Personalized neoadjuvant immunotherapy in stage III melanoma

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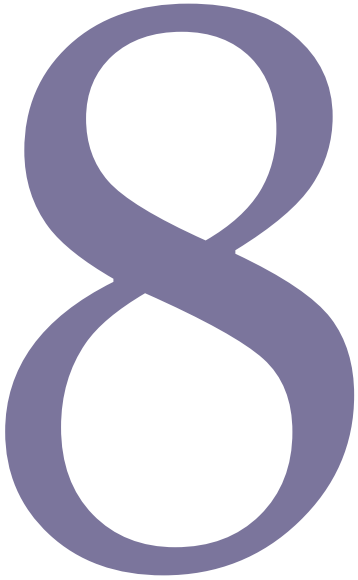

Biomarker-driven personalization of neoadjuvant immunotherapy in melanoma

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ABSTRACT

The introduction of immunotherapy has ushered a new era of anticancer therapy for many cancer types including melanoma. Given the increasing development of novel compounds and combinations and the investigation of these therapies in earlier stages of disease, there is a growing need in for biomarker-based treatment personalization. Stage III melanoma is one of the front-runners in the neoadjuvant immunotherapy field, facilitating quick biomarker identification by its immunogenic capacity, homogenous patient population and reliable efficacy readout.
In this review we will discuss potential biomarkers for response prediction to neoadjuvant immunotherapy, and how the neoadjuvant stage III melanoma platform could pave the way for biomarker identification in other tumor types.

Statement of significance

In accordance with the increasing rate of therapy-development, the need for biomarker-driven personalized treatments grows. Current landscape of neoadjuvant treatment and biomarker-development in stage III melanoma can function as a posterchild for these personalized treatments in other tumors, assisting in development of new biomarker-based neoadjuvant trials. This will contribute to personalized benefit-risk predictions to identify the most beneficial treatment for each patient.

INTRODUCTION

The introduction of immune checkpoint blockade (ICB) and targeted therapy has dramatically improved survival outcomes of patients with advanced melanoma [1-5]. In stage III and stage II (anti-PD1 only) melanoma these agents have shown to improve the relapse-free survival (RFS) in large adjuvant phase 3 trials [6-11], and have been included in the standard of care for stage III melanoma as adjuvant therapy after surgical resection of the disease. Adjuvant ipilimumab was the first checkpoint inhibitor in stage III melanoma demonstrating improved RFS and improved overall survival (OS) compared to placebo [8], but has not been implemented widely due to high toxicity rates (54% grade 3-4 immune-related adverse events, irAEs) [12]. Adjuvant nivolumab and pembrolizumab (PD-1 blockade) were better tolerated and yielded a higher RFS compared to ipilimumab [10,13] and interferon (IFN)α-2b [14] in resectable stage IIIB/C-IV melanoma, and compared to placebo [9] in resectable stage III melanoma, respectively. For patients with stage III BRAFV600E/K mutated melanoma specifically, targeted therapy with dabrafenib plus trametinib has been approved as adjuvant therapy for stage III-IV melanoma due to significant RFS improvement [7], although an indirect treatment comparison with adjuvant anti-PD1 indicated a more durable benefit with anti-PD1 [15,16]. Notwithstanding the different available adjuvant therapy options, still over 40% of patients develop a melanoma recurrence within 4 to 5 years after surgery and no OS benefit for adjuvant anti-PD1 antibodies nor BRAF/MEK inhibitors has been shown to date, illustrating the unmet need for more effective treatment modalities for stage III melanoma.

For immunotherapies, more efficacy could be achieved by its neoadjuvant application prior to surgery. Immune activation upon ICB requires a broad tumor neoantigen repertoire, increasing T cell receptor signaling and resulting in activation of different intra-tumoral and migrating tumor-recognizing T-cell clones. Multiple (pre-) clinical models have shown that expansion of these T cell clones in the peripheral blood is increased when the tumor is still present at the time of ICB initiation (neoadjuvant) as compared to after resection of the tumor and its microenvironment (adjuvant) [17-19]. In melanoma, an early phase I trial [20] showed that tumor clones present in the tumor and in peripheral blood expanded stronger in the peripheral blood after neoadjuvant versus adjuvant ICB. In addition, with neoadjuvant ICB more tumor present T cell clones that were not detectable at baseline in peripheral blood became detectable at week 6, which was associated with an improved RFS [20]. A recent phase 2 trial in melanoma also indicated the superiority of neoadjuvant ICB, as patients treated with neoadjuvant pembrolizumab had improved event-free survival (EFS) compared to the adjuvant treated patients [21]. Besides survival benefit, neoadjuvant administration of ICB offers several other advantages, such as reduction of the tumor burden resulting in enhanced resectability of the tumor lesions. Moreover, the possibility to readout the pathologic response provides early prognostic information, since a pathologic response has been shown to be a surrogate marker associated with EFS/RFS and OS after neoadjuvant ICB in stage III melanoma [22,23]. Therefore, this early response evaluation can guide further treatment decisions regarding e.g., extent of surgery, need for adjuvant therapy and intensity of the follow-up scheme. Finally, this fast efficacy readout, a homogeneous patient population and the availability of pre- and post-treatment tumor- and blood samples make the neoadjuvant therapy setting an ideal platform for biomarker

identification and the investigation of new therapies (Figure 1). A major concern that has been raised against neoadjuvant therapies is the risk of disease progression to unresectable disease. In current neoadjuvant trials in stage III melanoma, this risk varies between 2-17% [18,21,24,25], but an adjuvant trial observed progression in 18% of the patients within 7 weeks post-surgery [26], suggesting early progression is reflecting a more aggressive melanoma subtype or camouflaged stage IV disease. In these patients, surgery probably would not prevent progression nor improve OS, whereas patients were exposed to unneeded surgical morbidity. Neoadjuvant irAEs and their management might also hamper or complicate surgery, potentially resulting in more surgery-related adverse events or surgery delay, which has not been observed to date [25,27].

Due to the abovementioned advantages of neoadjuvant ICB, multiple new treatments and regimens are currently being tested in the neoadjuvant setting. The concept is not only tested in stage III melanoma, but has also expanded to the earlier stage II melanoma. However, it is currently unknown which patients would benefit from a more intense treatment schedule (e.g., combinations of anti-PD1 + anti-CTLA4/anti-LAG3/anti-TIGIT etc.) or a mild one (e.g., anti-PD1 monotherapy), which highlights the importance and need for biomarker-based personalization, selecting only patients that will benefit from that specific treatment. Stage III melanoma is currently one of the front-runners in biomarker-research in neoadjuvant ICB, serving as posterchild for ICB-treatment personalization for other stages and cancer types. This review discusses the current landscape of conducted and ongoing neoadjuvant immunotherapy trials in stage III melanoma, known and promising biomarkers, and our vision on the future of biomarker-driven personalized neoadjuvant immunotherapy in early-stage melanoma.

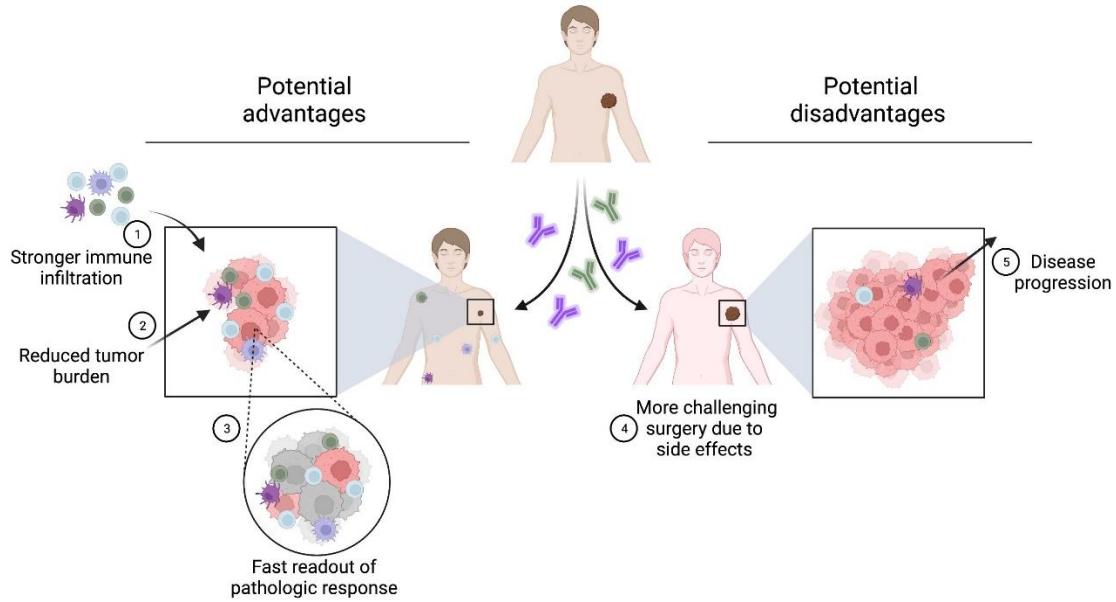

Figure 1 Neoadjuvant advantages and disadvantages.
Possible effects of neoadjuvant treatment include the following: (1) Induction of a broader and stronger immune reaction due to the presence of a more diverse tumor antigen repertoire. The presence of a greater intratumoral immune infiltration is correlated with pathologic response and prolonged EFS due to migration to possible micrometastasis in other parts of the body. (2) Reduction of the tumor burden can result in a better operable tumor. (3) Readout of pathologic response after treatment provides a fast reflection of the efficacy of the treatment. (4) The surgery could be more challenging due to side effects of ICB, or the surgery could be delayed due to side effects or the therapy for managing the side effects. (5) Disease progression results in more challenging surgery or an irresectable tumor. Created with BioRender.com.

THE NEW ERA OF NEOADJUVANT IMMUNOTHERAPY

The past and present: previous and ongoing neoadjuvant trials

During the last decade, neoadjuvant ICB has been investigated in several clinical trials in melanoma (Table 1). The first trial (2010) tested 2 cycles of neoadjuvant ipilimumab 10mg/kg 3 weekly followed by surgery in 35 patients with resectable stage III/IV melanoma, and observed a 1-year progression free survival (PFS) of 47% [28]. No patient achieved a complete pathologic response (pCR; 0% viable residual tumor), but in five (15%) patients only microscopic residual disease was found, which nowadays would be called a major pathologic response (MPR; ≤10% viable residual tumor) according to the INMC (International Neoadjuvant Melanoma Consortium) classification [29]. A subsequent trial testing the combination of neoadjuvant ipilimumab 3mg/kg (n=15) or 10mg/kg (n=15) in combination with high dose IFN-α in stage III melanoma, showed no difference in efficacy between the two arms (pCR rate 36% versus 29%, respectively), but a higher toxicity rate in the 10mg/kg arm. However, the combination with IFNα seemed to be superior to ipilimumab monotherapy in the previous trial with a 12-month PFS of 79% in the combination arm [30].

The phase 1b OpACIN trial was the first trial to prospectively compare neoadjuvant versus adjuvant ICB (2+2 cycles ipilimumab plus nivolumab neoadjuvant bracketing surgery, n=10, versus 4 cycles adjuvant after surgery, n=10) in stage III melanoma. Expansion of T cell clones in peripheral blood was higher at week 6 in the neoadjuvant arm compared to the 6 weeks adjuvant therapy, confirming the hypothesis of a stronger and broader immune activation upon neoadjuvant immunotherapy. Pathologic responses (<50% viable residual tumor) were observed in 7/9 (78%) patients of the neoadjuvant cohort, with a MPR rate of 62% [20]. After 4 years follow-up, RFS rates remained stable with 80% for the neoadjuvant and 60% for the adjuvant group [24]. The treatment regimen of ipilimumab 3 mg/kg and nivolumab 1mg/kg was, however, toxic with 90% grade 3-4 irAEs in both groups. This was an unexpected high toxicity rate, given that trial testing this combination in stage IV melanoma demonstrated toxicity rates of 55-59% [2,31]. Similar results were found in a trial comparing 9 weeks of neoadjuvant ipilimumab plus nivolumab (n=11) to nivolumab monotherapy (n=12), where combination therapy resulted in a higher efficacy (pCR rate 45% versus 25%, respectively), but again more grade 3-4 irAEs (73% versus 8%) were observed [18]. The trial was stopped prematurely because two patients in the nivolumab group developed stage IV disease in the neoadjuvant treatment period precluding surgery.

The monotherapy response rates were confirmed one year later by another trial testing one cycle (3 weeks) of neoadjuvant pembrolizumab followed by one year adjuvant pembrolizumab in stage III melanoma achieving a MPR rate of 30% [32]. However, the 2-year RFS rate was only 63%, suggesting that one cycle of anti-PD1 might be too short for durable tumor control or combination therapies (e.g., with ipilimumab) are needed for treating tumors with unfavorable characteristics [10].

We therefore adhered to the neoadjuvant combination of ipilimumab and nivolumab, and designed the multicenter OpACIN-neo in order to find a dosing scheme with less toxicity and preserved efficacy. Neoadjuvant ipilimumab 1mg/kg plus nivolumab 3mg/kg was found to be the most optimal scheme with a pathologic response rate of 77% and 20% grade 3-4 irAEs at week 12 [33]. After 3 years of follow-up a strong association between pathologic response and RFS was observed, with a RFS of 95% in

patients with a pathologic response compared to 37% in patients with a pathologic non-response (p<0.001) [23].

In the PRADO extension cohort of OpACIN-neo, 99 patients were included to receive 2 cycles neoadjuvant ipilimumab 1mg/kg plus nivolumab 3mg/kg. The pathologic response rate of 72% and 22% grade 3-4 irAEs during the first 12 weeks of therapy confirmed the efficacy of the previously found optimal dosing scheme. Additionally, response-directed personalized surgical and adjuvant treatment regimens were tested in this trial, based on the pathologic response in the index lymph node (ILN), which has previously shown to be representative for the whole lymph node bed [34]. In case of MPR, the therapeutic lymph node dissection (TLND) and adjuvant therapy were omitted. In patients with pathologic partial response (pPR; ≤50% viable tumor cells) and pathologic non-response (pNR; >50% viable tumor cells), treatment was escalated with a standard TLND, and patients with pNR received additional adjuvant BRAF/MEK inhibition or nivolumab. In the MPR group more local relapses were observed than expected (2-year RFS 93% compared to 97% in OpACIN-neo), but with similar rates of distant metastasis free survival (2-year DMFS 98% in PRADO versus 97% in OpACIN-neo), suggesting that salvage TLND for patients who developed local relapses after ILN resection was sufficient to prevent distant spread of the disease. For patients who achieved a pNR, 2-year RFS was improved by the addition of adjuvant therapy (76% compared to 36% in OpACIN-neo) [24,25].

Additional neoadjuvant combinations with anti-PD1 have already been tested. Pembrolizumab plus high dose IFN α−2b in patients with resectable stage III-IV melanoma showed a pCR rate of 43% and a 2-year RFS rate of 60% [35]. An indirect comparison with neoadjuvant ipilimumab plus nivolumab treatment demonstrated that pembrolizumab plus IFN yielded lower RFS rates and higher toxicity and discontinuation rates [24,33]. Whether this scheme could be attractive in a certain subgroup of patients, needs to be evaluated. A promising neoadjuvant combination in stage III melanoma is nivolumab plus relatlimab (anti-lymphocyte activating gene 3, LAG-3), which demonstrated a pathologic response rate of 70% including 57% pCR. The grade 3/4 toxicity rate of 26%, with 23% irreversible adrenal insufficiencies, was likely driven by the long adjuvant part of this treatment schedule, as no grade 3-4 irAEs were observed during the neoadjuvant part. A 2-year RFS rate of 92% was observed in patients with a pathologic response versus 55% in those without pathologic response, raising the question whether these excellent RFS rates could be preserved when omitting the adjuvant therapy in patients achieving an MPR, possibly making this scheme more tolerable [36].

In BRAF mutated patients, the phase II NeoTrio trial compared neoadjuvant pembrolizumab to sequential dabrafenib plus trametinib followed by pembrolizumab, and to the combination of dabrafenib plus trametinib and pembrolizumab. The triple combination therapy showed the highest pathologic response rate of 80% and pCR rate of 50%, but caused significant toxicity with 55% grade 3-4 irAEs compared to 25% in the sequential regime and 5% in the pembrolizumab monotherapy scheme. Although pathologic response rates were higher in the combination groups, EFS rates were similar [37].

In the DONIMI trial different combinations of neoadjuvant nivolumab +/- ipilimumab with the histone deacetylase inhibitor domatinostat where tested based on a baseline IFN-y signature algorithm. Addition of domatinostat did not improve the response upon ipilimumab plus nivolumab in patients with a low IFN-y signature in their baseline tumor

biopsy. In patients with a high IFN-γ signature nivolumab monotherapy seemed sufficient as 90% achieved a pathologic response in the nivolumab monotherapy group [38].

In the recently presented NIVEC trial that tested the addition of intratumoral oncolytic virus injection to neoadjuvant nivolumab, a high MPR of 65% was observed. The one-year EFS rate was 75% [39], which is comparable to anti-PD1 monotherapy [21]. Of note, only 8% grade 3-4 irAEs were reported. In a second trial testing another neoadjuvant oncolytic virus plus PD-1 blockade the EFS was also not superior to neoadjuvant pembrolizumab alone [40]. In this trial pembrolizumab in combination with anti-TIGIT indicated improved outcomes compared to the two other treatment-arms [40]. Yet, larger cohorts need to confirm this observation, as the cohorts were not perfectly balanced, e.g., with regard to BRAF mutation status.

The first larger randomized multicenter phase II trial (SWOG-S1801, NCT03698019) comparing neoadjuvant plus adjuvant pembrolizumab (n=154) versus adjuvant pembrolizumab (n=159) in resectable stage III/IV melanoma was presented last year. After a median follow-up of 14 months, the estimated 2-year EFS rate was superior for the neoadjuvant arm (72% versus 49%) [21], endorsing the theory that neoadjuvant checkpoint inhibition is able to induce a broader and stronger anti-tumor immune response [20,41].

Numerous clinical trials investigating neoadjuvant ICB in stage III melanoma are currently ongoing (Table 2). Worth highlighting are two company-driven platform trials (NCT05116202 and NCT04303169) testing novel checkpoint inhibitor combinations, often on a backbone of PD-1 blockade. Anti-TIGIT, anti-TIM-3, or cytokines like IL-2 or IL12, have been shown to be additive with anti-PD1+/- anti-CTLA-4 in several experimental models or early neoadjuvant trials [42-45] and are currently being tested or planned to be tested. The only phase 3 trial testing neoadjuvant ICB in melanoma is currently the NADINA trial (NCT04949113), which compares neoadjuvant ipilimumab plus nivolumab versus standard adjuvant nivolumab monotherapy. In line with the PRADO trial, patients with a MPR in the neoadjuvant arm do not receive adjuvant therapy, while patients without MPR do receive additional adjuvant therapy (nivolumab or dabrafenib plus trametinib). The first read-out is expected for the end of 2023.

The future: treatment personalization in the neoadjuvant setting

In order to maximize the risk-benefit ratio in a curative-intent situation, all efforts should be made to personalize treatment regimens. Personalized treatment regimens can not only improve RFS and OS, but also decrease toxicity and thereby improve the quality of life of patients. In addition, choosing the right therapy and limiting therapy-switching could also reduce health care costs and thus might enable such therapies for countries with less funded health care services.

The neoadjuvant therapy setting enables personalization in different phases of treatment. First, since previous trials have shown at pathologic response upon neoadjuvant ICB is a strong surrogate marker for long-term RFS, baseline biomarkers predictive for pathologic response could guide the choice of neoadjuvant treatment regimens, including mono- versus combination therapies. Second, early on-treatment (changes of) biomarkers might be used to adjust (i.e., intensify or abate) the neoadjuvant treatment regimen prior to surgery. And finally, the pathologic response upon the neoadjuvant treatment could direct the extent of surgery and omitting adjuvant treatment, as was already tested the PRADO trial [25].

Table 1 Neoadjuvant immunotherapy trials in stage III melanoma

Study	Phase	Disease stage	Patients (n)	Neoadjuvant therapy	Adjuvant therapy	MPR (%)	RFS/EFS (m)	Gr. 3/4 irAEs (%)
Tarhini et al. 2014 NCT00972933 *Monocenter*	I	III/IV	35	2x ipi 10mg/kg (q3w)	2x ipi 10mg/kg (q3w)	-	12m 47% (PFS)	-
Tarhini et al. 2018 NCT01608594 *Monocenter*	I	III	14	4x ipi 240mg + HDI^{neo} (q3w)	4x ipi 240mg + HDI^{adj} (q12w)	36% (pCR)	-	-
			14	4x ipi 800mg + HDI^{neo} (q3w)	4x ipi 800mg + HDI^{adj} (q12w)	29% (pCR)	-	-
Blank et al. 2018 OpACIN NCT02437279 *Monocenter*	I	III	10	none	2x ipi 240mg + nivo 80mg (q3w)	67%	24m 60% (RFS)	90%
			10	2x ipi 240mg + nivo 80mg (q3w)	none	-	24m 80% (RFS)	90%
Amaria et al. 2018 NCT02519322 *Multicenter*	II	III/IV	12	4x nivo 240mg (q2w)	13x nivo 240mg (q2w)	25% (pCR)	23m 58% (PFS)	8%
			11	3x ipi 240mg + nivo 80mg (q3w)		45% (pCR)	17m 82% (PFS)	73%
Huang et al. 2019 NCT02434354 *Monocenter*	I	III/IV	29	1x pembro 200mg	1 year pembro 200mg (q3w)	30%	24m 63% (RFS)	7%
Rozeman et al. 2019 OpACIN-neo NCT02977052 *Multicenter*	II	III	30	2x ipi 240mg + nivo 80mg (q3w)	none	70%	24m 90% (RFS)	43%
			30	2x ipi 80mg + nivo 240mg (q3w)		64%	24m 78% (RFS)	27%
			26	2x ipi 240mg (q3w) → 2x nivo 240mg (q3w)		47%	24m 83% (RFS)	54%
Long et al. 2022 Neo Trio NCT02858921 *Multicenter*	II	III BRAF V600 mutation-positive	20	2x pembro 200mg (q3w)	46w pembro 200mg (q3w)	40%	12m 80% (EFS)	30%
			20	dab 150mg bid + tram 2mg od (1 w) → 2x pembro 200mg (q3w)		30%	12m 80% (EFS)	25%
			20	dab 150mg bid + tram 2mg od (6w) + 2x pembro 200mg (q3w)		55%	12m 79% (EFS)	55%
Najjar et al. 2021 NCT02339324 *Multicenter*	I	III/IV	30	2x pembro 200mg + HDI^{neo} (q3w)	46w pembro 200mg + HDI^{adj} (q3w)	42%	24m 60% (RFS)	-
Reijers et al. 2022 PRADO NCT02977052 *Multicenter*	II	III	99	2x ipi 80mg + nivo 240mg (q3w)	*MPR and pPR*: none *pNR*: 11x nivo 480mg (q4w) or dab 150mg bid + tram 2mg od (46w)	61%	24m 85% (RFS)	22%
Long et al. 2022 NeoPeLe NCT04207086 *Moncenter*	II	III	20	2x pembro 200mg (q3w) + lenvatinib 20mg od (5w)	1 year pembro 200mg (q3w)	55%	12m 80% (EFS)	45%

Amaria et al. 2022 NCT02519322 *Multicenter*	II	III/IV	30	2x nivo 480mg + rela 160mg (q4w)	10x nivo 480mg + rela 160mg (q4w)	64%	24m (RFS)	82%	26%
Reijers et al. 2023 DONIMI NCT04133948 *Monocenter*	II	III IFN-γ high	10	2x nivo 240mg (q3w)	11x nivo 480mg (q4w) or 46w dab 150mg bid + tram 2mg od	80%	18m (RFS)	100%	0%
			10	2x nivo 240mg + doma 200mg bid, d1-14 (q3w)		60%	18m (RFS)	100%	20%
		III IFN-γ low	10	2x nivo 240mg + doma 200mg bid, d1-14 (q3w)		10%	18m (RFS)	80%	40%
			10	2x ipi 80mg + nivo 240mg + doma 200mg od, d1-14 (q3w)		40%	18m (RFS)	63%	20%
			4	2x ipi 80mg + nivo 240mg + doma 200mg bid, d1-14 (q3w)		25%			100 %
Patel et al. 2023 SWOG1801 NCT03698019 *Multicenter*	II	III/IV	313	none	18x pembro 200mg (q3w)	-	24m (EFS)	49%	14%
				3x pembro 200mg (q3w)	15x pembro 200mg (q3w)	21% (pCR)	24m (EFS)	72%	12%
Zijlker et al. 2023 NIVEC NCT04330430 *Monocenter*	II	III/IV	24	3x nivo 240mg (q2w) + 4x intralaesional T-VEC	9x nivo 480mg (q4w)	65% (MPR)	12m (EFS)	75%	8%
Dummer et al. 2023 KEYMAKER-U02C NCT04303169 *Multicenter*	I/II	III	15	1x pembro 400mg	Pembro 400mg (q6w)	40% (pCR)	18m (EFS)	78%	0%
			26	1x pembro 400mg + 5x gebasaxturev intratumoral (d1,3,5,8,22)		28% (pCR)	18m (EFS)	70%	12%
			25	2x pembro 200mg (q3w) + 2x vibostolimab 200mg (q3w)		38% (pCR)	18m (EFS)	85%	8%

Table 2 Ongoing neoadjuvant clinical trials

Identifier cinicaltrials.gov	Trial name	Neoadjuvant treatment arms	Phase
NCT04949113	Neoadjuvant Ipilimumab Plus Nivolumab Versus Standard Adjuvant Nivolumab in Macroscopic Stage III Melanoma (NADINA)	I: neoadjuvant ipilimumab and nivolumab	III
		II: adjuvant nivolumab	
NCT04207086	A Phase II Study of Neoadjuvant Pembrolizumab and Lenvatinib for Resectable Stage III Melanoma (Neo PeLe)	pembrolizumab, lenvatinib	II
NCT03554083	NeoACTIVATE: Neoadjuvant Therapy for Patients With High Risk Stage III Melanoma	I: atezolizumab, cobimetinib and vemurafenib	II
		II: atezolizumab and cobimetinib	
		III: atezolizumab and tiragolumab	
NCT03842943	Neoadjuvant Combination Immunotherapy for Stage III Melanoma	pembrolizumab and talimogene laherparepvec	II
NCT04139902	Neoadjuvant PD-1 Inhibitor Dostarlimab (TSR-042) versus Combination of Tim-3 Inhibitor Cobolimab (TSR-022) and PD-1 Inhibitor Dostarlimab (TSR-042) in Melanoma	I: dostarlimab	II
		II: dostarlimab and cobolimab	

NCT05289193	CD8+ T Cell Imaging During Pre-surgery Immunotherapy in People With Melanoma	ipilimumab and nivolumab	II
NCT04303169	Substudy 02C: Safety and Efficacy of Pembrolizumab in Combination With Investigational Agents or Pembrolizumab Alone in Participants With Stage III Melanoma Who Are Candidates for Neoadjuvant Therapy (MK-3475-02C/KEYMAKER-U02)	I: pembrolizumab II: pembrolizumab and vibostolimab III: pembrolizumab and gebasaxturev IV: pembrolizumab and MK-4830 V: pembrolizumab and favezelimab VI: pembrolizumab and ATRA	I/II
NCT04741997	Adjuvant Therapy Based on Pathologic Response After Neoadjuvant Encorafenib Binimetinib in Melanoma	encorafenib and binimetinib	I
NCT04013854	Adjuvant Treatment Determined By Pathological Response To Neoadjvuant Nivolumab	ipilimumab and nivolumab	II
NCT03567889	Efficacy of Daromun Neoadjuvant Intratumoral Treatment in Clinical Stage IIIB/C Melanoma Patients (NeoDREAM)	I: neoadjuvant daromun and adjuvant treatment II: adjuvant treatment	III
NCT04708418	A Study Evaluating Whether Pembrolizumab Alone or in Combination With CMP-001 Improves Efficacy in Patients With Operable Melanoma	I: pembrolizumab and CMP-001 II: pembrolizumab	II
NCT04331093	Neoadjuvant SHR-1210 Plus Apatinib for Resectable Stage III-IV Acral Melanoma	SHR-1210 and apatinib	II
NCT02938299	Neoadjuvant L19IL2/L19TNF- Pivotal Study (Pivotal)	I: L19IL2 and L19TNF II: adjuvant treatment	III
NCT05176470	Neoadjuvant Admin Autologous Tumor Infiltrating Lymphocytes and Pembrolizumab for Treatment of Advanced Melanoma Patients	lifileucel and pembrolizumab	I
NCT04401995	Study of TLR9 Agonist Vidutolimod (CMP-001) in Combination With Nivolumab versus Nivolumab	I: vidutolimod (CMP-001) and nivolumab II: nivolumab	II
NCT05116202	A Study Evaluating the Efficacy and Safety of Multiple Treatment Combinations in Patients With Melanoma (Morpheus-Melanoma)	I: nivolumab and ipilimumab	I/II

BIOMARKER-BASED TREATMENT PERSONALIZATION

The efficacy upon immunotherapy is the result of a complex interplay between the immune system, the tumor cells and their microenvironment (TME), including tumor antigen uptake, antigen presentation, activation of immune cells in the draining lymph node, homing to the tumor, and execution of immune-mediated tumor cell killing [46], previously summarized in the cancer immunogram [47]. Meanwhile, additional new biomarkers have been discovered for all disease-stages of melanoma, but for this review we will restrict ourselves to markers that we consider potentially relevant for the neoadjuvant treatment setting (Figure 2).

Tumor genomic biomarkers

Tumor mutational burden and neoantigens

The tumor mutational burden (TMB) is the number of somatic mutations harbored by tumor cells, which varies greatly across cancer types. TMB is often used as proxy for neoantigen burden in biomarker research, since recognition of tumor neoantigens is crucial for eliciting an anti-tumor immune response. Extensive research has demonstrated TMB to be a predictive biomarker for response and prolonged survival after ICB in different tumor types [48-51].

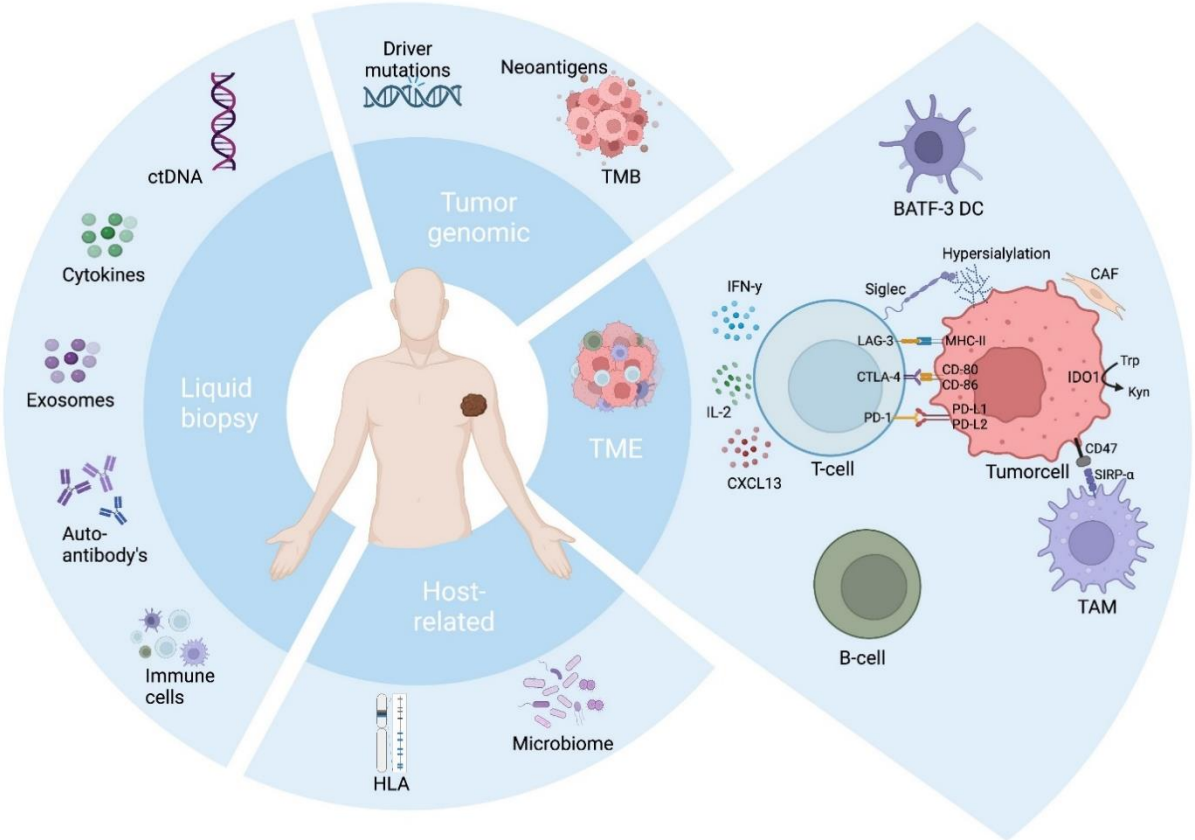

Figure 2 Predictive biomarkers neoadjuvant treated melanoma patients

TME, Tumor micro-environment; ctDNA, circulating tumor DNA; TMB, tumor mutational burden; DC, dendritic cell; IL, interleukin; IFN, interferon; CX3CL, CXC-chemokine ligand; CAF, cancer-associated fibroblast; IDO1, indoleamine 2,3-dioxygenase 1; Trp, tryptophan; Kyn, kynurenine; PD1, programmed death-1; PD-L1, programmed death-ligand 1; PD-L2, programmed death-ligand 2; LAG-3, lymphocyte activating gene-3; CTLA-4, Cytotoxic T-lymphocyte Antigen-4; MHC-II, major histocompatibility complex class II; TAM, Tumor-associated macrophages; HLA, human leukocyte antigens.

This has ultimately led to the tissue-agnostic FDA approval of TMB as a diagnostic biomarker for treatment with pembrolizumab of patients with unresectable or metastatic solid tumors harboring high TMB (≥10 mut/Mb) who progressed on prior therapies [52]. However, the implementation of TMB as a predictive biomarker is still facing significant hurdles [53-55]. The gold-standard method for TMB determination, whole-exome sequencing (WES), is expensive and time-consuming. Therefore, efforts are made to implement assays that can reliably extrapolate TMB from targeted panel-based sequencing data [56-58]. Several molecular diagnostic companies currently each developed their own methodologies to reliable replace TMB quantification by WES [59]. Moreover, the use of a fixed pan-cancer threshold of 10mut/Mb for the approval of pembrolizumab across solid cancer types limits the utility, as two retrospective analyses using this cutoff could not reproduce the predictive ability of TMB for response to ICB in all cancer types [55,60]. A TMB-cutoff of the highest 20% for each cancer type, showed a better association between TMB and improved OS after ICB in almost all cancer types [49]. Of note, TMB also variates strongly between subtypes of melanoma. For example, desmoplastic melanoma has shown to have a higher TMB than acral or mucosal melanoma [61,62], resulting in a higher response rate upon ICB in desmoplastic melanoma and lower response rates in acral and mucosal melanoma [63,64].

Neoantigen burden on its own might have an even stronger predictive value than TMB, since not all mutations give rise to neoantigens, and not all neoantigens are presented and/or recognized by immune cells [53,65]. Yet, so far, 'traditional' neoantigen predictions that mainly focused on peptide – MHC binding have shown no better prediction of response or survival to ICB as compared to TMB [51,66-68], indicating additional features defining the immunogenicity of neoantigens. For example, neoantigens derived from clonal mutations might elicit a more effective anti-tumor immune response than subclonal neoantigens [69]. Clonal TMB revealed to be a stronger predictor of ICB response compared to total TMB in a large meta-analysis [70]. Furthermore, traditional TMB analyses are mainly based on the calculation of nonsynonymous single-nucleotide variants (SNVs), whereas indel-mutations generate more 'foreign' neoantigens and are considered to be more immunogenic [70]. Somatic copy number alterations (SCNAs; changes to the chromosomal structure that results in gain or loss in copies of sections of DNA) can have a negative association with response or survival to ICB when occurring in antigen presentation genes or in other relevant immune pathways, since it has been speculated that SCNAs may interfere with neoantigen loading on MHCs or may result in loss of genes (e.g., HLA genes) that are needed for an immune response [71]. Finally, the differential agretopicity index (DAI), a metric that calculates the predicted MHC binding affinity of the 'wild-type' peptide relative to the mutated peptide, has outperformed TMB and neoantigen burden for survival prediction after ICB in different advanced melanoma and NSCLC cohorts [72].
In stage III melanoma patients treated with neoadjuvant ICB, a high TMB demonstrated to be associated with pathologic response and EFS (Table 3) [18,24,73]. Interestingly in the PRADO trial, TMB was only associated with response but not with EFS [73], possibly due to the addition of adjuvant therapy in non-responding patients. The different neoantigen/TMB subtypes have not been tested in the neoadjuvant melanoma setting, but could become important biomarkers extending or substituting sole TMB analyses in the future.

While there is no doubt on the potential value of TMB as predictive marker for neoadjuvant immunotherapy, further standardization, harmonization and cancer type-specific TMB cutoffs are warranted before broad implementation in the daily clinic.

Mutational signatures and DNA damage response pathways

Mutational processes caused by specific etiologies or exposures induce specific "mutation-signatures", which can represent biomarkers indicative of therapy response. In advanced melanoma, higher UV-mutational signature scores were predictive for response and survival after immunotherapy, in particular in patients with low to intermediate TMB in the tumor [74,75], suggesting that this can be a potential discriminator within TMB low cohorts. The predictive value is likely owed to an increased hydrophobicity of the neoantigens, hence increased immunogenicity due to better presentation on MHC molecules and better recognition by T cells [76,77]. The UV-signature has not yet been evaluated as predictive biomarker in the neoadjuvant melanoma setting.

Other signatures are less specific for melanoma, but therefore also might have implications in other cancer types. For example, the baseline Apolipoprotein B mRNA Editing Enzyme, Catalytic Polypeptide-like (APOBEC) signature was predictive for MPR in a trial testing neoadjuvant ipilimumab plus nivolumab in patients with head and neck squamous cell carcinoma (HNSCC) [78], and predictive for ICB responses in advanced NSCLC and urothelial carcinoma [79,80], but not in our melanoma cohorts. Also more prevalent in other tumor types, are alterations in the DNA mismatch repair pathway, leading to microsatellite instability (MSI). Five clinical trials in different tumor types showed durable responses to pembrolizumab in patients with MSI-high tumors, resulting in pembrolizumab being approved by the FDA for treatment of any advanced solid MSI-high tumor [52]. In a neoadjuvant ICB trial in MSI-high colorectal cancer (CRC), the pathologic response rate to neoadjuvant ipilimumab plus nivolumab was 99% [81]. If these pathologic responses translate into long-term EFS-benefit, one might envision that this biomarker could be the basis for omission of tumor resection and a less strict follow-up in MSI-high CRC.

Specific mutated genes

Genomic alterations of specific genes ("driver mutations") contribute to tumor growth by pathogenetic changes in cellular function, and may influence the ability of the tumor to bypass immune-surveillance. In stage IV melanoma, patients with a BRAF-mutated tumor had a significant improved survival upon combined ipilimumab plus nivolumab compared to nivolumab monotherapy, which was not observed in patients with a *BRAF* wild-type tumor [2]. In the neoadjuvant melanoma setting, two trials testing neoadjuvant ipilimumab plus nivolumab showed no significant difference in pathologic response [24,25]. Subgroup-analyses in the phase II SWOG-S1801 trial testing neoadjuvant pembrolizumab [21] indicate that both patients with a BRAF mutated and BRAF wild-type melanoma have a more favorable outcome upon neoadjuvant pembrolizumab. Whether this holds also true for ipilimumab plus nivolumab needs to be evaluated in the ongoing phase III NADINA trial [82]. Other mutations that have been associated with ICB-response/resistance and their potential immunogenicity in advanced melanoma (but have not been investigated in the neoadjuvant setting) are *NRAS*, *SERPINB3/SERPINB4*, *PTEN*, *BCLAF1* and *TP53* [83-86].

Table 3 Predictive biomarkers in stage III/IV melanoma patients treated with neoadjuvant immune checkpoint inhibition

Study	Neoad-juvant therapy	Origin biomarker	Biomarker	Method	Outcome	Association with outcome	Treatment arm
Tarhini et al. 2014 NCT00972933	IPI	Peripheral blood	IL-17	Multiplex	Grade 3 colitis	Positive	-
		Peripheral blood	IL-10	Multiplex	RFS	Negative	-
		Tumor	22-gene immune signature	RNA sequencing	RFS	Positive	-
Tarhini et al. 2018 NCT01608594	IPI + IFN	Peripheral blood	PBMC T cell clonalitiy	Immuno-sequencing	RFS	Negative	All
		Tumor	TIL clonality	Immuno-sequencing	RFS	Positive	All
Blank et al. 2018 OpACIN NCT02437279	NIVO + IPI	Tumor	IFN-γ gene signature[A]	RNA sequencing	RFS	Positive	All
		Peripheral blood	T cell clonality	T cell receptor (TCR) sequencing	RFS	Positive	All
		Tumor	PD-L1 expression	NanoString spatial microscopy	RFS	Positive	Neoadjuvant
		Tumor	CD3	NanoString spatial microscopy	RFS	Positive	Neoadjuvant
		Tumor	β2 microglobulin	NanoString spatial microscopy	RFS	Positive	Neoadjuvant
		Tumor	TMB	DNA sequencing	Pathologic response	No correlation	Neoadjuvant
Amaria et al. 2018 NCT02519322	NIVO + IPI	Tumor	TMB	DNA sequencing	RECIST response	Positive	All
		Tumor	CD8+ T cell infiltrate	IHC	RECIST response	Positive	All
		Tumor	PD-L1 expression	IHC	RECIST response	Positive	All
		Tumor	Lymphoid markers	IHC	RECIST response	Positive	All
		Tumor	CD45 immune markers including β2-microglobulin and B cell markers	Multiplex	RECIST response	Positive	All
Huang et al. 2019 NCT02434354	NIVO	Tumor	IFN-γ T-cell inflamed gene signature[B]	Nanostring ncounter	RFS	Positive	-
		Tumor	Increase in TILs	IHC	Pathologic response and RFS	Positive	-
		Tumor	Increase in Eomes expression	Flow cytometry	RFS	Positive	-
Rozeman et al. 2019 OpACIN-	NIVO + IPI	Tumor	TMB	DNA sequencing	Pathologic response and RFS	Positive	All

neo NCT02977 052		Tumor	IFN-γ gene signature[A]	Nanostring ncounter	Pathologic response and RFS	Positive	All
		Tumor	PD-L1 expression	IHC	Pathologic response	No correlation	All
		Peripheral blood	PD-L2	OLINK	Pathologic response	Negative	All
		Peripheral blood	VEGFR-2	OLINK	Pathologic response	Negative	All
		Peripheral blood	CX3CL1	OLINK	Pathologic response	Negative	All
Reijers et al. 2022 PRADO NCT02977 052	NIVO + IPI	Tumor	TMB	DNA sequencing	Pathologic response	Positive	-
		Tumor	IFN-γ gene signature[A]	Nanostring ncounter	Pathologic response and RFS	Positive	-
Amaria et al. 2022 NCT02519 322	NIVO + RELA	Tumor	LAG-3/PD-1 expression	Mass cytometry	Pathologic response	No correlation	-
		Tumor	CD45 cell frequency	Mass cytometry	Pathologic response	Positive	-
		Tumor	Decrease in M2-like macrophages	Mass cytometry	Pathologic response	Positive	-
		Peripheral blood	Increase in EOMES CD8+ T cells	Flow cytometry	Pathologic response	Positive	-
Reijers et al. 2023 DONIMI NCT04133 948	NIVO + IPI + DOM	Tumor	IFN-γ gene signature[A]	Nanostring ncounter	Pathologic response	Positive	All

Tumor immune microenvironment phenotype biomarkers

Immune cell presence and diversity

High rates of tumor infiltrating CD8+ T cells, CD4 T cells and FoxP3+ cells have been associated with response to neoadjuvant ICB in several melanoma and NSCLC trials [17-19,24,30,36]. Aside presence or density of these tumor infiltrating lymphocytes, their phenotype should also be considered, as expression of transcription factor TCF7, tumor reactivity marker CD39, or checkpoint PD-1 on the CD8 T cell surface have all showed an association with improved response to ICB [87-91].

Furthermore, since the activation and expansion of specific antigen-reactive T cell clones is required for an effective T cell response, the diversity and clonality of the intratumoral or peripheral T cell repertoire is also thought to be associated with ICB response [19,92,93]. A trial in patients with stage III melanoma demonstrated a higher T cell clonality and diversity in pre- and on-treatment tumor samples of patients with response to nivolumab monotherapy, while patients treated with ipilimumab and nivolumab showed a more diverse pattern of T cell clonality and diversity, lacking an association with response [18]. In the OpACIN trial, a lower productive T cell clonality in baseline tumor samples and lower number of newly detected T cell clones at week 6 in the peripheral blood were found in patients who relapsed after adjuvant or neoadjuvant ipilimumab plus nivolumab. Of note, neoadjuvant therapy induced a greater expansion of these T cells [20]. In line with this observation another group found that newly detected T cell clones in the TME itself, and not expansion of pre-existing T cell clones, was associated with response to PD-1 blockade in patients with basal of squamous cell carcinoma [94].

It is now generally considered that a diverse T-cell repertoire at baseline and a more clonal T-cell repertoire during therapy could predict improved response to ICB, but validation in larger cohorts in the neoadjuvant setting is needed.

Dendritic cells (DCs), in particular the basic leucine zipper transcription factor ATF-like 3 (BATF3) DCs, play an important role in cross-presenting antigens to CD8+ T-cells and attracting them into the tumor [95]. The role of this DCs subtype for outcome upon neoadjuvant immunotherapy is reflected by the Batf3+-DC-gene signature [96]. Patients with stage III melanoma were more likely to relapse after neoadjuvant or adjuvant ICB when they had a low expression of the Batf3+-DC-signature in their pre-treatment tumor biopsy [97]. In addition, CXC-chemokine ligand 9 (CXCL9) and CXCL10, which are mainly produces by BATF3+ DCs, recruit T-cells and B-cells into the TME [96,98]have also been associated with improved response to ICB in metastatic melanoma [32,98,99].
Indoleamine 2,3-dioxigenase 1 (IDO1) can suppress these DCs, but also NK- and T-effector- cells, by catalyzing tryptophan into kynurenine, and upregulating regulatory T-cells and neovascularization [100]. High IDO1 is associated with resistance to anti-PD1 in NSCLC [101]. Recently, a new anti-IDO/PD-L1 vaccine in combination with nivolumab showed promising results in patients with stage IV melanoma [102], potentially leading to the renaissance of IDO targeting. Whether IDO1 can function as a predictive biomarker for response in the neoadjuvant stage III melanoma setting needs to be evaluated.

Lymphoid formations (tertiary lymphoid structures; TLS) can be formed in non-lymphoid tissue upon chronic inflammation, but also in tumors. They induce influx of immune cells into the tumor and have been associated with improved prognosis in multiple cancers [103]. The ectopic lymphoid tissue consists of aggregates of immune-cells [103], B-cells in the TLS this have shown to be predictive for response to ICB in melanoma, renal cell carcinoma and sarcoma [18,104,105]. The chemokine CXCL13 is thought to be a major mediator in TLS formation and B-cells-attraction into the TLS [90,106]. CXCL13 has been identified as biomarker for response upon ICB in bladder cancer, potentially superior to PD-L1 expression or the IFN-y signature [106]. In melanoma CXCL13 was associated with improved RFS after neoadjuvant anti-PD1 [32]. Further analyses in larger cohorts are warranted to elucidate the relevance of this marker.

The tumor associated macrophages (TAMs) are important in multiple ways during the anti-tumor-immune response, and can be pro-inflammatory (M1-like macrophages) or anti-inflammatory (M2-like macrophages) [107,108]. A decrease in M2-like macrophages has been shown to be associated with pathologic response after neoadjuvant ICB in patients with stage III melanoma [36], suggesting that blocking the M2-like macrophage skewing could increase pathologic response after neoadjuvant treatment. Multiple approaches influencing macrophage-activity are currently being investigated [109], for example by re-polarization of the TAM into M1-like phenotype (e.g., by CSF1R inhibitors or CD40 agonists), inhibition of the tumor-promoting function (e.g., by TIM3 blockade), decreasing the their survival (e.g., by CSF1 inhibition), suppressing macrophage-recruitment (e.g., by CCL2/CCR2 inhibition), designing novel macrophages (e.g., by CAR-M), or by removing blockage of the phagocytosis [109].
Another example of improving the anti-tumor function of macrophages is targeting CD47 on tumors or its receptor signal receptor protein-alpha (SIRP-α) on the macrophages, which have been shown to mediate phagocytosis inhibition [110]. High

expression of CD47 or SIRP-α has been associated with impaired outcomes in multiple malignancies [111,112]. In preclinical models targeting the CD47 enhanced the tumor-cell phagocytosis by M1 and M2 macrophages [113] and dual targeting of PD1 and CD47 showed an increase in anti-tumor immune response [114], increasing the possible relevance of CD47 or SIRP-α being predictive biomarkers in the neoadjuvant ICB setting. Yet, so far, no data is available.

Inhibitory immune checkpoint expression

While various checkpoints have been extensively tested in the neoadjuvant melanoma setting, often on the backbone of anti-PD1 (Table 2), the use of checkpoint (ligand) expression as biomarker is restricted. The tumor expression of programmed death-ligand 1 (PD-L1; one of the two ligands of PD-1) has been approved as companion diagnostic for anti-PD1 therapy in several cancer types including NSCLC, HNSCC, urothelial carcinoma and triple negative breast cancer [52]. However, the results in melanoma are conflicting [115]. In stage III melanoma, some trials showed a significant association between PD-L1 and (pathologic) response or RFS after neoadjuvant treatment [18,20,25], whereas others did not find this association [33,36], making PD-L1 expression an unreliable marker for neoadjuvant therapy personalization. Expression of PD-1, CTLA-4, or LAG-3 have been shown to correlate with response upon targeting in late-stage disease, but data in the neoadjuvant space are pending or not convincing [36,116-120].

Finally, a currently under-examined mechanism of the cancer immune evasion is hyperglycosyliation and the binding of these glycans to immune-inhibitory sialic acid binding immunoglobulin type lectins (siglecs) [121-123]. In metastatic melanoma expression of siglec-3 and -7 binding sialoglycan ligands has been associated with anti-PD1 resistance [124]. We are currently investigating these siglecs as predictive biomarkers in our cohorts. Multiple new therapies interacting with this siglec-sialic acid axis are currently being tested [121], making hyperglycosyliation a promising new therapeutic target and increasing the relevance of the understanding of the role as a biomarker.

Inflammatory gene expression signatures

In contrast to the presence of single immune cell subsets or checkpoint molecules, immune gene expression signatures could represent a wider representation of an ongoing anti-tumor immune response within the tumor micro-environment. The 18-gene Tumor Inflammation Signature (TIS) represents an activated but suppressed adaptive anti-tumor immune response and was first described by Ayers et al. [125]. Higher expression of the TIS is strongly correlated with ICB response in multiple cancer types and independent of TMB [126,127]. The TIS-signature has been developed into a validated clinical assay, and could be used as a pan-tumor predictive biomarker.

In the neoadjuvant melanoma setting, a more confined signature described by Ayers and colleagues, called the 'preliminary IFN-γ signature', has been tested extensively. An IFN-γ signature algorithm proved indeed to be a predictive baseline biomarker for pathologic response and relapse in several neoadjuvant trials [24,25,38,73]. Combination of TMB and IFN-γ has shown to be highly predictive for pathologic response upon neoadjuvant ipilimumab plus nivolumab in stage III melanoma, with pathologic responses between 90-100% in high IFN-γ and high TMB in the baseline tumor biopsy compared to 39-42% in patients with low IFN-γ and low TMB [24,73]. Based on these

results, the DONIMI trial was the first trial to prospectively use an IFN-y signature algorithm for patient stratification to different neoadjuvant treatment regimens. Even though domatinostat did not show an additive effect, the trial did confirm the predictive value of the IFN-y signature with 14/20 (70%) MPR in the IFN-y high group and only 5/20 (25%) MPR in the IFN-y low group [38]. Moreover, evaluation of early on-treatment changes in the IFN-y signature indicated the relevance of the IFN- y signature algorithm for therapy adjustments during neoadjuvant immunotherapy. Patients with a low IFN-y signature in the baseline biopsy and a high IFN-y signature after one dose of ipilimumab and nivolumab (IFN-y signature low > high) had a pathologic response rate of 80%, while in patients who remained to have a low IFN-y signature (IFN-y signature low > low) in their on-treatment biopsy 0% achieved a pathologic response [38]. These results suggest that early on-treatment biopsies during neoadjuvant therapy can help identifying patients who will not benefit from the current neoadjuvant treatment options, and could benefit from an on-treatment escalation with novel combinations, like anti-PD-1 + anti-CTLA-4 + IL2 [45], anti-PD-1 +/- anti-CTLA-4 with intermitted BRAF/MEK inhibition [128], or anti-PD-1 +/- anti-CTLA-4 + anti-TIGIT or anti-LAG-3 [129,130].

The previously described association of the Batf3+-DC-gene signature with outcome upon neoadjuvant checkpoint inhibition, could be explained by insufficient CD4 help in tumors that don't respond to the treatment. Indeed, a low CD4/IL-2 signature in tumor material from neoadjuvant treated patients was associated with pNR, when IL-2 was added to tumor-fragments of patients with a pNR their profile changed to that of responding tumors [45]. Considering the idea of CD4 inducing an IL-12 driven DC maturation, one might postulate that addition of IL-12 might be effective in patients with a low CD4/IL2 or TIS/IFN-y signature in their tumor. In a study in 10 melanoma patients receiving a combination of neoadjuvant intra tumoral IL-12 and anti-PD-1, the researchers observed high pathologic response rates (MPR in 87%), suggesting addition of IL-12 could be beneficial in patients with a low inflammatory gene signature in their baseline biopsy [44].

Tumor stroma

The TME consists, aside tumor cells and immune cells, also of tumor stroma: connective tissue and vasculature exercising supportive functions and playing an important, underrated role in tumor growth, metastasis and therapeutic resistance [131].
A well investigated example is the cancer-associated fibroblast (CAF), which secrete extracellular matrix factors, promoting tumor growth, survival and migration and is able to form a network preventing intra-tumoral CD8 T-cell migration [132]. CAFs are associated with an impaired immune response and drug resistance after ICB [133]. UV radiation, the primary etiological factor for skin cancers as melanoma, causes change of the dermal fibroblasts into a CAF-phenotype. CAF activity is characterized by a six gene signature, this CAF-signature is predictive for response to anti-PD-1 in metastatic melanoma [134]. Targeting the CAFs could increase the response to the ICB, for example by inhibition of the CAF-activation via targeting TGB-β or CXCR-4 [135,136] or by reprogramming the CAFs with vitamin D/A receptor antagonists [136-138].
In addition, LRRC15 expression on the CAFs surrounding the tumor cells (demonstrated in the specific LRRC15+ CAF signature) is highly expressed in multiple tumor types and associated with poor response to anti-PD-L1 therapy in patient with bladder cancer and NSCLC [139]. The (LRRC15+) CAF-signature could serve as biomarker for new targeting

initiatives [140], even in early-stage melanoma, since the CAFs also play an important role in the primary melanomas.
Endothelial cells and pericytes in the tumor stroma play an important role in angiogenesis [131]. CAFs are thought to secrete vascular endothelial growth factor receptor (VEGFR) and to induce expression of leucine-rich alpha-2-glycoprotein 1 (LRG1) [141,142], both know to mediate tumor neo-angiogenesis and are associated with impaired responses to ICB [143-146]. In patients with stage III melanoma treated with neoadjuvant ipilimumab and nivolumab circulating VEGFR-2-levels are associated with pNR [24] and circulating LRG1 with relapse in patients with a pNR [24,147]. Whether patients with a high VEGFR-2 or LRG1 expression would benefit from for example lenvatinib (blocking VEGFR1-3) [148] or LRG1 targeting initiatives [149] should be further investigated.

Liquid biopsy biomarkers

Circulating tumor DNA (ctDNA)

Due to selective and static measurements such as tumor biopsies, sampling bias could result in default prediction of treatment response. Capturing the spatial and temporal complexity of the tumor is essential for response-prediction to ICB [150], which could be overcome by using repetitive liquid biopsies during treatment and follow up. Determination of the presence of microscopic residual disease after neoadjuvant systemic therapy and surgery might be the hallmark for decisions on subsequent adjuvant therapy indications, with circulating tumor DNA (ctDNA) being be the most powerful tool for detecting residual disease. The presence of pre- or post-surgery ctDNA has been associated with poor response to ICB in multiple cancer types such as melanoma and urothelial carcinoma [151-155]. In patients with stage III melanoma treated with adjuvant ICB, presence or increase of ctDNA post-surgery has been associated with decreased RFS and DMFS [155,156], indicating that there is still tumor present after surgery. Although the role ctDNA has not yet been confirmed in neoadjuvant ICB trials, we hypothesize that ctDNA could also assist in selection of adjuvant or neoadjuvant treatment, since it provides additional information to the IFN-γ signature and TMB [157]. In stage II colorectal cancer, researchers compared ctDNA-based adjuvant therapy (treating only the patients with detectable ctDNA-levels after surgery) with standard adjuvant therapy and found that fewer patients required adjuvant chemotherapy while relapse rates remained similar [158]. These findings imply that ctDNA could also serve as a marker for patient selection. This is currently being investigated in stage II melanoma in the DETECTION study (NCT04901988), treating stage IIB/C melanoma patients with an post-surgery elevated ctDNA with either adjuvant ICB or only at time of confirmed melanoma metastasis [159].
Next to being a biomarker on its own, ctDNA can also be used to determine the relative TMB (blood TMB), which has been shown to be reliable for predicting response to ICB in metastatic NSCLC [160-162]. In stage III melanoma this could provide an insight in the TMB at baseline, without requiring a baseline biopsy.

Circulating immune cells, cytokines and other small molecules

Post-treatment circulating PD1+ CD8+ T-cells have been shown to be predictive for ICB response in advanced metastatic NSCLC and for RFS in patients with stage III melanoma treated with adjuvant anti-PD1 [163,164]. Circulating PD-1 has indeed also been detected, using the OLINK assay, increasing strongly after neoadjuvant ipilimumab and nivolumab, but its baseline expression had no predictive value for response [24].

Upon neoadjuvant nivolumab and relatlimab in resectable stage III and IV melanoma post-treatment high rates of circulating eomesodermin (EOMES) expressing CD8 T cells were associated with favorable outcomes [36]. These EOMES+ CD8+ T cells are thought to play an important role in the tumor infiltration of CD8+ T cells and thus the anti-tumor immune response [165,166]. However, further research is needed to determine whether the CD8 T-cell EOMES-expression is useful as a baseline biomarker.

Extra-cellular vesicles, as for example exosomes, are thought to be important in the intercellular communication and to play an important role in tumor-progression and metastasis [167]. PD-L1 expressing exosomes have been postulated to mediate tumor mediated systemic immune suppression [168]. The preclinical work Poggi et al. showed that not PD-L1 expression on the tumor itself, but expression on exosomes, mediated PD-L1/PD-1 tumor immune escape [168]. Thus, tumors that express PD-L1 but produce exosomes to a lesser extent might be less susceptible to PD-L1/PD-1 blockade than tumors that do produce exosomes, possibly explaining the incongruences on tumor PD-L1 expression as biomarker for response to neoadjuvant ICB. Indeed, PD-L1 expressing exosomes have been shown to be associated with response to anti-PD1 [169] and to anti-CTLA-4 in metastatic melanoma [167], but need to be confirmed in larger cohorts and in the neoadjuvant setting.

Circulating cytokines have been proposed as another way of measuring the activity of the immune system. For example, high circulating IFN-y is associated with response in melanoma and NSCLC [170,171], compared to IL-6 and IL-8 that are associated with impaired response to ICB in multiple tumors as melanoma [172-176]. IL-6 is also thought to play an important role in the irAEs development [177,178]. In patients with stage III melanoma treated with neoadjuvant ipilimumab, high levels of IL-10 at baseline were associated with disease progression and high levels of IL-17 were associated with toxicity [179]. In patients treated with neoadjuvant ipilimumab and nivolumab circulating CX3CL1 was associated with non-response [24], but an independent confirmation-cohort missing. The predictive value of circulating cytokines and chemokines for response or toxicity should be further investigated.

Finally, an auto-antibody signature is developed from baseline serum auto-antibodies in patients with resectable stage III–IV melanoma treated with nivolumab, ipilimumab, or a combination of nivolumab and ipilimumab to predict the likelihood of recurrence but also the risk of developing significant toxicity [180]. The signatures for recurrence and toxicity showed little overlap, indicating a different pathophysiology [180]. To determine if auto-antibody biomarkers also apply to patients that received neoadjuvant treatment and if they can predict pathologic response, more research is required.

Host-related biomarkers

Human leukocyte antigen polymorphisms

Human leukocyte antigen (HLA) genes encode cell-surface proteins that are responsible for antigen presentation to T cells, and are known to be the most polymorphic in humans [181]. This variation is mainly located in the antigen-binding groove, altering the peptide-binding specificity of HLA molecules. A study in >1500 advanced cancer patients showed that a more diverse array of HLA-I molecules (i.e., maximal HLA-I heterozygosity at loci 'A', B' and 'C' versus homozygosity for at least one locus) was associated with

increased survival after ICB, possibly due to a broader presentation of tumor antigens to CD8+ T cells [182]. The combination of HLA heterozygosity and TMB enhanced the association with increased survival. Analysis of specific HLA-I super types showed that HLA-B44 was associated with improved survival and the HLA-B62 super type with decreased survival in patients with advanced melanoma [182]. Mechanisms interfering with the antigen-presenting pathway via the HLA system have been associated with resistance to ICB therapies. Examples are loss of heterozygosity (LOH) of HLA-I genes [182,183], downregulation of HLA-I expression [184] and mutations that disrupt the function of the B2-microglobulin (B2M) molecule that stabilizes the HLA-I complex [185]. To date, HLA heterozygosity, super types, and LOH have not been tested in the neoadjuvant melanoma setting due to too small cohorts, but if neoadjuvant immunotherapy becomes standard therapy HLA aberrations should be further investigated.

Intestinal microbiome

Over the past decades it has become evident that the gut microbiome has a complex and diverse role in many processes in the body, including alteration of the immune system and thus the anti-tumor immune response. A pro-tumor microbiome causes hyper-inflammation, altered cytokine levels and release of genotoxic chemicals such as carcinogens and mutagens, whereas an anti-tumor microbiome could increase immune surveillance, tertiary lymphoid structures and molecular mimicry [186]. The molecular mimicry between the tumor-associated antigens and the bacterial antigens of the microbiome increases the potential for anti-tumor T-cell-response [187].

In several studies, predominantly implemented in NSCLC and melanoma, it has been shown that the composition of the gut microbiome (and especially its diversity) impacts the sensitivity to ICB and the risk of irAEs [188-190]. Antibiotic use decreases this diversity, subsequently reducing response to ICB in melanoma, NSCLC and RCC [191-194]. In patients with stage III melanoma treated with neoadjuvant ipilimumab and nivolumab the *Ruminococcacae*-dominated microbiomes were associated with higher response-rates and lower toxicity compared to *Bacteriodoidacacae*-dominated microbiomes [195]. The prevalence of *Bacteriodoidacacae*-dominated microbiome was more prominent in Australia and the United States compared to the more frequent *Ruminococcacae*-dominated microbiome in the Netherlands [195], suggesting that certain patients could benefit from lifestyle interventions depending on geographical location. In line with this notion, a cross-cohort study identified a panel of species, including *Bifidobacterium pseudocatenulatum, Roseburia spp.* and *Akkermansia muciniphila*, associated with response to ICB, but no single species could be regarded as a fully consistent biomarker across studies [196]. Therefore, there is currently no clear-cut biomarker that could be used to predict response in neoadjuvant immunotherapy.

FUTURE BIOMARKER-DRIVEN TRIALS

In an era of rapidly growing knowledge of cancer evolution and resistance mechanisms, and increasing availability of anti-cancer specific therapies, the need for biomarker-driven personalized trials becomes more and more obvious. Due to its immunogenic properties, melanoma has been one of the most investigated cancer types regarding immunotherapies and is currently at the forefront of clinical neoadjuvant ICB research, providing optimal conditions for biomarker research.

In our opinion, biomarker research in the neoadjuvant melanoma setting should focus on baseline biomarkers identifying the most optimal neoadjuvant treatment compound or combination for each individual patient with regard to efficacy and toxicity. Naturally, each personalized therapy should aim for the highest probability of major pathologic response, facilitating de-escalation of subsequent surgical procedures and adjuvant treatment regimens since pathologic response has shown to be strongly associated with survival [22,25,197]. Extensive surgery or adjuvant therapies should be reserved for only those patients where the tumor does not respond despite of their personalized neoadjuvant treatment regimen. We speculate that this could even be pushed further, by omitting any form of surgery when a deep response is confirmed by imaging and/or multiple biopsies showing (near) complete pathologic responses.

In addition, we should focus also on toxicity, since the potential of immunotherapies is being explored in earlier stages of disease with longer life expectancies and a stronger emphasis on quality of life. Biomarker research for toxicity prediction is still in its infancy, but is expected to gain attention since more and more patients are being cured. Thus, efficacy and toxicity should be used as twin objective to guide patients' treatment decisions, balancing the probability of both pillars on each individual's situation, prognosis and preference (Figure 3). The ultimate goal for personalized biomarker-driven neoadjuvant therapy is a highly effective and minimal toxic systemic therapy for each individual patient with a short-term treatment duration and limited impact on quality of life.

Until we reach that goal, novel treatment compounds and treatment combinations should be primarily tested in patients that are thought to have a low chance on response upon the currently available therapies. The poor prognosis of these patents could justify the investigation of new combinations in these early-stage cancer patients, facilitating accelerated treatment innovation, instead of waiting for results of trials in heterogeneous late-stage cancer patient populations. Trials investigating these novel treatment combinations should be adaptive – with the possibility to stop early for futility – and fast and efficient in order to make optimal use of the relatively limited resources (patients, patient samples and time). An example is an adaptive umbrella trial where in one cancer type, multiple agents with specific molecular targets can be tested based on specific biomarkers. These multiple treatment arms can be implemented and adapted under a master protocol in order to enhance logistic and regulatory efficiency. Another example of an adaptive trial design has previously been proposed by our group: 'the Lombard street approach' [198], which focuses on identifying biomarkers that predict response upon a certain treatment, and using this biomarker in a subsequent trial to treat patients with favorable biomarkers with the identified therapy and patients with un-favorable biomarkers with new treatment regimens.

In conclusion, we believe that current biomarker-knowledge in the neoadjuvant melanoma field could serve as a posterchild and thus as a tool in treatment personalization for all stages of melanoma and other tumor-types, allowing all patients to receive the appropriate medication. The ultimate objective of all cancer research will continue to be curing every tumor without compromising the patient's quality of life.

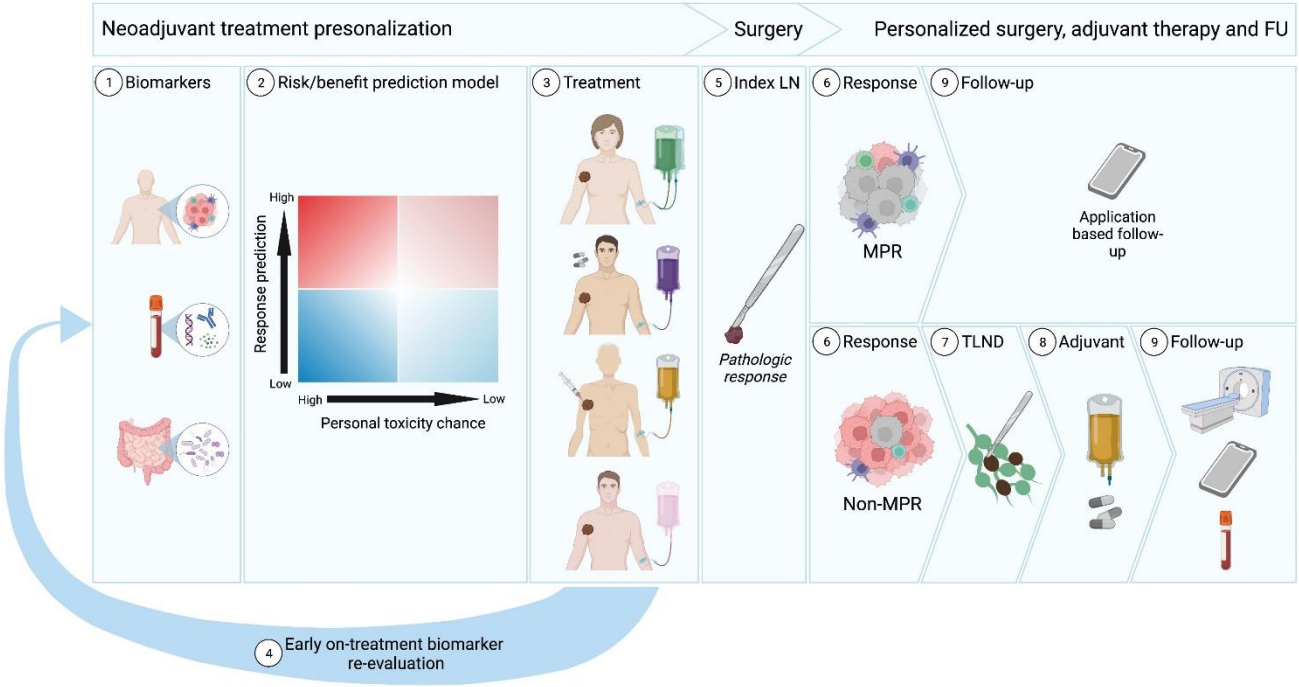

Figure 3 Biomarker-based neoadjuvant treatment personalization

This figure describes a biomarker-based personalized treatment-schedule: 1) Starting with the collection of biomarkers including tumor biopsies, liquid biopsies and the intestinal microbiome; 2) Based on the biomarkers a prediction can be made for expected response and toxicity, guiding in the decision for the most beneficial treatment for this patient; 3) The patient will start with the first dose of the most beneficial neoadjuvant treatment (immune checkpoint inhibition, targeted therapy or vaccinations); 4) Early on-treatment the biomarkers are re-evaluated and based on this evaluation treatment could be adjusted to another regimen (repeating step 1,2 and 3); 5) The patient will undergo an index lymph node (LN) procedure to determine the pathologic response; 6) In the response evaluation, most of the patients will achieve a major pathologic response (MPR; ≤ 10% vital residual tumor after neoadjuvant treatment), but still some patients will achieve no MPR ; 7) Based on the pathologic response, only the patients without a MPR will undergo a total lymph node dissection (TLND); 8) The same patients without a MPR will receive additional adjuvant therapy consisting of immune checkpoint inhibition or targeted therapy; 9) Follow up: patients with an MPR will be only followed by the use of applications, in patients without and MPR this follow up will be intensified by combining imaging, liquid biopsies and applications.

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