


Baseline and early functional immune response is associated with subsequent clinical outcomes of PD-1 inhibition therapy in metastatic melanoma patients

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ABSTRACT

Background Despite significant progress with antiprogrammed cell death protein 1 (PD-1) therapy, a substantial fraction of metastatic melanoma patients show upfront therapy resistance. Biomarkers for outcome are missing and the association of baseline immune function and clinical outcome remains to be determined. We assessed the in vitro nonspecific stimulation of immune response at baseline and during anti-PD-1 therapy for metastatic melanoma.

Methods Previously untreated metastatic melanoma patients received nivolumab and radiotherapy as part of the multicentric phase II trial NIRVANA (NCT02799901). The levels of Th1, Th2 and Th17 cytokines on in vitro non-specific stimulation of innate and adaptive immune cells were measured in patient sera before treatment, and at week 2 and week 6 after the beginning of the treatment, and correlated with tumorous response, progression-free survival (PFS) and occurrence of immune-related adverse events (irAEs). The results in melanoma patients were compared with those of a cohort of 9 sex and age-matched healthy donors.

Results Seventeen patients were enrolled in this ancillary study. Median follow-up was 16 months (2.2–28.4). The 12-month PFS rate was 67.7%. The incidence of irAEs of any grade was 58.8%. Without in vitro stimulation no differences in cytokines levels were observed between responders and non-responders. On in vitro stimulation, metastatic patients had lower Th1 cytokine levels than healthy donors at baseline for tumor necrosis factor- α and interferon- γ (IFN- γ) (1136 pg/mL vs 5558 pg/mL, $p < 0.0001$; and 3894 pg/mL vs 17 129 pg/mL, $p = 0.02$, respectively). Responders exhibited increasing cytokine levels from baseline to week 6. Non-responders had lower interleukin 17A (IL-17A) levels at baseline than responders (7 pg/mL vs 32 pg/mL, $p = 0.03$), and lower IFN- γ levels at week 6 (3.3 ng/mL vs 14.5 ng/mL, $p = 0.03$). A lower level of IL-17A at week 2 and a lower level of IFN- γ at week 6 correlated with worse PFS ($p = 0.04$ and $p = 0.04$ respectively). At baseline, patients who developed irAEs had higher IL-6 levels (19.3 ng/mL vs 9.2 ng/mL, $p = 0.03$) and higher IL-17A levels (52.5 pg/mL vs 2.5 pg/mL, $p = 0.009$) than those without irAEs.

Conclusions Our findings indicate that cytokine levels after in vitro non-specific stimulation could be a promising biomarker to predict the outcome of PD-1 inhibition therapy.

BACKGROUND

Immune checkpoint inhibitors (ICIs), based on the concept that cancer cells, can be targeted and eradicated by the immune system, have substantially improved clinical outcome in many types of cancer.¹ For the treatment of melanoma, ICIs are represented by two major classes of monoclonal antibodies. The first one is targeted against the cytotoxic T-lymphocyte-associated antigen-4 (ipilimumab) and the second one against the programmed cell death protein 1 (PD-1; nivolumab or pembrolizumab) or its ligand (PD-L1). Despite significant progress with these agents, a substantial fraction of patients (~50% depending on therapeutic regimen) will not respond or will escape to ICI due to innate or acquired resistance.² Moreover, severe immune-related adverse events (irAEs) that can sometimes be fatal can occur during immunotherapy.^{3 4} Therefore, biomarkers to predict the outcome of immunotherapy are strongly needed to guide individual treatment selection or sequence. To date, several studies aimed to identify biomarkers to predict outcome of ICI treatment, such as expression of PD-L1 within the tumor, tumor-infiltrating lymphocyte, T-cell receptor clonality, gene expression signature, peripheral blood markers and tumor mutational or neoantigen burden.^{5–9} Nevertheless, none of them can be considered as robust and reliable when selecting the patients who will benefit most from ICI treatment.

PD-1 blockade is believed to foster immune response by increasing Th1 and decreasing Th2 response,¹⁰ which may translate into an upheaval in cytokine concentrations such as Th1 pathway cytokines interferon- γ (IFN- γ) and interleukin 2 (IL-2), Th2 pathway cytokines IL-4 and IL-13, and Th17 pathway cytokine IL-17A.¹¹ However, since a boosted immune system can backfire on non-cancerous cells, checkpoint inhibition exposes patients to an array of inflammatory irAEs involving various organs such as skin or the digestive tract.^{3 12 13} Patients who respond to ICI are more prone to irAEs compared with non-responders.¹⁴⁻¹⁷

Tarhini *et al.* demonstrated that baseline circulating IL-17A without *in vitro* stimulation predicts toxicity.¹⁸ But the capability of an individual's immune system to respond to ICI and the factors that may predict the effectiveness of PD-1-blockade before treating patients remain to be determined. Functional tests provide a dynamic response and the individual's ability to respond to immunological stimulation^{19 20} and has never been evaluated in patients treated with anti-PD-1 therapy.

The objective of this exploratory study was to assess the immune response, measured by a functional assay, of patients treated with anti-PD-1 antibodies for metastatic melanoma and to correlate these measures to the response to treatment and the occurrence of irAEs.

PATIENTS, MATERIALS AND METHODS

Patients, study design and treatment

NIRVANA is an ongoing multicentric phase 2 non-randomized prospective trial (ClinicalTrials.gov number NCT02799901) exploring the association of nivolumab (240 mg every 2 weeks until disease progression, intolerable toxicity, or withdrawal of consent) with multisite hypofractionated radiotherapy (performed on week 2 and week 4) in adults with previously untreated advanced melanoma (stage III unresectable or IV) without brain metastases. Written informed consent was obtained from all patients. The primary outcome is the overall survival. Biomarker assessment is a prespecified exploratory outcome of the trial and safety is one of the secondary endpoints.

Herein, blood specimens for correlative studies were planned at baseline (day 0), week 2 and week 6 from 17 patients included from the Department of Dermatology in Nice University Hospital. The cytokine profile was analyzed before and after non-specific stimulation of innate and adaptive immune system and then correlated with tumor response rate, progression-free survival (PFS) and incidence of irAEs. Baseline lymphocyte and monocyte counts were gathered to adjust the baseline cytokines levels on the respective amount of producing cells.

The results in melanoma patients were compared with those of a cohort of 9 age-matched healthy donors (2:1).

Response, survival and toxicity assessments

PFS was defined as the time from the start of nivolumab to the date of disease progression or death, whichever occurred first. Patients who were alive without disease progression were censored at the date of their last disease assessment. Overall survival was defined as the time from the start of nivolumab to death from any cause.

In terms of tumor response, patients were classified as either responders (complete response, partial response, stabilization) or non-responders.

Disease progression and tumor response were evaluated using the response evaluation criteria in solid tumors (V.1.1) by radiologists at each participating institution.²¹ In case of tumor progression, in order to exclude pseudoprogression, a second CT scan was performed 4 weeks after the last evaluation.

Data on irAE incidence were collected and graded according to the US National Cancer Institute common toxicity criteria for adverse events (V.4.0).

Blood collection and cytokine assay

Blood samples were taken to assess cytokine levels on day 0, week 2, week 6, and within 2 weeks of any progression. The 1 mL of whole blood was collected and stimulated with immune ligands (anti-CD3 as T-cells stimulant, and R848 as TLR 7/8 agonist to stimulate Natural Killer cells and foster T-cells' response) on single lyophilized spheres (LyoSphere, Qiagen) within 8 hours from blood collection. Stimulated blood samples were incubated for 16 to 24 hours at 37°C and then centrifuged at 2000 to 3000 \times g for 15 min to harvest the stimulated serum. Stimulated serum was stored at -20°C until the analysis and freeze-thaw cycles were minimized to preserve the quality of the samples. Serum levels of cytokines were measured either using the ProcartaPlex Immunoassay Kit (Luminex, ThermoFisher) or with custom-designed cartridges Ella (ProteinSimple). We measured 11 cytokines (IL-17A, IL-6, IL-1 β , IFN-, IL-12p70, tumor necrosis factor- α (TNF- α), IL-10, IL-5, IL-4, IL-13 and GM-CSF) following the manufacturers' instructions.²²

Statistical analyses

For descriptive statistics, data are presented as mean and SD for continuous variables with Gaussian distribution, as median and IQR for continuous variables with non-Gaussian distribution, and as counts and percentages for categorical variables. The D'Agostino-Pearson normality test was used to determine if a variable had a Gaussian distribution or not. Groups of continuous values were compared by the Mann-Whitney test. Categorical variables were compared using χ^2 test. Median follow-up was estimated with Schemper method. Event-time distributions were estimated with the use of the Kaplan-Meier method. Log-rank test was used to compare survival data. Comparisons were two tailed and statistical significance was set at $p < 0.05$. To take into account the multiple testing problems, independent variables were sought with Spearman's correlation method. Bonferroni correction

was then applied to quantitative independent variables to adjust the statistical significance level for the comparisons of cytokines levels. Statistical analyses were performed using SPSS V.16.0 on Windows and GraphPad Prism V.7.0 (GraphPad Software).

RESULTS

Patients' characteristics and clinical outcomes

A total of 72 patients were included in the NIRVANA trial between April 2017 and June 2019. Eighteen patients were included in this ancillary study testing immune

response, among them one was excluded due to positive hepatitis B virus serology according to the study's non-inclusion criteria. Therefore, 17 patients were included in the current study. The median patient age was 72 years (IQR, 67.5–78.0), and a majority of the patients were male (n=13, 76.5%). Characteristics of patients are detailed in [table 1](#). Over a median follow-up of 16 months (2.2–28.4), two patients died, yielding a 12-month overall survival rate of 83.3%. The tumor control rate was 64.7% (tumor stabilization (n=6), partial response (n=2) or complete response (n=3)). The remaining six patients (35.3%)

Table 1 Demographic, clinical characteristics and cytokines levels with in vitro stimulation, at baseline

Characteristic	All (n=17)	Responders (n=11)	Non-responders (n=6)	P value
Median age (range) - years	72 (53–88)	73 (58–88)	72 (53–81)	0.84
Sex (n)				
Male	13	10	3	0.52
Female	4	1	3	
ECOG-PS status (n (%))				
0	10 (59)	8	2	0.28
1	5 (29)	2	3	
2	2 (12)	1	1	
Metastasis stage (n (%))				
M0, M1a, or M1b	13 (76)	8	5	>0.99
M1c	4 (24)	3	1	
LDH (n)				
≤ULN	15	11	4	0.11
>ULN	2	0	2	
BRAF status (n (%))				
Mutation	5 (29)	2	3	
No mutation	12 (71)	9	3	0.28
Immune-related adverse events (n)				
Yes	9	8	1	
No	8	3	6	0.04
Last observation carried forward (days)	540 (285; 735)	600 (180; 750)	360 (292; 810)	0.94
IL-1β/Monocytes (μg)	6740 (2434; 12572)	6740 (1689; 20266)	5724 (2744; 8514)	0.88
TNF-α/Monocytes (μg)	709 (261; 3373)	1320 (537; 3899)	2519 (371; 2519)	0.29
IL-6/Monocytes (μg)	11857 (6321; 38622)	24280 (7170; 39309)	8529 (5827; 23997)	0.43
IL-8/Monocytes (μg)	4643 (3012; 10859)	3916 (3076; 12219)	5370 (2819; 13909)	0.66
IFN-γ/Lymphocytes (μg)	2639 (843; 11479)	3039 (941; 10191)	1656 (651; 11864)	0.86
IL-12p70/Lymphocytes (μg)	39 (15; 97)	37 (16; 92)	42 (14; 125)	0.77
IL-2/Lymphocytes (μg)	60 (28; 129)	70 (42; 137)	34 (22; 98)	0.25
IL-17/Lymphocytes (μg)	0.57 (0.54; 0.71)	0.63 (0.56; 0.71)	0.57 (0.40; 0.57)	0.02*
IL-4/Lymphocytes (μg)	21 (12; 51)	25 (15; 63)	17 (11; 109)	>0.99
IL-5/Lymphocytes (μg)	26 (19; 57)	31 (22; 57)	21 (18; 72)	0.79
IL-13/Lymphocytes (μg)	18 (4; 78)	20 (11; 76)	8 (3; 93)	0.67

Each result of baseline cytokines levels was divided by the baseline monocytes or lymphocytes counts.

*Tend to be still statistically significant with Bonferroni adjustment ($p < 0.02$).

.ECOG-PS, Eastern Cooperative Oncology Group - performance status; IFN, interferon; IL, interleukin; LDH, lactate dehydrogenase; ; TNF, tumor necrosis factor; ULN, Upper limit normal.

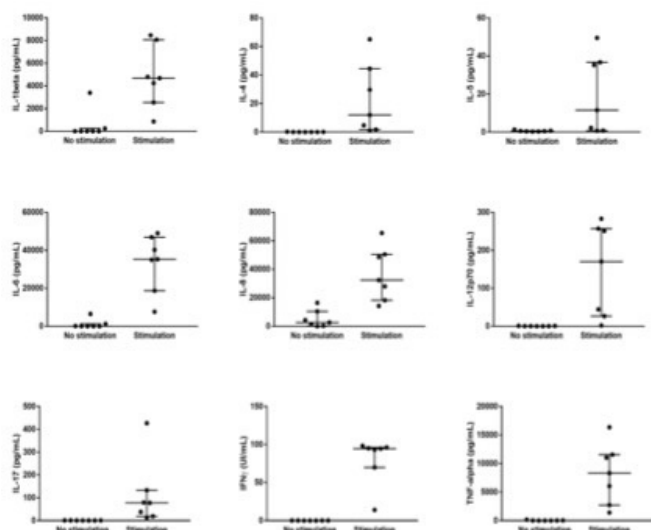


Figure 1 Effect of in vitro stimulation on the baseline levels of IL-1 β , IL-4, IL-5, IL-6, IL-8, IL-12p70, IL-17, IFN- γ and TNF- α in the seven first patients included. before stimulation, cytokines are mostly undetectable. IFN- γ , interferon- γ ; IL-8, interleukin 8; TNF- α , tumor necrosis factor- α .

presented immediate tumor progression. The 12-month PFS rate was 67.7%. The incidence of any grade of irAEs and grade 3–4 irAEs was 52.9% (n=9 patients) and 11.7% (n=2 patients), respectively.

To validate our conditions, we first test on the seven first inclusions, the cytokine level with and without in vitro stimulation. Without in vitro stimulation of immune cells, the levels of cytokines were mostly undetectable at baseline and did not differ between patients whereas differences could be observed after in vitro stimulation (figure 1).

We then compared inflammatory (IL-1 and IL-6) and Th1 (TNF- α and IFN- γ) baseline cytokine levels after in vitro stimulation from the 17 melanoma patients and 9 age and sex-matched healthy donors. Melanoma patients had significantly lower TNF- α , IFN- γ and IL-6 levels after in vitro stimulation compared with healthy donors (respectively $p < 0.0001$, $p = 0.002$, $p = 0.002$, figure 2).

Association between cytokines profiles after stimulation and PFS

We first explored the Th1 pathway: IFN- γ was significantly higher in responders compared with non-responders on week 6 ($p = 0.03$, figure 3A), while IL-2 was not statistically different ($p = 0.13$) between responders and non-responders (figure 3B). Moreover, PFS was significantly improved in patients with higher levels of IFN- γ at week 6 ($p = 0.04$, figure 3C).

We then explored Th17 pathway: IL-17A at baseline was significantly higher in responders compared with non-responders ($p = 0.03$), as well as on week 2 ($p = 0.005$) and week 6 ($p = 0.02$, figure 3D), while IL-6 was not significantly different in responders compared with non-at each time point (figure 3E). PFS was significantly improved in patients with higher levels of IL-17A at week 2 ($p = 0.04$,

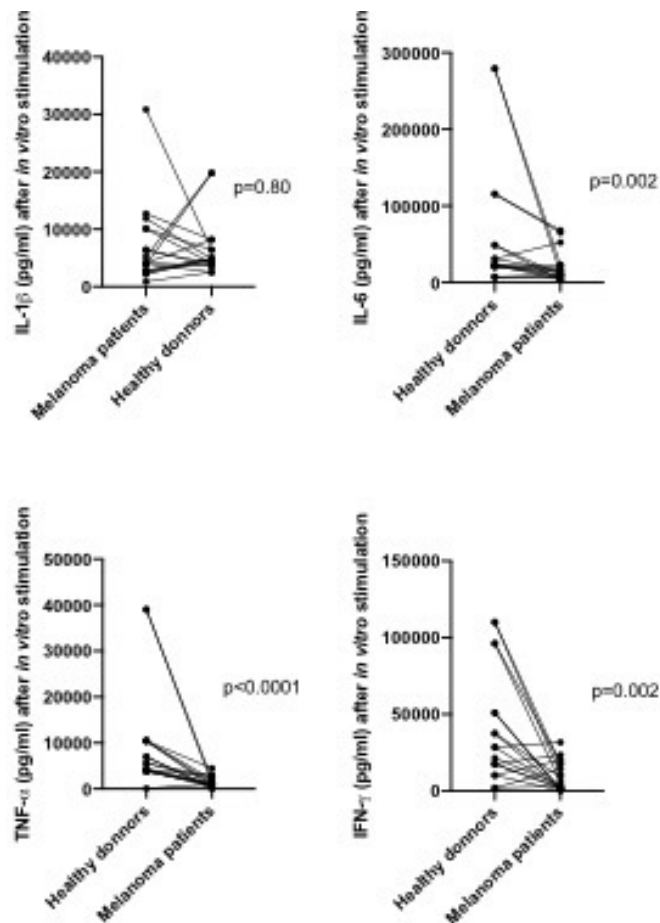


Figure 2 Comparison of baseline cytokines levels after stimulation in the 17 melanoma patients and in 16 age-matched healthy donors, showing that melanoma patients have significantly lower Th1 cytokines levels (TNF- α and IFN- γ). IFN- γ , interferon- γ ; IL-16, interleukin 16; TNF- α , tumor necrosis factor- α .

figure 3F). After adjustment on the baseline lymphocyte count, IL-17A was still significantly higher in responders compared with non-responders ($p = 0.02$).

Cytokines from Th2 pathway also exhibited an increase during follow-up: IL-4 and IL-5 were slightly but significantly higher in responders compared with non-responders on week 2 ($p = 0.049$ and $p = 0.048$ respectively, figure 3G,H) and PFS was significantly improved in patients with higher levels of IL-4 at week 2 ($p = 0.01$, figure 3I).

Clinical characteristics at baseline did not correlate with tumor response or PFS (table 1).

To adjust for multiple analysis problems, independence of variables was tested with Spearman's method. All tested cytokines were correlated with IL-17A levels, whereas age and lactate dehydrogenase were independent (online supplemental file 1). Bonferroni's correction was then applied to the statistical significance level, which yielded a corrected p at $p < 0.02$ (table 1).

Baseline cytokine levels of IFN- γ , IL-17A and IL-4 did not significantly differ when complete and partial responders

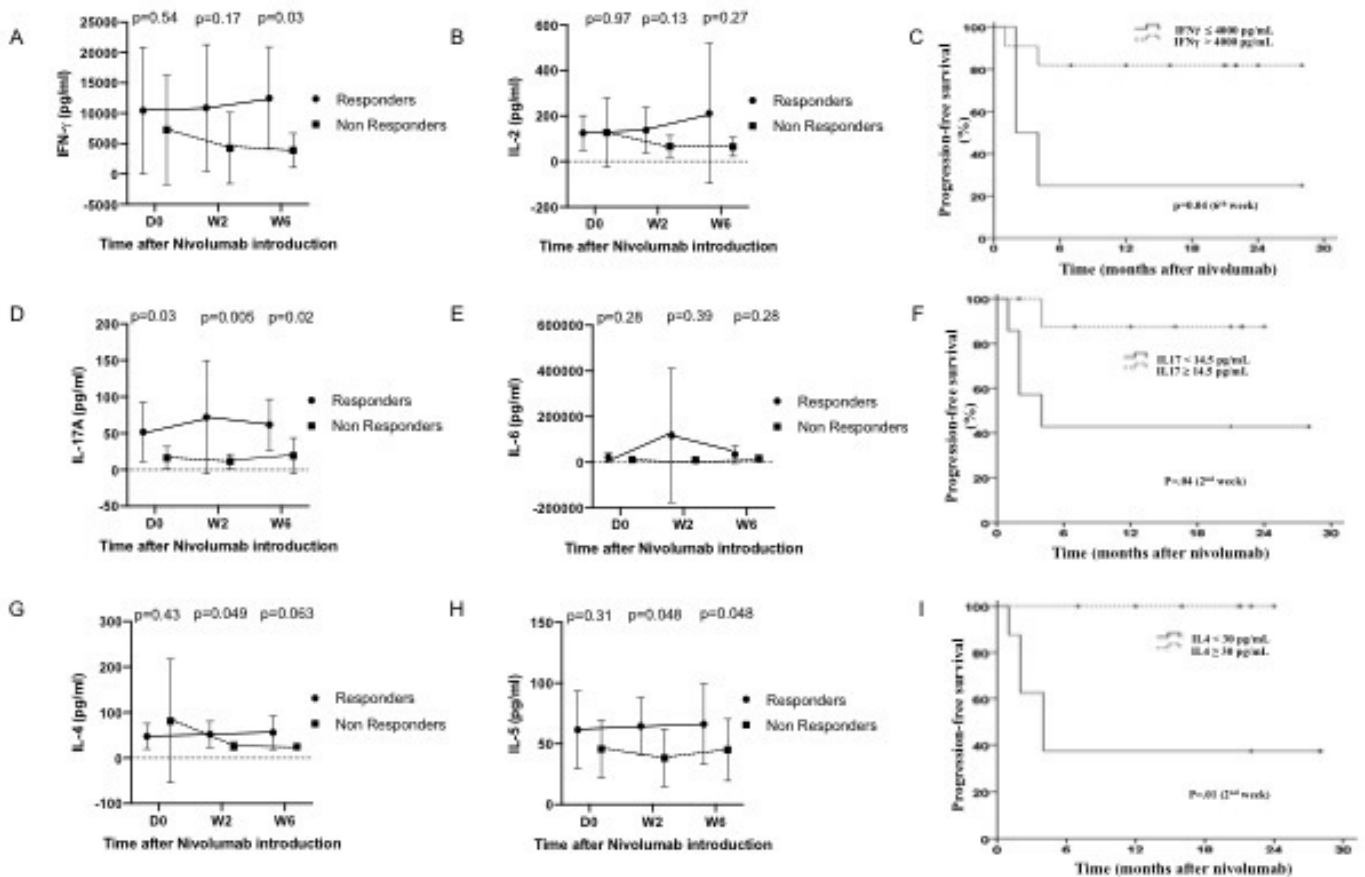


Figure 3 Cytokines levels of different immune pathways (Th1, Th2, Th17) after stimulation in responders and non-responders at baseline and during treatment with nivolumab (at week 2 and week 6). (A) IFN- γ levels in responders and non-responders at baseline and during treatment with nivolumab. IFN- γ levels become significantly higher in responders at week 6. (B) IL-2 levels in responders and non-responders at baseline and during treatment with nivolumab. Levels do not significantly differ between responders and non-responders. (C) Kaplan-Meier curves showing progression-free survival in patients with high and low IFN- γ levels at week 6. High IFN- γ levels at week 6 are associated with better progression free survival. (D) IL-17A levels in responders and non-responders at baseline and during treatment with nivolumab. IL-17A levels are significantly higher in responders at baseline as well as week 2 and week 6. (E) IL-6 levels in responders and non-responders at baseline and during treatment with nivolumab. Levels do not significantly differ between responders and non-responders. (F) Kaplan-Meier curves showing progression free survival in patients with high and low IL-17A levels at week 2. High IL-17A levels at week 2 are associated with better progression free survival. (G) IL-4 levels in responders and non-responders at baseline and during treatment with nivolumab. IL-4 levels become significantly higher in responders at week 2. (H) IL-5 levels in responders and non-responders at baseline and during treatment with nivolumab. IL-5 levels become significantly higher in responders at week 2. (I) Kaplan-Meier curves showing progression free survival in patients with high and low IL-4 levels at week 2. High IL-4 levels at week 2 are associated with better progression-free survival. IFN- γ , interferon- γ ; IL-6, interleukin 6; TNF- α , tumor necrosis factor- α .

were compared with stable patients and non-responders (online supplemental file 2).

Association between cytokine profile after stimulation and irAEs

IrAEs were significantly more frequent in responders than non-responders (81.8% vs 16.7% respectively, $p=0.04$), and were also identified as a factor associated with improved prognosis for 1-year PFS (88.9% vs 38.1%, $p=0.02$). Patients that presented irAEs had significantly higher IL-6 levels before treatment with nivolumab on Day 0 than patients without irAEs ($p=0.03$), and the levels remained higher during the course of nivolumab treatment on week 2 ($p=0.003$) and week 6 ($p=0.01$, figure 4). The same observation was made with IL-17A levels

($p=0.009$ on day-0; $p=0.04$ on week 2; $p=0.003$ on week 6, figure 4), whereas IL-6 levels were comparable between responders and non-responders at each point (data not shown). IFN- γ levels in patients with irAEs were significantly higher on week 2 ($p=0.018$) and week 6 ($p=0.01$) but not on day 0 ($p=0.25$, figure 4).

DISCUSSION

Our exploratory results suggest that a functional assay measuring cytokines after non-specific stimulation of immune cells appears as a promising tool to identify patients that could benefit most of a treatment based on checkpoint inhibition and those at highest risk of irAEs.

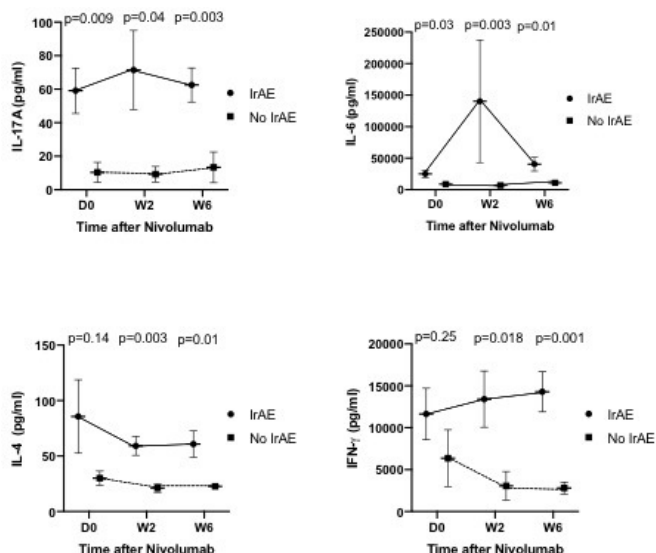


Figure 4 Cytokines (IL-17A, IL-6, IL-4, IFN- γ) levels after stimulation in patients with and without immune-related adverse events (irAE) at baseline and during treatment with nivolumab (at week 2 and week 6). IL-17A and IL-6 levels are consistently higher in patients with immune-related adverse events at each point, even at baseline. IL-4 and IFN- γ levels become both significantly higher in patients with immune related adverse events at week 2 and week 6. IFN- γ , interferon- γ ; IL-6, interleukin 6; TNF- α , tumor necrosis factor- α .

We have demonstrated in this cohort that patients with advanced melanoma at first line therapy are immunocompromised, with lower baseline Th1 cytokines levels (TNF- α and IFN- γ) compared with healthy donors. Furthermore, under treatment with ICI, responders exhibited a much stronger cytokine response than non-responders, reflecting successful activation of all inflammatory immune pathways (Th1, Th2, Th17). These findings confirm that response to ICI is in part determined by the preexisting ability of a patient's immune system to react when stimulated.²³ These results could only be observed after in vitro stimulation of immune cells.

The occurrence of irAEs was significantly higher in responders, and correlated with PFS, confirming the close pathophysiological link between tumor response and irAEs.¹⁴ Patients with irAEs had higher levels of IL-6, IL-17 and IFN- γ during treatment than the patients who did not present any irAEs. In fact, depending on the type of irAEs, Th1,²⁴ Th2²⁵ as well as Th17^{26 27} pathways may be implicated in predisposed patients. Knowing more precisely which immune pathway is specifically activated when an irAEs occurs could allow for a more targeted approach to its management, while limiting the impact on tumor response.

Our results highlight the importance of all immune pathways in the response against cancer and more specifically the pivotal role of the Th17 pathway that is the only one predicting at baseline both tumor response and irAE occurrence in our cohort. Moreover, this result stood out

even when the baseline IL-17A levels were related to the baseline lymphocyte counts. It has been demonstrated that IL-17A and/or Th17 cells may play a protective role in tumor immunity.^{11 28} In a case of skin and gastrointestinal irAEs induced by PD-1 inhibition with pembrolizumab, specific IL-17 blockade by secukinumab provided dramatic relief of the irAEs. Unfortunately, inhibition of the IL-17 pathway resulted in loss of ICI effectiveness and an upturn in tumor growth.²⁶ As demonstrated in our cohort, this observation suggests that Th17 pathway is implicated in both the effectiveness of ICI and is the trigger of irAEs.

Our study has several limitations. This is a monocentric cohort of predominantly male patients. All patients were treated for melanoma, which hinders any hasty extrapolation to other types of cancer pending further dedicated studies. Patients also received radiotherapy with barely known specific effects on immunity.²⁹ Multiple testing problems does not allow us to draw definite conclusions regarding the minimally significant variations of the cytokines belonging to the Th2 pathway and the study was not powered enough to find different baseline cytokine levels between responders, stable patients, and non-responders. Variation in cytokines levels after stimulation could partly reflect the concentration of lymphocytes and monocytes: this is why we correlated the cytokines levels with the lymphocyte or monocyte counts to mitigate this potential confounding factor. Furthermore, thresholds for cytokines levels were not defined a priori for PFS assessment. Our results on different cytokines levels are not independent and the size of the cohort is relatively limited precluding any multivariate analysis, which warrants further confirmation of our exploratory results in larger cohorts. This is likely why the classical prognosis factors of melanoma could not predict tumor response and PFS. Nevertheless, it is all the more striking that baseline IL-17A level reached significance in spite of a small number of subjects enrolled. This result must be confirmed on a validation cohort.

A functional immune assay underscores the implication of Th1, Th2 and Th17 pathways in the response to checkpoint inhibition and in the pathophysiology of irAEs. There was no significant variation of the cytokines levels before stimulation which shows the importance of the in vitro stimulation. The advantages of such assay are its simplicity and that it can be easily extended to other types of cancers as a ready to use tool using non-specific tumorous antigens for lymphocytic activation but detecting anergic lymphocytes thus identifying immunocompromised patients.

For the first time, we demonstrate a correlation between PFS, irAEs, and the Th17 immune pathway implicated in autoimmunity. These findings pave the way to an individualized tailored approach for the treatment of advanced malignancies: (1) combination of an anti-PD-1 with another ICI in case of poor immune reactivity/function; (2) restoration of immune function in immunocompromised patients before ICI and (3) identification

of cytokine levels associated with tumorous response with limited irAEs. Further studies should confirm in larger cohorts the predictive values of IL-17A and other cytokines in the response to checkpoint inhibition and the occurrence of irAE.

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Contributors BS-P, JD and HM designed the study. AG, MC and VB carried out experiments. BS-P, AG, JD, HM and VB analysed and interpreted the data. BS-P, JD, KZ and LB performed statistical analysis. BS-P, AG, JD, HM, MC, CR-C, LT, AP-G and TP provided medical oversight. AG, BS-P, JD and HM drafted and revised the manuscript. All authors approved the final version of the manuscript.

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