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Suitability of tumor-associated antibodies as predictive biomarker for response to immune checkpoint inhibitors in patients with melanoma: a short report

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ABSTRACT

In 2019, Fässler *et al* showed in this journal that the presence of tumor-associated antibodies correlated with response to immune checkpoint inhibitor treatment in patients with metastatic melanoma. The results of this study suggested that tumor-associated antibodies directed against melanocyte-differentiation antigens and the cancer-germline antigen NY-ESO-1 should be further investigated as candidate biomarkers for response to immune checkpoint inhibitors. The aim of the current study was to validate and extend these previous findings. Therefore, we examined the correlation between serum levels of tumor-associated antibodies and tumor response after treatment with immune checkpoint inhibitors in patients with metastatic melanoma.

All patients included in this prospective study were diagnosed with advanced stage melanoma and treated with nivolumab or pembrolizumab monotherapy. Blood samples were collected before and during treatment. Serum levels of tumor-associated antibodies against the melanocyte differentiation antigen Melan-A and the cancer germline antigens NY-ESO-1, MAGE-C2, MAGE-A6 and ROPN1B were measured at baseline and during treatment. Differences between responders and non-responders were assessed using the Mann-Whitney U-test, and differences between different overall survival categories with the Kruskal-Wallis test. P values ≤0.05 were considered significant.

Serum samples of 58 patients with advanced melanoma with long-term follow-up (>3 years) were collected. In contrast to the findings of Fässler *et al*, for all antibodies tested, we found no significant differences between serum levels of responders and non-responders before or during treatment with immune checkpoint inhibitors. In addition, no significant differences were found in serum levels of tumor-associated antibodies for different overall survival groups.

Although our study included a larger and more mature cohort of patients with longer follow-up, we could not externally validate the findings of Fässler *et al*. In addition, we were not able to identify other cancer germline antigens as predictive biomarkers of response to immune checkpoint inhibitors in patients advanced melanoma. Based on the results of the present study, clinical applicability of tumor-associated antibodies directed against tumor antigens as predictive biomarkers for immune checkpoint inhibitors in patients with advanced melanoma is not feasible.

INTRODUCTION

Cutaneous melanoma is a malignant tumor of the skin derived from melanocytes that accounts for around 3% of all malignant skin cancers. The introduction of immune checkpoint inhibitors (ICIs) for the treatment of (metastatic) melanoma has significantly improved the survival outcomes for patients with melanoma.[1] However, only a subset of patients gain long-term clinical benefit, while many patients experience severe and even lifelong toxicity from treatment with ICIs.[2] For a substantial number of patients with advanced stage melanoma, there is still a need for new therapies. The introduction of new agents[3] for patients with melanoma will be accompanied by uncertainties regarding the most suitable treatment for individual patients. Predictive biomarkers of response to ICI treatment may guide the individualized treatment strategy for patients with advanced stage melanoma.

Melanoma is known for its immunogenic properties, as shown by the rate of spontaneous regression,[4 5] the durable tumor responses after treatment with ICIs[1] and the use of adoptive T cell therapies[6] and tumor-infiltrating lymphocytes[7] as treatment. In addition, immunogenicity is illustrated by the presence of tumor-associated antibodies,[8] which are directed against tumor antigens. For melanoma, tumor antigens can be

divided into melanocyte differentiation antigens (MDAs), for example, glycoprotein 100 (gp100), tyrosinase and Melan-A/MART-1, and cancer germline antigens (CGAs), such as members of the MAGE family and NY-ESO-1. These tumor antigens are known for their ability to induce spontaneous cellular and humoral immune responses in patients with melanoma, while expression of CGAs is normally silenced in adult tissue.[4 8 9] Therefore, (immune responses against) these antigens are often targets for the development of new immunotherapy-based treatments.[10–13]

Fässler *et al* showed in this journal that the presence of tumor-associated antibodies prior to treatment initiation correlated with response to ICI treatment in two cohorts of patients with metastatic melanoma, consisting of 20 and 21 patients, respectively.[14] Serum concentrations of antibodies directed against both CGAs and MDAs were compared between responders and non-responders before and during treatment with ICIs. Based on the results of this study, it was suggested that tumor-associated antibodies directed against different MDAs and the CGA NY-ESO-1 should be further explored as candidate (surrogate) biomarkers for response to ICIs.[14]

Here, we examined the correlation between the presence of tumor-associated antibodies and clinical outcome after ICI treatment in patients with metastatic melanoma. First, the aim was to validate the results of Fässler *et al*, in an independent cohort of patients with metastatic melanoma, to determine whether clinical implementation as a predictive biomarker could be feasible. To this end, our antigen selection partly overlapped with the selection of Fässler *et al*, allowing for the development of harmonized ELISA-based antibody detection and direct comparison of the results with the previous cohorts.[14] Moreover, an additional number of antigens were selected to expand research on the predictive value of tumor-associated antibodies as a surrogate marker for tumor response to ICI treatment.

MATERIALS AND METHODS

Patient and sample collection

All patients selected for this study were diagnosed with advance stage melanoma and treated with nivolumab or pembrolizumab monotherapy. Patients were prospectively included in the MULTOMAB trial (MEC2016-011) (International Clinical Trials Registry Platform (ICTRP), NTR7015) (see online supplemental appendix 2) after providing written informed consent. All patients were treated at the Erasmus University Medical Center in Rotterdam, the Netherlands. Blood samples were collected prospectively before and during ICI treatment. Patients who started anti-PD-1 treatment before May 2018 were included to ensure long-term (ie, at least 3 years) follow-up data were available. Only patients from whom blood was collected at different time points both before and during treatment were included. The on-treatment samples were withdrawn between 1 and 3 months after treatment initiation. To analyze whether differences in blood serum concentrations of tumor-associated antibodies were affected by treatment type, that is, pembrolizumab or nivolumab, treatment details were collected and potential differences between these two treatment types were examined. To determine response to ICI treatment, the best overall response was measured according to RECIST V.1.1.[15] Responders were defined as patients having complete response (CR) or partial response (PR) according to RECIST V.1.1.[15] Moreover, survival data was collected. Overall survival (OS) was defined as the time from initiation of ICI treatment to death (from any cause). To analyze differences in OS, data were categorically divided into the following groups: short-term survivors (OS <1 year), patients with an intermediate OS (1–3 years), and long-term survivors (OS >3 years).

Tumor antigen selection

To determine the tumor antigen selection and to develop the different ELISAs, the methods and results of the study by Fassler *et al*[14] were applied exactly for the current study. Most significant results in the previous study were found for the MDA Melan-A and a CGA NY-ESO-1.[14] To validate and reproduce these results, these antigens were both included in the current study and ELISAs were developed according to the same conditions and protocols of the previous study[14] (for detailed information regarding the ELISA development, see section 'Detection of antibodies against tumor antigens').

The selection of the remaining antigens for the current study was based on the potential relevance of antibodies directed against CGAs, as the most important results in the study by Fässler *et al* were shown for the CGA NY-ESO-1.[14] CGAs are known for their promotion of oncogenic processes and CGAs have often been associated with tumor evolution and clinical outcome.[9 10 16–18] This allows for the identification of potential predictive biomarkers and possible targets for new oncological treatments. In addition, the majority of CGAs are known to have high expression in metastatic melanoma.[19] Therefore, the following antigens were additionally selected for the current analysis: MAGE-C2, MAGE-A6 and ROPN1B.

Detection of antibodies against tumor antigens

For a direct comparison with the results of Fässler *et al*, the ELISA conditions were reproduced for both Melan-A and NY-ESO-1. Since the background signal of the antibodies directed against Melan-A was high, different dilutions were again tested in healthy control samples. Finally, our tested dilutions led to the same conditions as stated in the paper by Fässler *et al*, resulting in the application of harmonized ELISAs for both Melan-A and NY-ESO-1 as compared with Fässler *et al*.[14] Maxisorp 96-well clear polystyrene flat-bottom ELISA plates (ThermoFisher Scientific, Massachusetts, USA) were coated overnight at 4°C with recombinant tumor antigens Melan-A (Abcam, Cambridge, UK), NY-ESO-1 (Lifespan Biosciences, Seattle, Washington, USA), MAGE-A6 (Abnova,

Taipei, Taiwan), MAGE-C2 (Abnova, Taipei, Taiwan) and ROPN1B (Abnova, Taipei, Taiwan) diluted in 0.1M carbonate buffer (pH 9.5) (see online supplemental appendix 1).[14] The plates were washed six times with phosphate-buffered saline (PBS) (pH 7.4). Afterwards, non-specific binding was blocked with 5% non-fat dry milk (Santa Cruz Biotechnology, Dallas, TX) in PBS and incubated for 2 hours at room temperature, followed by six wash cycles with PBS. The patient sera were diluted in 5% non-fat dry milk/PBS (according to online supplemental appendix 1) and incubated for 2 hours at room temperature, followed by six wash cycles with PBS. The peroxidase-conjugated anti-human IgG (ELITECH group, Spankeren, The Netherlands) (1:2500) was incubated for 2 hours at room temperature, and followed by six wash cycles with PBS. The substrate solution consists of orthophenylenediamine (0.5 mg/mL; Sigma-Aldrich, St. Louis, Missouri, USA) in 0.1M citrate buffer (pH 5.6), containing 0.08% H_2O_2 (Sigma-Aldirch, St. Louis, MO) and the plates were incubated for 30 min in the dark at room temperature. Afterwards, the reaction was stopped using 1.25M H_2SO_4. The optical density (OD) was read at 492 nm with an automatic ELISA plate reader (BioTek, Winooski, Vermont, USA).

Statistical analysis

Significance between two groups was determined using Mann-Whitney U-test and differences between multiple groups by using the Kruskal-Wallis test. GraphPad Prism V.5.0 software (GraphPad Software, San Diego, California, USA) was used for all statistical analysis. P values of 0.05 or less were regarded as significant.

RESULTS

In total, serum samples of 58 patients with advanced melanoma were collected. Baseline characteristics of the patients are summarized in table 1. Overall, 33 (57%) patients were male, median age was 63.5 years (interquartile rang 52.5–72 years) and for most patients anti-PD-1 treatment was the first treatment line in metastatic setting. In total, 30 patients (52%) had response to treatment (PR or CR), 19 (33%) of patients had progressive disease and 8 (14%) had stable disease. Median OS was 576 days (27 days–not reached). At 3 years since treatment initiation, 34 (58%) patients were still alive, while 12 (21%) patients died within 1 year after treatment initiation.

Table 1 Clinical patient characteristics

Variable—n (%)	Total group (n=58)
Sex	
Male	33 (57)
Female	25 (43)
Age	
Median age in years (IQR)	63.5 (52.5–72)
BRAF status	
Mutated	28 (48)
Non-mutated	29 (50)
Unknown	1 (2)
Prior systemic treatment	
No	50 (86)
Yes	8 (14)
Type of anti-PD-1 treatment	
Nivolumab	34 (59)
Pembrolizumab	24 (51)
Presence of brain metastases at treatment initiation	
No	30 (52)
Yes	11 (19)
Unknown	17 (29)
LDH	
≤ ULN	31 (53)
>1 × ULN	22 (38)
>2 × ULN	3 (5)
Unknown	2 (3)
Best overall response to treatment	
Complete response	14 (24)
Partial response	16 (28)
Stable disease	8 (14)
Progressive disease	19 (33)
Non-evaluable	1 (2)
Overall survival (OS)	
OS <1 year	12 (21)
OS 1–3 years	12 (21)
OS >3 years	34 (58)

LDH, lactate dehydrogenase levels; ULN, upper limit of the normal range.

Tumor-associated antibodies in responders and non-responders at baseline

Serum levels for all antibodies directed against the different CGAs and Melan-A were measured at baseline, i.e. before initiation of ICI treatment (figure 1A–E). Although for most patients antibody levels could be detected above the set quantification limit, the concentrations were very variable. In addition, serum levels for the antibody directed against Melan-A (figure 1A) were higher compared with the levels of CGA-directed antibodies, that is, NY-ESO-1 MAGE-A6, MAGE-C2 and ROPN1B, respectively (figure 1B–E). For all antibodies tested, no significant differences were found between the serum levels of responders and non-responders before start of treatment. To determine whether differences in antibody concentrations could predict survival outcomes, differences in serum levels between the OS groups were also examined (figure 1A–E). No significant differences

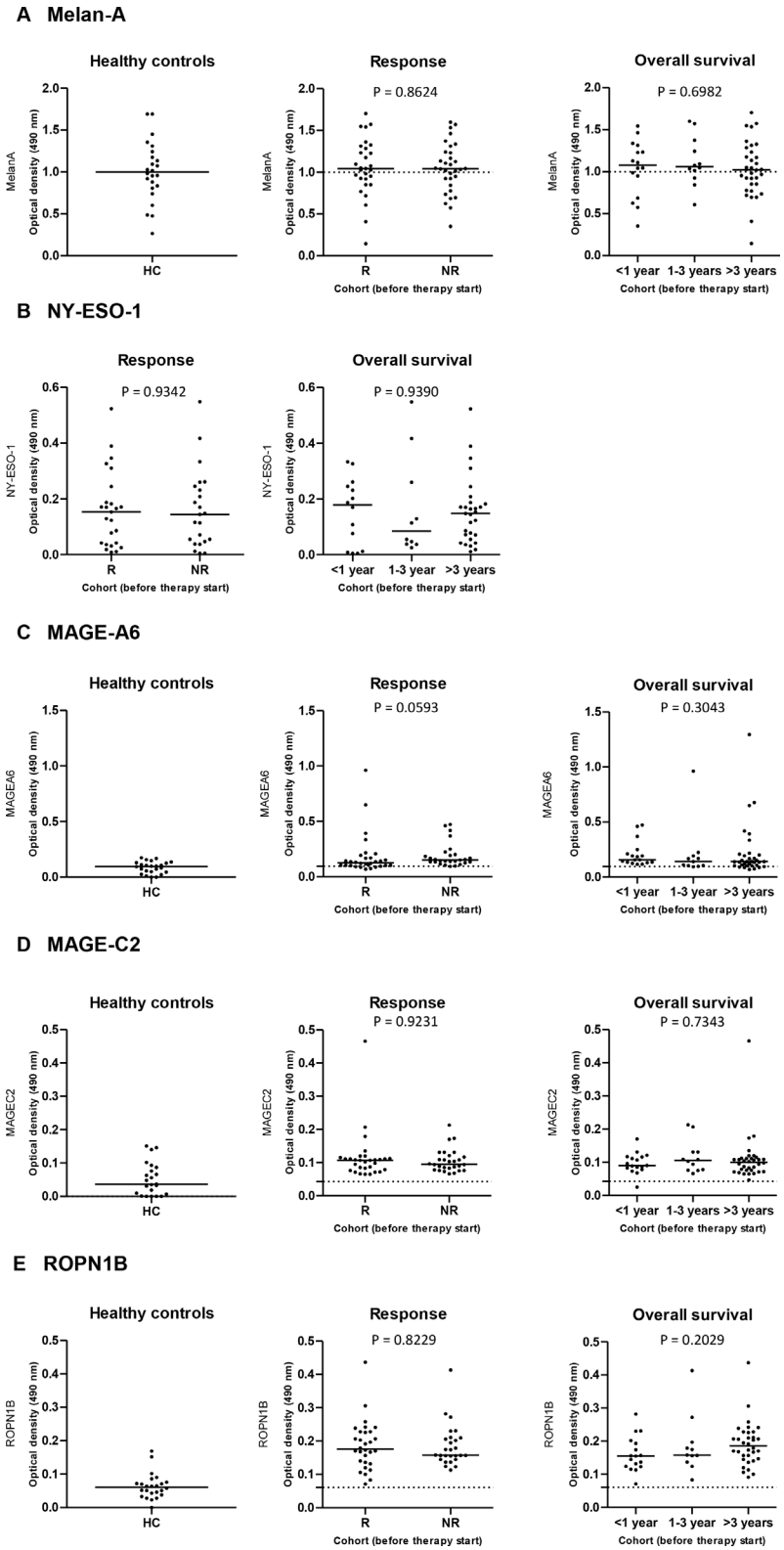

Figure 1 No difference in serum levels of tumor-associated antibodies at baseline. The figures show the optical density (OD) values of the different tumor-associated antibodies in serum of healthy controls, responders (R), non-responders (NR) and the different overall survival (OS) subgroups, that is, OS <1 year, OS 1–3 years and OS >3 years, respectively. The dotted lines represent the median serum levels of the healthy controls for the different antibodies. The following antibodies were measured: Melan-A (A), NY-ESO-1 (B), MAGE-A6 (C), MAGE-C2 (D) and ROPN1B (E). Differences between responders and non-responders were tested for significance using Mann-Whitney U test and differences between the overall survival groups by the Kruskal-Wallis test. P values are indicated in the different graphs. For all antibodies tested, no significant differences were found between the serum levels of different patient cohorts before treatment start. Figures were created with GraphPad Prism V.5.0 software.

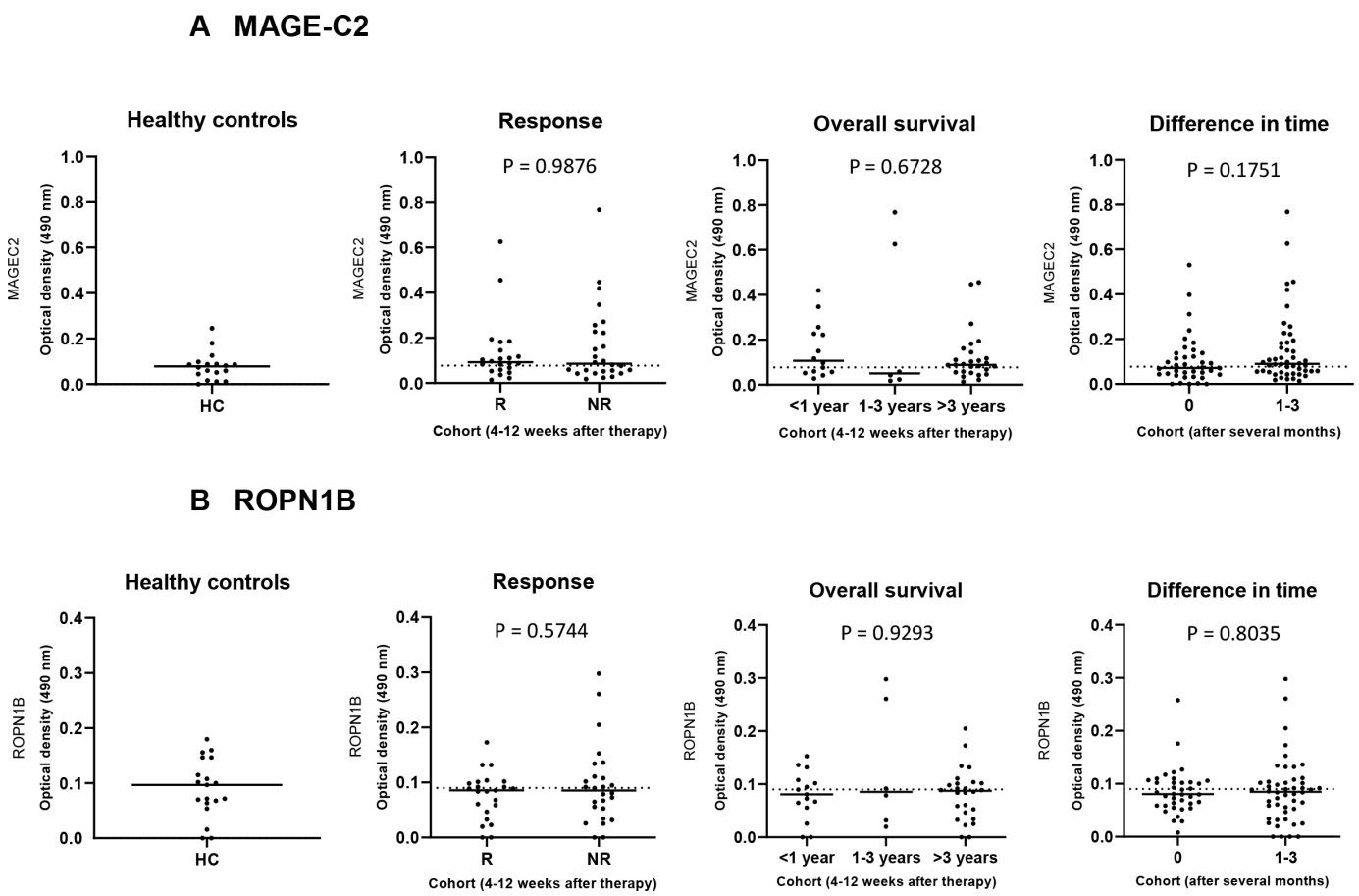

Figure 2 No differences in serum levels of tumor-associated antibodies during treatment with immune checkpoint inhibitors (ICIs). The figures show the optical density (OD) values of antibodies against MAGE-C2 (A) and ROPN1B (B) during treatment with immune checkpoint inhibitors in serum of healthy controls, responders (R), non-responders (NR) and the different overall survival (OS) subgroups, that is, OS <1 year, OS 1–3 years and OS >3 years, respectively. Blood samples for the on-treatment measurements were withdrawn 1–3 months after treatment initiation. The dotted line represents the median serum levels of the healthy controls for MAGE-C2 and ROPN1B, respectively. Differences between responders and non-responders and different time points were tested for significance using Mann-Whitney U test and differences between the OS groups by the Kruskal-Wallis test. P values are indicated in the different graphs. For both MAGE-C2 and ROPN1B, no significant differences were found between the serum levels of different patient cohorts during treatment. The figures were created with GraphPad Prism V.5.0 software.

were found for the different OS groups at baseline (figure 1A–E).

Tumor-associated antibody concentrations during treatment with ICIs

In the study by Fässler *et al*,[14] some antibody levels changed over time, resulting in differences between responders and non-responders. Therefore, in the current study, blood was also collected during treatment with ICIs to determine whether the antibody levels changed during treatment. Figure 2 shows the OD values of antibodies against MAGE-C2 (figure 2A) and ROPN1B (figure 2B) during ICI treatment. No significant differences were found between responders and non-responders, even when the different OS groups were compared. To determine whether the antibody levels changed over time, serum levels at baseline and during treatment were compared. However, no time differences could be detected for the antibodies directed against MAGE-C2 (figure 2A) or ROPN1B (figure 2B) between responders and non-responders nor in the different OS groups.

Last, although nivolumab and pembrolizumab are considered interchangeable in daily clinical practice, tumor-associated antibody levels could be selectively affected by the choice of drugs. As shown in figure 3, no significant antibody differences were found in the sera of patients treated with either nivolumab or pembrolizumab.

DISCUSSION

This study showed that antibody levels directed against the MDA Melan-A and against the CGAs NY-ESO-1, MAGE-C2, ROPN1B and MAGE-A6 cannot be used as predictive markers for the response to treatment with ICIs. More specific, no significant differences in the serum concentrations of these tumor-associated antibodies between responders and non-responders were demonstrated at

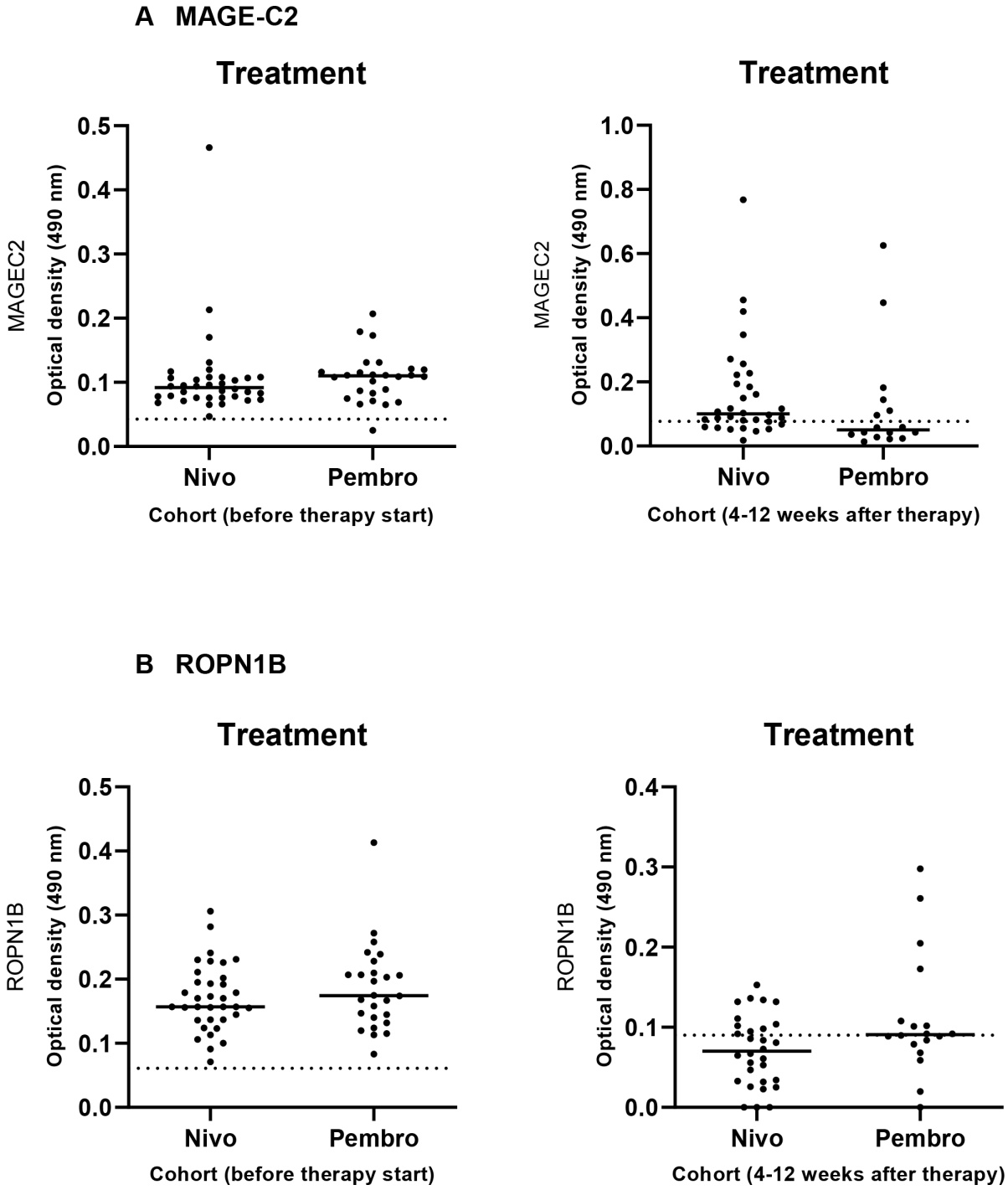

Figure 3 No differences in serum levels of tumor-associated antibodies in patients receiving different immune checkpoint inhibitors (ICIs). The figures show the optical density (OD) values of antibodies against MAGE-C2 (A) and ROPN1B (B) in serum of patients receiving either nivolumab or pembrolizumab. Antibodies against MAGE-C2 (A) and ROPN1B (B) were measured before and during treatment, that is, 1–3 months since treatment initiation. Figures were created with GraphPad Prism V.5.0 software.

baseline or during treatment. A homogeneous cohort of patients with metastatic melanoma was included for this study and long-term follow-up data were available for all patients. Therefore, it was possible to assess whether differences in serum antibody concentrations resulted in long-term OS differences. However, no differences in antibody concentrations at baseline or during treatment were found between the OS groups, that is, between short-term and long-term survivors.

Our results are in contrast with the study of Fässler *et al*, published in this journal in 2019, which demonstrated the potential of two of these antibodies as a surrogate marker for response to ICI treatment.[14] Because we included a larger, more homogeneous and more mature cohort of

patients with melanoma with longer follow-up, less significant results were not expected. Importantly, the antigen selection of the current study partly overlaps, but also differs significantly from the study by Fässler *et al*.[14] As the most promising results were found for NY-ESO-1 in their study, we decided to mostly include CGAs, while in their study mainly MDAs were included. Although this might explain some of the differences in the results, the promising results which were found in the previous study for NY-ESO-1[14] could not be reproduced in the current study.

One of the limitations of the current study is that only circulating antibodies have been taken into account, and the antigen expression of the tumor tissue was not examined. Previous studies have identified the expression of CGAs in tumor tissue as a poor prognostic marker and these antigens may play a role in tumor metastasis.[16–18] The circulating antibody levels might depend on the level of antigen expression of the tumor. In addition, the expression of CGAs or MDAs in tumor tissue has previously been correlated with tumor burden[19] and tumor burden has been associated with decreased OS rates.[20] Subsequently, it is conceivable that higher serum concentrations are associated with poorer response to treatment and decreased OS. For advanced melanoma, tumor burden is known to be associated with lactate dehydrogenase level (LDH) levels.[21] Since patients with variable LDH levels were included in the current study, this study cohort is representative of a real-world population of patients with melanoma with differences in tumor burden.

Antibodies directed against tumor antigens have been extensively studied in the past.[10 22] Immune responses against such antigens are often exploited for the development of new immunotherapy-based treatments,[23–25] for example, for therapeutic vaccines or adoptive T- cell treatment.[13] The results of the current study do not imply that these tumor antigens are not suitable as targets for new anticancer immunotherapies, since we did not investigate T-cell responses. However, based on the results of the current study, the clinical applicability of tumor-associated antibodies directed against tumor antigens as a predictive biomarker for ICI treatment does not seem feasible. This study emphasizes the importance of external validation of predictive biomarker studies, in order to determine their relevance for clinical practice.

Contributors Study concept and design: KdJ, AJ, DK, RD, RHJM, MWJS and AAMVdV. Acquisition, analysis or interpretation of data: KdJ, SV, CK, MWJS and AAMVdV. Statistical analysis: SV, CK and MWJS Drafting of the manuscript: KdJ, SV, CK, MWJS and AAMVdV. Critical revision of manuscript for important intellectual content: all authors.

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Patient consent for publication Consent obtained directly from patient(s)

Ethics approval This study involves human participants and was approved by Medical Ethical Committee at the Erasmus Medical Center.Protocol ID: MEC2016-011. Participants gave informed consent to participate in the study before taking part.

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