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Prognostic and predictive aspects of the tumor immune microenvironment and immune checkpoints in malignant pleural mesothelioma

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32 **List of abbreviations**

33

34 APC	Antigen presenting cell
35 FFPE	Formalin-fixed paraffin embedded
36 IFN-γ	Interferon-gamma
37 IHC	Immunohistochemistry
38 IL	Interleukin
39 LAG-3	Lymphocyte activation gene-3
40 MPM	Malignant Pleural Mesothelioma
41 NSCLC	Non-small-cell lung cancer
42 PD-1	Programmed death-1
43 PD-L1	Programmed death-ligand 1
44 TAM	Tumor associated macrophage
45 TGF	Transforming growth factor
46 TILs	Tumor infiltrating lymphocytes
47 TIM-3	T-cell immunoglobulin mucin-3
48 TME	Tumor microenvironment
49 Treg	Regulatory T-cell

50

Abstract

Malignant pleural mesothelioma (MPM) is an aggressive cancer with a poor prognosis and an increasing incidence, for which novel therapeutic strategies are urgently required. Since the immune system has been described to play a presumed role in protection against MPM, characterization of its tumor immune microenvironment (TME) and immune checkpoints can identify new immunotherapeutic targets and their predictive and/or prognostic value. To characterize the TME and the immune checkpoint expression profile we performed immunohistochemistry (IHC) on formalin-fixed paraffin embedded (FFPE) tissue sections from 54 MPM patients (40 at time of diagnosis, 14 treated with chemotherapy). We stained for PD-1, PD-L1, TIM-3, LAG-3, CD4, CD8, CD45RO, granzyme B, FoxP3 and CD68. Furthermore, we analyzed the relationship between the immunological parameters and survival, as well as response to chemotherapy. We found that TIM-3, PD-1 and PD-L1 were expressed on both immune and tumor cells. Strikingly, PD-1 and PD-L1 expression on tumor cells was only seen in unpretreated samples. No LAG-3 expression was observed. CD45RO expression in the stroma was an independent negative predictive factor for response on chemotherapy, while CD4 and TIM-3 expression in lymphoid aggregates were independent prognostic factors for better outcome. Our data propose TIM-3 as a promising new target in mesothelioma. Chemotherapy influences the expression of immune checkpoints and therefore further research on the best combination treatment schedule is required.

Introduction

Malignant pleural mesothelioma (MPM) is an aggressive and fatal cancer that is causally associated with previous asbestos exposure in most afflicted patients. Although a rare disease, MPM incidence has been increasing in recent years and this trend is expected to continue over the next decades. This is mainly due to the ongoing asbestos consumption in developing countries, as well as the long latency period between exposure to asbestos and disease onset. [1] Palliative platinum-antifolate chemotherapy has a significant but moderate impact on patients' outcome, resulting in a median overall survival of about one year compared to the 8-10 months observed for chemotherapy-naïve patients. [2-4] Based on its poor prognosis and increasing incidence, novel therapeutic strategies for MPM are required.

The discovery of immune checkpoints such as cytotoxic T-lymphocyte antigen-4, programmed death-1 (PD-1), T-cell immunoglobulin mucin-3 (TIM-3) and lymphocyte activation gene-3 (LAG-3), introduced a new era in targeted cancer therapy. Several monoclonal blocking antibodies have already shown promising results in different cancer types. [5] Their rationale is to reactivate silenced immune responses by neutralizing the so-called immune checkpoints, which are proteins that induce immune cell exhaustion and tolerance. Characterization of the tumor immune microenvironment (TME) could be of great value to unravel these silenced immune responses. Expression of programmed death-ligand 1 (PD-L1) on tumor cells and TILs has been described in literature. [6-11] However, only one series described PD-1 expression on tumor infiltrating lymphocytes (TILs) [8] and nothing has been reported yet on TIM-3 and LAG-3 in human MPM tissue.

Chemotherapy influences the TME, including the expression of immune checkpoints. An upregulation of PD-1 and/or PD-L1 after chemotherapy for leukemia, thymic epithelial tumors and ovarian cancer has been reported. [12-15] Identification of the effect of chemotherapy on the TME in MPM can guide the rational design of combination strategies of immune checkpoint inhibition with chemotherapeutics. [16-18]

We investigated the expression of TIM-3 and LAG-3 in human MPM tumor tissue using immunohistochemistry (IHC), along with several other immune cell markers of the TME,

100 and addressed their potential role as targets for immunotherapy. In order to elucidate the
101 effect of chemotherapy on the TME, we compared tissue sections from unpretreated and
102 chemotherapy pretreated patients. We furthermore analyzed the prognostic and predictive
103 value of different immunological parameters.
104

105 ## Results

106 ### Clinicopathological features of the MPM patient cohort

107 The clinicopathological characteristics of our MPM cohort are summarized in Table 1. All 54
108 patients were diagnosed between 2000 and 2015. Forty samples were taken at the time
109 of diagnosis and fourteen samples were treated with chemotherapy (detailed information
110 on the pretreated samples see table S1). The unpretreated samples consisted of 9 biphasic,
111 26 epitheloid and 5 sarcomatoid cases, while the pretreated samples comprised 1 biphasic
112 and 13 epitheloid cases. The median age of the unpretreated patients was 69 years and
113 63 years for the pretreated patients. In both groups, patients were predominantly male.
114 At the time of last follow-up, 22% of the unpretreated and 36% of the pretreated patients
115 were still alive. Except for age (p=0.043), no significant differences were found between
116 the clinicopathological parameters of both groups.

117 ### Immune composition of MPM tissue samples and biomarker identification

118 The tissue sections were analyzed for the presence of lymphocytes, lymphoid aggregates
119 and stroma (Table 2). Lymphocytic infiltration was found in all tissue samples (figure 1).
120 Samples from pretreated patients showed more infiltration than from unpretreated ones.
121 A stromal score of 1 was observed in more than half of the unpretreated samples while the
122 majority of the pretreated samples had a stromal score of 2 or 3. Lymphoid aggregates
123 were present in more than half of the unpretreated and pretreated samples (65% and
124 71%, respectively). Germinal centers within the aggregates were seen in around one third
125 of the samples (27% unpretreated, 30% pretreated).
126 Tissue sections were stained for 6 different immune cell markers (Table 2; Fig. 2 A-E).
127 Percentages of TILs in the stroma ranged from 20% to 80%. CD4+ and CD8+ cells showed

128 a strong intensity in the TILs and in hot spots of the lymphoid aggregates (Fig. 2 A). CD8+
129 TILs were present in all samples and were the predominant cell type of the immune
130 infiltrate over CD68+ cells, CD45RO+ cells and CD4+ TILs, with 70% of the unpretreated
131 and 57% of the pretreated samples showing CD8+ expression on more than 50% of the
132 lymphocytes.
133 Although present to a lesser extent than CD8+ TILs, CD68 expression on histiocytes and
134 macrophages was also seen in all samples (Fig. 2 B). The majority of samples had less
135 than 50% CD68+ cells. Similar observations were made for CD45RO, a marker for effector
136 and memory T cells (Fig. 2 C). Both CD68 and CD45RO expression in the stroma were
137 significantly correlated with stromal presence of CD4+FoxP3+ cells (R=0.41, p=0.002;
138 R=0.27, p=0.046). Results of a multivariate analysis revealed that an increase in CD45RO
139 expression on stromal lymphocytes was significantly associated with a lower likelihood of
140 partial or complete response to chemotherapy [odds ratio (OR)=0.06, p=0.008; Fig. 3 A;
141 Table S2].
142 CD4+ TILs in the stroma were seen in 75% of the unpretreated and 71% of the pretreated
143 samples. After multivariate analysis the presence of CD4+ lymphocytes in the lymphoid
144 aggregates was a significant good prognostic factor [risk ratio (RR)=0.13, p=0.014; Fig. 4
145 A; Table S3]. For each increase of CD4 expression with one category, there is a lower risk
146 of death. No significant differences were found between the unpretreated and pretreated
147 samples. A subset of the CD4+ cells was also FoxP3+ (Fig. 2 D) with a range from 1% till
148 50% CD4+FoxP3+ positive cells in the samples (data not shown). Those CD4+FoxP3+
149 cells were positively correlated with the presence of CD4+ TILs in the stroma [correlation
150 coefficient (R)=0.52, p<0.001].
151 Moderate positivity for granzyme B was observed in the cytoplasm of immune cells in the
152 stroma (mainly plasma cells and mast cells [19,20]) of less than half of the samples (33%
153 unpretreated, 43% pretreated) (Fig. 2 E).

154

155 **Immune checkpoint expression in MPM tissue**

156 A cytoplasmic granular staining with a moderate to strong intensity was observed for PD-L1
157 (Fig. 2 F). Four samples (3 unpretreated and 1 pretreated) also showed membrane
158 staining. PD-L1 was seen on TILs in the stroma, in lymphoid aggregates and in germinal
159 centers of both unpretreated and pretreated samples. Significant differences for PD-L1
160 expression in the stroma were observed according to the different histological subtypes.
161 Sarcomatoid histology showed more PD-L1 expression than the epitheloid and biphasic
162 subtypes ($p<0.001$, $p=0.008$; data not shown). Strikingly, PD-L1 expression on tumor cells
163 was only detectable in unpretreated tumor samples. 28% of the unpretreated samples with
164 PD-L1+ tumor cells also had PD-L1+ TILs in their stroma (Table 3). Presence of PD-1+
165 tumor cells and CD8+ TILs was seen in 40% of those samples. CD4+FoxP3+ cells were
166 seen in the majority of samples that had PD-1+ tumor cells and CD4+ TILs (Table 3).
167 PD-1 expression was localized in the cytoplasm showing membrane accentuation with a
168 moderate intensity (Fig. 2 G). A strong intensity was found on lymphocytes with in the
169 germinal centers of lymphoid aggregates. PD-1 was seen on TILs of both unpretreated and
170 pretreated samples (65% and 71% respectively), while the expression on tumor cells was
171 found only in 10% of the unpretreated samples. In 10% of those samples, expression of
172 PD-1 on both tumor cells and TILs was observed (Table 3). CD8+ TILs together with PD-
173 1+ tumor cells were also seen in 10% of the unpretreated samples (Table 3). The same
174 percentage was found for the expression of CD4 and the co-expression of CD4 and FoxP3
175 in unpretreated samples (Table 3). According to a univariate analysis the presence of PD-
176 1+ TILs in the stroma was associated with smaller likelihood of response after
177 chemotherapy (OR=0.50, $p=0.0514$; Fig. 3 B; Table S2). PD-1+ TILs were positively
178 correlated with CD4+FoxP3+ and granzyme B+ cells in the stroma ($R=0.36$, $p=0.008$;
179 $R=0.3$, $p=0.014$). 62% of the unpretreated samples with PD-1+ TILs also had PD-L1+ TILs
180 and 50% of those samples showed PD-L1+ tumor cells (data not shown). In contrast, only
181 30% of the pretreated samples with PD-1+ TILs also had PD-L1+ TILs (data not shown).
182 No PD-L1+ tumor cells were observed in the pretreated samples. After univariate analysis,
183 high expression of PD-1 in the aggregates was a good prognostic factor (RR=0.70,

184 p=0.029) associated with a lower risk of death than the lower expression category (Fig. 4
185 B; Table S3). No significant differences were found between the unpretreated and
186 pretreated samples. We observed that PD-1, PD-L1 and TIM-3 were expressed in lymphoid
187 aggregates but only PD-1 and PD-L1 were also expressed in their germinal centers.
188 TIM-3 scoring was based on the cytoplasmic (Fig. 2 H) and membrane staining of cells.
189 However, often a weak nuclear staining was seen in more than half of the tumor cells in
190 the tissue section, possibly indicating the translocation of TIM-3 proteins from the
191 cytoplasm to the nucleus. TIM-3 expression was found on tumor cells in unpretreated and
192 pretreated samples (40% and 36%, respectively). It was also expressed on TILs and on
193 plasma cells in the stroma however, less often in pretreated samples compared to the
194 unpretreated (29% vs 40%). The presence of TIM-3+ tumor cells in combination with
195 CD8+ TILs was most observed, followed by the expression of PD-1+ TILs in combination
196 with TIM-3+ tumor cells (Table 3). A strong correlation was found between the presence
197 of TIM-3+ TILs and PD-1+ TILs in the stroma (RR=0.48, p<0.001). TIM-3+ lymphocytes
198 were found in lymphoid aggregates of more than half of the unpretreated and pretreated
199 samples (54% and 60%) and were correlated with both TIM-3+ TILs and CD4+ TILs in the
200 stroma (RR=0.64, p<0.001; RR=0.42, p=0.010). Expression of TIM-3 on lymphocytes in
201 the aggregates was found to be an independent good prognostic factor after multivariate
202 adjustment (Fig. 4 C; Table S3). Overall survival was better for patients with high TIM-3
203 expression in their aggregates (RR=0.47, p=0.002). No significant differences were found
204 between the unpretreated and pretreated samples.
205 All samples were negative for LAG-3 (Fig. 2 I), in contrast to the control sample showing
206 cytoplasmic staining with strong intensity on lymphocytes (Fig. 2 J).
207

208 **Discussion**

209 In this series, we report a comprehensive description of the TME in MPM. In summary, we
210 are the first to describe the presence of TIM-3 and absence of LAG-3 expression in MPM
211 tissue, as well as PD-1 expression on MPM tumor cells. PD-1 and CD45RO expression in
212 the stroma were associated with worse response to chemotherapy. After multivariate

213 analysis stromal CD45RO expression remained a negative predictive factor for response to
214 chemotherapy. Expression of PD-1, CD4 and TIM-3 in lymphoid aggregates were good
215 prognostic factors after univariate analysis. CD4 and TIM-3 expression in lymphoid
216 aggregates remained independent good prognostic factors after multivariate adjustment.
217 PD-L1 expression was observed in 68% of the unpretreated samples using a cut off value
218 of ≥1%. Differences with other series results [7,9-11,21] might be due to the use of different
219 antibody clones, sample sizes or cut off values. Teng et al. [22] described a classification of
220 tumors into 4 groups based on their pretreatment PD-L1 expression status and the
221 presence of TILs, that might be used to predict a patient's response to anti PD-1/PD-L1
222 blockade. In our own MPM cohort TILs were present in all unpretreated samples. 40% of
223 the tumor samples can be classified as type I (PD-L1+TILs+), while the others are type IV
224 (PD-L1-TILs+). Type I tumors with adaptive immune resistance have been described to be
225 the most likely type to benefit from anti PD-1/PD-L1 therapy [23], suggesting that 40% of
226 our unpretreated MPM patients would respond to this checkpoint blockade. It is suggested
227 that other suppressors might be present in the type IV TME leading to immune tolerance,
228 thus targeting other suppressive pathways might offer an alternative treatment approach
229 for these types of cancer. We saw that PD-L1 on tumor cells was not always expressed
230 simultaneously with PD-L1 on TILs and other stromal components, which is in concordance
231 with the findings in other tumor types. [23] Presence of both PD-1+ and PD-L1+ TILs was
232 observed in unpretreated and pretreated samples. This might reflect a potential
233 immunosuppressive microenvironment created by the interaction between PD-1 and PD-
234 L1.
235 PD-1 was expressed to the same extent on immune cells in unpretreated and pretreated
236 samples. We found PD-1 expression on TILs in 65% of the unpretreated samples, which is
237 in line with the 62% reported by of Combaz-Lair et al. in MPM. [8] Our data are the first to
238 report PD-1 expression on TILs in the stroma as a negative predictive factor associated
239 with worse response on chemotherapy in MPM. This is in line with the finding of Zhang et
240 al. that large B-cell lymphoma patients with low PD-1 expression on T cells are more likely
241 to respond to chemotherapy. [24] Results derived from an immunodeficient mesothelioma

242 mouse model suggest that the effect of pemetrexed is mediated through activation of
243 CD8+ T cells, rather than direct killing of tumor cells. [25] Since more PD-1 expression can
244 point at exhausted CD8+ T cells, this might decrease the antitumor efficiency of
245 pemetrexed. PD-1 on lymphocytes in aggregates on the other hand was a prognostic factor
246 for better overall survival. Percentages of positive lymphocytes in the aggregates ranged
247 from 1% to 50%, stained with a weak to moderate intensity suggesting these are activated
248 PD-1+^{LOW} cells. It has been described that these cells are still functional, able to secrete
249 IFNγ, resulting in activation of other immune cells that play a role in the antitumor
250 response. [26] We observed PD-1 expression on tumor cells in unpretreated but not in
251 pretreated samples, which has not been described in other cancer types so far. Since a
252 rather low number of pretreated samples was used in our series, future studies including
253 larger validation cohorts are needed to draw any meaningful conclusions.
254 Although until now TIM-3 expression has been predominantly shown on T-cells, our data
255 demonstrate TIM-3 expression also on MPM tumor cells which is consistent with findings in
256 melanoma, NSCLC and renal cell carcinoma. [27-29] While PD-1 was only expressed on tumor
257 cells in 10% of the unpretreated samples, TIM-3 expression was observed on tumor cells
258 in both unpretreated and pretreated samples. More unpretreated samples had TIM-3+ TILs
259 compared to the pretreated, taken into consideration that our number of pretreated
260 samples is rather low. TIM-3 blockade shows promising results in vitro and in vivo in
261 several cancer types [30,31], but nothing has been described for mesothelioma so far. We are
262 the first to report TIM-3 expression in lymphoid aggregates as an independent prognostic
263 factor associated with better overall survival in MPM. Our data support further research on
264 TIM-3 as a target of new treatment strategies of MPM and advocate to prioritize clinical
265 translation of TIM-3 above LAG-3, which has not been detected in our tumor samples. In
266 this context, patients are currently being recruited for a phase 1 trial of an anti-TIM-3
267 blocking antibody in patients with solid tumors (NCT02817633, *ClinicalTrials.gov*).
268 The effect of chemotherapy on tumoral PD-L1 expression has previously been investigated
269 in several cancer types. Still, data about the influence of chemotherapy on the TME remain
270 contradictory. For this series, samples of only 14 pretreated patients were at our disposal

271 and thus no strong conclusions can be drawn based on our results. In our hands, PD-L1
272 expression on tumor cells was observed only in unpretreated samples, which is in contrast
273 with recent studies that have been presented at the AACR and ASCO annual meetings.
274 Preliminary data from the KEYNOTE-028 [6] and the JAVELIN [32] trial in mesothelioma indicate
275 that PD-L1 expression can be seen irrespective of prior chemotherapy treatment. However,
276 similar to our own observation a downregulation of PD-L1 following chemotherapy
277 treatment has also been noted in other cancer types, such as non-small cell lung cancer
278 (NSCLC) and breast cancer. [33,34] Expression of PD-L1 on the cell surface has been
279 associated with the activation of the PI3K/Akt signaling pathway and PD-L1 has been
280 described to be a downstream target of Akt. [35] Ghebeh et al. [34] reported a significant
281 downregulation of PD-L1 on the surface of breast cancer cell lines after doxorubicin
282 treatment which was accompanied by an upregulation of PD-L1 in the nucleus. They saw
283 that the redistribution of PD-L1 from the cell surface to the nucleus was associated with a
284 translocation of phosphorylated Akt from the membrane to the nucleus. They also reported
285 that inhibition of Akt partially decreased PD-L1 expression on the surface, findings that are
286 supported by Latswika et al. [35] Further research on the effect of chemotherapeutics on Akt
287 signalling is warranted in order to unravel the underlying mechanisms that might be
288 responsible for PD-L1 downregulation.
289 Compared to the unpretreated, fewer pretreated patients had TIM-3+ TILs in their stroma,
290 which is in line with findings from Zhang et al. in diffuse large B-cell lymphoma. [24] A stromal
291 immune cell score of 3 was more often found for pretreated samples, suggesting that
292 chemotherapy causes an increase in immune infiltration. This is in concordance with
293 findings in other tumor types, showing that cisplatin promotes recruitment and proliferation
294 of effector immune cells. [36] Future studies with a larger number of samples are required to
295 unravel the best treatment schedule to combine chemotherapy with immunotherapy.
296 Although data about the prognostic role of TILs in MPM are controversial [37-40], it is clear
297 that these cells are important for antitumor immunity. Like TIM-3 after multivariate
298 adjustments CD4 expression on lymphocytes in lymphoid aggregates was demonstrated to
299 be a good independent prognostic factor, with better overall survival observed for patients

300 with more CD4+ lymphocytes in their aggregates, confirming the findings of Yamada et al.
301 in mesothelioma. [39] Also in NSCLC CD4+ TILs have been described as a positive prognostic
302 factor. [41-43]
303 CD4+ TILs play an important role in antitumor immunity. Via secretion of several
304 immunoregulatory cytokines, such as interferon-gamma (IFN-γ) and interleukin-2, they
305 provide help for priming and proliferation of CD8+ TILs and activate natural killer cells.
306 CD4+ TILs also express CD40-ligand on their surface which binds to CD40 expressed by
307 antigen presenting cells (APC). [44-46] This interaction between causes activation of APC, that
308 also contribute in priming of CD8+ TILs. Taken together, CD4+ TILs have both a direct
309 and indirect impact on the generation of a T cell-mediated antitumor response.
310 In our series, the two immune-related parameters with prognostic significance after
311 multivariate adjustments (CD4+ TILs, PD-1, TIM-3) are all situated in the lymphoid
312 aggregates, suggesting these are important structures influencing a patient's outcome. We
313 observed CD4, CD8 and CD45RO expression in lymphoid aggregates, suggesting that these
314 structures might function as a site for the generation of antitumor adaptive immune
315 responses, as also suggested by Pagès et al. [47] This would imply that reactivation of
316 antitumor T-cell responses might occur in lymphoid aggregates, resulting in a favorable
317 prognosis, and that these aggregates show functional similarity with tertiary lymphoid
318 structures, in which T and B lymphocytes are segregated into two adjacent regions
319 surrounded by high endothelial venules. [48,49]
320 A subset of the CD4+ cells in the tissue sections was also FoxP3+. These double positive
321 cells were found in 80% of the unpretreated and 56% of the pretreated samples with CD4+
322 TILs in the stroma, suggesting that cisplatin and/or pemetrexed have a negative effect on
323 the number of CD4+FoxP3+ cells, which might be regulatory T cells (Treg). This idea is
324 supported by data from Wu et al. [50] who reported a decreased number of Tregs in a
325 mesothelioma mouse model after treatment with cisplatin. We found a positive correlation
326 between Tregs and PD-1+, PD-L1+ and CD4+ TILs in the stroma. The more activation of
327 the PD-1/PD-L1 pathway, the less FoxP3 transcription is controlled, eventually resulting in
328 an increased amount of CD4+FoxP3+ cells. [30] However, FoxP3 expression is also described

329 in activated non-suppressive T cell populations, so it is not a 100% specific Treg marker.
330 [51] More CD4+FoxP3+ cells in the stroma were also associated with more CD68+
331 macrophages in the stroma, suggesting that the latter affect the adaptive immune
332 response by secreting several molecules that lead to recruitment and stimulation of CD4+
333 T cells, as previously described by Solinas et al. [52] CD4+FoxP3+ cells were negatively
334 correlated with CD8+ TILs in the stroma, as also found for CD4+ and CD8+ TILs: the more
335 CD4+ TILs, the more CD4+FoxP3+ cells and the less CD8+ TILs. As observed for CD8+
336 TILs, CD45RO+ memory T cells were also seen in the stroma of all samples. The latter
337 were significantly associated with response to chemotherapy. More specifically, our data
338 show CD45RO expression in the stroma to be an independent negative predictive factor.
339 MPM patients with many CD45RO+ T cells had a higher likelihood of non-responding to
340 chemotherapy compared to those with few CD45RO+ T cells, suggesting that CD45RO is
341 an interesting predictive marker for response to chemotherapy in MPM patients.
342 Stromal expression of CD45RO was significantly correlated with the presence of
343 CD4+FoxP3+ cells in the stroma. Under the assumption that the latter are Tregs, derived
344 from CD45RO+ memory T cells, the negative predictive value of CD45RO expression might
345 be explained by a Treg-mediated suppression of the immune cells that are recruited after
346 cisplatin and pemetrexed treatment. [25,53] In addition, a significant correlation was also
347 found for CD4+FoxP3+ cells and CD68+ macrophages in the stroma (R=0.410, p=0.002),
348 as stated at page 6. Tumor associated macrophages (TAMs) in mesothelioma have been
349 described to be of the tumor promoting M2-phenotype (data presented by L. Coussens at
350 the 13th International Mesothelioma Interest Group meeting in May 2016, Birmingham UK).
351 [54,55] M2 macrophages secrete several molecules, such as IL-10 and transforming growth
352 factor-bèta (TGF-β) that results in down-regulation of adaptive immunity for example via
353 stimulation and recruitment of Tregs. [52] The latter explains our correlation observed for
354 Treg and macrophages in the stroma.
355

356 **Conclusion**

357 In conclusion, we report that the immune composition of the TME and expression of
358 immune checkpoints and their ligands is strongly patient dependent. In future research it
359 would be interesting to investigate the within patient dynamics of the TME in a prospective
360 study. Our results point to TIM-3 as a promising new target in mesothelioma. Regarding
361 combination of chemotherapy with immunotherapy, larger validation cohorts are needed
362 to determine the best treatment schedule.

363

364 ## Materials & Methods

365 ### Patient selection and samples

366 Formalin fixed paraffin embedded (FFPE) tissue samples from 54 MPM patients were
367 retrieved from the tumor biobank at the Antwerp University Hospital. Of those, 40 patients
368 were unpretreated and 14 were pretreated with standard chemotherapy (platinum and
369 pemetrexed). Exclusion criteria for the untreated group were: prior surgery or
370 chemotherapy treatment and tissue samples older than 10 years. The latter exclusion
371 criterion was also used for the pretreated group. Response was assessed with modified
372 RECIST criteria and confirmed after at least 4 weeks. [56] All the tissue samples had been
373 obtained through surgical biopsy, fixed in 4% formaldehyde for 6-18h and paraffin
374 embedded on a routine basis. This retrospective study has been approved by the Ethics
375 Committee of the Antwerp University Hospital/University of Antwerp.

376 ### Immunohistochemistry

377 Five µm-thick sections were prepared from FFPE tissue blocks. Prior to staining, the
378 sections were baked in an oven for one hour at 60°C to facilitate attachment and to soften
379 the paraffin. IHC was carried out on a Benchmark Ultra XT® autostainer (Ventana Medical
380 Systems Inc.) with standard antigen retrieval methods (CC1, pH 8.0; Ventana, #950-124).
381 The UltraView or OptiView DAB detection kit (Ventana, #760-500 or #760-700) and, in
382 case of double stainings for CD4/CD8 and CD4/FoxP3, the UltraView universal alkaline
383 phosphatase red detection kit (Ventana, #760-501) were used according to the
384 manufacturer's instructions. Sections were counterstained with hematoxylin as part of the
385 automated staining protocol. After staining, slides were washed in reaction buffer
386 (Ventana, #950-300), dehydrated in graded alcohol, cleared in xylene, mounted with
387 Quick-D Mounting Medium (Klinipath, #7280) and coverslipped. IHC staining protocols
388 from the manufacturer's datasheets were used for the following antibodies from Ventana:
389 anti PD-1 (clone NAT105, #760-4895), anti CD4 (clone SP35, #790-4423), anti CD8 (clone
390 SP57, #790-4460), anti CD45RO (clone UCHL-1, #790-2930). The protocols for PD-L1
391 (clone SP142, 1:50, Spring Bioscience, #7309457001), CD68 (clone KP-1, 52' CC1, 12'

392 antibody incubation, Ventana, #790-2931) and granzyme B (polyclonal, 32' antibody
393 incubation, Ventana, #760-4283) were slightly adapted from the datasheet. This was also
394 the case for the antibodies used from Abcam® (Abcam®): anti LAG-3 (clone 11E3, 1:100,
395 36' CC1, 60' antibody incubation, #ab4065), anti TIM-3 (polyclonal, 1:500, 52' CC1, 28'
396 antibody incubation, #185703) and anti FoxP3 (clone 236A/E7, 1:50, 36' CC1, #ab20034).
397 Positive controls were included in each staining run. Human placenta was included as
398 positive control for endogenous PD-L1, while human tonsil was used as positive control for
399 the other markers. We looked at the presence of lymphocytes, lymphoid aggregates (score
400 0= 0 aggregates; 1= 1-5 aggregates; 2= 5-10 aggregates; 3= >10 aggregates) and the
401 density of the stroma (score 0= scarce; 1= low density; 2= intermediate density; 3= high
402 density [57]). A lymphoid aggregate was defined as 50 or more lymphocytes clustered
403 together. Expression of each marker in the tissue was divided into five categories (0=
404 <1%; 1= 1-5%; 2= 5-10%; 3= 10-50%: 4= >50%). All sections were scored by two
405 observers, of whom one pathologist. Samples were considered to be positive in case of
406 ≥1% positive cells [7,8] with specific staining of any intensity (0= no expression, 1= weak,
407 2= moderate, 3= strong) and any distribution (membrane and/or cytoplasm) [7]. These
408 criteria were used for IHC scoring of all the different markers. An Olympus BX41
409 microscope was used for scoring of the tissue sections. Pictures were made using the Leica
410 acquisition software v4.

411 **Statistics**

412 Spearman correlation coefficients were calculated to investigate the association between
413 the expression of immune cell markers and immune checkpoints on MPM tissue samples.
414 Overall survival was assessed from the date of diagnosis to the date of last contact or
415 decease. Survival rates were visualized using Kaplan-Meier curves. The influence of
416 immunological and clinicopathological parameters on survival was assessed using Cox
417 proportional hazards models. Variables that showed significance (10%) in univariate
418 analyses were considered for the multivariate model. We used tests based on the scaled
419 Schoenfeld residuals to assess the proportional hazards assumption. The association of

immunological and clinicopathological variables with response to chemotherapy was analysed using logistic regression on 32 of 54 samples (treatment or response data not available for the other samples). Variable selection was performed by assessing significance on the 10% level in univariate analyses. Depending on the number of selected variables, forward or backward model building was used in the multivariate models. P-values ≤0.05 were considered statistically significant. All statistical analyses were performed using R statistical software version 3.2.2.

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Conflict of interest

The authors have no conflicts of interest to declare.

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621

622 ## Figure legends

623

624 **Figure 1. Lymphocyte infiltration of MPM tissue samples.** Pictures were taken from
625 slides of the 2 different MPM patients **(A)** and **(B)**. The lymphocyte infiltration of both
626 tissue samples is representative for those in our overall MPM cohort. The images show the
627 presence of lymphoid aggregates, CD4+ (red) and CD8+ (brown) lymphocytes in the
628 tumoral stroma and in between the tumor cells. Tumor cells are round, epitheloïd and
629 pleiomorfic with patient **(A)**, while they are smaller with less pleiomorphisme with patient
630 **(B)**. Original magnification: left column pictures 100x; right column pictures 200x.

631 **Figure 2. Immunohistochemical staining patterns of different immunomarkers in**
632 **MPM tissue. (A)** CD4+ (red) and CD8+ (brown) lymphocytes in stroma; **(B)** CD68+
633 histiocytes stained with strong intensity in the cytoplasm surrounding negative tumor cells;
634 **(C)** CD45RO+ lymphocytes in stroma with moderate to strong intensity;**(D)** CD4+FoxP3+
635 lymphocytes in stroma with brown nuclear staining for FoxP3 and red cytoplasmic staining
636 for CD4; **(E)** stromal cells expressing granzyme B in their cytoplasm surrounding negative
637 tumor cells; **(F)** PD-L1+ tumor cells with membrane accentuation; **(G)** PD-1+ tumor cells
638 stained with strong intensity in the cytoplasm;**(H)** tumor cells and lymphocytes in stroma
639 with strong TIM-3 staining in the cytoplasm; **(I)** Absence of LAG-3 staining in MPM tissue;
640 **(J)** LAG-3+ lymphocytes in human tonsil showing strong cytoplasmic staining. All tissue
641 sections were counterstained with hematoxylin (blue color). Original magnification 1000x.

642 LA, lymphoid aggregate; TC, tumor cells; TIL, tumor infiltrating lymphocytes; ST, stroma.

643 **Figure 3. Predicted probability plots for the significant predictive factors CD45RO**
644 **and PD-1.** Plot of response on chemotherapy (y-axis) versus: **(A)** CD45RO expression on
645 lymphocytes in the stroma (x-axis), and **(B)** PD-1 expression on lymphocytes in the stroma
646 (x-axis). Observations within our cohort are represented by the empty dots (right y-axis).
647 The full dots show the observed chance on partial/complete response within each
648 expression category. The curve depicts the estimated probability of partial/complete
649 response based on a univariate logistic regression model (p=0.017, p=0.0514; left y-axis).

650 After multivariate adjustments CD45RO expression on stromal lymphocytes remained an
651 independent good predictive factor (p=0.0076).

652 **Figure 4. Kaplan-Meier overall survival according to CD4, PD-1 and TIM-3**
653 **expression in the lymphoid aggregates.** Univariate analysis showed prognostic
654 significance for: **(A)** CD4 (p=0.008), **(B)** PD-1 (p=0.029), and **(C)** TIM-3 (p=0.001)
655 expression in the lymphoid aggregates. After multivariate adjustments CD4 and TIM-3
656 expression in the aggregates remained independent good prognostic factors (p=0.015 and
657 p=0.002).

658

659 **Tables**

660 **Table 1. Clinicopathological parameters of unpretreated and chemotherapy**

661 **pretreated mesothelioma patients.**

Characteristics	unpretreated (n,%)	pretreated (n,%)	p-value
Number of samples (N)			
	40	14	
Age (years)			0.043
Median	69	63	
Range	42-81	45-73	
Sex			0.890
Male	34 (85%)	11 (79%)	
Female	6 (15%)	3 (21%)	
Histological subtype			0.151
Epitheloid	26 (65%)	13 (93%)	
Sarcomatoid	5 (13%)	0	
Biphasic	9 (22%)	1 (7%)	
Smoker			0.502
No	7 (18%)	4 (29%)	
Yes	24 (60%)	6 (42%)	
No data	9 (22%)	4 (29%)	
Professional asbestos exposure			0.464
No	10 (25%)	1 (7%)	
Yes	23 (57%)	8 (57%)	
No data	7 (18%)	5 (36%)	
Survival			0.412
Alive	9 (22%)	5 (36%)	
Dead	31 (78%)	9 (64%)	
Laterality			0.812
Left	11 (28%)	5 (36%)	
Right	29 (72%)	9 (64%)	
Surgery			0.114
No surgery	33 (82%)	11 (79%)	
Diagnostic VATS	3 (8%)	1 (7%)	
P-D	1 (2%)	2 (14%)	
EPP	3 (8%)	0	
Stage			0.814
I-II	13 (33%)	6 (43%)	
III-IV	22 (55%)	7 (50%)	
No data	5 (12%)	1 (7%)	
Hemoglobin (g/dL)			0.272
< 14.6 (low)	5 (12%)	9 (64%)	
≥ 14.6 (high)	35 (88%)	4 (29%)	
No data	0	1 (7%)	
White blood cell count (x10^{3}cells/µL)			0.745
< 15.5 (low)	37 (93%)	13 (93%)	
≥ 15.5 (high)	3 (7%)	0	
No data	0	1 (7%)	
Platelet count (x10^3 cells/µL)			1.000
< 400 (low)	26 (65%)	9 (64%)	
≥ 400 (high)	14 (35%)	4 (29%)	
No data	0	1 (7%)	
Neutrophil / lymphocyte ratio			0.542
< mean★	16 (40%)	6 (43%)	
≥ mean★	24 (60%)	6 (43%)	
No data	0	2 (14%)	

662

663 ★, mean of unpretreated samples = 3.3; mean of pretreated samples = 3.2. EPP, Extrapleural

664 Pneumonectomy; P-D, Pleurectomy-Decortication; VATS, Video Assisted Thoracoscopic Surgery.

665 **Table 2. Expression of immune checkpoints and immune cell markers in FFPE tissue from unpretreated and pretreated MPM**

666 **patients.**

% samples	CD8+	CD68+	CD45RO+	CD4+	CD4+ FoxP3+	Granzyme B+	PD-1+	PD-L1+	TIM-3+	LAG-3+
Unpretreated										
TOTAL (N=40)	100	100	100	85	70	33	75	68	63	0
Tumor cells (n=40)	0	0	0	0	0	0	10	40	40	0
Immune cells in stroma (n=40)	100	100	100	75	60	33	65	53	40	0
Lymphocytes in lymphoid aggregates (n=26)	100	0	96	88	50	11	69	31	54	0
Germinal centers within lymphoid aggregates (n=7)	0	0	0	0	0	0	86	29	0	0
Prereated										
TOTAL (N=14)	100	100	100	100	79	43	71	57	79	0
Tumor cells (n=14)	0	0	0	0	0	0	0	0	36	0
Immune cells in stroma (n=14)	100	100	100	71	50	43	71	29	29	0
Lymphocytes in lymphoid aggregates (n=10)	100	0	100	100	80	0	70	60	60	0
Germinal centers within lymphoid aggregates (n=3)	0	0	0	0	0	0	33	100	0	0

667

668 Percentages of all positive samples are shown per marker. Expression on tumor cells, immune cells in the stroma (lymphocytes, macrophages, histiocytes,

669 plasma cells) and lymphocytes in lymphoid aggregates and in germinal centers within lymphoid aggregates is depicted separately.

670

671 **Table 3. Within patient combined expression of PD-1, PD-L1 and TIM-3 on tumor cells and TIL subtypes.**

% unpretreated samples		Tumor cells			% treated samples		Tumor cells
		PD-1+	PD-L1+	TIM-3+			TIM-3+
TILs	PD-1+	10	28	33	TILs	PD-1+	21
	PD-L1+	10	28	23		PD-L1+	7
	TIM-3+	5	15	23		TIM-3+	7
	CD8+	10	40	40		CD8+	36
	CD4+	10	30	33		CD4+	29
	CD4+FoxP3+	10	28	30		CD4+FoxP3+	21

672

673 Percentages of unpretreated and chemotherapy pretreated samples showing tumoral expression of PD-1, PD-L1 or TIM-3 together with the T cell

674 markers, PD-1, PD-L1 or TIM-3 on TILs.