Baseline β-catenin, programmed death-ligand 1 expression and tumour-infiltrating lymphocytes predict response and poor prognosis in BRAF inhibitor-treated melanoma patients

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Abstract  Background: The activation of oncogenic Wnt/β-catenin pathway in melanoma contributes to a lack of T-cell infiltration. Whether baseline β-catenin expression in the context of tumour-infiltrating lymphocytes (TILs) and programmed death ligand-1 (PD-L1) overexpression correlates with prognosis of metastatic melanoma patients (MMPs) treated with mitogen-activated protein kinase, MAPK inhibitor (MAPKi) monotherapy, however, has not been fully clarified.

Patients and methods: Sixty-four pre-treatment formalin-fixed and paraffin embedded melanoma samples from MMP treated with a BRAF inhibitor (n = 39) or BRAF and MEK inhibitors (n = 25) were assessed for presence of β-catenin, PD-L1, cluster of differentiation (CD)8, CD103 and forkhead box protein P3 (FOXP3) expression by immunohistochemistry, and results were correlated with clinical outcome. Quantitative assessment of mRNA
transcripts associated with Wnt/β-catennin pathway and immune response was performed in 51 patients.

**Results:** We found an inverse correlation between tumoural β-catennin expression and the level of CD8, CD103 or forkhead box protein P3 (FOXP3) positivity in the tumour microenvironment (TME). By multivariate analysis, PD-L1 <5% (odds ratio, OR 0.12, 95% confidence interval, CI 0.03–0.53, p = 0.005) and the presence of CD8+ T cells (OR 18.27, 95%CI 2.54–131.52, p = 0.004) were significantly associated with a higher probability of response to MAPKi monotherapy. Responding patients showed a significantly increased expression of mRNA transcripts associated with adaptive immunity and antigen presentation. By multivariate analysis, progression-free survival (PFS) (hazards ratio (HR) = 0.25 95%CI 0.10–0.61, p = 0.002) and overall survival (OS) (HR = 0.24 95%CI 0.09–0.67, p = 0.006) were longer in patients with high density of CD8+ T cells and β-catennin <10% than those without CD8+ T cells infiltration and β-catennin ≥10%.

**Conclusion:** Our findings provide evidence that in the context of MAPKi monotherapy, immune subsets in the (TME) and gene signature predict prognosis in MMPs.

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1. **Introduction**

In patients with metastatic melanoma (MMPs), BRAF inhibitor (BRAFi) monotherapy has shown overall survival (OS) benefit over chemotherapy, and its combination with MEK inhibitor (MEKi) has recently been shown to improve progression-free survival (PFS) and OS, compared with BRAFi monotherapy [1–3]. However, almost 20% of patients do not respond to therapy due to intrinsic or acquired resistance mediated by hyperactivation of receptor serine/threonine kinases, MAP kinase and alternative phosphatidylinositol 3-kinase/Akt (PI3-K/Akt) signalling and interactions with the tumour microenvironment (TME) [4].

In addition to these established molecular mechanisms of resistance, there is growing evidence that the therapeutic efficacy of BRAFi monotherapy relies on additional factors involved in tumour–host interactions, including the enhancement of melanoma antigen expression and the increase in immune response against tumour cells [5,6]. Preclinical data show that oncogenic BRAF contributes to immune evasion, as targeting this mutation may increase melanoma immunogenicity [7]. Within the first 2 weeks of BRAF inhibition, the expression of immunomodulatory molecules on the tumour cell surface, such as programmed death-ligand 1 (PD-L1) and its receptor programmed death-1 (PD-1) on T lymphocytes, is increased [5,6]. In vitro data and results obtained in experimental animals identify PD-L1 as a potential mechanism that contributes to reinforcing BRAFi resistance through the modulation of host immune responses [8].

We recently demonstrated that in BRAFi-treated MMPs, PD-L1 expression and the absence of tumour-infiltrating mononuclear cells (TIMCs) were significantly associated with shorter PFS and suggested them as independent prognostic factors for melanoma-specific survival [9]. By multivariate analysis, absence of tumoural PD-L1 staining and the presence of TIMCs were associated with a better response to treatment [9]. Hence, understanding the molecular mechanisms that contribute to the control of antitumour T-cell response could guide the development of novel therapeutic strategies, particularly for those patients with poor T-cell infiltration. Of interest, the intrinsic activation of oncogenic Wnt/β-catennin pathway in melanoma cells has been implicated in attenuating T-cell infiltration in a mouse model of spontaneous melanoma [10].

In the present study, we evaluated in a homogeneous series of 64 MMPs treated with either BRAFi monotherapy or a combination of BRAFi with MEKi, the expression of tumour-associated β-catennin and of series of parameters indicative of an inflamed TME, including PD-L1, TIMC, CD8+ effector T cells, CD103+ cells and FOXP3+ regulatory T cells. These morphological findings were supported by study of the expression of specific genes involved in the Wnt/β-catennin pathway and immunity. Finally, the impact of these biomarkers were analysed in relation to overall response rate (ORR), PFS and OS. Our work provides evidence of therapeutic and prognostic relevance of tumour-associated β-catennin overexpression, the density of CD8+ effector T cells, and the differential expression of genes involved in the Wnt/β-catennin pathway and immunity in BRAFV600 mutated MMPs treated with BRAFi, alone or in combination with MEKi.

2. **Materials and methods**

2.1. **Patient characteristics**

The cohort was identified by inspecting the electronic database of all melanoma patients treated at the Papa Giovanni XXIII Cancer Center Hospital, Bergamo,
Italy, from July 2011 to October 2014. We retrieved data concerning clinical outcome and BRAFi treatment from patients enrolled in compassionate or expanded access use protocols of BRAFi in our institution since July 2011. The local Ethics Committee approved the study protocol. The study was conducted in compliance with the World Medical Association Declaration of Helsinki.

Patients who were enrolled in this study were treated within the (1) vemurafenib and dabrafenib compassionate use programme or the (2) therapeutic and expanded access use of dabrafenib with or without trametinib or vemurafenib according to clinical practice. For patients included in the vemurafenib compassionate programme use, the inclusion criteria were an Eastern Cooperative Oncology Group performance status (ECOG-PS) 0 inclusion criteria were an Eastern Cooperative Oncology Group performance status (ECOG-PS) 0–2, as well as normal hepatic (serum bilirubin <1.5 mg/dl), renal (serum creatinine <1.5 mg/dl) and bone marrow (leucocyte count >4000/ll, platelet count >100 000/ll) functions. For the other patients, exclusion criteria were a rapid deteriorating medical condition, with severe liver or renal failure, QTc >500 mS and ECOG-PS 4.

Information on age, gender, medical condition, with severe liver or renal failure, QTc >500 mS and ECOG-PS 4. Information on age, gender, histopathology, and surgical and medical treatment were retrieved for each patient, as well as data on tumour RR, PFS and OS.

The study cohort included 64 consecutive cutaneous MMPs referred to Papa Giovanni XXIII Hospital, Bergamo, between July 2011 and April 2015. Data on treatment and survival were collected prospectively. This patients’ cohort represents part of the series previously reported [9].

Accuracy in histopathological classification was confirmed by medical records and/or review of pathology material. The study protocol was approved by the local Ethics Committee. tumour stage, assessed according to the American Joint Committee on Cancer (AJCC) TNM (Tumour, Node, Metastasis) staging system classification (VII edition). Clinical response to BRAF MEK inhibition was assessed by RECIST v1.1 criteria [11].

Tumour assessments were conducted according to RECIST version 1.1 at baseline, week 12, and every 12 weeks until disease progression or death, according to the internal guidelines at the Papa Giovanni XXIII Melanoma Unit, Bergamo.

For RT-PCR analyses, patients with either complete or partial response (CR and PR, respectively) were considered responders (R) while patients with progressive disease (PD) were considered non-responders. Patients with stable disease were not included in the final RT-PCR analysis.

2.2. Tissue samples

Formalin-fixed, paraffin-embedded (FFPE) tumour tissues were retrieved from pathologic archives. Melanoma samples were obtained from patients treated with BRAFi and combination of BRAFi and MEKi before starting treatment. The cohort included both primary melanomas (n = 20) and metastatic tumours (n = 44), including subcutaneous, lymph node and visceral metastases. Haematoxylin-Eosin (H&E)-stained sections were assessed for location and density of TIMCs, and intratumoural immune cell infiltration was scored as: absent (0), focal (1+), moderate (2+) and marked (3+), as previously reported [9]. Only true intratumoural mononuclear immune cells, rather than perivascular inflammation, was recorded. Tabulated for statistical analysis, only cases with moderate and marked TIMC were considered TIMC positive.

2.3. Immunohistochemistry

Representative 4-µm thick FFPE sections of melanoma samples were selected for immunohistochemical analysis. PD-L1 immunohistochemical analysis was performed as previously reported [9]. Additional primary antibodies used in this study include: rabbit monoclonal CONFIRM anti-CD8 (clone SP57 ready to use Ventana, Tucson, Arizona); rabbit monoclonal anti-CD103, dilution 1:500 (integrin αE, clone EPR4133 [2], Abcam, Cambridge UK); mouse monoclonal anti-β-catenin (clone 14 ready to use, Ventana) and rabbit monoclonal anti-FOXP3, dilution 1:80 (clone SP97, Abcam) on a Ventana BenchMark ULTRA immunostainer (Ventana Medical Systems, Tucson, AZ). The Ventana staining procedure included pre-treatment with cell conditioner 1 followed by antibody incubation. For all antibodies, signals were developed with Universal Red Detection Kit. After the staining run was complete, the tissue sections were counterstained with haematoxylin. Sections of tonsil were used as positive control. Negative controls were performed by substituting the primary antibody with a mouse-rabbit Serum (Normal, Dako, Carpinteria, CA). The control sections were treated in parallel with the samples. For β-catenin in primary tumours, normal epidermal keratinocytes were taken as positive internal control.

Isotype control staining for selected antibodies was performed, specifically, isotype control IgG1 staining for mouse monoclonal β-catenin and FOXP3, isotype control IgG for rabbit monoclonal CD8 and CD103 (Fig. 1S).

PD-L1 tumour staining intensity was scored as follows: 0 (no staining), 1+ (weak or equivocal staining), 2+ (moderate staining), or 3+ (strong staining), as previously reported [9]. In the final tabulation, tumour staining for PD-L1 positivity was defined as at least 5% of tumour cells showing 2+ or 3+ membrane staining.

Cytoplasmic, membranous and nuclear β-catenin tumoural staining subcellular localisation was assessed separately, as previously described in melanoma [12–14] and quantified for intensity (weak, moderate, strong) and for the proportion of tumour cells showing unequivocal staining. In primary melanomas, the immunoreactivity was assessed both in the whole tumour as
well as in the distinctive growth phases (radial and vertical growth phases). Score density of CD8+, CD103+ and FOXP3+ immune cells in intratumoural and peritumoural location was assessed as follows: 0: absent; 1+: mild (<10%); 2+: moderate (10–50%); 3+: severe (50–100%).

Immunohistochemical scoring was performed in a blinded fashion by an experienced melanoma pathologist (DM). Immunostained sections were initially assessed at low magnification to select the areas with highest density of positive cells at peritumoural and intratumoural location. Assessment of lymphocytes’ score density was then compared with evaluation obtained by image analysis.

2.4. Digital image acquisition and analysis

Anti-CD8 immunostained slides were scanned using D-Sight platform (A. Menarini Diagnostics, Florence, Italy) using 20× magnification. An algorithm based on quantification of CD8+ cells was developed processing RGB (red, green, blue) images and counting nuclei by haematoxylin stain (blue) and fast red as counterstain, respectively. In brief, the first step of the image analysis was the colour deconvolution applying a flexible method of separation and quantification of immunohistochemical staining by means of target colours in the image. The true colour information was evaluated and separated on the predefined and expected stains.

Thus, we evaluated haematoxylin and fast red stains on reference images, assessed by decomposing the absorbance values of stain mixtures into absorbance values of single stains using the colour deconvolution algorithm. The second step was based on a binarisation of the fast red image component reached by the first step using a recursive isodata threshold [15]. In the final step a watershed segmentation of the binary mask was applied in order to separate cells cluster and to identify properly CD8+ cells and to quantify their density (cells/mm²) knowing the calibration used during the image acquisition.

The number of fast red CD8+ cells was calculated in four different high power magnification fields of 10⁻³ mm². The algorithm calculated the density of CD8+ cells (cells/mm²) using D-Sight platform. The total number of CD8+ cells was calculated as the mean of each high power magnification fields. CD8 expression was determined using two read-outs that were independent of each other to account for tumour heterogeneity: the assessment of CD8+ cells cell density (number of positive cells per mm²) and score density (number of positive cells per number of nucleated cells).

2.5. Double immunofluorescence

Representative 4-µm thick FFPE sections of melanoma samples were selected for immunofluorescence analysis. Immunofluorescence staining was performed according to standard procedures. Briefly, after antigen retrieval (ethylene diamine tetra-acidic acid (EDTA) solution pH 9.0, Dako, Milan, Italy) for 20 min at 98 °C, sections were incubated with the following primary antibodies: CD103 dilution 1:500 (integrin αE, clone EPR4133 [2], Abcam, Cambridge UK), CD11c dilution 1:100 (Mouse Monoclonal Antibody, Novoceastra, Leica Microsystems, Milan, Italy), human leucocyte antigen-antigen D related (HLA-DR) dilution 1:1000 (Mouse monoclonal, clone TAL1B5, Abcam, Cambridge UK) and CD141 dilution 1:25 (Thrombomodulin/CD141 Antibody clone [QBEND/40], Thermo Fisher, Rockford, USA) and CD8 dilution 1:50 (Mouse monoclonal, Abcam, Cambridge UK) diluted in fresh blocking solution (phosphate-buffered saline (PBS), pH 7.4, 5 mg/ml bovine serum albumin and 2.5% normal goat serum) and applied 1 h at room temperature. Sections were then incubated for 2 h in the dark with the appropriate fluorescent secondary antibody (polyclonal AlexaFluor 488 fluorescein isothiocyanate (FITC)-conjugated, and polyclonal AlexaFluor 594 tetramethylrhodamine isothiocyanate (TRITC)-conjugated, Invitrogen, Milan, Italy) diluted 1:600 in blocking solution (PBS, pH 7.4, 5 mg/ml bovine serum albumin and 2.5% normal goat serum).

Sections were cover-slipped using a water-based mounting medium with 4’6’-diamidino-2-phenylindole (DAPI) (Abcam, Cambridge, UK). The analysis of negative controls (non-immune serum) was simultaneously performed in order to exclude the presence of non-specific immunofluorescent staining, cross-immunostaining, or fluorescence bleed-through. Tissues were visualised and digital images were captured using an Olympus BX51 (Olympus s.r.l., Milan, Italy).

2.6. RNA extraction

Total RNA was extracted from 52 formalin-fixed paraffin-embedded (FFPE) metastatic melanoma specimens using the high pure FFPE RNA isolation kit (Roche Life Science, product no: 06650775001). Of the 52 total samples processed, two were not further analysed by RT-PCR due to insufficient RNA quantity. The FFPE samples analysed contained at least 40% of tumour cells.

2.7. Quantitative RT-PCR for measurement of selected mRNA along Wnt pathway and immune pathways

The theoretical and practical aspects of real-time quantitative PCR have been described previously in detail [16]. Quantitative values were obtained from the cycle number (Ct value) at which the increase in the fluorescence signal associated with exponential growth of PCR products started to be detected by the laser detector of the ABI Prism 7900 Sequence Detection
System (Perkin—Elmer Applied Biosystems, Foster City, CA), using PE Biosystems analysis software according to the manufacturer’s manuals.

The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (i.e. lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of an endogenous RNA control gene, namely RPLPO (Genbank accession NM_001002.3), which encodes the ribosomal protein that is a component of the 60S subunit. Each sample was normalised on the basis of its RPLPO content. Results, expressed as N-fold differences in target gene expression relative to the RPLPO gene and termed “Ntarget”, were determined as $N_{\text{target}} = 2^{\Delta C_{\text{sample}}}$, where the $\Delta C_{\text{t}}$ value of the sample is determined by subtracting the Ct value of the target gene from the Ct value of the RPLPO gene. The Ntarget values of the samples were subsequently normalised such that the median of the 27 melanoma samples N target values was 1. cDNA synthesis, and PCR conditions were as described [16].

Primers for RPLPO and the target genes were designed with the assistance of Oligo 6.0 computer program (National Biosciences, Plymouth, MN). To avoid amplification of contaminating genomic DNA, one of the two primers was placed at the junction between two exons or on two different exons. Agarose gel electrophoresis was used to verify the specificity of PCR amplicons. The nucleotide sequences of the oligonucleotide primers for the examined genes are listed in Supplementary Table 1S.

2.8. Statistical analysis

PFS was defined as the time from the date of start of the treatment to the date of progression or death from any cause, whichever comes first. Patients who did not register progression of the disease or die as of the date of analysis were censored at their last disease assessment date. OS was defined as the time from the date of start treatment to the date of death from any cause. ORR was defined as the proportion of patients with a complete or partial response to treatment. Survival curves were estimated with the Kaplan—Meier method. PFS and OS were analysed by means of Cox regression model and results were expressed as hazard ratios (HR) with their 95% confidence intervals (95%CI). ORR was analysed by means of logistic regression models and expressed as odds ratios (OR) with their 95%CI. Clinical variables with a p value <0.05 at the univariate analysis and all immunohistochemical variables were considered for the multivariate model. The covariates were selected for the final model by means of a backward selection procedure.

β-catenin was tested as a continuous or as a dichotomous variable using the median value as cut-off (10%). CD8+ T cells were analysed in three groups: CD8 negative (−) including scores 0/1+; patients with overexpressed (3+) intratumoural CD8+ cells were considered as CD8+ high, while patients with medium/low (score 2+/1+) levels of intratumoural CD8+ and overexpressed peritumoural CD8+ T cells were considered in the CD8+ low group.

The same rule was applied for CD103 to create three groups. FOXP3+ T cells were considered in three groups: FOXP3+− were considered patients with scores 0/1+, patients with overexpression and medium levels (3+/2+) intratumoural FOXP3+ cells were considered FOXP3+ high while patients with low (1+) levels of intratumoural FOXP3+ and overexpression/medium or low levels of peritumoural FOXP3+ were considered in the FOXP3+ low group.

Combining β-catenin expression and CD8+ T cell infiltration, patients were categorised in three groups: group 1: patients with lack of CD8+ T cells and β-catenin ≥10%; group 2: patients with lack of CD8+ T cells and β-catenin <10% or patients with high density of CD8+ T cells and β-catenin ≥10%; group 3: patients with high density of CD8+ T cells and β-catenin <10%.

Spearman correlation coefficient was used to describe the correlation between β-catenin expression and TIMC or CD8/CD103/FOXP3 T cells positivity. Concordance between the CD8+ TIL read-out in intratumoural and peritumoural location performed at the microscope on immunostained sections and automated counting upon digital image acquisition was tested by linear regression and described by $I^2$. All tests were two-sided. Statistical significance was set at <0.05 for each analysis. Statistical analyses were carried out using SAS version 9.2 (SAS Institute, Cary, NC).

Relationships between molecular parameters (at the RNA level), and the clinical, biological and pathological parameters, were analysed through chi-squared and Kruskal—Wallis tests.

3. Results

3.1. Patients and treatments

Of the 64 patients studied about half (56.2%) were male, median age was 56 years old (ranging from 21 to 86) (Table 2S). All patients harboured metastatic melanoma, with 53% having stage M1c visceral metastatic disease (34/64). Twenty-six patients (41%) received vemurafenib, 13 (20%) received dabrafenib and 25 (39%) received combination of dabrafenib with trametinib. Two patients received ipilimumab before treatment with targeted therapy; while after the discontinuation of BRAFi, subsequent anticancer therapy was administered to 20 patients (31%). Eleven patients received ipilimumab at progression; the remaining cases received dacarbazine or fotemustine. Neither pembrolizumab nor nivolumab was available for commercial use in Italy at the time of the present clinical study.
3.2. Immunohistochemical evaluation of β-catenin expression in melanoma samples

β-catenin expression was evaluated in this cohort in the last available metastatic sample before starting BRAFi therapy in 44 patients. Pre-treatment biopsies in metastatic sites were obtained at a median time of 2 months (range 1–6 months). In 19 cases, β-catenin was evaluated in the primary melanomas due to lack of metastatic tissue samples availability. In the remaining patient, β-catenin immunohistochemical staining was not assessable due to diffuse and strong melanin pigmentation in melanoma cells.

Different patterns of subcellular localisation of β-catenin expression were visualised in melanoma cells, including cytoplasmic, membranous, and nuclear, in different combinations and with variable intensity (Fig. 2S). Cytoplasmic localisation of variable intensity (irrespective of nuclear or membranous staining) was included in statistical analysis. The median value of cytoplasmic β-catenin expression was 10%, a cut-off taken to discriminate low from high expression samples. Cytoplasmic positivity was observed in 42/63 cases (66.6%). Cytoplasmic β-catenin staining ≥5% of tumour cells was observed in 39/63 cases (62%). Cytoplasmic β-catenin staining ≥10% of tumour cells was observed in 34/63 cases (54%). Nuclear β-catenin staining was detected in 11/63 (17.5%) cases and was always associated with cytoplasmic staining. Membranous staining (without cytoplasmic or nuclear staining) was observed in 19/63 cases (30.1%).

Of note, 17 (40.5%), 2 (4.7%) and 23 (54.8%) of the cytoplasmic β-catenin-positive cases were identified in patients with stage M1a, M1b and M1c respectively.

3.3. Correlation between β-catenin expression and overall tumour-infiltrating mononuclear cell density

Among 64 MMPs, 30 showed absent/focal TIMCs, while 34 were associated with at least moderate TIMCs within tumour cells. To explore a potential correlation between β-catenin expression and the presence of TIMCs, we compared the degree of β-catenin expression with the presence of associated immune infiltrates. We found an inverse correlation between β-catenin expression and TIMCs (Spearman correlation coefficient = −0.34, p-value 0.007).

Representative images of β-catenin expression in absence or presence of TIMCs are illustrated in Fig. 1.

3.4. Expression analysis of genes involved in the Wnt/β-catenin pathway and in adaptive immunity

In order to further confirm protein expression data, we selected 31 responders (R) and 10 non-responders (NR) patients and quantified by RT-PCR 12 mRNA transcripts of WNT5A, frizzled receptor (FZD)1, FZD3, FZD8, adenomatous polyposis coli (APC), catenin (cadherin-associated protein), beta 1 (CTNNB1), AXIN1, AXIN2, transcription factor 7 (TCF7), TCF7L2, MSX2, and SOX9 that are involved in Wnt/β-catenin pathway (Fig. 2A, Table 3S) and 14 mRNA
transcripts of CD3E, CD4, CD8A, chemokine (C-X-C motif) ligand (CXCL)1, CXCL2, CXCL9, CXCL10, CXCL12, interferon alpha (IFNA)1, IFNAR1, granzyme A (GZMA), perforin 1, pore forming protein (PRF1), forhead box protein P3 (FOXP3), and thrombomodulin (THBD) that are involved in the immune response (Fig. 2B, Table 3S). There was a trend \( P \leq 0.42, \text{not significant} \) towards an increased expression of FZD8 in NR patients. Interestingly, mRNA transcripts of CD8A, FOXP3, and THBD were significantly \( p < 0.05 \) by Kruskal–Wallis test upregulated in R versus NR patients. We observed no difference in expression of the mRNA transcripts analysed in ten patients with stable disease (data not shown). Finally, there was no statistical difference in the expression of lymphoid enhancer-binding factor (LEF)-1 and c-Jun transcripts in R versus NR patients (Fig. 3S).

3.5. Correlation between \( \beta \)-catenin, PD-L1 expression, TIMC, CD8\(^+\) T cells and overall response to MAPK inhibitors

Treatment outcome was available for all patients and included 6 (9.4\%) complete responses (CRs); 36 (56.2\%) partial responses (PRs); 11 (17.2\%) stable disease (SD); and 11 (17.2\%) progressive disease (PD). Results of univariate and multivariate logistic model assessing the effect of the presence of \( \beta \)-catenin, PD-L1 expression and TIMC on response to treatment are reported in Tables 4S and 5S. At multivariate analysis, PD-L1 \( \geq 5\% \) (OR 0.13, 95%CI 0.03–0.54, \( p = 0.005 \)), the presence of TIMC (OR 18.27, 95%CI 1.67–36.75, \( p = 0.009 \)) (Table 5S, Fig. 4S) were significantly associated with a lower and a higher probability of response to treatment, respectively (Table 5S, Fig. 4S). Furthermore, after adjusting for stage, PD-L1 and \( \beta \)-catenin expression, the density of CD8\(^+\) T cells (OR 18.27, 95%CI 2.54–131.52, \( p = 0.004 \)) was significantly associated with a higher probability of response to treatment. The probability of response was higher in patients with high density of CD8\(^+\) T cells and \( \beta \)-catenin \(<10\%\) than those without CD8 T cells infiltration and \( \beta \)-catenin \(\geq10\%\) (OR 10.70, 95%CI 1.64–69.80, \( p = 0.013 \)) (Table 5S).

Immunohistochemical analyses according to response to treatment are reported in Table 6S.

3.6. CD8\(^+\), CD103\(^+\) and FOXP3\(^+\) intratumoural and peritumoural lymphocytes in melanoma samples

Representative images of CD8\(^+\), CD103\(^+\) and FOXP3\(^+\) immune cells in intratumoural and peritumoural melanoma samples are shown in Fig. 5S. Table 7S summarises the results of the densities of CD8\(^+\), CD103\(^+\) and FOXP3\(^+\) lymphocytes’ densities across the entire cohort. There was a significant concordance between the CD8\(^+\) tumour-infiltrating lymphocytes (TILs) read-out in intratumoural (\( p < 0.001, I^2 = 91\% \)) and peritumoural location (\( p = 0.02, I^2 = 100\% \)) performed at the microscope on immunostained sections and automated counting upon digital image acquisition (Fig. 6S).

To further clarify the nature of the CD103\(^+\) immune cells, we assessed by double immunofluorescence costaining of CD103 and CD8 and confirmed that the majority of CD103\(^+\) cells represent a subset of CD8\(^+\) TILs, indicating CD103 as a marker of effector memory T cells resident within tissues (Fig. 7S). Additional double immunofluorescence analyses also indicated that the majority of CD103\(^+\) cells did not coexpress CD11c,
HLA-DR, CD141 (Fig. 8S) confirming that in this context CD103 is mostly upregulated in CD8+ T cells following antigen stimulation and only a minority, if any, of the CD103+ cells may represent dendritic cells or activated CD4+ T lymphocytes.

### 3.7. Association of β-catenin expression with CD8+, CD103+ and FOXP3+ T cells

Among 64 patients, 32 (51.6%) were CD8-, 13 (21.0%) were CD8+ low, while 17 (27.4%) were CD8+ high. Due to technical reasons CD8 status was not determined in two patients. To explore a potential association between β-catenin expression and the presence of CD8+, CD103+ and FOXP3+ T cells, we compared the expression level of β-catenin with the expression to the presence of tumour-infiltrating immune cells. We found an inverse correlation between β-catenin expression and the level of CD8, CD103 or FOXP3 markers (Spearman correlation coefficient $r = -0.41$, $p = 0.001$; $r = -0.31$, $p = 0.016$; $r = -0.29$, $p = 0.023$ for CD8+, CD103+ and FOXP3+ respectively).

### 3.8. Correlation between β-catenin, PD-L1 expression, TIMC, CD8+ T cells and outcome

At a median follow-up of 30 months, 46 (71.9%) patients had registered progression of disease and 37 (57.8%) had died. Among 64 patients, 32 (51.6%) were CD8-, 13 (21.0%) were CD8+ low, while 17 (27.4%) were CD8+ high. Due to technical reasons CD8 status was not determined in two patients. To explore a potential association between β-catenin expression and the presence of CD8+, CD103+ and FOXP3+ T cells, we compared the expression level of β-catenin with the expression to the presence of tumour-infiltrating immune cells. We found an inverse correlation between β-catenin expression and the level of CD8, CD103 or FOXP3 markers (Spearman correlation coefficient $r = -0.41$, $p = 0.001$; $r = -0.31$, $p = 0.016$; $r = -0.29$, $p = 0.023$ for CD8+, CD103+ and FOXP3+ respectively).

#### Table 1

Univariate Cox regression models for progression-free survival and overall survival.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Progression-free survival</th>
<th>Overall survival</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Hazard ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Stage (M1c versus M1b/M1a)</td>
<td>1.95</td>
<td>(1.08–3.55)</td>
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<tr>
<td>Therapy (combined versus monotherapy)</td>
<td>1.88</td>
<td>(1.02–3.45)</td>
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<td>PD-L1 (≥5% versus &lt;5%)</td>
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<td>(0.32–1.12)</td>
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<tr>
<td>TIMC (pos versus neg)</td>
<td>1.87</td>
<td>(1.03–3.39)</td>
</tr>
<tr>
<td>β-catenin (≥10% versus &lt;10%)</td>
<td>0.69</td>
<td>(0.39–1.23)</td>
</tr>
<tr>
<td>CD8-</td>
<td>Reference</td>
<td></td>
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<tr>
<td>CD8+ (low levels)</td>
<td>0.98</td>
<td>(0.48–2.01)</td>
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<tr>
<td>CD8+ (high levels)</td>
<td>0.45</td>
<td>(0.21–0.97)</td>
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<tr>
<td>PD-L1 (≥5% versus &lt;5%)</td>
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<td>(0.32–1.12)</td>
</tr>
<tr>
<td>TIMC (pos versus neg)</td>
<td>1.87</td>
<td>(1.03–3.39)</td>
</tr>
<tr>
<td>β-catenin (≥10% versus &lt;10%)</td>
<td>0.69</td>
<td>(0.39–1.23)</td>
</tr>
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<td>0.98</td>
<td>(0.48–2.01)</td>
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<tr>
<td>PD-L1 (≥5% versus &lt;5%)</td>
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<td>TIMC (pos versus neg)</td>
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TIMC: tumour-infiltrating mononuclear cells; 95%CI: 95% Confidence Interval; CD8+β-catenin group 1: CD8+/CD8+ (low levels) and β-catenin ≥10%; CD8+β-catenin group 2: CD8+ (high levels) and β-catenin ≥10% or CD8-/CD8+ (low levels) and β-catenin <10%; CD8+β-catenin group 3: CD8+ (high levels) and β-catenin <10%.

4. Discussion

The development of targeted therapy and immunotherapy, as effective therapeutic strategies in melanoma, has been pursued independently and in parallel over the past 10 years [1,2,17–20]. Justification for individual monotherapy, and not combined therapy, in part derived from the assumption that the two approaches have distinct molecular targets: oncogenic MAPK pathway for BRAFi and MEKi and tumour-infiltrating immune cells for immune modulating agents, including anti-cytotoxic T-lymphocyte antigen 4 (anti-CTLA4) and anti-PD-1/L1.
antibodies. However, biological and clinical evidence suggests that targeting simultaneously or sequentially the two therapeutic pathways could produce better outcomes. V-raf murine sarcoma viral oncogene homologue B1, the oncogenic BRAF controls melanoma proliferation, differentiation and survival, and induces T-cell suppression by secreting inhibitory cytokines [7] or through membrane expression of coinhibitory molecules, such as the PD-1 ligands PD-L1 or PD-L2 [21]. Furthermore, enhanced expression of melanoma antigens enriched T-cell infiltration within 14 days post BRAFi therapy has been consistently reported [5,6].

Based on this strong interrelationship, our group previously reported that TIMC and PD-L1 predict prognosis in MMPs treated with BRAFi [9]. Here, we expanded the initial results by: (1) including the immunophenotype of TME, (2) evaluating the immune signature and (3) studying the correlation between β-catenin and various TME features. In the present study, the prognostic role of TIMC assessed by simple evaluation of H&E staining was not confirmed. However, specific, well-characterised immune cells predicted prognosis. High levels of CD8+ T cells independently predicted PFS and OS, after adjusting for well-known prognostic factors such as stage and PS. Furthermore, after adjusting for stage, PD-L1 and β-catenin expression, the density of CD8+ T cells was significantly associated with a higher probability of response to treatment and a longer PFS, suggesting that absence of high density CD8+ T cells is associated with both primary and secondary resistance to MAPKi.

It has been reported that CD8+ T cells are associated with the natural history of different primary cancers [22,23], and with cancer outcome after PD-1 blockade [24]. There is also evidence that melanomas with pre-existing CD8+ T cells could derive long-term prognosis by immunocheckpoints inhibitors [24]. Our data suggest that patients receiving MAPKi obtain similar long-term benefit. Hence, these immunological features should not be used to select a specific therapeutic strategy. Current data suggest that melanoma patients with a specific phenotypes do better regardless of the treatment applied. Nevertheless, heterogeneity of mononuclear cells in the microenvironment and the complex

![Fig. 3. Kaplan–Meier curves for progression-free survival (A) and overall survival (B) according to CD8+ T cell infiltration. Kaplan Meier curves for progression-free survival (C) and overall survival (D) according to a combined scoring which includes β-catenin expression combined to CD8+ T cell infiltration. Three groups are reported: Group 1) Patients with lack of CD8+ T cells and β-catenin ≥10% Group 2) Patients with lack of CD8+ T cells and β-catenin <10% or patients with high density of CD8+ T cells and β-catenin ≥10%; Group 3) Patients with high density of CD8+ T cells and β-catenin <10%.

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characterisation of lymphocyte phenotype support functional and translational studies incorporating longitudinal blood and tumour sampling for target validation and biomarker discovery [25].

In our cohort, we investigated the role of CD103+ cells, the majority of which are CD8+ T cells. This represents a subgroup of tissue-resident memory T cells, which express the inhibitory checkpoints PD-1 and TIM-3, indicating a preexisting antigen-specific immune activation associated with a T-cell-inflamed tumour environment [26]. In our series CD103+ C8+ cells were associated with a higher probability of response to MAPKi. In line with this observation, the responding patients showed increased expression of transcripts involved in adaptive immunity (CDS8A, FOXP3, and PFR1) and antigen presentation (THBD). The THBD gene encodes for thrombomodulin (CD141 or BDCA-3), a membrane protein expressed on the surface of several cell types, including a subset of human dendritic cells (DCs), characterised by high expression of toll-like receptor 3, production of interleukin (IL)-12p70 and interferon (IFN)-beta, and superior capacity to induce cytotoxic T-lymphocyte responses [27].

Recently, Spranger et al. [10] provided the first evidence that tumour-intrinsic β-catenin pathway activation promotes the exclusion of T cells and CD103 dendritic cells via chemokine (C-C motif) ligand 4 (CCL4) inhibition. The inverse relationship between active β-catenin signalling and T-cell infiltration in both transgenic melanoma mouse models and human melanoma samples provided a first insight into a potential new mechanism of immune resistance [10]. LEF1 down-expression and β-catenin modulation cause acquired MAPKi-resistance [28]. Most melanomas with acquired MAPKi-resistance displayed a significant CD8 T-cell depletion and exhaustion [28]. Similarly, β-catenin activation could modulate innate BRAFi sensitivity melanoma cell lines [29].

Our data, while confirming an inverse correlation between β-catenin expression and CD8+ T cells or CD8+/CD103+ T cells in TME, do not support any prognostic role of the sole β-catenin in terms of PFS or OS in MMPs receiving MAPKi. However, different results were obtained when β-catenin was evaluated together with T-cells infiltration. When CD8 and β-catenin scores were combined, the response rate improved as indicated by the fact that PFS and OS were longer in patients with higher CD8+ T cell density and lower (<10%) β-catenin than either those without CD8+ T cells infiltration and higher β-catenin ≥10% or those with high density of CD8+ T cells and β-catenin ≥10%. These results suggest that a composite score better predicted long-term prognosis of MMPs treated with MAPKi monotherapy. However, whether β-catenin pathway modulation could represent a potential therapeutic target for improving therapeutic responsiveness and long-term outcome should be investigated in ad hoc clinical trials. Nevertheless, evaluation and scoring of β-catenin is a semiquantitative exercise, as no standard criteria for such parameter have been adopted in melanoma. Furthermore, variable β-catenin subcellular localisation (membranous versus cytoplasmic or nuclear) has been reported in primary and metastatic melanoma, partially due to differences in the design of the clinical studies or melanoma sample collection (frozen versus FFPE versus melanoma cells) [30].

Harmonisation of scoring methods and further validation are needed to increase the likelihood of identifying the significance of β-catenin assessment in clinical practice.

The present study has several strengths, including: (1) the strong evidence for a robust relationship between BRAFi on the one hand and immune-markers in the
microenvironment and clinical outcome on the other; (2) homogeneous treatment with BRAFi alone or BRAFi/MEKi in combination, within a single centre of patients enrolled over the last 4 years; (3) evaluation of β-catenin and CD8+, CD103 and FOXP3 performed in most cases (40/64, 62%) in the last metastatic samples before starting MAPKi treatment to better reflect the actual immunological status of the patient cohort; (4) microscopic evaluation of CD8 staining on immunostained sections confirmed by automated counting upon digital image acquisition, which allows more quantitative and reliable results of the immune infiltrate; and (5) quantitative assessment of mRNA transcripts associated with Wnt/β-catenin pathway and immune response. There are also limitations, including: (1) this is a single-institution, retrospective study, and a validation dataset is needed; (2) in our cohort, 60% of patients received BRAFi and 40% the combination of BRAFi/MEKi, which is now known to improve RR, PFS and OS, as compared to BRAFi alone treatment [1–3]. Nevertheless, after adjusting for treatments (BRAFi alone or combination of BRAFi and MEKi), the prognostic role of CD8 T-cell infiltration remained statistically significant.

In summary, our present data support the hypothesis that a specific tumour immune infiltrate and selected gene expression by tumour cells predict response and outcome of MMPs in the presence of BRAF inhibition. Because MMPs lacking a preexisting immune response are at higher risk of developing early disease progression and worse prognosis, a different therapeutic strategy should be proposed for these patients. Whether priming the immune response or enriching tumours with effector T cells (by vaccines, adoptive T-cell therapy and/or β-catenin modulation) may result in a better outcome should be evaluated in ad hoc designed, prospective clinical studies.

Conflicts of interest statement

None declared.

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Appendix A. Supplementary data

Supplementary data related to this article can be found at http://dx.doi.org/10.1016/j.ejca.2017.03.012.

References


