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PD-L1 expression by tumor cell-lines: a predictive marker in melanoma

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Abstract

Prognostic biomarkers for melanoma patients after lymph node resection are of clinical relevance and could thus enable the identification of patients who therefore would most benefit from adjuvant treatment. The aim of this work was to determine, using an in vitro model, whether immune-related biomarkers such as MHC-class I and II, melanoma associated antigens, IDO1 and PD-L1, could also be relevant to predict the risk of relapse of stage III melanoma patients after lymph node resection.

We established tumor cell lines from metastatic lymph nodes of 50 melanoma patients. The expression of investigated biomarkers was determined on untreated and IFN- γ treated melanoma cell lines using flow cytometry. Among the selected biomarkers, the IFN- γ induced expression of PD-L1 and IDO1 was associated with an increased risk of relapse ($p=0.0001$ and $p=0.013$, respectively) and was also associated with death for IDO1 ($p=0.0005$). In the future, this immunologic signature could permit the identification of patients at higher risk of relapse, and justifying an adjuvant treatment using immunotherapy.

Key words: metastatic melanoma, prognostic markers, survival, melanoma cell-line, flow cytometry

Abbreviations:

AJCC: American Joint Committee on Cancer

CTA: cancer testis antigens

DNA: desoxyribonucleic acid

GS: gene signature

H&E: hematoxylin & eosin

HMW-MAA: high molecular weight-melanoma associated antigen

IDO1: indoleamine 2,3 dioxygenase

LN: lymph node

MAA: melanoma associated antigens

mAb: monoclonal antibody

MAGE-A: melanoma-associated antigen-A

MCSP: melanoma chondroitin sulfate proteoglycan

MHC: major histocompatibility complex

NSCLC: non-small cell lung cancer

NY-ESO-1: New York esophageal squamous cell cancer-1

OS: overall survival

P: progression

PCR: polymerase chain reaction

PD-L1: programmed death ligand 1

RC: complete response

RECIST: response evaluation criteria in solid tumor

RFS: relapse free survival

RP: partial response

SSM: superficial spreading melanoma

St: stabilization

TRP2: tyrosinase related protein 2

Introduction

Malignant melanoma is the most aggressive cutaneous malignancy with 132,000 new cases occurring worldwide each year (World Health Organization, Skin Cancers) and an annual 3-7% increase in the incidence rate for Caucasians [1].

Despite the therapeutic revolution brought by innovative treatments such as targeted therapies (BRAF inhibitors), or immune checkpoint inhibitors (i.e. PD-1/PD-L1 or CTLA-4/B7 antibodies), the identification of biomarkers capable of predicting patient prognosis after lymph node resection are still of clinical relevance. Furthermore, new treatments are arriving in the adjuvant setting for stage III melanoma, increasing the interest to select patients with higher risk of relapse [2].

Consequently, attention has been focused on finding biomarkers to identify patients most likely to respond to a specific cancer therapy. Thus, tumor gene expression profiling is a powerful technique for identifying prognostic gene signatures (GSs) [3,4]. Predictive GSs have also been reported in colorectal [5] and gastric cancers [6]. Moreover, both prognostic and predictive GSs have been identified in non-small cell lung cancer (NSCLC) and breast cancer [7,8].

Recently, a 84-gene GS associated with clinical response to MAGE-protein immunizations has been identified in metastatic melanoma [9]. These results were confirmed in resected NSCLC [10]. It was the evidence that clinical response resulting from a cancer immunotherapeutic treatment may be associated with an immune biomarker signature in two different settings (metastatic and adjuvant) and in two tumor types (melanoma and NSCLC). The genes differentially expressed were genes involved in antigen processing, MHC-class I and II, T-cell markers such as CD3 and CD8 and chemokines such as CCL5, CXCL9 and CXCL10.

Meanwhile, a 46-gene GS with strong overrepresentation of immune response genes was identified in 79 stage III melanoma patients, suggesting that BRAF mutation, NRAS mutation, and the absence of an immune-related expressed gene profile predicted poor outcome in stage III melanoma patients [11]. The genes differentially expressed were CCL5, CXCL9, CXCL10, MHC-class II, MAGE-C2, CD2, CD3 and CD8.

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4 The aim of this work was to assess whether modulation of some biomarkers expressed by melanoma
5 cell-lines, obtained from metastatic regional lymph nodes (LN) and exposed to IFN- γ , could be an in
6 vitro prognostic tool useful to identify stage III melanoma patients with a higher risk of relapse and a
7 shorter overall survival. For this purpose we analyzed expression of some immune-related biomarkers
8 (MHC-class I and II) and melanoma associated antigens. We also tested expression of PD-L1 and
9 IDO1, two biomarkers known to be modulated by IFN- γ and recently identified as enabling melanoma
10 cells to escape immune destruction despite antitumor responses [12-15].
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For Review Only

Materials and methods

Melanoma patients

A total of 50 stage IIIb (AJCC 2007) melanoma patients were included in this study (table 1). For each patient, a melanoma cell-line was obtained from a fragment of a metastatic lymph node (LN). Written informed consent was provided by all patients and the study was approved by the Ethics Committee of Pays de La Loire and Health Authorities (France). The 1964 Declaration of Helsinki protocols and its later amendments or comparable ethical standards were applied in the present study.

Establishment of melanoma cell-lines

Melanoma cell-lines were established for the 50 tumor samples as previously described by us [16] [17] and by others [18]. Briefly, fresh tumor samples were minced into small tumor pieces in wells of 24-well plates (NUNC) with RPMI medium supplemented with 10% fetal calf serum (FCS). Plates were placed at 37°C in a humidified incubator with 5% CO₂ and observed under a light microscope every week and sub-cultured when necessary.

Experiments on melanoma cell lines

For each cell-line, a total of 500 000 cells per well of a 6-well plate were seeded in 3 ml of culture medium with or without 500U/ml recombinant IFN- γ (Tebu, Le Perray en Yvelines, France), in duplicate. After 48 hours of incubation, cells were washed, detached from the wells using PBS-EDTA (Lonza, Levallois, France) and processed for flow cytometry.

Antibodies and flow cytometry

For membranous staining, 0.2 x 10⁶ cells were stained for 30 minutes at 4°C protected from light according to manufacturer's instructions with mouse anti-human MCSP (melanoma chondroitin sulfate proteoglycan) (Miltenyi Biotec, #130-091-252, dilution 1/40, Bergisch Gladbach, Germany), mouse anti-human B7H1 (PD-L1) (ebioscience, #12-5983-73, dilution 1/10), HLA-I primary antibody recognizing HLA-A, -B, -C or HLA-II primary antibody recognizing HLA-DP, -DQ, -DR (BD

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4 Biosciences, respectively #555551 and #555557, dilution 1/100, Le Pont de Claix, France) as
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6 previously described [19].

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8 For intracellular staining, 0.2×10^6 cells were rinsed twice in DPBS, permeabilized using
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10 fixation/permeabilization buffer set (eBioscience, #00-8333-56, Paris, France) according to the
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12 manufacturer's instructions and stained with mouse anti-MAGE-A (melanoma-associated antigen-A)
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14 (Tebu, #sc-20034, dilution 1/40, recommended for detection of MAGE-A1, 2,3, 4, 6, 10 and 12),
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16 mouse anti-NY-ESO-1 (New York esophageal squamous cell cancer-1) (Tebu, #sc-53869, 1/40),
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18 mouse anti-tyrosinase (Tebu, #sc-20035, 1/40), mouse anti-Melan-A (Tebu, #sc-20032, 1/40) or
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20 mouse anti-gp100 (Tebu, #sc-59305, 1/40) Abs for 30 minutes at 4°C, or with mouse anti-human
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22 IDO1 mAb (Bio-Rad AbD Serotec, #OBT2037G, dilution 1/200) as previously described [19].

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24 Melanoma cell-lines were gated according to their forward and size scatter characteristics. A minimum
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26 of 10^4 viable cell gated events were acquired on a FACScalibur flow cytometer and data were analyzed
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28 using the Cell Quest Pro software (Becton Dickinson, Grenoble, France). A 10% increase of the
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30 proportion of tumor cells stained with a given antibody after IFN- γ treatment compared to the
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32 proportion of tumor cells before IFN- γ treatment was considered as a significant increase, a 10%
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34 decrease in this proportion as a significant decrease. A difference comprised between -5% and -10% in
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36 the expression of a given biomarker after IFN- γ treatment compared to before IFN- γ treatment was
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38 considered as a moderate decrease.

39 40 41 42 *Statistical analysis*

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44 Each biomarker was assessed on melanoma cell-lines with and without IFN- γ stimulation.

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46 Cox models were developed for both situations: biomarkers only from treated melanoma cell-lines and
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48 biomarkers only from untreated melanoma cell-lines. All models were adjusted on clinico-pathological
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50 informations such as the Breslow index, capsular breaking, and number of invaded nodes, BRAF and
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52 NRAS mutations. For these 2 situations, the outcome was the impact of biomarkers on patient relapse-
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54 free survival (RFS) and overall survival (OS). RFS was calculated as the time interval between
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56 lymphadenectomy and the relapse or death and OS was calculated as the time interval between
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4 lymphadenectomy and the death whatever the cause. Regarding RFS, if death was caused by
5 melanoma, relapse has occurred earlier and if death is not from melanoma, it is considered as a
6 censoring event. Regarding OS, both deaths from melanoma and from another cause were considered.
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8 Multivariate Cox model was developed including all biomarkers and clinic-pathological informations
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10 such as the Breslow index, capsular breaking, and number of invaded nodes, BRAF and NRAS
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12 mutations. The variable selection method of the multivariate model was based on the Akaike
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14 Information Criterion [Akaike H. A new look at the statistical model identification. IEEE Trans
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16 Automat Contr 1974; 19: 716-723] (AIC). The threshold of significance was set at 5% in bilateral
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18 situation. The R 3.11 statistical software was used for all analyses. All quantitative data were scaled.
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Results

A total of 50 stage IIIb (AJCC 2007) melanoma patients were included in this study. The main clinical features of these patients are summarized in table 1 and detailed clinical characteristics of the 50 patients are summarized in table 2. Mean age was 51.9 ± 11.6 years (median: 53; min.-max.:27-72), with 35 men and 15 women. Median event-free survival was 8 months and median survival was 19.5 months.

Expression levels of selected biomarkers by untreated and IFN- γ treated melanoma cell-lines

No untreated melanoma cells expressed PD-L1 (median 0.36% positive cells) and most untreated melanoma cells did not express IDO1 (median 37% positive cells); this expression was greatly induced in vitro by IFN- γ (median 76.1% positive cells for PD-L1 and 56% for IDO1) (figure 1 and 2, table 3 and supplementary table 1).

Most untreated melanoma cells expressed MCSP, gp100, Melan-A and tyrosinase and also MHC-class I and MHC-class II antigens (table 3 and supplementary table 1). Upon IFN- γ treatment, MHC-class II expression was increased, whereas MCSP and MHC-class I expression was not modified and gp100, Melan-A and tyrosinase expression was moderately decreased (table 3 and supplementary table 1). Half of untreated melanoma cells expressed NY-ESO-1 and MAGE-A antigens and their expression was decreased upon IFN- γ in vitro stimulation (table 3 and supplementary table 1).

Significant associations between selected biomarkers and RFS

Univariate analysis

None of the clinical features was significantly associated with RFS (for Kaplan Meier curves, see supplementary figure 1). No relationship was observed between the 10 selected biomarkers in untreated melanoma cell-lines and RFS except for MAGE-A expression (p=0.021) and also no relationship between biomarkers in IFN- γ treated melanoma cell-lines and RFS except for tyrosinase (p=0.012) (supplementary table 2 and supplementary figure 1 for Kaplan Meier curves).

Multivariate analysis

Biomarkers in untreated melanoma cell-lines

The expression of MCSP in untreated melanoma cell-lines was associated with an improved RFS (p=0.026) (supplementary table 3). The other biomarkers were not significantly associated with RFS.

Biomarkers in IFN- γ treated melanoma cell-lines

The induction of PD-L1 and IDO1 expression in IFN- γ treated cell-lines was significantly associated with a decreased RFS (respectively p=0.0001 and p=0.013) (supplementary table 4). Regarding melanoma associated antigens (MAA), the decreased expression of tyrosinase in IFN- γ treated cell-lines was associated with an improved RFS (p=0.0013), whereas the decreased expression of NY-ESO-1 in IFN- γ treated cell-lines, was significantly associated with a decreased RFS (p=0.0005). The unmodified expression of MCSP upon IFN- γ stimulation was associated with an improved RFS (p=0.01). The other biomarkers were not significantly associated with RFS.

Other clinico-biological markers

NRAS mutation status was associated with an improved RFS (p=0.0015) (supplementary table 4). The number of invaded LNs was significantly associated with a poor RFS (p=0.0175). The other clinical markers were not significantly associated with RFS.

Significant associations between selected biomarkers and OS

Univariate analysis

We observed that none of the clinical features was significantly associated with OS (for Kaplan Meier curves, see supplementary figure 2). No relationship was observed between the 10 selected biomarkers in untreated melanoma cell-lines and OS except for MAGE-A and NY-ESO-1 (respectively p=0.0073 and p=0.044). In addition, no relationship was identified between these biomarkers in IFN- γ treated melanoma cell-lines and OS (supplementary table 5 and supplementary figure 2 for Kaplan Meier curves).

Multivariate analysis

Biomarkers in untreated melanoma cell-lines

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4 The expression of tyrosinase was associated with an improved OS ($p=0.003$) (supplementary table 6).

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6 The other biomarkers were not significantly associated with OS.

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8 *Biomarkers in IFN- γ treated melanoma cell-lines*

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10 The induction of IDO1 expression by IFN- γ was significantly associated with a decreased OS
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12 ($p=0.0005$) (supplementary table 7). The decreased expression of gp100 and MAGE-A by IFN- γ was
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14 associated with an improved OS (respectively $p=0.007$ and $p=0.023$) whereas the decreased expression
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16 of Melan-A by IFN- γ was significantly associated with a decreased OS ($p=0.028$). The unmodified
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18 expression of MCSP upon IFN- γ stimulation was associated with an improved OS ($p=0.02$). The other
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20 biomarkers were not significantly associated with OS.

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22 *Other clinico-biological markers*

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24 High Breslow index and capsular breaking were significantly associated with a poor OS (respectively
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26 $p=0.03$ and $p=0.0026$) (supplementary table 7). The other clinical markers were not significantly
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28 associated with OS.
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Discussion

In the present work, we conducted a multivariate analysis of clinical and pathological determinants of outcome and several immune-related biomarkers, melanoma associated antigens, and two important biomarkers of immune relevance in melanoma: IDO1 and PD-L1.

First, we observed that most untreated melanoma cell-lines did not express IDO1. Moreover, the induction of IDO1 expression by IFN- γ treatment was significantly associated with both decreased RFS and OS, making IDO1 the only biomarker associated with both RFS and OS in this study. IDO1 is an intra-cellular enzyme that degrades tryptophan to kynurenine, inducing a local immunosuppression through deficiency in this essential amino acid, which is needed for the activity of T-cells [20]. There is evidence that IFN- γ stimulates IDO1 gene transcription in many cell types [21,22]. In melanoma, IDO1 has been shown to be increased in both primary and metastatic lesions and its expression correlated with increased invasiveness and disease relapse rate [12,13]. We recently reported an induction of PD-L1 expression by melanoma cells when co-cultivated with IDO1 expressing fetal fibroblasts [23]. We assumed that this induction of PD-L1 by IDO1 may contribute to inhibit the efficacy of adoptive cell therapy using TILs, by inducing inactivation of lymphocytes, decreasing both their survival and proliferation. This way, IDO1 appears as a molecule that may play a major role in immune tolerance induction, in melanoma microenvironment.

We also observed that untreated melanoma cells did not express PD-L1. Interestingly, the induced expression of PD-L1 in IFN- γ treated cell-lines was significantly associated with a decreased RFS. It is well known that many malignant cells express PD-L1, either constitutively or after IFN- γ induction [24,25]. PD-L1 expression has been described as having multiple prognostic significances, depending on the tumor type. It has been correlated with decreased survival in ovarian, pancreatic, and renal cell carcinomas [26-28], and has been associated with an improved survival in patients with Merkel cell [29], breast [30], and cervical carcinomas [31]. Conflicting reports also exist regarding its significance in melanoma [14,15]. However, all these studies were conducted on tumor tissue samples and not on in vitro tumor cell-lines.

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4 Regarding melanoma associated antigens (MAA), gp100/PMEL, Melan-A/MART-1 and tyrosinase,
5 we observed that their expression was moderately decreased upon IFN- γ treatment of melanoma cell-
6 lines. Variable responses after IFN stimulation on MAA expression had already been reported in the
7 literature, ranging from moderate induction to suppression [32]. Moreover, upon IFN- γ stimulation, we
8 observed that the decreased expression of tyrosinase was associated with an improved RFS and the
9 decreased expression of gp100 to an improved OS whereas the decreased expression of Melan-A was
10 associated with a decreased OS. Our conflicting results regarding the predictive value of MAA
11 perfectly illustrates the fact that data on this subject are controversial [33-35]. Finally, we report that
12 the unmodified MCSP expression in IFN- γ treated melanoma cell-lines was related to both increased
13 RFS and OS. MCSP/HMW-MAA is highly expressed in more than 85% of melanomas [36] and is
14 thought to contribute to the malignant phenotype of melanoma cells via enhancement of their
15 spreading, invasion, and migration [37]. To our knowledge, no data exist to date regarding the
16 predictive or prognostic value of MCSP expression level in melanoma.

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30 Regarding cancer testis antigens (CTA), we observed here that the decreased expression of MAGE-A
31 was associated with an improved OS, whereas the decreased expression of NY-ESO-1 was associated
32 with a diminished RFS. This result is consistent with our previous study reporting that the expression
33 of MAGE-A1 and MAGE-A3 but in contradiction for NY-ESO-1 which was significantly associated
34 with a higher RFS in stage III melanoma patients [38]. However in the present work we used flow
35 cytometry to determine the expression level of tumor associated antigens on melanoma cell-lines,
36 whereas in our work published in 2009, we used a semi-quantitative RT-PCR analysis on melanoma
37 tissue specimens. In primary cutaneous melanoma, Svobodova et al reported that the median RFS of
38 patients with the three CTA (MAGE-A1, MAGE-A4 and NY-ESO-1) positive tumors was
39 significantly reduced compared to those of patients with CTA-negative tumors [39].

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50 We finally observed that NRAS mutation status was associated with an improved RFS. However,
51 controversial data exist in the literature on this subject. For instance, Ekedahl et al observed a trend
52 showing a better prognosis for patients with NRAS-mutant tumors compared with BRAF V600E-
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4 mutant tumors [40], although other studies reported an association between NRAS mutation and worse
5 prognosis in stage IV melanoma [41] but also in stage III melanoma [11].

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8 One of the limitations of our work is that it is not always possible to obtain melanoma cell-lines and
9 the delay for obtaining it. In our experience, we managed to obtain cell-lines for 80% of our patients.

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12 The median time necessary to obtain the cell-lines was 2 months after complete lymph node resection.

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14 This is not so long as compared to inclusion in a clinical trial. Furthermore, the adjuvant treatment
15 could be initiated before obtaining the cell line, and changed when the data become available.

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18 In conclusion, our results suggest that it is possible to identify a dynamic prognostic immunological
19 profile of melanoma cells, for stage III melanoma patients with lymph node involvement. Melanoma

20 cell-lines treated with IFN- γ could be an interesting tool to identify stage III melanoma patients with a
21 higher risk of relapse. Thus, we propose that the modulation of PD-L1 and IDO1 expression in

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24 melanoma cell-lines incubated with IFN- γ could help screening patients most at risk of relapse and
25 shorter overall survival. These biomarkers have a prognostic value on RFS and OS. In the future, this

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28 immunological profile of melanoma cells could help to identify stage III melanoma patients with a
29 higher risk of relapse and thus justifying an adjuvant treatment. Today, this last point is highly relevant

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32 because of new treatments costs, but also adverse events occurrence, that can persist despite stopping
33 treatment.

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Conflict of interest

The authors declare that they have no conflict of interest.

Author contributions

AC Knol and MC Pandolfino performed experiments

AC Knol, A.Khammari and B.Dréno conceived and designed the research study

A.Khammari and MG Denis contributed essential reagents/materials/analysis tools

AC Knol, MC Pandolfino P and JM Nguyen analysed the data

AC Knol and B.Dréno wrote the paper

All authors reviewed and commented critically drafts of the manuscript for important intellectual content and gave final approval to submit for publication.

References

1. Parkin DM, Bray F, Ferlay J, Pisani P: Estimating the world cancer burden: Globocan 2000. *Int J Cancer* 2001;94:153-156.
2. Eggermont AM, Chiarion-Sileni V, Grob JJ, Dummer R, Wolchok JD, Schmidt H, Hamid O, Robert C, Ascierto PA, Richards JM, Lebbe C, Ferraresi V, Smylie M, Weber JS, Maio M, Bastholt L, Mortier L, Thomas L, Tahir S, Hauschild A, Hassel JC, Hodi FS, Taitt C, de Pril V, de Schaetzen G, Suciú S, Testori A: Prolonged Survival in Stage III Melanoma with Ipilimumab Adjuvant Therapy. *N Engl J Med* 2016;375:1845-1855.
3. Kratz JR, He J, Van Den Eeden SK, Zhu ZH, Gao W, Pham PT, Mulvihill MS, Ziaei F, Zhang H, Su B, Zhi X, Quesenberry CP, Habel LA, Deng Q, Wang Z, Zhou J, Li H, Huang MC, Yeh CC, Segal MR, Ray MR, Jones KD, Raz DJ, Xu Z, Jahan TM, Berryman D, He B, Mann MJ, Jablons DM: A practical molecular assay to predict survival in resected non-squamous, non-small-cell lung cancer: development and international validation studies. *Lancet* 2012;379:823-832.
4. van 't Veer LJ, Dai H, van de Vijver MJ, He YD, Hart AA, Mao M, Peterse HL, van der Kooy K, Marton MJ, Witteveen AT, Schreiber GJ, Kerkhoven RM, Roberts C, Linsley PS, Bernards R, Friend SH: Gene expression profiling predicts clinical outcome of breast cancer. *Nature* 2002;415:530-536.
5. Baker JB, Dutta D, Watson D, Maddala T, Munneke BM, Shak S, Rowinsky EK, Xu LA, Harbison CT, Clark EA, Mauro DJ, Khambata-Ford S: Tumour gene expression predicts response to cetuximab in patients with KRAS wild-type metastatic colorectal cancer. *Br J Cancer* 2011;104:488-495.
6. Kim HK, Choi IJ, Kim CG, Kim HS, Oshima A, Yamada Y, Arai T, Nishio K, Michalowski A, Green JE: Three-gene predictor of clinical outcome for gastric cancer patients treated with chemotherapy. *Pharmacogenomics J* 2012;12:119-127.
7. Albain KS, Barlow WE, Shak S, Hortobagyi GN, Livingston RB, Yeh IT, Ravdin P, Bugarini R, Baehner FL, Davidson NE, Sledge GW, Winer EP, Hudis C, Ingle JN, Perez EA, Pritchard KI, Shepherd L, Gralow JR, Yoshizawa C, Allred DC, Osborne CK, Hayes DF: Prognostic and predictive value of the 21-gene recurrence score assay in postmenopausal women with node-positive, oestrogen-receptor-positive breast cancer on chemotherapy: a retrospective analysis of a randomised trial. *Lancet Oncol* 2010;11:55-65.
8. Zhu CQ, Ding K, Strumpf D, Weir BA, Meyerson M, Pennell N, Thomas RK, Naoki K, Ladd-Acosta C, Liu N, Pintilie M, Der S, Seymour L, Jurisica I, Shepherd FA, Tsao MS: Prognostic and predictive gene signature for adjuvant chemotherapy in resected non-small-cell lung cancer. *J Clin Oncol* 2010;28:4417-4424.
9. Kruit WH, Suciú S, Dreno B, Mortier L, Robert C, Chiarion-Sileni V, Maio M, Testori A, Dorval T, Grob JJ, Becker JC, Spatz A, Eggermont AM, Louahed J, Lehmann FF, Brichard VG, Keilholz U: Selection of immunostimulant AS15 for active immunization with MAGE-A3 protein: results of a randomized phase II study of the European Organisation for Research and Treatment of Cancer Melanoma Group in Metastatic Melanoma. *J Clin Oncol* 2013;31:2413-2420.
10. Ulloa-Montoya F, Louahed J, Dizier B, Gruselle O, Spiessens B, Lehmann FF, Suciú S, Kruit WH, Eggermont AM, Vansteenkiste J, Brichard VG: Predictive gene signature in MAGE-A3 antigen-specific cancer immunotherapy. *J Clin Oncol* 2013;31:2388-2395.
11. Mann GJ, Pupo GM, Campain AE, Carter CD, Schramm SJ, Pianova S, Gerega SK, De Silva C, Lai K, Wilmott JS, Synnott M, Hersey P, Kefford RF, Thompson JF, Yang YH, Scolyer RA: BRAF mutation, NRAS mutation, and the absence of an immune-related expressed gene profile predict poor outcome in patients with stage III melanoma. *J Invest Dermatol* 2013;133:509-517.

12. Brody JR, Costantino CL, Berger AC, Sato T, Lisanti MP, Yeo CJ, Emmons RV, Witkiewicz AK: Expression of indoleamine 2,3-dioxygenase in metastatic malignant melanoma recruits regulatory T cells to avoid immune detection and affects survival. *Cell Cycle* 2009;8:1930-1934.
13. Chevolet I, Speeckaert R, Haspelslagh M, Neyns B, Kruse V, Schreuer M, Van Gele M, Van Geel N, Brochez L: Peritumoral indoleamine 2,3-dioxygenase expression in melanoma: an early marker of resistance to immune control? *Br J Dermatol* 2014;171:987-995.
14. Hino R, Kabashima K, Kato Y, Yagi H, Nakamura M, Honjo T, Okazaki T, Tokura Y: Tumor cell expression of programmed cell death-1 ligand 1 is a prognostic factor for malignant melanoma. *Cancer* 2010;116:1757-1766.
15. Taube JM, Anders RA, Young GD, Xu H, Sharma R, McMiller TL, Chen S, Klein AP, Pardoll DM, Topalian SL, Chen L: Colocalization of inflammatory response with B7-h1 expression in human melanocytic lesions supports an adaptive resistance mechanism of immune escape. *Sci Transl Med* 2012;4:127ra137.
16. Pandolfino MC, Saiagh S, Knol AC, Dreno B: Comparison of three culture media for the establishment of melanoma cell lines. *Cytotechnology* 2010;62:403-412.
17. Gervois N, Heuze F, Diez E, Jotereau F: Selective expansion of a specific anti-tumor CD8+ cytotoxic T lymphocyte clone in the bulk culture of tumor-infiltrating lymphocytes from a melanoma patient: cytotoxic activity and T cell receptor gene rearrangements. *Eur J Immunol* 1990;20:825-831.
18. Halaban R, Ghosh S, Duray P, Kirkwood JM, Lerner AB: Human melanocytes cultured from nevi and melanomas. *J Invest Dermatol* 1986;87:95-101.
19. Zuliani T, Saiagh S, Knol AC, Esbelin J, Dreno B: Fetal fibroblasts and keratinocytes with immunosuppressive properties for allogeneic cell-based wound therapy. *PLoS One* 2013;8:e70408.
20. Munn DH, Shafizadeh E, Attwood JT, Bondarev I, Pashine A, Mellor AL: Inhibition of T cell proliferation by macrophage tryptophan catabolism. *J Exp Med* 1999;189:1363-1372.
21. Mancuso R, Hernis A, Agostini S, Rovaris M, Caputo D, Fuchs D, Clerici M: Indoleamine 2,3 Dioxygenase (IDO) Expression and Activity in Relapsing-Remitting Multiple Sclerosis. *PLoS One* 2015;10:e0130715.
22. Taylor MW, Feng GS: Relationship between interferon-gamma, indoleamine 2,3-dioxygenase, and tryptophan catabolism. *Faseb J* 1991;5:2516-2522.
23. Frenard C, Knol AC, Lemoigne M, Khammari A, Dreno B: Effect of indoleamine 2,3-dioxygenase expressed by foetal fibroblasts on melanoma cells. *Exp Dermatol* 2016;25:822-824.
24. Chen L: Co-inhibitory molecules of the B7-CD28 family in the control of T-cell immunity. *Nat Rev Immunol* 2004;4:336-347.
25. Dong H, Strome SE, Salomao DR, Tamura H, Hirano F, Flies DB, Roche PC, Lu J, Zhu G, Tamada K, Lennon VA, Celis E, Chen L: Tumor-associated B7-H1 promotes T-cell apoptosis: a potential mechanism of immune evasion. *Nat Med* 2002;8:793-800.
26. Giraldo NA, Becht E, Pages F, Skliris G, Verkarre V, Vano Y, Mejean A, Saint-Aubert N, Lacroix L, Natario I, Lupo A, Alifano M, Damotte D, Cazes A, Triebel F, Freeman GJ, Dieu-Nosjean MC, Oudard S, Fridman WH, Sautes-Fridman C: Orchestration and Prognostic Significance of Immune Checkpoints in the Microenvironment of Primary and Metastatic Renal Cell Cancer. *Clin Cancer Res* 2015;21:3031-3040.
27. Hamanishi J, Mandai M, Iwasaki M, Okazaki T, Tanaka Y, Yamaguchi K, Higuchi T, Yagi H, Takakura K, Minato N, Honjo T, Fujii S: Programmed cell death 1 ligand 1 and tumor-infiltrating CD8+ T lymphocytes are prognostic factors of human ovarian cancer. *Proc Natl Acad Sci U S A* 2007;104:3360-3365.

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- 3
- 4 28. Nomi T, Sho M, Akahori T, Hamada K, Kubo A, Kanehiro H, Nakamura S, Enomoto K, Yagita H, Azuma M, Nakajima Y: Clinical significance and therapeutic potential of the programmed death-1 ligand/programmed death-1 pathway in human pancreatic cancer. *Clin Cancer Res* 2007;13:2151-2157.
- 5
- 6
- 7
- 8 29. Lipson EJ, Vincent JG, Loyo M, Kagohara LT, Luber BS, Wang H, Xu H, Nayar SK, Wang TS, Sidransky D, Anders RA, Topalian SL, Taube JM: PD-L1 expression in the Merkel cell carcinoma microenvironment: association with inflammation, Merkel cell polyomavirus and overall survival. *Cancer Immunol Res* 2013;1:54-63.
- 9
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- 12 30. Wimberly H, Brown JR, Schalper K, Haack H, Silver MR, Nixon C, Bossuyt V, Puzstai L, Lannin DR, Rimm DL: PD-L1 Expression Correlates with Tumor-Infiltrating Lymphocytes and Response to Neoadjuvant Chemotherapy in Breast Cancer. *Cancer Immunol Res* 2015;3:326-332.
- 13
- 14
- 15
- 16 31. Karim R, Jordanova ES, Piersma SJ, Kenter GG, Chen L, Boer JM, Melief CJ, van der Burg SH: Tumor-expressed B7-H1 and B7-DC in relation to PD-1+ T-cell infiltration and survival of patients with cervical carcinoma. *Clin Cancer Res* 2009;15:6341-6347.
- 17
- 18
- 19 32. Hofbauer GF, Geertsen R, Laine E, Burg G, Dummer R: Impact of interferons on the expression of melanoma-associated antigens in melanoma short-term cell cultures. *Melanoma Res* 2001;11:213-218.
- 20
- 21
- 22 33. de Vries TJ, Smeets M, de Graaf R, Hou-Jensen K, Brocker EB, Renard N, Eggermont AM, van Muijen GN, Ruiter DJ: Expression of gp100, MART-1, tyrosinase, and S100 in paraffin-embedded primary melanomas and locoregional, lymph node, and visceral metastases: implications for diagnosis and immunotherapy. A study conducted by the EORTC Melanoma Cooperative Group. *J Pathol* 2001;193:13-20.
- 23
- 24
- 25 34. Takeuchi H, Kuo C, Morton DL, Wang HJ, Hoon DS: Expression of differentiation melanoma-associated antigen genes is associated with favorable disease outcome in advanced-stage melanomas. *Cancer Res* 2003;63:441-448.
- 26
- 27
- 28 35. Welinder C, Pawlowski K, Szasz AM, Yakovleva M, Sugihara Y, Malm J, Jonsson G, Ingvar C, Lundgren L, Baldetorp B, Olsson H, Rezeli M, Laurell T, Wieslander E, Marko-Varga G: Correlation of histopathologic characteristics to protein expression and function in malignant melanoma. *PLoS One* 2017;12:e0176167.
- 29
- 30
- 31 36. Kitago M, Koyanagi K, Nakamura T, Goto Y, Faries M, O'Day SJ, Morton DL, Ferrone S, Hoon DS: mRNA expression and BRAF mutation in circulating melanoma cells isolated from peripheral blood with high molecular weight melanoma-associated antigen-specific monoclonal antibody beads. *Clin Chem* 2009;55:757-764.
- 32
- 33
- 34 37. Campoli MR, Chang CC, Kageshita T, Wang X, McCarthy JB, Ferrone S: Human high molecular weight-melanoma-associated antigen (HMW-MAA): a melanoma cell surface chondroitin sulfate proteoglycan (MSCP) with biological and clinical significance. *Crit Rev Immunol* 2004;24:267-296.
- 35
- 36
- 37 38. Vourc'h-Jourdain M, Volteau C, Nguyen JM, Khammari A, Dreno B: Melanoma gene expression and clinical course. *Arch Dermatol Res* 2009;301:673-679.
- 38
- 39 39. Svobodova S, Browning J, MacGregor D, Pollara G, Scolyer RA, Murali R, Thompson JF, Deb S, Azad A, Davis ID, Cebon JS: Cancer-testis antigen expression in primary cutaneous melanoma has independent prognostic value comparable to that of Breslow thickness, ulceration and mitotic rate. *Eur J Cancer* 2010;47:460-469.
- 40
- 41 40. Ekedahl H, Cirenajwis H, Harbst K, Carneiro A, Nielsen K, Olsson H, Lundgren L, Ingvar C, Jonsson G: The clinical significance of BRAF and NRAS mutations in a clinic-based metastatic melanoma cohort. *Br J Dermatol* 2013;169:1049-1055.
- 42
- 43
- 44 41. Jakob JA, Bassett RL, Jr., Ng CS, Curry JL, Joseph RW, Alvarado GC, Rohlf ML, Richard J, Gershenwald JE, Kim KB, Lazar AJ, Hwu P, Davies MA: NRAS mutation status is an independent prognostic factor in metastatic melanoma. *Cancer* 2012;118:4014-4023.
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Table 1: Main clinico-pathological features of the 50 melanoma patients

Variable	Overall population (50)
Age (years)	51,9 years (+/-11,6)
≥ 50 years	30
<50 years	20
Gender	
Male	35
Female	15
Stage of melanoma	IIIb
Death	
Yes	38
No	12
Adjuvant treatment	
TILs + IL-2	23
IFN-alpha	12
IL-2	10
None	5
Breslow	2,74 (mean) 2 (median)
<1,5mm	21
>1,5mm	29
Missing data	0
Ulceration	
Yes	14
No	17
Missing data	19
Number of metastatic lymph nodes	2 (mean and median)
>1	28
1	22
Capsular breaking	
Yes	31
No	19
Missing data	0
Mean progression free survival	8 months
Mean overall survival	19,5 months
Mutations	40
<i>BRAF</i>	25
<i>NRAS</i>	15
<i>KIT</i>	0

Table 3: Median expression of selected biomarkers by untreated melanoma cell-lines and IFN- γ stimulated melanoma cell-lines

	untreated melanoma cells	IFN- γ treated melanoma cells
PD-L1	0,4%	76,1%
IDO1	37,0%	56,1%
MHC-I	90,2%	84,3%
MHC-II	67,7%	79,8%
gp100	68,5%	63,3%
Melan-A	74,5%	65,8%
tyrosinase	74,5%	58,2%
NY-ESO-1	52,5%	37,9%
MAGE-A	43,0%	26,8%
MCSP	91,3%	88,6%

Figure 1: Median expression of PD-L1 and IDO1 in untreated melanoma cell-lines and IFN- γ stimulated melanoma cell-lines

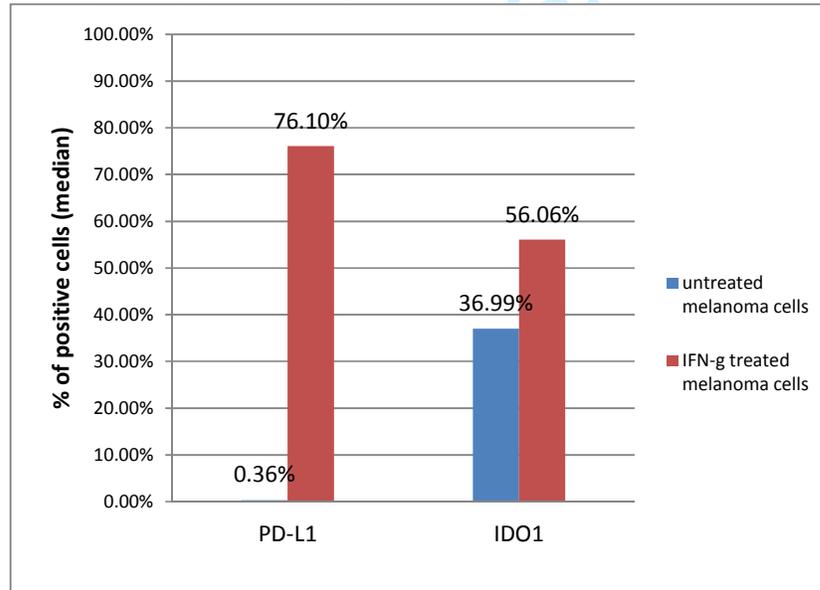
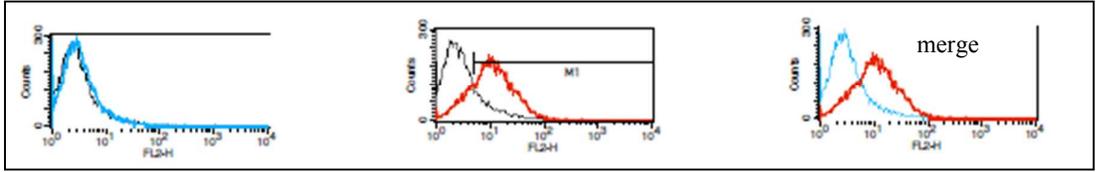
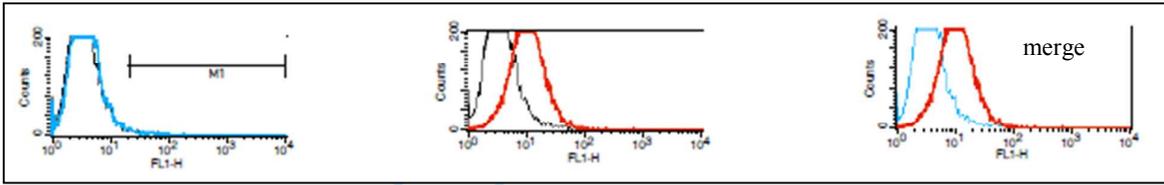


Figure 2: Representative pictures of flow cytometric analyses for PD-L1 (2A) and IDO1 (2B)

Blue line: untreated melanoma cells, red line: IFN- γ treated melanoma cells, black line: isotypic control



2A Membranous PD-L1 expression



2B Intracellular IDO1 expression

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Figure legends

Figure 1: Median expression of PD-L1 and IDO1 in untreated melanoma cell-lines and IFN- γ stimulated melanoma cell-lines

Figure 2: Representative pictures of flow cytometric analyses for PD-L1 (2A) and IDO1 (2B)

Blue line: untreated melanoma cells, red line: IFN- γ treated melanoma cells, black line: isotypic control

Supplementary figure 1: Kaplan Meier curves for RFS

Supplementary figure 2: Kaplan Meier curves for OS

Table 1: Main clinico-pathological features of the 50 melanoma patients

Table 2: Detailed clinico-biological characteristics of the 50 melanoma patients

Table 3: Median expression of selected biomarkers in untreated melanoma cell-lines and IFN- γ stimulated melanoma cell-lines

Supplementary table 1: Cytometric analysis of the 50 melanoma cell-lines (data are expressed as % of positive cells)

Supplementary table 2: Univariate model for RFS including biomarkers from both untreated and IFN- γ treated melanoma cell-lines

Supplementary table 3: Cox model for RFS including only biomarkers from untreated melanoma cell-lines

Supplementary table 4: Cox model for RFS including only biomarkers from IFN- γ treated melanoma cell-lines

Supplementary table 5: Univariate model for OS including biomarkers from both untreated and IFN- γ treated melanoma cell-lines

Supplementary table 6: Cox model for OS including only biomarkers from untreated melanoma cell-lines

Supplementary table 7: Cox model for OS including only biomarkers from IFN- γ treated melanoma cell-lines

	Patients code	Stage	BRAF/NRAS mutation testing FFPE tumor sample	% tumor cells in FFPE block	BRAF/NRAS mutation testing autologous tumor cell-line	Cell-line passage	Date of birth	Gender	Date of primitive tumor excision	Breslow	Breslow >1.5mm	Ulceration	Capsular breaking	Date of LN excision	Adjuvant treatment	Age at LN excision	Number of invaded LNs	Date of progression	Age at progression	Type of progression	Date of death	Age at death	Date of last news
1																							
2	1	III	p.Q61R	65	p.Q61R	ND	30/06/1941	M	28/11/1990	0,5	0	NA	1	21/12/1994	TIL + IL-2	53,00	5	10/07/1995	54,03	metastasis	07/01/1996	54,52	NA
3	2	III	p.Q61H	85	p.Q61H	P33	01/12/1952	M	01/12/1994	7,5	1	1	1	01/02/1995	TIL + IL-2	42,00	3	no	NA	NA	no	NA	10/05/2016
4	3	III	p.V600E	90	p.V600E	P5	20/02/1946	M	NK/NK/1994	3,4	1	1	1	16/02/1995	IL-2	48,00	1	22/01/1996	49,92	metastasis	02/05/1997	51,20	NA
5	4	III	p.V600K	50	p.V600K	P6	23/12/1939	M	02/07/1994	0,98	0	NA	1	16/03/1995	IL-2	55,00	2	13/06/1995	55,47	regional	29/08/1996	56,68	NA
6	5	III	wt	85	wt	P7	03/03/1939	M	23/02/1995	2,36	1	NA	0	30/03/1995	IL-2	56,00	5	06/06/1995	56,26	metastasis	24/06/1995	56,31	NA
7	6	III	wt	60	wt	P6	11/08/1944	M	13/07/1994	3,68	1	1	0	22/06/1995	TIL + IL-2	50,00	6	25/09/1995	51,12	metastasis	09/01/1996	51,41	NA
8	7	III	p.Q61R	90	p.Q61R	P4	23/02/1941	F	15/11/1993	1	0	NA	0	20/07/1995	IL-2	54,00	3	25/01/1996	54,92	metastasis	25/01/1996	54,92	NA
9	8	III	wt	70	wt	P15	24/03/1946	M	22/03/1995	0,8	0	NA	0	05/10/1995	TIL + IL-2	49,00	2	no	NA	NA	no	NA	10/05/2016
10	9	III	p.V600E	80	p.V600E	P10	19/07/1945	M	12/12/1995	3,4	1	NA	1	12/12/1995	IL-2	50,00	1	22/02/1996	50,60	regional	05/01/1997	51,47	NA
11	10	III	p.V600E	75	p.V600E	P10	02/04/1947	M	12/12/1995	3,9	1	1	0	25/04/1996	IL-2	49,00	2	10/06/1996	49,19	local	30/12/2000	53,75	NA
12	11	III	p.V600E	80	p.V600E	P6	08/10/1930	M	15/04/1997	9	1	NA	1	17/10/1996	TIL + IL-2	66,00	2	23/03/1997	66,46	metastasis	15/04/1997	66,52	NA
13	12	III	p.V600E	60	p.V600E	P4	07/11/1945	M	15/05/1996	2	1	0	0	23/07/1997	TIL + IL-2	51,00	1	no	NA	NA	no	NA	10/05/2016
14	13	III	wt	60	wt	P14	29/04/1937	F	19/03/1996	1,04	0	NA	1	11/09/1997	TIL + IL-2	60,00	2	no	NA	NA	no	NA	10/05/2016
15	14	III	p.V600E	50	p.V600E	P7	22/03/1978	F	03/03/1997	1,44	0	1	1	17/10/1997	IL-2	19,00	1	no	NA	NA	no	NA	10/05/2016
16	15	III	p.V600R	>90	p.V600R	P6	24/04/1925	M	14/01/1997	3,9	1	1	0	13/11/1997	TIL + IL-2	72,00	3	no	NA	NA	27/06/2000	75,18	NA
17	16	III	p.V600E	80	p.V600E	P8	01/11/1963	M	NK/01/1996	3,2	1	NA	1	19/02/1998	TIL + IL-2	34,00	1	07/08/1998	34,77	metastasis	17/06/1999	35,62	NA
18	17	III	p.V600E	80	p.V600E	P12	07/06/1974	M	17/04/1998	1,2	0	NA	1	17/04/1998	IL-2	23,00	1	13/10/1998	24,35	metastasis	15/12/1998	24,52	NA
19	18	III	p.Q61L	60	p.Q61L	P12	03/04/1940	M	25/18/1997	1,65	1	1	1	30/04/1998	TIL + IL-2	58,00	7	27/07/1998	58,31	locale	06/10/1998	58,51	NA
20	19	III	wt	45	wt	P7	09/11/1956	M	20/01/1998	1,35	0	1	0	23/07/1998	IL-2	41,00	1	no	NA	NA	no	NA	10/05/2016
21	20	III	p.V600E	65	p.V600E	P5	14/02/1949	F	31/07/1997	6	1	1	1	03/09/1998	IL-2	49,00	2	21/01/2000	50,93	local	07/01/2002	52,90	NA
22	21	III	p.V600E	80	p.V600E	P9	13/08/1952	F	29/06/1998	8	1	0	1	16/09/1998	TIL + IL-2	46,00	2	11/05/1999	46,74	metastasis	20/01/2000	47,44	NA
23	22	III	p.Q61R	80	p.Q61R	P13	03/03/1931	M	12/07/2001	1,9	1	NA	0	30/01/2003	TIL + IL-2	71,00	1	24/06/2005	74,31	regional	20/03/2006	75,05	NA
24	23	III	p.V600E	50	p.V600E	P40	14/11/1954	M	01/05/2003	0,88	0	0	1	19/06/2003	TIL + IL-2	48,00	1	23/07/2004	49,69	metastasis	30/12/2004	50,13	NA
25	24	III	wt	>90	wt	P5	12/06/1944	F	17/05/2004	2,45	1	1	0	15/05/2004	TIL + IL-2	59,00	1	21/07/2004	60,11	regional	09/04/2005	60,82	NA
26	25	III	p.Q61K	60	p.Q61K	P6	24/05/1959	F	15/07/1996	1	0	0	0	05/08/2004	TIL + IL-2	45,00	1	no	NA	NA	no	NA	10/05/2016
27	26	III	p.V600E	50	p.V600E	P7	07/03/1933	F	04/11/2003	0,331	0	0	0	22/11/2004	TIL + IL-2	71,00	1	25/01/2006	72,89	regional	04/03/2006	72,99	NA
28	27	III	p.V600E	85	p.V600E	P9	23/09/1932	F	22/09/1989	1,2	0	NA	1	18/02/2005	TIL + IL-2	72,00	1	18/11/2005	73,15	local	no	NA	10/05/2016
29	28	III	p.Q61R	60	p.Q61R	P11	22/02/1942	F	04/12/2001	1,04	0	0	0	03/06/2005	TIL + IL-2	63,00	1	no	NA	NA	07/07/2009	67,37	NA
30	29	III	p.Q61R	80	p.Q61R	P7	31/08/1952	M	23/06/2005	6	1	1	0	23/09/2005	IFN-alpha	53,00	2	23/04/2008	55,64	metastasis	06/04/2009	56,60	NA
31	30	III	wt	90	wt	P4	27/08/1946	M	03/06/2005	>3	1	1	0	07/10/2005	TIL + IL-2	59,00	1	07/04/2006	59,61	metastasis	10/10/2006	60,12	NA
32	31	III	p.V600E	95	p.V600E	P9	02/10/1960	M	29/09/1998	1,2	0	0	0	21/11/2005	TIL + IL-2	45,00	1	no	NA	NA	no	NA	10/05/2016
33	32	III	wt	90	wt	P8	10/10/1950	M	05/07/2005	0,8	0	0	1	10/03/2006	TIL + IL-2	55,00	1	23/06/2006	55,70	regional	03/03/2007	56,39	NA
34	33	III	p.Q61R	90	p.Q61R	P10	02/03/1942	F	NK/12/1997	1,8	1	NA	1	10/03/2006	None	64,00	2	no	NA	NA	no	NA	10/05/2016
35	34	III	p.Q61L	75	p.Q61L	P6	30/03/1955	M	22/08/2005	2	1	NA	1	05/05/2006	None	51,00	1	24/11/2006	51,66	reg	16/07/2008	53,30	NA
36	35	III	wt	<5	wt	P5	05/06/1950	M	27/06/2005	4	1	0	1	21/06/2006	IFN-alpha	56,00	6	11/09/2006	56,27	metastasis	20/08/2007	57,21	NA
37	36	III	p.V600E	35	p.V600E	P10	11/02/1943	M	30/12/2004	1,45	0	0	1	17/11/2006	IFN-alpha	63,00	3	04/05/2007	64,22	local	05/10/2008	65,65	NA
38	37	III	p.V600E	90	p.V600E	P7	14/12/1970	F	05/12/2005	2	1	0	1	15/12/2006	None	36,00	1	29/01/2007	36,13	metastasis	28/04/2007	36,37	NA
39	38	III	p.V600E	85	p.V600E	P7	13/01/1954	M	24/03/1995	4	1	NA	1	26/01/2007	IFN-alpha	53,00	4	11/09/2007	53,66	regional	04/07/2008	54,47	NA
40	39	III	p.V600E	60	p.V600E	P7	07/12/1957	F	28/09/2006	4,8	1	1	1	30/03/2007	IFN-alpha	49,00	4	28/12/2007	50,06	metastasis	03/06/2008	50,49	NA
41	40	III	p.Q61K	80	p.Q61K	P6	13/01/1948	F	30/04/2007	9	1	NA	1	19/07/2007	IFN-alpha	59,00	2	18/03/2010	62,18	locale	25/05/2011	63,36	NA
42	41	III	p.V600E	80	p.V600E	P7	12/12/1970	M	17/03/2006	0,6	0	NA	1	30/08/2007	IFN-alpha	36,00	3	19/11/2007	36,94	regional	27/03/2008	37,29	NA
43	42	III	p.V600K	95	p.V600K	P7	22/08/1944	M	09/10/2006	2,4	1	0	0	13/12/2007	IFN-alpha	63,00	2	23/09/2008	64,09	regional	no	NA	10/05/2016
44	43	III	p.V600E	50	p.V600E	P7	16/10/1951	M	19/02/2007	0,36	0	NA	1	13/12/2007	IFN-alpha	56,00	>1	07/04/2008	56,48	metastasis	20/07/2008	56,76	NA
45	44	III	p.Q61K	85	p.Q61K	P6	04/08/1959	M	23/10/2007	5,51	1	0	0	08/02/2008	None	48,00	1	11/08/2008	49,02	locale	10/06/2011	51,85	NA
46	45	III	p.V600E	85	p.V600E	P8	10/03/1959	M	19/02/2007	2,7	1	0	0	15/02/2008	TIL + IL-2	48,00	1	19/06/2008	49,28	regional	03/08/2008	49,40	NA
47	46	III	p.Q61R	10	p.Q61R	P7	25/09/1947	M	26/08/2007	2,9	1	0	1	25/02/2008	IFN-alpha	60,00	3	31/12/2008	61,27	metastasis	11/01/2010	62,30	NA
48	47	III	wt	85	wt	P4	25/05/1980	M	02/03/2006	1,3	0	0	1	12/03/2008	IFN-alpha	27,00	8	29/04/2008	27,93	regional	no	NA	10/05/2016
49	48	III	p.Q61K	40	p.Q61K	P6	31/01/1948	M	04/08/2003	1,39	0	NA	1	19/05/2008	None	60,00	2	21/08/2008	60,56	reg + meta	06/02/2009	61,02	NA
50	49	III	p.V600E	60	p.V600E	P8	20/11/1962	F	02/05/2008	4,83	1	1	0	11/07/2008	IFN-alpha	45,00	2	17/07/2009	46,66	local	16/07/2010	47,85	NA
51	50	III	p.Q61R	90	p.Q61R	P5	21/06/1954	M	05/06/2007	0,34	0	0	1	02/12/2009	TIL + IL-2	55,00	1	26/08/2010	56,18	metastasis	03/11/2010	56,37	NA

Table 2 : Detailed clinico-biological characteristics of the 50 melanoma patients

Patients code	CMH I		CMH II		MCSF		gp100		NYESO1		Melan-A		tyrosinase		MAGes		PD-L1		IDO1		
	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	untreated	+ IFN	
1	91,26%	86,05%	68,65%	85,83%	88,98%	87,83%	93,63%	85,81%	61,66%	0,33%	86,12%	78,36%	76,57%	58,02%	39,20%	0,38%	71,46%	79,42%	53,72%	81,94%	
2	83,11%	96,71%	19,88%	88,15%	62,90%	78,36%	89,12%	89,36%	77,16%	72,92%	78,02%	75,84%	77,07%	79,88%	62,17%	54,58%	0,00%	84,87%	0,00%	0,00%	
3	93,74%	88,99%	92,21%	92,65%	96,87%	90,59%	80,20%	62,77%	38,82%	1,98%	89,76%	58,20%	88,18%	52,72%	78,17%	0,00%	0,00%	77,71%	80,22%	67,38%	
4	79,96%	77,55%	16,40%	19,85%	89,59%	87,45%	80,39%	83,34%	75,35%	65,79%	73,55%	81,11%	80,68%	81,41%	18,39%	31,02%	82,16%	78,83%	74,01%	64,31%	
5	81,57%	86,50%	59,29%	73,84%	96,57%	92,50%	74,23%	75,06%	62,81%	55,69%	86,16%	75,48%	51,45%	36,52%	0,40%	1,01%	86,72%	80,59%	53,45%	51,43%	
6	100,00%	100,00%	0,00%	91,10%	95,48%	95,90%	0,00%	100,00%	80,38%	57,39%	94,77%	96,13%	100,00%	63,36%	42,65%	49,14%	100,00%	100,00%	0,00%	0,00%	
7	92,78%	83,97%	85,18%	79,79%	0,00%	0,00%	61,16%	71,34%	0,00%	0,00%	57,54%	51,62%	66,55%	48,21%	28,79%	14,22%	0,00%	72,64%	0,00%	16,27%	
8	66,39%	37,34%	88,88%	84,86%	94,12%	95,46%	54,97%	78,65%	33,61%	4,71%	81,61%	84,86%	43,95%	31,08%	25,70%	11,99%	2,39%	2,44%	40,58%	24,72%	
9	82,47%	76,40%	70,49%	63,54%	1,18%	0,57%	0,57%	0,35%	0,29%	0,28%	0,43%	0,30%	0,35%	0,11%	0,40%	0,26%	76,72%	82,74%	1,22%	3,42%	
10	78,11%	77,04%	39,26%	80,43%	94,61%	92,26%	53,44%	35,76%	72,25%	0,24%	66,43%	49,03%	51,04%	11,09%	36,51%	0,90%	0,14%	71,95%	0,84%	64,88%	
11	85,54%	83,02%	59,99%	70,43%	59,89%	53,37%	0,15%	0,03%	0,81%	0,20%	0,20%	0,45%	0,27%	0,54%	1,03%	1,06%	0,14%	57,10%	7,22%	56,37%	
12	95,93%	93,62%	78,56%	94,46%	97,35%	93,24%	87,75%	80,99%	70,51%	24,28%	79,65%	55,21%	77,08%	68,60%	53,81%	27,76%	0,00%	29,97%	41,70%	70,14%	
13	76,99%	71,73%	0,99%	61,94%	86,65%	86,14%	60,05%	66,21%	55,75%	44,88%	65,42%	69,56%	74,80%	75,09%	53,25%	17,12%	18,26%	62,41%	52,26%	44,35%	
14	72,22%	61,31%	61,93%	75,97%	81,88%	71,41%	3,52%	1,41%	0,31%	0,69%	2,42%	0,48%	0,19%	1,79%	1,48%	0,20%	0,37%	80,57%	78,98%	56,30%	7,56%
15	92,68%	85,90%	83,57%	72,04%	50,04%	36,15%	58,45%	62,23%	1,24%	2,21%	60,57%	58,81%	1,89%	60,20%	1,60%	1,88%	66,86%	66,68%	15,09%	64,88%	
16	100,00%	100,00%	45,37%	73,21%	95,37%	95,39%	100,00%	100,00%	97,93%	69,07%	99,75%	97,62%	97,15%	100,00%	100,00%	52,45%	100,00%	46,36%	0,00%	49,04%	
17	96,48%	92,16%	76,92%	94,29%	98,93%	98,28%	47,60%	72,19%	0,23%	0,23%	59,95%	65,52%	0,17%	64,95%	0,20%	70,58%	0,34%	88,14%	77,75%	39,82%	
18	84,10%	67,12%	69,85%	76,55%	90,46%	86,80%	83,82%	58,42%	91,97%	51,92%	91,20%	69,97%	78,70%	47,96%	28,39%	27,28%	39,66%	83,26%	0,62%	3,49%	
19	93,64%	93,45%	91,50%	86,32%	94,54%	92,31%	38,51%	42,99%	69,63%	60,88%	71,26%	59,90%	83,08%	87,01%	94,06%	91,43%	15,88%	4,63%	37,75%	55,75%	
20	89,09%	81,74%	23,72%	66,01%	92,54%	91,42%	62,07%	63,06%	50,14%	46,13%	70,60%	55,75%	74,82%	62,25%	60,95%	37,28%	0,08%	57,71%	37,74%	43,83%	
21	89,52%	88,26%	70,76%	68,07%	86,35%	84,92%	82,51%	70,72%	87,11%	26,03%	87,87%	53,02%	89,11%	70,21%	80,48%	8,34%	0,00%	77,66%	77,68%	25,11%	
22	98,50%	95,53%	99,20%	97,84%	98,43%	97,36%	64,49%	86,74%	84,90%	84,33%	98,08%	82,08%	84,33%	77,52%	51,11%	73,43%	0,00%	80,59%	89,74%	82,58%	
23	85,95%	96,18%	60,46%	85,06%	97,15%	97,38%	90,43%	89,81%	51,81%	41,50%	91,82%	89,35%	81,25%	82,73%	43,30%	55,05%	49,22%	75,30%	89,38%	80,33%	
24	83,43%	84,58%	84,20%	85,14%	1,60%	2,82%	0,20%	0,19%	0,13%	0,04%	0,19%	0,05%	0,49%	0,22%	0,23%	0,07%	0,06%	76,71%	80,25%	84,30%	
25	94,27%	95,88%	96,36%	97,84%	79,16%	83,78%	93,39%	93,68%	54,59%	47,99%	90,48%	90,58%	90,08%	84,05%	54,15%	47,94%	0,76%	86,21%	91,95%	94,18%	
26	0,00%	100,00%	1,59%	84,84%	83,83%	80,16%	0,00%	0,00%	92,91%	86,89%	85,21%	58,13%	100,00%	100,00%	60,24%	25,16%	85,21%	80,00%	100,00%	100,00%	
27	96,52%	90,15%	93,90%	88,89%	98,81%	97,49%	66,58%	58,02%	88,01%	82,79%	90,03%	83,62%	78,68%	71,13%	83,67%	76,32%	0,00%	91,45%	0,00%	0,00%	
28	92,85%	95,14%	73,73%	89,43%	91,92%	89,85%	17,89%	34,46%	78,57%	69,10%	74,53%	69,47%	7,07%	13,47%	30,06%	31,72%	87,76%	92,85%	76,00%	75,04%	
29	86,01%	87,31%	54,80%	54,93%	63,03%	46,43%	80,54%	79,88%	52,12%	52,51%	73,09%	70,13%	48,82%	34,15%	38,38%	21,17%	0,84%	1,12%	11,32%	24,83%	
30	96,04%	69,27%	88,11%	79,83%	59,52%	42,58%	0,00%	0,00%	6,35%	0,26%	0,00%	0,00%	0,22%	0,07%	0,25%	0,26%	0,05%	83,39%	22,34%	58,27%	
31	72,15%	64,23%	73,70%	70,44%	82,13%	77,96%	74,46%	72,04%	70,33%	77,29%	66,00%	65,99%	67,84%	78,29%	61,39%	0,65%	0,89%	0,60%	28,70%	0,00%	
32	71,17%	74,60%	56,74%	47,23%	54,77%	57,33%	75,40%	72,04%	50,67%	48,81%	61,21%	65,08%	56,58%	57,42%	0,21%	53,31%	0,50%	52,77%	1,39%	43,08%	
33	87,71%	83,58%	92,10%	90,21%	94,59%	88,32%	74,94%	72,66%	52,87%	33,08%	73,43%	51,69%	88,09%	58,42%	59,00%	29,64%	0,13%	87,99%	56,22%	71,59%	
34	91,24%	83,25%	66,77%	92,38%	94,69%	91,37%	90,71%	88,16%	52,91%	36,19%	89,66%	69,69%	86,05%	52,83%	100,00%	100,00%	0,00%	58,78%	0,00%	100,00%	
35	96,67%	88,89%	84,90%	99,42%	99,42%	99,07%	69,45%	44,35%	64,80%	62,17%	82,36%	77,51%	72,61%	57,11%	55,41%	57,69%	0,10%	92,18%	66,58%	86,93%	
36	75,92%	69,91%	66,59%	79,77%	94,43%	95,90%	71,01%	39,98%	19,09%	8,93%	72,73%	70,61%	83,13%	61,75%	48,36%	36,21%	0,11%	0,15%	26,94%	51,19%	
37	100,00%	100,00%	85,29%	98,09%	99,35%	98,01%	0,00%	100,00%	97,00%	86,88%	94,41%	91,51%	100,00%	100,00%	100,00%	0,00%	100,00%	100,00%	100,00%	100,00%	
38	93,33%	83,90%	70,50%	58,99%	95,60%	94,78%	80,17%	77,95%	40,31%	68,62%	71,69%	74,36%	74,21%	70,80%	47,04%	63,92%	69,05%	65,95%	1,05%	71,48%	
39	91,87%	80,98%	44,58%	75,00%	90,65%	88,84%	0,33%	0,37%	0,22%	0,52%	0,21%	0,58%	0,19%	0,33%	0,59%	0,38%	90,84%	88,20%	78,88%	0,00%	
40	100,00%	83,37%	1,46%	18,02%	90,64%	85,22%	96,56%	94,75%	90,14%	100,00%	90,67%	83,84%	95,19%	89,44%	82,09%	78,33%	0,00%	100,00%	0,00%	0,00%	
41	87,55%	63,13%	70,68%	52,19%	94,49%	93,40%	52,41%	54,94%	0,35%	0,28%	73,77%	64,41%	0,28%	0,26%	0,39%	0,38%	0,09%	75,48%	14,24%	37,28%	
42	100,00%	100,00%	0,00%	70,85%	97,54%	98,21%	0,00%	100,00%	99,26%	95,39%	99,64%	97,80%	100,00%	0,00%	100,00%	49,97%	0,00%	0,00%	0,00%	0,00%	
43	80,52%	73,36%	90,07%	86,10%	69,52%	85,03%	37,22%	14,12%	10,20%	1,13%	14,12%	21,68%	50,25%	0,88%	0,41%	2,45%	60,42%	70,98%	66,42%	0,00%	
44	90,78%	88,30%	87,65%	86,36%	95,64%	89,10%	69,73%	63,29%	47,98%	39,66%	74,42%	51,70%	91,09%	74,22%	79,22%	53,59%	0,40%	80,62%	93,53%	87,60%	
45	81,90%	76,21%	23,68%	54,15%	90,48%	84,34%	7,12%	60,06%	9,11%	2,04%	29,08%	51,46%	8,89%	5,10%	1,98%	2,17%	0,12%	68,14%	0,28%	65,56%	
46	100,00%	100,00%	58,65%	98,88%	99,78%	97,96%	0,00%	53,09%	0,68%	0,00%	100,00%	0,00%	84,47%	58,51%	100,00%	0,00%	100,00%	100,00%	0,00%	100,00%	
47	91,59%	91,44%	31,44%	25,62%	93,51%	93,44%	48,20%	50,97%	24,48%	18,01%	85,77%	85,47%	23,36%	28,58%	41,55%	11,49%	82,99%	74,84%	36,23%	23,45%	
48	76,48%	55,87%	0,71%	1,28%	0,46%	0,18%	70,58%	78,56%	23,22%	42,88%	63,26%	61,89%	22,78%	10,58%	18,58%	26,25%	0,06%	42,56%	0,20%	36,65%	
49	47,43%	39,34%	48,12%	46,07%	38,78%	34,46%	52,07%	49,00%	0,17%	0,27%	44,57%	41,36%	1,76%	2,13%	0,31%	0,25%	0,15%	39,81%	0,26%	0,35%	
50	90,95%	81,93%	31,19%	67,12%	63,16%	57,52%	79,85%	81,49%	81,01%	10,50%	88,97%	74,74%	81,95%	85,84%	75,08%	68,61%	0,03%	79,85%	69,28%	87,99%	

39

40 **Supplementary table 1** : Cytometric analysis of the 50 melanoma cell-lines (data are expressed as % of positive cells)

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	Untreated cell-lines	IFN- γ treated cell-lines
CMH I	0,886	0,514
CMH II	0,313	0,324
MCSP	0,703	0,363
gp100	0,837	0,48
Melan-A	0,378	0,613
tyrosinase	0,136	0,0121
NY-ESO-1	0,115	0,4
MAGE-A	0,021	0,15
IDO1	0,0653	0,769
PD-L1	0,334	0,673

Supplementary table 2 : univariate model for RFS including biomarkers from both untreated and IFN- γ treated melanoma cell-lines (data are expressed as p-values)

	HR	lower .95	upper .95	p-value
MCSP	0.985	0.971	0.998	0.0259
NRAS mutation	0.506	0.245	1.045	0.0655
Number of invaded nodes	1.365	1.083	1.720	0.0083
Capsular breaking	1.824	0.875	3.804	0.1088

Supplementary table 3: Cox model for RFS including only biomarkers from untreated melanoma cell-lines

	HR	lower .95	upper .95	p-value
MHC class II	0.986	0.968	1.004	0.1329
MCSP	0.970	0.948	0.993	0.0099
NY-ESO-1	1.035	1.015	1.056	0.0005
tyrosinase	0.978	0.965	0.991	0.0013
PD-L1	1.032	1.015	1.049	0.0001
IDO1	1.017	1.004	1.031	0.0130
NRAS	0.216	0.084	0.554	0.0015
Number of invaded nodes	1.337	1.052	0.554	0.0175

Supplementary table 4: Cox model for RFS including only biomarkers from IFN- γ -treated melanoma cell-lines

	Untreated cell-lines	IFN- γ treated cell-lines
CMH I	0,73	0,372
CMH II	0,78	0,435
MCSP	0,212	0,543
gp100	0,694	0,768
Melan-A	0,201	0,147
tyrosinase	0,0645	0,0797
NY-ESO-1	0,0439	0,331
MAGE-A	0,00727	0,0679
IDO1	0,533	0,137
PD-L1	0,618	0,428

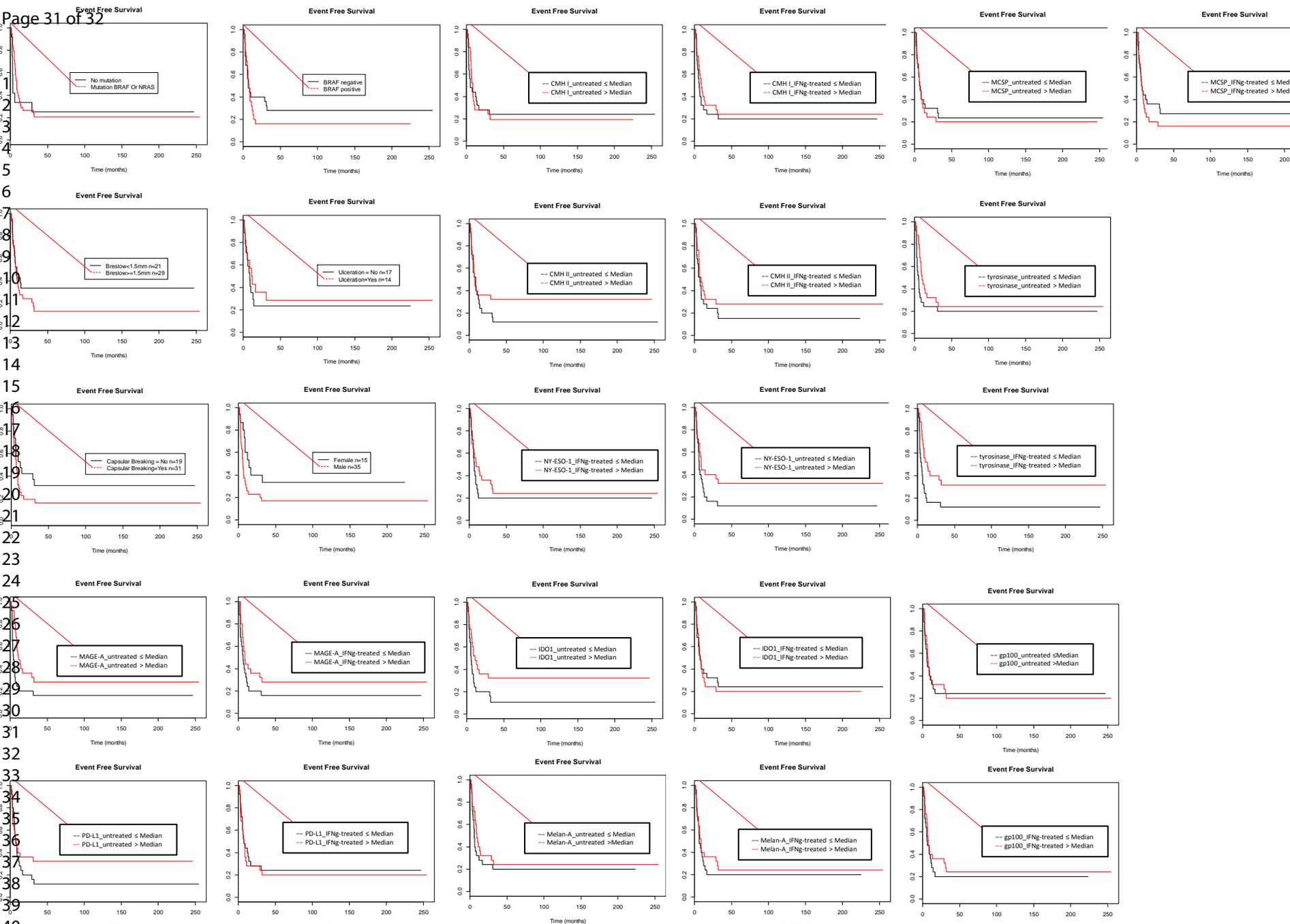
Supplementary table 5 : univariate model for OS including biomarkers from both untreated and IFN- γ treated melanoma cell-lines (data are expressed as p-values)

	HR	lower .95	upper .95	p-value
tyrosinase	0.985	0.975	0.995	0.0031
Capsular breaking	2.476	1.126	5.444	0.0242

Supplementary table 6: Cox model for OS including only biomarkers from untreated melanoma cell-lines

	HR	lower .95	upper .95	p-value
MCSP	0.981	0.964	0.997	0.0201
gp100	0.975	0.958	0.993	0.0070
Melan-A	1.025	1.003	1.048	0.0282
MAGE-A	0.984	0.971	0.998	0.0230
IDO1	1.025	1.011	1.039	0.0005
Breslow	1.218	1.020	1.456	0.0298
Capsular breaking	3.656	1.575	8.491	0.0026

Supplementary table 7: Cox model for OS including only biomarkers from IFN- γ -treated melanoma cell-lines



Supplementary figure 1 : Kaplan Meier curves for RFS

