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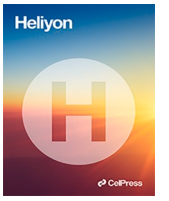

Research article

Immune mechanisms and predictive biomarkers related to neoadjuvant immunotherapy response in stage III melanoma

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ABSTRACT

The treatment for stage III melanoma has advanced significantly, nevertheless, a substantial proportion of patients experience relapse. Neoadjuvant immune checkpoint blockade has emerged as a promising approach, allowing early micrometastatic disease treatment, reduction of tumor burden before surgery, and enhanced tumor-specific T-cell responses. However, not all patients respond to treatment, highlighting the need for understanding immune mechanisms behind failure and identification of predictive markers. Here we performed a robust evaluation of systemic and tumoral immune profiles in a well-defined cohort of advanced melanoma patients treated with immune checkpoint inhibitors. Elevated CTACK and CXCL9 chemokines pre-treatment suggested their potential as predictive tools for treatment response. Furthermore, CD95 expression in $CD8^+$ T lymphocytes surfaced as a favorable prognostic indicator, while PD-1, CD161, and PD-L2 exhibited correlations with worst outcomes. These findings shed light on the intricate interplay between immune markers and melanoma response to neoadjuvant immune checkpoint therapy, offering insights into personalized treatment strategies.

1. Introduction

The survival outcomes for patients with clinical stage III melanoma have clearly improved with the current standard of care consisting of surgery followed by one year of adjuvant anti-PD-1 or BRAF/MEK inhibitors therapies. Despite these benefits, the relapse rates in the phase III adjuvant trials involving these drugs remain as high as 40 % at 2 years in this population [1–3].

As expected, neoadjuvant therapy with these new treatments has been studied as an alternative for this scenario. Beyond the well-known advantages associated with this strategy (such as early treatment of micrometastatic disease, reduction of tumor burden before

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surgery, and determination of the efficacy within the individual patient), neoadjuvant immune checkpoint blockade seems to be more effective in inducing a stronger and broader tumor-specific T cell response compared to the same regimen in the adjuvant setting, where the majority of tumor antigens were removed by upfront surgery [4,5].

A pooled analysis of the main neoadjuvant trials for patients with clinical stage III disease (almost all of them small non-randomized phase II trials) from the International Neoadjuvant Melanoma Consortium (INMC) has shown relapse-free survival of 75–80 % at 2 years with neoadjuvant immunotherapy, which is numerically higher than what is observed in the pivotal adjuvant trials [6]. Noteworthy, a significant association between pathologic response and important clinical outcomes, such as relapse-free and overall survival, was established. Patients treated with neoadjuvant immunotherapy who achieved a pathological complete response (pCR) had 96 % of relapse-free survival at 2 years compared to 64 % for patients who did not [7].

The possible use of pathological response as a surrogate endpoint for survival might contribute to the progress in this scenario in several manners. Patients with excellent response might be spared from the adjuvant part of therapy, which means less exposure to the risk of adverse effects and a reduction in financial costs. On the other hand, patients with poor response might have their treatment reprogrammed (eg. change treatment to adjuvant BRAF/MEK inhibitor if BRAF V600 muted). Another important gain is to accelerate the approval of registration studies for novel drugs by using pCR as a valid end-point, especially considering the large number of combinatorial strategies being studied and the lower incidence of melanoma compared to other tumors.

Despite all the benefits related to neoadjuvant immunotherapy, at least one-third of patients do not respond to this strategy, therefore, presenting the risk of disease progression that may result in unresectable disease or higher surgical morbidity. Moreover, the risk of immune-related adverse events can compromise or delay surgery and be long-lasting [8,9].

Thus, understanding immune mechanisms behind response outcome and identifying patients who will benefit from neoadjuvant therapy, even before it starts, is essential. Immunomodulatory biomarkers may have the potential to predict pathological response, provide insights into mechanisms of disease resistance and foresee the risk of immune toxicity.

2. Material and methods

2.1. Patients

All patients were treated under routine clinical care at AC Camargo Cancer Center, São Paulo, Brazil. The diagnosis, treatment and monitoring of patients was carried out regardless of the decision to include the patient in this study. All patients signed a written informed consent form for inclusion in this project. All procedures were in accordance with the Brazilian CNS #466 Resolution and have been previously approved by the local ethics committee, under protocol CAAE #80177417.0.0000.5432. Patients aged 18 years or over, of both sexes, histologically diagnosed with stage III melanoma and treated with neoadjuvant immunotherapy with both pembrolizumab monotherapy (anti-PD-1) for 3–4 cycles or nivolumab plus ipilimumab (anti-PD-1 plus anti-CTLA-4) for 2 cycles before surgery were included. The study excluded: patients with a previous diagnosis of another invasive cancer within 5 years of the inclusion in the current study, patients using antibiotic therapy, corticosteroids, immunosuppressive drugs or NSAIDs (non-steroidal anti-inflammatory drugs), and patients who received any previous treatment for the current neoplasm, including surgery, radiotherapy, immunotherapy, target therapy or some experimental therapy. Patients were classified as responder or non-responder based on pathology analysis. Complete response (no viable tumor cells) or major response (less than 10 % of viable tumor cells) were considered as responder. Any percentage of residual tumor cells between 10 and 100 % were considered as non-responder. This study also evaluates radiological response, as an exploratory analysis, using RECIST 1.1 criteria. The response evaluation occurred after 2 cycles of nivolumab and ipilimumab or 3–4 cycles of pembrolizumab monotherapy, right before the surgery, and aimed to compare with pathological response.

2.2. Tissue samples

Fresh tissue samples from metastatic lymph nodes were collected at the time of surgery, and the tissue was dissociated using BD MediMachine system in the presence of collagenase and nucleotidase, and frozen in freezing media composed of DMSO, fetal bovine serum (FBS) and culture media using standard procedures including controlled freezing at −80 °C, followed by storage in liquid nitrogen.

2.3. Blood samples

Peripheral blood was collected in EDTA tubes at the time of diagnosis, before starting the treatment. Whole blood aliquots were evaluated by flow cytometry. Blood samples were centrifuged within 2–4 h after collection and the plasma was frozen immediately at −80 °C. Remained blood was diluted 1:1 with PBS, PBMC (peripheral blood mononuclear cells) were purified using a Ficoll gradient and cryopreserved in FBS/DMSO.

2.4. Flow cytometry

PBMC or dissociated cells from metastatic lymph nodes were thawed, confirmed for viability using trypan blue exclusion, and incubated in culture medium overnight at 37 °C/5 % CO_2. After overnight incubation, cells were washed and resuspended in stain buffer for incubation with the appropriate collection of antibodies (Table S1). The cells were fixed and permeabilized to investigate

intracellular markers using the BD Pharmingen Transcription Factor Buffer Set (BD Biosciences). All panels were developed with BD Biosciences, tested, and confirmed before use in this study to generate compensation matrixes and other instrument settings which were maintained for every sample. At least 200,000 gated cells were acquired using the BD FACSymphony A5 flow cytometer. Analyses were performed using FlowJo software version 10.8.

2.5. Soluble factors multiplex assay

We measured 48 substances in the patients' plasma to evaluate the soluble immune profile, including inflammatory and anti-inflammatory mediators, and growth factors. We used the Bio-Plex Pro Human Cytokine Screening Panel kit on the Bio-Plex platform (Bio-Rad) according to manufacturer's instructions. The following proteins were measured: basic FGF, CTACK (CCL27), eotaxin (CCL11), G-CSF, GM-CSF, GRO-α (CXCL1), HGF, IFN-α2, IFN-γ, IL-1α, IL-1β, IL-1ra, IL-2, IL-2Ra, IL-3, IL-4, IL-5, IL-6, IL-7, IL-8, IL-9, IL-10, IL-12p40, IL- 12p70, IL-13, IL-15, IL-16, IL-17A, IL-18, IP-10 (CXCL10), LIF, MCP-1 (CCL2), MCP-3 (CCL7), M-CSF, MIF, MIG (CXCL9), MIP-1α (CCL3), MIP-1β (CCL4), β-NGF, PDGF-BB, RANTES (CCL5), SCF, SCGF-α, SDF-1α (CXCL11), TNF-α, TNF-β, TRAIL, VEGF-A.

2.6. Statistical analysis

Gaussian distribution was evaluated by Shapiro-Wilk test. For comparisons between groups, Mann-Whitney-Wilcoxon test or Student's *t*-test were used when appropriate. For analysis of soluble markers, we applied the false discovery rate correction. Heatmap and PCA analysis was performed using ClustVis Beta web tool [10] and multivariate ROC curve analysis was performed based on support vector machines (SVM) algorithm using MetaboAnalyst 5.0 web toll. To assess the accuracy of markers as predictors of response, receiver operating characteristic (ROC) curves were generated, followed by simple or multiple logistic regression. All analyses were performed using GraphPad Prism software version 8.4. p-values <0.05 were considered statistically significant.

3. Results and discussion

3.1. Patient characteristics

Eleven patients with clinical stage III melanoma treated with neoadjuvant immune checkpoint inhibitor were included in this analysis (Table 1). There were 8 males and 3 females, with a median age of 57.6 years (range, 36.2–78.2 years). The most frequent location was in axilla (n = 5), followed by supra and infraclavicular region (n = 3). The majority of patients had nodal disease (n = 10). Only one patient was included due to palpable in-transit metastasis. Among those 10 patients with nodal disease, eight presented only 1 enlarged lymph node detected by imaging (18F-FDG PET-CT). In the other two cases, one had 2 and the other had 3 enlarged lymph nodes. The median largest diameter of the target lesions was 25 mm (range, 12–40 mm). All patients had disease confirmation by fine-

Table 1
Patient characteristics.

Case	Neoadjuvant treatment	Response	BRAFs tatus	Site (nodal chain)	N lymph nodes	Pathologicalr esponse (viable tumor cells)	Pre treatment largest diameter	Pre surgical largest diameter
1	NIVO 3 + I PI1 2 cycles	Responder	WT	Infra clavicular	1	0 %	27 mm	20 mm
2	NIVO 3 + I PI1 2 cycles	Responder	NA	Axillary	1	0 %	40 mm	51 mm
3	NIVO 3 + I PI1 2 cycles	Responder	V600E	Axillary	1	0 %	25 mm	18 mm
4	NIVO 3 + I PI1 2 cycles	Responder	V600E	Axillary	3	5 %	20 mm 24 mm 27 mm	10 mm 10 mm 5 mm
5	NIVO 3 + I PI1 2 cycles	Responder	V600K	Supra clavicular	1	0 %	29 mm	21 mm
6	NIVO 3 + I PI1 2 cycles	Responder	Inconclusive	In transit metastasis *	3	0 % 5 % 10 %	10 mm 7 mm 5 m	10 mm 6 mm 5 m
7	NIVO 3 + I PI1 2 cycles	Responder	V600E	Axillaryi nguinal	2	0 % 0 %	27 mm 12 mm	24 mm 12 mm
8	NIVO 3 + I PI1 2 cycles	Non-responder	WT	Supra clavicular	1	40 % Sarcoidosis	36 mm	10 mm
9	NIVO 3 + I PI1 2 cycles	Non-responder	V600E	Axillary	1	60 %	20 mm	34 mm
10	Anti-PD1 x 4 Cycles	Non-responder	V600E	Intraparotid	1	100 %	20 mm	28 mm
11	Anti-PD1 x 3 Cycles	Non-responder	V600E	Inguinal	1	100 % 100 %	20 mm	22 mm 42 mm

needle aspiration before treatment. Regarding BRAF status, 7 patients were BRAF V600 mutated, 2 BRAF wild-type, 1 tested inconclusive and another one was not tested so far. Nine patients received neoadjuvant nivolumab 3 mg/kg plus ipilimumab 1 mg/kg every 3 weeks (2 cycles in 8 patients and 4 cycles in one patient) and 2 patients received neoadjuvant anti-PD-1 monotherapy with pembrolizumab 2 mg/kg every 3 weeks (2 cycles in one patient and 3 cycles in another one). The choices of the treatment regimens were made in tumor board rounds and reflected the opinion about the case at that time. The median duration of neoadjuvant treatment was 6 weeks (range, 6–12 weeks). All patients underwent complete lymphadenectomy (resection of in-transit metastasis with clear margins in one case) at the preplanned surgery time point. No serious surgery-related adverse events were detected.

3.2. Image and pathological responses assessment

Among the 10 patients with measurable disease, 2 achieved partial response, 4 stable disease, and 4 progressive disease using RECIST 1.1 criteria. Considering standard uptake values (SUV) measures of 18F-FDG analysis, five patients had an increase in the maximum SUV values compared to baseline, 5 had a reduction and one could not have its values measured. Regarding pathological assessment, which is considered the gold standard to determine responders and non-responders, seven patients achieved complete or major pathological response (64 %) and, therefore, were classified as responders. The 4 remaining cases (non-responders) had percentages ranging from 40 to 100 % of viable tumor cells. One of these non-responder patients presented a sarcoidosis-like reaction in association with the tumor. There was a poor correlation between RECIST evaluation and pathological response. Among 7 patients with complete or major response, an increase in the largest lymph-node diameter was observed in 2 cases. One out of 4 non-responder patients had a decrease in the lymph-node diameter.

3.3. Chemokines CTACK and CXCL9 predict response to therapy in melanoma patients

Neoadjuvant immunotherapy for melanoma patients is a recent therapeutic proposal and the mechanisms related to the control of the disease are still poorly understood. In addition, it is essential to predict which patients could benefit from this therapy. Thus, we collected plasma from patients before starting immunotherapy cycles (time 0) and measured 48 cytokines and chemokines by a multiplex assay. We observed that responder patients present higher concentrations of CTACK (cutaneous T cell-attracting chemokine) and CXCL9 (Fig. 1A and B), two chemokines important for an inflammatory response. CTACK/CCL27 is a chemokine produced by various cells, including keratinocytes in the skin. Its primary function is to attract specific immune cells, primarily T lymphocytes (T cells), to the skin. In the context of melanoma, CTACK can help recruit T cells to the site of the tumor in the skin and it was associated with longer progression-free survival for melanoma patients [11]. In a similar way, CXCL9, also known as monokine induced by gamma interferon (MIG), plays a significant role in the immune response, acting in the recruitment of cytotoxic cells to the tumor microenvironment [12,13]. Immunotherapies such as checkpoint inhibitors (e.g., anti-PD-1 or anti-CTLA-4 antibodies) work by reactivating T cells within the tumor. CXCL9 may influence the efficacy of these therapies by affecting the infiltration and activity of T

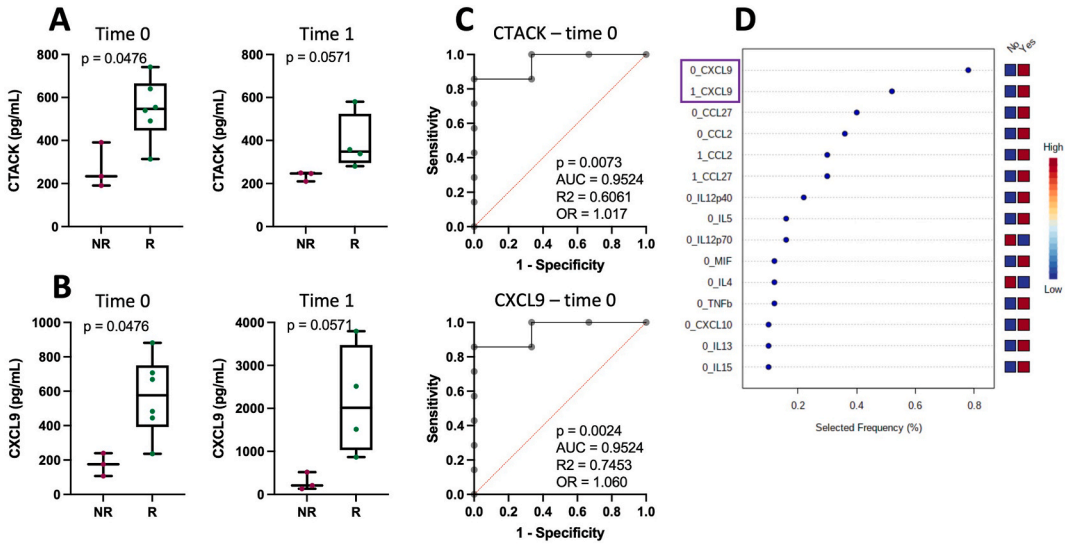

Fig. 1. Chemokines CTACK and CXCL9 predict response to neoadjuvant immunotherapy in melanoma patients. Plasma samples from patients with melanoma collected before (time 0) or three weeks after (time 1) treatment start were analyzed by BioPlex assay. After 3 months from the start of the treatment, pathological response was evaluated and patients were segregated into groups of non-responders (n = 3, pink symbols, NR) or responders (n = 6, green symbols, R). Plasma concentrations of (A) CTACK and (B) CXCL9. (C) The accuracy of CTACK (top panel) or CXCL9 (bottom panel) to predict response was assessed using univariate ROC curve analysis. p-value, AUC, Tijur's R squared and odds ratio are shown after simple logistic regression analysis. (D) Multivariate ROC curve analysis performed based on support vector machines (SVM) algorithm. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

cells within the tumor. Levels of both chemokines remained elevated in responder patients even after 3 weeks of starting the treatment (time 1). The power of these soluble factors to predict patient response was assessed by logistic regression. Fig. 1C shows that CTACK and CXCL9 were able to predict patients' response to immunotherapy, with high specificity and sensitivity, as shown by the ROC curves. The 48 soluble analytes were also submitted to multivariate ROC curve analysis and CXCL9, evaluated at both time 0 and time 1, proved to be the factor with the best predictive power (Fig. 1D). This was followed by CTACK/CCL27, evaluated at time 0, also with good predictive power. It has previously been shown that patients with metastatic melanoma responding to anti-PD-1 immunotherapy

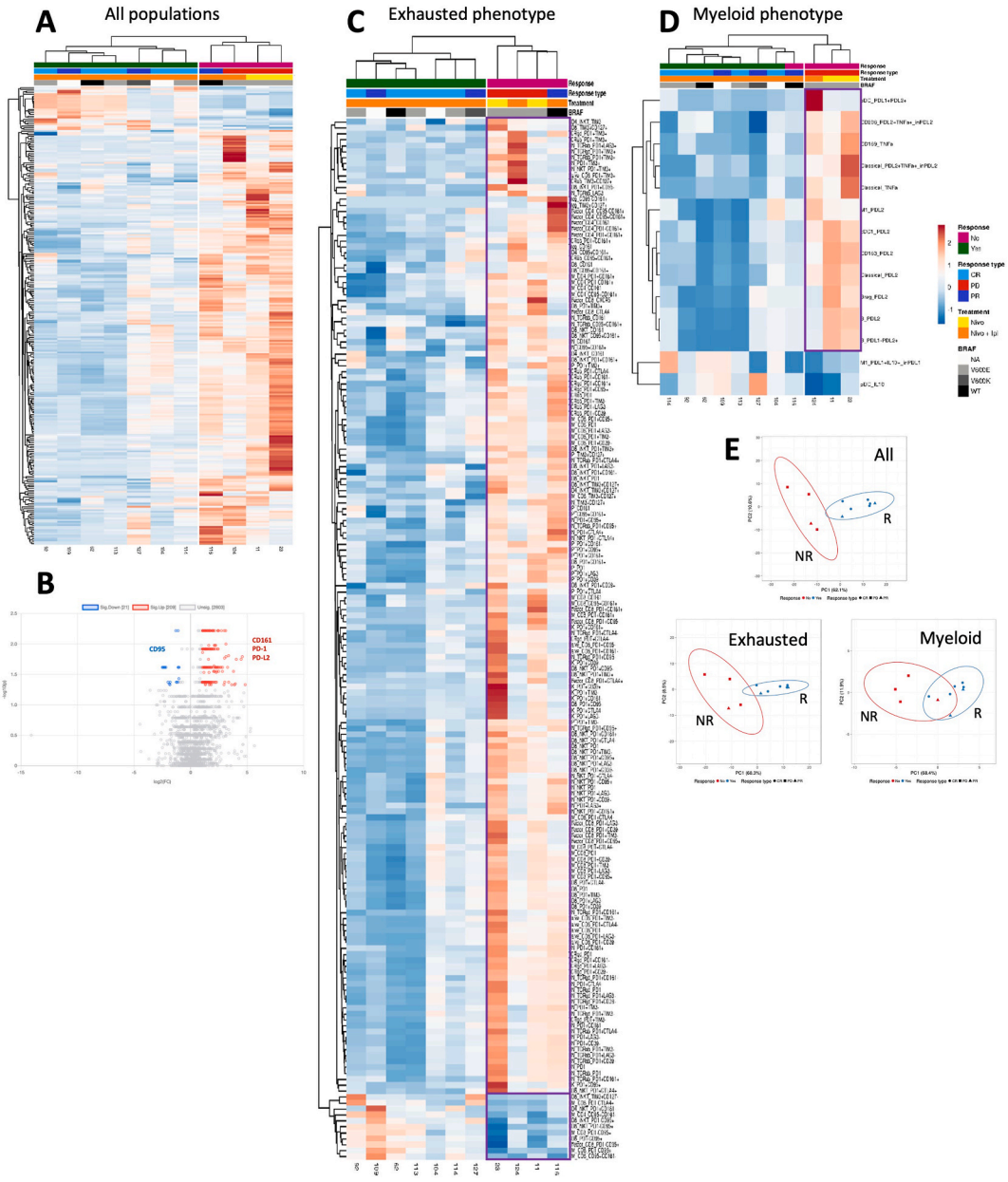

Fig. 2. High dimensional flow cytometry data allows segregation of responder and non-responder patients to neoadjuvant immunotherapy. PBMC from samples collected before starting the treatment were evaluated by flow cytometry. Patients were segregated into groups of non-responders (n = 4, pink symbols, NR) or responders (n = 7, green symbols, R). A total of 3033 leukocyte subpopulations was defined by supervised gating analysis. (A) Heatmap clustering of responder and non-responder patients using 230-top subpopulations with differential frequencies between these groups ($p < 0.05$). (B) Volcano plot to leukocyte subpopulations significantly downregulated (blue) or upregulated (red) in non-responder patients, including the markers more present in these cell populations. (C) Heatmap clustering of responder and non-responder patients using 175-top subpopulations with exhausted phenotypes differentially expressed , or (D) 14-top myeloid subpopulations differentially expressed in these groups ($p < 0.05$). (E) Principal component analysis (PCA) plots using the same parameters as that used in the heatmaps. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

have higher concentrations of CXCL9 during treatment, but not at baseline [14]. CTACK has not yet been tested as a possible biomarker for response, however, CXCL9 has been identified as a predictor in a cohort of patients with melanoma, mostly metastatic, undergoing first or second lines of treatment with anti-PD-1 [15]. Our study shows that higher levels of CXCL9 before first line neoadjuvant therapy begins in stage III melanoma patients is associated with response.

3.4. CD95 expression in $CD8^+$ T lymphocytes is a good prognostic marker for therapeutic response of melanoma patients

In the search for immune cell populations characteristic of responders or non-responders, we performed high dimensional flow cytometry using PBMC collected before the beginning of the treatment. Using a supervised analysis of flow cytometry data, we defined a total of 3033 leukocyte subpopulations, covering their activation/exhausted states, functional potential, and memory status.

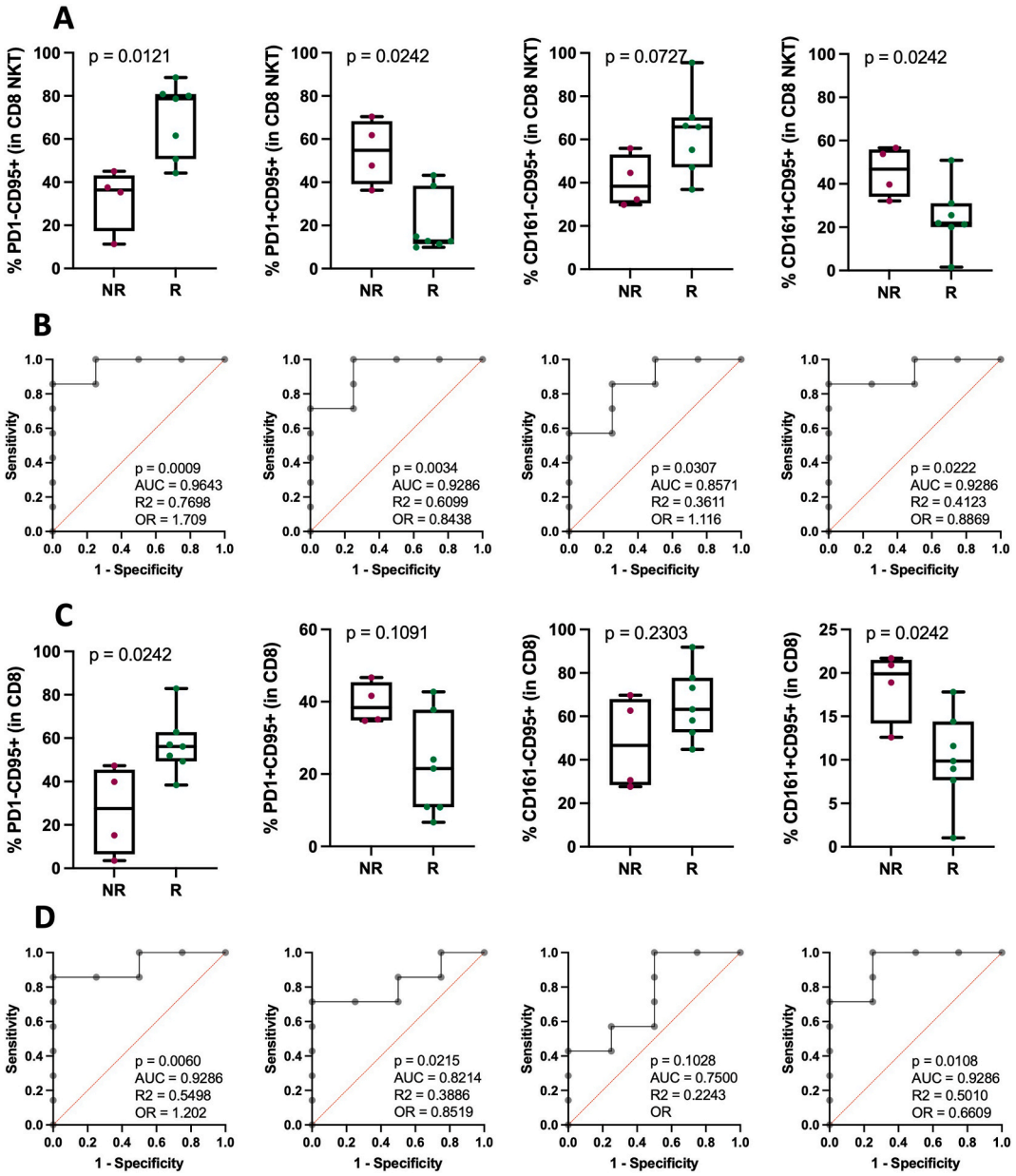

Fig. 3. CD95 expression in $CD8^+$ NKT or $CD8^+$ T lymphocytes are good prognostic markers for therapeutic response to neoadjuvant immunotherapy in melanoma patients. PBMC staining and segregation of patients was performed as described in Fig. 2. Frequencies of PD-1-$CD95^+$, PD-1+$CD95^+$, CD161-$CD95^+$ or CD161 + $CD95^+$ cells in subpopulations of (A) $CD8^+$ NKT or (B) $CD8^+$ T lymphocytes in PBMC from melanoma patients. (B and D) The accuracy of these subpopulations to predict response was assessed using univariate ROC curve analysis. p-value, AUC, Tijur's R squared and odds ratio are shown after simple logistic regression analysis.

Approximately 200 subpopulations with differential frequencies between responder and non-responder patients generated a heatmap graph with a perfect segregation of these two groups of patients (Fig. 2A). Likewise, we generate heatmaps by separating cell populations between T lymphocytes with an exhausted phenotype and B lymphocytes and myeloid cells, and the segregation of patients remained strong (Fig. 2C and D). Furthermore, a volcano plot showed populations downregulated or upregulated in non-responder

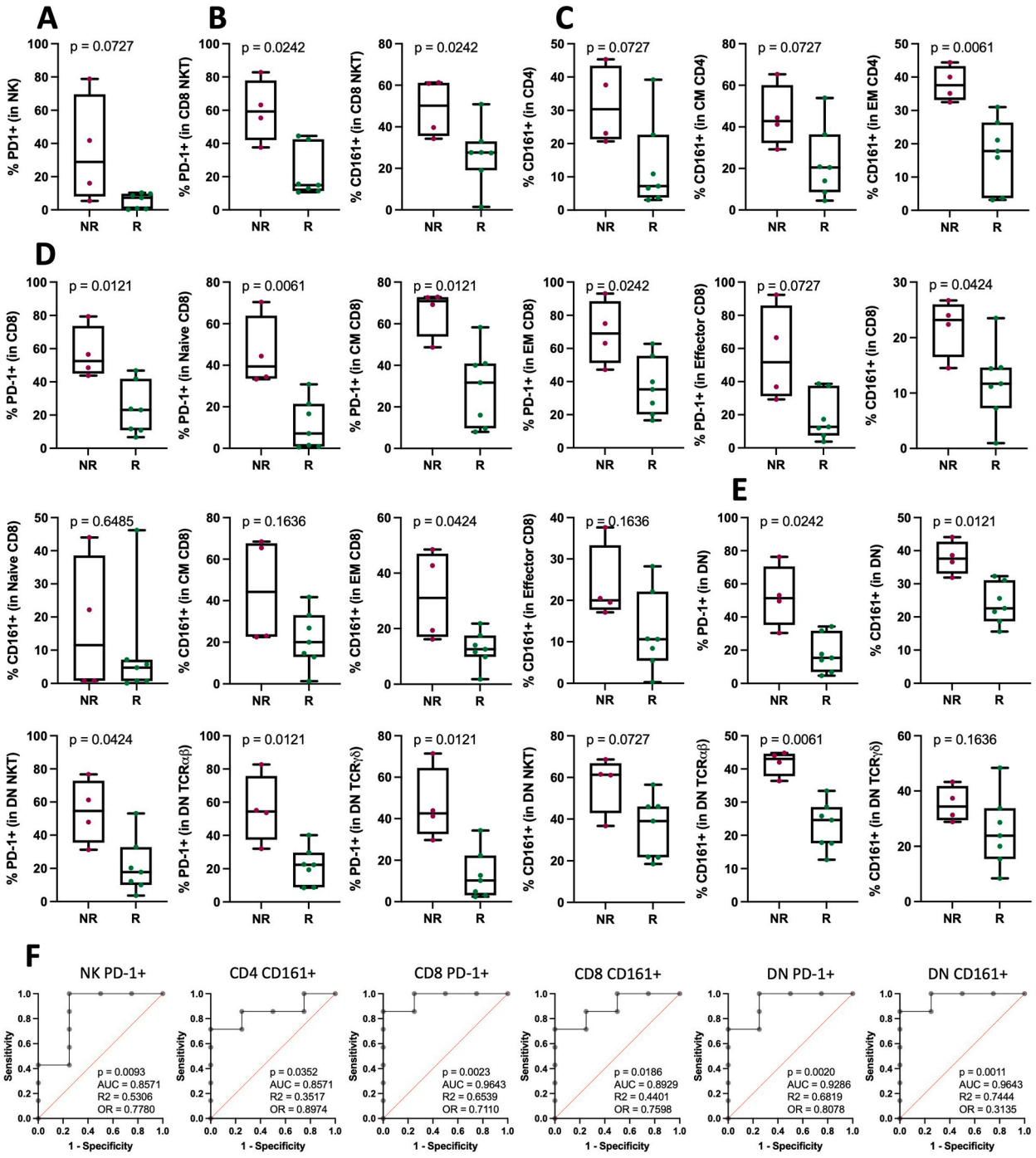

Fig. 4. PD-1 and CD161 are associated with worse prognosis for melanoma patients submitted to neoadjuvant immunotherapy. PBMC staining and segregation of patients was performed as described in Fig. 2. Frequencies of PD-1+ or CD161+ cells in subpopulations of (A) NK or (B) CD8+ NKT cells, (C) $CD4^+$, (D) $CD8^+$ or (E) double-negative T lymphocytes. (F) The accuracy of PD-1+ or CD161+ cells in subpopulations of NK cells or $CD4^+$, $CD8^+$ or double-negative T lymphocytes to predict response was assessed using univariate ROC curve analysis. p-value, AUC, Tijur's R squared and odds ratio are shown after simple logistic regression analysis. DN = double-negative, CM = central memory, EM = effector memory.

patients (Fig. 2B). CD95 (Fas) was the main marker present in populations downregulated in non-responder patients before therapy began. On the other hand, CD161, PD-1 and PD-L2 were markers found in many cell populations upregulated in non-responders. Finally, principal component analysis (PCA) plots using the same parameters as the heatmaps showed a clear segregation of responder and non-responder patients (Fig. 2E).

Focusing on a good prognostic marker according to our previous analysis, our results showed that responder patients had a higher frequency of $CD95^{+}$ cells in CD8+ NKT (Fig. 3A) or CD8+ T (Fig. 3C) lymphocyte populations. CD95 is a death receptor, but it can be also involved in T cell activation and proliferation [16,17]. Interestingly, when CD95 was co-expressed with PD-1 or CD161, the association was reversed, and these molecules proved to be characteristic of lymphocytes from non-responder patients. Moreover, CD95 expression on CD8+ NKT or CD8+ T lymphocytes was also a good predictor of response, as shown by the ROC curve, with an AUC between 0.7500 and 0.9643 (Fig. 3B and D).

3.5. PD-1 and CD161 are associated with a worse prognosis for melanoma patients submitted to neoadjuvant immunotherapy

PD-1 is a classic marker of cellular exhaustion, which if expressed in T lymphocytes can impair their activation and effector activities, including during tumor control [18,19]. CD161 is an inhibitory receptor of NK cells, but can also be expressed on lymphocytes, and its presence was described in some tumors, including melanoma. The role of CD161 in tumor progression is controversial, but a recent study suggests that the blockade of CD161 enhances T cell-mediated killing of glioma cells in vitro and in vivo, suggesting a potential role for targeting CD161 in cancer immunotherapy [20,21]. We also evaluated the expression of PD-1 or CD161, independent on the co-expression with other activation/exhaustion markers, on diverse cell populations and, in general, both proteins were more expressed in cells of patients refractory to treatment, as shown for NK cells (Fig. 4A), CD8+ NKT lymphocytes (Fig. 4B), CD4+ T

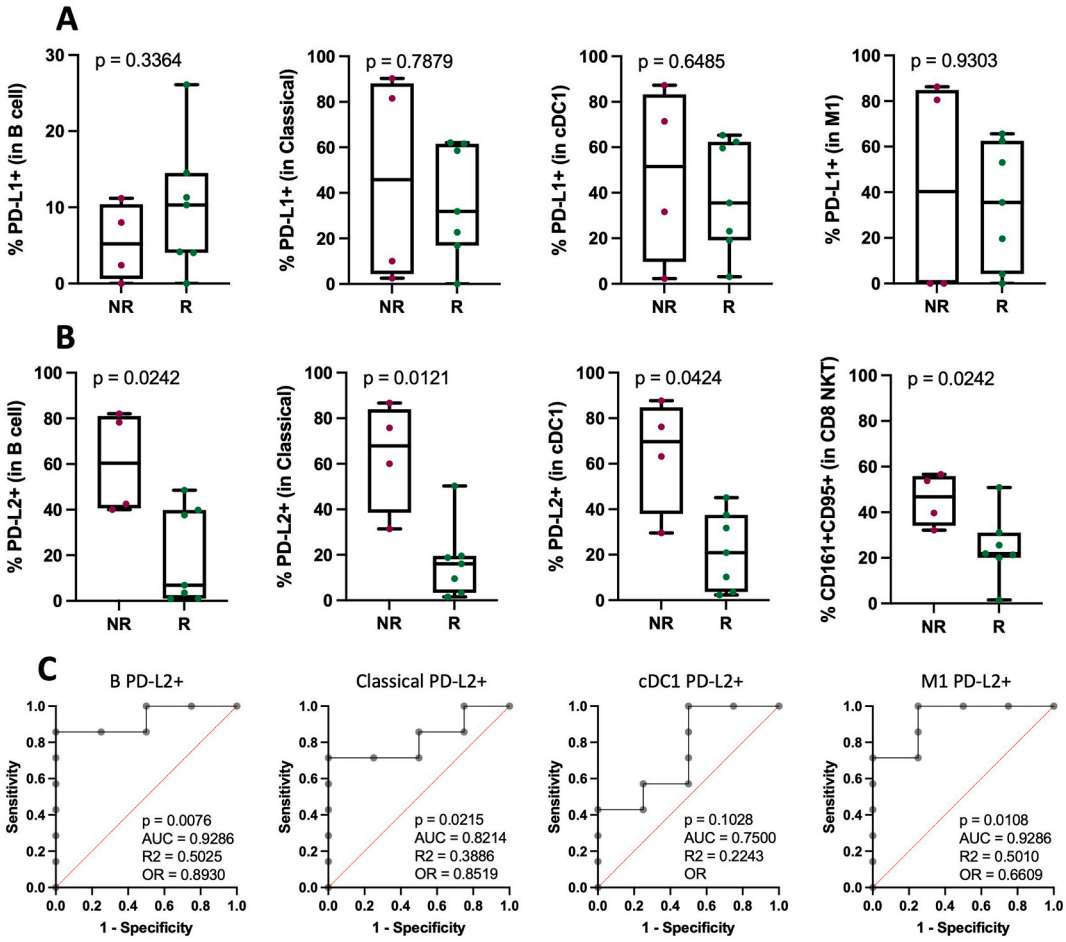

Fig. 5. PD-L2 expression in B lymphocytes or myeloid cells is a poor prognostic marker for therapeutic response to neoadjuvant immunotherapy in melanoma patients. PBMC staining and segregation of patients was performed as described in Fig. 2. Frequencies of (A) PD-L1+ or (B) PD-L2+ cells in populations of B lymphocytes, classical monocytes, type 1 conventional dendritic cells (cDC1) or M1 macrophages. (C) The accuracy of PD-L2+ cells in populations described before to predict response was assessed using univariate ROC curve analysis. p-value, AUC, Tijur's R squared and odds ratio are shown after simple logistic regression analysis.

lymphocytes, especially memory cells (Fig. 4C), CD8+ T lymphocytes, independent of their memory status (Fig. 4D), and double-negative T lymphocytes, independent of their TCR chains (Fig. 4E). It is important to note that the decreased PD-1 expression in responders was not due to competition between the antibody used for treatment and the antibody used for flow cytometry, as the clones taget different epitopes. We also assessed the predictive value for these specific immune cell populations. Our data show that the lower expression of PD-1 or CD161 on these cells was able to predict response of melanoma patients undergoing neoadjuvant immunotherapy (Fig. 4F).

3.6. PD-L2 expression in B lymphocytes or myeloid cells is a poor prognostic marker for therapeutic response of melanoma patients

Several groups are trying to discover a biomarker predictor of response to cancer therapy, and despite some contradictory results, PD-L1 is the best described so far [22–24]. We evaluated the expression of PD-L1 in B lymphocytes and specific populations of monocytes, macrophages and dendritic cells, especially associated with anti-tumor response, and found no difference in expression between responders and non-responders melanoma patients (Fig. 5A). In contrast, the expression of PD-L2, another ligand for PD-1, was greater in the same cells from non-responder patients (Fig. 5B), suggesting that it would indeed be associated with a worse outcome after a neoadjuvant therapy. PD-L2, like PD-L1, is a cell surface protein that plays a role in regulating the immune response. While both PD-L1 and PD-L2 interact with the same receptor on immune cells called PD-1, their roles in immune regulation and their expression in tumors can differ. In general, PD-L1 is more commonly associated with its inhibitory role in tumors. Tumor cells and certain immune cells within the tumor microenvironment can express PD-L1, and when PD-L1 on these cells binds to PD-1 on T cells, it can suppress the immune response [22,25]. This interaction can help tumors evade the immune system and promote immune tolerance, allowing the tumor to grow unchecked. On the other hand, the role of PD-L2 in tumors is less clear and not as extensively studied as PD-L1. PD-L2 is expressed on a subset of immune cells, such as dendritic cells and macrophages, as well as on some tumor cells. PD-L2's function in tumors and its impact on the immune response are still areas of active research. While PD-L2 can also interact with PD-1 on T cells, the precise effects of this interaction in the tumor microenvironment may vary depending on factors such as the type of cancer, the specific immune cells involved, and the overall immune context. Some studies have suggested that PD-L2 may have a weaker inhibitory effect on T cells compared to PD-L1, but it can still contribute to immune regulation [25,26]. Again, we generated ROC curves to assess the accuracy of PD-L2 as a predictor of therapeutic failure, and we found that its higher expression in B lymphocytes, classical monocytes or M1 macrophages predicted a worse outcome for melanoma patients (Fig. 5C).

3.7. Expression of inhibitory markers in PBMC from non-responder melanoma patients is associated with an exhausted profile in the metastatic tumor lymph node microenvironment

In order to investigate the association between the expression of inhibitory markers in circulating immune cells (PBMC) present in patients refractory to treatment to a loss of the local anti-tumor immune response, we evaluated the cellular immune profile in the metastatic lymph nodes of 6 patients (3 responders and 3 non-responders) submitted to two immunotherapy cycles, with tissue

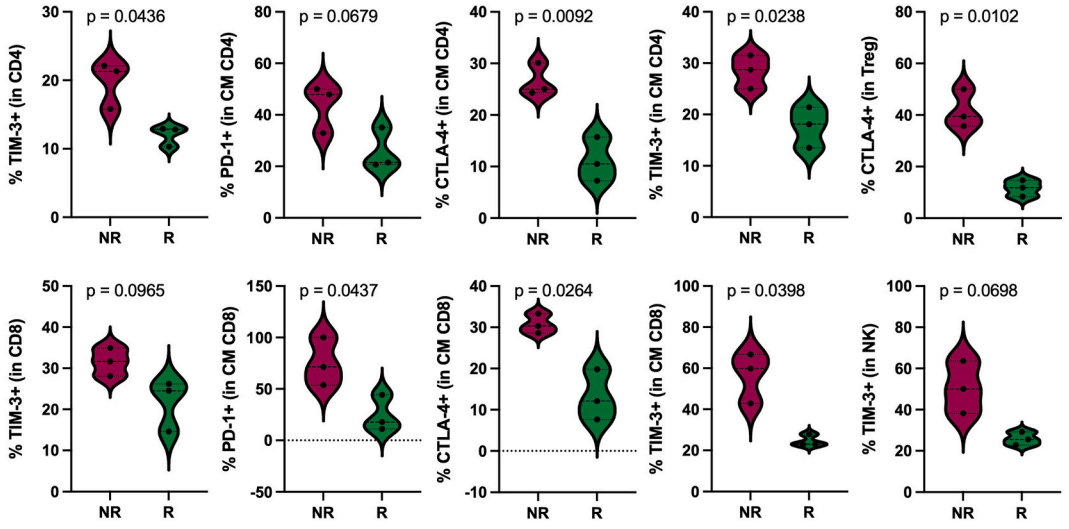

Fig. 6. Exhausted profile in the metastatic tumor lymph nodes is associated with expression of inhibitory markers in PBMC from non-responder melanoma patients. Melanoma patients were submitted to neoadjuvant checkpoint inhibitors-based immunotherapy and tumor samples (metastatic lymph nodes) were collected at the time of surgery, after 1–2 cycles of immunotherapy. The tissue samples from 3 non-responder (pink, NR) and 3 responder (green, R) patients were dissociated and suspension of cells were stained for flow cytometry analysis. Expression of TIM-3, PD-1 or CTLA-4 was evaluated in NK cells, $CD4^+$, $CD8^+$ or regulatory T lymphocytes. CM = central memory. (For interpretation of the references to colour in this figure legend, the reader is referred to the Web version of this article.)

samples collected at the time of surgery. Despite the small number of patients, our data showed a clear segregation between responders and non-responders with respect to the expression of PD-1, TIM-3 and CTLA-4, markers of inhibition/exhaustion profile, on the surface of NK cells or CD4+ or CD8+ T lymphocytes (Fig. 6), suggesting that the exhausted profile in metastatic tumor lymph nodes reflects that seen in PBMC from non-responder melanoma patients.

4. Conclusion

In conclusion, we suggest the pivotal role of CTACK and CXCL9 chemokines and critical immune cellular markers for predicting the response to neoadjuvant therapy in melanoma patients. These markers performed better than the traditional RECIST 1.1 radiological evaluation in terms of pathological response prediction and, when evaluated in peripheral blood collectively, serve as valuable indicators of the complex immune landscape within the tumor microenvironment. However, it is essential to acknowledge that while these findings are promising, further validation and standardization are needed to ensure their robust clinical applicability. Considering the limitation of our study in using a small sample size cohort, continued research efforts in larger and more diverse patient populations are warranted to refine the predictive potential of these biomarkers. Nevertheless, the accumulating evidence suggests that the assessment of these markers holds significant promise in guiding treatment decisions for melanoma patients undergoing neoadjuvant therapy and sheds light on immunotherapy response mechanisms.

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Declarations

The work has been approved by the local ethics committee at A.C.Camargo Cancer Center under protocol CAAE #80177417.0.0000.5432.

Data sharing

The data that support the findings of this study are available from the corresponding author, KJG, upon reasonable request.

CRediT authorship contribution statement

Amanda Braga Figueiredo: Writing – review & editing, Writing – original draft, Validation, Methodology, Investigation, Formal analysis, Data curation. **Milton José de Barros e Silva:** Writing – review & editing, Writing – original draft, Methodology, Investigation, Conceptualization. **Guilherme Ferreira de Britto Evangelista:** Writing – review & editing, Formal analysis. **Nayane Alves de Lima Galdino:** Writing – review & editing, Formal analysis. **Larissa de Melo Kuil:** Writing – review & editing, Resources, Data curation. **Iasmim Polido Santos:** Writing – review & editing, Methodology, Data curation. **Kátia Luciano Pereira Morais:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Clara Maciel Cavalcanti:** Writing – review & editing, Methodology, Formal analysis, Data curation. **Luciana Facure Moredo:** Writing – review & editing, Methodology, Data curation. **João Pedreira Duprat Neto:** Writing – review & editing, Methodology, Investigation. **Kenneth J. Gollob:** Writing – review & editing, Writing – original draft, Validation, Supervision, Resources, Project administration, Methodology, Investigation, Funding acquisition, Conceptualization.

Declaration of competing interest

The authors declare the following financial interests/personal relationships which may be considered as potential competing interests: Kenneth Gollob reports financial support, administrative support, article publishing charges, and equipment, drugs, or supplies were provided by GSK via the academic grant between FAPESP and GSK. Other authors, declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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Appendix A. Supplementary data

Supplementary data to this article can be found online at https://doi.org/10.1016/j.heliyon.2024.e32624.

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