Long-term survival after neoadjuvant low-dose ipilimumab plus high-dose nivolumab in resectable stage III melanoma: the 5-year survival-update and biomarker analysis from the PRADO-trial

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Abstract

Neoadjuvant ipilimumab plus nivolumab has become standard therapy for stage III melanoma based on the NADINA trial, though long-term data are lacking. In the phase 2 PRADO cohort of OpACIN-neo (NCT02977052), 99 patients with stage III macroscopic melanoma received this regimen.

We report first-time 5-year survival data: 71% event-free survival, 74% relapse-free survival, 79% distant metastasis-free survival, and 86% overall survival. Ongoing grade 1-2 immune-related adverse events occurred in 69% of patients alive, predominantly vitiligo and hypothyroidism.

Major pathologic response (MPR), high tumor mutational burden (TMB), high interferon-gamma signature (IFNg), and PD-L1 expression ≥1% were associated with favorable outcomes. Combined high TMB, IFNg, and PD-L1 expression yielded 100% MPR and 100% 5-year event-free survival, while triple low expression had only 18% MPR and 41% event-free survival.

Our findings demonstrate favorable long-term outcomes for patients with an MPR and identify TMB, IFNg, and PD-L1 as promising baseline biomarkers.

Introduction

During the last decade, neoadjuvant immune checkpoint blockade (ICB) has been widely investigated in many different cancer types[1-8]. This paradigm shift was driven by the hypothesis that the presence of more (neo)antigens prior to surgery results in a stronger and broader clonal expansion of tumor-specific T cells induced by neoadjuvant ICB. Results from early preclinical and clinical studies supported this hypothesis, suggesting superior outcomes for neoadjuvant compared to adjuvant ICB[9-13]. The randomized phase II SWOG-1801 and phase III NADINA trials in patients with macroscopic resectable stage III melanoma[1,2] subsequently confirmed these findings, showing superior clinical outcomes in those who received neoadjuvant ICB compared to those who received adjuvant ICB only.

The SWOG-1801 trial evaluated pembrolizumab (anti-PD1, 200 mg every 3 weeks) given as 3 neoadjuvant cycles followed by 15 adjuvant cycles versus 18 cycles of adjuvant-only treatment. The neoadjuvant approach demonstrated superior outcomes with an estimated 2-year event-free survival (EFS) of 72% compared to 49% for adjuvant-only treatment[2]. The NADINA trial showed even greater benefits with an estimated 18-month EFS of 81% in patients treated with 2 cycles of neoadjuvant nivolumab (anti-PD1) plus ipilimumab (anti-CTLA4) followed by a response-directed adjuvant treatment regimen as compared to 54% in patients treated with 12 cycles of adjuvant nivolumab[1,14].

Identifying patients likely to respond to treatment could enable personalized therapy approaches, to improve responses and minimalize toxicity. In stage IV melanoma, tumor mutational burden (TMB) and Programmed Cell Death Ligand 1 (PD-L1) expression at baseline are associated with favorable treatment responses [15], in stage III melanoma the interferon gamma gene signature (IFNg) and TMB, but not PD-L1 expression, are associated with pathological response and EFS[16].

Neoadjuvant ICB allows for pathologic response evaluation, a robust surrogate marker for relapse free survival (RFS) and distant metastasis free survival (DMFS)[14,17-19]. However, to date, all patients undergo potentially morbid surgery (therapeutic lymph node dissection; TLND) for pathologic response evaluation. The pathologic response in the largest lymph node metastasis at baseline (index lymph node; ILN) has shown to be representative of the pathologic response in the total lymph node bed[20,21], suggesting that an initial removal of the ILN could tailor further personalized treatment or follow-up.

The PRADO trial was the first trial in melanoma prospectively testing a surgical de-escalation approach, by marking and removing only the ILN for pathologic response evaluation after neoadjuvant immunotherapy[22].

We have previously reported that neoadjuvant ipilimumab plus nivolumab induced a pathologic response rate (≤50% residual viable tumor) of 72% (71/99 patients), including major pathologic response (MPR; ≤10% residual viable tumor) in 61% (60/99 patients), confirming the efficacy of this neoadjuvant regimen reported in the OpACIN-neo trial[13,22]. Of interest, the final pathological response analysis of the larger NADINA trial reported precisely the same MPR rate of 61% based on examination of the TLND specimen[14].

Early data from PRADO suggested that patients achieving a MPR in the ILN, can safely omit both TLND and adjuvant therapy, resulting in superior quality of life without increasing the individual risk of relapse at 3-years of follow-up [18,22]. Recently a large, pooled analysis from various studies and real-world patient databases confirmed the excellent outcomes for patients achieving an MPR, although these studies predominantly included TLND specimens [23]. However, patient outcome data beyond 3 years after neoadjuvant ipilimumab plus nivolumab, especially for those with an MPR in which subsequent surgery and adjuvant therapy were omitted, are lacking.

Therefore, we report the 5-year update of the PRADO trial including survival, long-term toxicity, and the first biomarker analyses, and the 5-year survival-outcomes of the OpACIN-neo trial. In addition, we will be comparing the long-term outcomes to the previously conducted OpACIN-neo trial, where the same inclusion-criteria were handled. In OpACIN-neo patients were treated with different dosages of neoadjuvant ipilimumab + nivolumab, without personalized surgery or adjuvant treatment [13].

Results

At the time of data-cutoff of 06/01/2025, the median follow-up was 60 months (interquartile range, IQR: 60 - 64 months), with a minimum follow-up of 31 months for all patients alive (*n*=83).

Survival outcomes

At data cut off, 29/99 (29%) patients had an event (defined as disease progression resulting in inoperability, locoregional recurrence, distant metastases, or death, whichever comes first): six patients

became inoperable due to progression before surgery, 23/92 (25%) patients had a locoregional recurrence after surgery, 18/92 (20%) had a distant metastasis after surgery, and 16/99 (16%) patients died, of whom 14 died from melanoma. One patient died of a cause not related to disease progression or toxicity and 1 patient died of an unknown cause without signs of disease progression. Patient-selection (99 patients versus 92 patients) for survival follow-up is displayed in **Extended Data Fig. 10a**.

The estimated 5-year EFS, relapse free survival (RFS), distant metastasis free survival (DMFS), overall survival (OS) and melanoma specific survival (MSS) of patients treated in the PRADO trial were 71% (95% confidence interval (CI): 63-81%), 74% (95% CI: 65-84%), 79% (95% CI: 71-89%), 86% (95% CI: 79-93%) and 88% (95% CI: 81-94%) **(Fig. 1a-e)**. The median EFS, RFS, DMFS, OS and MSS had not been reached at time of data cutoff. In OpACIN-neo similar estimated 5-year survival rates were observed, without significant differences between the three treatment-arms in that study **(Extended Data Fig. 4a-e & Extended Data Fig. 5a-d).**

When PRADO patients were grouped by pathologic response according to the International Neoadjuvant Melanoma Consortium (INMC) criteria[24] **(Supplemental Table 1),** the estimated 5-year RFS, DMFS and MSS were 86% (95% CI: 76-97%), 91% (95% CI: 83->99%) and 98% (95% CI: 95->99%) for patients with an MPR, 55% (95% CI: 32-94%), 55% (95% CI: 32-94%) and 64% (95% CI: 41->99%) for patients with a pathologic partial response (pPR; >10% and ≤50% residual viable tumor) and 48% (95% CI: 30-75%), 57% (95% CI: 40-83%) and 80% (95% CI: 64->99%) for patients with a pathologic non-response (pNR; >50% residual viable tumor) **(Fig. 1f-h)**. Baseline characteristics per pathologic response group are displayed in **Supplemental Table 2.**

For patients with an MPR survival was improved as compared to patients without MPR, with 5-year RFS rates being 86% versus 50% (log-rank *p*-value<0.0001), DMFS 91% versus 56% (log-rank *p*-value<0.0001) and MSS 98% versus 74% (log-rank *p*-value=0.00034). These outcomes were similar to the outcomes observed in the OpACIN-neo cohort, with the exception for patients achieving a pPR, where these patients had higher landmark EFS, RFS, DMFS and MSS in the OpACIN-neo trial compared with PRADO. Neither group received adjuvant therapy **(Fig. 1f-h & Extended Data Fig. 4f-h)**. When dividing MPR into pathologic complete response (pCR; 0% viable tumor cells) and near-pCR (>0%-≤10% viable tumor cells), similar estimated 5-year survival-outcomes are observed in PRADO and in OpACIN-neo (data not shown).

When searching for potential explanations of the outcome differences observed for the pPR patients from the PRADO vs OpACIN-neo cohorts, we compared their baseline characteristics **(Supplemental Table 3)**. We found that a larger proportion of patients in the PRADO pPR cohort were from Europe (10/11 in PRADO versus 5/12 in OpACIN-neo), no patients had an ulcerated tumor (0/10 in PRADO versus 5/12 in the OpACIN-neo; *p*-value=0.016) and a larger proportion of patients had a low tumor mutational burden (TMB; 100% in PRADO versus 44% in OpACIN-neo, *p*-value=0.026). There were no differences in measurable tumor burden (sum of diameter of target lesion and number of lymph nodes on imaging), *BRAF*-mutation status, frequency of grade ≥3 immune related adverse events (irAEs), use of prednisolone, or second line immunosuppression **(Supplemental Table 3)**.Three patients with a pPR in

PRADO did not undergo a TLND, two patients refused additional surgery, and one patient was suspected to have stage IV disease, all three patients did not have disease progression at time of cutoff.

BRAF-mutation status

In PRADO, patients with a *BRAFV600E/K*-mutant melanoma had worse EFS than patients with *BRAF*-wildtype melanoma, with 5-year EFS of 60% versus 80%, respectively **(Fig. 2a)** (log-rank *p*-value=0.011 and Hazard Ratio of 2.63 (95% CI: 1.24-5.57, *p*=0.0118)). All patients with a distant metastasis on imaging at time of surgery had a *BRAF*-mutant melanoma. No significant differences between BRAF-mutant and BRAF-wildtype melanoma were found for RFS (80% versus 64%, log-rank *p*-value=0.12) and DMFS (84% versus 72%, log-rank *p*-value=0.23), though survival curves suggested a differential effect over time, complicating interpretation **(Fig. 2b,c)**. No differences for OS (86% versus 84%, log-rank *p*-value=0.79) and MSS (90% versus 84%, log-rank *p*-value=0.3) were observed **(Fig. 2d,e)**. In addition, presence of a *BRAFV600E/K* mutation in the tumor was not predictive for RFS, DMFS and OS in the univariable Cox-regression analysis **(Fig. 3, Extended Data Fig. 2, Extended Data Fig. 3)**.

In the OpACIN-neo cohort, only numerical differences in EFS, RFS and DMFS were observed according to the *BRAF* mutational status, and no differences in OS and MSS **(Extended Data Fig. 6a-e)**.To investigate whether this difference was driven by the different doses of ipilimumab used in the OpACIN-neo cohort we divided the patients into different subgroups based on their *BRAF* mutational status (mutant versus wildtype) and treatment-arm (arm A (ipilimumab 3 mg kg^{-1} plus nivolumab 1 mg kg^{-1}) versus arm B (ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1})) **(Extended Data Fig. 6f,g)**. Even though thesubgroups become too small to draw any conclusions, there is a slight trend towards a better response to higher dose of ipilimumab in patients with a *BRAF*-mutant melanoma **(Extended Data Fig. 6f,g)**.

Safety

In total, irAEs ≥ grade 3 occurred in 30% of the PRADO study population **(Extended Data Fig. 1).** Ongoing grade 1-2 irAEs occurred in 69% of the patients alive at data cut-off and were predominantly vitiligo and hypothyroidism **(Extended Data Fig. 1).**

Subsequent therapies

Six out of 60 (10%) patients in the MPR group had disease recurrence **(Fig. 4)**, including1/12 (8%) patients with a near-pCR and 5/47 (11%) patients with a pCR. Four of these six patients had a local recurrence that occurred within 20 months and were treated with surgery combined with BRAF-targeted therapy (*n*=1), anti-PD1 (*n*=2), or systemic targeted therapy alone (*n*=1). Two out of the six patients in the MPR group had a distant metastasis as first recurrence, 60 and 61 months after registration **(Fig. 4)**. One of these patients had local progression in the jejunum that was treated with BRAF-targeted therapy, followed by surgery. The other patient had multiple metastatic sites including brain, and received re-induction with ipilimumab + nivolumab, however received only one dose due to toxicity and died soon thereafter.

Five out of the 11 (45%) patients achieving pPR had disease recurrence. One patient had a local recurrence as first recurrence, which was surgically removed. Later the patient developed a distant metastasis and received high dose ipilimumab plus nivolumab followed by radiotherapy. Four out of the five patients with disease recurrence from the patients that achieved a pPR, had a distant metastasis at time of first recurrence. One elderly patient only received palliative radiotherapy at time of recurrence and died later of disease progression. The other patients were treated with surgery (*n*=3) or radiotherapy combined with anti-PD1 (*n*=1) (**Fig. 4)**.

Ten out of the 21 (52%) patients who achieved a pNR upon the neoadjuvant regimen had disease recurrence. Three of these eleven patients had a local recurrence and were treated with surgery +/- radiotherapy or T-VEC. Seven patients had a distant metastasis at time of the first recurrence and were treated with targeted therapy (*n*=4), lenvatinib plus pembrolizumab (*n*=1) or no systemic treatment (*n*=2) **(Fig. 4)**. Of the two patients who did not receive any systemic treatment at time of progression, one received radiotherapy but later opted for best supportive care at time of further disease progression, and the other patient did not receive systemic due to previous toxicities.

Six patients had a distant metastasis before surgery. Four out of these six patients received subsequent targeted therapy as first treatment after progression, one patient had long-term response to this subsequent targeted therapy. Four out of six patients with distant metastasis before surgery were treated with a combination of ipilimumab and nivolumab (*n*=2 had a long-term response) before or after treatment with targeted therapy **(Fig. 4)**.

Single biomarker analysis

RNA sequencing data of the baseline biopsy is available in 80/99 (81%) patients **(Extended Data Fig. 10)**. Patients with a high IFNg score (*n*=39) had improved EFS (5-year rate 81% versus 54%; log-rank *p*-value=0.0039), RFS (81% versus 57%; log-rank *p*=0.044) and DMFS (86% versus 63%; log-rank *p*-value=0.031) compared to patients with a low IFNg score (*n*=41) **(Fig. 5a,g)** (RFS and DMFS not shown), but not OS (90% versus 70%; log-rank *p*-value=0.19) and MSS (92% versus 80%; log-rank *p*-value=0.22) **(Fig. 5b,g)** (MSS not shown). In OpACIN-neo similar trends were observed, with higher 5-year EFS, RFS, DMFS, OS and MSS in patients with a high IFNg score, though no significant log-rank test results **(Extended Data Fig. 7)** (RFS, DMFS and MSS not shown).

Whole exome sequencing from the baseline biopsies is available in 75/99 (76%) PRADO patients. No statistically significant differences were observed in EFS (5-year rate 75% versus 58%; log-rank *p*-value=0.1), RFS (76% versus 60%; log-rank *p*-value=0.3), DMFS (80% versus 67%; log-rank *p*-value=0.45), OS (84% versus 81%; log-rank *p*-value=0.88) and MSS (90% versus 81%; log-rank *p*-value=0.45) when comparing patients with a high TMB (*n*=32) to patients with a low TMB (*n*=43) **(Fig. 5c,d,h)** (RFS, DMFS and MSS not shown). In OpACIN-neo, significant differences in EFS (5-year rate 86% versus 66%; log-rank *p*-value=0.032), DMFS (89% versus 69%; log-rank *p*-value=0.022), OS (96% versus 80%; log-rank *p*-value=0.014) and MSS (100% versus 79%; log-rank *p*-value=0.012) and numerical differences in RFS

(86% versus 69%; log-rank *p*-value=0.061) were observed comparing patients with a high TMB to patients with a low TMB **(Extended Data Fig. 7)** (RFS, DMFS and MSS not shown).

In 76 patients, the PD-L1 expression on tumor cells (TPS score) was determined in the baseline biopsy. Patients with a high PD-L1 expression of ≥1% (*n*=43) had a significantly improved EFS (5-year rate 88% versus 60%; log-rank *p*-value=0.0036), RFS (87% versus 64%; log-rank *p*-value=0.045), OS (94% versus 79%; log-rank *p*-value=0.049) and MSS (97% versus 81%; log-rank *p*-value=0.029) compared to patients with a PD-L1 expression of <1%, respectively (*n*=33) **(Fig. 5e,f,i)** (RFS and MSS not shown). No significant differences were found in DMFS (5-year rate 90% versus 73%; log-rank *p*-value=0.097) between the groups. In OpACIN-neo, only small differences in 5-year rates and no significant log-rank test results were observed for EFS (85% versus 76%; log-rank *p*-value=0.29), RFS (85% versus 78%; log-rank *p*-value=0.36), DMFS (85% versus 83%; log-rank *p*-value=0.49), OS (96% versus 88%; log-rank *p*-value=0.12) and MSS (96% versus 89%; log-rank *p*-value=0.25) when comparing outcomes of patients with a high PD-L1 expression of ≥1% to patients with a PD-L1 expression of <1% **(Extended Data Fig. 7)** (RFS, DMFS and MSS not shown).

To investigate whether the missing PD-L1 expression of patients with an event influenced the predictive value of PD-L1 expression, we performed imputation by replacing these missing values by all low or all high. Although PD-L1 expression remained predictive for RFS, DMFS and OS **(Supplemental Table 4)**, the unavailability of the PD-L1 expression in some patients might have resulted in an overestimation of the differences observed in our cohort.

Combined biomarker analyses

Next, we analyzed the contribution of the most prominent baseline markers when combining them to a multiparameter baseline biomarker. In 61 patients the IFNg signature score, TMB and PD-L1 expression were available, and were distributed as displayed in **Fig. 6a**. As expected, having all three parameters low was associated with the lowest MPR rates (18%; 95% CI: 4-43%), while having all three parameters high was associated with a 100% chance for MPR (95% CI: 72-100%) (**Fig. 6b**).

Patients with MPR had a higher IFNg signature score (**Fig. 6c)** and a higher TMB (**Fig. 6d)**. The IFNg signature score did not correlate with the TMB **(Fig. 6e)**. We did not include PD-L1 as this was a non-numeric variable.

In line with the response data patients with a triple high score (*n*=11) had the longest 5-year EFS (100%) while patients with triple low (*n*=17) had the shortest (41%) **Fig. 6f**. The AUC curves of these two scenarios are shown in **Fig. 6g.**

Similar survival-trends were observed in the OpACIN-neo trial, as in the patients with triple high (*n*=3) 100% (95% CI: 29-100%) had MPR and an 100% estimated 5-year EFS and the patients with triple low (*n*=11) had a 27% (95% CI: 6-61%) MPR-rate and 55% estimated 5-year EFS **(Extended Data Fig. 8a-e)**

As clinical grade TMB evaluation is a costly procedure, we also analyzed the biomarker-combination of IFNg and PD-L1 expression alone. Patients with a low IFNg score and a low PD-L1 expression (*n*=22) had MPR rate of 27% **(Fig. 7b)** and 5-year EFS of 48% **(Fig. 7a, c)**,while patients with a high IFNg score and a high PD-L1 expression (*n*=21) had MPR rate of 86% **(Fig. 7a)** and a 5-year EFS of 91% **(Fig. 7d).** The AUC curves for these biomarker doublets are shown in **Fig. 7e and f** and showed only slightly lower AUC compared to the model using all three biomarkers **(Fig. 6g)** (AUC of 0.745 in double low versus 0.756 in triple low; and an AUC of 0.692 in double high versus 0.712 in triple high).

Similar results were observed in the OpACIN-neo cohort with MPR in 47% of patients with a low IFNg score and a low PD-L1 (*n*=19) and 100% of the patients with high IFNg score and a high PD-L1 (*n*=7). However, and in contrast to the PRADO cohort, only numerical differences were observed in EFS according to the combination of IFNg score and PD-L1 status **(Extended Data Fig. 9a-d)**.

Other parameters associated with RFS, DMFS or OS

Differences in baseline clinical parameters, grade of toxicity, immunosuppressives used, and baseline biomarkers for patients with or without a recurrence observed during follow-up are displayed in **Table 1**. Univariable Cox regression analysis results for RFS, DMFS and OS are displayed in **Fig. 3**, **Extended Data Fig. 2** and **Extended Data Fig. 3**. Given the relatively small number of events observed for these endpoints, we highlighted parameters with either statistical significance (i.e. p-value < 0.05) or an estimated HR ≤0.5 or ≥2. Selected variables according to these criteria were pathologic response (improved RFS and DMFS for MPR patients compared to pPR or pNR), lymph node location (improved DMFS and OS for neck compared to axilla), use of prednisolone (poorer OS when used), use of second line immunosuppressants (shorted DMFS and OS when used), IFNg (prolonged RFS, DMFS and OS if high IFNg), and PD-L1 (improved RFS, DMFS and OS if ≥1%). More patients with a *BRAF*-mutant melanoma had a recurrence, but presence of a *BRAF* mutation in the tumor was not predictive for RFS, DMFS and OS in the univariable Cox-regression analysis **(Table 1, Fig. 3, Extended Data Fig. 2, Extended Data Fig. 3)**. These associations remain similar in size and/or statistical significance when adjusting for other parameters, one at a time, in multivariable analyses.

The conclusions remained the same when performing these analyses on multiply imputed data.

Discussion

In this five-year update of the PRADO-trial we present the first long-term survival outcomes of patients treated with neoadjuvant low-dose (1 mg kg^{-1}) ipilimumab and standard-dose (3 mg kg^{-1}) nivolumab, followed by response driven adjuvant therapy. These phase 2 study data are of interest as they may indicate what to expect from the long-term follow-up of the phase III NADINA trial [1]. Additionally, these results might support re-imbursement initiatives for this neoadjuvant treatment approach in various countries.

In line with several previous reports [14,17-19], achieving MPR after neoadjuvant ICB remains a robust surrogate marker for long-term outcome. Of interest, our results indicate that it might be safe to de-escalate surgery in patients with MPR in the ILN [18,22], as the long-term outcomes in PRADO are comparable to that of the OpACIN [19] and OpACIN-neo trial cohorts where patients all underwent TLND. The OMIT (NCT06754904) and MSLT-3 (NCT07049276) trials will deliver additional prospective/randomized data for omitting TLND in patients with MPR in the ILN.

In contrast to earlier observed good outcomes for patients with a pPR in neoadjuvant ICB trials [16,23,25], in PRADO, these patients had worse outcomes than patients who had a pNR. Patients with a pNR had adjuvant therapy and pPR did not in PRADO. When comparing patients with a pPR from PRADO to patients with a pPR in OpACIN-neo[13,22], different outcomes (higher EFS, RFS, DMFS and MSS landmarks in OpACIN-neo) are not likely driven by differences in patient-selection, measurable tumor burden, adverse event management or a difference in measured residual viable tumor at surgery. Differences in neoadjuvant or surgical regimen might drive these different outcomes, as higher doses of ipilimumab (in arm A and C OpACIN-neo) could induce better outcomes in these patients, particularly those with *BRAF* mutant melanoma [26,27]. Another cause might be the differences in surgical regimen for patients who are treated with one surgery (TLND) in OpACIN-neo and two surgeries (ILN and TLND) in PRADO, as this might induce an immunosuppressive state [28,29]. Larger cohorts are needed to confirm these findings, as very few patients have a pPR within each trial.

We observed no ongoing grade 3-4 irAEs at five years follow-up, suggesting that this regimen has a manageable safety profile. However, potentially quality-of-life-impairing irAEs with hypothyroidism or adrenal insufficiency requiring life-long hormone replacement therapy were observed in 22% and 7% of patients in the total cohort and 25% and 8% in patients with MPR. In addition, we observed that the use of prednisolone and second line immunosuppressants remained associated with a worse OS in the multivariable analysis. This association was also observed in patients with advanced melanoma treated with ipilimumab +/- nivolumab, where peak dose prednisolone or second-line immunosuppression for irAEs was associated with impaired survival in a retrospective analysis [30-32]. Future research and prospective trials should prioritize identifying patients in whom neoadjuvant treatment could be de-escalated, for example to anti-PD1 monotherapy, to prevent unnecessary side effects.

In PRADO, the lower EFS in patients with a *BRAF*-mutant melanoma was pre-dominantly driven by the patients developing distant metastasis before surgery. RFS and DMFS of patients with a *BRAF*-mutant melanoma are better or similar compared to patients with a *BRAF*-wild type melanoma in short term, most likely due to the 1 year of adjuvant dabrafenib plus trametinib in the patients with a pNR, but this is lost in years thereafter. Similar trends were observed in the patients treated with the same regimen (low-dose ipilimumab plus normal dose nivolumab) in OpACIN-neo. Our results suggest that patients with a *BRAF*-mutant melanoma might benefit from high-dose neoadjuvant ipilimumab, which is in line with what has previously been observed in stage IV melanoma [26,27,33]. OS and MSS of patients with a *BRAF*-mutant or a *BRAF* wild type melanoma are comparable, which is most likely due to more options in subsequent

treatment-lines. If confirmed in the NADINA trial [1] the *BRAF* mutation status should be investigated further as a baseline biomarker for treatment escalation.

Not all patients achieve good outcomes on this regimen. Some patients would benefit from alternative/intensified neoadjuvant schemes to achieve equally promising long-term outcomes as those observed in patients with MPR. In line with previous trials [16,34], the 10-gene IFNg gene signature [35] was predictive for MPR and long-term EFS. In contrast to what was observed in OpACIN-neo [16], PD-L1 expression was predictive and TMB was not predictive for long-term survival-outcomes. This inconsistency of TMB and PD-L1 as baseline biomarkers, warrants further analyses in larger (real world) cohorts [23]. Critically, other factors including neoadjuvant regimen, extent of surgery (ILN versus upfront TLND) and adjuvant therapy (type and/or in all patients or response driven) should be concurrently analyzed to understand the impact of each of these factors and their interaction with tissue biomarkers.

The three tested baseline biomarkers (TMB, IFNg, and PD-L1) might be helpful tools in the future to identify patients who are not likely to respond to this regimen at baseline and include them into innovative neoadjuvant trials. For example, our previous DONIMI trial (NCT04133948)[34] showed that this baseline biomarker can be analyzed within clinically relevant timeframes to support neoadjuvant treatment decision-making. Another example is the NeoIReNi trial, that will use baseline biomarkers to enrich for those patients predicted to have non-MPR (NCT06999980). In addition, these promising baseline biomarkers should also be investigated in patient cohorts treated with other neoadjuvant treatments (e.g. anti-PD1 monotherapy, high-dose anti-CTLA4, anti-LAG3 or anti-TIGIT), since such analyses could help in personalizing neoadjuvant therapy for macroscopic melanoma further. The PRADO trial, and especially its biomarker subgroup analyses are limited by low patient numbers. Large, pooled analyses might deliver more significant data, that should be subsequently confirmed in prospective trials.

In conclusion, this survival-update of PRADO shows promising long-term survival outcomes for patients treated with neoadjuvant ipilimumab and nivolumab, especially in patients who achieve MPR in the index lymph node. For patients without MPR, alternative neoadjuvant and adjuvant schemes are needed, and baseline biomarkers like the IFNg signature and PD-L1 expression might be promising biomarkers that could help identify these patients for escalation of therapy. Our data opens the possibility for biomarker-based personalization and response-driven personalization of surgery and adjuvant treatment.

Methods

Study design and participants

The PRADO extension cohort from OpACIN-neo is an investigator-initiated phase II trial, testing 2 courses neoadjuvant ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}, followed by ILN response-based surgery (omitting the additional TLND in patients achieving MPR) and adjuvant therapy in patients achieving a pNR in the ILN (nivolumab for patients with a *BRAF*-wildtype and dabrafenib + trametinib for

patients with a *BRAF*-mutant melanoma until week 52 +/- local radiotherapy). The detailed eligibility criteria, trial design, ethical approval and the first responses (pathologic responses, EFS, RFS, DMFS and OS) have been published earlier[22]. The trial enrolled participants in Australia at Melanoma Institute Australia (MIA) and at several Dutch centers [Netherlands Cancer Institute (NKI), Leiden University Medical Center (LUMC), Erasmus Medical Center (EMC), University Medical Center Utrecht (UMCU), and University Medical Center Groningen (UMCG)].

Pathologic response at week 6 was assessed according to INMC guidelines [24] and was categorized into MPR (≤10% residual viable tumor, which included patients with complete response (pCR; 0% residual viable tumor) and near-pCR (1 – ≤10% residual viable tumor), pPR (>10 – ≤50% residual viable tumor) or pNR (>50% residual viable tumor). Follow-up consisted of a radiologic assessment with CT or PET-CT, physical examination and laboratory testing every 12 weeks for 2 years post-surgery, and in year 3, 4 and 5 according to institute standards. Patients experiencing progression prior to surgery or recurrence after surgery, remained in the trial and were further followed for DMFS (in case of local recurrence only) and OS.

Endpoints of the PRADO trial were pathologic response rate upon neoadjuvant two cycles ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}, to confirm the efficacy of arm B of the OpACIN-neo trail, and 24-month RFS of patients achieving MPR or pNR, to determine whether the TLND could be safely omitted in patients achieving MPR and to determine efficacy of adding adjuvant treatment in patients with a pNR. Secondary endpoints were short-term grade 3–4 irAEs, comparing surgical adverse events, radiologic response, DMFS, EFS, OS, health related quality of life, ongoing long-term irAEs, and biomarker analyses.

The data cutoff for collection of the data presented here was on January 6, 2025.

Validation cohort

The investigator-initiated, randomized, phase II OpACIN-neo trial is used as a validation cohort in this manuscript, as the PRADO trial was designed as an extension cohort of the OpACIN-neo. The detailed eligibility criteria, trial design, ethical approval and the first responses (pathologic responses, EFS, RFS, DMFS and OS) are described in earlier publications [13,16,19]. Patients were randomized 1:1:1 to receive either two cycles of ipilimumab 3 mg kg^{-1} plus nivolumab 1 mg kg^{-1} every 3 weeks (arm A), two cycles of ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1} every 3 weeks (arm B) or two cycles of ipilimumab 3 mg kg^{-1} every 3 weeks, directly followed by two cycles nivolumab 3 mg kg^{-1} every 2 weeks (arm C); all patients had a therapeutic lymph node dissection planned in week 6. None of the patients received any adjuvant treatment.

DNA and RNA sequencing and analyses

From 81/99 (81%) baseline patient samples declared to be sufficient fresh frozen pretreatment tumor material (≥30% tumor cells on an H&E-stained cryostat frozen section) RNA and DNA were isolated. The RNA and DNA were simultaneously isolated with the AllPrep DNA/RNA/miRNA Universal isolation kit

(Qiagen, 80224) using the QIAcube. Furthermore, germline DNA was isolated from peripheral blood mononuclear cells using AllPrep DNA/RNA/miRNA Universal isolation kit (Qiagen, 80224) to be able to filter out single-nucleotide polymorphisms when determining TMB. In total, baseline biopsy material of *n*=80 samples for RNA-sequencing and *n*=75 samples for DNA-sequencing were available for analysis. For OpACIN-neo, a detailed description of the tissue processing and analysis of the RNA and DNA sequencing has been described in detail elsewhere [16].

Messenger RNA (mRNA) sequencing and whole-exome sequencing of the PRADO samples were performed by CeGaT. Initial sample quality control was performed with Qubit dsNDA BR or HS(Thermo Fisher) for the DNA sequencing data and Qubit RNA (Thermo Fisher) & Bioanalyzer RNA (Agilent) for the RNA sequencing . Strand-specific libraries were generated using the TruSeq Stranded mRNA sample preparation kit (Illumina) and sequenced with 2x 100bp reads on the NovaSeq 6000 system. Demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.20). Adapters were trimmed with Skewer (version 0.2.2) [36]. The quality of FASTQ files was analyzed with FastQC (version 0.11.5-cegat). FASTQ files were mapped to the human reference genome (GRCh38.v82) using STAR (version 2.7.3a) with default settings [37]. Raw count data generated with HTseq-count (version 0.12.4) [38] were normalized for sequencing depth and RNA composition using the median of ratio's method implemented in DESeq2 (version 1.40.1) [39] and log2-transformed. For the downstream analysis the data were analyzed using R (version 4.3.0). Log2-transformed normalized gene expression data was gene-wise median centered by subtracting each element of a row with the median of that row to improve Pearson's correlation distances.

The IFNg signature score was determined by calculating the average expression z-score of the 10 genes that compose the IFNg signature (STAT1, CXCL9, CXCL10, HLA-DRA, GZMA, PRF1, IDO1, CXCL11, CCR5, IFNG)[35]. Due to the batch-effect between the RNA-sequencing data from PRADO and OpACIN-neo separate cutoffs were determined. The optimal cutoff for the PRADO cohort was calculated by the maximize-metric function of cutpointr (version 1.1.2), using major pathologic response (MPR) status as the binary outcome. For OpACIN-neo the previously calculated cutoff for IFNg was used in this manuscript [16].

Exome libraries were generated using 50ng with the Twist Human Core Exome Plus (Twist Biosciences). The libraries were sequenced with 2 x 100-bp reads on a NovaSeq 6000 System according to the manufacturer's protocols, with a sequence quality Q30 value of 90%. Data were analyzed in CeGaT exome analysis pipeline. Briefly, de-multiplexing of the sequencing reads was performed with Illumina bcl2fastq (version 2.20). The quality of FASTQ files was analyzed with FastQC (version 0.11.5-cegat) and multiQC (version 1.12)[40]. We used the nf-core/sarek pipeline v3.5.1 [41] to analyze tumor and normal FASTQ files for the identification of Single Nucleotide Polymorphisms (SNPs) and small insertions and deletions (INDELs). The whole exome sequencing data were first preprocessed using FASTP. Subsequent alignment of the reads to the human reference genome (GRCh38) was performed using Burrows-Wheeler Aligner (BWA-MEM). Duplicate reads were marked, and base quality scores recalibrated using

GATK MarkDuplicates, GATK BaseRecalibrator, and GATK ApplyBQSR. SNPs and small INDELs were called using MuTect2, and variants were annotated with Ensembl VEP. For further details, including the specific versions of each tool, we refer to the Sarek documentation.

Using the VEP-annotated VCF files, we calculated the Tumor Mutational Burden (TMB) with cyvcf2 (version 0.31.1) in Python (version 3.12.5). Variants were filtered to include only those that passed the MuTect2 call, and having a Variant Allele Frequency of at least 0.05 (5% variant read depth). The TMB was calculated by summarizing the total number of non-synonymous mutations in the filtered VCF per patient. Mutations ≤ 2 were excluded from the analysis. The optimal TMB cut-off was identified with the maximize_metric method in cutpointr (v 1.1.2), using major pathologic response (MPR) status as the binary outcome. The TMB of the PRADO and OpACIN-neo samples by using this pipeline and the optimal TMB cut-off calculated based on the patients treated in OpACIN-neo arm B and PRADO, as they were treated with the same neoadjuvant regimen (neoadjuvant ipilimumab 1 mg kg^{-1} plus nivolumab 3 mg kg^{-1}).

Anti-PDL1

FFPE blocks of baseline tumor samples were used for PD-L1 immunohistochemistry staining using the BenchMark Ultra autostainer (Ventana Medical Systems). Briefly, paraffin sections were cut at 3 µm, heated at 75°C for 28 minutes and deparaffinized in the instrument with EZ prep solution (Ventana Medical Systems). Heat-induced antigen retrieval was carried out using Cell Conditioning 1 (CC1, Ventana Medical Systems) for 48 minutes at 95°C. Anti-PD-L1 mAb (clone 22C3, DAKO) was used in an 1:40 dilution for PD-L1 staining. Bound antibody was visualized using the OptiView DAB Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin II and Bluing Reagent (Ventana Medical Systems). After staining slides were scanned with the P1000 (Sysmex) system. An experienced pathologist who was blinded to clinical outcome determined the tumor proportion score (TPS; the percentage of tumor cells with complete or partial membranous staining at any intensity out of all tumor cells) using Slide Score (www.slidescore.com). The TPS was classified as being <1%, 1-50%, >50% or not evaluable (due to pigmentation or little to no tumor cells).

Statistical analyses

Differences in patient characteristics were assessed using Mann-Whitney's test and Fisher's exact test. Patients with an event before surgery were excluded from RFS and DMFS analysis **(Extended Data Fig. 10)**. Associations between baseline parameters and survival endpoints were estimated using univariable and multivariable Cox regression models. Pathologic response was measured post-baseline and hence not considered for OS analyses. Immune related adverse events, use of prednisolone and second line immunosuppressants were modelled as time-dependent covariates.

Patients from the biospecimen cohort were included for survival-analysis of the different biomarker subgroups (EFS, RFS, DMFS, OS and MSS). One patient that did not undergo surgery (*n*=1) was excluded from the subgroup-analysis of patients with RNA-sequencing data, DNA-sequencing data, PD-L1

expression and pathologic response available. A detailed overview is demonstrated in **Extended Data Fig. 10.** Spearman's correlation coefficient was estimated for pairs of biomarkers.

The reverse Kaplan-Meier method was used for estimating median follow-up time. The Kaplan-Meier method was used to generate EFS, RFS, DMFS, OS and MSS curves and to calculate the 5-year landmark survival estimates for the clinical cohort, also stratified by pathologic response and biomarker values. Overall comparisons in survival outcomes across the subgroups were tested using the log-rank test. Univariable Cox regression analyses were performed in forest plots, and relevant characteristics were further analyzed with multivariable Cox regression. Proportionality of hazards was assessed using Schoenfeld residual plots. Imputation of missing data by best- and worst-case scenarios was performed as sensitivity analysis, as well as using multivariate imputation by chained equations. No adjustments for multiplicity were performed. Statistical analyses were performed using R software (version 4.2.2).

Declarations

Data availability

RNA-sequencing and DNA-sequencing data generated during the study will be deposited in the European Genome-phenome Archive (EGA) for PRADO under the accession codes EGAS50000000268 (DNA) or EGAS00001007601 (RNA). For OpACIN-neo under the accession codes EGAS00001004832 (DNA) or EGAS00001004833 (RNA). To minimize the risk of patient re-identification, de-identified individual patient-level clinical data are available under restricted access. Upon scientifically sound request, data access can be obtained via the NKI's scientific repository at repository@nki.nl, which will contact the corresponding author (C.U.B.). Data requests will be reviewed by the institutional review board of the NKI and will require the requesting researcher to sign a data access agreement with the NKI.

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Author contributions

C.U.B. designed the trial and had written the protocol. The final amendment of the PRADO extension was written by E.A.R. together with C.U.B. in 20th Workshop on 'Methods in Clinical Cancer Research' (Zeist, Netherlands). A.C.J.v.A. and G.V.L. reviewed the trial protocol. I.L.M.R., A.M.M., R.P.M.S., J.M.V., W.v.H., E.A.R., E.K., A.A.M.v.d.V., K.P.M.S., H.E., G.A.P.H., J.A.v.d.H., D.J.G., A.J.W., J.M.L., W.M.C.K., C.Z., A.Bruining, A.A.M., T.E.P., K.F.S., S.Chong, A.S., J.B.A.G.H., A.v.A., G.V.L., and C.U.B. have included and treated patients, and collected clinical data. A.T.A., L.G.G.-O. and A.v.d.W. contributed to central and local data management. M.G. was a clinical project manager of the trial. S.Cornelissen performed DNA and RNA isolations. A.Broeks coordinated and contributed to translational laboratory logistics and immunohistochemistry and molecular laboratory work. P.D. and J.R. performed the bioinformatics analysis. A.J.C., R.V.R., R.A.S. and B.v.d.W. reviewed and scored the pathology of all cases. L.L.H. and M.Y.L. performed the statistical analyses. L.L.H. and C.U.B. wrote the first draft of the manuscript. All authors interpreted the data, reviewed the manuscript and approved the final version.

Declaration of Interest

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 BMS, MSD, Novartis, Roche, Pierre-Fabre and QBiotics. R.P.M.S. has received honoraria for advisory board participation from MSD and Clinical Laboratories Pty Ltd. W.J.v.H. has received speakers honorarium regeneron, Sanofi, MSD, Belpharma, novartis, reports an advisory role Belpharma and has received a research grant from Amgen. E.K.A. has consultancy/advisory relationships with Delcath, Immunocore, and Lilly, and has received research grants unrelated to this paper from Bristol Myers Squibb, Delcath, Novartis, and Pierre-Fabre. These grants are unrelated to current work and are paid to the institute. A.A.M.v.d.V., received travel fees from Ipsen and consultancies fees (all paid to the institute) from BMS, MSD, Ipsen, Eisai, Pfizer, Novartis, Sanofi, Roche and Pierre Fabre. K.P.M.S. has consultancy/advisory relationships with Abbvie, Sairopa, has received research funding TigaTx, Bristol Myers Squibb, Philips, Genmab, Pierre Fabre, and has received honoraria from Bristol Myers Squibb (all paid to the institute). H.E. has received institutional research grants from SkyLineDx, BMS, Novartis and Pierre Fabre, speaker honorarium from Janssen, not personal speaker honorarium but to the hospital from BMS and Novartis, and served as expert board for Pierre Fabre and BMS. G.A.P.H consultancy/advisory relationships with Amgen, Bristol-Myers Squibb, Roche, MSD, Novartis, Sanofi, Pierre Fabre and has received research grants from Bristol-Myers Squibb, Seerave (all paid to the institute). S. Ch'ngreceives fees for professional services provided to MSD and SkylineDx. J.B.A.G.H. reports an advisory role for Achilles Therapeutics, AstraZeneca, BioNTech, Bristol-Myers Squibb, Immunocore, Instil Bio, Iovance Biotherapeutics, Ipsen, Molecular Partners, MSD Oncology, Neogene Therapeutics, Novartis, Roche/Genentech, Sanofi, Sāstra, Third Rock Ventures and T-Knife; has received

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Table

Table 1. Characteristics for patients with or without recurrence observed at 5 years follow-up

	All (*n*=99)	Recurrence (*n*=27)	No Recurrence* (*n*=72)
Baseline characteristics			
Continent (%)			
Europe	65 (66)	20 (74)	45 (63)
Australia	34 (34)	7 (26)	27 (38)
Age (median (IQR))	58.0 [51.0-69.5]	54.0 [51.0, 69.5]	60.0 [52.0, 69.3]
Sex (%)			
Men	65 (66)	16 (59)	49 (68)
Women	34 (34)	11 (41)	23 (32)
T-stage (%)			
T1-2	43 (43)	12 (44)	31 (43)
T3-4	41 (41)	11 (41)	30 (42)
Tx	2 (2)	2 (7)	0 (0)
MUP	13 (13)	2 (7)	11 (15)
Ulceration (%)			
No	58 (72)	14 (64)	44 (75)
Yes	23 (28)	8 (36)	15 (25)
Unknown	18	5	13
***BRAF*-mutation (%)**			
Wildtype	52 (54)	8 (30)	44 (63)
V600E/K mutant	45 (46)	19 (70)	26 (37)
Unknown	2	0	2
Location of the lymph node (%)			
Neck			
Axilla	24 (24)	4 (14)	20 (28)
Groin	39 (39)	9 (33)	30 (42)
	36 (36)	14 (52)	22 (31)
Number of positive lymph nodes on PET-CT (%)			

1			
>1-3	57 (58)	15 (56)	42 (58)
>3	33 (33)	9 (33)	24 (33)
	9 (9)	3 (11)	6 (8)
Sum of diameter target lesions, mm (median, IQR)	25.0 [18.0, 33.0]	26.0 [19.0, 31.5]	23.5 [18.0, 34.0]
LDH (median [IQR])	186.0 [163.0, 215.5]	192.0 [172.5, 206.5]	184.0 [161.5, 216.0]
Response			
Pathologic response *(%)			
MPR	60 (61)	6 (22)	54 (75)
pPR	11 (11)	5 (19)	6 (8)
pNR	21 (21)	10 (37)	11 (15)
Distant metastasis	6 (6)	6 (22)	0 (0)
Not evaluable	1 (1)	0 (0)	1 (1)
Toxicity			
Grade ≥3 irAE(%)	30 (30)	9 (31)	21 (30)
Prednisone (%)	50 (51)	16 (55)	34 (49)
Second line immunosuppressives (%)	11 (11)	4 (14)	7 (10)
Subsequent surgery/therapy			
ILN (%)	94 (95)	24 (89)	70 (97)
TLND (%)	33 (33)	16 (59)	17 (24)
Adjuvant (%)			
Nivolumab	7 (7)	3 (11)	4 (6)
BRAF + MEK inhibitors	10 (10)	5 (19)	5 (7)
Radiotherapy (%)	8 (8)	5 (19)	3 (4)
Biomarkers			
IFNg signature score (mean (SD))	0.37 [-7.28, 7.65]	-1.84 [-9.75, 0.37]	2.08 [-4.17, 9.16]
IFNg (%)			

Low	41 (51)	19 (76)	22 (40)
High	39 (49)	6 (24)	33 (60)
unknown	19	2	17
Number of nonsynomous mutations (mut/mb; median [IQR])	9.12 [5.61, 17.73]	8.50 [4.56, 12.31]	10.73 [6.80, 22.26]
TMB labels (%)			
Low	43 (57)	19 (70)	24 (50)
High	32 (43)	8 (30)	24 (50)
unknown	23	2	21
PDL1 (%)			
<1%	43 (57)	17 (85)	26 (46)
≥1%-<50%	26 (34)	3 (15)	23 (41)
≥ 50%	7 (9)	0 (0)	7 (13)
unknown	23	7	16
PDL1 (%)			
<1%	43 (57)	17 (85)	26 (46)
≥1%	33 (43)	3 (15)	30 (54)
unknown	23	7	16

Data are median (IQR) or n (%). Percentages may not sum up to 100 because of rounding. Percentages are derived of the patients per column with known parameters.

*The patients that died from non-melanoma related cause, were included in the group of patients with “no recurrence” since these patients did not have any signs of recurrence before death.

IQR, interquartile range; MUP, melanoma of unknown primary; LDH, Lactate dehydrogenase; ILN, index lymph node; TLND, therapeutic lymph node dissection; MPR, major pathologic response; pPR, pathologic partial response; pNR, pathologic non-response; irAE, immune related adverse events; IFNg, interferon gamma gene signature; TMB, tumor mutational burden; PD-L1, Programmed Cell Death Ligand 1;

Figures

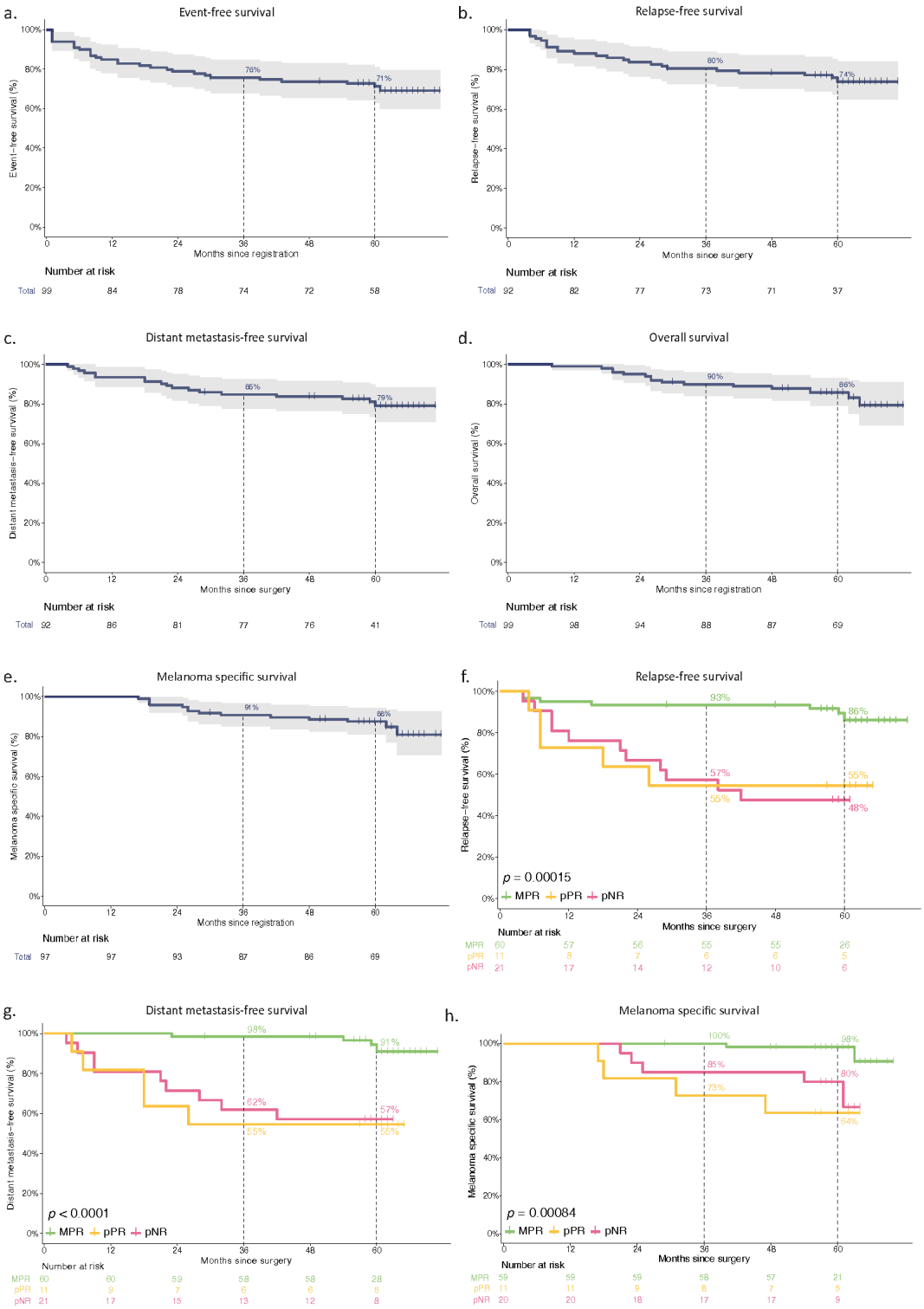

Figure 1

5-year survival analysis for all patients and per pathologic response category

(A) Event free survival for all patients (*n*=99), (B) Relapse-free survival for all patients (*n*=92), (C) Distant metastases-free survival for all patients (*n*=92), (D) Overall survival for all patients (*n*=99), (E) Melanoma Specific survival for all patients (from registration to melanoma related death) (*n*=96), (F) Relapse-free

survival per pathologic response category (*n*=92), (G) Distant metastases-free survival per pathologic response category (*n*=92), (H) Melanoma Specific survival per pathologic response category (from surgery to melanoma related death) (*n*=89).

MPR, major pathologic response (green); pPR, pathologic partial response (orange); pNR, pathologic non-response (red).

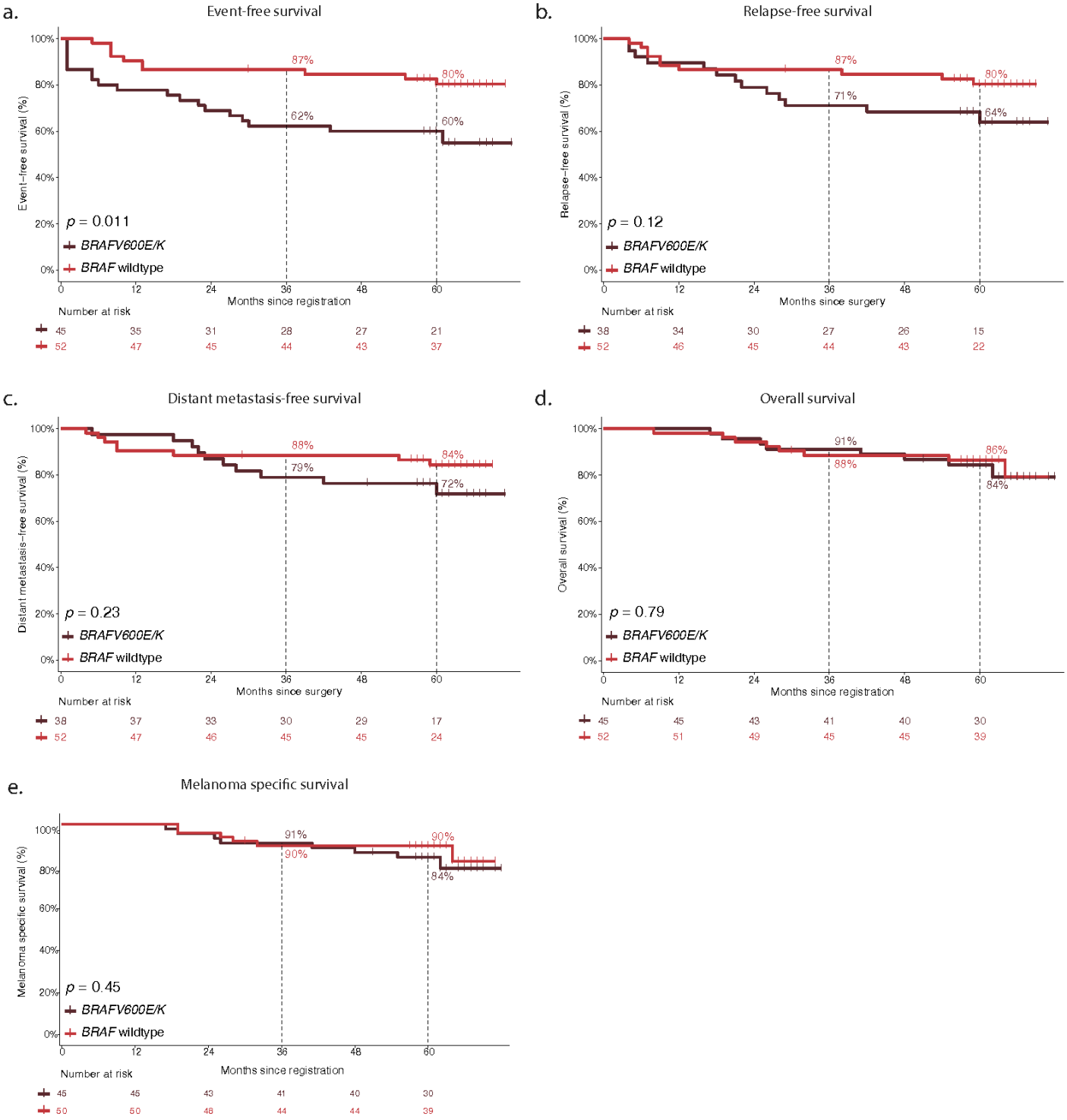

Figure 2

5-year survival analysis of patients with or without *BRAF*mutation

A) Event free survival *BRAF*-mutant (brown; *n*=45) versus *BRAF*-wildtype (red; *n*=52), (B) Relapse-free survival *BRAF*-mutant (brown; *n*=38) versus *BRAF*-wildtype (red; *n*=52), (C) Distant metastases-free survival *BRAF*-mutant (brown; *n*=38) versus *BRAF*-wildtype (red; *n*=52), (D) Overall survival *BRAF*-mutant (brown; *n*=45) versus *BRAF*-wildtype (red; *n*=52).

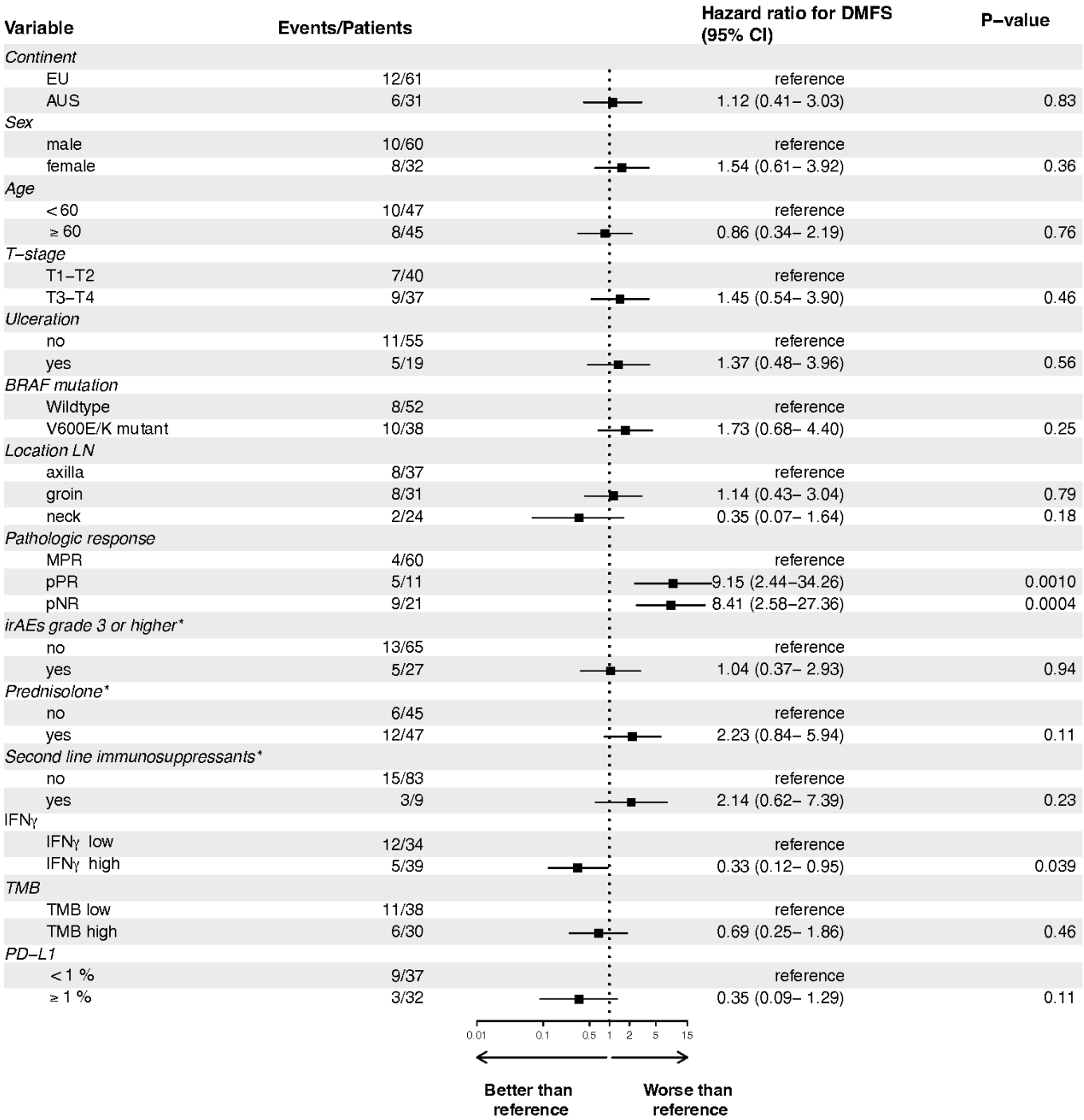

Figure 3

Forest Plot of Hazard Ratios for distant metastasis free survival by baseline variables

Forest plot of risk for distant metastasis calculated from surgery (*n*=92) using Cox-regression analysis expressed in hazard-ratios per variable, with a 95% confidence interval and *p*-value compared to the reference variable.

* Time-dependent variable

EU, Europe; AUS, Australia; LN, lymph node; MPR, major pathologic response; pPR, pathologic partial response; pNR, pathologic non-response; irAE, immune related adverse events; IFNg, interferon gamma gene signature; TMB, tumor mutational burden; PD-L1, Programmed Cell Death Ligand 1; CI, confidence interval.

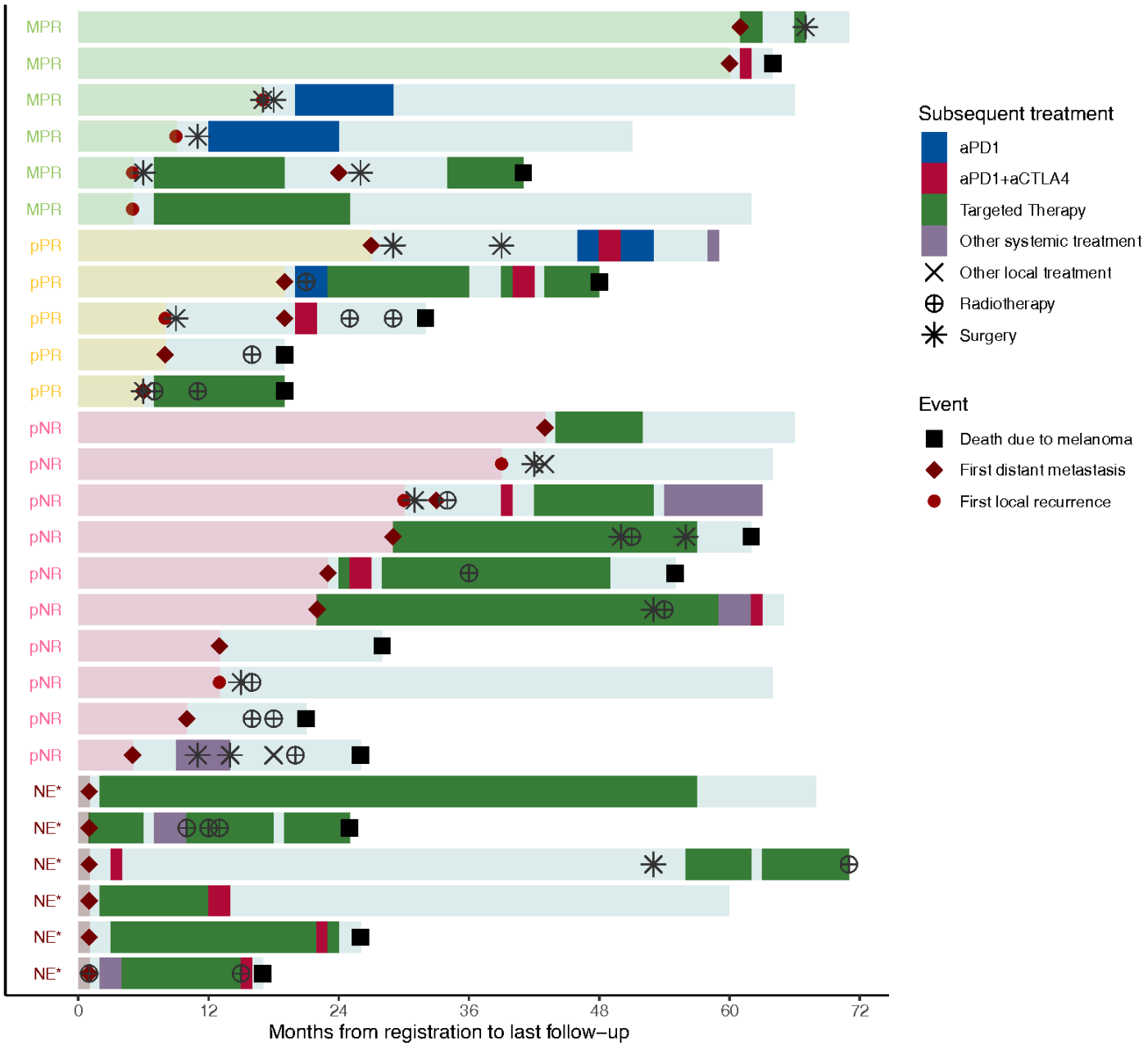

Figure 4

Swimmer plot of time to recurrence and subsequent therapies in patients who recurred (*n*=27)

The time from registration to recurrence, distant metastasis and subsequent therapies are displayed from all patients with a recurrence.

*due to progressive disease before surgery

Other systemic treatments (purple) include RO7284755(*n*=1), IMC F106C (*n*=1), Antibody Drug Conjugate (*n*=1) and TIL-therapy alone (*n*=1), TIL combined with anti-PD1 (*n*=1), Lenvatinib plus Pembrolizumab(*n*=1), and anti-LAG3 plus anti-PD1 (*n*=1). Other local treatments include ruthenium brachytherapy (*n*=1) and T-VEC (*n*=1).

MPR, major pathologic response (green); pPR, pathologic partial response (orange); pNR, pathologic non-response (red); NE, non-evaluable due to progressive disease before surgery (dark red); aPD1, anti-PD1; aCTLA4, ant-CTLA4; aLAG3, anti-LAG3.

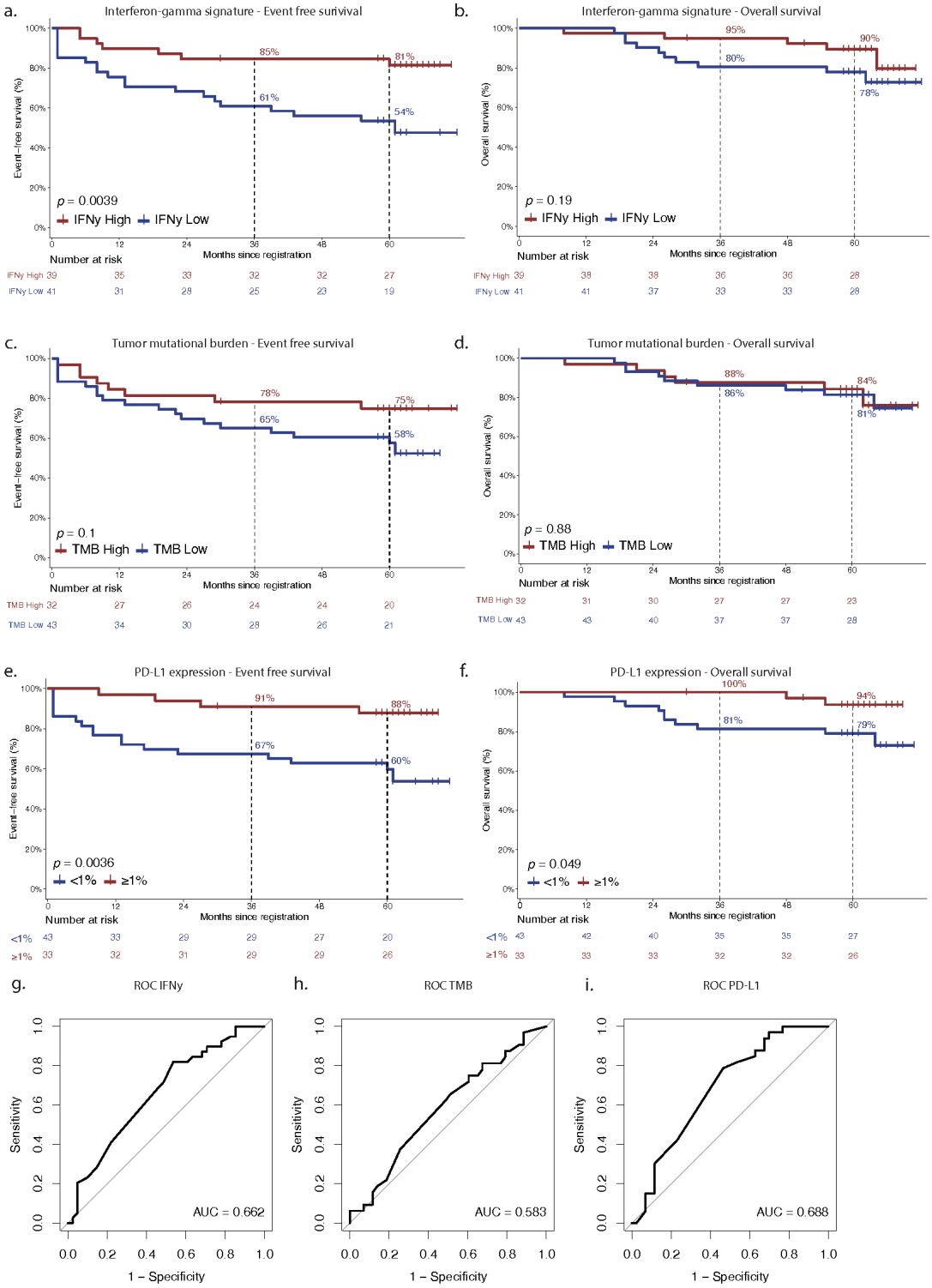

Figure 5

Biomarker analysis of the interferon gamma gene signature (IFNg), tumor mutational burden (TMB) and PD-L1 expression

(A) 5-year Event-Free Survival based on the IFNg score (high (red; *n*=39) vs low (blue; *n*=41)), (B) 5-year Overall Survival based on the IFNg score (high (red; *n*=39) vs low (blue; *n*=41)). Patients were included

when RNA sequencing data was available (*n*=80); (C) 5-year Event-Free Survival based on the TMB (high (red; *n*=32) vs low (blue; *n*=43)), (D) 5-year Overall Survival based on the TMB (high (red; *n*=32) vs low (blue; *n*=43)). Patients were included when WES data was available (*n*=75); (E) 5-year Event-free Survival based on the PD-L1 expression (<1% (blue; *n*=43) vs ≥1% (red; *n*=33)), (F) 5-year Event-free Survival based on the PD-L1 expression (<1% (blue; *n*=43) vs ≥1% (red; *n*=33)), Patients were included when PD-L1 expression data was available (*n*=76), (G) Prediction of an event based on IFNghigh versus low using an ROC curve (AUC=0.662), (H) Prediction of an event based on TMB high versus low using an ROC curve (AUC=0.583), (I) Prediction of an event based on PD-L1 high versus low using an ROC curve (AUC=0.688).

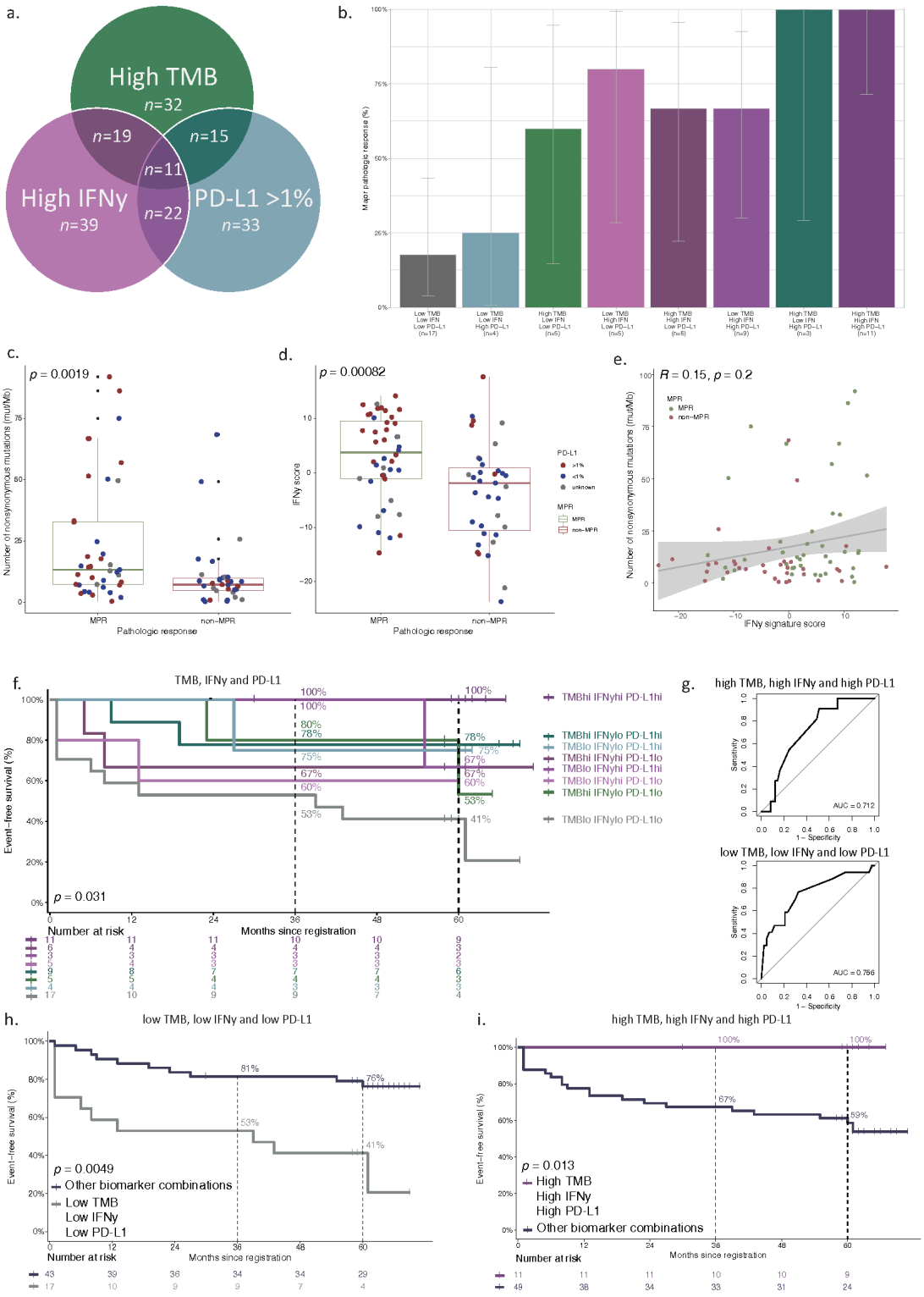

Figure 6

Combination of biomarkers

(A) VENN-diagram of patients with a high IFNg(purple), high TMB (green) and/or high PD-L1 (≥1%, blue), (B) Barplot of patients with a major pathologic response (including 95% confidence interval) according to the combination of biomarkers: low TMB, low IFNg and low PD-L1 (grey; *n*=17); low TMB, low IFNg and

high PD-L1 (blue; *n*=4); high TMB, low IFNg and low PD-L1 (green; *n*=5); low TMB, high IFNg and low PD-L1 (pink; *n*=5); high TMB, high IFNg and low PD-L1 (medium purple; *n*=6); low TMB, high IFNg and high PD-L1 (light purple; *n*=9); high TMB, low IFNg and high PD-L1 (cyan; *n*=3); high TMB, high IFNg and high PD-L1 (dark purple; *n*=11); Patients were included when a pathologic response information, WES, RNA sequencing and PD-L1 expression data was available (*n*=61), (C) Boxplot of the IFNg gene signature score of patients achieving MPR versus non-MPR, colored by PD-L1 expression (<1% (blue) vs ≥1% (red), (D) Boxplot of the TMB of patients achieving MPR versus non-MPR, colored by PD-L1 expression (<1% (blue) vs ≥1% (red) (E) Correlation plot for TMB and IFNg, (F) 5-year Event-Free Survival according to TMB, IFNg and PD-L1 as described at B, (G) Prediction of an event using ROC curves for low TMB, low IFNg and low PD-L1 versus the patients with other biomarker combinations (AUC=0.756) and for high TMB, high IFNg and high PD-L1 versus the patients with other biomarker combinations (AUC=0.712), (H) 5-year Event-Free Survival of patients with a low IFNg, low TMB and low PD-L1 (*n*=17) versus the patients with other biomarker combinations (*n*=43), (I) 5-year Event-Free Survival of patients with a high IFNg, high TMB and high PD-L1 (*n*=11) versus the patients with other biomarker combinations (*n*=49).

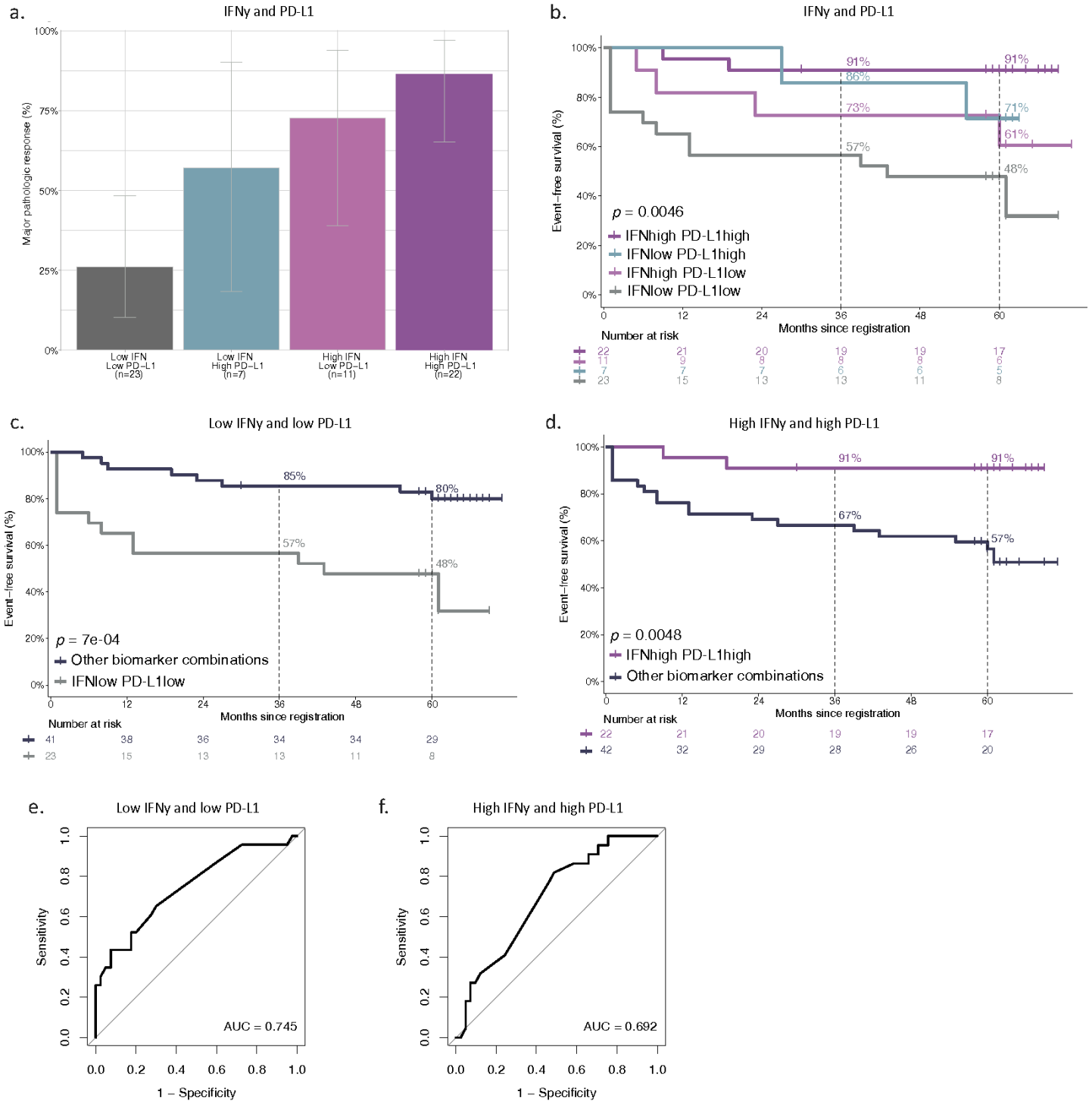

Figure 7

Combination of IFNg and PD-L1

(A) Barplot of pathologic response rate (including 95% confidence interval) according to IFNg score and high PD-L1 (≥1%), resulting in multiple subgroups: low IFNg and low PD-L1 (grey; *n*=23); low IFNg and high PD-L1 (blue; *n*=7); high IFNg and low PD-L1 (pink; *n*=11); high IFNg and high PD-L1 (light purple;

n=22); Patients were included when a pathologic response information, RNA sequencing and PD-L1 expression data was available (*n*=61), (B) 5-year Event-Free Survival according to IFNg and PD-L1 as described at A, (C) 5-year Event-Free Survival of patients with a low IFNg and low PD-L1 (grey; *n*=22) versus the patients with other biomarker combinations (dark blue; *n*=42), (D) 5-year Event-Free Survival of patients with a high IFNg and high PD-L1 (light purple*; n*=23) versus the patients with other biomarker combinations (dark blue; *n*=41), (E) Prediction of an event using an ROC curve for low IFNg and low PD-L1 versus the patients with other biomarker combinations (AUC=0.745), (F) Prediction of an event using an ROC curve for high IFNg and high PD-L1 versus the patients with other biomarker combinations (AUC=0.692).

Supplementary Files

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