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PREDICTIVE BIOMARKER OF LONG TERM SURVIVAL IN MESOTHELIOMA PATIENTS TREATED WITH IMMUNE CHECKPOINT INHIBITORS

Scientific disciplinary sector: MED/03 – Medical Genetics

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1. Abstract

Background: Targeting immune-checkpoint inhibitors (ICIs) has proven effective in a variety of tumor types. Primary and secondary resistance to treatment is emerging as a major limitation of ICIs therapy, but little data are available on efficacy of re-treatment in immune checkpoint blockade (ICB)-resistant subjects. The identification of biomarkers predictive of response to ICI and re-treatment of ICI-resistant patients are currently under investigations as promising tools in clinical practice.
Aim of this study is to evaluate the efficacy, safety and clinical activity of re-treatment with tremelimumab and durvalumab in malignant mesothelioma (MM) patients who developed resistance to these agents and to investigate the role of tumour mutational burden (TMB) as a predictive biomarker of response in the phase II NIBIT-MESO-1 study.

Methods: Eligible patients for re-treatment *per* the NIBIT-MESO-1 protocol were those who completed four dosing cycles of tremelimumab combined with durvalumab achieving partial response (PR) or stable disease (SD) followed by progressive disease (PD) during the maintenance with only durvalumab or the follow-up phase. Subjects who met the re-treatment criteria received tremelimumab (1 mg/Kg i.v.) and durvalumab (20 mg/Kg i.v.) every four weeks (Q4W) for four doses ("*re-induction pha*se"), followed by durvalumab (20 mg/Kg, i.v.) Q4W for additional nine doses ("*maintenance phase of re-treatment*"). The evaluated endpoints were objective response rate (ORR), disease control rate (DCR), *per* immune-related (ir)-modified RECIST criteria, overall survival (OS) and safety. A *post-hoc* analysis was conducted to evaluated tumor mutational burden (TMB) on all patients enrolled, whose paraffin tumor sample was available before starting treatment. Median values of TMB were calculated and used as a cut-off to divide patients in equal number groups for comparisons with survival. Survival times were analyzed with the Kaplan-Meier method and differences between curves were evaluated with the log-rank test. P values < 0.05 were considered as significant.

Results: Seventeen (42.5%) of the 40 patients enrolled in the NIBIT-MESO-1 study met the criteria for re-treatment and received therapy. Among them, 8 (47%) completed the *re-induction* phase, 7 (41.2%) went on maintenance phase of re-treatment, and 1 (5%) passed to follow-up phase. At data cut-off, April 30, 2020, all 17 patients were discontinued during re-treatment because of PD, and 13 (73%) received additional lines of therapy (chemotherapy or immunotherapy). Seven (41%) of the 17 re-treated subjects had an ir-SD, while no ir-ORR was observed. From the start of re-treatment to a median follow-up of 24 months, median OS (mOS) was 12.5 months. Grade 1-2 ir-adverse events (AEs) occurred in 6 (35%) re-treated patients, were most frequently dermatological and reversible *per protocol* guidelines; no grade 3-4 ir-AEs were observed. At a median follow-up of 46 months, mOS of re-treated patients was significantly (p=0.01) higher (25.6 months, 95% CI: 9.6-41.6) as compared to the 23 subjects who were not re-treated (9.9 months, 95% CI: 7.7-12.1). In a *post-hoc* analysis on the 28 patients for whom tumour tissue before treatment was available, TMB values higher than the median cut-off of 8.3 mutations *per* Mb were associated with a higher mOS compared with lower TMB, but this difference was non-significant (p=0.06). Moreover, when patients were additionally stratified for ICI re-treatment (n=13), there was a significant difference in survival between those with a TMB higher than 8.3 mutations *per* Mb and those with lower TMB values in the re-treated cohort (1256 days *vs* 528 days; p=0·02).

Conclusions: Re-treatment with tremelimumab and durvalumab of MM patients who developed resistance to therapy in the NIBIT-MESO-1 study seems to be clinically effective and safe in a sizeable proportion of re-treated subjects, suggesting its potential application in the clinical practice. Further studies on a larger sample will be needed to validate the predictive role of TMB, either as an independent predictive biomarker or associated with others potential predictive immune biomarkers.

2. Introduction

Malignant mesothelioma (MM) is an aggressive tumor that arises primarily from the surface serosal cells of the pleura (65-70%), peritoneum (30%), tunica vaginalis, testis, and pericardium (1%-2%).[1] Over 80% of MM are diagnosed in men and are associated with occupational asbestos exposure, altought non-occupational exposure related tumors were detected.[2] After inhalation, asbestos fibers can enter in the periphery of lung and the pleura, inducing local chronic inflammation and other carcinogenic processes. From the initial exposure to asbestos to the beginning of the symptoms there is often a time interval ranging from 20 to 40 years ("*latency period*"), that seems to have an inverse relationship with duration or degree of abestos exposure.[3]

The diagnosis of MM is often delayed, due to a long latent period and to a non-specific clinical presentation. Diagnosis is usually made by thoracoscopy with multiple biopsies to obtain histological examination and immunocytochemical analysis. There are three major histological subtypes, known as epithelioid (approximately 60% of all diagnosed MM), sarcomatoid (around 20%) and biphasic type with both epithelioid and sarcomatoid characteristics.[4] The 8th edition of the American Joint Committee on Cancer (AJCC), effective as of January 2018, is recommended for clinical staging of malignant pleural mesothelioma (MPM).[5]

The therapeutic management of MM is complex and should be evaluated by an expert multidisciplinary team composed by radiologist, oncologist, surgeon and radiotherapist. This cancer is often diagnosed in late stage and has a bad prognosis: median survival of un-treated cases is 6-12 months with less than 5% 5-year survivors. In fact, most patients die within one year of the diagnosis.[6] The main factors contributing to this poor survival data are the lack of effective therapeutic options and the chemoresistance of this cancer.

Treatment options include surgery, radiation therapy and systemic treatment. Unfortunately, few patients are elegible for surgical procedure, because only a minority of them meets the criteria for a total radical surgery (microscopically complete resection). Although no standard type of surgery (i.e., extra pleural pneumonectomy or pleurectomy/decortication in MPM) is validated, there is compelling evidence that a

selected subgroup of MM patients can benefit from a surgery-based multimodal approach.

Clinical trials on complementary treatments to improve the cytoreductive efficacy of surgery are ongoing. In this regard a series of phase I/II clinical trials relating neo-adjuvant immunotherapy prior to surgery have been started in MPM but as these trials are still running the results are not yet mature.[7]

Radiotherapy alone is not considered for treatment of MPM because of its lung toxicity and of the lack of evidence on its impact on survival, so the irradiation of MPM patients is principally used as palliative measure to alleviate pain induced by the infiltration of the chest wall. The use of radiation therapy for localized asymptomatic recurrences is not clearly described in the literature.[8]

Indeed, the front-line systemic treatment for unresectable mesothelioma patients has not significantly changed since 2003, and still consists in the platinum plus pemetrexed regimen, based on phase III trial EMPHACIS.[9] Far from being satisfactory, the median overall survival (mOS) of these patients remains approximately 12-16 months,[9,10] increasing up to 18 months when utilized in combination with bevacizumab in highly selected mesothelioma patients; however, this combination regimen is not routinely applied in most countries.[10] In the non-randomized, phase II STELLAR study, tumor treating fields in combination to standard first-line chemotherapy demonstrated to improve the OS of unresectable MPM compared to historical data, thus receiving the approval by the Food and Drug Administration (FDA) in 2019.[11]

Several studies about immune checkpoint inhibithrs (ICIs) in front-line setting have been completed or are ongoing. In the phase 3 Checkmate-743 trial, first-line nivolumab (human IgG4 monoclonal antibody that blocks programmed cell death protein-1 "PD-1") plus ipilimumab (human monoclonal IgG1κ antibody against the cytotoxic T-lymphocyte antigen-4 "CTLA4") significantly improved survival of patients with MPM compared to pemetrexed plus platinum chemotherapy (mOS 18.1 *vs* 14.1 months, HR=0.74, 96·6% CI:0·60-0·91; *p*=0.002); the 2-year OS rates were 41% for nivolumab plus ipilimumab and 27% for chemotherapy combinations. On October 2, 2020, based on the results of this study, the combination of ICIs was approved by FDA as first-line therapy for patients with unresectable MPM.[12,13]

The therapeutic landscape for pre-treated mesothelioma patients is even more dramatic, as no agents have been so far approved in this setting.[14] In this dismal scenario, promising results are emerging by targeting ICIs, in particular when employed in a combination regimen.[15] Indeed, despite promising results have been reported from targeting PD-1/PD-ligand-(L)1 as monotherapy in early studies, the large randomized PROMISE-Meso study failed to demonstrate an improvement in survival compared to chemotherapy in pre-treated patients (HR=1.12, 95% CI:0.74-1.69; *p*=0.59), even after adjusting data for cross-over.[15,16] NIBIT-MESO-1 was the first clinical study to demonstrate encouraging signs of efficacy from treatment with the anti-cytotoxic T lymphocyte antigen (CTLA)-4 tremelimumab combined with the anti-PD-L1 durvalumab in first or second-line: 27.5% (95% CI: 14.6-43.9) and 65% (95% CI: 48.3-79.4) of patients achieved an immune-related (ir)-objective response (ir-ORR) and an ir-disease control (ir-DCR), respectively; mOS was 16.6 months (95% CI: 13.1-20.1).[17] Similar results have been subsequently reported in the phase 2 studies MAPS-2 and INITIATE, in which ipilimumab plus nivolumab were administered in pre-treated MPM patients. In MAPS-2 trial mOS was 11.9 months for the single therapy with nivolumab and 15.9 months for the combination; in INITIATE trial mOS was not reached (NR) with an estimated mOS to exceed 12.7 months.[18,19]

Immune checkpoint inhibitors have revolutionized the treatment of oncological patients, yielding longlasting responses and improved survival. However, also in MM, as observed in other cancers, a subset of patients who initially respond to immunotherapy, later relapse and develop therapy resistance (termed "secondary or acquired resistance"), whereas others do not respond at all (termed "primary resistance"). Primary and acquired resistance to immune check blockade (ICB) are important clinical barriers to further improving outcomes of patients, and the known mechanisms underlying each involve various components of the tumoral immune cycle, and interactions between multiple signaling molecules and pathways. Due to this complexity, current knowledge on resistance mechanisms is still incomplete. Overcoming therapy resistance requires a thorough understanding of the mechanisms underlying immune evasion by tumors (Figure 1).[20,21,22]

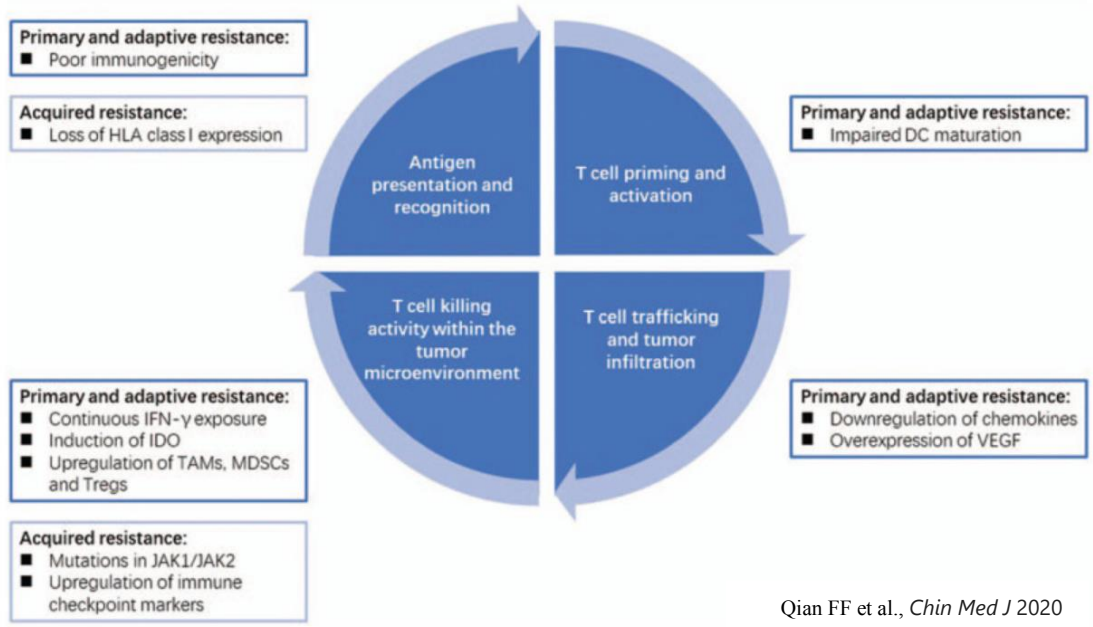

Figure 1: Mechanisms of resistance to immune checkpoint blockade therapies.

Immune response against cancer. Various immune escape mechanisms presenting at each of these stages can result in primary or acquired resistance to immunotherapy. DC: Dendritic cells; HLA: Human leukocyte antigen; IDO: Indoleamine 2,3-dioxygenase; IFN-γ: Interferon-γ; JAK1/JAK2: Janus kinases 1 and 2; MDSCs: Myeloid-derived suppressor cells; TAMs: M2-like tumor-associated macrophages; Tregs: Regulatory T cells; VEGF: Vascular endothelial growth factor.

In the literature there are little data on the safety and efficacy of re-treatment in immune checkpoint blockade-resistant patients. After acquiring resistance to several therapeutic regimens, ICI re-challenge is one of the therapeutic options for patients with recurrent disease. Unfortunately, re-treatment has been clinically effective in only a small number of patients and there was no information on its activity in mesothelioma prior to conducting and maturing the data from the NIBIT-MESO-1 trial.[23,24]

There are no validated predictive biomarkers for immunotherapy and so their identification would be mandatory for driving the selection of patients that could benefit for ICB treatment and for the effectiveness of ICIs re-challenge.[25]

The TMB acts as an indirect measure of tumor-derived neoantigens and emerges as a potential biomarker for ICI patient stratification. TMB is defined as the total number of DNA mutations per megabases (mut/Mb) in a tumor specimen. Among others, non-synonymous mutations have the potential to generate neoantigens recognized by the host's immune system, leading to an incresed tumor immunogenicity and, thus, to a more effective antitumor immune response. With a higher number of mutations detected, and

consequentially an increase in the number neo-epitopes, it is more likely that one or more of those neoantigens could trigger a T cell response, leading to improved clinical response to immunotherapy. A series of clinical trials showed that high TMB confers high response rate and sustained response to ICB therapy in many types of cancer such as melanoma and non small cell lung cancer (NSCLC), wich typically have high mutation burden owing to the mutagenic effect of ultraviolet light and tobacco smoke, respectively. Unfortunately, the strategy to define the total number of mutations present in a tumor specimen have still some drawbacks. A key aspect is how the mutations are detected since TMB could vary depending on the specific technique adopted to calculate it (whole-genome sequencing/WGS, whole-exome sequencing/WES, targeting sequencing on multigene panels). Moreover, there is no clear consensus for the TMB cutoff for patien stratification (though, the recent tissue agnostic FDA approval for pembrolizumab defined a high TMB value as greater than 10 mut/Mb). Finally, TMB is still not an ideal biomarker for immunotherapy, as responses are also observed in low TMB patients like in MM.[26,27]

3. Aim of the Study

A promising therapeutic strategy that appears to be clinically relevant in different cancers is the combination of ICIs. However, despite well documented success of these drugs in a variety of tumors, responses typically occur only in a proportion of patients also in MM, and secondary resistance frequently occurs. For this reason, there is an urgent requirement for the identification of different potential treatment strategies to overcome resistance to immunotherapy, such as the re-treatment with the same immunotherapeutic drugs, but limited evidence is currently available in literature. To answer this unmet clinical need, this study evaluated efficacy and safety of re-treatment with tremelimumab and durvalumab in MM patients who developed secondary resistance in the phase II NIBIT-MESO-1 trial.

Moreover, the identification of predictive biomarkers of ICIs efficacy is needed to enable more selective use of these drugs. In this regard, evidence suggest that TMB may serve as a predictive biomarker for immunotherapy in various solid cancers, so the predictive role of TMB (defined as the number of somatic mutations per megabase of interrogated genomic sequence) was evaluated in a "*post-hoc*" analysis in treated and re-treated patients, who had available tumour tissue before start of treatment in the NIBIT-MESO-1 study.

4. Materials and Methods

4.1. NIBIT-MESO-1 study design and patient population

This work evaluated the follow-up analysis of patients enrolled in the NIBIT-MESO-1 study. TMB assessment was performed in the subset of patients who had available tumor tissue sample collected before start of treatment.[17]

The NIBIT-MESO-1 was an open-label, single arm, phase 2 study, sponsored by the Italian Network of Bio-Immunotherapy (NIBIT) Foundation. Inclusion criteria for this study were: age of 18 years or older, histologically confirmed unresectable pleural or peritoneal mesothelioma, refusal or progression during or after one platinum-based chemotherapy regimen, presence of measurable disease (assessed at baseline by CT or MRI) according to the modified Response Evaluation Criteria in Solid Tumors [RECIST] for pleural mesothelioma or RECIST 1.1 for peritoneal mesothelioma), a life expectancy of 3 months or more (as judged by clinicians), adequate organ function, and an Eastern Cooperativ Oncology Group (ECOG) performance status (PS) of 1 or less.

From 30 October 2015 to 12 October 2016, 40 MM patients with the following features were enrolled and treated in NIBIT-MESO-1 study: median age 64 years (range 53-71); male (n=29) and female (n=11); EORTC prognostic score good (n=30) or poor (n=10); MPM (n=38) or peritoneal mesothelioma (n=2); histology epithelioid (n=32), biphasic (n=5), sarcomatoid (n=2) or undefined (n=1); stage disease III (n=11) or IV (n=29) according to 7th edition of the AJCC; first-line setting (n=12) and second-line setting (n=28) (Table 1). All patients received *induction* treatment with tremelimumab at dose 1 mg/Kg i.v. every four weeks (Q4W) and durvalumab at 20 mg/Kg i.v. Q4W for four doses. *Induction* treatment was followed by maintenance treatment ith only durvalumab 20mg/kg i.v. for nine doses, or until confirmed progression of disease (PD), unacceptable toxicity, investigator's decision, or patient's withdrawal of consent. Tumor assessment was performed at baseline and every 12 weeks and was evaluated *per* ir-modified RECIST and mRECIST for patients with MPM and *per* ir-RECIST version 1.1 and RECIST version 1.1 for patients with peritoneal MM.[28,29] Patients who

relapsed during maintenance treatment or follow-up had the option to restart combination treatment, if eligible *per* protocol (Figure 2).

Characteristic	Study population (n=40)
Sex	
Male	29 (72.5%)
Female	11 (27.5%)
Median age, years	64 (53-71)
Histology	
Epithelioid	32 (80%)
Sarcomatoid	2 (5%)
Biphasic	5 (12.5%)
Undefined	1 (2.5%)
Site	
Pleural	38 (95%)
Peritoneal	2 (5%)
Disease stage	
III	11 (27.5%)
IV	29 (72.5 %)
Setting	
First line	12 (30%)
Second line	28 (70%)
EORTC Prognostic score	
Good	30 (75%)
Poor	10 (25%)

Table 1: Baseline characteristics of patients enrolled and treated with tremelimumab plus durvalumab in NIBITMESO-1 study.
Data are number (%), or median (IQR). EORTC=European Organization for Research and Treatment of Cancer.

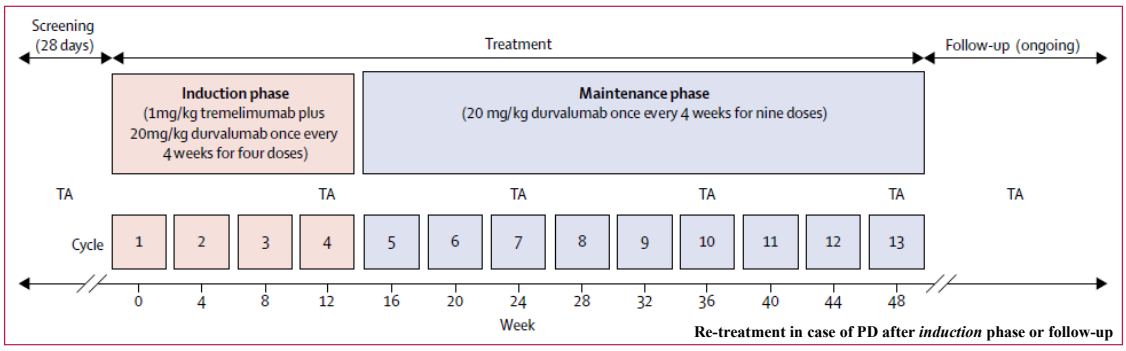

Figure 2: NIBIT-MESO-1: study design.
Patients were re-treated in cases of disease progression during maintenance treatment or follow-up. Tumour assessments were done roughly every 12 weeks. TA=tumour assessment, PD=progression disease.

For the follow-up study, patients were eligible for *re-treatment* if they had completed 4 cycles of tremelimumab plus durvalumab and had obtained a partial response (PR) or stable disease (SD), followed by PD during maintenance with durvalumab for 9 doses or during the follow-up phase. Additional eligibility criteria were ECOG PS of 0 or 1; life expectancy of ≥ 3 months (according to physicians judge); adequate organ function (blood, kidney, heart and liver); and measurable disease, defined as at least one lesion that could be accurately assessed at baseline by CT scan or MRI that was suitable for repeated measurements, according to the modified RECIST criteria for MPM and RECIST 1.1 for peritoneal MM. [28,29] Patients eligible for *re-treatment* received the same schedule of treatment used in the NIBIT-MESO-1 study (Figure 2).

No further *re-treatment* was permitted in case of PD. Radiological total-body tumour assessments (brain, neck, chest, abdomen, and pelvis CT scan) were undertaken at re-screening and every 12 weeks during *re-treatment* phase. The endpoints analyzed in this work were: ir-ORR, evaluated according to ir-modified RECIST or ir-RECIST 1.1 criteria for patients with pleural or peritoneal MM, respectively; ir-DCR, immune-related progression-free survival (ir-PFS), OS, and safety. In a *post-hoc* analysis a comparison was made between median survival of patients re-treated with ICIs versus those not re-treated. Adverse events were recorded according to the National Cancer Institute Common Terminology Criteria for Adverse Events (NCI-CTCAE) version 4.0. No dose reduction was allowed. All treatment-related toxicity had to be resolved to grade 1 or lower before giving the next dose of the combination or maintenence durvalumab. Patients were permanently discontinued from the study if two consecutive doses were missed because of ongoing treatment-related toxicity.

Re-treatment as a part of the NIBIT-MESO-1 study was already approved by the University Hospital of Siena's independent Ethics Committee and was done in accordance with the ethical principles of the Declaration of Helsinki and the International Conference on Harmonization of Good Clinical Practice. All participants or their legal representatives provided written informed consent before enrolment in *re-treatment*.

4.2. **DNA extraction and next-generation sequencing**

Formalin fixed and paraffin-embedded (FFPE) tumor tissue sections at baseline were available from 28 patients with MM enrolled in the NIBIT-MESO-1 study (of whom 13 patients entered the ICI *re-treatment* phase). To assess TMB, in this *post-hoc* analysis, genomic DNA was isolated from the above reported samples using the GeneRead DNA FFPE Kit (Qiagen, Hilden, Germany) and following the manufacturer's instructions.
DNA concentrations were measured with the Qubit 2.0 Fluorometer with Qubit dsDNA HS (High Sensitivity) Assay Kit (Life Technologies, Carlsbad, CA, USA). Next-generation sequencing analyses were performed using the Ion GeneStudio S5 System with the Comprehensive Cancer Panel that includes a total of 409 cancer-related genes arranged in four primer pools.[30] Libraries were generated starting from 10 ng of DNA per primer pool for a total of 40 ng of input DNA, using the Ion AmpliSeq Library Kit Plus, barcoded with Ion Xpress Barcode Adapters (Life Technologies) and purified with Agencourt Ampure XP Beads (Beckman Coulter Life Sciences, Indianapolis, IN, USA). The obtained PCR amplicons were diluted to a final concentration of 70 pM and pooled together; emulsion PCR and Chip (Ion 540) loading steps were performed with the Ion Chef Instrument. Sequencing of libraries was done with the Ion S5TM System (Thermofisher, Monza Italy). Sequencing data were processed with the Ion Torrent platform-specific pipeline software (Torrent Suite, V5.2.1). Ion Reporter Software V5.10 and Integrative Genome Viewer software) were employed for variant annotation and reads visualizations, respectively. TMB, the total number of non-synonymous somatic mutations (variants that alter the amino acid sequence of a protein) per coding area of a tumor genome, was measured in mutations *per* megabase (Mb). TMB values were directly calculated by the Ion Reporter™ Software through the specific panel analysis workflow.

4.3. **Statistical analysis**

The Kaplan-Meier method was utilized to estimate median follow-up (reverse method), mOS, and mPFS, as well as survival rates, with two-sided 95% confidence intervals

based on a normal approximation. Survival curves were compared using the log-rank test.

The restricted mean survival time (RMST) was considered an average survival time-dependent measure and was typically calculated over a defined period that has adequate follow-up (in our case from the beginning of the treatment to four years of follow-up).[31] Median OS was the period from the start of the treatment to which half of the patients in each group are still alive and was calculated at a 4-years follow-up (30 April 2020).

Two-sided 95% confidence intervals for ir-ORR and ir-DCR were estimated by an exact probability method. ORR was considered as the proportion of patients in the study whose tumor was totally or partially reduced by treatment (the sum of complete responses with no detectable evidence of a tumor, and partial responses with a decrease in tumor size, over a specified time). DCR was evaluated as the percentage of patients who achieved complete response (CR), PR, and SD to a therapeutic intervention in the clinical trial.

Median values related to available TMB of all enrolled patients were calculated and used as cut-offs to divide the patients into two groups of equal number for the analysis. Kaplan-Meier curve, followed by Log-rank (Mantel-Cox) test were used to compare groups defined by this candidate biomarker for the OS of treated and re-treated MM patients, and these statistical analyses were carried out using the Prism-8 software (GraphPad). All other statistical analyses used IBM-SPSS version 23.0. For all data the significance level of p value was considered 0.05.

5. Results

At data cut-off, April 30, 2020, five (13%) of 40 patients enrolled in the NIBIT-MESO-1 study were alive, and thirtyfive (88%) patients had died because of PD. With a median follow-up of 52 months (IQR 49-53), the mOS was 16.5 months (95% CI 13.7-19.2) (Figure 3).

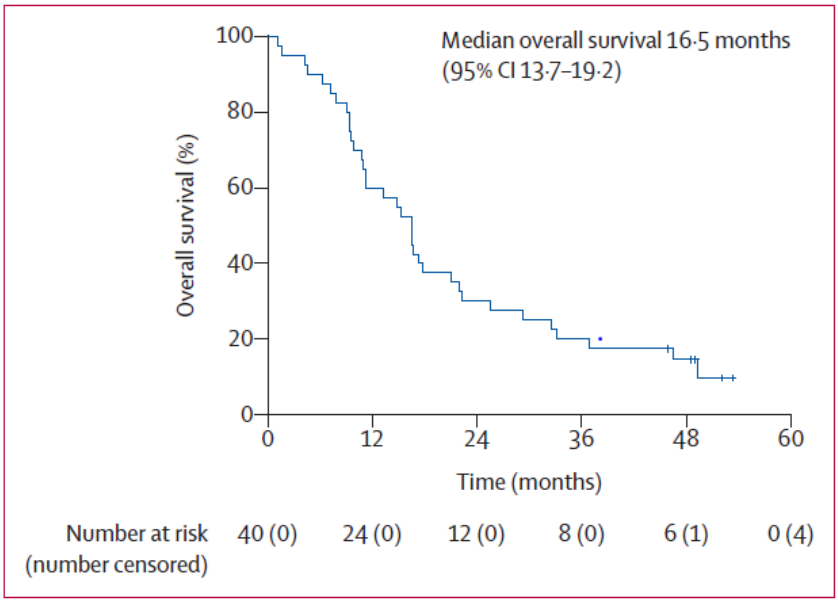

Figure 3: Kaplan–Meier curve of overall survival in patients with mesothelioma (n=40) treated with tremelimumab plus durvalumab in NIBIT-MESO-1 study.
Vertical lines indicate censored observations.

Survival was 20% (eight of 40 patients) at 36 months and 15% (six of 40 patients) at 48 months. Seventeen (43%) of 40 patients who had PD met the criteria for undergoing to *re-treatment* with tremelimumab and durvalumab (Table 2; Figure 4). Among these 17 patients who had a mPFS of 11.3 months (95% CI 9.0-13.5) at baseline in NIBIT-MESO-1 up to the point of starting *re-treatment*, eight (47%) patients progressed during the initial maintenance phase in NIBIT-MESO-1 prior to *re-treatment*, and nine (53%) experienced PD during the first follow-up phase. Between Sept 6, 2016, and March 29, 2019, the 17 patients who received *re-treatment* had a median of three doses (range 1-4) of tremelimumab and four doses (range 1-13) of durvalumab. Eight (47%) of 17 patients completed the *re-induction* phase with tremelimumab and durvalumab, of

whom seven (41%) began the *re-treatment* maintenance phase with only durvalumab and one (5%) entered the follow-up phase.

Characteristic	**Study population for *re-treatment* (n=17)**
Sex	
Male	11 (65%)
Female	6 (35%)
Median age, years	65 (49-69)
Histology	
Epithelioid	14 (82%)
Biphasic	3 (18%)
Site	
Pleural	16 (94%)
Peritoneal	1 (6%)
Previous treatment	
One*	4 (23%)
Two**	13 (77%)
ECOG performance status	
0	10 (59%)
1	7 (41%)

Table 2: Baseline characteristics of patients re-treated with tremelimumab and durvalumab in NIBIT-MESO-1 study.

*Data are n (%), or median (IQR). *First-line treatment with tremelimumab and durvalumab in the NIBITMESO-1 study. **First line treatment with platinum plus pemetrexed; second-line treatment with tremelimumab and durvalumab in the NIBIT-MESO-1 study. ECOG=Eastern Cooperative Oncology Group.*

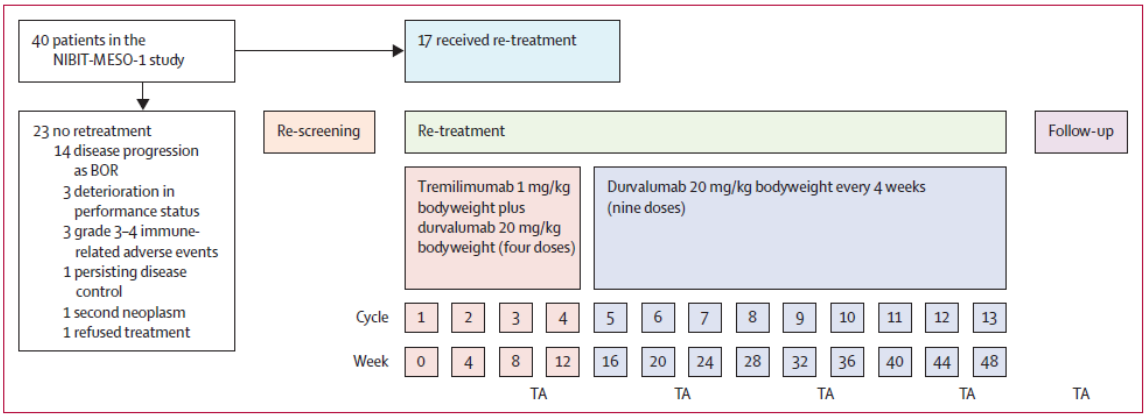

Figure 4: Patient disposition and schedule of **re-treatment** with tremelimumab and durvalumab in NIBIT-MESO-1 study

BOR=best overall response. TA=tumour assessment.

Overall, seven (41%) of 17 patients included in the *re-treatment* study achieved ir-SD (disease control), which lasted over 11 months in three patients. No ir-ORR responses were observed. Four (24%) of 17 patients were treatment-naïve when they were first enrolled in the NIBITMESO-1 study. Nine (53%) of 17 re-treated patients had a partial response as best overall response (BOR) with the first course of therapy in the NIBITMESO-1 study; of these, six (67%) had SD and three (33%) had PD as BOR with *re-treatment* in the follow-up study. The remaining eight (47%) patients had SD as BOR with the first course of treatment, of whom one (13%) achieved SD as BOR at *re-treatment* and seven (87%) had PD as BOR at *re-treatment*.

At median follow-up of 24 months (IQR 22.0-25.0) starting from *re-treatment*, the median ir-PFS of the 17 patients who were re-treated was 3.5 months (95% CI 3.2-3.8) and the mOS was 12.5 months (0.0-25.8). Survival at 12 months was 52.9% (nine of 17 patients), at 18 months was 35.3% (six of 17), and 24 months was 23.5% (four of 17). At data cut-off, April 30, 2020, all 17 patients who received *re-treatment* had discontinued treatment because of PD; 13 (76%) patients received subsequent chemotherapy or immunotherapy. Twenty-three (58%) of 40 patients enrolled in the NIBIT-MESO-1 study were not eligible for ICI *re-treatment* because of: PD as BOR (14 [35%] of 40 patients); deterioration of clinical condition PD-related (3 [8%]); grade 3-4 toxicity that required permanent treatment discontinuation (three [8%]); persisting disease control (one [3%]); occurrence of a second cancer (one [3%]); and refused ICI *re-treatment* (one [3%]). Of these 23 patients who did not receive ICI *re-treatment*, fifteen (38%) received further chemotherapy.

A *post-hoc* analysis was performed to compare the survival of 17 ICI re-treated patients with survival of the 15 patients who did not qualify for ICI *re-treatment* but received additional chemotherapy. At a median follow up of 48 months (IQR 46.0-49.0) from the start of NIBIT-MESO-1, the mOS of patients who recieved *re-treatment* was significantly higher than for patients who recieved chemotherapy (25.6 months [95% CI 9.6-41.6] *vs* 11.0 months [9.4-12.6]; p=0·009) (Figure 5). Furthermore, RMST analysis demonstrated a survival time of 21.3 months (95% CI 16.3-26.2) for all 40 patients enrolled in the original study, 29.3 months (22.1-36.5) for the 17 re-treated patients included in the follow-up study, and 16.7 months (9.8–23.5) for patients (n=23) who did not receive ICI *re-treatment*.

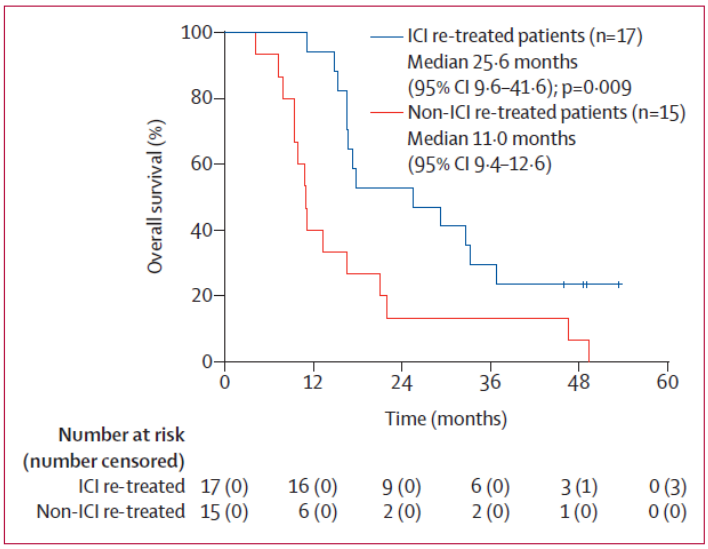

Figure 5: Kaplan–Meier curve of overall survival of patients re-treated with tremelimumab and durvalumab versus non-ICI re-treated patients who received additional chemotherapy (post-hoc analysis). *Vertical lines indicate censored observations. ICI=immune checkpoint inhibitor.*

Overall, *re-treatment* with tremelimumab and durvalumab was well-tolerated. Six (35%) of 17 patients experienced grade 1-2 ir-AEs, the most common of which were dermatological, and generally manageable and reversible *per protocol* guidelines. No grade 3-4 ir-AEs were observed (Table 3).

	Grade 1-2	**Grade 3-4**
Any immune-related adverse events	6 (35%)	0
Dermatological (rash, pruritus, erythema multiforme, psoriasis)	4 (24%)	0
Gastrointestinal (diarrhoea, nausea, vomiting)	2 (12%)	0
Pancreatic (amylase increase, lipase increase)	1 (6%)	0
Pneumonitis	2 (12%)	0
Articular and muscle pain	1 (6%)	0
Decrease appetite	1 (6%)	0

Table 3: Summary of immune-related adverse events in patients re-treated in NIBIT-MESO-1 study. *Data are n of patients (%). Population included n=17 patients.*

To study the predictive role of TMB as potential biomarker for NIBIT-MESO-1 patients, a *post-hoc* analysis was performed associating TMB with OS. Twenty-eight patients for whom tumour tissue before treatment was available were stratified based on

the median value (8.3 mutation *per* Mb) of TMB in two groups. Results demonstrated that patients with TMB higher than the median value had an improved mOS than patients with TMB lower than the median value (938 days vs 451 days; p=0.06), although this difference did not reach statistical significance (Figure 6).

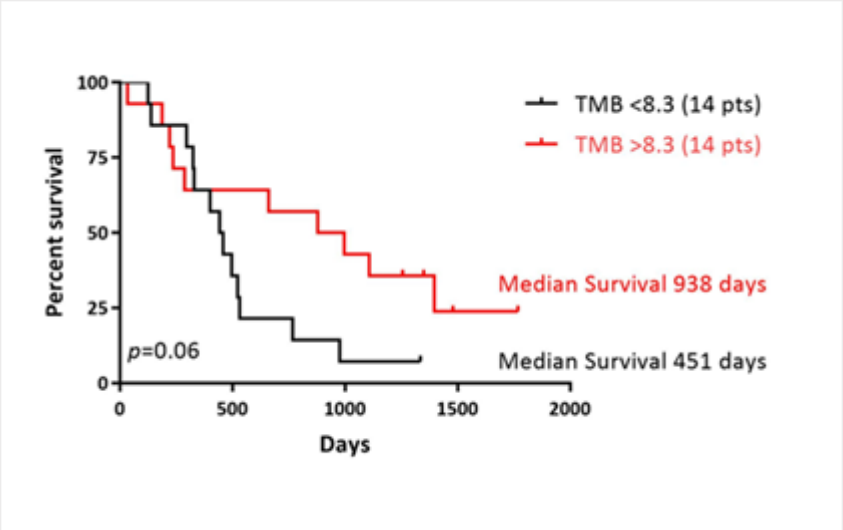

Figure 6: Kaplan–Meier cumulative survival analyses according to TMB (*post-hoc* analysis)**.** *Kaplan–Meier overall survival for all patients in the NIBIT-MESO-1 study with samples available for TMB analysis. Vertical lines indicate censored observations. Overall survival of patients stratified by median TMB of less than 8.3 per Mb (n=14) and more than 8.3 mutations per Mb (n=14).*

Interestingly, differences in OS became significant (p=0.001) when patients were additionally stratified based on their *re-treatment* or not with tremelilumab plus durvalumab (Figure 7.A). Additional analyses were performed to better understand the predictive role of TMB in re-treated compared to not re-treated patients. Results demonstrated a significant (p=0.02) advantage in OS for patients with a TMB > 8.3 *per* Mb that underwent ICI *re-treatment* (1256 days vs 528 days) (Fig 7.B). No difference in OS between not re-treated patients with a TMB > 8.3 *per* Mb (311 days) compared to those < 8.3 *per* Mb (288 days) was observed (Fig 7.C).

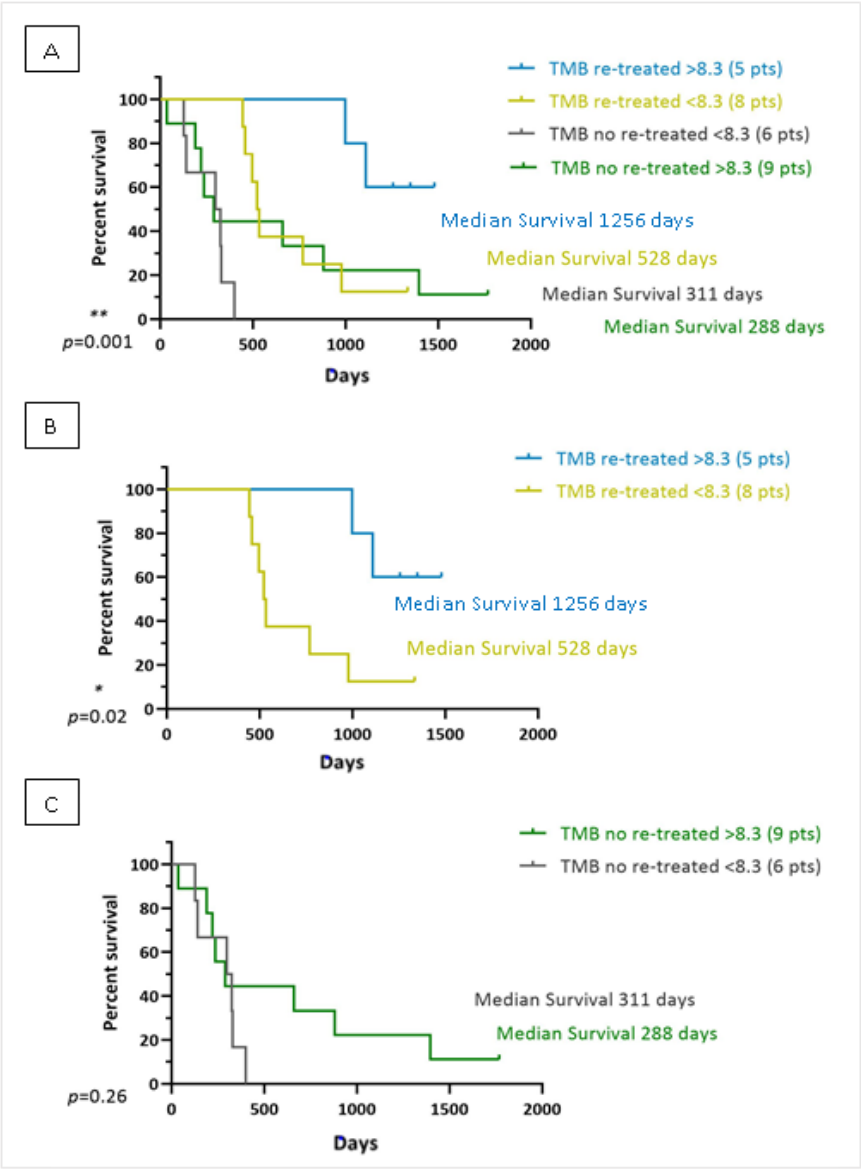

Figure 7: Kaplan–Meier cumulative survival analyses according to TMB, stratified according to re-*treatment* (*post-hoc* analysis).

(A) Kaplan–Meier overall survival for all patients with samples available for TMB analysis, stratified by median TMB (< and > 8.3 per Mb) and ICIs re-treatment and ICIs no-retreatment (n=28). (B) Overall survival of re-treated patients stratified by TMB value of 8.3 (n=13). (C) Overall survival of no-retreated patients stratified by TMB value of 8.3 (n=15). Vertical lines indicate censored observations. TMB=tumour mutational burden.

6. Discussion

No literature data are available regarding such a long follow-up in MM patients treated with co-targeting of the immune checkpoints CTLA-4 and PD-L1. From this updated survival analysis, it emerged that the combination of tremelimumab plus durvalumab is associated with durable survival at 36 and 48 months, in 20% and 15% of patients with MM, respectively. This result supported the hypothesis that simultaneously targeting multiple immune checkpoints might be an effective strategy to induce long-term survival in this population, similarly to what already demonstrated for other tumour types like melanoma and lung cacer. Furthermore, first-line combination treatment with anti-PD-1 and anti-CTLA-4 mAbs has been shown to significantly improve OS compared with platinum-based chemotherapy for patients with MPM. Consequentially, this association therapy has already obtained FDA approvation and it will become the globally approved standard of treatment soon.[11]

Little evidence is available on the therapeutic efficacy of *re-treatment* with ICIs in cancer patients with PD after initial clinical benefit (PR or SD) on these agents. This analysis was conducted to prospectively investigate the efficacy and safety of ICIs *re-treatment* in ICIs-refractory patients with MM.

Immunotherapy re-challenge can be applied to patients who progress after terminating a prior course of ICIs treatment in absence of PD. In this situation, ICIs re-challenge at the time of acquired resistance may reboost the expansion of memory T cells against the tumor, which, in turn, could help to restore sensitivity to treatment.[32] The benefits of ICIs re-challenge in this context have been retrospectively addressed in patients who were subjected to the anti-PD-L1 agent durvalumab within a phase I/II study conducted across multiple tumor types. Out of 1022 initial screenated patients, 168 individuals completed the planned 1 year of treatment without PD, of whom 70 were retreated with durvalumab upon relapse. Of all the latter, eight patients (11.4%) responded to ICIs re-challenge (PR), while forty-two patients (60%) experienced SD. Notably, out of the eight responding patients, five had responded to prior durvalumab, while on the total population of patients, the greatest benefit in terms of either PR or SD was noted in individuals who had a treatment-free interval since prior durvalumab ≥ 6 months as

compared with < 6 months (87% vs 48%). However, although this study suggested a correlation between a long treatment-free interval and augmented sensitivity to ICIs re-challenge, it should be noted that the maximum duration of prior durvalumab was only 1 year, which should be considered suboptimal based on CheckMate-153.[33] The results from CheckMate-153 represent the first insights from a randomized trial evaluating the impact of stopping treatment with a PD-1/PD-L1 inhibitor at 1 year *vs* continuing treatment in patients with advanced, previously treated, NSCLC, demostrating a significant improvement in PFS and OS for patients who continued nivolumab compared to those who did not.[34]

In this work, objective response was not observed, ICIs *re-treatment* resulted in disease stabilisation in 41% of patients, specially in those who had achieved an objective response during the first course of treatment of the NIBIT-MESO-1 study. However, the mPFS (11.3 months) of patients who had *re-treatment* compared favourably with the mPFS (8 months) observed for all patients in the first course of therapy in the NIBIT-MESO-1 study. It was interesting to evidence the impact on OS in *post-hoc* analysis, which was significantly improved in patients who were re-treated with tremelimumab and durvalumab compared with those who were not re-treated and received other additional chemotherapy (25.6 months *vs* 11 months). Supporting the efficacy of ICI-*re-treatment*, RMST analysis identified a mean survival time of 29.3 months for ICIs re-treated patients *vs* 16.7 months for patients who did not receive ICIs *re-treatment*. Although this analysis was conducted on a non-randomized study and the size of the re-treated cohort was small, the results suggest that ICI *re-treatment* might restore long-lasting tumor control in 24% of ICIs-refractory MM patients. This approach could be particularly relevant in previously treated MM patients for whom no active treatments are currently available. However, it must be acknowledged that in this follow-up study, patients who were eligible for *re-treatment* were also those who benefitted (i.e., PR or SD) from the initial therapy with ICIs and had no primary resistance to the same drugs.

To date, we do not have a solid basis and knowledge on the rationale for *re-treatment* with ICIs, as all available data are mainly based on retrospective studies on small subgroups of patients treated with mAb anti-CTLA-4, ipilimumab or with anti-PD-1/PD-L1 mAbs in larger studies. In an extended access program (EAP) with ipilimumab, 55% of patients with advanced melanoma who were re-treated regained disease control and

42% were alive 2 years after the first *induction* dose of ipilimumab. [35]

In KEYNOTE-006 trial, thirteen patients with metastatic melanoma, previously treated with pembrolizumab (anti-PD-1 mAb) for two years, were re-treated at disease progression with the same drug. Seven (54%) of 13 re-treated patients achieved an ORR.[36] In other reports, re-challenge with anti-PD-1 or PD-L1 mAbs has been associated with ORR of 25-27% and SD rates of 18-75% in several tumour types, including melanoma, NSCLC, colorectal cancer, urothelial cancer, and breast cancer. [37,38,39] In accordance with the results of this research, two further studies in patients with NSCLC reported primarily SD (with a single PR among 50 re-treated patients) as the best response to anti-PD-1 mAb re-challenge.[40,41]

Re-treatment with tremelimumab and durvalumab was associated with a good tolerability profile; no grade 3-4 ir-AEs occurred in re-treated patients. These findings are consistent with safety data for this combination in the NIBIT-MESO-1 study, as well as in other tumour types, and support further exploration of *re-treatment* with tremelimumab and durvalumab without safety concerns.

To conclude, the exact duration of ICIs treatment and the benefit of ICIs re-challenge for advanced cancers are still poorly defined. While a duration of at least two years for ICIs treatment has been suggested, the evidence on the benefit of ICIs re-challenge has been mainly obtained from retrospective studies. Taken together, the available data indicate that ICI *re-treatment* is clinically active in some patients regardless of tumour histology, and merits further investigation in dedicated larger prospective studies. At the present time, a parallel-group phase II clinical trial is being conducted in order to test re-challenge with pembrolizumab in patients with acquired resistance after stopping treatment (*NCT03526887*).

Finally, in another situation, ICIs re-challenge can be applied to patients who progressed during treatment or within 12 weeks of termination of immunotherapy. In this clinical scenario, ICIs re-challenge should be pursued only within clinical trials and several studies are ongoing to address different strategies of re-challenge such as: retreatment after intervening chemotherapy (*NCT03526887*), re-challenge with the addition of an anti-CTLA-4 agent to an anti-PD-1 drug (*NCT03262779*), re-treatment with continuation of anti-PD-1 agent beyond progression with the concomitant administration of a cytotoxic agent not previously administered (*NCT03041181*), re-challenge with the

addition of a targeted therapy to an ICI (*NCT03334617, NCT03829332*). Importantly, most of the mentioned clinical trials include a mandatory re-biopsy at baseline to select the most appropriate treatment and/or understand the molecular mechanisms underlying resistance to ICB treatment.

ICIs have revolutionized the management of oncological patients and have led to extraordinary improvements in ORR and OS. However, immunotherapy is efficacy only for a minority of patients, which therefore should be selected using relevant predictive biomarkers like TMB.

A growing research activity on the study of TMB, focused on non-synonymous somatic mutations in the tumor exome or per Mb DNA, demonstrated that cancers which harbor more mutations, such as melanoma and lung cancers (NSCLC and SCLC), have a greater likelihood of response to ICB treatment.[42,43,44] This could be speculated to be related to a higher probability of generating immunogenic neoantigens that might promote the expansion of functional tumour-specific T cells and eventually improve antitumor immune responses.[45] Moreover, the description of a high prevalence of insertions/deletions (indels) mutations and their association with response to ICB treatment in renal cell carcinoma, pointed out the importance of specific types of genomic alterations in tumours for the generation of immunogenic neoantigens. In this context, the recent identification of a low TMB in MPM coupled with the discrete rate of response of MPM patients to ICB treatment, strongly prompt an in deep characterization of genomic alterations in MPM useful for the identification of predictive biomarkers to ICB response.[46,47]

TMB is a continuous variable and variability of TMB (ranging from 0.001/Mb to more than 1000/Mb) has been observed across and within cancer types. Findings indicate that some tumor types have less variability in TMB, such as lung and head and neck cancers, and some have greater variability such as colon, bladder, and uterine cancers.[48]

The calculation of TMB has different methods, depending on the assay adopted. The whole-exome sequencing (WES)-TMB assays typically consider non-synonymous somatic mutations in the analysis, while next-generation sequencing (NGS) panels have generally taken a more comprehensive approach, such as FundationOne CDx, which includes synonymous and non-synonymous single-nucleotide variants (SNVs) for improved assay sensitivity, and indels mutationts per area of coding genome sampled,

but excludes known and likely oncogenic driver events and germline SNPs. Currently there is no standard for TMB evaluation and the TMB Harmonization Project is aimed to standardize TMB calculation and reporting.[49]

On June 16th, 2020, TMB ≥ 10 mutations [muts]/Mb was approved by FDA to select for pembrolizumab treatment in adult and pediatric patients with unresectable or metastatic solid tumors, regardless of histology. Foundation One CDx assay (Foundation Medicine, Inc.) was also approved as a companion diagnostic test.[50]

The FDA approved pembrolizumab for all solid tumors based on the KEYNOTE-158 study, which demonstrated durable responses in patients with a TMB ≥ 10 muts/Mb (TMB-high cohort). KEYNOTE-158 was a multi-cohort study that was limited to 10 malignancies. On further review of the TMB-high cohort, nearly 90% of enrolled participants had either NSCLC (n=34), cervical cancer (n=16), endometrial cancer (n=15), vulvar cancer (n=15), or anal cancer (n=14). Clinical benefit was not consistently demonstrated across all tumor types, which suggests that tumor origin, and by extension, tumor immune microenvironment, may influence clinical response. Whereas patients with TMB-H endometrial cancer had an ORR of 47%, patients with TMB-H anal cancer had an ORR of only 7%. Of note, patients with non-TMB-H anal cancer treated with pembrolizumab had a higher response rate (11%), suggesting that TMB, at least in this tumor histology, did not predict benefit. Low enrollment into other disease cohorts further limits the generalizability of study results.[51]

Despite the FDA approval, many variable factors remain before the implementation of TMB as a biomarker in clinical practice. These include the following: different tumor types biologically have different TMB; tissue type (FFPE tissue will artificially have more mutations than fresh frozen tissue); identification of therapies whose response is best informed by TMB status; robust definition of a predictive TMB cut-off, possibly cancer-specific; acceptable sequencing panel size and design; and the need for robust technical and informatic rigor to generate precise and accurate TMB measurements across different laboratories.

An additional ongoing challenge is the identification of biomarkers to enable selection of patients with MM who will derive most benefit from ICIs therapy and from ICIs *re-treatment*. In this work, the role of TMB was evaluated in treated and re-treated patients, an association between baseline TMB higher than the median population value and

improved survival in the whole patient population, was observed, although the results did not reach statistical significance. Interestingly, the advantage in survival for patients with higer TMB appeared to be even more evident in patients who received *re-treatment*. While these findings need to be interpreted with caution, being preliminary, and derived from a *post-hoc* analysis of a small number of patients, a high mutation load at baseline seems to identify patients with MM who are most likely to benefit from ICI therapy and *re-treatment* with these agents. This latter finding is intriguing and needs to be further explored, ideally comparing tumor biopsies at disease progression with archival samples, though its feasibility in mesothelioma is clearly limited by the very invasive procedures it would require. Nonetheless, the ability of TMB to identify patients with a favourable outcome to ICIs, including those who are suitable candidates for re-challenge, warrants prospective investigation in a larger study. Anti-tumour immunotherapy with ICB in patients with MM has yet to reach its full potential. There is considerable need to improve treatment outcomes and to overcome resistance to this class of immunotherapy. New ways of using these agents, including optimising the duration of ICIs treatment or re-challenge, are under active investigation, and will hopefully improve the clinical efficacy of ICIs against this still fatal and difficult-to-treat disease. Despite the limitations of this study, including the small number of patients who received ICIs *re-treatment* and the non-randomised study design, which prevent drawing firm conclusions, the clinical results support the efficacy and safety of ICIs *re-treatment* in patients who become refractory to inital ICIs treatment, warranting further investigation for potential application of *re-treatment* in the clinical practice. Finally, effective prediction of response to ICI therapy will likely require integration of TMB with a pannel of other potential biomarkers, including tumor genomic driver alterations, tumor-immune milieu, and other features of the host immune system.

7. Conclusion

Immunotherapies are revolutionizing therapeutic concepts which are changing the standard of care for cancer treatment, by converting the tumor from a fatal disease to a chronic one and often ensuring a long-term tumor control.

The potential of immunotherapy is evolving, even thanks to the good performance of the combination of multiple immunotherapeutic agents.

It is now recognized that the cancer-immune interaction is influenced by a complex set of tumor genetic and epigenetic factors, as well as by host genomic and environmental factors, which, acting together, govern the strength and timing of the anticancer response. Because of the complexity of the immune response and tumor biology, single analyte biomarkers are not very informative. Thanks to the rapid progress in technology, today we can measure the factors affecting the cancer-immune interaction using technology platforms that separately measure the different types of potentially informative analytes (DNA, RNA, and proteins). The current fundamental challenges in immuno-oncology translational research remain the amount of informative data available from small clinical samples and how to integrate data easily and in a timely fashion into biologically and clinically actionable information.[52]

The complexity and heterogeneity of the interaction between the immune system and tumor cells, particularly in the tumor microenvironment (TME), underlies the immune status (i.e., immunologically responsive, or immunologically ignorant) of each individual tumor for every patient. Biomarker-based research is an essential approach to understand both intrinsic and extrinsic tumor escape mechanisms and therefore improve the treatment process. As in other cancers, the current search for other potential predictive biomarkers for immunotherapy in MM includes assessments of tumor infiltration by immune cells, microsatellite instability (MSI), TMB and neo-antigens, whole genome sequencing and gut microbiota evaluation. ICIs appear to be more effective in tumors with higher mutational loads (TMB).

The best understanding of genomic alterations in MM could be of use to find biomarkers for predicting response to ICIs. MPM is a disease primarily associated with exposure to the carcinogen asbestos. Consistent with this carcinogenic exposure,

cytogenetic analyses identified multiple recurrent structural chromosomal abnormalities in this malignancy, but preliminary whole exome sequencing analyzes identified a low mutational burden in MPM.[53]

Despite this notion, a discrete percentage of MPM patients treated with ICIs have a good clinical outcome. Some of these patients who initially respond to immunotherapy, experiment relapse because of secondary resistance to the treatment.

To date, there is no gold standard strategy to overcome ICIs resistances and no validated predictive biomarker of response for these therapies has been identified.

ICIs re-challenge after an intervening treatment in patients who progressed during immunotherapy should be regarded as investigational and the enrollment in prospective clinical trials should be highly encouraged, expecially in lack of valid therapeutic alternatives. Notwithstanding, in case of PD occurred during or after the end of ICIs treatment, only the understanding of the molecular mechanisms involved in ICIs resistance would help to find novel thearapeutic targets, either immune or biological ones, and eventually facilitate the development of new therapeutic weapons. More widely, the refining of new tailored therapeutic strategies could extend the proportion of patients potentially benefiting from immunotherapy re-challenge, eventually included in combinatorial approaches with other classes of drugs. It is important to underline that it would be advisable to desing new studies in which biopsy may be available before starting treatment while a mandatory baseline ribiopsy should by always requested to select the most appropriate treatment and/or understand the molecular mechanisms underlying resistance to ICIs.

The challenge of the future will be the identification of a comprehensive panel of biomarkers that can accurately select among MM patients the best candidates for treatment/*re-treatment* with ICIs.

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