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Personalized response-directed surgery and adjuvant therapy after neoadjuvant ipilimumab and nivolumab in high-risk stage III melanoma: the PRADO trial

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Neoadjuvant ipilimumab and nivolumab induces high pathologic response rates (pRRs) in clinical stage III nodal melanoma, and pathologic response is strongly associated with prolonged relapse-free survival (RFS). The PRADO extension cohort of the OpACIN-neo trial (NCT02977052) addressed the feasibility and effect on clinical outcome of using pathologic response after neoadjuvant ipilimumab and nivolumab as a criterion for further treatment personalization. In total, 99 patients with clinical stage IIIb–d nodal melanoma were included and treated with 6 weeks of neoadjuvant ipilimumab 1 mg kg^{-1} and nivolumab 3 mg kg^{-1}. In patients achieving major pathologic response (MPR, ≤10% viable tumor) in their index lymph node (ILN, the largest lymph node metastasis at baseline), therapeutic lymph node dissection (TLND) and adjuvant therapy were omitted. Patients with pathologic partial response (pPR; >10 to ≤50% viable tumor) underwent TLND only, whereas patients with pathologic non-response (pNR; >50% viable tumor) underwent TLND and adjuvant systemic therapy ± synchronous radiotherapy. Primary objectives were confirmation of pRR (ILN, at week 6) of the winner neoadjuvant combination scheme identified in OpACIN-neo; to investigate whether TLND can be safely omitted in patients achieving MPR; and to investigate whether RFS at 24 months can be improved for patients achieving pNR. ILN resection and ILN-response-tailored treatment were feasible. The pRR was 72%, including 61% MPR. Grade 3–4 toxicity within the first 12 weeks was observed in 22 (22%) patients. TLND was omitted in 59 of 60 patients with MPR, resulting in significantly lower surgical morbidity and better quality of life. The 24-month relapse-free survival and distant metastasis-free survival rates were 93% and 98% in patients with MPR, 64% and 64% in patients with pPR, and 71% and 76% in patients with pNR, respectively. These findings provide a strong rationale for randomized clinical trials testing response-directed treatment personalization after neoadjuvant ipilimumab and nivolumab.

Adjuvant immune checkpoint inhibition (CPI) and BRAF/MEK-targeted therapies after therapeutic lymph node dissection (TLND) have improved relapse-free survival (RFS) in patients with clinical stage III nodal melanoma. Despite these improvements, approximately 40–50% of patients have a relapse within 3–5 years after TLND[1–3]. Preclinical and early clinical trial data suggest that neoadjuvant CPI leads to superior anti-tumor immunity and survival benefit compared to adjuvant CPI[4,5]. Similarly to stage IV melanoma, the combination of anti-CLTA-4 and anti-PD-1 appears to be superior to anti-PD-1 monotherapy in the neoadjuvant setting[6,7]. Previous clinical trials (OpACIN (NCT02437279) and OpACIN-neo (NCT02977052)) testing neoadjuvant ipilimumab (anti-CTLA-4) plus nivolumab (anti-PD-1) in stage III melanoma demonstrated high pathologic response rates (pRRs; 74–78%) and a strong association between pathologic response and RFS, with 94–100% of responding patients remaining free of relapse at 2 years[5,7–9]. Similarly, long-term benefit was observed upon complete response to CPI in stage IV melanoma, even after cessation of CPI[10–12].

The association between response and survival; the observed ongoing responses after cessation of therapy in stage IV melanoma; and the substantial morbidity from TLND[13–16] that impairs

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health-related quality of life (HRQoL)[17,18] raised the question of whether TLND could be safely omitted in patients with major pathologic response (MPR, ≤10% viable tumor) after neoadjuvant CPI. Furthermore, we hypothesized that the addition of adjuvant systemic therapy ± adjuvant radiotherapy in patients with pathologic non-response (pNR) (>50% viable tumor) might reduce the relapse rate as compared to non-responding patients from previous neoadjuvant trials who did not receive adjuvant therapy[5,8].

In two previous studies, we demonstrated that the pathologic response in the index lymph node (ILN, the largest lymph node metastasis at baseline) was a reliable indicator of the response to neoadjuvant ipilimumab and nivolumab in the entire TLND specimen of stage III nodal melanoma[19,20].

This multicenter phase 2 PRADO expansion cohort (NCT02977052) of the OpACIN-neo trial investigated the role of assessing pathologic response in only the ILN to determine subsequent management, including TLND and adjuvant therapy. After baseline marker placement in the ILN, patients were treated with two cycles of ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1} in week 0 and week 3, followed by ILN resection at week 6. Patients achieving MPR in the ILN did not undergo subsequent TLND or adjuvant treatment. Patients with pPR (>10–≤50% viable tumor) underwent TLND without adjuvant treatment, whereas patients with pNR (>50% viable tumor) underwent TLND and adjuvant nivolumab (*BRAF* wild-type tumors) or BRAF/MEK inhibitors (*BRAF*$^{V600E/K}$-mutated tumors) for 52 weeks ± local radiotherapy (Fig. 1a). Co-primary endpoints were pRR, 24-month RFS for patients achieving MPR and 24-month RFS for patients achieving pNR.

We report the first results from PRADO, including the efficacy and safety of neoadjuvant ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}; the feasibility of ILN resection and pathologic assessment; the effects of TLND and/or adjuvant therapy omission on morbidity and HRQoL; and the 24-month survival data after response-driven tailored treatment.

Results

In the PRADO trial, 99 patients with clinical stage III nodal melanoma and measurable disease according to Response Evaluation Criteria in Solid Tumors (RECIST) version 1.1 were enrolled between November 2018 and January 2020 (Fig. 1b). Median age was 58 years; 65 (66%) patients were male; 45 (45%) patients had a *BRAF*^{V600} mutation; and 42 (42%) patients had more than one fluorodeoxyglucose-positive lymph node on positron emission tomography (PET)–computed tomography (CT) at baseline (Table 1). The ILN was marked pre-treatment using ultrasound guidance with a magnetic seed (53%), nitinol marker (34%), radioactive I-125 seed (9%) or hydrogel marker (4%) (Extended Data Fig. 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), with a minimum follow-up of 23.4 months for all patients alive.

Immunotherapy-related adverse events. In total, 89 (90%) patients received two scheduled treatment cycles, whereas ten (10%) patients received only one cycle due to immunotherapy-related adverse events (irAEs) (Fig. 1b). Grade 3–4 irAEs within the first 12 weeks were observed in 22 patients (22%; 95% confidence interval (CI): 14–32%) (Table 2). The most prevalent grade 3–4 irAEs were increased alanine transaminase (ALT) levels ($n=7$, 7%), increased aspartate aminotransferase (AST) levels ($n=6$, 6%) and diarrhea/colitis ($n=5$, 5%/$n=4$, 4%). No treatment-related deaths were observed. Grade 3–4 irAEs occurred in 30 (30%) patients up to the data cutoff, with increase of serum lipase levels being the most prevalent grade 3–4 toxicity ($n=9$, 9%) (Supplementary Table 1). Two patients with adjuvant therapy and six patients without adjuvant therapy developed their grade 3–4 irAE beyond week 12.

Radiologic response. At 6 weeks after the start of neoadjuvant CPI, pre-surgical CT showed a RECIST version 1.1 radiologic response in 45 (45%, 95% CI: 35–56%) patients and stable disease in 38 (38%) patients, resulting in a disease control rate of 84% (Fig. 2a); three patients (3%) were evaluated at later time points than as per protocol due to irAEs. Radiologic progression occurred in 13 (13%) patients, including seven (7%) patients with regional disease progression only who underwent the ILN resection according to protocol and six (6%) patients with distant metastases (of whom four had regional progressive disease and two had stable disease on CT).

Feasibility of ILN resection after neoadjuvant CPI. Of the 93 patients without distant metastases at week 6, 90 underwent a resection of the ILN; two proceeded direct to TLND (which was also delayed) due to grade 3–4 irAEs; and one did not undergo any surgery due to irAEs (Fig. 1b and Supplementary Table 2). Additionally, four of the six patients with distant metastases still underwent the ILN resection for regional control, resulting in a total of 94 of 99 patients (95%) undergoing the ILN resection (Fig. 1b). Grade 3–4 irAEs caused delays in the ILN resection in three patients (Supplementary Table 2).

Histopathologic assessment demonstrated that the marked ILN was successfully resected (that is, the marker was in the resection specimen) during the ILN resection in 90 of 94 (96%) patients at first attempt (Supplementary Table 2). In two patients, the ILN was resected during secondary surgery after it was noticed that the marked ILN was missing from the initial resected specimen. Additional lymph nodes other than the ILN (median 1, range 1–6) were resected in 38 (40%) patients, mainly due to localization in front of, or adjacent to, the ILN (Supplementary Table 2).

Pathologic response. Pathologic response was assessed based on the resection specimen of the ILN resection, except for the two patients who only underwent TLND and had no ILN resection due to irAEs (Fig. 1b). Response percentages were calculated over the total cohort of 99 patients. Pathologic responses were observed in 71 of 99 (72%; 95% CI: 62–80%) patients, including 48 (49%) with pathologic complete response (pCR) and 12 (12%) with near-pCR, resulting in an MPR rate of 61% (96% CI: 50–70%) (Fig. 2b). Partial responses were found in 11 (11%) patients. Thus, the radiologic response rate (45%) underestimated the pRR (72%), similarly to findings in previous trials[6,8,21] (Extended Data Fig. 2). Exploratory analyses showed that pathologic response was not associated with tumor burden at baseline or other demographics (Fig. 2c). In addition, no association was found between maximum-grade irAEs during the first 12 weeks and pathologic response (Supplementary Table 3). PD-L1 expression in baseline tumor biopsies was associated with pathologic response; the pRR was 56% in tumors with <1% PD-L1-expressing tumor cells, 92% in tumors with 1–50% PD-L1-expressing tumor cells and 100% in tumors with >50% PD-L1-expressing tumor cells ($P=0.004$) (Fig. 2c).

Response-directed tailored treatment. Based on the pathologic response assessment in the ILN, TLND was omitted in 59 of the 60 patients who achieved MPR at week 6. One patient underwent TLND despite having a near-pCR due to the presence of extranodal extension and viable tumor in the ILN surgical margins (Fig. 1b and Supplementary Table 2). Additionally, two patients with MPR had additional lymph nodes resected during follow-up surgery due to radiologically suspected residual disease on postoperative imaging (these additional lymph nodes showed pCR in both patients). Eight of the 11 patients with pPR underwent TLND (Fig. 1b and Supplementary Table 4); two patients refused TLND; and one patient had no TLND because of suspected distant metastases that later were diagnosed as pulmonary sarcoid-like reaction. All 19 patients who had pNR in the ILN and no distant metastases at

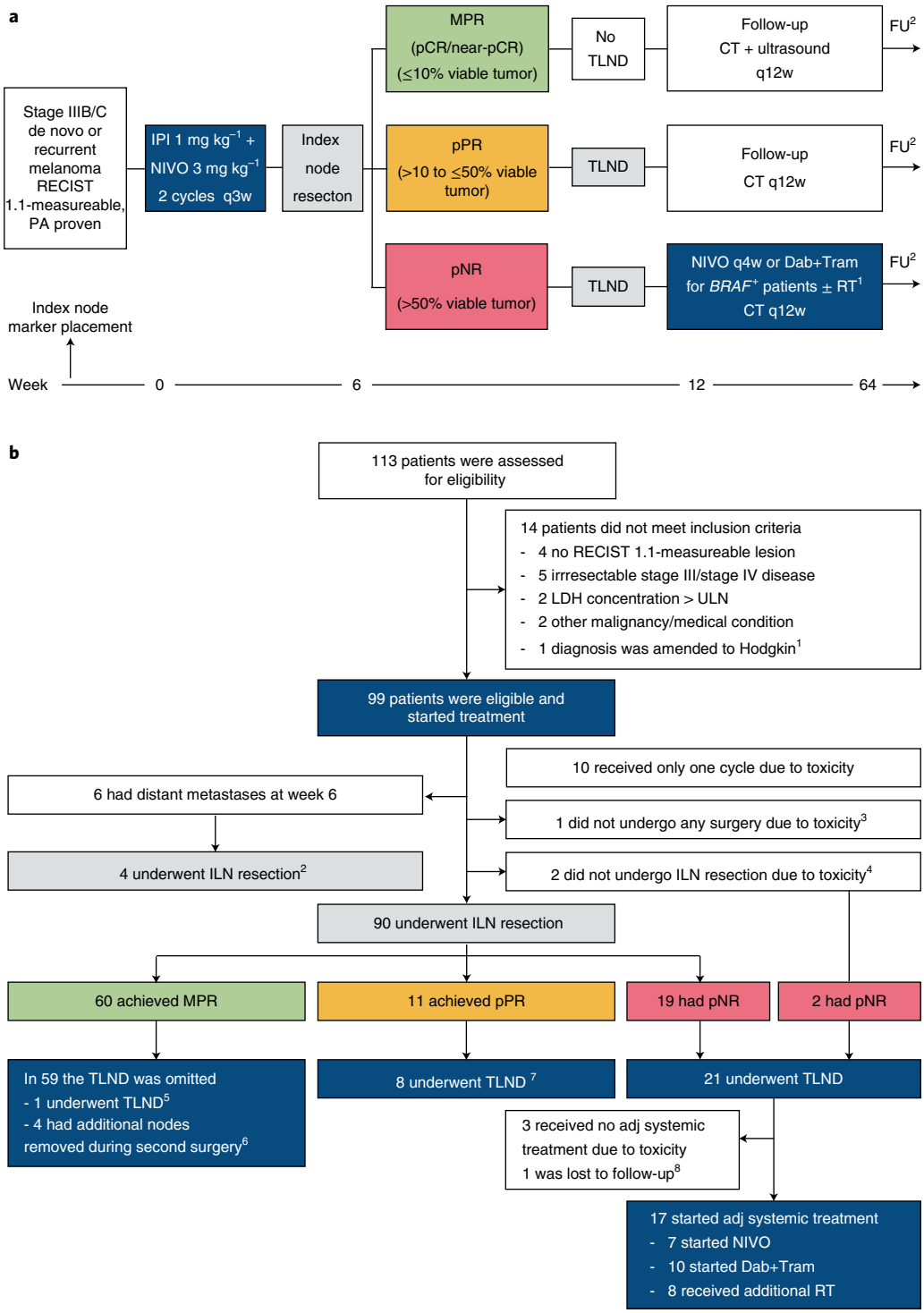


Fig. 1 | Study scheme and flowchart of PRADO. a, Study design of the PRADO trial. (1) Adjuvant radiotherapy according to patient and physician decisions and (2) according to institute standards. **b**, Flow chart of the PRADO trial. [1]For one patient, the diagnosis of melanoma was amended to Hodgkin lymphoma based on his pre-treatment tumor biopsy after inclusion into the trial. This patient went off study and was excluded from the data analyses. [2]Four patients with distant metastases underwent the ILN procedure for regional control ($n=3$) or because the distant metastases on CT were retrospectively identified after the ILN procedure ($n=1$). [3]One patient developed a myelitis transversa-like syndrome leading to constipation and colon perforation. This patient had a radiologic response. [4]Two patients had their ILN resected during TLND; their pathologic response assessment is based on the TLND. [5]One patient had a TLND despite achieving near-pCR due to viable tumor in the ILN margins, including extranodal extension. [6]Two patients had extra lymph nodes removed in a second surgery because of remaining suspect lymph nodes on postoperative CT scan. Second surgery showed no viable tumor. Two other patients had minor secondary surgery performed for removal of the marked ILN. [7]Two patients refused to undergo TLND after achieving pPR, and one patient did not have a TLND due to a pulmonary sarcoid-like reaction that was initially regarded as progressive disease. [8]All patients were *BRAF* wild-type; three patients did not receive adjuvant nivolumab due to an immunotherapy-related colitis, cholangitis and arthritis; and one patient was lost to follow-up. adj, adjuvant; Dab, dabrafenib; FU, follow-up; IPI, ipilimumab; NIVO, nivolumab; PA, pathology; RT, radiotherapy; Tram, trametinib; ULN, upper limit of normal.

Table 1 | Baseline characteristics of PRADO

Characteristic	Total cohort ($n=99$)	
Institute		
NKI	52	(53%)
MIA	34	(34%)
LUMC	5	(5%)
EMC	4	(4%)
UMCU	3	(3%)
UMCG	1	(1%)
Age, years (median, IQR)	58	(51.5–69.5)
Sex		
Men	65	(66%)
Women	34	(34%)
ECOG performance status score		
0	94	(95%)
1	5	(5%)
Primary tumor stage		
T1a/b	17	(17%)
T2a/b	26	(26%)
T3a/b	20	(20%)
T4a/b	21	(21%)
Tx	2	(2%)
Unknown primary	13	(13%)
Ulceration of primary tumor		
Yes	23	(23%)
No	58	(69%)
Unknown	18	(18%)
Location of affected lymph node		
Neck	24	(24%)
Axilla	39	(39%)
Groin	36	(36%)
Number of positive lymph nodes on PET-CT		
1	57	(58%)
>1–3	33	(33%)
>3	9	(9%)
Sum of diameter target lesions, mm (median, IQR)	25	(18–33)
Previous treatment		
Sentinel node procedure	24	(24%)
Lymph node dissection	2	(2%)
BRAF$^{V600E/K}$ mutation		
Yes	45	(45%)
No	43	(43%)
VE1 negative[a]	9	(9%)
Unknown	2	(2%)
LDH < ULN	97	(98%)

Data are median (IQR) or *n* (%). Percentages may not sum up to 100 because of rounding. [a]For these patients, the presence of *BRAF*^{V600E} mutation was assessed only by VE1 staining and negative (they achieved MPR, and no tumor material was left for formal testing). ECOG, Eastern Cooperative Oncology Group; ULN, upper limit of normal.

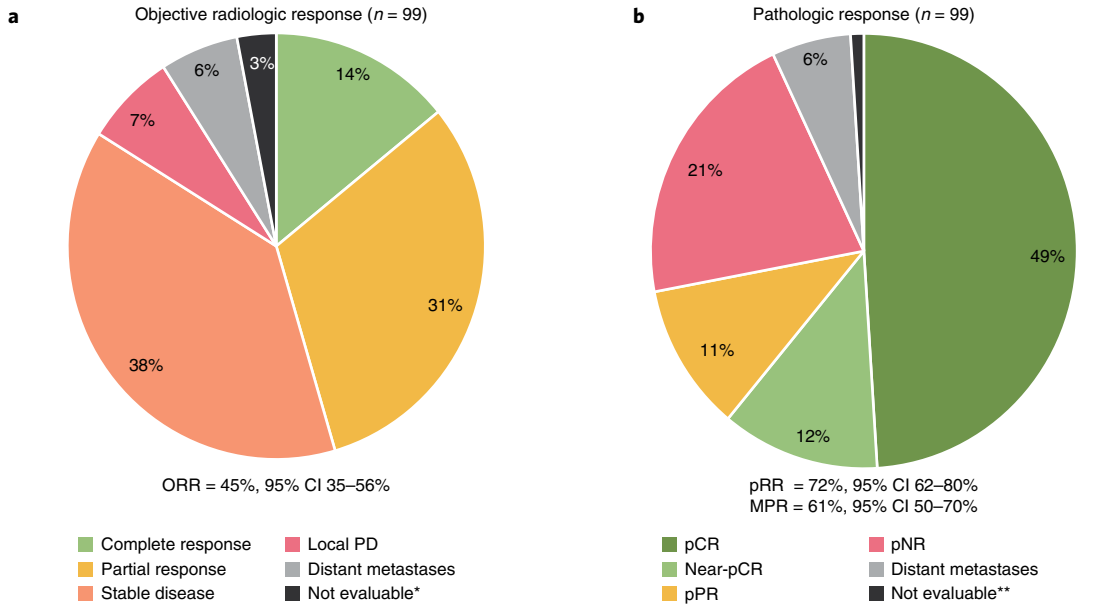
Table 2 | Immunotherapy-related adverse events within the first 12 weeks

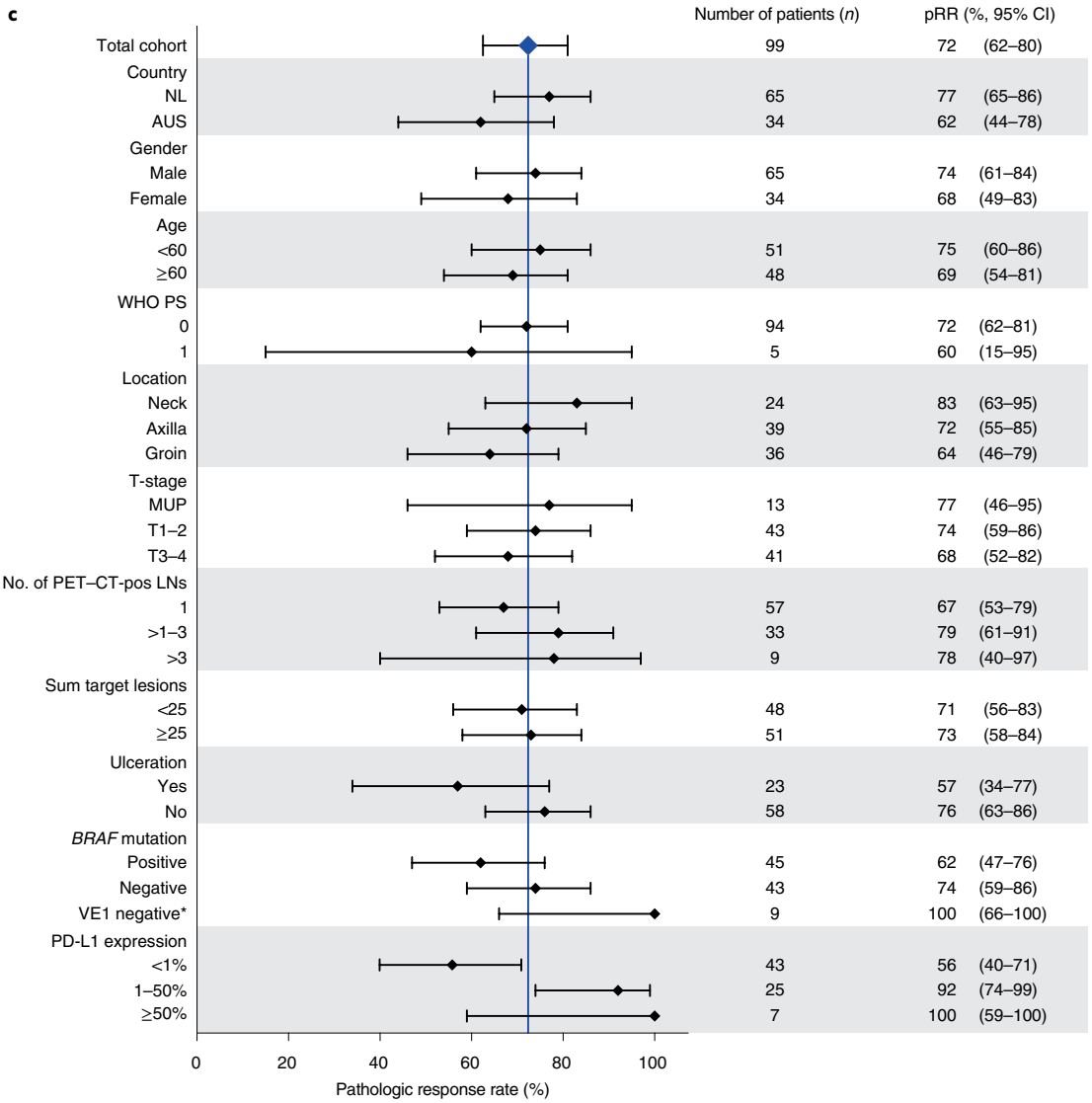
Immunotherapy-related adverse events	Total cohort ($n=99$)					
	Grade 1–2		Grade 3–4		Total	
Total number of patients with at least one adverse event[a]	74	(75%)	22	(22%)	96	(97%)
Fatigue	54	(55%)	0		54	(55%)
Rash	47	(47%)	3	(3%)	50	(51%)
Pruritus	28	(28%)	0		28	(28%)
Hyperthyroidism	23	(23%)	0		23	(23%)
ALT increased	15	(15%)	7	(7%)	22	(22%)
Diarrhea	17	(17%)	5	(5%)	22	(22%)
AST increased	13	(13%)	6	(6%)	19	(19%)
Nausea	18	(18%)	1	(1%)	19	(19%)
Dry mouth	17	(17%)	0		17	(17%)
Arthralgia	16	(16%)	0		16	(16%)
Hypothyroidism	16	(16%)	0		16	(16%)
Headache	12	(12%)	1	(1%)	13	(13%)
Myalgia	10	(10%)	0		10	(10%)
Infusion related reaction	8	(8%)	0		8	(8%)
Serum lipase increased	5	(5%)	3	(3%)	8	(8%)
Dry skin	7	(7%)	0		7	(7%)
Fever	7	(7%)	0		7	(7%)
Colitis	2	(2%)	4	(4%)	6	(6%)
Creatine kinase increased	5	(5%)	1	(1%)	6	(6%)
Dry eye	6	(6%)	0		6	(6%)
Dyspnea	5	(5%)	0		5	(5%)
Serum amylase increased	3	(3%)	1	(1%)	4	(4%)
GGT increased	1	(1%)	1	(1%)	2	(2%)
Myocarditis	0		2	(2%)	2	(2%)
Cholangitis	0		1	(1%)	1	(1%)
Functional decline[b]	0		1	(1%)	1	(1%)
Myelitis transversa-like syndrome	0		1	(1%)	1	(1%)

Data are *n* (%). Immunotherapy-related adverse events that occurred in more than 5% of patients and all grade 3–4 events are displayed in the table. Within the first 12 weeks, no grade 5 adverse events were observed. [a]Some patients had more than one event. [b]Functional decline possibly caused by corticosteroid induced-myopathy. GGT, gamma-glutamyltransferase.

week 6 on radiologic imaging underwent TLND (Supplementary Table 4). Two additional patients who did not undergo the ILN procedure due to irAEs underwent TLND that showed pNR (Fig. 1b). Of these 21 patients with pNR, 17 were treated with adjuvant systemic therapy (seven patients received adjuvant nivolumab and ten were treated with adjuvant BRAF/MEK inhibition), whereas the four remaining patients did not receive adjuvant nivolumab due to irAEs ($n=3$, all *BRAF* wild-type) or were lost to follow-up ($n=1$). Eight patients received concurrent adjuvant radiotherapy (Fig. 1b).

TLND omission resulted in reduced morbidity and better HRQoL. For all patients who underwent TLND in the PRADO trial, the median time from the start of neoadjuvant CPI to TLND was 9.6 weeks (range, 8.1–22.1 weeks). TLND was delayed in five (16%) patients due to irAEs ($n=4$) or because there was no on-time theater slot available ($n=1$) (Supplementary Table 2). No unexpected surgical complications were observed. A significantly lower surgery-related adverse event rate according to Common


a
Objective radiologic response (n = 99)
14%
31%
38%
7%
6%
3%
ORR = 45%, 95% CI 35–56%
Complete response
Partial response
Stable disease
Local PD
Distant metastases
Not evaluable*
b
Pathologic response (n = 99)
49%
12%
11%
21%
6%
pRR = 72%, 95% CI 62–80%
MPR = 61%, 95% CI 50–70%
pCR
Near-pCR
pPR
pNR
Distant metastases
Not evaluable**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Number of patients (n)
pRR (%, 95% CI)
Total cohort 99 72 (62–80)
Country
NL 65 77 (65–86)
AUS 34 62 (44–78)
Gender
Male 65 74 (61–84)
Female 34 68 (49–83)
Age
<60 51 75 (60–86)
≥60 48 69 (54–81)
WHO PS
0 94 72 (62–81)
1 5 60 (15–95)
Location
Neck 24 83 (63–95)
Axilla 39 72 (55–85)
Groin 36 64 (46–79)
T-stage
MUP 13 77 (46–95)
T1–2 43 74 (59–86)
T3–4 41 68 (52–82)
No. of PET–CT-pos LNs
1 57 67 (53–79)
>1–3 33 79 (61–91)
>3 9 78 (40–97)
Sum target lesions
<25 48 71 (56–83)
≥25 51 73 (58–84)
Ulceration
Yes 23 57 (34–77)
No 58 76 (63–86)
BRAF mutation
Positive 45 62 (47–76)
Negative 43 74 (59–86)
VE1 negative* 9 100 (66–100)
PD-L1 expression
<1% 43 56 (40–71)
1–50% 25 92 (74–99)
≥50% 7 100 (59–100)
0
20
40
60
80
100
Pathologic response rate (%)

Fig. 2 | Radiologic and pathologic response. a, Objective radiologic response (ORR) of all patients in the PRADO trial ($n=99$) after 6 weeks of neoadjuvant ipilimumab plus nivolumab. *Three patients were not evaluable for radiologic response at week 6 due to irAEs. **b**, Pathologic response of the ILN of all patients in the PRADO trial ($n=99$) based on INMC criteria. **One patient did not have any surgery due to irAEs. **c**, Forest plot of data for all patients ($n=99$). pRRs with 95% CIs are displayed according to demographic, clinical and tumor characteristics. The 95% CIs were calculated using the Clopper–Pearson method. *All patients of whom only a VE1 staining was available achieved MPR, and no tumor material was left for formal testing. VE1, a monoclonal antibody against mutant $BRAF^{V600E}$ protein. AUS, Australia; NL, The Netherlands; LN, lymph node; MUP, melanoma of unknown primary; PD, progressive disease; WHO PS, World Health Organization performance status.

Terminology Criteria for Adverse Events (CTCAE) version 4 was observed in patients who only underwent ILN resection ($n=61$) as compared to patients who underwent both ILN resection and subsequent TLND ($n=31$) (46% versus 84%, $P<0.001$) (Fig. 3a and Extended Data Fig. 3a). Similarly, ILN-only patients had significantly lower Clavien–Dindo classification grades at week 12 than ILN+TLND patients ($P<0.001$) (Fig. 3b). Undergoing TLND was significantly associated with the presence of surgery-related adverse events, but the use of high-dose ($\geq 1\,mg\,kg^{-1}$) steroids within the first 12 weeks after the start of neoadjuvant CPI was not significantly associated with surgical morbidity (Supplementary Table 5).

Longitudinal HRQoL outcomes were compared between patients with MPR ($n=60$, of whom most underwent ILN resection only) (Extended Data Fig. 3b) and patients with non-MPR ($n=31$, most underwent ILN and TLND). Differences in scores were calculated while adjusting for age, gender, adjuvant treatment and relapse status (no/yes). Overall, patients with MPR scored significantly better on several HRQoL functioning domains than patients with non-MPR, including physical functioning, role functioning, global functioning, social functioning, the European Organisation for Research and Treatment of Cancer (EORTC) Quality of Life Questionnaire-Core 30 (QLQ-C30) summary score and the melanoma (surgery) subscales. The biggest differences were detected at week 12, and all differences were clinically relevant (Fig. 3c, Extended Data Fig. 4 and Supplementary Table 6). Moreover, patients with MPR reported a lower symptom burden than patients with non-MPR with respect to fatigue and insomnia, with the biggest differences at week 12 (Fig. 3c, Supplementary Table 6 and Extended Data Fig. 4). After 2 years, significantly and small clinically relevant differences in scores were still present for physical functioning, fatigue and insomnia. Patients with non-MPR reported clinically important deterioration regarding physical and role functioning, fatigue and pain at week 12, nausea at week 36 and financial difficulties at week 48 and 60 (data not presented)[22]. Except for emotional functioning and insomnia, none of the HRQoL parameters was significantly different between both groups at week 6 (post-neoadjuvant CPI and pre-surgery) (Supplementary Table 7). To evaluate the effect of the patient's knowledge of his/her pathologic response on HRQoL outcomes, additional analyses were performed comparing the MPR to pPR and pNR subgroups (Extended Data Fig. 5). Statically significant and clinically relevant adjusted differences were observed in MPR versus pPR and MPR versus pNR patients. Because both the MPR and pPR patient groups were informed that they had a good prognosis (based on results from previous neoadjuvant trials[7]), the extent of surgery is likely to be an important contributing factor to the differences in HRQoL outcomes between patients with MPR and non-MPR.

Survival outcomes. After a median follow-up of 28.1 months (IQR, 25.0–33.8 months), median RFS (Fig. 4a), event-free survival (EFS) (Fig. 4c), distant metastasis-free survival (DMFS) (Fig. 4d) and overall survival (OS) (Fig. 4f) were not reached for the total cohort, with 24-month estimates being 85% (95% CI: 78–92%), 80% (95% CI: 72–88%), 89% (95% CI: 83–96%) and 95% (95% CI: 91 –99%), respectively.

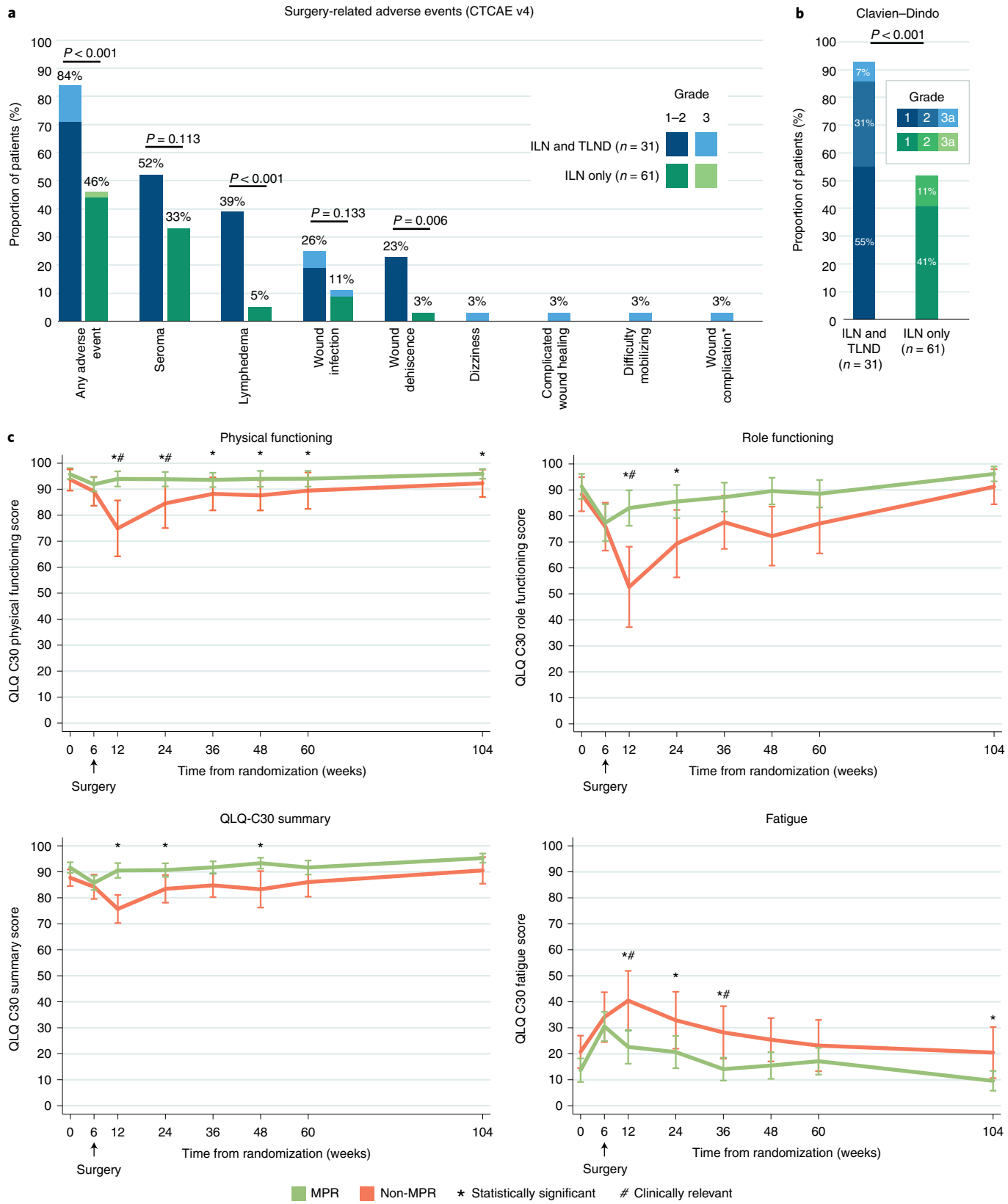
The estimated 24-month RFS rate for the patients achieving MPR was 93% (95% CI: 87–>99%) (Fig. 4b). Four of the 60 patients with MPR developed a regional recurrence (three pCR and one near-pCR in the ILN) (Supplementary Table 8). One of these patients developed later M1a disease, 23.5 months after ILN resection and 19.3 months after regional recurrence, resulting in a 24-month DMFS rate of 98% (95% CI: 94–>99%) for the MPR group (Fig. 4e). Notably, all four patients had two or more PET-positive lymph nodes at baseline and harbored a $BRAF^{V600E/K}$ mutation (versus 43% and 30% in MPR patients without relapse) (Supplementary Table 8). In total, 28 patients with MPR had two or more PET-positive lymph nodes at baseline, resulting in a recurrence rate of 4 of 28 (14%) in this group (Supplementary Table 9). Three of the four patients were treated by surgery followed by adjuvant therapy (nivolumab $n=2$, dabrafenib+trametinib $n=1$), and the fourth patient refused extended surgery and started BRAF/MEK inhibition with ongoing radiologic complete response. The subcutaneous lesions of the patient with M1a disease were surgically removed.

Of the 11 patients with pPR, four patients had recurrence, resulting in a 24-month RFS rate of 64% (95% CI: 41–99%) (Fig. 4b). Three patients developed distant recurrence, and the fourth patient developed regional followed by distant recurrence, yielding a 24-month DMFS rate of 64% (95% CI: 41–99%) (Fig. 4e). The patient and tumor characteristics of these patients are listed in Supplementary Table 10.

Fig. 3 | Effect of ILN procedure on surgical morbidity and HRQoL. a, Surgery-related adverse events of patients undergoing an ILN procedure only ($n=61$) versus those undergoing subsequent TLND ($n=31$) according CTCAE version 4.03. Only adverse events that occurred in three or more patients or were grade ≥3 are displayed in the figure. *P* values were calculated using Fisher's exact test. *The wound complication consisted of vacuum-assisted closure dressing of the wound and electrolyte monitoring. **b**, Clavien–Dindo classification at week 12 of patients undergoing ILN procedure only ($n=61$, green bar) versus patients undergoing subsequent TLND ($n=31$, blue bar). The *P* value was calculated using the linear-by-linear association test for ordinal data. **c**, Curves showing the unadjusted mean HRQoL scores of patients with MPR ($n=60$, green line) versus patients without MPR ($n=31$, orange line). Error bars indicate the 95% CI. The differences in mean HRQoL scores between patients with MPR and non-MPR (see also Supplementary Table 5) were adjusted for age, gender, adjuvant treatment and relapse status (no/yes). The adjusted score differences were interpreted in terms of statistical significance using a linear mixed-effects model with a two-tailed *P* value ($P<0.05$) and by clinical relevance according to the guideline of Cocks et al.[32]. Statistically significant adjusted differences were marked with *, and clinically relevant differences were marked with # (Supplementary Table 5). Results were considered clinically relevant if the adjusted difference in mean scores between the two groups was at least 'medium' and clinically irrelevant if differences in mean scores were 'trivial or small'. Questionnaire compliance rates in the MPR and non-MPR groups were 87% versus 97% at baseline, 98% versus 94% at week 6, 90% versus 81% at week 12, 88% versus 81% at week 24, 92% versus 84% at week 36, 85% versus 68% at week 48, 80% versus 77% at week 60 and 87% versus 61% at week 104 (year 2).

Six of 21 patients in the pNR group developed a melanoma recurrence ($n=5$) or died ($n=1$, recurrence status unknown) within the first 2 years after surgery, yielding a 24-month RFS rate of 71% (95% CI: 55–94%) (Fig. 4b). Relapses were regional ($n=1$), distant ($n=3$) or synchronous regional and distant ($n=1$), resulting in a 24-month DMFS rate of 76% (95% CI: 60–97%) (Fig. 4e).

At the data cutoff, recurrences were observed in two of seven patients treated with adjuvant nivolumab (24-month RFS rate 71%), in three of ten patients with adjuvant BRAF/MEK inhibition



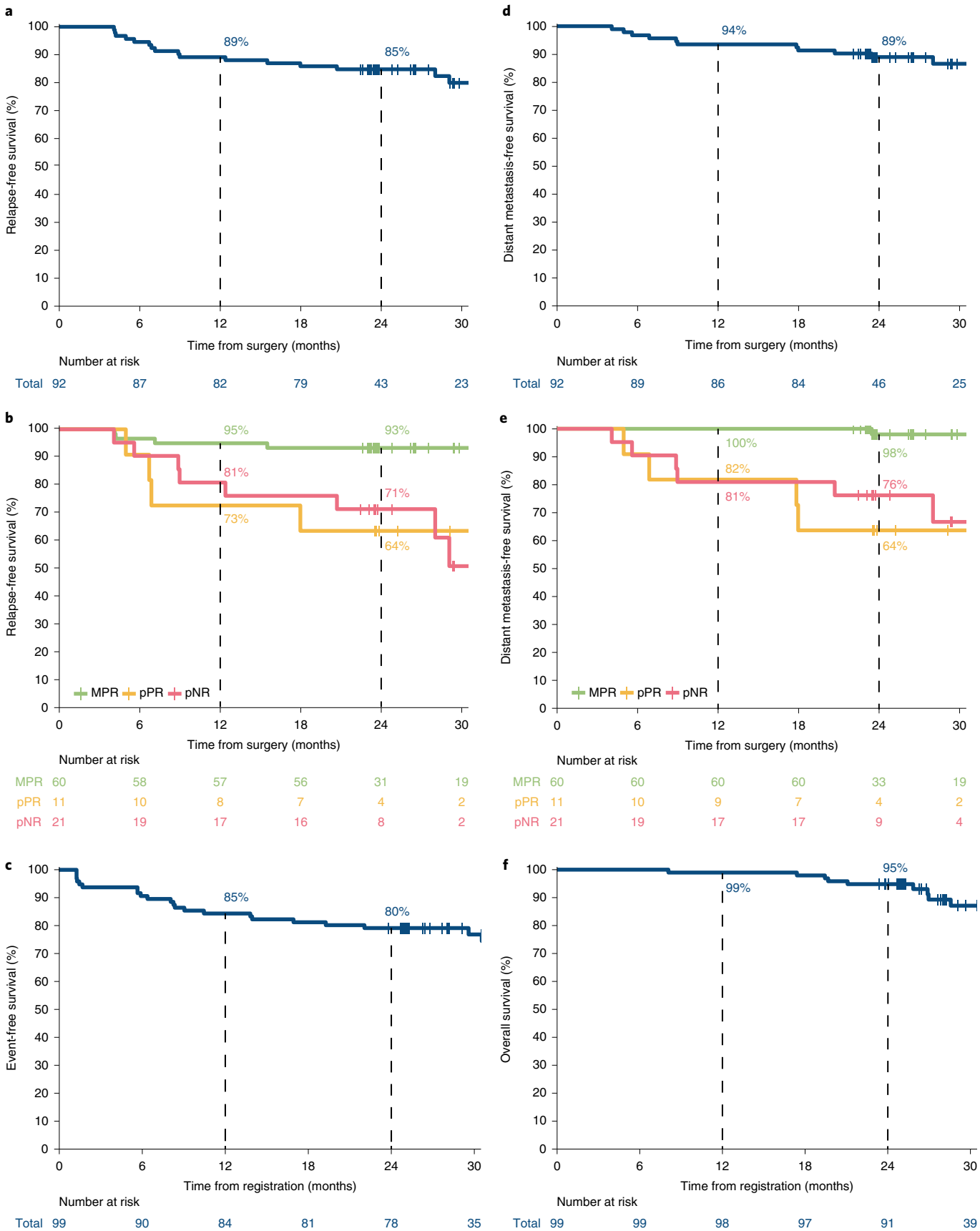


Fig. 4 | Survival outcomes. a, RFS for all patients who underwent surgery ($n=92$), six patients who progressed to stage IV disease before surgery and one patient who did not undergo surgery because of irAEs were excluded. **b**, RFS of the PRADO trial by pathologic response subgroup. Patients had MPR ($n=60$, green line), pPR ($n=11$, yellow line) or pNR ($n=21$, red line). **c**, EFS for the total population of the PRADO trial ($n=99$). **d**, DMFS for all patients who underwent surgery ($n=92$). **e**, DMFS of the PRADO trial by pathologic response subgroup. **f**, OS for the total population of the PRADO trial ($n=99$).

(24-month RFS rate 90%) and in two of three patients without systemic adjuvant therapy (24-month RFS rate 33%) (Extended Data Fig. 6). Of note, two patients with MPR in the ILN and one with pNR developed a new primary melanoma during follow-up. This was not counted as an event in the survival analyses.

Discussion

To our knowledge, PRADO is the first trial to demonstrate that the ILN procedure is feasible and enables response-directed tailored treatment. This approach enabled de-escalation of treatment (omission of TLND and adjuvant therapy) in most patients achieving MPR in their ILN (59 of 60), resulting in decreased morbidity and better HRQoL for these patients. Their 24-month RFS and DMFS rates were 93% and 98%, respectively, indicating that the response-driven tailored treatment did not impair their outcomes. In addition, our findings might be a first step in future efforts on reduction of health services use and costs for the treatment of stage III melanoma.

PRADO also confirmed the clinical outcomes observed in our prior neoadjuvant OpACIN-neo trial. The latter trial demonstrated that two cycles of ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1} was the most favorable neoadjuvant treatment schedule, with pRR of 77% and 20% grade 3–4 irAEs within the first 12 weeks of treatment[8]. In the current PRADO trial, we observed pRR of 72% and 22% grade 3–4 irAEs within the first 12 weeks after neoadjuvant ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}, confirming the efficacy and safety of this treatment regimen for clinical stage III nodal melanoma.

Furthermore, we found that the implementation of the ILN resection in our neoadjuvant CPI treatment regimen was safe and feasible. The marked ILN was retrieved in 96% of cases, indicating that all four evaluated markers (magnetic Memaloc marker, nitinol UltraCor Twirl marker, radioactive I-125 seed and hydrogel marker) are suitable for identifying and removing the ILN. Broad experience with image-guided marker placement for locating axillary lymph nodes has already been gained with breast cancer surgery after neoadjuvant chemotherapy. Marker placement is regarded as safe and simple with high detection rates[23,24]. Individual institutional or surgeon preference, experience and availability of localization technique should direct the choice for the preferred marker. Delay or cancellation of the ILN procedure due to irAEs occurred in only a small subset of patients (6%). However, one needs to note that (high-dose) steroids were no contraindication for surgery in our participating institutes[25].

One of the co-primary endpoints of PRADO—the 24-month RFS in the MPR group—was not met based on the predefined measure of feasibility. The trial protocol stated that the null hypothesis could not be rejected in case of more than one recurrence in the MPR group, which was based on a 24-month RFS rate of 97% for responders (≤50% viable tumor) in OpACIN-neo[9]. Four patients with MPR had recurred at the data cutoff, resulting in a 24-month RFS rate of 93%. Nevertheless, only one patient developed distant metastasis (M1a disease 23.5 months after ILN resection). The other three patients developed only regional recurrences, enabling salvage TLND followed by adjuvant systemic therapy. None of these patients had additional recurrences at the data cutoff.

Similar results regarding distant metastasis were seen in OpACIN-neo, a trial in which all patients underwent TLND after neoadjuvant CPI. In this trial, 52 of 86 (60%) patients achieved MPR, and after a median follow-up of almost 4 years only one MPR patient had developed distant metastasis (M1d disease, 8.3 months after surgery)[9]. Based on previous large datasets that indicate that the vast majority of relapses occur within the first 12 months after surgery[2,3], our data on RFS and DMFS in the MPR group of PRADO can be considered relatively mature. Therefore, and in our view, immediate TLND might be safely omitted in patients achieving MPR in the ILN.

In contrast to earlier reports showing similar RFS in patients with pPR and MPR[7,9], pPR patients in PRADO had a worse outcome. Although not being able to exclude a sampling error due to the low patient number, this observation suggests that pPR patients should not be treated like MPR patients and might benefit from adjuvant therapy. This is supported by the RFS outcomes of patients with pNR who received additional adjuvant therapy in this trial. With only 29% of patients with pNR who had developed a melanoma recurrence at 2 years, their RFS was improved compared to the 65% of non-responding patients from OpACIN-neo who developed a recurrence[9]. Thus, PRADO suggested not only that treatment de-escalation is safe in patients with MPR but also that treatment escalation in non-responding patients might improve their outcome.

With more patients treated by the ILN approach after neoadjuvant CPI, one might define MPR patients with a higher chance for melanoma recurrence in the future. Notably, all four patients with MPR who recurred in PRADO had two or more PET-positive lymph nodes at baseline. We previously showed that the ILN response was highly concordant with the pathologic response of the entire tumor bed, supporting the current PRADO study design[20]. However, we also reported on two cases (out of 82 patients) showing a pathologic response in their ILN but also a non-response in a small non-index node that did not alter the pathologic response subgroup for the entire TLND tumor bed. This indicates the presence of less CPI-responsive tumor subclones in a minority of patients[20]. We speculate that such CPI-resistant tumor clones might have been the reason for the development of recurrences in these MPR patients, and that TLND in such patients improves their outcome. Currently, we are investigating genetic and transcriptomic differences between the ILN and recurrent node metastases to gain insights into potential mechanisms of resistance to neoadjuvant CPI.

TLND omission significantly reduced surgical morbidity and was associated with better HRQoL. These data are in line with work on surgical morbidity from randomized trials and single-arm studies comparing morbidity and cancer control between completion lymph node dissection and observation after a positive sentinel lymph node biopsy in patients with melanoma[14–16,26–28]. The fact that adjuvant therapy and relapse status were included in the mixed-effects model, and differences in HRQoL outcomes were observed in patients with MPR and pPR, indicate that the differences in HRQoL outcomes are likely to be attributed to the different extent of surgery. Additional factors contributing to the lower physical functioning status and higher fatigue scores after 2 years in non-MPR patients might be the two sequential surgeries and anesthesia within a short time period (±3 weeks)[17,18,29].

This trial is limited by the small sample sizes per pathologic subgroup, especially for patients with pPR and pNR, impeding definitive conclusions on survival outcomes after response-tailored treatment. Moreover, PRADO did not randomize TLND versus the ILN approach or response-tailored adjuvant therapy versus adjuvant therapy, allowing for only indirect comparisons to historical cohorts from previous neoadjuvant and adjuvant studies. The non-randomized study design also did not allow for a strict comparison of HRQoL between patients with and without TLND.

The randomized phase 3 NADINA trial (NCT04949113) currently investigates standard TLND followed by adjuvant nivolumab versus neoadjuvant ipilimumab plus nivolumab followed by TLND and adjuvant nivolumab or BRAF/MEK inhibition (in non-MPR patients only) in clinical stage III melanoma. NADINA includes, unlike most previous neoadjuvant immunotherapy trials, patients with in-transit metastases. Two small case series have shown that the pathologic response after neoadjuvant CPI in in-transit metastases or locally advanced primary tumors is concordant with the response in the lymph node metastases[30,31]. Another randomized phase 2 trial (SWOG S1801, NCT03698019) in clinical stage III–IV melanoma

compares neoadjuvant plus adjuvant pembrolizumab versus adjuvant pembrolizumab. Although these trials will define the benefit of neoadjuvant systemic CPI in melanoma versus adjuvant anti-PD-1, a large randomized trial analyzing the ILN approach versus TLND is pending.

Online content

Any methods, additional references, Nature Research 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/s41591-022-01851-x.

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Methods

Study design and participants. The PRADO trial included patients who were 18 years of age or older with histologically confirmed resectable stage III nodal melanoma and at least one node measurable according to RECIST version 1.1 (≥15 mm short axis). Normal lactate dehydrogenase (LDH) levels and a World Health Organization performance status score of 0 or 1 were required. Major exclusion criteria were prior treatment with CPI targeting CTLA-4/PD-1/PD-L1, BRAF±MEK inhibition or radiotherapy, a history of in-transit metastases within 6 months before inclusion and a history of autoimmune diseases. Full inclusion and exclusion criteria can be found in the appendix (protocol pages 41–43). Patients were enrolled in Australia at the Melanoma Institute Australia (MIA, Sydney) and in the Netherlands at the Netherlands Cancer Institute (NKI, Amsterdam), Leiden University Medical Center (LUMC), Erasmus Medical Center (EMC, Rotterdam), University Medical Center Utrecht (UMCU) and University Medical Center Groningen (UMCG). The 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 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 were treated with two cycles ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1} after placement of a marker in the ILN (largest melanoma-containing lymph node at baseline). Removal of the marker lymph node (ILN procedure) was planned after 6 weeks. Patients who achieved pCR or near-pCR (both together termed MPR) in their ILN did not undergo TLND nor received any adjuvant treatment. Patients with pPR underwent TLND without adjuvant treatment, and patients with pNR underwent TLND plus adjuvant systemic treatment (nivolumab or dabrafenib+trametinib) for 52 weeks with or without local radiotherapy (Fig. 2a). Enrollment continued until a minimum of 50 patients had achieved MPR in their ILN.

Randomization and blinding. In the PRADO trial, there was no randomization and no blinding.

Treatment and assessments. Before initiation of neoadjuvant treatment, the ILN was marked using ultrasound guidance. Different markers were used depending on the participating sites' preference, including a magnetic Memaloc marker, nitinol UltraCor Twirl marker, hydrogel marker and radioactive I-125 seed. The ILN procedure was scheduled after 6 weeks from the start of CPI, during which only the marked ILN was planned to be resected. Additional radiologically suspected or biopsy-proven lymph nodes other than the ILN were allowed to remain in situ and were planned to be resected (only in case of pPR or pNR in the ILN) during TLND, which was planned after 9 weeks (range, 7–12 weeks) from the start of CPI. Treatment of irAEs of the neoadjuvant CPI with steroids was no contraindication to proceeding to surgery.

Patients were treated until the end of the treatment schedule, unacceptable toxicity or withdrawal of consent. Discontinuation criteria due to irAEs are described in the appendix (trial protocol pages 57–58). Permanent discontinuation of CPI due to irAEs did not preclude patients from undergoing the ILN procedure or TLND. All treatment-related adverse events and laboratory values were recorded and graded by the investigators according to the CTCAE version 4.03. Surgery-related morbidity was also graded according to the Clavien–Dindo classification. Laboratory assessments were performed at baseline and at week 3, week 6, week 9 and week 12. Radiologic tumor assessments by CT were done at baseline, in week 6 before the ILN procedure and in week 12. Radiologic responses were assessed using RECIST version 1.1 guidelines by the radiologists at the participating centers without central review. Patients who progressed to stage IV disease went off study according to the protocol and were treated according to standard of care.

The pathologic responses were centrally revised by experienced pathologists (B.A.v.d.W., A.J.C. and R.A.S. at MIA or NKI) according to International Neoadjuvant Melanoma Consortium (INMC) guidelines[33]. Pathologic responses of the ILN were categorized as being pCR (0% viable tumor), near-pCR (1–≤10% viable tumor), pPR (>10–≤50% viable tumor) or pNR (>50% viable tumor). Subsequent response-tailored treatment was based on the pathologic response of the ILN, except for two patients who only underwent TLND and no ILN resection due to irAEs. The pathologic responses of non-index nodes that were resected during the ILN procedure and TLND are shown in Supplementary Table 4. Starting at week 12, patients without pathologic response received adjuvant treatment with nivolumab or BRAF/MEK inhibition for 52 weeks ± local radiotherapy in parallel. All patients were assessed for recurrence of disease by radiologic assessment with CT or PET–CT, physical examination and laboratory testing for every 12 weeks until development of distant metastases, death, lost to follow-up or withdrawal of consent for up to 2 years after surgery and in years 3, 4 and 5 according to institute standards. The data cutoff for collection of survival and toxicity data was 7 February 2022.

Baseline tumor PD-L1 expression analysis was performed centrally (NKI) on formalin-fixed, paraffin-embedded tumor sections with an automated laboratory-validated immunohistochemistry assay, using the 22C3 antibody on a Ventana platform. PD-L1 expression was determined by the tumor proportion score (the percentage of tumor cells with complete or partial membranous staining at any intensity).

HRQoL. HRQoL scores were assessed by use of the EORTC QLQ-C30 and melanoma (surgery)-specific questions of the Functional Assessment of Cancer Therapy-Melanoma (FACT-M). The HRQoL assessments took place before treatment (at baseline), at week 6 (post-neoadjuvant CPI, pre-surgery) and at weeks 12, 24, 36, 48, 60 and 104 (year 2). The data cutoff for collection of HRQoL data was 1 February 2022. Missing items from the EORTC QLQ-C30 were imputed according to EORTC guidelines. More information on the questionnaires can be found in the Supplementary Materials (page 16).

Endpoints. The primary objective of the PRADO trial was to confirm the pRR of the most favorable treatment arm of OpACIN-neo (arm B: two cycles ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}). Co-primary objectives were to investigate whether TLND could be safely omitted in patients achieving MPR in the ILN and whether RFS of patients with pNR could be prolonged by adding adjuvant treatment. Primary endpoints were pRR and 24-month RFS in patients achieving MPR and pNR.

Secondary endpoints were grade 3–4 irAE rate during the first 12 weeks after CPI initiation, radiologic response rate, DMFS, EFS, OS, ongoing long-term irAEs, comparison of surgical morbidity between marked ILN resection and TLND, HRQoL and biomarker analyses. For definitions of pathologic response and survival endpoints, see Supplementary Table 11.

Statistical analyses. *Sample size and power.* 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 patients with melanoma were accrued. The first objective of the trial was to confirm the pRR (pCR, near-CR and pPR) of the most favorable treatment schedule from OpACIN-neo (arm B). A pRR of 55% was considered unacceptable, and 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 MPR (pCR or near-pCR) and (2) improvement of the RFS rate at 24 months for patients with pNR by adding adjuvant treatment. 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% CI, applied to Kaplan–Meier estimate of RFS at 24 months, using beta product confidence procedure (BPCP)[34]. For at least 50 patients who were expected to achieve MPR and assuming 24-month 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 two or more years of follow-up, the lower boundary of the one-sided 95% BPCP CI would exceed 90% if no more than one 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 two or more years of follow-up, the lower boundary of the one-sided 95% BPCP CI would exceed the 20% if no more than 13 recurrences occurred. No interim analyses were planned for PRADO. However, if one relapse in the MPR patient cohort was observed before the end of patient inclusion in the trial, the Data and Safety Monitoring Board and Bristol Myers Squibb would be immediately informed and the further procedure of the trial discussed. If, at any moment, there were two relapses, the trial would be amended by reintroducing TLND in this cohort.

Response, toxicity and survival. For the PRADO trial, analyses on pathologic response, radiologic response and irAEs were performed in all patients with melanoma who received at least one dose of the study drug. For pathologic response, patients were not evaluable if they did not undergo any surgery due to irAEs, and patients with stage IV disease at week 6 were allocated to the 'distant metastases' subgroup independent of undergoing the ILN procedure or not. Patients were not assessable for radiologic response if they had not been radiologically evaluated for response at week 6. The pathologic and radiologic responses as well as adverse events were summarized as proportions of the total cohort with the two-sided 95% CI calculated using the Clopper–Pearson method. For analyses on surgical-related toxicity, patients who underwent only the ILN resection ($n=61$) were compared to patients who underwent the ILN resection followed by TLND and those who proceeded immediately to TLND ($n=29$ and $n=2$, respectively). Patients who underwent no surgery ($n=3$) or a small secondary surgery for removal of some additional lymph nodes ($n=4$) were excluded from the analysis. CIs for difference in proportions of patients with surgical toxicity were calculated using the asymptotic method, and the provided P values come from Fisher's exact test. The P value for differences in the Clavien–Dindo classification was calculated by linear-by-linear association test for ordinal data. Odds ratios and P values for the association between TLND and steroid use on the presence of surgical morbidity were calculated using a

multivariate logistic regression model. Survival outcome curves (RFS, EFS, DMFS and OS) for the total cohort and pathologic response subgroups were estimated using Kaplan–Meier methodology. CIs were calculated using the Greenwood formula and log transformation. Analyses were performed using R (version 3.5.1) and SPSS Statistics (version 27). This trial is registered with ClinicalTrials.gov (NCT02977052) and is ongoing for survival analysis.

HRQoL. HRQoL outcomes were evaluated using mixed-effects linear regressions for longitudinal data with patient-specific intercepts and an autoregressive covariance matrix structure. All models were adjusted for MPR status (no-MPR/MPR), age (measured in years), gender (female/male), adjuvant treatment (no/yes, measured as a time-dependent variable), relapse status (no/yes, measured as a time-dependent variable) and time (baseline and weeks 6, 12, 24, 36, 48, 60 and 104). Additionally, for comparison of outcomes between MPR and no-MPR at specific time points, interaction terms between MPR status and time were added to the models. Coefficients of all covariates were considered as fixed effects. Results were interpreted in three ways: (1) statistically significant difference was defined with a two-sided P value ≤ 0.05; (2) medium to large differences were defined as clinically relevant according to the guideline of Cocks et al.[32]; and (3) domain-specific thresholds were used to identify functional impairments and symptoms that limit patients' daily life[22]. All analyses were conducted using STATA version 15.1 software (StataCorp).

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

Data availability

To minimize the risk of patient re-identification, de-identified individual patient-level clinical data are available under restricted access. Upon a scientifically sound request, data access can be obtained via the NKI's scientific repository at repository@nki.nl, which will contact the corresponding author (C.U.B.). 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.

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Author contributions

C.U.B. designed the trial and wrote the trial protocol. E.A.R. provided additional input and wrote the amendment for the PRADO extension cohort during the 20th Workshop on 'Methods in Clinical Cancer Research' (Zeist, Netherlands). G.V.L. and A.v.A. reviewed the protocol. A.H.B. wrote the HRQoL part of the protocol. I.L.M.R., A.M.M., J.M.V., R.P.M.S., T.P., E.A.R., E.K., A.A.M.v.d.V., K.P.M.S., G.A.P.H., W.M.C.K., W.J.v.H., J.A.v.d.H., D.J.G., M.W.W., A.J.W., C.L.Z., J.M.L., K.F.S., S.C., J.S., A.S., A.v.A., G.V.L. and C.U.B. recruited and treated patients and collected data. A.J.C., R.V.R., R.A.S. and B.A.v.d.W. reviewed and scored the pathology of all cases. N.M.J.v.d.H., I.L.M.R., M.G., L.V.v.d.P.-F., A.H.B. and C.U.B. collected and interpreted data on HRQoL. K. Sikorska and I.L.M.R. performed statistical analysis of the clinical data. A.H.B. and K.J. performed the HRQoL statistical analyses. A.T.A. and L.G.G.-O. contributed to central and local data management. R.Z. and M.G. were clinical project managers of the trial. I.L.M.R., A.H.B. and C.U.B. 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 manuscript, and no medical writer was involved at any stage of the preparation of this manuscript. A.M.M. has served on advisory boards for Bristol Myers Squibb (BMS), Merck Sharp & Dohme (MSD), Novartis, Roche, Pierre Fabre and QBiotics. R.P.M.S. has received honoraria for advisory board participation from MSD, Novartis and Qbiotics and speaking honoraria from BMS and Novartis. E.K. received honoraria for consultancy/advisory relationships (all paid to the institute) from BMS, Novartis, Merck and Pierre Fabre and received research grants not related to this paper from BMS. 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, Roche, 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. W.J.v.H. received compensation for advisory roles (all paid to the institute) from BMS, Amgen and Sanofi. D.J.G. received compensation for advisory roles (all paid to the institute) from Amgen and Novartis. M.W.W. received compensation for advisory roles (all paid to the institute) from Novartis. A.J.S. has served on an advisory board for QBiotics and received fees for professional services from Eli Lily Australia. J.B.A.G.H. received compensation (all paid to the institute) for advisory roles from AIMM, Amgen, BioNTech, BMS, GlaxoSmithKline, Ipsen, MSD, Merck Serono, Molecular Partners, Neogene Therapeutics, Novartis, Pfizer, Roche/Genentech, Sanofi, Seattle Genetics, Third Rock Ventures and Vaximm; stock option ownership of Neogene Therapeutics; and institutional research funding from Amgen, BioNTech, BMS, MSD and Novartis. B.A.v.d.W. has served on the advisory board for BMS. A.v.A. had served on advisory boards and received consultancy honoraria (all paid to the institute) for Amgen, BMS, Novartis, MSD, Merck-Pfizer, Pierre Fabre, Sanofi, Sirius Medical and 4SC and received research grants (all paid to the institute) from Amgen and Merck-Pfizer. R.A.S. has received fees for professional services from F. Hoffmann-La Roche, Evaxion, Provectus Biopharmaceuticals Australia, Qbiotics, Novartis, MSD, NeraCare, Amgen, BMS, Myriad Genetics and GlaxoSmithKline. A.H.B. has received a research grant from BMS. G.V.L. is consultant advisor for Aduro, Amgen, Array Biopharma, Boehringer Ingelheim, BMS, Evaxion, Hexal AG (Sandoz Company), Highlight Therapeutics, MSD, Novartis, Oncosec, Pierre Fabre, Provectus, QBiotics and Regeneron Pharmaceuticals. 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 other authors declare no conflicts of interest.

Additional information

Extended data is available for this paper at https://doi.org/10.1038/s41591-022-01851-x.

Supplementary information The online version contains supplementary material available at https://doi.org/10.1038/s41591-022-01851-x.

Correspondence and requests for materials should be addressed to Christian U. Blank.

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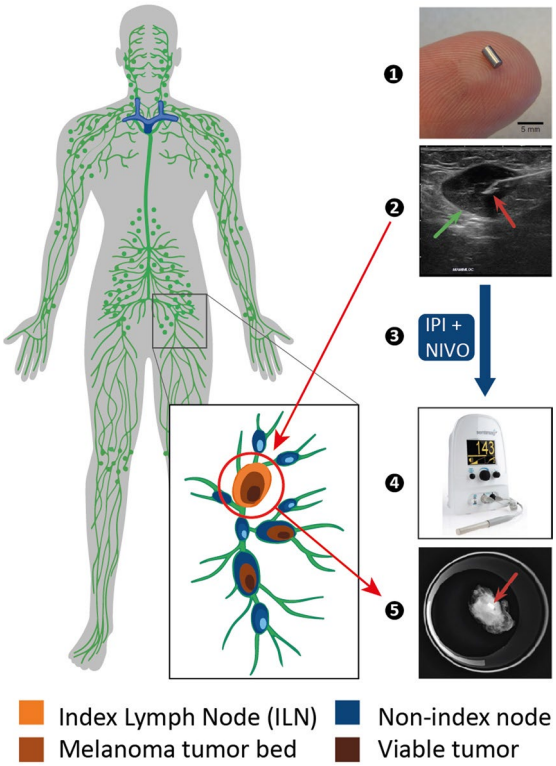
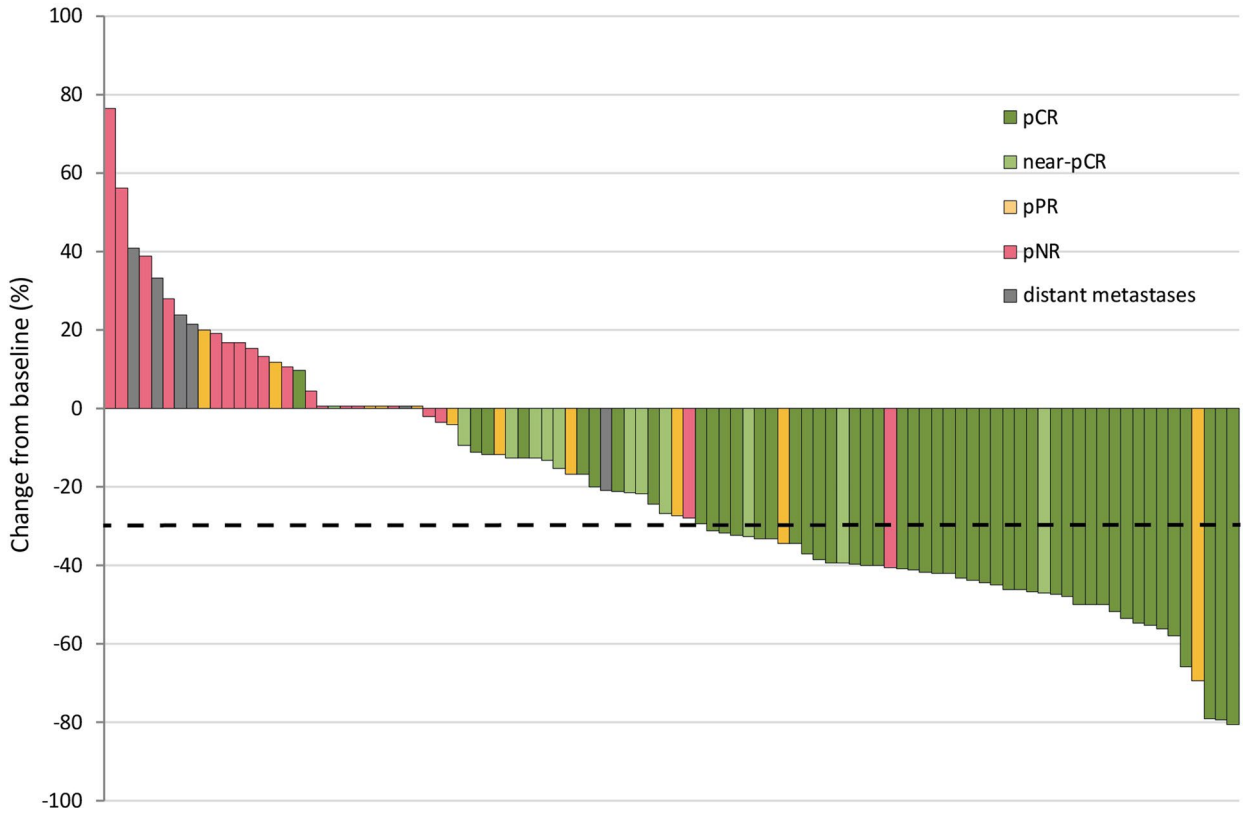
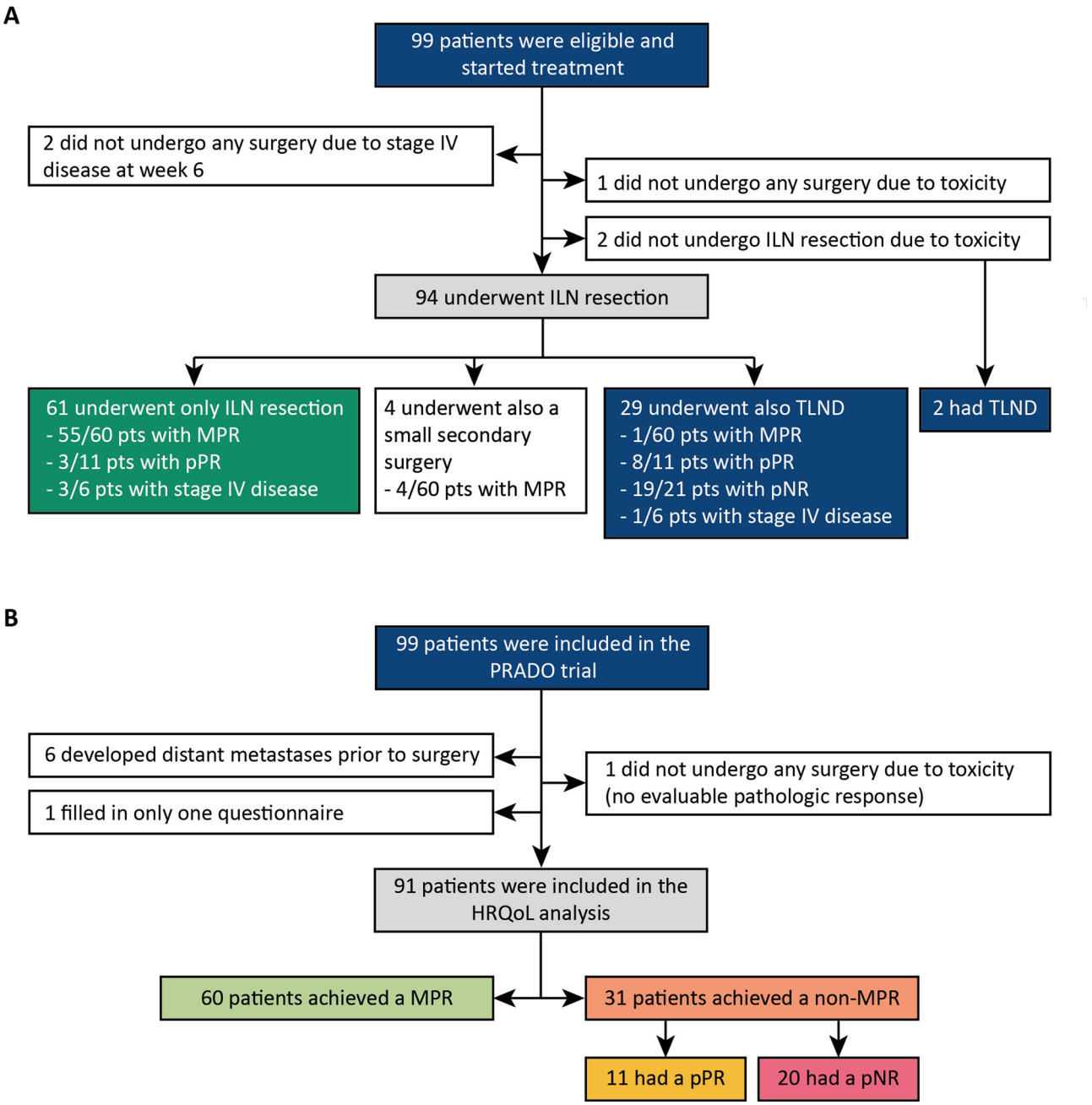


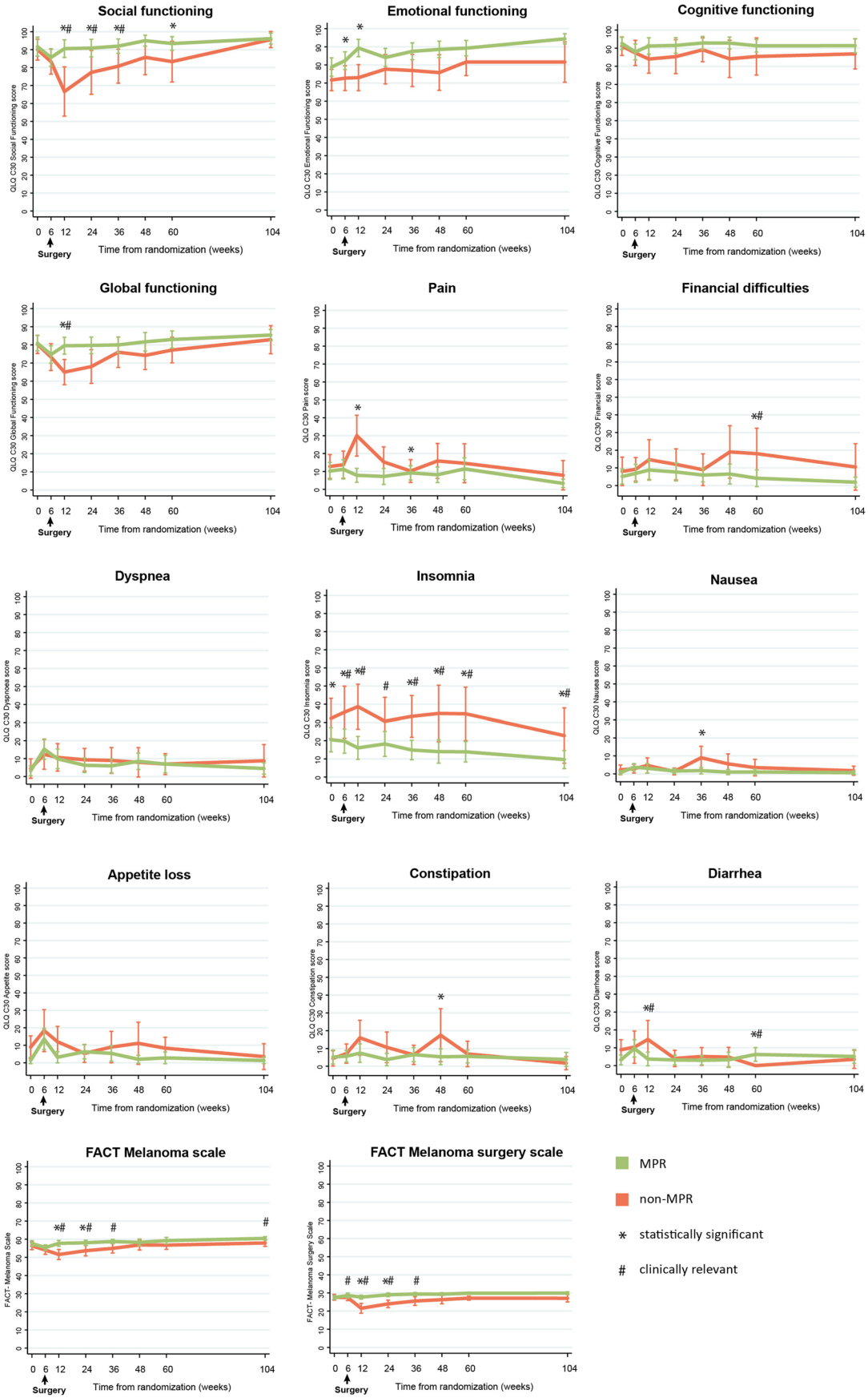
Extended Data Fig. 1 | Marker placement in the ILN. Schematic overview of magnetic seed placement in the ILN and retrieval of the ILN during the ILN procedure. (1) Magnetic seed, (2) Ultrasound image of positioning of the needle tip (red arrow) in the ILN (green arrow) before implantation of the magnetic seed, (3) Two cycles of ipilimumab plus nivolumab are given after the magnetic seed is implanted, (4) Magnetic detector (*Endomag Sentimag®*) used during surgery for seed detection, (5) Postoperative specimen X-ray with magnetic seed (red arrow) in situ. This image has been adapted from *Schermers B, Br J Surg, 2019*[19].



Extended Data Fig. 2 | Objective radiologic response underestimates pathologic response. Waterfall plot of the radiologic change in target lesions (in %) between baseline and week 6 of all PRADO patients with evaluable CT-scan ($n = 96$). Colours indicate the responses as pCR (dark green), near-pCR (light green), pPR (yellow), pNR (red) and distant metastases (grey). The dotted line indicates the cutoff for RECIST version 1.1 radiologic response.

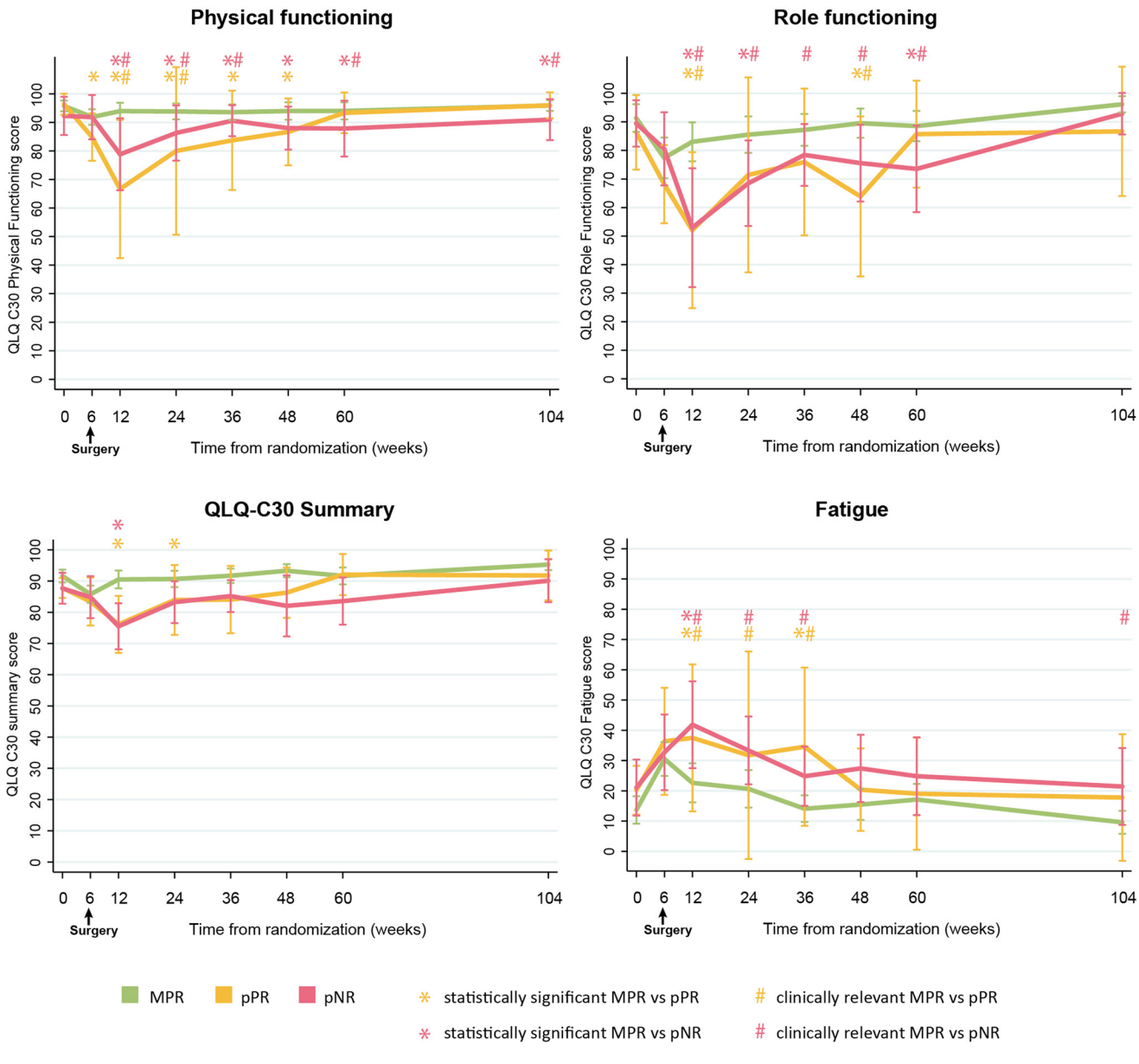


Extended Data Fig. 3 | Flowchart for patient inclusion for surgical morbidity and HRQoL analyses. a, Flow chart of patient inclusion for surgical morbidity analyses. For information regarding the execution of the ILN resection and TLND, see also Supplementary Table 2. **b**, HRQoL analyses of the PRADO trial.

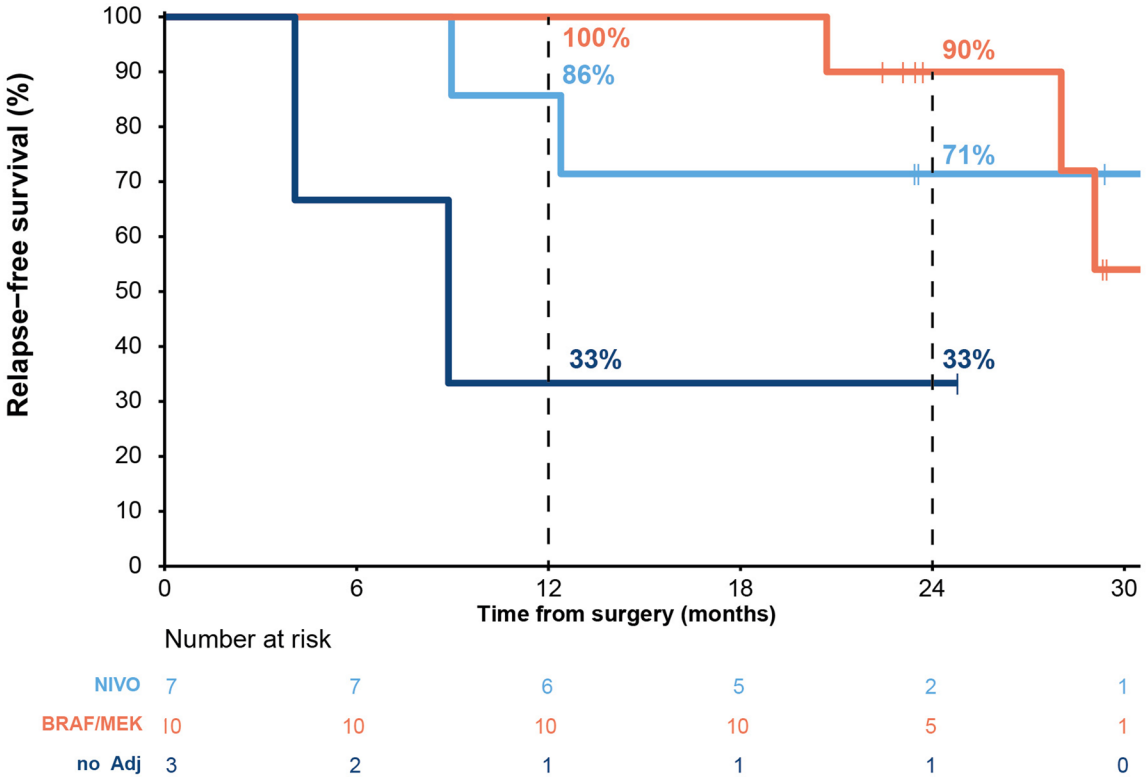


Extended Data Fig. 4 | See next page for caption.

Extended Data Fig. 4 | Effect of pathological response and treatment on HRQoL outcomes. Curves showing the unadjusted mean HRQoL scores of patients with MPR ($n = 60$, green line) versus patients without MPR ($n = 31$, orange line). Error bars indicate the 95% CI. The differences in mean HRQoL scores between patients with MPR and non-MPR (see also Supplementary Table 5) were adjusted for age, gender, adjuvant treatment and relapse status (no/yes). The adjusted score differences were interpreted in terms of statistical significance using a linear mixed effect model with a two tailed *P* value ($P < 0.05$), and by clinical relevance according to the guideline of Cocks et al[32]. Statistically significant adjusted differences were marked with * and clinically relevant differences were marked with # **(**Supplementary Table 5**)**. Results were considered clinically relevant if the adjusted difference in mean scores between the two groups was at least 'medium' and clinically irrelevant if differences in mean scores were 'trivial or small'. Questionnaire compliance rates in the MPR and non-MPR group were 87% vs 97% at baseline, 98% vs 94% at week 6, 90% vs 81% at week 12, 88% vs 81% at week 24, 92% vs 84% at week 36, 85% vs 68% at week 48, 80% vs 77% at week 60 and 87% vs 61% at week 104 (year 2).



Extended Data Fig. 5 | HRQoL comparison between patients with MPR, pPR and pNR. Curves showing the unadjusted HRQoL scores between patients with MPR ($n=60$, green line), pPR ($n=11$, yellow line) and pNR ($n=20$, red line). Error bars indicate the 95% CI. The differences in mean HRQoL scores between patients with MPR versus pPR and MPR versus pNR were adjusted for age, gender, adjuvant treatment and relapse status (no/yes). The adjusted score differences were interpreted in terms of statistical significance using a linear mixed effect model with a two tailed P value ($P<0.05$), and by clinical relevance according to the guideline of Cocks et al[32]. Statistically significant adjusted differences were marked with * and clinically relevant differences were marked with #. Results were considered clinically relevant if the adjusted difference in mean scores between the two groups was at least 'medium' and clinically irrelevant if differences in mean scores were 'trivial or small'.



Extended Data Fig. 6 | RFS by adjuvant treatment. RFS of patients with pNR from the PRADO trial by adjuvant therapy. Patients were treated with adjuvant nivolumab ($n=7$, light blue line), adjuvant BRAF/MEK inhibition ($n=10$, orange line) or no adjuvant therapy ($n=3$, dark blue line). The patient who was lost to follow-up was excluded.

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Reporting Summary

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☐	☒ For null hypothesis testing, the test statistic (e.g. *F*, *t*, *r*) with confidence intervals, effect sizes, degrees of freedom and *P* value noted *Give P values as exact values whenever suitable.*
☒	☐ 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 including 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	Statistical analyses of the PRADO study were performed using R (version 3.5.1). The Health-related quality of life analyses were performed using STATA (version 15.1, StataCorp, College Station, Texas, USA).

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 Research guidelines for submitting code & software for further information.

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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 surgical morbidity between patients who underwent only the ILN procedure and patients who also subsequently underwent TLND, patients who underwent no surgery (n=3) or who underwent a small secondary surgery for removal of some additional lymph nodes (n=4) were excluded from the analysis. For the comparison of health-related quality of life between patients with MPR and no-MPR, patients were excluded if they had distant metastases prior to surgery (n=6, patients went off study after six weeks), if they did not undergo any surgery (n=1), or if they filled in only one questionnaire (n=1).
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. Replication of tumor biopsy analyses is limited due to precious tumor material; a standard automated lab-validated immunohistochemistry assay was followed for PD-L1 staining.
Randomization	PRADO used a single arm trial design with personalized response-directed surgery and adjuvant therapy following neoadjuvant checkpoint inhibition, thus randomization is not relevant to the study.
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

We require information from authors about some types of materials, experimental systems and methods used in many studies. Here, indicate whether each material, system or method listed is relevant to your study. If you are not sure if a list item applies to your research, read the appropriate section before selecting a response.

Materials & experimental systems

n/a	Involved in the study
☒	☐ Antibodies
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☒	☐ Palaeontology and archaeology
☒	☐ Animals and other organisms
☐	☒ Human research participants
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☒	☐ Dual use research of concern

Methods

n/a	Involved in the study
☒	☐ ChIP-seq
☒	☐ Flow cytometry
☒	☐ MRI-based neuroimaging

Human research participants

Policy information about studies involving human research participants

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.

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 (and for the health-related quality of life analyses this was Februari 1st, 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.

Article

Neoadjuvant relatlimab and nivolumab in resectable melanoma

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Relatlimab and nivolumab combination immunotherapy improves progression-free survival over nivolumab monotherapy in patients with unresectable advanced melanoma[1]. We investigated this regimen in patients with resectable clinical stage III or oligometastatic stage IV melanoma (NCT02519322). Patients received two neoadjuvant doses (nivolumab 480 mg and relatlimab 160 mg intravenously every 4 weeks) followed by surgery, and then ten doses of adjuvant combination therapy. The primary end point was pathologic complete response (pCR) rate[2]. The combination resulted in 57% pCR rate and 70% overall pathologic response rate among 30 patients treated. The radiographic response rate using Response Evaluation Criteria in Solid Tumors 1.1 was 57%. No grade 3–4 immune-related adverse events were observed in the neoadjuvant setting. The 1- and 2-year recurrence-free survival rate was 100% and 92% for patients with any pathologic response, compared to 88% and 55% for patients who did not have a pathologic response ($P = 0.005$). Increased immune cell infiltration at baseline, and decrease in M2 macrophages during treatment, were associated with pathologic response. Our results indicate that neoadjuvant relatlimab and nivolumab induces a high pCR rate. Safety during neoadjuvant therapy is favourable compared to other combination immunotherapy regimens. These data, in combination with the results of the RELATIVITY-047 trial[1], provide further confirmation of the efficacy and safety of this new immunotherapy regimen.

Patients with locoregionally advanced, resectable melanoma have a high risk of relapse and death from melanoma[3]. Specifically, patients with clinically detected nodal disease have a risk of melanoma-specific mortality that could be as high as 75%[3]. Although current adjuvant therapy decreases the risk of recurrence by about 50% (BRAF-targeted therapy hazard ratio (HR) 0.49, single agent PD-1 HR approximately 0.54)[4,5], there has yet to be confirmation of the impact on overall survival[4,6]. In an attempt to intensify therapy beyond single agent anti-PD-1, the Checkmate-915 trial was designed to investigate if the addition of ipilimumab to nivolumab in the adjuvant setting improved recurrence-free survival (RFS) compared to nivolumab alone. The combination of ipilimumab and nivolumab did not improve RFS (HR 0.92) and it significantly increased toxicity (grade 3–4 adverse events (AEs) 43%, compared to 23% for single agent anti-PD-1)[7], indicating that intensification of adjuvant therapy with ipilimumab and nivolumab in the adjuvant setting is not the optimal approach for improving recurrence outcomes.

Neoadjuvant therapy offers several advantages over upfront surgery and adjuvant therapy, including potential for improvement in clinical outcomes and understanding molecular and immunological mechanisms of treatment response and resistance[8–13]. Additionally, neoadjuvant immunotherapy has demonstrated ability in preclinical models and in human samples to increase expansion of antigen-specific T cells due to the presence of tumour at the time of treatment compared to

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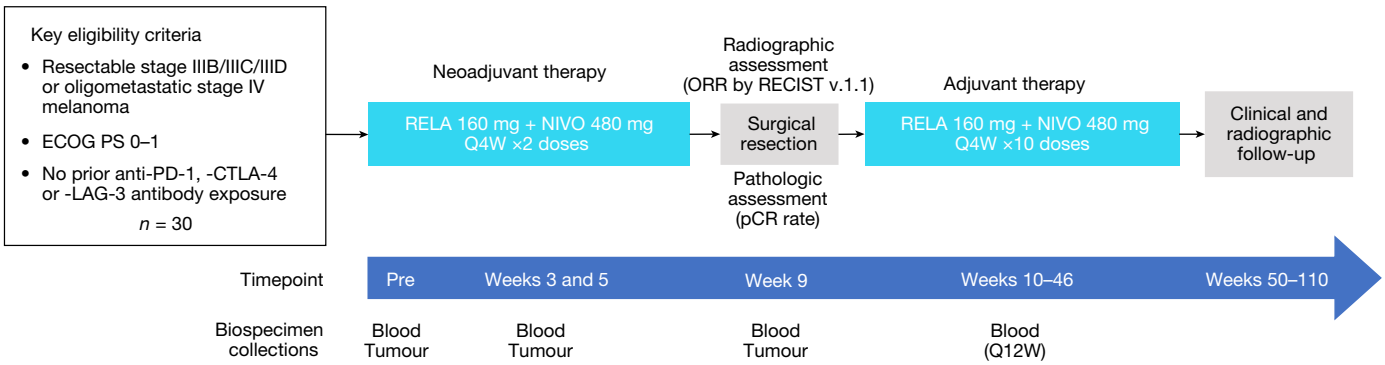

Fig. 1 | Study design. Eligible patients receive two doses of relatlimab 160 mg with nivolumab 480 mg intravenously every 4 weeks (Q4W) in the neoadjuvant setting and then have repeat imaging for calculation of RECIST response. Surgery takes place at week 9 for evaluation of pathologic response. Patients receive up to ten doses of relatlimab 160 mg and nivolumab 480 mg every 4 weeks in the adjuvant setting and are followed for 2 years for evidence of recurrence. Blood and tumour are collected during screening, at weeks 3, 5 and at time of surgery at week 9. Blood is collected every 12 weeks (Q12W) in the adjuvant setting. ECOG PS, Eastern Cooperative Oncology Group Performance Status; RELA, relatlimab; NIVO, nivolumab; ORR, objective response rate; RECIST, Response Evaluation Criteria in Solid Tumors.

the expansion seen when the same immunotherapy is administered in the adjuvant setting[14,15]. The neoadjuvant setting also offers the opportunity to intensify therapy with combinations for a short pre-operative course, allowing for a direct estimate of therapeutic efficacy and the ability to inform adjuvant therapy decisions.

One potential limitation of neoadjuvant immunotherapy is delay in curative-intent surgery if grade 3/4 immune-related adverse events (IRAEs) occur during treatment. For example, neoadjuvant administration of 2–3 doses of ipilimumab 3 mg kg^{-1} + nivolumab 1 mg kg^{-1} was associated with 73–90% grade 3/4 toxicities, which led to surgical delays in approximately 27% of patients[15,16]. The OpACIN-NEO trial compared two doses of neoadjuvant therapy with different dosing strategies of ipilimumab and nivolumab. This study demonstrated that ipilimumab 1 mg kg^{-1} with nivolumab 3 mg kg^{-1} showed an at least equivalent pCR rate (57%) to the ipilimumab 3 mg kg^{-1} + nivolumab 1 mg kg^{-1} regimen (47%), but with a lower (20% versus 40%) incidence of grade 3/4 toxicities[17]. These data highlight the goal of identifying new regimens that enhance pathologic responses and reduce risk of recurrence with improved toxicity profiles.

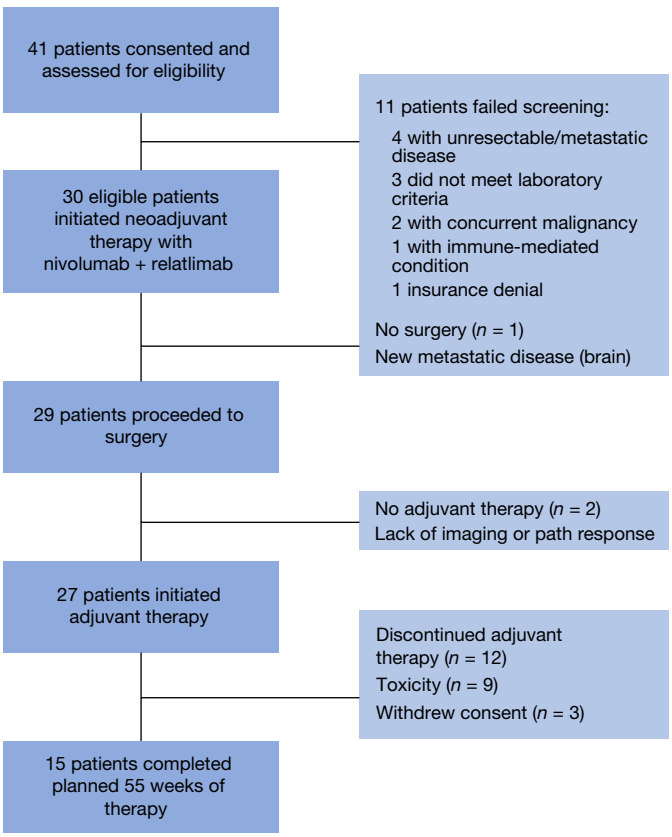

Fig. 2 | Consort diagram and patient disposition. A total of 41 patients were screened for protocol and there were 11 screen failures and 30 patients were eligible to initiate therapy. After completion of neoadjuvant therapy, one patient developed distant metastases and did not proceed to surgery. Twenty-nine patients proceeded to surgery and 17 patients (57%) achieved a pCR. Twenty-seven patients initiated adjuvant therapy and 15 went on to complete entire duration of treatment. path, pathologic.

The lymphocyte-activation gene 3 (LAG-3) regulates an inhibitory immune checkpoint limiting T cell activity and is a marker for T cell exhaustion[18,19]. Relatlimab is a human IgG4 LAG-3-blocking monoclonal antibody that restores the effector function of exhausted T cells and has been investigated in both checkpoint inhibitor-naïve (NCT03470922)[1] and refractory metastatic melanoma (NCT01968109)[20]. In the randomized phase 2/3 RELATIVITY-047 study, the combination of relatlimab with nivolumab in patients with treatment-naïve unresectable stage III or stage IV metastatic melanoma demonstrated significant improvement in progression-free survival compared to single agent nivolumab (HR 0.78 (95% confidence interval (CI), 0.64–0.94)). Moreover, the combination was well tolerated with 21.1% of patients experiencing grade 3/4 treatment-related AEs[1]. Given its efficacy and favourable toxicity profile, this combination therapy received US Food and Drug Administration approval for use in patients with metastatic melanoma on 18 March 2022.

Our group previously published our experience of a randomized, investigator-initiated clinical trial of either single agent nivolumab (240 mg intravenously every 2 weeks up to four doses) or nivolumab 1 mg kg^{-1} with ipilimumab 3 mg g^{-1} (intravenously every 3 weeks up to three doses) in the neoadjuvant setting[16]. In this trial, we concluded that although neoadjuvant single agent nivolumab was safe (8% grade 3/4 toxicities), its efficacy was modest (25% pCR rate). Although the combination of nivolumab with ipilimumab was effective with a 45% pCR rate, the toxicity was prohibitively high with 73% grade 3/4 toxicities[16]. Given these data and the early closure of the study due to suboptimal performance of both treatment arms, our team sought to evaluate new immunotherapy combinations with the intention of preserving pathologic response while minimizing toxicities. We opened a new arm to this existing prospective clinical trial to determine pCR rate, safety and efficacy of the relatlimab and nivolumab combination in patients with resectable clinical stage III or oligometastatic stage IV melanoma (Clinicaltrial.gov number NCT02519322) (Fig. 1). Here we report the clinical results and immune profiling of this neoadjuvant therapy combination.

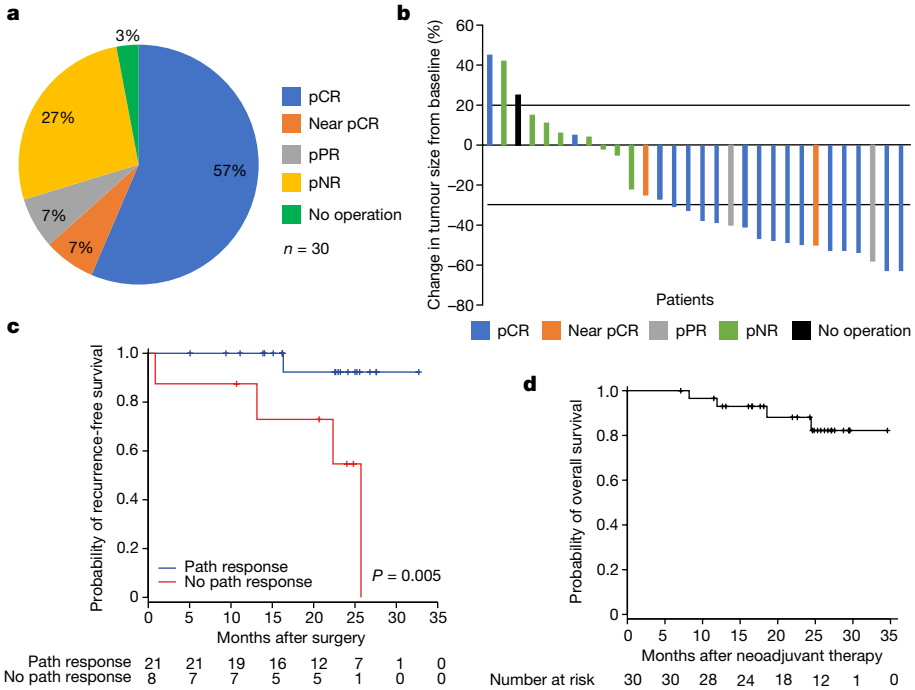

Fig. 3 | Response data and long-term outcomes. a, Breakdown of pathologic responses for the 29 patients who underwent surgery as interpreted by the guidelines of the INMC. Result details (values in chart rounded): no operation, 1 of 30 patients (3.33%);pCR, 17 of 30 patients (56.67%); near pCR, 2 of 30 patients (6.67%);pPR, 2 of 30 patients (6.67%);pNR, 8 of 30 patients (26.67%). **b**, Waterfall plot of neoadjuvant response as per RECIST 1.1 criteria with colour coding indicating pathologic response. pCR indicates lack of viable tumour. Near pCR indicates greater than 0% but less than or equal to 10% viable tumour, pPR is greater than 10% to less than or equal to 50% viable tumour and pNR is greater than 50% viable tumour. **c**, Probability of being relapse-free based on any pathologic response versus no pathologic response. **d**, Overall survival curves for the entire cohort.

Patient characteristics

From 19 September 2018 to 23 September 2020, 41 patients were consented and 30 passed screening evaluations and were treated at MD Anderson Cancer Center and Memorial Sloan Kettering Cancer Center. The most common reasons for screen failure included lack of resectable disease as determined by multidisciplinary review (n = 4 patients) and laboratory values outside the specified criteria (n = 3 patients) (Fig. 2).

The median age of treated patients was 60 (range 35–79) and 63% of patients were male (Extended Data Table 1). Melanoma clinical stage was 60% stage IIIB, 26% IIIC, 7% IIID and 7% M1A by the American Joint Committee on Cancer 8th edition criteria[3]. Thirty-three per cent of patients had de novo clinical stage III or oligometastatic stage IV melanoma, and 67% had prior melanoma surgery. Only 17% of patients had *BRAF*-mutated melanoma, probably due to enrolment on a competing neoadjuvant trial specific for patients with *BRAF*-mutated disease. Only one patient had prior systemic therapy (BRAF and MEK inhibition). The median target lesion sum of diameters was 26 mm (Extended Data Table 1).

Patient disposition

Of the 30 treated patients, 29 were able to receive the planned two doses of neoadjuvant relatlimab and nivolumab. One patient received only one dose due to asymptomatic troponin elevations with concern for myocarditis, which was eventually determined to not be attributable to neoadjuvant immunotherapy after the patient underwent myocardial biopsy and was able to proceed safely to surgery. One patient did not proceed to surgery due to development of distant metastatic disease during neoadjuvant therapy. Of the 29 patients that underwent surgery, 27 patients proceeded to surgery as scheduled at week 9; one patient was delayed due to the aforementioned myocarditis toxicity concern and one patient was delayed due to SARS-CoV2 pandemic-related hospital surgery restrictions. Twenty-seven patients proceeded with adjuvant therapy and two patients elected to not proceed with adjuvant therapy due to suboptimal pathologic and imaging response. Fifty-six per cent of patients completed the entire duration of protocol therapy, 33% of patients discontinued adjuvant therapy due to toxicity and 11% of patients withdrew consent during adjuvant therapy (Fig. 2). Currently, all patients are off protocol therapy.

Clinical activity

Of the 30 patients enroled, 29 patients underwent surgery (97%), 17 (57%; 95% CI, 37–75%) achieved pCR, two (7%) near pCR (defined as greater than 0% but less than or equal to 10% viable tumour), two (7%) partial pathologic response (pPR; defined as greater than 10% to less than or equal to 50% viable tumour) and eight (27%) no pathologic response (pNR; defined as greater than 50% viable tumour) (Fig. 3a). A major pathologic response (pCR + near pCR) was achieved in 63% of patients and any pathologic response (pCR + near pCR + pPR) in 70% of patients[2].

The radiographic overall response rate was 57% (all partial responses (PRs); 33% had stable disease (SD) and 10% had progressive disease (PD) (Fig. 3b)) in the intention-to-treat population. Pathologic response was frequently disconcordant with radiographic response at 8 weeks. For example, of the 19 patients who achieved major pathologic response (pCR and near pCR), one patient had radiographic PD, three had SD and 15 had PR. Of the eight patients with pNR, only one had radiographic PD and seven had SD. In the 16 patients with tumour sum of diameters at the median or higher (at least 26 mm), there was a mix of Response Evaluation Criteria in Solid Tumors (RECIST; 6% PD, 38% SD, 56% PR) and pathologic responses (38% pNR, 6% pPR, 6% near pCR, 50% pCR), indicating that baseline tumour burden did not correlate directly with pathologic or radiographic response.

With a median follow-up of 24.4 months (range 7.1–34.6 months) for the 30 treated patients, 1- and 2-year event-free survival rates (time

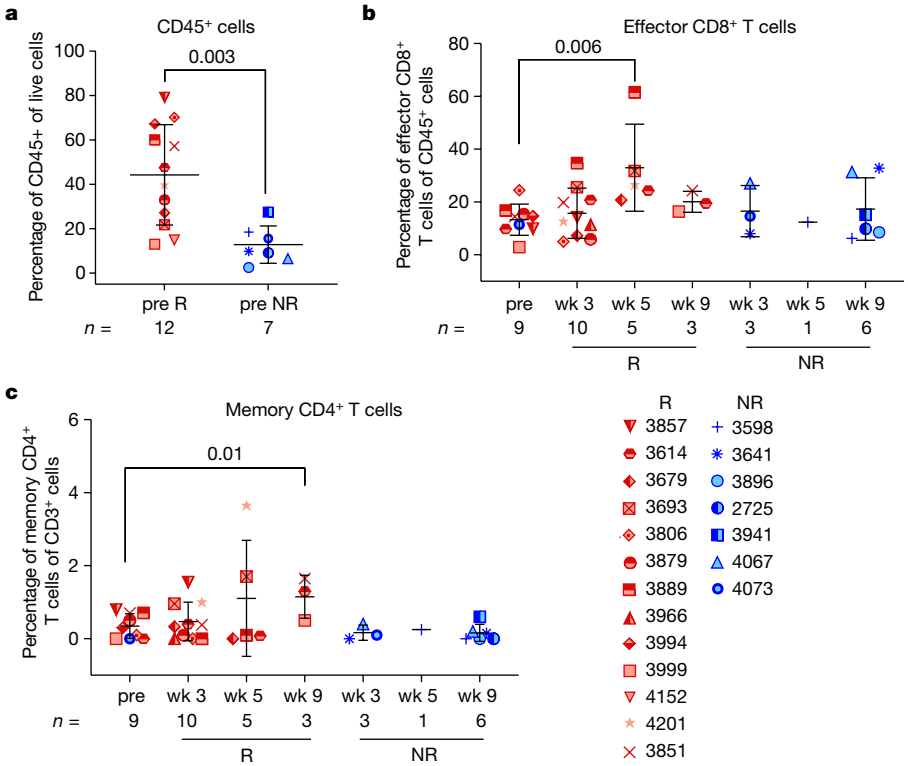

Fig. 4 | Correlative analyses in tumour specimens. Tumour tissue samples harvested from patients at baseline, and post relatlimab and nivolumab treatment were analysed in a single experiment by CyTOF (**a**–**c**). **a**, Frequency of CD45$^+$ cells was assessed through manual gating. **b**, Frequency of an effector CD8$^+$ T cell subset (CD3$^+$CD8$^+$CD45RO^{low}) in unsupervised clustering is shown. **c**, Frequency of a memory CD4$^+$ subset (CD45RO$^+$ICOS$^+$ TCF7$^+$BTLA$^+$CD28$^+$TIGIT$^+$) was determined by unsupervised clustering. Data shown in **a**–**c** are mean ± s.d., and *n* values are indicated in the figure. *P* values shown in each graph were calculated by two-tailed unpaired *t*-test, with no multiple comparisons. Red indicates pathologic responders; blue, non-responders. CyTOF, mass cytometry; NR, non-responder; R, responder; wk, week.

from treatment initiation to recurrence in all patients) were 90% and 81%, respectively (Extended Data Fig. 1). The 1- and 2-year RFS rates (time from surgery to recurrence in patients that underwent surgery) were 97% and 82%, respectively (Extended Data Fig. 2a). The 1- and 2-year RFS rates were 100% and 91% for patients with pCR, compared to 92% and 69% for those without pCR ($P = 0.10$) (Extended Data Fig. 2b). The 1- and 2-year RFS rates were 100% and 92% for patients with any pathologic response, compared to 88% and 55% for those without a pathologic response ($P = 0.005$) (Fig. 3c). The 1- and 2-year overall survival rates for all patients were 93% and 88% (Fig. 3d).

Of the three patients with RECIST PD to neoadjuvant therapy, one patient developed distant metastases (brain) and did not undergo surgery. The two other RECIST PD patients appeared to progress locally in the involved nodal basin only, and complete surgical resection was achieved for both. One of these patients did not proceed with adjuvant therapy due to pNR and patient/physician decision; the other achieved a pCR, proceeded with adjuvant therapy and completed protocol therapy without disease recurrence (Fig. 2). Two patients (both pNR) experienced local recurrence in soft tissue adjacent to site of prior surgical resection at 3 and 14 months after completion of all ten doses of adjuvant therapy. One patient with pCR reportedly experienced unconfirmed disease progression in the brain and passed away 14 months after surgery.

Safety

There were no grade 3/4 IRAEs during the 8 weeks of neoadjuvant therapy (Extended Data Table 2). Twenty-six per cent of patients developed grade 3/4 IRAEs in the adjuvant setting (from week 9 and beyond) (Extended Data Table 2). Overall, 33% of patients elected to discontinue adjuvant therapy due to any toxicity (most commonly transaminitis). Although there were asymptomatic troponin elevations, no patients experienced symptomatic troponin elevations, myocarditis or other cardiac toxicity attributable to study medications as assessed by cardiology consultation. The most frequent IRAE was secondary adrenal insufficiency (23%), with none of the patients experiencing adrenal recovery to date.

Correlative studies

Biomarker analysis focused on characterizing immune cell subsets in the tumour microenvironment and peripheral blood was performed by mass cytometry (CyTOF) and flow cytometry. LAG-3 and PD-1 levels in baseline tumour samples did not correlate with pathologic response (Extended Data Fig. 3). In tumours, the frequency of CD45$^+$ cells was higher in pretreatment samples of responders, defined as patients with less than 50% tumour viability at surgery, compared to pretreatment samples of non-responders (NRs; greater than or equal to 50% tumour viability) (Fig. 4a) by CyTOF. Unsupervised clustering identified an effector CD8$^+$ T cell subset (CD8$^+$CD45RO^{low}) and a memory CD4$^+$ T cell subset (CD4$^+$CD45RO$^+$TCF7$^+$CD28$^+$BTLA$^+$TIGIT$^+$) that were increased in posttreatment tumour specimens versus pretreatment in patients with favourable response (Fig. 4b,c). The increases in these cell populations were not appreciated in the NR patient group, although it should be noted that the number of evaluable specimens was low in this group (Fig. 4b,c). By contrast, the frequency of an M2-like macrophage subset decreased in tumours after treatment in patients with favourable response (Extended Data Fig. 4a). In blood, there was a trend for increased EOMES$^+$CD8$^+$ T cells in patients with favourable versus non-favourable response after treatment, with largest differences seen at week 5 posttreatment (Extended Data Fig. 4b).

Discussion

In patients with resectable clinical stage III or oligometastatic stage IV melanoma, neoadjuvant relatlimab with nivolumab resulted in high pCR rate (57%; 95% CI, 37–75%) and improvement in the 2-year RFS rate in patients who achieved any pathologic response compared to those without a pathologic response ($P = 0.005$). The lower limit CI (37%) exceeded the minimum target of 30% in the study design. This regimen was tolerated well in the neoadjuvant setting, with 26% grade 3 toxicities noted with continued dosing in the adjuvant setting. In patients with pathologic response, increased immune cell infiltration was identified at baseline and decreased M2 macrophages were demonstrated over the course of neoadjuvant therapy.

The first two randomized arms of this trial evaluated both single agent nivolumab and the combination of ipilimumab 3 mg kg^{-1} and nivolumab 1 mg kg^{-1}. Twenty-seven per cent of patients treated with ipilimumab 3 mg kg^{-1} and nivolumab 1 mg kg^{-1} required surgical delays of 1–10 weeks due to need for steroids and prolonged steroid taper[16]. With no grade 3/4 IRAEs observed in the neoadjuvant setting and no confirmed toxicity-related surgical delays, the combination of nivolumab and relatlimab now provides complementary information and demonstrates a highly effective regimen with manageable toxicities in the neoadjuvant setting.

Although there were no grade 3/4 IRAEs in the neoadjuvant setting, 26% grade 3/4 toxicities were experienced in the adjuvant setting. The most common IRAE observed was secondary adrenal insufficiency. As 33% of patients discontinued therapy before the planned full year of treatment, due to toxicity, it raises questions of whether continued dosing in the adjuvant setting is necessary following pathologic response to neoadjuvant therapy. Additionally, none of the patients who stopped therapy early due to toxicity have experienced a recurrence event. There is not clear consensus on the need for the adjuvant phase of therapy within neoadjuvant trials, with completed or ongoing trials including complete omission of any adjuvant therapy, use of adjuvanttherapy only in poor responders or adjuvant therapy to complete 1 year of treatment[8,15–17,21–23], Additionally, the use of adjuvant therapy can certainly affect the RFS and can cloud the interpretation of neoadjuvant therapy data. Understanding the contribution of adjuvant immunotherapy following immunotherapy in the neoadjuvant setting to clinical benefit remains an active area of research interest.

The historic dogma in neoadjuvant chemotherapy emphasized pCR as the critical end point correlating with the most durable clinical outcomes[11–13]. This was similarly appreciated in the International Neoadjuvant Melanoma Consortium (INMC) pooled analysis of neoadjuvant BRAF/MEK inhibitor use in patients with clinical stage III melanoma, showing that achieving a pCR, but not a pPR, correlated with improved RFS[9,22,23]. Although the pCR end point may still be appropriate for neoadjuvant chemotherapy or molecularly targeted therapy, our data provide further evidence that in the context of neoadjuvant immunotherapy in melanoma, any pathologic response (less than 50% viable tumour) is associated with favourable long-term clinical outcomes (Fig. 3c)[9,16,17,21]. Similar patterns of improved clinical responses with any pathologic response are being appreciated in neoadjuvant immunotherapy trials across solid tumours[24–26].

Although baseline LAG-3 and PD-1 levels in tumour samples did not correlate with response, we observed increased frequencies of memory $CD4^+$ and effector $CD8^+$ T cells in the posttreatment tumour specimens of patients with favourable treatment response. These findings are concordant with previous studies in which responses to anti-PD-1 were associated with higher $CD8^+$ T cells[15–17,21,27,28]. Furthermore, we observed a reduction in M2-like macrophages with treatment only in the patients that achieved a pathologic response, possibly serving as a target to further improve responsiveness to this regimen, and/or to further evaluate in other studies of nivolumab plus relatlimab[29]. Analysis of longitudinal peripheral blood specimens by flow cytometry revealed higher frequency of $EOMES^+CD8^+$ T cells in posttreatment samples of responding patients, suggesting $CD8^+$ T cells expressing EOMES could contribute to tumour regression. This supports a potentially critical role of EOMES for antitumour activity of $CD8^+$ T cells, as previously described[30]. These data indicate that a higher frequency of total immune cell infiltration, as well as increased specific effector $CD4^+$ and $CD8^+$ T cell subsets, with a concomitant decrease in suppressive myeloid cells in the tumour microenvironment, correlate with clinical response to this regimen in the neoadjuvant setting. It should be noted that the number of usable samples in the NR patients was low, which limits comparative correlative analyses in this study.

We acknowledge that the study is limited by its small sample size and that these results are preliminary, based on findings at two academic research institutions. However, the cohort evaluated in this study ($n = 30$) is largely similar to the individual arms in the OpACIN-NEO study and to other single-arm neoadjuvant immunotherapy trials[17,21,23–26]. With a median follow-up of 24 months, we also acknowledge that additional follow-up is needed to fully assess clinical impact and the durability of responses. However, this initial data is encouraging, and the pooled analyses of melanoma neoadjuvant trials support the importance of pathologic response rates as an early predictor of durable benefit[9]. Similarly, additional translational studies beyond the scope of this manuscript are planned, including RNA sequencing for broad assessment of additional immune signatures and populations that have been implicated in immunotherapy resistance[28,31].

In summary, neoadjuvant relatlimab and nivolumab is a highly active regimen that achieves a 70% pathologic response rate with a favourable safety profile in patients with high-risk, resectable clinical stage III or oligometastatic stage IV melanoma. These data are complementary to the RELATIVITY-047 study in patients with unresectable metastatic melanoma, and together further support the promise of this new combination immunotherapy regimen in this disease.

Online content

Any methods, additional references, Nature Research 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/s41586-022-05368-8.

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Methods

Patients

Eligible patients were 18 years or older with clinical stage III or oligometastatic (less than three organ sites with metastases) stage IV melanoma with lesions that were measurable by RECIST 1.1 (ref. [32]). Resectable clinical stage III melanoma was defined as clinically detectable, RECIST-measurable lymph node disease with or without regional in-transit or satellite metastases and without distant metastases. Resectability of stage III and IV disease was verified via multidisciplinary conference. Patients with recurrent melanoma or de novo American Joint Committee on Cancer 8th edition[3] clinical stage III or IV disease were considered eligible, and all melanoma subtypes, including uveal, mucosal or acral, were eligible for enrolment. All patients had Eastern Cooperative Oncology Group performance status of 0 or 1 with normal organ function and no contra-indication to surgery. Patients requiring active immunosuppressive therapy, or who had active autoimmune or infectious disease, or with uncontrolled cardiovascular disease or ongoing concurrent malignancy were excluded.

Study design

This investigator-initiated, prospective study was conducted at two academic medical centres in the United States. Patients received two intravenous fixed doses of relatlimab 160 mg with nivolumab 480 mg at 4-week intervals. Surgery was planned 9 weeks after treatment initiation. Patients were given up to ten doses of the combination starting 4–6 weeks after surgery to complete a total of 12 doses. Patients were followed for 2 years postsurgery for any evidence of disease recurrence (study design details are provided in Fig. 1).

The primary end point was determination of pCR (defined as no viable tumour upon pathologic evaluation at surgery) rate[2]. For this exploratory biomarker study, a pathologic response rate of 30% was suggested for patients treated with this combination. Assuming this true pCR rate, the probability of at least 5 out of 30 patients experiencing a pCR is 0.97. Secondary end points included RECIST 1.1 overall response rate, safety, RFS, event-free survival, overall survival and correlation of immune profiling with response.

All patients were monitored for AEs according to the National Cancer Institute Common Terminology Criteria for Adverse Events, v.4.03 (ref. [33]). Due to concern for myocarditis based on prior relatlimab studies[1,20], patients were required to have cardiac troponin testing, in addition to assessment of blood counts, electrolytes, liver and kidney function before each scheduled infusion. All patients underwent baseline tumour staging (either computed tomography or positron-emission tomography-computed tomography of body and magnetic resonance imaging of brain) within 28 days of treatment initiation and again during week 8 for determination of RECIST response. Scans were performed every 3 months in the postoperative setting for up to 2 years after surgery. Core needle biopsy was performed within 28 days of treatment initiation and at weeks 3 and 5 for correlative research. Blood was collected at time of treatment initiation, weeks 3, 5, 9 and then every 12 weeks in the postoperative setting for up to 2 years (Fig. 1). Surgical resection was completed at week 9 per institutional standards and per the guidelines of the INMC[8,10]. Pathologic review of surgical resection specimens was performed by a small group of dermatopathologists who assessed the specimens according to the practices outlined by the INMC[2]. pCR was defined as no viable tumour, near pCR as greater than 0% but less than or equal to 10% viable tumour, pPR as greater than 10% to less than or equal to 50% viable tumour and pNR as greater than 50% viable tumour.

Study oversight

The study was conducted in accordance with the clinical trial protocol and Good Clinical Practices Guidelines as defined by the International Conference on Harmonization and the Declaration of Helsinki. The study was approved by the institutional review boards of MD Anderson Cancer Center and Memorial Sloan Kettering Cancer Center. All patients provided informed consent for participation in the clinical trial. The study was designed by investigators at MD Anderson Cancer Center and the manuscript was written by the authors in its entirety. Trial monitoring was by the Investigational New Drugs office at MD Anderson Cancer Center. Study drugs were supplied by Bristol-Myers Squibb.

Statistical analyses

RFS time was computed from surgery date to date of progression/recurrence or death (if died without progression/recurrence). Event-free survival time was computed from start of treatment to date of progression/recurrence or death (if died without progression/recurrence). Patients alive at the last follow-up date who did not experience progression/recurrence were censored. Patients who died without experiencing progression/recurrence were censored. Overall survival time was computed from start of neoadjuvant therapy to last known vital status. Patients alive at the last follow-up date were censored. The Kaplan–Meier method was used to estimate the outcome measures, and group differences were evaluated using the log-rank test. All statistical analyses were performed using SAS v.9.4 for Windows.

Correlative studies

Blood and tumour were collected at the timepoints shown in Fig. 1. Cells were isolated and prepared from peripheral blood and tumour tissues for flow cytometry and CyTOF analyses as per the specifications below.

Isolation and preparation of cells from peripheral blood and tissues. Whole blood was collected in tubes containing sodium heparin (BD Vacutainer), resuspended in phosphate-buffered saline (PBS), layered atop Ficoll (StemCell Technologies) and centrifuged at 800*g* for 25 min. The interface peripheral blood mononuclear cells (PBMCs) were harvested and washed twice with PBS and centrifuged at 500*g* for 10 min. Fresh tumour tissue was dissociated with GentleMACS system (Miltenyi Biotec). PBMC and tumour specimens destined for CyTOF analysis were stained for viability with 5 μmol l^{-1} cisplatin (Fluidigm, now Standard Biotools) in PBS containing 1% bovine serum albumin (BSA) and then washed three times. All specimens were resuspended in AB serum with 10% (vol/vol) dimethyl sulfoxide for storage in liquid nitrogen until downstream assays were performed.

Flow cytometry staining and analysis

Flow cytometry analysis was performed on PBMCs (see Extended Data Table 3 for antibodies used in flow cytometry). Single-cell suspensions were stained with 16 fluorescent primary antibodies and live/dead dye. Specimens were analysed using the BD LSRFortessa ×20 cytometer and BD FACSDiva acquisition software v.8.0.1 (BD Biosciences), and downstream analyses were performed manually using FlowJo software v.10.5.3 (BD). See Extended Data Fig. 5 for flow cytometry sequential gating/sorting strategies.

Mass cytometry staining and analysis

CyTOF analyses were performed on tumour specimens as well as PBMCs (see Extended Data Table 4 for antibodies used in CyTOF analysis). Single-cell suspensions were assayed with 41 antibodies, plus Ir DNA-intercalator and cisplatin. Antibodies were either purchased preconjugated from Fluidigm or purchased purified and conjugated in-house using MaxPar X8 Polymer kits (Fluidigm, now Standard Biotools). Briefly, samples were thawed and stained with cell surface antibodies in PBS containing 5% goat serum and 1% BSA for 30 min at 4 °C. Samples were then washed in PBS containing 1% BSA, fixed and permeabilized according to the instructions of the manufacturers using the FoxP3 staining buffer set (eBioscience), before being incubated with intracellular antibodies in permeabilization buffer for 30 min at 4 °C. Samples were washed and incubated in Ir intercalator (Fluidigm,

now Standard Biotools) and stored at 4 °C until acquisition, generally within 12 h. Immediately before acquisition, samples were washed and resuspended in water containing EQ 4 element beads (Fluidigm, now Standard Biotools). Samples were acquired on a Helios mass cytometer (Fluidigm, now Standard Biotools).

FCS files were preprocessed in R (R Foundation for Statistical Computing (https://www.R-project.org/)) using a CyTOF package (Premessa, Parker Institute for Cancer Immunotherapy (https://github.com/ParkerICI)) and gated manually in FlowJo (BD). Data were then exported as FCS files for downstream analysis and arcsinh transformed using a coefficient of 5 [x_transformed = arcsinh(x/5)]. To visualize the high-dimensional data in two dimensions, the t-Distributed Stochastic Neighbor Embedding dimension reduction algorithm was applied, using all channels besides those used to manually gate the population of interest (for example, CD45 or CD3). Clustering analysis was performed in R using the FlowSOM and ConsensusClusterPlus packages[34].

Graphics and statistics

Graphs were created and statistical analyses performed using GraphPad Prizm v.9.2 (GraphPad Software, LLC).

Reporting summary

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

Data availability

Data supporting the findings of this study have been provided to *Nature* through direct deposition.

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Competing interests R.N.A.: research funding from Bristol-Myers Squibb, Iovance, Merck and Novartis; consulting role for Bristol-Myers Squibb, Iovance and Novartis. M. Postow: consulting fees from Aduro, Array BioPharma, Bristol-Myers Squibb, Eisai, Incyte, Merck, NewLink Genetics, Novartis and Pfizer; honoraria from Bristol-Myers Squibb and Merck; institutional support from Array BioPharma, AstraZeneca, Bristol-Myers Squibb, Infinity, Merck, Novartis and RGenix. M.I.R.: clinical research funding from Amgen; consulting/advisory board member role for Amgen, Castle BioSciences, Merck and Novartis. I.C.G.: research funding from Bristol-Myers Squibb, Merck and Pfizer; consulting role for Bristol-Myers Squibb and Novartis. J.L.M.: honoraria for Bristol-Myers Squibb and Roche; consultant for Merck. M.K.W.: advisory boards for Adagene, Bristol-Myers Squibb, Castle Biosciences, EMD-Serono, ExiCure, Merck, Pfizer and Regeneron. J.E.G.: consultant and/or advisory role; Merck and Regeneron. A.N.S.: research funding from Bristol-Myers Squibb, Checkmate Pharmaceuticals, Foghorn Therapeutics, Immunocore, Novartis, Pfizer, Polaris, Targovax and Xcovery; advisory board for Bristol-Myers Squibb, Immunocore and Novartis. A.D.: research funding from Apexigen, Idera and Nektar; consulting for Apexigen, Idera, Memgen, Nektar and Pfizer. S.P.P.: research funding from Bristol-Myers Squibb, Ideaya and Provectus; consulting honoraria from Cardinal Health, Castle Biosciences and Merck. M.A.D.: consultant to ABM Therapeutics, Apexigen, Array, Bristol-Myers Squibb, Eisai, GlaxoSmithKline, Pfizer, Roche/Genentech, Novartis, Sanofi-Aventis and Vaccinex; PI of research grants to GlaxoSmithKline, MD Anderson by Roche/Genentech, Merck, Myriad, Oncothyreon and Sanofi-Aventis. J.P.A.: consulting or stock ownership or advisory board for Achelois, Adaptive Biotechnologies, Apricity, BioAtla, BioNTech, Candel Therapeutics, Codiak, Dragonfly, Earli, Enable Medicine, Hummingbird, ImaginAb, Jounce, Lava Therapeutics, Lytix, Marker, PBM Capital, Phenomic AI, Polaris Pharma, Time Bioventures, Trained Therapeutix and Venn Biosciences. P.S.: consulting or stock ownership or advisory board for Achelois, Adaptive Biotechnologies, Affini-T, Apricity, BioAtla, BioNTech, Candel Therapeutics, Catalio, Codiak, Constellation, Dragonfly, Earli, Enable Medicine, Glympse, Hummingbird, ImaginAb, Infinity Pharma, Jounce, JSL Health, Lava Therapeutics, Lytix, Marker, MedImmune, Oncolytics, PBM Capital, Phenomic AI, Polaris Pharma, Sporos, Time Bioventures, Trained Therapeutix and Venn Biosciences. J.A.W.: compensation for speaker's bureau and honoraria from Bristol-Myers Squibb, Dava Oncology, Gilead, Illumina, Imedex, MedImmune, Omniprex, PeerView and Physician Education Resource; consultant/advisory board member for AstraZeneca, Biothera Pharmaceuticals, Bristol-Myers Squibb, GlaxoSmithKline, Merck, Micronoma, Novartis and Roche/Genentech. C.A.: consulting fees from Iovance. H.A.T.: research funding from GlaxoSmithKline; research funding and consulting honoraria from Bristol-Myers Squibb, Genentech, Merck and Novartis; consulting for Boxer, Eisai, Iovance, Karyopharm and Pfizer. All other authors report no conflicts of interest.

Additional information

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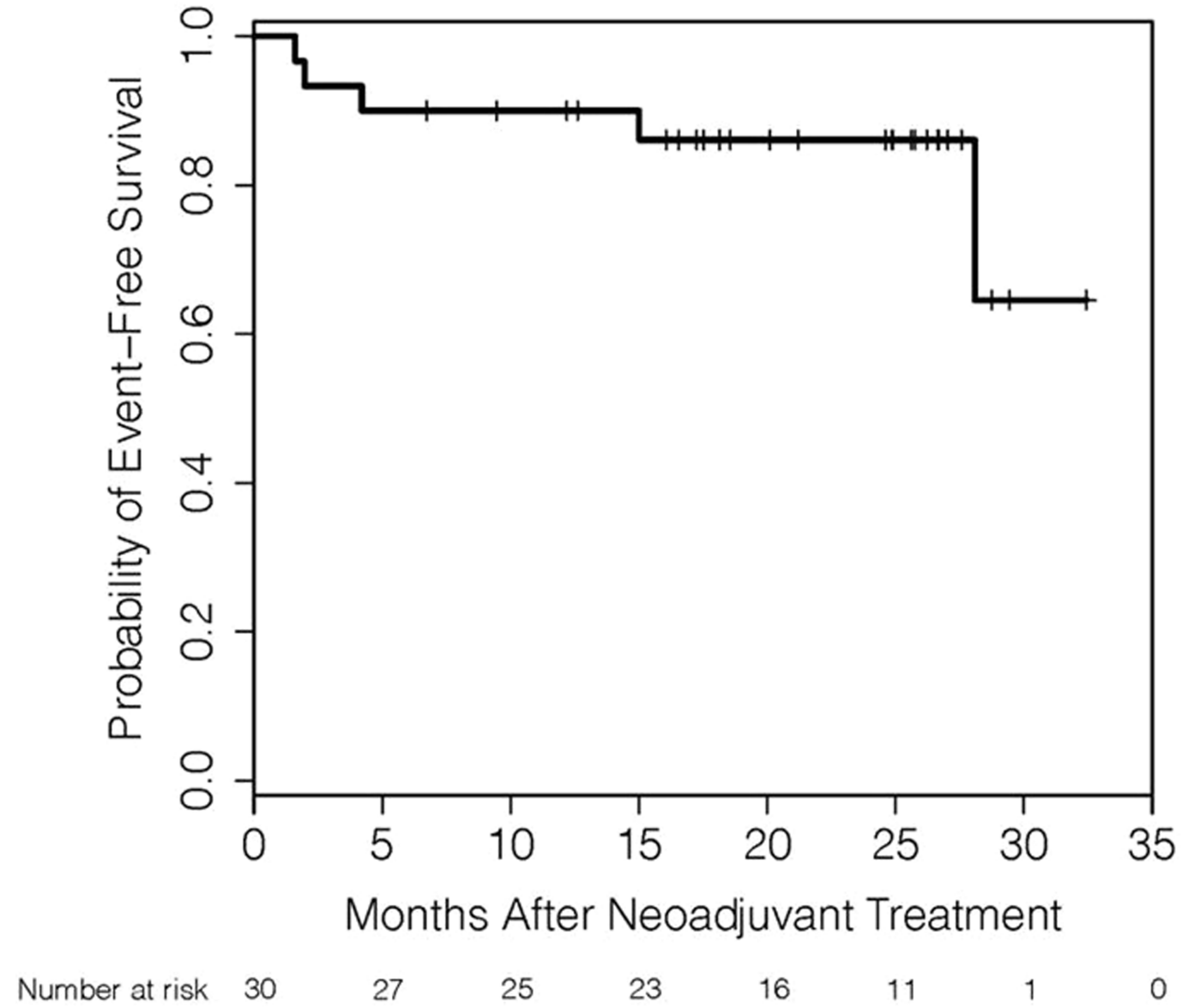
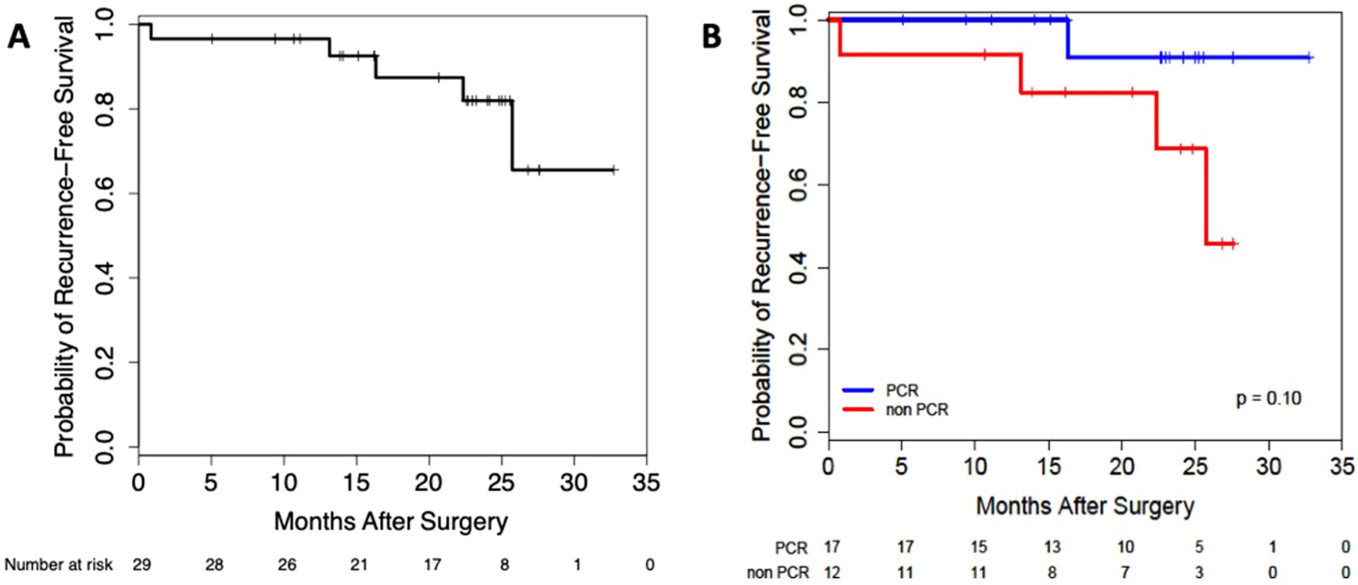
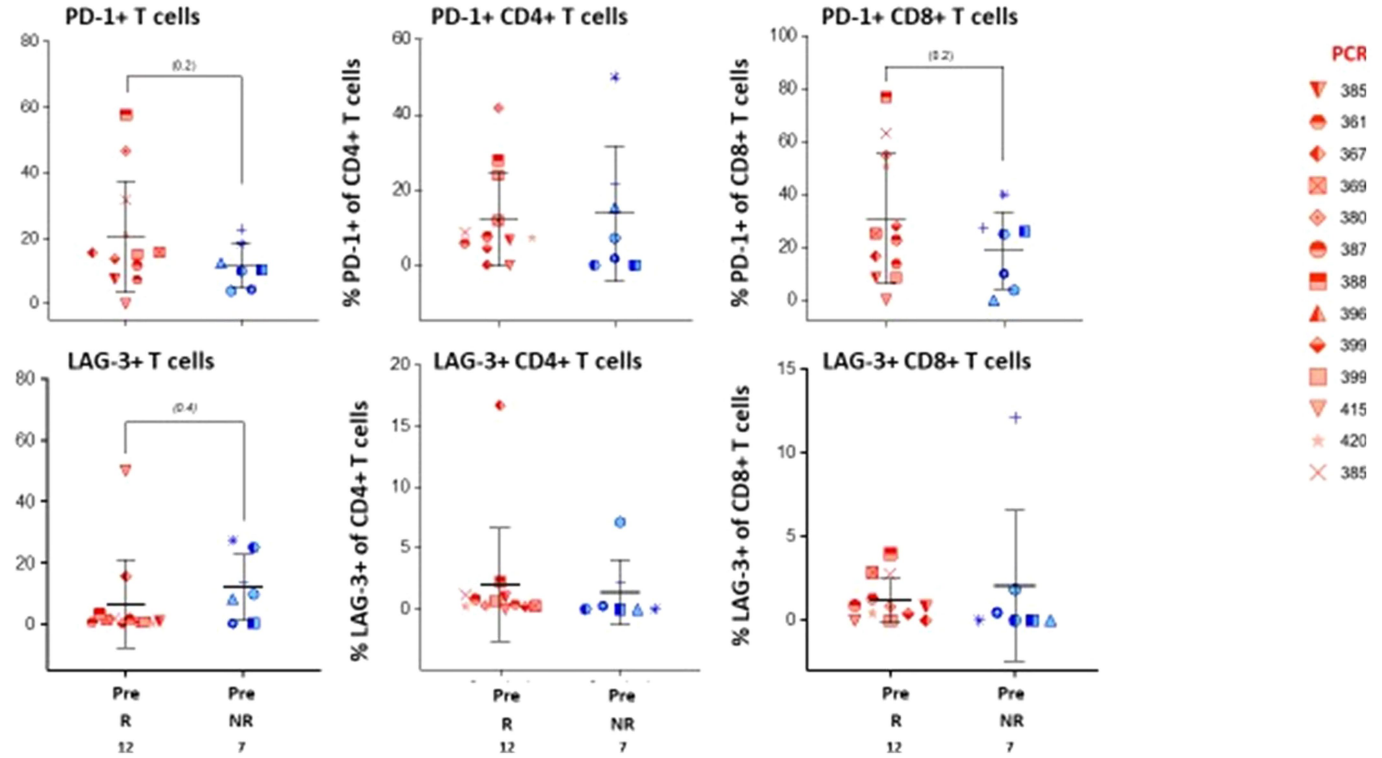


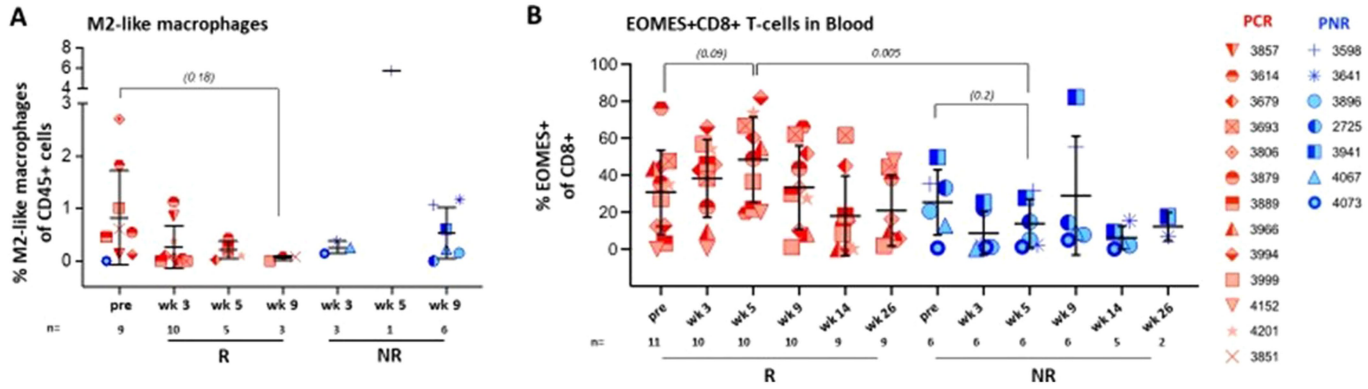
Extended Data Fig. 1 | Probability of being event-free for all patients who received study treatment.



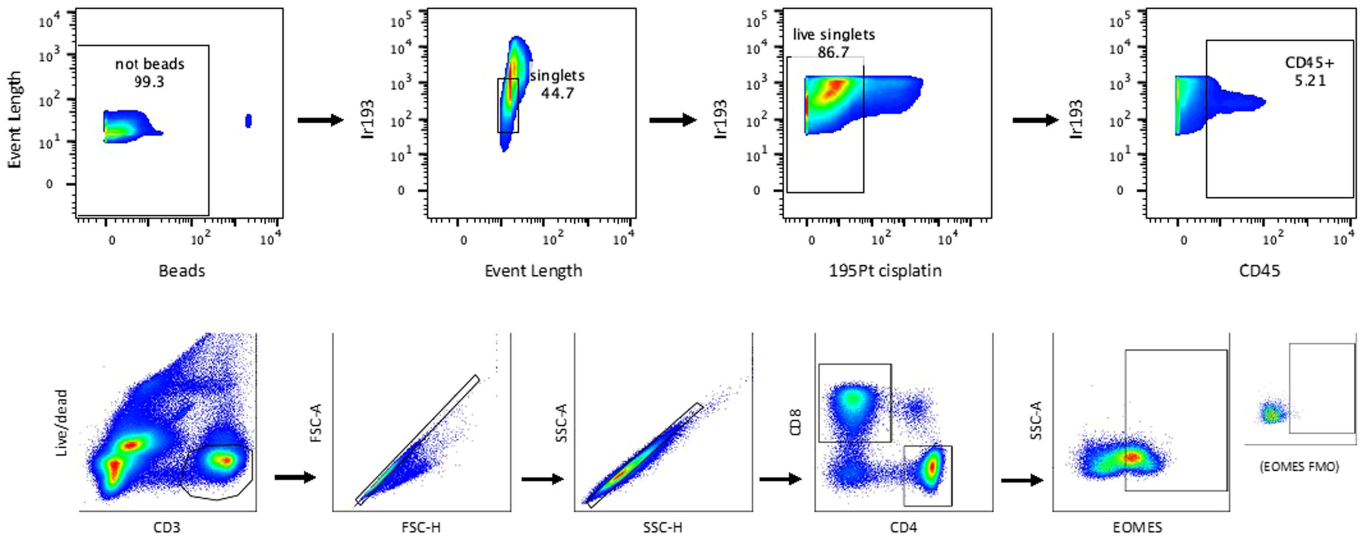
Extended Data Fig. 2 | Probability of being recurrence-free. A) Probability of being recurrence-free for all patients who underwent surgery. **B**) Probability of being recurrence-free based on pathologic complete response versus non-pathologic complete response ($P = 0.10$).



Extended Data Fig. 3 | PD-1 and LAG-3 levels in baseline tumour. Tumour infiltrating immune cells were assayed via CyTOF and analysed by manual gating for frequency of **A**) PD-1 and **B**) LAG-3 levels in T cells prior to treatment. Red, pathologic responders; blue, pathologic non-responders. Data are mean +/− SD; *P* values where shown were determined by two-tailed unpaired *t*-test, with no multiple comparisons. *n* values for each group/timepoint are indicated in each graph.



Extended Data Fig. 4 | M2-like macrophages in tumour and EOMES+ CD8+ T cells in blood. A) Frequency of an M2-like macrophage subset (CD68+ HLA-DR+ CD14+ VISTA+ CD163+ CD45RO+ PD-L1+) was determined by unsupervised clustering of CyTOF data from a single experiment. **B**) Frequency of EOMES+ CD8+ T cells. PBMCs isolated from blood samples were analysed by flow cytometry from a single experiment. Data are mean +/− SD; *P* value was determined by two-tailed unpaired *t*-test, with no multiple comparisons. *n* values for each group/timepoint are indicated in each graph. Red indicates pathologic responders; blue, non-responders.



Extended Data Fig. 5 | Gating schema for manual analysis of CyTOF data from tumour and blood specimens. A) Tumours were mechanically dissociated and cells were stained with immune cell-specific antibodies. Specimens were assayed on the Helios mass cytometer via CyTOF Software. Cytometer data were then prepared for manual and unsupervised analyses via FlowJo software. Major cell populations were identified manually and reported. An example of one patient specimen is shown above for reference. **B**) Gating schema for flow cytometric analysis of blood specimens. Peripheral blood mononuclear cells from patient specimens were stained along with FMO (fluorescence minus one) controls and assayed via a BD LSRFortessa cytometer and BD FACSDiva acquisition software. Data were analysed via FlowJo software as described above. Briefly, live CD3+ singlets were identified and gated into T cell lineages, and those lineages analysed for frequency of each of eight phenotypic markers (BCL6, BLIMP1, CD27, CD28, cMYC, EOMES, ICOS, Ki67) as defined by FMO (fluorescence minus one) specimens. An example of one phenotypic marker (EOMES) in one patient specimen is shown above for reference.

Extended Data Table 1 | Baseline patient characteristics

	Total cohort (n=30)
Age	
Median (range)	60 (35-79)
Gender	
Female / Male	11 (37%) / 19 (63%)
ECOG PS	
0 / 1	28 (93%)/ 2 (7%)
Clinical stage*	
IIIB	18 (60%)
IIIC	8 (26%)
IIID	2 (7%)
IV M1a	2 (7%)
LDH above upper limit of normal	3 (10%)
BRAF V600E/K mutated	5 (17%)
Pretreatment status	
Prior surgery	20 (67%)
Prior systemic therapy	1 (3%)
Median target lesions sum of diameters	26 (13-76 mm)

Extended Data Table 2 | Immune-related adverse events during neoadjuvant and adjuvant therapy

Immune Related Adverse Events	Neoadjuvant Treatment (n=30)		Adjuvant Treatment (n=27)	
	Grade 1-2 (%)	Grade 3-4 (%)	Grade 1-2 (%)	Grade 3-4 (%)
Adrenal insufficiency	1 (3%)	0	3 (11%)	3 (11%)
Increased ALT/AST	3 (10%)	0	8 (30%)	2 (7%)
Increased alkaline phosphatase	0	0	2 (7%)	1 (4%)
Anemia	3 (10%)	0	8 (30%)	0
Anorexia	1 (3%)	0	4 (15%)	0
Arthralgia	0	0	3 (11%)	2 (7%)
Troponin increase	3 (10%)	0	3 (11%)	0
CPK increase	2 (7%)	0	1 (4%)	1 (4%)
Creatinine increase	2 (7%)	0	4 (15%)	0
Diarrhea	0	0	4 (15%)	0
Hypothyroidism	2 (7%)	0	6 (22%)	0
Fatigue	5 (17%)	0	7 (26%)	0
Hyponatremia	3 (10%)	0	5 (19%)	2 (7%)
Infusion reaction	2 (7%)	0	0	0
Myalgia	0	0	4 (15%)	0
Nausea	1 (3%)	0	4 (15%)	0
Rash	5 (17%)	0	5 (19%)	0

Extended Data Table 3 | Antibodies for flow cytometry analysis

Antibody	Clone	Vendor	Catalog #
CD45RO	UCHL1	BioLegend	304218
CD28	CD28.2	eBioscience	47-0289-42
CD62L/L-selectin	DREG-56	BioLegend	304828
Yellow Live/Dead	n/a	Life Technologies	L34959
CD45RA/PTPRC	HI100	BioLegend	304136
CD197/CCR7	G043H7	BioLegend	353230
CD8a	RPA-T8	eBioscience	58-0088-42
CD3	UCHT1	BD Biosciences	562280
CD27	O323	eBioscience	15-0279-42
CD4	SK3	eBioscience	35-0047-42
CD278/ICOS	ISA-3	eBioscience	25-9948-42
Eomes	WD1928	eBioscience	50-4877-42
BLIMP-1	646702	R&D Systems	IC36081G
BCL-6	K112-91	BD Biosciences	562198
T-bet	4B10	BioLegend	644817
Ki-67	Ki-67	BioLegend	350516
cMyc	9E10	R&D Systems	IC3696P

Extended Data Table 4 | Antibodies for CyTOF analysis

Antibody	Clone	Vendor	Catalog Number
CD45	HI30	Fluidigm	3089003B
CD19	HIB19	BioLegend	302247
CD4	RPA-T4	BioLegend	300541
CD8	RPA-T8	BioLegend	301053
CD163	GHI/61	BioLegend	333602
CD14	M5E2	BioLegend	301843
CCR7	G043H7	BioLegend	353237
PD-1	EH12.2H7	BioLegend	329941
Eomes	WD1928	eBioscience	14-4877-82
CD1c	L161	BioLegend	331502
CD11c	3.9	Fluidigm	3146014B
T-bet	4B10	BioLegend	644825
CD16	3G8	Fluidigm	3148004B
LAG-3	874501	R&D	MAB23193
PD-L1	MIH1	eBioscience	14-5983-82
CD123	6H6	Fluidigm	3151001B
TCR$\gamma\delta$	11F2	Fluidigm	3152008B
ICOS	ISA-3	eBioscience	14-9948-82
TIGIT	MBSA43	Fluidigm	3154016B
CD45RA	H100	Fluidigm	3155011B
CD86	IT2.2	Fluidigm	3156008B
LAG-3	11C3C65	BioLegend	369302
CD161	HP3G10	Fluidigm	3159004B
CD141	AD5-14H12	Miltenyi Biotec	130-090-694
CTLA-4	14D3	Fluidigm	3161004B
FOXP3	PCH101	Fluidigm	3162011A
CRTH2	BM16	Fluidigm	3163003B
CXCR5	RF8B2	Fluidigm	3164029B
TCF7	7F11A10	BioLegend	655202
TIM3	F38-2E2	BioLegend	345019
BTLA	J168-540	BD	624084
CD73	AD2	Fluidigm	3168015B
CCR10	314305R	R&D Systems	MAB3478R-100
CD3	UCHT1	BioLegend	300437
CD68	Y1/82A	Fluidigm	3171011B
CD28	CD28.2	BioLegend	302937
Granzyme B	GB11	Fluidigm	3173006B
Ki67	Ki67	BioLegend	350523
CD45RO	UCHL1	BioLegend	304239
CD56	NCAM16.2	Fluidigm	3176008B
HLA-DR	L243	Fluidigm	custom

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☐	☒	A full description of the statistical parameters including central tendency (e.g. means) or other basic estimates (e.g. regression coefficient) AND variation (e.g. standard deviation) or associated estimates of uncertainty (e.g. confidence intervals)
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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	Clinical data collection via MDACC Prometheus Data Collection System. Flow cytometry data collection software: BD FACSDiva software, version 8.0.1 for flow cytometry (Becton Dickinson & Company) CyTOF data collection software: CyTOF Software v 7.0.8493 for mass cytometry (Fluidigm, now Standard Biotools)
Data analysis	Clinical data analysis by SAS 9.4 by Windows (Copyright © 2002-2012 by SAS Institute Inc., Cary, NC) FlowJo v.10.5.3 (Becton Dickinson & Company); WorkFlow script for unsupervised clustering (Nowicka et al F1000 Research 2017), running on R version 3.5.2 (R Foundation for Statistical Computing) R Foundation for Statistical Computing

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☒	☐ Animals and other organisms
☐	☒ Human research participants
☐	☒ Clinical data
☒	☐ Dual use research of concern

Methods

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

Antibodies

Antibodies used	The anti PD-1 antibody nivolumab and anti LAG-3 antibody relatlimab were provided by the study sponsor Bristol-Myers Squibb as part of their investigational supply of agents. Relatlimab should be stored at 2°C to 8°C (36oF to 46oF) with protection from light. Do not freeze the drug product. Relatlimab is to be administered combined with nivolumab in the same bag as a 60 minute IV infusion through a 0.2/1.2-µm pore size, low-protein-binding polyethersulfone membrane in-line filter at the protocol-specified doses. The Relatlimab and nivolumab injection can be diluted with 0.9% sodium chloride injection (normal saline), to protein concentrations no lower than 1.33 mg/mL. Detailed instructions for drug product dilution and administration are provided in the pharmacy manual for the clinical study
Validation	These antibodies were provided as part of BMS's investigational study supply

Human research participants

Policy information about studies involving human research participants

Population characteristics	Clinical stage III melanoma with resectable disease. Patients aged 18 and over, ECOG PS 0-1, normal organ function with no contra-indications to surgery.
Recruitment	Patients were enrolled at MD Anderson and Memorial Sloann Kettering in the Melanoma Clinics. Patients were offered either clinical trial enrollment or standard of care therapies. Patients were provided copies of the study informed consent document and were fully aware of risks prior to trial enrollment. As patients needed to fulfill inclusion criteria of trial, this could have caused selection bias.
Ethics oversight	IRB of MDACC and MSKCC provided ethics oversight. An informed consent statement was included in the Methods/Study Oversight section

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

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	NCT02519322
Study protocol	Available upon request
Data collection	9/19/2018 - 9/23/2020 for enrollment, patients followed for at least 1 year after date of last enrollment. Data was stored in a secure database sponsored by MD Anderson Cancer Center and was able to be accessed by staff at MSKCC for direct data input.
Outcomes	The primary outcome was assessment of pathologic response following neoadjuvant therapy as per the criteria of the INMC which is agreed upon pathologic response criteria utilized in melanoma neoadjuvant studies. Secondary outcomes including RECIST response, safety, RFS, EFS, and OS are standard outcome criteria utilized in neoadjuvant studies to describe characteristics of response. Correlation of immune profiling with response was exploratory in nature and dependent upon results of correlative studies.

Flow Cytometry

Plots

Confirm that:

☒ The axis labels state the marker and fluorochrome used (e.g. CD4-FITC).

☒ The axis scales are clearly visible. Include numbers along axes only for bottom left plot of group (a 'group' is an analysis of identical markers).

☒ All plots are contour plots with outliers or pseudocolor plots.

☒ A numerical value for number of cells or percentage (with statistics) is provided.

Methodology

Sample preparation	Sample preparation details can be found in the Methods section of the manuscript. Isolation and preparation of cells from peripheral blood and tissues Whole blood was collected in tubes containing sodium heparin (BD Vacutainer), resuspended in PBS, layered atop Ficoll (StemCell Technologies) and centrifuged at 800 × g for 25 minutes. The interface peripheral blood mononuclear cells (PBMC) were harvested and washed twice with PBS and centrifuged at 500 × g for 10 minutes. Fresh tumor tissue was dissociated with GentleMACS system (Miltenyi Biotec). PBMC and tumor specimens destined for CyTOF analysis were stained for viability with 5 µmol/L cisplatin (Fluidigm) in PBS containing 1% BSA and then washed 3x. All specimens were resuspended in AB serum with 10% (vol/vol) DMSO for storage in liquid nitrogen until downstream assays were performed.
Instrument	BD LSRFortessa x20 flow cytometer (Becton Dickinson & Company); Helios mass cytometer (Fluidigm, now Standard Biotools)
Software	Acquisition Software: BD FACSDiva software, version 8.0.1 for flow cytometry (Becton Dickinson & Company); CyTOF Software v 7.0.8493 for mass cytometry (Fluidigm, now Standard Biotools) Analysis Software: FlowJo v.10.5.3 (Becton Dickinson & Company); WorkFlow script for unsupervised clustering (Nowicka et al F1000 Research 2017), running on R version 3.5.2 (R Foundation for Statistical Computing)
Cell population abundance	Sorting was not performed. Bulk tumor cells were procured, the immune fraction enriched via buffy layer, and immune cell-specific detection antibodies were used for cytometry analysis.
Gating strategy	Please see Extended Data Figure 5 to review the Flow Cytometry and CyTOF gating strategies.

☒ Tick this box to confirm that a figure exemplifying the gating strategy is provided in the Supplementary Information.

rapid communications

Lifileucel, a Tumor-Infiltrating Lymphocyte Therapy, in Metastatic Melanoma

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abstract

PURPOSE Effective treatment options are limited for patients with advanced (metastatic or unresectable) melanoma who progress after immune checkpoint inhibitors and targeted therapies. Adoptive cell therapy using tumor-infiltrating lymphocytes has demonstrated efficacy in advanced melanoma. Lifileucel is an autologous, centrally manufactured tumor-infiltrating lymphocyte product.

METHODS We conducted a phase II open-label, single-arm, multicenter study in patients with advanced melanoma who had been previously treated with checkpoint inhibitor(s) and BRAF ± MEK targeted agents. Lifileucel was produced from harvested tumor specimens in central Good Manufacturing Practice facilities using a streamlined 22-day process. Patients received a nonmyeloablative lymphodepletion regimen, a single infusion of lifileucel, and up to six doses of high-dose interleukin-2. The primary end point was investigator-assessed objective response rate (ORR) per RECIST, version 1.1.

RESULTS Sixty-six patients received a mean of 3.3 prior therapies (anti–programmed death 1 [PD-1] or programmed death ligand 1 [PD-L1]: 100%; anticytotoxic T-lymphocyte-associated protein-4: 80%; BRAF ± MEK inhibitor: 23%). The ORR was 36% (95% CI, 25 to 49), with two complete responses and 22 partial responses. Disease control rate was 80% (95% CI, 69 to 89). Median duration of response was not reached after 18.7-month median study follow-up (range, 0.2-34.1 months). In the primary refractory to anti–PD-1 or PD-L1 therapy subset, the ORR and disease control rate were 41% (95% CI, 26 to 57) and 81% (95% CI, 66 to 91), respectively. Safety profile was consistent with known adverse events associated with nonmyeloablative lymphodepletion and interleukin-2.

CONCLUSION Lifileucel demonstrated durable responses and addresses a major unmet need in patients with metastatic melanoma with limited treatment options after approved therapy, including the primary refractory to anti–PD-1 or PD-L1 therapy subset.

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ASSOCIATED CONTENT

See accompanying editorial on page 2640

Data Supplement

Protocol

Author affiliations and support information (if applicable) appear at the end of this article.

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INTRODUCTION

The treatment of advanced (unresectable or metastatic) melanoma with immune checkpoint inhibitors (ICI) and targeted oncogenic pathway inhibition with BRAF and MEK inhibitors has improved patient outcomes.[1-7] Forty percent to 65% of patients with advanced melanoma have primary resistance to ICI.[8-11] Of those with initial disease control, 30%-40% develop acquired resistance.[8,12] Approximately 15% to 20% of *BRAF* V600 mutation-positive patients fail to respond to targeted therapy initially,[13] and only 22% remain progression-free at 3 years.[14] Although primary resistance is lower in patients treated with programmed death 1 (PD-1) blocking antibody plus anticytotoxic T-lymphocyte–associated protein 4 (CTLA-4) therapy, 36% of patients discontinue therapy because of treatment-emergent adverse events (TEAEs), with 88% developing immune-related adverse events (irAEs), many of these being persistent.[10] Patients progressing after anti–PD-1 therapy, anti–PD-1 plus anti–CTLA-4 therapy, and targeted agents have limited options.[15-17] Only 4%-10% of these patients have objective responses to chemotherapy, with a limited median overall survival (OS) of 7 months.[15,16,18,19] There are no treatment options with approval based on data from patients with advanced melanoma who have progressed after

CONTEXT

Key Objective

This study evaluated the efficacy and safety of lifileucel, a one-time, autologous tumor-infiltrating lymphocyte (TIL) product, in patients with metastatic melanoma who had progressed on standard immune checkpoint inhibitors (ICI) and targeted therapies (if applicable), who otherwise have limited treatment options. Notably, chemotherapy post-ICI shows poor response rates (4%-10%).

Knowledge Generated

Sixty-six patients received lifileucel infusion with $> 1 \times 10^9$ TIL cells. Lifileucel was efficacious with an objective response rate of 36%, and a median duration of response that is not reached at 18.7-month median study follow-up.

Relevance

Lifileucel represents a significant improvement in the treatment of advanced melanoma, particularly in the post-ICI patient population, which is an expanding population. The study contributes to the advancement in TIL therapy through a centrally standardized manufacturing approach for autologous TIL, allowing broader patient access.

one line of ICI therapy (for *BRAF* wild-type tumors), or two lines of therapy (for *BRAF* V600 mutation-positive tumors). In addition, patients recurring with advanced melanoma after adjuvant anti–PD-1 therapy for high-risk disease represent an emerging unmet need.[20-22]

Adoptive cell therapy with tumor-infiltrating lymphocytes (TIL) offers a potential therapeutic option for metastatic melanoma, although it has not been studied extensively in the ICI era.[23-25] TIL are enriched with polyclonal T cells with diverse antigen specificity.[26] Extraction of a fragment of tumor followed by ex vivo expansion removes TIL from the hostile tumor microenvironment and reduces the immunosuppressive effects of intratumoral regulatory T cells. Expansion of TIL ex vivo rejuvenates the cells, yielding billions of such cells to be infused back into the patient. Melanoma is characterized by a high mutational burden[27] and highly individualized neoantigens.[28] A cellular therapy product that can address the broad nature of neoantigens and the unique array from each patient would lead to the possibility of a tailored response. Lifileucel (LN-144) is an autologous TIL therapy that uses tumor-tissue T cells capable of recognizing tumor antigens and being expanded ex vivo while maintaining the heterogeneous repertoire of T cells, using a centralized manufacturing process. We report the safety and efficacy of lifileucel, a one-time cellular therapy, in patients with advanced melanoma who have progressed on ICI and BRAF inhibitors (if *BRAF* V600 mutation-positive).

METHODS

Trial Conduct

The study was approved by the institutional review board at each site and was conducted in accordance with the Declaration of Helsinki and Good Clinical Practice guidelines of the International Conference on Harmonization. All patients provided written informed consent. The study was designed and sponsored by Iovance Biotherapeutics, Inc. All authors discussed, analyzed, and interpreted the results, and vouch for the accuracy and completeness of the data analyses and adherence to the Protocol (online only). All authors contributed to this study and the writing of the manuscript. Professional medical writing or editorial assistance was paid for by the sponsor.

Patients and Study Design

The parent study (ClinicalTrials.gov identifier: NCT02360579) consisted of multiple cohorts (Data Supplement, online only). Cohort 2 data are reported here. Patients were enrolled from April 2017 to January 2019 at 26 sites (see the Data Supplement for the investigator list).

Patients had unresectable or metastatic melanoma (stage IIIC or IV) with confirmed radiologic progression. Patients must have progressed following one or more prior systemic therapies including a PD-1–blocking antibody and if *BRAF* V600 mutation-positive, a BRAF ± MEK inhibitor. Key eligibility criteria are detailed in the Data Supplement. Patients with a history of irAEs were eligible, as outlined in the Data Supplement.

At least one resectable lesion (or aggregate of lesions) measuring a minimum of 1.5 cm in diameter postresection was required. Resected tumor was processed in a protocol-specified manner and shipped to a Good Manufacturing Practice facility in the provided tumor procurement kit. The optimized manufacturing conditions involved a centralized 22-day process, resulting in a cryopreserved product (Data Supplement). Lifileucel (LN-144) was shipped to the clinical sites after meeting prespecified release criteria. Patients received a nonmyeloablative lymphodepleting (NMA-LD) regimen with cyclophosphamide (60 mg/kg) once daily for 2 days followed by fludarabine (25 mg/m^2) once daily for 5 days. A single infusion of lifileucel (1×10^9 – 150×10^9 cells) was thawed and administered after approximately 24 hours from the last dose of fludarabine. A

TABLE 1. Patient Demographics and Baseline Characteristics

Characteristic	Cohort 2 (N = 66)
Median age, years (range)	55 (20-79)
Sex, No. (%)	
Male	39 (59)
Female	27 (41)
Melanoma stage at study entry	
IIIC	9 (14)
IV	57 (86)
Prior therapies, No. (%)	
Mean No. of prior therapies (SD)	3.3 (1.69)
Anti–PD-1 or PD-L1[a]	66 (100)
Anti–CTLA-4[b]	53 (80)
Anti–PD-1 plus CTLA-4 combination	34 (52)
BRAF± MEK[c]	15/17 (88)
IL-2	7 (11)
Surgery	65 (99)
Radiotherapy	34 (52)
Progressive disease for at least one prior therapy, No. (%)	
Anti–PD-1 or PD-L1[d]	65/66 (99)
Anti–CTLA-4	41/53 (77)
Primary refractory to prior anti–PD-1 or anti–PD-L1, No. (%)	42 (64)
Patients with baseline liver lesions, No. (%)	23 (35)
Patients with baseline brain lesions, No. (%)	7 (11)
Patients with baseline liver and/or brain lesions, No. (%)	28 (42)
Baseline ECOG score, No. (%)	
0	37 (56)
1	29 (44)
BRAF status, No. (%)	
Mutated V600	17 (26)
Wild type	45 (68)
Unknown	3 (5)
Other	1 (2)
Baseline LDH, No. (%)	
≤ ULN	39 (59)
1-2 × ULN	19 (29)
> 2 × ULN	8 (12)
PD-L1 status, No. (%)	
TPS ≥ 5%	24 (36)
TPS < 5%	23 (35)
Missing	19 (29)
Target lesion sum of diameter	
≥ 70 mm, No. (%)	40 (61)
Mean (SD), mm	106 (71)
No. of target and nontarget lesions (at baseline)	
> 3, No. (%)	51 (77)
Mean (SD)	6 (2.7)
Median (range)	5 (2-14)
Median time from stop of anti–PD-1 or PD-L1 to TIL infusion (range), months	4.8 (1.6-56.5)

(continued in next column)

TABLE 1. Patient Demographics and Baseline Characteristics (continued)

Abbreviations: CTLA-4, cytotoxic T-lymphocyte–associated protein 4; ECOG, Eastern Cooperative Oncology Group; IL, interleukin; LDH, lactate dehydrogenase; PD-1, programmed cell death protein 1; PD-L1, programmed death-ligand 1; SD, standard deviation; TIL, tumor-infiltrating lymphocytes; TPS, tumor proportion score; ULN, upper limit of normal.

[a]Includes pembrolizumab, nivolumab, durvalumab, and atezolizumab.

[b]Includes ipilimumab and tremelimumab.

[c]One patient received only BRAF inhibitor. Two patients were enrolled under an earlier protocol version that did not require *BRAF* V600 mutation-positive patients to receive BRAF ± MEK inhibitors. Percentage is calculated based on number of patients who were *BRAF* V600E- or V600K-mutated and received a BRAF inhibitor (dabrafenib or vemurafenib) ± a MEK inhibitor (trametinib or cobimetinib).

[d]One patient discontinued anti–PD-1 therapy because of toxicity and then progressed on interval therapy before enrollment.

short course of bolus interleukin (IL)-2 (600,000 IU/kg) was infused every 8-12 hours for up to six doses, starting within 3-24 hours of completing lifileucel infusion.

End Points and Assessments

The primary objective was to evaluate the efficacy of a single infusion of lifileucel in patients with unresectable or metastatic melanoma using investigator-assessed objective response rate (ORR) by RECIST v1.1.[29] Secondary end points included duration of response (DOR), disease control rate (DCR), OS, and safety. Efficacy assessments started at week 6. Subsequent efficacy, adverse event (AE), and serious AE (SAE) assessment schedules are outlined in the Data Supplement.

Statistical Analysis

The analyses for efficacy and safety were conducted on the full analysis set (FAS), defined as patients from cohort 2 who received lifileucel that met manufacturer's specifications, including a cell dose 1×10^9 – 150×10^9. The planned sample size was 60 based on estimation of ORR using the maximum half-width of the two-sided 95% CI of < 13.2% when ORR is expected to be 20%-50%. This was considered meaningful, assuming that the historical response rate of similar patients after chemotherapy is 10%.[15,30] The FAS consisted of 66 patients because of rapid enrollment.

The ORR was analyzed as a binomial proportion with two-sided 95% CI estimated based on the Clopper-Pearson

TABLE 2. Efficacy Outcomes by Investigator Assessment

Response (RECIST v1.1)	Cohort 2 (N = 66)
ORR, No. (%) (95% CI)	24 (36) (25 to 49)
DCR, No. (%) (95% CI)	53 (80) (69 to 89)
Best overall response, No. (%)	
CR	2 (3)
PR	22 (33)
SD	29 (44)
PD	9 (14)
Nonevaluable	4 (6)
Median DOR, months (range)	Not reached (2.2-26.9+)

NOTE. +, censored.

Abbreviations: CR, complete response; DCR, disease control rate; DOR, duration of response; ORR, objective response rate; PD, progressive disease; PR, partial response; SD, stable disease.

exact method. Time-to-event efficacy end points were estimated using the Kaplan-Meier product limit method, and two-sided corresponding 95% CIs were based on log-log transformation. Safety data were reported descriptively. All statistical analyses were conducted using the Statistical Analysis System (SAS) version 9.4.

RESULTS

Patients and Treatment

Seventy-eight patients underwent tumor resection. Sixty-six patients received lifileucel (LN-144) infusion with $> 1 \times 10^9$ but $< 150 \times 10^9$ TIL cells and comprised the FAS. Three patients either did not receive TIL or received $< 1 \times 10^9$ TIL cells, whereas nine patients could not be treated because of other causes (Data Supplement). Table 1 details the demographics and baseline characteristics. Patients had received a mean of 3.3 lines of prior therapies (range, 1-9 lines). All patients had received prior anti–PD-1 or anti–programmed death ligand 1 (PD-L1) therapy, and 53 (80%) had received prior anti–CTLA-4 therapy. Fifty-two percent of the patients had received concurrent CTLA-4 plus PD-1 blockade. Notably, 99% had progressed on prior anti–PD-1 or PD-L1 therapy, and 77% had progressed on prior anti–CTLA-4 therapy. Overall, 42 patients (64%) had a best response of progressive disease to initial anti–PD-1 or PD-L1 therapy (primary refractory subset). Of the 17 patients who were *BRAF* V600 mutation-positive, 88% had received BRAF ± MEK inhibitors. Forty patients (61%) had a baseline target lesion sum of diameters (SOD) ≥ 70 mm, 51 (77%) patients had more than three target and nontarget lesions at baseline, and 27 (41%) had baseline lactate dehydrogenase levels higher than institutional upper limit of normal. Overall, patients had a high tumor burden at

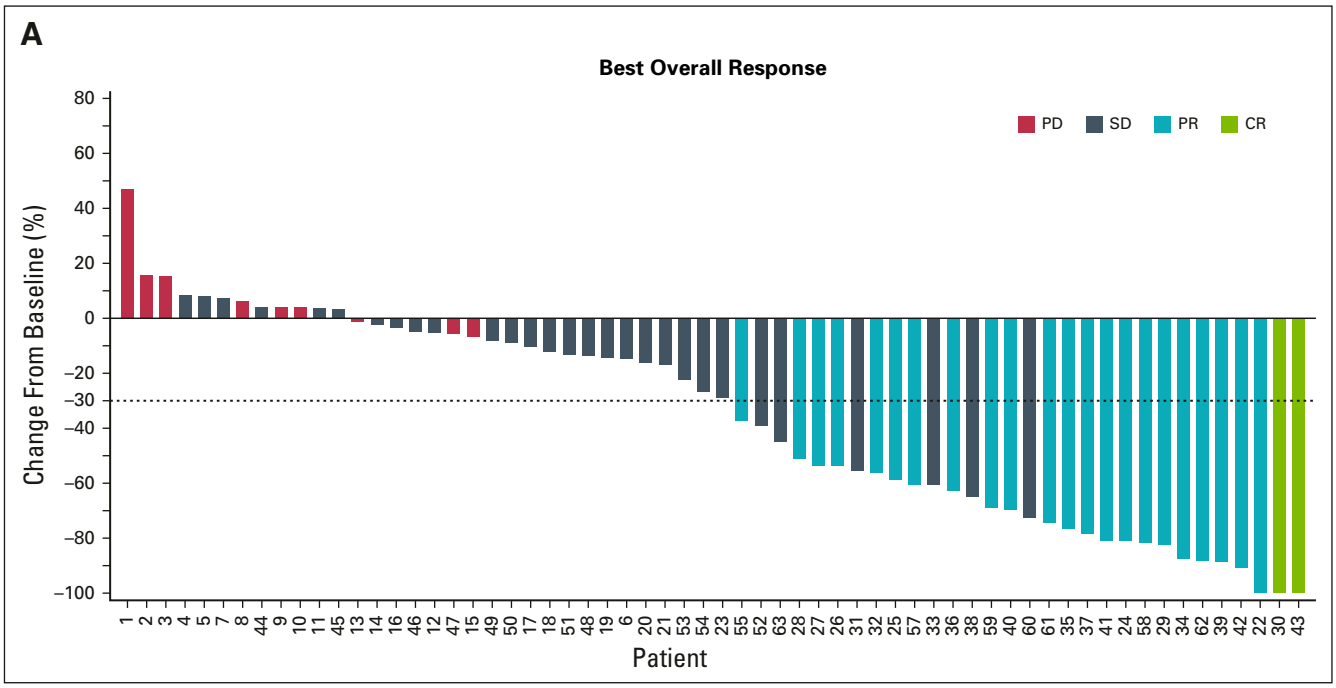

FIG 1. Change in tumor burden of target lesions, response by subgroup, and response assessment in individual patients. (A) Waterfall plot depicting BOR as assessed by investigator and the best change from baseline in the SOD of the target lesions (per RECIST v1.1 criteria) in the FAS. A change of −100% from baseline is presented for CR assessment that includes lymph node lesions that resolved to < 10 mm. The horizontal dashed line indicates a 30% reduction in the tumor burden in the target lesions. Twelve patients had an increase in the SOD of the target lesions, whereas 50 patients had a decrease in the SOD of the target lesions. Thirty patients (two CR, 22 PR, and six SD) had > 30% reduction in the SOD of the target lesions. Three patients had no post-TIL assessments because of early death. One patient had no post-TIL assessment because of start of new anticancer therapy before day 42. (continued on next page)

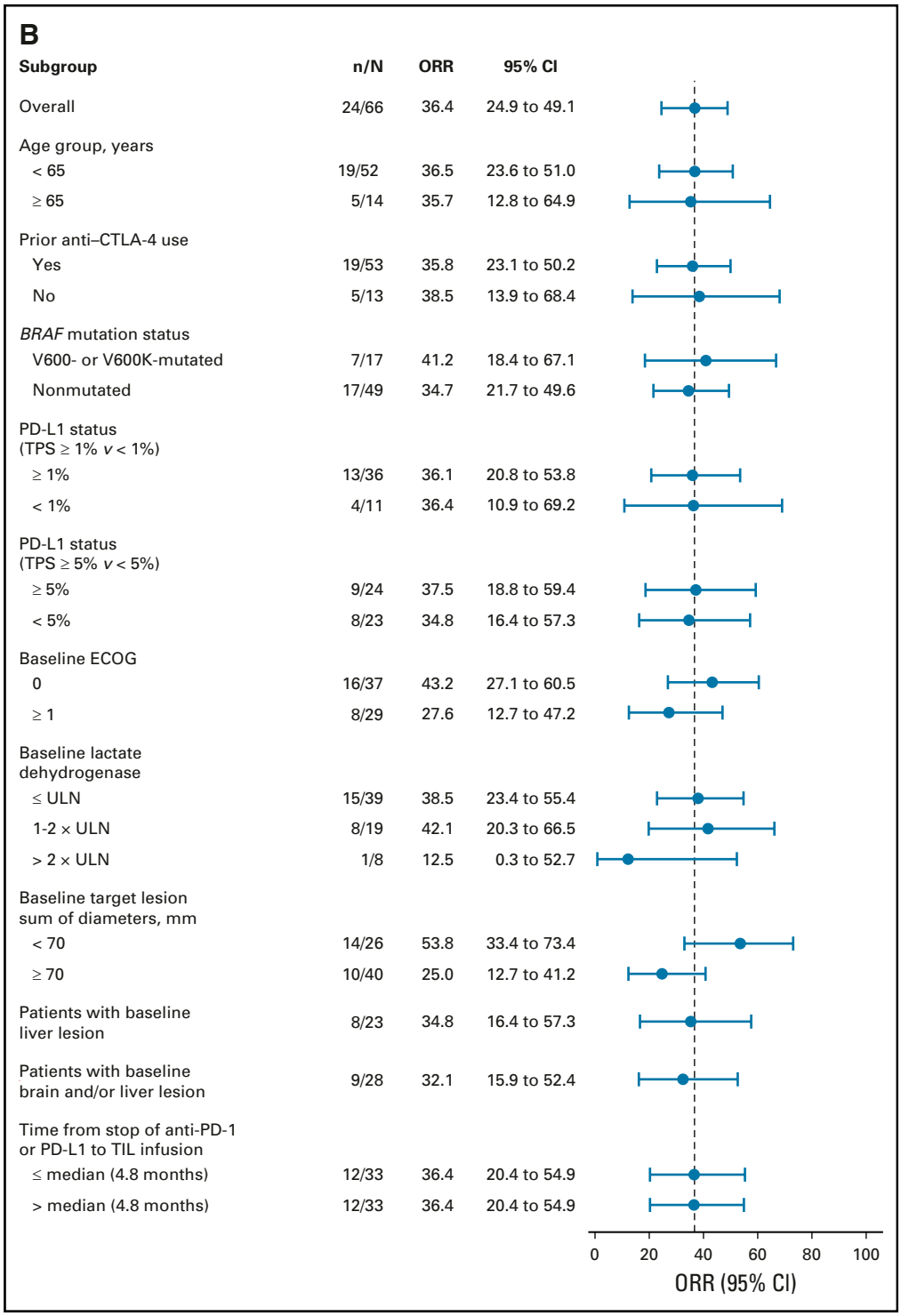

FIG 1. (Continued). (B) Forest plot for ORR (FAS) by subgroup per investigator assessment using the RECIST v1.1 criteria. 95% CI is calculated using the Clopper-Pearson Exact test. (continued on next page).

baseline (mean SOD for the target lesions: 106 mm); 28 patients (42%) had liver and/or brain lesions at baseline.

The harvested tumor was collected from a variety of sites, such as skin, lymph nodes, liver, lung, peritoneum, musculoskeletal sites, breast, and other organs. The median number of cyclophosphamide and fludarabine doses were 2 (range, 1-2) and 5 (range, 2-5), respectively. The mean number of TIL cells infused was 27.3×10^9 (range, 1.2×10^9 to 99.5×10^9). The median number of IL-2 doses administered was 5.5 (range, 1-6).

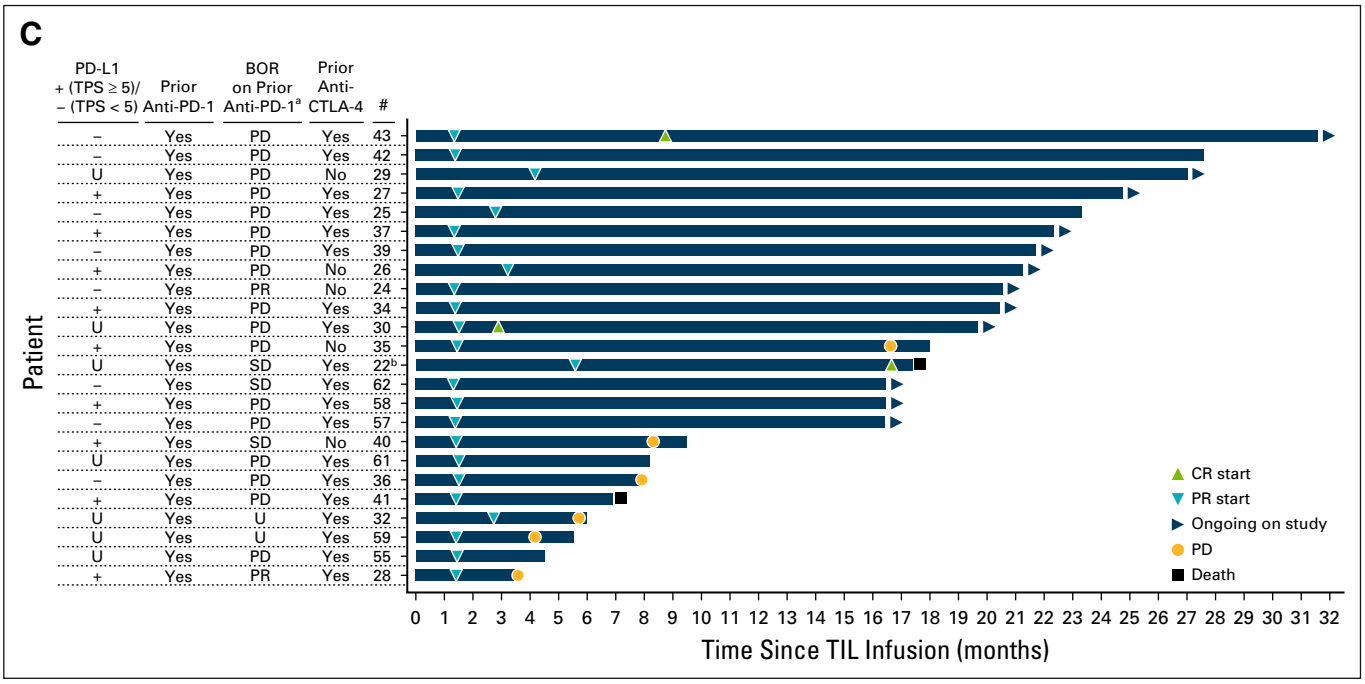

FIG 1. (Continued). (C) Swimmer's plot showing time to first response, duration of response, and time on efficacy assessment in confirmed responders by investigator per RECIST v1.1 criteria. Among 24 responders, 12 (50%) showed ongoing response to lifileucel, six (25%) had progressed, two (8%) had died, three (13%) started a new anticancer therapy, and one patient discontinued assessment because of relocation. [a]BOR is best overall response on prior anti-PD-1 immunotherapy. [b]For patient 22, a CR was not confirmed; therefore, the BOR with lifileucel for this patient was PR. Causes of death: patient 22: possible pulmonary embolism; patient 41: failure to thrive. BOR, best overall response; CR, complete response; CTLA-4, cytotoxic T-lymphocyte–associated protein 4; ECOG, Eastern Cooperative Oncology Group; FAS, full analysis set; ORR, objective response rate; PD, progressive disease; PD-1, programmed death 1; PD-L1, programmed death-ligand 1; PR, partial response; SD, stable disease; SOD, sum of diameters; TIL, tumor-infiltrating lymphocytes; TPS, tumor proportion score; U, unknown; ULN, upper limit of normal.

Efficacy

Sixty-six patients received a lifileucel infusion of $\geq 1 \times 10^9$ TIL cells. At the data cutoff of April 23, 2020 (median follow-up of 18.7 months [range, 0.2-34.1 months]), the investigator-assessed ORR was 36% (95% CI, 25 to 49) and the DCR was 80% (95% CI, 69 to 89) (Table 2), with 2 (3%) complete responses (CRs), 22 (33%) partial responses (PRs), and 29 (44%) patients showing stable disease (SD). Sixty-two patients (94%) had a baseline and at least one postbaseline radiologic assessment. Of the four patients in the FAS who did not undergo postbaseline assessment, three had died of disease, and one received an additional line of systemic therapy; all were considered as not evaluable for best overall response. Of the evaluable patients, 50 (81%) had a reduction in tumor burden (Fig 1A). Data Supplement details the percentage change in target SOD from baseline over time in patients who achieved a confirmed response. Response to lifileucel was observed regardless of age, prior anti–CTLA-4 use, *BRAF* mutation status, PD-L1 status as measured by tumor proportion score, baseline Eastern Cooperative Oncology Group performance status, tumor burden (assessed by lactate dehydrogenase elevation above upper limit of normal and target lesion SOD at baseline), presence of liver and/or brain lesions at baseline, and timing of prior PD-1 therapy (Fig 1B).

Median time from lifileucel infusion to best response was 1.4 months (range, 1.3-8.7 months). Time to response for individual patients is illustrated in Figure 1C; 19 of 24 patients achieved response by the time of first planned assessment (6 weeks after lifileucel infusion). Only 25% of patients had progressed after achieving a response. The median DOR has not been reached (95% CI, 11.8 months to not reached) (Fig 2A) with a 1-year DOR of 69% (95% CI, 46 to 84). The median OS was 17.4 months (95% CI, 11.0 to not reached; Fig 2B). Of the patients who had SD and PR or CR, 38% and 92% patients, respectively, had an OS $\geq$ 1 year. Progression-free survival for the FAS is shown in the Data Supplement, and duration of SD in individual patients is outlined in the Data Supplement.

An efficacy analysis was performed for the primary-refractory subset (42 patients primary refractory to anti–PD-1 or PD-L1 therapy). The ORR was 41% (95% CI, 26 to 57), with 2 CRs (5%) and 15 PRs (36%), and the DCR was 81% (95% CI, 66 to 91). Seventeen (41%) of these patients had SD, and five (12%) had progressive disease; three patients were nonevaluable. Median DOR was not reached for this subpopulation.

Thirty-four (52%) patients received anti–PD-1 plus anti–CTLA-4 combination therapy, either as frontline (n = 15, 23%), or after failing frontline therapy (n = 19, 29%). The

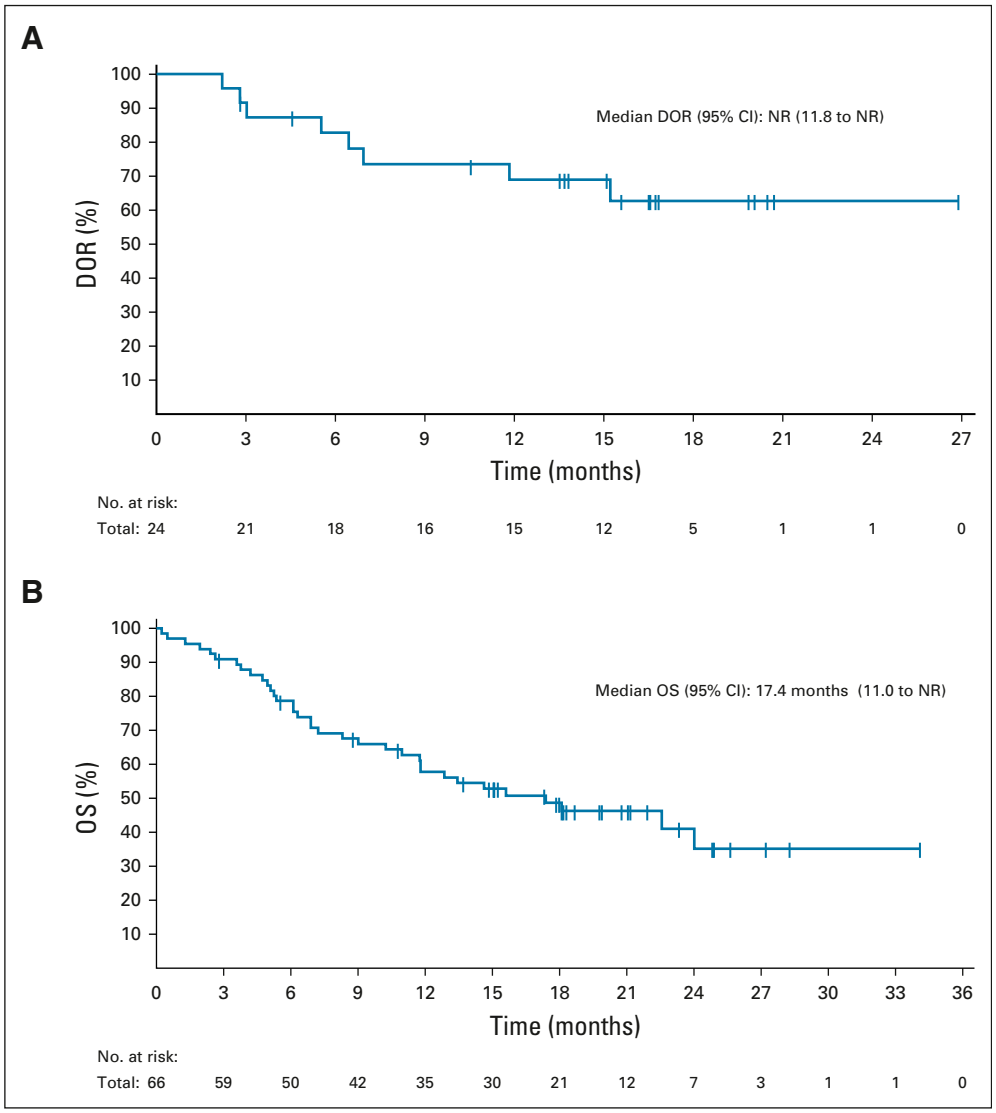

FIG 2. (A) The Kaplan-Meier curve for DOR in confirmed responders who achieved a PR or better. The DOR is measured from the time point at which the initial measurement criteria are met for a PR or CR, whichever occurred first, until the first date that PD or death occurred. (B) The Kaplan-Meier curve for OS in the full analysis set. OS was defined as the time (in months) from the start date of lifileucel infusion to death because of any cause. Patients who were alive at the time of data cutoff had their event times censored on the last date of their known survival status. The median OS was 17.4 months (95% CI, 11.0 to NR), with 1-year OS of 58% (95% CI, 45 to 69). CR, complete response; DOR, duration of response; NR, not reached; OS, overall survival; PD, progressive disease; PR, partial response.

ORRs for lifileucel in these two subsets were 33% (5/15) and 32% (6/19), respectively. The ORRs for lifileucel in patients with primary resistance (n = 17) or acquired resistance (n = 11) to anti–PD-1 plus anti–CTLA-4 combination therapy were 35% (6/17) and 27% (3/11), respectively. Details of these patients who responded to lifileucel are outlined in the Data Supplement.

Exploratory analyses of product-specific characteristics, including levels of phenotypic markers of T-cell lineage, memory subset, youth, activation or exhaustion, or trafficking (Data Supplement), did not demonstrate association with response. Tumor burden reductions were seen across the continuum of cell doses (Data Supplement).

Safety

All patients experienced at least one TEAE, with the most common (≥ 30%) grade 3 or 4 TEAEs being thrombocytopenia (82%), anemia (56%), febrile neutropenia (55%), neutropenia (39%), hypophosphatemia (35%), leukopenia (35%), and lymphopenia (32%) (Table 3), consistent with the toxicity profile of NMA-LD and IL-2. Fatal TEAEs occurred in two patients—1 death was because of intra-

TABLE 3. TEAEs Occurring in ≥ 20% of Patients

	Cohort 2 (N = 66)		
Preferred Term	**Any Grade**	**Grade 3 or 4**	**Grade 5**
No. of patients reporting at least one TEAE, No. (%)	66 (100)	64 (97)	2 (3)[a]
Thrombocytopenia	59 (89)	54 (82)	0
Chills	53 (80)	4 (6)	0
Anemia	45 (68)	37 (56)	0
Pyrexia	39 (59)	11 (17)	0
Neutropenia[b]	37 (56)	26 (39)	0
Febrile neutropenia	36 (55)	36 (55)	0
Hypophosphatemia	30 (46)	23 (35)	0
Leukopenia[b]	28 (42)	23 (35)	0
Fatigue	26 (39)	1 (2)	0
Hypotension	24 (36)	7 (11)	0
Lymphopenia[b]	23 (35)	21 (32)	0
Tachycardia	23 (35)	1 (2)	0
Alopecia	19 (29)	0	0
Increased AST	19 (29)	0	0
Decreased appetite	19 (29)	1 (2)	0
Diarrhea	19 (29)	1 (2)	0
Hypokalemia	17 (26)	2 (3)	0
Hypoxia	17 (26)	10 (15)	0
Peripheral edema	17 (26)	1 (2)	0
Rash	17 (26)	3 (5)	0
Hypocalcemia	16 (24)	3 (5)	0
Hypomagnesemia	16 (24)	0	0
Increased weight	16 (24)	1 (2)	0
Increased ALT	15 (23)	2 (3)	0
Nausea	15 (23)	0	0
Increased blood alkaline phosphatase	14 (21)	2 (3)	0
Dyspnea	14 (21)	3 (5)	0
Hypoalbuminemia	14 (21)	3 (5)	0
Maculopapular rash	14 (21)	6 (9)	0
Vomiting	14 (21)	0	0
Constipation	13 (20)	0	0
Pruritus	13 (20)	0	0

Abbreviations: AE, adverse event; TEAE, treatment-emergent adverse event; TIL, tumor-infiltrating lymphocytes.

[a]One death was because of intra-abdominal hemorrhage reported as possibly related to TIL, and one was because of acute respiratory failure assessed as not related to TIL by the investigator.

[b]All patients had grade 4 laboratory abnormality per the Common Terminology Criteria for Adverse Events v4.03 for leukopenia, neutropenia, and lymphopenia during the treatment-emergent period. Only clinically significant laboratory abnormalities per investigators were reported as AEs.

abdominal tumor hemorrhage reported as possibly related to TIL, and one was because of acute respiratory failure assessed as not related to TIL by the investigator. The incidence of TEAEs, including grade 3 or 4 TEAEs, decreased rapidly over time (Fig 3) with no lifileucel-related SAEs reported after 6 months, and no recurrence of irAEs related to prior ICI. Tumor harvest AEs related to surgery are outlined in the Data Supplement.

DISCUSSION

Treatment options for patients with advanced melanoma who progress after treatment with ICI and BRAF ± MEK inhibitors are limited. Cytotoxic chemotherapy has shown poor response rates,[15,16,18,19] with a limited median OS of 7 months.[15] Many of the patients in our study had exhausted all approved therapy (mean lines of prior therapy, 3.3). The encouraging antitumor activity of lifileucel observed in our study addresses a major unmet need in patients with advanced melanoma after progression on ICI, and targeted agents if indicated.

Lifileucel, a one-time cellular therapy, represents a significant improvement in the treatment of advanced melanoma, particularly the current post-ICI patient population. First, lifileucel demonstrated an ORR of 36%, meeting the study primary end point in a patient population that had failed frontline anti–PD-1 therapy, the current standard of care. This is noteworthy because prior TIL therapy studies were conducted in the pre-ICI era, or enrolled a very small population of patients who had received prior anti–PD-1 therapy.[23-25,31] A previous cohort 2 analysis has demonstrated a high concordance rate of 89.4% between the Independent Review Committee–assessed and investigator-assessed ORR.[32] Second, at a median 18.7-month follow-up, the median DOR has not been reached, emphasizing the durability of lifileucel responses in a heavily pretreated post-ICI patient population with a high baseline tumor burden. Third, the efficacy of lifileucel was equivalent agnostic of PD-L1 status, *BRAF* mutation status, or prior anti–CTLA-4 therapy. Lifileucel was efficacious in the subset of patients who were primarily refractory to anti–PD-1/PD-L1 therapy, demonstrating an ORR of 41% and a DCR of 81% in this subgroup. Furthermore, lifileucel demonstrated similar ORR in patients who received anti–PD-1 plus anti–CTLA-4 combination as a frontline therapy (33%) or after failing frontline therapy (32%).

TIL recognize multiple tumor-specific neoantigens,[33] which may be required for response in solid tumors with high mutational burden. Removal from the hostile microenvironment and ex vivo expansion enable TIL to evade a broad array of immunosuppressive mechanisms. Indeed, both downregulation of PD-1 expression and restored functionality were reported for ex vivo expanded TIL.[34,35] By

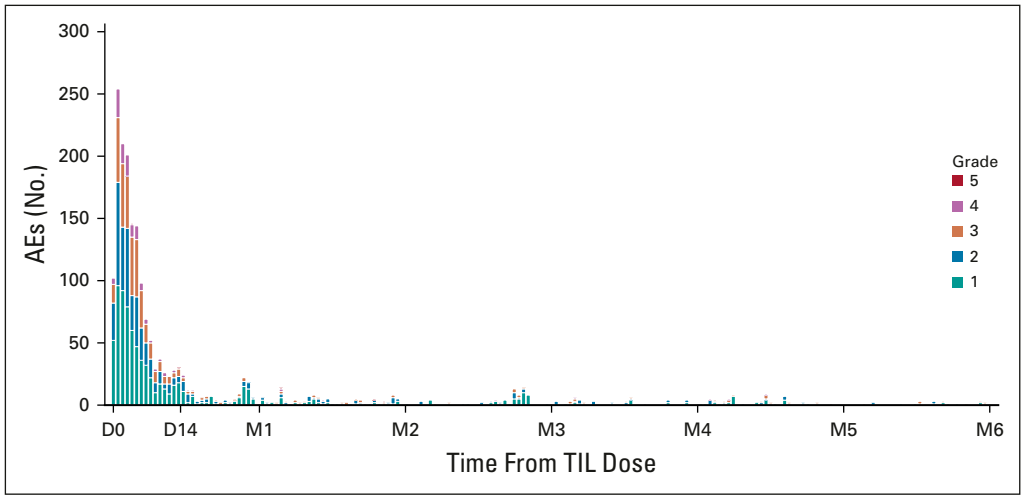

FIG 3. AEs over time. The distribution of onset of AEs starting from lifileucel infusion until 6 months postinfusion is shown. A TEAE was defined as any AE with onset after start of lifileucel through day 30 postinfusion. All occurrences of AEs were counted if a patient experienced a new onset of the same AE at different timepoints. If multiple records were reported on the electronic case report form because of toxicity grade decrease of the same AE that had not resolved, then the event was counted once with the highest grade reported. Overall, 24 AEs were reported post month 6 until data cutoff date, which are not shown in the histogram. No SAEs related to lifileucel were reported post month 6. AE, adverse event; D, day; M, month; SAE, serious adverse event; TEAE, treatment-emergent adverse event; TIL, tumor-infiltrating lymphocytes.

contrast, ICI target only a limited number of pathways in situ. Additionally, in vitro culture results in large-scale expansion of TIL, potentially increasing the number of tumor-specific T cells available for tumor targeting after adoptive transfer. The T cells comprising the TIL product are recovered directly from the tumor tissue, a site enriched for T-cell clones that are able to recognize patient-specific tumor antigens.[36,37] As a result, a polyclonal product is obtained that has the potential to target multiple relevant antigens, addressing (1) the ability to identify the unique spectrum of patient-specific tumor antigens[38]; (2) the heterogeneous nature of solid tumors[39]; and (3) immune escape through antigen loss.[40] Finally, substantial fractions of TIL-derived T cells were shown to persist for at least 6 weeks,[41] consistent with the memory phenotype of the majority of the T cells comprising the product.[35] These varied mechanisms and TIL properties likely contribute to the antitumor efficacy of lifileucel.

The tumors were harvested with minimal surgical morbidity, although 58% were extranodal or nonskin/subcutaneous lesions. A small subset of enrolled patients (12%) could not be treated because of progression, death, or other causes.

TEAEs occurred during or immediately after NMA-LD or IL-2 administration and were generally transient, with no new lifileucel-associated SAEs reported after 6 months. Although patients were hospitalized for NMA-LD and IL-2 administration, lifileucel is a one-time cellular therapy with durable responses, as demonstrated by an ongoing response in 50% of responders at a median follow-up of 18.7 months. In addition, the safety profile indicates that lifileucel is a viable option for patients who are not eligible for ICI because of prior significant irAEs, as it is not associated with recrudescence of irAEs.

Single-center studies conducted at NCI[23,31] have been important in laying the groundwork for TIL therapy in patients with advanced melanoma but were limited to a few centers with dedicated on-site cell therapy facilities. Although lifileucel centralized manufacturing required shipping of the tumor samples, TIL could be manufactured in 96% of patients. The present multicenter study constitutes a significant advance by successfully demonstrating the feasibility of a centrally standardized manufacturing approach for TIL therapy, which allows for broadened patient access, whereas cryopreservation of lifileucel provides flexibility in treatment scheduling in the real-world clinical setting.

In summary, lifileucel, a first-in-class centrally manufactured autologous TIL cell therapy, was efficacious and demonstrated durable responses in heavily pretreated patients and represents a potential new standard of care for patients with advanced melanoma following failure of ICI and targeted therapy. Patients with advanced melanoma who have failed anti–PD-1 therapy (and BRAF ± MEK inhibitors if *BRAF* V600 mutation-positive), irrespective of baseline tumor characteristics, should be considered for the one-time lifileucel therapy as second-line therapy (third-line if *BRAF* V600 mutation positive) if they have performance status and organ function adequate for administration of lymphodepleting chemotherapy and a shortened course of IL-2. The US Food and Drug

Administration has granted lifileucel a Regenerative Medicine Advanced Therapy designation, Orphan Drug designation, and a Fast Track designation for advanced melanoma. Based on these encouraging results, an additional cohort has been fully enrolled, using Independent Review Committee–assessed ORR for registration purposes. Given the favorable risk-benefit profile of lifileucel, its role earlier in the disease course and in combination with ICI is being investigated in melanoma, as well as additional studies in other metastatic solid malignancies.

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Lifileucel, a Tumor-Infiltrating Lymphocyte Therapy, in Metastatic Melanoma

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Travel, Accommodations, Expenses: Bristol Myers Squibb, Pfizer, Novartis, Roche/Genentech, AstraZeneca, Boston Biomedical, Incyte, GlaxoSmithKline, Pierre Fabre, Merck Serono

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Patents, Royalties, Other Intellectual Property: PCT/US2019/059598 for Expansion of TILs Utilizing AKT Pathway Inhibitors. PCT/US2019/012729 for Processes for Generating TIL Products Enriched for Tumor Antigen-specific T-cells. PCT/US2019/029286 for Gene Editing of Tumor Infiltrating Lymphocytes and Uses of Same in Immunotherapy. PCT/US2019/065892 for Methods of Expanding Tumor Infiltrating Lymphocytes Using Engineered Cytokine Receptor Pairs and Uses Thereof. PCT/US2019/012733 for Processes for Generating TIL Products Enriched for Tumor Antigen-Specific T-Cells. PCT/US2020/013095 for System and Methods for Monitoring Adoptive Cell Therapy Clonality and Persistence. PCT/US2020/063767 for Processes for the Production of Tumor Infiltrating Lymphocytes (TILs) and Methods of Using the Same. PCT/US2020/057135 for Gene Editing of Tumor Infiltrating Lymphocytes and Uses of Same in Immunotherapy. One patent application is nonpublic, for which Iovance declines to furnish any information
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Speakers' Bureau: Bristol Myers Squibb
Research Funding: Amgen, Bristol Myers Squibb, Castle Biosciences, Checkmate Pharmaceuticals, Immunocore, Iovance Biotherapeutics, Novartis, Merck

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Patents, Royalties, Other Intellectual Property: University of Louisville US Patents

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