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Abstract

Occupational and environmental asbestos exposure is the main determinant of malignant pleural mesothelioma (MPM), however, the mechanisms by which its fibres contribute to cell toxicity and transformation are not completely clear. Aberrant DNA methylation is a common event in cancer but epigenetic modifications involved specifically in MPM carcinogenesis need to be better clarified.

To investigate asbestos-induced DNA methylation and gene expression changes, we treated Met5A mesothelial cells with different concentrations of crocidolite and chrysotile asbestos (0.5 ÷ 5.0 μg/cm^2, 72 hours incubation).

Overall, we observed 243 and 302 differentially methylated CpGs (≥10%) between the asbestos dose at 5 μg/cm^2 and untreated control, in chrysotile and crocidolite treatment, respectively. To examine the dose-response effect, Spearman's correlation test was performed and significant CpGs located in genes involved in migration/cell adhesion processes were identified in both treatments.

Moreover, we found that both crocidolite and chrysotile exposure induced a significant up-regulation of *CA9* and *SRGN* (log-2 fold change > 1.5), previously reported as associated with a more aggressive MPM phenotype.

However, we found no correlation between methylation and gene expression changes, except for a moderate significant inverse correlation at the promoter region of *DKK1* (Spearman rho=-1, P-value=0.02) after chrysotile exposure.

These results describe for the first time the relationship between DNA methylation modifications and asbestos exposure. Our findings provide a basis to further explore and validate asbestos-induced DNA methylation changes, that could influence MPM carcinogenesis and possibly identifying new chemopreventive target.

Introduction

Asbestos refers to a class of six different silicate fibrous minerals that occur naturally in the environment, and are classified by the International Agency for Research on Cancer (IARC) as "carcinogenic to humans (Group 1)" (Arsenic, metals, fibres, and dusts 2012).

Exposure to asbestos can cause lung benign diseases (pleural plaques and thickening), or tumour, including lung cancer and mesothelioma (Mirabelli et al. 2008; Pistolesi and Rusthoven 2004; Sen 2015). The risk in term of health is related to both fibre types and inhaled dose. Amphibole asbestos (amosite and crocidolite), that is longer and insoluble within the range of pH encountered in an organism, appears to be more carcinogenic and mutagenic than chrysotile (Kagan 2013).

The pathogenic mechanisms by which asbestos contributes to the development of malignant pleural mesothelioma (MPM) have long been studied but still they remain incompletely characterized. Several studies demonstrated both clastogenic and cytotoxic effects of asbestos fibres (Msiska et al. 2010; Vaslet et al. 2002). Phagocytosis of fibres by macrophages and oxidoreduction reactions on fibre surfaces are known to generate genotoxic reactive oxygen species (ROS) which in turn result in DNA damage and oxidative stress within cell, leading to genetic alterations in MPM (Burmeister et al. 2004; Pietruska et al. 2010).

Crocidolite is the most pathogenic type of asbestos in the causation of human mesothelioma. For this reason, a large number of studies investigated its toxicity on mesothelial cells and profiled gene expression after exposure in benign and neoplastic cells (Shukla et al. 2009; Singhal et al. 2003). On the other hand, chrysotile accounts for approximately 95% of all asbestos used worldwide and, although it has a less carcinogenic capacity, several studies clearly demonstrated that chrysotile exposure can cause mesothelioma (Tweedale 2002). *In vivo* and *in vitro* studies demonstrated that both chrysotile and crocidolite induce oxidative stress and the production of local inflammatory mediators such as cytokines and growth factors. This leads to a reactive microenvironment characterized by inflammation and proliferation of mesothelial cells ultimately inducing MPM or other asbestos-related diseases (Nymark et al. 2007; Trevisan et al. 2016). Mesothelial cells exposed to crocidolite were reported to have an extensive altered expression of genes involved in integrin-mediated signalling pathways, DNA damage repair, and cell cycle regulation (Acencio et al. 2015; Wang et al. 2011). Although it is widely acknowledged that fibrous geometry, surface and chemical composition, as well as durability, are important features in the development of asbestos-associated diseases (Boulanger et al. 2014), it is still unclear how these factors might contribute to cell toxicity and transformation.

Genomic alterations are clearly associated with MPM oncogenesis (Sekido 2008) but also epigenetic changes can play an important role in cancer development (Das and Singal 2004). Several studies demonstrated that epigenetic changes are significant contributors of MPM development and indicated a significant association between promoter methylation of tumour suppressor genes and asbestos exposure (Vandermeers et al. 2013). However, it remains unclear whether a dose response exists between exposure and methylation changes extent.

This study was conducted to investigate how DNA methylation and whole gene expression patterns are altered when MeT5A human mesothelial cells are exposed *in vitro* to crocidolite and chrysotile asbestos at different doses.

Methods

Cell cultures

MeT5A commercial cell line (SV-40 transformed human non-malignant mesothelial cell line, ATCC CRL-9444) was authenticated by microsatellite analysis using the PowerPlex kit (Promega Corporation, Madison, WI;). MeT5A cells were cultured in RPMI 1640 medium (Gibco-Thermo Fisher Scientific, MA, USA) supplemented with 10% foetal

bovine serum (FBS) (Gibco) and 1% penicillin/streptomycin (Gibco) and maintained at 37°C in a humidified atmosphere containing 5% CO_2.

Crocidolite and chrysotile treatments

Semiconfluent cells (3.5×10^5) were plated in 150-mm–diameter Petri dishes. Twenty-four hours later, crocidolite or chrysotile asbestos fibres were added at different doses (0.5 µg/cm^2, 1.0 µg/cm^2, 2.5 µg/cm^2, 5.0 µg/cm^2) and cells were incubated at 37 °C, 5% CO_2 for 72 hours, which is an interval time that proved suitable to investigate asbestos-induced DNA damage (Burmeister et al. 2004; Mossman and Landesman 1983). Untreated cells were used as control and each experiment was performed in triplicate (Fig. 1).

Union International Contre le Cancer (UICC) crocidolite and chrysotile fibres were sonicated at 100 W for 30 s (Labsonic Sonicator; Sartorius Stedim Biotech S.A., Aubagne, France) to dissociate fibre bundles before addition to cell cultures, allowing better suspension in the culture medium. To keep constant the concentration of asbestos, medium was not changed during the incubation. After 72 hours, both adherent and floating cells were collected for DNA and RNA extraction.

DNA methylation array

Genomic DNA was extracted from Met5A cells using GenElute Mammalian Genomic DNA Miniprep Kit (Sigma Aldrich). DNA integrity was checked by standard electrophoresis on a 1% agarose gel in TBE 0.5X buffer. NanoDrop 8000 Spectrophotometer (Thermo Fisher Scientific Inc., MA, USA) was used to assess DNA purity and concentration. The EZ-96 DNA Methylation-Gold Kit (Zymo Research Corp., CA, USA) was utilized for the bisulphite conversion of 500 ng of genomic DNA for each sample, according to manufacturer's standard protocol. The HumanMethylation450 BeadChip (Illumina Inc., San Diego, CA, USA) was used to assess the methylation status of more than 485,000 individual CpG loci at a genome-wide resolution.

Gene expression array

Total RNA from asbestos-treated MeT5A cells was isolated by phenol/chloroform method (Trizol, Thermo Fisher Scientific Inc.) according to manufacturer's instructions. RNA was suspended in DNA nuclease-free water (Sigma Aldrich, Poole, UK) and its integrity and amount were determined by capillary electrophoresis (2100 Bioanalyzer, Agilent Technologies, Santa Clara, CA, USA). Gene expression arrays were performed using the HumanHT-12 v4 Expression BeadChip, targeting more than 31,000 annotated genes with more than 47,000 probes derived from the National Center for Biotechnology Information Reference Sequence (NCBI RefSeq Release 38; November 7, 2009).

Technical Validation by qPCR

RNA samples used in gene expression arrays were re-analysed by quantitative PCR (qPCR) for the technical validation of three selected genes (Carbonic anhydrase 9, *CA9*; Serglycin, *SRG*;, Dickkopf WNT Signaling Pathway Inhibitor 1 *DKK1*. See results section for details). One microgram of total RNA was used to synthesize complementary DNA, using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems), according to the manufacturer's protocol. The TaqMan Gene Expression Master Mix and TaqMan Gene Expression Assays (*DKK1*, Hs00183740_m1; *CA9*, Hs00154208_m1; *SRGN*, Hs01004159_m1, Applied Biosystems) were used for qPCR by 5' Nuclease assay on a 7900HT Real-Time PCR System (Applied Biosystems). Each sample was run in triplicate. The Abelson murine leukemia viral oncogene homolog 1 (*ABL1*; Hs00245443_m1) and hypoxanthine phosphoribosyltransferase 1 (*HPRT1*; Hs02800695_m1) genes were used as reference for relative quantitation.

All the qPCR experiments comply with the Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines (Bustin et al. 2009).

Statistical Analysis

Raw data from gene expression and DNA methylation arrays were quality-checked using respectively Lumi and Methylumi packages of the open source R statistical environment (version 3.1.0. 2014).

Gene expression levels and methylation levels were compared for each biological triplicate, and the outliers were filtered out with Grubbs' test. After outlier removal, mean values for each remaining triplet or pair measurements were used for the following analyses.

DNA Methylation: the average methylation value at each CpG locus, or average "beta value", was computed as the ratio of the intensity of the methylated signal over the total signal (unmethylated + methylated). Thus, beta values represent the percentage of methylation at each CpG locus, ranging from 0 to 1.

We took into account only the BeadChip probes with a detection P-value < 0.01 and with missing beta values in less than 20% of the samples. We included in our analysis only CpGs identified by probes that mapped uniquely to the genome, and with no genetic variation annotated at the CpG site or in the probe sequence.

Methylation changes between untreated control and highest dose treatment were computed as difference between mean beta values at each CpGs. Signals with at least delta-beta value $\geq|0.10|$ were considered differentially methylated and retained into the analyses, then the identified CpGs were tested for correlation among the 5 treatment points (different doses) with Spearman rank correlation.

Besides single CpG analysis, we investigated differentially methylated regions (DMRs) using DMRforpairs (Rijlaarsdam et al. 2014). This tool defines genomic regions using local probe density and quantifies differential methylation patterns between unique samples including pairwise comparison of samples.

Gene expression: differential expression analysis was performed using Limma package (R 3.1.0, lmFit and ebayes functions) in order to highlight those genes that were differentially expressed in cells treated with the maximum dosage of crocidolite or chrysotile compared to the untreated cells. Deregulated genes were considered for further analysis when FDR-adjusted P-value < 0.05, and the absolute value of the log fold change (log FC) > |1|.

To examine the association between DNA methylation and the changes in mRNA levels at the corresponding gene related to different asbestos doses, Spearman rank correlation was employed. Only correlations with statistically significant Spearman coefficient rho ± 1 and P-value < 0.05 were shown and further commented.

All statistical analyses were conducted using the open source software R (R 3.1.0).

Gene enrichment analysis of differentially methylated and differentially expressed genes

In order to identify asbestos-associated biological processes, a gene ontology (GO) functional enrichment analysis was performed for the differentially methylated and differentially expressed genes using the Biological Networks Gene Ontology tool (BiNGO, version 3.0.2) (Maere et al. 2005) in the open source bioinformatic platform Cytoscape 2.8.0 (http://www.cytoscape.org/) with a threshold of P-value <0.05.

Results

Effect of crocidolite and chrysotile on DNA methylation profiles

To evaluate the effect of crocidolite and chrysotile on genome-wide methylation we only considered CpG sites with methylation changes higher than 10% (delta-beta value $\geq|0.10|$) between the highest asbestos dose and untreated controls.

A graphic depiction of methylation changes between treated and untreated cells, for each type of asbestos, is provided as heatmap (Supplementary Figure 1).

Chrysotile. Overall, we identified 243 differentially methylated CpGs, with only few of them (N=5) showing marked changes (>20%) between the chrysotile dose at 5 µg/cm^2 and untreated control. The greatest differences took place in intergenic regions (Supplementary Table 1a).

The BiNGO tool was used for functional annotation clustering of the 201 genes corresponding to the 243 differentially methylated CpGs. The most represented ontologies were "Homophilic cell adhesion", "Cell-cell adhesion" and "Biological adhesion" (Table 1).

Spearman's correlation test was performed to examine the dose-response effect of chrysotile on DNA methylation profiles of the 243 differentially methylated CpGs among the 5 treatments at different doses. Four CpG sites corresponding to three genes (*TNR, PAX2, CRYL1*) showed a significant positive chrysotile dose-response effect on DNA methylation, while six CpGs, located in *LHX8*, *LRRC66*, *LMOD2*, *TMEM132D*, *BCAS3* genes, displayed a significant negative correlation with dose (Table 2).

Crocidolite. Overall, 302 differentially methylated sites were found in 214 genes. In this case, the most marked changes in methylation levels (>20%) were observed in gene-regions (Supplementary Table 1b), of the following genes: hypermethylation occurred at the promoter of *HBM*, *ZNF260*, *CBX6* genes and in the body of *SLAIN1* while hypomethylation has been observed for *LPIN1*, *SORBS1*, *EIF4A1*, *SENP3*, *LOC728743* and *BCL2* genes.

For eleven CpGs located in *LAMC1, MAGI3, UBE2E3, LAMB4, MAD1L1, NRXN2, GABRG3*, and *ACO2* genes, we observed a positive correlation (Spearman rho=1, P-value=0.02) between methylation changes and dose in the MeT5A cells exposed to increasing concentration of crocidolite. On the other hand, seven probes in *PPA2, PRKG1, ODZ4, APOH, ADCK4,* and *ITPKC* genes exhibited a negative correlation (Spearman rho=-1, P-value=0.02), (Table 3). Gene ontology analysis was used to highlight significant functions, but no biological process was found with BiNGO analysis.

Overall, the methylation sites modulated by asbestos exposure were different between the two treatments. However, 26 CpG sites were modified by both treatments, with 15 of them with the same direction change (Supplementary Figure 2).

No significant DMRs were identified using DMRforPairs when comparing the dose at 5 µg/cm^2 and untreated controls for both treatments.

Effect of crocidolite and chrysotile on gene expression profile

Comparative analysis of the asbestos effects on MeT5A revealed significant changes for 1,788 transcripts between the maximum chrysotile dose (5 µg/cm^2) and untreated cells, and 3994 transcripts in crocidolite-treated cells (P-value < 0.02) (data not shown). We further restricted the analysis to the up- and down-regulated genes with log 2 –fold change > |1| (Fig. 3a, 3b), that were investigated separately using BiNGO tool. Seventy genes up-regulated after chrysotile treatment (Supplementary Table 2) were significantly enriched in several biological processes, including "Response to chemical stimulus", "Response to temperature stimulus", "Developmental process", "Negative regulation of biological process", "Regulation of cell migration", "Regulation of cell communication", "Response to hypoxia" (Supplementary Table 3a). Forty-eight down-regulated genes after chrysotile treatment were enriched in only one GO term ("Response to virus") (Supplementary Table 3b).

In crocidolite treated cells, a total of 567 differentially expressed genes were found (log2 fold change ≥|1|, P-value<0.02), 190 of them up- regulated and 377 down-regulated (Supplementary Table 4). Although no biological

process has been enriched analysing the down-regulated genes, we recognized several genes involved in DNA damage response and cell cycle regulation, such as *ATM*, *RAD51*, *MBD4*, *GTSE1*, *CDKN2B*, *E4F1*, *EXO5*, *DDB1*, *PCMT1*, *INIP*, *N4BP2*, *XRCC2*. The ontological analysis of the genes up-regulated after crocidolite exposure showed that genes involved in metabolic and biosynthetic processes are highly represented (Supplementary Table 5).
Gene expression profiling identified nine genes that showed expression changes after both treatments. More specifically, *KCTD12*, *PRAGMIN*, *HNRNPU*, *TMEM106A*, *ZNF483* were down-regulated and *NEU1*, *SNORD3A*, *SRGN*, *CA9* were up-regulated in both treatments (Fig. 2).

Correlation between differentially methylated sites and gene expression of the corresponding gene
Spearman correlation was performed to assess the relationship between differentially methylated loci and the expression levels of related genes. Considering all differentially methylated loci after chrysotile exposure, a significant correlation with mRNA expression levels (P-value=0.02, Spearman rho=-1) emerged for only one CpG located at the transcriptional start site of *DKK1* gene. Although we observed a subtle decrease in DNA methylation between the highest dose of chrysotile and untreated control (0.1%), the change in expression levels was three times higher (Supplementary Table 2). Conversely, we did not observe any significant relationship between methylation and expression levels in crocidolite-treated cells.

Real-time PCR
We focused on *DKK1*, *CA9* and *SRGN* genes and validate their gene expression after asbestos exposure with a qPCR assay. The selection of these genes is explained as follow: 1) *DKK1* is the only gene for which the methylation levels of a promoter CpG correlated with expression levels after chrysotile treatment; 2) *CA9* and *SRGN* genes were altered in both asbestos treatments with a log2 fold change > 1.5.
Real-time PCR confirmed the results of microarray (Supplementary Table 6).

Discussion
In the present study, new genes and pathways potentially associated with asbestos exposure were identified after an integrated analysis of DNA methylation and gene expression profiles in MeT5A cell line treated with crocidolite and chrysotile.
DNA methylation is one of the most extensively studied epigenetic modification and has been reported to play a key role during mesothelial malignant transformation (Tomasetti et al. 2017). A growing body of evidence supported cellular epigenetic dysregulation as a critical mode of action for asbestos in the induction of MPM (Christensen et al. 2008). However, a genome-wide study on differentially methylated genes following asbestos exposure is lacking.
Overall, we observed slight changes of DNA methylation in MeT5A cells in both crocidolite and chrysotile treatments. Interestingly, most of the genes with differentially methylated loci after treatment have a function in the regulation of cellular matrix and adhesion, that represent direct mechanisms for mesothelial infiltration and injury, facilitating epithelial-to-mesenchymal transition (EMT) in MPM. This finding may suggest an involvement of methylation changes as potential modulators of asbestos-induced pleural injury.
After crocidolite treatment, the strongest hypermethylated single-CpG was in the promoter region of Haemoglobin Subunit Mu (HBM) gene. In previous studies, elevated levels of α- and β-Globin in cancer cells resulted to play a cytoprotective role against oxidative insults (Li et al. 2013). Even though we did not find a correlation between *HBM* promoter methylation and gene expression, we cannot rule out a possible role of HBM as a potential marker of asbestos exposure.

By analysing differentially methylated signals modulated in a dose-dependent manner by crocidolite exposure, we identified genes coding molecules involved in cell adhesion, such as Neurexin 2 (*NRXN2*), Laminin Subunit Gamma 1 (*LAMC1*) and Beta 4 (*LAMB4*) genes. The expression changes in cell adhesion-related molecules are known to be accompanied with EMT in MPM and the process of cell adhesion has been shown to be important both in the initial spreading and in the progression of the tumour (Kato et al. 2017).

Among differentially expressed genes, most of the biological processes enriched in crocidolite-treated cells included metabolic, cellular biosynthetic and translation processes, adding new potential pathways modulated by asbestos exposure with respect to those reported in previous microarray studies in MeT5A cells (Nymark et al. 2007). The results are compatible with neoplastic transformation of mesothelial cells, which need special metabolic requirements for their development, including hypoxic microenvironment (Dang and Semenza 1999).

An increased expression of adhesion molecules was previously described in endothelial cells after crocidolite and chrysotile exposure (Treadwell et al. 1996). Evidence showed that continuous exposure to chrysotile asbestos causes alterations of cytoskeletal molecules (Maeda et al. 2013).

Also in chrysotile treatment, functional annotation and clustering analysis of the differentially methylated genes highlighted an enrichment for genes involved in cell adhesion, such as in “Homophilic cell adhesion”, “Cell adhesion” and “Biological adhesion”. Even if we detected low correlations with gene expression changes, for the upregulated genes there was a significant enrichment for GO terms “Regulation of cell migration”, “Regulation of chemotaxis” and “Positive regulation of cell migration”, suggesting a chrysotile-induced cellular transformation mechanisms at both DNA methylation and gene expression levels.

Moreover, we observed a dose-response relationship with DNA methylation especially in genes involved in cell adhesion (*TNR*, *LRRC66*) and reorganization of the actin cytoskeleton (*BCAS3*).

Aberrant regulation of *BCAS3* gene was previously reported in breast and brain malignancies (Schulte et al. 2013). This molecule is involved in the control of cell migration and angiogenesis by facilitating crosstalk between cytoskeletal elements, especially during tumour progression (Jain et al. 2012).

Interestingly enough, a consistent hypermethylation was detected for cg18437039 in an intergenic regions, located 3,7 kb upstream to *RPS15*, which binds MDM2, inhibits degradation of MDM2 and p53, cause apoptosis and cell cycle arrest in G2-phase (Daftuar et al. 2013). In MPM, MDM2–p53 interaction is deregulated, driving mesothelioma progression and aggressiveness (Urso et al. 2016).

Differential methylation was also found in *EDNRB*, *SOX2*, *PDGFB* and *ASCL2* genes, previously described as having altered methylation patterns in MPM tissue and thus to be possibly used as markers to distinguish MPM from lung adenocarcinoma (Christensen et al. 2009).

Several genes resulting up-regulated after chrysotile exposure (*CD68, SLC2A1, CD44, AXL, CA9, FOSL1, NRP1, THBS2, ADM, DIO2, DKK1, F3, GREM1,* and *TPBG),* were already found deregulated in MPM cell lines, or involved in MPM development (Capkova et al. 2014; Lee et al. 2004; Nymark et al. 2007).

Our data indicate in general a different gene expression profile of crocidolite versus chrysotile fibres , with common patterns of expression observed for few genes.

Among them we identified *CA9*, that encodes for a membrane-bound enzyme with catalytic activity at the extracellular membrane surface and involved in pH regulation, which is necessary for survival of hypoxic cells (Klier et al. 2016). Interestingly enough, MPM is characterized by hypoxic areas that can promote tumour progression and survival (Nabavi et al. 2016). We can thus speculate a possible role of CA9 in the adaptation of mesothelial cells to hypoxic conditions, contributing to neoplastic transformations. As a matter of fact, *CA9* is overexpressed in aggressive tumours

in which rapid growth limits available oxygen (Patard et al. 2008). The higher expression of *CA9* in MPM tissue and its reliability as biomarker has been already described by Ramsey and colleagues (Ramsey et al. 2012).

SRGN gene, whose expression is also deregulated after both treatments, encodes for a proteoglycan residing both in the extracellular matrix and intracellular compartments. SRGN is highly secreted in cancer-associated fibroblast (CAFs) (Tyan et al. 2012) and may promote EMT. An increased expression of *SRGN* induces an increase of matrix metalloproteinase-2 (*MMP2)* expression in myeloma (Skliris et al. 2013). Further experiments will allow to better understand the relationship between these two genes in asbestos-related diseases, being MMP2 a typical marker of EMT in mesothelioma (Fassina et al. 2012). *In vitro* and *in vivo* studies have proven that *SRGN* can promote motility, invasion, and metastasis of cells via induction of the mesenchymal molecule vimentin (Li et al. 2011), strongly associated with MPM (Bonotti et al. 2017).

In our study, only one gene (*DKK1)* showed a significant negative correlation between DNA methylation and gene expression, and limited to chrysotile treatment. This gene, previously found overexpressed in human MPM (Melotti et al. 2010), resulted up-regulated also in our technical validation only in chrysotile-treated cells. In human mesenchymal stem cells, the inhibition of Wnt signaling with recombinant soluble DKK1 induces spontaneous transformation and tumorigenicity (Rijlaarsdam et al. 2014). DKK1 has been proposed as a potential marker of MPM: the inhibitory effect of DKK1 would maintain primary MPM cell cultures in an undifferentiated, "stem-like" cellular state, ultimately leading to malignant transformation in MPM (Melotti et al. 2010).

We are aware of some weaknesses in the present study. Given the difficulties in obtaining sufficient primary human mesothelial cells to study the complex response to asbestos, we employed MeT5A human mesothelial cell line. Transformed cells may not be entirely comparable to normal mesothelial cells since *in vitro* experiments can never precisely reflect the conditions and mechanisms of *in vivo* models (for example, interactions between inflammatory cells and extra-cellular matrix are missing). Furthermore, MeT5A are SV40-immortalized cells and SV40 infection is known to decrease sensitivity of human mesothelial cell lines to toxicity induced by asbestos. This effect may contribute to gene expression and epigenetic changes. However, Met5A cells are widely used to assess the carcinogenic mechanisms induced by asbestos fibres and the efficient control with untreated cells and careful analysis of the raw data should account for these limitations and minimize false positive results.

Conclusion

In this study, we provided the first extensive DNA methylation profiling of MeT5A human mesothelial cells exposed to asbestos fibres with the aim to discover potential associations between asbestos exposure and mesothelial methylation patterns changes.

Our data revealed different patterns of methylation changes induced by crocidolite and chrysotile fibres. A global view based on molecular interactions in asbestos-treated cells suggested an intricate network of genes involved in the regulation of cell migration and cell adhesion. We reported novel genes putatively affected by crocidolite and chrysotile exposure, leading to new scenarios in the asbestos-induced carcinogenesis.

These results may contribute to better understand how asbestos exposure could influence the aetiology of MPM, giving new insights to be investigated in more details in other experimental settings in order to link results to human MPM carcinogenesis and possibly identifying also new chemopreventive targets.

Conflict of interest The authors declare that they have no conflict of interest.

Figure Captions

Fig. 1 Overall study design. MeT5A semiconfluent cells were plated in 150-mm–diameter Petri dishes. Twenty-four hours later, crocidolite or chrysotile asbestos fibres were added at different doses (0.5 µg/cm^2, 1.0 µg/cm^2, 2.5 µg/cm^2, 5.0 µg/cm^2) and cells were incubated for 72 hours. Untreated cells were used as control and each experiment was performed in triplicate

Fig. 2 Volcano plots of differentially expressed transcripts in (a) crocidolite- and (b) chrysotile-treated MeT5A cells. In red, probes with a log 2 –fold change > |1|; in blue, the common differentially expressed genes between the two treatments.

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