




# Interferon-gamma signature as prognostic and predictive marker in macroscopic stage III melanoma

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## ABSTRACT

**Background** A substantial proportion of patients with macroscopic stage III melanoma do not benefit sufficiently from adjuvant anti-PD-1 therapy, as they either recur despite therapy or would never have recurred. To better inform adjuvant treatment selection, we have performed translational analyses to identify prognostic and predictive biomarkers.

**Patients and methods** Two cohorts of patients with macroscopic stage III melanoma from an ongoing biobank study were included. Clinical data were compared between an observation cohort (cohort 1) and an adjuvant intention cohort (cohort 2). RNA sequencing for translational analyses was performed and treatment subgroups (cohort 1A and cohort 2A) were compared for possible biomarkers, using a cut-off based on the treatment-naïve patients. In addition, two validation cohorts (Melanoma Institute Australia (MIA) and University Medical Centre Utrecht (UMCU)) were obtained.

**Results** After a median follow-up of 26 months of the 98 patients in our discovery set, median recurrence-free survival (RFS) was significantly longer for the adjuvant intention cohort (cohort 2, n=49) versus the observation cohort (cohort 1, n=49). Median overall survival was not reached for either cohort, nor significantly different. In observation cohort 1A (n=24), RFS was significantly longer for patients with high interferon-gamma (IFN $\gamma$ ) score (p=0.002); for adjuvant patients of cohort 2A (n=24), a similar trend was observed (p=0.086). Patients with high B cell score had a longer RFS in cohort 1A, but no difference was seen in cohort 2A. The B cell score based on RNA correlated with CD20<sup>+</sup> cells in tumor area but was not independent from the IFN $\gamma$  score. In the MIA validation cohort (n=44), longer RFS was observed for patients with high IFN $\gamma$  score compared with low IFN $\gamma$  score (p=0.046), no difference in RFS was observed according to the B cell score. In both the observation (n=11) and the adjuvant (n=11) UMCU validation cohorts, no difference in RFS was seen for IFN $\gamma$  and B cell.

**Conclusions** IFN $\gamma$  has shown to be a prognostic marker in both patients who were and were not treated with adjuvant therapy. B cell score was prognostic but did not improve

## WHAT IS ALREADY KNOWN ON THIS TOPIC

⇒ Previous studies have examined the prognostic and predictive performance of clinicopathologic features such as Breslow thickness and ulceration to improve the stratification for adjuvant therapy. However, sensitive and specific tissue biomarkers have yet to be identified in stage III melanoma.

## WHAT THIS STUDY ADDS

⇒ Both the interferon-gamma (IFN $\gamma$ ) and B cell scores have prognostic value in stage III melanoma, but a single strong predictive biomarker of adjuvant anti-PD-1 was not determined.

## HOW THIS STUDY MIGHT AFFECT RESEARCH, PRACTICE, OR POLICY

⇒ These results provide a rationale for further investigations of both prognostic and predictive biomarkers of adjuvant anti-PD-1 therapy in stage III melanoma.

accuracy over IFN $\gamma$ . Our study confirmed RFS benefit of adjuvant anti-PD-1 for patients with macroscopic stage III melanoma.

## INTRODUCTION

Until recently, the standard of care for patients with macroscopic stage III melanoma has been surgery only. Due to the high risk of recurrence, adjuvant therapies (eg, interferon alpha-2b) have been investigated over the past decade, many without demonstrating a significant benefit to patients. Recently, both immune checkpoint inhibition (ICI) and BRAF-targeted therapies have been shown to improve recurrence-free survival (RFS) and are now a standard treatment for patients with resected stage III melanoma.<sup>1–5</sup> However, approximately 80% of patients do not benefit sufficiently from these adjuvant therapies. This issue is presented by two

subsets of patients: first, patients who recur despite adjuvant systemic therapy and second, patients who would never have recurred after surgery (and therefore do not require adjuvant treatment). In terms of the number needed to treat, on average five patients need adjuvant therapy in order to prevent one recurrence in stage III melanoma, illustrating the issue of overtreatment, unnecessary toxicity, and high healthcare costs.

Selection of adjuvant therapy is currently based on the American Joint Committee on Cancer (AJCC) staging system.<sup>6</sup> Patients with stage IIIA (sentinel node tumor burden >1 mm), IIIB, and IIIC melanoma, according to the AJCC 7th edition, were included in the randomized clinical trials demonstrating improved outcomes for patients treated with adjuvant systemic therapy compared with placebo or ipilimumab.<sup>1–3</sup> Previous studies have examined the prognostic and predictive performance of additional clinicopathologic features, for example, age, Breslow thickness, ulceration, number of positive lymph nodes, and extracapsular extension, to improve the stratification for adjuvant therapy.<sup>7–10</sup> However, sensitive and specific tissue biomarkers have yet to be identified in stage III melanoma. Adding tissue biomarkers to these clinical characteristics could lead to a more personalized approach by a more adequate selection of patients, whereby patients are selected to receive adjuvant therapy based on their risk of recurrence and the expected reduction in risk of recurrence from such therapy.

Unraveling the immunologic characteristics of the tumor microenvironment associated with recurrence may reveal insight into possible biomarkers.<sup>11–12</sup> Interferon-gamma (IFN $\gamma$ ) plays an important role in the antitumor response in the tumor microenvironment, and signatures related to IFN $\gamma$  signaling were evaluated as possible biomarkers in previous studies. These studies have shown the IFN $\gamma$  signature to be predictive of response in both patients with advanced melanoma treated with anti-PD-1 therapy and patients with stage III melanoma treated with combination ICI in a neoadjuvant setting.<sup>13–15</sup> Therefore we hypothesized that the IFN $\gamma$  signature could also be useful as a prognostic and/or predictive marker in patients treated with adjuvant therapy.

The aim of this study was to identify biomarkers prognostic and predictive of recurrence after surgery for macroscopic stage III melanoma, both in patients who did and who did not receive adjuvant systemic therapy, in order to predict in whom adjuvant therapy should be omitted, either because of a very low risk of recurrence or due to a lack of benefit.

## PATIENTS AND METHODS

### Patients

Patients were selected from an ongoing institutional database and biobank study (collecting tumor material from patients with macroscopic stage III melanoma) at the Netherlands Cancer Institute (NKI). Patients included between October 2017 and June 2020 were eligible for

selection, when naïve to systemic therapy, complete resection of macroscopic stage III melanoma was performed and sufficient tumor material was available for RNA isolation. Exception for complete resection was made for patients with in-transit metastases who underwent an isolated limb perfusion (ILP), alone or combined with lymph node dissection (LND). Patients with missing data on adjuvant therapy or follow-up were excluded.

Approval and reimbursement of adjuvant systemic therapy in the Netherlands started in December 2018, resulting in two cohorts of similar high-risk patients: prior to availability of adjuvant therapy (observation cohort 1) and thereafter (adjuvant treatment cohort 2) (online supplemental figure S1). Clinical data regarding patient and tumor characteristics, adjuvant therapy, and outcome were collected with a median follow-up of 24 months for both cohorts.

As shown by the screening failures in randomized trials evaluating adjuvant therapy,<sup>16</sup> a proportion of patients will not receive adjuvant systemic therapy due to an early recurrence that occurs between surgery and start of adjuvant therapy. These patients were present in our study, as they represent patients faced in daily clinical practice and were therefore included in the clinical data analyses. However, in terms of translational research, analyses were performed for treatment groups: patients who did not (observation cohort) versus who did receive adjuvant therapy (cohort 1A and 2A for RNA sequencing data, cohort 1B and 2B for PD-L1 data, respectively). To perform an equal comparison, patients with early recurrence were excluded from these translational analyses in both groups (online supplemental figure S1). The definition of early recurrence is described in online supplemental methods.

### Tumor samples

Tumor samples were derived from surgical resection material in most patients and in some patients from biopsies prior to surgical procedures (eg, ILP). Per biobank protocol both fresh-frozen (FF) and formalin-fixed paraffin-embedded (FFPE) samples were stored, if feasible. From FF samples that contained sufficient tumor material based on the pathologist's scoring (at least 30% tumor cells of H&E stained cryostat frozen section), RNA was isolated using the AllPrep DNA/RNA/miRNA universal isolation kit (QIAGEN, 80224) on the QIAcube, according to the manufacturer's protocol. Details on RNA sequencing can be found in the online supplemental methods.

Immunohistochemistry (IHC) of FFPE tumor samples was performed on a BenchMark Ultra (PD-L1 clone 22C3) or a Discovery Ultra (CD20-CD3 double stain) automated stainer (Ventana Medical Systems). Detailed methods of IHC are described in online supplemental methods. PD-L1 expression was scored by a pathologist in a blinded fashion: a score of >1% was considered to indicate PD-L1 positivity.<sup>1–2</sup> Image analysis of CD20-CD3 double-stained samples was performed using HALO software (Module

Multiplex IHC v3.0.3). A MiniNet AI classifier was used to define tissue into tumor and non-tumor. Color thresholds were set for CD20<sup>+</sup> B cells (yellow), CD3<sup>+</sup> T cells (purple), and Melanin and used in analysis for reliable detection. Cells were segmented using hematoxylin as a nuclear detection.

### Validation cohorts

To validate the findings from our cohort, we performed analyses on two validation cohorts.

#### Validation cohort MIA

A cohort of 44 patients treated with adjuvant anti-PD-1 at Melanoma Institute Australia (MIA) between May 2015 and December 2018 was selected from a retrospective discovery cohort of patients with available resected stage III melanoma tissue.<sup>17</sup> FFPE tumor samples were collected for research purposes with Sydney Local Health District Human Ethics Review Committee approval (Protocol no X15-0454 & 2019/ETH06874+X17-0312 & HREC/11/RPHA/32) and informed patient consent from the MIA Biospecimen Tissue Bank. RNA sequencing was performed at MIA, which is described in online supplemental methods. Raw data were made available, on which the same analyses were performed as in the initial cohort.

#### Validation cohort University Medical Centre Utrecht (UMCU)

The second validation cohort of 25 patients consisted of patients with melanoma treated at the UMCU. This cohort, similar to the Netherlands Cancer Institute (NKI) study population, consisted of patients who did (n=11) and did not (n=11) receive adjuvant treatment after complete resection of stage III melanoma and patients with early recurrence (n=3). FFPE tumor samples were transported to the NKI, RNA was isolated and sent to CeGaT for sequencing. The same protocols for isolation, sequencing, and analyses were performed as on the initial cohort. In this validation cohort, not all non-adjuvantly treated patients were from the preadjuvant treatment era (which is the case in the original study cohort). Patients who decided not to undergo adjuvant treatment despite available adjuvant therapy were included in this cohort.

### Statistical analyses

#### Clinical data

Clinical data were analyzed using IBM SPSS Statistics, V.27. Baseline and treatment characteristics were compared between patients who did not receive adjuvant systemic therapy (observation cohort 1) and patients who intended to receive adjuvant therapy (cohort 2). Follow-up, RFS, and overall survival (OS) were analyzed using Kaplan-Meier estimates and a log-rank test was used to compare cohorts. Follow-up, RFS, and OS were defined as the time between surgery at the time of inclusion in the biobank and recurrence or death, respectively. Patients not experiencing an event were censored at the time of last follow-up. Cox regression analyses were carried out; correlation was assessed by Spearman's rho test.

### RNA sequencing analyses

The previously defined IFN $\gamma$ ,<sup>15</sup> Danaher immune cell,<sup>18</sup> and micro-environment cell population (MCP counter)<sup>19</sup> gene expression signatures were analyzed. Cut-offs were calculated based on receiver operating characteristic (ROC) curves, using only observation patients (including patients with an early recurrence) to exclude a treatment effect on risk of disease recurrence. For the immune cell populations of both the Danaher and MCP-counter, z-score of the immune subsets was compared between the treatment groups. Bar and dot plots were generated in GraphPad Prism (V.9.0.2).

### RESULTS

The 98 patients in our discovery set were grouped into two cohorts: cohort 1 included 49 patients who did not receive adjuvant systemic therapy (observation); and cohort 2 included 49 patients who received adjuvant therapy, although some patients did not due to recurrence prior to the planned start (adjuvant intention) (online supplemental figure S1). Patients in cohort 2 were younger (p=0.027), more often had a tumor harboring a BRAF mutation (p=0.031) and a normal lactate dehydrogenase (LDH) level (p=0.009). Also, these patients more frequently underwent an LND only, instead of an LND combined with surgical treatment of in-transit metastases (p=0.002) (table 1). Patients were well balanced between the cohorts for sex and Breslow thickness. In both cohorts, the vast majority of patients had AJCC 8th edition stage IIIC melanoma and S100B levels below the upper limit of normal.

In cohort 2, 10 patients (20%) developed a recurrence before start of adjuvant therapy, and therefore 39 patients received at least one dose of adjuvant therapy. One patient initially started adjuvant BRAF-targeted therapy due to temporarily adjusted logistics following the outbreak of the COVID-19 pandemic, but switched to anti-PD-1 because of toxicity. All other patients received adjuvant anti-PD-1. The median time between surgery until the start of adjuvant therapy was 9 weeks (IQR 8–12) (online supplemental table S1). At data cut-off, all patients had ceased adjuvant therapy. The main reason for cessation was end of treatment (56%), followed by recurrence (26%). Due to the outbreak of the COVID-19 pandemic, 15 patients (39%) had a break in their adjuvant regimen, skipping one or two cycles of treatment.

Median follow-up of the total patient cohort was 26 months (95% CI 23 to 28). After a median follow-up of 28 months (95% CI 26 to 30) in cohort 1, median RFS was 6 months (95% CI 4 to 8). After a median follow-up of 22 months (95% CI 19 to 25), the median RFS of 23 months (95% CI not reported (NR)) was significantly longer in cohort 2 (p=0.006) (figure 1A). At 24 months, the RFS rate was 19% for cohort 1 and 49% for cohort 2 (p=0.008). Univariable Cox regression analyses for RFS (online supplemental table S2) demonstrated, besides adjuvant therapy, significance for type of surgery and

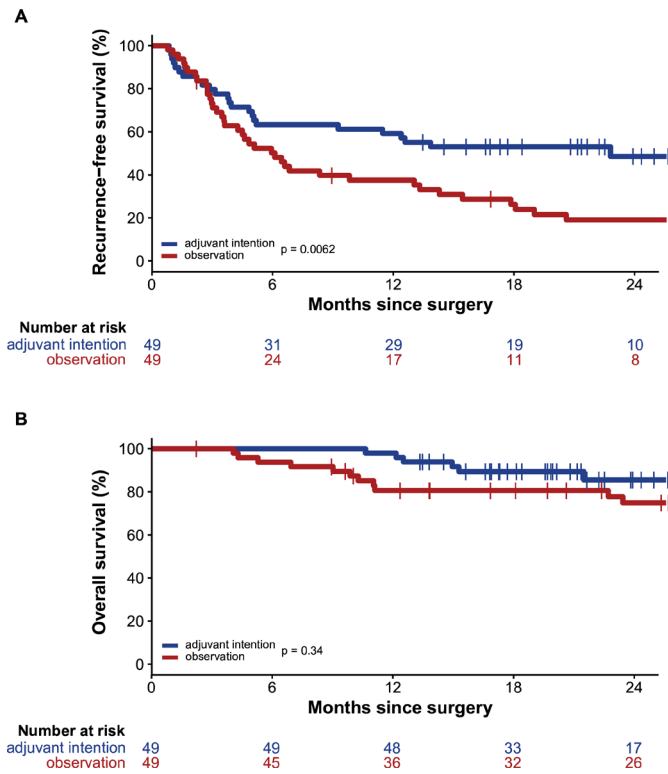
**Table 1** Baseline characteristics of all patients intended for adjuvant therapy versus observation

	All patients (N=98)	Observation cohort 1 (n=49)	Adjuvant intention cohort 2 (n=49)	P value
Age				<b>0.027</b>
Median	63	69	59	
IQR	54–73	54–76	53–70	
Sex				0.306
Male	57 (58)	31 (63)	23 (47)	
Female	41 (42)	18 (37)	26 (53)	
Site primary				0.055
Extremities	50 (51)	31 (63)	19 (39)	
Trunk	41 (42)	15 (31)	26 (53)	
Head and neck	2 (2)	0	2 (4)	
Acral	1 (1)	0	1 (2)	
Mucosal	2 (2)	2 (4)	0	
MUP	2 (2)	1 (2)	1 (2)	
Breslow thickness				0.369
≤1.0 mm	7 (7)	2 (4)	5 (10)	
1.01–2.0	25 (26)	13 (27)	12 (25)	
2.01–4.0	37 (38)	21 (43)	16 (33)	
>4.0	21 (21)	8 (16)	13 (27)	
Unknown	8 (8)	5 (10)	3 (6)	
Ulceration				0.067
No	53 (54)	21 (43)	32 (65)	
Yes	29 (30)	17 (35)	12 (25)	
Unknown	16 (16)	11 (22)	5 (10)	
Stage (AJCC 8th edition)				0.718
IIIB	14 (14)	5 (10)	9 (18)	
IIIC	78 (80)	41 (84)	37 (76)	
IIID	4 (4)	2 (4)	2 (4)	
Unknown	2 (2)	1 (2)	1 (2)	
Mutation status				<b>0.031</b>
BRAF	59 (60)	24 (49)	35 (71)	
NRAS	24 (25)	12 (25)	12 (25)	
cKIT	1 (1)	1 (2)	0	
No driver mutations	10 (10)	8 (16)	2 (4)	
Unknown	4 (4)	4 (8)	0	
Type of surgery				<b>0.002</b>
LND	54 (55)	18 (37)	36 (74)	
ITM	20 (20)	13 (27)	7 (14)	
LND+ITM	10 (10)	6 (12)	4 (8)	
ILP±LND	14 (14)	12 (25)	2 (4)	
S100b				0.905
≤ULN	81 (83)	39 (80)	42 (86)	
>ULN	12 (12)	6 (12)	6 (12)	
Unknown	5 (5)	4 (8)	1 (2)	
LDH				<b>0.009</b>
≤ULN	85 (87)	38 (78)	47 (96)	
>ULN	6 (6)	6 (12)	0	
Unknown	7 (7)	5 (10)	2 (4)	

Reported as number (%), percentages may not total 100 due to rounding.

P<0.05 is statistically significant and set in bold.

ILP, isolated limb perfusion; IQR, Interquartile Range; ITM, in-transit metastasis; LND, lymph node dissection; MUP, melanoma of unknown primary; ULN, upper limit of normal.



**Figure 1** Survival curve. (A) Recurrence-free survival for patients intended for adjuvant therapy versus observation. (B) Overall survival for patients intended for adjuvant therapy versus observation.

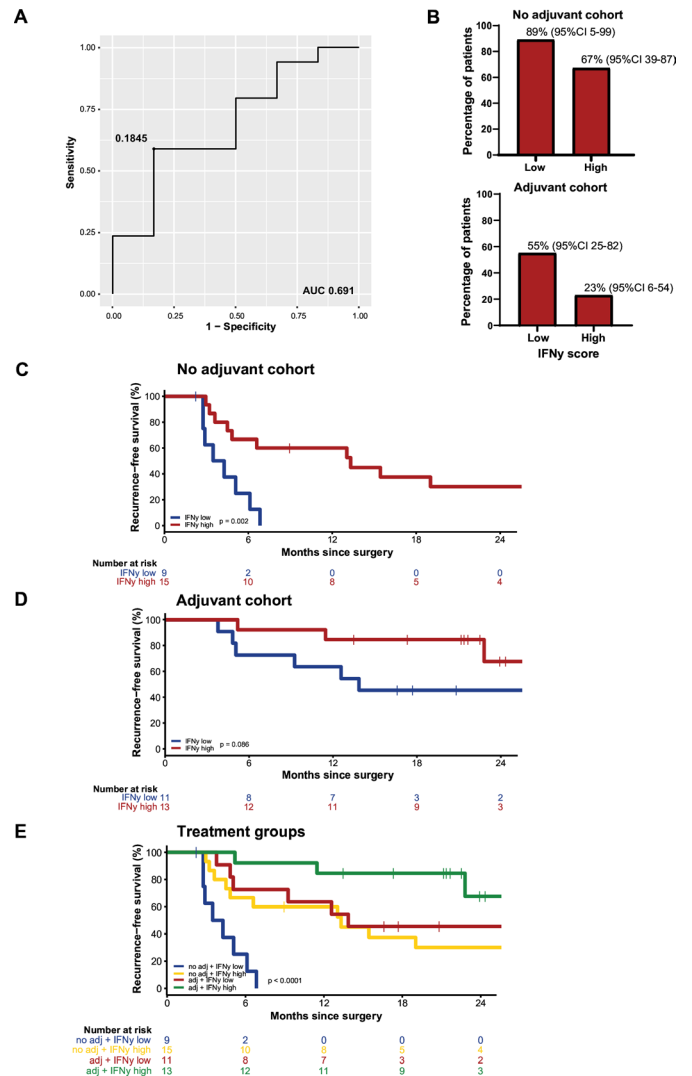
S100b levels at baseline, but not for LDH levels. In multi-variable analyses, S100b and adjuvant therapy remained significant contributors.

Median OS was not reached in both cohorts (figure 1B). At 12 months, there was a significant difference in OS rate in favor of adjuvant therapy (81% vs 98%,  $p=0.006$ ). The 24 months OS rate was 75% vs 86% in cohort 1 and cohort 2, respectively ( $p=0.158$ ).

### IFN $\gamma$ score

RNA sequencing data were available for the subset of patients in the discovery set of whom RNA was of sufficient quality for sequencing: 24 observation patients (cohort 1A) and 24 adjuvant treatment patients (cohort 2A) (online supplemental figure S1). To perform an equal comparison, patients with early recurrence were excluded from these translational analyses in both groups. Of the patients in cohort 1A, 18 patients had a recurrence, compared with 9 patients in cohort 2A. Baseline characteristics for these cohorts are described in online supplemental table S3.

The optimal cut-off for the IFN $\gamma$  score was determined at 0.1845 with a ROC curve, with an area under the curve (AUC) of 0.691 (figure 2A). In cohort 1A, 9 patients had low IFN $\gamma$  score of whom 8 (89%, 95% CI 5 to 99) had a recurrence, and 15 had a high IFN $\gamma$  score of whom 10 (67%, 95% CI 39 to 87) had disease recurrence. In cohort 2A, recurrences occurred in 6/11 patients (55%, 95% CI 25 to 82) with low IFN $\gamma$  score and in 3/13 (23%, 95%



**Figure 2** Interferon-gamma (IFN $\gamma$ ). (A) ROC curve determining the cut-off for the IFN $\gamma$  score. (B) Bar plots with percentage of patients with recurrence, split for low and high IFN $\gamma$  score. (C) Recurrence-free survival (RFS) for patients with low versus high IFN $\gamma$  score in the no adjuvant cohort. (D) RFS for patients with low versus high IFN $\gamma$  score in the adjuvant cohort. (E) RFS for patients with low versus high IFN $\gamma$  score for both the no adjuvant and adjuvant cohort. AUC, area under the curve; ROC, receiver operating characteristic.

CI 6 to 54) patients with high IFN $\gamma$  score (figure 2B). In the patients with an early recurrence, more patients had a low IFN $\gamma$  score (12 vs 4 with a high IFN $\gamma$  score) (online supplemental figure S2).

In both cohort 1A (median follow-up of 27 months) and cohort 2A (median follow-up of 22 months), a longer RFS for patients with high IFN $\gamma$  scores was observed, although not statistically significant in the adjuvant cohort (figure 2C,D). RFS was significantly improved by adjuvant systemic therapy compared with observation, both in patients with high and especially in patients with low IFN $\gamma$  score ( $p<0.001$ ) (figure 2E). Patients with low IFN $\gamma$  score had a median RFS of 4 months (95% CI 2 to 5) in the observation cohort, which increased to 13 months

(95% CI 2 to 25) in patients receiving adjuvant therapy. In patients with high IFN $\gamma$  scores, median RFS was 14 months (95% CI NR) versus not reached for patients receiving adjuvant therapy.

IFN $\gamma$  score was an independent prognostic parameter, as it did not correlate with other known prognostic clinical factors such as T stage, staging according to AJCC 8th edition, Breslow thickness, ulceration, and S100B and LDH levels at day of surgery (online supplemental table S4). In addition, the observed differences between the cohorts for age, mutation status, and surgical treatment were not correlated with IFN $\gamma$  score either.

### Immune cell infiltration

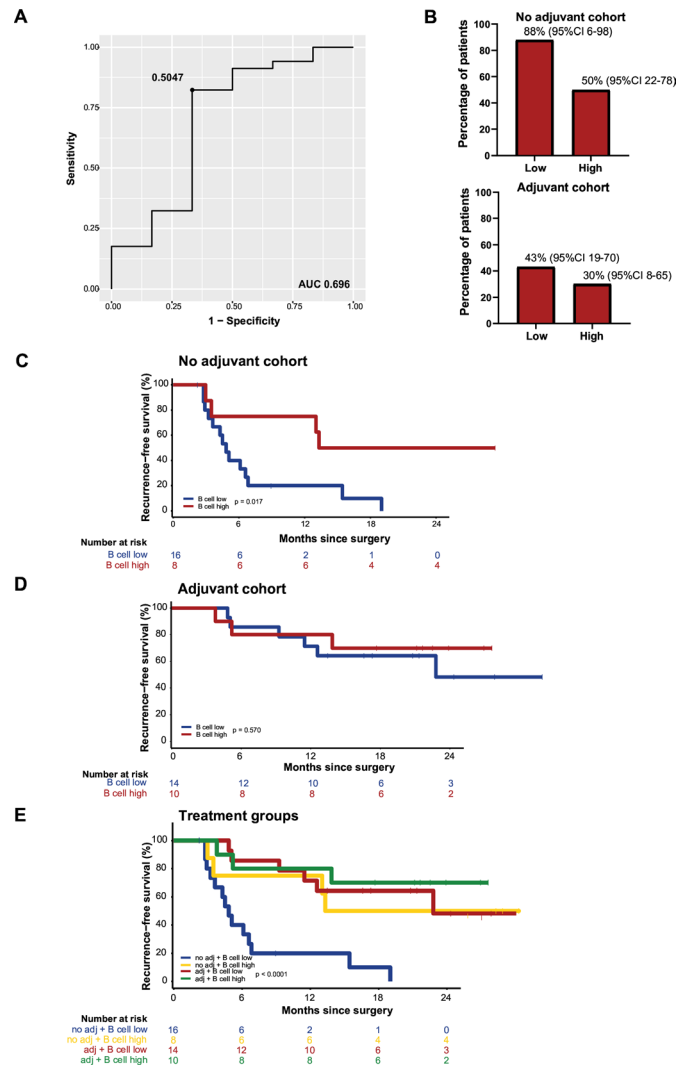
The subsets of immune cells for both the Danaher signature<sup>18</sup> and MCP counter<sup>19</sup> did not show significant differences between patients with and without a recurrence within the two treatment cohorts 1A and 2A (online supplemental figures S3 and S4). However, full cohorts 1 and 2, including the patients with an early recurrence, demonstrated a difference ( $p=0.014$ ) for the B cell subset within the Danaher signature. Therefore, we decided to expand analyses with the B cell score.

### B cell score

Similar to the IFN $\gamma$  score, we determined the optimal cut-off at 0.5047 for a low and high B cell score using a ROC curve, with an AUC of 0.696 (figure 3A). In cohort 1A, 16 patients with a low B cell score were identified, of whom 14 (88%, 95% CI 6 to 98) had a recurrence versus 4/8 patients (50%, 95% CI 22 to 78) with a high B cell score. In cohort 2A, 6/14 patients (43%, 95% CI 19 to 70) with a low B cell score had a recurrence, versus 3/10 (30%, 95% CI 8 to 65,) of the patients with a high B cell score (figure 3B). In the patients with an early recurrence, more patients had a low B cell score (10 vs 6 with a high B cell score) (online supplemental figure S5).

In cohort 1A, RFS was longer in patients with a high B cell score (figure 3C). In cohort 2A, there was no difference in RFS (figure 3D). For patients of cohort 1B, median RFS was 5 months (95% CI 4 to NR) in patients with low and 13 months (95% CI 13 to NR) in patients with high B cell score. Patients with low B cell score had a median RFS of 23 months (95% CI 13 to NR) versus not reached for patients with a high score receiving adjuvant therapy (figure 3E).

To validate if the B cell score based on RNA sequencing data corresponded with B cell presence in the tumor, an IHC CD3/CD20 staining was performed and scored on 46/48 samples used for RNA sequencing (2 samples were not scored due to lack of tumor). In cohort 1A ( $n=23$ ), the percentage CD20<sup>+</sup> cells in tumor region of patients with recurrence was lower, but higher in the stroma region. In cohort 2A ( $n=23$ ), there was a minimal difference in percentage CD20<sup>+</sup> cell in the tumor region, but this was higher for patients without recurrence in the stroma region (online supplemental figure S6A). The B cell score correlated strongly with CD20 staining, the



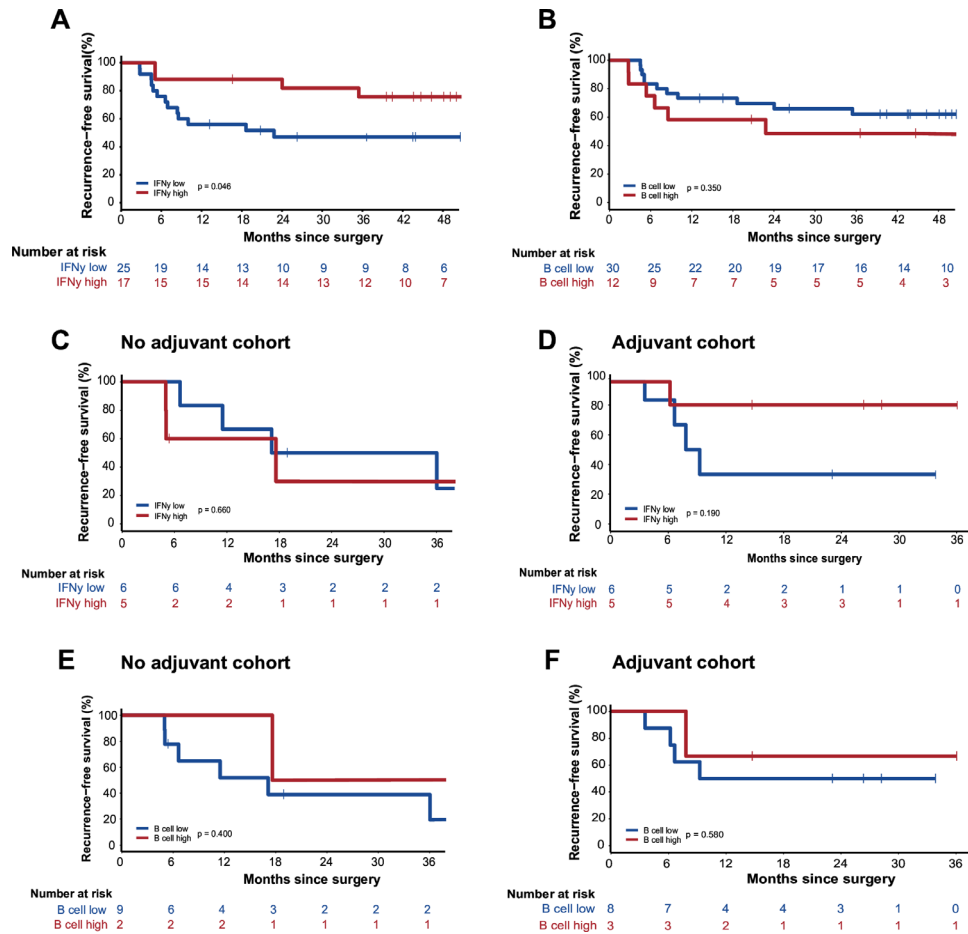
**Figure 3** B cell. (A) ROC curve determining the cut-off for the B cell score. (B) Bar plots with percentage of patients with recurrence, split for low and high B cell score. (C) Recurrence-free survival (RFS) for patients with low versus high B cell score in the no adjuvant cohort. (D) RFS for patients with low versus high B cell score in the adjuvant cohort. (E) RFS for patients with low versus high B cell score for both the no adjuvant and adjuvant cohort. AUC, area under the curve; ROC, receiver operating characteristic.

strongest correlation ( $p<0.001$ ) was seen for the CD20<sup>+</sup> cells in the tumor area (online supplemental figure S6B).

The Danaher T cell score correlated strongly ( $p<0.001$ ) with the CD3 staining as well (online supplemental figure S6C). As the B cell score and IFN $\gamma$  score were strongly correlated ( $p<0.001$ ), these are not independent markers for recurrence (online supplemental figure S6D).

### Validation cohorts

We used two validation cohorts to further test our determined cut-offs. Validation cohort MIA consisted of 44 patients treated with adjuvant therapy: 17 had a high and 27 had a low IFN $\gamma$  score, 36 had a high and 8 had a low B cell score. After a median follow-up of 46 months (95% CI 44 to 53), RFS was significantly longer for patients



**Figure 4** Validation cohorts. (A) Recurrence-free survival (RFS) of patients of validation cohort MIA with a low versus high IFN $\gamma$  score. (B) RFS of patients of validation cohort MIA with a low versus high B cell score. (C) RFS of patients of validation cohort UMCU with a low versus high IFN $\gamma$  score in the no adjuvant cohort. (D) RFS of patients of validation cohort UMCU with a low versus high IFN $\gamma$  score in the adjuvant cohort. (E) RFS of patients of validation cohort UMCU with a low versus high B cell score in the no adjuvant cohort. (F) RFS of patients of validation cohort UMCU with a low versus high B cell score in the adjuvant cohort. IFN $\gamma$ , interferon-gamma; MIA, Melanoma Institute Australia; UMCU, University Medical Centre Utrecht.

with high IFN $\gamma$  score compared with those with a low score ( $p=0.046$ ) (figure 4A), but no difference in RFS was observed according to the B cell score (figure 4B).

For validation cohort UMCU, in line with our own cohort, three patient groups were determined: patients not receiving adjuvant therapy ( $n=11$ ), patients receiving adjuvant therapy ( $n=11$ ), and patients with early recurrence ( $n=3$ ). 11 patients had high and 14 had a low IFN $\gamma$  score, 7 had high and 18 had a low B cell score. In both the no adjuvant and the adjuvant cohort, no difference in RFS was seen for the IFN $\gamma$  (figure 4C,D) or B cell score (figure 4E,F).

### PD-L1

PD-L1 staining was performed and scored on 73 tumor samples of the 98 patients in the discovery set; 28 from observation cohort 1 (cohort 1B), 28 from adjuvant treatment cohort 2 (cohort 2B), and 16 patients with an early recurrence (online supplemental figure S1). Samples of 24 patients were not scored due to either not enough tumor in the stained slide or too much pigment present

for reliable scoring. Baseline characteristics are displayed in online supplemental table S5.

For patients who received adjuvant therapy (cohort 2B), 23 (82%) had a high and 5 (18%) had a low PD-L1 score. Fewer patients with a positive PD-L1 score developed a recurrence compared with patients with a negative PD-L1 score (30%, 95% CI 14 to 53 vs 80%, 95% CI 30 to 99). In cohort 1B, this was not as distinct: 71% (95% CI 48 to 88) of 21 patients with PD-L1 positive score developed a recurrence versus 86% (95% CI 42 to 99) of 7 patients with PD-L1 negative score (online supplemental figure S7A). In cohort 1B, 21 patients (75%) had high and 7 (25%) had low PD-L1 score. In the patients with an early recurrence, more patients scored negative for PD-L1 (9 vs 7 patients with PD-L1 positive score) (online supplemental figure S7B).

After a median follow-up of 28 months (95% CI 25 to 30), median RFS did not differ for patients with PD-L1 positive score versus PD-L1 negative score in cohort 1B ( $p=0.285$ ) (online supplemental figure S7C). In cohort 2B, RFS was significantly longer ( $p=0.032$ ) for patients

with PD-L1 positive tumors (median RFS not reached), versus 13 months (95% CI 0 to 29) for PD-L1 negative tumors (online supplemental figure S7D). Both PD-L1 low and PD-L1 high cohorts had an improved RFS when receiving adjuvant therapy in the treatment cohorts (online supplemental figure S7E).

## DISCUSSION

Our study, focusing on identifying biomarkers for adjuvant treatment selection in macroscopic stage III melanoma, demonstrated IFN $\gamma$  to be of prognostic value. The lack of predictive value, however, limits its sole use in clinical decision making.

Furthermore, our study demonstrated a significant RFS benefit for adjuvant anti-PD-1 therapy. These real-world data confirm the improved RFS seen with pembrolizumab in the EORTC-1325/Keynote-054 and nivolumab in the Checkmate-238 trials.<sup>1,2</sup> Additionally, our study showed a significantly better 1-year and numerically higher (although not statistically significant) 2-year OS rate for adjuvant therapy, in slightly imbalanced cohorts with a possible selection bias due to the retrospective nature of the study. This is in line with data from the EORTC-1325/Keynote-054 and Checkmate-238 trial, which as of last analyses have not shown an OS benefit.<sup>20</sup>

As reflected in the number needed to treat five for adjuvant anti-PD-1, there is a clear need to identify patients with a higher risk of disease recurrence and a higher chance of benefit of anti-PD-1 prior to commencing adjuvant therapy, as well as those with lower risk and lower benefit. Accurate biomarkers would enable patient selection, so only patients truly in need and with high chance of treatment benefit would receive adjuvant therapy. Furthermore, patients destined to recur despite adjuvant anti-PD-1 therapy could be directed to alternative adjuvant therapies such as BRAF/MEK inhibitors or novel clinical trials, while those would never recur after surgery regardless of any adjuvant therapy can be spared treatment and the risk of toxicities altogether.

PD-L1 has previously been shown to correlate with response and outcome in advanced melanoma and non-small-cell lung cancer, therefore PD-L1 was analyzed in our cohorts.<sup>21-26</sup> PD-L1 did show a predictive value in our study, although this should be interpreted with caution as the PD-L1 low group consisted of very few patients. Our results are in line with previous data: in the EORTC-1325/Keynote-054 trial, in both the PD-L1 positive and negative subgroups, adjuvant pembrolizumab demonstrated a significantly longer RFS than placebo.<sup>1</sup> In the Checkmate-238 trial, RFS was longer for patients with PD-L1  $\geq 1\%$  treated with either adjuvant nivolumab or adjuvant ipilimumab.<sup>2</sup> Expression of PD-L1 is a controversial biomarker, as both trials demonstrated activity of ICI in PD-L1 low tumors. Moreover, the assessment of PD-L1 is heterogeneous because of different assays used, different cut-offs, and the staining is subject to interobserver variability.

We have investigated the IFN $\gamma$  signature as it is known to be predictive of response of neoadjuvant combination ICI in macroscopic stage III melanoma.<sup>13-15</sup> In the discovery cohort, IFN $\gamma$  was an independent prognostic marker in both patients who were and were not treated with adjuvant anti-PD-1 therapy, as patients with low IFN $\gamma$  score have an inferior RFS compared with patients with high IFN $\gamma$  score in both cohorts. Comparable, in the COMBI-AD trial, an IFN $\gamma$  signature score above median was prognostic for prolonged RFS in both patients treated with adjuvant dabrafenib plus trametinib and placebo.<sup>27</sup> In the Checkmate-915, patients receiving adjuvant nivolumab $\pm$ ipilimumab with an IFN $\gamma$  score above median had a longer RFS than patients with an IFN $\gamma$  score below median.<sup>28</sup> Additionally, the Checkmate-238 trial demonstrated favorable RFS and OS outcomes in IFN $\gamma$  high tumors in both patients treated with adjuvant nivolumab or ipilimumab.<sup>29</sup> In the Checkmate-76K trial of adjuvant nivolumab versus placebo in resected stage IIB/C melanoma, IFN $\gamma$  was shown to be both prognostic (IFN $\gamma$  high associated with prolonged RFS regardless of therapy) and predictive (IFN $\gamma$  high associated with prolonged RFS within the nivolumab group).<sup>30</sup> We are the first, however, to use a cut-off based on a cohort of patients with untreated stage III melanoma and to study the risk of disease recurrence, uninfluenced by treatment effects, since above mentioned trials did not use the cut-off of the placebo group in treatment groups. Our data suggest that patients with low IFN $\gamma$  score benefit from adjuvant therapy especially: without adjuvant therapy, nearly all develop a disease recurrence within 6 months of surgery, and with adjuvant treatment RFS is improved to the level of IFN $\gamma$  high patients not receiving adjuvant therapy. This was also shown in the COMBI-AD trial.<sup>27</sup> However, both the IFN $\gamma$  low and high groups in our cohort have RFS benefit from adjuvant anti-PD-1 therapy, which makes IFN $\gamma$  a prognostic and less a predictive biomarker. In the neoadjuvant OpACIN-neo trial, the IFN $\gamma$  signature did show strong predictive value as it was associated with pathologic response to neoadjuvant ipilimumab plus nivolumab, and responding patients had a significantly longer RFS than patients without pathologic response.<sup>14</sup>

As IFN $\gamma$  was a strong prognostic factor, we explored possible additional biomarkers. B cells were the strongest marker we could find of the subsets of immune cells defined by Danaher<sup>18</sup> and MCP counter,<sup>19</sup> but the IFN $\gamma$  and B cell scores were strongly correlated with one another in our cohort. Thus, adding B cell analyses to the IFN $\gamma$  score did not provide an additional predictive effect. It has been shown that B cells can facilitate an antitumor response by releasing pro-inflammatory cytokines, such as IFN $\gamma$ .<sup>31</sup> This could partly explain the correlation found.

Unfortunately, the validation cohorts lacked similar patient cohorts or patient numbers to validate our data. Validation cohort MIA did show a longer RFS in IFN $\gamma$  high patients but consisted only of patients who had received adjuvant therapy. B cell score was not a significant



prognostic factor in this cohort. Validation cohort UMCU did have similar patient groups, but the patient numbers were very low and no differences were seen in RFS for both IFN $\gamma$  and B cell scores. Additionally, this validation cohort included patients deciding not to receive adjuvant therapy, despite availability. This may be based on, for example, comorbidities or risk of recurrence, and may therefore partly explain differences in results.

To our knowledge, our study is the first to use biomarkers with a cut-off identified on a patient population naïve for systemic therapy, in both patients treated with and without adjuvant systemic therapy in the setting faced in daily clinical practice. However, limitations of our study are the retrospective design, the limited patient numbers both in the original dataset, and even more distinctly, in the validation cohorts. Due to the retrospective design, cohorts were defined by the timeframe in which patients were treated, rather than by randomization. This may account for the imbalances between the treatment cohorts as previously described and therefore limits the definitive conclusions drawn from our study.

Another important observation is the rate of early recurrences after surgery, 20% within 12 weeks, usually on the first postoperative scan prior to commencement of adjuvant systemic therapy. This is in line with the previous observations of Bloemendal *et al*<sup>16</sup> and is an important consideration when comparing data from neoadjuvant and adjuvant trials, as none of the adjuvant trials have included these early recurrences, because they were considered screen failures. All patients with such aggressive biology are included in neoadjuvant trials and this makes the results of the neoadjuvant SWOG-1801 trial that much more impressive.<sup>32</sup>

In conclusion, we demonstrated that both the IFN $\gamma$  and B cell scores have prognostic value in stage III melanoma, but we failed to find a single strong predictive biomarker of response. Our study confirmed the high rate of early recurrences in patients with high-risk stage III melanoma, who are intended to start adjuvant therapy. We confirmed the RFS benefit of adjuvant anti-PD-1 in melanoma versus observation and showed at least a numerical OS benefit when looking at all patients.

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**Contributors** JMV, SAB, ACJvA, and CB designed this study. JMV and SAB collected patient and translational data, analyzed and interpreted clinical and translational data, and wrote the first draft of the manuscript. WvH, YMS, MWJMW, and ACJvA included patients in the biobank study. JSW, WAMB, AMM, KPMS, and GVL provided patient data and material of the validation cohorts. PD performed bioinformatics analyses. AB was responsible for the RNA isolations and performance of the staining of the biopsies. RE scored the CD3/CD20 stainings, JS scored the PD-1 stainings. All authors interpreted the data, reviewed the manuscript, and approved the final version. ACJvA and CB functioned as guarantor.

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## Supplemental methods

### Early recurrence

Early recurrence was defined as recurrence <12 weeks after surgery in patients not treated with adjuvant therapy and as recurrence before planned start of adjuvant therapy in patients intended to receive adjuvant therapy. This cut-off was based on our institutional guidelines stating that adjuvant therapy should start <12 weeks after surgery. Additionally, this was supported by data from our cohort (the 75th percentile of time from surgery date to start adjuvant therapy is 11.7 weeks), and the EORTC 1325 trial with a median of 11.3 weeks to start of adjuvant therapy (personal communication, EORTC).

### RNA sequencing

Transcriptome sequencing was performed by CeGaT GmbH (Tübingen, Germany). Transcriptome libraries were generated using the KAPA RNA HyperPrep with RiboErase (HMR), according to manufacturer's instructions. The libraries were sequenced with 2x 100-bp reads on a NovaSeq 600 system according to manufacturer's protocols, with a sequence quality with a Q30 value of >93%.

Data were analyzed in CeGaT transcriptome analysis pipeline. Briefly, demultiplexing of the sequencing reads was performed with Illumina bcl2fastq (2.20). Adapters were trimmed with Skewer (version 0.2.2).<sup>33</sup> The quality of FASTQ files was analyzed with FastQC (version 0.11.5-cegat).<sup>34</sup> Plots were created using ggplot2 in R (version 3.6.1).<sup>35</sup>

FASTQ files were mapped to the human reference genome (Homo.sapiens.GRCh38.v82) using STAR(2.6.0c)<sup>36</sup> with default settings. Count data generated with HTseq-count<sup>37</sup> was analyzed with DESeq2.<sup>38</sup> For the total dataset, centering of normalized gene expression was performed by subtracting the row means and scaling by dividing the columns by the standard deviation.

### Immunohistochemistry

Immunohistochemistry (IHC) of the FFPE tumor samples was performed on a BenchMark Ultra (PD-L1 stain) or a Discovery Ultra (CD20-CD3 double stain) automated stainer (Ventana Medical Systems). Briefly, paraffin sections were cut at 3 µm, heated at 75°C for 28 minutes and deparaffinized 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 (PD-L1) or 32 minutes (CD20-CD3 double staining).

The PD-L1 clone 22C3 (DAKO) was detected using 1/40 dilution, 1 hour at RT (DAKO). 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). Both tumor and

immune cells were scored separately. Membranous coloring was used to determine PD-L1 positivity, immune cells were in addition scored as positive when cytoplasmic coloring was present. Scoring was performed on the online platform SlideScore ([www.slidescore.com](http://www.slidescore.com)).

For the double staining CD20 (yellow) followed by CD3 (purple) the CD20 was detected in the first sequence using clone L26 (1/800 dilution, 32 minutes at 37°C, Agilent/DAKO). CD20 bound antibody was visualized using Anti-Mouse NP (Ventana Medical Systems) for 12 minutes at 37°C followed by Anti-NP AP (Ventana Medical Systems) for 12 minutes at 37°C, followed by the Discovery Yellow detection kit (Ventana Medical Systems). In the second sequence of the double staining procedure CD3 was detected using clone SP7 (1:100 dilution, 32 minutes at 37°C, Thermo Scientific) and visualized using Anti-Rabbit HQ (Ventana Medical Systems) for 12 minutes at 37°C followed by Anti-HQ HRP (Ventana Medical Systems) for 12 minutes at 37°C, followed by the Discovery Purple Detection Kit (Ventana Medical Systems). Slides were counterstained with Hematoxylin and Bluing Reagent (Ventana Medical Systems). After staining, the slides were scanned with the P1000 (Sysmex).

#### **RNA sequencing of validation cohort MIA**

Haematoxylin and eosin-stained sections were assessed by a clinical pathologist and samples with >100 viable tumor cells were included for downstream analysis. The tumor location was circled for DNA/RNA microdissection. RNA was extracted using the AllPrep DNA/RNA FFPE kit (#80234, Qiagen). Quality control was assessed through RNA quantity using a Qubit 4 Fluorometer (#Q33238, Invitrogen) and quality via the TapeStation (Agilent). Samples with less than 50% RNA fragments longer than 200 nucleotides (DV200) were re-extracted using the High Pure FFPE RNA isolation kit (06650775001, Roche) before NGS library preparation.

Libraries were generated using the TruSeq RNA Exome kit (#20020189, Illumina) and quality assessed via the TapeStation system to measure the quality and quantity of libraries. Libraries with a DV200 of <50% were not sequenced. Sequencing was performed on a NovaSeq instrument (NovaSeq S2 2x100bp, Illumina) and FASTQ files generated.

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## SUPPLEMENTARY APPENDIX

Supplement to: 'Interferon-gamma signature as prognostic and predictive marker in macroscopic stage III melanoma'

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**Table S1.** Characteristics of patients who received adjuvant therapy in discovery cohort 2

	N	%
<b>Type of adjuvant therapy</b>		
Anti-PD-1 <sup>a</sup>	39	100
<b>Time to start adjuvant therapy (in weeks)</b>		
Median	9.0	
IQR	7.9-11.7	
Range	4.0-23.1 <sup>b</sup>	
<b>Treatment ongoing</b>		
No	39	100
<b>Time on adjuvant treatment</b>		
Median	10	
IQR	3-10	
Range	0-12	
<b>Reason stop end of adjuvant therapy</b>		
End of treatment	22	56
Toxicity <sup>c</sup>	6	15
Recurrence	10	26
Other <sup>d</sup>	1	3
<b>COVID break</b>		
No	24	62
Yes <sup>e</sup>	15	39

Characteristics of adjuvant therapy, reported for the 39 patients who received at least 1 dose of adjuvant therapy.

Reported as number (%), percentages may not total 100 due to rounding.

<sup>a</sup> One patient participated in a trial and was randomized between either anti-PD-1 monotherapy, or combination anti-PD-1 and anti-CTLA-4. <sup>b</sup> One patient started with targeted therapy, due to COVID, but switched to anti-PD-1 due to toxicity. <sup>c</sup> One patient was first treated with targeted therapy due to logistic challenges following the COVID-19 pandemic, then proceeded with adjuvant anti-PD-1. <sup>d</sup> One patient skipped two cycles due to COVID break, did not receive last cycle due to abscess in appendectomy scar. <sup>e</sup> COVID break: eight patients completed 10 cycles instead of 12, 1 patient received 11 cycles and 1 patient had a delayed start of treatment.

**Table S2.** Cox regression analyses

	Crude HR (95% CI)	P value	Adjusted HR (95% CI)	P value
<b>Age</b>	1.01 (0.99-1.03)	0.513		
<b>Sex</b>	1.46 (0.88-2.44)	0.143		
<b>Mutation status</b>				
BRAF	1.62 (0.39-6.75)	0.505		
NRAS	1.52 (0.35-6.65)	0.578		
cKIT	2.82 (0.25-31.37)	0.398		
No driver mutations	2.16 (0.46-10.19)	0.330		
Unknown	ref			
<b>Type of surgery</b>				
LND	<b>0.31 (0.21-0.81)</b>	<b>0.010</b>	0.69 (0.34-1.43)	0.325
ITM	0.61 (0.28-1.33)	0.212	0.98 (0.44-2.23)	0.975
LND + ITM	0.59 (0.23-1.50)	0.266	0.81 (0.31-2.09)	0.661
ILP ± LND	ref		ref	
<b>S100b</b>	<b>2.39 (1.23-4.63)</b>	<b>0.010</b>	<b>2.64 (1.29-5.38)</b>	<b>0.008</b>
<b>LDH</b>	1.85 (0.66-5.19)	0.243		
<b>Adjuvant therapy</b>	<b>0.25 (0.14-0.45)</b>	<b>&lt;0.001</b>	<b>0.25 (0.13-0.48)</b>	<b>&lt;0.001</b>

HR: hazard ratio; CI: confidence interval; LND: lymph node dissection; ITM: in-transit metastasis; ILP: isolated limb perfusion.



**Table S3.** Baseline characteristics of patients of whom RNA sequencing data is available

		Observation cohort 1A		Adjuvant treatment cohort 2A	
		Recurrence	No recurrence	Recurrence	No recurrence
		N=18	N=6	N=9	N=15
<b>Age</b>					
	Median	71	73	55	61
	Range	55-77	57-82	45-63	54-73
<b>Sex</b>					
	Male	11 (61)	3 (50)	4 (44)	7 (47)
	Female	7 (39)	3 (50)	5 (56)	8 (53)
<b>Site primary</b>					
	Extremities	13 (72)	3 (50)	4 (44)	8 (53)
	Trunk	4 (22)	3 (50)	5 (56)	5 (33)
	Head&neck	0	0	0	1 (7)
	Acral	0	0	0	1 (7)
	Mucosal	1 (6)	0	0	0
	MUP	0	0	0	0
<b>Breslow thickness</b>					
	≤1.0mm	1 (6)	0	0	3 (20)
	1.01-2.0	4 (22)	1 (17)	2 (22)	2 (13)
	2.01-4.0	6 (33)	4 (80)	5 (56)	4 (27)
	>4.0	5 (28)	0	1 (11)	6 (40)
	Unknown	2 (11)	1 (17)	1 (11)	0
<b>Ulceration</b>					
	No	6 (33)	3 (50)	6 (67)	11 (73)
	Yes	7 (39)	2 (33)	1 (11)	3 (20)
	Unknown	5 (2)	1 (17)	2 (22)	1 (7)
<b>Stage (AJCC 8<sup>th</sup> edition)</b>					
	IIIB	2 (11)	1 (17)	2 (22)	4 (27)
	IIIC	16 (89)	4 (67)	6 (67)	10 (67)
	IIID	0	0	0	1 (7)
	Unknown	0	1 (17)	1 (11)	0
<b>Mutation status</b>					
	BRAF	9 (50)	4 (67)	6 (67)	9 (60)
	NRAS	6 (33)	1 (17)	1 (11)	6 (40)
	cKIT	1 (6)	0	0	0
	No driver mutations	1 (6)	0	2 (22)	0
	Unknown	1 (6)	1 (17)	0	0
<b>Type surgery</b>					
	LND	8 (44)	2 (33)	7 (78)	11 (73)
	ITM	2 (11)	2 (33)	0	3 (20)
	LND + ITM	2 (11)	1 (17)	2 (22)	1 (7)
	ILP ± LND	6 (33)	1 (17)	0	0
<b>Type sample</b>					
	LND	12 (67)	3 (50)	9 (100)	12 (80)
	ITM	6 (33)	3 (50)	0	3 (20)
<b>S100b</b>					
	≤ULN	14 (78)	6 (100)	7 (78)	14 (93)
	>ULN	2 (11)	0	1 (11)	1 (7)
	Unknown	2 (11)	0	1 (11)	0
<b>LDH</b>					
	≤ULN	16 (89)	4 (67)	8 (89)	15 (100)
	>ULN	1 (6)	2 (33)	0	0
	Unknown	1 (6)	0	1 (14)	0

Baseline characteristics of patients of whom RNA sequencing data is available, reported per cohort for patient with and without recurrence.

Reported as number (%), percentages may not total 100 due to rounding.

IQR: interquartile range; MUP: melanoma of unknown primary; LND: lymph node dissection; ITM: in-transit metastasis; ILP: isolated limb perfusion; ULN: upper limit of normal; LDH: lactate dehydrogenase.

**Table S4.** Correlation IFN $\gamma$  with clinical parameters in all patients of whom RNA sequencing data is available

	<b>Pearson coefficient</b>	<b>P value</b>	<b>Spearman coefficient</b>	<b>P value</b>
<b>T stage</b>	0.140	0.295	0.154	0.248
<b>Stage (AJCC 8<sup>th</sup> edition)</b>	0.021	0.871	0.095	0.455
<b>Breslow thickness</b>	-0.182	0.151	-0.063	0.621
<b>Ulceration</b>	-0.042	0.744	-0.020	0.877
<b>S100B surgery</b>	0.169	0.181	-0.031	0.811
<b>S100B adjuvant</b>	0.223	0.307	0.234	0.282
<b>LDH surgery</b>	-0.020	0.875	-0.073	0.568
<b>LDH adjuvant</b>	0.141	0.522	0.176	0.423
<b>Age</b>	-0.021	0.868	-0.009	0.946
<b>Mutation status</b>	-0.035	0.783	0.003	0.979
<b>Type of surgery</b>	-0.111	0.382	-0.125	0.326

Correlation of interferon-gamma (IFN $\gamma$ ) with known prognostic clinical parameters.

LDH: lactate dehydrogenase.

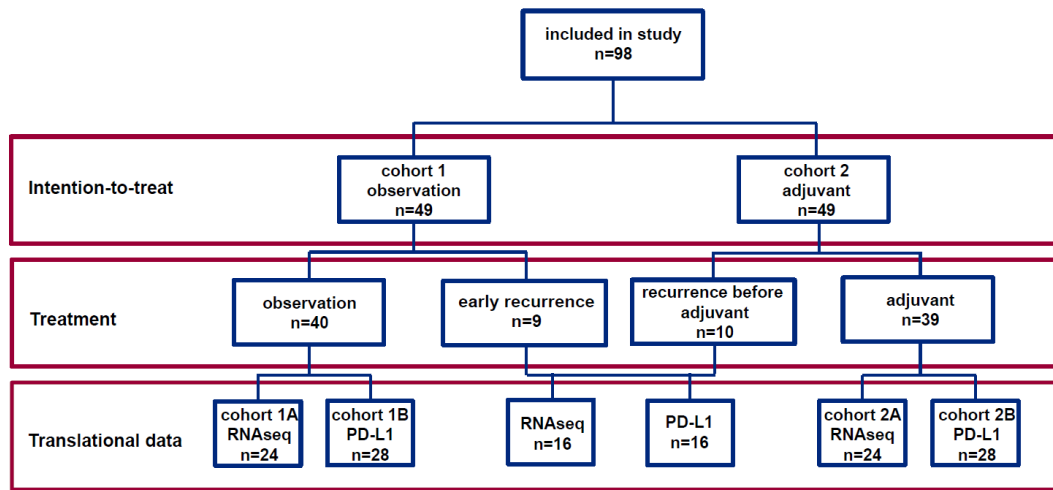
**Table S5.** Baseline characteristics of patients of whom PD-L1 expression data is available

		Observation cohort 1B		Adjuvant treatment cohort 2B	
		Recurrence	No recurrence	Recurrence	No recurrence
		N=21	N=7	N=11	N=17
<b>Age</b>					
	Median	67	75	59	61
	Range	51-74	73-81	55-70	54-71
<b>Sex</b>					
	Male	9 (43)	4 (57)	5 (46)	7 (41)
	Female	12 (57)	3 (43)	6 (55)	10 (59)
<b>Site primary</b>					
	Extremities	14 (67)	3 (43)	4 (36)	7 (41)
	Trunk	5 (24)	4 (57)	7 (64)	9 (53)
	Head&neck	0	0	0	0
	Acral	0	0	0	1 (6)
	Mucosal	1 (5)	0	0	0
	MUP	1 (5)	0	0	0
<b>Breslow thickness</b>					
	≤1.0mm	1 (5)	0	0	3 (18)
	1.01-2.0	5 (24)	2 (29)	1 (9)	4 (24)
	2.01-4.0	10 (48)	3 (43)	5 (6)	4 (24)
	>4.0	3 (14)	1 (14)	3 (27)	6 (35)
	Unknown	2 (10)	1 (14)	2 (18)	0
<b>Ulceration</b>					
	No	7 (33)	2 (29)	5 (45)	10 (59)
	Yes	7 (33)	3 (43)	3 (27)	6 (35)
	Unknown	7 (33)	2 (29)	3 (27)	1 (6)
<b>Stage (AJCC 8<sup>th</sup> edition)</b>					
	IIIB	1 (5)	0	2 (18)	4 (24)
	IIIC	20 (95)	6 (86)	8 (73)	12 (71)
	IIID	0	0	0	1 (6)
	Unknown	0	1 (14)	1 (9)	0
<b>Mutation status</b>					
	BRAF	12 (57)	3 (43)	8 (73)	11 (65)
	NRAS	5 (24)	0	2 (18)	6 (35)
	cKIT	1 (5)	0	0	0
	No driver mutations	2 (10)	2 (29)	1 (9)	0
	Unknown	1 (5)	2 (29)	0	0
<b>Type surgery</b>					
	LND	8 (38)	2 (29)	8 (73)	12 (71)
	ITM	6 (29)	2 (29)	1 (9)	3 (18)
	LND + ITM	1 (5)	2 (29)	2 (18)	1 (6)
	ILP ± LND	6 (29)	1 (4)	0	1 (6)
<b>Type sample</b>					
	LND	12 (57)	4 (57)	10 (1)	14 (82)
	ITM	9 (43)	3 (43)	1 (9)	3 (18)
<b>S100b</b>					
	≤ULN	15 (71)	6 (86)	9 (82)	16 (94)
	>ULN	4 (19)	0	2 (18)	1 (6)
	Unknown	2 (10)	1 (14)	0	0
<b>LDH</b>					
	≤ULN	15 (71)	4 (57)	10 (91)	17 (100)
	>ULN	3 (14)	2 (29)	0	0
	Unknown	3 (14)	1 (14)	1 (9)	0

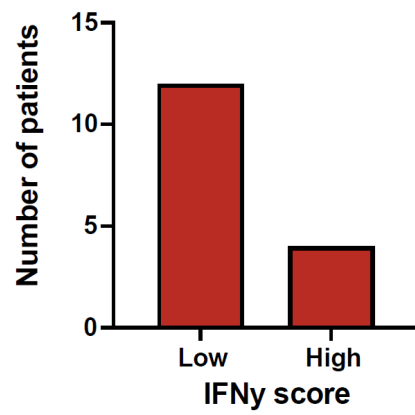
Baseline characteristics of patients of whom PD-L1 expression data is available, reported per cohort for patients with and without recurrence.

Reported as number (%), percentages may not total 100 due to rounding.

IQR: interquartile range; MUP: melanoma of unknown primary; LND: lymph node dissection; ITM: in-transit metastasis; ILP: isolated limb perfusion; ULN: upper limit of normal; LDH: lactate dehydrogenase.

**Figure S1.** Flow diagram of patient selection

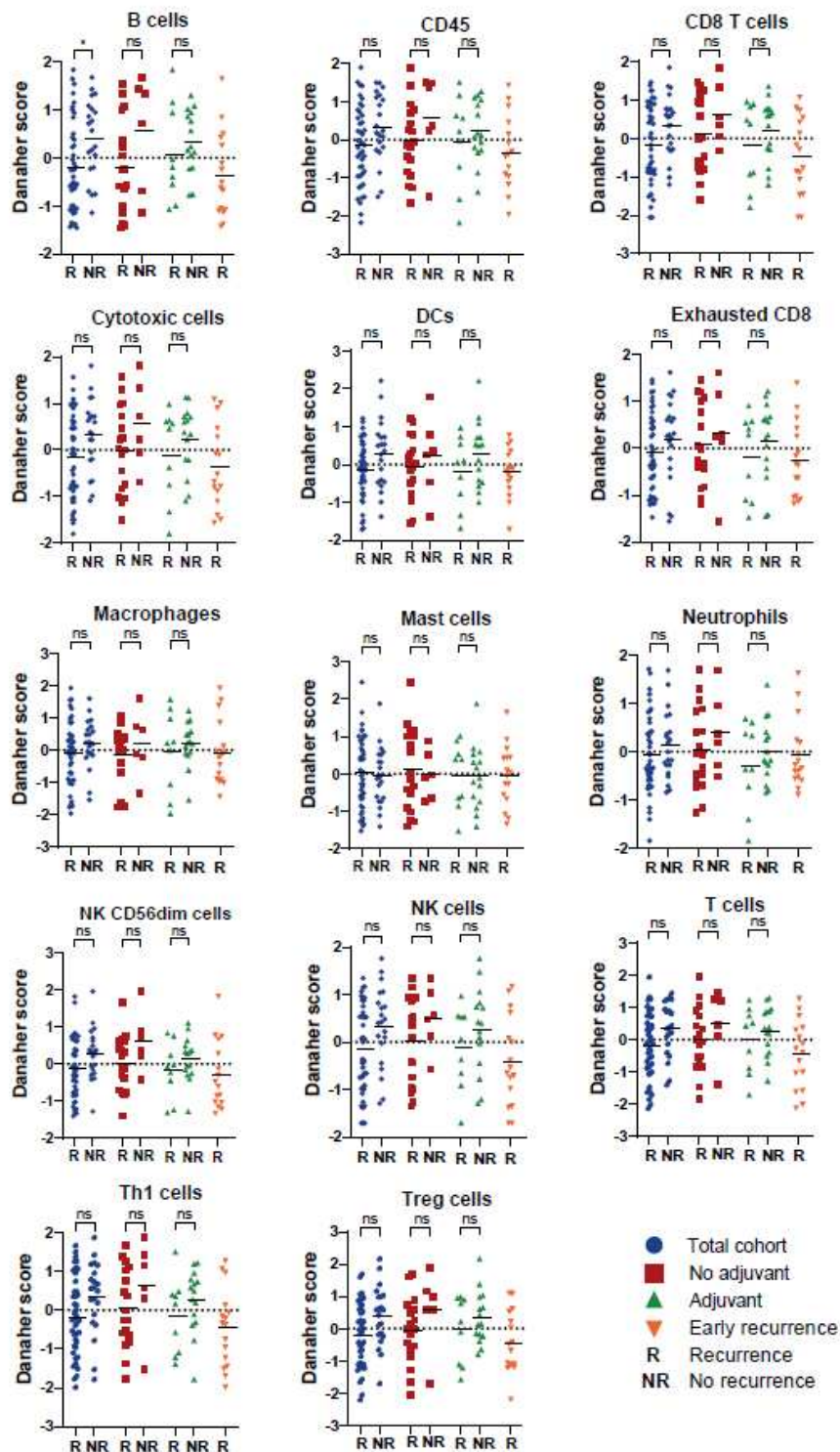
RNAseq: RNA sequencing.

**Figure S2.** Interferon-gamma score

Bar plots with number of patients with an early recurrence with low and high IFN $\gamma$  score.

IFN $\gamma$ : interferon-gamma.

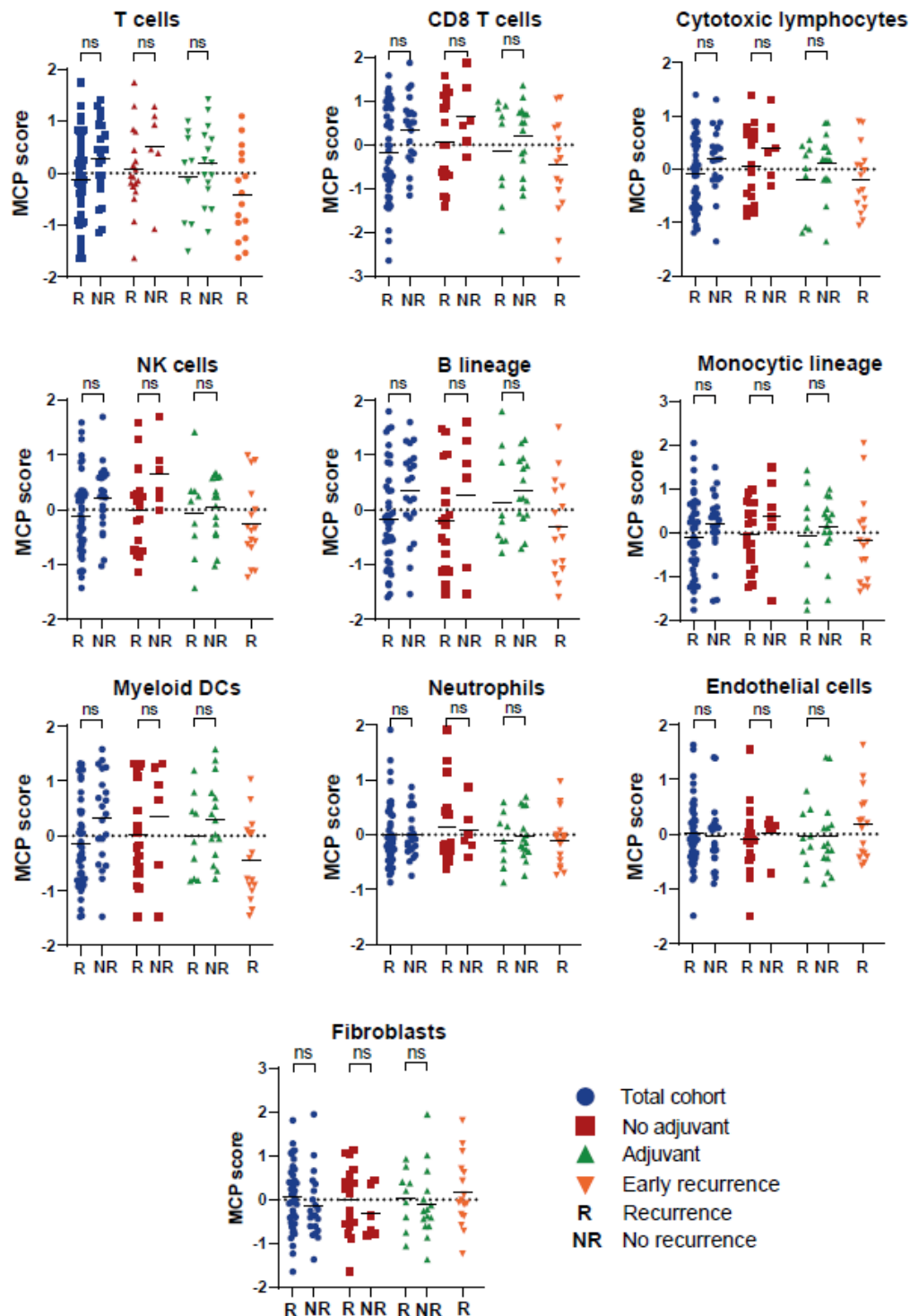
Figure S3. Overview of immune subsets of Danaher



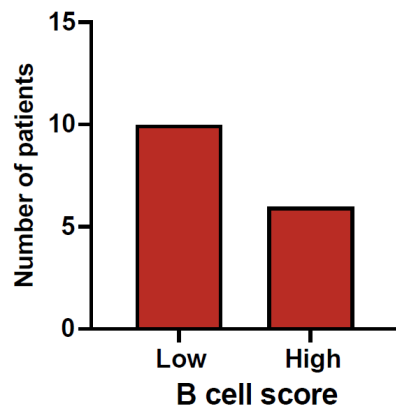
\*: significant difference ( $P < 0.005$ ); ns: not significant difference. Immune subsets as defined by Danaher et al. Gene expression markers of tumor infiltrating leukocytes. *J Immunother Cancer* 2017.



Figure S4. Overview of immune subsets of the MCP counter

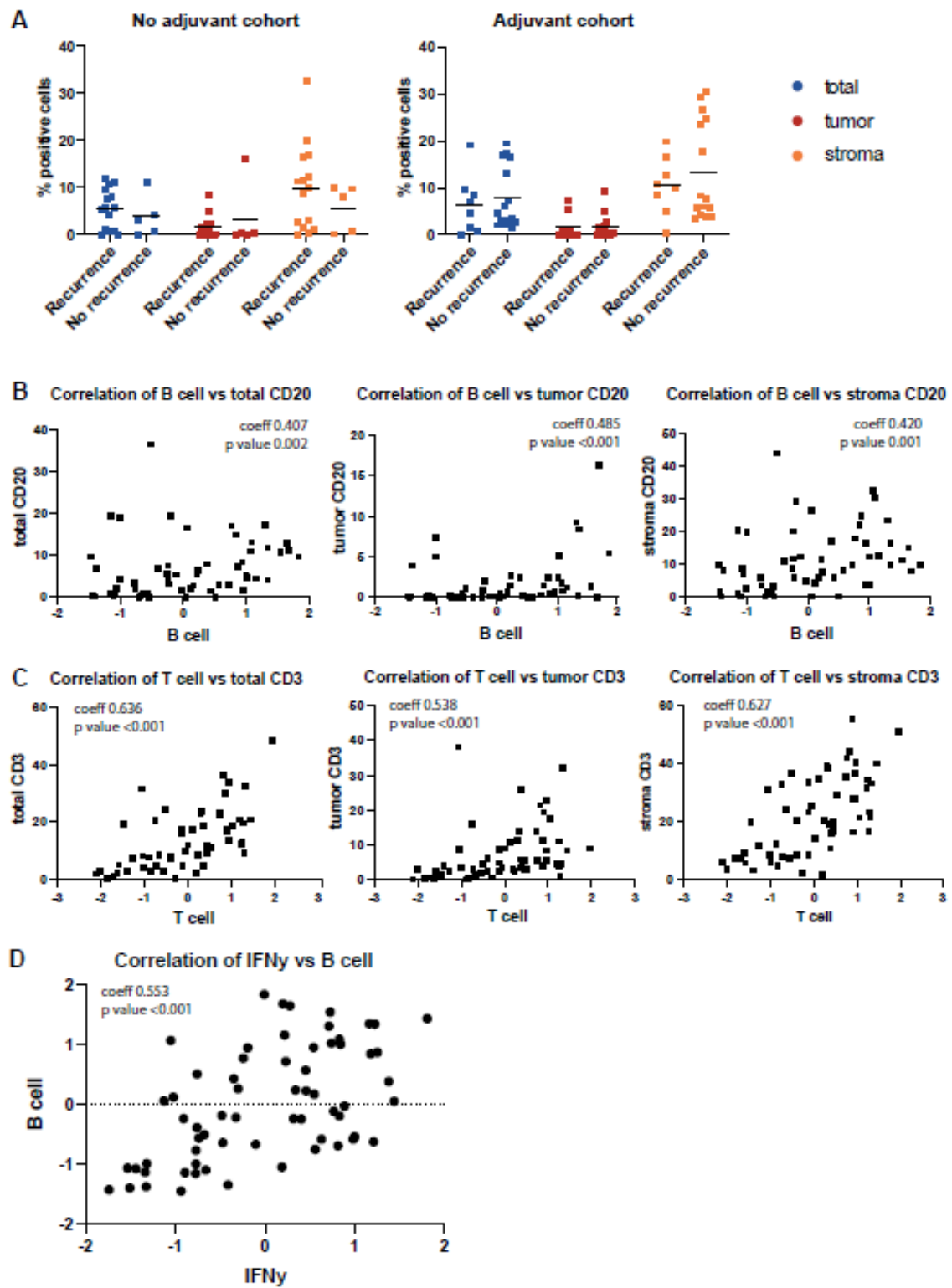


\*: significant difference ( $P < 0.005$ ); ns: not significant difference. Immune subsets as defined by Becht et al. Estimating the population abundance of tissue-infiltrating immune and stromal cell populations using gene expression. *Genome Biol* 2016.

**Figure S5. B cell score**

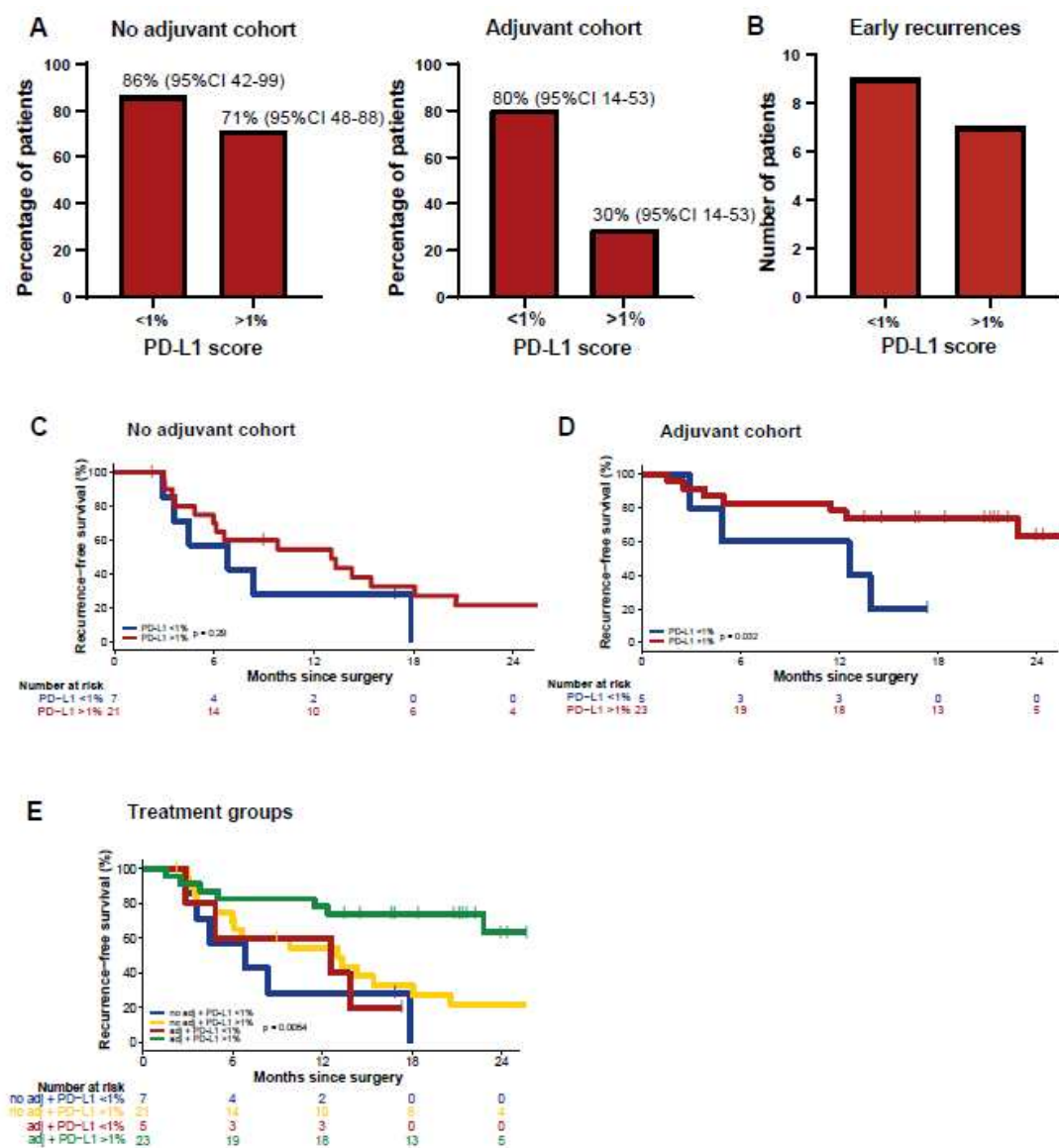
Bar plots with number of patients with an early recurrence with low and high B cell score.

Figure S6. CD20 and CD3 staining



- A. Dot plots percentage CD20<sup>+</sup> cells of patients with and without recurrence reported for total sample, tumor and stroma area.
  - B. Correlation of B cell score based on RNAseq compared to percentage CD20<sup>+</sup> cells for the total samples, tumor and stroma area.
  - C. Correlation of T cell score based on RNAseq compared to percentage CD3<sup>+</sup> cells for the total samples, tumor and stroma area.
  - D. Correlation of B cell score compared to IFN $\gamma$  score.
- RNAseq: RNA sequencing.

Figure S7. PD-L1 staining



- A. Bar plots with percentage of patients with recurrence, split for a low and high PD-L1 score.
- B. Bar plots with number of patients with an early recurrence with low and high PD-L1 score.
- C. Recurrence-free survival for patients with a low versus a high PD-L1 score in the no adjuvant cohort.
- D. Recurrence-free survival for patients with a low versus a high PD-L1 score in the adjuvant cohort.
- E. Recurrence-free survival for patients with a low versus a high PD-L1 score for both the no adjuvant and adjuvant cohort.