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Neoadjuvant immune checkpoint blockade (ICB) outperforms adjuvant ICB for treatment of stage IIIB–D melanoma, but potential biomarkers of response, such as interferon-gamma (IFNγ) signature and tumor mutational burden (TMB), are insufficient. Preclinical studies suggest that emotional distress (ED) can negatively affect antitumor immune responses via β-adrenergic or glucocorticoid signaling. We performed a post hoc analysis evaluating the association between pretreatment ED and clinical responses after neoadjuvant ICB treatment in patients with stage IIIB–D melanoma in the phase 2 PRADO trial (NCT02977052). The European Organisation for Research and Treatment of Cancer scale for emotional functioning was used to identify patients with ED (n = 28) versus those without (n = 60). Pretreatment ED was significantly associated with reduced major pathologic responses (46% versus 65%, adjusted odds ratio 0.20, P = 0.038) after adjusting for IFNγ signature and TMB, reduced 2-year relapse-free survival (74% versus 91%, adjusted hazard ratio 3.81, P = 0.034) and reduced 2-year distant metastasis-free survival (78% versus 95%, adjusted hazard ratio 4.33, P = 0.040) after adjusting for IFNγ signature. RNA sequencing analyses of baseline patient samples could not identify clear β-adrenergic- or glucocorticoid-driven mechanisms associated with these reduced outcomes. Pretreatment ED may be a marker associated with clinical responses after neoadjuvant ICB in melanoma and warrants further investigation. ClinicalTrials.gov registration: NCT02977052.

Adjuvant systemic treatment with immune checkpoint blocking antibodies pembrolizumab or nivolumab (anti-PD-1 antibodies), or the BRAF/MEK-inhibitors dabrafenib plus trametinib, have become standard therapies for high-risk stage III melanoma. Although these therapies improved relapse-free survival (RFS), none has shown a statistically significant overall survival benefit so far, even after 5 years follow-up[1–3]. Neoadjuvant immune checkpoint blockade (ICB) has been shown to be superior to adjuvant therapy with regard to RFS and event-free survival

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(EFS) in a phase 1 and a randomized phase 2 trial[4,5]. Several neoadjuvant trials testing neoadjuvant pembrolizumab monotherapy, and especially the combination of neoadjuvant ipilimumab (anti-CTLA-4 antibody) plus nivolumab, have shown high pathologic response rates, RFS, EFS and overall survival (OS) in this high-risk melanoma population[6,7].

The PRADO trial (NCT02977052) is currently the largest completed trial testing neoadjuvant ipilimumab plus nivolumab and the only neoadjuvant immunotherapy trial in melanoma that collected extensive quality-of-life data[7]. This trial confirmed the high pathologic response rates of its predecessor OpACIN-neo trial, achieved by neoadjuvant ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}. In total, 71 of 99 (72%) patients achieved a pathologic response (≤50% viable tumor) in their index lymph node, including 60 (61%) patients with a major pathologic response (MPR; ≤10% viable tumor), allowing for de-escalation of surgery and adjuvant therapy in the latter patient group. This response-directed treatment personalization was associated with superior quality-of-life outcomes. Two-year RFS and distant metastasis-free survival (DMFS) rates remained high for the MPR patients (93% and 98%, respectively)[7].

Molecular biomarkers like tumor mutational burden (TMB) or interferon-gamma (IFNγ) signature have previously been associated with favorable outcomes following neoadjuvant treatment with ipilimumab plus nivolumab, also confirmed in PRADO[8,9]. However, biomarker analyses based on tumor genomics (for example, TMB), transcriptomics (for example, IFNγ signature and other RNA signatures) or tumor immunohistochemistry (for example, PD-L1 expression) do not fully explain ICB efficacy. Recently, more comprehensive views on optimizing ICB therapies have raised the importance of exploring the complexity of ICB responses in relation to environmental, behavioral and psychological factors such as dietary habits, physical activity and psychological distress[10]. In particular, the role of emotional distressED; a negative reaction to stressful factors) is receiving increasing attention, because it affects a large proportion of cancer patients including those with melanoma[11,12]. Emerging evidence from preclinical studies suggests that ED negatively affects tumor progression and antitumor immune responses, mediated by β-adrenergic or glucocorticoid signaling resulting from the sympathetic nervous system or hypothalamic–pituitary–adrenal axis activation[13–17]. With regard to antitumor immune responses, preclinical data have shown that stress can decrease the number and/or effector function of several immune-supportive cells, including $CD8^+$ T cells, its effector cytokines and $CD4^+$ T cells[18–22], and increase the amount of immunosuppressive cells, for example, regulatory T cells[23,24]. ICB efficacy is dependent on these immune-supportive cells, and depletion or neutralization of for example $CD8^+$ and $CD4^+$ cells diminishes this efficacy[25]. As a result, several preclinical models have shown that stress impairs ICB therapy[19–21,26].

We sought to investigate the association between baseline (pretreatment) ED and pathological response and RFS in this post hoc analysis of the PRADO trial, and to explore the role of stress pathways by investigating levels of stress hormones, adrenergic- and glucocorticoid-associated gene pathways, and immune cell populations in the potential association between ED and outcomes.

Results

Patients and baseline characteristics

In the PRADO trial, 99 patients were included, 88 of whom (89%) completed the baseline health-related quality-of-life (HRQoL) questionnaire. All these patients were included in the current analysis and were defined as having ED at baseline before therapy ($n = 28$) or having no ED at baseline ($n = 60$) (Extended Data Fig. 1). There were no significant differences in baseline tumor and clinical patient characteristics between the two cohorts (Table 1). At data cutoff (7 February 2022), the median follow-up from date of registration was 28.1 months (interquartile range (IQR) 25.0–33.8).

ED is associated with impaired MPR rates

Patients with ED were less likely to achieve an MPR after neoadjuvant ipilimumab plus nivolumab compared with patients without ED (46% versus 65%) (Fig. 1a). The overall pathologic response rate was similar between both groups (71% versus 72%, respectively) because patients with ED had a higher rate of partial pathologic responses (>10% to ≤50% viable tumor) compared with patients without ED (25% versus 7%). Baseline clinical and tumor characteristics did not significantly differ between the group with ED ($n = 28$) and the group without ED ($n = 60$) (Table 1). To evaluate the association between ED and MPR we used univariable and multivariable logistic regression analysis. MPR was observed in 52 of 88 patients. Univariable logistic regression analysis showed that a high baseline IFNγ signature (odds ratio (OR) 5.77, 95% confidence interval (CI) 2.03–16.38, $P < 0.01$) and high TMB (OR 10.77 95% CI 3.06–37.93, $P < 0.01$) were significantly associated with MPR (Table 2). Furthermore, although not statistically significant, ED (OR 0.47, 95% CI 0.19–1.16, $P = 0.10$), age (OR per 10 years 1.35, 95% CI 0.99–1.83, $P = 0.05$), sex (OR 2.04, 95% CI 0.97–5.96, $P = 0.06$) and *BRAF* mutation status (OR 0.44, 95% CI 0.18–1.09, $P = 0.08$) showed an indication of an effect. Because of a substantial amount of missing data (45%) in the variables, we used multiple imputation (45 imputed datasets) to increase the power of the complete case analyses. Baseline and clinical characteristics of the complete cases ($n = 48$) were comparable to the noncomplete cases ($n = 88$) (Extended Data Table 1), and results from univariable logistic regression analysis in the imputed dataset were similar (Extended Data Table 2). Based on the results of the univariable analysis in the nonimputed and imputed datasets, ED, age, sex, *BRAF* mutational status, IFNγ signature and TMB were selected as candidate predictors for multivariable logistic regression analysis. From this, the multivariable model including ED, IFNγ signature and TMB minimized the corrected Akaike information criterion (AICc) (Extended Data Table 3). Using these same steps for variable selection in each of the 45 imputed datasets, the model with predictors ED, IFNγ signature and TMB was selected in 44 of them. Multivariable analysis in the nonimputed dataset on complete cases ($n = 64$) showed a statistically significant association between ED and MPR (adjusted odds ratio (aOR) 0.20, 95% CI 0.04–0.92, $P = 0.04$), while adjusting for IFNγ signature (aOR 7.93, 95% CI 2.08–30.29, $P < 0.01$) and TMB (aOR 16.39, 95% CI 3.28–81.92, $P < 0.01$) (Table 2). Multivariable analysis in the imputed dataset ($n = 88$) led to similar results (Extended Data Table 2).

When checking for associations between other clinically impaired European Organisation for Research and Treatment of Cancer (EORTC) QLQ-C30 metrics and MPR, an association between clinically impaired insomnia and MPR (OR 0.24, 95% CI 0.08–0.77, $P = 0.02$) was found. However, insomnia was significantly associated with more ED ($\chi^2$12.30, $P < 0.01$) and is therefore not reported separately.

ED is associated with reduced RFS and DMFS

We have previously shown that pathologic response is a strong surrogate marker for RFS and DMFS[8,27]. Therefore, we analyzed whether ED was also associated with long-term outcomes of the patients. After a median follow-up of 28.0 months (95% CI 25.2–31.0), 19 patients had progressed before surgery ($n = 6$) or relapsed after surgery ($n = 13$). Patients with ED at baseline had a 2-year EFS rate of 71% compared with 83% for patients without ED (Fig. 1b). RFS and DMFS analyses were not possible for patients who had progressive disease before surgery ($n = 6$). These patients were excluded from RFS and DMFS analyses resulting in 82 patients in these cohorts. In EFS analyses these six patients were included.

The 2-year RFS was lower in the group of patients with ED compared with those without ED (74% versus 91%) (Fig. 1c). Likewise, patients with ED also had a significantly reduced DMFS (2-year DMFS 78% versus 95%) (Fig. 1d). To evaluate the association between ED and RFS/DMFS, univariable and multivariable Cox regression analyses were performed. Univariable Cox regression analysis on RFS showed a

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Table 1 | Baseline clinical and tumor characteristics of patients with and without ED (*n*=88)

	ED (*n*=28)	No ED (*n*=60)	*P*
Country, *n* (%)			0.30[a]
The Netherlands	20 (71)	36 (60)	
Australia	8 (29)	24 (40)	
Sex, *n* (%)			0.18[b]
Male	16 (57)	43 (72)	
Female	12 (43)	17 (28)	
Age, median (IQR)	55 (46–70)	61 (52–72)	0.40[c]
Primary tumor stage, *n* (%)			0.49[a]
T1a/b	7 (25)	9 (15)	
T2a/b	9 (32)	15 (25)	
T3a/b	3 (11)	15 (25)	
T4a/b	5 (18)	10 (17)	
Unknown Tx	1 (4)	1 (2)	
Unknown primary (MUP)	3 (11)	10 (17)	
Ulceration of primary tumor, *n* (%)			0.78[a]
Yes	7 (25)	13 (22)	
No	16 (57)	35 (58)	
Unknown	10 (18)	12 (20)	
Location of affected LN, *n* (%)			0.62[b]
Neck	6 (21)	15 (25)	
Axilla	10 (36)	26 (43)	
Groin	12 (43)	19 (32)	
Number of positive LN on PET–CT, *n* (%)			0.58[b]
1	15 (54)	37 (62)	
>1–3	11 (39)	17 (28)	
>3	2 (7)	6 (10)	
Sum of diameter target lesions, mm (median, IQR)	26 (19–36)	24 (18–33)	0.50[d]
BRAF V600E/K mutation, *n* (%)			0.85[b]
Positive	11 (39)	26 (43)	
Negative	13 (47)	28 (47)	
Unknown	4 (14)	6 (10)	
IFNγ signature, *n* (%)			0.80[b]
High	11 (39)	21 (35)	
Low	12 (43)	26 (43)	
Unknown	5 (18)	13 (22)	
TMB, *n* (%)[b]			0.31[b]
High	10 (36)	14 (23)	
Low	12 (43)	29 (48)	
Unknown	6 (21)	17 (28)	

[a]Fisher's exact test. [b]Chi-square test. [c]Independent samples *t*-test. [d]Wilcoxon rank-sum test. Missing/unknown values were not taken into account when calculating *P* values and proportions. LN, lymph node; MUP, melanoma of unknown primary; PET–CT, positron emission tomography–computed tomography.

significantly increased hazard of relapse/death for patients with ED versus those without ED (hazard ratio (HR) 3.78, 95% CI 1.23–11.56, $P = 0.02$) (Table 3). Other variables of interest showed no significant association with RFS, although IFNγ signature showed an indication of an effect (HR 0.35, 95% CI 0.09–1.34, $P = 0.13$). Univariable Cox regression analysis on DMFS also showed a significantly increased hazard of distant relapse/death for patients with ED versus those without ED (HR 5.20, 95% CI 1.34–20.13, $P = 0.02$) (Table 4). In line with RFS analyses, other variables of interest also showed no significant association with DMFS, whereas IFNγ signature showed an indication of an effect (HR 0.27, 95% CI 0.06–1.29, $P = 0.10$). Based on the a priori assumption that IFNγ is a predictor of response and survival in this patient group, we included ED and IFNγ in multivariable analyses. Multivariable analysis in the non-imputed dataset on complete cases ($n = 64$) showed a statistically significant association between ED and RFS (adjusted hazard ratio (aHR) 3.81, 95% CI 1.11–13.16, $P = 0.04$), while adjusting for IFNγ signature (aHR 0.35, 95% CI 0.09–1.33, $P = 0.12$) (Table 3). Furthermore, a statistically significant association was found between ED and DMFS (aHR 4.38, 95% CI 1.09–17.55, $P = 0.04$), while adjusting for IFNγ signature (aHR 0.26, 95% CI 0.05–1.25, $P = 0.09$) (Table 4). Similar to the MPR analyses, there were 45% missing values in the possible candidate predictors for survival analyses. Multiple imputation (45 imputations) was done and Cox regression analyses were repeated in the imputed dataset. Results after multiple imputation were similar for both RFS (Extended Data Table 4) and DMFS (Extended Data Table 5).

Overall, multivariable survival analyses were hampered by the small number of events. Proportional hazard assumptions were not violated in both RFS and DMFS models.

Effects of ED on tumor microenvironment

We next sought to identify the underlying mechanisms by which ED is associated with impaired outcomes after ICB. Stress causes the release of stress hormones like glucocorticoids, adrenaline and noradrenaline[13,14], with an implied association with inflammation, immune evasion and reduced immunotherapy efficacy in animal models[14,15,18–21,26]. Therefore, we assessed the baseline cortisol levels, hormone signaling pathway gene expressions and their potential effects in baseline blood samples and tumor biopsies of our patients.

Data on baseline cortisol levels in peripheral blood samples were available for 26 patients with ED and 53 patients without ED. Because cortisol blood levels follow a strong circadian rhythm, and patients had their baseline blood samples drawn at different times of the day, we analyzed the association between ED status and cortisol levels while adjusting for time of blood withdrawal using a linear regression model. Although numerically higher baseline cortisol levels were observed between patients with ED versus those without ED when adjusting for time of blood withdrawal (mean difference 39.9 nmol l^{-1}, $P = 0.079$), this difference was only marginal and seemed mainly driven by a few patients with ED having high cortisol levels early in the morning (Extended Data Fig. 2a,b). We next assessed whether there were differences in the baseline peripheral blood composition between both patient cohorts. When analyzing absolute counts of several immune cell subsets (for example, lymphocytes, neutrophils, monocytes), immune cell ratios (for example, neutrophil-to-lymphocyte ratio, monocyte-to-lymphocyte ratio), the systemic immune–inflammation index and C-reactive protein, no differences were found between patients with and without ED (Extended Data Table 6), and no association with MPR (Extended Data Table 7).

Because most animal models studying the association between stress and antitumor immune responses focused on changes in the tumor, we finally analyzed the tumor microenvironment. Bulk RNA sequencing data of baseline tumor samples were available for 70 patients ($n = 23$ patients with ED, $n = 47$ patients without ED). Gene set enrichment analysis (GSEA) using gene sets based on the Gene Ontology database and focusing on adrenergic and glucocorticoid signaling pathways showed no significantly upregulated pathways in patients with versus without ED or vice versa (Extended Data Fig. 2c). Comparison of genes associated with inflammation showed no differences in gene expression of cyclooxygenase-2 (prostaglandin endoperoxide synthase 2), prostaglandin E2 and interleukin-6 in the tumor (Extended

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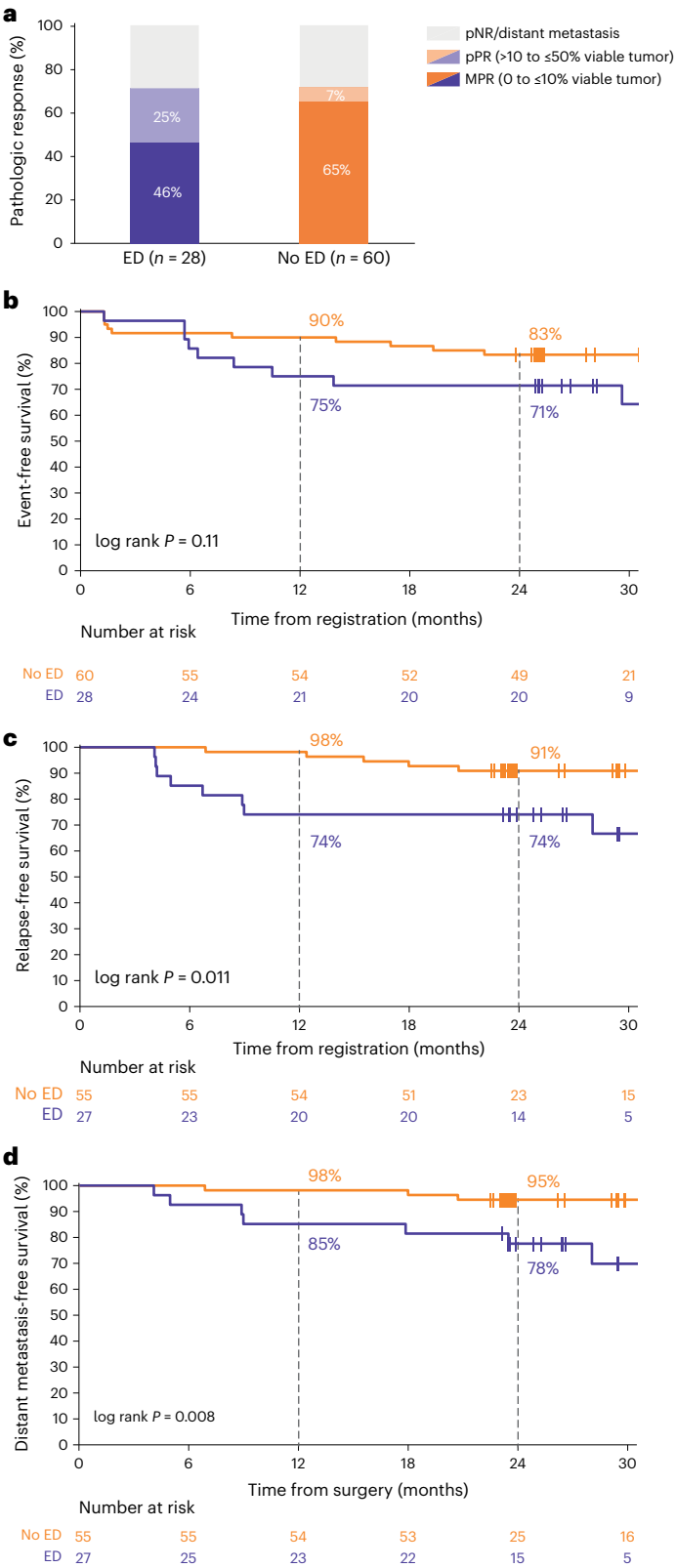

Fig. 1 | Pathologic response and survival of PRADO according to ED status. **a**, Pathologic response of the patients in the PRADO trial with ED ($n = 28$) and without ED ($n = 60$) based on International Neoadjuvant Melanoma Consortium criteria. **b**, EFS for all patients in this analysis ($n = 88$) by ED status. **c**, RFS for all patients without event before surgery ($n = 82$) by ED status. **d**, DMFS for all patients without event before surgery ($n = 82$) by ED status. **a**–**d**, Patients were emotionally distressed (purple) or not emotionally distressed (orange) at baseline. **b**–**d**, *P* values were calculated using a two-tailed log-rank test. pPR, partial pathologic response.

Data Fig. 3a–c). Furthermore, using the Microenvironment Cell Populations (MCP) counter method, we found no statistically significant differences in abundance of the eight immune cell and two stromal cell populations in the tumor between the two ED cohorts (Fig. 2a). In particular, no significant difference in $CD8^+$ T cell ($P = 0.3522$) and cytotoxic lymphocyte ($P = 0.6000$) abundance was found.

Considering that preclinical evidence has shown that β2-adrenergic signaling reduced mainly the effector $CD8^+$ tumor-infiltrating lymphocyte frequency rather than the total $CD8^+$ T cell population[21], we next assessed factors associated with $CD8^+$ T cell activity. We showed that the baseline IFNγ signature was not significantly different between patients with and without ED (Table 1 and Fig. 2b). Investigation in T cell activity markers yielded conflicting results, with certain activation markers showing a significant or numerical reduction in patients with ED (for example, CD69, $P = 0.0257$; HLA-DR, $P = 0.0625$; CD25, $P = 0.1084$), whereas other markers were unaffected (for example, PD1, $P = 0.54$; CD44, $P = 0.53$) (Fig. 2c–e and Extended Data Fig. 3d–j). In conclusion, no clear β-adrenergic- or glucocorticoid-driven mechanisms associated with reduced outcomes after ICB in patients with ED could be identified.

Discussion

In our homogeneous PRADO patient cohort of stage III melanoma patients treated with neoadjuvant ICB, we found that patients with ED before treatment had a reduced MPR (46% versus 65%) and significantly impaired 2-year RFS (74% versus 91%) and 2-year DMFS (78% versus 95%) compared with patients without ED and after adjusting for the previously identified baseline biomarkers (IFNγ signature and TMB).

Our findings are supported by two other more heterogeneous patient cohorts that tested ICB in patients with advanced nonsmall cell lung cancer[28,29]. Both Bi et al.[29] and Wu et al.[28] found that the objective response rate (ORR) was significantly lower in the group of patients with psychological distress compared with those without psychological distress (ORR 6.0% versus 18.5%, $P < 0.05$ and ORR 35.9% versus 63.6%, $P = 0.033$, respectively). Moreover, both studies demonstrated a significantly reduced progression-free survival (PFS) in patients with psychological distress (HR for nondistressed group 0.34, 95% CI 0.19–0.59, $P < 0.001$ (ref. 29) and HR for distressed group: 2.71, 95% CI: 1.06–6.90, $P = 0.037$ (ref. 28)), and according to Wu et al.[28] psychological distress remained the only independent predictor for PFS in multivariable Cox regression analyses[28,29].

Next to these small clinical trials, emerging evidence from animal studies suggests that stress can assist tumor growth and metastasis of numerous cancer types via adrenergic or glucocorticoid signaling and their corresponding receptors on tumor and immune cells[14–17], and that stress may influence the efficacy of anticancer therapies. More specifically, studies have shown that stress affects the tumor and its microenvironment by promoting tumor cell initiation[30,31], proliferation and metastasis[32,33], angiogenesis[34,35], inflammation[13,36] and immune evasion[18–23,37–42].

Besides the direct effects of stress on tumor progression, one may argue that stress could also be a consequence of poor tumor characteristics that impair prognosis (for example, large tumor burden, paraneoplastic syndromes, inflammation). However, in our patients with ED versus those without ED, tumor characteristics were not different at baseline. Therefore, the association of impaired outcome upon ICB in patients with ED led us to hypothesize that the effects of stress on the immune cell compartment (rather than tumor cell-associated effects) primarily drove these differences in response and survival.

Indeed, previous work has shown that β-adrenergic and glucocorticoid receptors are expressed on immune cells and regulate antitumor immune responses[15,17,43,44]. In murine models, stress induced suppressed natural killer (NK) cell cytotoxicity against tumor cells[45], which was mainly driven by β-adrenergic signaling[37], whereas endogenous or exogenous corticosterone suppressed NK cell cytotoxicity

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Table 2 | Univariable and multivariable logistic regression analysis of MPR in total patient population including missing values

	Univariable			Multivariable[a]	
	N_{MPR}/N_{total}	OR (95% CI)	*P*	aOR (95% CI)	*P*
Emotional distress					
No ED	13/28	Reference		Reference	
ED	39/60	0.47 (0.19–1.16)	0.10	0.20 (0.04–0.92)	0.04
Country					
Australia	20/32	Reference			
The Netherlands	32/56	0.80 (0.34–1.95)	0.62		
Age (per 10 years)	52/88	1.35 (0.99–1.83)	0.05		
Sex					
Female	13/29	Reference			
Male	39/59	2.40 (0.97–5.96)	0.06		
T stage					
T1/2	23/40	Reference			
T3/4	19/33	1.00 (0.39–2.55)	1.00		
MUP	10/13	2.46 (0.59–10.34)	0.22		
Ulceration					
No	29/51	Reference			
Yes	11/20	0.93 (0.33–2.63)	0.89		
Number of positive LN					
1	29/52	Reference			
>1	23/36	1.40 (0.59–3.36)	0.45		
BRAF V600E/K					
Negative	26/41	Reference			
Positive	16/37	0.44 (0.18–1.09)	0.08		
IFNγ signature					
Low	13/38	Reference		Reference	
High	24/32	5.77 (2.03–16.38)	<0.01	7.93 (2.08–30.29)	<0.01
TMB					
Low	13/41	Reference		Reference	
High	20/24	10.77 (3.06–37.93)	<0.01	16.39 (3.28–81.92)	<0.01

N_{MPR}, number of patients with MPR; N_{total}, number of patients in subgroup. [a]Final multivariable model included only patients without missing data (n=64).

Table 3 | Univariable and multivariable Cox regression analysis of RFS in patients in total patient population including missing values

	Univariable			Multivariable[a]	
	$N_{relapse}/N_{total}$	HR (95% CI)	*P*	aHR (95% CI)	*P*
Emotional distress					
No ED	8/27	Reference		Reference	
ED	5/55	3.78 (1.23–11.56)	0.02	3.86 (1.13–13.21)	0.03
Country					
Australia	5/30	Reference			
The Netherlands	8/52	0.87 (0.28–2.71)	0.82		
Age (per 10 years)	13/82	1.15 (0.78–1.71)	0.47		
Sex					
Female	5/27	Reference			
Male	8/55	0.75 (0.25–2.31)	0.63		
T stage					
T1/2	5/37	Reference			
T3/4	6/30	1.59 (0.49–5.22)	0.44		
MUP	2/13	1.18 (0.36–6.10)	0.84		
Ulceration					
No	8/48	Reference			
Yes	3/17	1.07 (0.55–2.07)	0.85		
Number of positive LN					
1	7/48	Reference			
>1	6/34	1.20 (0.40–3.59)	0.74		
BRAF V600E/K					
Negative	6/41	Reference			
Positive	7/31	1.62 (0.54–4.82)	0.39		
IFNγ signature					
Low	8/32	Reference		Reference	
High	3/32	0.35 (0.09–1.34)	0.13	0.35 (0.09–1.33)	0.12
TMB					
Low	7/36	Reference			
High	4/23	0.96 (0.28–3.29)	0.95		

$N_{relapse}$, number of patients with relapse event. [a]Final multivariable model included 64 patients for complete case analysis.

mostly secondarily to the NK-suppressing impact of epinephrine and/or prostaglandins[38]. Moreover, psychological stress and exposure to glucocorticoids were shown to inhibit the maturation of immature dendritic cells (DCs) in vivo and in vitro, subsequently affecting the priming of T helper 1 (T_H1) cells and $CD8^+$ T cells and reducing IFNγ production in the tumor microenvironment[18,39]. Yang et al. demonstrated that stress-induced elevated endogenous glucocorticoid signaling or exogenous glucocorticoids repressed type I IFN responses in DCs and $IFN\gamma^+$ T cell activation via upregulation of the expression of glucocorticoid-inducible factor TSC22d3 (ref. 19). $CD8^+$ T cell cytotoxicity was shown to be impaired by glucocorticoid signaling or adrenergic signaling, demonstrating an increase of terminally exhausted T cells expressing checkpoints PD-1, TIM-3 and LAG-3, an increase of pro-inflammatory cytokines (IL-10), and decrease of effector cytokines (IFNγ, IL-2, tumor necrosis factor)[20–22]. Of note, Bucsek et al. showed that β2-adrenergic signaling reduced the frequency of effector $CD8^+$ tumor-infiltrating lymphocytes, but had no effect on the total $CD8^+$ tumor-infiltrating lymphocyte population[21].

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Table 4 | Univariable and multivariable Cox regression analysis of DMFS in patients in total patient population including missing values

		Univariable		Multivariable[a]	
	N_{dm}/N_{total}	HR (95% CI)	*P*	aHR (95% CI)	*P*
Emotional distress					
No ED	7/27	Reference		Reference	
ED	3/55	5.20 (1.34–20.13)	0.02	4.38 (1.09–17.55)	0.04
Country					
Australia	5/30	Reference			
The Netherlands	5/52	0.49 (0.14–1.73)	0.27		
Age (per 10 years)	10/82	1.10 (0.71–1.69)	0.67		
Sex					
Female	4/27	Reference			
Male	6/55	0.74 (0.21–2.62)	0.64		
T stage					
T1/2	4/37	Reference			
T3/4	5/30	1.58 (0.42–5.87)	0.50		
MUP	1/13	0.72 (0.08–6.43)	0.77		
Ulceration					
No	6/48	Reference			
Yes	3/17	1.22 (0.61–2.43)	0.58		
Number of positive LN					
1	7/48	Reference			
>1	3/34	0.54 (0.14–2.09)	0.37		
BRAF V600E/K					
Negative	5/41	Reference			
Positive	5/31	1.30 (0.38–4.50)	0.68		
IFNγ signature					
Low	7/32	Reference		Reference	
High	2/32	0.27 (0.06–1.29)	0.10	0.26 (0.05–1.25)	0.09
TMB					
Low	6/36	Reference			
High	3/23	0.81 (0.20–3.26)	0.77		

dm, distant metastasis; N_{dm}, number of patients with distant metastasis event. [a]Final multivariable model included 64 patients for complete case analysis.

Next to a decreased number or functions of immune-supportive cells, increased amounts of immunosuppressive cells or functions have also been reported. Psychological stress or a depressed and anxious mood caused a shift from T_H1 to T_H2 cytokine production within the tumor microenvironment of a colon cancer mouse model and ovarian patients, respectively[40,46]. Similar results were observed following β2-adrenergic receptor activation via (nor)epinephrine or pharmacological agonists[47,48] and glucocorticoid signaling[49]. In addition, chronic psychological stress and β2-adrenergic signaling caused an increase in regulatory T cells and myeloid-derived suppressor cells and enhanced their suppressive activity[23,24,42].

Finally, these stress-induced effects on immune cell populations and their effector function have shown to influence immunotherapies, with impaired efficacy of (prophylactic) cancer vaccines[18,50], IL-2 immunotherapy[51] or ICB treatment[19–21,26] in preclinical models.

In our work, the exact mechanism of action driving the differences in ICB outcomes in our melanoma patients remains elusive, because no clear β-adrenergic- or glucocorticoid-driven pathways were identified. β-Adrenergic- or glucocorticoid-associated pathways as measured in the tumor by GSEA were not significantly associated with ED. Yet, we acknowledge that single-cell RNA sequencing rather than bulk RNA sequencing would have been more appropriate for deciphering the effects of stress on the tumor-infiltrating immune cell compartment, because tumor-specific immune cells comprise only a small proportion of the total cell population in a biopsy and (minor) immune modulatory effects might therefore have been missed[52,53]. We also did not identify changes in immune cell abundance using the MCP counter method. This is in line with preclinical data suggesting no changes in the frequency of $CD8^+$ tumor-infiltrating lymphocytes between propranolol-treated mice and controls, although the relative number of effector $CD8^+$ tumor-infiltrating lymphocytes was significantly elevated[21]. Indeed, we also found some activation markers to be higher in patients without ED. Planned additional analyses encompassing single-cell spatial imaging may provide additional insights in the future.

Although the importance of screening and psychosocial care for cancer patients has long been recognized[54], our findings suggest yet another reason: if it is confirmed in an independent cohort that ED before treatment negatively affects the effectiveness of ICB, this would be an additional reason to systematically screen for ED in patients and evaluate (non)pharmacological interventions that could potentially improve patient outcomes after neoadjuvant ICB.

Pharmacologic blockade of adrenergic signaling by the nonspecific β-blocker propranolol has been shown to significantly and synergistically improve tumor control by anti-PD1 checkpoint blockade in two mice models exposed to mild cold stress[21]. Of note, CD8 T cell depletion reversed this positive synergistic effect. Similar results were observed in another melanoma-bearing mouse model treated with anti-PD1 with or without IL-2 in combination with propranolol[55]. Blockade of β-adrenergic receptors by propranolol also improved the efficacy of HPV16-derived E7 peptide vaccination in an HPV16 E6/E7-driven mouse tumor model[50]. In line with these animal data, a retrospective analysis of the phase 3 trial investigating the adjuvant anti-PD1 inhibitor pembrolizumab versus placebo in high-risk stage III melanoma indicated that patients receiving β-blockers had a larger RFS improvement with adjuvant pembrolizumab compared with those without β-blockers, although the differential prognostic importance did not reach statistical significance[56]. Importantly, propranolol in combination with pembrolizumab has been prospectively tested in a phase 1 dose-finding study in patients with irresectable stage IIIC/IV melanoma (NCT03384836). First results showed that the combination is safe, and preliminary data suggested a synergistic antitumor activity with an objective response observed in seven of nine (78%) patients and median PFS and OS not reached after a median follow-up of 15.6 months[57]. Such a combination could also be tested in the neoadjuvant setting with pathologic response as fast efficacy readout, randomizing patients with ED to either immunotherapy alone or immunotherapy plus propranolol.

The combination of, for example, propranolol with short- and long-term nonpharmacological psychological interventions (both before and after surgery) might also be appropriate, although current available data do not provide insight into potential synergistic effects. Such nonpharmacological supportive care interventions could include mindfulness training, psychoeducation, cognitive behavioral therapy and/or exercise therapy[58–60]. A recent systematic review of

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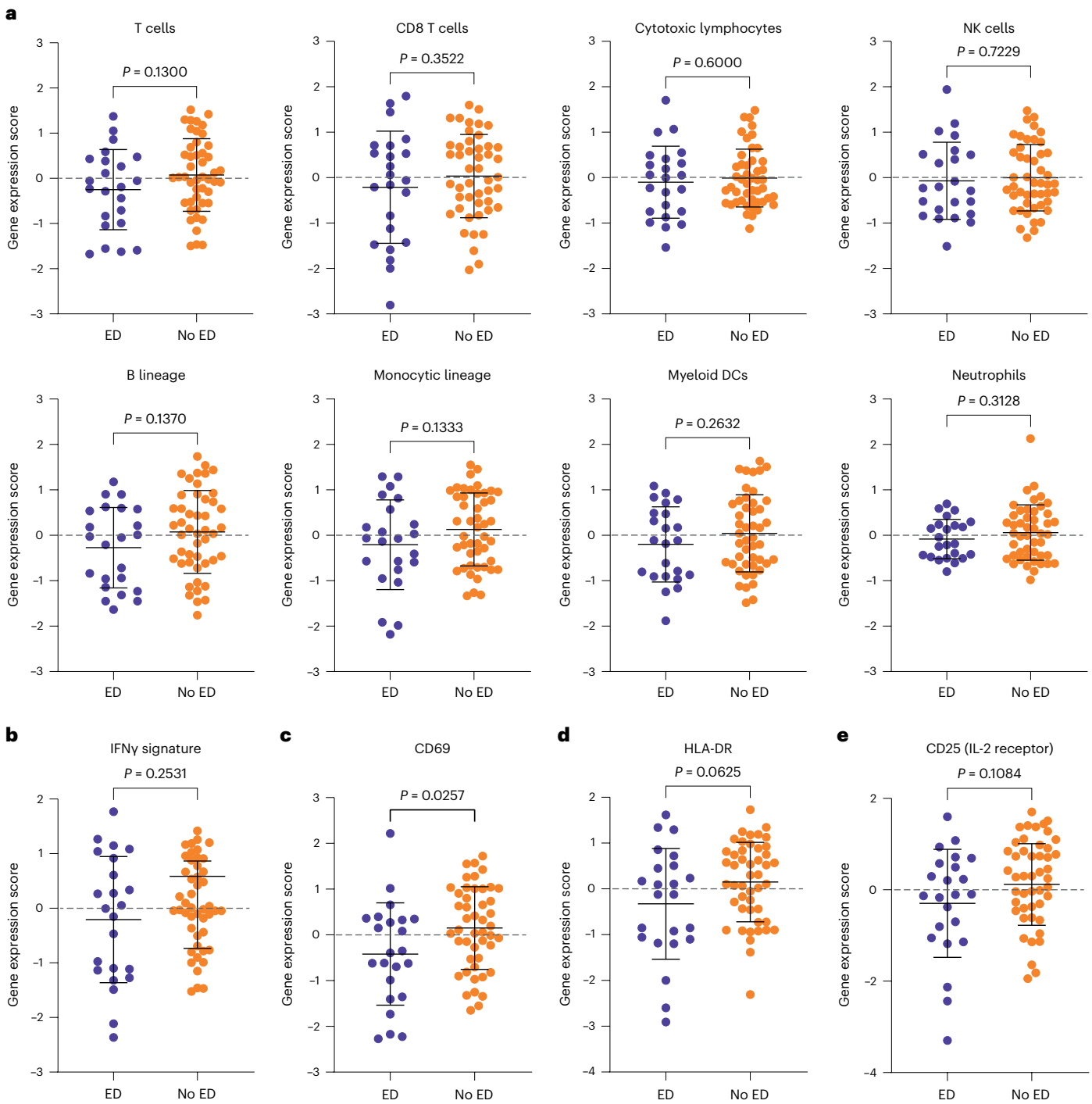

Fig. 2 | Effects of ED on immune cell populations in the tumor microenvironment. a, Differences in immune cell populations between patients with and without ED assessed by the MCP counter method. **b**, IFNγ signature at baseline based on RNA sequencing. **c**–**e**, Activation markers CD69 (**c**), HLA-DR (**d**) and CD25 (**e**) as measured by RNA sequencing. **a**–**e**, Patients with available RNA sequencing data ($n = 23$ patients with ED, $n = 47$ patients without ED) were included. *P* values were calculated using two-tailed unpaired Student's *t*-test. Bars represent mean ± s.d.

nonpharmacological interventions and their effect on stress reduction reported that mindfulness interventions, among others, appear to be effective and appropriate for patients with cancer or survivors, also because they can be taught quickly, self-administered and implemented easily outside medical settings[61].

To our knowledge, our work is the first showing a potential negative association between ED and outcome upon neoadjuvant ICB in early-stage melanoma. It is limited by its relatively small sample size and the absence of an independent confirmation cohort, because the PRADO trial was our first neoadjuvant trial to include quality-of-life assessments. Furthermore, assessment of the ED status before therapy could be improved by the addition of an extra assessment time point or questions inquiring about ED over a longer period. Our finding that insomnia is associated with MPR can be explained by the strong association between insomnia and ED. From the literature it is well known that emotional functioning and insomnia cluster and are interrelated[62]. Furthermore, insomnia is measured as sleeping disturbances with a sole question in the EORTC QLQ-C30. The EORTC QLQ-C30's single-item

sleep scale is less reliable than more comprehensive sleep scales, but large sample sizes can compensate for this[63,64]. In this study, we do not have this large sample size. We therefore decided not to report this separately, but it adds to the value of stress-reducing interventions before start of therapy. Data from the ongoing phase 3 NADINA trial (NCT04949113) comparing neoadjuvant ipilimumab plus nivolumab versus adjuvant nivolumab address some of these limitations, and may be used to confirm our results in the future. In this trial, 420 patients with stage III melanoma and broadly comparable inclusion criteria to those of PRADO will be included, and quality-of-life assessments (additionally including the Cancer Worry Scale and Hospital Anxiety and Depression Scale) are performed at baseline, during treatment and follow-up.

Thus, this study can only be seen as hypothesis-generating because of the abovementioned limitations and also because no exact mechanism of action could be identified. Nevertheless, our observation is in line with animal experiments indicating a role for adrenergic signaling in regulating the antitumor immune response, a retrospective analysis in a similar early-stage melanoma cohort treated with adjuvant ICB and a phase 1 prospective trial evaluating propranolol with ICB. Therefore, if confirmed in another large cohort like the NADINA trial, these data could be the rationale for testing adrenergic receptor blockade and/or psychological interventions in stage III melanoma patients with ED treated with neoadjuvant ICB, for example, in a randomized phase 2 trial treating patients with ICB alone, ICB plus propranolol or ICB plus nonpharmacological interventions.

Online content

Any methods, additional references, Nature Portfolio reporting summaries, source data, extended data, supplementary information, acknowledgements, peer review information; details of author contributions and competing interests; and statements of data and code availability are available at https://doi.org/10.1038/s41591-023-02631-x.

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Methods

Study design and study population

The PRADO trial (NCT02977052) enrolled 99 patients with nodal stage IIIB–D melanoma between November 2018 and January 2020. Inclusion and exclusion criteria have previously been described[7].

Patients were enrolled by local investigators in Australia at the Melanoma Institute Australia (Sydney) and in the Netherlands at the Netherlands Cancer Institute (Amsterdam), Leiden University Medical Center, Erasmus Medical Center (Rotterdam), University Medical Center Utrecht and University Medical Center Groningen. Patients were registered using ALEA software. The medical ethics review committee of the Netherlands Cancer Institute and ethical committees at the Melanoma Institute Australia approved the trial. The trial was conducted in accordance with the protocol and Good Clinical Practice Guidelines as defined by the International Conference on Harmonization and the Declaration of Helsinki. All participating patients provided written informed consent before enrollment.

Patients received two cycles of ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1} followed by resection of the index lymph node at week 6, and were subsequently treated with a pathologic response-directed approach; in patients who achieved an MPR (0 to ≤10% viable tumor) the therapeutic lymph node dissection (TLND) was omitted, patients with pPR (>10% to ≤50% viable tumor) underwent TLND and patients with pathologic non-response (pNR) (>50% viable tumor) underwent a TLND plus adjuvant systemic treatment (nivolumab or dabrafenib + trametinib) for 52 weeks with or without local radiotherapy.

Endpoints

Primary endpoints of the PRADO trial were the pathologic response rate and 2-year RFS in patients achieving MPR and pNR. Secondary endpoints were systemic treatment-related toxicity and surgical toxicity, radiologic response rate, DMFS, EFS, OS, HRQoL and biomarker analyses. The current analysis focusing on ED in particular was not described in the protocol and is a post hoc analysis.

Pathologic response was assessed according to International Neoadjuvant Melanoma Consortium guidelines[65]. RFS and DMFS were defined as the time between date of index lymph node resection and date of local (only RFS) or distant melanoma recurrence, or death due to any cause, whichever occurred first. EFS was defined as the time between date of registration and date of melanoma progression (to irresectable stage III or stage IV disease), local or distant melanoma recurrence or death due to any cause, whichever occurred first.

Emotional distress

HRQoL was measured by the EORTC QLQ-C30 questionnaire at baseline, week 6, week 12, week 24, week 36, week 48, week 60 and week 104 (ref. 66). One of the domains in the EORTC QLQ-C30 is emotional functioning, which is measured with four items: "Did you feel tense?", "Did you worry?", "Did you feel irritable?" and "Did you feel depressed?". After transformation, scores ranged from 0 to 100, with higher scores indicating better emotional functioning[67]. Subgroups of patients with good (emotional functioning score >71) or deteriorated (emotional functioning score ≤71) functioning were defined according to established clinically relevant thresholds and referred to as no ED and ED groups, respectively[68,69]. The emotional functioning subscale of the EORTC QLQ-C30 covers both anxiety and depression and it has previously been shown that it is robust to assess psychological distress in different types of cancer patients[70–73]. Furthermore, the EORTC QLQ-C30 emotional functioning subscale is an appropriate screening measure when compared with more in-depth questionnaires such as the Hospital Anxiety and Depression Scale[70]. In 2016, the EORTC has established 'clinical importance' thresholds for the EORTC-C30 to identify symptoms and functional health problems that require a healthcare professional's attention, to increase the interpretability of the questionnaire results and foster its use in daily clinical practice and clinical research. Based on these thresholds (with good sensitivity and specificity[68,69]), the definition of ED has been defined. Patients were excluded from analyses when the baseline questionnaire was not completed. For completeness, other threshold-based functioning and symptom scales, as measured by the EORTC QLQ-C30 at baseline, were checked for their association with pathological response.

Collection of blood and tumor samples

Blood samples for toxicity evaluation were collected at baseline and at each subsequent study visit. Blood samples for isolation of plasma and peripheral blood mononuclear cells were collected at baseline, week 6, week 12 and relapse. Pretreatment tumor biopsies were taken from a lymph node metastasis and were immediately snap frozen or formalin fixed and paraffin embedded.

Peripheral blood analyses

Hematologic measurements were performed locally at each institute and included the absolute measurement of immune cells such as lymphocytes and granulocytes, platelets and C-reactive protein in 10^9 per liter. Based on previous studies, systemic inflammation status was assessed by calculating the neutrophil-to-lymphocyte ratio, platelet-to-lymphocyte ratio, monocyte-to-lymphocyte ratio and systemic immune–inflammation index[74]. Measurement of baseline cortisol levels was also performed at local institutes in μmol l^{-1} or nmol l^{-1}, and collected with corresponding times of blood withdrawal.

RNA and DNA sequencing and analyses

RNA and DNA were isolated from patients with sufficient tumor material based on the pathologist's scoring (≥30% tumor cells on an H&E-stained cryostat frozen section of the tumor sample). RNA and DNA were simultaneously isolated from fresh frozen pretreatment tumor sections (10 μm) with the AllPrep DNA/RNA/miRNA Universal isolation kit (Qiagen, catalog no. 80224) using the QIAcube, according to the manufacturer's protocol. Germline DNA was isolated from peripheral blood mononuclear cells using the AllPrep DNA/RNA/miRNA Universal isolation kit (Qiagen, catalog no. 80224) to be able to filter out single nucleotide polymorphisms when determining TMB. In total, data of $n = 70$ and $n = 65$ patients with known baseline ED status were available for RNA and DNA sequencing analyses, respectively.

Messenger RNA sequencing was performed by CeGaT. Initial sample quality control was performed with Qubit RNA (Thermo Fisher) and Bioanalyzer RNA (Agilent). Strand-specific libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina) according to the manufacturer's instructions, and sequenced with 2× 100 base pair (bp) reads on the NovaSeq 6000 system. Demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (v.2.20). Adapters were trimmed with Skewer (v.0.2.2)[75]. The quality of FastQ files was analyzed with FastQC (v.0.11.5-cegat)[76]. FastQ files were mapped to the human reference genome (GRCh38.v82) using STAR (v.2.7.3a) with default settings[77]. Count data generated with HTseq-count (v.0.12.4)[78] were analyzed with DESeq2 (v.1.38.2)[79]. For downstream analysis the data were analyzed using R (v.4.2.2). Centering of the normalized gene expression data for each dataset was performed by subtracting the row means and scaling by dividing the columns by the standard deviation. Next, the previously defined IFNγ signature[80], MCP counter (using MCPcounter v.1.1.0)[81] and activation markers were analyzed. GSEA (using fgsea v.1.24.0) was performed to identify gene sets from the Gene Ontology database (v.2023.1, accessed on 17 March 2023) (https://doi.org/10.5281/zenodo.2529950) that were significantly enriched in patients with ED compared with patients without ED. The ranking of the genes was computed with apeglm (v.3.16)[82]. The normalized enrichment scores and other statistics were computed by fgsea with minimum gene set size of 6 and 1,000 permutations.

Whole-exome sequencing was also performed by CeGaT. Exome libraries were generated using the Twist Human Core Exome Plus (Twist Biosciences), according to the manufacturer's instructions. The libraries were sequenced with 100-bp reads on a NovaSeq 6000 system according to the manufacturer's protocols, with a sequence quality Q30 value of >90%. Data were analyzed in CeGaT exome analysis pipeline. Briefly, demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (v.2.20). Adapters were trimmed with Skewer (v.0.2.2). The quality of FastQ files was analyzed with FastQC (v.0.11.5-cegat). Subsequent FastQ files were aligned to the human reference genome (GRCh38) using Burrows–Wheeler aligner (v.0.7.12), followed by marking of duplicate reads by the MarkDuplicates tool in Picard (v.1.140). Subsequently, base quality scores were recalibrated using BaseRecalibrator in GATK (v.4.0.6.0) and single-nucleotide variants were called using MuTect2 in GATK39. The TMB was calculated by summarizing the total number of nonsynonymous, somatic mutations per sample with minimal variant allele frequency of 0.05 (5%).

Statistical analysis

Differences between variables of interest in patients with or without ED were checked using Pearson's chi-squared test (Fisher's exact test in case of sparse data) for categorical variables, and the independent samples *t*-test or Wilcoxon rank-sum test, depending on distributional assumptions, for continuous variables. The cutoffs for IFNγ signature and TMB high and low status have been previously assessed on the total PRADO cohort using a receiver operating characteristic curve. The cutoff for IFNγ signature (mean score of the ten genes) was 0.054 and for TMB was 424 nonsynonymous mutations.

We applied univariable and multivariable logistic regression models that investigated the association between all baseline and clinical baseline variables and MPR. Multivariable logistic regression analysis included ED and the known baseline biomarkers predictive for response and survival, IFNγ and TMB. Additional clinical and baseline variables with $P < 0.10$ in the univariable regression analysis were also considered for inclusion in the multivariable model. AICc was used to compare all possible models combining the aforementioned predictors and select the one with the lowest AICc. Similarly, associations between ED and survival outcomes (EFS, RFS, DMFS) were examined using univariable and multivariable Cox proportional hazard regression models. For RFS and DMFS analyses, patients who had an event before surgery were also excluded, resulting in $n = 82$ patients. Multivariable Cox regression analyses were done including ED and IFNγ as predetermined predictor. For TMB, no association with survival has been found in the existing literature[9], and given the limitations in the number of events available for the survival analyses in our study, it was excluded from the multivariable regression analysis. The Kaplan–Meier method was used to generate EFS, RFS and DMFS curves, and Cox regression analyses for overall comparisons across ED status were done, displaying estimated HRs and their corresponding 95% CIs.

The reverse Kaplan–Meier method was used for computing median follow-up time. Schoenfeld residuals were inspected to verify model assumptions. The association between ED status and baseline cortisol levels was examined while adjusting for time of blood withdrawal, using a linear regression model with cortisol as outcome variable and ED status and draw time as independent variables.

Information on all candidate predictors was not always available in our dataset. As part of sensitivity analyses, Markov Chain Monte Carlo multiple imputation was done, assuming data were missing at random. This assumption was considered plausible because missing data were often related to the availability of suitable tumor biopsy material for genetic analysis rather than to the missing values themselves or to unobserved characteristics. Because there were approximately 45% incomplete cases in the data, we generated 45 imputed datasets. Descriptive and Cox/logistic regression analyses were performed using Stata v.15.1 software (StataCorp) or IBM SPSS Statistics v.27. Kaplan–Meier plots and RNA sequencing analyses were performed in GraphPad Prism (v.9.3.0), R (v.4.2.2) and R Studio (v.1.2.1335) using the packages lubridate (v.1.9.1), dplyr (v.1.0.10), ggplot2 (v.3.4.0), survival (v.3.4.0), survminer (v.0.4.9), DESeq2 (v.1.38.2), tidyverse (v.1.3.2), biomaRt (v.2.54), Biobase (v.2.58.0), RColorBrewer (v.1.1-3), heatmap.3 (github/lwaldron/LeviRmisc/), fgsea (v.1.24.0), mMCPcounter (v.1.1.0) and immunedeconv (v.2.1.0). Dot plots and bar plots were generated in GraphPad Prism (v.7.03).

Reporting summary

Further information on research design is available in the Nature Portfolio Reporting Summary linked to this article.

Data availability

RNA sequencing data generated during the study will be deposited in the European Genome-phenome Archive (EGA) under the accession code EGAS00001007601. To minimize the risk of patient re-identification, de-identified individual patient-level clinical data are available under restricted access. Upon scientifically sound request, data access can be obtained via the Netherlands Cancer Institute's (NKI) scientific repository at repository@nki.nl, which will contact the corresponding author (L.V.v.d.P.-F.). Data requests will then be reviewed by the institutional review board of the NKI and will require the requesting researcher to sign a data access agreement with the NKI.

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Author contributions

L.V.v.d.P.-F. designed the clinical emotional distress analysis. C.U.B. designed the clinical trial and wrote the trial protocol. G.V.L. reviewed the protocol. I.F., I.L.M.R., M.G., A.M.M.M., E.K., A.A.M.v.d.V., K.P.M.S., G.A.P.H., G.V.L. and C.U.B. recruited and treated patients and/or collected data. A.B. coordinated patient tumor sample processing and biobanking. I.F. and I.L.M.R. performed statistical analysis of the clinical data. P.D. performed RNA sequencing analyses. I.F., I.L.M.R., C.U.B. and L.V.v.d.P.-F. wrote the first draft of the manuscript. All authors interpreted the data, reviewed the manuscript and approved the final version.

Competing interests

No author has received financial support for the work on this paper, and no medical writer was involved at any stage of the preparation of this paper. I.L.M.R. and P.D. report financial interest in Signature Oncology and will receive some possible revenues if the IFNγ signature is being developed as a clinical companion diagnostic. A.M.M.M. has served on advisory boards for Bristol Myers Squibb (BMS), Merck Sharp & Dohme (MSD), Novartis, Roche, Pierre Fabre and QBiotics. E.K. received honoraria for consultancy/advisory relationships (all paid to the institute) from BMS, Novartis, Merck, Lilly and Pierre Fabre, and received research grants not related to this paper from BMS, Pierre Fabre and Delcath. A.A.M.v.d.V. received compensation for advisory roles and honoraria (all paid to the institute) from BMS, MSD, Merck, Roche, Eisai, Pfizer, Sanofi, Novartis, Pierre Fabre and Ipsen. K.P.M.S. received compensation for advisory roles and honoraria (all paid to the institute) from BMS, MSD, Novartis, Pierre Fabre and Abbvie, and received research funding from Novartis, TigaTx and BMS. G.A.P.H. received compensation for consulting and advisory roles (all paid to the institute) from Amgen, Roche, MSD, BMS, Pfizer, Novartis and Pierre Fabre, and received research grants (paid to the institute) from BMS and Seerave. G.V.L. is consultant advisor for Agenus, Amgen, Array Biopharma, AstraZeneca, Boehringer Ingelheim, BMS, Evaxion, Hexal AG (Sandoz Company), Highlight Therapeutics, Innovent Biologics, MSD, Novartis, Oncosec, PHMR Ltd, Pierre Fabre, Provectus, QBiotics and Regeneron. C.U.B. reports receiving compensation for advisory roles from BMS, MSD, Roche, Novartis, GlaxoSmithKline, AstraZeneca, Pfizer, Eli Lilly, Genmab, Pierre Fabre and Third Rock Ventures, and receiving research funding from BMS, MSD, Novartis, 4SC and NanoString. Furthermore, C.U.B. reports to be co-founder of Immagene BV. All compensations and funding for C.U.B. were paid to the institute, except for Third Rock Ventures and Immagene. The remaining authors declare no competing interests.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-023-02631-x.

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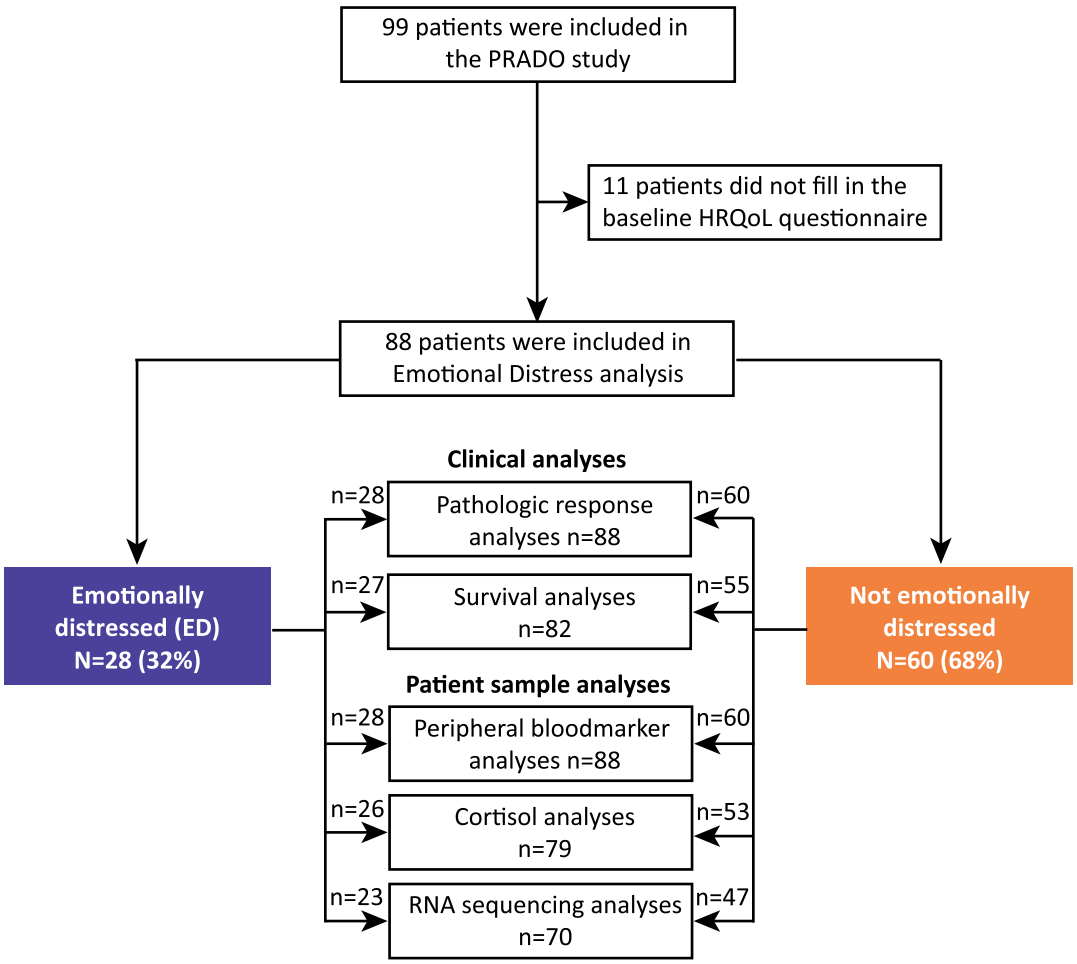

Extended Data Fig. 1 | Flowchart for the Emotional Distress analyses of the PRADO trial. Patients were defined as having emotional distress (emotional functioning score≤71) or no emotional distress (emotional functioning score >71) according to established clinically relevant thresholds using the EORTC QLQ-C30 questionnaire. *HRQoL = health-related quality of life.*

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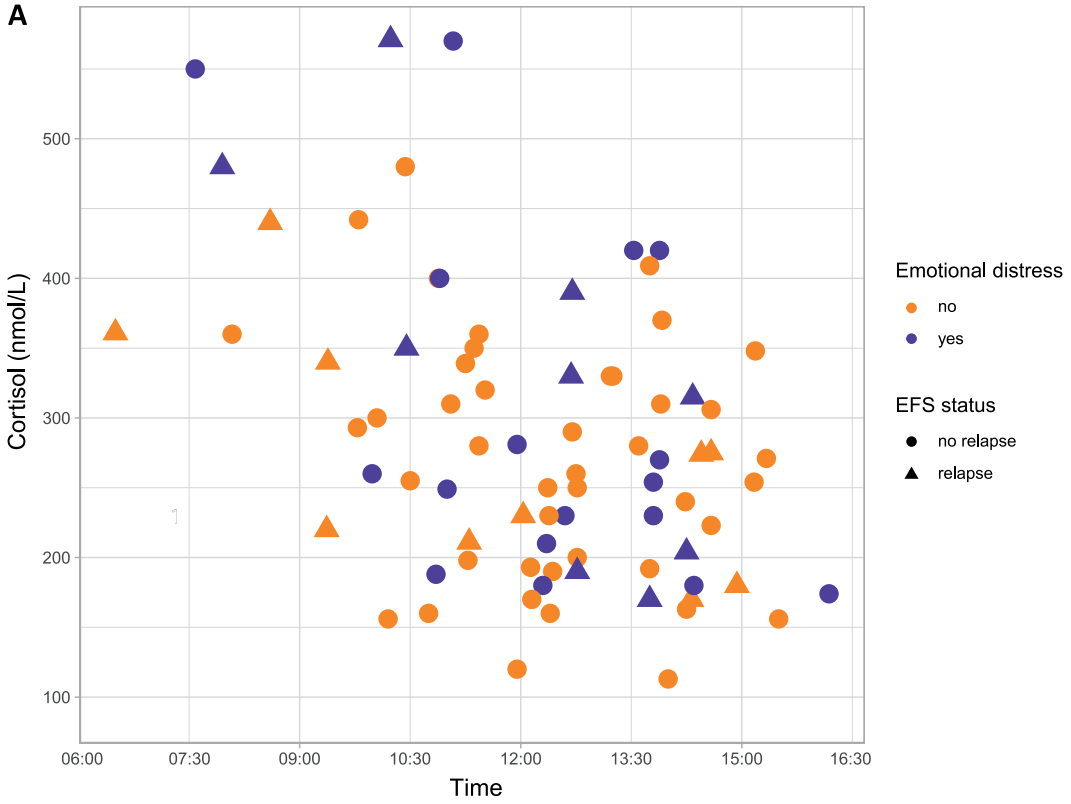
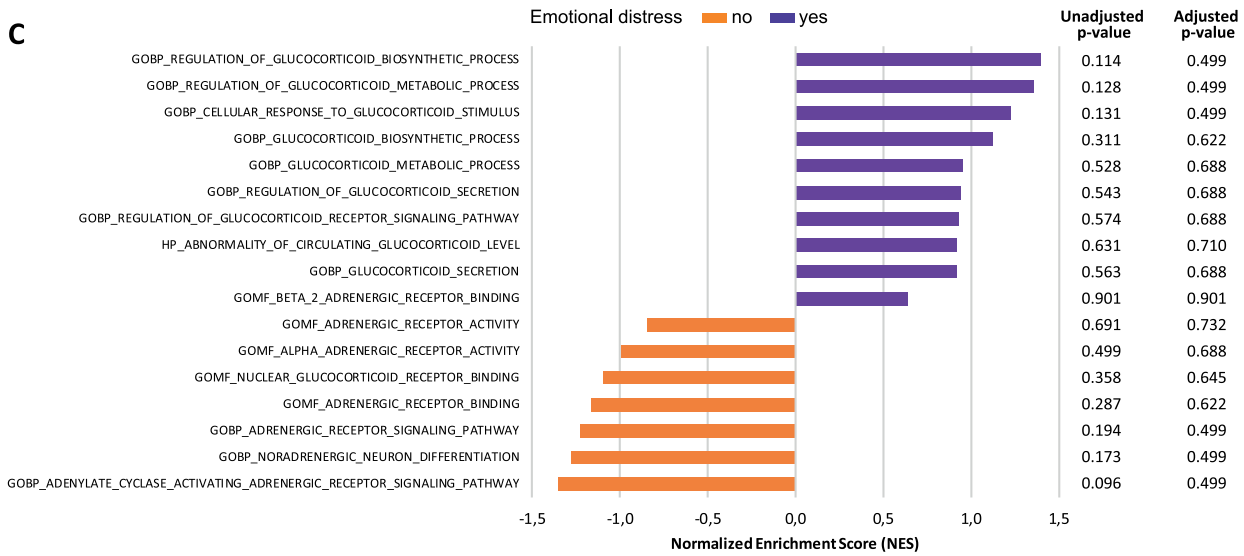
A



B

Linear regression analysis for cortisol (nmol/L)			
Variable	Coefficient	95% CI	P-value
Emotional Distress			
Yes	39.88	-4.73 – 84.48	0.079
No	*Reference*	*NA*	*NA*
Time blood withdrawal			
<10:00 h	145.78	72.16 – 219.41	0.000
10:00-12:00 h	81.73	22.00 – 141.46	0.008
12:00-14:00 h	31.80	-24.73 – 88.32	0.226
>14:00 h	*Reference*	*NA*	*NA*

C



Extended Data Fig. 2 | See next page for caption.

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Extended Data Fig. 2 | Baseline cortisol levels in patients with and without ED. a, Cortisol levels in nmol/L as measured in the peripheral blood at baseline of patients with emotional distress (n = 26, purple) and without emotional distress (n = 53, orange). Patients with unknown baseline cortisol levels or unknown time of blood withdrawal were excluded. **b**, Two-tailed linear regression analysis (n = 79 patients) showing the association between cortisol levels and emotional distress status or time of blood withdrawal. **c**, Gene set enrichment analysis (n = 70 patients) using gene sets based on the Gene Ontology database showing the normalized enrichment score (NES) and corresponding unadjusted two-sided p-value of adrenergic and glucocorticoid-associated pathways. Orange bars indicate enrichment of pathways in patients without ED (n = 47), and purple bars indicate enrichment of pathways in patients with ED (n = 23). No corrections for multiple testing were performed.

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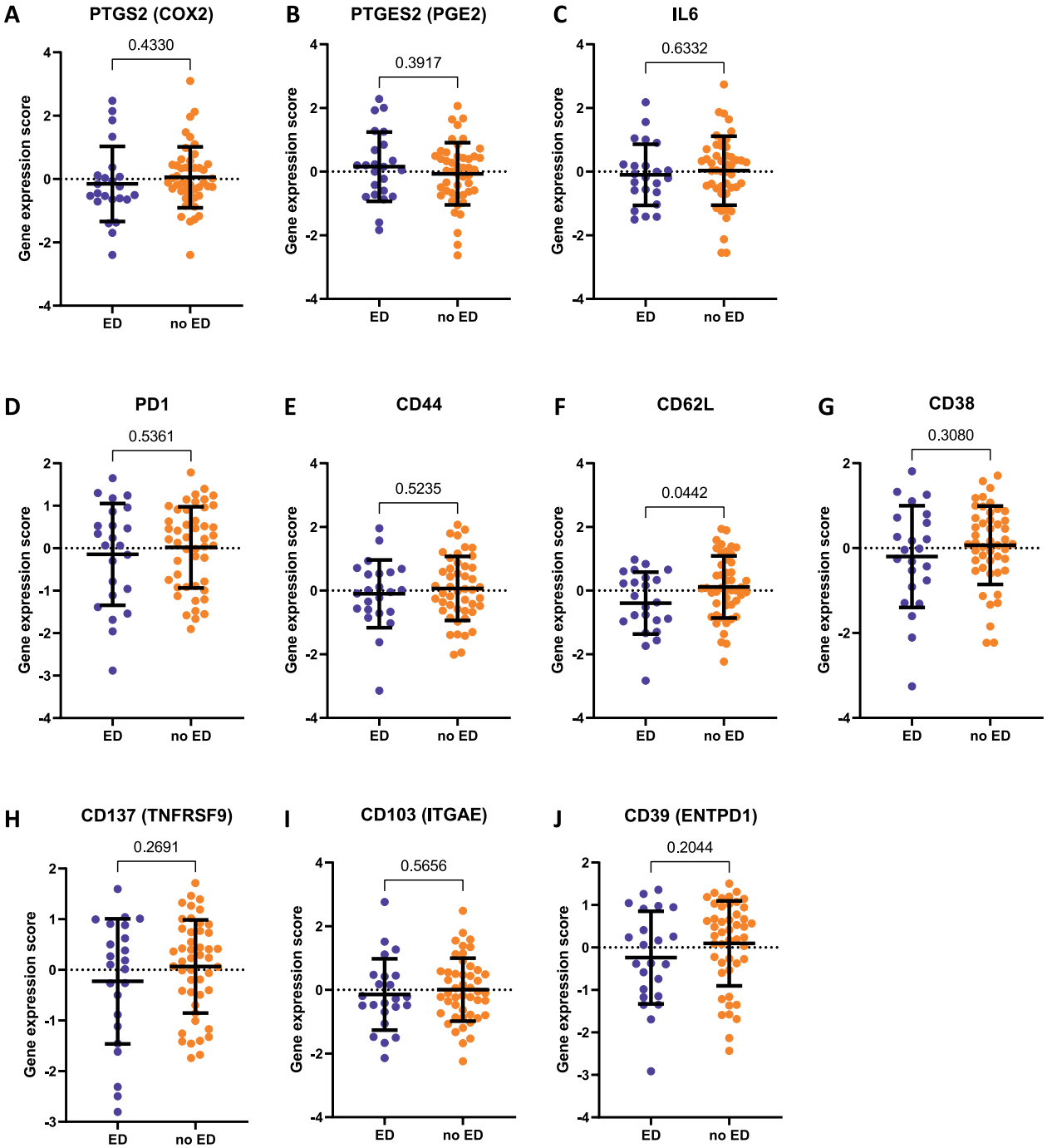

Extended Data Fig. 3 | Inflammation and T cell activation markers in patients with and without ED. a-c, Comparison of genes associated with inflammation: COX2 (PTGS2), prostaglandin E2 (PGE2) and IL6 in the tumor. **d-j**, Comparison of T cell activation markers as measured by RNA sequencing. **a-j**, Patients with available RNA sequencing data (n = 23 patients with ED, n = 47 patients without ED) were included. P-values were calculated using two-tailed unpaired Student's t-test. Bars represent mean +/− S.D.

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Extended Data Table 1 | Baseline clinical and tumor characteristics of patients without missing data in any of the variables (n = 48)

	ED (n=16)	No ED (n=32)	p-value
Country, N (%)			0.29[1]
NL	14 (87)	23 (72)	
AUS	2 (13)	9 (28)	
Sex, N (%)			0.12[2]
Male	9 (56)	25 (78)	
Female	7 (44)	7 (22)	
Age, median (IQR)	59 (49-69)	58 (46-66)	0.81[3]
Primary tumor stage, N (%)			0.70[1]
T1a/b	5 (31)	7 (22)	
T2a/b	5 (31)	8 (25)	
T3a/b	3 (19)	11 (34)	
T4a/b	3 (19)	6 (19)	
Unknown primary (MUP)	0 (0)	0 (0)	
Ulceration of primary tumor, N (%)			1.00[1]
Yes	11 (69)	22 (69)	
No	5 (31)	5 (31)	
Location of affected LN, N (%)			0.95[1]
Neck	2 (12)	6 (19)	
Axilla	7 (44)	13 (41)	
Groin	7 (44)	13 (40)	
Number of positive LN on PET-CT, N (%)			0.69[1]
1	9 (56)	18 (56)	
>1-3	6 (38)	9 (28)	
>3	1 (6)	5 (16)	
Sum diameter target lesions, mm (median, IQR)	26 (18-31)	23 (18-32)	0.95[4]
***BRAF V600E/K* mutation, N (%)**			0.84[2]
Positive	10 (62)	19 (59)	
Negative	6 (38)	13 (41)	
IFN-γ signature, N (%)*			0.41[2]
High	9 (56)	14 (44)	
Low	7 (44)	18 (56)	
TMB, N (%)[2]*			0.48[2]
High	5 (31)	7 (22)	
Low	11 (69)	25 (78)	

Abbreviations: ECOG: Eastern Cooperative Oncology Group, ED: Emotional distress, IFN-γ: interferon gamma, LN: lymph node, PET-CT: positron emission tomography–computed tomography, TMB: tumor mutational burden. P-values were calculated using two-tailed [1] Fisher's exact test, [2] Chi-square test, [3] independent samples t-test and [4] Wilcoxon rank-sum test

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Extended Data Table 2 | Univariable and multivariable logistic regression analysis of MPR in total patient population with imputed data (n = 88)

	Univariable		Multivariable	
	OR (95% CI)	p-value	aOR (95% CI)	p-value
Emotional distress				
No ED	*Ref*		*Ref*	
ED	0.49 (0.17-1.42)	0.10	0.21 (0.05-0.84)	**0.03**
Country				
AUS	*Ref*			
NL	0.80 (0.33-1.95)	0.62		
Age (per 10 years)	1.35 (1.00-1.82)	0.05		
Sex				
Female	*Ref*			
Male	2.40 (0.97-1.31)	0.06		
T-stage				
T1/2	*Ref*			
T3/4	1.00 (0.39-2.55)	0.99		
MUP	2.46 (0.59-10.28)	0.22		
Ulceration				
No	*Ref*			
Yes	1.06 (0.64-1.75)	0.83		
Number of positive lymph nodes				
1	*Ref*			
>1	1.40 (0.59-3.35)	0.45		
BRAF V600E/K				
Negative	*Ref*			
Positive	0.42 (0.18-1.06)	0.07		
IFN-γ signature				
Low	*Ref*		*Ref*	
High	5.75 (1.91-17.29)	**<0.01**	7.54 (1.95-29.96)	**<0.01**
TMB				
Low	*Ref*		*Ref*	
High	11.47 (3.15-41.68)	**<0.01**	17.12 (3.49-83.93)	**<0.01**

Indicated p-values are two-tailed. Abbreviations: aHR: adjusted hazards ratio, CI: confidence interval, ED: emotional distress, HR: hazards ratio, IFN-γ: interferon-gamma, Nrelapse: number of patients with relapse event, Ntotal: number of patients in subgroup, TMB: tumor mutational burden

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Extended Data Table 3 | Comparison of multiple models with ED/MPR and preselected (stronger) predictors in terms of corrected AIC

Predictors in multivariable model	AICc
ED IFN-gamma TMB	65.488
ED age IFN-gamma TMB	66.726
ED *BRAF* IFN-gamma TMB	67.855
ED sex IFN-gamma TMB	67.87
ED age sex IFN-gamma TMB	69.193
ED age *BRAF* IFN-gamma TMB	69.21
ED sex *BRAF* IFN-gamma TMB	70.335
ED age IFN-gamma	70.565
ED age sex BRAF IFN-gamma TMB	71.777
ED IFN-gamma	72.3
ED age *BRAF* IFN-gamma	72.898
ED age sex IFN-gamma	72.939
ED *BRAF* IFN-gamma	73.936
ED sex IFN-gamma	74.485
ED age sex *BRAF* IFN-gamma	75.363
ED TMB	76.08
ED sex *BRAF* IFN-gamma	76.265
ED sex TMB	78.252
ED age TMB	78.287
ED *BRAF* TMB	78.366
ED age sex TMB	80.584
ED sex *BRAF* TMB	80.636
ED age *BRAF* TMB	80.677
ED	81.957
ED age sex *BRAF* TMB	83.072
ED age	86.224
ED *BRAF*	87.051
ED sex	87.565
ED age *BRAF*	88.016
ED age sex	88.253
ED sex *BRAF*	88.965
ED age sex *BRAF*	90.206

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Extended Data Table 4 | Univariable and multivariable Cox regression analysis of RFS in total patient population with imputed data (n = 82)

	Univariable		Multivariable	
	HR (95% CI)	p-value	aHR (95% CI)	p-value
Emotional distress				
No ED	*Ref*		*Ref*	
ED	3.78 (1.23-11.55)	**0.02**	3.82 (1.24-11.82)	**0.02**
Country				
AUS	*Ref*			
NL	0.87 (0.28-2.71)	0.81		
Age (per 10 years)	1.15 (0.78-1.71)	0.47		
Sex				
Female	*Ref*			
Male	0.76 (0.25-2.31)	0.63		
T-stage				
T1/2	*Ref*			
T3/4	1.59 (0.49-5.21)	0.44		
MUP	1.18 (0.23-6.11)	0.84		
Ulceration				
No	*Ref*			
Yes	1.04 (0.55-1.93)	0.91		
Number of positive lymph nodes				
1	*Ref*			
>1	1.20 (0.40-3.60)	0.74		
BRAF V600E/K				
Negative	*Ref*			
Positive	1.70 (0.57-5.10)	0.34		
IFN-γ signature				
Low	*Ref*		*Ref*	
High	0.46 (0.13-1.56)	0.22	0.45 (0.13-1.61)	0.22
TMB				
Low	*Ref*			
High	0.76 (0.22-2.67)	0.66		

Patients with an event prior to surgery (n=6) were excluded from this analysis. Indicated p-values are two-tailed.

Abbreviations: aHR: adjusted hazards ratio, CI: confidence interval, ED: emotional distress, HR: hazards ratio, IFN-γ: interferon-gamma, Nrelapse: number of patients with relapse event, Ntotal: number of patients in subgroup, TMB: tumor mutational burden

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Extended Data Table 5 | Univariable and multivariable Cox regression analysis of DMFS in total patient population with imputed data (n = 82)

	Univariable		Multivariable	
	HR (95% CI)	p-value	aHR (95% CI)	p-value
Emotional distress				
No ED	*Ref*		*Ref*	
ED	5.20 (1.34-20.13)	**0.02**	5.42 (1.38-21.24)	**0.02**
Country				
AUS	*Ref*			
NL	0.49 (0.14-1.73)	0.27		
Age (per 10 years)	1.09 (0.71-1.69)	0.67		
Sex				
Female	*Ref*			
Male	0.74 (0.21-2.61)	0.64		
T-stage				
T1/2	*Ref*			
T3/4	1.58 (0.42-5.86)	0.50		
MUP	0.72 (0.08-6.42)	0.77		
Ulceration				
No	*Ref*			
Yes	1.14 (0.57-2.25)	0.71		
Number of positive lymph nodes				
1	*Ref*			
>1	0.53 (0.14-2.09)	0.37		
BRAF V600E/K				
Negative	*Ref*			
Positive	1.39 (0.40-4.76)	0.61		
IFN-γ signature				
Low	*Ref*		*Ref*	
High	0.35 (0.08-1.52)	0.16	0.33 (0.08-1.46)	0.15
TMB				
Low	*Ref*			
High	0.64 (0.15-2.66)	0.54		

Patients with an event prior to surgery (n=6) were excluded from this analysis. Indicated p-values are two-tailed.

Abbreviations: aHR: adjusted hazards ratio, CI: confidence interval, ED: emotional distress, HR: hazards ratio, IFN-γ: interferon-gamma, Nrelapse: number of patients with relapse event, Ntotal: number of patients in subgroup, TMB: tumor mutational burden

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Extended Data Table 6 | Baseline median blood cell ratios and inflammation markers in ED vs No ED cohorts (n = 88)

	Median (IQR)		
	ED (n=28)	No ED (n=60)	p-value*
Basophiles	0.1 (0.05-0.1)	0.07 (0.02-0.1)	0.24
Eosinophiles	0.1 (0.1-0.2)	0.1 (0.1-0.2)	0.41
Neutrophiles	4.7 (3.5-5.7)	4.4 (3.5-5.4)	0.71
Lymphocytes	1.8 (1.4-2.1)	1.8 (1.4-2.2)	0.98
Platelets	270.5 (239.5-324.0)	259.5 (214.0-302.0)	0.12
CRP	2.0 (1.0-3.2)	1.5 (1.0-3.0)	0.55
NLR	2.6 (2.0-3.6)	2.4 (1.9-3.3)	0.81
PLR	158.0 (116.3-211.9)	153.6 (120.4-180.5)	0.43
SII	694.7 (444.0-1163.1)	621.9 (477.6-834.5)	0.33

*Two-tailed Mann-Whitney test. Abbreviations: CRP: C-reactive protein, ED: emotional distress, IQR: interquartile range, NLR: neutrophil-to-lymphocyte ratio, PLR: platelet-to-lymphocyte ratio, SII: systemic inflammation index

https://doi.org/10.1038/s41591-023-02631-x

Extended Data Table 7 | Logistic regression analysis of inflammation markers and association with MPR in total patient group (n = 88)

	Univariate	
	OR (95% CI)	p-value
logBasophiles	1.10 (0.63-1.92)	0.75
logEosinophiles	0.87 (0.43-1.74)	0.69
logNeutrophiles	0.90 (0.24-3.38)	0.88
logLymphocytes	1.04 (0.25-4.31)	0.96
logPlatelets	0.35 (0.05-2.49)	0.29
logCRP	0.68 (0.43-1.09)	0.11
logNLR	0.92 (0.32-2.63)	0.88
logPLR	0.63 (0.18-2.22)	0.47
logSII	0.75 (0.30-1.85)	0.53

Indicated p-values are two-tailed. Abbreviations: ED: emotional distress, IFN-y: interferon-gamma, TMB: tumor mutational burden, NLR: neutrophil/lymphocyte ratio, OR: odds ratio, PLR; platelet/lymphocyte ratio, SII: systemic inflammation index.

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n/a	Confirmed
☐	☒ The exact sample size (*n*) for each experimental group/condition, given as a discrete number and unit of measurement
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☐	☒ The statistical test(s) used AND whether they are one- or two-sided *Only common tests should be described solely by name; describe more complex techniques in the Methods section.*
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☒	☐ For Bayesian analysis, information on the choice of priors and Markov chain Monte Carlo settings
☒	☐ For hierarchical and complex designs, identification of the appropriate level for tests and full reporting of outcomes
☒	☐ Estimates of effect sizes (e.g. Cohen's *d*, Pearson's *r*), indicating how they were calculated

Our web collection on statistics for biologists contains articles on many of the points above.

Software and code

Policy information about availability of computer code

Data collection	The clinical data was collected and processed in electronic case report forms (eCRF) using Tenalea (version 18.1) at the Netherlands Cancer Institute, Melanoma Institute Australia, Leiden University Medical Centre, Erasmus Medical Center, University Medical Centre Utrecht and University Medical Centre Groningen. Quality of Life data was collected via questionnaires and also entered in the eCRF.
Data analysis	Descriptive and regression analyses were performed using STATA version 15.1 software (StataCorp) or IBM SPSS Statistics version 27 (IBM Inc., New York, NY). Kaplan Meier plots and RNA sequencing analyses were performed in GraphPad Prism (version 9.3.0), R (version 4.2.2) and R Studio (version 1.2.1335) using the packages lubridate (version 1.9.1), dplyr (version 1.0.10), ggplot2 (version 3.4.0), survival (version 3.4.0), survminer (version 0.4.9), DESeq2 (version 1.38.2), tidyverse (version 1.3.2), biomaRt (version 2.54), Biobase (version 2.58.0), RColorBrewer (version 1.1-3), heatmap.3(github/lwaldron/LeviRmisc/), fgsea (version 1.24.0), mMCPcounter (version 1.1.0), immunedeconv (version 2.1.0). Dot plots and bar plots were generated in GraphPad Prism (version 7.03).

For manuscripts utilizing custom algorithms or software that are central to the research but not yet described in published literature, software must be made available to editors and reviewers. We strongly encourage code deposition in a community repository (e.g. GitHub). See the Nature Portfolio guidelines for submitting code & software for further information.

Data

Policy information about availability of data

All manuscripts must include a data availability statement. This statement should provide the following information, where applicable:

- Accession codes, unique identifiers, or web links for publicly available datasets
- A description of any restrictions on data availability
- For clinical datasets or third party data, please ensure that the statement adheres to our policy

RNA-sequencing data generated during the study will be deposited in the European Genome-phenome Archive (EGA) under the accession code EGAS0000XXXXXXX. To minimize the risk of patient re-identification, de-identified individual patient-level clinical data are available under restricted access. Upon scientifically sound request, data access can be obtained via the NKI's scientific repository at repository@nki.nl, which will contact the corresponding author (L.V.v.d.P.F.). Data requests will be reviewed by the institutional review board of the Netherlands Cancer Institute (NKI) and will require the requesting researcher to sign a data access agreement with the NKI.

Research involving human participants, their data, or biological material

Policy information about studies with human participants or human data. See also policy information about sex, gender (identity/presentation), and sexual orientation and race, ethnicity and racism.

Reporting on sex and gender	Data on biological sex (female or male) was collected and reported in the manuscript: in total 66% of patients were male and 33% were female. These frequencies are comparable to other studies reporting a higher percentage of male subjects with metastasized stage III melanoma [Leeneman, EJC, 2021; Tarhini, Future Oncology, 2018]. Non-significant baseline differences in sex were observed in the emotional distress subgroup, with 57% male patients in the ED group and 72% male patients in the no-ED group. A bordeline significant association was found for sex in univariable analysis of MPR (male vs female OR 2.40, 95% CI 0.97-5.96, p=0.06). However, when using the corrected Akaike Information Criterion for variable selection, sex was not included in the multivariable analysis. Our trial was not designed/powered to prospectively test differences in terms of neoaduvant ICB efficacy based on biological sex. No significant association between sex and survival was found. No data on gender has been collected.
Reporting on race, ethnicity, or other socially relevant groupings	No data on race, ethnicity, or other socially relevant groupings were collected.
Population characteristics	Resectable stage III melanoma patients with one or more measurable lymph node metastases (according to RECIST v1.1) that can be biopsied, no history of in-transit metastases within the last 6 months, naïve for CTLA-4/PD-1/PD-L1 immunotherapy, and more than 18 years old. Of all the patients included the median age was 58 years and 66% was male, 95% had an ECOG performance status of 0, 2% had an elevated LDH level at baseline, and 45% had a BRAF V600 mutation.
Recruitment	All patients with clinically detected stage III nodal melanoma who were treated in one of the participating centers and were deemed eligible for the study were invited to participate. Patients were recruited by either surgical oncologists or medical oncologist from the melanoma cancer clinics in the Netherlands Cancer Institute, Melanoma Institute Australia, Leiden University Medical Centre, Erasmus Medical Center, University Medical Centre Utrecht and University Medical Centre Groningen. Patients were generally referred to the participating study centers by outside hospitals. No specific bias in recruitment was identified.
Ethics oversight	Medical ethics review committee of the Netherlands Cancer Institute and ethical committees at Melanoma Institute Australia approved the trial. The trial was conducted in accordance with the ICH Harmonized Tripartite Guideline for Good Clinical Practice and the principles of the Declaration of Helsinki. All patients provided written informed consent.

Note that full information on the approval of the study protocol must also be provided in the manuscript.

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Life sciences study design

All studies must disclose on these points even when the disclosure is negative.

Sample size	During designing of the trial we planned to enroll 100-110 patients. Eventually 99 melanoma patients were accrued. The first goal of the study was to confirm the pathologic response rate (pCR, near-CR, pPR), of the most favorable treatment schedule from Opacin-neo (arm B). A pathologic response rate of 55% was considered unacceptable, while we expected 70% of patients to respond to treatment. An exact test for one proportion has 85% power to test this hypothesis at the two-sided alpha level of 0.05. At least 65 responders were required, which implies the actual significance level of 0.043.

Co-primary objectives of the PRADO cohort were to assess 1) whether it is safe to omit TLND in patients achieving a MPR (pCR or near-pCR) and 2) improvement of the RFS rate at 24 months for patients with pNR by adding adjuvant treatment. We expected no recurrences within 24 months in patients achieving MPR, and RFS at 24 months of 90% or less would be considered unsafe. Power calculation for this objective was performed via simulations accessing the lower bound of the one-sided 95% confidence interval, applied to Kaplan-Meier estimate of RFS at 24 months, using beta product confidence procedure (BPCP)29. With at least 46 patients achieving MPR and followed for 24 months, this method has 80-90% power to test H0: 90% versus HA: >90%. In total, 21 patients were included in the pNR group. The BPCP method has 81% power to reject RFS at 24 months of 20% at one-sided alpha of 0.05 in case of improvement of the RFS at 24 months to 45%

Data exclusions

100 patients were registered in the study and 1 patient was excluded after registration because the diagnosis of melanoma was amended to Hodgekin based on the baseline biopsy of the lymph node. Inclusion and exclusion criteria were prespecified in the study protocol (protocol p.41-43).
For the comparison of patients with and without emotional distress, patients were excluded if they had not filled in a baseline HRQoL questionnaire (n=11), resulting in a total population of n=88 patients.

Replication

Replication of the clinical results is not applicable as this manuscript reports results of a phase II clinical trial. Pathological responses were centrally revised by experienced pathologists from the Netherlands Cancer Institute and Melanoma Institute Australia.
With regard to analyses on tumor biopsy samples or blood samples (eg. RNA sequencing data), replication of the analyses was limited due to the limited amount of patients and precious patient materials. It is stated in the manuscript that confirmation of the results by data from future patient cohorts (eg from the NADINA trial) is warranted.

Randomization

PRADO used a single arm trial design with personalized response-directed surgery and adjuvant therapy following neoadjuvant checkpoint inhibition. Since PRADO was used as extension cohort of OpACIN-neo to confirm the efficacy and toxicity of the most optimal neoadjuvant treatment schedule (ipilimumab 1mg/kg + nivolumab 3mg/kg), no randomization to different treatment arms was applied.

Blinding

The trial was nog randomized and all patients were allocated to receive the same neoadjuvant treatment. Blinding was therefore not performed.

Reporting for specific materials, systems and methods

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Methods

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☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Clinical data

Policy information about clinical studies

All manuscripts should comply with the ICMJE guidelines for publication of clinical research and a completed CONSORT checklist must be included with all submissions.

Clinical trial registration

This trial is registered with ClinicalTrials.gov (NCT02977052)

Study protocol

The full trial protocol can be found in the supplementary appendix.

Data collection

Patients were enrolled between November 2018 and January 2020 in the Netherlands Cancer Institute, Melanoma Institute Australia, Leiden University Medical Centre, Erasmus Medical Center, University Medical Centre Utrecht and University Medical Centre Groningen.

The data cut-off in the current manuscript was Februari 7th, 2022.
Clinical data was collected through an eCRF by the clinical trial department of the Netherlands Cancer Institute. Clinical data was analyzed by the department of biostatistics at the Netherlands Cancer Institute.

Outcomes

The primary objective of the PRADO trial was to confirm the pathologic response rate of the most favorable treatment arm of OpACIN-neo (Arm B: 2 cycles ipilimumab 1mg/kg plus nivolumab 3mg/kg). Co-primary objectives were to investigate whether a TLND could be safely omitted in patients achieving a pCR/near-pCR in the ILN, and whether RFS of patients with a pNR could be prolonged by adding adjuvant treatment. Primary endpoints were pathologic response rate and 2-year RFS of patients with MPR and pNR.

When designing the trial, we planned to enroll 100-110 patients with the goal to include at least 50 patients with MPR. This goal was earlier achieved, so that eventually 99 melanoma patients were accrued. A pathological response rate of 55% was considered

unacceptable, while we expected 70% of patients to respond to treatment. An exact test for one proportion has 85% power to test this hypothesis at the two-sided alpha level of 0.05. At least 65 responders were required, which implies the actual significance level of 0.043.
Our assumption was that no recurrences within 24 months in patients achieving MPR would occur, and RFS at 24 months of 90% or less would be considered unsafe. Power calculation for this objective was performed via simulations accessing the lower bound of the one-sided 95% confidence interval, applied to Kaplan-Meier estimate of RFS at 24 months, using beta product confidence procedure (BPCP). For at least 50 patients that were expected to achieve MPR and assuming 24-months RFS under the alternative hypothesis of 98%, there was 75.5% power, and under the alternative hypothesis of 99% there was 91.5% power. With 60 MPR patients having ≥2 years follow-up, the lower boundary of the one-sided 95% BPCP confidence interval would exceed 90% if no more than 1 recurrence occurred.
In total, 21 patients were included in the pNR group. The BPCP method has 81% power to reject RFS at 24 months of 20% at one-sided alpha of 0.05 in case of improvement of the RFS at 24 months to 45%. With 21 pNR patients having ≥2 years follow-up, the lower boundary of the one-sided 95% BPCP confidence interval would exceed the 20% if no more than 13 recurrences occurred.

Secondary objectives were confirmation of the toxicity rate of OpACIN-neo arm B (defined as the grade 3-4 immunotherapy-related adverse event rate during the first 12 weeks), radiologic response rate, distant metastases free survival, event-free survival, overall survival, ongoing toxicity at 3 years, comparison of surgical morbidity between patients undergoing only the ILN procedure and ILN procedure plus subsequent TLND, health-related quality of life, and biomarker analyses.