Iron signature in asbestos-induced malignant pleural mesothelioma: A population-based autopsy study

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ABSTRACT

Malignant pleural mesothelioma (MPM) is an aggressive cancer with poor prognosis. The development of MPM is frequently linked to inhalation of asbestos fibers. A genetic component of susceptibility to this disease is suggested by the observation that some individuals develop MPM following lower doses of asbestos exposure, whereas others exposed to higher quantities do not seem to be affected. This hypothesis is supported also by frequent reports of MPM familial clustering. Despite the widely recognized role of iron (Fe) in cellular asbestos-induced pulmonary toxicity, the role of the related gene polymorphisms in the etiology of MPM has apparently not been evaluated. Eighty-six single-nucleotide polymorphisms (SNPs) of 10 Fe-metabolism genes were examined by exploiting formalin-fixed paraffin-embedded postmortem samples from 77 patients who died due to MPM (designated AEM) and compared with 48 who were exposed to asbestos but from died in old age of cause other than asbestos (designated AENM). All subjects showed objective signs of asbestos exposure. Three SNPs, localized in the ferritin heavy polypeptide, transferrin, and hephaestin genes, whose frequencies were distributed differently in AEM and AENM populations, were identified. For ferritin and transferrin the C/C and the G/G genotypes, respectively, representing intronic polymorphisms, were significantly associated with protection against MPM and need to be considered as possible genetic markers of protection. Similarly, the C/C hephaestin SNP, a missense variation of this multicopper ferroxidase encoding gene, may be related, also functionally, with protection against MPM. In conclusion, it is proposed that three Fe metabolism-associated genes, significantly associated with protection against development of MPM, may serve as protective markers for this aggressive tumor.

Malignant pleural mesothelioma (MPM) is an aggressive cancer, generally refractory to therapy and characterized by poor prognosis. The development of MPM is frequently linked to inhalation of asbestos fibers, with a long latency period (30–40 years) from the beginning of exposure to the clinical onset of the disease (Bianchi et al., 1997; Bianchi and Bianchi, 2007).

An important mechanism resulting in pulmonary toxicity induced by asbestos is thought to be the one mediated through redox cycling of fiber-bound and bioavailable iron (Fe) (Aust et al., 2000; 2011; Ghio et al., 2004; Liu et al., 2013; Chew and Toyokuni, 2015). The lungs are continuously exposed to Fe-containing pollutants from the atmosphere and employ a number of mechanisms to control potential oxidative stress, all of which result in the coordinated transport of the metal. The transport of non-transferrin-bound (and non-lactoferrin-bound) Fe with the obligate reduction of Fe^{3+} (e.g., by duodenal cytochrome b and other ferrireductases), followed by translocation through a metal carrier protein (divalent metal transporter-1, DMT-1) and incorporation of metal into intracellular ferritin, represents a major means for Fe detoxification in the lung. The storage of the metal in ferritin limits its accessibility to catalytically active metal and protects pulmonary tissue against generation of oxidative stress (Turi et al., 2004). This coordinated handling of Fe in the lung appears to be disrupted by asbestos

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exposure. Experimental evidence showed that Fe homeostasis is disrupted following asbestos exposure in humans and in murine animal models (Ghio et al., 2008, 2009, Jiang et al., 2012).

Asbestos fibers seem to perturb Fe homeostasis by functioning as an inappropriate chelating factor complex to host Fe, presenting it in a catalytically reactive state to generate an oxidative stress (Ghio et al., 2004). Asbestos bodies (AB), namely, ferruginous bodies with asbestos fibers at their core, testify to the capacity of asbestos to accumulate Fe (Kane, 2003; Mossman et al., 2011). After complexing with the fiber surface, Fe might still participate in catalysis of oxidants (Ghio et al., 2004, 2008), thus triggering a vicious cycle that prompts a cascade of cell signaling, transcriptional factor activation, and mediator release, resulting in clinical manifestations that culminate in inflammatory, fibrotic, and neoplastic disease (Mossman et al., 2011).

Although mesothelioma has been considered for many years the paradigm of environmentally determined cancers, the presence of a genetic component in the etiology of this disease has been proposed, mostly based on evidence that only a minority of asbestos-exposed subjects develop MPM (5–17% of heavily exposed individuals) (Neri et al., 2008). Further, some subjects develop malignant mesothelioma following lower doses of asbestos exposure, whereas others exposed to higher quantities apparently fail to suffer from this disease (Carbone et al., 2012). This observation, together with the frequent reports of MPM familial clustering (Bianchi et al., 2004; Ascoli et al., 2007; Ugolini et al., 2008; Testa et al., 2011; de Klerk et al., 2013), suggests a role for genetic susceptibility in this disease.

In an attempt to identify the genetic risk factors that contribute to development of MPM, a number of studies analyzed the polymorphic genes that are active during various steps of xenobiotic and oxidative metabolism (Hirvonen et al., 1995, 1996; London et al., 1995; Neri et al., 2005, 2006, 2008; Landi et al., 2007; Gemignani et al., 2009; Betti et al., 2009; Murakami et al., 2012), in DNA repair processes (single-nucleotide polymorphisms [SNPs] in XRCC1 and XRCC3) (Dianzani et al., 2006; Gemignani et al., 2009), and in interleukin (IL)-1ß–driven inflammatory responses (inflammasome complex) (Girardelli et al., 2012; Borelli et al., 2015). Among the genetic polymorphisms associated with enhanced risk of MPM, only GSTM1 (glutathione *S*-transferase Mu 1, *GSTM1*) null genotype and two variant alleles of x-ray repair complementing defective repair in Chinese hamster cells 1, *XRCC1* and *XRCC3*, displayed increased risks in a subset of studies (Neri et al., 2008). Despite the widely recognized role of Fe in cellular asbestos-induced pulmonary toxicity, the contribution of Fe-metabolism gene polymorphisms to the etiology of mesothelioma has not apparently been evaluated thus far.

Recent advances in high-throughput techniques have made it possible to genotype a large number of SNPs and perform exploratory studies at whole-genome level, generally known as genome-wide association studies (GWAS), but thus far, no known genetic variants in Fe metabolism genes have been found to be associated with enhanced risk of mesothelioma (Matullo et al., 2013; Cadby et al., 2013). The aim of this study was to investigate the association between Fe-metabolism gene variants and risk of developing MPM in a population-based autopsy pilot study.

Materials and methods

Population-based autopsy study

This research was conducted in the Monfalcone (northeastern Italy), a small industrial town with large shipyards. This town is characterized by high incidence of asbestos-related mesothelioma (Bianchi et al., 1993) and by high prevalence of pleural plaques in the necropsy population (Bianchi et al., 1991). The autopsies were performed between 1980 and 2000 at the Hospital of Monfalcone and reviewed for the presence of MPM as well as objective signs of asbestos exposure, such as presence of pleural plaques and lung asbestos body content, and age at the time of death. The presence of pleural plaques, total area of which bears a direct relationship with intensity of asbestos exposure (Bianchi et al, 1991), was determined at necropsy by careful examination of the thoracic cavities for asymptomatic focal thickenings of the pleura, considered the hallmark of

asbestos exposure. Pleural plaques were classified into three groups: (1) small, (2) moderate, and (3) large, based on their size, as previously reported (Bianchi et al., 1997).

One way of assessing past exposure to asbestos in tissue samples from mesothelioma patients is to determine lung burden of asbestos bodies (AB), qualitatively as presence of AB in routine lung sections obtained at necropsy and/or quantitatively. The quantification of AB was performed after chemical digestion of lung tissue using the Smith–Naylor method (Smith and Naylor, 1972) and was expressed as number of AB per gram dry lung tissue (number AB/g). The diagnosis of malignant pleural mesothelioma was based on (or confirmed by) necropsy by histological examination.

Study population: subjects exposed to asbestos who died for MPM (Asbestos Exposed MPM = AEM)

Inclusion criteria were that subjects (n = 77) died from MPM (asbestosis free) with objective signs confirming asbestos exposure: pleural plaques and/or presence of AB in routine lung sections. For most subjects (94.8%), quantification of lung AB and a documented occupational history (78%) of asbestos exposure were also available.

Reference population: individuals exposed to asbestos who died of other causes (Asbestos Exposed Non-MPM = AENM)

Exposed subjects with the highest MPM-free follow-up were selected as controls in this study. Inclusion criteria were subjects (n = 48) with objective signs confirming asbestos exposure (presence of AB in routine lung sections and pleural plaques class 2 or above), who did not develop pleural or peritoneal malignant mesothelioma, lung carcinoma, or lung asbestosis, and died of other causes after the age of 75 years. The 75 years cutoff in the reference population was selected on the basis of previously reported data on latency periods (defined as the time interval between first exposure to asbestos and death), types of occupational exposure, and age at time of death, obtained from a series of MPM from the same geographic area (Bianchi et al., 2007). This study was approved by the ethics committee of the Hospital of Monfalcone.

Healthy subjects population

Healthy blood donor subjects (n = 190) from the same geographic area of AEM and AENM (European-Caucasian from Friuli Venezia Giulia region, 127 males and 63 females, median age ± SD = 45 ± 9.6, range = 20–67 years) were enrolled at the Centro Trasfusionale of Maggiore Hospital, Trieste, Italy. Informed consent was obtained from all participants in the study, and the project was approved by the IRCCS Burlo Garofolo Ethical Committee (CIB prot. L.-1055 n. 118/10). DNA was extracted from whole blood using the "salting out" procedure, following standard protocols (Miller et al, 1988).

Postmortem samples

Histological specimens from all autopsy cases were stored in the archives of the Department of Pathological Anatomy of the Hospital of Monfalcone. Genomic DNA of study and reference samples was extracted from formalin-fixed and paraffin-embedded (FFPE) archived autopsy heart tissue slices as previously described (Borelli et al., 2015).

The myocardial tissue of all autopsy cases was always free of neoplastic cells and selected to avoid bias due to somatic alterations in tumor tissue, such as loss of heterozygosity. At the time of DNA extraction, tissue blocks ranged in age from 15 to 35 years, with a mean age ± SE of 25.7 ± 0.5 years. Samples were all fixed in 10% buffered formalin but the exact fixation times and conditions are not known. Forty to 50 slices with a thickness of 5–7 μm were cut from the same paraffin block. A new blade was used for each patient sample to avoid cross-contamination. Gloves were worn at all times.

DNA extraction from FFPE-tissue samples

Genomic DNA was extracted using the QIAsymphony DSP DNA mini kit (QIAGEN,

Hilden Germania) and eluted in 50 µl TE buffer. Quantitative and qualitative evaluation of extracted DNA was performed using NanoDrop ND-1000 (Thermo Scientific, Wilmington, DE) and agarose gel electrophoresis. DNA was subsequently diluted to the final concentration of 50 ng/µl.

Genetic analysis in AEM and AENM

Eighty-six SNPs (cSNPs and Tag SNPs) selected were screened using the SNP Browser 4.0 software (Life Technology, Foster City CA), able to find optimal sets of SNP for association studies by visualizing SNP in their genomic context using linkage disequilibrium maps and putative haplotype blocks. The selected SNP are located in 10 genes of Fe metabolism, namely, Scavenger Receptor Class A, Member 5 (*SCARA5*), cytochrome *b* reductase 1 (*CYBRD1*), solute carrier family 11 (proton-coupled divalent metal ion transporter), member 2 (*SLC11A2*), solute carrier family 40 (Fe-regulated transporter) member 1 (*SLC40A1*), hepcidin antimicrobial peptide (*HAMP*), hephaestin (*HEPH*), transferrin (*TF*), transferrin receptor (TFRC) ferritin light polypeptide (*FTL*), and ferritin heavy polypeptide 1 (*FTH1*). Table 1 shows the gene and SNP list. Genetic analysis was performed using the Veracode Chips and Bead Array technology on the iScan system (Illumina San Diego, CA), following the manufacturer protocols.

HEPH, TF *and* FTH1 *Polymorphisms analysis in healthy blood donors*

The polymorphisms were genotyped using fluorogenic TaqMan SNPs genotyping assays and TaqMan Genotyping Master Mix on an ABI7900HT Fast Real-Time PCR (polymerase chain reaction) instrument (Applied Biosystems–Life Technologies, Carlsbad, CA) following manufacturer instructions. *HEPH* rs3747359 and *TF* rs2715631 polymorphisms were analyzed using predeveloped SNP genotyping assays (C__27476246_20 and C____148065_20, respectively); in the case of *FTH1* rs76059597 polymorphism a custom TaqMan SNP genotyping assay was developed (Life Technologies, Foster City, CA). Allelic discrimination was done both manually and automatically with the SDS detection software version 2.1 (Life Technologies, Foster City, CA).

In silico analysis with bioinformatic tools

A number of computational methods have been developed based on evolutionary principles to predict the effect of coding variants on protein function, including SNPnexus (http://snp-nexus.org), Polyphen (http://genetics.bwh.harvard.edu/pph2), and SITF (http://sift.jcvi.org/) software packages. The 1000 Genomes database (www.1000genomes.org) was used to retrieve *FTH1* (rs76059597), *TF* (rs2715631), and *HEPH* (rs3747359) frequencies in the European population.

Statistical analysis

Adherence was to the Hardy–Weinberg equilibrium for both AEM and AENM groups. Allelic and genotypic differences between the groups were assessed using chi-squared test with Yates's continuity correction, whereas odds ratio (OR) and 95% confidence intervals (95% CI) were estimated using Woolf's method. These calculations were performed for different genetic models as described in Lewis and Knight (2012). All calculations were conducted using a Microsoft Excel spreadsheet with a *p*-value threshold of .0167 to determine statistical significance after Bonferroni's correction.

Results

Study and control populations

Analyses were carried on autopsy samples from selected subjects with confirmed asbestos exposure who died of MPM (AEM, $n = 77$), or for individuals also exposed to asbestos but who died from other causes not asbestos related (AENM, $n = 48$).The AEM and AENM main characteristics are summarized in Table 2. The most evident difference is the higher proportion of males between both groups. The AEM group comprised 66 men and 11 women aged between 44 and 87 years (mean age ± SE: 69.5 ± 1.2 years). Pleural

Table 1. Selected Polymorphisms of Genes Involved in the Iron Metabolism.

Accession number	Gene_symbol	rs_ID	cSNP/Tag SNP	MAF	Chromosome	Position*
NM_173833.4	SCARA5	rs2726986	Tag SNP	0.47	8	27763607
		rs11993701	Tag SNP	0.45	8	27744762
		rs870819	Tag SNP	0.24	8	27761130
		rs11774579	Tag SNP	0.24	8	27740449
		rs2280931	Tag SNP	0.22	8	27728042
		rs13281095	Tag SNP	0.28	8	27742959
		rs4732617	Tag SNP	0.18	8	27829251
		rs2726975	Tag SNP	0.44	8	27756254
		rs2726940	Tag SNP	0.17	8	27823868
		rs2726959	Tag SNP	0.20	8	27749344
		rs17390967	Tag SNP	0.23	8	27758750
		rs2685390	Tag SNP	0.33	8	27850161
		rs1001866	Tag SNP	0.49	8	27836417
		rs4582511	Tag SNP	0.25	8	27748041
		rs2685325	Tag SNP	0.23	8	27775117
		rs17393091	Tag SNP	0.18	8	27825560
		rs6558023	Tag SNP	0.40	8	27731779
		rs2685372	Tag SNP	0.46	8	27759126
		rs9650427	Tag SNP	0.41	8	27844622
		rs11780951	Tag SNP	0.16	8	27840616
		rs11786962	Tag SNP	0.23	8	27839246
		rs17058207	cSNP	0.11	8	27767231
		rs17058374	cSNP	<0.01	8	27824039
NM_018474.3	NCRNA00153	rs6137287	Tag SNP	0.20	20	21180259
NM_024843.3	CYBRD1	rs10455	Tag SNP	0.26	2	172411273
		rs1047255	Tag SNP	0.13	2	172412008
		rs7585194	Tag SNP	0.34	2	172412616
		rs11684782	Tag SNP	0.40	2	172405989
		rs6734372	Tag SNP	0.22	2	172388804
		rs12692966	Tag SNP	0.48	2	172395221
		rs17554	Tag SNP	0.23	2	172403369
		rs6759240	Tag SNP	0.37	2	172380798
		rs960748	Tag SNP	0.5	2	172379936
NM_000617.2	SLC11A2	rs224575	Tag SNP	0.42	12	51419621
		rs224589	Tag SNP	0.26	12	51399050
NM_014585.5	SLC40A1	rs994227	Tag SNP	0.29	2	190432467
		rs1123110	Tag SNP	0.42	2	190444199
		rs2304704	cSNP	0.45	2	190430177
NM_021175.2	HAMP	rs75448148	Tag SNP	0.04	19	35771623
		rs75714555	Tag SNP	0.01	19	35771728
		rs10421768	Tag SNP	0.16	19	35772899
		rs66868858	Tag SNP	0.20	19	35771850
		rs10414846	Tag SNP	0.16	19	35772471
		rs74887182	Tag SNP	<0.01	19	35773676
		rs12608868	Tag SNP	0	19	35774097
		rs8101606	Tag SNP	0.16	19	35774556
NM_139011.2	HFE	rs2071303	Tag SNP	0.36	6	26091336
		rs2794719	Tag SNP	0.40	6	26088890
		rs1572982	Tag SNP	0.45	6	26094367
NM_000096.3	CP	rs16861593	Tag SNP	0.22	3	148899389
		rs1523513	Tag SNP	0.44	3	148910521
		rs11709714	Tag SNP	0.39	3	148931980
		rs772908	Tag SNP	0.19	3	148923656
		rs7652826	Tag SNP	0.47	3	148938950
		rs16861582	Tag SNP	0.32	3	148896430
		rs701755	Tag SNP	0.12	3	148902714
NM_138737.2	HEPH	rs5964499	Tag SNP	0.28	X	65411527
		rs3747359	cSNP	<0.01	X	65417725
		rs5919015	Tag SNP	0.25	X	65382685
NM_001063.2	TF	rs381165	Tag SNP	<0.01	3	133476852
		rs2715631	Tag SNP	0.24	3	133482889
		rs8177224	Tag SNP	0.33	3	133474003
		rs1880669	Tag SNP	0.50	3	133483696
		rs8177213	Tag SNP	0.32	3	133472227

(*Continued*)

Table 1. (Continued).

Accession number	Gene_symbol	rs_ID	cSNP/Tag SNP	MAF	Chromosome	Position*
NM_003234.2	TFRC	rs13072608	Tag SNP	0.36	3	195800267
		rs714602	Tag SNP	0.38	3	195793979
		rs9855973	Tag SNP	0.2	3	195794788
		rs3817672	cSNP	0.42	3	195800811
		rs4927866	Tag SNP	0.2	3	195796049
		rs41295879	cSNP	0.01	3	195791240
		rs9852079	cSNP	<0.01	3	195792423
		rs41301381	cSNP	<0.01	3	195798320
NM_013240.3	N6AMT1	rs1153266	Tag SNP	0.40	21	30011018
NM_138765.3	FTL	rs73577717	Tag SNP	0.04	19	49467998
		rs77396842	Tag SNP	0.01	19	49469412
		rs55945473	Tag SNP	0.08	19	49470200
		rs77100029	Tag SNP	<0.01	19	49470425
NM_002032.2	FTH1	rs1801621	Tag SNP	0.058	11	61731727
		rs75281081	Tag SNP	0.08	11	61731880
		rs17156609	Tag SNP	0.017	11	61731977
		rs12270214	Tag SNP	<0.01	11	61732598
		rs77202794	Tag SNP	0.02	11	61733250
		rs76059597	Tag SNP	0.01	11	61733349
		rs76178964	Tag SNP	0.02	11	61733894
		rs195154	Tag SNP	0.008	11	61734028
		rs2073588	Tag SNP	0.058	11	61736411

*Position based on Ensembl_75 (GRCh37.p13) database.

Table 2. Characteristics of Selected Malignant Pleural Mesothelioma (MPM) Cases and Controls in a Necropsy Series, Monfalcone Area, 1980–2000.

	Study group (AEM)	Controls (AENM)
Gender		
Men	66	45
Women	11	3
Age at death, years (mean ± std. error)	69.5±1.2	80.1±0.6
Pleural plaques		
Absent	10	0
Class1	15	0
Class2	24	27
Class3	28	21
Lung asbestos bodies counts (number/g dry lung tissue)		
0–999	13	0
1000–9999	22	0
10,000–99,999	28	21
100,000–1,000,000	10	13
Not available	4	14
Total	77	48

plaques were not observed in 10 out of 77 cases, all routine lung sections were positive for AB, and AB isolated from the lungs, in almost all cases (73 out of 77), ranged between 100 and 876,000 AB/g, in agreement with previously reported data (Bianchi et al., 1993). For most subjects (78%) of the AEM group, occupational data were available and confirmed occupational asbestos exposure. A majority of male patients worked in shipbuilding (61%), while female patients had histories of asbestos exposure at home, through laundering work clothes of family members employed in workplaces that were contaminated by asbestos.

The AENM group comprised 45 men and 3 women aged between 75 and 88 years (mean age ± SE: 80.1 ± 0.6 years) who died of causes other than asbestos-related diseases (15 neoplastic, 33 nonneoplastic). The presence of AB in routine lung sections and pleural plaques of class 2 or above was an inclusion criterion. Asbestos bodies isolated from lungs in most cases (34 out of 48 cases) ranged between 16,000 and 994,000 AB/g, well above the 1000-AB/g threshold for establishing occupational exposure (Casali et al., 2015). On this basis, and even if data confirming occupational exposure were available only for 23 out of 48 cases, data indicate that the subjects of the AENM group were older with heavier asbestos exposure than AEM subjects, which suggests a type of "asbestos-resistant population," suitable for comparison with AEM.

Genetic analysis in study and reference populations

Among the 86 SNP localized in genes involved in Fe metabolism and analyzed in the context of

increased mesothelioma risk, 3 SNP were identified localized in *FTH1* (rs76059597), *TF* (rs2715631), and *HEPH* (rs3747359) genes, whose frequencies were distributed differently in the 77 subjects exposed to asbestos who died of MPM (AEM) and in the 48 individuals exposed to asbestos who died of other causes (AENM); allele and genotype frequencies were in Hardy–Weinberg equilibrium (HWE) in AEM but not AENM, as summarized in Tables 3, 4, and 5.

The rs76059597 SNP (Table 3) is located in an intronic region of the *FTH1* gene. This gene encodes the heavy subunit of ferritin, involved in the storage of Fe in a soluble and nontoxic state, which is notably the main component of AB coating (Borelli et al., 2007). The presence of the C allele conferred protection against development of mesothelioma, as it was significantly more frequent in AENM than in AEM subjects. The frequency of the C allele in AENM recorded in this

Table 3. Distribution on Allelic and Genotype Frequencies for the Three *FTH1* (rs76059597) SNPs in Subjects Exposed to Asbestos Who Died of MPM (AEM) and Individuals Exposed to Asbestos Who Didn't Develop MPM and Died After Age 75 Years of Other Causes (AENM).

FTH1 rs76059597	AEM (77)	AENM (48)	OR (CI 95%)	*p* Value	Healthy controls (190)	1000 Genomes EUR TSI allele frequencies
T	138 (0.89)	43 (0.45)	Ref.		366 (0.96)	0.97
C	16 (0.11)	53 (0.55)	0.09 (0.05–0.18)	3.9×10^{-14}*	14 (0.04)	0.03
Co-dominant genetic model						
T/T	62	18	Ref.	6.7×10^{-10}*	176	
T/C	14	7	0.58 (0.2–1.66)	0.46	14	
C/C	1	23	0.01 (0–0.1)	5.3×10^{-10}*	0	
Dominant genetic model						
T/T	62	18	Ref.			
T/C + C/C	15	30	0.15 (0.06–0.33)	2.8×10^{-6}*		
Recessive genetic model						
T/T + T/C	76	25	Ref.			
C/C	1	23	0.01 (0–0.11)	5.6×10^{-10}*		
Over-dominant genetic model						
T/T + C/C	63	41	Ref.			
T/C	14	7	1.3 (0.48–3.5)	0.78		

Note. Healthy controls (blood donors) and 1000 Genomes allele frequencies (for European Italian Toscani, EUR TSI) are also reported. Asterisk indicates statistically significant; OR, odds ratio; CI, confidence interval; Ref, reference allele or genotype (the most frequent in the population analyzed).

Table 4. Distribution on Allelic and Genotype Frequencies for the *TF* (rs2715631) SNP in Subjects Exposed to Asbestos Who Died of MPM (AEM) and Individuals Exposed to Asbestos Who Didn't Develop MPM and Died After Age 75 Years of Other Causes (AENM).

TF rs2715631	AEM (77)	AENM (48)	OR (CI 95%)	*p* Value	Healthy controls (190)	1000 Genomes EUR TSI allele frequencies
T	117 (0.76)	45 (0.47)	Ref.		286 (0.75)	0.77
G	37 (0.24)	51 (0.53)	0.28 (0.16–0.48)	5.4×10^{-6}*	94 (0.25)	0.23
Co-dominant genetic model						
T/T	46	14	Ref.	1.2×10^{-4}*	108	
T/G	25	17	0.45 (0,19–1,06)	0.1	70	
G/G	6	17	0.11 (0.04–0.32)	6.1×10^{-5}*	12	
Dominant genetic model						
T/T	46	14	Ref.			
T/G + G/G	31	34	0.28 (0.13–0.6)	1.7×10^{-3} *		
Recessive genetic model						
T/T + T/G	71	31	Ref.			
G/G	6	17	0.15 (0.06–0.43)	2.7×10^{-4} *		
Over-dominant genetic model						
T/T + G/G	52	31	Ref.			
T/G	25	17	0.88 (0.41–1.87)	0.88		

Note. Healthy controls (blood donors) and 1000 Genomes allele frequencies (for European Italian Toscani, EUR TSI) are also reported. Asterisk indicates statistically significant; OR, odds ratio; CI, confidence interval; Ref, reference allele or genotype (the most frequent in the population analyzed).

Table 5. Distribution on Allelic and Genotype Frequencies for the *HEPH* (rs3747359) SNP in Subjects Exposed to Asbestos Who Died of MPM (AEM) and Individuals Exposed to Asbestos Who Didn't Develop MPM and Died After Age 75 Years of Other Causes (AENM).

HEPH rs3747359	AEM (77)	AENM (48)	OR (CI 95%)	*p* Value	Healthy controls (190)	1000 Genomes EUR TSI\ allele frequencies
G	152 (0.99)	61 (0.63)	Ref.		380 (1)	1
C	2 (0.01)	35 (0.37)	0.02 (0.01–0.1)	1.1×10^{-13}*	0 (0)	0
Co-dominant genetic model						
G/G	75	25	Ref.	4.2×10^{-9}*	190	
C/G	2	11		5.7×10^{-5}*	0	
C/C	0	12	0.06 (0.01–0.29)	9.8×10^{-6}*	0	
Dominant genetic model						
G/G	75	25	Ref.			
C/G + C/C	2	23	0.03 (0.01–0.13)	3.01×10^{-9}*		
Recessive genetic model						
G/G + C/G	77	36	Ref.			
C/C	0	12		6.9×10^{-5}*		
Over-dominant genetic model						
G/G + C/C	75	37	Ref.			
C/G	2	11	0.09 (0.02–0.43)	9.05×10^{-4}*		

Note. Healthy controls (blood donors) and 1000 Genomes allele frequencies (for European Italian Toscani, EUR TSI) are also reported. Asterisk indicates statistically significant; OR, odds ratio; CI, confidence interval; Ref, reference allele or genotype (the most frequent in the population analyzed).

study is higher than the one reported in the 1000 Genome project for the European Italian (EUR TSI, Italian Toscani) population (AENM 0.55 vs 0.03 EUR TSI). When examining genotypes distribution, the CC genotype was significantly associated with protection against MPM, in accordance with a recessive genetic model. A significant difference was also observed between AEM and AENM in accordance with codominant CC genotype and dominant T/C + C/C genotypes.

The SNP rs2715631 (Table 4) is situated in an intronic region of the *TF* gene. This gene encodes transferrin, a glycoprotein involved in Fe transport from intestine, reticulo-endothelial system, and liver parenchymal cells to all proliferating cells in the body.

The presence of the G allele conferred protection against mesothelioma, with the G allele being significantly more frequent in AENM compared to AEM. In addition, in this case, the frequency of the G allele in AENM was higher than the one reported in the 1000 Genome project for the European Italian (EUR TSI, Italian Toscani) population (AENM 0.53 vs 0.23 EUR TSI). Similarly, the GG genotype association with protection against MPM was consistent with a codominant GG genotype genetic model. Significant differences within AEM and AENM were also obtained when considering recessive GG genotype and dominant T/G + G/G genetic models.

The SNP rs3747359 (Table 5) is a coding SNP (cSNP) with a minimum allele frequency (MAF) <0.01 as reported in the 1000 genomes data of the gene *HEPH*, encoding hephaestin, a multi-copper oxidase protein family involved in the transport of dietary Fe from epithelial cells into the circulatory system. The presence of the C allele is associated with protection against development of mesothelioma. The *HEPH* C/G and C/C genotype also conferred protection against MPM, following a dominant genetic model. Significant differences in the distribution of rs3747359 genotypes between AEM and AENM were observed considering recessive CC genotype and codominant CC genotype genetic models.

In order to evaluate in silico the potential functional effects of three SNP associated with protection against developing MPM, SNP Nexus was used to analyze the two *FTH1* rs76059597 and *TF* rs2715631 intronic polymorphisms; Polyphen and SITF software packages were employed for the analysis of the HEPH rs3747359 cSNP. SNP Nexus analysis revealed no apparent regulatory element for *FTH1* rs76059597 and *TF* rs2715631 intronic polymorphisms. A possible damaging effect (SITF score 0.01 and Polyphen score 0.708) was observed for the *HEPH* rs3747359 missense variant. In order to evaluate the possible effect of the number of AB found in patients with AEM or AENM, our genetic results were corrected for this covariant but found no confounding effect (data not shown).

Genetic analysis in healthy blood donors

The three polymorphisms were also genotyped in 190 healthy blood donors from the same geographic area of AEM and AENM, also sharing with them the same ethnic background (European Caucasian): *FTH1* rs76059597 and *TF* rs2715631 were in Hardy–Weinberg equilibrium in the population assessed. The *HEPH* rs3747359 polymorphism was monomorphic for the G allele in all the samples determined; similarly, the *FTH1* rs76059597 polymorphism presented a high presence of T/T genotype (93%), and no C/C genotype was detected; and finally, for the *TF* rs2715631 polymorphism the T/T genotype (57%) was predominant, followed by heterozygous G/T genotype (37%) and homozygous G/G genotype (6%). The three polymorphisms frequencies were comparable to those reported in the 1000 Genomes project for European Italian Toscani (see Tables 3, 4, and 5 for the distribution of allele and genotype frequencies of *HEPH* rs3747359, *FTH1* rs76059597, and *TF* rs2715631 in the healthy blood donors).

Discussion

This study employed, for the first time, postmortem paraffin-embedded tissue samples to study genetic susceptibility to mesothelioma. In such diseases as MPM, rare and with a long latency period, a number of problems may occur, and the design of a genetic susceptibility investigation might be affected by several possible biases (Puntoni et al., 2003). A long follow-up period is necessary to have a statistically adequate number of subjects with MPM, and asbestos exposure may be objectively assessed by the presence of pleural plaques and lung AB content only postmortem.

Previous genetic studies were affected by various sources of heterogeneity, such as remarkable differences in exposure assessment (Neri et al., 2008). For this reason a population-based autopsy study was designed in which exposure might be evaluated and quantitated by investigating objective signs of exposure (Bianchi et al., 2001). The low number of analyzed subjects is due to the restrictive enrollment criteria applied in order to possess a population study of individuals with similar exposure to asbestos who did or did not develop MPM. The measures employed to restrict as much as possible the biases related to the selection of control population make our study unique.

Formalin-fixed and paraffin-embedded (FFPE) specimens, stored in hospital archives for years or even decades, are an invaluable source of diagnostic material that may be used for genomic analysis and translational studies. Historically, FFPE samples were not considered a viable source for molecular analysis as nucleic acids may be markedly modified by protein–nucleic acid and protein–protein cross linking. However, in recent years, methods and protocols for extraction of nucleic acids (DNA, mRNA, miRNA) and proteins from FFPE tissue samples have improved enormously (Klopfleisch et al., 2011), and single nucleotide polymorphisms (SNPs) may now be assayed using DNA isolated from archival FFPE samples.

Genetic analysis was performed on AEM and AENM to identify variations possibly associated with susceptibility to develop MPM. Significant information was attained for 3 out of the 86 analyzed SNPs located in genes involved in Fe metabolism. The role of Fe-metabolism gene polymorphisms in etiology of mesothelioma has not apparently been previously evaluated, and *FTH1* rs76059597, *TF* rs2715631 SNPs, and *HEPH* rs3747359 variations were not reported in GWAS studies performed on patients with mesothelioma (Matullo et al., 2013; Cadby et al., 2013).

FTH1 rs76059597 and *TF* rs2715631 SNPs are intronic polymorphisms, without any known functional significance, either predicted in silico or reported in the literature. Based on our present findings, one can only consider them as being associated with a marker of protection from MPM. However, as reported in the results, the frequencies of *FTH1* C and *TF* G alleles are higher in AENM individuals compared not only to AEM but also to the general EUC TSI population, as reported in 1000 Genomes data, as well as in European Caucasian healthy blood donors from the same geographic area of AEM and AENM (Friuli Venezia Giulia). Although through a mechanism that has yet to be established, one might postulate a true protective effect against MPM for rs76059597 and rs2715631 SNPs, since

only in AENM were the *FTH1* C and *TF* G alleles markedly more frequent. This higher frequency might justify lack of HWE in AENM.

Resequencing analysis of both genes and in vitro evaluation of the genetic variations might confirm or refute the role of FTH1 and TF in the pathogenesis of mesothelioma.

The *HEPH* rs3747359 missense variant was significantly associated with protection from MPM, with the C allele and CC genotype being more frequent in AENM than AEM. In silico analysis with Polyphen and SIFT software predicted possibly damaging and deleterious effects, respectively. This SNP induces the Asp/His aminoacidic nonsynonymous substitution at position 601 of the HEPH protein. At present no genomic database or literature information is available for the *HEPH* rs3747359 missense variant, which previously was not identified with clinical phenotypes. Even if further functional analyses are required to better understand the role of this variation, it is interesting to note that, despite being extremely rare, with a global MAF of 0.00197, it was frequently detected only in AENM. Therefore it is conceivable that *HEPH* rs3747359 may play a role in protection from MPM. The higher frequency of the C allele in AENM, compared to both AEM and the general EUR TSI population as well as to the healthy blood donors from Friuli Venezia Giulia, again may account for deviation from HWE.

Hephaestin knockout mice (HEPH KO) showed significant Fe deficiency in liver and spleen, while an accumulation of Fe was found in various brain regions (Jiang et al., 2015). To the best of our knowledge, the effect of mutations in the HEPH gene in humans has not apparently been reported yet. Further, *FTH1, TF*, and *HEPH* polymorphisms are reported for the first time correlated with protection from developing MPM.

The failure of GWAS studies to detect these associations may be ascribed to the AENM population used in these studies and to how asbestos exposure was assessed. In GWAS studies, the control populations are members of general population cohort studies or healthy subjects or patients hospitalized for nonneoplastic/nonrespiratory conditions (Matullo et al., 2013), or in a cohort of asbestos-exposed individuals with a malignancy-free follow-up of over 21 years (Cadby et al., 2013), which may not be sufficient with a latency period for MPM of 30–40 years (Bianchi et al., 1997). Further, in these studies asbestos exposure was always assessed on the basis of individual occupational history only.

The main limitation of our genetic study is represented by the low number of enrolled cases, due to the extremely restrictive sample inclusion criteria of the study subjects. This selection was the result of the choice to create an "asbestos-resistant exposed population" sample that might be suitably compared with the MPM group. Based on the findings obtained from this highly selected population of asbestos-resistant exposed subjects (AENM), it is possible that an involvement of the *HEPH* rs3747359 variation serves as a protective factor against development of MPM. There are some indications regarding the possible role of FTH1 and TF in the modulation of susceptibility to MPM, but these observations need to be further investigated.

The availability of genetic markers of individual susceptibility might help identify subjects who are at higher risk of developing MPM within an asbestos-exposed population, and to implement new strategies of primary and secondary prevention. Finally, this pilot study provides new and valuable insight into the mechanisms of Fe homeostasis in asbestos-related diseases, emphasizing the importance of studying Fe metabolism for the development of new therapeutic approaches (Toyokuni, 2011; Chew and Toyokuni, 2015).

In conclusion, a significant association was found between selected Fe-metabolism associated genes and susceptibility to develop MPM. It is not possible to describe a functional scenario, since there are no predicted "in silico" role for *FTH* and *TF* SNP, and little is known regarding the role of hephaestin in the airways. However, in addition to confirming our findings on a larger number of autoptic samples, it is planned to start a prospectic genetic analysis on a population of living (presently healthy) heavily asbestos-exposed subjects, for the purpose of assessing, based on the findings reported in this study, their risk of developing MPM.

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