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Blood cell DNA methylation biomarkers in preclinical malignant pleural mesothelioma: The EPIC prospective cohort

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Abstract

Malignant pleural mesothelioma (MPM) is a rare and aggressive cancer mainly caused by asbestos exposure. Specific and sensitive noninvasive biomarkers may facilitate and enhance screening programs for the early detection of cancer. We investigated DNA methylation (DNAm) profiles in MPM prediagnostic blood samples in a case-control study nested in the European Prospective Investigation into Cancer and nutrition (EPIC) cohort, aiming to characterise DNAm biomarkers associated with MPM. From the EPIC cohort, we included samples from 135 participants who developed MPM during 20 years of follow-up and from 135 matched, cancer-free, controls. For the discovery phase we selected EPIC participants who developed MPM within 5 years from enrolment ($n = 36$) with matched controls. We identified nine differentially methylated CpGs, selected by 10-fold cross-validation and correlation analyses: cg25755428 (*MRI1*), cg20389709 (*KLF11*), cg23870316, cg13862711 (*LHX6*), cg06417478 (*HOOK2*), cg00667948, cg01879420 (*AMD1*), cg25317025 (*RPL17*) and cg06205333 (*RAP1A*). Receiver operating characteristic (ROC) analysis showed that the model including baseline characteristics (age, sex and PC1wbc) along with the nine MPM-related CpGs has a better predictive value for MPM occurrence than the baseline model alone, maintaining some performance also at more than

Abbreviations: AUC, area under curve; DNAm, DNA methylation; EPIC, European Prospective Investigation into Cancer and nutrition; EWAS, Epigenome Wide Association Study; miRNA, microRNA; MM, malignant mesothelioma; MPM, malignant pleural mesothelioma; ROC, receiver operating characteristic.

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For affiliation refer to page 734

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5 years before diagnosis (area under the curve [AUC] < 5 years = 0.89; AUC 5-10 years = 0.80; AUC >10 years = 0.75; baseline AUC range = 0.63-0.67). DNAm changes as noninvasive biomarkers in prediagnostic blood samples of MPM cases were investigated for the first time. Their application can improve the identification of asbestos-exposed individuals at higher MPM risk to possibly adopt more intensive monitoring for early disease identification.

KEYWORDS

cancer biomarkers, DNA methylation, mesothelioma, prospective nested case-control study

What's new?

Malignant pleural mesothelioma (MPM), though rare, is an aggressive cancer chiefly caused by exposure to asbestos. Here, the authors describe a distinctive DNA methylation profile that can help clinicians identify people who are at higher risk of developing MPM. Using samples from 135 individuals who developed MPM and 135 cancer-free controls, they identified nine sites that had methylation differences between the cancer cases and the controls. This biomarker profile could help screen asbestos-exposed individuals for MPM and improve early detection.

1 | INTRODUCTION

Malignant mesothelioma (MM) is an aggressive cancer of the serous membranes with an increasing incidence worldwide. MM shows a latency period for up to 40 years and its prognosis is poor, with a median survival of about 12 months from diagnosis.¹ At the time of clinical diagnosis, disease is usually unresectable and chemotherapy only vaguely improves prognosis, compared to best supportive care. Currently, the diagnosis of malignant pleural mesothelioma (MPM) is particularly challenging, with no established tissue or soluble biomarkers in clinical practice. The diagnosis of mesothelioma at early stages might be a promising opportunity to improve prognosis over time. Today, most of the MPM studies are focused on biomarkers research, including genetic and epigenetic ones.^{2,3}

Exposure to asbestos fibres is a major risk factor for MPM, lung cancer and other non-neoplastic conditions, such as asbestosis and pleural plaques.⁴ However, several studies have shown that polymorphisms in genes involved in xenobiotic and oxidative metabolism or in DNA repair processes may modify individual susceptibility to disease after exposure to asbestos.⁵ The risk of developing MPM is slightly increased among people exposed to asbestos with a positive history of familial cancers.⁶

Several blood-based potential MPM biomarkers have been reported recently in retrospective studies, including peripheral blood DNA methylation (DNAm) variation and microRNAs (miRNAs)⁷: the finding of asbestos fibres in extrapulmonary tissues, translocated through lymphatic and blood flows and causing continuous exposure of blood cells to asbestos, supports the search for epigenetic changes in blood cell DNA.⁸

Epigenetic mechanisms may modulate gene expression without altering the DNA sequence itself. DNAm is a crucial type of epigenetic modification of DNA, by which a methyl group covalently bonds to the C5 position of cytosine in 5'-C-phosphate-G-3' (CpG)

dinucleotides; ultimately DNAm regulates gene transcription activity and may modulate key biological functions.⁹

Systematic studies of genome-wide DNAm profiles at single CpG level associated with MPM in prospective cohorts are lacking. Most studies so far used biological samples collected at or after diagnosis,¹⁰ that may limit application for early disease detection of the identified biomarkers due to reverse causality.

We previously identified DNAm biomarkers in blood taken at MPM diagnosis able to discriminate between MPM and non-MPM asbestos-exposed individuals, assuming that DNAm profile of blood cells may contribute to identify early changes associated with MPM development.¹¹ More recently, we identified one single-CpG signal in *FKBP5* gene associated with asbestos-exposure as a biomarker of MPM and MPM survival.^{12,13}

In the current study we investigated, for the first time, the relationship between DNA methylation profiles in prediagnostic blood samples and MPM, in a case-control study nested in the prospective European Prospective Investigation into Cancer and nutrition (EPIC) cohort.¹⁴

Our aim was to identify predictive biomarkers of MPM development in asbestos-exposed individuals by DNAm analysis in blood samples.

2 | MATERIALS AND METHODS

2.1 | Study design and population

EPIC is a multicentre cohort study, conducted in 23 centres across 10 European countries, aiming at investigating the aetiological role of biological, lifestyle and environmental factors in cancer and other chronic diseases. Overall, 521 468 healthy participants were enrolled in EPIC, and followed up on an ongoing basis.¹⁴ In the frame of EPIC

cohort, 135 participants developed MPM (EPIC-Meso) after a mean follow-up of 8.3 years (range 0.5-18.8 years). The disease was classified by ICD-10 diagnosis codes (ICD, International Statistical Classification of Diseases, Injuries and Causes of Death) as C38.4 (malignant neoplasm of pleura) in all participants. Cancer endpoint data is based on the latest round of follow-up received from the EPIC centres and centralised at IARC between 2014 and 2016.

Prediagnostic DNAm profiles of their blood samples, taken at enrolment, were compared to those of matched controls with no oncological diagnosis.

As one case did not pass the data quality control, the final dataset included 134 cases and 134 matched controls (dataset “MESO_ALL,” Table 1). Controls were 1:1 matched to the cases by sex, age at enrolment (± 1.5 years), study centre and asbestos-exposure (see Section 2.2).

Cases were divided into three subgroups (Table 1, MESO_ALL Cases) based on the time between blood sample collection and MPM diagnosis: Group 1, $n = 36$ participants who developed MPM within 5 years; Group 2, $n = 40$ participants who developed MPM from 5 to 10 years from the recruitment; and Group 3, $n = 58$ participants who were diagnosed with MPM more than 10 years after recruitment. Group 1 cases and matched controls (MESO_5YRS) were further analysed to identify differential methylation, and its trend with respect to the time before MPM diagnosis (Table 1, MESO_5YRS Cases).

2.2 | Asbestos-exposure assessment

Occupational information was available from the baseline EPIC questionnaire. It included the occupation at enrolment and data on ever working up to the time of enrolment in 52 at-risk occupations. No information was available on duration of employment and time of first employment. A semi-quantitative job-exposure matrix (JEM) was developed by expert epidemiologists as previously described,¹⁵ assigning an “exposure probability” and an “exposure intensity” score to each of the 52 EPIC occupational categories. Both scores were expressed over a numerical scale with four levels: 0 “no exposure,” 1 “low,” 2 “intermediate,” 3 “high” and multiplied to generate an Exposure Index (EI). Various exposure metrics have been derived from the EI, as reported in Table S1. For the present study, we used a categorical variable with three levels defined as “no exposure” (EI = 0), “low exposure” (EI = from 1 to 3) and “high exposure” (EI ≥ 4).

2.3 | DNA methylation

DNAm levels were measured in DNA from buffy coat collected at enrolment, using the Infinium Methylation EPIC Bead Chip (>850 000 methylation sites, Illumina, San Diego, California). Laboratory methods for DNA extraction, BeadChip processing, methylation levels measurement and data quality controls were previously described.¹¹

TABLE 1 Sample characteristics within the two EPIC-Meso subgroups analysed: MESO_ALL and MESO_5YRS groups

	MESO_ALL		MESO_ALL cases			MESO_5YRS			MESO_5YRS cases		
	Cases	Controls	>10 years	5-10 years	<5 years	Cases	Controls	3-5 years	1.5-3 years	< 1.5 years	
N (%)	134 (50)	134 (50)	58 (43.3)	40 (29.9)	36 (26.7)	36 (50)	36 (50)	14 (38.9)	13 (36.1)	9 (25)	
Sex (%**)											
M	107 (79.9)	107 (79.9)	47 (81)	30 (75)	30 (83.3)	30 (83.3)	30 (83.3)	12 (85.7)	10 (76.9)	8 (88.9)	
F	27 (20.1)	27 (20.1)	11 (19)	10 (25)	6 (16.7)	6 (16.7)	6 (16.7)	2 (14.3)	3 (23.1)	1 (11.1)	
Age (mean, SD)	57.2, 6.9	57.2, 6.9	56.4, 5.8	57.6, 8.6	58.2, 6.6	58.2, 6.6	58.2, 6.7	59.1 (7.3)	55.7 (6.5)	60.5 (4.9)	
Asbestos exposure (%**)											
0 (no)	21 (15.7)	21 (15.7)	7 (12.1)	8 (20)	6 (16.6)	6 (16.6)	6 (16.6)	2 (14.3)	2 (15.4)	2 (22.2)	
1 (medium)	28 (20.9)	28 (20.9)	12 (20.7)	5 (12.5)	11 (30.6)	11 (30.6)	11 (30.6)	3 (21.4)	6 (46.2)	2 (22.2)	
2 (high)	43 (32.1)	43 (32.1)	21 (36.2)	13 (32.5)	9 (25)	9 (25)	9 (25)	3 (21.4)	4 (30.8)	2 (22.2)	
NA	42 (31.3)	42 (31.3)	18 (31)	14 (35)	10 (27.8)	10 (27.8)	10 (27.8)	6 (42.9)	1 (7.7)	3 (33.3)	

Note: The description of MESO_ALL cases and MESO_5YRS cases divided by years from recruitment to MPM diagnosis is included. Percentages were calculated in relation to the whole subgroup (*) or the N individuals of each column (**).

2.4 | DNA methylation bioinformatics analyses

The average methylation value at each CpG locus, that is, average “beta (β) value” ranging from 0 to 1, was computed as the ratio of the intensity of the methylated signal over the total signal (unmethylated + methylated).

Data were processed using ChAMP Bioconductor package (v2.22) filtering out the BeadChip probes with a detection P value $>.01$ and with <3 beads in at least 5% of samples per probe; non-CpG sites SNP-related and CpGs located in chromosome X and Y were excluded. The remaining signals were quality controlled, normalised and batch effect corrected using ComBat algorithm.

After quality controls 725 050 CpGs were used in the following analyses.

DNA methylation regional differences were calculated by ChAMP approach (champ DMR function).

2.5 | White blood cells estimate

In order to evaluate differences in white blood cells (WBCs) proportion between cases and controls, we estimated WBC subtype percentages from genome-wide methylation data.¹⁶ For each individual, we extracted WBC subtype percentages, estimated based on genome wide methylation data. We estimated the frequencies of B cells, CD8+ and CD4+ T cells, natural killer cells, granulocytes and monocytes using methylation profiles, both in MESO_ALL and MESO_5YRS datasets. WBCs differences between cases and controls were tested with Wilcox test R function.

2.6 | Statistical analyses

All statistical analyses were conducted using the open source software Rv4.1.0.

Epigenome-wide differential methylation on MESO_ALL dataset (134 cases and 134 controls, Table 1) was tested by a multiple regression model (R function *glm*).

Cross-validation analysis was conducted on the subset of the original group consisting of the 36 participants who developed MPM within 5 years from the blood sample collection and 36 matched controls (dataset “MESO_5YRS”), in order to improve the estimate of the mean model performance. Cross validation was carried out mixing and splitting the dataset randomly, summarising the goodness of the model using the sample of model evaluation scores. Each run consisted of an Epigenome Wide Association Study (EWAS) analysis done on 80% of the samples ($n = 58$), with the option of maintaining comparable proportions of matched cases and controls in the two groups; we conducted a total of 10 cycles. As at false discovery rate (FDR)-level we did not obtain any statistically significant

differentially methylated signal between cases and controls (possibly due to the small sample size population), we focused on those signals that showed an |effect size| $> 10\%$ and a nominal $P \leq .05$ in at least 8 out of 10 runs.

All the analyses, including trend test, were adjusted for age at blood collection, sex and WBC's first principal component (PC1wbc; R *prcomp* function), which takes into account 66% of the variance. WBC subtypes were estimated as previously described.¹⁷

A Pearson correlation test was performed to evaluate the association between two CpG sites, to select only one of the two correlated signals (selecting correlating values of $|\rho| > 0.7$, $P \leq .05$). For each correlated couple, we selected the CpG signal with the higher effect size.

Multiple regression models were done with *glm* R function, correlations with *cor.test* R function, ROC analyses with *ROCR* and *pROC* libraries.

3 | RESULTS

A flow diagram of participants and relative analyses performed in the present study is represented in Figure S1.

3.1 | Differential methylation analysis

The baseline characteristics of the study participants are shown in Table 1.

Leukocyte DNAm was used to quantify different leukocyte sub proportions applying an implemented deconvolution pipeline for high-resolution immune profiling.¹⁶ In MESO_ALL cases we observed a slight difference in neutrophils, that were higher than controls (Wilcoxon test $P = .04$, Table S2) and a weak decrease of CD4+ memory cells (Wilcoxon test $P = .02$, Table S2). In MESO_5YRS we did not observe WBC differences between cases and controls (Table S3). However, although we expected a larger deviation of WBCs close to diagnosis, in both participant groups we did non observe any trend of WBCs numbers with the time to diagnosis (data not shown).

We first focused the DNAm analysis on 36 participants who developed MPM within 5 years from recruitment, vs 36 controls matched by sex, age, study centre and asbestos-exposure level (MESO_5YRS), under the hypothesis that the most significant changes in blood should happen closer to MPM diagnosis, due to the already ongoing carcinogenic process.

In the cross-validation analysis we identified 22 differentially methylated CpGs with nominal $P \leq .05$ and |effect size| $> 10\%$ (Table 2). Unsupervised hierarchical clustering heatmap of DNA methylation of the 22 CpGs is shown in Figure S2.

We observed a very high correlation between DNA methylation levels of CpGs located in the same gene (Table S4), allowing us to select only one CpG for each gene, with a final list of nine significant CpGs (highlighted in bold in Table 2).

TABLE 2 Description of the 22 differentially methylated CpGs identified by 10-fold cross-validation EWAS analysis: number of runs for which conditions of significance exist, their genomic localization, methylation levels (β -values) in cases and controls, effect size range in cross-validation runs and the P value range among runs are indicated

CpG ID	Runs (n) with eff.size >10% (abs value) and P -value <.05	CHR	MAPINFO	Gene name	Relation to gene	β -value MPM cases (n = 36)	β -value MPM controls (n = 36)	Effect size min (min as abs value)	Effect size max (max as abs value)	P -value min	P -value max
cg23870316	10	8	2 216 155			0.85	0.69	0.146	0.180	.0001	.002
cg20389709	10	2	10 184 303	KLF11	5'UTR; TSS200; Body	0.12	0.28	-0.123	-0.197	.013	.00005
cg13498216	8	2	10 184 305	KLF11	5'UTR; TSS200; Body	0.08	0.21	-0.106	-0.144	.00004	.002
cg06417478	9	19	12 876 846	HOOK2	Body	0.26	0.44	-0.159	-0.253	.002	.048
cg23899408	10	19	12 877 188	HOOK2	Body	0.27	0.43	-0.141	-0.226	.043	.001
cg16474696	9	19	13 875 014	MRI1	TSS1500	0.33	0.2	0.108	0.166	.0006	.022
cg25755428	10	19	13 875 111	MRI1	TSS1500	0.41	0.24	0.134	0.234	.0001	.023
cg25317025	9	18	47 019 823	RPL17	TSS1500	0.55	0.44	0.103	0.138	.003	.033
cg00667948	9	13	100 651 721			0.88	0.76	0.106	0.135	.007	.045
cg01879420	9	6	111 194 645	AMD1	TSS1500; 5'UTR	0.17	0.29	-0.107	-0.145	.00003	.004
cg18434912	8	6	111 194 786	AMD1	TSS1500; 5'UTR	0.2	0.3	-0.103	-0.139	.0002	.010
cg06205333	8	1	112 161 618	RAP1A	TSS1500	0.63	0.76	-0.109	-0.174	.002	.023
cg04282082	9	9	124 988 720	LHX6	Body	0.67	0.51	0.140	0.244	.0003	.045
cg11479503	8	9	124 989 052	LHX6	Body	0.84	0.72	0.117	0.161	.00002	.002
cg21469772	9	9	124 989 294	LHX6	Body	0.74	0.59	0.124	0.224	.00002	.025
cg13571460	9	9	124 989 337	LHX6	Body	0.72	0.58	0.109	0.199	.00002	.024
cg05136264	9	9	124 989 408	LHX6	Body	0.73	0.6	0.109	0.193	.0001	.036
cg05037505	8	9	124 989 550	LHX6	Body	0.5	0.4	0.106	0.145	.0006	.019
cg15124400	9	9	124 989 839	LHX6	1st Exon; 5'UTR; Body	0.78	0.6	0.162	0.259	.0002	.021
cg13862711	9	9	124 989 915	LHX6	Body	0.74	0.54	0.174	0.279	.0001	.017
cg04622888	9	9	124 990 010	LHX6	TSS200; Body	0.7	0.55	0.141	0.217	.0006	.026
cg03363289	9	9	124 990 165	LHX6	Body	0.45	0.32	0.121	0.193	.0002	.021

Note: Signals selected after correlation analysis are highlighted in bold.

TABLE 3 ROC analysis for each of the nine statistically differentially methylated CpGs, performed on all MESO_5YRS cases (n = 36) and all the asbestos-exposed controls (n = 71)

CpG ID	Gene name	AUC MODEL 0	AUC MODEL 1	DeLong's P
cg01879420	AMD1	0.66	0.73	.1
cg06417478	HOOK2	0.66	0.69	.42
cg20389709	KLF11	0.66	0.74	.08
cg13862711	LHX6	0.66	0.71	.2
cg25755428	MRI1	0.66	0.71	.2
cg06205333	RAP1A	0.66	0.67	.51
cg25317025	RPL17	0.66	0.67	.52
cg23870316		0.66	0.75	.02
cg00667948		0.66	0.71	.12

Note: Model 0: age, sex, PC1wbc; model 1: age, sex, PC1wbc, nine CpGs methylation levels. Statistically significant results (DeLong's $p < 0.05$) are highlighted in bold.

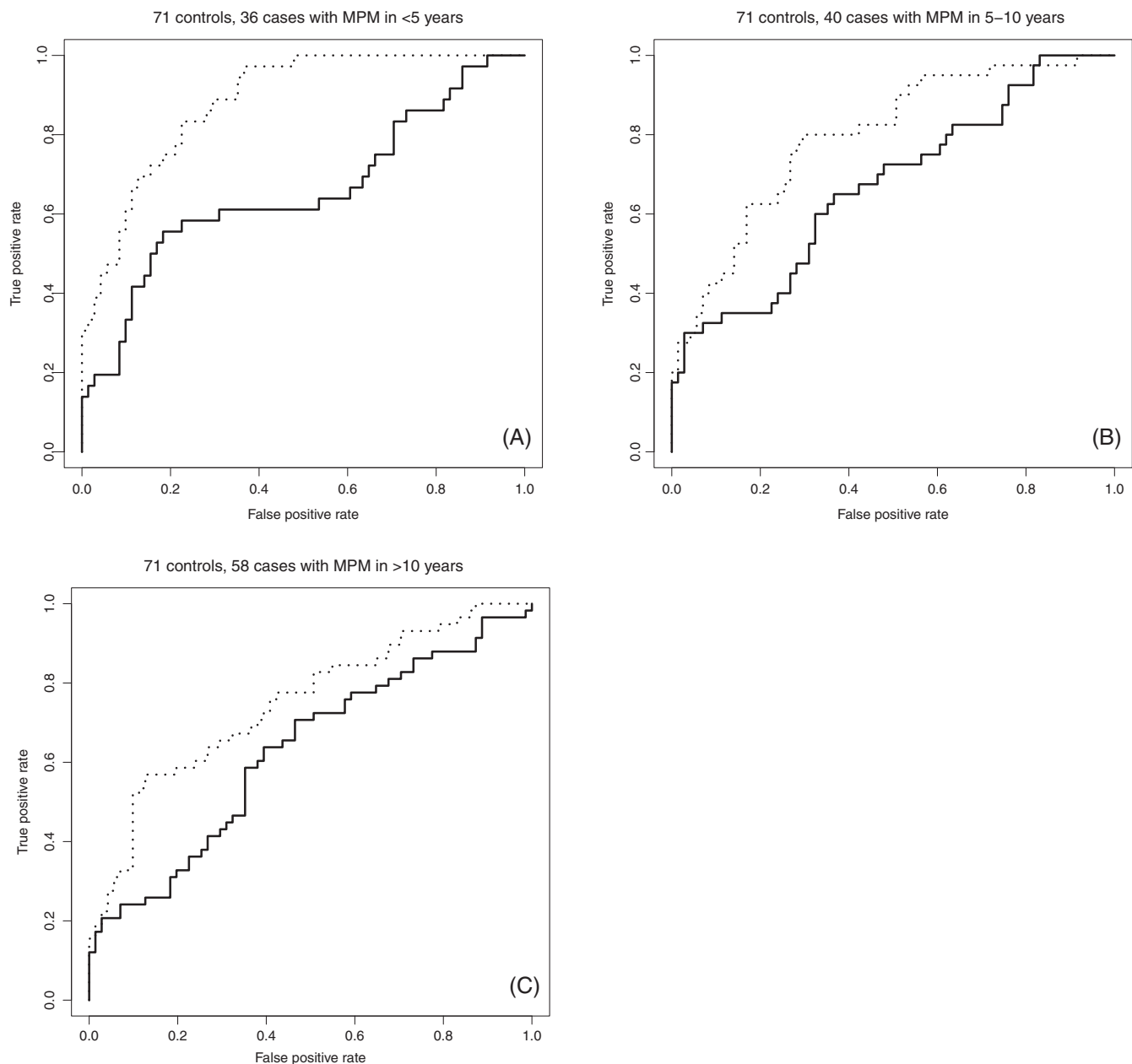


FIGURE 1 ROC curves including model 0 (dotted line): age, sex, PC1wbc; and model 1 (solid line): age, sex, PC1wbc, 9 CpGs methylation levels (cg01879420, cg06417478, cg20389709, cg13862711, cg25755428, cg06205333, cg25317025, cg23870316, cg00667948) on the following subgroups of MESO_ALL: (A) 107 participants, including 71 asbestos-exposed controls and 36 participants developing MPM within 5 years from recruitment (AUC model 0 = 0.656, AUC model 1 = 0.885, De-Long's $P = 6.44 \times 10^{-5}$); (B) 111 participants, including 71 asbestos-exposed controls and 40 participants who developed MPM in 5–10 years from recruitment (AUC model 0 = 0.671, AUC model 1 = 0.795, De-Long's $P = .018$); (C) 129 participants, including 71 asbestos-exposed controls and 58 participants who developed MPM more than 10 years from recruitment (AUC model 0 = 0.627, AUC model 1 = 0.747, De-Long's $P = .018$). AUC, area under the curve. [Correction added after first online publication on 29 November 2022. In Figure 1, the legend keys in the graphs have been removed.]

Regional differences in MESO_5YRS cases and controls were analysed with no statistically significant results (Table S5). Looking at DMRs with a mean of the estimate $> |0.5|$, we confirmed DMRs in genes already identified in Table 2: LHX6 (16 CpGs), HOOK2 (4 CpGs) and RAP1A (3 CpGs).

The association between methylation of 22 CpGs listed in Table 2 and asbestos-exposure was evaluated both in cases and in controls. To do this, we performed a Trend test, adjusted for age, sex and PC1wbc in MESO_5YRS individuals (26 cases and 26 controls with

exposure assessment). However, we did not observe any statistically significant results (Table S6).

EWAS analysis performed on the whole population of 268 participants (MESO_ALL) showed three signals with nominal $P \leq .05$ and $|\text{effect size}| > 10\%$: cg04131969 (*MYADML* gene, effect size = 0.11, $P = .004$) was hypermethylated in cases compared to controls, while cg17939448 (*FAM47E* gene, effect size = -0.10 , $P = .001$) and cg01201512 (*NINJ2* gene, effect size = -0.10 , $P = .006$) were hypomethylated.

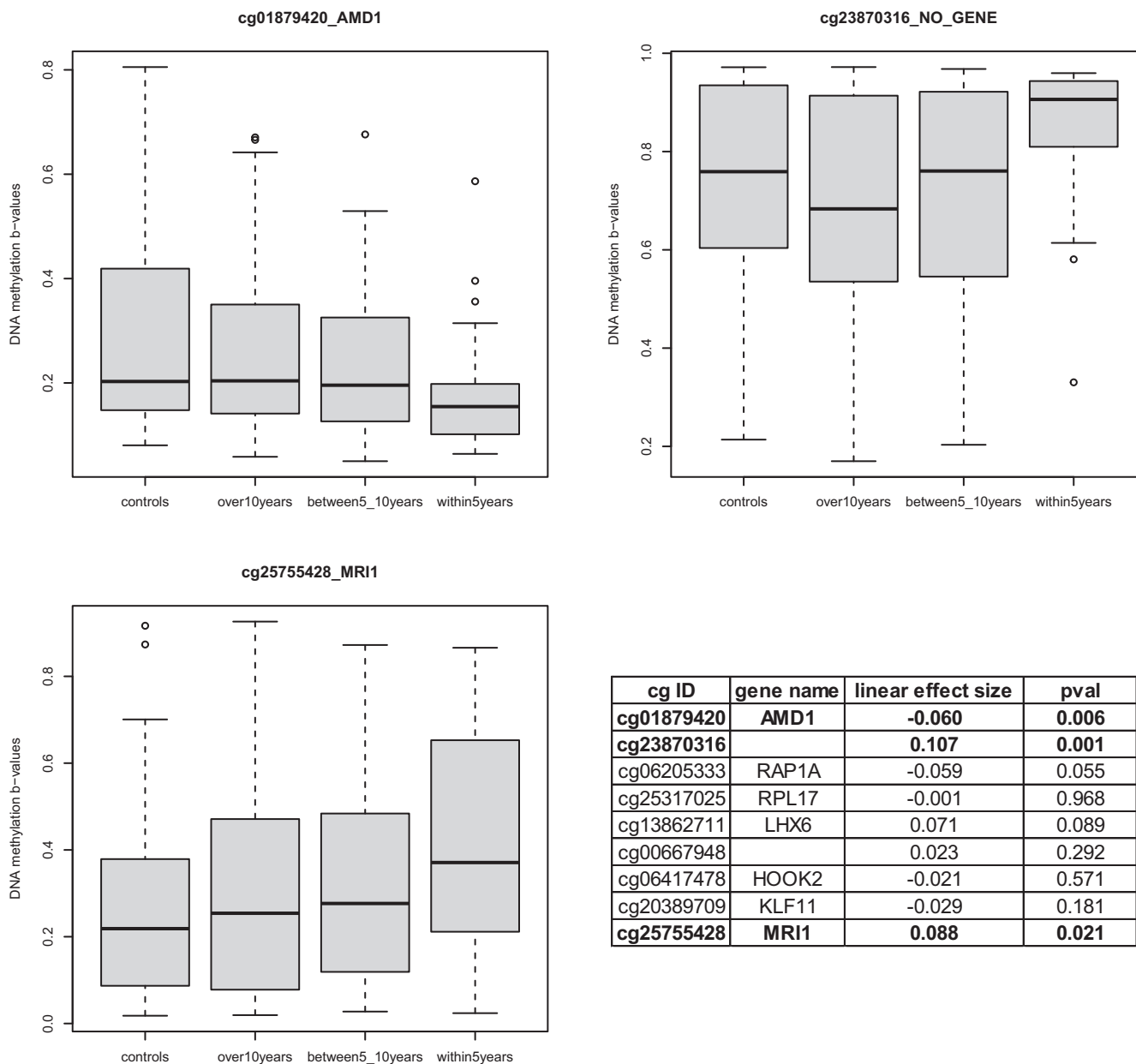


FIGURE 2 Trend test analysis of nine differentially methylated CpGs on MESO_ALL cases divided into groups by time from recruitment to diagnosis, controlling for age, gender and PC1wbc: >10 years (n = 58), between 5 and 10 years (n = 40), <5 years (n = 36). The table shows trend test P values among groups of cases. Boxplots of significant CpGs are shown on the right, including also DNAm values of the control group (n = 71). [Correction added after first online publication on 29 November 2022. In Figure 2, the table was missing and has been added to the figure.]

3.2 | Receiver operating characteristic curves of DNA methylation signals

To evaluate improvement in discrimination between cases and controls when including single CpG methylation levels in the analysis (nine CpGs obtained by cross-validation analysis and selected after correlation analysis), we compared two receiver operating characteristic (ROC) analyses: model 0 includes age, sex, PC1wbc and model 1 includes age, sex, PC1wbc and single CpG methylation levels.

ROC analyses (1 for each of the 9 CpGs identified in MESO_5YRS) were performed on a larger population of

107 participants, including the 71 tumour-free asbestos-exposed (asbestos-exposure = 1 or 2) participants from MESO_ALL and 36 participants who developed MPM within 5 years from recruitment from MESO_5YRS (Table 3). One DNAm signal was statistically significant: cg23870316 (AUC model 0 = 0.66; AUC model 1 = 0.75; De-Long's P = .02). However, DNA methylation level of cg23870316 is not able to discriminate cases from controls in participants recruited more than 5 years before MPM diagnosis (Table S7).

To investigate a potential extension of results to longer time windows, we also carried out ROC analysis with all the nine identified CpGs, to discriminate asbestos-exposed controls with no diagnosis of

MPM during follow up ($n = 71$, asbestos-exposure = 1 or 2) from participants who developed MPM within 5 years after enrolment ($n = 36$, Figure 1A), within 5 to 10 years ($n = 40$, Figure 1B) and over 10 years ($n = 58$, Figure 1C). We performed ROC analysis including model 0: age, sex, PC1wbc; and model 1: age, sex, PC1wbc, 9 CpGs methylation levels. De-Long's test showed a statistically significant discrimination between the two groups in model 1 compared to model 0 in all the subgroups: within 5 years from MPM (AUC model 0 = 0.66, AUC model 1 = 0.89, De-Long's $P = 6.4 \times 10^{-5}$), from 5 to 10 years (AUC model 0 = 0.67, AUC model 1 = 0.80, De-Long's $P = .018$) and > 10 years (AUC model 0 = 0.63, AUC model 1 = 0.75, De-Long's $P = .018$), showing a good performance also when the participants were recruited >10 years before MPM diagnosis.

ROC analysis with the nine CpG was also performed excluding the 20 asbestos-exposed controls belonging to the MESO_5YRS dataset and already used in discovery. Thus, ROC curves were performed including model 0: age, sex, PC1wbc; and model 1: age, sex, PC1wbc, nine CpGs methylation levels on 51 asbestos-exposed controls and 36 MESO_5YRS cases, obtaining similar results (AUC model 0 = 0.70; AUC model 1 = 0.87; De-Long's $P = .004$). Moreover, to exclude asbestos-exposure effect in case-control discrimination, we performed also the same ROC analysis with model 0 (age, sex, PC1wbc, asbestos-exposure) and model 1 (age, sex, PC1wbc, asbestos-exposure, nine CpGs methylation levels), confirming previous results (AUC model 0 = 0.73; AUC model 1 = 0.86; De-Long's $P = .02$).

3.3 | Biomarkers trend with time to diagnosis

We performed a trend test to evaluate the changes of the nine differentially methylated CpGs in association with the time to diagnosis. MESO_5YRS MPM cases were divided into participants who developed MPM within 1.5 years from sample collection ($n = 9$), participants who developed MPM after 1.5 and before 3 years ($n = 13$) and participants who developed MPM after 3 and before 5 years ($n = 14$) (MESO_5YRS cases, Table 1) and the trend test was performed comparing these three groups. We did not observe any statistically significant trend (Table S8). On the other hand, three of these signals showed a statistically significant linear trend in MESO_ALL cases divided by time from recruitment to diagnosis as described in Table 1 (Figure 2): cg01879420 in *AMD1* gene ($P = .006$), cg25755428 in *MRI1* gene ($P = .021$) and cg23870316 ($P = .001$). Nevertheless, EPIC participants who were diagnosed with MPM a longer time from enrolment showed DNAm levels very similar to control participants, as expected (Figure 2).

3.4 | DNAm diagnostic biomarkers changes in prediagnostic samples

We aimed to assess whether previously reported DNAm biomarkers in MPM patients at diagnosis^{11,13} are also informative in DNA blood samples taken up to 5 years before MPM diagnosis. To do this, we

performed a multiple regression model on MESO_5YRS dataset of DNAm biomarkers previously identified as differentially methylated in a case/control study (Table S9). Cg01521397 in *TAF4* gene body was statistically significantly hypomethylated in EPIC-Meso who developed MPM within 5 years from recruitment compared to matched controls ($P = .01$). However, any of these CpGs showed statistically significant differences in individuals closer to MPM diagnosis (data not shown) compared to the other participants.

4 | DISCUSSION

In the present study, we identified nine DNAm biomarkers in blood preclinical samples from MPM patients. The panel of the nine signals was able to discriminate cases from controls with the highest performance within 5 years before diagnosis, and maintaining some discriminating power up to 10 years before MPM symptoms. Generally, the diagnosis of cancer at early stages, when clinical symptoms have not yet occurred, appears to be a promising opportunity to improve therapeutic outcomes. Treatment at early stages together with newly developed therapies could possibly lead to improvement in overall survival of patients with MM.¹⁸ We focused the DNAm data analysis mainly on individuals who were diagnosed with MPM within 5 years from the blood sample collection, with the purpose to identify early changes of tumour-related DNAm. A few previous studies measured blood biomarkers several years before MPM diagnosis, but comparison with them is hard due to different time from measurements to MPM diagnosis, most of them shorter than those of EPIC.^{16,19} Except for mesothelin,¹⁹ no other biomarkers have been evaluated in a sufficiently large prospective cohort study with serial prediagnostic samples of MM cases so far. Morrè and colleagues, observed that ENOX2 protein transcript variants characteristic of malignant mesothelioma were present in serum 4 to 10 years in advance of clinical symptoms.²⁰

Among the 22 differentially methylated signals, we identified five genes in which more than one CpG were differentially methylated: *LHX6*, *MRI1*, *HOOK2*, *AMD1* and *KLF11*.

In particular, *LHX6* gene contained 10 hypermethylated CpGs (Figure S3 shows a gene-based visualisation of DM CpGs in *LHX6* gene), two of which were located in transcriptionally active gene regions (eg, TSS200, 1st exon). Methylation-mediated inactivation of *LHX6* (LIM Homeobox domain 6) has been shown in lung,²¹ head and neck,²² cervical²³ and pancreatic²⁴ tumours. The mechanism by which *LHX6* acts as a tumour suppressor gene is related to the interference with the Wnt/ β -catenin pathway,²⁵ which is severely involved also in MPM progression.²⁶ Moreover, *LHX6* is a target gene of miR-214, and upregulated *LHX6* gene expression was observed to be related to downregulation of miR-214 in non-small-cell lung cancer.²⁷ The role of miR-214 has also been evaluated in mesothelioma, and previous studies in human samples showed a down-regulation of miR-214 expression.²⁸

Although it is already known that *LHX6*-cg13862711 hypermethylation in tissue is associated with several types of cancer (eg, in

breast, kidney and upper aerodigestive tract; <https://cancer.sanger.ac.uk/cosmic>), the epigenetic regulation of *LHX6* in blood cells of preclinical samples needs further investigation.

Two hypomethylated signals in cases compared to controls in the *KLF11* 5'UTR/TSS200 region were identified. Krüppel-like factors (KLFs) form a highly conserved family of zinc finger transcription factors, and play important roles in the progression of human malignant tumours, such as breast cancer and colon cancer.²⁹ Recently, high *KLF11* expression was associated with poor prognosis of glioma.³⁰

In *MRI1* gene we identified two hypermethylated signals located in the promoter region (TSS1500) in cases compared to controls. *MRI1* codes for an enzyme (methylthioribose-1-phosphate isomerase) that catalyses the interconversion of methylthioribose-1-phosphate (MTR-1-P) into methylthioribulose-1-phosphate (MTRu-1-P). In addition to the catalytic activity, *MRI1* promotes cell invasion and signal transmission in response to RhoA activation in cancer cells, and for this reason is also called “Mediator of RhoA-dependent Invasion” (MRDI).³¹ Elevated expression of the encoded protein is associated with metastatic melanoma and this protein promotes melanoma cell invasion independently of its enzymatic activity.³¹

In the gene body of Hook Microtubule Tethering Protein 2 (*HOOK2*) we identified two hypomethylated CpGs. Several studies focused on differentially methylated regions in *HOOK2*, showing its implication in several pathological conditions, as in diabetes, obesity, polycystic ovary syndrome and cardiometabolic diseases.³²⁻³⁴ In particular, the deregulation of cg06417478 DNAm was shown as associated with diabetes,³⁴ while DNAm changes in cg23899408 were identified in blood as a prediction marker in liver diseases.³⁵ Deregulation of both CpGs was associated with colon adenocarcinoma in patients with metabolic syndrome.³⁶

Other deregulated CpGs were found in Adenosylmethionine Decarboxylase 1 (*AMD1*) gene. This gene encodes S-adenosylmethionine decarboxylase 1, an important intermediate enzyme in polyamine biosynthesis. The polyamines spermine, spermidine and putrescine are highly regulated in cellular proliferation and tumour promotion.³⁷ Multiple alternatively spliced transcript variants have been identified, also in association with cancer survival (eg, non-small cell lung cancer).³⁸ Several studies have demonstrated that *AMD1* differential expression is implicated in cancer, such as chronic myeloid leukaemia (CML)³⁹ and B-cell non-Hodgkin's lymphoma.⁴⁰

Finally, two other genes contain significant differentially methylated CpG sites: *RPL17* (cg25317025) and *RAP1A* (cg06205333).

RPL17 encodes a ribosomal protein that is a component of the large 60S subunit, called 60S ribosomal protein L17.⁴¹ A variety of extra-ribosomal functions were recently recognised for ribosomal proteins, including the regulation of immune signalling, tumorigenesis and cellular development.⁴² *RPL17* expression has been reported to be associated with breast⁴³ and liver⁴⁴ cancer.

RAP1A deregulated CpG is located in the promoter region (TSS1500). *RAP1A* encodes a small GTPase member of the Ras family. Its encoded protein may be involved in signalling pathways that affect cell proliferation, adhesion and may play a role in tumour malignancy.^{45,46} Inactivation of *RAP1* by bisphosphonates treatment has

been observed in several pathologies, including MM: growth inhibition of MM cells by zoledronic acid has been shown both in vitro and in vivo, and the described effect is related to *RAP1* unprenylation.^{47,48}

Besides the seven genes described above, we also identified two deregulated CpGs in noncoding regions (cg23870316 and cg00667948). In this regard, the cg23870316 is the unique DNAm signal which was statistically significant in discriminating prospective cases from controls. This CpG is located in the 8p23.2 DNA region with scanty information in the literature.

The nine differentially methylated CpGs together were not only able to discriminate cases within 5 years from MPM diagnosis, but they also partly maintained their discrimination power also for cases who were diagnosed with MPM in a range of 5 to 10 years or more than 10 years from sample collection. However, the significance (DeLong's test) decreases moving away from the time of diagnosis, showing that the prediagnostic potential of these biomarkers is limited to time periods closer to diagnosis.

In summary, our results identified nine differentially methylated CpGs that could be used as MPM biomarkers to identify early changes in preclinical individuals (ie, within 5 years before diagnosis). Interestingly, the identified CpGs are located on genes already known for their potential role as tumour biomarkers.

Regarding the estimate of WBC subpopulations, we applied a new reference-based deconvolution analysis of peripheral blood DNA methylation data developed by Salas et al, which include memory and naive cells from cytotoxic and helper T cells and B cells and parse the granulocyte subtypes into neutrophils, eosinophils and basophils.¹⁶ Although we did not obtain statistically significant results in MESO_5YRS dataset, we observed a statistically significant difference between preclinical cases and controls in CD4+ memory T cells and neutrophils in MESO_ALL dataset, indicating a different immunological response in individuals who developed MPM compared to controls. The reduction of estimated CD4+ memory lymphocytes in cases suggests a weaker adaptive immune system and is compatible with the possible occurrence of functional changes in cellular subpopulations in MPM, while an increase of neutrophils could correlate with the recent interest for their cancer-promoting effects: an elevated neutrophil-to-lymphocyte ratio is considered a prognostic indicator for cancer patients, in particular in mesothelioma, although its value remains debated.⁴⁹ However, the WBCs differences that we estimated did not change in relation to the time to diagnosis, and are so minimal that it could need further insights.

We previously observed DNAm biomarkers in MPM patients^{11,13}; among them only cg01521397 in *TAF4* gene is differentially hypomethylated in preclinical samples (as well as in MPM patients), but with any significant changes closer to MPM diagnosis. The meaning of this results is interesting in the context of biomarkers reverse causality interpretation, discriminating biomarkers related to the presence of MPM from those associated to prediagnostic biological changes. However, if reverse causality accounted for our findings, we would expect the cg01521397 DNAm-MPM association to become stronger closer to the time of diagnosis and this does not seem to be the case.

Furthermore, we are aware of the limitations of our study. First of all the lack of a replication in an independent cohort. Small sample size-related problems are especially common in the study of preclinical samples of rare diseases. For this reason, cross-validation represents a common and efficient solution when the available data are limited. In addition, when validation with a separate dataset is not feasible, cross-validation allows to use all the data for training and to reuse it for validation. The procedure of splitting data at every run offers an efficient quality forecast and we were also able to make predictions on all our data. Moreover, each run is independent from the others, avoiding overestimation of the generalisation of the model by/through developing a new one with each CV cycle. The cross-validation approach is widely used, representing a very powerful tool: reduces bias, improves the use of the data, avoiding the overfitting problem, especially with small datasets, produces robust and unbiased performance estimates regardless of sample size.⁵⁰ However, this limitation needs to be considered interpreting results.

The use of whole blood samples to examine the DNAm levels, which may not directly reflect the status of the target tissue, is another limit of the study. However, the analysis of preclinical samples with the main goal to monitor high MPM risk asbestos-exposed individuals should rely on a noninvasive biosample such as blood.

The coverage at disease relevant genes by CpG sites included in the DNAm Infinium Methylation EPIC beadchip array used in the study could be another limitation, as it investigates only 30% of the human methylome. In comparison, whole-genome bisulphite sequencing is able to capture more than 28 million CpGs, but the feasibility remains low for the population-based EWAS due to high cost and large genomic DNA input requirements to compensate for degradation during DNA bisulphite treatment.

The large EPIC cohort is a unique and very valuable resource, as the prospective nature of the study is more suitable to stratify high-risk individuals on the basis of genetic and epigenetic profiles in combination with other biomarkers and clinical risk factors. Moreover, since MPM diagnosis takes place only in the late stage of the disease, prospective cohort studies may be optimal for investigation of early MPM-related changes in the asymptomatic phase.

The identified DNAm biomarkers could be associated with germline mutations analysis in the follow-up of asbestos-exposed individuals with MPM genetic predisposition. Based on published studies from our group and others, germline mutations in several genes may increase MPM susceptibility even with low levels of asbestos-exposure, and possibly predict the response to standard treatments.^{51,52} Moreover, the detection of *BRCA*-mutated or *BAP1*-mutated MPM in a very early phase of tumour development could address a personalised therapy approach with PARP-inhibitors or immune checkpoint inhibitors.⁵³

Our study identified potential blood DNAm changes in preclinical MPM individuals. If further replicated in other studies, these signals could represent potential circulating biomarkers, promising for early MPM detection.

AUTHOR CONTRIBUTIONS

The work reported in the article has been performed by the authors, unless clearly specified in the text. **Alessandra Allione**: conceptualization, methodology, writing—original draft, project administration; **Clara Viberti**: formal analysis, data curation, writing—original draft; **Ilaria Cotellessa**: formal analysis, data curation, writing—original draft; **Chiara Catalano**: investigation; **Elisabetta Casalone**: conceptualization, methodology, project administration; **Giovanni Cugliari**: formal analysis, data curation; **Alessia Russo**: methodology; **Simonetta Guarrera**: methodology; **Dario Mirabelli**: data curation; **Carlotta Sacerdote**: resources, data curation; **Marco Gentile**: resources, data curation; **Fabian Eichelmann**: resources, data curation; **Matthias B. Schulze**: resources, data curation, writing—review & editing; **Sophia Harlid**: resources, data curation; **Anne Kirstine Eriksen**: resources, data curation; **Anne Tjønneland**: resources, data curation; **Martin Andersson**: resources, data curation; **Martijn E. T. Dollé**: resources, data curation; **Heleen Van Puyvelde**: resources, data curation; **Elisabete Weiderpass**: resources, data curation; **Miguel Rodriguez-Barranco**: resources, data curation; **Antonio Agudo**: resources, data curation; **Alicia K. Heath**: resources, data curation; **María-Dolores Chirlaque**: resources, data curation; **Thérèse Truong**: resources, data curation; **Dzevka Dragic**: resources, data curation; **Gianluca Severi**: resources, data curation; **Sabina Sieri**: resources, data curation; **Torkjel M. Sandanger**: resources, data curation; **Eva Ardanaz**: resources, data curation; **Paolo Vineis**: conceptualization; **Giuseppe Matullo**: conceptualization, supervision, project administration, funding acquisition.

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CONFLICT OF INTEREST

The authors declare no financial competing interests.

DATA AVAILABILITY STATEMENT






Array data has been deposited at the European Genome-phenome Archive (EGA), which is hosted by the EBI and the CRG, under accession number EGAS00001006432. Further information about EGA can be found on <https://ega-archive.org> “The European Genome-phenome Archive of human data consented for biomedical research” (<http://www.nature.com/ng/journal/v47/n7/full/ng.3312.html>).

Other data that support the findings of our study are available from the corresponding author upon request.

ETHICS STATEMENT

The study was approved by the Institutional Review Board of IARC and the ethics committees in the participating countries. All participants gave their written informed consent to participate in the EPIC study. The present study was carried out in accordance with the ethical principles of the Declaration of Helsinki.

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SUPPORTING INFORMATION

Additional supporting information can be found online in the Supporting Information section at the end of this article.

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