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REVIEW ARTICLE

Recent Progress in Mutation-driven Therapy, Immunotherapy and Combination Therapy for the Treatment of Melanoma

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Abstract: With increases in our understanding of the human genome and immune system, the treatment armamentarium for melanoma has benefitted from the development and approval of BRAF inhibitors, MEK inhibitors, immune checkpoint modulators *via* cytotoxic T-lymphocyte antigen-4 blockade, and PD-1 and PD-L1 inhibitors. These advances, however, have raised questions about combination therapy, the optimal sequential use of these agents, the limited assessment of response using traditional metrics, and the optimal selection of the population to be treated. In this review we summarize recent breakthroughs and then itemize the development of newer agents, potential prognostic and predictive biomarkers, resistance mechanisms, and strategies of combination therapy. We also emphasize the multifaceted attributes of immunotherapy in terms of durable responses and long-term survival that paradoxically necessitate further research into the underlying mechanisms and longer patient follow-up.

Keywords: Melanoma, BRAF inhibitors, MEK inhibitors, CTLA-4 inhibitors, PD-1 inhibitors, PD-L1 inhibitors, mutation-driven therapy, immunotherapy.

1. INTRODUCTION

Every year, 132,000 new cases of melanoma are diagnosed around the globe, and 48,000 people die from advanced disease [1, 2]. Advanced melanoma has been treated with traditional chemotherapy and immunotherapy, but responses have been minimal [3]. In the last two decades, however, unprecedented advances in research have led to the discovery of targeted drugs, immunotherapeutic agents and other biological agents. The introduction of BRAF and MEK inhibitors, CTLA-4 inhibitors, and new chemotherapy agents and explorations into biomarkers have also showed great promise for improving patient outcomes [4]. Although BRAF and MEK inhibitors have resulted in considerable responses, expedited by a refined understanding of the genetic defects that drive melanoma, especially mutations in the mitogen-activated protein kinase (MAPK) and related pathways, resistance to these agents inevitably develops, mediated at least in part through NRAS pathways. Solid strategies are urgently needed to address this issue.

As melanoma is among the most immunogenic cancers, intense research has focused on inducing an antitumoral immune response with various agents targeting the checkpoints CTLA-4, PD-1 and PD-L1 [5]. These strategies have shown major benefits and produced durable responses. With the advent of effective targeted therapies and more effective immunotherapy, the pivotal challenge remains how to optimally incorporate and sequence these drugs to maximize survival, given the lack of validated clinical decision-making tools to maximize long-term benefits.

This review will discuss the latest advances in the treatment of melanoma and outline results from recent clinical trials of combination of MAPK inhibitors and immunotherapy and will discuss issues to be considered in developing a personalized treatment algorithm.

2. MUTATION-DRIVEN THERAPY

A variety of oncogenic aberrations including loss of cycle control, activating mutations and amplifications enhance proliferation, produce resistance to apoptosis and facilitate distant dissemination of melanoma [6]. The mitogen-activated protein kinase (MAPK) pathway is a pivotal, well-established circuit that is constitutively activated in more than 90% of melanomas, driving unregulated cellular proliferation and unrestrained tumor growth through a number of genes implicated in melanocytic differentiation such as MITF [7-9]. MAPK pathway defects comprise activating mutations of serine/threonine-specific kinases including RAF (rapidly accelerated fibrosarcoma), specifically BRAF (v-raf murine sarcoma viral oncogene homolog B1), RAS (rat sarcoma), especially the G-protein NRAS, MEK (mitogen-activated protein kinase kinase)/ERK (extracellular signal-

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related kinase) and the receptor tyrosine kinase KIT [10]. Some melanomas lack these mutations but carry other MAPK pathway anomalies including NF1 and H-RAS or display phosphorylated ERK without a known driver mutation [10]. This network of kinases has long attracted interest from melanoma researchers, as some mutations, particularly BRAF, NRAS and MEK, appear to be associated with specific clinical presentations as well as worse prognoses in metastatic disease [11-13].

2.1. Vemurafenib

Somatic mutations involving BRAF occur in 40-50% of advanced melanoma cases [14, 15]. Eighty percent of these mutations involve a substitution of glutamine for valine at the 600th amino acid position (V600E). BRAF V600K mutations account for most of the remainder; V600R, V600D, V600M, V600E', L597 and K601E mutations are quite rare [9, 16-18].

The discovery of V600E mutation featuring oncogenic addiction and the characteristics of the serine/threonine kinase domain has led to successful new therapeutic opportunities, as it is an extremely powerful predictor of response to a BRAF inhibitors [13]. Vemurafenib (PLX4032 [Zelboraf]) was engineered as a highly specific and potent BRAF V600E mutation inhibitor *in vitro,* and early clinical trials showed objective response rates (ORRs) around 53 to 81%, including complete response in 10% of cases [19-22]. A confirmative phase III trial (BRIM-3) comparing vemurafenib with dacarbazine reported an ORR of 59% with vemurafenib in BRAF V600E mutant melanoma. Overall survival (OS) at 6 months was higher with vemurafenib than dacarbazine (84% *vs*. 64%), with a median OS of 9.7 months for the group receiving chemotherapy and 13.6 months for the group receiving vemurafenib (hazard ratio (HR) 00.7). Median progression-free survival (PFS) was also longer (5.9 *vs.* 1.6 months) [23]. This trial was the first to demonstrate major survival benefits with the use of targeted therapy in the treatment of metastatic melanoma, leading to FDA approval of vemurafenib in 2011 as first-line agent in this setting [4]. Vemurafenib has also shown similar activity in patients who have V600K mutations, and data from preclinical studies suggest efficacy in the less common V600 mutations and perhaps also in the rare L597 mutations [18, 22, 24].

Although treatment with vemurafenib appears to be generally well tolerated, its adverse effects are not insignificant: they include fatigue, arthralgia, edema, nausea and prolonged QT interval. Notably, 20% of patients receiving vemurafenib develop troublesome keratoacanthomas and cutaneous squamous cell carcinomas, usually in the first 2 or 3 months after starting therapy [25, 26]. This is probably due to the fact that in non-melanoma cells not harboring the BRAF mutations, the MAPK pathway may be paradoxically stimulated by BRAF inhibitors through a CRAF-dependent mechanism [27]. In addition, it appears that activated MAPK signaling may unmask or even accelerate the development of secondary malignancies. Selective de novo primary melanomas with wild-type BRAF were noticed to occur at a higher rate in patients receiving treatment with a BRAF inhibitor, and there have been increasing reports of gastrointestinal adenomatous polyps and RAS-mutant cases of chronic myelomonocytic leukemia [28-30]. Interestingly, selective BRAF inhibition does not appear to be the primary driver of these malignancies but rather, it heightens their clinical manifestations.

2.2. Dabrafenib

Dabrafenib (GSK2118436) is the second specific BRAF V600 inhibitor found to have high activity against BRAF mutant melanoma xenografts and cell lines in preclinical studies as well as in early-phase clinical trials [31]. Objective response rates were recorded in 53-69% of patients with documented V600E and V600K mutations. In BREAK-3, a randomized phase III trial comparing dabrafenib with dacarbazine in either BRAF V600E or V600K mutant metastatic melanoma, there were significant improvements in ORRs (53% *vs*. 19%) and PFS (5.3 *vs*. 2.7 months) in the dabrafenib arm [32]. However, this trial allowed patients to cross over to dabrafenib upon progression and did not have sufficient power to detect an overall survival benefit. Additionally, BREAK-3 recorded early responses in the subset of participants with brain metastases. This important observation was confirmed by a small phase III trial (BREAK-MB), where 39% of newly diagnosed patients with brain metastases from V600E BRAF-mutant melanoma who had not yet received treatment demonstrated significant ORRs (95% confidence interval (CI) 28.0-51.2), as did 30% of patients with brain metastases whose cancer progressed after failure of local treatment (95% CI 19.9-43.4) [33].

The side effect profiles of dabrafenib and vemurafenib slightly differ. For example, the rate of cutaneous squamous cell carcinomas and keratoacanthomas is lower with dabrafenib (6-19%) than with vemurafenib. This difference might be due to the fact that dabrafenib exhibits a higher affinity and selectivity for BRAF mutations with less cross-reactivity to the CRAF pathway, enhancing MAPK inhibition. In 2013, the US Food and Drug Administration (FDA) approved dabrafenib as a single agent for the treatment of BRAF-mutated unresectable or metastatic melanoma [32].

It is noteworthy to mention that patients with BRAF V600-wild type melanoma can not only be predicted not to respond to BRAF inhibitors, but some reports also suggest that these drugs may enhance tumor growth and facilitate metastases in these patients [21, 34]. Additional research is needed to refine our understanding of the molecular roles played by BRAF mutations and BRAF inhibitors.

2.3. MEK Inhibitors

MEK is the downstream effector of BRAF. *In vitro* studies have shown that MEK inhibition represses the growth of melanoma cells and enhances apoptosis in most BRAF mutated melanoma, but less so in NRAS mutant disease [12, 35, 36]. MEK inhibitors remain clinically effective in patients pretreated with a BRAF inhibitor and in those who have not received BRAF inhibitors but have been pretreated with immunotherapy and/or chemotherapy [37].

2.3.1. Trametinib

Trametinib (GSK1120212) is a potent, highly specific inhibitor of MEK1/MEK2 and the best-studied MEK inhibitor in the clinical setting. Early-phase trials demonstrated efficient MAP kinase signaling inhibition with tolerable side

effects [38]. METRIC was a phase III trial that randomly assigned 322 patients with advanced melanoma in a 2:1 ratio to receive either trametinib (2 mg/day orally) or chemotherapy (dacarbazine or paclitaxel) [39]. In this trial, all patients had either the V600E or V600K mutation in their melanomas. One-third of patients had previously received chemotherapy and another third had previously received immunotherapy, but prior BRAF inhibitor therapy was not permitted. Of note, crossover to trametinib was allowed in patients whose disease progressed on chemotherapy. The primary endpoint was PFS: 4.8 months for trametinib and 1.5 months for chemotherapy (hazard ratio [HR] = 0.54, 95% CI 0.33–0.63). Despite a crossover rate of 47% to secondary treatment with trametinib, the OS was significantly better with trametinib at 6 months of follow-up (81% *vs.* 67%, HR for death 0.54, 95% CI 0.32-0.92). Side effects included diarrhea, peripheral edema, acneiform rash and blurred vision. The so-called central serous retinopathy (CSR), which is a rare but well-described cause of blurred vision in patients on MEK inhibitors, was not reported with this study [40]. Squamous cell skin cancers were not seen with trametinib. Seven percent of patients had a decrease in ejection fraction and ventricular dysfunction after extended use of trametinib. On the basis of the results of the METRIC trial, the FDA approved trametinib in June 2013 for the treatment of patients with advanced melanoma containing a BRAF V600E or V600K mutation [41].

2.3.2. Other MEK Inhibitors

MEK162 is another specific inhibitor of MEK1/MEK2 and the first to demonstrate potential activity in patients with advanced NRAS mutant melanoma [42]. In a phase II study of 71 patients with advanced melanoma and either a V600 BRAF mutation or an NRAS mutation (41 and 30 cases, respectively), objective partial responses were observed in 8 of 41 patients (20%) with the BRAF mutation and 6 of 30 patients (20%) with the NRAS mutation, at a median follow-up of three months. In addition, the combined rates of objective response plus stable disease were 52 and 63% for those with the BRAF and NRAS mutations, respectively, leading to the conclusion that MEK162 has only modest activity in both BRAF and NRAS mutant disease [43].

Selumetinib (AZD6244) also inhibits MEK1/MEK2. It was compared with temozolomide in a randomized phase II trial that involved 200 unselected patients with previously untreated, unresectable stage III or IV melanoma [44]. The study found no significant difference in PFS, the primary endpoint of the trial. Additionally, in a retrospective analysis, only five of 45 patients (11%) with a BRAF V600E mutation had an objective tumor response. In a second randomized phase II trial, selumetinib combined with dacarbazine was compared with dacarbazine alone in 91 patients with melanoma harboring a BRAF mutation. The combination significantly improved PFS without a gain in OS, leading to the conclusion that selumetinib has limited activity in advanced melanoma [45].

2.4. Mechanisms of Resistance and Rationale for Combination Therapy Inhibitors

A major concern for treatment with BRAF or MEK inhibitors is a short median duration of response of only 6–7 months, and the development of resistance is inevitable [22, 25, 32]. Furthermore, around 15% of treatment-naive patients will not respond at all and will experience rapid clinical progression, while nearly all other patients will ultimately relapse [8]. Preclinical and the initial clinical data have revealed several critical resistance mechanisms at different levels of the kinase pathway, including mutation of NRAS, an upstream kinase in the MAPK pathway; expression of a shortened form of the BRAF V600-mutated molecule; activation of downstream kinase in MAPK including MEK1/2; as well as aberrations in pathways other than MAPK, such as PI3K/AKT/mTOR and CDK4 [46-49].

In the last few years, a number of basic studies reported on the finding of an adaptive metabolic process at least partly explaining the limited efficacy of BRAF inhibitors [50-52]. In their cell lines studies, Haq *et al.* showed that BRAF activation suppresses oxidative phosphorylation and mitochondrial biogenesis, mediated by the oncogenic melanocyte lineage-specification transcription factor *MITF* and the mitochondrial master regulator PGC1α [50]. These 2 molecules are interdependent while MITF appears to be a direct target of BRAF. Consequently, treatment with BRAF inhibitors stimulates PGC1α pathway leading to addictive gains in diverse mitochondrial functions mostly oxidative phosphorylation. Moreover, PGC1α expression in this subset of tumors clearly defines an aggressive behavior with increased mitochondrial capacity and resistance to oxidative stress. This observation hypothesized that BRAF-mutant melanoma patients may potentially benefit from the therapeutic combination of mitochondrial inhibitors (like biguanides) and BRAF inhibitors [50].

On the other hand, Vazquez *et al.* report functional reduction of glycolysis with increased oxidative phosphorylation in BRAF-mutant melanomas with PGC1α expression, enabling escape of reactive oxygen species and promoting survival under oxidative stress conditions [52]. Based on these reports and on the fact that AMPK is an established target of biguanides, Yuan *et al.* investigated the cotreatment of phenformin and vemurafenib in cell lines of mice with BRAF-mutant melanoma [53]. Compared with monotherapy, the combination resulted in inhibition of survival, tumor regression and more importantly in delaying development of resistance to vemurafenib. These findings remain to be validated by well-designed clinical trials with translational studies.

Finally, as BRAF inhibition has previously shown to suppress glycolysis through a large network of transcription factors involved in the metabolism [50-52], Parmenter *et al.* further demonstrated that simultaneous inhibition of BRAF and glycolysis overcomes resistance to BRAF inhibitor therapy in melanoma cells [54]. This observation largely impacts the interpretation of Positron Emission Tomography (PET) imaging with fluoro-2-deoxyglucose (FDG) uptake in the setting of metastatic melanoma patients treated with BRAF inhibitors. In the setting of melanoma, glycolytic as well as non-glycolytic pathways are important for survival [55, 56]. Consequently, blood supply and oxygenation might differ between cells as well as between the center and periphery of the tumor, a finding further fueled by treatment with BRAF inhibitors. Indeed, while FDG PET concludes a theoretically good radiological response to this treatment, the actual findings at the clinical or visceral levels might be those of dis-

ease progression. The same principle applies to the serum glycolytic enzyme lactate dehydrogenase (LDH), often used as prognostic factor in metastatic melanoma [57].

To conclude, there are opportunities for new targeted treatments to be developed to delay or even overcome the development of resistance through any of these postulated mechanisms.

2.5. Combination Therapies with MEK Inhibitors

The optimal inhibition of the MAPK pathway with BRAF and MEK inhibitors through concomitant or sequential use of these agents has not yet been determined, mainly because more needs to be learned about the development and evolution of resistance [48, 58]. Molecular studies suggest that treatment failure ensues when melanoma cells become largely dependent on anti-BRAF agents for survival and proliferation, or because of the reconstitution of phosphorylated MEK and ERK, the downstream targets of BRAF [59, 60]. It has been postulated that promising antitumoral activity could be obtained by combining selective BRAF and MEK targeting agents [59, 61]. This strategy has been explored in a phase I/II open-label study of 443 patients with BRAF V600E or V600K mutant metastatic melanoma [59]. The combination regimen using full doses of both dabrafenib and trametinib significantly prolonged PFS compared with dabrafenib alone (median of 11. 4 months *vs.* 7.3 months, hazard ratio, 0.56; 95% CI, 0.46 to 0.69; P<0.001) and significantly increased the proportion of patients alive and progression-free at one year of follow up (41% *vs.* 9%). The objective response rate was 64% in the combination-therapy group and 51% in the vemurafenib group (P<0.001). The investigators conducted a preplanned interim overall survival analysis after 77% of the total number of expected events occurred. At 12 months, the overall survival rate was 72% (95% confidence interval [CI], 67 to 77) in the group of patients who received combination therapy and 65% (95% CI, 59 to 70) in the group of patients who received vemurafenib (hazard ratio for death in the combination-therapy group, 0.69; 95% CI, 0.53 to 0.89; P=0.005) [62]. The expected but tolerated toxicities with MEK inhibitors occurred, including fever, acneiform rash, peripheral edema, ocular events and, less commonly, decreased cardiac ejection fraction. Notably, the combination strategy appeared to reduce the rate of skin toxicities (7% and 2% at 2 and 1 mg/kg of trametinib, respectively) compared with the monotherapy group (19%), seemingly by interrupting the paradoxical MAP kinase activation and blocking reactivation of ERK caused by BRAF inhibitors [26]. Following these promising results, three randomized phase III trials have been initiated in BRAF-mutant melanoma, and combined BRAF and MEK inhibition has outperformed, by far, single-agent BRAF inhibition in all three studies (Table **1**) [62-64]. As a result, the combination of dabrafenib with trametinib earned FDA approval in 2014 for treatment in this setting [62-64].

Additionally, a three-arm phase III trial (NCT01909453) is currently comparing LGX818 (a BRAF inhibitor) plus MEK162 with vemurafenib vx LGX818 monotherapy for patients with locally advanced unresectable or metastatic BRAF V600-mutant melanoma. To summarize, the partnership of BRAF and MEK inhibitors has proved to be the first oncogene targeted therapy combination to reduce a major toxicity of single-agent treatment while delaying or overcoming primary resistance. Nevertheless, treatment failure universally ensues, and the mechanisms underlying this failure remain to be confirmed [65-68]. Blockade of MEK1/2 with selective agents such as trametinib, selumetinib or MEK 162 has not generated clinically relevant response rates in comparison with selective BRAF inhibitors and has failed when patients experienced disease progression on a BRAF inhibitor [37, 42, 45]. An inverse sequential strategy entailing upstream inhibition of advanced melanoma with a MEK inhibitor followed by a BRAF inhibitor upon progression has been retrospectively explored, surprisingly demonstrating long disease control rates. This treatment strategy appears feasible in patients with V600 BRAF mutant melanoma, resulting in a median time to progression of 8.9 months, a number approaching that achieved with combination therapy with BRAF and MEK inhibitors (median PFS 9.4 months) [59, 69].

Finally, other reports suggested that intermittent scheduling of BRAF or MEK inhibitors may overcome resistance and warrants further investigation [58, 70]. The success of all of the above strategies suggests that resistance mechanisms following treatment with BRAF and MEK inhibitors may differ. However, in the context of approved immunotherapies and advances in preclinical studies, the role of this combination therapy remains uncertain. Oncologists may also opt to use single agents in certain situations because of reimbursement issues and financial limitations.

3. IMMUNOTHERAPY

3.1. Interleukin-2

The immune system plays a key role at different stages in the progression of melanoma. After the earliest studies on mice showed that high-dose interleukin-2 (IL-2), an essential cytokine that triggers T-cell proliferation and function, had promising antitumor activity, early trials of immunotherapy used high-dose IL-2 to improve outcomes in patients with advanced disease [71, 72]. Subsequently, some patients showed spontaneous tumor regression in primary but not metastatic tumors without any systemic intervention, suggesting a potential immunomodulatory effect of IL-2 on melanoma cells [73, 74].

The FDA approved the use of high-dose IL-2 in 1998 for the treatment of advanced melanoma. An analysis of eight separate phase II trials of IL-2 over nine years demonstrated an overall response rate of 16%, with 44% of the responding patients surviving for longer than five years (ranging from 1.5 to more than 122 months) [3, 75]. Nevertheless, a randomized phase III trial of 93 patients yielded an ORR of only 6% [76]. Moreover, the durable responses achieved in some patients are hindered by the severe toxicities of the drug, which affect several systems (skin, gastrointestinal tract, cardiovascular, respiratory, renal and nervous systems) [77].

In summary, the relatively extended IL-2 treatment experience provides indisputable evidence that durable clinical benefit in melanoma greatly depends on immune system modulation, mostly by T cells. This knowledge has provided a solid platform on which to build further improvements in immunotherapy.

Table 1. Summary of efficacy and toxicity endpoints in phase III trials comparing combined treatment with BRAF and MEK inhibitors *versus* treatment with a single BRAF inhibitor.

Combination	Population	Response Rate (%)	Progression-free Survival (Months) Primary Endpoint	Median Overall Survival (Months)	Major Toxicities
Vemurafenib + cobimetinib Co-BRIM *vs.* Vemurafenib	N=495 treatment-naïve patients with unresectable locally advanced or metastatic BRAF V600 mutant melanoma	68 *vs.* 45 (favoring combination)	9.9 *vs.* 6.2 months HR 0.51 [95% CI 0.39–0.68]; $p < 0.001$ (favoring combination)	Not reached HR 0.65 [95% CI 0.42–1.00]; $p = 0.046$ 9-month survival rates of 81% *vs.* 73% (favoring combination)	Nonsignificant higher incidence of adverse events and study-drug discontinuation. Less cutaneous SCC with the combination
Dabrafenib + trametinib *vs.* Dabrafenib alone Combi-d	N=423 treatment-naïve patients with unresectable stage IIIC or IV, BRAF V600E or V600K mutant melanoma	67 *vs.* 51 (favoring combination)	11 *vs.* 8.8 (favoring combination)	Median overall survival was 25·1 months (95% CI 19·2–not reached) in the dabrafenib and trametinib group *versus* 18·7 months (15·2–23·7) in the dabrafenib only group (hazard ratio [HR] 0·71, 95% CI 0·55–0·92; p=0·0107). Overall survival was 74% at 1 year and 51% at 2 years in the dabrafenib and trametinib group *versus* 68% and 42%, respectively, in the dabrafenib only group	Similar rates of adverse events. More dose modifications in the combination group. Less cutaneous SCC in the combination group (2% *vs.* 9%). More pyrexia in the combination group (51% *vs.* 28%)
Dabrafenib + trametinib *vs.* vemurafenib Combi-v	N=704 treatment-naïve patients with BRAF V600 mutant metastatic melanoma	64 *vs.* 51 (favoring Combination)	11.4 *vs.* 7.3 HR 0.56 [95% CI 0.46–0.69]; $p < 0.001$ (favoring combination)	Not reached *vs.* 17.2, HR 0.69 [95% CI 0.53–0.89]; $p = 0.005$ 72% *vs.* 65% at 12 months, $p = 0.005$. (favoring combination)	Similar rates of adverse events and study-drug discontinuations. Less cutaneous SCC and keratoacanthoma in the combination (1% *vs.* 18%)

Note: CI, confidence interval; HR, hazard ratio; SCC, squamous cell carcinoma.

3.2. Checkpoint Inhibitors

Cancer in general and melanoma in particular is a complex disease with unpredictably evolving cellular heterogeneity and loss/escape of physiological regulatory processes (so-called immune checkpoints). In advanced melanoma, the host's anti-tumor immune response is greatly suppressed by mechanisms inducing immune tolerance. The tumor microenvironment therefore plays a substantial role in facilitating melanoma cell survival, proliferation and progression. Restoring the immune system's ability to control melanoma's survival and growth has been the subject of intense research. The most interesting idea has been to overcome the suppression of host responses to tumor-associated antigens by restoring physiologic immunomodulatory mechanisms that represent immune checkpoints. The currently available checkpoint inhibitors target either the cytotoxic T-lymphocyte-associated antigen 4 (CTLA-4), the programmed death-1 (PD-1) or the programmed death ligand-1 (PD-L1).

3.3. Blockade of Cytotoxic T Lymphocyte Antigen-4 (CTLA4) Receptors

CTLA4, also known as CD152, belongs to the CD28:B7 immunoglobulin superfamily and is normally expressed at the surface of helper T cells and at lower levels on the surface of naïve effector T cells and is involved in the functioning of regulatory T cells. While CTLA4 is related in structure to the T-cell co-stimulatory protein CD28, and the two molecules bind to CD80 (B7-1) as well as to CD86 (B7-2) on antigen-presenting cells (APCs), CTLA4 transmits an inhibitory signal to T-cells, whereas CD28 transmits a stimulatory signal; CTLA4 also displays greater binding affinity for CD80 and CD86 [78, 79]. Following stimulation of naïve T cells through the T-cell receptor (TCR), CTLA-4 traffics to the surface membrane and competes with CD28 for B7, acting as an "off" switch of T-cell receptor signaling on APCs and establishing a key co-stimulatory pathway [80, 81]. Therefore, CTLA-4 is a first critical checkpoint physiologically destined to escape autoimmunity and to restore tolerance to self-antigens [82]. Multiple experiments in mice

showed that *in vivo* inoculation of CTLA4-specific inhibitors led to rejection of established tumors [83]. The development of CTLA4-specific human monoclonal antibodies as immunotherapeutic agents rapidly followed, especially in the field of melanoma [84].

3.3.1. Ipilimumab

Ipilimumab (Yervoy) is a first-in-class recombinant human immunoglobulin G1κ monoclonal antibody that binds to CTLA4 and inhibits the interaction of CTLA4 with CD80 or CD86, thereby blocking the co-stimulatory inhibition of the immune system, enhancing T-cell activation and proliferation [85]. A significant, unprecedented survival benefit with ipilimumab was shown in two large randomized, double-blind controlled phase III trials, MDX010-20 and CA184-024 [86, 87].

In the MDX010-20 trial, the safety and efficacy of ipilimumab was evaluated in 676 patients with advanced unresectable or metastatic melanoma. The study population included those whose disease had progressed after failure of one or more conventional therapies. Notably, this study exclusively enrolled HLA-A*0201-positive patients, a specific MHC molecule to which the gp100 peptide vaccine is presented. Eligible patients were assigned in a 3:1:1 fashion to receive ipilimumab in combination with an experimental gp100 peptide vaccine (403 patients), ipilimumab alone (137 patients) or gp100 alone (136 patients). The primary endpoint was OS in the ipilimumab plus gp100 arm compared with the gp100 arm. Secondary endpoints included OS in the ipilimumab plus gp100 arm compared with the ipilimumab arm, OS in the ipilimumab arm compared with the gp100 arm, and ORRs and duration of response in each of the trial's arms. Ipilimumab significantly improved OS from a median of 6.4 months in the gp100 alone arm to 10.0 months in the ipilimumab plus gp100 arm and 10.1 months in the ipilimumab alone arm. Interestingly, the ORR was as low as 1.5% in the gp100 alone arm *versus* 5.7% in the ipilimumab plus gp100 arm and 10.9% in the ipilimumab alone arm. The median duration of response was 11.5 months in the ipilimumab plus gp100 arm and has not been reached in the ipilimumab alone or gp100 alone arms.

In the CA184-024 study, treatment-naïve patients with advanced melanoma received ipilimumab 10 mg/kg plus DTIC (dacarbazine) or DTIC plus placebo [87]. The median OS was 11.2 and 9.1 months, respectively (HR 0.72; $p < 0.001$). It's a milestone analysis that we carried out to evaluate the 5-year survival rate of treatment-naïve patients with advanced melanoma who were treated with ipilimumab plus dacarbazine *vs*. placebo + dacarbazine in a phase III trial. The 5-year survival rate was found to be 18.2% (95% CI, 13.6% to 23.4%) for patients who received therapy with ipilimumab plus dacarbazine *versus* 8.8% (95% CI, 5.7% to 12.8%) for patients who received therapy with placebo plus dacarbazine ($P = .002$). A plateau in the survival curve began at approximately 3 years. In patients who survived for at least 5 years and continued to receive ipilimumab, grade 3 or 4 immune-related adverse events were recorded only in the skin. Of note, the combination arm was limited by frequent hepatotoxicity leading to discontinuation of ipilimumab in some patients [88].

The results of these two large phase III clinical trials and other studies underline the concept that a subset of patients with metastatic melanoma treated with ipilimumab can achieve remissions of at least 5 years. Interestingly, a study of extended ipilimumab treatment in patients who had participated in four phase II trials (CA184-025) found a significant 5-year survival of 16.5% to 17.0% for patients receiving ipilimumab 3 mg/kg, rising to 17.6% to more than 49% for patients receiving ipilimumab 10 mg/kg [89]. A meta-analysis of pooled survival data from phase II and III ipilimumab trials included 1861 patients with metastatic or locally advanced, unresectable melanoma and reported a 3-year OS of 22% [90]. Importantly, durable responses, even off-treatment, were followed through a plateau in the Kaplan–Meier curve beginning at 3 years after initiation of therapy and extending to as long as 10 years.

These practice-changing results and the limitations of treatment options for metastatic melanoma generated strong interest in ipilimumab, which obtained FDA and European Medicines Evaluation Agency approval in 2011 for first-line use in this setting [91].

This substantial advance in the treatment of metastatic melanoma is hindered by multiple challenges. On one hand, classic Response Evaluation Criteria In Solid Tumors (RECIST) responses are rarely seen and largely do not apply. They mostly occur either after prolonged therapy or after initial evidence that disease has progressed whether in existing lesions or with the detection of new metastases [86, 92, 93]. On the other hand, considerable morbidity secondary to serious immune-related adverse events occurs in 10–20% of patients, including endocrinopathies, colitis, hepatitis, dermatitis and neurotoxicity [86, 94, 95]. Although often manageable and reversible by corticosteroids, these toxicities might lead to death in a number of cases, limiting the survival benefit acquired by the whole population treated with ipilimumab.

3.3.2. Tremelimumab

Tremelimumab, another monoclonal antibody directed against CTLA-4, also showed promising activity in phase I and II clinical studies in previously treated patients with metastatic melanoma [96, 97]. This has led to a subsequent open-label phase III trial (A3671009) in which previously untreated patients with advanced melanoma were randomly assigned to receive either tremelimumab or standard chemotherapy (dacarbazine or temozolomide) [98, 99]. In this study, the primary endpoint was OS. The 1-year survival rate reached 50% for tremelimumab and the median survival was 12.02 months, compared with 10.45 months for chemotherapy. When a second interim analysis was conducted, the log rank test-statistic ($p = 0.729$) crossed the prespecified O'Brien-Fleming futility boundary and the decision was made to close the trial because of futility. Nevertheless, despite a prolongation in response duration among those treated with tremelimumab and the fact that most responses were durable, the difference in OS was not statistically significant [99]. This result has been attributed in part to the trial's eligibility criteria and the open-label strategy of treatment, as well as the access to an alternate anti-CTLA4.

Despite these discouraging results and three reported treatment-related deaths in the tremelimumab cohort, another

trial investigated the efficacy and safety of the combination of tremelimumab and interferon-2b *versus* interferon-2b alone in patients with metastatic melanoma [100]. The results were disappointing, as the best recorded ORR was 24%, with only four complete and five partial responses and a median OS of 21 months [100]. On the basis of the final analysis of the clinical data obtained from these two trials and the futility of responses, tremelimumab was abandoned as a potential treatment option for advanced melanoma.

3.4. Programmed Death 1 Protein (PD-1) and Programmed Death Ligands (PD-L)

Similar to CTLA-4, PD-1 is an immune-inhibitory receptor belonging to the CD28: B7 superfamily of immunoglobulins and is expressed on CD4+ and CD8+ T-cells chronically exposed to antigen stimulation, as well as B and natural killer cells [101-104]. PD-1 binds to their two known ligands, PD1-L1 (B7-H1) and PD1-L2 (B7-DC) [105-107]. While this interaction has a physiologic role in establishing self-immune tolerance and suppressing autoimmunity, the neoplastic cells exploit this mechanism to prevent the immune system from rejecting the tumor, thereby facilitating immune evasion [108]. In advanced melanoma, PD-L1 is expressed on approximately 40–50% of malignant cells and has been found to have limited expression in visceral tissues except at the surface of respiratory and placental cells [105, 109, 110]. Paradoxically, PD1-L2 expression is restricted to immune cells; however, less is known about its role in antitumor immunity and immune tolerance to tumor antigens.

The engagement of PD-1 by PD-L1 down-regulates T-cell function, leading to inhibition of the effector function of tumor-specific CD8+ T-cells and it is associated with deletion/apoptosis, inhibition of proliferation, or down-regulation of cytokine expression (IL-2, IFN) [111, 112].

The expression of PD-L1 on the cell surface of multiple tumor types including ovarian cancer, renal cell carcinoma and melanoma has been regarded as a potential prognostic biomarker whereby high tumoral expression correlates with poorer outcomes [113-115]. A number of studies have revealed that PD-L1 expression in human melanocytic cells strongly correlates with the presence of tumor-infiltrating lymphocytes (TILs). Indeed, 98% of PD-L1 expressing tumors were associated with TILs while 28% of PD-L1 negative tumors had evidence of TILs. Of further interest, interferon-γ, a primary inducer of PD-L1 expression, was found at the interface of PD-L1 (+) tumors and TILs and was absent in PD-L1 (-) tumors; this suggests that TILs trigger their own inhibition by releasing cytokines that drive the expression of tumor PD-L1. Consistent with this hypothesis, OS of patients with PD-L1 (+) metastatic melanoma was significantly longer than that of patients with PD-L1 (-) metastatic melanoma [116]. A considerable number of anti-PD-1 (nivolumab, pembrolizumab) and anti-PD-L1 (MPDL3280A, MEDI4736, others) monoclonal antibodies have been engineered to date and some have been approved and made available for clinical application after promising results in several trials.

3.4.1. Nivolumab

Nivolumab (Opdivo, BMS) is a fully humanized immunoglobulin G4 (IgG4) monoclonal antibody engineered to target the PD-1 protein. The first clinically promising results came from a phase I/II study of 296 patients with a range of heavily pretreated malignancies, including 107 patients with advanced melanoma [108]. The highest dose of nivolumab that has been tested is 10 mg/kg, but no maximum tolerated dose has been identified [117]. After one year of further follow-up of the melanoma cohort, updated efficacy data showed a response rate of 31% for the entire group with an additional 7% having stable disease. At the pharmacodynamic level, the binding of nivolumab to its receptor was found to be dose independent over a large range of tested dose levels, and receptor saturation was maintained for several months even after discontinuation of the drug. This might explain the presence of durable responses: the median response was 24 months [117]. Long-term follow-up of patients who received nivolumab demonstrated unprecedented survival rates of 61% at 1 year and 44% at 2 years; the median OS in this heavily pretreated population was 16.8 months. Grade 3 or 4 treatment-related adverse events, of which three involved pulmonary toxicity, were observed in 32 (14%) of the 296 patients enrolled in the entire study [108]. Of note, the patterns, severity, and frequency of nivolumab-related adverse events were quite similar across all of the doses that were tested; however, the 10 mg/kg dose level was associated with higher grade 3/4 drug-related adverse events, leading to the decision to discontinue treatment. The overall incidence of immune-related side effects reached approximately 20%, with the most common being skin rash, pruritus and diarrhea. Other immune-related adverse events include pneumonitis, vitiligo, an increase of thyroid-stimulating hormone, hepatotoxicity and infusion reactions.

Further testing of nivolumab was done in another phase I trial of treatment-naïve patients and those refractory to ipilimumab, in combination with a peptide vaccine to melanoma antigens gp100, NY-ESO-1, and MART-1 [118]. The ORR was 25%. In ipilimumab-naïve patients, the ORR was 24% compared with 26% in ipilimumab-refractory patients. Additionally, the two groups had similar amounts of complete response (CR) with partial response (PR) + stable disease (SD) responses, 45% for ipilimumab-naïve patients and 47% for ipilimumab-refractory patients. These results strongly suggest that although CTLA-4 and PD-1 receptors coexist on the cell surface, they seem to play non-overlapping roles in modulating the adaptive immune response. While CTLA-4 works early in establishing an immune response by regulating the proliferation and migration of T-cells to the tumor, PD-1 and its ligand PD-L1 regulate the activation and proliferation of T-cells at the tumor site [81, 83].

These promising results led to a larger open-label phase III clinical trial comparing nivolumab with standard cytotoxic chemotherapy (dacarbazine or carboplatin/paclitaxel), in patients with metastatic melanoma whose disease progressed on ipilimumab or BRAF inhibitors (when applicable). In this study, the nivolumab arm again had a higher ORR (32%) than the chemotherapy group (11%). Interestingly, 36 of 38 responding patients maintained their responses for over 6 months of follow-up, indicating a potential for durable remissions with nivolumab [119]. In the first-line setting, 418 treatment-naïve patients who had metastatic melanoma but not a BRAF mutation were randomly assigned in a large

double-blind phase III trial (CheckMate 066) to receive nivolumab or cytotoxic chemotherapy (dacarbazine) in a 1:1 ratio [120]. The primary endpoint of this study was overall survival. Nivolumab was found to be significantly better in terms of response rate (40% *vs.* 13.9%), median PFS (5.1 months *vs.* 2.2 months) and 1-year overall survival (72.9% *vs.* 42.1%), translating to a 58% decreased risk of death with nivolumab. The median OS was not reached in the group receiving nivolumab while it was 10.8 months in the group receiving dacarbazine. Additionally, CheckMate 066 demonstrated that nivolumab produces high response rates with a rapid time to response (median of 2.1 months), which is equivalent to that of dacarbazine. Of further interest, the survival benefit was recorded across all prespecified subgroups, including those stratified by PD-L1 status (positive staining defined as at least 5% of tumor cells showing cell-surface PD-L1 staining of any intensity in a section containing at least 100 tumor cells that could be evaluated) [120].

Treatment-related adverse events of any grade were quite similar in the nivolumab cohort and the dacarbazine group (74.3% and 75.6%, respectively), with fatigue, nausea and pruritus being the most common. Interestingly, grade 3 or 4 side effects were reported less frequently in the nivolumab group than in the dacarbazine group (11.7% *vs.* 17.6%). Dacarbazine was chosen for the comparator arm because in many countries it represented the standard first-line treatment option until the concurrent approval of the combination of ipilimumab and dacarbazine for patients with metastatic melanoma not harboring a BRAF mutation [87]. Of note, although CheckMate 066 only recruited patients with melanoma with wild-type BRAF, earlier studies demonstrated that nivolumab is active regardless of the patient's BRAF mutation status [118, 119]. On the basis of these findings and in view of the limited treatment options for patients without BRAF mutations, the FDA approved nivolumab in December 2014 for the treatment of metastatic melanoma, either as a first-line option regardless of BRAF status or for patients who had previously been treated with ipilimumab and, if applicable, a BRAF inhibitor.

3.4.2. Pembrolizumab

Pembrolizumab (MK-3475, Lambrolizumab) is a high-affinity selective IgG4/kappa humanized monoclonal antibody devised to arrest the engagement of PD-1 and its ligands, PD-L1/PD-L2. Pembrolizumab was first evaluated in KEYNOTE-001, an open-label phase I dose escalation study that evaluated its safety and efficacy in 411 patients with various advanced solid tumors, including 294 patients with advanced melanoma. The patients with melanoma were further divided into ipilimumab-naïve (179) and ipilimumab-pretreated (115) groups [121, 122]. In this study, the three dose levels, 1 mg/kg, 3 mg/kg and 10 mg/kg, were well tolerated. The lowest dose with full potential for antitumor activity was 2 mg/kg given every 3 weeks. At a median follow-up of 14.8 months, the ORR was 33% and the complete response rate was 13.5% (95% CI, 8.2-20.5). Among patients who received the treatment as first-line therapy, the ORR was 45.1% (95% CI, 36.5-54.0) and the complete response rate was 14%. A high ORR was seen in patients with *BRAF V600* mutations and wild-type *BRAF*. Over the long term, there was a median PFS of 13.8 months and a median OS of 31.1 months in the entire cohort. The median duration of response was 28.2 months [123]. Unsurprisingly, the highest dose (10 mg/kg every 2 weeks) was associated with the greatest number of adverse events. Common low-grade events attributed to pembrolizumab were fatigue, rash, pruritus, and diarrhea. Grade 3 and 4 drug-related side effects occurred in 13% of patients.

More recently, results of KEYNOTE-002, a randomized international phase II trial assessing the efficacy and safety of two pembrolizumab doses compared with chemotherapy, became available, with a primary endpoint of PFS assessed as per RECIST v1.1 [124]. This study involved 540 patients with ipilimumab-refractory melanoma, either BRAFV600 wild-type or mutant-positive, previously treated with a BRAF inhibitor or MEK inhibitor or both, randomly assigned to receive pembrolizumab 2 mg/kg (180 patients), or pembrolizumab 10 mg/kg (181) or chemotherapy (179). At 6 months, PFS was significantly higher in the pembrolizumab arms, at 34% (95% CI 27–41) in the 2 mg/kg group, 38% (95% CI 31–45) in the 10 mg/kg group and 16% (95% CI 10–22) in the chemotherapy group. However, this study was not sufficiently powered to evaluate the equivalence of the two pembrolizumab doses. Taken together with the findings of KEYNOTE-001, the results published so far do not support one pembrolizumab dosing regimen over another [122, 125].

Despite the longer exposure of patients to chemotherapy, pembrolizumab appeared to be better tolerated, with fewer treatment-related adverse events, the most common being fatigue (1% *versus* 5%), edema and myalgia. Grade 3 and 4 adverse events were observed in 11% of patients in the pembrolizumab 2 mg/kg arm, 14% in the pembrolizumab 10 mg/kg arm and 26% in the chemotherapy arm. The usual immune-related adverse events including hypopituitarism, hypothyroidism, colitis, pneumonitis, hepatitis and nephritis occurred infrequently in the pembrolizumab arms and were mostly of grade 1 or 2 severity, generally treated with immunosuppressive therapy or treatment interruptions. Interestingly, no cases of new-onset type 1 diabetes mellitus were recorded. Finally, pembrolizumab also scored higher than chemotherapy on the scales of health-related quality of life, adding further real strength to its benefits. These results supported the FDA's accelerated approval of pembrolizumab for treatment of this population with a poor prognosis. Nevertheless, results of longer follow-up of patients in KEYNOTE-002 and other studies must be awaited to accurately determine the response duration.

KEYNOTE-002 is the largest randomized, controlled trial of an anti-PD-1 or anti-PD-L1 drug for ipilimumab-refractory advanced melanoma reported to date. The potential for further disease regression with PD-1 inhibitors after progression on ipilimumab may reflect the diverse mechanisms by which anti-CTLA-4 and anti-PD-1 therapies modulate the T-cell mediated immune response [124]. Indeed, a number of studies conducted at the molecular level have revealed that CTLA-4 blockade increases the peripheral T-cell repertoire thereby highly recruiting lymphocytes to infiltrate the tumor and broaden the immune response, while PD-1 inhibition activates intra-tumoral T-cell proliferation without a noticeable effect on the peripheral immune repertoire, thereby inhibiting adaptive immune resistance [126-134].

While ipilimumab was associated with major survival benefits in a number of studies, grade 3 or 4 adverse events, mostly immune-related, were observed in 23% of patients [86, 87, 135]. In KEYNOTE-006, an international, randomized, controlled, phase III study, 834 patients who had advanced melanoma were assigned in a 1:1:1 ratio to receive pembrolizumab (10 mg/kg) every 2 weeks or every 3 weeks or four doses of ipilimumab (3 mg/kg) every 3 weeks [136]. Primary endpoints were progression-free and overall survival. The estimated 6-month PFS rates were 47.3% for pembrolizumab every 2 weeks, 46.4% for pembrolizumab every 3 weeks and 26.5% for ipilimumab. Twelve-month survival rates were estimated at 74.1%, 68.4% and 58.2%, respectively, translating to a decrease in the relative risk of disease progression or death by 42% with the two pembrolizumab regimens, as well as a decrease in the relative risk of death by 31 to 37%. These benefits were extended to most of the analyzed subgroups except for an evident survival advantage in patients with PD-L1-negative melanoma, an observation that, according to the KEYNOTE-006 investigators, further supports use of pembrolizumab over ipilimumab.

It should be noted that patients who tested positive for PD-L1 constituted less than 20% of the recruited population. Additionally, the response rates were significantly better with pembrolizumab treatment every 2 weeks (33.7%) and every 3 weeks (32.9%), compared with ipilimumab treatment (11.9%). Responses appeared to be durable in all cohorts, being ongoing in 89.4%, 96.7% and 87.9% of patients, respectively, after a median follow-up of 7.9 months. Interestingly, the two pembrolizumab regimens showed similar efficacy; the absence of a dose-response relationship is consistent with results of KEYNOTE-001 and KEYNOTE-002 [125, 137-139].

The rates of grade 3-5 treatment-related adverse events were lower in the pembrolizumab groups (13.3% and 10.1%) than in the ipilimumab group (19.9%). Of note, patients harboring BRAF V600 mutations and having high lactate dehydrogenase (LDH) and/or symptomatic and/or rapidly progressive disease were not enrolled in KEYNOTE-006 if they had not previously received anti-BRAF targeted therapy because BRAF-targeted agents may induce a rapid clinical response in this group of patients [25]. Questions about the optimal sequencing of immunotherapy and BRAF or MEK inhibitors remain to be answered.

The KEYNOTE-006 investigators concluded by questioning the role of combination immunotherapy or even single-agent treatment for patients with minimal disease progression or mixed responses. The search for prognostic and predictive biomarkers to improve the selection of patients with advanced melanoma continues, and translational studies are ongoing.

4. COMBINATION THERAPY IN BRAF-MUTANT MELANOMA

The number of patients achieving complete responses with a BRAF inhibitor therapy is negligible, reaching a maximum of 5-7% of cases in the studies with the longest reported follow-up. Long-term survival data for patients treated with a single-agent BRAF inhibitor remain scarce, and experts believe this strategy has great potential to delay progression without producing long-term cures. A clear advantage of this therapy is the dramatic and rapid responses induced by BRAF inhibitors in a high percentage of cases of BRAF-mutant melanoma) despite an acquired resistance, and this has raised a number of questions about the optimal time and place of immunotherapy in the sequence of treatment [21]. Given the progress made with immune checkpoint inhibitors and the currently available results in terms of response rates and OS, a major dilemma has emerged with respect to whether the combination and/or sequential use of immune checkpoint inhibitors and targeted therapy would be more clinically relevant. In terms of clinical response, evidence to guide sequencing decisions remains scant, being limited to ad hoc analyses of small subsets of patients in large studies or analyses comparing data from across studies or using data from compassionate access programs or retrospective data [140, 141]. While we await pivotal randomized studies and in the absence of information on molecular mechanisms, treatment decisions will need to continue to be made by combining or sequencing agents with variable mechanisms of action and clinical profiles and non-overlapping toxicities (Table **2**).

4.1. Combined Blockade of BRAF and CTLA-4 Inhibitors

Both ipilimumab and vemurafenib have demonstrated improved OS and response rates in phase III trials. By itself, ipilimumab induces slow and modest but long-term responses and produces durable stable disease in only a few patients, while vemurafenib classically leads to transient responses but impressively rapid tumor regression [22, 142].

A considerable amount of data suggests that immunotherapy and BRAF inhibition may act synergistically [143]. Preclinical findings suggest that selective BRAF inhibition (through vemurafenib or analogs) or inhibition of the MAPK pathway does not affect lymphocyte function but instead improves T-cell viability and function and recognition of melanoma antigens and decreases production of the immunosuppressive IL-10, thereby boosting immune recognition of tumor-specific antigens through enhanced antigen presentation [144-149]. Furthermore, BRAF inhibition can stimulate the expression of melanoma-associated antigens, limits MAPK-driven immunosuppression, and induces tumoral infiltration by cytotoxic T cells [145, 150-152]. This suggests that a BRAF inhibitor given with immunotherapy could strongly sensitize melanoma cells, thereby enhancing response rates and reducing the odds of the development of resistance.

To examine this issue, Wilmott *et al.* evaluated the change in cytokine and chemokine levels in the serum of patients before and during therapy with MAPK inhibitors. Early in treatment, meaningful increases in IFNγ and TNFα were detected, denoting a heightened Th1 T-cell mediated response. This is of particular pertinence as this immune response has been previously found to predict improved responses to anti-PD1 therapy [153]. Moreover, Cooper *et al.* reported that adding a CTLA-4 antibody to a BRAF inhibitor early in the treatment scheme increases the ratio of cytotoxic T-cells to regulatory T-cells (FOXP3) in patients' tumor cells [154]. They also found that combining a BRAF inhibitor and an anti-PD1 or an anti-PDL1 in mice significantly

stimulates the production of IFNγ, TNFα and the cytotoxic T-cell activation marker granzyme B, while mice treated with BRAF inhibitor monotherapy showed no increase in granzyme B, suggesting functional inhibition in the T cells.

Paradoxically, the phase I/II trial of concurrent ipilimumab and vemurafenib therapy was prematurely terminated because of the dose-limiting hepatotoxicity of both drugs [155]. Furthermore, the combination of dabrafenib and ipilimumab in a phase I study resulted in bowel perforation in several patients when trametinib was added [156]. One might then argue that these drugs may be more tolerable when used one after the other (in either order) rather than in combination. For patients with high tumor burden, a synergistic partnership might be that a BRAF or MEK inhibitor could be used upfront to debulk tumors through a rapid response, and then an immunomodulatory therapy could be used to eradicate any residual melanoma cells [157, 158]. In a single-institution study of 28 patients with BRAF V600-mutated metastatic melanoma treated with vemurafenib or dabrafenib followed by ipilimumab, 43% of patients experienced rapid disease progression and died before completing all four induction doses of ipilimumab; their median OS was 5.7 months. The 57% of patients in whom there was slower disease progression and who were able to complete induction therapy with ipilimumab had a significantly longer median OS (18.6 months) [140]. These findings suggest that concomitant treatment with ipilimumab and a BRAF inhibitor may be more convenient than sequential therapy for patients with poor prognostic factors. More recently, Ackerman *et al.* reported that patients who received a BRAF inhibitor before ipilimumab had rapid progression with negligible response to immunotherapy [141].

While some preclinical data have suggested that use of an upfront BRAF inhibitor treatment can induce infiltration of the tumor by immune cells, this phenomenon seems transient and is paradoxically followed by an increase in regulatory T cells (FOXP3), markers of T-cell exhaustion (like TIM3 and PD-1), and expression of immunosuppressive ligands, particularly PD-L1 [145, 151, 154]. Interestingly, while response to treatment is due to the fact that an intratumoral immune reaction builds up on the BRAF inhibitor, the tumor itself acquires a novel immunosuppressive apparatus facilitated by resistance to the BRAF inhibitor, resulting in failure of immunotherapy. However, retrospective data from clinical trials suggest that use of a BRAF inhibitor following progression on ipilimumab is effective [159]. Although this finding is biased because of the retrospective nature of the data and the ad hoc nature of the analyses, it appears that response rates of ipilimumab are preserved but resistance to BRAF inhibitors constitutes a challenge. The sequential use of these two agents will probably have little impact on durable response rates, and upfront use of BRAF inhibitors may surprisingly neutralize the success of immunomodulators in this setting.

Another challenge is that when a patient initially responds to a BRAF inhibitor then develops a rapid progression, the patient may not have enough time to respond to subsequent immunotherapy [160]. On the basis of published studies including BRIM-3 and subgroup analyses of clinical trials, a number of negative prognostic indicators have been used to guide treatment decisions in various situations [25]. These include older age, poor performance status, male gender and elevated LDH levels (reflecting higher tumor burden) [161, 162]. Although these analyses suggest that upfront use of BRAF inhibitors may provide greater clinical benefit to patients with negative prognosticators, the question is whether we can confidently choose the optimal treatment plan based solely on baseline factors.

While we await larger studies, decisions regarding sequential therapy remain constrained by the patient's clinical status and disease burden. It has not been established whether sequential or concurrent therapy is preferable, nor has the optimal choice of BRAF inhibitor partner, since targeted inhibitors possess variable, sometimes unpredictable, "off-target" adverse effects.

4.2. Combined Blockade of BRAF and PD-1 Inhibitors

Immune checkpoint inhibitors have been demonstrated to be agents of hope for the treatment of advanced melanoma [163]. Similar to CTLA-4 inhibitors, their clinical activity is independent of BRAF status, and their safety profile, their relatively more rapid and higher response rates and their impressive PFS are superior to those of ipilimumab. Ten to 14 days of BRAF inhibition leads to differentiation of melanoma antigens with a considerable intratumoral infiltration of clonal CD8+ T-cells [7, 145, 151]. Interestingly, this is accompanied by downregulation of IL-6, IL-8, IL-1α and vascular endothelial growth factor (VEGF), with increased levels of PD-1 and PD-L1 levels, caused probably by IFN-γsecreting T-cells [133, 145, 164, 165]. PD-L1 expression would then be considered as a pathway of resistance to BRAF inhibition, as reported by Jiang *et al.* in cell lines [166].

The combination of a BRAF inhibitor with an antibody to PD-1 could therefore appear to be an appealing and potent strategy. More importantly, even patients with a high tumor burden and rapid disease progression can still derive clinical benefit [152]. The choice of the appropriate kinase inhibitor will supplement the immunostimulatory function of an immune checkpoint inhibitor with further immunosensitizing effect. This alliance might potentially ensure a long-term immune response and robust tumor eradication. A phase Ib, open-label study is evaluating the safety and pharmacology of MPDL3280A, an antibody to PD1, in combination with vemurafenib in patients with previously untreated BRAF V600-mutated metastatic melanoma (NCT01656642).

4.3. Combined CTLA-4 and PD-1/PD-L1 Blockade

Preclinical data suggest that dual blockade of pathways involved in immunotherapy has a synergistic antitumor effect, compared with inhibition of either pathway alone [167, 168]. Frequent observations that immune cells classically express multiple immune checkpoints with non-overlapping and complementary mechanisms of action strongly support this suggestion [169, 170]. Numerous phase I clinical trials combining ipilimumab and nivolumab have recorded tumor reduction rates of greater than 80% in approximately 40% of patients, including complete responses, with 75% to 79% of patients being alive at 2 years [171-173]. The adverse events profile resembles that of ipilimumab monotherapy, with earlier and more frequent occurrence and severity of adverse

Table 2. Summary of some combination therapy clinical trials.

Agent	Trial Name or Sponsor	Phase	Study Design	Patients' Settings	Status	Endpoints	ClinicalTrials.gov Identifier
Vemurafenib + Ipilimumab + DNE3 (PI3K-Akt inhibitor)		Phase I/II	Open Label	First-line for BRAF-mutant Metastatic Melanoma	Terminated	Safety/Efficacy Study OS (Phase II)	NCT02095652
Vemurafenib/Cobimetinib With or Without Bevacizumab	Melanoma Research Foundation Breakthrough Consortium	Phase II	Randomized Open label	Up to 3rd-line for BRAF-mutant Metastatic Melanoma or unresectable stage IIIc and clearly progressive.	Recruiting	Safety/Efficacy Study. Maximum tolerated dose Median PFS, OS, RR Correlative studies.	NCT01495988
LGX818 Plus MEK162 *Versus* Vemurafenib and LGX818 Monotherapy	COLUMBUS	Phase III	2-part, randomized, open label, multi-center, parallel group	Stage IIIB, IIIC, or IV, BRAF-mutant, Naïve untreated patients or patients who have progressed on or after prior first line immunotherapy.	Recruiting	PFS, OS, ORR, DCR	NCT01909453
Vemurafenib and cobimetinib administered alone and in combination	Sidney Kimmel Comprehensive Cancer Center	Phase III	Randomized Open-Label,	Treatment-naïve, unresectable stage III or stage IV, BRAFV600E/K mutant melanoma	Recruiting	Safety and tolerability. Antitumor response	NCT02427893
Vemurafenib+Cobimetinib	REPOSIT	Phase II	Open label Multicenter	Treatment-naïve, BRAF-mutant , unresectable Stage IIIc or Stage IV melanoma	Recruiting	PFS, Response Monitoring and Resistance Prediction With PET and Tumor Characteristics	NCT02414750
Vemurafenib+Cobimetinib	CONVERCE	Phase II	Open label	BRAF Mutated Melanoma With Brain Metastasis not treated with BRAF or MEK inhibitors.	Not yet recruiting	Complete or partial intracranial response rate ORR, OS, PFS	NCT02537600
Vemurafenib and High-dose Interferon Alfa-2b	12-107	Phase I/II	Open label Non-Randomized	1st-line for unresectable stage III or stage IV, BRAFV600E/K mutant melanoma	Recruiting	Safety, efficacy OS, PFS	NCT01943422
Vemurafenib		Phase II	single-arm, multicenter, Open-label	BRAF-mutant advanced melanoma	Recruiting	Kinetics and Effects on Intratumoral and Host Immunity. Implications for Combination With Immunotherapy	NCT01813214
Vemurafenib		Phase II	Single-arm open label	stage IV or unresectable stage III melanoma with documented BRAF V600 mutation	Recruiting	Effects on The Innate and Adaptive Immune Systems	NCT01942993

(Table 2) contd….

Agent	Trial Name or Sponsor	Phase	Study Design	Patients' Settings	Status	Endpoints	ClinicalTrials.gov Identifier
Vemurafenib		Phase II	Open-label, Single-arm, Multicenter	Stage IV BRAF-mutant melanoma with measurable brain metastases.	Ongoing not recruiting	Safety, Efficacy, OS, PFS	NCT01378975
Vemurafenib	ZeSS	Cohort	Observational, prospective	BRAF-V600 mutant Unresectable or Metastatic Melanoma Treated With Vemurafenib	Recruiting	Safety over 2 years	NCT01990248
Vemurafenib and Metformin		Phase I/II	Open-label, Interventional	1st-line for unresectable Stage IIIC and Stage IV BRAF-mutant melanoma	Recruiting	Safety, efficacy, OS, ORR,	NCT01638676
Vemurafenib+Helper peptide vaccine		Phase I/II	Non-randomized, Open-label	Measurable stage IIIB, IIIC, or IV melanoma. Patients with 3 or less brain metastases are included.	Not recruiting yet	Safety, Efficacy, Immunogenicity (infiltration of T cells into metastases pre and post-vaccination) of a Helper Peptide Vaccine Plus BRAF Inhibition (Mel61), PFS	NCT02382549
Vemurafenib+ White blood cell therapy (TIL, Aldesleukin, fludarabine, cyclophsophamide)		Phase I	Non-randomized Open label	BRAF-mutant measurable metastatic melanoma. 3 or less brain metastases are allowed.	Ongoing not recruiting	Safety	NCT01585415
Vemurafenib+ IL-2+Interferon		Phase I/II	Open label	Advanced or metastatic BRAF-mutant melanoma	Ongoing not recruiting	Safety, Efficacy, PFS	NCT01603212
Vemurafenib+ IL-2 (aldesleukin)	COMBAT	Phase II	Interventional Open label	Advanced or metastatic BRAF-mutant melanoma, may have received prior immunotherapy excluding IL-2.	Ongoing not recruiting	Safety, Efficacy ORR, OS, biomarkers	NCT01754376
Vemurafenib + Lymphodepletion = Adoptive Cell Transfer & High Dose IL-2		Phase II	Interventional Open label	Treatment-naïve or treated advanced or stage IV BRAF-mutant melanoma	Recruiting	Safety, Efficacy ORR, PFS,	NCT01659151
Vemurafenib + Decitabine	ML28604	Phase I/II	Open label	Any patient with metastatic V600EBRAF positive melanoma (any site) regardless of prior treatment.	Currently recruiting	Safety Efficacy	NCT01876641
Vemurafenib + Fotemustine	BeyPro1	Phase II	Interventional Open Label	Patients with unresectable Stage IV BRAF V600 positive **melanoma** who recurred while in treatment with **Vemurafenib**	Ongoing but not recruiting	PFS, Toxicity, OS, DCR, Time to progression of brain metastases	NCT01983124
Vemurafenib + TIL		Phase I/II	Open label	Unresectable stage III or IV melanoma, BRAF mutant, excluding brain metastases.	Recruiting	Safety Efficacy ORR, PFS, OS	NCT02354690

(Table 2) contd….

Agent	Trial Name or Sponsor	Phase	Study Design	Patients' Settings	Status	Endpoints	ClinicalTrials.gov Identifier
Vemurafenib+ PEG-Interferon	VEMU-PLINT	Phase I/II	Interventional Open label	Untreated and pretreated (no more than 1 treatment) patients with stage unreseactable IIIb-IV melanoma, BRAF mutant	Recruiting	Adverse events ORR	NCT01959633
Vemurafenib *vs.* Vemurafenib+ Cobimetinib	coBRIM	Phase III	Randomized double-blind Placebo-controlled	Previously untreated BRAFV600 mutant unresectable stage IIIc or IV melanoma.	Ongoing but not recruiting	PFS, OS, Safety, pharmacokinetics Quality of life	NCT01689519
Vemurafenib + Cobimetinib	CoBRIM-B	Phase II	Open label	BRAF-mutant melanoma with at least one measurable intracranial target lesion	Not recruiting yet	Safety, Efficacy ORR	NCT02230306
Vemurafenib+ Trientine		Phase I	Pilot Open label	Unresectable stage IIIC or IV BRAF mutant melanoma. Stable, treated brain metastases are allowed	Ongoing not recruiting	Safety, OS, PFS, ORR	NCT02068079
XL888 + Vemurafenib		Phase I	Multi-cohort Open label	Unresectable BRAF Mutated Stage III/IV Melanoma, treatment-naïve or previously treated.	Currently recruiting.	Efficacy and Safety. PFS, OS, ORR	NCT01657591
Vemurafenib + GDC-0973		Phse Ib	Interventional Non-randomized Open label	BRAFV600E mutant Previously Treated (But not with BRAF or MEK Inhibitors) or Previously Untreated for Advanced/Unresectable or stage IV Melanoma or Those Who Have Progressed After Treatment With Vemurafenib	Ongoing not recruiting	Efficacy Safety Dose escalation, PFS, OS, ORR	NCT01271803
Atezolizumab + vemurafenib or vemurafenib + cobimetinib		Phase Ib	Non-randomized Open label Multicenter Multi-cohort	Previously Untreated BRAFV600-Mutation Positive Metastatic Melanoma	Currently recruiting	Safety and pharmacology, dose-escalation	NCT01656642
PD- 0332991+ Vemurafenib	**OPTIMUM**	Phase I/II	Open label Interventional	Patients Suffering Metastatic **Melanoma** With BRAFv600 Mutated and CDKN2A Loss and Expression of Rb and Treated by **Vemurafenib**	Currently recruiting	Tumor Molecular Pharmacodynamic Evaluation and Pharmacokinetics	NCT02202200
Dabrafenib + trametinib		Phase II	Open label	BRAF V600E/K mutant Stage IIIC or Stage IV melanoma, who have failed at least 2 prior systemic treatment including a BRAF inhibitor and ipilimumab.	recruiting	ORR, PFS, OS, Adverse events	NCT02296996

(Table 2) contd….

Agent	Trial Name or Sponsor	Phase	Study Design	Patients' Settings	Status	Endpoints	ClinicalTrials.gov Identifier
IMCgp100 + Durvalumab (MEDI4736) or Tremelimumab or Durvalumab + Tremelimumab		Phase Ib/II	Randomized multi-center Open label	Advanced refractory cutaneous melanoma	Not yet open for recruitment	ORR, PFS, OS	NCT02535078
PLX3397 + Pembrolizumab		Phase I/II	Open label	Relapsed or refractory patients with advanced melanoma	Currently recruiting	Dose escalation, Safety, ORR	NCT02452424
Nivolumab + Dabrafenib and/or Trametinib		Phase I	Non-randomized Open label	Naïve or pre-treated metastatic melanoma or unresectable Stage III with BRAF V600E/K or NRAS mutations.	Not yet recruiting	Safety/ efficacy	NCT02357732
MEDI4736 + Dabrafenib + Trametinib or + Trametinib Alone		Phase I/II	Non-randomized Open label	IIIc (unresectable) or Stage IV BRAF V600E or V600K mutation-positive (cohort A) or mutation-negative (cohorts B and C)	Recruiting	Safety/ Tolerability	NCT02027961
Pembrolizumab (MK-3475) + Trametinib + Dabrafenib	MK-3475-022 KEY-NOTE-022	Phase I/II	Randomized double-blind	Advanced or metastatic cutaneous melanoma	Recruiting	Safety PFS, ORR	NCT02130466
Ipilimumab With or Without Dorgenmeltucel-L (HyperAcute Melanoma)		Phase II	Randomized Open label	Metastatic progressive refractory melanoma.	Recruiting	Safety Efficacy	NCT02054520
Intralesional BCG followed by Ipilimumab		PhaseI	Non-Randomized Open label	stage III (unresectable) or stage IV melanoma	Recruiting	Safety Efficacy Immunogenicity	NCT01838200
Nivolumab *versus* Nivolumab + Ipilimumab	**ABC**	Phase II	Randomized Open label	Stage 4 melanoma with at least 1 definitive brain metastasis that is ≥ 5mm and ≤40mm measurable per RECIST version 1.1 guidelines.	Recruiting	Intracranial response rate, ORR, PFS, OS	NCT02374242
Helper Peptide Vaccine (6MHP)+ Ipilimumab	**Mel62; 6PAC**	Phase I/II	Open label	Advanced or metastatic melanoma	Recruiting	Safety, Immunogenicity, Clinical Activity	NCT02385669
Nivolumab + Ipilimumab		Phase II	Open Label	Metastatic uveal melanoma	Recruiting	ORR, PFS	NCT01585194
Abraxane + Ipilimumab		Phase II	Open label	Refractory advanced melanoma previously treated but not with ipilimumab	Recruiting	PFS	NCT01827111
Intratumoral CAVATAK (Coxsackievirus A21) + Ipilimumab		Phase I	Open label	Advanced or metastatic melanoma	Recruiting	Safety Efficacy	NCT02307149

(Table 2) contd….

Agent	Trial Name or Sponsor	Phase	Study Design	Patients' Settings	Status	Endpoints	ClinicalTrials.gov Identifier
Stereotactic Radiation Therapy + Ipilimumab		Phase II	Open label	Melanoma metastatic to visceral organs	Recruiting	PFS, OS, ORR	NCT02107755
Ipilimumab + All-Trans Retinoic Acid		Phase II	Randomized Open label	Metastatic melanoma patients who are ipilimumab candidates	Not yet open for recruitment	Safety Efficacy	NCT02403778
Ipilimumab + HF 10 (Replication-competent HSV-1 Oncolytic Virus)		Phase II	Open label	Advanced or metastatic melanoma with non-visceral lesions.	Recruiting	ORR, Best overall response rate, PFS, adverse events	NCT02272855

events. A double-blind phase II study of 142 patients with treatment-naïve metastatic melanoma randomly allocated patients to receive ipilimumab combined with either nivolumab or placebo [174]. The ORR, the primary endpoint of the study, was 61% in the combination group whereas it was 11% in the ipilimumab-monotherapy group, with confirmed complete responses in 22% of patients in the combination group and none in the ipilimumab-monotherapy group. The median duration of response and the median PFS were not reached with the combination arm. Grade 3 or 4 drug-related adverse events were recorded in 54% of the patients receiving the combination therapy *versus* 24% of the patients receiving ipilimumab monotherapy. These results were confirmed in a large phase III study of 945 patients (CheckMate 067): the median PFS was 11.5 months with nivolumab plus ipilimumab, 2.9 months with ipilimumab and 6.9 months with nivolumab [175]. Notably, for patients with PD-L1 positive tumors, the median PFS reached 14.0 months in the combination group and in the nivolumab arm, while patients with PD-L1-negative tumors had a PFS of 11.2 months with the combination therapy and 5.3 months with nivolumab alone. These results, although encouraging and potentially practice-changing, open room for further questioning with regard to the undeniable toxicity, deaths, and as-yet-unproven survival gain with the combination immunotherapy. There is a pressing need to establish and validate biomarkers to better predict treatment outcomes, especially since even a proportion of patients who withdrew from the combination therapy continued to respond [132, 176].

FUTURE PERSPECTIVE

Capitalizing on the unprecedented breakthroughs discussed in this paper, the treatment of advanced melanoma will continue to evolve, with the goal of long-term disease control and possibly cure. All available data suggest that combination therapy, whether concurrent or sequential, is the best strategy for the foreseeable future. However, the efficacy and safety of such treatments must be confirmed in large-scale clinical trials. Additionally, more insights into the tumor microenvironment as well as immunologic regulators will probably improve our ability to decode the intertumoral and intratumoral molecular heterogeneity of melanoma. This advanced knowledge will inform decision-making about the best agents, the best combinations and the best timing of therapy.

CONFLICT OF INTEREST

The authors confirm that this article content has no conflict of interest.

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