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Genomic profiling of tissue and blood predicts survival outcomes in patients with resected pleural mesothelioma

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ABSTRACT

Purpose: Pleural mesothelioma (PM) is an aggressive tumor still considered incurable, in part due to the lack of predictive biomarkers. Little is known about the clinical implications of molecular alterations in resectable PM tissues and blood. Here, we characterized genetic alterations to identify prognostic and predictive biomarkers in patients with resected PM.

Experimental Design: Targeted next-generation sequencing was performed in retrospective pleural tumor tissue and paired plasma samples from stage IB-IIIB resected PM. Association between prognosis and presence of specific mutations was validated *in silico*.

Results: Thirty PM tissues and paired blood samples from 12 patients were analyzed. High tissue tumor mutational burden (TMB) (>10 mutations/Mb), tissue median minor allele frequency (MAF) (>9 mutations/Mb), and blood TMB (>6 mutations/Mb), tissue *KMT2C, PBRM1, PKHD1,EPHB1* and blood *LIFR* mutations correlated with longer disease-free survival and/or overall survival. High concordance (>80%) between tissue and blood was found for some mutations.

Conclusions: Tissue TMB and MAF, blood TMB, and specific mutations correlated with outcomes in patients with resected PM and should be further studied to validate their role as prognostic biomarkers and potentially predictive factors for combinations with immune-checkpoint inhibitors. This suggest that molecular profiling could identify longer survivors in patients with resected PM.

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Background

Pleural mesothelioma (PM) is an aggressive malignancy that represents a highly unmet medical need. PM is still considered incurable and often portends a median survival in the one year range [1]. Diffuse PM is classified in three major subtypes (epithelioid, sarcomatoid, and biphasic mesothelioma) [2], and histology is the most relevant factor for selecting therapeutic approaches, including cytoreductive surgery and systemic treatments [3,4]. One of the most peculiar characteristics of PM is the highly heterogenous behavior of this disease, with a selected subgroup of mesothelioma patients benefitting from a surgery-based multimodal approach [5]. With the exception of the still under discussion role of germline *BAP1* mutations [6,7], there is a lack of molecular predictive and prognostic biomarkers with therapeutic value.

Most patients with PM present at advanced stages [8,9], where surgery is not an option. However, for patients at early stages, resection has clearly shown to improve the median overall survival (OS) of those with epithelioid (from ~15 to ~21 months) and biphasic tumors (from ~9 to ~15 months), demonstrating heterogeneity of outcomes between different histologies [9,10]. Over the last two decades, treatment of advanced PM has little changed since the approval of cisplatin/pemetrexed [1]. Recently, the combination of immune-checkpoint inhibitors (ICIs) nivolumab plus ipilimumab [11] has replaced cisplatin/pemetrexed approach as the standard of care for first line therapy in patients with improved outcomes in non-epithelioid and epithelioid PM [12]. However, no predictive biomarker for immunotherapy in PM has been identified to date and no targeted therapies have demonstrated efficacy in this disease. Thus, there is an urgent need for better understanding the histologic and molecular heterogeneity of this disease, in particular in patients undergoing resection, which could help identify novel biomarkers to stratify patients and select the most suitable for novel targeted therapies and/or immunotherapy-based combinations increasing the outcomes. This will definitely allow PM to move beyond the outdated concept of "*one size fits all*" and finally enter in the era of precision oncology.

Few studies have explored genomic alterations in tissues from resected PM while none evaluated them in plasma circulating tumor DNA (ctDNA). Tissue and blood tumor mutational burden (TMB) have been investigated as predictive and prognostic biomarkers for treatment with ICIs in several tumor types [13–16]. However, the role of tissue and blood TMB in PM remains still elusive. In the present study, we aimed to perform a comprehensive molecular analysis of both tumor and cell-free DNA (cfDNA) samples in patients with resected stage I-IIIB PM to identify the biological factors that might predict the outcome of patients with early-stage PM undergoing surgery.

Methods

Patients and study design

We retrospectively analyzed tissue and blood samples from patients with resected stage IB-IIIB PM from the National Mesothelioma Virtual Registry and Tissue Bank USA obtained by regional and national referrals. Blood and tissue samples were collected at the time of extended pleurectomy decortication after informed consent was obtained under IRB approval (HP-00094701). Inclusion criteria included individuals over 18 years old with diagnosis of PM determined to be eligible for lung sparing surgery by a multidisciplinary mesothelioma treatment team while patients unable or unwilling to provide consent for sample collection were excluded. A series of 30 pleural-based tumor specimens from 30 patients were collected. Eighteen matching plasma samples were collected and 12 of them passed DNA quality controls and were analyzed.

Genomic analysis

Nucleic acids extraction

For tissue specimens, experienced internal pathologists (P.P, G.T) morphologically evaluated hematoxylin and eosin–stained slides to verify the presence of at least 10% of neoplastic cells. Four 5 μm tissue slides were used to manually extract genomic DNA (gDNA) with the Mini Amp kit (Qiagen, Hilden, Germany). Briefly, after proteinase K digestion, gDNA was further purified following manufacturer instructions. Finally, nucleic acids were eluted in 30 μl of nuclease-RNAse free water (Thermo Fisher Scientific, Waltham, Massachusetts). For plasma samples, cfDNA was directly purified from a total of 1.5 mL aliquoted plasma using the QIAsymphony DSPVirus/Pathogen Midi Kit (Qiagen) on QIAsymphony platform (Qiagen) following a standardized internal workflow [17]. Finally, cfDNA was eluted in a final volume of 60μl nuclease-RNAse free water.

Nucleic acids quantification

Overall, gDNA was quantified on the TapeStation 4200 microfluidic platform by using a genomic ladder and buffer (Agilent Genomic ScreenTape, Agilent Technologies) and the Genomic ScreenTape device (Agilent Technologies) following manufacturer's recommendations. In detail, 1 μl of extracted cfDNA was combined with 10 μl of Genomic buffer on the corresponding Screen Tape. Data analysis was performed on a proprietary software. DNA amount (ng/μl) and fragment length (in terms of DNA Integrity Number) were inspected and annotated.

Next-Generation Sequencing analysis

A comprehensive molecular analysis of 409 genes mainly involved in cancer development and progression was carried out by using Oncomine™ Tumor Mutation Load Assay (Thermofisher Scientific, USA) on the Ion S5™ plus platform (Thermofisher Scientific) for tissue and liquid biopsy specimens [18]. Briefly, 10 ng of extracted gDNA was manually amplified following manufactured thermal conditions. Similarly, the maximum volume available for library amplification (6 μl) was adopted for cfDNA samples. Amplified libraries were diluted at 60 pM and pooled together for template preparation on Ion Chef system (Thermofisher Scientific) by adopting Ion 550 Kit—Chef (Thermofisher Scientific) following manufacturer instructions. Finally, pooled libraries were automatically loaded on 550™ chip (Thermofisher Scientific). Data were inspected with a default pipeline on the Ion Reporter torrent Suite (v.5.0.2.1; Thermofisher Scientific). In details, TMB was calculated as the total number of non-synonymous mutations per Mb. In addition, visual inspection was carried for detected mutations by using Genome Browser v.2.0.7 software (Golden Helix INC, MT, USA). Only variants that harbored $\geq 5\times$ allele coverage and quality score ≥ 20 within an amplicon coverage of at least 500 $\times$ and 1000X alleles for gDNA and cfDNA specimens, respectively, were called. Finally, the frequency of each mutant allele was recorded. Median minor allele frequency (MAF) from somatic mutations was calculated by comparing tissue MAF$>$ 50% Next-Generation Sequencing (NGS) data with corresponding plasma specimens.

In silico analysis

Analysis of the impact of mutational status or expression levels of genes of interest which showed association with the survival in the Cox univariate analysis was analyzed *in silico* in different cohorts of primary PM, including all stages (Stage I-IV). First, these mutations were evaluated in the TCGA cohort of patients with PM (n = 87) from the *cBioPortal* platform (https://www.cbioportal.org/). Second, the TCGA dataset of PM (containing 84 patients with primary tumors) was evaluated in the bioinformatic platform Survival Genie [19]. In this analysis,

the median value of expression of the genes of interest was used to divide the dataset in low vs high expression groups. Third, RNA expression levels were also evaluated in 59 patients with PM from the E-MTAB-6877 dataset available in the ArrayExpress repository. CEL files were retrieved and normalized using the RMA algorithm (Bioconductor affy package). Similarly, expression values of the available probes for *KMT2C* (Probe ID:17064603), *PBRM1* (Probe ID:16954856), *PKHD1* (Probe ID:17019935), *LIFR* (Probe ID:16984224 and 16995500) were log2 transformed and median centered.

Statistical analysis

Statistical analyses were performed using the SPSS Statistics software, Version 22.0 (IBM Corp., Armonk NY, US) while graphs were represented using the GraphPad Prism Version 8.4 (Graph-Pad Software Inc., San Diego CA, US) and R software (version 3.4.0, R Foundation for Statistical Computing, Vienna, Austria). Non-parametric test evaluated differences between variables. Survival risks were determined by Kaplan–Meier (log-rank test) and Cox Proportional-Hazards Regression with backward stepwise selection for the multivariate model. A first multivariate analysis was elaborated in all patients where tissue mutations and TMB along with clinical variables were included (n = 30). Then, a second analysis was performed in those with tissue MAF and blood mutations were also available (n = 12). Two-tailed p values < 0.05 were considered statistically significant.

Results

Clinical features

Tumor specimens were collected from the 30 PM patients as well as matching blood samples available in 12 (40.0%) of them. Patients had a median age of 68 years, asbestos exposure was reported in 66.7% of patients, and 46.7% of patients presented stage IIIB tumors. Additional clinical information can be found in Table 1**.** Clinical characteristics were correlated with the number of mutations, TMB and presence of the most commonly mutated genes in the tissues (Figure 1). No association was found between the presence of alterations in these genes and stage or histology. Higher frequency of *EPHB1* and *BLM* mutations was found in females ($p = 0.048$ & $p = 0.038$), *BAP1* mutations were associated with asbestos exposure ($p = 0.012$), and *PKHD1* was more commonly mutated in younger patients (<65 years) ($p = 0.001$).

Table 1
Patient clinical and prognostic characteristics:

Characteristics		Number of patients (%)
Gender	Men	23 (76.7%)
	Women	7 (23.3%)
Age (years)	Median (range)	68 (35–85)
Smoking habits	Current	2 (6.7%)
	Former	9 (30.0%)
	Never	19 (63.3%)
Asbestos exposure	Yes	20 (66.7%)
	No	10 (33.3%)
Laterality	Right PM	19 (63.3%)
	Left PM	11 (37.5%)
Histology	Epithelioid	25 (83.4%)
	Sarcomatoid	1 (3.3%)
	Biphasic	4 (13.3%)
ECOG	0	14 (46.7%)
	1	10 (33.3%)
	2	1 (3.3%)
	NA	5 (16.7%)
Stage	IB	5 (16.7%)
	II	3 (10.0%)
	IIIA	8 (26.7%)
	IIIB	14 (46.7%)
Neoadjuvant treatment	Yes	7 (23.3%)
	No	23 (76.7%)
Adjuvant treatment	Yes	14 (46.7%)
	No	16 (53.3%)
Disease free	Yes	6 (20.0%)
	No	24 (80.0%)
DFS (months)	Median (range)	9.5 (0–48.4)
Death	Yes	18 (60.0%)
	No	12 (40.0%)
OS (months)	Median (range)	18.0 (1–48.4)

When comparing tissue vs blood samples, higher number of total and somatic mutations as well as higher TMB were found in the tissue, while median MAF showed higher levels in the blood (Supplementary Figure 1). Median TMB in tissue was 7.56 mutations/Mb versus median 7.14 mutations/Mb found in the blood. The number, type of alterations, and their chromosomal distribution can be found in Supplementary Figure 2.

Mutations present in tissue

The most common mutations detected in tissue are represented in Figure 1 and Table 2. *EPHB1* alterations were present in 29 (93.3%) patients with c.2589G>A being the most commonly mutated gene. The second most common tissue alteration was *KTM2C*, found in 27 (90.0%) patients with c.850–91A>G as the most common alteration. Information about the function, type, and location of these specific alterations and their occurrence in blood samples can be found in Table 2. We evaluated the presence of co-mutations between these genes (Supplementary Figure 3). Interestingly, *BAP1* mutations presented co-occurrence, or positive correlation, with mutations in *PBRM1, PKHD1, and CSMD3,* while negative correlation with those in *LIFR* ($p < 0.05$). Moreover, *EPHB1* mutations co-occurred with *MTR* and *ARID1A* mutations ($p < 0.05$). Patients with mutated *EPBH1* presented higher number of mutations in tissue and patients with mutated *PBRM1*, *BAP1*, or *PKHD1* had higher number of total and also somatic mutations in tissue ($p < 0.05$).

Mutations present in blood

High prevalence of mutations in most of these genes was also found in blood, such as *COL1A1*, *THBS1*, *EPHB1*, *MTR, LIFR, PBRM1,* or *KMT2C*, as well as mutations in other genes such as *ZNF384*, *PLEKHG5*, and *MDM4* which showed low prevalence in tissues (Supplementary Table 1**)**. No association between these mutations and the clinical characteristics was found. Some most common blood and tissue specific mutations were highly co-occurrent, being genes such as *COL1A1*, *THBS1*, *EPHB1*, *MTR,* or *PBRM1,* altered in > 80% of the patients (Figure 2). In the blood, mutations such as *COL1A1*, *THBS1*, or *ZNF384* co-occurred as they were found in all cases (Supplementary Figure 4).

TMB, MAF, and specific mutations in tissue and blood predict outcomes

At the last follow-up of these patients, 6 (20%) remained disease-free and 12 (40%) were alive, median disease-free survival (DFS) was 9.5 months (range: 0 – 48.4) and median OS was 18 months (range: 0.1 – 48.4). No significant differences in survival were found in patients with epithelioid versus non-epithelioid (Supplementary Table S2). Along with the clinical characteristics, different cutoff for the levels of tissue and blood TMB and MAF were analyzed (Figure 3). We observed that longer DFS and OS were associated with higher tissue TMB (≥10 mutation/ Mb), blood TMB (≥6) and higher median tissue MAF (≥9) (Figure 3 **& 4**). No statistically significant cutoff for DFS or OS was found based on blood median MAF levels. Then, individual tissue and blood mutations were studied. As a result, patients with tissue mutations in *KMT2C*, *PBRM1*, or *PKHD1* showed longer DFS ($p < 0.05$), showing a superiority of more than 10 months in median DFS. In terms of OS, patients with tissue mutations in *PKHD1 or EPHB1* showed more than 14 months longer median OS (Figure 4) (Supplementary Table S3).

The multivariate analysis for DFS in the 30 patients revealed that

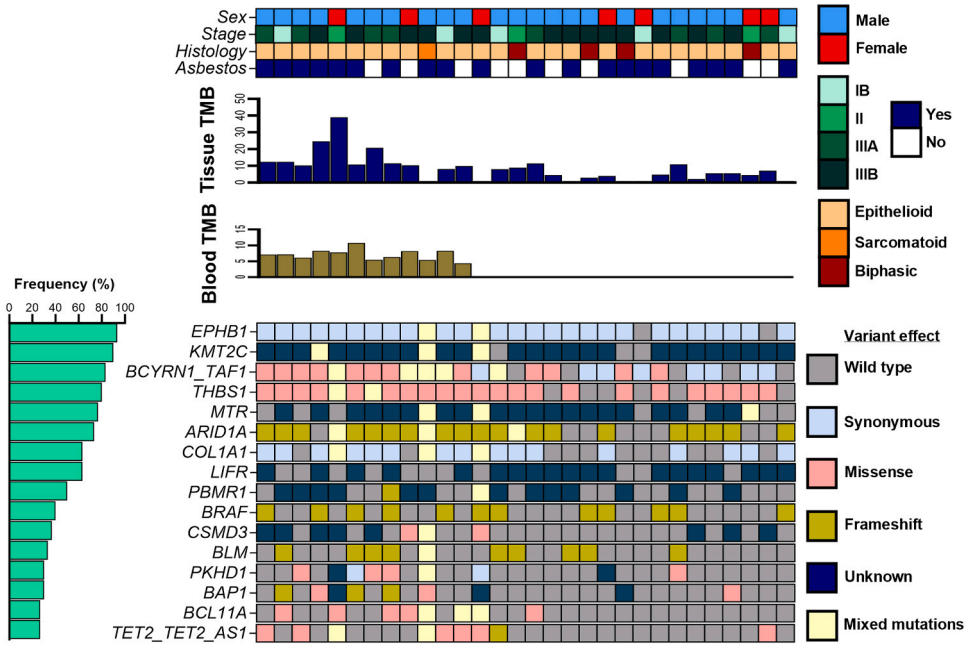

Fig. 1. Landscape of clinical and molecular characteristics in patients with PM. Clinical characteristics, tissue and blood tumor mutational burden (TMB), and most frequent alterations in the tissue. TMB is expressed in mutations per megabase.

Table 2
Most commonly mutated genes in tissues:

Mutated gene	Prevalence	Most common mutation	Function, type, and location	Prevalence+
EPHB1	28 (93.3%)	c.2589G>A	p.Lys863= , SNV synonymous, exonic (3q22.2)	27 (90.0%)
KMT2C	27 (90.0%)	c.850–91A>G	p.?, SNV unknown, intronic (7q36.1)	26 (86.7%)
BCYRN1_TAF1	25 (83.3%)	c.455A>G	p.Asp152Gly, SNV missense, exonic & intronic (2p21 & Xq13.1)	16 (53.3%)
THBS1	24 (80.0%)	c.2646G>T	p.Gln882His, SNV missense, exonic (15q14)	24 (80.0%)
MTR	23 (76.7%)	c.669+32_669+ 33insGTCT	p.?, indel unknown, intronic (1q43)	23 (76.7%)
ARID1A	22 (73.3%)	c.3429delG	p.Gln1145ArgfsTer16, indel frameshift deletion, exonic (1p36.11)	22 (73.3%)
COL1A1	19 (63.3%)	c.1149C>T	p.Gly383= , SNV synonymous, exonic (17q21.33)	17 (56.7%)
LIFR	19 (63.3%)	c.143–11_143–10delGT	p.?, indel unknown, intronic (5p13.1)	17 (56.7%)
PBRM1	15 (50.0%)	c.4576+3_4576+ 4insAGG	p.?, indel unknown, intronic (3p21.1)	14 (46.7%)
BRAF	12 (40%)	c.87_88delCGinsGC, c.87delC	[Ala29= ;Gly30Arg], frameshift deletion, exonic (7q34)	10 (33.3%)
CSMD3	11 (36.7%)	c.4723T>C	p.Tyr1575His, missense, exonic (8q23.3)	8 (26.7%)
BLM	10 (33.3%)	c.1025delT	p.Leu342CysfsTer7, frameshift deletion, exonic (15q26.1)	9 (30.0%)
PKHD1	9 (30.0%)	c.7184T>A	p.Phe2395Tyr, SNV missense, exonic (6p12.3-p12.2)	2 (6.7%)
BAP1	9 (30.0%)	c.*60C>T	p.?, SNV unknown, UTR_3 (3p21.1)	1 (3.3%)
BCL11A	8 (26.7%)	c.499C>A	p.Pro167Thr, SNV missense, exonic (2p16.1)	7 (23.3%)
TET2_TET2_AS1	8 (26.7%)	c.3083T>A	p.Met1028Lys, missense, exonic (4q24)	6 (20.0%)

+ Prevalence of the most common mutation.

higher tissue TMB is an independent predictive factor associated with better outcomes (HR: 0.34, $p = 0.045$) being modulated by tissue mutations in *PBRM1* (Supplementary Figure 5A). Similarly, in the multivariate analysis for OS, patients with higher tissue TMB shower better OS as well as those under 65 years old (HR: 0.28, $p = 0.033$ & HR: 0.15, $p = 0.029$, respectively). The independent analysis of the 12 patients where tissue and blood samples were available revealed that tissue median MAF was the predictor for DFS (HR: 0.30, $p = 0.030$) (Supplementary Figure 5B) and higher tissue TMB and presence of *LIFR* mutation in blood were independently associated with longer OS (HR: 0.40, $p = 0.010$ & HR: 0.07, $p = 0.016$, respectively) (Supplementary Figure 5B).

Predictive role of specific mutations in tissue are validated in silico

We analyzed *in silico* the association between the *KMT2C*, *PBMR1*, *LIFR*, *PKHD1*, and *EPHB1* mutations or RNA levels and the outcomes in publicly available databases of patients with different stages of PM (Stage I-IV). Very limited data was reported on DFS in these databases and validation analyses could only be performed for OS. First, the TCGA dataset including 87 patients, 82% of them on early stages, with median OS of 21.7 months, showed a mutation frequency of *KMT2C* (1.2%), *PBMR1* (5.7%), *LIFR* (0%), *PKHD1* (3.4%), and *EPHB1* (1.2%). None of these mutations showed an association with the OS ($p > 0.05$). Second, the TCGA dataset including 84 patients with PM reported expression of these genes and classified patients into high/low expression by the median value, having 42 patients in each group. As a result, we found that patients with low *PKHD1* presented shorter OS ($p = 0.003$) and those with low *LIFR* showed higher OS ($p = 0.05$) (Supplementary Figure 6A). Third, we analyzed the 59 patients with PM included in the E-MTAB-6877 dataset that also analyzed RNA expression levels of some of these genes and classified patients by the median value. In this cohort, low levels of *PBRM1* were associated with longer OS ($p = 0.044$) (Supplementary Figure 6B).

Discussion

The prognosis of patients with PM remains dismal due to multiple

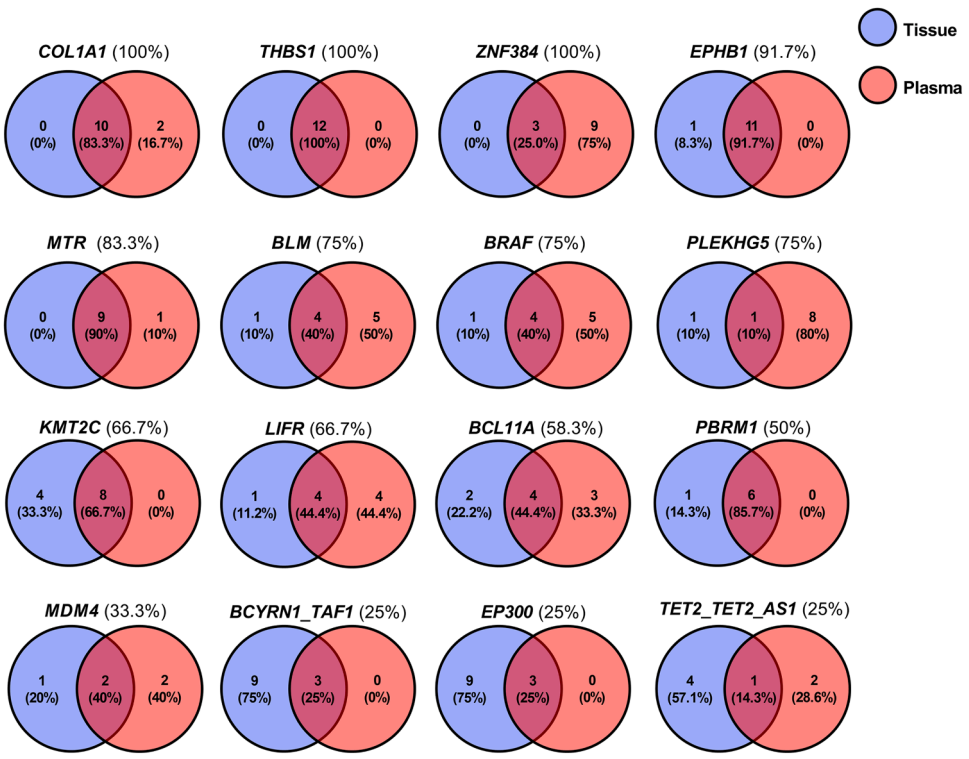

Fig. 2. Co-mutations in paired tissue and plasma samples: The most common mutated genes in plasma are ordered by their mutation presence in the 12 patients with plasma availability (%). Presence of the mutation in tissue is included in blue, red indicates presence in plasma and combination shows number and percentage of patients with presence in both.

factors including the aggressive nature of the disease, limited treatment options, and lack of predictive and prognostic biomarkers that could stratify patients into risk groups. Few studies have analyzed the genomic landscape of patients with PM, [7,8,20,21] regardless of tumor stage and treatments. These studies focused exclusively on tissue, with very limited data extracted from liquid biopsy strategies, such as the DNA methylation changes in blood cells [22] and none specifically on resected PM. Tissue samples may carry a potential bias due to the intra-tumoral heterogeneity that hinders biomarker discovery, but which liquid biopsy could overcome. To the best of our knowledge, we present here the first study to combine tissue and blood DNA sequencing for the identification of biomarkers in patients with PM undergoing resection.

We found that patients with high tissue TMB (≥10 mutations/Mb) and high tissue MAF (≥9 mutations/Mb) had better outcomes. Even if the association of survival outcomes and TMB is still controversial due to the lack of standardization of sequencing platforms and cut-off values used among other variables, several studies have demonstrated that tissue TMB ≥ 10 mutations/Mb has been one of the classical cut-off used in solid tumors including PM [21,23]. Moreover, levels of TMB with ≥ 10 mutations/Mb have been associated with better response to immunotherapy [24]. We observed a median tissue TMB of 7.56 mutations/Mb, which is similar to the median 8.3 mutations/Mb observed in other studies in mesothelioma [25]. Interestingly, 36.7% patients presented high tissue TMB (≥10 mutations/Mb), a high value in comparison with other studies where only 1–2% of patients showed high tissue TMB [21,26] or where the median TMB was 3.1 mutations/Mb [27]. This way, PM has been traditionally considered a tumor with low TMB when using whole genome sequencing, however, other techniques, such as mate-pair sequencing, have revealed higher values that could be more associated with the modest response to ICIs observed in PM [28]. Of particular interest is the observed association of high blood TMB (≥6 mutations/Mb) and better outcomes, suggesting a potential role of liquid biopsy in these patients. To date, no other studies in PM have analyzed the predictive role of blood TMB. However, even if it is not fully comparable, our results concur with others trials in metastatic lung cancer which showed that high blood TMB is associated with longer OS after immunotherapy [29]. Despite technical aspects such as sample collection, timepoints, cut-offs, or gene panels need to be standardized and larger prognostic studies need to validate these associations, tissue and blood TMB are promising prognostic biomarkers that could be used in the regular routine follow-up of patient with PM.

We identified recurrent tissue mutations in *BAP1*, a tumor suppressor gene involved in DNA damage repair commonly mutated in PM. Mutations in this gene had similar prevalence in our cohort to previously reported studies (30% and 27%, respectively) [7,20]. In particular, we found higher frequency of *BAP1* mutations in patients with asbestos exposure, which concurs with the previously described predisposition to asbestos-related mesothelioma in patients with mutated *BAP1*. Mutations in other chromatin-remodeling genes were found with high prevalence and associated with survival of these patients. *KMT2C* showed a mutation rate of 90% compared to the 2–18% observed in previous studies of PM and NSCLC [21,30], and potential association with longer DFS. Little is known about this specific mutation in PM, however, it has been associated with a better response to ICIs in NSCLC patients, even in those with low TMB, suggesting that it could be used as a predictive biomarker for this treatment [30,31]. *PBRM1* mutations also showed high prevalence (50%) in comparison to the previously reported 2–9.1% occurrence in PM [20,21], but similar to the reported 46% in other types of mesotheliomas [32,33]. Patients with *PBRM1* mutated tumors presented better outcomes which has previously been described in patients with renal carcinomas [34,35]. Interestingly, we identified mutations in *PKHD1* in 30% of the patients, which were associated with better DFS and OS. This has not been explored in mesotheliomas, but was found to be protective against colorectal carcinoma [36]. On the other hand, we observed that patients with mutations in *KMT2C* presented higher TMB and those with mutated *PBRM1, BAP1*, or *PKHD1* had higher number tissue mutations, which could be explained by their role at maintaining

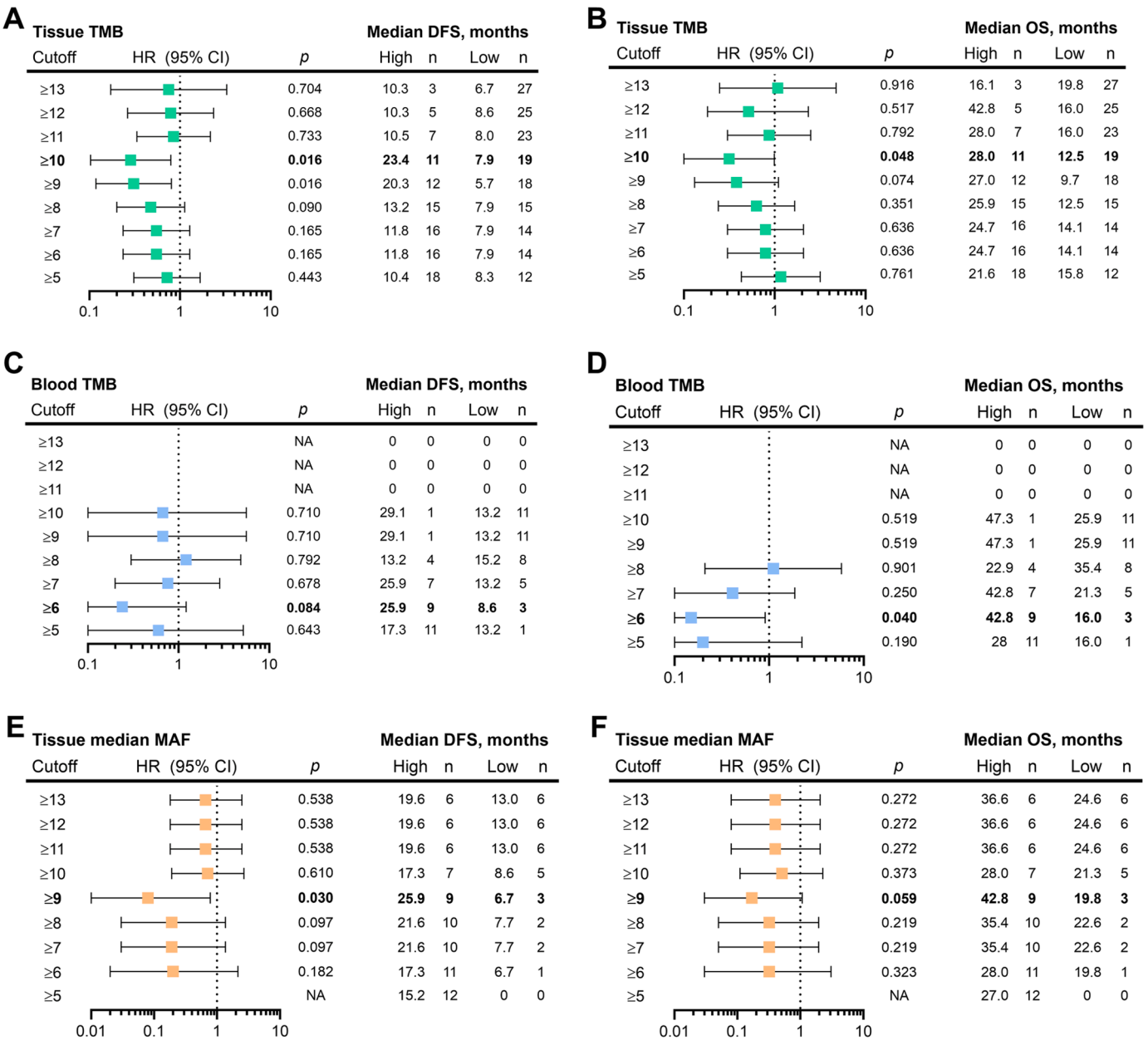

Fig. 3. Tissue and blood TMB and MAF as predictors for survival. These graphs represent the Hazard Ratio (HR), 95% confidence interval (CI), p value, median value, and number of patients in the high and low group based on the different potential cutoffs. Cutoffs for TMB in tissue for DFS (A) and OS (B); TMB in blood for DFS (C) and OS (D); and MAF in tissue for DFS (E) and OS (F). Selected cutoffs are presented in bold characters. No significant cutoff was found for blood median MAF as it was not represented.

genomic instability. The *in-silico* validation in the limited available datasets of patients with PM showed that mutations of these were not associated with the outcome in the TCGA dataset containing 87 patients. On the contrary, the other two datasets that contained expression levels of these genes suggested that *PKHD1*, *LIFR*, and *PBRM1* were associated with the OS of patients with PM.

The multivariate analysis of our entire cohort revealed that tissue TMB was an independent predictive biomarker associated with longer DFS and OS. The DFS was also improved by mutations in *PBRM1* while lower than 65 years of age was an independent factor for longer OS. Interestingly, mutations in *PKHD1,* associated with better outcomes in the univariate analysis as mentioned above, were more frequent in younger patients (<65 years). The analysis of the patients with available blood data revealed that, along with high tissue TMB, blood mutation in *LIFR* was associated with longer OS. *LIFR* encodes a transmembrane cytokine receptor involved in several tumor mechanism such as DNA damage responses, tumor growth, angiogenesis, and metastasis and its overexpression has been associated with shorter survival in solid tumors. Moreover, it modulates the tumor immune microenvironment by the regulation of CXCL9 in tumor-associated macrophages and the prevention of CD8 + T cell tumor-infiltration, which hinders the effect of anti-PD1 therapy. LIFR targeting could be a potential combinatory treatment with immunotherapy and blood *LIFR* analysis could work as a biomarker for prediction to immunotherapy resistance in this type of patients [37]. While the co-occurrence of this mutation in tissue and blood was only 44.4%, *KMT2C* or *PBRM1* showed an 67% and 85.7% co-occurrence, respectively, where the blood was able to identify the mutational status of the tumor in most cases. The high co-occurrence and frequency in tissues and blood manifest the promising role of these mutations as clinical parameters in PM. Moreover, we confirmed

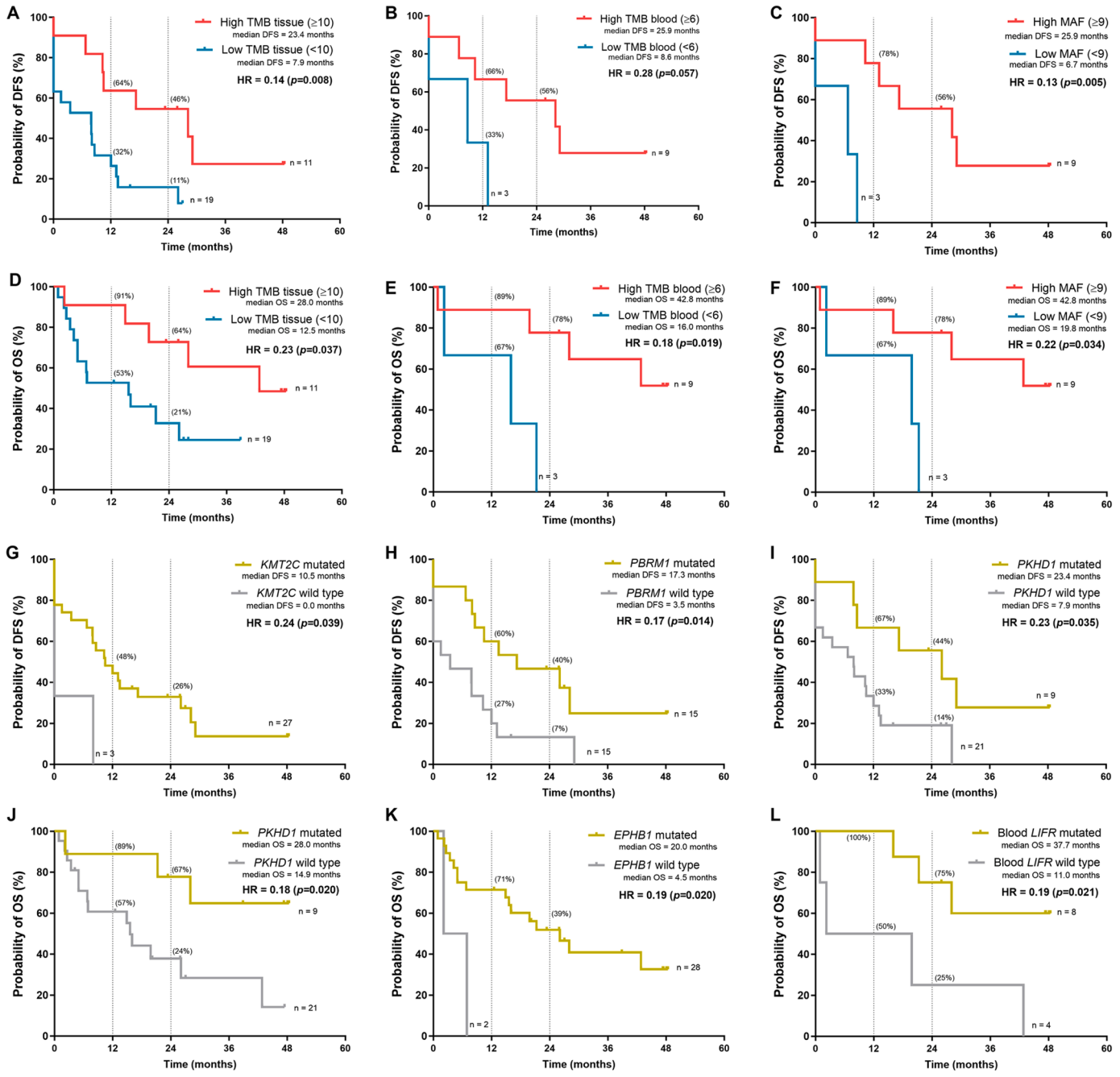

Fig. 4. Tissue and blood TMB, *high tissue MAF, and specific tissue and blood mutations* reveal longer DFS and/or OS. (A-F) Higher tissue TMB, blood TMB, and high tissue MAF correlated with longer DFS and OS. (G-L) Patients with tissue mutations in *KMT2C*, *PBRM1* & *PKHD1*, *EPHB1* and blood *LIFR* experienced better outcomes in terms of DFS and/or OS. The displayed percentages (%) show the patients free of recurrence or alive at 12 and 24 months.

the feasibility for the detection of plasma ctDNA mutations by NGS and the potential use of blood as a tissue surrogate in patients with early PM. This ratifies the results of the only previous study analyzing tissue and plasma in 14 patients with PM, however, they did not evaluate early stages [38].

We found no association between tissue *BAP1* mutational status and outcomes in concordance to previous observations in PM [7]. However, in concordance with previous reports, BAP1 mutation was highly correlated with *PBRM1,* another tumor suppressor gene located in the 3p21 [21], but also with *PKHD1*, which were associated with the outcomes. This could suggest that *BAP1* mutational status could still be of importance in future studies in PM based on its role as a deubiquitinating enzyme and modulating gene transcription [7,20]. Epithelioid PM usually present better prognosis than non-epithelioid [10]. However, we found no significant differences in the outcomes between these subtypes in our cohort patients. This could be related to low number of non-epithelioid patients enrolled (16.6%) but also to the reported differences in administration of neo- and adjuvant treatments.

As abovementioned, our results showed high levels of TMB in PM in comparison to other studies, which could be linked to a larger variety of tumor specific neoantigens and a better efficacy with ICIs [20]. High tissue and blood TMB levels were associated with better outcomes and these could be also potentially used to predict the response to ICIs [39]. Moreover, *KMT2C or PBRM1* mutations have also described as modulators of the immune response and should be investigated for a potential role as biomarkers for immunotherapy combinations [30,31,40].

Nonetheless, we consider our study hypothesis generating research and recognize its limitations, including the small sample size and heterogeneity in stages, histology, and treatments in our cohort, the availability of blood samples in only 40% of the cases, the only available limited mutation or RNA level *in silico* validation, and the lack of a real patient validation cohort. Another potential limitation is the high TMB values observed in these patients which could be associated to the high frequency of gene alterations not previously described as common in patients with PM. Thus, larger studies in early PM are needed to validate these mutations and their clinical relevance. Moreover, future studies focusing on the novel combinations with ICIs are warranted.

Taken together, our findings provide novel insight on the genomic landscape present in tissues and blood from patients with early PM and candidate biomarkers for the prediction of the survival after surgery and potentially to treatment combinations with ICIs.

Ethics approval and consent to participate

Patients samples were collected after providing informed consent and the study was approved by IRB (HP-00094701). The study was performed in accordance to the Declaration of Helsinki.

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CRediT authorship contribution statement

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Declaration of Competing Interest

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Appendix A. Supporting information

Supplementary data associated with this article can be found in the online version at doi:10.1016/j.ejca.2023.113457.

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