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A Study To Estimate the Anti-Tumor Activity And Identify Potential Predictors of Response in Patients with Advanced Mucosal or Acral Lentiginous Melanoma Receiving Standard Nivolumab in Combination with Ipilimumab followed by Nivolumab Monotherapy

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A Study To Estimate the Anti-Tumor Activity And Identify Potential Predictors of Response in Patients with Advanced Mucosal or Acral Lentiginous Melanoma Receiving Standard Nivolumab in Combination with Ipilimumab followed by Nivolumab Monotherapy

The Melanoma Research Foundation Breakthrough Consortium (MRFBC)

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SCHEMA

Patients with advanced or metastatic mucosal melanoma (cohort A) and acral lentiginous melanoma (cohort B) eligible for treatment with nivolumab in combination with ipilimumab followed by nivolumab therapy will submit tissue blocks from tumors of malignant melanoma for histopathology review and immunohistochemistry analysis at GU-LCCC. Pretreatment blood will be drawn and stored in the MRFBC Virtual Repository at each participating institution. At the end of participation, samples will be sent to GU-LCCC for processing and storage. An optional pretreatment biopsy of an accessible tumor lesion will be performed in a subset of enrolled patients. Patients will receive nivolumab in combination with ipilimumab according to the standard FDA approved treatment regimen. Commercial drugs will be used in this protocol and will not be supplied by the study.

1.0 INTRODUCTION

1.1 Background

In 2014, it has been estimated that over 76,000 new patients with melanoma were diagnosed in the United States and over 9,700 succumbed to their disease. Over the past several decades, a significant increase in the incidence of melanoma has been observed. The annual increase in the incidence rate varies between regions throughout the world, but in general, the increase in the Caucasian population has been approximately 3% to 7% per year. The number of deaths due to melanoma has also increased in populations with light skin complexions throughout the world in the past four decades (1).

1.2 Mucosal Melanoma (MCM)

Primary mucosal melanoma represents approximately 1.3% of all melanoma diagnoses in the U.S. (2). While the incidence of cutaneous melanoma has increased over the past several years, the incidence of MCM remains relatively unchanged. Additionally, the pathogenesis of MCM is poorly understood in part due to its rarity, the endodermal origin of the mucosa and relative paucity of mucosal melanocytes compared to cutaneous tissues (3). Although *BRAF* mutation is a common somatic alteration in cutaneous melanomas, being documented in approximately 50% of tumors, it is relatively infrequent (3%) in MCM. On the other hand, 10-22% of patients with MCMs have tumors containing activating *KIT* mutations or amplifications (4-7). While vigilant dermatologic evaluation can lead to early detection and facilitate early treatment of patients with cutaneous melanoma, MCM often presents at a late stage, with a higher propensity for deep invasion and distant metastases. MCM is a heterogeneous group of diseases that is comprised of primary tumors arising in the head and neck (55.4%), anorectal (23.8%), vulvovaginal (18%), and urinary track (2.8%). Each subtype demonstrates distinct natural history

1.3 Acral Lentiginous Melanoma (ALM)

ALM is an uncommon variant of melanoma with an estimated incidence between 1-7% of all melanomas (8). The tumor most commonly arises from palmoplantar area and less commonly in subungual and mucous membrane areas. While the incidence is relatively low in Caucasians, ALM is more common in Hispanic, Asian and African populations, representing up to 50-70% of all melanomas in these groups (8-10). Somatic *KIT* aberration was found in 36% of ALM, with the majority being enhancement or amplification of KIT expression (6). Although somatic *BRAF* mutation rate has been reported to be up to 15-20%(11, 12), these results are likely confounded by non-acral lentiginous cutaneous melanoma at acral sites. In fact, a small Spanish study found that 87% of ALMs contain somatic alterations including *AURKA*, *CCND1*, *CDK4*, *NRAS* as well as somatic copy number variations, but no somatic *BRAF* mutations. A larger pool of samples is required to determine the nature of the molecular changes in ALM and their

clinical relevance (13).

1.4 Treatment of Metastatic Melanoma

Approximately half of patients with cutaneous melanoma have tumors with activating mutations in BRAF and another 20% have mutations in NRAS making these tumors sensitive to selective BRAF and/or MEK inhibitors. However, such small molecule inhibitors have limited to no use in patients with MCM or ALM where such mutations are rare. In contrast, activating *KIT* mutations are present in the tumors of 10-22% of patients with MCM and a much smaller percentage of patients with ALM. Further, while therapy with imatinib, a tyrosine kinase inhibitor, can inhibit a subset of activating *KIT* mutations, this treatment only produces an overall response rate of approximately 40%, a median duration of response of 4.7 months (2.6-27.1 months), and a median progression-free survival of 10-15 weeks (5, 14, 15) in such patients.

The use of biochemotherapy combinations has been extensively evaluated, as well. The results of the largest available Phase III trial comparing cisplatin, vinblastine, and dacarbazine (CVD) with concurrent rhIL-2 and interferon-α versus CVD alone, showed that biochemotherapy had slightly higher objective response rates (ORR) and significantly longer median progression-free survival (PFS) than CVD alone, but failed to show an improvement in median overall survival (OS). Given the lack of activity together with its associated toxicity and complexity, biochemotherapy combinations are generally not recommended for patients with metastatic melanoma (16).

Reported ORR using chemotherapy combination in the second-line setting has been approximately 10%. For example, in a Phase III randomized study, the ORR reported for a paclitaxel and carboplatin combination was 11%, without any impact on OS. Addition of bevacizumab to cisplatin and paclitaxel in a randomized Phase II study also did not meet the primary end point in PFS and OS(17, 18).

Recently, treatment options for patients with advanced melanoma have expanded greatly with the FDA approval in 2011-2013 of the CTLA4 blocking antibody, ipilimumab, and the highly selective inhibitors of *BRAF*V600E, vemurafenib and dabrafenib. In addition, the MEK inhibitor, trametinib, also received FDA approval in 2013 for the treatment of patients with BRAFV600E mutant melanoma. In addition the combination of dabrafenib and trametinib received FDA approval in 2014 based on a randomized Phase II study in which the combination showed superior efficacy relative to dabrafenib alone (19). Finally, the combination of vemurafenib + cobimetinib (a MEK inhibitor) received FDA approval in late 2015 based on an phase III trial showing the combination was superior to vemurafenib alone.

1.5 PD-1 inhibitor monotherapy in Metastatic Melanoma

Nivolumab (BMS-936558; anti-PD-1 monoclonal antibody) is a fully human monoclonal immunoglobulin (Ig) G4 antibody that binds to the PD-1 cell surface membrane receptor, a

negative regulatory molecule expressed by activated T and B lymphocytes. Inhibition of the interaction between PD-1 and its ligands promote immune responses and antigen-specific T cell responses to both foreign and self antigens. PD-1 receptor blockade by nivolumab is a new approach for immunotherapy of tumors. Results from a Phase 1/2 study (CA209-003) indicate that nivolumab is active in multiple tumor types including advanced melanoma, non-small cell lung carcinoma (NSCLC) and renal cell carcinoma (RCC) (20-22).

Pembrolizumab (MK-3475) is a selective humanized monoclonal antibody (mAb) of the IgG4/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2. The variable region sequences of a mouse antihuman PD-1 antibody were attached into a human IgG immunoglobulin with a stabilizing S228P Fc alteration, where the IgG4 subtype does not engage Fc receptors or activate complement, therefore avoiding cytotoxic effects of the antibody when it binds to the T cells that are intended to activate. Results from a Phase 1 study (Keynote-001) demonstrated that pembrolizumab is active in patients with advanced melanoma (23)

In CA209003 (MDX1106-03), the clinical activity of nivolumab was demonstrated in a variety of tumor types and across a range of doses (0.1 mg/kg, 0.3 mg/kg, 1 mg/kg, 3 mg/kg and 10 mg/kg). As of the clinical cut-off date of 05-Mar-2013, a total of 306 subjects with melanoma, RCC, and NSCLC have been treated with nivolumab. All subjects initiated treatment at least one year prior to analysis. A response of either CR or PR, as determined by investigator assessed tumor evaluations based on modified RECIST 1.0, has been reported at all dose levels. Among 107 patients with advanced melanoma who received nivolumab, the objective response rate was 33/107 (31% [95% CI 22, 41]). Responses occurred at each dose level, with 6/17 (35%), 5/18 (28%), 11/35 (31%), 7/17 (41%), and 4/20 (20%) melanoma subjects responding at 0.1, 0.3, 1, 3, and 10 mg/kg, respectively (24).The 1-, 2-, and 3-year survival rates were 63%, 48%, and 41%, respectively, based on Kaplan Meier estimates. The median overall survival (OS) was 17.3 months (95% CI: 12.5, 36.7). Overall survival at 3 mg/kg dose was 20.3 months (95% CI: 7.2, NE [not estimable]. The PFS rate in subjects with melanoma at 48 weeks was 38% [95% CI: 28, 47]) and at 96 weeks 29% [95% CI: 20, 39]. Median PFS was 3.7 months in all melanoma subjects (95% CI: 1.9, 9.3) and 9.7 months (95% CI:1.8, 16.4) for subjects treated at the 3 mg/kg dose level. In CA209037, the clinical activity of nivolumab was compared with investigator's choice of chemotherapy (ICC), dacarbazine or paclitaxel with carboplatin, in a 2:1 randomized, phase 3 trial in a total of 405 participants with unresectable or metastatic melanoma who progressed after ipilimumab, and BRAF inhibitor if they had BRAF (V600) mutation. With a minimum follow-up of 24 weeks, confirmed objective response rate was 31.7% (38/120) in nivolumab group versus 10% (5/47) in ICC group. At the time of the primary analysis, 87% (33/38) of nivolumab response were continuing on treatment without progression. Median progression-free survival was 4.7 months (95%CI 2.3-6.5) for the nivolumab group and 4.2months (2.1-6.3) for the ICC group (HR 0.82; 99.99% CI 0.32-2.05) (25)

Similarly, In KEYNOTE-002 (26), a randomized cross-over phase 2 trial in subjects with advanced melanoma who had confirmed disease progression after ipilimumab and if BRAF (V600) mutant positive, previous treatment with a BRAF or MEK inhibitor or both, 180 patients

was randomly assigned to pembrolizumab 2mg/kg, 181 patients to pembrolizumab 10mg/kg, and 179 patients to chemotherapy of choice (paclitaxel plus carboplatin, paclitaxel alone, carboplatin alone, dacarbazine, or temozolomide). With median follow-up duration of 10 months, the study showed improvement of median progression-free survival with hazard ratios of 0.57 (95%CI 0.45-0.73) for pembrolizumab 2mg/kg and 0.5 (95%CI 0.39-0.64) for 10mg/kg compared with chemotherapy, with 6-months PFS of 34% and 38% in pembrolizumab 2mg/kg and 10mg/kg respective, compared with 16% in chemotherapy control. Objective response rate was 21% and 25% in pembrolizumab 2mg/kg and 10mg/kg respectively, in comparison to 4% in chemotherapy control.

1.6 Combined Checkpoint Inhibitors in Metastatic Melanoma

Immune checkpoint blockade is a rapidly advancing therapeutic approach in the field of immuno-oncology, and treatment with investigational agents targeting this mechanism has induced regressions in several types of cancer. CTLA-4and PD-1 receptor are two important cellular targets that play complementary roles in regulating adaptive immunity. Whereas PD-1 contributes to T-cell exhaustion in peripheral tissues, CTLA-4 inhibits at earlier points in T-cell activation. In preclinical models, combined blockade of PD-1 and CTLA-4 achieve more pronounced antitumor activity than blockade of either pathway alone (27).

Ipilimumab is a fully humanized IgG1 monoclonal antibody (mAb) binding to the anti-cytotoxic T-cell lymphoma-4 antigen (CTLA-4). Ipilimumab is an approved therapy for metastatic melanoma (Yervoy® Prescribing Information, 2011) and has demonstrated improved overall survival as monotherapy and in combination with dacarbazine (28, 29). Ipilimumab has been studied in combination with multiple standard of care (SOC) therapies including chemotherapy for squamous and non-squamous NSCLC and radiotherapy for hormone resistant prostate cancer. Phase 3 studies are ongoing in NSCLC, small cell lung carcinoma (SCLC), and prostate carcinoma.

Ipilimumab has been shown to produce two-year survival of 24% and prolonged median overall survival in two randomized phase III trials (30, 31). A composite analysis of 12 clinical studies confirmed the potential long-term survival impact of ipilimumab. In this series, 1,257 patients were pretreated and 604 were previously untreated for metastatic disease. The dose of ipilimumab was 3 mg/kg for 965 patients and 10 mg/kg for 706 patients. The median overall survival for the whole patient population was 11.4 months. Most importantly, the survival curve reached a plateau of 22% at 3 years, which extended to 10 years, and it was independent of the dose. The most frequently reported adverse events (AEs) related to ipilimumab were immune-related, which could be severe and/or long lasting (28).

In the Phase 1 dose escalation study CA209-004, the combination of nivolumab and ipilimumab has been studied in subjects with unresectable or metastatic melanoma. In this study, a safe dose level for the combination of ipilimumab and nivolumab was established for the treatment of advanced melanoma. An objective response rate of 53% (9/17) was observed for patients treated with 3 mg/kg ipilimumab plus 1 mg/kg nivolumab administered every 3 weeks for four doses (induction) and subsequently continued every 12 weeks for up to eight doses (32).

In a Phase 2 double-blind randomized study, 142 patients with previously untreated advanced melanoma were randomized in a 2:1 ratio to either receive both ipilimumab (3mg/kg) and nivolumab (1mg/kg) every 3 weeks followed by nivolumab (3mg/kg), or ipilimumab (3mg/kg) and placebo every 3 weeks followed by placebo every 2 weeks (CA209-069), an objective response rate (ORR) of 61% (44/72) was observed in patients treated with the combination versus 11% (4/37) in ipilimumab monotherapy, with complete responses reported in 22% (16/72) in the combination group, and no patients in the ipilimumab monotherapy group. With a minimum follow-up period of 11 months, median PFS and median duration of response were not reached in the combination group, compared with mPFS of 4.4 months with ipilimumab monotherapy. Grade 3 or 4 AEs was higher in the combination group than with ipilimumab monotherapy (54% vs. 24%).(33)

In a Phase III double-blinded randomized study, 945 patients with previously untreated advanced or metastatic melanoma were randomized in a 1:1:1 ratio to receive nivolumab monotherapy, nivolumab in combination with ipilimumab, or ipilimumab monotherapy. While the study was not formally powered to detect statistical difference between the nivolumab monotherapy and the combination group, rather to compare the efficacy between nivolumab monotherapy and the combination independently to ipilimumab monotherapy group, ORR was observed in 43.7% (38.1%-49.3%) in nivolumab monotherapy, 57.6% in the combination group, and 19% in ipilimumab monotherapy. With a median follow-up ranging from 12.2 to 12.5 months, a median PFS of 6.9 months (95%CI:4.3-9.5), 11.5 months (95%CI: 8.8-16.7), and 2.9 months (95%CI:2.8-3.4) were observed in nivolumab monotherapy, nivolumab plus ipilimumab combination, and ipilimumab monotherapy respectively. Grade 3 or 4 treatment-related AEs occurred in 16.3% of nivolumab monotherapy, 55% in the combination, and 27.3% in the ipilimumab monotherapy group. (34) The result from CA209-069 has led to FDA approval of Nivolumab/Ipilimumab combination for *BRAF*-wild type advanced melanoma.

1.7 Immunotherapy in Metastatic MCM and ALM

Immunotherapy with HD-IL-2 produces durable benefit in 10% of patients with metastatic cutaneous melanoma. Little data is available about the value of HD IL-2 in patients with MCM and ALM. A multicenter, retrospective study in 2013 evaluating 33 patients with advanced MCM who were treated with ipilimumab found a treatment response rate roughly comparable to that seen in patients with cutaneous melanoma and a durable response rate of 6.7%(4.2-10.9%) (35). Another retrospective study of 71 subjects with advanced MCM who received ipilimumab as a part of European Expanded Access Program also demonstrated similar objective response rate of 12%, with a median progression-free survival of 4.3 months (95%CI:3.4-5.2)(36).

In a recent multi-institutional retrospective analysis of 60 patients with advanced acral and mucosal melanoma treated with anti-PD1 inhibitor monotherapy (nivolumab or pembrolizumab), including patients from published prospective trials, expanded access program and standard treatment (following pembrolizumab approval in 2014), with 77% of patients received prior ipilimumab treatment, objective response rate was 32%(15-54%) in ALM, and 23% (10-40%) in MCM respectively, comparable to the response rate in cutaneous melanoma. Half of patients (8/16) with objective response developed relapsed disease with median duration

of response of 12.9 months (95%CI: 2.1-15.9), and median progression-free survival of 3.9 months (unpublished MSKCC data; In Press)

Despite the potential treatment benefit, majority of patients with metastases from these rare variant melanomas either do not respond or only have only a transient response and therefore require additional or improved therapeutic options.

In a recent pooled analysis of two phase I (CheckMate 003 and 038) and three phase III studies (CheckMate 037, 066, and 067) that enrolled 86 patients with mucosal melanoma (among 889 treated patients) to receive nivolumab monotherapy, at median follow-up of 9.2months (0.3-62.5), a median PFS of MCM was 3.0 months (95% CI: 2.2-5.4) compared to 5.1 months (95% CI:3.9-6.1) for all treated patients. ORR was 23.3% (95% CI: 14.8-33.6) compared with 35.9% (95% CI: 32.7-39.1) for all treated patients, with CR in ~6% in both group. Median duration of response was not reached (NR) in mucosal melanoma group, and was 22 months (95% CI: 22-NR)in all treated patients. Data on the activity of the ipilimumab + nivolumab combination in patients with ALM and MCM remains lacking and therefore remains to be determined.

1.8 Correlative Studies

1.8.1 Biomarkers in Melanoma

Reliable predictive biomarkers for the use of immune checkpoint inhibitors are needed to identify pretreatment those patients most likely to respond and early on treatment assays could help identify mechanisms of tumor response and resistance necessary to improve therapy.

1.8.2 Mutational Burden in Metastatic Melanoma

Malignant melanoma contains the highest prevalence of somatic mutation per megabase, especially high base-pair mutation rate attributable to UV exposure, in comparison to other cancers. The highly variable prevalence of somatic mutations among each cancer genome, between 0.001 per megabase (Mb) to more than 400 per Mb, is likely contributed by known mutagenic exposure, mismatch repair machinery defects, or the duration of cellular damage/replication error of passenger mutations between the fertilized egg to the time of samples analysis in cancers arise from self-renewing tissues (37, 38).

High mutational burden correlated with overall survival in patients with cutaneous melanoma treated with ipilimumab (39) or lung cancer treated with anti-PD1 (40).

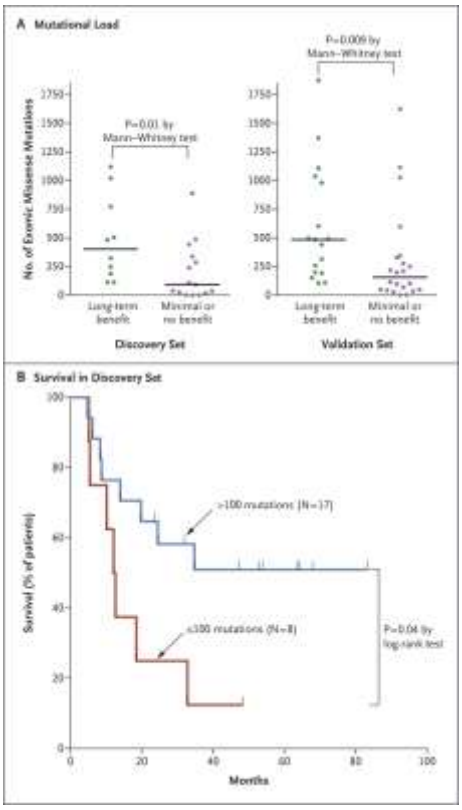

Figure 1 Mutational Landscape of Tumors According to Clinical Benefit from Ipilimumab Treatment. Panel A shows the mutational load (number of nonsynonymous mutations per exome) in the discovery and validation sets, according to status with respect to a clinical benefit from therapy. Panel B depicts the Kaplan–Meier curves for overall survival in the discovery set for patients with more than 100 nonsynonymous coding mutations per exome and patients with 100 or fewer mutations(39).

Although the predictive markers research and discovery in cutaneous melanoma has made great progress, there is a paucity of data on the biomarkers predictive of response in patients with MCM and ALM receiving checkpoint inhibitor therapy and very little data in any patient population receiving combination CTLA4 and anti-PD1 inhibitor therapy. Furney et.al observed an average of twelve to sixty-six non-synonymous single nucleotide variants (SNV) in whole exome sequencing of both MCM and ALM (41, 42), at least 5-10 fold less mutational load than typically seen in cutaneous melanomas. Paucity of data from next-generation whole gnome/exome sequencing in MCM and ALM, with less than 10 study subjects from each disease, limits analytical power and is subjected to ascertainment bias.

A preliminary study on whole exome sequencing of anorectal, nasopharyngeal and vulvovaginal MCM demonstrated unique mutational pattern between MCM and cutaneous melanoma. Additionally, analysis of each MCM subgroup could also distinguish each subset of MCM by different mutational profiles (Gorden et al; SMR 2015)

1.8.3 Oncogenic Driver Mutation in MCM and ALM

The Cancer Genome Atlas project (TCGA) has described an important genomic landscape of cutaneous melanoma through next-generation analysis of DNA, RNA and proteins of 333 primary and/or metastatic melanoma and categorized both genomic and transcriptomic landscape signature of cutaneous melanoma with distinct genomic and transcriptomic alterations. However, no patient with MCM or ALM was included in the study of 331 patients in the TCGA analysis. Previous report has suggested *MUC2, MUC4, TNR, MED1, C17orf74, VN1R4* and *ZNF717* as potential driver mutations in ALM, beside *BRAF*, *TP53* and *KIT,* while authors did not detect new recurrent SNV besides *KIT, PTEN* and *TPR* in MCM cohort(41).

1.8.4 Tumor Infiltrating Lymphocytes in Metastatic Melanoma

Extensive studies on metastatic colorectal cancer has demonstrated that a new scoring system based on the two lymphocytes populations, CD3+/CD45RO+, CD3+/CD8+, or CD8+/CD45RO+, as well as density of immune cells infiltrates at the center of the tumor and its invasive margin, described as Immunoscore, could accurately separate a group of patients with high Immunoscore with improved DFS, and OS from those with low Immunoscore where the histopathological staging system cannot (43-45). Increased number of pre-treatment CD8+ T lymphocytes, and PD-L1 expression at the invasive tumor margin of advanced cutaneous melanoma metastases also predicted improved treatment outcome in patients receiving single agent therapy with the PD-1 inhibitor, pembrolizumab (46, 47).

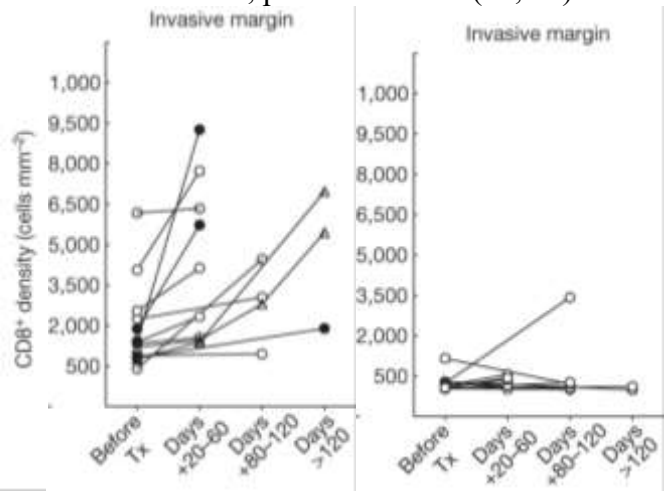

Figure 2. $CD8^+$-cell density at the invasive margin in samples from all responders (**left**; *n* = 13) and progressors (**right**; *n* = 12) who received a biopsy before and during treatment. Filled circle indicates complete response; open circle indicates partial response; triangle indicates delayed response(47).

Further, there appeared to be an association between tumor response and clonality of the immune infiltrate based on ImmunoSEQ assays (Adaptive Biotechnologies). These findings suggested that response to single agent anti-PD1 therapy was related to the presence within the tumor of clones of tumor antigen-specific CD8 T cells primed to be unleashed by PD1/PDL1 blockade.

1.8.5 Tumor's Neoantigen Epitopes Discovery in Metastatic Melanoma

Because antigen presentation via MHC class I molecule as well as cytotoxic T-cell recognition

and binding is essential for immune checkpoint inhibitors to function, computational analyses from whole exome sequencing have generated putative mutant and non-mutant peptides from each non-synonymous missense mutation. An algorhythmic computational method was able to predict that the somatic neoantigen epitope formed from mutant peptides, bound patient-specific MHC class 1 molecules with higher affinity (≤500nM) and strength.

Further, a number of immunogenic peptides were discovered among patients who achieved long-term clinical benefit. The number of 9- to 10- amino acid immunogenic neoantigens with (≤500nM binding affinity for HLA class I molecules (using patient-specific nonsynonymous mutation and HLA types) are also associated with clinical benefit to CTLA-4 monoclonal antibody therapy(39, 48). Interestingly, while both neoantigen loads and mutational burden are independently correlated with clinical benefit to CTLA-4 monoclonal antibody, both mutational load and neoantigen load were strongly correlated to each other (Spearman rho = 0.97, $p<0.001$) (48). Although prior study proposed a panel of shared neoantigen peptides among subjects with long-term clinical benefit from CTLA-4 monoclonal antibody (39), subsequent studies failed to identify recurrent or shared neoantigens or neoantigen epitopes that could predict response to CTLA-4 monoclonal antibody therapy. Instead, majority of neoantigen epitopes identified through exome sequencing appears to be private events without obvious shared recurrent domains among responders (48).

Based on preliminary analysis of low mutational burden in MCM And ALM, it is important to assess whether MCM and ALM low mutational burden translate into low number of neoantigen loads, and whether predicted neoantigen peptides burden in variant melanomas have any clinically meaningful association with clinical benefits to combined immune-checkpoint inhibitor therapy or not.

1.8.6 Gene Expression Profiling in Metastatic Melanoma

High-throughput gene expression profiling has recently discovered several important findings in patients with melanoma.(49-53). Next-generation sequencing (NGS) technology using RNA sequencing (RNA-Seq) provided unbiased determinations of RNA transcript enrichment and detection of novel transcripts with high sensitivity and specificity. It also gave broader dynamic range of detection and allowed detection of rare and low-abundance, yet potentially important, transcripts from the RNA samples. Information on gene expression profiling including the level of expression, landscape of transcript isoform variants, and the enrichment of specific RNA isoforms affect melanoma tumorigenesis, organ-specific metastasis, malignant transformation and inform intratumoral heterogeneity (54-56). This information is unobtainable by standard exome or genome sequencing alone. Additionally, Van Allen demonstrated that prediction of neoantigen peptides based on WES alone overestimated 6320 neoantigens without RNA-sequencing data, and missed 166 neoantigen that are expressed in the tumor, likely due to post-transcriptional RNA processing. Moreover, the authors found that a large proportion of neoantigen predicted by WES are expressed at negligible levels in the patients' tumors as determined by the TCGA database(48).

While considerable information from RNA-Seq expression and methylation data are available

for cutaneous melanoma from the Cancer Genome Atlas (TCGA) database and other publicly available domains, including remarkable sub-classification of cutaneous melanoma according to their transcriptomic expression levels and patterns (57), there is no transcriptomic information on MCM and ALM gene expression available in literature.

1.8.7 Gut Microbiota and Immune Checkpoint Inhibitor Therapy

Recent breakthrough studies have demonstrated that, in mouse models, certain intestinal microbiomes, i.e. Bacteriodes fragilis and Bifidobacterium sp. altered the response of cutaneous melanoma in mouse model to the anti-PD-1 and anti-CTLA-4 monoclonal antibodies. Further studies into Bifidobacterium demonstrated that the bacteria alters dendritic cell activity, which led to improvement of tumor-specific CD8+ T cell activity, and delays in melanoma growth (58, 59). It is not clear whether intestinal microbiomes in variant melanoma have similar or distinct immunomodulatory effects against tumor and affect the activity of immune checkpoint blockades.

1.8.8 Circulating Cell-Free Tumor Mutant DNA in Metastatic Melanoma

Successful techniques of circulating cell-free tumor-specific mutant DNA recovery have been established with various detection methods, from restriction enzyme digestion and selective priming of mutant alleles (60), BEAMing (Beads, Emulsion, Amplification, and Magnetics) (61), and droplet digital PCR (ddPCR) (62) in cutaneous melanoma with known driver *BRAF*, *NRAS* or *KIT* mutations. However, identifiable driver mutations are detected in only 10-30% of variant melanoma, identification of potential circulating tumor-specific mutant DNA as circulating biomarkers in variant melanoma whose tumors are often identified as *BRAF/NRAS/KIT*-wild type is an unmet need.

1.9 Study Rationale

Immunotherapy with HD-IL-2 has produced durable benefit in 10% of patients with metastatic cutaneous melanoma. The antitumor activity of IL-2 has been limited at least in part by immunosuppressive and immune-regulatory forces within the tumor microenvironment. Antibodies against CTLA4 (e.g. ipilimumab), PD1 and its ligand (PD-L1) produced long-term benefit in approximately 20-40% of patients with advanced melanoma. In addition, PD1 pathway blockers have shown anti-tumor activity in a variety of advanced solid tumors, including renal cell carcinoma, non-small cell and small cell lung carcinoma, bladder cancer, head and neck cancer, Merkel cell carcinoma and a rapidly expanding list of other tumors. In addition, the combination of ipilimumab with the anti-PD1 antibody, nivolumab, has shown tumor responses in up to 60% of patients with advanced melanoma (34). These findings have led to FDA approval of ipilimumab and nivolumab as an indication for treatment of patients with advanced melanoma and nivolumab for treatment of patients with advanced non-small cell lung carcinoma, with approvals of nivolumab and other PD1 pathway blockers either alone or in combination anticipated in other cancer types in the near future. While these data are exciting, few if any patients enrolled to this study had metastatic MCM or ALM. Only two retrospective analyses evaluated the efficacy of ipilimumab in patients with MCM with the overall objective response rate of 6-10% (35, 36), with no prospective immunotherapy studies conducted in MCM or ALM-specific population. Therefore the activity of the ipilimumab + nivolumab combination in these

subsets or patients remains unknown

Reliable predictive biomarkers for the use of immune checkpoint inhibitors are needed to identify pretreatment those patients most likely to respond and early on in treatment assays could help identify mechanisms of tumor response and resistance necessary to improve therapy. Although tumor PD-L1 expression in tumor confers higher treatment response rate, responses to nivolumab or nivolumab + ipilimumab alone were noted in 55% and 41% of patients, respectively, with PD-L1- tumors (63). Therefore, more reliable predictive biomarkers are needed.

Recently, extensive studies on metastatic colorectal cancer have demonstrated that a new scoring system as well as density of immune cells infiltrates at the center of the tumor and its invasive margin, described as Immunoscore, could accurately separate a group of patients with high Immunoscore with improved DFS, and OS from those with low Immunoscore where the histopathological staging system cannot. A recent study has also demonstrated relationship between degree of pre-treatmentCD8+ tumor infiltrating lymphocytes (TILs) infiltration and PD-L1 expression at the invasive margin of the advanced cutaneous melanoma and improved long-term clinical benefits in patients with advanced melanoma who received Pembrolizumab monotherapy (47). Further, there appeared to be an association between tumor response and clonality of the immune infiltrate based on a next-generation sequencing method used to evaluate T-cell receptor rearrangement pre- and in response to checkpoint inhibitor therapy. Also, high mutational burden correlated with overall survival in patients with cutaneous melanoma treated with ipilimumab or lung cancer treated with anti-PD1. However, the biology of MCM and ALM are distinct from cutaneous melanoma at multiple levels as mentioned in Section 1.2.1 &1.2.2. Consequently, the utility of predictive biomarkers developed for cutaneous melanoma remains unknown

1.10 Research Hypothesis

Treatment with nivolumab in combination with ipilimumab in patients with advanced MCM will result in an objective response rate of >25% with 90% confidence.

2.0 **OBJECTIVES**

Primary Objectives

2.1 To assess objective response rate (ORR, defined as complete response [CR] + partial response [PR] per investigator-assessed RECIST 1.1 criteria in patients with mucosal melanoma (MCM)

Secondary Objectives

2.2 To assess objective response rate (ORR, defined as complete response [CR] + partial response [PR] per investigator-assessed RECIST 1.1 criteria in patients with acral lentiginous melanoma (ALM)

2.3 To determine progression-free survival (PFS), and overall survival (OS) in each cohort

2.4 To determine whether pre-existing immune cells infiltrate, as well as Ki-67 and PD-L1 expressing cells at the invasive tumor margin correlate with clinical response to a combination of CTLA-4 and PD-1 blocking therapy

2.5 To evaluate subject's cancer genomic landscape using whole exome sequence profiling, identify the mutational pattern, and the frequency of somatic mutations and their relationship to tumor response in MCM and ALM, as well as subject's cancer genomic landscape using whole exome sequence profiling and utilize bioinformatic tools to translate mutations in exomes to identify specific driver mutations in MCM and ALM

Exploratory Objective

2.6 To evaluate tumor's putative neoantigen epitopes from le exome sequencing data and determine binding affinity to MHC class I molecules as a potential predictor of response to combined checkpoint inhibitors treatment

2.7 To determine clonal preservation, expansion, and selection of T-cell receptor rearrangement of tumor infiltrating lymphocytes before and after treatment with combined ipilimumab/nivolumab therapy as a correlative biomarker of treatment response

2.8 To identify MCM and ALM gene expression profiling pattern associated with treatment response or resistance to combined checkpoint inhibitors treatment

2.9 To identify relationship of specific gut microbiota in MCM and ALM with CD8+T cell function and anti-tumor immunity

2.10 To identify circulating cell-free nucleic acids and its pattern that might be associated with combined checkpoint inhibitor treatment response/resistance in order to further narrow the application of combined checkpoint inhibitors to those who will benefit the most

3.0 SELECTION OF PATIENTS

3.1 Eligibility Criteria

3.1.1 Patients must have histologically confirmed MCM or ALM that is metastatic or unresectable.

3.1.2 Patients must have measurable disease and be eligible to receive nivolumab in combination with ipilimumab treatment per institutional guidelines.

3.1.3 Patients must have a tissue block (or 26 unstained slides) available with adequate tumor to perform multiplex immunohistochemistry and nucleic acids analyses (i.e. whole exome sequencing) Patients with only a previous fine-needle aspirate are ineligible for enrollment.

3.1.4 Patients must be willing to donate a small amount of whole blood prior to treatment and during treatment for laboratory analysis.

3.1.5 Patients must give informed consent prior to initiation of therapy.
3.1.6 Patients must be ambulatory with good performance status (ECOG 0 or 1)

3.2 Ineligibility Criteria

3.21 Patients who do not have available tissue for immunohistochemistry and nucleic acids analyses.
3.22 Patients who have received prior immunotherapy for unresectable or metastatic disease.
3.23 Patients with evidence of active brain metastases, or active leptomeningeal disease are ineligible. Patients with a history of brain metastases must have completed treatment (i.e. surgery or radiation) prior to enrollment..
3.24 Patients with inadequate tissue for analysis.

4.0 REGISTRATION PROCEDURES

All patients must be registered with the Georgetown CRMO (Clinical Research Management Office) before enrollment to study. Prior to registration, eligibility criteria must be confirmed with the Georgetown University Multi-Site Study Coordinator.

All study documents will be redacted by the local site to remove Protected Health Information as defined by HIPAA, and will be labeled with subject initials and number. These materials will be uploaded by the local site to a secure password-protected cloud-based Georgetown Box portal. Access to Box will be provided by Georgetown-LCCC staff, who will receive and review the source documents and confirm eligibility. The local site will receive written notification from Georgetown-LCCC regarding eligibility.

To register a patient, the patient registration form should be completed by the research nurse or data manager and faxed or emailed to the Coordinating Center Attn:Multi- Site coordinator: fax-[redacted] along with the source documentation for the eligibility review.

5.0 STUDY DESIGN

5.1 Protocol Summary

Eligible patients will receive treatment with nivolumab in combination with ipilimumab followed by nivolumab monotherapy following the FDA approved schedule. Patients will have a diagnosis of mucosal or acral lentiginous melanoma confirmed by a pathologist at each participating institution. Baseline unstained tumor slides will be collected. Some patient will have optional fresh biopsies at week#6. Pretreatment blood will be drawn, Peripheral blood mononuclear cells (PBMCs), and serum,will be separated.. Stool will be collected at baseline and at

3 weeks into treatment. All tissues, peripheral blood, and stool samples will be stored separately in frozen storage units within the MRFBC Virtual Tissue Repository until shipped to GU-LCCC.

5.2 Duration of Therapy and Response Assessment

All patients will receive nivolumab administered IV combined with ipilimumab administered IV every 3 weeks for 4 treatment cycles per standard of care (Induction) then continue with nivolumab administered IV per standard of care until progression, intolerable toxicity, or a maximum of 48 doses, whichever comes first (Maintenance). Patients exhibiting complete response (CR) should continue nivolumab monotherapy at least 12 weeks beyond documentation of CR, if possible. Patients will be treated within participating sites within the MRFBC. Commercial drugs will be used in this protocol and will not be supplied by the study.

Patients will be evaluated for response at approximately 12 weeks following the initiation of treatment and then approximately every 12 weeks. Response will be evaluated by the study investigators using the Response Evaluation Criteria in Solid Tumors (RECIST) criteria ,v1.1. (See appendix C). Following completion of therapy, patients with evidence of SD, PR or CR will have CT scans every 3 months for 2 years, then every 6 months during year 3 and then yearly thereafter for years 4 and 5. Attention will be paid to the time of disease progression. Both responding and non-responding patients will be followed until death.

6.0 PATIENT MONITORING AND DATA SAFETY MONITORING

As this is a tissue collection protocol in patients receiving standard of care therapy, patient management will occur through the standard operating procedures of the individual participating institution in general accordance with the FDA approved regimen.

Case Report forms documenting 1) patient demographic and tumor characteristics, 2) extent of treatment, 3) SAEs, 4) efficacy parameters and 5) specimen collection forms will be filled out for each patient.

The Principal Investigator and the Co-Investigators will discuss accrual, unusual toxicities and any other protocol issues including safety monitoring at their regular institution-based disease group meetings and on monthly investigator teleconferences. organized by the MRFBC,.

The Georgetown Lombardi Comprehensive Cancer Center will be responsible for the data and safety monitoring of this multi-site trial. As this study is an investigator initiated study utilizing FDA approved drugs for which the PI does not hold the IND it is

considered a moderate risk study which requires real-time monitoring by the PI and study team and semi-annual reviews by the LCCC Data and Safety Monitoring Committee (DSMC)
All Severe Adverse Events (SAEs) are required to be reported to the Multi-Site Coordinator Office at G-LCCC for review by the DSMC. (see below)

In addition, all SAEs are required to be reported to the local IRB per IRB policy. Based on SAEs, the IRB retains the authority to suspend further accrual pending more detailed reporting and/or modifications to further reduce risk and maximize the safety of participating patients.

Additionally, all SAEs will be reported to BMS Worldwide Safety using BMS SAE forms. Please refer to additional details in Appendix E. The sponsor/investigators are required to reconcile SAEs reported in the clinical database with SAE cases transmitted to BMS Global Pharmacovigilance (GPV&E); [redacted]. BMS requests this to be done quarterly and prior to the database lock or final data summary.
A summary of the process for the sponsor/investigator is listed below:

- Sponsor/Investigator sends a request to BMS GPV&E for a "GPV&E reconciliation report". Requests for reconciliation should be sent to [redacted] The request should provide the BMS protocol ID, study title and PI, and sponsor/investigator protocol ID.
- BMS will send a report back to the sponsor/investigator. The data elements listed on the GPV&E reconciliation report will contain information the investigator can use for individual case identification. Cases on the list from BMS GPV&E should be compared to the SAE cases in the clinical database.

If the sponsor/investigator determines a SAE case was not transmitted to BMS GPV&E, the case should be sent immediately to BMS

Progress on the trial and the toxicities experienced will be reviewed by the G-LCCC Data and Safety Monitoring Committee every 6 months from the time the first patient is enrolled on the study. Results of the DSMC meetings will be forwarded to the G-LCCC IRB with recommendations regarding need for study closure.

A written copy of the DSMC recommendations will be given to the trial PI and the Georgetown IRB. If the DSMC recommends a study change for patient safety or efficacy reasons the trial PI must act to implement the change as expeditiously as possible. In the unlikely event that the trial PI does not concur with the DSMC recommendations, then the G-LCCC Associate Director (AD) of Clinical Research must be informed of the reason for the disagreement. The trial PI, DSMC Chair, and the LCCC AD for Clinical Research will be responsible for reaching a mutually acceptable decision about the study and providing details of that decision to the IRB. Confidentiality must be preserved during these discussions. However, in some cases, relevant data may be shared with

other selected trial investigators and staff to seek advice to assist in reaching a mutually acceptable decision.

If a recommendation is made to change a trial for reasons other than patient safety or efficacy, the DSMC will provide an adequate rationale for its decision. If the DSMC recommends that the trial be closed for any reason, the recommendation will be reviewed by the Associate Director for Clinical Research at G-LCCC. Authority to close a trial for safety reasons lies with the IRB, with the above described input from DSMC and the AD for Clinical Research.

7.0 EVALUATION AND MANAGEMENT OF TOXICITY

As this is a tissue collection protocol in patients receiving standard of care therapy, formal documentation of physician evaluation and management of toxicity is not necessary. However, it is suggested that patients with life-threatening or persistent, severe toxic reactions to either checkpoint inhibitors receive no further treatment with either agent.

8.0 CRITERIA FOR REMOVAL FROM STUDY

As this is a tissue collection protocol, there is no specific criteria for removal from the study with one notable exception; if the patient withdraws consent. Otherwise, patients will continue to be followed clinically and information such as date of progression and date of death will be documented.

9.0 LABORATORY STUDIES

The goal of this protocol are to acquire pretreatment tissue and blood from patients receiving standard of care nivolumab/ipilimumab combination for advanced MCM or ALM. Studies will be performed on both blood and tissue samples. Samples will be processed and stored at the Virtual Tissue Repository at each site for later batch shipping to the specified lab for analysis. Please refer to the Laboratory Manual in Appendix H for a complete instructions for specimen collection, processing, and storage. The tissue, blood and stool specimen shipment form can be found in Appendices A and B.

9.1 The materials required for this protocol are:

Reports and Forms Required

- MRFBC Pathology Material Submission Form
- A copy of the surgical pathology report.

Biological Material Submission

1. Available hematoxillin and eosin stained slides for pathology review
2. Tumor tissues from a primary melanoma excisional biopsy specimen or from a metastatic lesion
    a. A paraffin-fixed, formalin-embedded tumor block, or
    b. 16 unstained slides at 10micron and 10 unstained slides at 5 micron
3. Blood samples (see Section 9.3)
4. Stool samples (see Section 9.4)
5. (Optional) On-treatment fresh tumor biopsies at baseline and at 6-8 weeks of therapy

NOTE: An informed consent MUST be signed prior to the submission of any material for any correlative study, including mandatory diagnostic reviews.

NOTE: Appendix A and B must be submitted with each specimen submission at the end of the study. All samples, sample treatments, and collection time points are to be indicated on the form(s). Appendix A and B should accompany the samples to the central laboratory. Appendix C must be submitted with on-treatment biopsy specimens.

9.2 Studies to be performed on tissue

All participants will have either formalin-fixed, paraffin embedded (FFPE) tissue or fresh tumor biopsy collected at baseline. Fresh tumor biopsies will be obtained from safely accessible locations. Preferred biopsies include excisional or punch biopsy; 14-16 gauge core needle biopsies are acceptable if these are not feasible.

Please refer to Laboratory Manual for detailed instructions on specimen collection, processing, storage and shipment.

Baseline tumor specimen from each patient will be divided for three different biomarker analyses (i) Tumor Whole Exome Sequencing (WES) for analysis of mutational burden, mutational pattern, prediction of tumor neoantigen peptides that react to patient's Major Histocompatibility (MHC) class I molecules requires 8 unstained slides at 10microns thickness. (ii) Immunohistochemistry study and quantification of immune cells and PD-L1+ cells infiltration at the invasive tumor margin requires 10 unstained slides at 5 microns thickness. (iii) Baseline assessment of T-cell receptor (TCR) rearrangement of TILs using next-gen sequencing technique requires 8 unstained slides at 10-micron thickness. After the initial analysis, tumor blocks from each participant will be stored at the site in MRFBC Tissue Bank for potential later investigation.

Approximately 25% of patients enrolled in this protocol, n=15 (10 patients from MCM cohort and 5 patients from ALM cohort) are anticipated to have on-treatment fresh biopsies from safely accessible location during weeks 6, 7 or 8 into treatment. Each on-treatment biopsy will be

evaluated for CD8+, PD-L1+, and Ki-67+ cells at the invasive margin as a predictive biomarker of response (ii) TCR rearrangement in response to a combined immune checkpoint inhibitor therapy.

9.2.1 Pathology Review

A total of 26 unstained FFPE tumor tissue will be stored at each institution after enrollment. At the end of the study, all samples, including on-treatment biopsy samples, at participating institution will be batched and shipped to the Histopathology Shared Resource at the G-LCCC. Pathologic diagnosis will undergo central review by study dermatopathologist. A provided Haematoxillin & Eosin stained slide will be used to confirm physical characteristics of tumor samples and eligibility for the correlative studies. The following criteria are required for molecular analyses based on the TCGA Biospecimen Qualification Criteria:
1. Tumor nuclei are presented in ≥60% of the slide
2. Tumor necrosis is ≤ 20%
3. In the case of frozen tissue submission, the samples must be continuously frozen with less than 30 minutes between devascularization and freezing.

9.2.2 Tumor Whole Exome Sequencing

At the end of the study, unstained slides will be batched and shipped to Dr. William Robinson at University of Colorado where exome sequencing will be performed.

9.2.3 Assessment of Tumor's Mutational Burden and Driver Mutations

Analysis will utilize matched paired of (i) tumor, and (ii) normal adjacent stromal tissue from pre-treatment samples. Tissue dissection will be done by means of macrodissection. Paired normal tissue will serve as control and reference sequence for each patient's tumor and differentiation of germline mutations/polymorphisms from true somatic mutations. Tumor and normal stromal tissue will undergo DNA extraction according to standard FFPE DNA extraction protocol. DNA fragmentation, DNA library preparation, exome enrichment, automated cluster generation, paired-end sequencing, and base calling will be performed at University of Colorado at the end of the study.

Human exome sequencing from gDNA will include initial DNA sample QC using NanoDrop and Qubit. Exome sequences will be captured using the Agilent SureSelect Exome enrichment kit, along with associated QC, and libraries constructed using standard Illumina library preparation. Samples will be sequenced on an Illumina HiSeq2000 using PE100-125bp read length chemistry, designated 400x average coverage for the cancer and 200x for normal exomes with approximately 9-11 Gb output. Standard exome bioinformatic analysis will include mapping and alignment (BAM file), QC, coverage, sequence variation (SNPs/Indel report (.VCF file)), VCF annotations, functional prediction, and population frequency. Deliverable will include raw data FASTq and analysis files (.VCF)

Data cleanup, variant discovery, annotation, and evaluation will be performed at University of Colorado. Mutational load will be quantified as the number of non-synonymous somatic mutations per megabase (MB). Raw and annotated exome sequencing data will be available at MRFBC Cloud library for future data mining and comparison.

9.2.4 Assessment of MCM and ALM Gene Expression Profiling

Analysis will utilized matched paired of (i) tumor, and (ii) normal adjacent stromal tissue from pre-treatment and (optional) on-treatment samples. Tissue dissection will be done by means of macrodissection. Paired normal tissue will serve as internal control for each patient's tumor and tumor and normal stromal tissue will undergo RNA extraction according to standard FFPE DNA&RNA extraction protocol. This process will be performed simultaneously with DNA extraction using the same starting FFPE or frozen tissue material. Poly(A) enrichment, RNA fragmentation, library preparation, automated cluster generation, paired-end sequencing, base calling and mapping will be performed. Following RNA-sequencing, raw data will undergo standard bioinformatic analysis. BAM files will be converted to merged, demultiplexed FASTQs. Paired-end reads will be mapped to the UCSC hg19 human transcriptome using specific algorithm to allow alignment of sequence with single base changes due to point mutations. Expression level of genes will be quantified using Li et. al algorithm or equivalent method ($E_{i,j}=\log_2(TPM_{i,j}/10+1)$, where $TPM_{i,j}$ refers to transcript-per-million (TPM) for gene i in sample j, as calculated by RSEM(64) v1.2.3 in paired-end mode) Gene expression profiling analysis will be performed at MD Anderson Cancer Center under a direction of Drs. Michael Davies and Alexander Lazar at the end of the study.

9.2.5 Analysis of putative tumor neoantigen epitopes

Sequence annotation and variant data from each patient at pre-treatment baseline will be used for computational analysis to validate neoantigen peptides-MHC class I binding prediction algorithm. The prediction will generate a set of candidate T-cell epitopes that could bind to patient's MHC class I alleles, and determine binding affinity between patient's specific neoantigen epitopes and patient's MHC class I alleles *in silico*.

The analysis will be performed by Dr. Ming T. Tan at department of Biostatistics & Bioinformatics, Georgetown University-Lombardi Comprehensive Cancer Center.

Human Leukocyte Antigen Testing

High-resolution Human leukocyte antigen (HLA) typing information from each participant will be extracted from whole exome sequencing data obtained from whole blood (genomic DNA). The analysis will be performed at University of Colorado (Dr. William Robinson) at the end of the study.

Please refer to Laboratory Manual for detailed instruction on specimen collection, processing, storage and shipment.

9.2.6 Assessment of Tumor Immune Cells Infiltrates and PD1+ Cells at Invasive Margin

Planned analyses include immunohistochemistry (IHC) for PD-1, PD-L1+, CD28, CD3, CD4, CD8, CD45RO, FoxP3, Granzyme B, CD14, CD20, DAPI, Pan Melanoma Cocktail (HMB45, MART-1, Tyrosinase), and Ki-67+ expression, and quantification of immune cells infiltrates, and co-inhibitory/co-stimulatory marker-positive cells at the invasive tumor margin by multiplex immunohistochemistry and digital pathology system. The study will be performed at G-LCCC under a direction of Drs. Suthee Rapisuwon and Deborah Berry. Additional IHC markers may be added based on available information from MRFBC Consensus information.

Please refer to Laboratory Manual for detailed instruction on specimen collection, processing, storage and shipment.

9.2.7 Assessment and Monitoring of T-Cell Receptor Rearrangement

Planned analyses include assessment of TILs T-cell receptor β-chain genes using ImmunoSeq® platform. The analyses will assess the baseline TCR recombination (unique TCR sequences, clonal frequency distribution, and TCR diversity) between variable (V), joining (J), and diversity (D) segments that result in unique sequences in the antigen-binding site, as well as the effect of combined immune checkpoint inhibitor therapy on V(D)J rearrangement of TCR. Specimens for TCR rearrangement assay will be batched and sent from G-LCCC to Adaptive Biotechnology for analyses at the end of the study. Data analysis will be performed by Dr. Ming Tan at G-LCCC.

Please refer to Laboratory Manual for detailed instruction on specimen collection, processing, storage and shipment.

9.2.8 Shipping Procedures

At the end of study, fax the tissue specimen shipment form (Appendix A and Appendix C) in advance to attn: Deborah Berry All tissue samples are to be shipped overnight to:
G-LCCC Histopathology Shared Resource (HTSR)-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.
Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]

Materials are to be shipped SUNDAY THROUGH THURSDAY only. Do not ship samples the day before a Holiday.

9.3 Studies to be performed on blood

Peripheral blood samples will be collected on all patients prior to initiation of checkpoint inhibitors therapy and during treatment.

Serum will be used in circulating nucleic acids analyses. One additional 10ml tube of blood at each specimen collection step has been incorporated into the protocol to exclusively store as a part of MRFBC Virtual Repository and for future research.

At the conclusion of the therapy for each patient, specimens should be batched and transported to G-LCCC-Histopathology Shared Resource (HTSR) c/o Deborah Berry at the provided address for assessment of circulating cell-free nucleic acids. Circulating nucleic acid analyses will be done under the direction of Dr. Suthee Rapisuwon at GU-LCCC.

Please refer to Laboratory Manual for detailed instruction on specimen collection, processing, storage and shipment.

9.3.1 Assessment of Pretreatment and Serial Levels of Circulating Cell-Free Nucleic Acids

All patients will have plasma and serum collected at pre-treatment, every 6 weeks on-treatment, and at PD. Guidelines for isolation of plasma and serum are outlined in Section 9.3. Each sample will be linked to the corresponding tumor and labeled with non-identifiable coding system designated for each tumor.

Information on mutant DNA enriched in each tumor will be extracted from WES analyses. The analysis will assess circulating cell-free mutant DNA from the top most enriched mutations in each patient's tumor, in addition to a panel of cancer related genes. Circulating cell-free mutant DNA analysis will be performed on Illumina HiSeq system with TruSeq Nano Sample Preparation Kit (or equivalent system) to detect and quantify the circulating mutation load per ml of serum. The frequency of circulating mutant DNA will be assessed at baseline, at 6 weeks interval, and at RECIST-defined disease progression. The kinetics of circulating cell-free mutant DNA compared to baseline will be compared with radiographic response (per RECIST v.1.1) to establish the relationship between circulating mutant DNA burden and response to combined immune-checkpoint blockade.

Serum samples from each patient may be used to analyze for circulating non-coding RNA (i.e. miRNA) and exosomal substrates (i.e. protein, miRNA, snRNA)

We will attempt to establish predictive circulatory biomarker that could separate responders from non-responders in MCM and ALM cohorts

9.3.2 Sample Submission Schedule

- Blood samples will be taken ≤ 7 days prior to treatment of checkpoint inhibitors.
- **Two** 10ml Cell-Free DNA BCT® tubes (Streck™; Omaha, NE), **two** 10ml CPT™ Mononuclear cell preparation tube and **one** 10ml serum separators (red-top

SST) will be drawn at pre-treatment.

- **Two** 10ml Cell-Free DNA BCT® tube (Streck™; Omaha, NE) One 10ml CPT™ Mononuclear cell preparation tube and **One** 10ml serum separators (red-top SST) will be drawn every 6 weeks during treatment and at the time of disease progression.

9.3.3 Sample Preparation Guidelines

A batch of Cell-Free DNA BCT® tubes will be distributed to each participating site as the patients are being registered to the study. Each site will provide their own CPT™ Mononuclear cell preparation tube and serum separators (red-top SST) tubes. All samples will be processed locally at each participating institution according to the laboratory manual (see Section 9.3). PBMCs, serum and plasma will be stored at -80°C freezer at each participating institution. Specimen will be batched and shipped on dry ice overnight to G-LCCC HTSR (attn.Deborah Berry) at the end of the study. Specimen will be stored under MRFBC Mucosal & Acral Melanoma Tissue Bank.

9.3.4 Shipping Procedures

Fax the blood and stool specimen shipment form (Appendix B) in advance to [redacted] attn. Deborah Berry.

At the conclusion of the treatment, all samples are to be batched and shipped on dry ice overnight to G-LCCC-HTSR attn. Deborah Berry. All samples are to be shipped overnight to:

G-LCCC Histopathology Shared Resource (HTSR)-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.
Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]

Materials are to be shipped SUNDAY THROUGH THURSDAY only. Do not ship samples the day before a Holiday.

9.3.5 Collection

All blood samples will be stored in the MRFBC Virtual Specimen Repository and then batch shipped to G-LCCC.

9.4 Study to be performed on Stool

In this study, collection of stool will occur at baseline (within 7 days prior to starting treatment up to within 24 hours of the first treatment), at 3weeks into the treatment (+/- 5 days), and at the time of disease progression or study conclusion (+/- 7 days), as indicated on Appendix E.

At the conclusion of the therapy for each patient, specimens should be batched and transported to GU-LCCC-Tissue Culture Shared Resource (TCSR) c/o Dionyssia Clagett at the provided address in Appendix B. Gut microbiota analyses will be done under the direction of Drs. Jennifer Wargo and Michael Davies at MD Anderson Cancer Center.

Please refer to Laboratory Manual for detailed instruction on specimen collection, processing, storage and shipment.

9.4.1 Effects of Gut Microbiota and Anti-Tumor Activities and Effects of Combined Immune Checkpoint Inhibitor Therapy on Gut Microbiota in MCM and ALM

At the end of the study, genomic DNA will be isolated from fecal samples using QIAamp DNA Stool Mini Kit (Qiagen), or equivalent brand, following the manufacturer's instruction. Targeted qPCR system will be applied using either Taqman technology (for systems targeting all bacteria domain and Bacteroidetes/Prevotella group) or SYBRGreen (for different Bacteroides species) Quantitative –PCR will be performed using ABI PRISM 7300 qPCR system or equivalent.

9.4.2 Sample Submission Schedule

Stool samples will be collected at the following time points

- Less than 5 days prior to treatment with combined checkpoint inhibitors.
- Three weeks or C1W4 (+/- 5 days) of therapy
- At the time of disease progression or at the end of the study

9.3.3 Sample Preparation Guidelines

All samples will be processed locally at each participating institution according to the laboratory manual (see Section 9.4). Stool samples in Omnigene®-Gut kits will be stored at -80°C freezer at each participating institution. Specimen will be batched and shipped on dry ice overnight to GU-LCCC HTSR (attn. Deborah Berry) at the end of the study first, then batched & shipped to Dr. Jennifer Wargo at MD Anderson Cancer Center.

9.3.4 Shipping Procedures

Fax the blood and stool specimen shipment form (Appendix B) in advance to [redacted] attn. Deborah Berry.

At the conclusion of the treatment, all samples are to be batched and shipped on dry ice overnight to G-LCCC-HTSR attn. Deborah Berry. All samples are to be shipped overnight to:

G-LCCC Histopathology Shared Resource (HTSR)-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.
Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]

Materials are to be shipped SUNDAY THROUGH THURSDAY only. Do not ship samples the day before a Holiday.

10.0 STATISTICAL CONSIDERATIONS

10.1 Design and Objectives

All patients who meet eligibility criteria and enroll in the trial will receive the same nivolumab in combination with ipilimumab followed by nivolumab monotherapy regimen following the FDA approved schedule. Patients whose tissue block is not evaluable, either due to failure to be received by the GU-LCCC MRFBC Mucosal and Acral Lentiginous Melanoma Tissue Bank or due to insufficient tumor on the block, will be replaced.

The primary objective of this trial is to determine if treatment with nivolumab in combination with ipilimumab would produce ORR greater than historical 23% response rate in patients with MCM who received anti-PD1 monotherapy. Secondary objectives are to determine ORR, PFS and OS in each cohort, evaluate MCM and ALM genomic landscape by using whole exome sequence profiling and identify frequency of somatic mutations and relationship to tumor response, identify specific driver mutation in MCM and ALM. Exploratory objectives are to evaluate whether pre-existing immune cell infiltrates and tumors at the invasive tumor margin could predict clinical response to the combination of nivolumab and ipilimumab, as previously suggested in cutaneous melanoma, evaluate tumor putative neoantigen epitopes and determine binding affinity to MHC class I molecules as a potential predictor of response to combined checkpoint inhibitors treatment. The study will also explore relationship of specific local microbiota in MCM and ALM and response or resistance to the combination therapy. The final exploratory objective is to determine clonal preservation, expansion, and selection of TCR rearrangement of TILs before and during treatment with combined immune checkpoint inhibitors therapy as a predictive biomarker for treatment response.

The primary endpoint is best overall response (complete or partial response as defined in Section 10.3.1); patients will be followed for progression-free and overall survival.

Patients who enroll in this trial but do not receive at least one dose of nivolumab in combination with ipilimumab, or those for whom tissue is not submitted (see section 9.2) and therefore cannot be classified into a predictive factor group, will be deemed unevaluable for the primary endpoint of the study; available information on these patients will be described in the final report.

10.2 Sample Size Considerations

The primary endpoint is objective response rate (CR+PR) as defined earlier in the metastatic MCM population. ORR in the ALM populations will be exploratory. The response rate with current standard therapy is approximately 23-25%(65). Secondary clinical endpoints include, median PFS and OS, 6 month landmark PFS and 1 year landmark OS. Biomarker exploratory endpoints will include: 1) the relationship of CD8 T cell score at the invasive margin and tumor response for each malignancy and 2) the relationship with tumor mutational frequency using whole exome sequencing and response. Bioinformatic tools will be used in an attempt to identify recurring driver mutations in MCM (as well as ALM).

The sample size is based on the primary endpoint objective response rate (ORR). The null hypothesis that the ORR is at most 25% will be tested against a one-sided alternative. If the ORR is less than 25%, we will consider this therapy not worth pursuing. If it is more than 25%, the therapy is considered worth pursuing. With 39 patients, the study would have 90% power at a significance level of 10% if the true ORR is 45%. To take into consideration a potential non-evaluable or dropout rate of 20%, 49 patients with MCM (cohort A) will need to be enrolled into this study. The exact binomial calculation is used.

Given the less frequent occurrence of metastatic melanoma involving ALM subtypes, we will plan to enroll only 24 patients with ALM (cohort B). This should allow the accrual to both cohorts to finish at roughly the same time. Analysis of activity Cohorts B and the biomarker data will be primarily descriptive and exploratory.

Patients who enroll in this trial but do not receive the immune checkpoint inhibitor combination, or those for whom tissue is not submitted (see section 9.0) and therefore cannot be classified into a predictive factor group, will be deemed unevaluable for the primary endpoint of the study; available information on these patients will be described in the final report.

The overall accrual rate is anticipated to be 2-4 patients per month on each cohort, based on past experience among each institution in the Melanoma Research Foundation Breakthrough Consortium , resulting in expected accrual duration of about 20-24 months. The trial's primary analyses are planned to occur at 6 months after each patient has received the treatment.

10.3 Statistical Analysis for Correlative Studies

10.3.1 Best Overall Response

The proportion of patients achieving a response (best overall response; complete or partial response as per RECIST v1.1 criteria (outlined in appendix C) and exact binomial 95% CI will be calculated. Objective response rates (complete response + partial response) will be calculated separately for each cohort.

Among all enrolled patients, additional hypothesis-generating analyses will be undertaken to explore whether somatic mutational loads, difference in driver mutation profiles, level of pre-treatment CD8+ TIL and PD-L1 expressing cells at the invasive tumor margin, presence of tumor specific neoantigen, TCR clonality, gene expression profiles and enrichment of specific gut microbiota might be associated with responsiveness in order to further refine the optimal population for treatment with ipilimumab and nivolumab combination in MCM and ALM.

Categorical factors and continuous factors will be compared between responders and non-responders using Fisher's exact tests and two-sample Wilcoxon rank sum test respectively.

10.3.2 Time to Event

Progression-free survival, response interval and overall survival will be summarized using the Kaplan-Meier method.

Progression-free survival is defined as the time from the date of treatment initiation until the date that disease progression criteria are met or the date death without progression, or is censored at the date of last disease assessment without evidence of progression.

Response interval is defined among patients achieving a complete or partial response, from the date that response is achieved until the date that disease progression criteria are met or death without progression, or is censored at the date of last disease assessment without evidence of progression.

Overall survival is calculated from the date of treatment initiation to the date of death, or censored at date of last contact.

The association between somatic mutational load and time to event outcome in MCM and ALM can be assessed using log rank tests. Kaplan-Meier curves by mutation status will be presented.

10.3.3 Toxicity

The toxicity of nivolumab in combination with ipilimumab is well established in the melanoma patient population (9-11). As this treatment represents standard of care for this patient population, a formal analysis of toxicity is not considered as an endpoint for this study. As such, only the incidence of severe adverse events (immune related AEs) will be collected at the end of each patient’s treatment course. A single page form will be provided to collect this summary

information. (see Section 7).

11.0 RECORDS TO BE KEPT

The original signed consent form will be kept with the patient's other study documentation (e.g., the hospital or clinic chart, the case report form).

A data manager will be assigned to this protocol to ensure that data is collected and reviewed on a continuous basis.

The following forms will be submitted to the electronic data capture (eDC) system:

Forms:

1) Patient Registration Form
2) **On-Study Form** (within 2 weeks after initiation of treatment)
3) **Measurement Form** (at baseline and then every 12 weeks) until treatment completion. For patients with persistent SD or response at time of treatment discontinuation, subsequent imaging will be every 3 months until 2years from treatment initiation, then every 6 months during year 3, and then yearly thereafter for years 4 and 5 per standard of care)
4) **Treatment Form (within 4 weeks of decision to stop treatment for each subject) Documenting the number of doses of ipilimumab and nivolumab combination and nivo monotherapy**
5) **Toxicity Form listing Grade 3, 4 SAEs and Grade 2-4 iRAEs on each subject (within 12 weeks of decision to stop treatment)**
6) **Follow-up Form** (every 3 months)
7) **Pathology** (submitted along with pathology specimens at end of patient treatment)
8) **Off-Treatment Form** (within 2 weeks after treatment terminates)

12.0 PATIENT CONSENT AND PEER JUDGMENT

All institutional, NCI, FDA, state, and Federal regulations concerning informed consent and peer judgment will be fulfilled.

13.0 PUBLICATION PLAN

We anticipate that upon completion of this trial, the subsequent manuscript reporting the results will be published in the Journal of Clinical Oncology, or similar publication, within 12 months of the completion of the study analysis. Correlative science may be reported in a separate manuscript.

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Appendix A

MRFBC MUCOSAL AND ACRAL MELANOMA
TISSUE SPECIMEN SHIPMENT FORM

Instructions: Send overnight Sunday through Thursday to:

G-LCCC HTSR-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.
Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]

PROTOCOL:
Case No.: 00__ __ __ 01 – GU LCCC

Subject Initials __ __ __ (F,M,L) Site #:_____

Contact Name: _____

Contact Phone: _____

Date of Shipment: _____

Shipping Conditions: _____
(indicate dry ice, room temp, etc)
Sample Sent: _____
(indicate total amount: i.e. one core biopsy)
Site of Biopsy: _____
(indicate primary vs. metastasis and location)

Surgical Path Report Enclosed: YES NO

Formalin-Fixed Paraffin Embedded Biopsy: YES NO

Frozen Tissue Biopsy (on dry ice): YES NO

Shipping Notes:
Please fax form in advance to Bridget Haley
Please include a copy of the form in the shipping container.

Specimen Received by: _____
Date/Time Received: _____

Appendix B
MRFBC MCM and ALM Protocol
BLOOD & STOOL SPECIMEN SHIPMENT FORM
Instructions: Send overnight Sunday through Thursday to:
G-LCCC HTSR-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.
Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]

PROTOCOL:
Case No.: 00__ __ __ 01 – GU_LCCC

Subject Initials __ __ __ (F,M,L) Site #:_____

Contact Name: _____

Contact Phone: _____

Date of Shipment: _____

Type of Specimen BLOOD STOOL
(circle one)
Time point on study: _____
(E.g. Screening, C2D1, etc)
Shipping Conditions: Dry Ice_____
Sample Sent:

YES NO

Shipping Notes:
Please fax form in advance to [redacted], ATTN: Bridget L. Haley, RN
Please include a copy of the form in the shipping container.

Specimen Received By: _____

Date/Time Received: _____

Appendix C

(OPTIONAL) MRFBC MUCOSAL AND ACRAL MELANOMA
ON-TREATMENT
TISSUE SPECIMEN SHIPMENT FORM

Instructions: Send overnight Sunday through Thursday to:

G-LCCC Histopathology Shared Resource (HTSR)-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.
Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]

PROTOCOL:
Case No.: 00__ __ __ 01 – GU LCCC

Subject Initials __ __ __ (F,M,L) Site #:_____

Contact Name: _____

Contact Phone: _____

Date of Shipment: _____

Shipping Conditions: _____
(indicate dry ice, room temp, etc)

Date of Biopsy: _____

Sample Sent: _____
(indicate total amount: i.e. one core biopsy)

Site of Biopsy: _____
(indicate primary vs. metastasis and location)

Surgical Path Report Enclosed: YES NO

Formalin-Fixed Paraffin Embedded Biopsy: YES NO

Frozen Tissue Biopsy (on dry ice): YES NO

Shipping Notes:
Please fax form in advance to Bridget Haley

Please include a copy of the form in the shipping container.
Specimen Received by: _____
Date/Time Received: _____

Appendix D
RECIST v1.1 Response Criteria

Definitions of measurable, non-measurable, target and non-target lesions and objective response criteria based on the REVISED (January, 2009) RECIST 1.1 criteria. This Appendix is for reference only. The complete RECIST 1.1 criteria are found at: www.recist.com.

1. Definition of Measurable and Non-Measurable Lesions

Measurable disease. Measurable lesions are defined as those that can be accurately measured in at least one dimension (longest diameter to be recorded) as ≥20 mm by chest x-ray, as ≥10 mm with CT scan, or ≥10 mm with calipers by clinical exam. All tumor measurements must be recorded in millimeters (or decimal fractions of centimeters).

Note: Tumor lesions that are situated in a previously irradiated area might or might not be considered measurable. If the investigator thinks it appropriate to include them, the conditions under which such lesions should be considered must be defined in the protocol.

Malignant lymph nodes. To be considered pathologically enlarged and measurable, a lymph node must be ≥15 mm in short axis when assessed by CT scan (CT scan slice thickness recommended to be no greater than 5 mm). At baseline and in follow-up, only the short axis will be measured and followed.

Non-measurable disease. All other lesions (or sites of disease), including small lesions (longest diameter <10 mm or pathological lymph nodes with ≥ 10 to <15 mm short axis), are considered non-measurable disease. Bone lesions, leptomeningeal disease, ascites, pleural/pericardial effusions, lymphangitis cutis/pneumonitis, inflammatory breast disease, and abdominal masses (not followed by CT or MRI), are considered as non-measurable.

Note: Cystic lesions that meet the criteria for radiographically defined simple cysts should not be considered as malignant lesions (neither measurable nor non-measurable) since they are, by definition, simple cysts.

'Cystic lesions' thought to represent cystic metastases can be considered as measurable lesions, if they meet the definition of measurability described above. However, if non-cystic lesions are present in the same patient, these are preferred for selection as target lesions.

Target lesions. All measurable lesions up to a maximum of 2 lesions per organ and 5 lesions in total, representative of all involved organs, should be identified as target lesions and recorded

and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter), be representative of all involved organs, but in addition should be those that lend themselves to reproducible repeated measurements. It may be the case that, on occasion, the largest lesion does not lend itself to reproducible measurement in which circumstance the next largest lesion which can be measured reproducibly should be selected. A sum of the diameters (longest for non-nodal lesions, short axis for nodal lesions) for all target lesions will be calculated and reported as the baseline sum diameters. If lymph nodes are to be included in the sum, then only the short axis is added into the sum. The baseline sum diameters will be used as reference to further characterize any objective tumor regression in the measurable dimension of the disease.

Non-target lesions. All other lesions (or sites of disease) including any measurable lesions over and above the 5 target lesions should be identified as non-target lesions and should also be recorded at baseline. Measurements of these lesions are not required, but the presence, absence, or in rare cases unequivocal progression of each should be noted throughout follow-up.

2. Methods of measurements
All measurements should be taken and recorded in metric notation using a ruler or calipers. All baseline evaluations should be performed as closely as possible to the beginning of treatment and never more than 4 weeks before the beginning of the treatment.

The same method of assessment and the same technique should be used to characterize each identified and reported lesion at baseline and during follow-up. Imaging-based evaluation is preferred to evaluation by clinical examination unless the lesion(s) being followed cannot be imaged but are assessable by clinical exam.

Clinical lesions: Clinical lesions will only be considered measurable when they are superficial (e.g., skin nodules and palpable lymph nodes) and ≥10 mm diameter as assessed using calipers (e.g., skin nodules). In the case of skin lesions, documentation by color photography, including a ruler to estimate the size of the lesion, is recommended.

Chest x-ray: Lesions on chest x-ray are acceptable as measurable lesions when they are clearly defined and surrounded by aerated lung. However, CT is preferable.

Conventional CT and MRI: This guideline has defined measurability of lesions on CT scan based on the assumption that CT slice thickness is 5 mm or less. If CT scans have slice thickness greater than 5 mm, the minimum size for a measurable lesion should be twice the slice thickness. MRI is also acceptable in certain situations (e.g. for body scans).

Use of MRI remains a complex issue. MRI has excellent contrast, spatial, and temporal resolution; however, there are many image acquisition variables involved in MRI, which greatly impact image quality, lesion conspicuity, and measurement. Furthermore, the availability of MRI is variable globally. As with CT, if an MRI is performed, the technical specifications of the scanning sequences used should be optimized for the evaluation of the type and site of disease.

Furthermore, as with CT, the modality used at follow-up should be the same as was used at baseline and the lesions should be measured/assessed on the same pulse sequence. It is beyond the scope of the RECIST guidelines to prescribe specific MRI pulse sequence parameters for all scanners, body parts, and diseases. Ideally, the same type of scanner should be used and the image acquisition protocol should be followed as closely as possible to prior scans. Body scans should be performed with breath-hold scanning techniques, if possible.

PET-CT: At present, the low dose or attenuation correction CT portion of a combined PET-CT is not always of optimal diagnostic CT quality for use with RECIST measurements. However, if the site can document that the CT performed as part of a PET-CT is of identical diagnostic quality to a diagnostic CT (with IV and oral contrast), then the CT portion of the PET-CT can be used for RECIST measurements and can be used interchangeably with conventional CT in accurately measuring cancer lesions over time. Note, however, that the PET portion of the CT introduces additional data which may bias an investigator if it is not routinely or serially performed.

Ultrasound: Ultrasound is not useful in assessment of lesion size and should not be used as a method of measurement. Ultrasound examinations cannot be reproduced in their entirety for independent review at a later date and, because they are operator dependent, it cannot be guaranteed that the same technique and measurements will be taken from one assessment to the next. If new lesions are identified by ultrasound in the course of the study, confirmation by CT or MRI is advised. If there is concern about radiation exposure at CT, MRI may be used instead of CT in selected instances.

Endoscopy, Laparoscopy: The utilization of these techniques for objective tumor evaluation is not advised. However, such techniques may be useful to confirm complete pathological response when biopsies are obtained or to determine relapse in trials where recurrence following complete response (CR) or surgical resection is an endpoint.

Tumor markers: Tumor markers alone cannot be used to assess response. If markers are initially above the upper normal limit, they must normalize for a patient to be considered in complete clinical response. Specific guidelines for both CA-125 response (in recurrent ovarian cancer) and PSA response (in recurrent prostate cancer) have been published [JNCI 96:487-488, 2004; J Clin Oncol 17, 3461-3467, 1999; J Clin Oncol 26:1148-1159, 2008]. In addition, the Gynecologic Cancer Intergroup has developed CA-125 progression criteria which are to be integrated with objective tumor assessment for use in first-line trials in ovarian cancer [JNCI 92:1534-1535, 2000].

Cytology, Histology: These techniques can be used to differentiate between partial responses (PR) and complete responses (CR) in rare cases (e.g., residual lesions in tumor types, such as germ cell tumors, where known residual benign tumors can remain).

The cytological confirmation of the neoplastic origin of any effusion that appears or worsens during treatment when the measurable tumor has met criteria for response or stable disease is

mandatory to differentiate between response or stable disease (an effusion may be a side effect of the treatment) and progressive disease.

FDG-PET: While FDG-PET response assessments need additional study, it is sometimes reasonable to incorporate the use of FDG-PET scanning to complement CT scanning in assessment of progression (particularly possible 'new' disease). New lesions on the basis of FDG-PET imaging can be identified according to the following algorithm:

a. Negative FDG-PET at baseline, with a positive FDG-PET at follow-up is a sign of PD based on a new lesion.
b. No FDG-PET at baseline and a positive FDG-PET at follow-up: If the positive FDG-PET at follow-up corresponds to a new site of disease confirmed by CT, this is PD. If the positive FDG-PET at follow-up is not confirmed as a new site of disease on CT, additional follow-up CT scans are needed to determine if there is truly progression occurring at that site (if so, the date of PD will be the date of the initial abnormal FDG-PET scan). If the positive FDG-PET at follow-up corresponds to a pre-existing site of disease on CT that is not progressing on the basis of the anatomic images, this is not PD.
c. FDG-PET may be used to upgrade a response to a CR in a manner similar to a biopsy in cases where a residual radiographic abnormality is thought to represent fibrosis or scarring. The use of FDG-PET in this circumstance should be prospectively described in the protocol and supported by disease-specific medical literature for the indication. However, it must be acknowledged that both approaches may lead to false positive CR due to limitations of FDG-PET and biopsy resolution/sensitivity.

Note: A 'positive' FDG-PET scan lesion means one which is FDG avid with an uptake greater than twice that of the surrounding tissue on the attenuation corrected image.

3. Tumor response evaluation

3.1 Assessment of overall tumor burden and measurable disease

To assess objective response, it is necessary to estimate the overall tumor burden at baseline and use this as a comparator for subsequent measurements. Only subjects with measurable disease (defined as the presence of at least 1 measurable lesion) at baseline should be included. If the measurable disease is restricted to a solitary lesion, its neoplastic nature should be confirmed by cytology/histology.

3.2 Baseline documentation of "Target" and "Non-Target" lesions

All measurable lesions up to a maximum of 5 lesions total (and maximum of 2 lesions per organ) representative of all involved organs should be identified as target lesions and will be recorded and measured at baseline. Target lesions should be selected on the basis of their size (lesions with the longest diameter) and their suitability for accurate repetitive measurements (either by imaging techniques or clinically). A sum of the longest diameter (LD) for all target lesions will be calculated and reported as the baseline sum LD. The baseline sum LD will be used as reference to further characterize the objective tumor response of the measurable dimension of the disease.

All other lesions (or sites of disease) including pathological lymph nodes should be identified as non-target lesions and should also be recorded at baseline. Measurements are not required and these lesions should be followed as “present,” “absent,” or “unequivocal progression.”

4. Response Criteria

4.1 Evaluation of target lesions
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Complete Response (CR):	Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm.
Partial Response (PR):	At least a 30% decrease in the sum of LD of target lesions, taking as reference the baseline sum LD.
Progressive Disease (PD):	At least a 20% increase in the sum of LD of target lesions, taking as references the smallest sum of LD recorded since the treatment started or the appearance of 1 or more new lesions.
Stable Disease (SD):	Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as references the smallest sum LD since treatment started.

4.2 Evaluation of non-target lesions

Complete Response (CR):	Disappearance of all target lesions. Any pathological lymph nodes (whether target or non-target) must have reduction in short axis to <10 mm (the sum may not be “0” if there are target nodes) Disappearance of all non - target lesions and normalization of tumor marker level. All lymph nodes must be non-pathological in size (<10 mm short axis)
Non-Complete Response (non-CR)/Non- Progressive Disease (non- PD):	Persistence of 1 or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits
Progressive Disease (PD):	Unequivocal progression of existing non-target lesions defined as: Overall level of substantial worsening in non-target disease such that, even in presence of SD or PR in target disease, the overall tumor burden has increased sufficiently to merit discontinuation of therapy In the absence of measurable disease, change in non- measurable disease comparable in magnitude to the increase that would be required to declare PD for measurable disease. Examples include an increase in a pleural effusion from ‘trace’ to ‘large’, an increase in lymphangitic disease from localized to widespread Target Lesions: >20% increase in the SLD taking as reference the smallest SLD recorded since the treatment started (nadir) and minimum 5 mm increase over the nadir

Although a clear progression of “non target” lesions only is exceptional, in such circumstances,

the opinion of the treating physician should prevail and the progression status should be confirmed later by the review panel (or study chair).

4.3 Evaluation of best overall response

The best overall response is the best response recorded from the start of the treatment until disease progression/recurrence (taking as reference for progressive disease the smallest measurements recorded since the treatment started). The patient's best response assignment will depend on the achievement of both measurement and confirmation criteria.

For Patients with Measurable Disease (i.e., Target Disease)

Target Lesions	Non-Target Lesions	New Lesions	Overall Response	Best Overall Response when Confirmation is Required*
CR	CR	No	CR	≥4 wks. Confirmation**
CR	Non-CR/Non-PD	No	PR	≥4 wks. Confirmation**
CR	Not evaluated	No	PR	
PR	Non-CR/Non-PD/not evaluated	No	PR	
SD	Non-CR/Non-PD/not evaluated	No	SD	documented at least once ≥4 wks. from baseline**
PD	Any	Yes or No	PD	no prior SD, PR or CR
Any	PD***	Yes or No	PD	
Any	Any	Yes	PD	

* See RECIST 1.1 manuscript for further details on what is evidence of a new lesion.

** Only for non-randomized trials with response as primary endpoint.

*** In exceptional circumstances, unequivocal progression in non-target lesions may be accepted as disease progression.

Note: Patients with a global deterioration of health status requiring discontinuation of treatment without objective evidence of disease progression at that time should be reported as "symptomatic deterioration." Every effort should be made to

document the objective progression even after discontinuation of treatment.

For Patients with Non-Measurable Disease (i.e., Non-Target Disease)

Non-Target Lesions	New Lesions	Overall Response
CR	No	CR
Non-CR/non-PD	No	Non-CR/non-PD*
Not all evaluated	No	not evaluated
Unequivocal PD	Yes or No	PD
Any	Yes	PD
* 'Non-CR/non-PD' is preferred over 'stable disease' for non-target disease since SD is increasingly used as an endpoint for assessment of efficacy in some trials so to assign this category when no lesions can be measured is not advised		

5. Confirmatory measurement/Duration of response

5.1 Confirmation

The main goal of confirmation of objective response is to minimize the risk of overestimation of the response rate. This aspect of response evaluation is particularly important in non- randomized studies where response is the primary endpoint. In this setting, to be assigned a status of PR or CR, changes in tumor measurements must be confirmed by repeat studies that should be performed no less than 4 weeks after the criteria for response are first met. In the case of SD, follow-up measurements must have met the SD criteria at least once after study entry at a minimum interval of 6 weeks.

6. Specifications for radiological imaging

These notes are recommendations for use in clinical studies and as such these protocols for computed tomography (CT) and magnetic resonance imaging (MRI) scanning may differ from those employed in clinical practice at various institutions. The use of standardized protocols allows comparability both within and between different studies, irrespective of where the examination has been undertaken.

6.1 Chest X-ray

Measurement of lesions surrounded by pulmonary parenchyma is feasible, but not preferable as the measurement represents a summation of densities. Furthermore, there is poor identification of new lesions within the chest on X-ray as compared with CT. Therefore, measurements of pulmonary parenchymal lesions as well as mediastinal disease are optimally performed with CT of the chest. MRI of the chest should only be performed in extenuating circumstances. Even if IV contrast cannot be administered (for example, in the situation of allergy to contrast), a non-contrast CT of the chest is still preferred over MRI or chest X-ray.

6.2 CT

CT scans of the thorax, abdomen and pelvis should be contiguous throughout the anatomical

region of interest. As a rule of thumb, the minimum size of the lesion should be no less than double the slice thickness. Lesions smaller than this are subject to significant "partial volume" effects and such a lesion may appear to have "responded" or "progressed" on subsequent examinations, when in fact they remain the same size. This minimum lesion size for a given slice thickness at baseline ensures that any lesion appearing smaller on subsequent examinations will truly be decreasing in size.
The type of CT scanner is important regarding the slice thickness and minimum sized lesion. For spiral (helical) CT scanners, the minimum size of any given lesion at baseline may be 10mm, provided the images are reconstructed contiguously at 5 mm intervals. For conventional CT scanners, the minimum sized lesion should be 20 mm using a contiguous slice thickness of 10 mm.

The fundamental difference between spiral and conventional CT is that conventional CT acquires the information only for that particular slice thickness scanned, which is then expressed as a 2 dimensional representation of that thickness or volume as a grey scale image. The next slice thickness needs to be scanned before it can be imaged and so on. Spiral CT acquires the data for the whole volume imaged, typically the whole of the thorax or upper abdomen in a single breath hold of about 20-30 seconds. To view the images, a suitable reconstruction algorithm is selected, by the machine, so the data are appropriately imaged. As suggested above, for spiral CT, 5 mm re-constructions can be made thereby allowing a minimum sized lesion of 10mm.
Spiral CT is now the "standard" in most hospitals involved in cancer management in U.S., Europe, and Japan, so the comments related to spiral CT are pertinent. However, some institutions involved in clinical studies will have conventional CT, but the number of these scanners will decline as they are replaced by spiral CT.

Other body parts, where CT scans are of different slice thickness, (such as the neck, which are typically of 5 mm thickness) or in the young pediatric population, where the slice thickness may be different, the minimum sized lesion allowable will be different. However, it should be double the slice thickness. The slice thickness and the minimum sized lesion should be specified in the study protocol.
In subjects in whom the abdomen and pelvis have been imaged, oral contrast agents should be given to accentuate the bowel from other soft tissue masses. This is almost universally undertaken routinely.

Intravenous contrast agents should also be given, unless contra-indicated for medical reasons, such as allergy. This is to accentuate vascular structures from adjacent lymph node masses and to help enhance liver and other visceral metastases. Although in clinical practice its use may add little, in the context of a clinical study where objective response rate based on measurable disease is the endpoint, unless an intravenous contrast agent is given, a significant number of otherwise measurable lesions will not be measurable. In subjects in whom the disease is apparently restricted to the periphery of the lungs, for example, the use of intravenous contrast agents appears unnecessary, but the aim of a clinical study is to ensure lesions are truly resolving, and there is no evidence of new disease at other sites scanned, e.g., small metastases in the liver.

The method of administration of intravenous contrast agents is variable. Rather than try to institute rigid rules regarding methodology of administration of contrast agents and the volume injected, it is appropriate to suggest that an adequate volume of a suitable contrast agent should be given such that the metastases are demonstrated to best effect and a consistent method is used on subsequent examinations for any given subject.

All images from each examination should be included and not "selected" images of the apparent lesion. This is to ensure that if a review is undertaken, the reviewer can satisfy him/herself that no other abnormalities coexist. All window settings should be included, particularly in the thorax where lung and soft tissue windows should be considered.

When measuring lesions, lesions should be measured on the same window setting on each examination. It is not acceptable to measure a lesion on lung windows on 1 examination, then on soft tissue settings on the next. In the lung, it does not really matter whether lung or soft tissue windows are used for intra-parenchymal lesions, provided a thorough assessment of nodal and parenchymal disease has been undertaken and the target lesions are measured as appropriate using the same window settings for repeated examinations throughout the study.

6.3 MRI

MRI is a complex issue. MRI is entirely acceptable and capable of providing images in different anatomical planes. It is important therefore that when it is used lesions must be measured in the same anatomical plane using the same imaging sequences on subsequent examinations. MRI scanners vary in the images produced. Some of the factors involved include the magnet strength (high field magnets require shorter scan times, typically 2-5 minutes), the coil design and subject cooperation. Wherever possible, the same scanner should be used. For instance, the images provided by a 1.5T scanner will differ from those using a 0.5T scanner. Although a comparison can be made, it is not ideal. Moreover many subjects with advanced malignancy are in pain, so their ability to remain still for the duration of a scan sequence, in the order of 2-5 minutes is limited. Any movement during the scan time leads to motion artifacts, degradation of image quality such that the examination will probably be useless.

For these reasons, CT is at this point in time the imaging modality of choice.

The same imaging modality must be used throughout the study to measure disease. Different imaging techniques have differing sensitivities, so any given lesion may have different dimensions at any given time if measured with different modalities. It is therefore not acceptable to interchange different modalities throughout a study and use these measurements. It must be the same technique throughout.

6.4. Ultrasound

Ultrasound examinations should not be used in clinical studies to measure tumor regression or progression because the examination is necessarily subjective. It is not available for independent review at a later date, it has to be assumed that the hard copy films available represent a true and accurate reflection of events, which may or may not be the case. Furthermore, if, for example, the only measurable lesion is in the para-aortic region of the abdomen, and if gas in the bowel

overlies the lesion, the lesion will not be detected because the ultrasound beam cannot penetrate the gas. This subject will then become a protocol violator.

Appendix E.
Bristol-Myer Squibb Worldwide Safety Adverse Event Reporting

All Serious Adverse Events (SAEs) that occur following the subject's written consent to participate in the study through 100 days of discontinuation of dosing must be reported to BMS Worldwide Safety

If the BMS safety address is not included in the protocol document (e.g. multicenter studies where events are reported centrally), the procedure for safety reporting must be reviewed/approved by the BMS Protocol Manager. Procedures for such reporting must be reviewed and approved by BMS prior to study activation.

The BMS SAE form should be used to report SAEs. If the BMS form cannot be used, another acceptable form (i.e CIOMS or Medwatch) must be reviewed and approved by BMS. The BMS protocol ID number must be included on whatever form is submitted by the Sponsor/Investigators.

Following the subject's written consent to participate in the study, all SAEs, whether related or not related to study drug, are collected, including those thought to be associated with protocol-specified procedures. The investigator should report any SAE occurring after these time periods that is believed to be related to study drug or protocol-specified procedure.

For studies with long-term follow-up periods in which safety data are being reported, include the timing of SAE collection

- In accordance with local regulations, BMS will notify investigators of all reported SAEs that are suspected (related to the investigational product) and unexpected (i.e., not previously described in the IB). In the European Union (EU), an event meeting these criteria is termed a Suspected, Unexpected Serious Adverse Reaction (SUSAR). Investigator notification of these events will be in the form of an expedited safety report (ESR).
    - Other important findings which may be reported by the as an ESR include: increased frequency of a clinically significant expected SAE, an SAE considered associated with study procedures that could modify the conduct of the study, lack of efficacy that poses significant hazard to study subjects, clinically significant safety finding from a nonclinical (eg, animal) study, important safety recommendations from a study data monitoring committee, or sponsor decision to end or temporarily halt a clinical study for safety reasons.
    - Upon receiving an ESR from BMS, the investigator must review and retain the ESR with the IB. Where required by local regulations or when there is a central

IRB/IEC for the study, the sponsor will submit the ESR to the appropriate IRB/IEC. The investigator and IRB/IEC will determine if the informed consent requires revision. The investigator should also comply with the IRB/IEC procedures for reporting any other safety information.

In addition, suspected serious adverse reactions (whether expected or unexpected) shall be reported by BMS to the relevant competent health authorities in all concerned countries according to local regulations (either as expedited and/or in aggregate reports).

Serious Adverse Event Collection and Reporting

Following the subject's written consent to participate in the study, all SAEs, whether related or not related to study drug, must be collected, including those thought to be associated with protocol-specified procedures. All SAEs must be collected that occur within 100 days of discontinuation of dosing.

All SAEs must be collected that occur during the screening period. If applicable, SAEs must be collected that relate to any protocol-specified procedure (eg, a follow-up skin biopsy). The investigator should report any SAE that occurs after these time periods that is believed to be related to study drug or protocol-specified procedure.

SAEs, whether related or not related to study drug, and pregnancies must be reported to BMS within 24 hours. SAEs must be recorded on BMS or an approved form; pregnancies on a Pregnancy Surveillance Form.

SAE Email Address: [redacted]

SAE Facsimile Number: [redacted]

If only limited information is initially available, follow-up reports are required. (Note: Follow-up SAE reports should include the same investigator term(s) initially reported.)

If an ongoing SAE changes in its intensity or relationship to study drug or if new information becomes available, a follow-up SAE report should be sent within 24 hours to the BMS (or designee) using the same procedure used for transmitting the initial SAE report.

All SAEs should be followed to resolution or stabilization.

The Sponsor/Investigator will ensure that all SAEs in the clinical database are reported to BMS and any applicable health authority during the conduct of the study. This reconciliation will occur at least quarterly and be initiated by the sponsor/investigator. Sponsor/investigator will request a reconciliation report from: [redacted] During reconciliation, any events found to not be reported previously to BMS must be sent to [redacted]

Appendix F

Suggested Schedule of Events

Assessment 6 wks = 1 cycle	Baseline	Induction			Maintenance	End of Treatment	Disease Progression	Follow-up/ Safety Follow-up
	Within 4 weeks	Cycle 1 Day1	Cycle 1 Day 22	Cycle 2 Day 1	Cycle 3 and after Day 1			
Informed consent	x							
Assignment identification numbers	x							
Medical history and demographics[e]	x							
Complete physical exam	x							
Weight	x							
Height	x							
ECOG performance status	x					x	x	x
Hematology&Chemistry lab[c,d]	x		x	x	x	x	x	
Adverse events			x	x	x	x	x	x
Imaging Studies[a]	x[a]				x[a]			x[a]
Submission of Archival tumor for review	x							
Fresh tumor biopsy				x[b]				
Peripheral blood correlates	x	x		x	x			
Microbiota correlates		x	x				x	

a. Non-CNS Imaging should include a chest, abdomen and pelvis CT. Imaging of other sites should be obtained when clinically indicated. If follow up scans

indicate partial or complete response, a repeat confirmatory scan should be obtained within 12 weeks (but no sooner than 6 weeks). Baseline imaging should be done within 4 weeks prior to registration, then every 12 weeks if patient is < 2 years from study entry, then every 6 months (+/- 4 weeks) until 5 years from study entry

b. Fresh tumor biopsy is expected in 25% of patients (N=25; 10 MCM and 5 ALM patients) to consent to on-treatment biopsies. Please refer to appendix G for detail. This can be collected within weeks 6-8.
c. Hematology Labs should include CBC with differential (hemoglobin, hematocrit, white blood cells, platelets, neutrophils, lymphocytes, eosinophils and monocytes)
d. Chemistry Labs should include albumin, amylase, lipase, BUN, creatinine, ALT, AST, LDH, serum alkaline phosphatase, direct and total bilirubin, glucose, total protein, sodium, potassium, chloride, HCO3, calcium, uric acid and CPK. Labs should also include TSH and free T4 if TSH is elevated. Sites should follow their institutional guidelines for hematology and Chemistry labs.
e. Updated history and limited physical examination are recommended to be performed every 3 weeks for the first two cycles, then on the first day of subsequent cycles.

Appendix G

Specimen Submission Guidelines

Baseline tumor: tumor block or 26 unstained FFPE slides from primary or met; preferred excisional or punch (if not possible 14-16 gauge core needle)

- Each pt: at least 1 single tumor block or **16** unstained slides at 10 micron thickness plus **10** unstained slides at 5 micron thickness for 4 different baseline correlative studies
- Fresh tumor will be split for processing as frozen and FFPE
  - For excisional & punch bx, approx 2/3 embedded in OCT, placed on dry ice and stored at -80c. Remaining 1/3 processed for FFPE
  - For core needle bx, 3-4 14-16 gauge cores embedded in OCT and at least 2 14-16 gauge cores processed for FFPE

Correlative Study	**Sample required at each time point**	**Baseline**	**6-8 wks [1] after tx**	**Every 6 wks during tx[2]**	**Disease Progression**	**Shipping/ Storage for Future Use[3]**
Baseline correlative Studies on Tissues	Tumor block or **16** unstained FFPE tumor tissue slides at 10-micron	XX				Ship to GU-LCCC-HTSR (Deborah Berry) at the end of the study. Leftover specimens (if any) stored at GU-LCCC-HTSR & placed in MRFBC VR for future use
	Tumor Block or **10** unstained FFPE tumor tissue slides at 5-micron	XX				
(Optional) On-treatment correlative Studies on Tissues	1.Frozen tumor embedded in OCT at-80c 2. FFPE tumor block		XX			Ship to GU-LCCC-HTSR (Deborah Berry) at the end of the study. Leftover specimens (if any) stored at GU-LCCC-HTSR & placed in MRFBC VR for future use

1 Expect 25% of patients (N=15; 10 MCM and 5 ALM patients) to consent to on-treatment biopsies
2 Blood samples will be taken ≤ 7 days prior to treatment
3 Specimens should be batched shipped to GU-LCCC-GESR at the conclusion of therapy for each patient. (at the end of the trial)

Correlative Study	**Sample required at each time point**	**Baseline**	**3 wks [4] after tx**	**Every 6 wks during tx[5]**	**Disease Progression or at the end of study**	**Shipping/ Storage for Future Use[6]**
High-resolution HLA typing	CPT™ Mononuclear cell preparation tube blood	XX; 10ml				Ship to GU-LCCC-HTSR (Deborah Berry) at the end of the study.
Blood Correlates	Blood in Cell-Free DNA BCT® tubes (Streck™) -Red-top blood -CPT™ Mononuclear cell preparation tube blood	XX[7] 20ml blood in Cell-Free DNA BCT® tubes; 10ml red-top blood, 10ml CPT™ tube blood (collect within 7 days prior to treatment)		XX[8] 20ml blood in Cell-Free DNA BCT® tube; 10ml red-top blood; 10ml CPT™ tube blood	XX[9] 20ml blood in Cell-Free DNA BCT® tube; 10ml red-top blood; 10ml CPT™ tube blood	Ship to GU-LCCC-HTSR (Deborah Berry) at the end of the study.; Leftover specimens (if any) stored at GU-LCCC-GESR & placed in MRFBC VR for future use
Microbiota Correlates	Stool in Omnigene.Gut collection tube	XX (collect ≤ 7 days prior to Rx up to within 24 hours of receiving first dose)	XX (collect ≤ 5 days prior to Rx)		XX (+/- 7 days)	Ship to GU-LCCC-HTSR (Deborah Berry) at the end of the study.

All samples should be shipped overnight to:

GU-LCCC Histopathology Shared Resource (HTSR)-MRFBC Mucosal & Acral Melanoma Tissue Bank
c/o Deborah Berry, Ph.D
Georgetown-Lombardi Comprehensive Cancer Center
3900 Reservoir Road N.W.

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4 Expect 25% of patients (N=15; 10 MCM and 5 ALM patients) to consent to on-treatment biopsies

5 Blood samples will be taken ≤ 7 days prior to treatment

6 Specimens should be batched shipped to GU-LCCC-GESR at the conclusion of therapy for each patient. (at the end of the trial)

7 -9 Red-top tubes will be subsequently processed into serum, which will be aliquotted into 1ml aliquots & stored at -80C.
Cell-free DNA BCT® tube (Streck™) will be subsequently processed into plasma (which will be aliquotted into 1ml aliquots & stored at -80C) and PBMC. Please refer to the protocol guideline in blood component preparation

Pre-Clinical Science Bldg., LR-10
Washington, DC 20007
[redacted]
Materials are to be shipped SUNDAY THROUGH THURSDAY only. Do not ship samples the day before a Holiday