELC, *n*=57) using, as a reference, another set of autopsy samples from asbestos-exposed individuals which died of asbestos-unrelated diseases (AE, *n*=48). AEM and AELC populations were in Hardy-Weinberg equilibrium (HWE) for all the genetic variants analyzed. However, AE population was not in HWE for the *FTH1* SNP, while for all the other variants considered it was in HWE.

*Asbestos-exposed individuals who developed Mesothelioma (*AEM*)*

The AEM population included 6 women and 46 men, with a mean age of 74,2 ± 8,8 years at the time of death, all having evidence of pleural plaques, the majority of samples presenting grade 2 or above (59,6%). Moreover, AB lung burden was determined for all cases, ranging from 108 to 950.00 AB/gr of dry tissue, median 6400 AB/gr, thus well above the threshold for occupational exposure (1000 AB/gr) (Casali et al., 2015). Occupational data were available for 49 out of 52 cases (94,3%) and validated asbestos exposure due to working condition: 34 cases were employed in the shipyard while the additional 11 cases in shipyard-related professions. Domestic exposure was also observed in four cases, being wives of shipyard workers.

*Asbestos-exposed individuals who developed Lung Cancer (*AELC*)*

Only men belonged to AELC population with a mean age of 73,9 ± 8,7 years at the time of death; pleural plaques were present in 51 out of 57 cases (89,5 %) with the majority of samples showing grade 2 or above (75,5%). Also, in this population, AB lung burden was determined in the majority of samples (48 out of 57) demonstrating exposition well above the occupational threshold, with a median of 3600 AB/gr dry tissue (min = 60, maximum = 275.000), a result confirmed by occupational data,

available in 52 out of 57 cases, showing that the majority of cases were employed in shipyard (44 cases) and shipyard-related professions (7 cases), while for only one case employment was not directly linked to occupational asbestos exposure, being the patient a carpenter not directly working on the shipyard.

Smoking habit was available for 48 cases out of 57 (84,2%): 46 cases were smokers (S) and only 2 cases were non-smoker (N). Due to the small number of never smoker samples, we were unable to subdivide the AELC population based on smoking habits. Likewise, due to the small number of samples, we were unable to subdivide both the MPM and the AELC population based on pleural/lung cancer histological subtype, an interesting aspect that need further investigation.

*Asbestos-exposed individuals (*AE*)*

The reference AE population is composed by 45 men and 3 women, mean age 80.1 ± 0.6 years at the time of death; cause of death is not asbestos-related. These subjects were also based on the ~~with~~ presence of AB and of pleural plaques of class 2 or above. Quantitation of AB lung burden in all cases indicated that asbestos exposure was above the threshold for occupational exposure (AB/g dry lung tissue from 16,000 and 994,000). Occupational data were available for only 32 out of 48 individuals with 27 patients being employed in shipyard and 5 in shipyard-related professions, but exposure and longevity data indicate that AE, in spite of having been exposed to asbestos, did not develop mesothelioma or lung cancer, so we enroll them as a control for genetic testing on AEM and AELC, as previously shown (Crovella et al., 2016).

Genetic analysis

We firstly tried to replicate the findings obtained in Crovella and colleagues (2016) for iron-related genes and in Borelli and coworkers (2015) for inflammasome-related genes in a new population of asbestos-exposed individuals who developed mesothelioma (AEM). Subsequently we analyzed, for the

first time, a population of exposed individuals who developed lung cancer (AELC), using, as reference, individuals exposed to asbestos who did not develop asbestos-related diseases (AE), aiming to assess if a common pathogenic mechanism existed, shared by AEM and AELC.

Iron-related genes

HEPH gene

The first genetic variant examined in this study is rs3747359 in *HEPH* gene. It is a coding SNP (cSNP) with a minimum allele frequency (MAF) < 0,01, as reported by 1000 genomes data (http://www.internationalgenome.org/); *HEPH* gene is located on X chromosome. The protein encoded by this gene, hephaestin, is a multi-copper oxidase, involved in iron transport across the epithelial cells to the circulatory system. Being *HEPH* localized on X chromosome and not in the pseudoautosomal region, we compared allele frequencies, counting the chromosomes, according to Clayton et al., (2009), (i.e males contribute for one observation and females for two). For the analysis to be statistically solid, Hardy-Weinberg Equilibrium must be valid, as it is in our samples (p=0.14 for AE and p=0.76 for Chi-square test). Moreover, to avoid sex bias effects, allele frequencies between male and female must be equal, as it is the case for our samples (p= 0.06 for AE and p= 1 for AEM with Fisher exact test comparing the frequencies). Taking into account these considerations, we compared the allelic frequencies: the C allele was statistically more frequent in AE population than in AEM thus possibly being associated with protection against mesothelioma (O.R. = 0.06 95% CI = 0.003 – 0.3); $p=8.5x10^{-5}$, Fisher Exact test, power $\beta = 0.99$), increasing the probability by 5.6% of not to develop this disease Moreover, assessing the genetic frequencies, we observed a statistically significant difference in C hemizygotes in AE population, being this genotype more frequent in this population in respect to AEM; besides the C/C genotype was also more frequent in the control population (Figure 1) (O.R. = 0.01 95% CI = 0.00 – 0.05 $p=2.68x10^{-18}$ Fisher Exact test, power $\beta = 0.99$). Genetic model of inheritance was

also analyzed, considering males as homozygote as in Clayton et al., (2009), and statistical analysis indicates a dominant model of inheritance (Supplementary Table 1) (G/G vs G/C+C/C O.R = 0.04 95% CI = 0.0004 – 0.2 p=2.6 $X10^{-7}$, power β = 0.99). We decided to further expand our findings examining AELC population in comparison to AE (control) population. Being AELC population composed only of males, is not possible to verify HWE and equal allelic frequency between sexes; indeed, being the AE population fulfilling those criteria, we decided to confront allelic distribution in these samples, finding a significant statistical difference of C allele presence in AE population, associating with protection against LC (O.R. = 0.03 95% CI 0.005 -0.12, p=$6.1x10^{-12}$, Fisher Exact test, power β = 0.99). Indeed, presence of C allele increase of 2.9% the probability of not developing lung cancer. Observing the genetic distribution between the two population it is also possible to note the higher frequency of C allele and C/C genotypes in AE with respect to AELC (O.R: = 0.03 95% CI 0.01 – 0.13, p =$2.69x10^{-18}$, Fisher Exact test, power β = 0.99) (Figure 2). Analysis of inheritance models indicates also in this case a dominant model (G/G vs G/C+C/C O.R = 0.04 95% CI = 0.01 – 0.16; p=6.3 $X10^{-8}$, power β = 0.99) (Supplementary table 2).

TF gene

We then analyzed one variant in *TF* gene, which encodes transferrin, a protein involved in iron transport; the SNP rs2715631, situated in an intronic region, has been reported as involved in the protection from mesothelioma, as previously shown in Crovella et al. (2016). Allele G was significantly more frequent in AE population in comparison with AEM or AELC (O.R = 0.22 95% CI =0.12-0.47 p=3.5 X 10^{-6}, Fisher Exact test for AEM vs AE, power β = 0.93 Supplementary Table 3) (O.R. = 0.15 95% CI = 0.07 – 0.31 p=3.9 X 10^{-9}, Fisher Exact test for AELC vs AE, power β = 0.99 Supplementary Table 4). Therefore, the presence of G allele increases the probability of not developing pleural mesothelioma 18.1% and 13.01% considering lung cancer. Analyzing genotype distribution in

AEM vs AE (Figure 3) it is possible to observe a major frequency of G/G individuals in AE control population, suggesting a protective role of this genotype against mesothelioma. Statistical analysis showed a dominant model of inheritance for protective alleles (T/T vs T/G+G/G OR = 0.22; 95% CI = 0.09-0.52 p = 3.3 $x10^{-4}$; Fisher Exact test, power $\beta = 0.99$) (Supplementary Table 3).

Furthermore, observing genotype frequencies in AELC vs AE populations we noticed major frequency of G/G individuals in the control population (Figure 4). Statistical analysis indicated a co-dominant model of inheritance for the protective alleles (T/T vs T/G O.R. = 0.21 95% CI = 0.08 – 0.56 p = 1.7 X 10^{-3} Fisher Exact test, power $\beta = 0.99$, and T/T vs G/G O.R. = 0.06 95% CI = 0.01 – 0.22 p= 2.9 X 10^{-6} Fisher Exact test, power $\beta = 0.99$) (Supplementary Table 4).

FTH1 gene:

The third gene examined in this study is *FTH1* selected since one SNP, rs76059597, located in an intronic region, has been described as involved in the protection against mesothelioma in asbestos-exposed individuals (Crovella et al., 2016). *FTH1* encodes ferritin heavy subunit, a protein involved in Fe-storage and one of the main components of AB coating(Borelli et al., 2007). In AEM population allele C was significantly less frequent in comparison with AE, confirming previous results(Crovella et al., 2016)(O.R. = 0.1 95% CI=0.05–0.22 p= 2.5X 10^{-11}, Fisher Exact test, power $\beta = 0.99$; Supplementary Table 5) . Furthermore, also in AELC population, C allele was less present in affected individuals (O.R. = 0.13 95% CI = 0.07 – 0.25 p=2.3 X 10^{-10}, Fisher Exact test, power $\beta = 0.99$; Supplementary Table 6); these results indicated that with the presence of this allele the probability of developing either mesothelioma or lung cancer is reduced by 9.1% and 11.5% respectively.

Genotypes distribution in AEM vs AE population also showed the major prevalence of C/C genotype in AE (control) in respect to AEM population, confirming its possible protective role (Figure 5). Indeed, statistical analysis of genotype distribution suggested a dominant model of inheritance further

indicating its protective role (T/T vs T/C+C/C OR = 0.15, 95% CI = 0.06-0.35, p-value from Fischer Exact test =1.4 X 10^{-5} , power $\beta = 0.99$) (Supplementary Table 5).

Likewise, for the other genes, we analyzed also genotypes distribution in AELC vs AE populations observing the higher prevalence of C/C individuals in AE control population, again suggesting a protective role of this genotype (Figure 6). Statistical analysis indicated a dominant model of transmission (T/T vs C/T+C/C, OR = 0.22, 95% CI = 0.09-0.47, p-value for Fisher Exact test =3.1 x10^{-4}, power $\beta = 0.99$) (Supplementary Table 6)

Inflammation-related genes

Having replicated and expanded previous results on iron-signature genes, we decided to test also if these genetic variants in inflammasome genes could influence the risk of developing mesothelioma or lung cancer. The proteins coded by *NLRP1* and *NLRP3* genes are involved in innate immunity and inflammation; in response to damage-associated signals and pathogens these proteins catalyze the assembly of inflammasome complex, which activates and cleaves caspase-1, leading to secretion of IL-1β, a key mediator of inflammation (Hayward, Mathur, Ngo, & Man, 2018). Our research group has previously examined the role of different SNPs in *NLRP1* and *NLRP3* genes, finding no significant difference in allelic distribution between the AEM and AE(Borelli et al., 2015).

NLRP1 gene

When analyzing rs12150220 alleles distribution in the AEM population, no significant differences were found in comparison with AE population, thus indicating no involvement of this *NLRP1* inflammasome SNP in mesothelioma development (Table 3, top). When considering the AELC group in the entire population, no statistical differences have been observed in the distribution of *NLRP1* SNP rs12150220 A and T allele when compared with AE reference (Table 3, top).

To complete our analysis, we then assessed allelic distribution of another *NLRP1* SNP, rs6502867, previously analyzed in Borelli et al., (2015), pertaining to a different haploblock than rs12150220 (Borelli et al., 2015). Even in this occasion, we found no statistical differences in allele distribution both between AEM and AE and between AELC and AE populations, further confirming our previous results for AEM group and expanding our observation to AELC patients (Table 3, bottom)

NLRP3 gene

The involvement of inflammasome in susceptibility to develop malignant pleural mesothelioma (MPM), considering its capacity to sense asbestos fibers has been already reported (Dostert et al., 2008; Hillegass et al., 2013; Brooke T. Mossman et al., 2013). Activation of inflammation appears to be crucial for the transition from mesothelial cell to fibroblast cell, induced by asbestos fibres, and NLRP3 protein has been proposed as the key mediator of this process (Thompson, MacPherson, Beuschel, & Shukla, 2017). We had previously shown that two *NLRP3* gene SNPs (rs10754558 and rs35829419) did not associate with mesothelioma predisposition(Borelli et al., 2015); we thus sought to replicate our previous findings analyzing both *NLRP3* SNPs formerly assessed. As indicated in Table 4, both *NLRP3* SNPs alleles showed no statistically significant differences considering either AEM vs AE and AELC vs AE groups.

Discussion

The presence of genetic risk factor affecting mesothelioma development is demonstrated by epidemiological analysis, where is evident that only a minority of asbestos-exposed subjects develop such pleural neoplasm (5–17% of heavily exposed individuals) (Neri et al., 2008). Furthermore, subjects only mildly exposed to asbestos can develop the disease whereas others, heavily exposed, apparently fail to become ill (Betti et al., 2018; Carbone & Yang, 2012)

However, being MPM a rare disease and with a long latency period, the design of genetic susceptibility studies can be affected by these two factors. Moreover asbestos exposure is not routinely quantitatively evaluated. Post-mortem FFPE tissue samples represent an important tool for uncovering genetic susceptibility to mesothelioma, since they allow the selection of individuals with similar exposure features and also well characterized as far as clinicopathological characteristics and occupational history is concerned.

In previous population-based autopsy studied we identified three Fe metabolism-associated genes, significantly associated with protection against MPM (Crovella et al., 2016), while no association was found for NLRP1 and NLRP3 polymorphisms (Borelli et al., 2015). Here, we confirmed our previously published results (Crovella et al., 2016) by employing a different set of samples, further strengthening the role of iron metabolism gene in mesothelioma susceptibility. We confirmed the protective role of *HEPH* coding SNP (rs3747359) as well as of the two non-coding SNP, either in *FTH* or in *TF* (rs2715631 and rs76059597 respectively). Previous *in silico* analysis (Crovella et al., 2016) has suggested a damaging effect C substitution in *HEPH* gene might produce on hephaestin protein function, while it was not possible to determine the putative functional impact releated to the non-coding SNPs. Indeed, we also tested other databases (such as SROOGLE (http://sroogle.tau.ac.il/) for change in splice sites, or SSC profiler(http://mirna.imbb.forth.gr/SSCprofiler.html) to check for miRNA genes, without reaching conclusive results.

We also confirmed that *NLRP1* gene is not involved in mesothelioma development, finding no association between the pathology and rs12150220 SNP, in contrast to previous results from Girardelli and coworkers (2012). Allelic frequencies obtained in the present study (A=0,56 and T=0,44) are in accordance with the ones obtained either in Borelli et al., (2015) (A=0,52 and T=0,58) and Crovella et al., (2017) (A=0,54 and T=0,46), together with genotype frequencies, further strengthening our conclusions. Moreover, allelic frequencies obtained in the present and previous studies, are similar to

the ones reported by 1000Genomes project (http://www.internationalgenome.org/home) for the European (A=0,56 and T=0,44) and Tuscan (A=0,55 and T=0,45) populations, indicating the substantial concordance of our analysis with the general Italian population. We also confirmed that polymorphisms in another inflammation-related gene, NLRP3, are not linked to MPM development, corroborating previous results from Borrelli et al., (2015).

The availability of novel samples was not limited to mesothelioma patients but also included individuals who died of lung cancer, another asbestos-related disease for which, to date, the impact of individual genetic variations is still not well understood and also less studied.

Inflammation and disruption of iron homeostasis are hallmarks of asbestos exposure affecting specifically the lung tissue and inflammation is a key player in lung cancer development; it is mediated by activation of both inflammasome platforms mediated by NLRP1 and NLRP3, which are expressed and activated in various lung cancer cell lines although at different levels (Kong et al., 2015). However we found that, as for MPM, SNPs at NLRP1 and NLRP3 genes seem not to be involved in LC development. Interestingly, for all tested iron related-SNPs, we found the same results obtained with mesothelioma samples, suggesting that a common protection mechanism could operate. Iron metabolism is crucial for cells and the whole organism survival, and precise homeostasis of this element is required. It is the first time that a correlation between genetic variations in some iron-metabolism gene and lung cancer in asbestos exposed individuals is observed, highlighting the role of this element in the development of lung cancer.

There is substantial evidence that cigarette smoke, the major risk factor for lung cancer (Le Calvez et al., 2005), causes iron dysregulation (Zhang, Butler, & Cloonan, 2019) as well as asbestos, a lower risk factor (Kamp, 2009). Asbestos exposure can induce lung cancer independently, or synergistically with smoking (International Agency for Research on Cancer & Weltgesundheitsorganisation, 2012) but the interaction between asbestos and smoking has been found to be approximately multiplicative

(Markowitz, Levin, Miller, & Morabia, 2013). Unfortunately, due to the very small number of non smokers samples in LC population (2 of 57), we were unable to subdivide it based on smoking habits. Anyway, since our AELC study population was almost composed by smokers, we can hypothesize that asbestos and smoking exert a multiplier effect in inducing iron dysregulation. Iron could represent the link between exposure to pollutants (cigarette smoke, asbestos…) and related lung diseases. This interesting topic will need further investigation in a novel population of AELC with participants equally distributed between never, former, and current smokers.

Recently, a screening of a cohort of mesothelioma patients with a family history of cancer showed the presence of *BAP1* (*BRCA associated protein* 1) mutations in a minority of patients (Ohar et al., 2016), a result further confirmed by two whole-genome studies accomplished in different geographic areas (Betti et al., 2017; Bueno et al., 2016). Mutations in *BAP1* associate with different types of cancer, supporting the concept that a common mutation can trigger diverse malignancies.

External pollutants, such as asbestos and cigarette smoke, increase the iron loads in the lungs (Ghio et al., 2008; Pascolo et al., 2016), induce oxidative stress and inflammation, tipping the point towards cancer development. In this context, alterations of genes involved in iron metabolism could either increase or decrease the toxicity of this metal thus possibly either inducing or protecting from the neoplastic transformation. We have found the same protective genetic variants in mesothelioma and in lung cancer, suggesting a common "protection profile" rather than a common "risk profile" as already reported for *BAP1*.

Our study confirms the previous results obtained in individuals exposed to asbestos which developed mesothelioma and extends these findings to individuals who developed lung cancer. As indicated above, these results suggest the presence of a common mechanism in developing asbestos-related disease, that needs further investigation. It possible to define the iron-metabolism pathway as central in pleural mesothelioma and lung cancer development, with hephaestin playing a pivotal role; expression

of this protein has been described as a prognostic marker in renal cancer and glioma, being its low expression correlating with increased survival (Uhlen et al., 2017)(The Human protein Atlas: www.proteinatlas.org). In our study, a possible hypofunctional protein is correlated with protection against cancer development, thus its ferroxidase activity could be correlated with neoplastic transformation, i.e. via ROS increase, modulated by the other proteins linked to iron metabolism; this pathway needs further functional confirmation. The availability of well-characterized populations in regards to asbestos exposure, that has been objectively quantitated during necroscopic examination, and the availability of occupational data, allowed us to precisely select individuals with comparable levels of asbestos exposure. Numerous genetic studies (as an example Wei et al., 2012) lack an exact quantitation of asbestos exposure, referring only to self-reported exposure or occupational data. Moreover, although based on a limited number of individuals, power analysis confirms distribution in AE population of possible "protective" alleles in iron metabolism genes, in respect to both AEM and AELC populations, giving further strength to our results. Further research into a specific mesothelioma "genetic signature" would be needed, also with the help of Next Generation Sequencing, to broaden our knowledge of the genetic landscape causative of pleural mesothelioma and lung cancer.

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Declaration of interest

All authors declare to have no conflict of interest.

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Figure Captions:

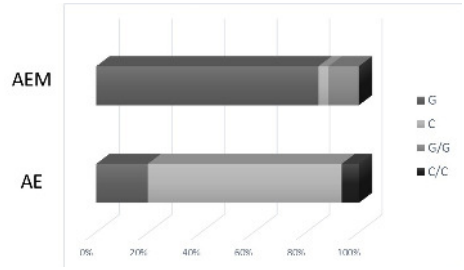

Figure 1: rs3747359 -*HEPH* gene- Genetic frequencies in AE (control) and AEM (mesothelioma-affected) populations.

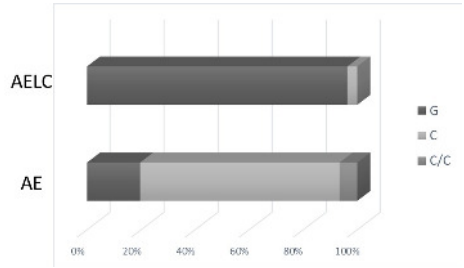

Figure 2: rs3747359 -*HEPH* gene- Genetic frequencies in AE (control) and AELC (Lung Carcinoma-affected) populations.

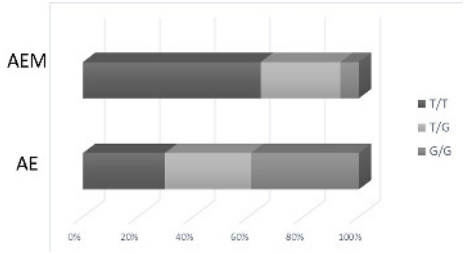

Figure 3: rs2715631 -*TF* gene- Genetic frequencies in AE (control) and AEM (mesothelioma-affected) populations.

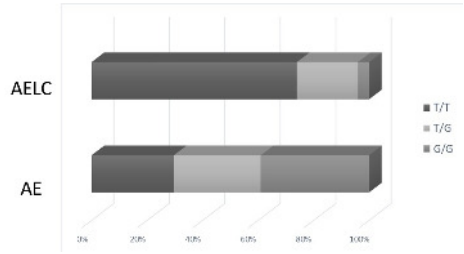

Figure 4: rs2715631 -*TF* gene- Genetic frequencies in AE (control) and AELC (Lung Carcinoma-affected) populations.

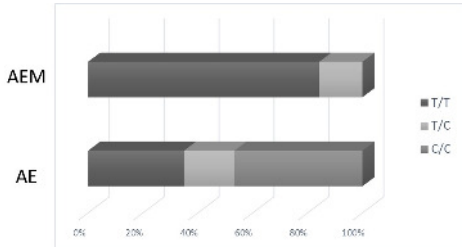

Figure 5: rs76059597 -*FTH1* gene- Genetic frequencies in AE (control) and AEM (mesothelioma-affected) populations.

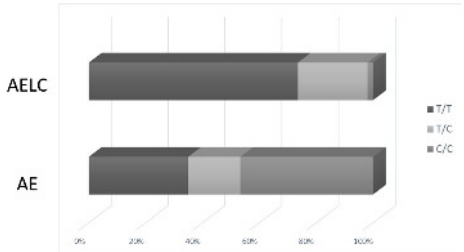

Figure 6: rs76059597 -*FTH1* gene- Genetic frequencies in AE (control) and AELC (Lung Carcinoma-affected) populations.

Table 1: Clinicopathological characteristics of AEM population	
	AEM
All cases, *n*	52
Mean age (years ± S.D.)	74,2 ± 8,8
Gender, *n*(%)	
Female	6 (11,6%)
Male	46 (88,4%)
Histology, *n*(%)	
Epithelioid Mesothelioma	23 (44,2%)
Sarcomatoid Mesothelioma	10 (19,2%)
Biphasic Mesothelioma	16 (30,8%)
N.A.	3 (5,8%)
Asbestos exposure (Asbestos bodies counts) (n/g dry tissue)	
0-999	9 (17,2%)
1000-9999	17 (33,2%)
10.000-99.999	14 (26,8%)
100.000-1.000.000	6 (11,4%)
N.A.	6 (11,4%)
Ialine plaques, *n*(%)	
Absent	10 (19,2%)
Grade 1	11 (21,2%)
Grade 2	26 (50%)
Grade 3	5 (9,6%)
Occupation Type, *n*(%)	
Maritime	4 (7,69%)

Builder	3 (5,77%)
Shipyard	34 (64,38 %)
Domestic	4 (7,69 %)
Other	4 (7,69 %)
N.A.	3 (5,77 %)

Table 1: Characteristics of selected Asbestos-Exposed individuals which developed Pleural Mesothelioma (AEM) in Monfalcone Area (2009-2015).

Table 2: Clinicopathological characteristics of AELC population	
	AELC
All cases, *n*	57
Mean age (years ± S.D.)	73,9 ± 8,7
Gender, *n*(%)	
Female	0 (0%)
Male	57 (100%)
Histology, *n*(%)	
Adenocarcinoma	14 (24,6 %)
Squamous cell carcinoma	7 (12,3)%
Small cell carcinoma	3 (5,3%)
Large cell carcinoma	4 (7,0 %)
N.A.	29 (50,8%)
Smoking Status, *n* (%)	
Smokers (S)	46 (80,7 %)
Non-smokers (N)	2 (3,5 %)
N.A.	9 (15,8%)
Asbestos exposure (Asbestos bodies counts) (n/g dry tissue)	
0-999	13 (22,8%)
1000-9999	20 (35,1%)
10.000-99.999	11 (19,3%)
100.000-1.000.000	5 (8,8%)
NA	8 (14,0%)
Ialine plaques, *n* (%)	
Absent	2 (3,5%)
Grade 1	6 (10,5%)

Grade 2	31 (54,4%)
Grade 3	12 (21,1 %)
N.A.	6 (10,5 %)
Occupation Type, *n*(%)	
Maritime	4 (7,0 %)
Builder	3 (5,3 %)
Shipyard	44 (77,2%)
Other	1 (1,8 %)
N.A.	5 (8,8 %)

Table 2: Characteristics of selected Asbestos-Exposed individuals which developed Lung Cancer (AELC) in Monfalcone Area (2009-2015).

Table 3: *NLRP1* polymorphisms genetic counts

	NLRP1			
	AEM	AE	OR (CI 95%)	P value
rs12150220	n=52 (Freq.)	n=48 (Freq.)		
A/A	18 (0.35)	14 (0.29)	Ref.	
A/T	23 (0.44)	21 (0.43)	0.85 (0.33 – 2.15)	0.82
T/T	11 (0.21)	13 (0.27)	0.66 (0.22 – 1.94)	0.59
	AELC	AE	OR (CI 95%)	P value
rs12150220	n=57 (Freq.)	n=48 (Freq.)		
A/A	20 (0.35)	14 (0.29)	Ref.	
A/T	24 (0.43)	21 (0.43)	0.8 (0.32-1.98)	0.65
T/T	13 (0.22)	13 (0.27)	0.7(0.24-1.99)	0.6
	AEM	EA	OR (CI 95%)	P value
rs6502867	n=52 (Freq.)	n=48 (Freq.)		
T/T	26(0.5)	27(0.57)	Ref.	
T/C	17(0.33)	16(0.33)	1.1(0.45-2.66)	1
C/C	9(0.17)	5(0.10)	1.83(0.54-6.84)	0.37
	AELC	EA	OR (CI 95%)	P value
rs6502867	n=57 (Freq.)	n=48 (Freq.)		
T/T	36(0.63)	27(0.57)	Ref.	
T/C	15(0.26)	16(0.33)	0.7 (0.29-1.68)	0.51
C/C	6(0.11)	5(0.10)	0.89(0.24-3.52)	1.0

Table 3: Distribution of Genotype Frequencies for NLRP1 polymorphism rs12150220 and rs6502867 in Asbestos-exposed individuals who developed Mesothelioma (AEM) or Lung Cancer (AELC) vs Asbestos-Exposed individuals (AE) OR (CI 95%): Odds Ratio with values covering 95% Confidence Interval. P value: p value from Fisher exact tests, asterisk indicates statistically significance.

Table 4: *NLRP3* polymorphisms genetic counts

	NLRP3			
	AEM	AE	OR (CI 95%)	P value
rs10754558	n=52 (Freq.)	n=48 (Freq.)		
G/G	14(0.27)	16(0.34)	Ref.	
G/C	27(0.52)	21(0.44)	1.45(0.58-3.72)	0.48
C/C	11(0.21)	11(0.22)	1.13(0.37-3.51)	1
	AELC	AE	OR (CI 95%)	P value
rs10754558	n=57 (Freq.)	n=48 (Freq.)		
G/G	16(0.28)	16(0.34)	Ref.	
G/C	28(0.49)	21(0.44)	1.32(0.53-3.29)	0.64
C/C	13(0.23)	11(0.22)	1.17(0.4-3.48)	0.79
	AEM	AE	OR (CI 95%)	P value
rs35829419	n=52 (Freq.)	n=48 (Freq.)		
C/C	46 (0.88)	46(0.96)		
C/A	6(0.12)	2(0.04)	2.83(0.59-22.32)	0.27
A/A	0(0)	0(0)	NA	1
	AELC	AE	OR (CI 95%)	P value
rs35829419	n=57 (Freq.)	n=48 (Freq.)		
C/C	52(0.91)	46(0.96)	Ref.	
C/A	5(0.09)	2(0.04)	2.1(0.41-17.01)	0.44
A/A	0(0)	0(0)	NA	1

Table 4: Distribution of Genotype Frequencies for NLRP3 polymorphism rs10754558 and rs35829419 in Asbestos-exposed individuals who developed Mesothelioma (AEM) or Lung Cancer (AELC) vs Asbestos-Exposed individuals (AE) OR (CI 95%): Odds Ratio with values covering 95% Confidence Interval. P value: p value from Fisher exact tests, asterisk indicates statistically significance.