NCI Protocol #: N/A

DF/HCC Protocol #: 16-577

TITLE: A randomized phase II study of eribulin mesylate with or without pembrolizumab for metastatic hormone receptor positive breast cancer

Coordinating Center: *DF/HCC and Dana-Farber/Partners Cancer Care (DF/PCC)*

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IND #: 132715
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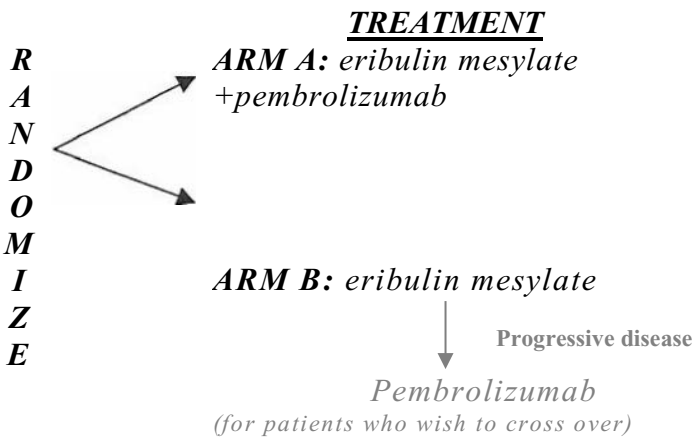
Protocol Type / Version # / Version Date: Amendment / Protocol Version 10 / October 18, 2018

SCHEMA

Disease:
Hormone-receptor positive metastatic breast cancer
(0-2 prior lines of chemotherapy for metastatic disease)

Drugs:
Eribulin mesylate
Pembrolizumab



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1. OBJECTIVES

1.1 Study Design

This is a randomized phase 2 open label study of eribulin mesylate +/- pembrolizumab for patients with metastatic hormone receptor (HR) positive breast cancer treated on 0-2 lines of chemotherapy. Eighty eight patients will be randomized (1:1) to eribulin mesylate with pembrolizumab (Arm A) or eribulin mesylate (Arm B). Patients randomized to eribulin mesylate monotherapy will have the option to receive pembrolizumab monotherapy at time of progression. Eribulin mesylate will be administered weekly on days 1 and 8 of a 21-day cycle. Pembrolizumab will be administered on D1 of each 21 day cycle. PD-L1 tumor positivity will not be used for patient selection. Scientific correlative questions will also be incorporated in this study.

1.2 Primary Objectives

To compare the progression free survival (PFS) of eribulin mesylate in combination with pembrolizumab to eribulin mesylate monotherapy among patients with metastatic HR positive breast cancer treated on 0-2 lines of chemotherapy. PFS is defined as the time from study randomization to disease progression per RECIST 1.1 or death due to any cause, whichever occurred first. Patients alive without disease progression are censored at the date of last disease evaluation.

1.3 Secondary Objectives

Efficacy objectives

1.3.1 To evaluate PFS per immune-related response criteria (irRECIST) among patients treated with eribulin mesylate in combination with pembrolizumab and eribulin mesylate monotherapy, respectively treated on 0-2 lines of chemotherapy

1.3.2 To evaluate objective response rate (ORR) among patients treated with eribulin mesylate in combination with pembrolizumab and eribulin mesylate monotherapy, respectively. ORR will beexamined using RECIST 1.1 and irRECIST criteria.

1.3.3 To evaluate clinical benefit rate (CBR) among patients treated with eribulin mesylate in combination with pembrolizumab and eribulin mesylate monotherapy, respectively. CBR will be examined using RECIST 1.1 and irRECIST criteria.

1.3.4 To evaluate duration of response (DOR) among patients treated with eribulin mesylate in combination with pembrolizumab and eribulin mesylate monotherapy, respectively. DOR will be examined using RECIST 1.1 and irRECIST criteria.

1.3.5 To evaluate overall survival (OS) of among patients treated with eribulin mesylate in combination with pembrolizumab and eribulin mesylate monotherapy, respectively OS will be examined using RECIST 1.1 and irRECIST criteria.

1.3.6 **Among patients randomized to Arm B:** To explore ORR, CBR, DOR, and PFS in patients receiving pembrolizumab after progression on eribulin mesylate monotherapy.

Safety objectives

1.3.7 To evaluate the safety and tolerability of eribulin mesylate in combination with pembrolizumab with eribulin mesylate among patients with metastatic HR positive breast cancer whose cancer progressed after at least 2 prior lines of chemotherapy for metastatic disease.

1.3.8 **Among patients randomized to Arm B:** To explore the safety and tolerability of pembrolizumab after progression on eribulin mesylate monotherapy.

1.4 Correlative Objectives

1.4.1 To characterize a broad array of immune markers in metastatic HR-positive breast tumors (characterization will be based on histology, protein expression, and mRNA expression)

1.4.2 To explore how different immunosuppressive and/or immune-stimulating immune marker profiles at baseline vary between patients with and without response (response assessed by RECIST 1.1 and immune-related response criteria)

1.4.3 To characterize serial changes in immune marker profile in peripheral blood mononuclear cells (PBMCs) over the course of the trial treatment

1.4.4 To explore whether induction of changes in the immunosuppressive and/or immune-stimulating immune marker profile in PBMCs vary between patients with and without response (response assessed by RECIST 1.1 and irRECIST criteria)

1.4.5 To investigate whether there is an immune marker in circulating PBMCs that corresponds to tumor infiltrating lymphocyte (TIL) percentage in baseline tumor

1.4.6 In the cohort of patients who have re-biopsy at progressive disease (PD): To characterize changes in a broad array of immune markers at time of progression (as characterized in aim 1.4.1) to time of progressive disease on trial therapy).

1.4.7 To collect blood to study cell-free DNA for comparison to tumor specimens.

1.4.8 To characterize the structure and function of the gut microbiome in patients with breast cancer prior to starting this clinical trial.

1.4.9 To determine whether pre-treatment characteristics of the structure and function of the gut microbiome in patients with breast cancer is associated with efficacy of pembrolizumab plus eribulin versus eribulin alone.

1.4.10 To characterize changes in the structure and function of the gut microbiome of patients with breast cancer after two cycles of therapy compared to baseline.

1.4.11 To determine whether changes in the overall diversity of the gut microbiome, estimated by the Shannon Index, of patients with breast cancer after two cycles of therapy regimens is associated with efficacy of pembrolizumab plus eribulin versus eribulin alone.

1.4.12 To determine if the abundance and functional profile of specific gut bacteria are associated with objective response to pembrolizumab plus eribulin.

1.4.13 To evaluate the functional pathways that may play a role as a predictive biomarker of response to pembrolizumab plus eribulin.

1.4.14 To explore whether the number and/or type of mutations identified using a next generation sequencing (NGS) panel is correlated with patient outcomes (PFS, ORR, CBR, and OS). This will be done on DFCI patients only.

2. BACKGROUND

2.1 Study Disease(s)

Globally, breast cancer is the most frequent female cancer and the leading cause of cancer death in women[1]. In the United States, over ~200,000 new breast cancer cases are expected/year and almost 40,000 deaths.[2, 3] In women, the lifetime probability of developing invasive breast cancer is one in eight overall.[4] HR-positive breast cancer (defined as estrogen receptor and/or progesterone receptor expressing tumors) accounts for 60-70% of breast cancer. Despite many advances in the adjuvant treatment of early-stage disease, 30-40% of women will develop systemic relapse in addition to the 6-10% of patients who present with de novo metastatic breast cancer,[5] and nearly all patients with metastatic breast cancer eventually succumb to their disease. Currently the median survival for patients with HR-positive tumors does not exceed 3 years. [6] Chemotherapy and endocrine therapy remain the backbone of systemic treatment for HR-positive tumors. More recently, the combination of endocrine therapy plus a molecular-targeted agents (such as CDK 4/6 inhibitors, mTOR) showed to be promising to overcome endocrine resistance.[7, 8]

Nevertheless, remains an urgent need to better understand the biology of human breast cancer, and to understand the interaction between tumor and host characteristics, treatment, and clinical outcomes and develop new treatment strategies.

2.2 The PD-1/PD-L1 pathway in cancer

The importance of intact immune surveillance in controlling outgrowth of neoplastic transformation has been known for decades. Accumulating evidence shows a correlation between tumor-infiltrating lymphocytes (TILs) in cancer tissue and favorable prognosis in various malignancies. In particular, the presence of CD8+ T-cells and the ratio of CD8+ effector T-cells / FoxP3+ regulatory T-cells seems to correlate with improved prognosis and long-term survival in many solid tumors.

The PD-1 receptor-ligand interaction is a major pathway hijacked by tumors to suppress immune control. The normal function of PD-1, expressed on the cell surface of activated T-cells under healthy conditions, is to down-modulate unwanted or excessive immune responses, including autoimmune reactions. PD-1 (encoded by the gene *Pdcd1*) is an Ig superfamily member related to CD28 and CTLA-4 which has been shown to negatively regulate antigen receptor signaling upon engagement of its ligands (PD-L1 and/or PD-L2). The structure of murine PD-1 has been resolved. PD-1 and family members are type I transmembrane glycoproteins containing an Ig Variable-type (V-type) domain responsible for ligand binding and a cytoplasmic tail which is responsible for the binding of signaling molecules. The cytoplasmic tail of PD-1 contains 2 tyrosine-based signaling motifs, an immunoreceptor tyrosine-based inhibition motif (ITIM) and an immunoreceptor tyrosine-based switch motif (ITSM). Following T-cell stimulation, PD-1 recruits the tyrosine phosphatases SHP-1 and SHP-2 to the ITSM motif within its cytoplasmic tail, leading to the dephosphorylation of effector molecules such as CD3ζ, PKCθ and ZAP70 which are involved in the CD3 T-cell signaling cascade. The mechanism by which PD-1 down

modulates T-cell responses is similar to, but distinct from that of CTLA-4 as both molecules regulate an overlapping set of signaling proteins. PD-1 was shown to be expressed on activated lymphocytes including peripheral CD4+ and CD8+ T-cells, B-cells, T regs and Natural Killer cells. Expression has also been shown during thymic development on CD4-CD8- (double negative) T-cells as well as subsets of macrophages and dendritic cells. The ligands for PD-1 (PD-L1 and PD-L2) are constitutively expressed or can be induced in a variety of cell types, including non-hematopoietic tissues as well as in various tumors. Both ligands are type I transmembrane receptors containing both IgV- and IgC-like domains in the extracellular region and contain short cytoplasmic regions with no known signaling motifs. Binding of either PD-1 ligand to PD-1 inhibits T-cell activation triggered through the T-cell receptor. PD-L1 is expressed at low levels on various non-hematopoietic tissues, most notably on vascular endothelium, whereas PD-L2 protein is only detectably expressed on antigen-presenting cells found in lymphoid tissue or chronic inflammatory environments. PD-L2 is thought to control immune T-cell activation in lymphoid organs, whereas PD-L1 serves to dampen unwarranted T-cell function in peripheral tissues. Although healthy organs express little (if any) PD-L1, a variety of cancers were demonstrated to express abundant levels of this T-cell inhibitor. PD-1 has been suggested to regulate tumor-specific T-cell expansion in subjects with melanoma (MEL). This suggests that the PD-1/PD-L1 pathway plays a critical role in tumor immune evasion and should be considered as an attractive target for therapeutic intervention.

The PD-1/PD-L1 pathway in breast cancer

Accumulating evidence suggests that the immune system can also control breast cancer. Recent data suggests that HR positive breast cancers are positive for PD-L1 expression in about 6% of cases, and positive for PD-1 expression in about 43% of cases.[9] Additionally, elevated mRNA expression of immune markers, including PD-L1, CTLA4, B7H-3, and ID01 was noted in the ER+HER2- luminal population. The expression of immune regulatory targets in HR positive breast cancer suggests that these patients may benefit from immunotherapeutic approaches. A correlation has been shown between tumor-infiltrating lymphocytes (TILs) in breast cancer tissue and improved prognosis.[10, 11] Additionally, unsupervised expression profiling of cancer-associated stroma revealed a gene signature predictive of good prognosis that was enriched for CD8+ T cell responses.[12]

2.3 Pembrolizumab

Pembrolizumab is a potent and highly 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. Keytruda™ (pembrolizumab) has recently been approved in the United States for the treatment of patients with unresectable or metastatic melanoma and disease progression following ipilumumab and, if BRAF V600 mutation positive, a BRAF inhibitor. Clinical data are derived from an ongoing, first-in-human phase I study (PN001, NCT01295827) to evaluate the safety and clinical activity of Pembrolizumab as a monotherapy, sponsored by Merck Sharp & Dohme. There are five parts to this study (Parts A-D and F) (Investigator's Brochure, 2014).

Part A was a 3+3 dose escalation study in subjects with solid tumors to evaluate safety, tolerability, pharmacokinetics (PK), and pharmacodynamics, and to determine a maximum

tolerated dose (MTD) or preliminary recommended phase 2 doses (RP2Ds). Doses were 1, 3, and 10 mg/kg every 2 weeks (Q2W); doses of either 2 mg/kg or 10 mg/kg were also administered every 3 weeks (Q3W). All 3 dose levels were well tolerated and no dose-limiting toxicities (DLTs) were observed; therefore, the MTD was not determined. The RP2D was determined by the sponsor based on safety, PK, and pharmacodynamic measurements, along with the strength of antitumor activity signals observed.

Pharmacokinetics
The half-life ($t_{1/2}$) of pembrolizumab is approximately 4 weeks and there is no indication of dose dependency or half-life in the three dose groups (1, 3, and 10 mg/kg) (Investigator's Brochure, 2014). The long $t_{1/2}$ supports a dosing interval of every 2 or 3 weeks.

There was a dose-related increase in exposure from 1 to 10 mg/kg. Serum concentrations of pembrolizumab were lower by a factor of approximately 5 in patients receiving 2 mg/kg Q3W than in those receiving 10 mg/kg Q3W. Steady-state trough concentrations were 20% greater in the patients receiving 10 mg/kg Q2W than in those receiving the same dose Q3W.

A population pharmacokinetic analysis has been performed using serum concentration time data from 476 patients. Within the resulting population PK model, clearance and volume parameters of pembrolizumab were found to be dependent on body weight. The relationship between clearance and body weight, with an allometric exponent of 0.59, is within the range observed for other antibodies and would support both body weight normalized dosing or a fixed dose across all body weights. Pembrolizumab has been found to have a wide therapeutic range based on the melanoma indication. The population PK evaluation revealed that there was no significant impact of tumor burden on exposure. In addition, exposure was similar between the NSCLC and melanoma indications. Therefore, there are no anticipated changes in exposure between different indication settings.

The choice of the 200 mg Q3W as an appropriate dose for the switch to fixed dosing is based on simulations performed using the population PK model of pembrolizumab showing that the fixed dose of 200 mg every 3 weeks will provide exposures that 1) are optimally consistent with those obtained with the 2 mg/kg dose every 3 weeks, 2) will maintain individual patient exposures in the exposure range established in melanoma as associated with maximal efficacy response and 3) will maintain individual patients exposure in the exposure range established in melanoma that are well tolerated and safe.

A fixed dose regimen will simplify the dosing regimen to be more convenient for physicians and to reduce potential for dosing errors. A fixed dosing scheme will also reduce complexity in the logistical chain at treatment facilities and reduce wastage.

Anti-Drug Antibodies (ADA) Data
The occurrence of ADA has been observed in less than 1% of the patients screened, indicating a low potential of pembrolizumab to elicit the formation of ADA. No impact of ADA on pembrolizumab exposure has been observed.

Efficacy

When treated with pembrolizumab monotherapy, the overall response rate (ORR) for ipilimumab (IPI)-treated patients with melanoma was 25%/27% according to the Response Evaluation Criteria in Solid Tumors (RECIST)/investigator-assessed irRECIST, respectively (Investigator's Brochure, 2014). The ORR for IPI-naïve patients with melanoma was 39%/43% by RECIST/investigator-assessed irRC, respectively. The majority of responses were seen in patients with melanoma by 16 weeks of therapy; however, some responses have been reported after 24 weeks or more of therapy with pembrolizumab. Responses can be delayed, and in some patients, a RECIST-defined progression followed by response has been observed.

The preliminary ORR for 38 patients with non-small cell lung cancer was 21%/24% by RECIST/investigator-assessed irRC, respectively (Investigator's Brochure, 2014).

Early findings for 27 patients with triple negative breast cancer showed a 18.5% ORR by RECIST.

Pharmacodynamics/Biomarkers
Pharmacodynamic data (IL-2 release assay) has suggested that peripheral target engagement is durable (>21 days).

PD-L1 is being investigated as a predictive biomarker for pembrolizumab treatment. At the 15th World Conference on Lung Cancer, Garon et al presented preliminary data on a subset of patients suggesting that higher levels of tumor PD-L1 expression are associated with increased clinical activity.[13] ORR by RECIST 1.1 occurred in 4 out of 7 patients with higher levels of PD-L1 expression (57%, 95% CI 18-90%) versus 2 out of 22 patients with lower levels of PD-L1 expression (9%, 95% CI 1-29%). These data are extremely preliminarily, and PD-L1 is not being used for patient selection.

Biomarkers to evaluate immune modulation and markers in the tumor microenvironment, such as T-cell infiltration, the baseline expression of markers of T-cell suppression FoxP3 or the immunoregulatory enzyme incoleamine 2,3-dioxygenase (IDO) in tumor biopsies, were associated with a high response rate in patients with advanced melanoma.[14, 15]

Safety data
The most frequent treatment-related adverse events (AEs) were fatigue, nausea, cough, pruritis, diarrhea, and rash (Investigator's Brochure, 2014). Most AEs were not considered serious. The most commonly-reported immune-related AEs were rash, pruritis, vitiligo, hypothyroidism, arthralgia, diarrhea, and pneumonitis.

Important identified risks include: pneumonitis, thyroid disorders (hypothyroidism and hyperthyroidism), colitis, diarrhea, hepatitis, nephritis, uveitis, rash/pruritis, and neuropathy.

2.4 Eribulin mesylate

Halichondrin B (Hal B) is a large polyether macrolide isolated from a marine sponge (*Halichondria okadai*).[16] It was shown to have anti-cancer activity, based on a microtubule destabilizing anti-mitotic mechanism of action.[16, 17] Although other cancer drugs, as vinca

alkaloids or taxanes, share this mechanism of action, interactions between Hal B and tubulin were found to be unique.[17-20] Eribulin mesylate induces mitotic block, secondary to tubulin polymerization inhibition. It sequesters tubulin into nonproductive aggregates, exerting impressive potent anti-cancer effects *in vitro* and *in vivo* studies.[21]

Summary of mechanism of action
In vitro studies have demonstrated that analogues of Hal B inhibit cell growth at subnanomolar concentrations in a wide variety of cancer cell types, such as breast, ovary, colon and melanoma.[21] Eribulin mesylate exerts its effects binding to the plus end of microtubules and leading to suppression of microtubule growth. It causes tubulin sequestration into nonproductive tubulin aggregates. Thereby, it inhibits tubulin polymerization and microtubule dynamics, interfering with normal mitotic spindle formation and blocking the prometaphase portion of mitosis.[22] These results in induction of irreversible cell cycle block at G2/M, point disruption of mitotic spindles, and cell death via apoptosis after prolonged mitotic blockage.[23]

Eribulin mesylate is a substrate for the P-glycoprotein drug efflux pump with reduced potency against cells expressing high levels of this pump, however it can retain *in vitro* efficacy against taxane-resistant cell lines due to β-tubulin mutations.[24] *In vivo,* eribulin mesylate has led to tumor regression and even eradication against several human cancer xenograft models, including breast cancer. [21]

Summary of pharmacokinetic (PK) profile and metabolism
Pre-clinical studies
In animal models (mice, rat and dogs), the pharmacokinetics (PK) of eribulin mesylate after intravenous administration is characterized by a rapid distribution phase, a large volume of distribution and a prolonged elimination phase (t½: 3.6 -6.9 hours in mice, 15.9- 27.9 hours in rats, 21.9-28.2 hours in dogs).
Eribulin mesylate also has low penetration in brain, likely related to its role as a substrate of the P-glycoprotein drug efflux pump. It also has high penetration in tissues such as the lung, bladder, renal cortex and medulla, liver, spleen, thyroid, stomach, and salivary gland.
Unchanged eribulin mesylate is the major circulating compound in plasma following its administration. Metabolism is a minor component of eribulin mesylate clearance, with minor metabolic changes occurring through cytochrome P450 3A4 (CYP3A4). Eribulin mesylate is eliminated primarily unchanged in feces.

Clinical Studies
In clinical studies, most of these findings were confirmed. Eribulin mesylate's PK is characterized by a rapid distribution phase, with a prolonged elimination phase after intravenous infusion. The disposition of eribulin mesylate follows a linear kinetics over the dose range studied (0.25mg/m^2- 4.0mg/m^2). It has low plasma clearance, with a mean clearance=1.16-2.42 L/hr/m^2, a large mean volume of distribution at steady state (43-114 L/m2) and a half life of elimination of 40 hours. The human plasma protein binding of eribulin mesylate occurs at concentrations of 100ng/mL to 1.000ng/mL and ranges from 49% to 65%.

Eribulin mesylate exposure after multiple doses is comparable to a single dose, with no accumulation of eribulin mesylate with weekly administration. As expected based on work in pre-clinical models, metabolism is a minor component of eribulin mesylate clearance. Metabolites represent less than 0.6% of parent compound in plasma. Renal elimination is also a minor route of eribulin mesylate excretion, with less than 10% of drug excreted unchanged in urine. Most of excretion of eribulin mesylate is fecal and unchanged. It is unknown if eribulin mesylate is excreted into human milk.
Eribulin mesylate does not induce or inhibit hepatic CYP3A4 activity at clinically relevant concentrations. Concomitant administration of ketoconazole, a CYP3A4 inhibitor, or rifampicin, a CYP3A4 inducer, had no effect on exposure to eribulin mesylate. Eribulin mesylate does not induce or inhibit CYP1A, CYP2C9, CYP2C19, CYP2D6 activity at clinically relevant concentrations.

Hepatic impairment may decrease the clearance of eribulin mesylate and prolong the elimination half-life, resulting in increased exposure to eribulin mesylate. As such, it is proposed that the eribulin mesylate dose in patients with moderate hepatic impairment should be adjusted. No studies for patients with severe hepatic impairment have been performed.[25]

Moderate renal impairment (CrCL 30-50 mL/min) may also impact the clearance of eribulin mesylate, resulting in increased exposure. As such, it is proposed that eribulin mesylate dose in patients with moderate renal impairment should be adjusted. No studies for patients with severe renal impairment have been performed.

Population PK analyses based on Phase 1 and 2 studies showed that eribulin meslyate's clearance is affected by body weight, serum albumin, alkaline phosphatase and bilirubin. The effects of age, gender, race and concomitant medications (CYP3A4 inhibitors and inducers) on clearance were not found to be significant.

Summary of toxicology data
A variety of nonclinical toxicology studies have been conducted to support the use of eribulin mesylate in humans. The findings from these studies are summarized below. No significant serious adverse events (AE) were observed in any preclinical safety studies with regard to central nervous, respiratory, or cardiovascular systems. Eribulin mesylate induced no significant reduction in nerve conduction velocity or peak nerve amplitude in caudal and digital nerves, in contrast to paclitaxel. The morphological changes in sciatic nerve and dorsal root ganglia were less severe in eribulin mesylate than those observed with paclitaxel. In animal models, eribulin mesylate induced markedly less neuropathy than paclitaxel.

Bone marrow toxicity appeared to be dose-limiting in both rats and dogs. Intestinal toxicity was also present in dogs. Other toxicities that were considered to be drug-related occurred in the lymphoid tissue, testes, and skeletal muscle. All observed toxicities (except testicular toxicity) were reversible in both dogs and rats. In repeated-dose toxicity studies in rats, testicular toxicity, thymic atrophy, bone marrow toxicity, and fiber degeneration of sciatic nerve were found.

Although the changes in testes and sciatic nerve were still present after a 14-day recovery period, other toxicities were reversible. Repeated-dose toxicity in dogs produced leukopenia, which was fully reversible in 14 days with compensatory extramedullary hematopoiesis. The chronic toxicity studies in rats and dogs were conducted over 6 months. In rats, bone marrow and testicular toxicity were the most important effects observed. Hypocellularity of bone marrow and a reduction in the weight of testes (correlating with hypocellularity of seminiferous epithelium with associated hypospermia/aspermia of the epididymides) were found. Increases in alanine aminotransaminase (ALT), aspartate aminotransaminase (AST), and cholesterol were also observed. In dogs, bone marrow toxicity mainly represented by leukopenia was observed. With regard to findings in the testes, this finding was limited to high doses of drugs and included decrease of testes weights and microscopic changes with mild to moderate hypocellularity of testis with hypospermia/aspermia of the epididymides. In addition, hypercellularity of bone marrow, lymphoid depletion in mesenteric lymph nodes and Peyer's patches, and thymic atrophy were noted. These studies suggest that male fertility may be compromised by eribulin mesylate treatment. Carcinogenicity studies have not been conducted with eribulin mesylate. Genotoxicity was evaluated both *in vitro* and *in vivo*. *In vitro,* results indicated that the effect was due primarily to chromosome segregation interference rather than chromosome breakage because eribulin mesylate was negative in the Ames test with or without S9 and was weakly positive in the L5178Y tk+/- mouse lymphoma mutagenesis assay. *In vivo,* in rats, dose-related increases of micronucleated- polychromatic erythrocytes (MN-PCEs) at either sampling time and dose-related decreases in the proportion of PCEs were observed, which may indicate some degree of bone marrow supression. In the intravenous embryo and fetal developmental studies in rats, eribulin mesylate mesylate was given to rats at doses of 0.01, 0.03, 0.10, and 0.15 mg/kg/day on gestational days 8, 10, and 12. External and/or soft tissue anomalies were noted at 0.15 mg/kg/day, indicating that eribulin mesylate has teratogenic potential.

Summary of safety data for eribulin mesylate (Also refer to Section 6)

Pivotal Phase III trial

In the phase III EMBRACE trial, AEs occurred in 99% of patients receiving eribulin mesylate, however serious AEs occurred in 25% and AEs leading to therapy discontinuation occurred in 13% of patients on study. The primary toxicities with eribulin mesylate were asthenia or fatigue (54%) neutropenia (52%). Grade 4 neutropenia lasting more than 1 week occurred in 12% of patients and F+N occurred at low incidence (5%). Patients with elevated ALT/AST or bilirubin experienced a higher frequency of grade 4 neutropenia or F+N. Peripheral neuropathy was the most common AE leading to discontinuation of therapy, occurring in 5% of patients. Neuropathy lasting more than one year occurred in 5% of the patients. Alopecia occurred in 45% of patients. Nausea occurred in 35% of patients, but it was grade 3 just in 1% of patients. Diarrhea occurred in 18% of cases; in all cases was grade 1 or 2. Dose interruptions, delays and reductions were undertaken in 6%, 49% and 29% in the eribulin mesylate group, respectively.[26]

Overall Adverse events and serious adverse events

Overall, in clinical studies, the most common treatment-related toxicities were hematological toxicity, mainly neutropenia, asthenia or fatigue, alopecia, nausea and peripheral neuropathy. Most AE were grade 1 or 2.

Peripheral neuropathy was an important AE leading to discontinuation of therapy. According to the investigator's brochure, the most frequently reported eribulin mesylate related AEs were asthenia/fatigue (65%), alopecia (60%), neutropenia (60%), nausea (44%), anemia (28%), pyrexia (23%), leucopenia (22%), anorexia (21%), constipation (19%), vomiting (18%), and peripheral neuropathy (5.5%; only grade 3). Grade 4 neutropenia occurred in 32% of patients, and F+N occurred in 5.5% of patients. The frequency of all other grade 3/4 AEs was less than 3%.

Summary of efficacy data for eribulin mesylate- Metastatic Breast Cancer
Phase II studies have observed a significant level of activity in heavily pretreated patients with advanced breast cancer. [25, 27, 28] The aim of the Phase II study, E7389-A001-201, was to determine the response rate of eribulin mesylate monotherapy in patients with advanced/MBC pre-treated with chemotherapy, including an anthracycline and a taxane. Here, an ORR of 11.5% (95% confidence interval [CI), 6-20%) and a CBR of 17.2% (95% CI, 10-27%) were achieved. The median duration of response was 5.6 months and the median OS was 9.0 months.[25, 27]
In addition, the Phase II study E7389-G000-211, determined the response rate of eribulin mesylate monotherapy in patients with advanced/MBC pre-treated with chemotherapy, including an anthracycline,taxane and capecitabine. ORR by an independent review was 9.3% (95% CI, 6.1-13.4%), and the CBR was 17.1%. Median duration of response was 4.1 months, and median OS was 10.4 months.[28] The Phase II study E7389-J081-221 was conducted to determine the efficacy and safety of eribulin mesylate in Japanese patients with advanced/MBC pre-treated with chemotherapy, including an anthracycline and a taxane. ORR by an independent review was 21.3 % and the CBR was 27.5%.[25] These Phase II studies led to the Phase III EMBRACE trial (Eisai Inc. MBC Study Assessing Physician's Choice vs. E7389, study E7389-G000-305), a multicentre, randomized, open label study. It included 762 locally advanced or MBC patients pre-treated with at least 2 and up to 5 chemotherapy regimens. Women were randomly assigned to receive eribulin mesylate (1.4 mg/m2 days 1 and 8 every 21 days) or any other chemotherapy by the physician's and patient's choice. The study met its primary objective, showing a significant increase in OS (Hazard Ratio (HR): 0.81, 95% CI, 0.66-0.99; p=0.041). Treatment with eribulin mesylate compared to alternative therapy significantly improved median OS (13.1 vs. 10.6 months, p=0.041). ORR was also improved in patients treated with eribulin mesylate mesylate (12% vs. 5%, p=0.002). CBR was 23% for eribulin mesylate and 17% for provider-choice therapy. [25-28]
Kaufam et al examined the efficacy of eribulin vs. capecitabine among patients with metastatic breast cancer treated up to second line of chemotherapy. In this trial ORRs by independent review was 11.0% (95% CI, 8.5% to 13.9%) for the eribulin arm and the median PFS was 4.1 months (95% CI, 3.5 to 4.3 months) for eribulin.[29]
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2.5 Rationale for the proposed study combination

Recent evidence suggests that some chemotherapeutic agents rely on the induction of anticancer immune responses, and that the innate and adaptive immune systems are critical in determining the efficacy of cytotoxic-based regimens. Based on this recent data, and based on the fact that tumor immune infiltrates have been demonstrated to have an impact on survival in breast cancer, we propose a study combining chemotherapy with anti-PD1 therapy in metastatic HR+ breast cancer.

Pembrolizumab is a humanized monoclonal antibody of the IgG4/kappa isotype designed to directly block the interaction between PD-1 and its ligands, PD-L1 and PD-L2. Pembrolizumab strongly enhances T lymphocyte immune responses in cultured cancer cells, and modulates the levels of IL-2 TNF-α, and IFN-ɣ. The antibody potentiates existing immune responses only in the presence of antigen. The most common adverse events include fatigue, rash, and pruritus.

There is strong rationale that chemotherapy can work synergistically with immune modulation[30]. For example, cyclophosphamide has been shown to deplete T regulatory cells and myeloid-derived suppressive cells, thereby enhancing antitumor immune response[31, 32]. The direct cytotoxicity of chemotherapy can also enhance cross presentation of tumor antigens, leading to augmented T cell responses directed at the tumor.[33] Moreover, cytotoxic chemotherapeutics, such as paclitaxel, have been shown to upregulate PD-L1 expression on breast cancer cells.[34] Additionally, administration of taxanes in the preoperative setting results in an increase in the levels of tumor-infiltrating lymphocytes (TILs) within the tumor parenchyma.[35] In the metastatic setting, docetaxel has been shown to increase levels of Th-1 associated cytokines (IL-2, IFN-γ) while decreasing negative inflammatory markers, such as tumor necrosis factor beta (TNF-β).

A recent study in the 4 T1 breast cancer mouse model demonstrated remarkable synergy between therapy with anti-PD-1 antibody and paclitaxel [36]. As opposed to paclitaxel monotherapy, the combination significantly suppressed tumor growth and 80% of the mice (4 of 5) survived tumor-free until day 90. Moreover, theses mice developed memory immune responses as demonstrated by their resistance to re-challenge with 4 T1 cells.

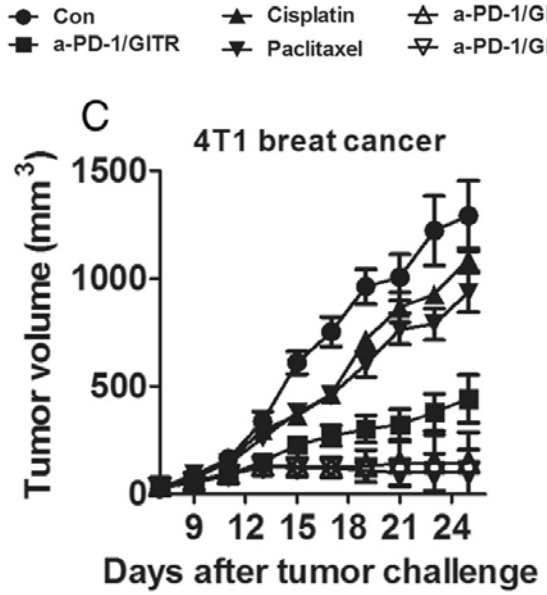

Figure 1: BALB/c mice were transplanted s.c. with 5 x 10^5 4 T1 cellson day 1 and were pretreated with a dose of paclitaxel (10mg/kg) on day 8, followed by 3 doses of anti-PD-1 antibody (250μg) on day 9, 12, and 16.

Eribulin mesylate is a synthetic analogue of halichondrin B, a product that is isolated from the marine sponge Halichondria okadai. Eribulin mesylate is designed to inhibit microtubule polymerization without affecting depolymerization. This results in apoptosis through an

irreversible mitotic block at the G2-M phases. In November 2010, the FDA approved eribulin mesylate as a treatment for patients with metastatic breast cancer following the administration of at least 2 regimens with an anthracycline and a taxane. This approval was based on data from the EMBRACE trial, which demonstrated a statistically significant improvement in overall survival for patients receiving eribulin mesylate compared with physician's choice of therapy.[26] In a second study, eribulin mesylate was compared to capecitabine in patients who had received 0-2 prior lines of chemotherapy for metastatic disease. The two studies enrolled 1864 patients, with 31.5% receiving eribulin mesylate as second-line therapy and 32.7% receiving treatment as third-line therapy. There was no statistically significant difference in PFS or OS between these two treatments.[29] Additionally, Dana-Farber has recently completed accrual of a first and second-line study of eribulin mesylate for patients with metastatic HR positive breast cancer.

This is a randomized phase 2 study of eribulin mesylate with or without pembrolizumab in patients with metastatic HR positive breast cancer. Eribulin mesylate will be administered weekly on days 1, 8 of a 21 day cycle. Pembrolizumab will be administered on D1 of each 21 day cycle. Patients who are randomized to eribulin mesylate monotherapy and develop progressive disease will be allowed to receive Pembrolizumab at time of progression.

2.6 Correlative Studies Background

2.6.1 Blood and Tissue Analysis

There is growing evidence that signatures of tumor-associated immunologic activity are seen in breast cancer and are significant prognostic predictors. Many different groups have demonstrated that the amount of tumor-infiltrating lymphocytes (TILs) in a tumor specimen, commonly assessed simply by histological evaluation of a standard hematoxylin and eosin-stained slide by a trained pathologist, is a significant predictor of both response to therapy and overall disease outcomes in the neoadjuvant and adjuvant settings.[37-42] Recently, more in-depth methods of immunologic profiling are being explored in breast cancer, for example mRNA expression of immune-activating and immunosuppressive factors, and these additional immune profiles also appear to have prognostic significance.[38]

However, the development of immune signatures in breast cancer that go beyond simple histology is at a very early stage. Additionally, much of the work done in this area has been in the adjuvant and neoadjuvant settings, with less known about the immunologic profile of metastatic disease. Given the promise of immune-based therapies in other solid malignancies such as melanoma and non-small cell lung cancer, with preliminary data demonstrating activity of immune checkpoint inhibition in advanced breast cancer patients,[43, 44] there is a great need to better characterize the immune profile of breast tumors across multiple disease subtypes, and at different points in the course of therapy. The bulk of our correlative science in this trial focuses on characterizing a broad array of immune markers in metastatic HR-positive breast cancer, investigating whether those markers predict disease response to therapy, and exploring how markers change from the start of treatment to the time of development of progressive disease.

In melanoma, the solid malignancy currently at the forefront of understanding the anti-tumor immune response, investigation into expression of immune mediators in the peripheral blood is

at an early phase.[45] As a correlative study to this trial, we will characterize the immune marker profile of peripheral blood mononuclear cells (PBMCs) in enrolled breast cancer patients. Additionally, given the demonstrated clinical significance of TILs in breast cancer specimens, we will investigate whether there is a peripheral marker whose level corresponds to TIL percentage. Lastly, we will evaluate whether there is a correlation between (a) changes in PBMC immune profiles and disease response to therapy. Evidence of a correlation would be of significant interest as it would suggest the potential presence of a predictive biomarker in the peripheral blood.

These correlative projects are made possible by collaboration with Drs. Scott Rodig and Evisa Gjinin, and Mariano Severgnini, all of whom are lab scientists with extensive experience with immune profiling in melanoma. Further details can be found in Section 9.

2.6.2 Microbiome Analysis

Breast Cancer (BC) is the most frequently diagnosed cancer and the second cause of cancer death in American women[57]. In the advanced setting, despite multiple available systemic therapies, virtually all patients will die from their disease. Thus, the exploration of new treatments, such as immune checkpoint inhibitors (ICI), including pembrolizumab, is imperative.

An increasing body of preclinical and clinical evidence suggests that breast cancer is an immunogenic malignancy[58]. It is now recognized that a fraction of breast tumors, mainly triple-negative breast cancer (TNBC), have substantial lymphocyte infiltration, and that this pathologic feature has prognostic implications[59]. Early clinical trials assessing the efficacy of PD-1/PD-L1 inhibitors given as monotherapy showed that only a small fraction of patients derive benefit from immunotherapy with an approximate 20% objective response rate (ORR) among patients with PDL1+ TNBC[60,61], and a 12% ORR among those with PDL1+ hormone receptor (HR)-positive BC[62]. Therefore, new research approaches combining therapeutic agents aiming to boost antitumor immunity, as well as developing predictive biomarkers of response, are needed to increase the rates of clinical success of immunotherapy in BC.

In this context, the gut microbiota has been recognized as a modulator of immune system development[63]. Healthy individuals have microbial populations in their intestinal tract that vary markedly in composition[64,65]. The diversity of intestinal microbiota represents a significant challenge to the host's immune defenses, which must balance immune tolerance of beneficial microbes with inflammatory responses against pathogens. Alterations in the gut microbiota and their resulting interactions with intestinal epithelieum and the host immune system are associated with many disease, including cancer[66]. Recently, two preclinical studies provided to ICI, raising the possibility that stool microbiota could be used as biomarker predictors of efficacy to immunotherapy[67,68]. Interestingly, postmenopausal women with breast cancer have altered composition and low diversity of their gut microbiota compared to healthy controls[69].

Identification of biomarkers that predict response to ICI-based therapies can spare *de novo* resistant patients from the unnecessary risks of immune-related adverse events. In addition, the identification of bacterial species associated with response could open new strategies to maximize the clinical benefit of cancer immunotherapy through the modulation of gut microbiota.

This correlative project is made possible by collaboration with the BWH/Harvard Cohorts Biorepository and an external lab vendor, Microbiome Dx. Further details can be found in Section 9.

2.6.3 Tumor Genomic Profile

In addition to the immune microenvironment, intrinsic tumor factors may be associated with response to immune checkpoint inhibitors. Although some of the mechanisms related to de novo or acquired resistance to ICI have been recently described, including loss of function in beta-2-microglobulin or defects in the interferon signaling pathway[Gao *et al.*, 2016, Zaretsky *et al.*, 2016], the knowledge of immune resistance remains largely unknown. Several gene/pathways have been described as possible candidates of having an immunosuppressive role in different advanced solid tumor, including MYC amplification[Casey *et al.*, 2016], activation in WNT-β-catenin pathway[Spranger *et al.*, 2015], activation in MAPK pathway, loss of PTEN[Li *et al.*, 2016, Peng *et al.*, 2016, George *et al.*, 2017]. On the other hand, few possible biomarkers of response to ICI have emerged, including mutational load[Snyder *et al.*, 2014, Rizvi *et al.*, 2015], tumor aneuploidy[Davoli *et al.*, 2017], mismatch repair defects[Le *et al.*, 2015], and BRCA2 mutation[Hugo *et al.*, 2016]. Notably, there is no data on genomic mechanisms of de novo resistance to anti-PD-1 therapy in patients with breast cancer.

Therefore, as a correlative study to this trial, we will to explore whether the number and/or type of mutations identified using a next generation sequencing (NGS) panel – OncoPanel - is correlated with patient outcomes (PFS, ORR, CBR, and OS). This tool is a cancer genomic assay to detect somatic mutations, copy number variations and structural variants in tumor DNA extracted from fresh, frozen or formalin-fixed paraffin-embedded samples. The OncoPanel assay surveys exonic DNA sequences of 447 cancer genes and 191 regions across 60 genes for rearrangement detection. DNA is isolated from tissue containing at least 20% tumor nuclei and analyzed by massively parallel sequencing using a solution-phase Agilent SureSelect hybrid capture kit and an Illumina HiSeq 2500 sequencer. The targeted NGS assay (OncoPanel) will be performed at the Center for Advanced Molecular Diagnostics (Department of Pathology, Brigham and Women's Hospital). This assay has been extensively validated and is used as a CLIA-approved clinical molecular test in our institution without any additional sequencing assays to validate the findings [Wagle *et al.*, 2012].

3. PARTICIPANT SELECTION

Eligibility will be assessed as part of the screening procedures for all patients.

3.1 Eligibility Criteria

3.1.1 Patients must have histologically or cytologically confirmed Stage IV invasive breast cancer. Patients without pathologic or cytologic confirmation of metastatic disease should have unequivocal evidence of metastasis from physical examination or radiologic evaluation.

3.1.2 Subjects must have at least one lesion that is not within a previously radiated field that is evaluable on computerized tomography (CT) or magnetic resonance imaging (MRI) scan per RECIST version 1.1.[46] If the subject's only evaluable disease is within a previously radiated field, it must have demonstrated progression since the time of radiation.

3.1.3 Participants must have HR positive, HER2-negative breast cancer (ER>1% and/or, PR>1%, HER2-negative per ASCO CAP guidelines, 2013 resulted on the primary tumor and/or a metastatic lesion).

3.1.4 Participants must have already received or been intolerant to at least two lines of hormonal therapies (including the adjuvant or metastatic setting) or be appropriate candidates for chemotherapy

3.1.5 Prior chemotherapy:
Participants are allowed to have received up to 2 prior lines of chemotherapy in the metastatic setting. If a prior chemotherapy was given for less than 1 cycle, it will not be counted as a prior line. The last dose of chemotherapy must be ≥14 days prior to initiation of study therapy. Participants should be adequately recovered from acute toxicities of prior treatment. No prior treatment with eribulin mesylate is allowed.

3.1.6 Prior biologic therapy: The last dose of biologic or investigational therapy must be ≥21 days prior to initiation of study therapy.

3.1.7 Prior hormonal therapy: Hormonal therapy must have been discontinued ≥14 days prior to initiation of study therapy. However, continuation of ovarian suppression is allowed.

3.1.8 Prior radiation therapy: Participants may have received prior radiation therapy in either the metastatic or early-stage setting. Radiation therapy must be completed ≥14 days prior to initiation of study therapy.

3.1.9 Prior targeted therapy: Targeted therapy must have been discontinued ≥ 14 days prior to initiation of study therapy.

3.1.10 Biphosphonates/Denosumab: Participants on bisphosphonates/denosumab may continue receiving bisphosphonate therapy during study treatment.

3.1.11 Participants must have an archival tumor sample available (1 block or 20 unstained slides). If no archival tissue is available, participants must be willing to undergo a research biopsy of their disease if it is safely accessible.

3.1.12 Age ≥ 18 years of age

3.1.13 ECOG performance status ≤2 (Karnofsky ≥60%, see Appendix A)

3.1.14 Participants must have normal organ and marrow function as defined below:

– absolute neutrophil count	≥1,500/mcL
– platelets	≥100,000/mcL
– hemoglobin	≥ 8 g/dl
– total bilirubin	≤1.5 × institutional upper limit of normal (ULN)
– AST(SGOT)/ALT(SGPT)	≤2.5 × institutional ULN (≤ 5 × institutional ULN with documented liver metastases,
– serum creatinine	≤1.5mg/dL or calculated GFR ≥60 mL/min
– INR/PT	≤1.5 times ULN unless participant is receiving anticoagulant therapy, as long as PT or PTT is within therapeutic range of intended use of anticoagulants
– aPTT/PTT	≤1.5 times ULN unless participant is receiving anticoagulant therapy, as long as PT or PTT is within therapeutic range of intended use of anticoagulants

3.1.15 The effects of eribulin mesylate and pembrolizumab on the developing human fetus are unknown. Pre-clinical data was suggestive of a teratogenic effect of eribulin mesylate. For these reasons women of child-bearing potential and men must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) for the duration of study participation and 4 months after the last dose of eribulin mesylate and/or pembrolizumab. Note: abstinence is acceptable if this is the usual lifestyle and preferred contraception for the subject.

- Should a woman become pregnant or suspect she is pregnant while she or her partner is participating in this study, the treating physician and principal investigator should be informed immediately.
- While on the study, women must not breastfeed.
- Subjects of childbearing potential are defined as those who have not been surgically sterilized and/or have had a menstrual period in the past year

3.1.16 Female subjects of childbearing potential, as defined above, must have a either a negative urine or a negative serum pregnancy test within seven (7) days of first dose of pembrolizumab. If a urine test is positive or cannot be confirmed as negative, a serum pregnancy test will be required.

3.1.17 Ability to understand and willingness to sign a written informed consent document.

3.2 Exclusion Criteria

3.2.1 Chemotherapy-related or radiation-related toxicities that have not resolved to Grade 1 severity or lower, except for stable sensory neuropathy (≤ Grade 2) and alopecia.

3.2.2 Participants who are receiving any other investigational agents.

3.2.3 Previous treatment with eribulin mesylate or any anti-PD-1, PD-L1, or PD-L2 agent or participation in any MK-3475 Merck studies.

3.2.4 History of allergic reactions attributed to compounds of similar chemical or biologic composition to eribulin mesylate or pembrolizumab.

3.2.5 Known brain metastases that are untreated, symptomatic, or require therapy to control symptoms. Participants with previously diagnosed brain metastases are eligible if they have completed treatment at least 4 weeks prior to registration, are neurologically stable and absence of new neurologic symptoms for the last 4 weeks prior to study entry, and have recovered from the effects of radiotherapy or surgery. Any corticosteroid use for brain metastases must have been discontinued without the subsequent appearance of symptoms for ≥2 weeks before the first study drug. Treatment for brain metastases may have included whole brain radiotherapy, radiosurgery, or a combination as deemed appropriate by the treating physician.

3.2.6 Uncontrolled intercurrent illness, including, but not limited to uncontrolled hypertension, unstable angina pectoris, uncontrolled cardiac arrhythmia, congestive heart failure-New York Heart Association Class III or IV, active ischemic heart disease, myocardial infarction within the previous six months, uncontrolled diabetes mellitus, gastric or duodenal ulceration diagnosed within the previous 6 months, chronic liver or renal disease, severe malnutrition or psychiatric illness/social situations that would limit compliance with study requirements.

3.2.7 Clinically significant electrocardiogram (ECG) abnormality, including a marked baseline prolonged QT/QTc ([QT interval/corrected QT interval], eg, a repeated demonstration of a QTc interval >500 ms).

3.2.8 Medcial condition that requires chronic systemic steroid therapy or on any other form of immunosuppressive medication. For example, participants with autoimmune disease that requires systemic steroids or immunosuppression agents should be excluded. Replacement therapy (eg., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency, etc.) is not considered a form of systemic treatment.

3.2.9 History or evidence of active, noninfectious pneumonitis that required treatment with steroids.

3.2.10 History of interstitial lung disease.

3.2.11 Participants known to be positive for the human immunodeficiency virus (HIV), Hepatitis B antigen (HepBsAg), or Hepatitis C virus (HCV) RNA. HIV-positive participants on combination antiretroviral therapy are ineligible because of the potential for pharmacokinetic interactions with Pembrolizumab and/or eribulin mesylate. In addition, these participants are at increased risk of lethal infections.

3.2.12 Individuals with a history of a second malignancy are ineligible except for the following circumstances. Individuals with a history of other malignancies are eligible if they have been disease-free for at least 5 years and are deemed by the investigator to be at low risk for recurrence of that malignancy. Individuals with the following cancers are eligible if diagnosed and treated within the past 5 years: cervical cancer *in situ*, and non-melanoma cancer of the skin. Patients with other cancers diagnosed within the past 5 years and felt to be at low risk of recurrence should be discussed with the study sponsor to determine eligibility.

3.2.13 Has received a live vaccine within 28 days of planned start of study therapy.

3.3 Inclusion of Women and Minorities

Both men and women of all races and ethnic groups are eligible for this trial.
The accrual targets will reflect the expected accrual over the life of the study.

3.4 Inclusion Criteria for Crossover Therapy

3.4.1 ECOG performance status ≤2 (Karnofsky ≥60%, see Appendix A)

3.4.2 Participants must have normal organ and marrow function as defined below:

- absolute neutrophil count ≥1,000/mcL
- platelets ≥75,000/mcL
- total bilirubin ≤1.5 × institutional upper limit of normal (ULN) (≤1.5 x institutional upper limit of normal (ULN) in patients with well documented Gilbert's Syndrome)
- AST(SGOT)/ALT(SGPT) ≤3.0 × institutional ULN (≤ 5.0 × institutional ULN with documented liver metastases,

3.4.3 Female subjects of childbearing potential, as defined in section 3.1.14, must have either a negative urine or a negative serum pregnancy test within seven (7) days of first dose of pembrolizumab. If a urine test is positive or cannot be confirmed as negative, a serum pregnancy test will be required.

3.4.4 Participants with accessible disease must be willing to undergo a research biopsy before beginning crossover therapy.

3.5 Exclusion Criteria for Crossover Therapy

3.5.1 Clinically significant electrocardiogram (ECG) abnormality, including a marked baseline prolonged QT/QTc ([QT interval/corrected QT interval], eg, a repeated demonstration of a QTc interval >500 ms).

3.5.2 History or evidence of active, noninfectious pneumonitis that required treatment with steroids.

4. REGISTRATION PROCEDURES

4.1 General Guidelines for DF/HCC Institutions

Institutions will register eligible participants in the Clinical Trials Management System (CTMS) OnCore. Registrations must occur prior to the initiation of protocol therapy. Any participant not registered to the protocol before protocol therapy begins will be considered ineligible and registration will be denied.

Participants who are randomized, but do not initiate study treatment will be replaced.

The following registration/randomization procedures should be followed:

- An investigator will confirm eligibility criteria and a member of the study team will complete the protocol-specific eligibility checklist.
- The eligibility checklist(s) and all pages of the consent form(s) will be faxed to the ODQ at 617-632-2295.
- The ODQ Registrar will (a) review the eligibility checklist, (b) register the participant on the protocol, and (c) randomize the participant.
- An email confirmation of the registration and/or randomization will be sent to the Overall PI, study coordinator(s) from the Lead Site
- Prior to beginning crossover therapy, the crossover eligibility section of the checklist should be completed and signed by the treating investigator and re-faxed to the ODQ at 617-632-2295. The ODQ registrar will review the eligibility checklist, change the subject's treatment arm, and notify the study team.

Following registration, participants may begin protocol therapy. Issues that would cause treatment delays should be discussed with the Overall Principal Investigator (PI). If a participant does not receive protocol therapy following registration, the participant's registration on the study must be canceled. Registration cancellations must be made in OnCore as soon as possible.
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4.2 Registration Process for DF/HCC Institutions

DF/HCC Standard Operating Procedure for Human Subject Research Titled *Subject Protocol Registration* (SOP #: REGIST-101) must be followed.

4.3 General Guidelines for Other Investigative Sites

Not applicable

4.4 Registration Process for Other Investigative Sites

Not applicable

5. TREATMENT PLAN

5.1 Treatment Regimen

This is a phase 2 study of treatment with eribulin mesylate +/- pembrolizumab, in metastatic HR-positive breast cancer. Eighty-eight participants will be randomized (1:1) to eribulin mesylate with pembrolizumab (Arm A) or eribulin mesylate (Arm B). Participants randomized to eribulin mesylate monotherapy will have the option to receive pembrolizumab monotherapy at time of progression but they must meet the criteria in section 5.2.3 and begin treatment within 2 months of progression to crossover. Eribulin mesylate will be administered on days 1 and 8 of each 21day cycle. Pembrolizumab will be administered on day 1 of each 21 day cycle. If treatment is delayed, all protocol required assessments will be delayed accordingly.

Treatment will be administered on an outpatient basis. Reported adverse events and potential risks are described in Section 7. Appropriate dose modifications are described in Section 6. Details of the regimen are described in Table 1/2. No investigational or commercial agents other than those described below may be administered with the intent to treat the participant's malignancy.
Treatment regimen is described in Table 1 (Arm A) and in Table 2 (Arm B).

Table 1: Regimen description- ARM A

Regimen Description					
Agent	**Premedication ; Precautions**	**Dose**	**Route**	**Schedule**	**Cycle Length**
Pembrolizumab	Not routinely necessary unless prior infusion reaction.	200mg- *iv*	IV over approximately 30 minutes (range: 25-40 minutes). Infuse prior to starting eribulin mesylate infusion.	Day 1, q3w	21 days (3 weeks)

Eribulin mesylate	No routine premedication required	1.4 mg/m^2 D1 and D8iv*	IV, 2-5 minutes. May be administered diluted in 100 mL normal saline.	Days 1 and 8, iv	21 day (3 weeks)

*Dose reductions may be made as per Tables 4-7. Further details about dose reductions can be found in Section 6.

Table 2: Regimen description- ARM B

Regimen Description

Agent	Premedications ; Precautions	Dose	Route	Schedule	Cycle Length
Eribulin mesylate	No routine premedication required	1.4 mg/m^2 * iv	IV, 2-5 minutes. May be administered diluted in 100 mL normal saline.	Days 1 and 8, iv *	21 day (3 weeks)

*Dose reductions may be made as per Tables 6-7. Further details about dose reductions can be found in Section 6.

Note: After progression patients who wish can receive pembrolizumab200mg, at a final concentration of 1 mg/mL to 10 mg/mL in NS or D5, every 21 days. Pembrolizumab should be administered as described in Arm A, pre-treatment criteria will also follow what is recommended in Arm A.

5.2 Pre-Treatment Criteria, Treatment, end off treatment and follow up visits

5.2.1 Cycle 1, Day 1

Criteria to treat at cycle 1 day 1:

- **Absolute neutrophil count ≥1500/** mcL
- **Platelets ≥100,000/** mcL
- **ALT and AST ≤2.5 x ULN in a patient with no documented liver metastases; ALT and AST ≤5.0 x ULN in a patient with documented liver metastases**
- **Total bilirubin ≤1.5 x ULN (2.0 x ULN in a patient with well documented Gilbert's syndrome)**

5.2.2 Day 8 and Day 1 of subsequent cycles (including C1D1 and beyond of Crossover)

Criteria to treat at day 8 and day 1 of subsequent cycles:

- **Absolute neutrophil count ≥1000/** mcL
- **Platelets ≥75,000/** mcL
- **ALT and AST ≤3.0 x ULN in a patient with no documented liver metastases; ALT and AST ≤5.0 x ULN in a patient with documented liver metastases**
- **Total bilirubin ≤1.5 x ULN (2.0 x ULN in a patient with well documented Gilbert's syndrome)**

5.3 Agent Administration

5.3.1 Pembrolizumab

Pembrolizumab will be administered by trained medical personnel at the investigational site. Treatment compliance will be monitored through documentation of study treatment administration in the subject's medical record.

Pembrolizumab will be administered in clinic on day 1 (+/- 3 days) of each cycle. It will be administered as a 30 minute IV infusion. Sites should make every effort to target infusion timing to be as close to 30 minutes as possible. However, given the variability of infusion pumps from site to site, a window of -5 minutes and +10 minutes is permitted (i.e., infusion time is 25-40min).

Pembrolizumab should be administered prior to eribulin mesylate administration. There should be no overlap in timing of the two administrations.

5.3.2 Eribulin mesylate

Eribulin mesylate will be administered by trained medical personnel at the investigational site. Treatment compliance will be monitored through documentation of study treatment administration in the subject's medical record.

Please refer to the FDA-approved package insert for eribulin mesylate for product information and a comprehensive list of adverse events.

Eribulin mesylate will be administered on Days 1 and 8 of each 21-day cycle Day 8 Eribulin mesylate administration may occur at DFCI Milford, as needed.

Eribulin mesylate will be dosed per institutional guidelines.

5.4 Discontinuation of pembrolizumab and Second Course Phase (Retreatment Period)

If pembrolizumab is stopped for toxicity, participants are permitted to continue on protocol therapy with eribulin mesylate alone.

Participants may elect to stop pembrolizumab and eribulin mesylate with SD, PR or CR after at least 27 weeks of treatment and having had at least two treatments with pembrolizumab after documentation of the SD or response.

Subjects who stop pembrolizumab in these conditions may be eligible additional pembrolizumab therapy if they progress after stopping study treatment. This retreatment is termed the Second Course Phase of this study and is only available if the study remains open and the subject meets the following conditions:

- Stopped initial treatment with pembrolizumab after attaining an investigator-determined confirmed SD, PR or CR according to RECIST 1.1, and

    1. Was treated for at least 27 weeks with pembrolizumab before discontinuing therapy

    2. Received at least two treatments with pembrolizumab beyond the date when the initial SD or response was declared

OR

- Had SD or response and stopped pembrolizumab treatment after 27 weeks of study therapy for reasons other than disease progression or intolerability.

Subjects who restart treatment will be retreated at the same dose and dose interval as when they last received pembrolizumab. Visit requirements are as outlined for subjects on the initial treatment phase of the trial.

5.5 Discontinuation of eribulin mesylate

If eribulin mesylate is stopped for toxicity, participants are permitted to continue on therapy with pembrolizumab alone.

5.6 General Concomitant Medication and Supportive Care Guidelines

5.6.1 Concomitant Medication Guidelines

Medications or vaccinations specifically prohibited in the exclusion criteria are not allowed during the ongoing trial. If there is a clinical indication for one of these or other medications or vaccinations specifically prohibited during the trial, discontinuation from trial therapy or vaccination may be required. The investigator should discuss any questions regarding this with the Merck Clinical team. The final decision on any supportive therapy or vaccination rests with the investigator and/or the subject's primary physician.

Acceptable Concomitant Medications

All treatments that the investigator considers necessary for a subject's welfare may be administered at the discretion of the investigator in keeping with the community standards of medical care. All concomitant medication will be recorded on the case report form (CRF) including all prescription, over-the-counter (OTC), herbal supplements, and IV medications and fluids. If changes occur during the trial period, documentation of drug dosage, frequency, route, and date may also be included on the CRF.

All concomitant medications received within 28 days before the first dose of trial treatment and 30 days after the last dose of trial treatment should be recorded. Concomitant medications administered after 28 days after the last dose of trial treatment should be recorded for SAEs as specified in Section 7.2.

Prohibited Concomitant Medications

Subjects are prohibited from receiving the following therapies during the Screening and Treatment Phase of this trial:

- Antineoplastic systemic chemotherapy or biological therapy
- Immunotherapy not specified in this protocol
- Chemotherapy not specified in this protocol
- Biologic or targeted agents not specified in this protocol
- Investigational agents other than pembrolizumab
- Radiation therapy
- Any systemically active oral, injected, or implanted hormonal method of contraception except for progesterone coated intrauterine devices (IUDs) that had been previously implanted.
- Estrogen replacement therapy.
- Live vaccines within 28 days prior to the first dose of trial treatment and while participating in the trial. Examples of live vaccines include, but are not limited to, the following: measles, mumps, rubella, varicella/zoster, yellow fever, rabies, BCG, and typhoid vaccine.
- Systemic glucocorticoids for any purpose other than to modulate symptoms from an event of clinical interest of suspected immunologic etiology. The use of physiologic doses of corticosteroids (10 mg prednisone by mouth daily or equivalent) is allowed..
- Care should be taken with concomitant use of strong CYP3A4 inhibitors/inducers (e.g., ketoconazole and itraconazole; see Appendix B) and eribulin mesylate. An alternate medication with no or minimal potential to inhibit CYP3A4 should be considered. If a strong CYP3A4 inhibitor is co-administered with eribulin mesylate, patients should be closely monitored for adverse reactions.

Subjects who, in the assessment by the investigator, require the use of any of the aforementioned treatments for clinical management should be removed from the trial. Subjects may receive other medications that the investigator deems to be medically necessary.

There are no prohibited therapies during the Post-Treatment Follow-up Phase.

5.6.2 Supportive Care Guidelines – general medications

The following treatments are permitted throughout the duration of the study treatment phase and during follow-up:

- Standard therapies for pre-existing medical conditions unless listed as prohibited therapy below. Any medication intended solely for supportive care (e.g., analgesics, anti-diarrheal, anti-depressants) may be used at the investigator's discretion. Antiemetics and anti-diarrheal medications should not be administered prophylactically before initial treatment with study drugs. At the discretion of the investigator, prophylactic antiemetic and anti-diarrheal medication(s) may be used as per standard clinical practice before subsequent doses of study drugs.
- Hematopoietic growth factors (e.g., G-CSF, granulocyte macrophage colony stimulating factor) may be used at investigator's discretion for the primary prophylaxis and/or management of treatment-emergent neutropenia and/or for secondary prophylaxis as per NCCN/European Society for Medical Oncology guidelines [47, 48] or local standard practice. However, treatment with granulocyte-colony stimulating factors will not permitted in cycle 1 unless the patient has febrile neutropenia and the physician considers its use as clinically indicated. It will be left to the treating physician choice from cycle 2.
- Bisphosphonate or denosumab therapy to be used in accordance with the approved labeled indication and/or nationally recognized treatment guidelines. Participants already receiving bisphosphonate/denosumab at the time of study entry can continue the treatment.
- Anticoagulants - Anticoagulation with heparin, heparin derivatives, and/or warfarin may be given at the discretion of the treating physician. Coagulation parameters should be checked at least once monthly, or more frequently at discretion of treating physician.
- Pain medications administered per standard clinical practice are acceptable while the patient is enrolled in the study.

Patients who experience toxicities should be treated symptomatically as clinically indicated. Medications that are considered necessary for the subject's welfare and that are not expected to interfere with the evaluation of study treatment or be restricted may be given at the discretion of the investigator. Ancillary treatments will be given as medically indicated.

5.7 Criteria for Taking a Participant Off Protocol Therapy

Duration of therapy will depend on individual response, evidence of disease progression and tolerance. In the absence of treatment delays due to adverse event(s), treatment may continue until one of the following criteria applies:

- Disease progression – in any subject who showed first radiologic evidence of progressive disease (PD) by RECIST 1.1 (see Section 11.1.4) and is deemed clinically stable, it is at the discretion of the investigator to continue treating the subject until progression is confirmed at least 4 weeks from the date of the first radiologic evidence of PD. Further details are described below.

- Participants who have attained a confirmed complete response (CR) that have been treated for at least 27 weeks with pembrolizumab and had at least two treatments with pembrolizumab beyond the date when the initial CR was declared. Concurrent discontinuation of Eribulin mesylate is at the discretion of the investigator. Subjects who then experience radiographic disease progression may be eligible for up to one year of additional treatment with pembrolizumab via the Second Course Phase (see below) at the discretion of the investigator if no systemic cancer treatment other than Eribulin mesylate was administered since the last dose of pembrolizumab, the subject meets the safety parameters listed in the Inclusion/Exclusion criteria, and the trial is open. Subjects will resume therapy at the same dose and schedule as at the time of initial discontinuation.

- Intercurrent illness that prevents further administration of treatment

- Unacceptable adverse event(s)

- Participant demonstrates an inability or unwillingness to comply with the medication regimen and/or documentation requirements

- Participant decides to withdraw from the protocol therapy

- General or specific changes in the participant's condition render the participant unacceptable for further treatment in the judgment of the treating investigator

Participants will be removed from the protocol therapy when any of these criteria apply. The reason for removal from protocol therapy, and the date the participant was removed, must be documented in the case report form (CRF) and recorded in the clinical trials management system (OnCore). Alternative care options will be discussed with the participant.

In the event of unusual or life-threatening complications, treating investigators must immediately notify the Overall PI, Sara Tolaney, MD MPH at 617-632-3800 or stolaney@partners.org.

Confirmation of Progressive Disease

Pembrolizumab, like other immunotherapeutic agents, may produce antitumor effects by potentiating endogenous cancer-specific immune responses. The response patterns seen with such an approach may extend beyond the typical time course of image responses seen with cytotoxic agents, and can manifest as a clinical response after an initial increase in tumor burden

or even the appearance of new lesions.

For any subject who showed first radiologic evidence of progressive disease (PD) by RECIST 1.1 (see Section 11.1.4) and is deemed clinically stable, it is at the discretion of the investigator to continue treating the subject until progression is confirmed at least 4 weeks from the date of the first radiologic evidence of PD. If progression is confirmed, the subject will be discontinued from study treatment. Otherwise, the subject will continue treatment and radiographic scans. Any subject who had initial radiologic progression and is deemed clinically unstable should be discontinued from both study drugs and no subsequent scan for confirmation is required.

Further details are as below:

For purposes of PFS assessment on this trial, in addition to radiographic assessment of tumor response or progression, the investigator should into account the clinical condition/stability of subjects.

- Clinically stable is defined by the following criteria:
    - Absence of signs and symptoms (including worsening of laboratory values) indicating disease progression
    - No decline in ECOG performance status
    - Absence of rapid progression of disease
    - Absence of progressive tumor(s) at critical anatomical sites (eg, cord compression) requiring urgent alternative medical intervention

Any subject who showed first radiologic evidence of progressive disease and is deemed clinically unstable should be discontinued from both study drugs and is not required to have repeat imaging for confirmation.

For a clinically stable subject with first radiologic evidence of progressive disease (ie, unconfirmed progression of disease), it is at the discretion of the investigator to continue treating the subject with the assigned treatment per protocol until progression of disease is confirmed on a subsequent scan at least 4 weeks later. If progression is not confirmed on the subsequent scan, the subject should continue to receive study treatment and have radiographic scans performed every 6 weeks if the patient has been on study for less than 24 weeks, or every 9 weeks for patients who have been on study greater than 24 weeks to monitor disease status. If radiologic progression is confirmed by subsequent scans, then the subject will be discontinued from study treatment. Exceptions may be considered to continue treatment in the presence of clinically stable or improved condition only after consultation with the principal investigator.

5.8 Duration of Follow Up

Participants removed from protocol therapy for unacceptable adverse event(s) will be followed until resolution or stabilization of the adverse event.

In patients who come off trial for a reason other than progressive disease, if possible during the follow-up period, scans should be performed every 9 weeks (if the patients is within 27 weeks of

initiation of study treatment) or every 12 weeks (if the patient is greater than 54 weeks from initiation of study treatment) to evaluate for disease progression.

All participants will be followed annually by phone or by medical record until death.

5.9 Criteria for Taking a Participant Off Study

Participants will be removed from study when any of the following criteria apply:

- Lost to follow-up
- Withdrawal of consent for data submission
- Death

The reason for taking a participant off study, and the date the participant was removed, must be documented in the case report form (CRF).

The research team submits a completed Off Treatment/Off Study form to ODQ when a participant comes off study. This form can be found on the ODQ website or obtained from the ODQ registration staff.

6. DOSING DELAYS/DOSE MODIFICATIONS

Dose delays and modifications will be made as indicated in the following section. The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for dose delays and dose modifications. A copy of the CTCAE version 4.0 can be downloaded from the CTEP website http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

Dosing interruptions are permitted in the case of medical / surgical events or logistical reasons not related to study therapy (e.g., elective surgery, unrelated medical events, patient vacation, and/or holidays). Subjects should be placed back on study therapy within 3 weeks of the scheduled interruption, unless otherwise discussed with the Sponsor. The reason for interruption should be documented in the patient's study record.

If there are dosing delays for any reason, all study assessments are to be delayed in the same fashion, such that that scans and other assessments occur in conjunction with cycles of treatment.

Eribulin mesylate doses may be delayed and/or reduced. No re-escalation will be allowed. Pembrolizumab may be delayed as a result of toxicities but there are no dose reductions allowed.

For toxicities in this Section, which are attributable to pembrolizumab alone, only pembrolizumab should be held as directed. It is permissible to continue eribulin mesylate despite discontinuation of pembrolizumab in these select cases. This will be left at physician discretion.

For toxicities in this Section, which are attributable to eribulin mesylate alone, it is permissible to continue pembrolizumab and is left to the treating investigator's discretion. If a decision to stop indefinitely eribulin mesylate is made, it is permissible to continue pembrolizumab despite discontinuation of eribulin mesylate in these select cases. This will be left at physician discretion.

6.1 Management of toxicities attributable to pembrolizumab

Adverse events (both non-serious and serious) associated with pembrolizumab exposure may represent an immunologic etiology. These adverse events may occur shortly after the first dose or several months after the last dose of treatment. Pembrolizumab must be withheld for drug-related toxicities and severe or life-threatening AEs as per Table 4 below.

Table 4: Dose modification guidelines for Pembrolizumab for drug-related adverse events

General instructions:

1. Corticosteroid taper should be initiated upon AE improving to less than or equal to Grade 1 baseline and continue to taper over at least 4 weeks.
2. For situations where pembrolizumab has been withheld, pembrolizumab can be resumed after AE has been reduced to less than or equal to Grade 1 or baseline and corticosteroid has been tapered. Pembrolizumab should be permanently discontinued if AE does not resolve within 12 weeks of last dose or corticosteroids cannot be reduced to ≤10 mg prednisone or equivalent per day within 12 weeks.
3. For severe and life-threatening irAEs, IV corticosteroid should be initiated first followed by oral steroid. Other immunosuppressive treatment should be initiated if irAEs cannot be controlled by corticosteroids.

Immune-related AEs	**Toxicity grade or conditions (CTCAEv4.0)**	**Action taken to pembrolizumab**	**irAE management with corticosteroid and/or other therapies**	**Monitor and follow-up**
Pneumonitis	Grade 2	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	• Monitor participants for signs and symptoms of pneumonitis • Evaluate participants with suspected pneumonitis with radiographic imaging and initiate corticosteroid treatment • Add prophylactic antibiotics for opportunistic infections
	Grade 3 or 4, or recurrent Grade 2	Permanently discontinue		
Diarrhea / Colitis	Grade 2 or 3	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	• Monitor participants for signs and symptoms of enterocolitis (ie, diarrhea, abdominal pain, blood or mucus in stool with or without fever) and of bowel perforation (ie, peritoneal signs and ileus). • Participants with ≥ Grade 2 diarrhea suspecting colitis should consider GI consultation and performing endoscopy to rule out colitis.
	Grade 4	Permanently discontinue		

				• Participants with diarrhea/colitis should be advised to drink liberal quantities of clear fluids. If sufficient oral fluid intake is not feasible, fluid and electrolytes should be substituted via IV infusion.
AST / ALT elevation or Increased bilirubin	Grade 2	Continue	• Consider administering corticosteroids (initial dose of 0.5 - 1 mg/kg prednisone or equivalent) followed by taper	• Monitor with liver function tests (consider weekly or more frequently until liver enzyme value returned to baseline or is stable
	Grade 3 or 4	Withhold	• Administer corticosteroids (initial dose of 1-2 mg/kg prednisone or equivalent) followed by taper	
Type 1 diabetes mellitus (T1DM) or Hyperglycemia	Newly onset T1DM or Grade 3 or 4 hyperglycemia associated with evidence of β-cell failure	Withhold	• Initiate insulin replacement therapy for participants with T1DM • Administer anti-hyperglycemic in participants with hyperglycemia	• Monitor participants for hyperglycemia or other signs and symptoms of diabetes.
Hypophysitis	Grade 2	Withhold	• Administer corticosteroids and initiate hormonal replacements as clinically indicated.	• Monitor for signs and symptoms of hypophysitis (including hypopituitarism and adrenal insufficiency)
	Grade 3 or 4	Withhold or permanently discontinue[1]		
Hyperthyroidism	Grade 2	Continue	• Treat with non-selective beta-blockers (eg, propranolol) or thionamides as appropriate	• Monitor for signs and symptoms of thyroid disorders.
	Grade 3 or 4	Withhold or permanently discontinue[1]		
Hypothyroidism	Grade 2-4	Continue	• Initiate thyroid replacement hormones (eg, levothyroxine or liothyroinine) per standard of care	• Monitor for signs and symptoms of thyroid disorders.
Nephritis and Renal dysfunction	Grade 2	Withhold	• Administer corticosteroids (prednisone 1-2 mg/kg or equivalent) followed by taper.	• Monitor changes of renal function
	Grade 3 or 4	Permanently discontinue		
Myocarditis	Grade 1 or 2	Withhold	• Based on severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other cause
	Grade 3 or 4	Permanently discontinue		
Infusion Reaction[2]	Grade 3 or 4	Permanently discontinue	• See Table 5	• See Table 5
All other immune-related AEs	Intolerable/ persistent Grade 2	Withhold		

	Grade 3	Withhold or discontinue based on the type of event. Events that require discontinuation include and not limited to: Gullain-Barre Syndrome, encephalitis	• Based on type and severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other causes
	Grade 4 or recurrent Grade 3	Permanently discontinue		
1. Withhold or permanently discontinue pembrolizumab is at the discretion of the investigator or treating physician. 2. See Table 5 for further guidance on all grades of pembrolizumab infusion reactions. **NOTE:** For participants with Grade 3 or 4 immune-related endocrinopathy where withhold of pembrolizumab is required, pembrolizumab may be resumed when AE resolves to ≤ Grade 2 and is controlled with hormonal replacement therapy or achieved metabolic control (in case of T1DM).				

Supportive care for pembrolizumab toxicity, particularly suspected immune-mediated toxicity
Subjects should receive appropriate supportive care measures as deemed necessary by the treating investigator. Suggested supportive care measures for the management of adverse events with potential immunologic etiology are outlined below.

Note that several courses of steroid tapering may be necessary as symptoms may worsen when the steroid dose is decreased. For each disorder, attempts should be made to rule out other causes such as metastatic disease or bacterial or viral infection, which might require additional supportive care. The treatment guidelines are intended to be applied when the investigator determines the events to be related to pembrolizumab.

Note: if after the evaluation the event is determined not to be related, the investigator is instructed to follow the guidance in Section 7 but does not need to follow the treatment guidance provided.

It may be necessary to perform conditional procedures such as bronchoscopy, endoscopy, or skin photography as part of evaluation of the event.

- **Management of Infusion Reactions**: Signs and symptoms usually develop during or shortly after drug infusion and generally resolve completely within 24 hours of completion of infusion. Table 5 below shows treatment guidelines for subjects who experience an infusion reaction associated with administration of pembrolizumab.

Table 5: Infusion Reaction Treatment Guidelines for pembrolizumab

NCI CTCAE Grade	Treatment	Premedication at subsequent dosing
Grade 1 Mild reaction; infusion interruption not indicated; intervention not	Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator.	None

NCI CTCAE Grade	Treatment	Premedication at subsequent dosing
indicated		
Grade 2 Requires infusion interruption but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDS, narcotics, IV fluids); prophylactic medications indicated for < =24 hrs	**Stop Infusion and monitor symptoms.** Additional appropriate medical therapy may include but is not limited to: IV fluids Antihistamines NSAIDS Acetaminophen Narcotics Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator. If symptoms resolve within one hour of stopping drug infusion, the infusion may be restarted at 50% of the original infusion rate (e.g., from 100 mL/hr to 50 mL/hr). Otherwise dosing will be held until symptoms resolve and the subject should be premedicated for the next scheduled dose. **Subjects who develop Grade 2 toxicity despite adequate premedication should be permanently discontinued from further trial treatment administration.**	Subject may be premedicated 1.5h (± 30 minutes) prior to infusion of pembrolizumab with: Diphenhydramine 50 mg po (or equivalent dose of antihistamine). Acetaminophen 500-1000 mg po (or equivalent dose of antipyretic).
Grades 3 or 4 Grade 3: Prolonged (i.e., not rapidly responsive to symptomatic medication and/or brief interruption of infusion); recurrence of symptoms following initial improvement; hospitalization indicated for other clinical sequelae (e.g., renal impairment, pulmonary infiltrates) Grade 4: Life-threatening; pressor or ventilatory support indicated	**Stop Infusion.** Additional appropriate medical therapy may include but is not limited to: IV fluids Antihistamines NSAIDS Acetaminophen Narcotics Oxygen Pressors Corticosteroids Epinephrine Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator. Hospitalization may be indicated. **Subject is permanently discontinued from further trial treatment administration.**	No subsequent dosing
Appropriate resuscitation equipment should be available in the room and a physician readily available during the period of drug administration.		

6.2 Management of toxicities attributable to Eribulin mesylate

Subjects should be carefully monitored for toxicity. If a treatment delay is required for eribulin mesylate toxicity, treatment with pembrolizumab is allowed. If toxicities recur, the dose of eribulin mesylate should be reduced. Once reduced, the dose of eribulin mesylate will not be re-escalated.

Dose adjustment recommendations for eribulin mesylate are shown in Tables 6 and delays are discussed below in Table 7.

If treatment with eribulin mesylate is withheld permanently, participants are allowed to continue to receive pembrolizumab therapy.

Table 6. Eribulin mesylate dose modifications

Starting dose	**1.4 mg/m2 (1 cycle = 21 days)**
1st dose reduction	1.1 mg/m2
2nd dose reduction	0.7 mg/m2

Table 7. Dose adjustments for Eribulin Mesylate

Adverse reaction/Toxicity	**Grade/Details**	**Eribulin mesylate dose modification**
ANC	< 500 cells/mm3 lasting > 7 days with or without use of growth factors	Hold eribulin mesylate until recovery to grade ≤ 2 and reduce by 1 dose level. Prophylactic growth factor support should be instituted for subsequent cycles.
	< 500 cells/mm3 lasting ≤7 days without use of growth factors	Hold eribulin mesylate until recovery to grade ≤ 2. Resume eribulin mesylate at the same dose. Growth factor support should be provided.
	< 500 cells/mm3 lasting ≤ 7 days in despite use of growth factors	Hold eribulin mesylate until recovery to grade ≤ 2, and reduce by 1 dose level. Continue ongoing prophylactic growth factor support for subsequent cycles.
	<1000 /mm3 with fever or infection without use of growth factors	Hold eribulin mesylate until recovery to grade ≤ 2. Resume eribulin mesylate at the same dose. Growth factor support should be provided.
	<1000 /mm3 with fever or infection despite use of growth factors	Hold eribulin mesylate until recovery to grade ≤ 2, and reduce by 1 dose level. Growth factor support should be provided.
	<1000 /mm3 without fever or infection	First occurrence: Hold eribulin mesylate until recovery to grade ≤ 2 then resume at the same dose. Growth factor support should be provided . If uncomplicated neutropenia (<1000 /mm3 without fever or infection) occurs/recurs despite growth factor support, then hold eribulin mesylate until recovery to grade ≤ 2. Eribulin mesylate may be then be resumed at the same dose with growth factor support, or it may be reduced by one dose level. If a patient misses two consecutive Day 8 eribulin mesylate doses due to uncomplicated neutropenia, then hold eribulin mesylate until recovery to grade ≤ 2 , reduce by 1 dose level, and consider

		prophylactic growth factor support for subsequent cycles.
Platelets	Grade 2	Hold eribulin mesylate until recovery to grade ≤ 2
	Grade 3	Hold eribulin mesylate until recovery to grade ≤ 2 then reduce by 1 dose level.
	Grade 4	Hold eribulin mesylate until recovery to grade ≤ 2 then reduce by 1 dose level.
Anemia	Grade 4	Hold eribulin mesylate until recovery to grade ≤ 2 then reduce by 1 dose level. PRBC transfusions are permitted to treat anemia on protocol.
Peripheral neuropathy	Grade 2	For intolerable (as determined by physician and patient) grade 2, decrease eribulin mesylate by one dose level.
	Grade 3	Hold eribulin mesylate until recovery to grade ≤ 2 then reduce by 1 dose level.
	Grade 4	Permanently discontinue eribulin mesylate
Non-hematologic toxicity	Grade 3, first occurrence	Hold eribulin mesylate until recovery to grade ≤ 2. Maximize supportive care measures. If symptom recovery occurs within 1 week, then eribulin mesylate may be resumed at the same dose if deemed appropriate by the investigator, otherwise, reduce by 1 dose level.
	Grade 3, despite maximal supportive measures	Hold eribulin mesylate until recovery to grade ≤ 2 then reduce by 1 dose level.
	Grade 4	Hold eribulin mesylate until recovery to grade ≤ 2 then reduce by 1 dose level.

ANC = absolute neutrophil count

- Toxicities graded in accordance with National Cancer Institute Common Toxicity Criteria for AEs, version 4.0
- Discontinuation should be considered if a dose reduction to <0.7 mg/m^2 is required.
- A minimum of 6 days between D1 and D8 of eribulin mesylate administration will be required and a minimum of 10 days between D8 and D22 (i.e. D1 of next cycle) will be required.
- If a patient does not meet criteria to receive Day 8 of eribulin mesylate, the investigator may decide to make up the dose on Day 15 of the cycle. If the Day 15 dose is given, the next cycle should not be initiated until 2 weeks later. If the Day 15 dose is held again, the next cycle may initiate the following week.
- Once a dose is reduced, it should not be re-escalated.
- Treatment with eribulin mesylate will be discontinued if participants require a delay greater than 3 weeks.
- Treatment with pembrolizumab is allowed to continue if eribulin mesylate is discontinued.

7. ADVERSE EVENTS: LIST AND REPORTING REQUIREMENTS

Adverse event (AE) monitoring and reporting is a routine part of every clinical trial. The following list of reported and/or potential AEs and the characteristics of an observed AE will determine whether the event requires expedited reporting **in addition** to routine reporting.

7.1 Adverse Events Lists

7.1.1 Adverse Event List(s) for pembrolizumab

In the pembrolizumab monotherapy trials (P001/P002, P012, P013, and P028, plus the P011 monotherapy arm), the overall incidence of AEs ranged from 83.0% (73 of 88 subjects in P012) to 100% (10 of 10 subjects in P011). The most commonly reported AEs included fatigue, diarrhea, decreased appetite, nausea, and anemia. The incidence of drug –related AEs (DRAEs) ranged from 39.8% (35 of 88 subjects in P013) to 80.0% (8 of 10 subjects in P011). The most commonly reported DRAEs across all studies were nausea, fatigue, and diarrhea. The incidence of Grade 3-5 DRAEs across studies ranged from 6.8% (6 of 88 in P013) to 12.0% (187 of 1562 subjects) in P001/P002. The most commonly reported Grade 3-5 DRAEs were anemia, alanine aminotransferase increased, and aspartate aminotransferase increased. Most subjects who experienced an AE continued in the study, with the incidence of AEs leading to discontinuation ranging from 1.9% (8 of 430 subjects in P028) to 12.3% (192 of 1562 subjects in P001/P002). The majority of AEs leading to discontinuation were not considered drug related. Discontinuations due to a DRAE were infrequent and ranged from 0% (no subjects in P011) to 4.5% (4 of 88 subjects in P013). The most commonly reported DRAEs leading to discontinuation were pneumonitis, alanine aminotransferase increased, and aspartate aminotransferase increased.

List of AEs considered expected:

- Endorcine disorders: Adrenal insufficiency, Hyperthyroidism, Hypophysitis, Hypopituitarism, Hypothyroidism, Secondary adrenal insufficiency, Thyroid disorder
- Eye disorders: Uveitis
- Gastrointestinal disorders: Abdominal pain, Colitis, Diarrhoea,Pancreatitis
- General disorders and administration site conditions: Asthenia, Pyrexia
- Hepatobiliary disorders: Autoimmune hepatitis, Hepatitis
- Infusion related reaction
- Metabolism and nutrition disorders: Diabetic ketoacidosis, Hyponatremia, Type 1 diabetes mellitus
- Musculoskeletal and connective tissue disorders: Arthralgia, Back pain, Myositis
- Nervous system disorders: Guillain-Barré syndrome
- Renal and urinary disorders: Nephritis
- Respiratory, thoracic and mediastinal disorders: Cough, Pneumonitis
- Skin and subcutaneous tissue disorders: Pruritis, Rash, Severe skin reaction, Vitiligo

7.1.2 Adverse Event List(s) eribulin mesylate

Most AEs on studies with eribulin mesylate are grade 1 or 2. Peripheral neuropathy was an important AE leading to discontinuation of therapy.

The most frequently reported eribulin mesylate related AEs were:

- asthenia/fatigue
- alopecia
- neutropenia
- nausea
- anemia
- pyrexia
- leucopenia
- anorexia
- constipation
- vomiting
- and peripheral neuropathy

7.2 Adverse Event Characteristics

- **CTCAE term (AE description) and grade:** The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. All appropriate treatment areas should have access to a copy of the CTCAE version 4.0. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site http://ctep.cancer.gov/protocolDevelopment/electronic_applications/ctc.htm.

- **For expedited reporting purposes only:**
  - AEs for the agent(s) that are listed above should be reported only if the adverse event varies in nature, intensity or frequency from the expected toxicity information which is provided.

- **Attribution** of the AE:
  - Definite – The AE *is clearly related* to the study treatment.
  - Probable – The AE *is likely related* to the study treatment.
  - Possible – The AE *may be related* to the study treatment.
  - Unlikely – The AE *is doubtfully related* to the study treatment.
  - Unrelated – The AE *is clearly NOT related* to the study treatment.

7.3 Expedited Adverse Event Reporting

Investigators **must** report to the Overall PI any serious adverse event (SAE) that occurs after the initial dose of study treatment, during treatment, or within 30 days of the last dose of treatment on the local institutional SAE form.

7.3.1 DF/HCC Expedited Reporting Guidelines

Investigative sites within DF/HCC and DF/PCC will report SAEs directly to the DFCI Office for Human Research Studies (OHRS) per the DFCI IRB reporting policy.

7.4 Expedited Reporting to the Food and Drug Administration (FDA)

The Overall PI, as study sponsor, will be responsible for all communications with the FDA. The Overall PI will report to the FDA, regardless of the site of occurrence, any serious adverse event that meets the FDA's criteria for expedited reporting following the reporting requirements and timelines set by the FDA.

7.5 Expedited Reporting to Hospital Risk Management

Participating investigators will report to their local Risk Management office any participant safety reports or sentinel events that require reporting according to institutional policy.

7.6 Expedited Reporting to Merck

A serious adverse event is any adverse event occurring at any dose or during any use of Merck's product that:

- Results in death;
- Is life threatening;
- Results in persistent or significant disability/incapacity;
- Results in or prolongs an existing inpatient hospitalization;
- Is a congenital anomaly/birth defect;
- Is a new cancer (that is not a condition of the study);
- Is associated with an overdose;
- Is an other important medical event

Any serious adverse event, or follow up to a serious adverse event, including death due to any cause other than progression of the cancer under study that occurs to any subject from the time the consent is signed through 90 days following cessation of treatment, or the initiation of new anti-cancer therapy, whichever is earlier, whether or not related to Merck product, must be reported within 24 hours to the Sponsor and within 2 working days to Merck Global Safety.

Non-serious Events of Clinical Interest will be forwarded to Merck Global Safety and will be handled in the same manner as SAEs.

Additionally, any serious adverse event, considered by an investigator who is a qualified physician to be related to Merck product that is brought to the attention of the investigator at any time outside of the time period specified in the previous paragraph also must be reported immediately to Merck.

SAE reports and any other relevant safety information are to be forwarded to the Merck

Global Safety facsimile number: +1-215-993-1220

A copy of all 15 Day Reports and Annual Progress Reports is submitted as required by FDA, European Union (EU), Pharmaceutical and Medical Devices agency (PMDA) or other local regulators. Investigators will cross reference this submission according to local regulations to the Merck Investigational Compound Number (IND, CSA, etc.) at the time of submission. Additionally investigators will submit a copy of these reports to Merck & Co., Inc. (Attn: Worldwide Product Safety; FAX 215 993-1220) at the time of submission to FDA.

All subjects with serious adverse events must be followed up for outcome.

7.6.1 Events of Clinical Interest (ECIs)

Selected non-serious and serious adverse events are also known as Events of Clinical Interest (ECI) and must be reported within 24 hours to the Sponsor and within 2 working days to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220).

For the time period beginning when the consent form is signed until treatment allocation/randomization, any ECI, or follow up to an ECI, that occurs to any subject must be reported within 24 hours to the Sponsor and within 2 working days to Merck Global Safety if it causes the subject to be excluded from the trial, or is the result of a protocol-specified intervention, including but not limited to washout or discontinuation of usual therapy, diet, placebo treatment or a procedure.

For the time period beginning at treatment allocation/randomization through 90 days following cessation of treatment, or 30 days following cessation of treatment if the subject initiates new anticancer therapy, whichever is earlier, any ECI, or follow up to an ECI, whether or not related to Merck product, must be reported within 24 hours to the Sponsor and within 24 hours to Merck Global Safety.

Events of clinical interest for this trial include:

1. Overdose of Merck product, as defined in Section 7.13.2 - Definition of an Overdose for This Protocol and Reporting of Overdose to the Sponsor, that is not associated with clinical symptoms or abnormal laboratory results.
2. An elevated AST or ALT lab value that is greater than or equal to 3X the upper limit of normal and an elevated total bilirubin lab value that is greater than or equal to 2X the upper limit of normal and, at the same time, an alkaline phosphatase lab value that is less than 2X the upper limit of normal, as determined by way of protocol-specified laboratory testing or unscheduled laboratory testing.*

*Note: These criteria are based upon available regulatory guidance documents. The purpose of the criteria is to specify a threshold of abnormal hepatic tests that may require an additional evaluation for an underlying etiology.

7.6.2 Definition of an Overdose of Pembrolizumab for This Protocol and Reporting of Overdose to Merck

For purposes of this trial, an overdose of pembrolizumab will be defined as any dose of 1,000 mg

or greater. No specific information is available on the treatment of overdose of pembrolizumab. Appropriate supportive treatment should be provided if clinically indicated. In the event of overdose, the subject should be observed closely for signs of toxicity. Appropriate supportive treatment should be provided if clinically indicated.

If an adverse event(s) is associated with ("results from") the overdose of a Merck product, the adverse event(s) is reported as a serious adverse event, even if no other seriousness criteria are met.

If a dose of Merck's product meeting the protocol definition of overdose is taken without any associated clinical symptoms or abnormal laboratory results, the overdose is reported as a non-serious Event of Clinical Interest (ECI), using the terminology "accidental or intentional overdose without adverse effect."

All reports of overdose with and without an adverse event must be reported within 24 hours to the Sponsor and within 2 working days hours to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220)

7.6.3 Reporting of Pregnancy and Lactation to Merck

Although pregnancy and lactation are not considered adverse events, it is the responsibility of investigators or their designees to report any pregnancy or lactation in a subject (spontaneously reported to them) that occurs during the trial.

Pregnancies and lactations that occur after the consent form is signed but before treatment allocation/randomization must be reported by the investigator if they cause the subject to be excluded from the trial, or are the result of a protocol-specified intervention, including but not limited to washout or discontinuation of usual therapy, diet, placebo treatment or a procedure.

Pregnancies and lactations that occur from the time of treatment allocation/randomization through 120 days following cessation of Sponsor's product, or 30 days following cessation of treatment if the subject initiates new anticancer therapy, whichever is earlier, must be reported by the investigator. All reported pregnancies must be followed to the completion/termination of the pregnancy. Pregnancy outcomes of spontaneous abortion, missed abortion, benign hydatidiform mole, blighted ovum, fetal death, intrauterine death, miscarriage and stillbirth must be reported as serious events (Important Medical Events). If the pregnancy continues to term, the outcome (health of infant) must also be reported.

Such events must be reported within 24 hours to the Sponsor and within 2 working days to Merck Global Safety. (Attn: Worldwide Product Safety; FAX 215 993-1220)

7.7 Expedited Reporting to Eisai

SAEs where the Overall PI considers a relationship to the eribulin therapy to be at least a reasonable possibility, will be reported to EISAI on a Medwatch 3500A form within one business days of the notification of the event. Serious adverse events (SAEs) that are not related to eribulin therapy and non-serious AEs will be provided to Eisai in the final study report and any interim reports provided.

Events **not** considered to be serious adverse events are hospitalizations for:

- routine treatment or monitoring of the studied indication, not associated with any deterioration in condition, or for elective procedures
- elective or pre-planned treatment for a pre-existing condition that did not worsen
- emergency outpatient treatment for an event not fulfilling the serious criteria outlined above and not resulting in inpatient admission
- respite care

The reports will be sent on MedWatch 3500A form to EISAI at the number/email listed below:

EISAI safety fax number (732-791-1111) Email: ESI_Safety@eisai.com

7.8 Routine Adverse Event Reporting

All Grade 2 or greater Adverse Events **must** be reported in routine study data submissions to the Overall PI on the toxicity case report forms. **AEs reported through expedited processes (e.g., reported to the IRB, FDA, etc.) must also be reported in routine study data submissions.**

8. PHARMACEUTICAL INFORMATION

A list of the adverse events and potential risks associated with the investigational and other agents administered in this study can be found in Section 7.1.

8.1 PEMBROLIZUMAB

Please refer to the Investigator's Brochure for detailed agent information, and to the FDA label for additional information.

8.1.1 Description

Pembrolizumab is a humanized monoclonal antibody of the IgG4/kappa isotype. Other name: MK-3475, Keytruda. Pembrolizumab blocks negative immune regulatory signaling by binding to the PD-1 receptor, inhibiting the interaction between PD-1 and its ligands.

The molecular weight of Pembrolizumab is 148.9-149.5 KDa.

8.1.2 Form

Clinical supplies will be manufactured and provided by Merck as summarized in Table 7.

Table 7: Product Description

Product Name & Potency	Dosage Form
Pembrolizumab 100 mg/ 4mL	Solution for Injection

8.1.3 **Storage and Stability**

Store intact vials between 2°C-8°C (36°F-46°F). Do not freeze. Protect from light by storing in the original box.

Stability testing of the intact vials is ongoing.

Administer prepared solutions immediately after preparation. If not administered immediately, prepared solutions may be stored refrigerated for up to 20 hours. PEMBROLIZUMAB solutions may be stored at room temperature for a cumulative time of up to 4 hours. This includes room temperature storage of liquid drug product solution in vials, room temperature storage of infusion solution in the IV bag, and the duration of infusion.

8.1.4 **Compatibility**

Compatible IV bag materials: PVC plasticized with DEHP, non-PVC (polyolefin), EVA, or PE lined polyolefin.

8.1.5 **Handling**

Qualified personnel, familiar with procedures that minimize undue exposure to themselves and the environment, should undertake the preparation, handling, and safe disposal of the chemotherapeutic agent in a self-contained and protective environment.

8.1.6 **Availability**

Pembrolizumab is an investigational agent and will be supplied free of charge from Merck.

8.1.7 **Preparation**

Pembrolizumab solution for infusion must be diluted prior to administration. Allow the required number of vials to equilibrate to room temperature. Do not shake the vials. Do not use if opaque or extraneous particulate matter other than translucent to white proteinaceous particles is observed. Do not use if discolored. To prepare the infusion solution add the dose volume of Pembrolizumab to an infusion bag containing 0.9& Sodium Chloride Injection, USP of 5% Dextrose Injection, USP. Gently invert the bag 10-15 times to mix the solution. The final concentration must be between **1 mg/mL to 10 mg/mL.**

8.1.8 **Administration**

Route of administration: IV infusion only. Do not administer as an IV push or bolus injection.

Method of administration: Infuse over approximately 30 minutes (range: 25-40 minutes) using an infusion set containing a low-protein binding 0.2 to 5 m in-line filter make of polyethersulfone or polysulfone. Infusion rate should not exceed 6.7 mL/min. A central line is not required however if a subject has a central venous catheter in place, it is recommended that it be used for the infusion. Do not co-administer drugs through the same infusion line. Following the infusion, flush the IV line with normal saline.

8.1.9 **Ordering**

Pembrolizumab will be obtained directly from Merck, the study sponsor.

8.1.10 **Accountability**

The investigator, or a responsible party designated by the investigator, should maintain a careful record of the inventory and disposition of the agent using the NCI Drug Accountability Record Form (DARF) or another comparable drug accountability form. (See the NCI Investigator's Handbook for Procedures for Drug Accountability and Storage.)

8.1.11 **Destruction and Return**

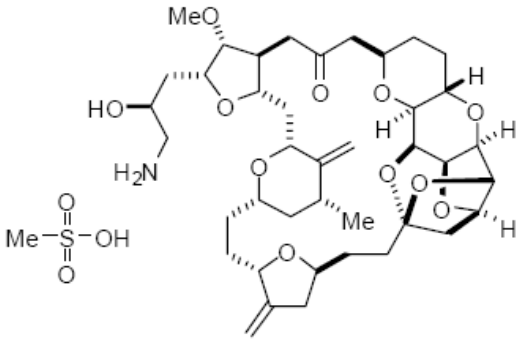
At the end of the study, unused supplies of Pembrolizumab should be destroyed according to institutional policies. Destruction will be documented in the Drug Accountability Record Form.

8.2 Eribulin mesylate

Please refer to the Investigator's Brochure for detailed agent information, and to the FDA label for additional information.

The chemical name for eribulin mesylate is 11,15:18,21:24,28-Triepoxy-7,9ethano-12,15-methano-9H,15H-furo[3,2-i]furo[2',3':5,6]pyrano[4,3-b][1,4]dioxacyclopentacosin-5(4H)-one,2[(2S)-3-amino-2-hydroxypropyl]hexacosahydro-3-methoxy-26-methyl-20,27-bis(methylene)-(2R,3R,3aS,7R,8aS,9S,10aR,11S,12R,13aR,13bS,15S,18S,21S,24S,26R,28R,29aS)-methanesulfonate (salt). It's molecular weight is: 826.00.

Structural formula:



8.2.1 **Form**
Eribulin mesylate is a sterile, ready-to-use, clear, colorless aqueous solution for i.v. administration.

Eribulin mesylate for i.v. injection will be supplied on an open-label basis by the sponsor in single-use vials. Each single-use vial contains 1 mg/2 mL of clear, colorless solution. Each single-use vial of eribulin mesylate is primarily packaged in a 5 mL nominal volume United States Pharmacopeia (USP) Type 1 Flint glass, stoppered with a FluroTec® plug stopper and sealed with an aluminum seal and a flip-off cap. Twelve labeled vials of eribulin mesylate are packaged in a labeled carton. Each of the 6 eribulin mesylate vials within a given labeled carton may be assigned individually to 6 different subjects or to the same subject, as required by the study site or as clinical supply inventory demands.

8.2.2 **Storage Conditions**
Eribulin mesylate must be stored in accordance with labeled storage conditions. Intact and unopened vials must be stored at ambient room temperature. **Do NOT refrigerate or freeze.** Shelf-life surveillance of the intact vials is ongoing. The 0.5 mg/mL solution has been shown to be stable in syringes at ambient temperature and ambient lighting for up to 4 hours, or under refrigeration for up to 24 hours. The drug is also stable at concentrations ranging from 0.005 ng/mL to 0.2 mg/mL when diluted in normal saline (0.9% sodium chloride [NaCl]) and kept refrigerated in syringes or i.v. bags for up to 48 hours at ambient temperature and ambient lighting, or under refrigeration. It is not compatible with solutions with dextrose.

Product vials that are opened and refrigerated between 2°C to 8°C (36°F – 46°F) must be used within 24 hours of being opened, and the remaining drug solution discarded.
Temperature monitoring for eribulin mesylate is required at the storage location to ensure that the study drug is maintained within an established temperature range. The investigator is responsible for ensuring that the temperature is monitored throughout the total duration of the trial and that records are maintained; the temperature should be monitored continuously by using either an in-house validated data acquisition system, a mechanical recording device such as a calibrated chart recorder, or by manual means, such that minimum and maximum thermometric values over a specific time period can be recorded and retrieved as required.

8.2.3 **Handling**

Qualified personnel, familiar with procedures that minimize undue exposure to themselves and the environment, should undertake the preparation, handling, and safe disposal of the eribulin mesylate in a self-contained and protective environment.

8.2.4 **Availability**

Eribulin mesylate is being provided by Eisai for this trial.

8.2.5 **Ordering**

Eribulin mesylate will be ordered directly from Eisai.

8.2.6 **Treatment Compliance**

Eribulin mesylate will be administered by trained medical personnel at the investigational site. Treatment compliance will be monitored through drug accountability documentation, as well as the recording of study treatment administration in the subject's medical record. In addition, all details pertaining to the administration of eribulin mesylate, including but not limited to date, actual dose, and start and end times of each dose, will be recorded on the Study Drug Administration page of the subject's eCRF. Clinical research associates (CRAs) will review treatment compliance during investigational site visits conducted during and at the completion of the study.

8.2.7 **Drug Supplies and Accountability**

Drug supplies and accountability will be as institutional policy.

The investigator, or a responsible party designated by the investigator, will maintain a careful record of the inventory and disposition of the agent (investigational or free of charge) using the NCI Drug Accountability Record or another comparable drug accountability form. (See the CTEP website at http://ctep.cancer.gov/protocolDevelopment for the "Policy and Guidelines for Accountability and Storage of Investigational Agents" or to obtain a copy of the drug accountability form.)

9. BIOMARKER, CORRELATIVE, AND SPECIAL STUDIES

All patients will be asked to provide archival tumor tissue (either paraffin blocks or 20 unstained slides, ideally 4 micron thickness). However, if archival tissue is not available or not evaluable, that will not be a basis to exclude the patient from any portion of the trial or the planned analysis. Archival tissue will be used for immune profiling assays to ascertain baseline values in patients who choose not to undergo baseline research fresh tissue biopsy or in whom there is insufficient tissue or failed testing of any of the planned assays from baseline fresh tissue biopsy.

If obtained, we plan to use baseline biopsy tissue to perform a number of immune profiling assays, detailed below, and characterize the samples based on histology (TILs), protein

expression, and mRNA expression. Additionally, specimens we will banked for possible future analysis.

Serial blood draws for correlative science are required on this trial; blood draws will be obtained every 3 weeks on treatment days prior to the infusion of study drugs, at the end-of-treatment visit in patients who go off study for progressive disease, and all efforts will be made to obtain a sample at the time of progressive disease from participants who went off study for anything other than progressive disease. We will perform flow cytometry on these samples to characterize protein expression of immune mediators as detailed in the lab manual. Circulating tumor DNA will be assessed as well.

An optional research biopsy will be collected for participants who have consented and have biopsy accessible tumor on arm B crossing over to pembrolizumab therapy.

An optional research biopsy at the time of progression will be collected for participants who consent and have biopsy accessible tumor. These biopsies will undergo the same characterization testing as described for baseline biopsies.

All patients will additionally be asked to provide a stool sample at three separate timepoints: prior to treatment, during treatment, and at the time of disease progression. A fourth collection may be requested from patients who experience grade ≥ 2 diarrhea after discussion with the PI. This collection is not required, but is strongly encouraged. These samples will be analyzed for microbiota content.

Please refer to the separate laboratory manual for additional correlative details including collection, processing, and shipping instructions.

9.1 Summary table: research tissue and blood specimen collection

Research Sampling	**Time point**	**Contents**
Blood	Cycle 1 Day 1	1-9 mL Streck Tube 5- 10mL green top tubes*
	Every Cycle Day 1	5- 10mL green top tubes *
	Restaging Visits Only	1-9mL Streck Tube
	Off Treatment for PD	1-9mL Streck Tube 5- 10mL green top tubes*
	Time of PD, in patients who came off-study for a reason other than PD (Optional)	1-9mL Streck Tube 5- 10mL green top tubes*
Fresh Tissue	Pre-treatment (Optional) – within 7 days of starting therapy	5-7cores
	Progression**	5-7cores
Archival Tissue	Anytime	1 block or 20, 4 micron unstained slides

Stool Sample	Pre-treatment (within 28 days of starting therapy)	Home Stool Kit (DNA Genotek)
	Cycle 3 Day 1 (within 14 days prior)	
	Disease Progression	
	At the time of grade ≥ 2 diarrhea (Optional)***	

* EDTA (purple top) tubes or CPT tubes may be used interchangeably with green top tubes
**Biopsy at the time of disease progression is optional for subjects on Arm A. Subjects on Arm B will be required to undergo a biopsy if they have accessible disease at the time of progression on Eribulin therapy, prior to initiating crossover treatment. Subjects on Arm B Crossover Therapy may choose to undergo an optional biopsy at the time of disease progression.
*** Collection at the time of grade ≥ 2 diarrhea is optional and should be discussed with the study PI.

9.2 Fresh Tissue Collection

9.2.1 Collection and handling of biopsy specimens

Biopsies are optional at baseline (within 7 days of starting therapy) and at the time of disease progression for subjects on Arm A and subjects on Arm B Crossover Therapy. There will be a required biopsy for subjects with accessible disease on Arm B who experience disease progression on Eribulin mesylate prior to initiating crossover therapy. Guidelines for biopsies from various metastatic sites can be found in Appendix C. Additional collection and processing instructions may be found in the separate laboratory manual.

Ideally five core biopsies will be obtained:

- Two cores should be placed in 10% neutral buffered formalin tube supplied by the study.
- One core should be placed in RNAlater
- Two cores should be placed in sterile DMEM

9.3 Procedures for obtaining blood and stool specimens for study

9.3.1 Blood Collection

Research blood collection is mandatory for all participants for flow cytometry and DNA isolation. The samples will be banked in the DF/HCC Clinical Trials Core Laboratory for these and future research purposes. These specimens will become the property of the DF/HCC.

Please refer to the separate laboratory manual for collection and processing instructions

9.3.2 Handling of Blood Specimens

All samples should be de-identified and labeled with the Participant initials, Participant Study ID number and date of collection and time point (e.g., “Baseline” or “Cycle 1” or “Progressive

Disease"). Please refer to the separate laboratory manual for collection and processing instructions.

- Green Top tubes:
  Five 10mL green top, purple top, or CPT tubes should be filled completely and should be processed within 3-4hrs of being drawn. Will be delivered or shipped, ambient overnight to Mariano Severgnini's lab. Please contact the lab ahead of time by approximately one week to notify of upcoming specimen drop off.

- Streck tubes:
  Fill the Streck tube completely and immediately mix by gentle inversion 8 to 10 times. Inadequate or delayed mixing may result in accurate results.

  Tube precautions:
  - DO NOT FREEZE OR REFRIGERATE TUBES as this could result in cfDNA breakage. Blood collected in the Streck tube can be stored for 14 days between 6-37 degrees Celsius.
  - Do not use tubes after expiration date.
  - Fill the tube completely; overfilling or underfilling of tubes will result in an incorrect blood-to-additive ratio and may lead to incorrect analytical results.

9.3.3 Stool Collection

All stool samples will be collected by each patient at home using a home-based kit with a pre-paid mailer that provides nearly equivalent metagenomic and metatranscriptomic data to state-of-the-art fresh-frozen sample-collection protocol. Patients will be asked to provide samples at the following timepoints:

- Baseline
- After two cycles of therapy
- At the time of disease progression
- Optional collection at the time of grade ≥ 2 diarrhea

Most kits will be provided to the patients at their clinic visits. If the study team is unable to provide the kits to the patients in clinic, they may be mailed to patients by members of the study team. All kits will contain a questionnaire for patients to complete and return with their samples regarding timing and conditions surrounding their stool sample.

Please refer to the separate lab manual for collection and processing instructions.

Samples will be stored at the BWH/Harvard Cohorts Repository and will be shipped out to an external lab vendor, Microbiome Dx, who will perform the analysis of the samples.

9.4 Sites Performing Correlative Studies

DFCI Center for Immuno-Oncology
DFCI Clinical Trials Core Laboratory

BWH/Harvard Cohorts Biorepository
Microbiome Dx – External Lab Vendor

9.5 Blood, tissue and stool banking

Any leftover blood, tissue, or stool will be banked in the lab of Mariano Severgnini or the DF/HCC Clinical Trials Core Laboratory, respectively, as per standard lab protocol, such that it can be used for additional or future analyses as needed.

9.6 Hypothesis for correlative objectives

9.6.1 Hypotheses

- We hypothesize that the immune marker profile in the peripheral blood will change over the course of eribulin mesylate +/- pembrolizumab therapy.
- We hypothesize that a larger increase in markers of immune activity in the peripheral blood will correlate with a better disease response as assessed on concurrent restaging scans, and in terms of best radiographic response at any time on trial.
  We hypothesize that an immune marker or composite of markers in the peripheral blood at baseline will correspond to TIL percentage in archival tumor tissue.
- We hypothesize that the immune activity associated with a breast tumor, as assessed both in tumor tissue and in circulating blood, will be less at the time of disease progression on therapy compared to the time of therapy initiation.
- We hypothesize that the structure and function of the gut microbiome differs among patients with breast cancer.
- We hypothesize that the structure and function of the gut microbiome before starting pembrolizumab and eribulin can be predictive of efficacy of this therapy, with a greater microbial diversity, estimated by Shannon index, being predictive of efficacy.
- We hypothesize that the structure and function of gut microbiome changes in response pembrolizumab-based therapy and can predict the likelihood of response to pembrolizumab.
- We hypothesize that the abundance and functional profile of specific gut bacteria is associated with response to pembrolizumab.

9.7 Planned assays for correlative objectives

All of the below-mentioned analyses may be altered based on novel developments in the field of cancer immune profiling at the time of correlative science. Additional markers or alternative technologies (based on scientific developments and/or novel technologies) may also be used, to explore potential prognostic or predictive candidate markers/panels or markers related to treatment benefit and/or safety, to improve diagnostic tests, or to understand breast cancer biology.

9.7.1 Tumor infiltrating lymphocyte (TIL) percentage and determination of lymphocyte predominant breast cancer (LPBC)

Paraffinized, hematoxylin and eosin-stained slides taken from two tissue planes will be derived from each biopsy and will be reviewed by certified pathologists. The extent of lymphocytic infiltrate in tumor tissue will be assessed, and stromal TIL percentage will be determined. More detailed guidelines for the quantification of stromal TILs in breast cancer can be found in the recommendations from the International TILs Working Group 2014.[31]

After assessment of the TIL percentage, the specimen may be categorized as lymphocyte predominant breast cancer (LPBC), defined as a tumor that contains >60% stromal lymphocytes, or non-LPBC.

9.7.2 Immunohistochemistry

Tissue will be collected and fixed by 10% neutral buffered formalin overnight, dehydrated, and paraffin embedded. Four micrometer-thick sections will be cut. The paraffin blocks and unstained slides will be stored at room temperature. All immunohistochemical staining will be performed in the Center for Immuno-Oncology Pathology Core at Dana-Farber/Harvard Cancer Center (DF/HCC) Specialized Histopathology Core.

Formalin fixed-paraffin embedded (FFPE) tumor slides will be prepared and H&E stained for assessment of TIL in pre- and post-treatment tumor samples. To identify subsets of different immune populations (effector/memory CD8 cells, T regulatory cells, dendritic cells, tumor associated macrophages, and Tie-2 expressing monocytes (TEM)), immunohistochemical (IHC) staining will be performed on FFPE tumor slices using some or all of the following antibodies:
Core set: CD8, PD-1, PD-L1, PD-L2
Others: CD3, CD4, CD25, FoxP3, Indoleamine 2,3 deoxygenase-1 (IDO1), CD11c, CD83, CD86, CD56, CD14, CD16, Tie2 (See also Appendix E)

Chen et al[37] describe a semi-quantitative scoring method, which is in accordance with typical biomarker scoring in anatomic and surgical pathology. Briefly, staining is scored according to intensity (0=no staining, 1=weak staining, 2=moderate staining, 3=strong staining), staining patter (M=predominantly cell membrane; C=predominantly cell cytoplasm), and the percentage of cells showing positive staining (0-100%). The semi-quantitative scoring is performed for: 1) the neoplastic tumor cells and 2) the non-neoplastic infiltrating immune cells. Significant discordant results have been rare during case evaluations.[37]

Further details of the immunohistochemical assay and assessment are described in the lab manual for this protocol.

It should be noted that the above staining protocols are based on standard methods used at the time of protocol writing. It is possible that at the time protein expression assays are conducted, novel and improved methods for staining will exist. In this case, we plan to use the best available, best validated experimental method available at the time.

9.7.3 Flow cytometry, genomic analysis of biopsy tissue

TILs will be isolated from the biopsy specimen and assessed by surface staining as described in the lab manual for this protocol.

Messenger RNA (mRNA) expression within tumor biopsy specimens will be assessed using the most current and informative methodologies at the time that correlative science is performed on all specimens. NanoString signatures and comprehensive RNA sequencing may be used. Potential genes of interest, based on prior immune profiling of breast tumors,[27] include CXCL9, CCL5, CD8ACD80, CXCL13, IGKC, CD21, IDO1, PD-1, PD-L1, PD-L2, CTLA4, and FOXP3. Additional DNA analysis, for example to assess mutational load and neoantigen burden, may also be performed.

9.7.4 Analysis of PBMCs

PBMCs will be generated as described in the lab manual for this protocol, and used to assess immune cell populations by flow cytometry.

9.7.5 Analysis of cell-free DNA

Blood will be collected at baseline, restaging visits and at time of progression for evaluation of cell-free DNA (cfDNA). The cfDNA will be banked in the DF/HCC Clinical Trials Core laboratory for future research purposes. The banked samples will be used to analyze DNA, RNA and protein in future studies.

9.7.6 Analysis of 16S rRNA gene sequencing

Microbial DNA is extracted using the Mag-Bind Universal Pathogen DNA Kit (Omega Bio-Tek). Briefly, 250 mg of the specimen is transferred to a deep-well plate for bead beating followed by DNA precipitation and purification following the manufacturer's instructions. Finally, DNA is eluted in 100 uls of Elution Buffer and stored at -80°C until further use. 16S sequencing libraries are generated by amplifying the v3-v4 hypervariable regions of the 16S gene in a polymerase chain reaction using primers F341and R785. Resulting amplicons are tagged with unique molecular barcodes that are later used to demultiplex sequencing reads into individual sample buckets. Libraries are loaded on a MiSeq flowcell and sequenced following Illumina's loading instructions. Sequence data are retrieved from the instrument by converting base call format files into fastq files for data processing purposes.

MicrobiomeDX uses BacPro™, a proprietary algorithm, to inspect and validate sequencing files by employing demultiplexing, trimming, merging, and quality filtering steps. Paired sequencing reads are merged using an overlap of 25 bp allowing for 10 base mismatches. Merged sequences are dereplicated and clustered in a de-novo fashion using VSEARCH, while filtering out sequence chimeras and singletons. Representative sequences from each cluster are mapped against the SILVA database at 99% sequence identity to obtain accurate taxonomic classifications and relative abundances. In parallel, feature tables are constructed to derive alpha diversity indices, and distance matrices are built to derive beta diversity indices. The BacPro™

pipeline generates a comprehensive report that includes alpha diversity scores describing community richness and evenness, taxonomic composition with relative abundances, and beta diversity metrics to determine the in-between sample differences based on the bacterial communities identified.

9.7.7 Shotgun sequencing and metabolic pathway reconstruction of stool samples

Stool samples from patients included in the trial 2 will be subjected to whole genome shotgun sequencing. Libraries will be constructed with Illumina barcodes from the TruSeq DNA Sample Prep kit (Illumina) and reagents from KAPA Library Preparation kit (Kapa Biosystems), and then sequenced on an Illumina MiSeq platform using 2_250 nucleotide paired-end sequencing, according to the manufacturer's instructions. Sequencing reads will be converted into relative abundances of microbial metabolic modules using HUMAnN35, the Human Microbiome Project metabolic reconstruction pipeline and mapped to the KEGG36. Relative species abundances will be calculated by the MetaPhlAn pipeline37.

10. STUDY CALENDAR

Consent may be obtained **28 days** prior to the start of protocol therapy. Baseline evaluations are to be conducted within **21 days** prior to start of protocol therapy (unless otherwise specified). If these screening assessments occur within 3 days before start of study treatment, they do not need to be repeated on Cycle 1 Day 1. Scans must be done within **28 days** prior to the start of protocol therapy.

As detailed in the Study Calendar, a negative pregnancy test in women of child-bearing potential (as defined in the eligibility criteria) must be documented within **7 days** prior to the start of protocol therapy.

If participants agree to a baseline tumor biopsy, this biopsy should be obtained within **7 days** before starting protocol therapy.

In the event that the participant's condition is deteriorating, laboratory evaluations should be repeated within 48 hours prior to initiation of the next cycle of therapy.

Assessments must be performed prior to administration of any study agent. Study assessments and agents should be administered within $\pm$ 3 days of the protocol-specified date, unless otherwise noted.

Day 8 assessments may be performed at DFCI Milford, as needed; however, due to correlative requirements, the Day 1 visits, scans, and/or biopsies may not occur at this location.

	Screening	C1		C2		C3		Cycle 4 and subsequent cycles		Crossover[a]	Off-Treatment[m]	Follow-Up
		D1	D8[s]	D1	D8[s]	D1	D8[s]	D1	D8[s]			
Arm A- Drug administration												
Pembrolizumab		X		X		X		X				
Eribulin mesylate		X	X	X	X	X	X	X	X			
Arm B- Drug administration												
Eribulin mesylate		X	X	X	X	X	X	X	X			
Pembrolizumab										X		
Informed consent	X											
Demographics	X											
Medical history	X									X		
Physical exam	X	X		X		X		X		X	X	
Vital signs	X	X	X	X	X	X	X	X	X	X	X	
Weight	X	X		X		X		X		X	X	
Performance status	X	X		X		X		X		X	X	
Concurrent medications	X	X----------X										
Adverse event evaluation		X----------X										
Hematology panel[b]	X	X	X	X	X	X	X	X	X	X	X	
Chemistry panel[c]	X	X	X	X	X	X	X	X	X	X	X	
Cortisol[t]	X			X		X		X				
TSH[d]	X			X				X		X		
Coagulation panel (PT/PTT)	X											
Pregnancy test[e]	X									X		
EKG	X									X		
Tumor Assessments[f, h]	X							X[g]				X[i]
Tumor biopsy	X[j]									X[k]	X[k]	
Archival tumor samples[l]	X											
Research Blood Collection		X		X		X		X		X	X[n]	X[o]
Research Stool Collection[q]	X					X					X	
Stool Questionnaire[r]	X					X					X	
Survival												X[p]

1 cycle = 21 Days

a. Participants on Arm B crossing over to Pembrolizumab monotherapy must meet the criteria provided in Section 3.4 and 3.5 to begin therapy with pembrolizumab.

b. Hematology to include: CBCA

c. Chemistries to include: Chloride, potassium, sodium, BUN, serum creatinine, phosphorus, calcium, albumin, total protein, alkaline phosphatase, ALT, AST, total bilirubin (NOTE: the frequency of checking magnesium levels is left up to the treating provider)

d. TSH collected at screening, C2D1, and every other cycle for participants receiving pembrolizumab. TSH is required at time of crossover.

e. In female participants of child-bearing potential (as defined in the eligibility criteria), a urine or serum pregnancy test must be performed within **7 days** prior to the first dose of study medication. If the urine test is positive or cannot be confirmed as negative, then a serum test is required. For female participants of child-bearing potential crossing over, a negative pregnancy test is also required within 7 days of starting crossover therapy.

f. Baseline tumor assessments must be done within 28 days prior to the start of therapy. Tumor assessments will be performed every 3 cycles (9 weeks) for the first 18 cycles (1 year). If after Cycle 18 scans (1 year), a participant has SD or better by RECIST the frequency of assessments may be reduced to every 4 cycles (12 weeks). If treatment is delayed, scans should continue to align with cycles of treatment, rather than weeks on treatment. Effort should be made to use the same type of imaging to assess measurable lesions at baseline and in follow-up. Confirmatory scans 4 weeks after documented response should be obtained

g. For any subject who showed first radiologic evidence of progressive disease (PD) by RECIST 1.1 (see Section 11.1.4) and is deemed clinically stable (see section 5.6), it is at the discretion of the investigator to continue treating the subject until progression is confirmed at least 4 weeks from the date of the first radiologic evidence of PD. If progression is confirmed, the subject will be discontinued from study treatment. Otherwise, the subject will continue treatment and radiographic scans. Any subject who had initial radiologic progression and is deemed clinically unstable should be discontinued from both study drugs and no subsequent scan for confirmation is required.

h. Tumor assessments should continue as scheduled for participants on Arm B who crossover to receive pembrolizumab.

i. During follow-up of patients who went off study for reasons other than PD, tumor assessments should continue every 9 to 12 weeks from the date of the last assessment until progressive disease is documented

j. Optional biopsy should be obtained within 7 days before starting protocol therapy. See Section 9, appendices, and lab manual for biopsy handling and processing instructions.

k. Optional tumor biopsy should be offered only in patients who go off study for progressive disease. See Section 9, appendices, and lab manual for biopsy handling and processing instructions. For patients randomized for the eribulin mesylate arm who wish to cross over to pembrolizumab arm, a biopsy at progression is mandatory for those whose disease is amenable for biopsy.

l. 1 block or 20 unstained slides should be collected. If archival tissue is not available, patients with safely accessible disease should have a baseline biopsy.

m. Off treatment visit is to occur within 30 days of final administration of study treatment. End of treatment assessments do not have to be repeated if the same assessments were performed within 7 days prior to the visit.

n. Research blood should only be collected on patients who come off treatment for progressive disease.

o. Optional blood collection for immune cells/cfDNA may be offered to patients who develop progressive disease during follow-up.

p. Survival status should be collected annually, either by review of the medical record or by phone call (see Section 5.8).

q. Baseline stool collection should be obtained within 28 days before starting protocol therapy. The C3D1 stool collection should be performed as close to C3D1 as possible, but may be collected up to 14 days prior. A sample will additionally be collected at the time of disease progression. An optional stool sample may be collected at the time of grade $\geq$ 2 diarrhea after discussion with the PI. Stool collections will not occur on Pembrolizumab Crossover therapy for patients randomized to Arm B. As these collections are for exploratory correlative purposes, failure to provide a sample at these timepoints will not constitute a protocol violation. See section 9 and/or lab manual for stool collection and processing instructions.

r. Each stool collection kit will contain a questionnaire for the patients to complete regarding the conditions surrounding their collection. These will be a part of the kit and are not to be administered in clinic. Failure to complete these questionnaires are the required or optional timepoints will not constitute a protocol violation.

s. D8 assessments may occur at DFCI Milford, as needed. Additionally, if a subject is on Arm A and discontinues Eribulin, D8 visits will not be required. D8 visits are also not required when Arm B subjects are receiving Crossover Pembrolizumab monotherapy.

t. For Arm A subjects, Cortisol blood draws will be required at baseline and every D1. For Arm B subjects, Cortisol blood draws will be drawn at baseline, as treatment arm is likely unknown, and then not again until all D1s of Crossover treatment. This will not be required when on Eribulin alone.

11. MEASUREMENT OF EFFECT

11.1 Antitumor Effect – Solid Tumors

Response and progression will be evaluated in this study using the new international criteria proposed by the Response Evaluation Criteria in Solid Tumors (RECIST) guideline (version 1.1) [*Eur J Ca* 45:228-247, 2009]. Changes in the largest diameter (unidimensional measurement) of the tumor lesions and the shortest diameter in the case of malignant lymph nodes are used in the RECIST criteria.

Pembrolizumab, like other immunotherapeutic agents, may produce antitumor effects by potentiating endogenous cancer-specific immune responses. The response patterns seen with such an approach may extend beyond the typical time course of image responses seen with cytotoxic agents, and can manifest as a clinical response after an initial increase in tumor burden or even the appearance of new lesions.

For any subject who showed first radiologic evidence of progressive disease (PD) by RECIST 1.1 (see Section 11.1) and is deemed clinically stable, it is at the discretion of the investigator to continue treating the subject until progression is confirmed at the next scheduled restaging (or with a confirmatory completed at least 4 weeks from the initial date of PD). If progression is not confirmed on the subsequent scan, the subject should continue to receive treatment and have radiographic scans performed according to the study calendar [approximately every 3 cycles (9 weeks) for the first 12 months and then every 4 cycles (12 weeks)]. If radiologic progression is confirmed, then the subject should be discontinued from all study treatment. If the treating investigator feels that the participant is clinically stable, demonstrates improved condition, or is clearly continuing to benefit from the treatment; the PI may approve the participant to continue to receive study treatment. In all participants, the date of progression will be documented as the first date progression was observed.

11.1.1 Definitions

Evaluable for Target Disease response. Only those participants who have measurable disease present at baseline, have received at least one cycle of therapy, and have had their disease re-evaluated will be considered evaluable for target disease response. These participants will have their response classified according to the definitions stated below. (Note: Participants who exhibit objective disease progression prior to the end of cycle 1 will also be considered evaluable.)

Evaluable Non-Target Disease Response. Participants who have lesions present at baseline that are evaluable but do not meet the definitions of measurable disease, have received at least one cycle of therapy, and have had their disease re-evaluated will be considered evaluable for non-target disease. The response assessment is based on the presence, absence, or unequivocal progression of the lesions.

11.1.2 Disease Parameters

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 or ≥10 mm with CT scan, MRI, or 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 are not considered measurable.

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/pulmonitis, inflammatory breast disease, abdominal masses (not followed by CT or MRI), and cystic lesions are all considered 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 participant, 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.

11.1.3 Methods for Evaluation of Disease

All measurements should be taken and recorded in metric notation using a ruler, calipers, or a digital measurement tool. 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 in 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 thickness is 5mm or less. If CT scans have slice thickness greater than 5 mm, the minimum size of 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.

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.

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.

MIBG (meta-iodobenzylguanidine).The following is recommended, to assure high quality images are obtained.

Patient preparation: Iodides, usually SSKI (saturated solution of potassium iodide), are administered to reduce thyroidal accumulation of free radioiodine, preferably beginning the day prior to injection and continuing for 3 additional days (4 days total). For infants and children, one drop t.i.d. is sufficient, for adolescents 2 drops t.i.d., and for adults 3 drops t.i.d. Participants and/or parents are asked about exposure to potential interfering agents. If none is noted, an indwelling intravenous line is established. The dose of MIBG is administered by slow intravenous injection over 90 seconds.

Images from the head to the distal lower extremities should be obtained.

I-123MIBG scintigraphy is performed to obtain both planar and tomographic images.

Planar: Anterior and posterior views from the top of the head to the proximal lower extremities are obtained for 10 minutes at 24 hours and occasionally at 48 hours

following injection of 10 mCi/1.7 square meters of body surface area (~150 μCi/kg, maximum 10 mCi). Anterior views of the distal lower extremities are adequate. A large field of view dual head gamma camera with low energy collimators is preferred.

SPECT: Most participants receiving I-123 MIBG also undergo SPECT at 24 hours, using a single or multi-headed camera with a low energy collimator. The camera is rotated through 360 degrees, 120 projections at 25 seconds per stop. Data are reconstructed using filtered back projections with a Butterworth filter and a cut off frequency of 0.2-0.5. SPECT/CT may be performed at institutions with this capacity.

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 data 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 from 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 participant 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.

11.1.3.1 Evaluation of 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.

Partial Response (PR): At least a 30% decrease in the sum of the diameters of target lesions, taking as reference the baseline sum diameters.

Progressive Disease (PD): At least a 20% increase in the sum of the diameters of target lesions, taking as reference the smallest sum on study (this includes the baseline sum if that is the smallest on study). In addition to the relative increase of 20%, the sum must also demonstrate an absolute increase of at least 5 mm. (Note: the appearance of one or more new lesions is also considered progressions).

Stable Disease (SD): Neither sufficient shrinkage to qualify for PR nor sufficient increase to qualify for PD, taking as reference the smallest sum diameters while on study.

11.1.3.2 Evaluation of Non-Target Lesions

Complete Response (CR): 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).

Note: If tumor markers are initially above the upper normal limit, they must normalize for a patient to be considered in complete clinical response.

Non-CR/Non-PD: Persistence of one or more non-target lesion(s) and/or maintenance of tumor marker level above the normal limits.

Progressive Disease (PD): Appearance of one or more new lesions and/or *unequivocal progression* of existing non-target lesions. *Unequivocal progression* should not normally trump target lesion status. It must be representative of overall disease status change, not a single lesion increase.

Although a clear progression of "non-target" lesions only is exceptional, the opinion of the treating physician should prevail in such circumstances, and the progression status should be confirmed at a later time by the review panel (or Principal Investigator).

11.1.3.3 Evaluation of New Lesions

The finding of a new lesion should be unequivocal (i.e. not due to difference in scanning technique, imaging modality, or findings thought to represent something other than tumor (for example, some 'new' bone lesions may be simply healing or flare of pre-existing lesions). However, a lesion identified on a follow-up scan in an anatomical location that was not scanned at baseline is considered new and will indicate PD. If a new lesion is equivocal (because of small size etc.), follow-up evaluation will clarify if it truly represents new disease and if PD is confirmed, progression should be declared using the date of the initial scan on which the lesion

was discovered.

11.1.3.4 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 Participants 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	
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: Participants 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 Participants 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

11.1.4 Duration of Response

Duration of overall response: The duration of overall response is measured from the time measurement criteria are met for CR or PR (whichever is first recorded) until the first date that recurrent or progressive disease is objectively documented (taking as reference for progressive disease the smallest measurements recorded since the treatment started, or death due to any cause. Participants without events reported are censored at the last disease evaluation).

Duration of overall complete response: The duration of overall CR is measured from the time measurement criteria are first met for CR until the first date that progressive disease is objectively documented, or death due to any cause. Participants without events reported are censored at the last disease evaluation.

Duration of stable disease: Stable disease is measured from the start of the treatment until the criteria for progression are met, taking as reference the smallest measurements recorded since the treatment started, including the baseline measurements.

11.1.5 Clinical Benefit rate

Clinical benefit rate: defined as CR, PR and stable disease (SD) $\geq$ 27 weeks.

11.2 Antitumor Effect – Hematologic Tumors

N/A

11.3 Other Response Parameters

11.3.1 Definition of Tumor Response Using Immune-Related Response Criteria (irRECIST)

The sum of the product of the longest diameter of lesions (SPD) at tumor assessment using the immune-related response criteria (irRECIST) for progressive disease incorporate the contribution of new measurable lesions. Each net Percentage Change in Tumor Burden per assessment using irRC criteria accounts for the size and growth kinetics of both old and new lesions as they appear.

11.3.1.1 Impact of New Lesions on irRECIST

New lesions in and of themselves do not qualify as progressive disease. However, their contribution to total tumor burden is included in the SPD which in turn feeds into the irRECIST criteria for tumor response. Therefore, new non-measurable lesions will not

discontinue any subject from the study.

11.3.1.2 Definition of Target Lesions Response Using irRECIST

- **irRECIST Complete Response (irRECIST CR):** Complete disappearance of all target lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.
- **irRECIST Partial Response (irRECIST PR):** Decrease, relative to baseline, or 50% or greater in the sum of the products of the two largest perpendicular diameters of all target and all new measurable target lesions (i.e., Percentage Change in Tumor Burden). Note: the appearance of new measurable lesions is factored into the overall tumor burden, but does not automatically qualify as progressive disease until the SBD increases by $\geq$25% when compared to SPD at nadir.
- **irRECIST Stable Disease (irRECIST SD):** Does not meet criteria for irRECIST RC or irPR, in the absence of progressive disease.
- **irRECIST Progressive Disease (irRECIST PD):** At least 25% increase Percentage Change in Tumor Burden (i.e. taking SPD of all target lesions and any new lesions) when compared to SPD at nadir.

11.3.1.3 Definition of Non-Target Lesions Response Using irRECIST

- **irRECIST Complete Response (irRECIST CR):** Complete disappearance of all non-target lesions. This category encompasses exactly the same subjects as "CR" by the mWHO criteria.
- **irRECIST Partial Response (irRECIST PR) or irRECIST Stable Disease (irRECIST SD):** Non-target lesion(s) are not considered in the definition of PR; these terms do not apply.
- **irRECIST Progressive Disease (irRECIST PD):** Increases in number or size of non-target lesion(s) does not constitute progressive disease unless/until the Percentage Change in Tumor Burden increases by 25% (i.e. the SPD at nadir of the target lesions increases by the required amount).

11.3.1.4 Definition of Overall Response Using irRECIST

Overall response using irRECIST will be based on these criteria:

- **Immune-Related Complete Response (irRECIST CR):** Complete disappearance of all tumor lesions (target an non-target) together with no new measurable/unmeasurable lesions for at least 4 weeks from the date of documentation of complete response.
- **Immune-Related Partial Response (irRECIST PR):** The sum of the products of the two largest perpendicular diameters of all target lesions is measured and captured as the SPD baseline. At each subsequent tumor assessment, the SPD of the two largest perpendicular diameters of all target lesions and of new measurable lesions are added together to provide the Immune Response Sum of Product Diameters (irRECIST SPD). A decrease, relative to baseline, of the irRECIST SPD compared to the previously SPD baseline of 50% or greater is considered an irRECIST PR.

- **Immune-Related Stable Disease (irRECIST SD):** irRECIST SD is defined as the failure to meet criteria for immune complete response or immune partial response, in the absence of progressive disease
- **Immune-Related Progressive Disease (**irRECIST **PD):** It is recommended in difficult cases to confirm PD by serial imaging. Any of the following will constitute PD:
  - At least 25% increase in the SPD of all target lesions over nadir SPD calculated for the target lesions.
  - At least 25% increase in the SPD of all target lesions and new measurable lesions (irRECIST SPD) over the baseline SPD calculated for the target lesions.

Criteria for determining overall response by irRECIST are summarized as follows:

Immune-Related Response Criteria Definitions

Target Lesion Definition	Non-Target Lesion Definition	New Measurable Lesions	New Unmeasurable Lesions	Percent change in tumor burden (including measurable new lesions when present)	Overall irRC Response
Complete Response	Complete Response	No	No	–100%	irCR
Partial Response	Any	Any	Any	≥ –50%	irPR
				<–50% to <+25%	irSD
				>+25%	irPD
Stable Disease	Any	Any	Any	<–50% to <+25%	irSD
				>+25%	irPD
Progressive Disease	Any	Any	Any	≥+25%	irPD

11.3.1.5 Immune-Related Best Overall Response Using irRECIST (irRECIST BOR)

irRECIST BOR is the best confirmed overall response over the study as a whole, recorded between the date of first dose until the last tumor assessment before subsequent therapy (except for local palliative radiotherapy for painful bone lesions) for the individual subject in the study. For the assessment of irBOR, all available assessments per subject are considered.

irRECIST CR or irRECIST PR determinations included in the irRECIST BOR assessment must be confirmed by a second (confirmatory) evaluation meeting the criteria for response and performed no less than 4 weeks after the criteria for response are first met.

12. DATA REPORTING / REGULATORY REQUIREMENTS

Adverse event lists, guidelines, and instructions for AE reporting can be found in Section 7.0 (Adverse Events: List and Reporting Requirements).

12.1 Data Reporting

12.1.1 Method

ODQ will collect, manage, and perform quality checks on the data for this study.

12.1.2 Responsibility for Data Submission

Investigative sites within DF/HCC or DF/PCC are responsible for submitting data and/or data forms to ODQ according to the schedule set by ODQ.

12.2 Data Safety Monitoring

The DF/HCC Data and Safety Monitoring Board (DSMB) will review and monitor study progress, toxicity, safety and other data from this study. The board is chaired by a medical oncologist from outside of DF/HCC and has external and internal representation. Information that raises any questions about participant safety or protocol performance will be addressed by the Overall PI, statistician and study team. Should any major concerns arise, the DSMB will offer recommendations regarding whether or not to suspend the study.

The DSMB will meet twice a year to review accrual, toxicity, response and reporting information. Information to be provided to the DSMB may include: participant accrual; treatment regimen information; adverse events and serious adverse events reported by category; summary of any deaths on study; audit results; and a summary provided by the study team. Other information (e.g. scans, laboratory values) will be provided upon request.

12.3 Multicenter Guidelines

N/A

12.4 Collaborative Research and Future Use of Data and Biospecimens

Tissue, blood, stool, bodily fluids, and other materials derived from these will be collected in this study to analyze genes, DNA, RNA, proteins and cells for the study's correlative endpoints and potential future research, utilizing new types of biomarker testing as it becomes available.

These samples and any data generated as a part of these clinical trials may be used for future research studies and may be provided to collaborating investigators both within and outside of the DF/HCC for either correlative endpoints or secondary use. Samples and data may be shared with outside non-profit academic investigators, as well as with for-profit pharmaceutical investigators or commercial entitites, with whom we collaborate. When samples or data are sent to collaborators and when any research is performed on them, all information will be identified with a code, and will not contain any PHI, such as name, birthday, or MRNs.

In order to allow the greatest amount of research to be performed on the specimens and information generated as a part of this trial, researchers in this study may share results of genetic sequencing with other scientists. De-identified specimen or genetic data may be placed into one of more publicly-accessible scientific databases, such as the National Institutes of Health's Database for Genotypes and Phenotypes (dbGaP). The results from the correlative research on this study will be shared with these public databases. Through such databases, researchers from around the world will have access to de-identified samples or data for future research. More detailed information, beyond the public database, may only be accessed by scientists at other research centers who have received special permission to review de-identified data.

13. STATISTICAL CONSIDERATIONS

13.1 Study Design/Endpoints

This is a randomized phase II open label study of eribulin mesylate +/- Pembrolizumab for patients with metastatic HR positive breast cancer treated with 0-2 line of chemotherapy. Eighty-eight patients will be randomized (1:1) to eribulin mesylate with pembrolizumab or eribulin mesylate. Patients randomized to eribulin mesylate monotherapy will have the option to receive pembrolizumab monotherapy at time of progression.

The primary endpoint is PFS, defined as the time from study randomization to disease progression per RESICT 1.1 or death due to any cause, whichever occurred first. Patients alive without disease progression are censored at the date of last disease evaluation. The primary objective is to compare PFS of patients randomized to receive eribulin mesylate in combination with pembrolizumab (Arm A) versus those randomized to receive eribulin mesylate monotherapy (Arm B).

Secondary endpoints include PFS per irRECIST criteria, ORR per RECIST 1.1 and irRECIST, DOR, CBR, OS, safety and tolerability. For the patients randomized to the eribulin monotherapy arm who elect to receive the combination after progression, we will also perform an exploratory analysis of all clinical endpoints for patients using measurements at progression on monotherapy as the baseline tumor assessment.

13.2 Sample Size, Accrual Rate and Study Duration

The target accrual is 88 patients (44 in eribulin mestlate alone arm and 44 in eribulin mesylate+pembrolizumab combination arm). The study has 83% power to distinguish a 3 month improvement in PFS from 4.1 to 7.1 months (HR = 0.58) with a one-sided alpha of 0.1. This statement of power assumes a final analysis when 70 PFS events are observed, and would be anticipated to occur after a constant accrual over 18 months with 6 months of additional follow-up. Further assumptions are a constant hazard of PFS and dropout such that 5% of patients are lost-to-follow-up at 1 year. This allows for one futility analysis at 50% information (35 PFS

events) and would stop early if the Z-statistics from the logrank test is less than 0 (i.e. observed HR from a Cox model > 1.0).

The expected accrual rate is 3-5 patients per month, and the accrual is expected to complete within 18-24 months.

13.3 Interim Monitoring Plan

A single interim analysis for futility is planned to minimize the likelihood of exposing study patients to an inactive regimen. The interim analysis will be performed at 50% information, when the 44th patient is evaluable for response. Stopping criteria were set considering Lan-Demets (O'Brien Fleming) beta-spending function, and shifting the boundary slightly to Z = 0 (from Z = 0.116) to correspond to no observed difference in the proportion of response or worse outcomes with the combination of Eribulin/Pembro. This boundary corresponds to a Bayesian predicted probability of 3.5% of concluding superiority at final analysis with a non-informative prior of treatment effects. Futility criteria are non-binding, and enrollment will not be suspended for patient follow-up and interim analysis. No early stopping for efficacy will be considered.

13.4 Analysis of Primary Endpoints

The primary analysis of the study will be the comparison of PFS per RECIST 1.1 between Arm A and Arm B using logrank test and group sequential methods to control the overall one-sided Type I error rate at 0.1.

The null and alternative hypotheses are defined using the hazard ratio (HR) of the Arm A to Arm B as:
H_0: HR $\leq$ 1
H_1: HR > 1

All inferences will be conducted in the intent-to-treat manner, and the survival function will be summarized using the Kaplan-Meier methods according to randomized treatment assignment. Final analysis will be performed after 70 PFS events are observed or at least 12 months after the last patient is enrolled on study, whichever occurs first.

13.5 Analysis of Secondary Endpoints

<u>Efficacy Endpoints</u>
All patients who initiated protocol therapy will be evaluated for PFS per irRECIST criteria, ORR, CBR, and OS. ORR and CBR will be evaluated using RECIST 1.1 and irRECIST criteria (as defined in Section 11.3). DOR will be evaluated among patients who had CR or PR. All these endpoints will be estimated separately in the two treatment arms.

The objective response rate (CR + PR) and immune-related response rate (irCR + irPR) will be reported by arm with 90% exact confidence intervals. Objective response rate and immune-related response rate will be compared between Arm A and Arm B, respectively using a Pearson

chi-squared test for difference in proportions with a one-sided α of 0.1.

Clinical benefit is defined as CR, PR or SD ≥ 27weeks. Clinical benefit will be calculated using RECIST 1.1 and irRECIST criteria, respectively. Clinical benefit rate (CR+PR+SD≥27 wks) per RECIST 1.1 and irRECISTcriteria will be reported respectively with 95% exact confidence intervals. CBR will be compared between two treatment arms using a Pearson chi-squared test for difference in proportions with a one-sided α of 0.1.

PFS per irRECIST and OS will be also analyzed using Kaplan–Meier product-limit estimatesand 95% confidence bands. PFS per irRECIST is defined as the time from study randomization to disease progression per irRECIST or death due to any cause, whichever occurred first. Patients alive without disease progression per irRECIST are censored at the date of last disease evaluation. OS is defined as the time from study randomization to death due to any cause. Patients will be censored at the date they were last known alive. The hazard ratio for each time-to-event endpoint will be estimated with 95% confidence intervals derived from the Cox proportional hazard model, but no hypothesis testing will be conducted.

DOR is defined as the time from CR or PR achieved until renewed disease progression is detected. DOR will be calculated per RECIST 1.1 and irRECIST criteria, respectively, and descriptive statistics will be used to summarize the intervals observed by arm.

In the group of patients treated with Pembrolizumab after progression on eribulin mesylate, CBR, PFS, OS, and DOR after they received Pembrolizumab will be estimated using binomial, Kaplan-Meier and descriptive statistics, respectively with 95% confidence intervals. For this group, PFS and OS will start from the time of initiating Pembrolizumab. Under an assumption that approximately two-thirds of patients elect to cross over (n=30), the maximum width to an exact confidence interval for a proportion will be 0.374.

Safety and tolerability

All patients will be evaluable for toxicity from the time of their first treatment with any study agent. Toxicity will be graded according to NCI CTCAE, Version 4.0. Toxicities will be summarized by maximum grade and by treatment arm. Incidence rate of each toxicity will be reported with 95% exact CI. The incidence rates of any grade 3+ toxicity will be compared between two arms using using a Pearson chi-squared test for difference in proportions with a one-sided α of 0.1..

13.6 Analysis of Correlative Science Endpoints

All analyses of correlative scientific endpoints are exploratory and hypothesis-generating. Any promising findings will be tested in future studies.

13.6.1 *Blood and tissue correlative science endpoints:*

- We will describe the presence and abundance of multiple immune markers in metastatic HR-positive breast tumors (characterization will be based on histology, protein

expression, and mRNA expression; see Section 9 for further details on characterization) using frequency tables and descriptive statistics (mean, standard deviation, median , and inter-quartile range).

- We will explore the correlation of immunosuppressive and/or immune-stimulating immune marker profiles at baseline to disease response to therapy (response assessed by RECIST 1.1 and irRECISTcriteria) using odds ratios and 95% confidence intervals.
- We will summarize serial changes in immune marker profile in peripheral blood mononuclear cells (PBMCs) from baseline (pre-trial therapy) using mean, standard deviation, median , and inter-quartile range.
- We will plot serial changes in the immunosuppressive and/or immune-stimulating immune marker profile in PBMCs by disease response to therapy (response assessed by RECIST 1.1 and irRECISTcriteria)
- We will explore an immune marker or set of markers in circulating PBMCs that corresponds to tumor infiltrating lymphocyte (TIL) percentage in baseline tumor
- In the cohort of patients who have re-biopsy at progressive disease (PD): We will summarize changes in a broad array of immune markers from baseline to time of progressive disease on trial therapy using mean, standard deviation, median , and inter-quartile range.

13.6.2 *Stool and microbiome correlative science endpoints:*

Overall, we plan to describe the landscape of gut microbiota in patients with BC who will receive pembrolizumab, and the changes in their gut microbiota after two cycles of pembrolizumab. Statistical analyses of intestinal microbiota samples will be performed using R Statistical Language (v3.1.1) and GraphPad Prism (version 6.0e) software packages. Unpaired Mann–Whitney rank sum test (two-tailed) will be used for comparisons of continuous variables between two groups. Bar plots will be used to represent the data's mean at the center values, with error bars to indicate standard deviation. In order to explore the association of response (objective response according RECIST 1.1 and progression-free survival) to baseline microbiota diversity, and changes from baseline in microbiota, inference will be based on Wilcoxon rank sum tests and estimates of predictive value along the continuous scales will be visualized using receiver operating characteristic (ROC) curves and reported with c-index and confidence intervals derived from variance estimates of Somers rank correlation. Unadjusted P-values will be considered significant for the Mann–Whitney rank sum test.

We will quantify microbiome features from amplicon, metagenome, metatranscriptome using established pipelines to identify strain-level taxonomic, functional gene, transcriptional, and microbially-mediated metabolite profiles associated with BC patients with and without immunotherapy70[-76]. We will use modified multivariate linear modeling to identify statistically significant features associated with outcomes. Statistical tests for association with these outcomes and covariates will be performed using the sparse generalized linear model MaAsLin, which provides random effects models for both log-Gaussian and zero-inflated negative binomial link functions. Computational workflows for these steps are implemented as AnADAMA2 (http://huttenhower.sph.harvard.edu/anadama) workflows, a reproducible data handling environment that captures all provenance during the analysis process.

13.6.3 *Tumor Genomic Profile correlative science endpoints:*

All analyses of Oncopanel in correlation with patient outcomes are exploratory and hypothesis-generating. Any promising findings will be explored in future studies.

13.7 Reporting and Exclusions

13.7.1 Evaluation of Efficacy

For this Phase II trial, the efficacy evaluable population is a modified intent-to-treat (ITT) population. The modified ITT population consists of all randomized patients who initiate protocol therapy, even if there are major protocol therapy deviations.

13.7.2 Evaluation of Safety

The safety population will be used in the safety data summaries. The safety population consists of all patients who took at least one dose of any randomized treatment and who have at least one post-baseline safety assessment. Note that a patient who had no adverse events constitutes a safety assessment. Patients who have received at least one dose of study drug but have no post-treatment safety data of any kind would be excluded.

14. PUBLICATION PLAN

The results should be made public within 24 months of reaching the end of the study. The end of the study is the time point at which the last data items are to be reported, or after the outcome data are sufficiently mature for analysis, as defined in the section on Sample Size, Accrual Rate and Study Duration. If a report is planned to be published in a peer-reviewed journal, then that initial release may be an abstract that meets the requirements of the International Committee of Medical Journal Editors. A full report of the outcomes should be made public no later than three (3) years after the end of the study.

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APPENDIX A PERFORMANCE STATUS CRITERIA

ECOG Performance Status Scale		**Karnofsky Performance Scale**	
Grade	Descriptions	Percent	Description
0	Normal activity. Fully active, able to carry on all pre-disease performance without restriction.	100	Normal, no complaints, no evidence of disease.
		90	Able to carry on normal activity; minor signs or symptoms of disease.
1	Symptoms, but ambulatory. Restricted in physically strenuous activity, but ambulatory and able to carry out work of a light or sedentary nature (*e.g.*, light housework, office work).	80	Normal activity with effort; some signs or symptoms of disease.
		70	Cares for self, unable to carry on normal activity or to do active work.
2	In bed <50% of the time. Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50% of waking hours.	60	Requires occasional assistance, but is able to care for most of his/her needs.
		50	Requires considerable assistance and frequent medical care.
3	In bed >50% of the time. Capable of only limited self-care, confined to bed or chair more than 50% of waking hours.	40	Disabled, requires special care and assistance.
		30	Severely disabled, hospitalization indicated. Death not imminent.
4	100% bedridden. Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair.	20	Very sick, hospitalization indicated. Death not imminent.
		10	Moribund, fatal processes progressing rapidly.
5	Dead.	0	Dead.

APPENDIX B STRONG CYP3A INDUCERS/INHIBITORS

The list provided below is not exhaustive. For a more comprehensive, frequently updated list, please visit: http://medicine.iupui.edu/clinpharm/ddis/table.aspx.

Medications that strongly inhibit CYP3A:
Amprenavir
Atazanavir
Boceprevir
Clarithromycin
Conivaptan
Delavirdine
Diltiazem
Erythromycin
Fosamprenavir
Indinavir
Itraconazole
Ketoconazole
Lopinavir
Mibefradil
Miconazole
Nefazodone
Nelfinavir
Posaconazole
Ritonavir
Saquinavir
Telaprevir
Telithromycin
Verapamil
Voriconazole
Grapefruit, grapefruit juice, or any product containing grapefruit

Medications that strongly induce CYP3A:
Carbamazepine
Felbamate
Nevirapine
Phenobarbital
Phenytoin
Primidone
Rifabutin
Rifampin
Rifapentin
St. John's wort

APPENDIX C GUIDELINES FOR COLLECTING RESEARCH BIOPSY TISSUE

Tissue specimens will be collected from metastatic lesions using standard institutional procedures. The amount of tissue collected may follow the guidelines listed below:

Skin/chest wall: A goal of 2 4-mm punch biopsies will be obtained.

Lymph node: A goal of 5-7 core biopsy specimens will be obtained using an 18-gauge needle.

Liver: A goal of 5-7core biopsy specimens will be obtained using an 18-gauge needle.

Lung: Because of the risk of pneumothorax associated with core needle biopsies of lung nodules, no core biopsies of lung nodules are mandated on this protocol, unless they are clinically indicated.

Bone: Because the yield of malignant tissue from bone biopsies tends to be relatively low, if a patient has another accessible site of disease (i.e. skin, lymph node, liver), that site should be biopsied preferentially. If bone is the only biopsy-accessible site, then a goal of 3-6 core biopsy specimens will be obtained using an 11-13 gauge needle.

Please note that the above are guidelines for the amount of tissue to be obtained, and are not meant to replace clinical judgment at the time the procedure is performed. Less than the goal quantity of tissue is accepted for each type of biopsy, and will be left to the clinical judgment of the physician performing the procedure.

If a patient is undergoing resection of a lesion for clinical reasons (i.e. wedge resection of a new lung lesion for confirmation of diagnosis or re-testing of hormone receptor or HER2 status; or, resection of a chest wall lesion; or, resection of a lymph node), then the patient may opt to have a portion of that tissue (roughly equivalent to the goal amount of tissue listed in the guidelines above, i.e. the equivalent of two 5-mm punch biopsies of the skin, or 3-6 18-gauge core biopsies) stored for research at the time of the procedure (provided that the tissue is processed as specified), in which case, the patient would not be required to undergo a separate research biopsy at baseline on this protocol.

Coded laboratory specimens will be stored in the Tumor Bank of the DFCI. These specimens will become the property of DFCI. Patients will be informed that their specimens may be used for research by investigators at DF/HCC and other approved collaborators. Shared specimens will be identified with a sample ID number; all patient identifying material will be removed.

Risks of Research Biopsy and Procedures for Minimizing Risk

Potential risks according to site are:
Skin/chest wall (punch biopsy):

- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, or infection

Lymph node, liver, or bone (core needle biopsy):

- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, damage to adjacent organs. Additional risks may be present if intravenous conscious sedation is required

Breast (core biopsy):

- Likely: local discomfort and minor bleeding.
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, pneumothorax, damage to adjacent organs.

Pleural fluid (thoracentesis):

- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, pneumothorax, damage to adjacent organs

Ascites fluid (paracentesis):

- Likely: local discomfort and minor bleeding
- Less likely: moderate or major bleeding, need for blood transfusion, hospitalization due to bleeding or other complications, infection, bowel perforation or damage to adjacent organs. In order to minimize the risk of a biopsy, only qualified personnel will perform these procedures.

Prior to the procedure, the physician performing the procedure will discuss the risks with each study participant, answer any questions, and obtain separate procedure consent. Patients will be evaluated for comorbidities or concomitant medications that may increase the risk of potential complications. For biopsies of lesions that are not superficial and clearly palpable, imaging studies such as CT or ultrasound will be used to guide the biopsy in order to minimize the risk of damage to adjacent structures. After lymph node biopsies, patients will be observed a minimum of 2 hours (range 2-4 hours) after the procedure, or according to standard institutional guidelines. After liver biopsies, patients will be observed a minimum of 4 hours (range 4-6 hours) after the procedure, or according to standard institutional guidelines. Less than the goal quantity of tissue is accepted for each type of biopsy, and will be left to the clinical judgment of the physician performing the procedure.

Risks of Anesthesia

Local Anesthesia

All biopsy procedures require local anesthesia using lidocaine, xylocaine, or related compounds. There is a small risk of an allergic reaction associated with these drugs. In order to minimize the risk of local anesthesia, only qualified personnel will perform the biopsy procedure. Patients will be queried if they have had previous allergic

reactions to local anesthetics.

Intravenous Conscious Sedation

Certain biopsy procedures, such as lymph node, liver, or bone biopsies, may require intravenous conscious sedation (IVCS). IVCS is a minimally depressed level of consciousness that retains the patient's ability to maintain a patent airway independently and continuously and respond appropriately to physical stimulation and verbal commands.

The risks of intravenous conscious sedation include: inhibition of the gag reflex and concomitant risk of aspiration, cardiopulmonary complications (myocardial infarction, cardiac arrhythmias, hypoxemia), and allergic reactions to the sedative or analgesic medications. These risks are small but real; for example, in a prospective study of 14,149 patients undergoing IVCS during upper gastrointestinal endoscopies, the rate of immediate cardiopulmonary events was 2 in 1000.[56] The 30-day mortality was 1 per 2,000 cases. In this study, there was a strong association between lack of monitoring and use of high-dose benzodiazepines with adverse outcomes. There was also an association between the use of local anesthetic sprays to the oropharynx and the development of pneumonia. In order to minimize the risk of intravenous conscious sedation, only qualified personnel will be responsible for conscious sedation. A minimum of two individuals will be involved in the care of patients undergoing conscious sedation—the physician performing the biopsy procedure, and the individual (M.D. or R.N.) who monitors the patients and his/her response to both the sedation and the procedure, and who is capable of assisting with any supportive or resuscitative measures. The room where the procedure utilizing IVCS takes place will have adequate equipment to provide supplemental oxygen, monitor vital signs, and maintain an airway should this be necessary. An emergency cart will also be immediately accessible to the room where the procedure is to take place, and emergency support services will be available on page. Patients will be screened and evaluated for their fitness to undergo conscious sedation by a trained physician. Patients with active cardiac disease are excluded from this study. No local anesthetic spray to the oropharynx will be necessary, given that endoscopy is not a planned procedure. Following the procedure, patients will be observed closely in the recovery room for a minimum of 2 hours.

General Anesthesia

Because of the higher risk of general anesthesia compared with local anesthesia or intravenous conscious sedation, biopsies that would require general anesthesia in order to be performed *are not permitted* on this protocol, unless they are being done for clinical reasons, and excess tissue that otherwise would have been discarded is then banked for the purpose of this protocol.

For Biopsies of Soft Tissue, Liver, Bone, Breast, Etc:

1. After biopsy is performed, the tissue mass is placed on a sterile gauze
2. Using forceps, separate the tumor tissue
3. Place 2 pieces (cores) of tumor tissue in each cassette (typically end up with 3 cassettes per biopsy); the last cassette will contain many small pieces of tumor tissue
4. Fill cassettes with OCT
    a. Completely cover tissue
    b. Limit the amount of bubbles
5. Place cassettes on dry ice and prepare for transport by limiting OCT leakage
6. Return samples to the lab and complete freezing of samples in OCT with dry ice (about 10 minutes freezing time)
7. Once samples are frozen, place in plastic bag; label bag with date, protocol number, patient number, and number of initials included
8. Store in –80C freezer

For Effusions and Ascites

1. Fluid sample should be split into two equal aliquots
2. One aliquot should be spun down into a pellet and snap frozen in an ETOH/dry ice bath or in liquid N2
3. One aliquot should be fixed and processed as a standard cell block.

Note: if the sample preparation is done by a clinical cytopathology laboratory, it is important to explain that the sample is for research purposes only and that no thin prep should be performed as this uses up a significant portion of the sample.

For Fine Needle Aspiration Samples

A goal of 3 passes:

1. One pass should be evacuated and rinsed directly into 2mL of room temperature Trizol for RNA analysis.
2. One pass should be evacuated and rinsed directly into 2mL of room temperature Trizol for DNA analysis.
3. One pass should be evacuated and rinsed directly into 10-20mL of RPMI to prepare a cell block.

APPENDIX D ANTIBODIES THAT CAN BE USED FOR IMMUNOHISTOCHEMISTRY IN CORRELATIVE STUDIES

IHC Biomarkers	Priority	Clone/ Cat #	Source	Host species	Dilution	Optimized?
PD-L1	1	9A11	G. Freeman	Mouse	1/125	Yes
PD-L2	1	9E 6	G. Freeman	Mouse	1/10000	Yes
PD-1	1	EH33	G. Freeman	Mouse	1/600	Yes
CD3	1	IS503	Dako	Rabbit	1/250	Yes
CD4	1	4B12	Vector Labs	Mouse	1/200	Yes
CD8	1	144B	Dako	Mouse	1/100	Yes
FOXP3	1	206D	BioLegend	Mouse	1:50	Yes
TIM3	1	AF2365	R&D Systems	Goat	1:50	Yes
LAG3	1	17B4	LifeSpan BioSc	Mouse	1/200	Yes
Tie2	2	AF313	R&D Systems	Goat	1/500	Yes
ANGPT2	2	sc-74403	Santa Cruz Bio	Mouse	1/200	Yes
IDO1	2	ab55305	Abcam	Mouse	1/100	No
CD38	3	SPC32	Abcam	Mouse	1/300	Yes
CD56	3	123C3	Dako	Mouse	1/100	Yes
CD14	3	ab49755	Abcam	Mouse	1/100	Yes
CD16	3	ab183354	Abcam	Rabbit	1/100	No
CD11c	3	EP1347Y	Abcam	Rabbit	1/500	Yes

APPENDIX E ANTIBODIES THAT MAY BE USED FOR FLOW CYTOMETRY IN CORRELATIVE STUDIES

Cell Type	Antibody	Color	Clone
T effector	CD4	FITC	SK3
		PC7	SK3
	CD62L	APC	DREG-56
	CD69	PE	FN50
T regs	CD4	FITC	SK3
		PC7	SK3
	CD25	PE	Bc96
		PC5	B1.49.9
	FOXP3	PE	PCH101
		FITC	PCH101
	CD127	APC	eBioRDR5
NK	CD3	FITC	UCHT1
		PC7	UCHT1
	CD56	PE	NCAM16.2
	CD57	PE	TB01
NKT	CD3	FITC	UCHT1
		PC7	UCHT1
	CD56	PE	NCAM16.2
	TCR a/b	APC	BW242/412
	CD314 (NKG2D)	PE	ON72
MDSC	HLA-DR	PC7	L243
		FITC	L243
	CD11b	FITC	Bear1
	CD14	APC	61D3
	CD33	PE	WM53
Cytotoxic	CD8	APC	BW135/80
		PE	BW135/80
	CD3	FITC	UCHT1
		PC7	UCHT1
Memory T	CD197 (CCR7)	PE	3D12
	CD45RO	FITC	UCHL1
	CD45RA	PC7	HI100
	CD4	FITC	SK3
		PC7	SK3
	CD8	APC	BW135/80
		PE	BW135/80
B cells	CD5	BV421	UCHT2
		FITC	UCHT2
	CD19	PC7	SJ25C1
		PE	SJ25C1
		APC	SJ25C1

	CD20	FITC	2H7
Classic Monocytes	CD14	APC	61D3
	CD16	FITC	eBioCB16
Dendritic	CD123	APC	6H6
	CD303a	FITC	201A
	CD11c	FITC	11-0116
	CD141	APC	M80
	CD1c	PE	L161
Macrophages	CD40	APC	5C3
Progenitors	CD34	PE	4H11
Intracellular Cytokines	IL-10	PE	JES3-9D7
	IL-17a	PercP Cy5	eBio64DEC17
	INFg	APC	B27
	TNFa	FITC	Mab11
Co-stimulatory and inhibitory markers	CD134 (OX40)	APC	ACT-35
	CD137 (4-1BB)	FITC	4B4
	CD154 (CD40L)	PercP 710	24-31
	CD223 (LAG3)	PercP 710	3DS223H
	CD252 (OX40L)	PE	11C3.1
	CD278 (ICOS)	FITC	ISA-3
	Tim-3	BV421	F38-2E2
	CD274 (PD-L1)	PE	MIH1
	CD279 (PD-1)	FITC	MIH4
		PE	MIH4
		APC	MIH4
	CD357 (GITR)	APC	621
	CD152 (CTLA-4)	efluor 660	14D3
Proliferation	Ki-67	FITC	20Raj1

APPENDIX F IMMUNOHISTOCHEMICAL STAINING ASSAYS

Design of immunohistochemical assay

The immunohistochemical assay for PD-L1 and PD-L2 is semi-quantitative while PD-1 stained slides will be scanned by an automated scanning microscope and quantitatively analyzed by Aperio image analysis system (Lecia Biosystems) after they are evaluated and positive cells are manually counted by a pathologist.

Standard EnVision two-step (indirect) staining method will be utilized. Four micrometer-thick sections will be cut, deparaffinized, rehydrated, and subjected to heat modified antigen retrieval in citrate buffer (pH 6) (Invitrogen) by steaming for 30 minutes. After cooling, tissue sections will be incubated with peroxidase block (DAKO, Carpinteria, CA) for five minutes, then serum free protein block (DAKO) for 20 minutes. Slides will be incubated at room temperature for one hour with a primary antibody. Antibodies will be diluted in Da Vinci Green Diluent (Biocare Medical, Concord, CA). EnVision™ anti-mouse HRP-labeled polymer (DAKO) will be applied to the sections for 30 minutes, followed by visualization using the chromogen 3,3-diaminobenzidine (DAKO). All the sections will then be counterstained with hematoxylin, dehydrated, mounted, and coverslipped. Positive and negative controls shall be included in each staining. Known positive stained Hodgkin Lymphoma (PD-L1), tonsil (PD-1), and melanoma (PD-L2) slides will be used as external control (separate slides). Stained slides will be stored at room temperature.

In a pilot study performed by our correlative scientists, immunoreactivity for PD-L1 was detected in the cytoplasm and membrane while PD-L2 and PD-1 expression was observed in the cytoplasm. Scoring for PD-L1 and PD-L2 will be semi-quantitative/ordered categorical. The percentage of the tumor cells staining positive for PD-L1 or PD-L2 and the intensity of the staining will be recorded (using the scale 0=no staining, 1=weak staining, 2=moderate staining, 3=intense staining). Absolute PD-1 positive cells will be counted under microscope lens x20 power field. Five representative areas will be chosen to count. The average number from 5 areas will be recorded and compared with data from image analysis.

For PD-1 staining, slides will be scanned by an automated scanning microscope and analyzed by Aperio image analysis system (Lecia Biosystems). Tumor areas will be marked by a pathologist to exclude non-neoplastic areas, such as stroma, normal epithelial, and necrotic regions. The software will be used to count the number of positive cells in each tissue. The percentage of PD-1 positive cells will be calculated. Data will be compared with that of manual counting by a pathologist to exclude tissue artifacts that cannot be recognized by computer image software.

Assay performance

Protocols of these three antibodies have been optimized and standardized to minimize staining variance. Positive control and negative controls were used and stained separately with each batch of slides. The IHC staining of three markers (PD-1, PD-L1, PD-L2) has been performed in two different labs by three different technicians on whole tissue sections of Hodgkin lymphomas, melanomas, lung cancers, and renal cell carcinomas. Three readers were involved, confirming the good reproducibility of the assay.

Thresholds of positivity

Tumor will be considered positive if >5% (PD-L1)[53] or >10% (PD-L2) of the tumor cell population demonstrates unequivocal staining. PD-1 positivity will be defined as >3% positive cells/high power field.[54] All IHC stained slides will be evaluated and scored by a pathologist. A subset of slides will be reviewed by a second pathologist to ensure concordance of interpretation.

APPENDIX G TIL ISOLATION FROM SOLID TUMORS

1. Prepare an enzyme solution of collagenase, hyaluronidase and deoxyribonuclease in advance:
    a. Dissolve collagenase (collagenase type I, cat#17100-017, Invitrogen) in DMEM at a concentration of 1 mg/ml
    b. Add hyaluronidase (hyaluronidase type V, cat#H6254, Sigma-Aldrich) to a final concentration of 1mg/ml (1,500 units/ml) and deoxyribonuclease (deoxyribonuclease I, type IV, cat#5025, Sigma-Aldrich) to a final concentration of 50 micrograms/ml (100 units/ml)
    c. Filter the solution with a 10 ml sterile syringe, a sterile 23G needle, and a sterile 0.2 μm filter.
2. Record the date and time of the start of TIL isolation.*
3. Dissect patient tumor sample into pieces as small as possible with sterile scissor or scalpel. **Note**: Mincing of tumor may be facilitated by lining up two scalpels in parallel.
4. Submerge the pieces of tumor in 5 - 10 ml prepared enzyme solution in a 50 ml conical tube.
5. Enzymatically digest tumor pieces in 37°C waterbath for one to two hours; every 15 min., vigorously shake the tube.
6. Put a sterile cell strainer (100 μm, 352360, BD Falcon) on a 50 ml conical tube, and pass the digested tumor solution through the strainer; the flowthrough will be collected in the 50 ml conical tube. Rinse the strainer and undigested tumor once with PBS.
7. Add 2-5 ml complete DMEM medium (with 10% FBS + 50 μg/ml gentamycin) into flowthrough to stop digestion.
8. Spin the tube at 1500 rpm for 5 min in a centrifuge at room temperature.
9. In the meantime, put undigested tumor tissue into a sterile 50 ml conical tube, and add 5-10 ml enzyme solution and continue with digestion from step 4.
10. Repeat step 3 to step 8, based on tissue digestion

NOTE: For samples with lot of red blood cells and/or undigested debris that has passed through the cell strainer, the following is recommended before proceeding to step 11:

A. Resuspend the cell pellet in 10 ml complete DMEM medium

B. Add 10 ml Ficoll Paque Plus (Cat# 17-1440-03; GE Healthcare) in a 50 ml conical tube.

C. Slowly and gently layer the digested tumor suspension onto the Ficoll Paque Plus.

D. Centrifuge the tube at room temperature at 1500 - 2000 rpm (1000 g) with slow acceleration and deceleration for 20 - 30 min.

E. Pipette off the interface between complete DMEM and the Ficoll Paque Plus (lower part), and

transfer the layer into a 50 ml conical tube. The bottom pellet will be composed of red blood cells and undigested debris.

F. Add 2 - 3X bed volume of PBS to dilute Ficoll.

G. Spin the tube at 1500 rpm for 5 min in a centrifuge at room temperature.

H. Aspirate supernatant and proceed to step 11.

11. Resuspend cell pellets in complete DMEM medium plus gentamycin 50ug/ml and combine cells-TIL into one sterile 50 ml conical tube..
12. Centrifuge at 1500 rpm for 5 min in a centrifuge at room temperature.
13. Aspirate off the supernatant and remove a small aliquot to record the cell count and viability, then place the tube on ice.
14. For each timepoint, collect the following parameters:
    a. Cell viability (%) before freezing*
    b. Total yield of TIL (x 10^6 cells/mL/vial) isolated prior to freezing*
15. Resuspend the cell suspension in pre-chilled PBMC freezing media (CTL-cryoABS kit, CTL cellular Technology)
    a. Transfer 1 ml aliquots of the cell suspension to a cryovial labeled with the **supplied Quintiles labels**. A minimum of one (1) cryovial should be obtained with a minimum concentration of cells at $1x10^6$ cells/mL/vial.
    b. For each cryovial prepared, please record the total # of cells in the cryovial*. If there are more than $2x10^6$ cells, then aliquot cells into as many cryovials as possible at a concentration of $1x10^6$ cells/ml/vial.
16. Store in 1 ml aliquots in cryovials at -80^0C in a slow freeze container. Leave undisturbed overnight or for a **minimum of 12 hrs and a maximum of 24 hrs.**
17. Transfer into liquid nitrogen for long-term storage. Record the time, date, and location that the samples* were placed in liquid nitrogen storage.
18. At the sponsor's request, samples should be batch shipped at the end of study. Please, follow the shipping instructions provided. Samples will be shipped on liquid nitrogen.

APPENDIX H FLOW CYTOMETRY PROCEDURES

Prep without Permeabilization

KEEP EVERYTHING ON ICE

1. Thaw cell vial in 37 degree water bath until completely thawed.
2. Resuspend cells in 50 ml of RPMI medium (Gibco, 11A75-093) + 10% FBS + 1X final Anti-Anti (Gibco, 15240-062) in a 50 ml conical tube (Corning, 430290).
3. Culture cells in 2 T-150 culture flasks (Corning, 431465) overnight (25 ml per flask)
    - In one flask, activate cells by adding 0.4 ml (whole vial) of Dynabeads Human T-Activator (Gibco, 1161D). Before adding beads to flask, wash beads according to manufacturer's protocol.
4. Incubate cells for 24 hours at 37 degrees with 5% CO_2
5. Remove cells and media from flask and filter through 70 micron cell filter (Biologix, 15-1070) into 50 ml conical tube.
6. Spin conical tubes for 5 min at 1800 rpm in a Sorvall Legend XTR centrifuge.
7. Make wash/blocking media: PBS +2.5% FBS (Gibco, 14040).
8. Vacuum media off pellet, resuspend pellet in calculated volume of wash/blocking media according to calculations from cell density + number of wells and tubes for 700,000 cells/tube in 100 µl.
9. Pipet 100 µl/ well of cells + wash/blocking media containing FcR Blocking Reagent (Milteny Biotec, 130-059-901) into v-bottomed plate (Costar, 3894) according to well map (let sit for 20-30 minutes on ice).
10. Spin plate at 1800 rpm for 5 minutes at 4C in Sorvall Legend XTR centrifuge
11. Mix antibody cocktails in flat bottomed plate (amount according to manufacturer specifications or from previously developed assays)
12. After plate with cells is finished spinning, aspirate liquid off pellet by carefully tilting the plate. Add appropriate antibody cocktails from flat bottomed plate according to well map after pipetting up and down to mix at least three times.
13. Let plate with cells + antibodies sit for 45 minutes on ice in the dark.
14. Spin plate as previously described in step 11
15. Aspirate off liquid by tilting plate and wash with 150 µl/well of wash/blocking media, pipetting up at down to mix at least 3 times (described in step 6)
16. Resuspend cells in 150 µl/well in wash/blocking media
17. Keep plate and single tubes (single color controls) on ice, in the dark or covered with aluminum foil until read by Fortessa LTS II (Beckton- Dickinson).

APPENDIX I GENERATION OF PBMCS

1. Pour blood from green-cap tubes (heparin treated tubes) into two 50 ml conical tubes (Corning, 430290).
2. Spin tubes at 1500 rpm for 10 min (Sorvall Legend XTR centrifuge).
3. Aspirate 2 ml plasma/tube and aliquot into 4 tubes mircocentrifuge tubes (Fisherbrand, 05-408-138)
4. Spin plasma at 3000 RPM for 5 minutes (Sorvall Legend Micro 21R centrifuge)
5. Aspirate plasma into Cryogenic tubes 2 ml plasma/ tube (Corning, 430488).
6. Dilute blood 1:1 with PBS. (Blood amount should not exceed 25 ml per tube.)
7. Take 2 new 50 ml conical tubes and add 12 ml ficoll-paque (Cat# 17144003; GE Healthcare) per tube.
8. Slowly and gently layer the diluted blood on top of the ficoll-paque of the tube with a maximum volume of 35 ml.
9. Centrifuge the tube at 1900 rpm for 20 min at room temperature with slow acceleration (#7) and deceleration (#7) (Sorvall Legend XTR centrifuge).
10. Remove the PBMC layer from between the upper layer (diluted plasma) and middle layer (ficoll-paque) and transfer into a 50 ml conical tube. The lower layer is composed of red blood cells.
11. Completely fill conical tube containing isolated PBMC with PBS, mixing well.
12. Count viable cells by mixing 10 µl Trypan Blue with 10µl PBMC/PBS dilution in a microcentrifuge tube. Load 10 µl of mixture onto Countess Cell Counting Chamber Slide (Invitrogen, C10283) and read with Countess Automated Cell Counter (Invitrogen).
13. Centrifuge the tubes containing PBMC/PBS mixture at 1500 rpm for 5 min at room temperature (Sorvall Legend XTR centrifuge).
14. Remove PBS, and resuspend PBMC pellet in appropriate amount of freezing solution so that there are approx 5×10^6 cells/cryo vial in 300-500 µl of Fetal Bovine Serum (heat inactivated) plus 15% DMSO.
15. Put vials in CoolCell container (Biocision Inc.) and transfer to -80C freezer overnight.
16. Transfer cells to liquid nitrogen tank