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ORIGINAL ARTICLE

Pathological response and tumour bed histopathological features correlate with survival following neoadjuvant immunotherapy in stage III melanoma

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Background: Guidelines for pathological evaluation of neoadjuvant specimens and pathological response categories have been developed by the International Neoadjuvant Melanoma Consortium (INMC). As part of the Optimal Neo-adjuvant Combination Scheme of Ipilimumab and Nivolumab (OpACIN-neo) clinical trial of neoadjuvant combination anti-programmed cell death protein 1/anti-cytotoxic T-lymphocyte-associated protein 4 immunotherapy for stage III melanoma, we sought to determine interobserver reproducibility of INMC histopathological assessment principles, identify specific tumour bed histopathological features of immunotherapeutic response that correlated with recurrence and relapse-free survival (RFS) and evaluate proposed INMC pathological response categories for predicting recurrence and RFS.

Patients and methods: Clinicopathological characteristics of lymph node dissection specimens of 83 patients enrolled in the OpACIN-neo clinical trial were evaluated. Two methods of assessing histological features of immunotherapeutic response were evaluated: the previously described immune-related pathologic response (irPR) score and our novel immunotherapeutic response score (ITRS). For a subset of cases ($n = 29$), cellular composition of the tumour bed was analysed by flow cytometry.

Results: There was strong interobserver reproducibility in assessment of pathological response ($\kappa = 0.879$) and percentage residual viable melanoma (intraclass correlation coefficient = 0.965). The immunotherapeutic response subtype with high fibrosis had the strongest association with lack of recurrence ($P = 0.008$) and prolonged RFS ($P = 0.019$). Amongst patients with criteria for pathological non-response (pNR, >50% viable tumour), all who recurred had ≥70% viable melanoma. Higher ITRS and irPR scores correlated with lack of recurrence in the entire cohort ($P = 0.002$ and $P \leq 0.0001$). The number of B lymphocytes was significantly increased in patients with a high fibrosis subtype of treatment response ($P = 0.046$).

Conclusions: There is strong reproducibility for assessment of pathological response using INMC criteria. Immunotherapeutic response of fibrosis subtype correlated with improved RFS, and may represent a biomarker. Potential B-cell contribution to fibrosis development warrants further study. Reclassification of pNR to a threshold of ≥70% viable melanoma and incorporating additional criteria of <10% fibrosis subtype of response may identify those at highest risk of recurrence, but requires validation.

Key words: metastatic melanoma, neoadjuvant, immunotherapy, pathological response

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INTRODUCTION

Patients with resectable, clinically detectable American Joint Committee on Cancer stage III melanoma historically had poor clinical outcomes.[1,2] Adjuvant treatment with immune checkpoint blockade or BRAF/MEK inhibitor combination targeted therapy is now regarded as standard of care following surgical resection for these high-risk melanoma patients, with an improvement in relapse-free survival (RFS) compared to placebo.[3,4] Importantly, despite adjuvant therapy and its improvements over placebo, nearly 50% of stage ≥IIIB melanoma recur.[5,6]

Pre-clinical and human translational research has suggested increased therapeutic efficacy of neoadjuvant over adjuvant immunotherapy in the treatment of metastatic malignancies in mice and humans.[7,8] In the management of a number of malignancies, including breast cancer, neoadjuvant systemic therapy is already standard practice.[9,10] Neoadjuvant therapy offers a number of advantages over adjuvant therapy. Firstly, it allows identification of both the extent and patterns of response, which could provide prognostic information and guide further management personalised to the patients' tumour response. Secondly, it provides interval access to pre- and post-systemic treatment tumour tissue, which can be analysed to derive mechanistic information on treatment response and resistance. Thirdly, it potentially downstages tumours and improves surgical resectability.[11] Therefore, the use of combination ipilimumab (an antibody against cytotoxic T-lymphocyte-associated protein 4) and nivolumab [an antibody against programmed cell death protein 1 (PD-1)] as well as nivolumab and pembrolizumab monotherapy have been interrogated in the neoadjuvant setting in a number of early-phase trials of patients with clinically detectable, RECIST-measurable, but resectable stage III melanoma.[8,12-15] In a pooled analysis of these studies,[16] the pathological complete response (pCR) rate was 37% (44% combination, 21% monotherapy). pCR, as well as any pathological response which includes pCR, near pathological complete response (near-PCR) (≤10% of viable tumour within the tumour bed) and partial pathological response (pPR) (≤50% and >10% of the treated tumour bed occupied by viable tumour cells), strongly correlated with improved RFS.[16] The association between pCR and improved long-term outcome is consistent with studies using BRAF/MEK inhibitor targeted therapy in melanoma and chemo(radio)therapy in other malignancies.[17-21]

However, patients who have International Neoadjuvant Melanoma Consortium (INMC)-defined pathological non-response (pNR ≥ 50% viable tumour cells[11,22]) are most at risk of recurrence (37% RFS at 2 years for immunotherapy).[16] Therefore, histopathological analysis of the surgical resection specimens, including patients with a pNR, could identify particular histological features and immunotherapeutic response patterns that might distinguish patients with a favourable outcome from patients most at risk of recurrence who may benefit from additional adjuvant therapy.

In all patients with clinically detectable stage III metastatic melanoma treated in the phase II Optimal Neoadjuvant Combination Scheme of Ipilimumab and Nivolumab (OpACIN-neo) trial (NCT02977052),[15] we analysed histopathological features of immunotherapeutic response, including our novel immunotherapeutic response score (ITRS) and the immune-related pathologic response (irPR) score,[23] and correlated the features with pathological response and clinical outcomes. The interobserver reproducibility of the pathological response and the patterns of immunotherapeutic response, as assessed by a range of pathologists, were also evaluated. Correlations between the determination of the extent of the pathological and radiological responses were also assessed. In order to gain additional insights into the biological underpinnings of features predicting or associated with a favourable response, we also correlated flow cytometric analysis of viable cell dissociates with pathological response and histopathological patterns of immunotherapeutic response.

PATIENTS AND METHODS

Patients

Eighty-six patients with RECIST-measurable and resectable stage III melanoma were enrolled into the phase II OpACIN-neo clinical trial[15] at three participating centres: Melanoma Institute Australia (MIA) ($n = 38$), The Netherlands Cancer Institute (NKI) ($n = 46$) and Karolinska University Hospital (KS) ($n = 2$). Patients were randomised to 6 weeks of neoadjuvant therapy in one of three different dosing schedules of ipilimumab and nivolumab, as previously described.[15] After 6 weeks of neoadjuvant therapy, all patients underwent therapeutic lymph node dissection (TLND) and the specimens were analysed, as outlined below.

Pathological assessment

Histopathological examination was carried out using routine haematoxylin and eosin (H&E) slides and scanned digital images. All slides from all cases were scored independently by specialised melanoma pathologists from MIA (RVR, CA and RAS) and NKI (BAvdW). The initial evaluation of all cases was carried out locally at either MIA, NKI or KS utilizing H&E glass slides. All cases were also independently reviewed at either MIA or NKI utilizing scanned whole images of H&E slides (accessed via cloud-based image storage) with pathologists blinded to the initial local assessment. Following independent evaluation of all cases, where there was a discrepancy in the pathological response category, these specimens were reanalysed and discussed by both pathology teams together until consensus was reached. The pathological response of each case was assessed by the four-tier system outlined by the INMC.[22] As prescribed in this system, the percentage of viable tumour is calculated as the percentage of the area of the total tumour bed occupied by tumour. In post-treatment lymph node resections, the tumour bed is defined as the area within the specimen

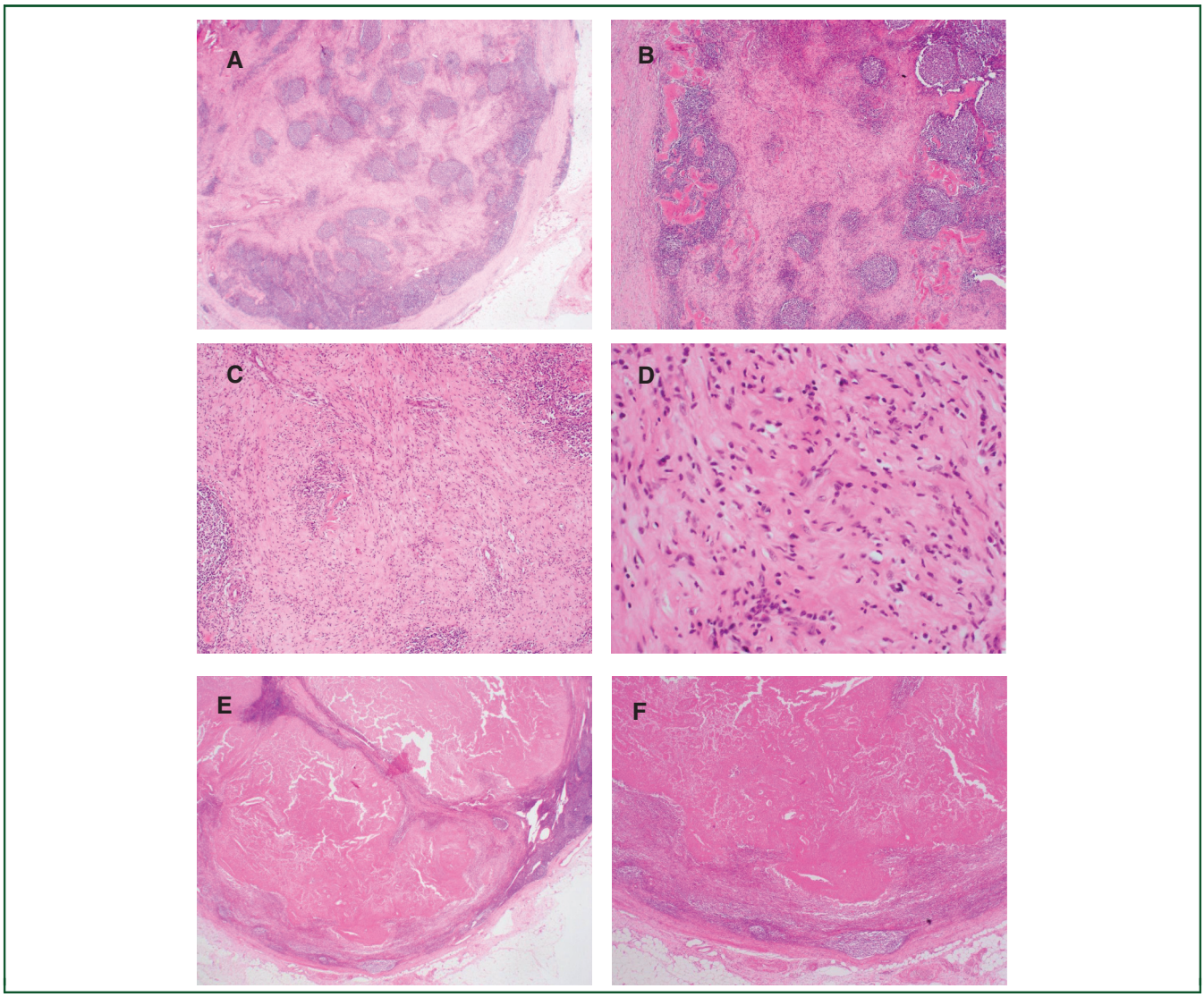

Figure 1. (A-L) Haematoxylin and eosin (H&E) slides showing different subtypes of immunotherapeutic pathological complete response (pCR) following neoadjuvant immunotherapy for stage III melanoma.
No viable tumour is present within any of the illustrated lymph nodes. (A-D) Fibrosis/fibroinflammatory stroma replacing lymph node parenchyma (A: ×20, B: ×40, C: ×100, D: ×400). (E-H) Necrosis replacing the lymph node (E: ×20, F: ×40, G: ×100, H: ×400). (I-L) Pigment-laden macrophages and necrosis replacing the lymph node (I: ×20, J: ×40, K: ×100, L: ×400).

occupied by viable tumour cells and/or the area of regressed tumour [as evidenced by aggregates and sheets of pigmented macrophages, fibrosis/fibroinflammatory stroma (FIS) and necrosis][11,22] (Figure 1 and Supplementary Figure S1, available at https://doi.org/10.1016/j.annonc.2021.03.006). pCR was defined as the complete absence of viable tumour within the treated tumour bed area, near-pCR as ≤10% of viable tumour within the tumour bed, pPR as ≤50% and >10% of the treated tumour bed occupied by viable tumour cells and pNR as >50% of the tumour bed occupied by viable tumour cells.[22] The relative proportion of each of the different immunotherapeutic response patterns (aggregates and sheets of pigmented macrophages/tumoural melanosis, fibrosis/FIS and necrosis) within the tumour bed was also calculated as a percentage of the total tumour bed area. Fibrosis/FIS was also subclassified into hyalinised and proliferative types, as previously defined.[23] Tumour-infiltrating lymphocytes (TILs) within any viable tumour were scored by density (0—absent, 1—mild, 2—moderate, 3—marked), as previously described.[24] The slides of 81 of the 83 cases were examined for other histopathological features of irPR[23,25,26] including: lymphoid aggregates (>50 lymphocytes clustered), tertiary lymphoid structures (TLSs) (lymphoid aggregate with a germinal centre and high endothelial venules), neovascularisation, granulomas, cholesterol clefts, multinucleated giant cells, neutrophils (0—absent, 1—focal, 2—diffuse), plasma cells (0—absent, 1—scattered, 2—focal clusters, 3—widespread clusters) and distribution of treatment response (peripheral, central, mixed). The recently described irPR score was also calculated.[23]

Immunotherapeutic response score

Since a number of features of the irPR score are either co-dependent variables or are features of the pathological

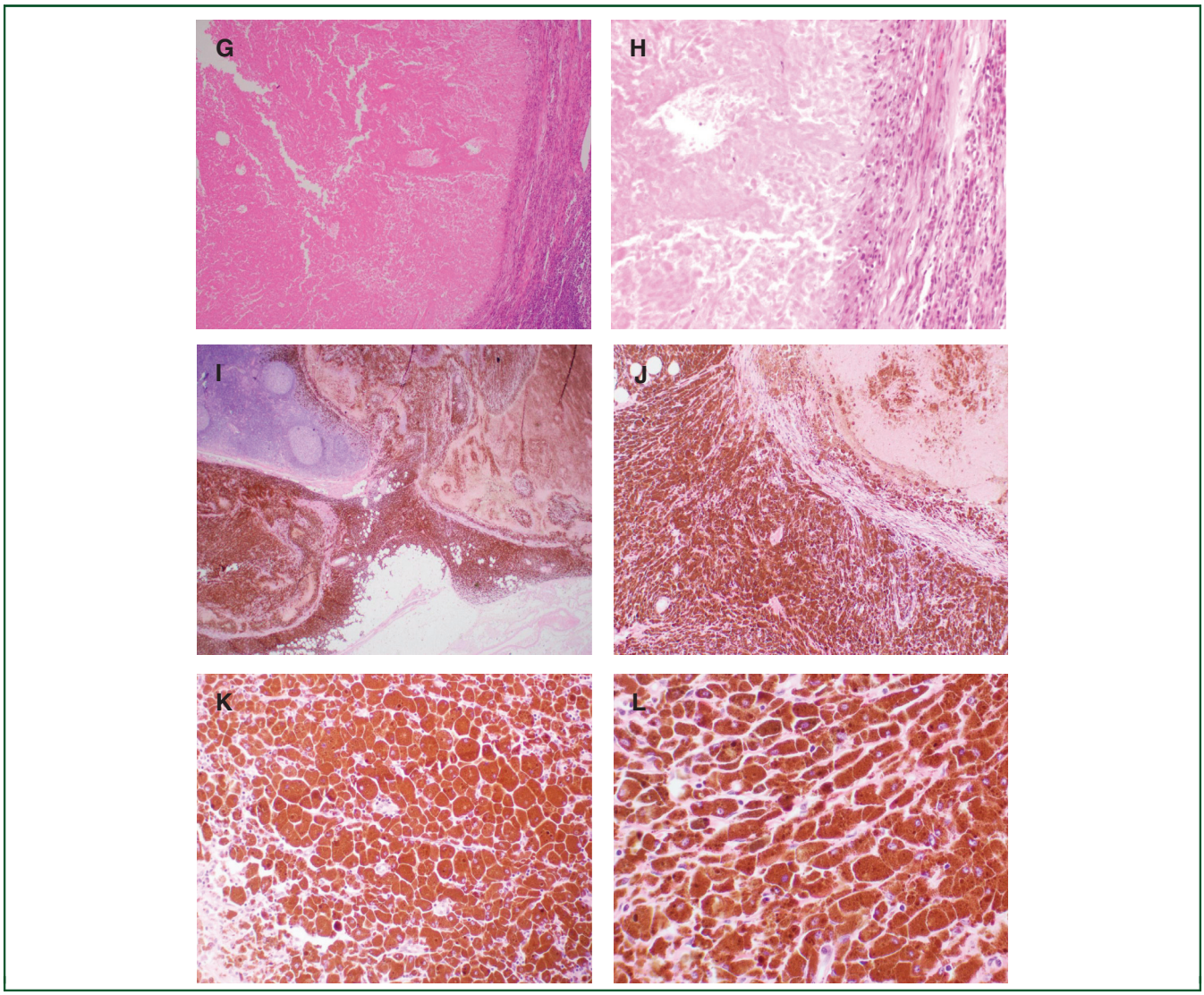

Figure 1. Continued.

pattern of response, a novel ITRS excluding co-dependent variables was also calculated. This score was derived by assigning one point for every histological feature of immune response present [peripheral distribution of treatment response, neovascularisation, lymphoid aggregates, TLSs (lymphoid aggregates with germinal centres and high endothelial venules), plasma cells, granulomas, cholesterol clefts, multinucleated giant cells, neutrophils], which do not form part of the pathological response category or the immunotherapeutic response subtype (Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2021.03.006).

Radiological assessment

Radiological response assessment of only the largest lymph node on computed tomography (CT) scan was carried out using three methods: RECIST (version 1.1),[27] the immune-related response criteria[28] and the measurement of long axis of the largest lymph node. CT scans were carried out at baseline before start of drug therapy, at week 6 before TLND, 12 weekly thereafter to year 3, and then 6 monthly as per standard at each institution. Further details of radiological assessment are presented in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2021.03.006.

Outcomes

The outcomes included the pathological response at week 6 (pCR, near-pCR, pPR, pNR), the radiological response at week 6 (pre-surgery) using RECIST 1.1,[27] the immune-related response criteria[28] as described above, the measurement of the long axis of the largest lymph node and RFS. RFS was calculated from the time of surgery to first recurrence or death.

The details of statistical analyses and flow cytometry are presented in Supplementary Methods, available at https://doi.org/10.1016/j.annonc.2021.03.006.

RESULTS

Patient population and treatment

Eighty-six patients with resectable but RECIST 1.1-measureable stage III melanoma were randomly assigned to one of the three dosing schedules of neoadjuvant ipilimumab and nivolumab, as previously described.[15] The baseline demographics of patients, tumour characteristics and radiological and pathological responses have been previously reported.[15] Of the 86 patients, 3 patients (3%) were not included in the pathological analysis as they did not have surgery, 1 due to a severe immune-related adverse event and 2 patients had distant metastasis by week 6.

Interobserver reproducibility of assessment of pathological response

The pathological response assessment for all specimens was independently carried out by experienced histopathologists at MIA and NKI. The number of slides examined per specimen at both centres ranged from 1 to 32 slides (mean = 6). The interobserver reliability of both the pathological response category ($\kappa = 0.879$) and percentage pathological response (intraclass correlation coefficient = 0.965) showed strong agreement. In seven specimens, there was discordance in the pathological response category (Table 1). A consensus pathological response category was adjudicated. In two of these cases, there was an underestimation of treatment effect by the pathologist reviewing scanned images resulting in two cases of pPR (confirmed on review) classified initially at one centre as pNR. A similar underestimation of treatment effect in the scanned images of a further case resulted in classification as pPR at one centre instead of near-pCR. In the remaining four cases, interpretation of very small foci of viable melanoma (<1% of tumour bed) caused initial misclassification between near-pCR and pCR. In five of the seven discordant cases, the over- or underestimation of viable melanoma was made by the pathologist interpreting scanned images.

Pathological response and correlation with radiological response and outcome

There was a highly significant correlation between the longest diameter of the target lesion on the week 6 CT scan and the largest dimension of the tumour bed at surgical excision as assessed by the pathologist ($R^2 = 0.87$) (Supplementary Figure S2A, available at https://doi.org/10.1016/j.annonc.2021.03.006). Pathological (pCR, near-pCR and pPR) and radiological (CR and PR) response categories, as defined by RECIST 1.1, following 6 weeks of neoadjuvant therapy, (Supplementary Table S1, available at https://doi.org/10.1016/j.annonc.2021.03.006) correlated on Fisher's exact test ($P < 0.001$). Further results are provided in Supplementary Materials, available at https://doi.org/10.1016/j.annonc.2021.03.006.

The follow-up period ranged from 6 to 30 months (median 17.7 months) and during this period, 12 of 83 patients recurred. Eleven of the 12 patients with recurrent disease had a pNR, whilst one patient had a pCR. RFS significantly correlated with pathological response ($P < 0.001$, Figure 2A). All pNR patients who subsequently recurred had ≥70% viable melanoma within their tumour bed (Figure 3) and had <30% reduction in radiological assessment of the lesions when using RECIST 1.1 (Supplementary Figure S2B, available at https://doi.org/10.1016/j.annonc.2021.03.006). The sensitivity and specificity of different thresholds of viable melanoma in predicting recurrence are shown in Supplementary Table S2, available at https://doi.org/10.1016/j.annonc.2021.03.006.

Table 1. Interobserver reproducibility in classification of pathological response categories between different sites in the OpACIN-neo clinical trial for clinically detectable stage III metastatic melanoma ($n = 83$)

MIA pathologists reported	NKI pathologist reported			
	pCR	Near-pCR	pPR	pNR
pCR	36	4	0	0
Near-pCR	0	11	0	0
pPR	0	1	10	0
pNR	0	0	2	19

MIA, Melanoma Institute Australia; near-pCR, near pathological complete response; NKI, The Netherlands Cancer Institute; OpACIN-neo, Optimal Neo-adjuvant Combination Scheme of Ipilimumab and Nivolumab; pCR, pathological complete response; pNR, pathological non-response; pPR, partial pathological response.

Histopathological immunotherapeutic response subtype and features of response and immune cell populations

The percentage of the different subtypes of pathological immunotherapeutic response (fibrosis/FIS, pigment-laden macrophages and necrosis) in relation to pathological response categories and percentage of viable tumour is presented in Figure 3. The area of tumour bed consisting of each subtype of immunotherapeutic response stratified by pathological response categories for all patients and the area of tumour bed consisting of each immunotherapeutic response subtype in pNR patients stratified by recurrence are presented in Supplementary Figure S3, available at https://doi.org/10.1016/j.annonc.2021.03.006. The mean of the tumour bed area occupied by fibrosis/FIS was significantly higher in patients who had a pathological response (pCR, near-pCR and pPR) compared with pNR ($P < 0.001$). A higher mean percentage area of fibrosis within the tumour bed also strongly correlated with a higher degree of overall pathological response in all patients with pCR/near-pCR compared with pPR and pNR cases ($P < 0.001$). The percentage of tumour bed consisting of pigment-laden macrophages and necrosis did not correlate with pathological response or the degree of response.

RFS stratified by each pathological immunotherapeutic response subtype (into high and low groups) is shown in Figure 2B-D for all cases and also for those who showed a pNR. Those patients with a high proportion of fibrosis/FIS within the tumour bed showed significantly improved RFS ($P = 0.019$). In addition, when just assessing the pNR patients, there was a trend, which fell just short of significance, towards longer RFS in those patients with a higher

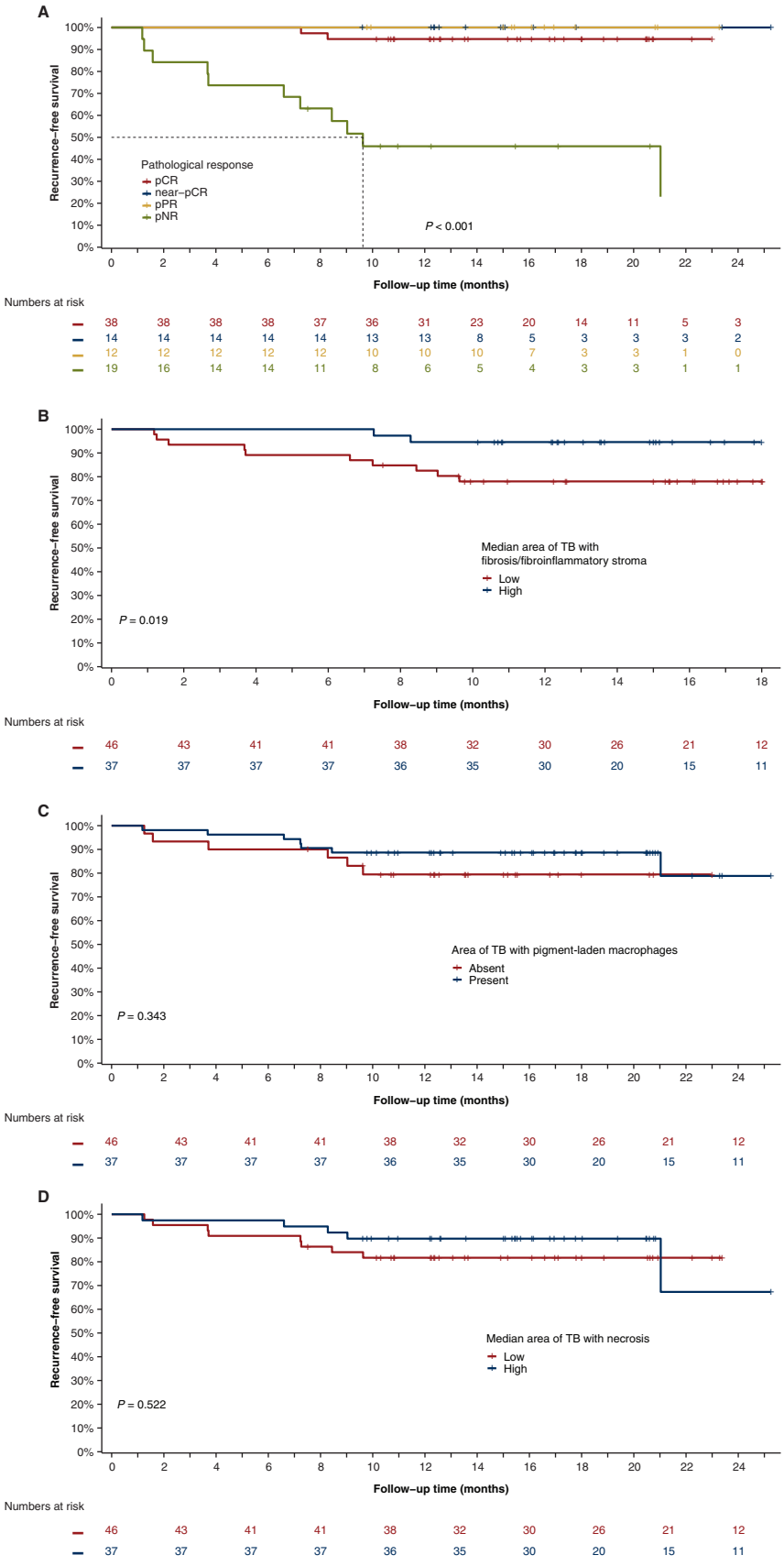

Figure 2. Recurrence-free survival following 6 weeks of neoadjuvant immunotherapy in the OpACIN-neo clinical trial for clinically detectable stage III metastatic melanoma. (A) Stratified according to pathological response category (pCR, pathological complete response; pPR, partial pathological response; pNR, pathological non-response). (B-G) Stratified by histological immunotherapeutic response subtype as a proportion of tumour bed (TB), in all patients (B-D) and patients with pNR (E-G). (B and E) Percentage area of TB with fibrosis/fibroinflammatory stroma; (C and F) TB with and without pigment-laden macrophages; (D and G) Percentage area of TBwith necrosis. One patient with pCR died unrelated to melanoma.

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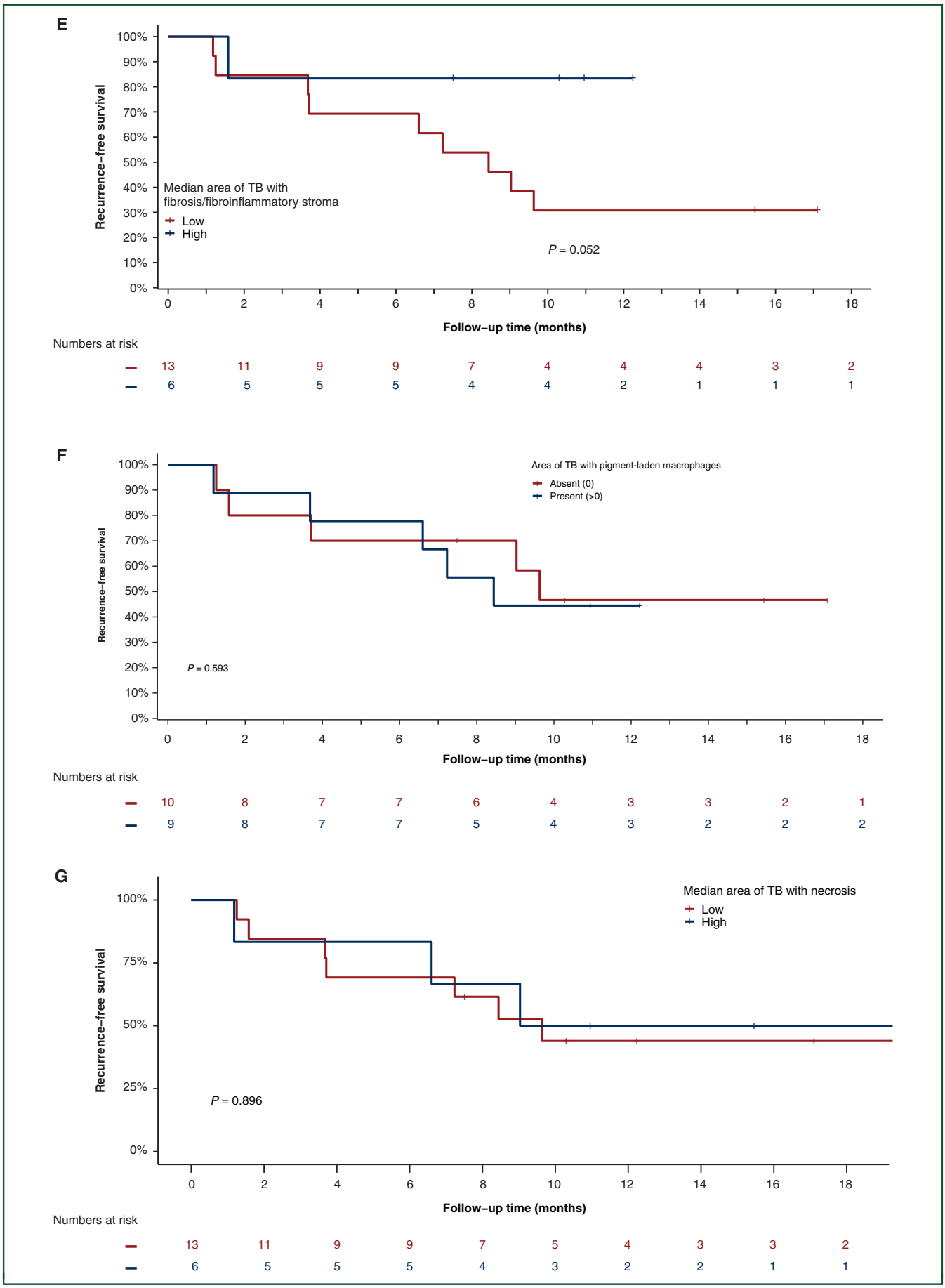

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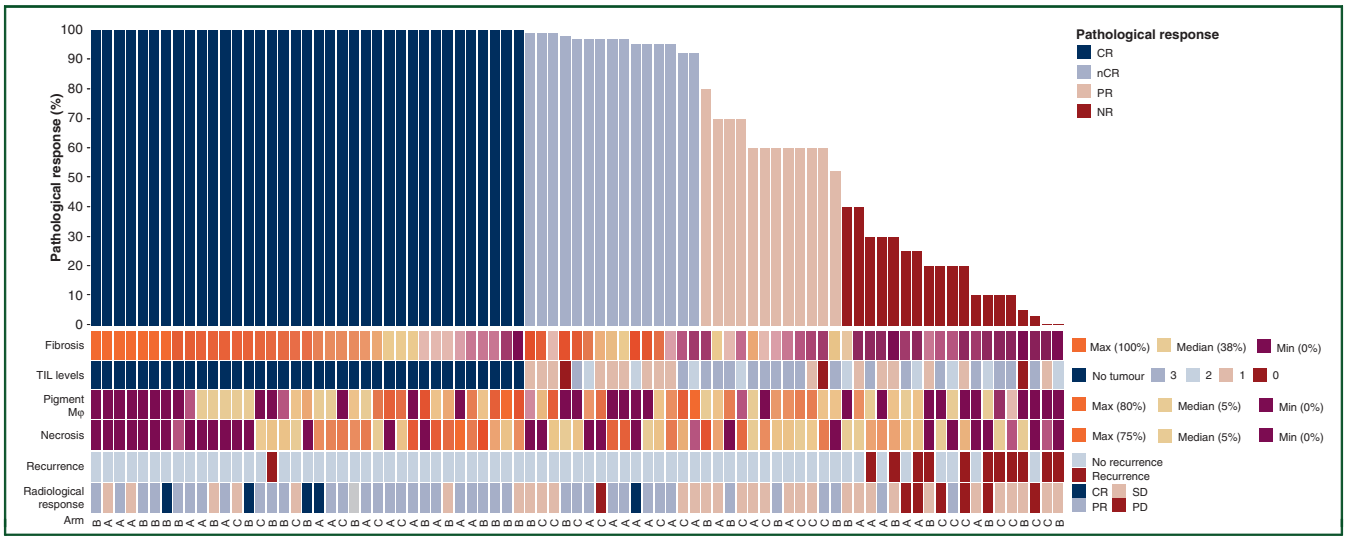

Figure 3. Heat map demonstrating each case with percentage of pathological response, different subtypes of pathological immunotherapeutic response (fibrosis, pigment-laden macrophages, necrosis) as a percentage of the tumour bed and density of tumour-infiltrating lymphocytes (TILs) within the viable tumour.
Cases that had recurred are also noted.
Pathological response = tumour bed area less % viable melanoma.
CR, complete response; nCR, near pathological complete response; pCR, pathological complete response; PD, progressive disease; pNR, pathological non-response; pPR, partial pathological response; PR, partial response; SD, stable disease.

degree of fibrosis/FIS within the tumour bed ($P = 0.052$). Of the 11 pNR patients who recurred, all but one patient had <10% fibrosis/FIS within their tumour bed. The other subtypes of pathological immunotherapeutic response and density of TILs showed no correlation with RFS for all patients or in the subgroup of pNR patients. On univariable logistic regression for the entire cohort (Table 2), the features which significantly correlated with lack of recurrence were increased percentage area of the tumour bed occupied by fibrosis ($P = 0.008$) [particularly hyalinised fibrosis ($P = 0.025$) compared with proliferative fibrosis], increased necrosis within the tumour bed ($P = 0.025$) and the presence of lymphoid aggregates in the tumour bed ($P = 0.003$). When just the pNR group was analysed, none of the variables examined were significantly associated with recurrence (Table 2). These findings were confirmed when the absolute recurrence rates were compared (Supplementary Table S4, available at https://doi.org/10.1016/j.annonc.2021.03.006). Further results are provided in Supplementary Materials, available at https://doi.org/10.1016/j.annonc.2021.03.006.

Immunotherapeutic response score

Because a number of features of the irPR are co-dependent variables, we developed a novel ITRS that was independent of the pathological response category and pathological immunotherapeutic response subtypes to provide an additional independent measure of the immune response. The number of histopathological irPR features present (not

Table 2. Pathological and radiological features associated with recurrence (univariate logistic regression) for all patients ($n = 83$) and also for the pathological non-response group ($n = 19$)

Variable[b]	Entire cohort ($n = 83$)			pNR subgroup ($n = 19$)		
	OR (95% CI)	*P* value	AUC (%)	OR (95% CI)	*P* value	AUC (%)
Percentage pathological response[a]	0.54 (0.40-0.74)	<0.001	87.9	0.52 (0.22-1.22)	0.132	63.6
Radiological response (RECIST 1.1)[a]	1.28 (1.04-1.58)	0.019	83.5	0.99 (0.82-1.19)	0.935	37.5
Radiological response (change in long axis)[a]	0.83 (0.59-1.16)	0.272	58.2	0.83 (0.52-1.33)	0.445	61.4
Radiological response (change in long axis × short axis)[a]	1.24 (1.07-1.44)	0.005	83.8	1.07 (0.90-1.27)	0.442	61.4
Percentage area of TB with viable melanoma[a]	0.73 (0.46-1.17)	0.190	46.7	1.94 (0.81-4.65)	0.138	62.5
Percentage area of TB with fibrosis/fibroinflammatory stroma[a]	0.94 (0.88-0.97)	0.008	89.6	0.19 (0.03-1.37)	0.098	76.1
Percentage area of TB with hyalinised fibrosis[a]	0.89 (0.79-0.96)	0.025	84.4	0.90 (0.75-1.09)	0.291	67.0
Percentage area of TB with proliferative fibrosis[a]	0.97 (0.90-1.01)	0.221	70.5	0.69 (0.47-1.02)	0.064	72.7
Percentage area of TB with necrosis[a]	0.89 (0.81-0.99)	0.025	81.3	1.18 (0.35-3.95)	0.793	44.3
Percentage area of TB with melanophages[a]	0.97 (0.92-1.02)	0.221	62.6	0.69 (0.18-2.65)	0.585	28.4
Neovascularisation	0.28 (0.06-1.36)	0.113	35.0	0.30 (0.02-4.06)	0.365	22.7
Lymphoid aggregates	0.12 (0.03-0.48)	0.003	55.4	0.37 (0.05-3.01)	0.353	30.7
Tertiary lymphoid structures	0.41 (0.10-1.64)	0.207	33.7	1.56 (0.12-20.85)	0.739	15.9
Plasma cell infiltrate (score 0/1 versus 2/3)	0.51 (0.14-1.86)	0.311	32.9	0.34 (0.05-2.26)	0.266	39.8

AUC, area under the curve; CI, confidence interval; OR, odds ratio; pNR, pathological non-response; TB, tumour bed.
[a] Based on 10% change.
[b] Presence of granulomas, cholesterol clefts, giant cells, neutrophils *P* values >0.97 for both cohorts. No patients recurred and no pNR patients had a peripheral pattern of response.

including the pathological immunotherapeutic response subtype, i.e. fibrosis, melanophages or necrosis) in the tumour bed of each case was scored to provide an ITRS (Supplementary Figure S4A-E, available at https://doi.org/10.1016/j.annonc.2021.03.006 and Supplementary Table S3, available at https://doi.org/10.1016/j.annonc.2021.03.006). The previously described irPR score was also recalculated (Supplementary Figure S4F-J, available at https://doi.org/10.1016/j.annonc.2021.03.006).[23] An ITRS and irPR score of 0 were each significantly associated with development of recurrence in the entire cohort ($P = 0.002$ and $P \leq 0.001$, respectively). In the pNR group, there was a trend for those with an ITRS of 0 to recur, but this association was not significant ($P = 0.26$). In contrast, in this same pNR cohort, an irPR score of 0 was significantly associated with recurrence ($P < 0.001$) as all patients who had not recurred had a score >0. The presence of an ITRS and irPR score of >0 was significantly associated with a higher degree of pathological response ($P < 0.0001$ for both scores) and an increased RFS ($P = 0.0001$ and $P = 0.001$, respectively).

Immune cell subsets in tumour bed correlate with immunotherapeutic response subtype

To investigate possible biological associations of the favourable fibrotic/FIS response subtype, we carried out flow cytometry analysis of cell dissociates from the tumour bed[29,30] in a subset of cases ($n = 29$). B lymphocytes represented a higher proportion of the immune cells (total CD45+ cells) in the tumour bed in patients with high tumour bed fibrosis compared to those with low fibrosis ($P = 0.046$, Supplementary Figure S5, available at https://doi.org/10.1016/j.annonc.2021.03.006). The presence of TLSs within the tumour bed also correlated with a higher proportion of B cells ($P = 0.03$). Further results are provided in Supplementary Materials, available at https://doi.org/10.1016/j.annonc.2021.03.006.

DISCUSSION

The findings in the OpACIN-neo trial are consistent with other neoadjuvant immunotherapy trials in metastatic melanoma where any pathological response is an excellent predictor for prolonged RFS. From all these trials, there appear to be two distinct groups within pNR patients, those who recur early and those who do not relapse. It is therefore of critical importance to predict those in this pNR group who are at a higher risk of recurrence.

All patients who recurred in the pNR groups had $\geq$70% viable melanoma and, when compared with the INMC cut-off of 50%, the sensitivity in predicting recurrence was unchanged but there was increased specificity. Whilst only a small number of patients recurred in this study, our data therefore suggest that a threshold of 70% viable melanoma may be a more informative threshold to classify patients treated with neoadjuvant immunotherapy as pNR, but this needs to be evaluated in a larger independent dataset. When using radiological assessment, all pNR patients who recurred had $<$30% (by RECIST 1.1) and $<$60% (immune-related response criteria) reduction in tumour size by radiological assessment of the involved target lymph nodes at the 6-week CT scan. Whilst two patients with pNR who did not recur showed a radiological response, further study is indicated to determine whether particular radiological measurements could also be combined with pathological assessment to more accurately identify which pNR patients are at a higher risk of recurrence.

We have demonstrated for the first time that there is excellent interobserver reproducibility of the assessment of pathological response categories and percentage of response using INMC criteria when different international pathology centres assessed the same surgical specimen. Minor discordances were identified predominantly in cases where digitally scanned images were analysed, which is a method not typically used in routine pathological practice. High interobserver reproducibility has been reported for pathological response assessment of the primary lesion following neoadjuvant immunotherapy in lung carcinoma,[25] but not, as far as we are aware, in melanoma or in the assessment of response in involved lymph nodes for any cancer type. Since pathological response is the primary endpoint for most neoadjuvant clinical trials, this finding is important and supports comparisons of datasets across different clinical trials of neoadjuvant therapies in melanoma.

In each of the specimens, we evaluated the features of the immunotherapeutic response subtype which we and others have observed and previously reported.[15,22,25,26] The extent of tumour bed comprising fibrosis/FIS appeared to represent a more effective immunotherapeutic response subtype than the other subtypes and correlated with a higher pathological response category, improved RFS in the entire cohort and also improved RFS within the pNR subgroup. Stein et al.[23] previously reported an association with proliferative, but not hyalinised, fibrosis in patients responding to immunotherapy in metastatic melanoma. Here we demonstrated that an increased percentage of either type of fibrosis within the tumour bed was associated with favourable outcomes; however, the degree of hyalinised fibrosis was significant in predicting outcome whilst proliferative fibrosis was not. These findings are concordant with those we have separately reported following neoadjuvant treatment with targeted therapies (dabrafenib and trametinib) where hyalinised fibrosis also correlated with improved RFS and proliferative type fibrosis was an adverse indicator of prognosis.[31] A similar association with fibrosis subtype of response has been reported in other malignancies following neoadjuvant immunotherapy[25] and chemotherapy.[32] These findings indicate that fibrosis/FIS immunotherapeutic response subtype, particularly hyalinised fibrosis, may represent a more effective type of immunotherapeutic response and a possible predictive biomarker that, when absent, may help identify those patients who may benefit from additional alternative treatments. Of note, in this study, in the pNR group, 10 of the 11 patients who recurred had $<$10% fibrosis within their tumour bed. Adding this threshold to the proposed pNR

threshold of ≥70% viable melanoma within the tumour bed increases the specificity but does decrease the sensitivity for predicting recurrence.

We developed a novel ITRS which assessed the number of histological features of irPR present in the tumour bed. Using both our ITRS and Stein et al.'s irPR score, a score of 0 strongly correlated with recurrence in the entire cohort and a score of >0 significantly correlated with pathological response and improved RFS. We do recognise that these calculations are only based on a reasonably small cohort of patients who recurred in this study and therefore a multivariate analysis could not be carried out. When more data are available from further studies, we hypothesise that the incorporation of proportion of viable melanoma, percentage of fibrosis in the tumour bed, a score of histological features of irPR and possibly the use of radiological response may help further accurately identify those patients at risk of recurrence following neoadjuvant immunotherapy.

One of the principal advantages of neoadjuvant therapy is the opportunity to analyse the treated lesion to determine mechanisms of response and resistance. Similar to previous studies,[33,34] we demonstrated a higher proportion of T lymphocytes in the tumour bed of patients with a pathological response compared to those with a pNR. However, in addition to T lymphocytes, an increase in plasma cells and TLSs has also been noted in neoadjuvant therapy-treated resection specimens[25] raising the possibility that other important cell populations might contribute to clinical response. Likewise, B-lymphocyte populations have been shown to predict the response of melanoma patients to anti-PD-1 in advanced melanoma.[35,36] Interestingly, we found significantly higher levels of B lymphocytes within tumour beds containing higher degrees of fibrosis and higher B-cell counts also corresponded with the presence of TLSs. These findings may indicate that a B lymphocyte, or an antibody-mediated process, contributes to this favourable pathological immunotherapeutic response subtype.

Whilst we have demonstrated important findings which could help guide management of patients in this emerging field, we do recognise limitations in our study. The sample size of the study is reasonably small and the follow-up period relatively short. Due to the size of the patient cohort, we were unable to perform a multivariate analysis to account for potential confounders and to validate the model using an independent dataset. Further studies are warranted to confirm the importance of the various histopathological features of immunotherapeutic response as predictors of recurrence. Further validation of our findings on a larger cohort of patients is required, which will be possible, as more data become available through further clinical trials.

In conclusion, the findings from our study have important implications for the neoadjuvant therapy field for stage III melanoma patients. Firstly, the high interobserver reproducibility of pathological assessment supports the feasibility of comparison across clinical trials. Secondly, whilst pathological and radiological response correlate, in a significant number of cases there is discordance between the two and our data support pathological assessment as the most robust primary endpoint for neoadjuvant trials. Thirdly, our data suggest that ≥70% viable melanoma is perhaps a more specific threshold for a designation of pNR following neoadjuvant immunotherapy than the previous INMC-defined 50%, but evaluation in larger independent datasets is required. Fourthly, the presence of a fibrosis/FIS pathological immunotherapeutic response, particularly of the hyalinised type, is a predictor of improved clinical outcomes and may serve as a potential biomarker. Fifthly, a scoring system of histological features of immune-related treatment response improves identification of patients at risk of recurrence. Finally, we highlight the possible important role that B lymphocytes may play in the pathogenesis of this favourable pathological immunotherapeutic response subtype, which warrants further study in expanded patient cohorts.

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