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Early View

Original article

Soluble PD-L1 is a potential biomarker of cutaneous melanoma aggressiveness and metastasis in obstructive sleep apnea patients

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Title: **Soluble PD-L1 is a potential biomarker of cutaneous melanoma aggressiveness and metastasis in obstructive sleep apnea patients**

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TAKE HOME MESSAGE

In patients with cutaneous melanoma, severe OSA increases serum levels of sPD-L1, which are associated with several indices of tumor aggressiveness and are an independent risk factor of sentinel lymph node metastasis.

ABSTRACT

Obstructive sleep apnea (OSA) up-regulates the programmed cell death-1 receptor and its ligand (PD-L1) pathway, potentially compromising immunosurveillance. We compared circulating levels of soluble PD-L1 (sPD-L1) in patients with cutaneous melanoma (CM) according to the presence and severity of OSA, and evaluated relationships with tumor aggressiveness and invasiveness.

In a multicenter observational study, 360 patients with CM underwent sleep studies, and serum sPD-L1 levels were assayed by ELISA. CM aggressiveness indices included mitotic rate, Breslow index, tumor ulceration, Clark level and tumor stage, and sentinel lymph node (SLN) metastasis was recorded as a marker of invasiveness.

sPD-L1 levels were higher in severe OSA compared to mild OSA or non-OSA patients. In OSA patients, sPD-L1 levels correlated with Breslow index and were higher in patients with tumor ulceration, advanced primary tumor stages or with loco-regional disease. The incorporation of sPD-L1 to the classic risk factors to SLN metastasis led to net improvements in the classification of 27.3%.

Thus, sPD-L1 levels are increased in melanoma patients with severe OSA and, in addition, might serve as a potential biomarker of CM aggressiveness and invasiveness in this group of subjects.

KEY WORDS: Sleep apnea; melanoma; intermittent hypoxia; immune system**;** biomarkers

INTRODUCTION

Obstructive sleep apnea (OSA) is a highly prevalent disorder affecting 10–50% of middle-aged subjects [1], characterized by recurrent episodes of partial or complete upper airway obstruction associated with intermittent hypoxia and sleep fragmentation. These alterations induce oxidative stress, sympathetic activation, and metabolic deregulation [2] and are associated with an increased risk of cardiovascular and neurocognitive consequences [3,4]. More recently, significant associations between OSA and cancer prevalence, incidence and mortality have emerged [5-8], and more specifically, a relationship has been demonstrated between OSA and the aggressiveness of cutaneous melanoma [8,9], the most lethal form of skin cancer, with a high tendency to rapidly metastasize [10].

A number of pathogenic mechanisms have been suggested to justify the possible correlation between OSA and cancer, ranging from the effects of oxidative stress on genetic malignant cell stability to the promotion of tumor angiogenesis [11,12]. Furthermore, evidence suggesting alterations in immune function induced by intermittent hypoxia and sleep fragmentation have become apparent, including deregulation of tumor associated macrophages and cytotoxic T cell lymphocyte activity [13-16], which could compromise immunosurveillance and favor both the viability and progression of tumor cells. In this contextual setting, we have recently described intermittent hypoxia-induced increases in the expression of the pathway of programmed cell death-1 (PD-1) receptor and its ligand (PD-L1) among cancer-free OSA patients, and such changes would be anticipated to reduce autologous T-cell proliferation and cytotoxic activity of CD8+ lymphocytes , as well as increases in the recruitment of myeloid-derived suppressor cells

[17], thereby providing biological plausibility to the increased incidence and aggressiveness of cancer as reported in these patients.

Of note, malignant tumor cells can also activate the PD-1/PD-L1 pathway to evade antitumoral immunity and promote malignant cell growth [18]. Recently, the soluble form of PD-L1 (sPD-L1) has been detected in the blood of patients with tumors [19], and elevated sPD-L1 levels have been associated with worse prognosis in certain types of cancer [20]. For these reasons, sPD-L1 could provide a potential diagnostic, therapeutic or prognostic biomarker in patients with malignant tumors [21]. However, no information is available on whether the presence of OSA increases sPD-L1 levels in patients with cancer, or whether such levels are related to adverse tumor evolution in apneic subjects.

In the present study, we hypothesized that serum levels of sPD-L1 in patients with melanoma would be affected and enhanced in a severity-dependent fashion by the concurrent presence and severity of OSA. In addition, we evaluated the correlation of sPD-L1 levels with the aggressiveness and invasiveness of cutaneous melanoma (CM), as inferred from a variety of well-established staging approaches as well as by the presence of sentinel lymph node (SLN) metastasis, is the latter being considered as the most important factor in terms of patient survival [22].

METHODS

Study subjects

In a multicenter, observational cross-sectional study performed in 29 teaching hospitals in Spain, newly diagnosed CM patients aged > 18 years were consecutively recruited from the dermatology or oncology units of each participating medical center [8,23]. Exclusion criteria included extra-cutaneous location of melanoma, daytime respiratory or heart failure, and current or previous use of home oxygen therapy, continuous positive airway pressure (CPAP) or noninvasive mechanical ventilation. The study was approved by the local ethics committees, and all participants signed an informed consent.

Dermatological evaluation

As previously described [24], patients were evaluated by a dermatologist at each hospital, and recordings of tumor location (head or neck, upper extremities, trunk, lower extremities, or acral) and clinical stage at diagnosis (categorized as localized [I-II] or loco-regional disease or distant metastasis [III-IV]) were performed. All tumors were surgically removed and the following pathologic features were determined: Breslow tumor thickness (in mm and categorized as ≤ 1, 1.01 to 2.00, 2.01 to 4.00, and > 4.00), ulceration (presence or absence), tumor mitotic rate (mitoses/mm^2), and Clark levels (level 1 or melanoma confined to the epidermis, level 2 or invasion into the papillary dermis, level 3 or invasion to the junction of the papillary and reticular dermis, level 4 or invasion into the reticular dermis, and level 5 or invasion into the subcutaneous fat) [25]. Based on tumor thickness, ulceration and mitotic rate, primary tumor categories were established, according to the American Joint Committee on

Cancer (AJCC) 8th edition melanoma staging system [25]. In addition, sentinel lymph node metastasis was recorded as a marker of CM invasiveness and indicator of poor prognosis [22]. Classic predictors for SLN status included Breslow index (in mm), presence of ulceration, mitotic index and Clark levels [26]. All dermatological and pathological evaluations were blinded to the sleep study and biochemical analysis findings.

Sleep study

All patients underwent overnight respiratory polygraphy within a maximum of 6 months after CM diagnosis at the sleep unit of each hospital. The validated portable devices provided continuous recording of oronasal flow and pressure, thoracic and abdominal respiratory movements, heart rate and arterial oxygen saturation (SaO_2). All scoring and readings were conducted manually by experienced and trained personnel. Apnea was defined as an interruption of oronasal flow of > 10 s. Hypopnea was defined as a 30-90% reduction in the oronasal airflow for > 10 s associated with an oxygen desaturation ≥ 3%. The apnea-hypopnea index (AHI) was defined as the number of apneas plus hypopneas per hour of recording, while tSat90 was defined as the percentage of recording time with SaO_2 < 90%. In addition, mean saturation, minimum saturation and oxygen desaturation index (ODI) were measured. According to the AHI, patients were divided into four groups: non-OSA (<5 h^{-1}), mild OSA (5-14.9 h^{-1}), moderate OSA (15-29.9 h^{-1}) and severe OSA (> 30 h^{-1}) [27].

Determination of serum levels of soluble PD-L1

Fasting venous blood samples were drawn between 08:00 and 09:00 h. The blood samples were centrifuged to separate serum, and all specimens were immediately aliquoted, frozen and stored at -80ºC. Serum sPD-L1 was concomitantly assayed using the human PD-L1 enzyme-linked immunosorbent assay (ab214565, Abcam, Cambridge, UK) following the manufacturer's instructions for all samples. Measurements for serum samples were done in duplicate. The detection limit of the assays was 2.91 pg/mL, while intra-assay and inter-assay variations were below 20%.

Statistical analysis

Continuous variables are expressed as mean ± standard deviation or median (interquartile range [IQR]), depending on their distribution, while categorical variables are reported as absolute numbers and percentages. Normality in the distribution of the data for each variable was explored using Shapiro-Wills and Skewness-Kurtosis tests. Differences between groups were analyzed by the chi-square test or Fisher exact test (categorical variables) and Kruskal-Wallis test (ordinary or non-normal metric variables).

Potential associations between anthropometric and sleep parameters with sPD-L1 levels were evaluated by Spearman's correlation. Those variables exhibiting statistically significant findings were then introduced into a multiple linear regression analysis to identify independent determinants of sPD-L1 levels. Stepwise methods were used to include or remove individual independent variables at each step, based on the probability of F (entry: 0.05; removal: 0.10).

Other aspects explored included residual standard deviation, changes in the distribution of the residuals and the homogeneity of the variance over the predictors.

Associations between anthropometric and sleep parameters, classic risk factors and sPD-L1 levels with SLN metastasis were analyzed by bivariate and forward stepwise multiple logistic regression. To assess whether the addition of sPD-L1 level to the classical aggressiveness markers improved the predictive power for invasion of SLN, we calculated the area under curve of the receiver operating characteristics curve (AUC-ROC) for the classical model with and without inclusion of sPD-L1 serum concentration. The equality of AUCs was assessed by the DeLong *et al* method [28]. Net reclassification index (NRI) and integrated discrimination improvement (IDI) were applied to quantify the improvement contributed by this approach [29].

All tests were two-tailed, and a statistical significance level of 0.05 was retained. Analyses were performed using the Statistical Package for the Social Sciences, version 13.0 software (SPSS Inc., Chicago, IL, USA) and MedCalc (www.medcalc.org).

RESULTS

Out of 476 eligible patients with a diagnosis of CM, 441 patients provided informed consent and were prospectively recruited. However, stored serum samples were not available for 81 patients, such that the remaining 360 patients were included in the current analysis (Figure 1). Globally, 172 patients were females; mean age ± SD was 57 ± 15 years; and mean body mass index was 27.4 ± 4.6 Kg/m^2. 317 patients (88.1%) had local melanoma extension, and evidence of ulceration was found in 63 (17.5%). The median (IQR) Breslow index was 0.81 (0.49-1.80)

mm, and the median (IQR) mitotic index was 1 (0-2) cells.mm^{-2}. The overall prevalence rates of mild (AHI < 5/h) and moderate-to-severe (AHI > 15/h) OSA were 30.3 and 33.1%, respectively. The demographic and clinical characteristics of the four study groups are shown in Table 1, and illustrates that as the severity of OSA increases, patients were increasingly more likely to be men, older and more overweight or obese.

sPD-L1 serum levels are elevated in CM patients with severe OSA

sPD-L1 serum levels showed significant differences among the study groups (p=0.035). When adjusted for sex, age, BMI and neck circumference, sPD-L1 levels were higher in severe OSA patients versus mild OSA or non-apneic patients (Figure 2).

In the overall analysis of the cohort, sPD-L1 levels exhibited direct proportional relationships with AHI, desaturation index and level of nocturnal hypoxemia, as well as with age, BMI and neck circumference (Table 2). However, upon introduction of all the variables that reached significance into a multivariate linear regression analysis, only AHI was retained as an independent variable, both in for the whole cohort (beta coefficient 0.983, 95%CI 0.509 to 1.457, p<0.001) and also for patients with OSA (beta coefficient 1.057, 95%CI 0.512 to 1.638, p<0.001).

sPD-L1 levels and melanoma aggressiveness in OSA patients

Serum levels of sPD-L1 were higher in OSA patients with loco-regional disease or metastasis than in patients with localized CM (96.6 [58.6-200.0] versus 71.5 [41.9-98.6] pg/mL, p=0.013). Moreover, in OSA patients, we found a positive correlation between the Breslow index and

sPD-L1 levels (Figure 3), with significant differences between the degree of CM thickness (Figure 4). Specifically, sPD-L1 levels were 60.2 (41.8-93.9) pg/mL in patients with CM thickness < 1 mm and 92.1 (76.2-134.9) pg/mL in patients with a thickness > 4 mm (p=0.019). Serum levels of sPD-L1 were also higher in CM patients with tumor ulceration when compared to those patients who did not have ulceration (86.7 [58.8-120.4] versus 71.5 [41.4-99.4] pg/mL, p=0.035). Finally, a progressive increase in sPD-L1 levels was observed among the successive primary tumor categories (Figure S1).

In contrast, no relation was found between sPD-L1 and mitotic index (Figure 3), and there were no differences in sPD-L1 levels between the Clark levels (70.2 [42.4-103.6] pg/mL in patients with level 2, 82.0 [47.5-99.8] pg/mL in those with level 3, 75.0 [37.8-113.8] pg/mL in those with level 4 and 101.7 [76.3-145.0] pg/mL in those with level 5; p=0.136).

Noteworthy, the levels of sPD-L1 of patients without OSA were not related to the Breslow or the mitotic indices (Figure S2), and they did not show differences depending on the presence of tumor ulceration, thickness degrees, Clark levels or primary tumor categories (Table S1).

sPD-L1 is a risk factor for sentinel lymph node metastasis in melanoma patients with OSA

In 248 patients with melanoma and OSA, SLN biopsy was obtained and showed evidence of metastasis in 30 (12.1%). In a bivariate regression analysis, SLN metastasis was significantly associated with young age, mitotic index, Breslow index, tumor ulceration, Clark level and serum levels of sPD-L1 (Table 3). SLN status was not associated with sex, BMI or any of the sleep study-derived parameters. However, a stepwise multivariate logistic regression analysis only retained four variables as independent risk factors for SLN metastasis, namely young age

(p=0.006), increased Breslow index (p=0.025) and Clark level (p=0.026), and high sPD-L1 levels (p=0.008) (Table 3).

For the individual prediction of positive SLN risk, an isolated sPD-L1 value had limited utility. The diagnostic accuracy of an optimal cut-off point (i.e., serum level > 74 pg/mL) only reached a sensitivity of 75.0% (95%CI, 53.0 to 89.4) and a specificity of 50.6% (95%CI, 42.7 to 58.6). However, when sPD-L1 levels were incorporated into the classical prediction model based on conventional risk factors (age, Breslow depth, mitotic rate and Clark index), the AUC-ROC significantly increased (p=0.024) from 0.837 (0.771-0.902) to 0.896 (0.844 to 0.947) (Figure 5). In turn, the incorporation of sPD-L1 to the classic risk model determined an IDI of 0.089 (0.037-0.141; p=0.001) and an NRI of 0.273 (0.263-0.283; p=0.036), indicating that the addition of sPD-L1 led to a net improvement in the classification of 27.3% of the cases.

DISCUSSION

The present study shows that severe OSA is associated with increases in serum levels of sPD-L1 in patients with CM. Moreover, we found that sPD-L1 levels in OSA patients are related to CM aggressiveness and that they are an independent risk factor for the presence of sentinel lymph node metastasis.

Similar to other molecules involved in immunoregulatory pathways, PD-L1 assumes two forms of expression, a membrane-bound form (mPD-L1) and a soluble form. Usually, the soluble moiety is generated by proteolytic cleavage of the membrane-bound form of co-stimulatory proteins or by translation of alternative spliced mRNA [21,30]. Although other sources of sPD-L1 cannot be excluded, it seems probable that the PD-L1 expressed on the immune and tumor cell

surface may provide the main source of the soluble PD-L1 form, since sPD-L1 is detectable in supernatants from mPD-L1+ cell lines, rather in those supernatants originating from mPD-L1- cell lines [31]. Thus, the increases in sPD-L1 levels should be viewed as putative confirmation of the OSA contribution to up-regulation of the PD-1/PD-L1 pathway, as recently described by our group [17].

Both in the whole cohort and in those patients who suffered from OSA, serum sPD-L1 levels were significantly correlated with age and with several polygraphic measures. The contribution of age was not novel since it has already been previously identified. Indeed, in normal subjects, serum levels of sPD-L1 increase with age, probably due to an increased activity of matrix metalloproteinases (MMPs), which would facilitate the cleavage of mPD-L1 [31]. With regards to sleep study-derived parameters, AHI emerged as the main determinant of sPD-L1 levels rather than some of the indices representing nocturnal hypoxemia, especially considering that increases in PD-1/PD-L1 expression seem to be dependent on intermittent hypoxia and are mediated by hypoxia-inducible factor transcriptional activity [17]. It is possible that oxidative stress elicited by OSA may also favor increased sPD-L1 levels, promoting the capacity of MMPs to cleave the extracellular fraction of mPD-L1. In fact, the link between oxidative stress and MMPs has been demonstrated in several experimental models [32], and it has been recently reported that the altered expression of certain MMPs and their tissue inhibitors in subjects with OSA [33,34] correlates with several oxidative stress parameters [35].

In OSA patients with CM, sPD-L1 levels were related to tumor aggressiveness and were higher in patients who present other poor prognostic indicators, such as the presence of tumor ulceration or higher tumor stage. It is particularly interesting to consider that sPD-L1 levels

show a directly proportional relationship with an indicator of tumor aggressiveness, such as the Breslow index, while they do not reach statistical significance with an indicator of the intrinsic properties of the tumor cells, such as mitotic rate. This could reflect the importance of the interaction between the characteristics of the tumor cells and the immune system to determine the aggressiveness of the neoplastic disease. In any case, and according to the AJCC staging system, Breslow thickness and ulceration are considered, along with SLN involvement, the most important prognostic factors in melanoma patients, while mitotic rate is recognized only as a prognostic factor in the subgroup of thin melanomas [26].

Our findings concerning the relationship between sPD-L1 levels and melanoma aggressiveness agree with previous findings in other types of malignant tumors. Several studies have revealed that sPD-L1 levels are related with a higher aggressiveness and poorer prognosis in patients with non-small cell lung cancer, lymphoma, multiple myeloma or renal cell carcinoma [21,36-38]. However, the mechanism by which sPD-L1 promotes tumor aggressiveness is more controversial. At present, it is still debatable whether sPD-L1 levels reflect only greater cellular expression of PD-L1 or if sPD-L1 can actually bind to PD-1, similar to mPD-L1, thereby promoting immunosuppression [21]. In any case, both options represent an up-regulation of the PD-1/PD-L1 pathway, which has recognized and well established effects on tumor aggressiveness and progression. The activation of the PD-1/PD-L1 pathway can lead to tumor immune escape through several mechanisms, including the downregulation of the effector phase of T-cell immune responses by elevating the threshold of T-cell activation, inhibiting T-cell proliferation, or by promoting T-cell apoptosis, as well as by enhancing immunosuppressive Treg cell function [36,39].

Surprisingly, in the patients of our study with melanoma but without OSA, no significant correlation emerged between sPD-L1 levels and tumor aggressiveness. This is particularly striking if one considers the aforementioned role of sPD-L1 in the promotion of immunity evasion and development and progression of several types of malignancies, including melanomas, as previously reported [40]. It is possible that the limited size of the group of patients with CM but without OSA and the narrow aggressiveness spectrum may account for this apparent discrepancy. However, we should also point out that the presence or absence of OSA was not systematically explored in previous studies. Moreover, no association has been reported between tumor PD-L1 expression detected by immunohistochemical analysis and sPD-L1 levels in patients with some non-CM tumors,. This suggests a major contribution of tumor microenvironment, including immune cells, to sPD-L1 generation [41]. Indeed, it has been recently demonstrated that PD-L1 is preferentially expressed on antigen-presenting cells, rather than on tumor cells, and plays an essential role in checkpoint blockade therapy [42].

In the management of patients with CM, the importance of SLN biopsy lies in both the detection of occult nodal metastasis and as an indicator of overall treatment prognosis. However, SLN biopsy selection criteria are still under discussion. Conventionally, SLN biopsy is performed according to the risk estimated by the Breslow index, presence of tumor ulceration and Clark level [43]. However, the prediction capacity is not optimal, and other factors such as age or mitotic index have also been proposed as predictors of SLN positivity [26,44].

Our study, which mainly involved patients with thin to intermediate thickness CM reached an SLN positivity rate (12.1%), which is similar to previous cohorts [26,45], and highlights the potential usefulness of sPD-L1 levels in patients with CM and OSA. Regarding classic risk factors,

our findings are consistent with those previously established in the literature. In a recent cohort of 147 patients with stage I and II cutaneous melanoma, the Breslow index, Clark level, mitotic rate and presence of ulceration were identified as independent predictors of SLN metastasis [26]. However, the Breslow index is traditionally considered the most important predictor of SLN metastasis. In fact, in an analysis of 3,460 patients with melanoma, multivariate analysis revealed young age and Breslow thickness as the only significant predictors, while mitotic rate and presence of ulceration were not retained in the model [45]. Although several studies found that ulceration or mitotic rate are associated with SLN metastasis [26,43,46-48], their role as independent predictors of SLN involvement has not been consistently established. Our findings suggest that increased sPD-L1 levels might also be a risk factor for SLN involvement in patients with CM and OSA, which would confer them utility as a potential biomarker in this group of patients.

However, the prediction capacity solely based on sPD-L1 levels is limited, such that use of sPD-L1 levels as a single risk factor is discouraged. However, the results of the present study show that incorporation of sPD-L1 into the classical risk models, together with the Breslow index, mitotic rate and presence of ulceration increases the predicting capacity of SLN metastasis in patients with CM and OSA.

The main strengths of this study are its prospective nature, multicenter character and the relatively large cohort size, as well as the homogenization and rigorous control of sleep studies performed by the Spanish Sleep Network. However, our study has several limitations that must be acknowledged. First, not all sleep variables potentially related to cancer, such as sleep fragmentation or even sleep habits or shift work, were documented and accounted for in our

analyses. Second, there was some temporal delay between the skin biopsy and the sleep study and the collection of serum samples. Although we have tried to minimize this time interval (which was not different between the study groups and never exceeded five months) and no changes in patient weight or any other potential confounding factors were identified, we cannot rule out that such factors were void of any influence in our results. Third, no biopsy tissues were available to determine PD-1 expression on the CM tumor cells. Fourth, our results only refer to patients with cutaneous melanoma, such that the relationship between sPD-L1 levels and the aggressiveness and invasiveness of other cancer types in patients with OSA should be cautiously extrapolated. Fifth, our study does not provide any information on the effect of treatment of OSA on sPD-L1 levels or their effect on melanoma aggressiveness, and therefore no therapeutic recommendations beyond those in place for OSA criteria can be formulated.

In summary, the present study shows that melanoma patients with severe OSA have increased serum levels of soluble PD-L1. The addition of a simple assay, such the one required for determination of this parameter in patients with CM and OSA, may provide a potential simple biomarker of aggressiveness and invasiveness in these patients. However, caution should be exercised not to make pathogenic inferences from our results, because, although sPD-L1 seems to be a marker of aggressiveness, our data do not show that elevated sPD-L1 levels are necessarily the cause of aggressiveness. Although it is attractive to speculate that high sPD-L1 levels might reflect the presence of greater underlying degrees of immunosuppression due to up-regulation of the PD-1/PD-L1 pathway, our data do not allow confirm this hypothesis. Both

melanoma aggressiveness and OSA could raise PD-L1 levels, but the higher levels in OSA may not necessarily worsen melanomas.

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Table 1. General characteristics of the study subjects*

	Non OSA patients	OSA patients			p
		Mild	Moderate	Severe	
N	132	109	70	49	-
Males	51 (39)	54 (50)	47 (67)	36 (73)	<0.001
Age, yr	44 (37-52)	59 (46-68)	67 (53-73)	69 (61-77)	<0.001
BMI, Kg/m^2	24.5 (22.9-27.2)	28.3 (25.1-30.8)	28.1 (25.8-30.8)	29.8 (26.9-34.4)	<0.001
Neck circumference, cm	35 (32-39)	38 (35-40)	39 (36-42)	41 (37-44)	<0.001
Smoking status					0.311
Never	62 (47)	60 (55)	33 (47)	29 (59)	
Current smoker	32 (24)	20 (18)	10 (14)	7 (14)	
Past smoker	38 (29)	29 (27)	27 (39)	12 (24)	
Melanoma location					0.606
Head or neck	16 (12)	16 (15)	8 (11)	12 (24)	
Arms	17 (13)	18 (17)	9 (13)	6 (12)	
Legs	34 (26)	33 (30)	17 (24)	9 (18)	
Trunk	60 (46)	35 (32)	32 (46)	21 (43)	
Acral	5 (4)	7 (6)	3 (4)	2 (4)	
Type of melanoma					0.043
Superficial spreading melanoma	98 (74)	83 (76)	48 (69)	26 (54)	
Lentigo maligna melanoma	7 (5)	5 (5)	3 (4)	10 (21)	
Acral lentiginous melanoma	7 (5)	6 (6)	3 (4)	3 (6)	
Nodular melanoma	17 (13)	13 (12)	15 (21)	8 (17)	
Other	3 (2)	2 (2)	1 (1)	1 (2)	
Breslow index					<0.001
< 1 mm	92 (70)	65 (60)	31 (44)	16 (33)	
1-2 mm	24 (18)	20 (18)	17 (24)	16 (33)	
2.1-4 mm	13 (10)	15 (14)	10 (14)	11 (24)	
>4 mm	3 (2)	9 (8)	12 (17)	6 (12)	
Ulceration	13 (10)	23 (21)	15 (21)	12 (25)	0.033
Mitotic index, $cells.mm^{-2}$	1 (0-2)	1 (0-2)	1 (0-4)	1 (0-3)	0.029
Disease stage					0.138
Localized melanoma	120 (92)	99 (92)	59 (86)	39 (81)	
Locoregional disease	11 (8)	10 (9)	11 (16)	10 (20)	
Chronic snore	63 (48%)	64 (59)	53 (76)	42 (86)	<0.001
Witnessed apneas	9 (7%)	16 (15)	23 (33)	22 (45)	<0.001
ESS score	5 (3-8)	6 (4-7)	6 (3-9)	7 (4-10)	0.064
AHI, h^{-1}	2.0 (0.7-3.0)	9.0 (7.0-12.0)	20.6 (17.7-25.0)	45.2 (36.2-55.8)	<0.001
DI, h^{-1}	0.8 (0.3-1.6)	5.1 (3.4-8.4)	12.4 (8.9-	32.4 (25.0-	<0.001

			15.3)	39.8)	
Mean nocturnal SpO_2, %	95 (94-96)	94 (93-95)	93 (92-95)	93 (92-93)	<0.001
Low nocturnal SpO_2, %	89 (87-92)	85 (81-88)	83 (79-87)	77 (69-82)	<0.001
$tSpO_2$ <90%, %	0.0 (0.0-0.2)	1.0 (0.1-3.5)	2.7 (0.5-5.9)	9.4 (4.6-17.9)	<0.001
Comorbidities					
Hypertension	17 (13)	41 (38)	32 (46)	28 (57)	<0.001
Dyslipidemia	24 (18)	39 (36)	32 (46)	22 (45)	<0.001
Depression	11 (8)	10 (9)	2 (3)	7 (14)	0.163
Cerebrovascular disease	1 (1)	2 (2)	2 (3)	5 (10)	0.006
Ischemic heart disease	0	2 (2)	6 (9)	2 (4)	0.004
Atrial fibrillation	1 (1)	2 (2)	4 (6)	5 (10)	0.008
Diabetes mellitus	9 (7)	8 (7)	12 (17)	13 (27)	0.001
sPD-L1 serum level, pg/ml	64.4 (35.2-84.8)	60.2 (40.9-85.9)	86.1 (51.2-115.6)	88.5 (50.2-159.5)	0.035

*Data are presented as median (interquartile range [IQR]) or n (%).
Abbreviations: BMI, body mass index; ESS, Epworth sleepiness score; AHI, apnea-hypopnea index; DI, desaturation index; SpO_2, oxygen saturation; $tSpO_2$<90% night time spent with oxygen saturation < 90%

Table 2. Anthropometric and sleep parameters related with sPD-L1 serum levels in the overall study subjects and OSA patients

	Overall patients				OSA patients		
	r	**95%CI**	**p**		**r**	**95%CI**	**p**
Age, yr	0.238	0.123 to 0.347	<0.001		0.224	0.083 to 0.356	0.002
BMI, Kg/m^2	0.135	0.017 to 0.250	0.026		0.033	-0.111 to 0.176	0.656
Neck circumference, cm	0.136	0.018 to 0.251	0.024		0.042	-0.103 to 0.185	0.573
ESS	0.085	-0.034 to 0.201	0.162		0.049	-0.096 to 0.192	0.508
AHI, h^{-1}	0.206	0.090 to 0.317	0.001		0.224	0.083 to 0.356	0.002
DI, h^{-1}	0.233	0.118 to 0.342	0.001		0.258	0.119 to 0.387	<0.001
Mean nocturnal SpO_2, %	-0.232	-0.341 to -0.117	<0.001		-0.230	-0.362 to -0.089	0.002
Low nocturnal SpO_2, %	-0.173	-0.286 to -0.056	0.004		-0.123	-0.262 to 0.021	0.095
$tSpO_2$ <90%, %	0.239	0.124 to 0.348	<0.001		0.180	0.037 to 0.316	0.014

Abbreviations: BMI, body mass index; ESS, Epworth sleepiness score; AHI, apnea-hypopnea index; DI, desaturation index; SpO_2, oxygen saturation; $tSpO_2$<90%, night time spent with oxygen saturation < 90%; r, Spearman correlation coefficient; CI, confidence interval

Table 3. Factors related to sentinel lymph node metastasis in patients with melanoma and obstructive sleep apnea

	Bivariate logistic regression analysis			**Stepwise multivariate logistic regression analysis**		
	Odds ratio	**95% CI**	**p**	**Odds ratio**	**95% CI**	**p**
Males	1.358	0.616 to 2.987	0.450	-	-	-
Age, yr	0.988	0.968 to 0.999	0.049	0.939	0.897 to 0.982	0.006
BMI, Kg/m^2	1.003	0.938 to 1.073	0.949	-	-	-
Current smoker	0.938	0.395 to 2.228	0.884	-	-	-
ESS	1.011	0.909 to 1.123	0.845	-	-	-
AHI, h^{-1}	1.009	0.989 to 1.031	0.376	-	-	-
DI, h^{-1}	1.013	0.996 to 1.029	0.130	-	-	-
Mean nocturnal SpO_2, %	1.158	0.932 to 1.439	0.184	-	-	-
Low nocturnal SpO_2, %	0.990	0.957 to 1.025	0.581	-	-	-
$tSpO_2$ <90%, %	0.983	0.949 to 1.019	0.345	-	-	-
Mitotic index, $cells.mm^{-2}$	1.155	1.074 to 1.241	<0.001	-	-	-
Breslow index, mm	1.479	1.270 to 1.723	<0.001	1.371	1.041 to 1.805	0.025
Presence of ulceration	5.909	2.646 to 13.196	<0.001	-	-	-
Clark level	4.961	2.690 to 9.152	<0.001	3.286	1.155 to 9.346	0.026
Serum sPD-L1, pg/ml	1.009	1.004 to 1.014	0.001	1.009	1.002 to 1.016	0.008

Abbreviations: BMI, body mass index; ESS, Epworth sleepiness score; AHI, apnea-hypopnea index; DI, desaturation index; SpO_2, oxygen saturation; $tSpO_2$<90% night time spent with oxygen saturation < 90%

LEGENDS OF FIGURES

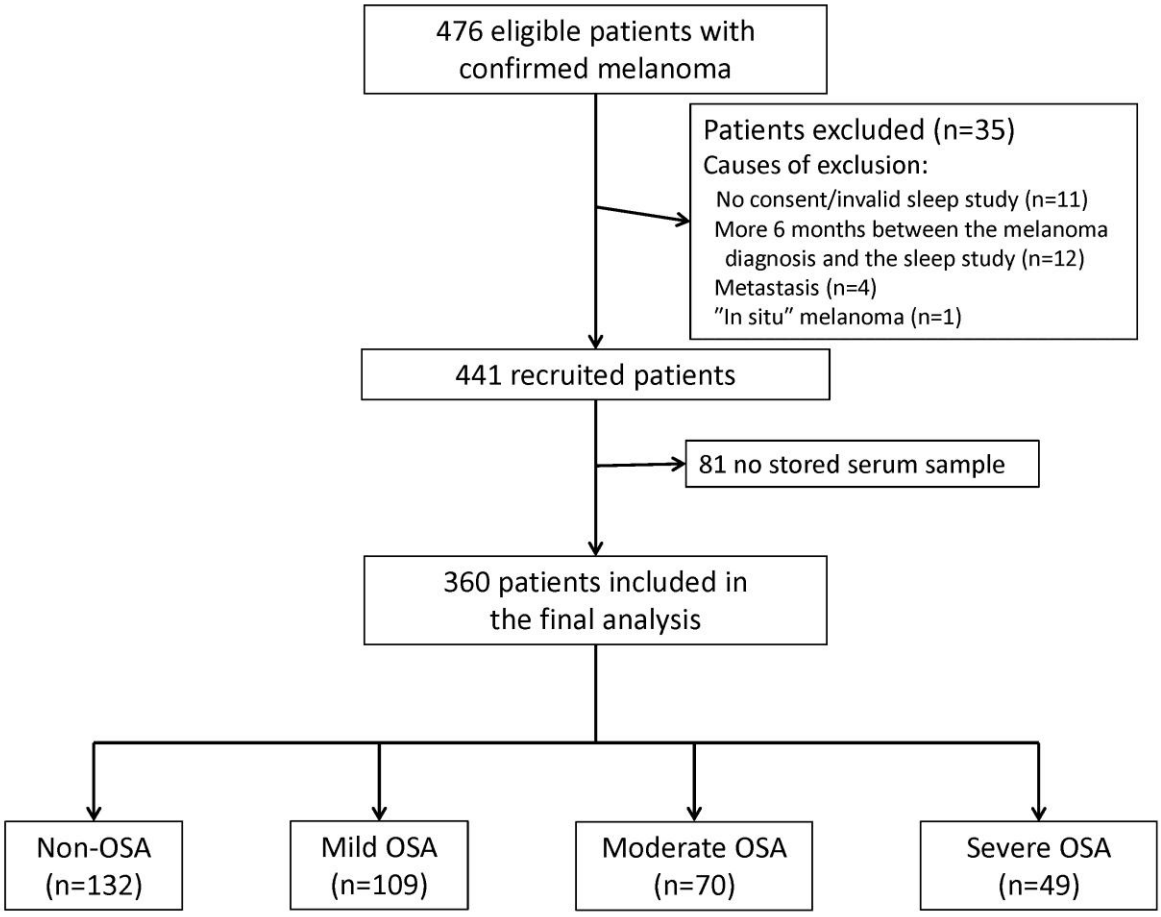
Figure 1. Flow chart of the study. OSA, obstructive sleep apnea

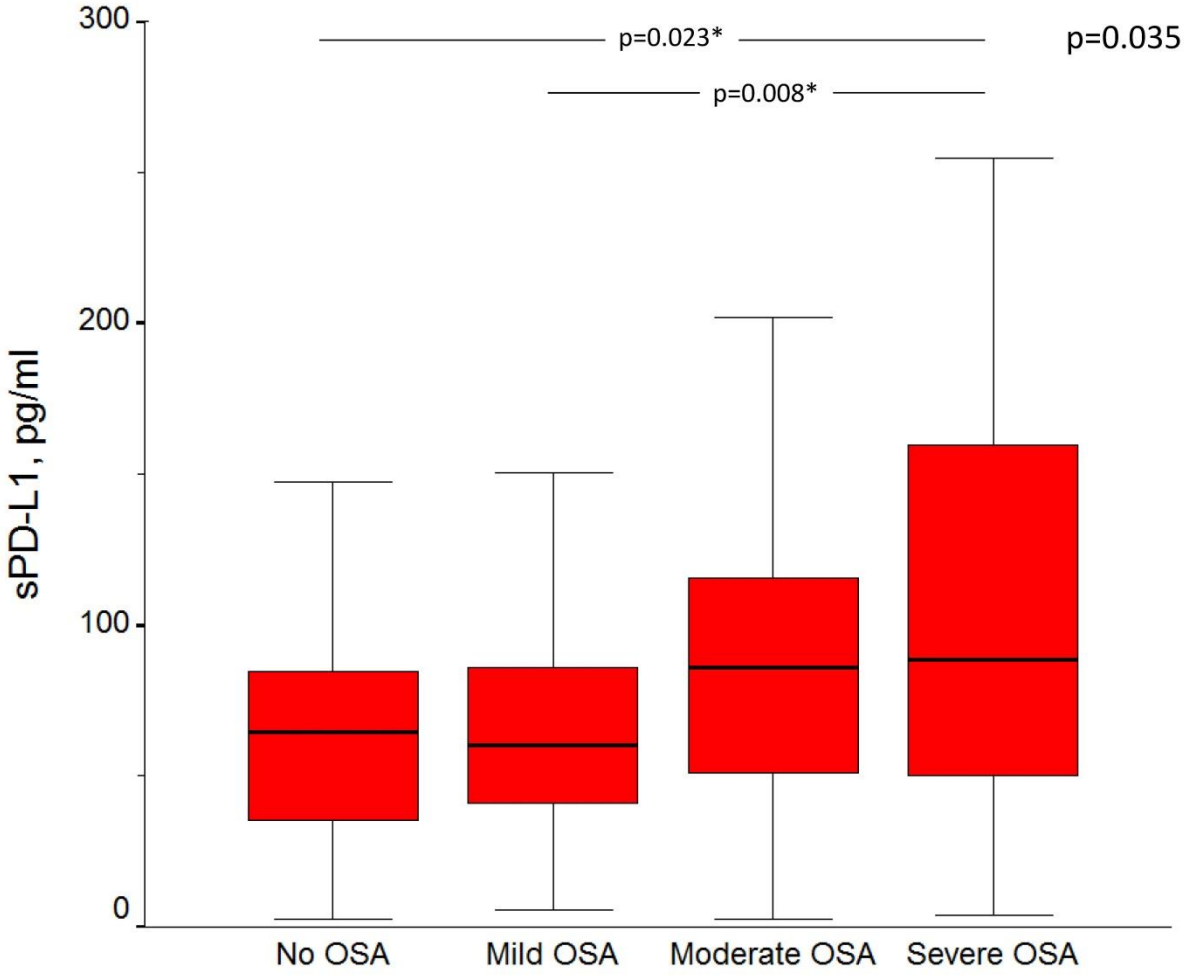
Figure 2. Box-and-whisker plots depicting the distribution of serum levels of soluble PD-L1 in cutaneous melanoma patients according to their apnea-hypopnea index.
The dark line in the middle of the boxes represents the median and the length of the box reflects the interquartile range (IQR). The T-bars represent maximum and minimum values. p values correspond to the comparisons between groups adjusted by sex, age, body mass index and neck circumference.

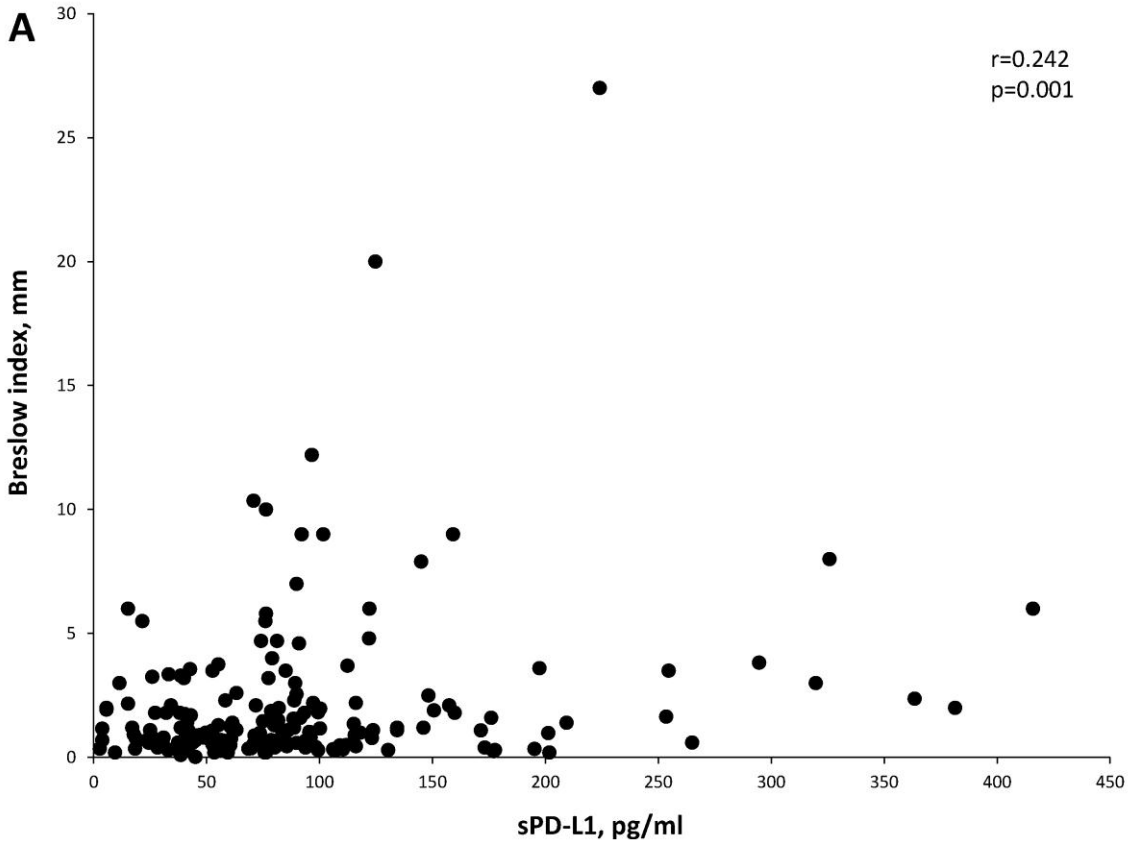
Figure 3. Relationship of the serum levels of soluble PD-L1 with the Breslow index (A) and the mitotic index (B) in patients with cutaneous melanoma and OSA

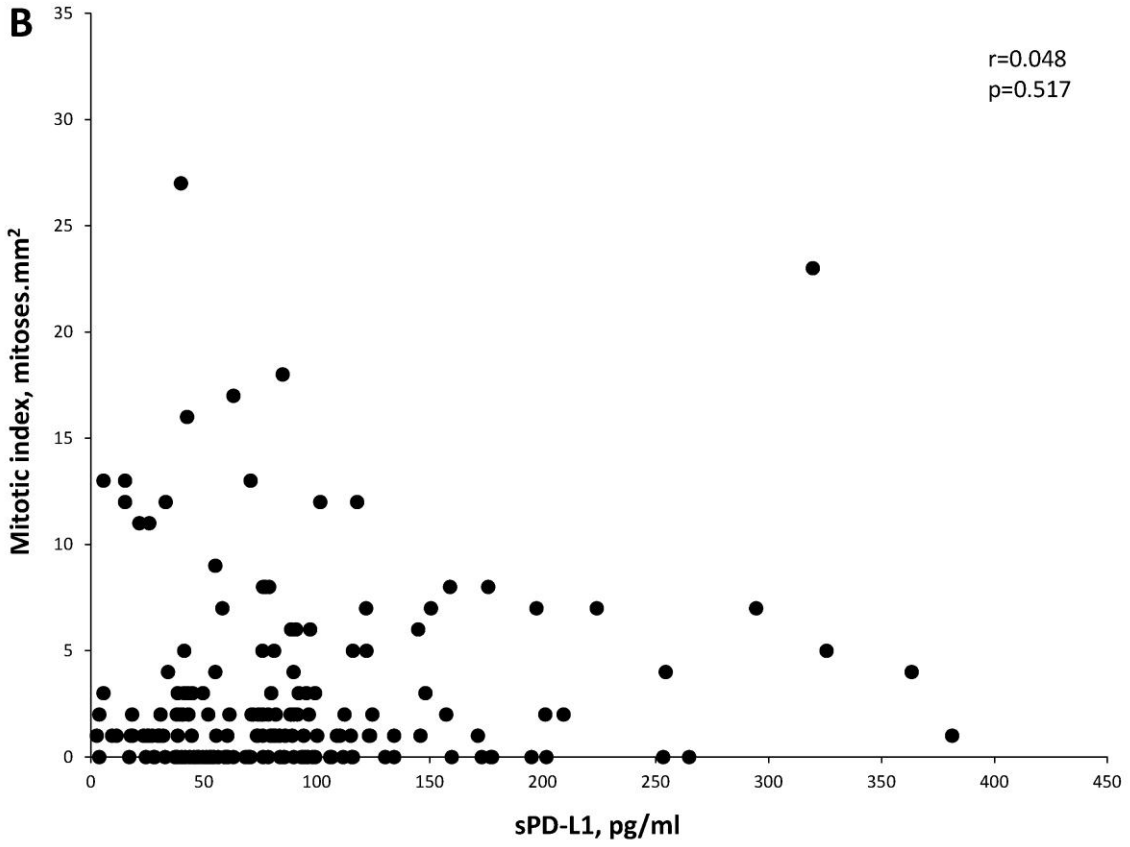
Figure 4. Comparison of the serum levels of soluble PD-L1 according to the cutaneous melanoma thickness in OSA patients. The dark line in the middle of the boxes indicates the median and the length of the box is the interquartile range (IQR). The T-bars correspond to maximum and minimum values. p-value corresponds to between-group comparison using the Kruskal-Wallis test.

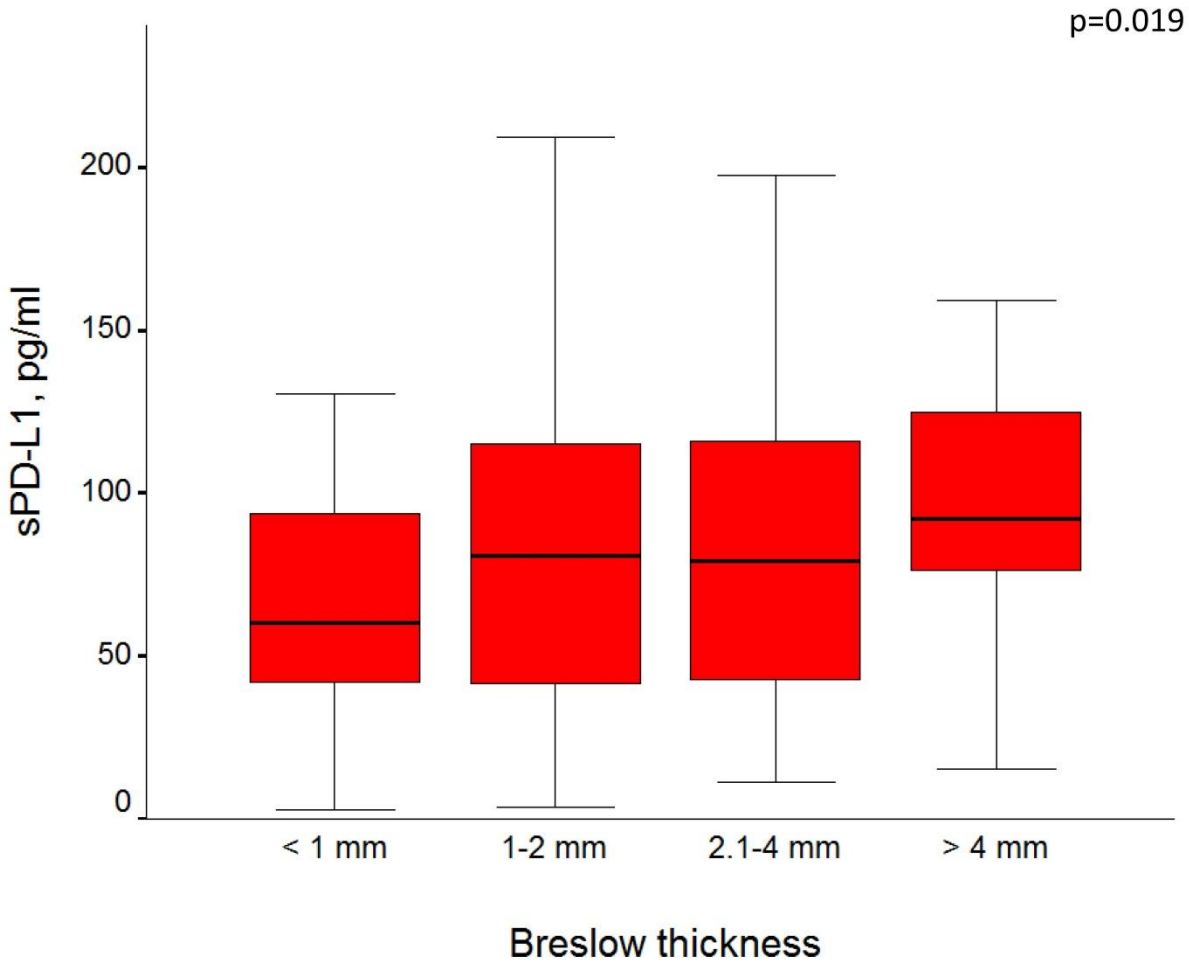
Figure 5. Receiver operating characteristic curves for prediction of sentinel lymph nodule metastasis using the conventional risk factors (age, mitotic rate, Breslow index and Clark levels) alone (green line) or together with the new risk model (which includes serum levels of soluble PD-L1) (red line).


476 eligible patients with confirmed melanoma
Patients excluded (n=35)
Causes of exclusion:
No consent/invalid sleep study (n=11)
More 6 months between the melanoma diagnosis and the sleep study (n=12)
Metastasis (n=4)
"In situ" melanoma (n=1)
441 recruited patients
81 no stored serum sample
360 patients included in the final analysis
Non-OSA (n=132)
Mild OSA (n=109)
Moderate OSA (n=70)
Severe OSA (n=49)


300
200
100
0
sPD-L1, pg/ml
p=0.023*
p=0.008*
p=0.035
No OSA
Mild OSA
Moderate OSA
Severe OSA


A
r=0.242
p=0.001
Breslow index, mm
sPD-L1, pg/ml
0
5
10
15
20
25
30
0
50
100
150
200
250
300
350
400
450


B
r=0.048
p=0.517
Mitotic index, mitoses.mm2
sPD-L1, pg/ml
0
5
10
15
20
25
30
35
0
50
100
150
200
250
300
350
400
450


p=0.019
sPD-L1, pg/ml
200
150
100
50
0
< 1 mm
1-2 mm
2.1-4 mm
> 4 mm
Breslow thickness

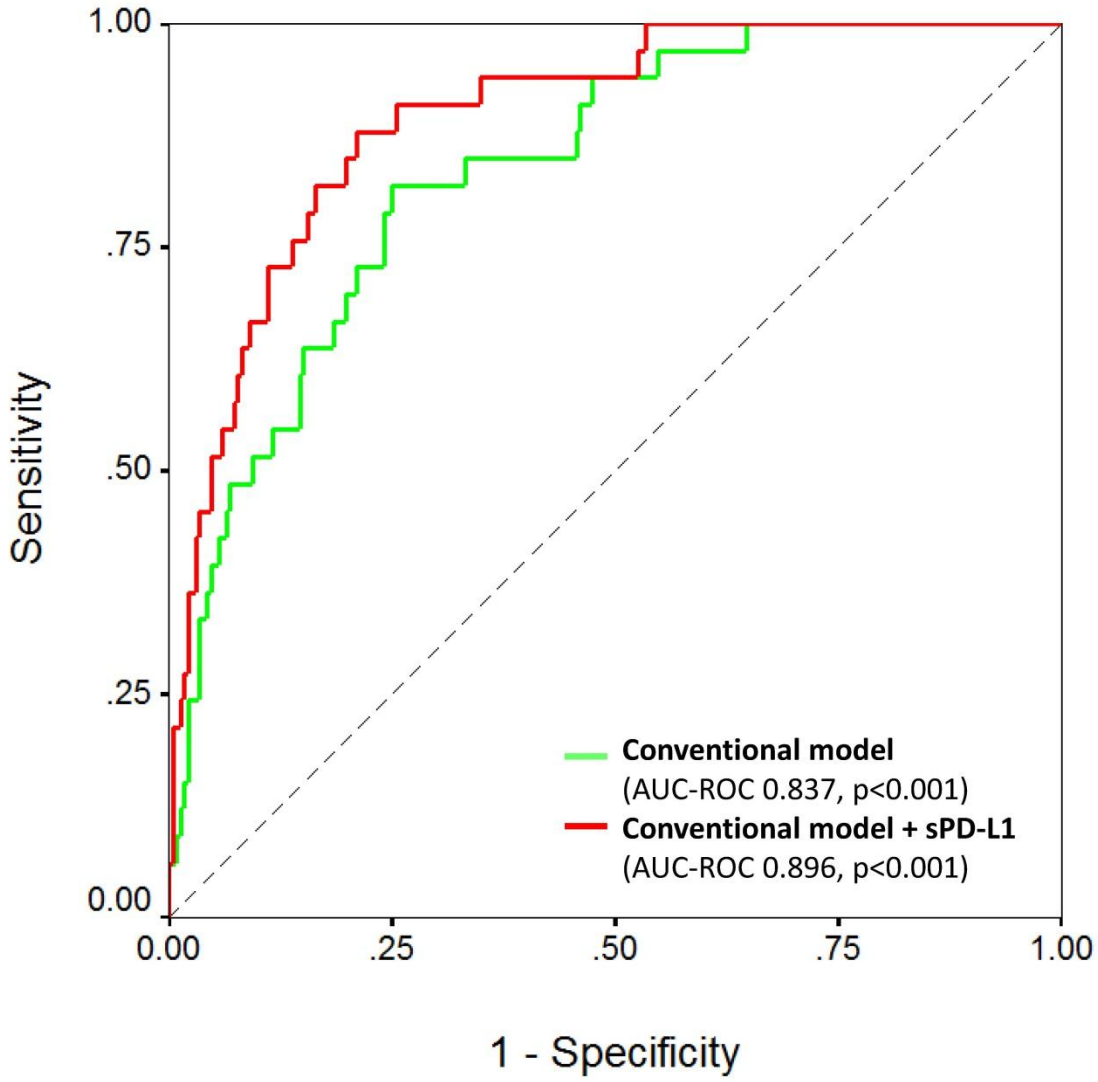

1.00
.75
.50
.25
0.00
Sensitivity
0.00
.25
.50
.75
1.00
1 - Specificity
Conventional model
(AUC-ROC 0.837, p<0.001)
Conventional model + sPD-L1
(AUC-ROC 0.896, p<0.001)

Table S1. Comparison of the serum levels of sPD-L1 according aggressiveness indices of melanoma in patients without sleep apnea*

		n	sPD-L1 level, pg/ml	p value†
Disease stage				0.568
	Localized melanoma	112	63.5 (33.3-83.1)	
	Loco regional disease	12	65.6 (57.9-92.1)	
Breslow index				0.818
	< 1 mm	87	63.5 (34.1-83.9)	
	1-2 mm	23	57.9 (41.6-82.5)	
	2.1-4 mm	11	70.4 (39.2-83.1)	
	>4 mm	3	111.2 (66.1-113.3)	
Tumor ulceration				0.587
	Yes	13	63.2 (53.2-91.2)	
	Non	111	65.0 (33.3-84.8)	
Clark levels				0.419
	Invasion into the papillary dermis	54	65.4 (44.7-86.7)	
	Invasion to the junction of the papillary and reticular dermis	47	65.6 (35.2-84.9)	
	Invasion into the reticular dermis	22	49.7 (22.3-71.2)	
Primary tumor categories				0.944
	T1a	51	58.9 (33.3-73.2)	
	T1b	36	65.0 (38.7-94.7)	
	T2a	19	61.5 (33.6-88.4)	
	T2b	4	57.9 (53.2-59.5)	
	T3a	8	71.2 (45.8-83.0)	
	T3b	3	65.6 (52.4-96.0)	
	T4a	0	-	
	T4b	3	111.2 (66.1-113.3)	

*Data are presented as mean ± SD, median (interquartile range [IQR])

†p values by the Kruskal-Wallis test.

FIGURES

Figure S1. Box plots representing the serum levels of soluble PD-L1 in the different primary tumor categories in patients with OSA. The dark line in the middle of the boxes is the median and the length of the box is the interquartile range (IQR). The T-bars represent maximum and minimum values. p-value corresponds to between-group comparison using the Kruskal-Wallis test.

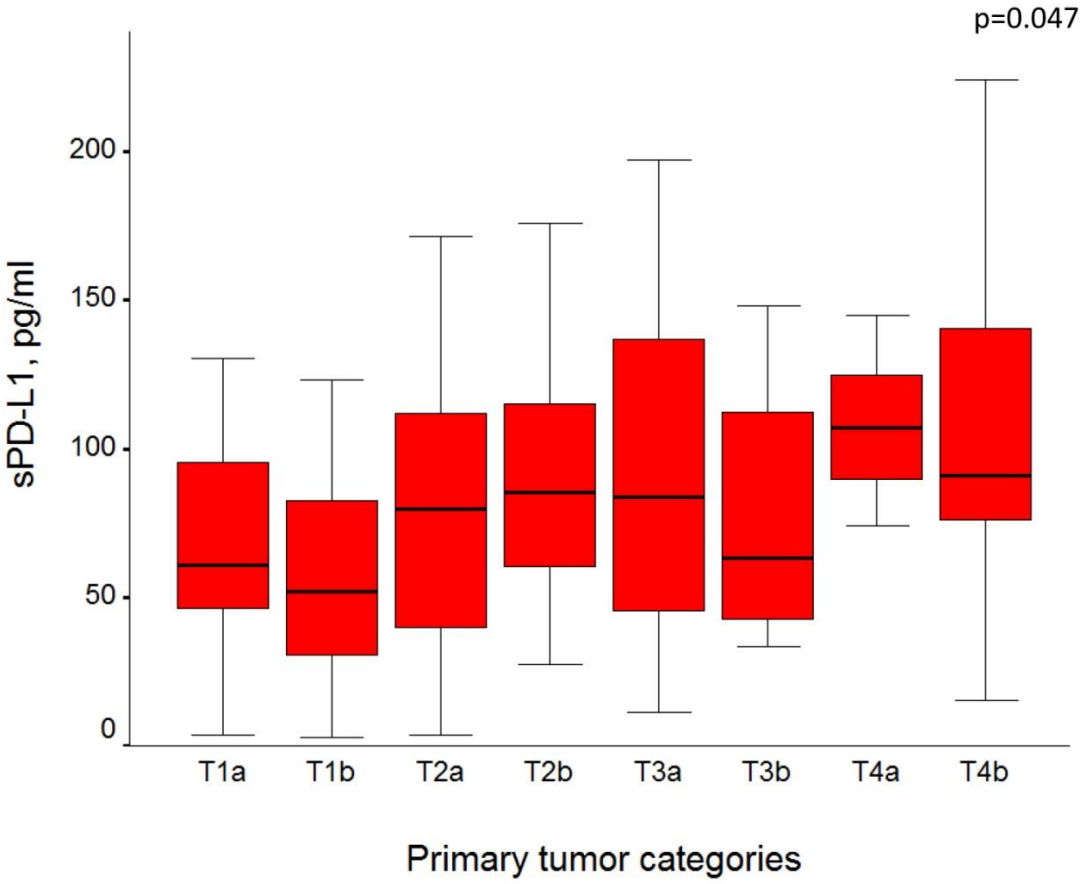


Figure S2. Relationship of soluble PD-L1 serum levels with the Breslow index (r=0.021, p=0.843) (A) and the mitotic index (r=0.071, p=0.5149 (B) in non-OSA patients with melanoma