IIT2017-07-HO-PEMBRORT

Preoperative Combination of Pembrolizumab and Radiation Therapy in Patients with Operable Breast Cancer

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Signature Page

The signature below constitutes the approval of this protocol and the attachments, and provides the necessary assurances that this trial will be conducted according to all stipulations of the protocol, including all statements regarding confidentiality, and according to local legal and regulatory requirements and applicable U.S. federal regulations and ICH guidelines.

Principal Investigator (PI) Signature	**Date**

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LIST OF ABBREVIATIONS

3D	3-dimensional
AE	Adverse Event
ALT	Alanine Aminotransferase
ANC	Absolute Neutrophil Count
ASCO-CAP	American Society of Clinical Oncology-College of American Pathologists
AST	Aspartate Aminotransferase
BUN	Blood Urea Nitrogen
C	Cycle
CBC	Complete Blood Count
CD	Cluster of differentiation
Cm	Centimeter
CMP	Comprehensive Metabolic Panel
CR	Complete Response
CT	Computed Tomography
CTCAE	Common Terminology Criteria for Adverse Events
CTL	Cytotoxic T-lymphocyte
CTLA	Cytotoxic T-lymphocyte-associated protein 4
CTV	Clinical target volume
D	Day
DNA	Deoxyribonucleic acid
DSMB	Data and Safety Monitoring Board
ECOG	Eastern Cooperative Oncology Group
ER	Estrogen Receptor
FDA	Food and Drug Administration
FISH	Fluorescence in-situ hybridization
FOXP3	Forkhead box P3
FSH	Follicle stimulating hormone
Gy	Gray
H&P	History & Physical Exam
HBsAg	Hepatitis B surface antigen
HCV	Hepatitis C virus
HER2	Human Epidermal Growth Receptor 2
HIV	Human immunodeficiency virus
HRT	Hormone replacement therapy
IB	Investigator's brochure
IDFS	Invasive Disease-Free Survival
IFN	Interferon
IHC	Immunohistochemistry
IL-2	Interleukin-2
IMRT	Intensity-modulated radiation therapy
irAE	Immune-related adverse event
IV	Intravenously

Kg	Kilogram
MerFISH	Multiplexed error-robust FISH
Mg	Milligram
mIHC	Multispectral immunohistochemistry
MK	Merck
MMG	Mammogram
Mo.	Month(s)
MRI	Magnetic resonance imaging
MTD	Maximum Tolerated Dose
N	Nodal status
NCI	National Cancer Institute
ORR	Objective Response Rate
PBMCs	Peripheral Blood Mononuclear Cells
PD	Progressive Disease
PD-L1	Programmed Death-Ligand 1
PFS	Progression Free Survival
PK	Pharmacokinetic
PR	Progesterone Receptor
PTV	Planning target volume
Q	Every
RNA	Ribonucleic acid
RT	Radiation Therapy
SAE	Serious Adverse Event
SBRT	Stereotactic body radiation therapy
SCOEPS	Safety Committee on Early Phase Studies
SD	Stable Disease
SEER	Surveillance, epidemiology, and end results program
SGOT	Serum Glutamic Oxaloacetic Transaminase
SOC	Standard of care
SPGT	Serum Glutamic Pyruvic Transaminase
SUSAR	Suspected Unexpected Serious Adverse Reaction
T	Tumor size
TCR	T-Cell Receptor
TIL	Tumor infiltrating lymphocyte
TNBC	Triple-Negative Breast Cancer
TNF-α	Tumor necrosis factor-alpha
ULN	Upper limit of normal
US	Ultrasound
W	Week
WBC	White Blood Cells
WBRT	Whole breast radiation therapy
WOCBP	Women of child-bearing potential
YR	Year

STUDY SCHEMA

This is a prospective, phase Ib/II study of the preoperative combination of pembrolizumab (pembro) and radiation therapy (RT) in patients with operable breast cancer. Patients with newly diagnosed, Stage I-III breast cancer who are planned for breast-conserving surgery and adjuvant whole breast radiation therapy will be recruited into one of 2 cohorts as per their biomarker profile as depicted below. The TNBC cohort will have 50 subjects (10 TNBC subjects for phase Ib and 40 TNBC subjects for phase II) and the HR+ cohort will have 10 subjects. Enrollment will start with the phase Ib TNBC cohort. The trial will be conducted in a Bayesian design, wherein safety as assessed by lack of delay in standard of care treatment will be assessed after 3, 6, and 9 patients have initiated standard of care treatment in each cohort. Standard of care treatment is defined as breast-conserving surgery or initiation of neoadjuvant systemic chemotherapy. If the phase Ib TNBC trial meets a pre-specified safety endpoint,enrollment in the phase II TNBC trial and HR+ cohort will take place.

TNBC Phase Ib Trial (N=10)

- ER/PR < 10%
- HER2-by ASCO-CAP
- T ≥ 2cm, any N

TNBC Phase II Trial (N=40)

- ER/PR < 10%
- HER2-by ASCO-CAP
- T ≥ 2cm, any N

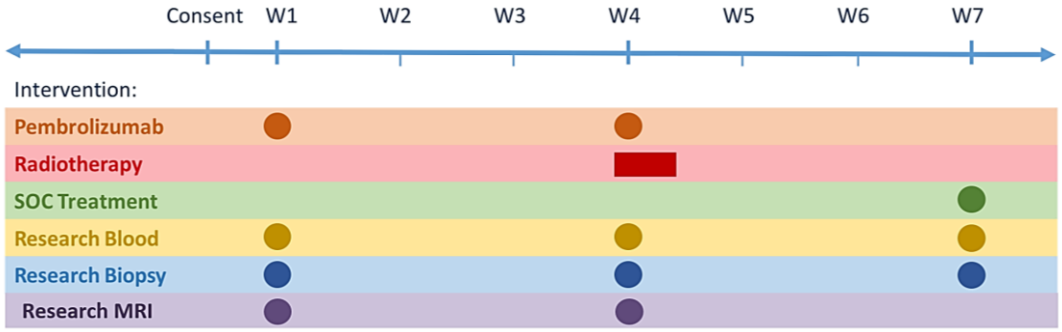
High-Risk HR+ (N=10)

- ER ≥ 10%
- HER2- by ASCO-CAP
- T at least 2cm, any N

High-risk defined by 2 out of 3:

- Grade II-III
- Ki-67 > 20%
- ER < 75%

Study treatment will consist of 2 doses of neoadjuvant pembro. The second dose will be given in combination with a RT boost. The primary objectives are: 1) to assess the feasibility of the preoperative combination of pembro and RT as defined by the number of patients who do not necessitate a delay in standard of care treatment, 2) to assess the change in tumor infiltrating lymphocyte (TIL) score with therapy. The secondary objectives are to describe the toxicity with preoperative pembro and RT up to 15 weeks after administration of pembrolizumab, to assess the invasive disease-free (iDFS) survival, and to assess the pathological complete response (pCR) rates.



- The first dose of pembro will be given on cycle 1, day 1 (C1D1). Each cycle will be 21 days. The second of pembro dose will be given on C2D1 (+/- 3 days).
- RT will be administered concurrently within one week of the second dose of pembro (C2D1 + 5 calendar days). 8Gy will be delivered in3 fractions over 3 to 5 business days (for a total dose of 24Gy).
- Standard of care treatment, including breast-conserving surgery or additional neoadjuvant systemic therapy, will be pre-set to start 8 weeks after study enrollment (+/- 5 days).
- Research blood and stool will be obtained after consent up to C1D1. It will also be obtained on C2D1 (+/-3 days) and at the time of initiation of standard of care treatment (+/-5 days).
- Research tumor biopsy will be performed after consent up to C1D1 (in the HR+ cohort, a diagnostic biopsy accepted if sufficient archived tissue is available) and on C2D1 (+/-3 days) prior to the initiation of radiation therapy. Biopsies will be processed for scSeq and tumor infiltrating

lymphocytes (TIL) analysis.

- Research tissue will also be obtained prior to the initiation of standard of care treatment at week 7 (+/-5 days). If standard of care treatment consists of surgery, surgical tissue will be used. If it consists of further neoadjuvant systemic therapy, another research biopsy will be conducted. Fresh tissue may also be procured at the time of surgery if the surgery occurs after completion of neoadjuvant systemic therapy. This will be determined at the discretion of the treating physician.

STUDY SUMMARY

Title	Preoperative Combination of Pembrolizumab and Radiation Therapy in Patients with Operable Breast Cancer
Protocol Number	IIT2017-07-HO-PEMBRORT
Phase	Pilot Study; Phase Ib/II
Methodology	Prospective therapeutic trial
Study Duration	Approximately 5 years
Study Center(s)	Cedars-Sinai Medical Center
Objectives	Primary Objectives: • To assess the feasibility of the preoperative combination of pembro and RT as defined by the number of patients who do not necessitate a delay in standard of care treatment • To assess the change in tumor infiltrating lymphocyte score with therapy Secondary Objective: • To describe the toxicity with preoperative pembrolizumab and RT up to 15 weeks after the last dose. • To assess invasive disease-free survival (iDFS) rates. • To assess the pathological complete response (pCR) rates
Number of Subjects	60
Diagnosis and Main Inclusion Criteria	Operable hormone receptor positive (HR+) or triple negative breast cancer (TNBC) with planned breast-conserving surgery and adjuvant whole breast radiation therapy
Study Product(s), Dose, Route, Regimen	Pembrolizumab 200mg IV every 3 weeks x 2 doses RT 8Gy x 3 fractions
Duration of administration	6 weeks
Reference therapy	None
Statistical Methodology	Bayesian design

1.0 BACKGROUND AND RATIONALE

1.1 Disease Background

With more than 1 million new cases diagnosed yearly worldwide, breast cancer is a global public health burden.[2] For most patients in the developed world, breast cancer is diagnosed at an early stage.[3] However, despite improvements with systemic therapies, approximately one-third of women with localized disease still develop distant metastases.[4] Over the past decade, molecular subtyping of breast cancer has identified intrinsic subtypes that may be at enhanced risk for both local and distant recurrence. In 2000, Perou et al first described the more modern concept of breast cancer subtypes[5]. They identified multiple breast tumor subgroups that could be defined by distinct expression patterns in genes related to proliferation, hormone receptor signaling, HER2 signaling and expression of basal epithelial markers. Today, there are 4 major subtypes (luminal A, luminal B, HER2-enriched and basal like/TNBC). Although these subtypes are most accurately defined by patterns of gene expression, characteristic immunohistochemistry patterns are commonly used as a surrogate in the literature.

The prognostic value of these breast tumor subtypes has been demonstrated in multiple datasets. In addition, the 8th edition of the American Joint Committee on Cancer (AJCC) Manual has incorporated biologic factors including tumor grade, ER, PR and HER2 status. In this new staging system, an Oncotype Dx Recurrence Score (RS)<11 categorizes patients with hormone receptor (HR) positive, HER2 negative tumors into the prognosis of Stage 1 breast cancer, regardless of the size of the primary tumor. Of the 4 major subtypes, basal like/TNBC has the worst prognosis with disproportionately poorer clinical outcomes compared to other subtypes[5,6]. Compared to Luminal A tumors, Luminal B tumors are characterized by higher tumor grade, larger size, and increased incidence of lymph node positivity. In such patients who remain at high risk for relapse with standard therapy, harnessing the immune system to recognize and fight early stage breast cancer prior to definitive surgical intervention has the potential to confer long-term immunity, and ultimately, cure.

Further, there is a growing body of evidence indicating that women with breast cancers of certain subtypes are ideally suited for immunotherapy trials. Specifically, dense lymphocytic infiltration has been observed in a significant proportion of TNBCs and HER2-enriched tumors, indicating an inherent interplay between the tumor and the immune system.[7,8] Although HR+/HER2- tumors are less likely to be infiltrated by CD8+ cytotoxic T-cells compared to TNBCs and HER2+ tumors, approximately 40% of these tumors do display a CD8+ infiltrate. It has been suggested that expression of the estrogen receptor results in decreased immunogenicity [9,10]. Thus, it is likely that the subset of HR+/HER2- tumors that display increased lymphocytic infiltrate are not as strongly driven by estrogen and belong to the Luminal B subtype. Thus, this study focuses on the immunogenicity of high-risk breast cancer subtypes that are likely to display a dense lymphocytic infiltration including TNBC and HR+/HER2- tumors that are more likely to be consistent with Luminal B subtypes, defined by 2 out of 3 of the following characteristics: grade II-III, Ki-67 > 20%, ER < 75%[11].

Locoregional therapies for patients with breast cancer consist of either mastectomy or lumpectomy followed by whole breast radiation therapy (WBRT). Both have been shown to be equivalent to mastectomy in terms of local control and overall survival.[12] Recently, radiation therapy (RT) has been described to have numerous immune-modulatory effects. RT-induced tumor cell death increases the supply of tumor-specific antigens, leading to the release of signaling molecules that attract immune cells to the tumor microenvironment and to the upregulation of programmed death-ligand 1 (PD-L1) on tumor cells.[13-15] Whether or not RT combined with PD-L1 blockade synergistically increases the tumor immunogenicity, compared to PD-L1 blockade alone, is the subject of this trial. Our study population will focus on women with immunogenic subtypes of breast cancer and/or those who are at high risk of recurrence. Our study design, which describes lead-in with administration of pembrolizumab alone prior to the addition of RT, will allow for examination of immune correlatives in a staged fashion and subsequent determination the efficacy of our strategy.

1.2 Rationale for Immune Augmentation in Breast Cancer

Thus far, results from early clinical trials of checkpoint inhibition in breast cancer have been relatively modest but encouraging. In ER+/HER2- patients with locally advanced or metastatic disease treated with pembrolizumab, an objective response rate (ORR) of 12% was achieved.[16] The Phase Ib JAVELIN study

reported a response rate of 4.8% among breast cancer patients treated with the anti-PD-L1 antibody avelumab overall and an ORR of 8.6% and 2.8% in TNBC and ER+ or PR+/HER2- subtypes, respectively.[17]

In PD-L1 positive TNBC patients, the Phase Ib trial KEYNOTE-012 showed a 18.5% ORR in 27 evaluable patients treated with pembrolizumab.[18] The median duration of response was not yet reached (15 to ≥ 47 weeks). Another Phase I trial using the PD-L1 antibody atezolizumab reported a response rate of 19% in 21 evaluable patients with PD-L1 positive metastatic TNBC.[19] The median duration of response was not yet reached (18 to ≥56 weeks). Of note, in both trials, only data from subjects that were PD-L1 positive by immunohistochemistry (IHC) were presented. The KEYNOTE-012 trial defined PD-L1 positivity as PD-L1 expression in the stroma or in ≥ 1% of tumor cells. The atezolizumab trial defined PD-L1 positivity as PD-L1 expression in ≥ 5% of infiltrating immune cells.

Recently, a Phase I expansion cohort of patients with metastatic TNBC demonstrated a 10% ORR in 112 evaluable patients treated with atezolizumab.[20] ORR was 26% among patients that received atezolizumab as a first line treatment, 4% for patients that received atezolizumab as second line treatment, and 8% among those that received atezolizumab as third line or greater treatment. Notably, all 11 patients who responded were alive after one and two years, while OS rates were 33% and 11% for non-responders after one year and two years, respectively.

1.3 Rationale for Combining Radiation Therapy and Immune Therapy

In-situ tumor destruction releases a large amount of tumor antigens. Antigen-presenting cells, such as dendritic cells, then take up these antigens in the periphery and migrate to lymph nodes where they activate CD4+ and CD8+ T-lymphocytes that recognize these tumor antigens. Immune augmentation via immune co-stimulatory molecules then permits the ensuing immune response to strengthen and destroy cancer systemically.[21,22] Thus, the anti-tumor immune response initiated by RT delivered to an intact tumor can be potentiated by combination with checkpoint blockade.

1.3.1 Preclinical Data

Multiple pre-clinical studies support the combination of RT with checkpoint inhibition. The combination of radiotherapy and anti-CTLA-4 antibody delayed growth of irradiated tumor, inhibited lung metastases, and improved survival in a 4T1 murine carcinoma model.[23] These findings were confirmed in a 9H10 model, in which combination therapy enhanced primary tumor regression and produced abscopal regression of non-irradiated lesions.[24] Another murine model demonstrated that the combination of radiotherapy and antibodies against CD137 and PD-1 was curative and associated with tumor antigen specific CD8+ T-cell infiltration.[25] Finally, Deng and colleagues showed that RT enhanced the effect of anti-PDL-1 in the TUBO breast cancer cell line, with reduced TUBO tumor growth after mice were re-challenged in the opposite flank, implying systemic immunity.[13]

1.3.2 Clinical Data

A systemic response to localized RT in combination with anti-CTLA-4 therapy, the so-called “abscopal effect”, was reported in a patient after disease progression with CTLA-4 blockade with ipilimumab alone.[14] Specifically, a right hilar lymph node and spleen metastases, which was not the target of RT, showed regression only after the patient received palliative RT after several months of anti-CTLA-4.

Based on the above phenomenon as well as pre-clinical data suggesting the synergism of RT with immune therapies, studies evaluating the efficacy of RT with checkpoint blockade in metastatic breast cancer are actively ongoing. In a phase II study evaluating pembrolizumab and RT in metastatic TNBC patients, 2 out of 5 evaluable patients had a response outside of the irradiated field.[26] Although assessing distant response is not one the endpoints of this protocol, which includes women with non-metastatic breast cancer, the study nevertheless provides proof-of-principle for the concept of systemic immune modulation by RT when added to checkpoint inhibition.

Once safety of the pembro-RT regimen has been established, the efficacy of the regimen remains to be tested. Current chemotherapy regimens (GeparSixto) report pathologic complete response

rates of approximately 50%[27] and in the metastatic setting addition of atezolizumab (PD-L1) to nab-paclitaxel (IMpasion130) showed increased PFS in triple negative breast cancer[28]. Based on these two recent observations, the phase II portion of this trial will test the efficacy of adding pembro-RT to neoadjuvant chemotherapy in early-stage breast cancer and determine the pathologic complete response rate in comparison to best available chemotherapy which is the current standard of care.

1.4 Whole Breast Radiation Plus Boost for Localized Breast Cancer

1.4.1 Rationale

A radiation boost targeted at the tumor bed is routinely administered to breast cancer patients as part of breast-conserving therapy. The rationale for a boost is based on the patterns of recurrence data that has demonstrated that the vicinity of the original index tumor is the most common site of local recurrence and therefore requires a higher tumoricidal dose from RT compared to the rest of the breast[29]. Several randomized controlled trials have shown that the addition of a boost to standardly fractionated whole breast RT (WBRT) in women with localized breast cancer led to an improvement in local control [30,31]. The largest of these trials, the EORTC 22822, included 5318 patients with stage I-II breast cancer who underwent lumpectomy + axillary lymph node dissection[30]. Following WBRT (50 Gy/25 fractions), patients were randomized to +/- 16 Gy boost. With a median of 17.8 years of follow-up, the cumulative incidence of local failure was 6.4%, 8.8%, and 12.0%, respectively, in boosted patients compared with 10.2%, 13.1%, and 16.4% in the non-boost group. The benefit of a boost was observed in all age groups.

A boost to the lumpectomy bed is most commonly delivered sequentially following the completion of whole breast RT. However, this is generally out of convention rather than due to scientific rationale. A boost may be equally efficacious, if prior to whole breast RT. In this protocol, we propose the delivery of a boost in the preoperative setting prior to lumpectomy, so that it may engage the immune system while the primary tumor remains intact, providing a rich supply of tumor antigens.

1.4.2 Safety of Preoperative RT

Radiation therapy is routinely administered preoperatively in other tumor types such as sarcoma and rectal cancer.[32,33] Studies have shown that RT can be safely administered to early stage breast cancer patients in the preoperative setting.[34,35] In a phase I dose-escalation trial of single-dose preoperative partial breast irradiation in early stage breast cancer patients, no acute dose-limiting toxicity was observed, no post-operative wound dehiscence was seen, and cosmetic results were favorable.[34] A dose escalation study of stereotactic body radiation therapy (SBRT) combined with neoadjuvant chemotherapy in breast cancer patients demonstrated that the combination was well tolerated with the MTD not reached in the study.[35] These data support administration of RT preoperatively in localized breast cancer patients.

1.4.3 Dose and Fractionation of RT with Immune Therapy

Multiple studies have examined the role of radiation dose and fractionation in generating systemic anti-tumor immune responses.[36] In murine models of RT, several groups have demonstrated enhanced production of circulating IFN-gamma expressing, tumor-specific CD8+ T cells with a single fraction of RT (15 Gy) delivered focally to tumors.[37,38] Dewan et al reported that a hypofractionated dose of 8 Gy x 3 fractions was more effective than single fraction therapy when combined with anti-CTLA-4 therapy and resulted in increased TILs.[24] Furthermore, in a 4T1 mammary carcinoma model, when CTLA-4 was combined with RT, two fractions of 12Gy each improved survival compared to single fraction.[23] In a phase I dose-escalation trial of single-dose preoperative partial breast irradiation in early stage breast cancer patients, expression of genes immune-modulatory genes was increased in a dose-dependent fashion with single fraction RT.[34]

Although there is no consensus regarding the optimal dose and fractionation of RT to utilize with immune therapies, it appears reasonable that higher total doses and that relative to single fraction, short, hypofractionated courses of fractionation generate greater antigen release, leading

to improved anti-tumor immune responses. In this study, we plan to use 8 Gy x 3 fractions on the basis of preclinical data from Dewan et al..[24]

1.5 Rationale for Pembrolizumab Dose Selection/Regimen

Pembrolizumab is an optimal immunotherapy agent to study, as this agent has recently been FDA-approved for use in multiple tumor types. It is therefore ready to be tested for efficacy in other disease sites and in combination with other treatments. It is currently in being tested in combination with RT for metastatic TNBC (NCT02730130).

An open-label Phase I trial was conducted to evaluate the safety and clinical activity of single agent MK-3475 (pembrolizumab).[39] The dose escalation portion of this trial evaluated three dose levels, 1 mg/kg, 3 mg/kg, and 10 mg/kg, administered every 2 weeks (Q2W) in subjects with advanced solid tumors. All three dose levels were well tolerated and no dose-limiting toxicities were observed. This first in-human study of MK-3475 showed evidence of target engagement and objective evidence of tumor size reduction at all dose levels (1 mg/kg, 3 mg/kg and 10 mg/kg Q2W). No MTD has been identified to date. Recent data from other clinical studies within the MK-3475 program has shown that a lower dose of MK-3475 and a less frequent schedule may be sufficient for target engagement and clinical activity.

PK data analysis of MK-3475 administered Q2W and Q3W showed slow systemic clearance, limited volume of distribution, and a long half-life (refer to IB). Pharmacodynamic data (IL-2 release assay) suggested that peripheral target engagement is durable (>21 days). This early PK and pharmacodynamic data provides scientific rationale for a Q2W and Q3W dosing schedule.

The rationale for further exploration of 2 mg/kg and comparable doses of pembrolizumab in solid tumors is based on: 1) similar efficacy and safety of pembrolizumab when dosed at either 2 mg/kg or 10 mg/kg Q3W in melanoma patients, 2) the flat exposure-response relationships of pembrolizumab for both efficacy and safety in the dose ranges of 2 mg/kg Q3W to 10 mg/kg Q3W, 3) the lack of effect of tumor burden or indication on distribution behavior of pembrolizumab (as assessed by the population PK model) and 4) the assumption that the dynamics of pembrolizumab target engagement will not vary meaningfully with tumor type.

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.

1.6 Correlative Studies

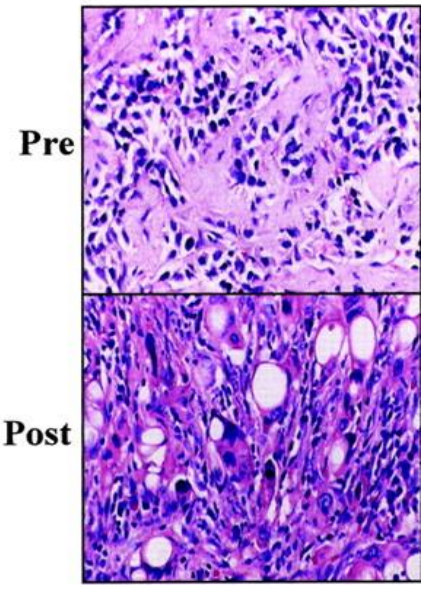
Tumor Tissue:

Infiltration of immune cells has predicted improved prognosis and response to therapy in many different tumor types including breast cancer.[40,41] The greatest clinical benefit is seen when TILs comprise at least 50-60% of the tumor.[40] While a baseline TIL score indicates the immunogenicity of a tumor, the influx of TILs with therapy indicates the engagement of the immune system as a result of therapy (Figure 1).[1]

In addition to the amount of lymphocytic infiltrate, the phenotype of that infiltrate has also been shown to be prognostic. Type 1 T-cells are associated with favorable prognosis. Type 1 $CD4^+$ T-helper cells facilitate antigen presentation and activation of $CD8^+$ cytotoxic T-cells (CTL), which are essential for tumor destruction.[42] On the other hand, Type 2 $CD4^+$ T-helper cells, including Forkhead box P3 (FOXP3) $CD4^+$ regulatory T-cells, contribute to a tumor environment that inhibits CTL function and promotes tumor growth.[43]

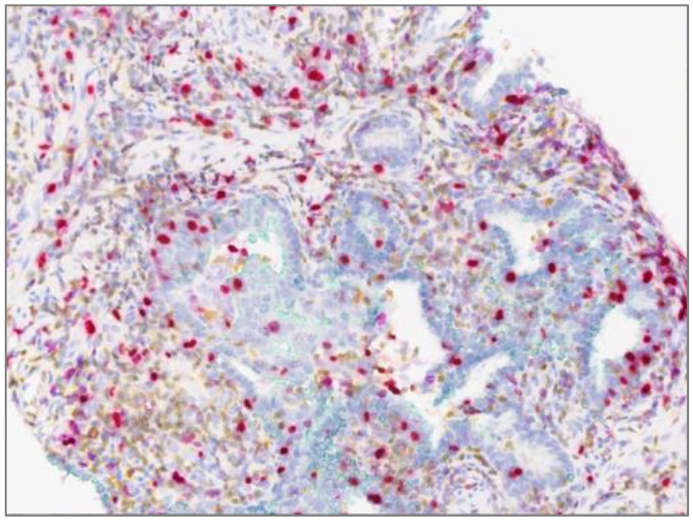
Multiplex IHC (mIHC) allows for multiple fluorescently tagged antibodies to be applied to paraffin-embedded tissue quantification of cell types including macrophages (CD163+), effector T-cells (CD3+CD8+), and regulatory T-cells (CD3+CD4+FOXP3+) (Figure 2). Assays can also be used to assess the activation and proliferation of TILs using markers such as Ki-67, PD-1, CTLA-4.

Figure 1



Increase in TILs in a breast cancer patient after treatment with neoadjuvant paclitaxel.[1]

Figure 2



Multispectral IHC to quantify immune cell subsets on a paraffin-embedded tumor slide. Slide courtesy of Dr. Stephen Shiao, study collaborator.

Epithelium/Pan-CK = green
T-cell/CD4 = yellow
T-cell/CD8 = purple
Macrophage = red
Nuclear hematoxylin = blue

Flow cytometry can similarly be used on fresh or frozen tissue to quantify cell types including effector T-cells (CD3+CD8+), and regulatory T-cells (CD3+CD4+FOXP3+) cells.

Activation of the immune system can be further assessed using sequencing technology. Using TCR sequencing analysis on extracted DNA from paraffin-embedded tissue, increase in shared clones suggests that therapy is enhancing proliferation of antigen experienced cells within the tumor.

Single cell gene expression profiling can be used to assess the transcriptome of individual cells. Innovative technology allows for the evaluation of the transcriptome in single cells within the tumor and

the tumor microenvironment.[44] Serial assessment of the transcriptome through the course of therapy can identify genes that are indicators and/or effectors of safety and response.

Whole Genome/Exome Sequencing: Frozen or FFPE tumor samples will be submitted for DNA extraction and whole genome/exome sequencing to assess the expression level of different genes within the tumor.

Whole Blood:

An increase in circulating cytokines is an indicator of systemic immune activation. Further, flow cytometry can also be performed on fresh or frozen blood to assess the composition of systemic immune cells. Similarly, TCR sequencing can be conducted on blood to assess the clonality of systemic immune cells.

Optional Research Imaging:

One of the most critical questions facing immunotherapy is how to determine which patients will respond. There is currently no biomarker for early response, however recent data indicates that DCE-MRI can assess inflammation and that this may be an early marker of the response to immunotherapy. MR imaging is incorporated to assess possible responses to treatment and may aide in the decision for additional therapy after completion of the experimental portion of the trial. The unique tissue characterization of MRI will be utilized and compared to tissue analysis from biopsy samples at the same timepoints; before, during and after neoadjuvant immunotherapy and radiation treatment.

Optional Fecal Samples for Analysis

Many factors have been shown to regulate the anti-tumor immune response and, in particular, the immune set point as determined by the basal immune responses. Recent evidence has found that most, if not all, immune responses are shaped by the interaction of the immune system with the microbiome.[45]

Several experimental models revealed that the efficacy of immune therapies including CpG and checkpoint inhibitors as well as immune-related toxicity can be influenced by the microbiome.[46] Thus, for this trial we plan to collect optional fecal samples for microbiome analysis as a potential biological correlate for efficacy and/or toxicity.

2.0 STUDY OBJECTIVES

2.1 Primary Objectives

2.1.1 To assess the feasibility of the preoperative combination of pembrolizumab and RT as defined by the number of patients who do not necessitate a delay in standard of care treatment

2.1.2 To assess the change in TIL score with preoperative pembrolizumab and RT.

2.2 Secondary Objectives

2.2.1 To describe the toxicity with preoperative pembrolizumab and RT up to 15 weeks after administration of pembrolizumab.

2.2.2 To assess invasive disease-free survival (iDFS) rates.

2.2.3 To assess the pathological complete response (pCR) rates.

2.3 Exploratory Objectives

2.3.1 To assess changes in intratumoral and serum lymphocyte composition and serum cytokines with therapy.

2.3.2 To explore T-cell diversity and clonality by deep sequencing.

2.3.3 To analyze the RNA transcriptome in tumor, intratumoral lymphocytes, and adjacent normal tissue.

2.3.4 To assess changes in the microbiome**.**

2.3.5 To assess changes in breast cosmesis.

2.3.6 To assess disease status by breast MR imaging

2.3.7 To assess the pathological complete response (pCR) rates

2.4 Endpoints

- **Primary Efficacy Endpoint: Feasibility**

  Feasibility will be defined by the number of patients who do not necessitate a delay in standard of care treatment. Since the combination of pembrolizumab and RT has not been tested in the preoperative setting in patients with potentially curative disease, it is necessary to establish whether such preoperative treatment can be safely given without delaying curative-intent treatment.

- **Co-Primary Efficacy Endpoint: Change in TILs**

  An increase in TILs is an indicator of immune system engagement. A lead in with pembrolizumab alone followed by the combination of pembrolizumab with RT will allow for serial assessment of TILs. This will establish the contribution of RT to the immune response generated by pembrolizumab.

- **Safety Evaluations**

  Immune related adverse events that result from checkpoint blockade can occur weeks after the drugs are administered. Therefore, adverse events will be recorded up to 15 weeks after administration of pembrolizumab and radiation therapy and graded according to CTCAE, Version 4.03. Immune related adverse events will also be documented for up to 1-year post treatment. When considering if an adverse event is immune related, a general principle is that differential diagnoses should be diligently evaluated according to standard medical practice. Non-inflammatory etiologies should be considered.

- **iDFS**

  iDFS will be defined as the time from completion of surgery to the first occurrence of the following events: invasive ipsilateral, local, regional, or distant recurrence, or death due to breast cancer.[60]

- p**CR**

  pCR will be defined as the absence of invasive disease in the breast and lymph nodes at the time of curative-intent surgery.

3.0 STUDY DESIGN

Study Design

This is a prospective, single-institution, Phase Ib/II study of the preoperative combination of pembrolizumab (pembro) and radiation therapy (RT) in patients with operable breast cancer (TNBC). The two cohorts will consist of 50 TNBC and 10 HR+ women each. Enrollment will start with the Phase Ib TNBC safety cohort. The trial will be conducted in a Bayesian design, wherein safety as assessed by lack of delay in standard of care treatment will be assessed after 3, 6, and 10 patients have initiated standard of care treatment. If at least 80% of the first 10 subjects in the TNBC cohort proceed with standard of care therapy without delay, the combination will be deemed safe in the cohort, and enrollment in the HR+ cohort, as well as continued enrollment in the TNBC cohort (40 subjects), will occur at that time.

Each cohort will be evaluated for delay in curative-intent standard of care treatment every time 3 patients complete study therapy and undergo standard of care treatment. At each of those timepoints, if the posterior probability that a patient experiences a delay in standard of care treatment exceeds 20%, the cohort will stop enrollment. In Bayesian statistics, the posterior probability of a random event or an uncertain proposition is the conditional probability that is assigned after the relevant evidence or background is taken into account. This design will assure that a cohort does not continue enrollment if early delays in standard of care treatment are seen. Enrollment will be carried out continuously and will not be stopped in order to evaluate the posterior probability of a delay. However, the number of patients undergoing assessment for a possible delay simultaneously will not exceed four in each of the 2 cohorts.

Study Population

Subjects with pathologically confirmed, localized (Stage I-III) breast cancer with a primary tumor size of at least 2cm for whom breast-conserving surgery and adjuvant whole breast RT is planned will be recruited into one of 2 cohorts, delineated by breast cancer subtype (TNBC or HR+). We anticipate that a low proportion of patients who are planning breast-conserving surgery may ultimately choose mastectomy. The receipt of a pre-operative boost should not preclude the ability to receive mastectomy with immediate reconstruction. The outcomes of these patients will be captured and followed on protocol.

The magnitude of TILs is reported to be higher in TNBC tumors than HR+/HER2- tumors.[47] It is postulated that TNBCs have a higher lymphocytic infiltrate due to higher mutational load, which enhances immunogenicity and results in increased TIL recruiting.[48] Clinical data with checkpoint blockade therapy supports the increased immunogenicity of TNBC and HER2+ tumors compared to HR+/HER2- tumors. For example, the Phase Ib JAVELIN study reported an ORR of 8.6%, 3.8%, and 2.8% in TNBC, HER2+, and ER+ or PR+/HER2- subtypes, respectively.[17] Due to the differences in intrinsic risk and baseline immunogenicity, the cohorts in this study are delineated by biomarker subtype.

As such, the cohorts include:

1) Cohort 1: TNBC patients with primary tumors measuring at least 2 cm

2) Cohort 2: High-risk, ER-positive and HER2-negative breast cancer patients with primary tumors measuring at least 2 cm

ER-positive and HER2-negative breast cancer will be defined by ER expression $\geq$ 10% by immunohistochemistry (IHC) and HER2-negativity per ASCO-CAP guidelines.[49] High-risk disease will be defined by the presence of at least 2 of the following 3 criteria: histologic grade II-III, Ki-67 > 20%, ER expression < 75% by IHC. TNBC will be defined by ER and PR expression < 10% by IHC and HER2-negativity by ASCO-CAP guidelines[49].

It is well known that systemic therapy activates the immune system.[50] Therefore, clear, discriminate assessment of the contribution of RT to tumor immunogenicity in each of the 2 subsets requires that this trial be conducted in the absence of prior therapy. We recognize the

importance of other standard of care therapy, including neoadjuvant systemic therapy in TNBC tumors.[51,52] Therefore, the standard of care curative-intent treatment will be set to start 8 weeks (+/-5 days) after trial initiation, and the primary endpoint is to assess delays in this pre-planned date.

Bleicher, et al reported increased breast cancer-specific mortality with delays from time of diagnosis to surgery of > 60 days in a retrospective cohort analysis from the SEER Medicare database.[53] This was particularly true in Stage I patients. In a similar retrospective analysis from the California Cancer Registry, Chavez-Macgregor, et al reported worse overall survival and breast cancer-specific survival in patients whose adjuvant chemotherapy was delayed beyond 90 days after surgery.[54] The planned standard of care treatments in this study are within the timeframes of these analysis. The Bayesian design of the trial will prevent ongoing recruitment into each of the cohorts if the probability of surgical delays exceeds pre-specified limits after 3, 6, and 9 patients undergo standard of care treatment in each cohort.

This study requires tumor samples collected at 3 different time points for each patient to complete the immune correlative analysis. Therefore, a minimum tumor size of 2 cm was chosen to maximize the probability of having sufficient tumor tissue available for analysis.

Study Intervention

Patients will receive pembrolizumab Q3W for 2 doses. The second dose will be given in conjunction with an RT boost, consisting of 8 Gy for 3 fractions. We estimate that approximately 10% of patients who are planned to undergo breast-conserving therapy may undergo mastectomy for reasons including positive margins and patient choice.[55] It is anticipated that receipt of a boost dose, which is directed to a small portion (<30%) of the total breast volume, will not preclude the ability to undergo mastectomy.

Approximately 3 weeks after the second dose of pembrolizumab, patients will initiate standard of care curative-intent treatment, consisting of breast surgery or neoadjuvant systemic treatment. Patients will have a pre-planned date for initiation of standard of care treatment scheduled approximately 8 weeks (+/- 5 days) after study initiation. **Research study appointments/interventions will not interfere with the pre-planned, standard of care treatment arrangements.**

Recruitment Plan

At the time of the initial surgical consultation at the Saul and Joyce Brandman Breast Center at Cedars-Sinai, medical oncology or radiation oncology consultation at the Samuel Oschin Comprehensive Cancer Institute at Cedars-Sinai, potentially eligible patients will be identified, informed consent forms provided, and initiation of standard of care treatment (breast conserving surgery or neoadjuvant systemic therapy) dates established. Potentially eligible patients will be evaluated by 1) A designated consenting medical oncologist and 2) consenting radiation oncologist for assessment of amenability to preoperative radiation. All available (per the standard of care) imaging (mammogram +/- breast ultrasound +/- breast MRI) as well as clinical breast exam will be reviewed to assess tumor size and determine feasibility of preoperative radiation boost delivery and thus, study participation. Whenever feasible, the RT evaluation will be arranged prior to the testing date for standard of care treatment so that baseline research bloods may be collected after the patient is confirmed eligible and together with the non-study pretreatment bloods. Prior to protocol intervention, consent will take place at the first available appointment with a consenting professional.

4.0 PATIENT ELIGIBILITY

4.1 Inclusion Criteria

Subjects must meet the following criteria at screening to be eligible to participate in the study.

4.1.2 Women age 18 years or older

4.1.3 Confirmed histologic diagnosis of invasive adenocarcinoma of the breast, and

4.1.4 ER, PR and HER2 testing (on outside or Cedars-Sinai biopsy report), and

4.1.5 High-risk, ER-positive and HER2-negative breast cancer patients. ER-positive disease is defined as ER≥10%, any PR and HER2-negative by ASCO CAP guidelines. High-risk disease will be defined by the presence of at least 2 of the following 3 criteria: histologic grade II-III, Ki-67 > 20%, ER expression < 75% by IHC)

4.1.6 TNBC patients (defined as ER<10%, PR<10%, HER2-neu 0-1+ by IHC or FISH-negative; or as per MD discretion)

4.1.7 Operable tumor measuring ≥2 cm in maximal diameter as measured by any available standard of care imaging (examples include mammogram, breast ultrasound, breast MRI, CT) and/or by clinical exam

4.1.8 Any nodal status

4.1.9 Multifocal disease is permitted; largest focus must measure ≥2 cm

4.1.10 Synchronous bilateral invasive breast cancer is permitted

4.1.11 No indication of distant metastases

4.1.12 Candidate for breast-conserving therapy

4.1.13 Tumor amenable to preoperative radiation therapy boost as determined by radiation oncologist

4.1.14 ECOG performance status score of 0 or 1

4.1.15 Screening laboratory values must meet the following criteria:

i. White blood cells (WBCs) ≥ 2000/μL
ii. Absolute neutrophil count (ANC) ≥ 1500/μL
iii. Platelets ≥ 100 x 10^3/μL
iv. Hemoglobin ≥ 9.0 g/dL
v. Serum creatinine ≤ 2 mg/dL (or glomerular filtration rate ≥ 40 ml/min)
vi. AST ≤ 2.5 x upper limit of normal (ULN)
vii. ALT ≤ 2.5 x ULN
viii. Total bilirubin within normal limits (except subjects with Gilbert's syndrome, who must have total bilirubin < 3.0 mg/dL)
ix. INR ≤ 1.5 x ULN unless participant is receiving anticoagulant therapy as long as PT or aPTT is within therapeutic range of intended use of anticoagulant(s)
x. Negative HIV screening test
xi. Negative screening tests for Hepatitis B and Hepatitis C.
Patients with positive results that do not indicate true active or chronic infection may enroll after discussion and consensus agreement by the treating physician and principal investigator.

4.1.16 Women of childbearing potential (WOCBP) must be using an acceptable method of contraception to avoid pregnancy throughout the study and for at least 4 months after the last dose of pembrolizumab in such a manner that the risk of pregnancy is minimized. See below for the definition of WOCBP.

4.1.17 WOCBP must have a negative urine or serum (preferred) pregnancy test within 14 days prior to the first dose of pembrolizumab. Note: Patients undergoing egg harvesting are exempt from being required to take a pregnancy test within 72 hours of C1D1 due to the likelihood of false positive results. These patients may have false positive results for at least two weeks and may be exempt from pregnancy testing requirements within 14 days of C1D1 and may proceed to treatment per investigator discretion.

Definition of WOCBP

Women of childbearing potential include any female who has experienced menarche and who has not undergone successful surgical sterilization (hysterectomy, bilateral tubal ligation, or bilateral oophorectomy) or is not postmenopausal. Post menopause is defined as:

- Amenorrhea ≥ 12 consecutive months without another cause and a documented serum follicle stimulating hormone (FSH) level >35 mIU/mL
- Women with irregular menstrual periods and a documented FSH level > 35 mIU/mL
- Women on hormone replacement therapy (HRT)

Women who are using oral contraceptives, other hormonal contraceptives (vaginal products, skin patches, or implanted or injectable products), or mechanical products such as an intrauterine device or barrier methods (diaphragm, condoms, spermicides) to prevent pregnancy, or are practicing abstinence or where their partner is sterile (e.g., vasectomy) should be considered to be of childbearing potential.

4.1.18 Women must not be breastfeeding.

4.1.19 Willingness to adhere to the study visit schedule and the prohibitions and restrictions specified in this protocol.

4.1.20 Willingness to undergo mandatory Week 4 research biopsy

4.1.21 Written informed consent obtained from subject and ability for subject to comply with the requirements of the study.

4.2 Exclusion Criteria

4.2.1 HER2-positive breast cancer defined as IHC3+ or IHC2+ with FISH≥2 AND copy number ≥4 OR FISH ≤2 AND copy number ≥6

4.2.2 Inflammatory breast cancer

4.2.3 Contraindication(s) to breast-conserving therapy

4.2.4 Contraindication to radiation therapy or planned partial breast irradiation

4.2.5 Patients with cosmetic breast augmentations, specifically sub glandular implants with altered breast tissue, at the time of diagnosis

4.2.6 Evidence of metastatic disease.

4.2.7 Diagnosis of immunodeficiency or is receiving chronic systemic steroid therapy (in dosing exceeding 10 mg daily of prednisone equivalent) or any other form of immunosuppressive therapy within 7 days prior to the first dose of study drug.

4.2.8 Known additional malignancy that is progressing or has required active treatment within the past 3 years. Note: Participants with basal cell carcinoma of the skin, squamous cell carcinoma of the skin, or carcinoma in situ (e.g. breast carcinoma, cervical cancer in situ) that have undergone potentially curative therapy are not excluded.

4.2.9 Medical history and concurrent diseases

- Active autoimmune disease that has required systemic treatment in the past 2 years (i.e. with use of disease modifying agents, corticosteroids or immunosuppressive drugs). Replacement therapy (eg., thyroxine, insulin, or physiologic corticosteroid replacement therapy for adrenal or pituitary insufficiency, etc.) is not considered a form of systemic treatment.
- History of (non-infectious) pneumonitis that required steroids or has current pneumonitis.
- Active infection requiring systemic therapy.
- Any serious or uncontrolled medical disorder that, in the opinion of the investigator, may increase the risk associated with study participation or study drug administration, impair the ability of the subject to receive protocol therapy, or interfere with the interpretation of study results.
- Known history of Human Immunodeficiency Virus (HIV) (HIV 1/2 antibodies).
- Known history of Hepatitis B (e.g., HBsAg reactive) or known active Hepatitis C (e.g., HCV RNA [qualitative] is detected).

4.2.10 Prohibited Treatments and/or Therapies

- Chronic use of immunosuppressants and/or systemic corticosteroids (used in the management of cancer or non-cancer-related illnesses). However, use of corticosteroids is allowed for the treatment of immune related Adverse Events (irAEs), or adrenal insufficiency.
- Live vaccines within 30 days prior to the first dose of study treatment and while participating in the study. Examples of live vaccines include, but are not limited to, the following: measles, mumps, rubella, varicella/zoster, yellow fever, rabies, BCG, and typhoid vaccine. Seasonal influenza vaccines for injection are generally killed virus vaccines and are allowed; however, intranasal influenza vaccines (eg, FluMist®) are live attenuated vaccines and are not allowed. Prior treatment with an anti-PD-1, anti-PD-L1, or anti-PD-L2 agent or with an agent directed to another stimulatory or co-inhibitory T-cell receptor (eg, CTLA-4, OX-40, CD137)
- Prior systemic anti-cancer therapy including investigational agents within 4 weeks prior to study start.
  Note: Participants must have recovered from all AEs due to previous therapies to ≤Grade 1 or baseline. Participants with ≤Grade 2 neuropathy may be eligible.

Note: If participant received major surgery, they must have recovered adequately from the toxicity and/or complications from the intervention prior to starting study treatment.

- Prior radiotherapy within 2 weeks of start of study treatment to sites outside the breast. Participants must have recovered from all radiation-related toxicities, not require corticosteroids, and not have had radiation pneumonitis.

- Currently participating in or has participated in a study of an investigational agent or has used an investigational device within 4 weeks prior to the first dose of study treatment.

  Note: Participants who have entered the follow-up phase of an investigational study may participate as long as it has been 4 weeks after the last dose of the previous investigational agent.

4.2.11 For subjects who agree to the research breast MRI sub-study: Four or more previous gadolinium contrast scans due to the risk of brain deposits following repeated use of gadolinium-based contrast agents.

5.0 TREATMENT PLAN

5.1 Treatment Dosage and Administration

5.1.1 Pembrolizumab

Subjects will receive pembrolizumab 200 mg as an IV infusion. Pembrolizumab will be administered as a 30-minute IV infusion (every effort should be made 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 30 minutes: -5 min/+10 min).

5.1.1.1 Pembrolizumab Agent

Pembrolizumab is manufactured by Merck. Please refer to investigator brochure and MK-3475 Drug Preparation Instructions' manual.

Table 1: Product Descriptions

Product Name & Potency	Dosage Form
Pembrolizumab 50 mg	Lyophilized Powder for Injection
Pembrolizumab 100 mg/ 4mL	Solution for Injection

The investigator shall take responsibility for and shall take all steps to maintain appropriate records and ensure appropriate supply, storage, handling, distribution and usage of investigational produce in accordance with the protocol and any applicable laws and regulations. Clinical supplies will be affixed with a clinical label in accordance with regulatory requirements.

5.1.2 RT Boost

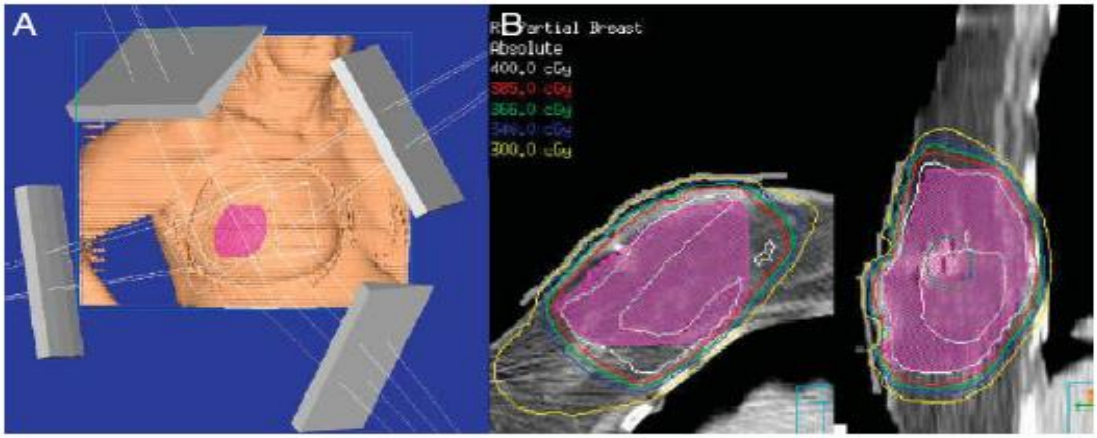
The Gross Target Volume (GTV) for the boost will be defined by preoperative imaging +/- clips placed into the tumor at the time of the biopsy. A planning target volume (PTV) will be created from the GTV as per departmental partial breast irradiation treatment planning guidelines. The PTV will be treated with 8 Gy x 3 fractions (24 Gy total) with external beam techniques. RT will

begin within 5 calendar days of the second dose of pembrolizumab.

A total dose of 24 Gy will be delivered over 3 fractions within a 3 to 5-day period. Doses of 8 Gy will be given once daily for 3 treatment days.

Figure 3

A: Beams-eye view of the boost directed to the PTV. B: Axial and coronal views of the PTV with isodose lines denoting the radiation doses delivered with partial breast irradiation.



5.1.2.1 Modality and Energy

Any combinations of photon beams of energy 6 MV or higher, with or without the addition of electrons of any energy, may be used for treatment, provided the dosimetric requirements of adequately treating the planning target volume (PTV) and homogeneity are met, although mixed energy (photons and electrons) is encouraged to minimize the exposure of the lung to radiation.

5.1.2.2 Patient Positioning and Simulation

The patient will be simulated in the supine positioning in a breast board or alpha cradle.

5.1.2.3 Target and Normal Structure Delineation

The radiation oncologist will identify the tumor by utilizing diagnostic MRI and mammographic imaging as the presence of clips in the breast. A radiopaque wire will be placed around the extent of the palpable breast tissue to delineate the whole breast volume. Axial non-contrast CT images will be obtained in 2.5 mm thick slices, superiorly from the angle of the mandible through the lung bases, except for the region of the tumor, where slice thickness will be narrowed to 1.25 mm to ensure precision in identifying the tumor. Following the initial scan, a review of the CT images will be performed to confirm the location of the primary tumor to be targeted. Tattoos will be placed in the same number and configuration as those for traditional whole breast treatments.

The radiation oncologist will outline the tumor, utilizing MRI or mammographic imaging to delineate the volume. A margin of 2 - 5 mm will be added superiorly, inferiorly, medially, and laterally to the tumor gross treatment volume (GTV) in order to create the PTV; the border of the PTV will be limited anteriorly to within 5 mm below the skin surface and posteriorly to the anterior chest wall, i.e. the anterior surface of the ribs. No attempt will be made to include the entire length of the incision scar.

The margins to be given around the excision volume will be determined in relation to the maximum diameter of the tumor GTV. If the maximum diameter of the tumor GTV is 4 cm or less, then up to 5 mm margins around it should be used. If the maximum diameter is larger than 4 cm, then 2 – 4 mm margins should be used. It is preferred the PTV does not exceed 30-35% of the

breast volume. Estimation of the PTV will be performed immediately after the simulation.

It is also required that 50% of the non-target breast tissue (ipsilateral breast volume minus the PTV) does not receive more than 50% of the prescribed dose. Additional margin beyond the PTV to the field edges will be added as required to achieve full dose (100%) to the PTV. Blocks or multileaf collimators should be used to define the treatment area.

5.1.2.4 Dose-Volume Histogram and Normal Tissue Constraints

The following structures should be contoured for all patients by the radiation oncologist:

1. Whole breast
2. Tumor CTV
3. Tumor PTV
4. Ipsilateral lung
5. Contralateral lung
6. Heart
7. Nipple

A dose-volume histogram (DVH) with volumes expressed as % total will be submitted and the dosimetry forms will be completed using the DVH data of the following:

1. Ipsilateral lung
2. Contralateral lung
3. Heart
4. Skin
5. PTV
6. Breast minus PTV

5.1.2.5 Treatment Dose

The dose delivered will be 8 Gy in 3 fractions (for a total of 24 Gy), given once daily over 3-5 days. Treatment may be started on any day of the week, but preferably on a Monday or Tuesday in order to allow for completion of RT prior to the weekend.

5.1.2.6 Prescription Point

The minimum isodose line that completely encompasses the PTV on the transverse, sagittal and coronal planes through the center of the PTV. Inhomogeneity corrections are to be used.

5.1.2.7 Time-Dose Considerations

- Dose per fraction: 24 Gy to the isodose line that completely encompasses the PTV
- Deliver one fraction per day to the isodose line that completely encompasses the PTV
- Total elapsed time: 3 to 5 days, unless there is a treatment break or interruption

5.1.2.8 Homogeneity and Reference Points

- The dose within the PTV must be within 100-125% of the prescribed dose
- The minimum isodose line that completely encloses the PTV on the transverse, sagittal and coronal planes through the center of the PTV.

5.1.2.9 Dose Restraints for Uninvolved Ipsilateral Breast, Lungs, Heart and Liver Volumes[57]

5.1.2.9.1 Ipsilateral Lung

- Volume receiving 20 Gy should not exceed 3%
- Volume receiving 20 Gy should not exceed 3%
- Volume receiving 20 Gy should not exceed 3%

5.1.2.9.2 Contralateral Lung

- Volume receiving 20 Gy should not exceed 3%
- Volume receiving 20 Gy should not exceed 3%

- Volume receiving 20 Gy should not exceed 3%

5.1.2.9.3 Heart

- Maximum dose constraint: 90% of Rx dose

5.1.2.9.4 Liver

- Maximum dose constraint: 90% of Rx dose
- 20% of the liver should not receive more than 5 Gy

5.1.2.9.6 Skin

- Maximum dose constraint: 12 Gy to a point dose
- No more than 1 cc of the skin should receive greater than 10 Gy

5.1.2.9.5 Treatment Technique

Treatment must be performed using CT-guided planning with the capability of performing true 3D reconstruction

5.1.2.10 Daily Imaging Guidance and Respiratory Motion Management

Radiation treatment will be delivered with multiple beams utilizing either intensity-modulated or 3D-conformal techniques. One of the following three techniques will be employed for accurate positioning of the patient on the treatment machine, for each treatment received. Each patient will only use one technique per treatment during the course of radiation.

1. Two orthogonal pair kilovoltage images can be acquired with the patient positioned for treatment, and registered to digitally reconstructed radiographs (DRRs) from the planning CT. Shifts will be determined based on registration to biopsy clips.

2. Cone beam (CBCT) imaging is a new technique that utilized X-ray imaging equipment that has recently been installed on the gantry of the radiation-treatment machines, manufactured by Varian Medical Systems, Palo Alto, CA. During CBCT scan, the treatment-machine gantry makes a full rotation while taking ~600 projection images. The projection images are then converted into axial slices, similar in nature to a CT scan. The time taken from the beginning of the CBCT scan to the generation of the axial slices usually takes several minutes. The use of the imaging equipment for the purpose of setting up patients for external-beam radiotherapy has received FDA approval. Further information regarding this approval can be accessed via https://www.accessdata.fda.gov/cdrh_docs/pdf4/K040192.pdf. The software used to control the imaging equipment for the CBCT scan is also FDA approved.

3. Align RT® employs two ceiling-mounted 3D camera units to produce high-resolution and accurate 3D surface data referenced to the treatment isocenter. At each treatment fraction, the system images the current patient position instantaneously. Align RT® is able to gate its image capture so that 3D data are acquired at a reproducible point in the breathing cycle. State-of-the-art surface-matching software registers these data to the reference surface within seconds. Couch shifts are displayed to show discrepancies between existing and ideal treatment positions. New coordinates for the optimal couch position are then displayed and may be applied. This system has been FDA approved. The use of AlignRT for positioning of patient for partial breast irradiation has been published by Bert[58] et al and Gierga[59] et al. Further information regarding this approval is available on the website: http://www.visionrt.com/wp-content/uploads/2016/04/AlignRTPlus-510kClearance-Jan-2013.pdf

Standard of Care Therapy

The decision to proceed to breast-conserving surgery versus neoadjuvant systemic therapy will be at the discretion of the treating physician. Patients who are planned for neoadjuvant systemic

therapy will initiate treatment after study treatment completion. Either after neoadjuvant systemic therapy if planned or after study treatment, all patients will proceed to breast-conserving surgery. After surgery, patients will undergo adjuvant systemic treatment consisting of chemotherapy and/or endocrine therapy, as per physician's choice.

Following breast-conserving surgery, whole breast RT (WBRT) will be delivered as per standard of care. Given the hypofractionated boost approach, a standard fractionation of 200 cGy x 25 fractions (5 days a week for 5 weeks) will be utilized. Patients will begin WBRT approximately 4-6 weeks postoperatively or following chemotherapy.

5.3 Method of Assigning Subjects to Treatment Groups

There is no randomization. Subjects will be assigned to one of two clinically relevant cohorts per their biomarker profile.

5.4 Toxicities and Dosing Delays/Dose Modifications

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. Therefore, early recognition and initiation of treatment is critical to reduce complications. Based on existing clinical study data, most irAEs were reversible and could be managed with interruptions of pembrolizumab, administration of corticosteroids and/or other supportive care. For suspected irAEs, ensure adequate evaluation to confirm etiology or exclude other causes. Additional procedures or tests such as bronchoscopy, endoscopy, skin biopsy may be included as part of the evaluation. Based on the severity of irAEs, withhold or permanently discontinue pembrolizumab and administer corticosteroids. Dose modification and toxicity management guidelines for irAEs associated with pembrolizumab are provided in Tables 2 and 3.

Pembrolizumab dose modifications will not be allowed. Pembrolizumab must be withheld for drug-related toxicities and severe or life-threatening AEs as per Table 2 and 3 below. To avoid delay in standard of care treatment, study treatment should be discontinued if toxicities preclude administration of the second dose pembrolizumab as planned. See Pembrolizumab Program Events of Clinical Interest Guidance Document for supportive care guidelines, including use of corticosteroids to treat immune related AEs.

Any patient who receives treatment on this protocol will be evaluable for toxicity.

Radiotherapy is given in a continuous course. It is not anticipated that a treatment break should be needed, as the development of acute skin-related or other side effects is extremely rare. If a break seems necessary due to the development of CTCAE version 4.0 Grade 4 skin or subcutaneous side effects during treatment, or must be given due to unavoidable medical necessity (i.e., intercurrent illness), the situation should be discussed with the treating radiation oncologist. In the event that a patient must miss a scheduled treatment for non-toxicity related reasons (i.e.: weather, sickness, family emergencies, etc) the treatment visit will be made up at the end.

Table 2: Dose Modification and Toxicity Management Guidelines for Immune-related AEs Associated with Pembrolizumab General instructions:

1. Corticosteroid taper should be initiated upon AE improving to Grade 1 or less 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 Grade 1 or 0 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. • 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.
	Grade 4	Permanently discontinue		

AST / ALT elevation or Increased bilirubin	Grade 2	Withhold	• Administer 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	Permanently discontinue	• 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 causes
	Grade 3 or 4	Permanently discontinue		

All other immune-related AEs	Intolerable/ persistent Grade 2	Withhold	• Based on type and severity of AE administer corticosteroids	• Ensure adequate evaluation to confirm etiology and/or exclude other causes
	Grade 3	Withhold or discontinue based on the type of event. Events that require discontinuation include and not limited to: Gullain-Barre Syndrome, encephalitis		
	Grade 4 or recurrent Grade 3	Permanently discontinue		

1. Withhold or permanently discontinue pembrolizumab is at the discretion of the investigator or treating physician.

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).

Table 3 Pembrolizumab Infusion Reaction Dose modification and Treatment Guidelines

NCI CTCAE Grade	Treatment	Premedication at Subsequent Dosing
Grade 1 Mild reaction; infusion interruption not indicated; intervention not indicated	Increase monitoring of vital signs as medically indicated until the participant is deemed medically stable in the opinion of the investigator.	None
Grade 2 Requires therapy or infusion interruption but responds promptly to symptomatic treatment (e.g., antihistamines, NSAIDs, narcotics, IV fluids); prophylactic medications indicated for ≤24 hrs	**Stop Infusion.** 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 participant is deemed medically stable in the opinion of the investigator. If symptoms resolve within 1 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 participant should be premedicated for the next scheduled dose. **Participants who develop Grade 2 toxicity despite adequate premedication should be permanently discontinued from further study drug treatment**	Participant may be premedicated 1.5h (± 30 minutes) prior to infusion with: Diphenhydramine 50 mg po (or equivalent dose of antihistamine). Acetaminophen 500-1000 mg po (or equivalent dose of analgesic).
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: Epinephrine** IV fluids Antihistamines NSAIDs Acetaminophen Narcotics Oxygen Pressors Corticosteroids Increase monitoring of vital signs as medically indicated until the participant is deemed medically stable in the opinion of the investigator. Hospitalization may be indicated. **In cases of anaphylaxis, epinephrine should be used immediately. **Participant is permanently discontinued from further study drug treatment.**	No subsequent dosing
Appropriate resuscitation equipment should be available at the bedside and a physician readily available during the period of drug administration. For further information, please refer to the Common Terminology Criteria for Adverse Events v4.0 (CTCAE) at http://ctep.cancer.gov		

5.4 Concomitant Medications/Treatments

Permitted Concomitant Medications

Investigators may prescribe concomitant medications or treatments (e.g., acetaminophen, diphenhydramine) deemed necessary to provide adequate prophylactic or supportive care except for those medications identified as “excluded” as listed below.

Excluded Concomitant Medications

Subjects must be instructed not to take any medications, including over-the-counter products, without first consulting with the investigator.

The following medications are considered exclusionary during the study. The Principal Investigator must be notified if a subject receives any of these during the study.

- Any investigational anticancer therapy.
- Any concurrent chemotherapy (except as per study), immunotherapy, or biologic therapy.
- Concurrent use of hormones for non-cancer-related conditions (e.g., insulin for diabetes and hormone replacement therapy) is acceptable.
- Immunosuppressive medications including, but not limited to systemic corticosteroids (>10 mg/day prednisone or equivalent), methotrexate, azathioprine, and tumor necrosis factor alpha (TNF-α) blockers. Use of immunosuppressive medications for the management of investigational product- related AEs, in subjects with contrast allergies is acceptable. In addition, use of inhaled and intranasal corticosteroids is permitted.

5.5 Other Modalities or Procedures/ Research vs. Standard of Care Procedures

A RT boost is a standard component of RT planning and usually follows WBRT after breast-conserving surgery. In this study, the boost will be administered preoperatively at a dose of 8 Gy for 3 fractions, to begin within 5 days of the second dose of pembrolizumab. RT will be performed using external beam ionizing radiation in accordance with institutional standard practice.

5.6 Duration of Study Participation

The study duration per subject will be up to 150 days, with up to 14 days of screening, up to 47 days on treatment, and 15 weeks of follow-up after the second pembrolizumab dose.

5.7 Removal of Patients from Protocol

Patients will be removed from the study when any of the criteria listed in Section 6.5 apply. Notify the Principal Investigator, and document the reason for study removal and the date the patient was removed on the Case Report Form. The patient should be followed-up per protocol.

5.8 Subject Replacement

Subjects who withdraw from the study treatment prior to starting or during the study intervention and fail to complete the intervention may need to be replaced at the discretion of the study PI. Subjects who complete the study intervention, but are not evaluable for the primary endpoint of tumor infiltrating lymphocytes will also be replaced at the discretion of the study PI.

6.0 STUDY PROCEDURES

6.1 Screening/Baseline Procedures

Assessments performed exclusively to determine eligibility for this study will be done only after obtaining informed consent. Assessments performed for clinical indications (not exclusively to determine study eligibility) may be used for baseline values even if the studies were done before informed consent was obtained.

Following consent, a research biopsy of the primary tumor will be performed on all patients in the TNBC cohort.

All screening procedures must be performed within 28 calendar days prior to the first dose of pembro (C1D1) unless otherwise stated. The screening procedures include:

6.1.1 Informed Consent

6.1.2 Medical history and record review

Relevant medical history, including history of current disease, other pertinent history, current medication use, and information regarding underlying diseases will be recorded at Screening.

6.1.3 Demographics

6.1.4 Review subject eligibility criteria

6.1.5 Complete physical exam including ECOG PS

6.1.6 Adverse event assessment

Baseline adverse events will be assessed. See Sections 6.2 and 7.0 for Adverse Event monitoring and reporting.

6.1.7 Standard Laboratory Evaluations Including:

Comprehensive metabolic panel (CMP): aspartate aminotransferase (AST), alanine aminotransferase (ALT), albumin, alkaline phosphatase (ALP), total bilirubin, calcium, creatinine, glucose, total protein, BUN, sodium, potassium, chloride, bicarbonate

Complete blood cell count with differential and platelet count (CBC)

Amylase, lipase, lactate dehydrogenase (LDH)

Prothombin time (PT), international normalized ratio (INR), partial thromboplastin time (aPTT)

HIV testing

Hepatitis serologies including hepatitis B surface antigen (HBsAg), hepatitis B core antibody (HBcAb) and hepatitis C antibody

6.1.8 Research-Related Laboratory Evaluation including:

Thyroid stimulating hormone (TSH), free T4, T3, ACTH, FSH/LH, prolactin, testosterone levels. Labs must be drawn, but do not require results to determine eligibility or treatment continuation.

6.1.9 EKG

6.1.10 Pregnancy Testing for WOCBP

Urine or serum (preferred) pregnancy testing within 14 days of first dose of pembrolizumamb for screening (C1D1) and also 72 hours prior to C1D1. If screening pregnancy test was done 72 hours of C1D1, it does not have to be repeated.

Note: In the setting of egg harvesting, the test for pregnancy looks for beta-HCG which is the agent used for triggering the egg harvest. Given the sensitivity of the test, it will remain positive for about two weeks. This will be true for all egg harvesting patients. Therefore, the 72-hour test is not required for patients undergoing egg harvesting because of the high likelihood of false positive results. These patients may have false positive results for at least two weeks and may be exempt from pregnancy testing requirements within 14 days of C1D1, and may proceed per investigator discretion.

6.1.11 Collection for correlative research tests including:

Blood (3 CPT tubes,1 red-top tube, and 1 green-top tube) for immune response studies

Core needle biopsy of primary tumor with 2 cores obtained for immune response studies

Optional fecal sample for microbiome analysis

6.1.12 Cosmesis Evaluation

Cosmesis will be scored by the treating physician using the four-tier Harvard Scale (Excellent, Good, Fair, Poor) to compare the treated breast to the control breast (Appendix C)
Digital photographs (frontal, with arms at side) will be recorded.

6.1.13 Optional breast MRI

6.2 Procedures During Treatment

Prior to Each Treatment Cycle

History and physical exam including vital signs and ECOG PS
Adverse event assessment

Duration (start and stop dates and times), severity/grade, outcome, treatment and relation to study drug and radiation therapy of adverse events will be recorded on the case report form (CRF).

Medication reconciliation
Labs: CBC & CMP

6.2.1 Prior to Cycle 2

Research tests including:

- Blood (3 CPT tubes, 1 red-top tube, and 1 green-top tube) for immune response studies.
- Core needle biopsy of primary tumor with 2 cores obtained for immune response studies.
- Optional breast MRI

- Optional fecal sample for microbiome analysis

6.2.2 Prior to standard of care treatment or at the time of surgery if that is the planned standard of care treatment

Research tests including:

- Blood (3 CPT tubes, 1 red-top tube, and 1 green-top tube) for immune response studies.
- Core needle biopsy of primary tumor with 2 cores obtained for immune response studies or surgical sample with wedge of tumor tissue and benign tissue obtained.
    - If subject receives neoadjuvant systemic therapy before proceeding to surgery as part of SOC treatment, fresh specimen may also be collected at the time of surgery. This will be determined at the discretion of the treating investigator.
- Optional breast MRI
- Optional fecal sample for microbiome analysis
- **Cosmesis Evaluation (#2 if surgery is planned prior to SOC treatment)**

  Cosmesis will be scored by the treating physician using the four-tier Harvard Scale (Excellent, Good, Fair, Poor) to compare the treated breast to the control breast.
- Digital photographs (frontal, with arms at side) will be recorded.

6.3 Follow-up Procedures

Patients will be followed at minimum every 3 weeks after completion of (or early withdrawal from) study treatment until week 19 after study initiation after which they will be followed every 6 months for 1 year post treatment. The assessments are listed below:

6.3.1 History and physical exam including vital signs and ECOG PS

6.3.2 Adverse event assessment:

AE collection will continue until week 19, after which time only immune related AE's listed in table 2 will be collected post treatment for 1-year post treatment as well as through chart reviews at these time points. For study patients who are receiving standard chemotherapy (usually at Weeks 7 -25) at their treating physician's office, AE collection may also be done via phone call and documented in the subject's study record.

6.3.3 Medication reconciliation

6.3.4 Cosmesis Evaluations

(#2 prior to surgery if after SOC treatment, #3 prior to whole breast radiation, approx. 4 weeks post-operative, #4 at 6 months post-op)

Cosmesis will be scored by the treating physician using the four-tier Harvard Scale (Excellent, Good, Fair, Poor) to compare the treated breast to the control breast.

Digital photographs (frontal, with arms at side) will be recorded.

6.3.5 Endocrine Toxicity Assessment

At one year +/- 2 months post-treatment follow-up, a full thyroid function panel (including TSH, free and total T3, and free T4) will be completed. Cortisol results from adrenal glands will also be obtained as part of this assessment. Results will be obtained if feasible or if already available in the medical chart.

6.3.6 Survival Status

Survival status assessment and any documentation of recurrence (if possible) will be conducted every 6 months for up to 5 years following the end of treatment. Study staff will record survival status and recurrence via medical record review, phone call, or at standard clinical visits.

6.4 Time and Events Table

	Screening ≤ 28 days[4]	**Week 1** (C1D1)	**Week 4** (C2D1)	**Week 7** (SOC Tx)	**Post Treatment Follow Up**[12] Q3W until week 19 & Q6 Mo.post Tx for 1 YR	**Long-Term Follow Up** Q6mos for up to 5 years post Tx
Pembrolizumab[1]		X	X			
Radiation Treatment[2]			X			
Initiation of Standard of Care Treatment[3]				X		
Eligibility Criteria	X					
Informed Consent	X					
Pathology Reviewed by Investigator	X					
Medical History	X					
EKG	X					
Height and weight	X					
History of present illness[5]	X	X	X	X	X	
Safety bloodwork[6]	X	X	X	X		
Full thyroid function panel (TSH, free and total T3 and free T4),	X[13]		X	X	X[16]	
Cortisol					X[16]	
ACTH, FSH/LH, prolactin, testosterone	X					
Amylase, lipase, LDH,	X					
PT/INR and aPTT	X					
HIV, Hepatitis B and C[7]	X					
Pregnancy test[8]	X	X				
Research bloods[9]	X		X	X		
Research tumor biopsy[10]	X		X	X	X	
Optional research breast MRI	X		X	X		
Optional fecal samples for microbiome analysis[11]	X		X	X		
Cosmesis Evaluation and Digital Photos[14]	X			X	X[14]	
Survival Status						X

1. Each cycle, 3 weeks (21 days) in duration, corresponds to 1 completed pembrolizumab treatment. Pembrolizumab will begin on C1D1. The second dose will be given on C2D1 (+/- 3 days.
2. RT will consist of 8Gy for 3 fractions. RT will be delivered concurrently with the second dose of pembrolizumab within 5 business days of C2D1
3. Initiation of standard of care treatment consisting of either breast-conserving surgery or neoadjuvant systemic therapy will begin 3 weeks after the second dose of pembrolizumab (+/- 5 days).

4. Procedures must be performed within 28 calendar days prior to C1D1.
5. A history of present illness (HPI) consisting of physical examination, vital signs (including temperature, pulse rate, respiratory rate, blood pressure), performance status assessment, medication reconciliation, and toxicity/adverse event assessment will occur at each visit, prior to study treatment administration if due.AE collection will continue until week 19, immune related AE collection (listed in table 2) will continue until 1 year post treatment
6. Safety bloodwork consisting of CBC, CMP, AST, ALT, albumin, alkaline phosphatase (AP), and total bilirubin will be obtained at the time of screening, on C2D1 (+/- 3 days), at the time of initiation of standard of care treatment (+/- 5 days). Bloodwork from screening to be used for C1D1 dosing if drawn within 14 days of C1D1.
7. Serology for HIV antibodies, HBsAg, HBcAb and hepatitis C antibody (unless previously tested negative).
8. Urine or serum pregnancy test for WOCBP. Serum is preferred to urine test; within 14 days of first dose of pembrolizumab for screening (C1D1) and also 72 hours prior to C1D1. If screening pregnancy test was done within 72 hours of C1D1, it does not have to be repeated. Patients undergoing egg harvesting are exempt from the 72-hour test.
9. Blood draws for research purposes performed after obtaining consent but prior to C1D1, then C2D1 (+/- 3 days), and at the time of initiation of standard of care treatment (+/- 5 days). A minimum of 3 CPT tubes, 1 red top tube, and 1 green top tube of blood will be required at all timepoints. All research blood draws will be performed in conjunction with safety labs in order to minimize the number of venipunctures.
10. Research tumor biopsy will be performed after consent at baseline, within 14 days prior to C1D1 (for the ER+ cohort, archived diagnostic biopsy is allowed if sufficient tissue available, including 20-5 µm unstained slides; for the TNBC cohort, a baseline research biopsy of fresh tumor is required), prior to C2D1 (+/- 5 days), and prior to Week 7(+/- 5 days) at the time of initiation of standard of care treatment. If standard of care treatment is surgery, surgical tissue will be used. If it consists of further neoadjuvant systemic therapy, a research biopsy will be obtained. If FFPE diagnostic tissue alone is accepted, flow cytometry and MerFISH at baseline will not be performed. If subject receives neoadjuvant systemic therapy before proceeding to surgery as part of SOC treatment, then fresh specimen collection may also be done at the time of surgery.
11. All fecal samples for microbiome analysis are optional. Fecal samples will be collected at baseline, within 14 days prior to C1D1, C2D1 (+/- 5 days), and at the time of initiation of standard of care treatment (+/- 5 days).
12. Follow up appointments will occur through week 19 after study initiation, at minimum every 3 weeks (+/- 5 business days) and continue every 6 months for 1-year post treatment. Patients receiving NAC may be seen more frequently (every 2 weeks) depending upon SOC treatment schedule.
13. Thyroid panel results from screening will be used for C1D1 dosing, if drawn within 14 days of C1D1.
14. Cosmesis Evaluation performed at baseline, prior to surgery, approximately 4 weeks postoperatively (prior to whole breast radiation), and 6-months postoperatively
15. Imaging will be performed as an optional sub-study after consent at baseline, within 14 days prior to C1D1, prior to C2D1 (+/- 5 days), and prior to Week 7(+/- 5 days) at the time of initiation of standard of care treatment. Timing to coincide with research biopsies.
16. Cortisol lab results along with full thyroid function panel will be obtained at one year (+/- 2 months) post-treatment follow-up, if feasible or available in medical chart.

6.5 Removal of Subjects from Study

Patients can be taken off the study treatment and/or study at any time at their own request, or they may be withdrawn at the discretion of the investigator for safety, behavioral or administrative reasons. The reason(s) for discontinuation will be documented and may include:

6.5.1 Patient voluntarily withdraws (follow-up permitted);

6.5.2 Patient withdraws consent (termination of treatment and follow-up);

6.5.3 Patient is unable to comply with protocol requirements;

6.5.4 Patient demonstrates disease progression (unless continued treatment with study drug is deemed appropriate at the discretion of the investigator);

6.5.5 Patient experiences toxicity that makes continuation in the protocol unsafe;

6.5.6 Treating physician determines continuation on the study would not be in the patient's best interest;

6.5.7 Patient becomes pregnant (pregnancy to be reported along same timelines as a serious adverse event);

6.5.8 Development of second malignancy (except for basal cell carcinoma or squamous cell carcinoma of the skin) that requires treatment, which would interfere with this study;

6.5.9 Lost to follow-up. If a research subject cannot be located to document toxicities after a period of up to 20 weeks following study enrollment or cannot be located to document survival after a period of 2 years, the subject may be considered "lost to follow-up." All attempts to contact the subject during the two years must be documented. This will be reviewed during an interim data monitoring visit.

6.6 Subject Withdrawal

Participants may discontinue study treatment at any time for any reason or be dropped from the study treatment at the discretion of the investigator should any untoward effect occur. In addition, a participant may be discontinued from study treatment by the investigator or the Sponsor if study treatment is inappropriate, the trial plan is violated, or for administrative and/or other safety reasons.

A participant must be discontinued from study treatment but continue to be monitored in the study for any of the following reasons:

- The participant or participant's legally acceptable representative requests to discontinue study treatment
- Confirmed radiographic disease progression
- Any progression or recurrence of any malignancy, or any occurrence of another malignancy that requires active treatment
- Unacceptable adverse experiences
- The participant has a medical condition or personal circumstance which, in the opinion of the investigator and/or sponsor, placed the participant at unnecessary risk from continued administration of study treatment.
- The participant has a confirmed positive serum pregnancy test (except for patients undergoing egg harvesting. See notes in Section 6.1.10 for further details)
- Noncompliance with study treatment or procedure requirements
- Recurrent Grade 2 pneumonitis
- The participant is lost to follow-up

7.0 ADVERSE EVENTS (AE)

7.1 Definitions

7.1.1 Adverse Event

An adverse event is any untoward medical occurrence in a patient receiving study treatment and which does not necessarily have a causal relationship with this treatment. An AE can therefore be any unfavorable and unintended sign (including an abnormal laboratory finding), symptom, or disease temporally associated with the use of an experimental intervention, whether or not related to the intervention.

7.1.2 Serious Adverse Events (SAE)

A “serious” adverse event is defined in regulatory terminology as any untoward medical occurrence that:

7.1.2.1 Results in death.
If death results from (progression of) the disease, the disease should be reported as event (SAE) itself.

7.1.2.2 Is life-threatening.
(the patient was at risk of death at the time of the event; it does not refer to an event that hypothetically might have caused death if it were more severe).

7.1.2.3 Requires in-patient hospitalization for ≥ 24 hours or prolongation of existing hospitalization.

7.1.2.4 Results in persistent or significant disability or incapacity.

7.1.2.5 Is a congenital anomaly/birth defect

7.1.2.6 Is an important medical event

Any event that does not meet the above criteria, but that in the judgment of the investigator jeopardizes the patient, may be considered for reporting as a serious adverse event. The event may require medical or surgical intervention to prevent one of the outcomes listed in the definition of “Serious Adverse Event “.

For example: allergic bronchospasm requiring intensive treatment in an emergency room or at home; convulsions that may not result in hospitalization; development of drug abuse or drug dependency.

7.1.3 Unanticipated Problem (UP)

Unanticipated problems include any incident, experience, or outcome that meets all of the following criteria:

- Unexpected (in terms of nature, severity, frequency) given (a) the research procedures that are described in the protocol-related documents, such as the IRB-approved research protocol and informed consent document; and (b) the characteristics of the subject population being studied;

- Related or possibly related to participation in the research (possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and

- Suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known to an individual or group of individuals (including research subjects, research staff, or others not directly involved in the research).

7.1.3.1 Evaluable for toxicity

All patients will be evaluable for toxicity from the time of their first treatment with study drug.

7.2 Severity of Adverse Events

All non-hematologic adverse events will be graded according to the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0. The CTCAE current version is available at http://ctep.cancer.gov/reporting/ctc.html.

7.3 Adverse Event Monitoring

The investigator or designee is responsible for ensuring that all adverse events (both serious and non-serious) observed by the clinical team or reported by the subject which occur after the subject has signed the informed consent are fully recorded in the subject's medical records. Source documentation must be available to support all adverse events.

Note: For study patients who are receiving standard chemotherapy (usually at Weeks 7 – 25) at their treating physician's office, AE collection may also be done via phone call and documented in the subject's study record.

A laboratory test abnormality considered clinically relevant (e.g., causing the subject to withdraw from the study, requiring treatment or causing apparent clinical manifestations result in a delay or dose modification of study treatment, or judged relevant by the investigator), should be reported as an adverse event.

The investigator or sub-investigator (treating physician if applicable) will provide the following for all adverse events (both serious and non-serious):

- Event term (as per CTCAE) - Descriptions and grading scales found in the NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting.
- Description of the event
- Date of onset and resolution
- Expectedness of the toxicity
- Grade of toxicity
- Attribution of relatedness to the investigational agent- (this must be assigned by an investigator, sub-investigator, or treating physician)
- Action taken as a result of the event, including but not limited to; no changes, dose interrupted, reduced, discontinued, etc. or action taken with regard to the event, i.e. no action, received conmed or other intervention, etc.

Outcome of event

All patients experiencing an adverse event, regardless of its relationship to study drug, will be monitored until:

- the adverse event resolves or the symptoms or signs that constitute the adverse event return to baseline
- any abnormal laboratory values have returned to baseline
- Week 19 for all AEs
- 1-year post treatment for all immune related AE's listed in table 2
- there is a satisfactory explanation other than the study drug for the changes observed, or death.

7.4 Potential AEs

Radiotherapy:

Toxicities may occur from radiation treatment. The type and risk of toxicity will depend on the presence of normal tissue structures in close proximity to the target. These normal tissue doses will be constrained by the treatment plan to deliver doses no more than those recommended in the Cedars-Sinai Department of Radiation Oncology guidelines.

The estimated risks of each type of toxicity are noted below, and include:

1. Grade 2-3 skin (erythema, dry or moist desquamation, patchy ulceration) - 10%
2. Grade 1-2 non-debilitating fatigue - 10%
3. Any grade brachial plexopathy - 5%
4. Grade 2-4 pneumonitis- 2-5%

Pembrolizumab:

Pembrolizumab is an FDA-approved agent on formulary for the treatment of metastatic melanoma, non-small cell lung cancer, head and neck squamous cell cancer, classical Hodgkin's lymphoma, primary mediastinal large B-cell lymphoma, urothelial carcinoma, microsatellite instability-high cancer, gastric cancer, cervical cancer, hepatocellular cancer, and Merkel cell carcinoma. It is investigational for use in breast cancer. A comprehensive list of toxicities may be found in Lexicomp and in the investigator's brochure. Principal side effects of pembrolizumab are listed in Table 4.

Table 4: Principal side effects of pembrolizumab

Cardiovascular	Peripheral edema
Central Nervous System	Fatigue, headache, chills, insomnia, dizziness, optic neuritis (rare), uveitis (rare)
Endocrine	Hyperglycemia, hyponatremia, hypoalbuminemia, hypertriglyceridemia, hypocalcemia, adrenal insufficiency (rare), hypophysitis (rare)
GI	Nausea, decreased appetite, constipation, diarrhea, vomiting, abdominal pain, pancreatitis (rare), increased serum AST, hepatitis (rare)
Hematologic	Anemia, hemolytic anemia (rare)
Neurological/Muscular	Arthralgias, limb pain, myalgia, back pain, arthritis (rare), Lambert-Eaton syndrome (rare), myositis (rare), partial epilepsy (rare), rhabdomyolysis

	(rare)
Respiratory	Cough, dyspnea, upper respiratory tract infection
Miscellaneous	Fever
Dermatologic	Exfoliative dermatitis (rare)
Renal	Interstitial nephritis (rare), nephritis (rare)

7.5 Supportive Care Guidelines

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. Where appropriate, these guidelines include the use of oral or intravenous treatment with corticosteroids as well as additional anti- inflammatory agents if symptoms do not improve with administration of corticosteroids. 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.

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

Management of Pneumonitis

- For Grade 2 events, treat with systemic corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks.
- For Grade 3-4 events, immediately treat with intravenous steroids. Administer additional anti-inflammatory measures, as needed.
- Add prophylactic antibiotics for opportunistic infections in the case of prolonged steroid administration.

Management of Diarrhea/Colitis

Subjects should be carefully monitored for signs and symptoms of enterocolitis (such as diarrhea, abdominal pain, blood or mucus in stool, with or without fever) and of bowel perforation (such as peritoneal signs and ileus).

- All subjects who experience 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. For Grade 2 or higher diarrhea, consider GI consultation and endoscopy to confirm or rule out colitis.
- For Grade 2 diarrhea/colitis that persists greater than 3 days, administer oral corticosteroids.
- For Grade 3 or 4 diarrhea/colitis that persists > 1 week, treat with intravenous steroids followed by high dose oral steroids.
- When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks.

Management of Type 1 diabetes mellitus (if new onset, including diabetic ketoacidosis [DKA]) or ≥ Grade 3 Hyperglycemia, if associated with ketosis (ketonuria) or metabolic

acidosis (DKA)

- For T1DM or Grade 3-4 Hyperglycemia
- Insulin replacement therapy is recommended for Type I diabetes mellitus and for Grade 3-4 hyperglycemia associated with metabolic acidosis or ketonuria.
- Evaluate patients with serum glucose and a metabolic panel, urine ketones, glycosylated hemoglobin, and C-peptide.

Management of Hypophysitis

- For Grade 2 events, treat with corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks. Replacement of appropriate hormones may be required as the steroid dose is tapered.
- For Grade 3-4 events, treat with an initial dose of IV corticosteroids followed by oral corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks. Replacement of appropriate hormones may be required as the steroid dose is tapered.

Management of Hyperthyroidism or Hypothyroidism

Thyroid disorders can occur at any time during treatment. Monitor patients for changes in thyroid function (at the start of treatment, periodically during treatment, and as indicated based on clinical evaluation) and for clinical signs and symptoms of thyroid disorders.

- Grade 2 hyperthyroidism events (and Grade 3-4 hypothyroidism):
    - In hyperthyroidism, non-selective beta-blockers (e.g. propranolol) are suggested as initial therapy.
    - In hypothyroidism, thyroid hormone replacement therapy, with levothyroxine or liothyronine, is indicated per standard of care.
- Grade 3-4 hyperthyroidism. Treat with an initial dose of IV corticosteroid followed by oral corticosteroids. When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks. Replacement of appropriate hormones may be required as the steroid dose is tapered.

Management of Hepatic toxicities

- For Grade 2 events, monitor liver function tests more frequently until returned to baseline values (consider weekly).
    - Treat with IV or oral corticosteroids
- For Grade 3-4 events, treat with intravenous corticosteroids for 24 to 48 hours.
- When symptoms improve to Grade 1 or less, a steroid taper should be started and continued over no less than 4 weeks.

Management of Renal Failure or Nephritis

- For Grade 2 events, treat with corticosteroids.
- For Grade 3-4 events, treat with systemic corticosteroids.
- When symptoms improve to Grade 1 or less, steroid taper should be started and continued over no less than 4 weeks.

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 shows treatment guidelines for subjects who experience an infusion reaction associated with administration of pembrolizumab.

Table 5: Pembrolizumab Infusion Reaction Treatment Guidelines

NCI CTCAE Grade	Treatment	Premedication at subsequent dosing
Grade 1 Mild reaction; infusion interruption not indicated; intervention not indicated	Increase monitoring of vital signs as medically indicated until the subject is deemed medically stable in the opinion of the investigator.	None
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 (MK- 3475) 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	Stop Infusion. Additional appropriate medical therapy may	No subsequent dosing

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	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.	
Appropriate resuscitation equipment should be available in the room and a physician readily available during the period of drug administration.		

7.6 Reporting Requirements for Adverse Events

7.6.1 Steps to Determine If an Adverse Event Requires Expedited Reporting

Step 1: Identify the type of adverse event using the NCI Common Terminology Criteria for Adverse Events (CTCAE VERSION 4.0).

Step 2: Grade the adverse event using the NCI CTCAE VERSION 4.0

Step 3: Determine whether the adverse event is related to the protocol therapy

Attribution categories are as follows:

- 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.

Note: This includes all events that occur within 90 days of the last dose of protocol treatment (pembrolizumab and RT). Any event that occurs more than 90 days after the last dose of treatment (pembrolizumab and/or RT) and is attributed (possibly, probably, or definitely) to the agent(s) must also be reported accordingly.

Step 4: Determine the prior experience of the adverse event.
Expected events are those that have been previously identified as resulting from administration of the agent. An adverse event is considered unexpected, for expedited reporting purposes only, when either the type of event or the severity of the event is not listed in:

- the current known adverse events listed in the Agent Information Section of this protocol;
- the drug package insert;
- the current Investigator's Brochure

7.6.2 Expedited Reporting

7.6.2.1 The Principal Investigator must be notified by study staff or co-investigators within 24 hours of learning of any serious adverse events, regardless of attribution, occurring during the study or within 30 days of the last administration of the study drug.

Phone Number for Expedited Reporting:
Stephen Shiao, MD, PhD
310-423-2836

Alternate Phone Number for Expedited Reporting:
Reva Basho, MD
832-423-8255

Serious Adverse Event deemed to be related to the protocol and on-study deaths, including death of a research subject unless the death is expected (e.g. due to disease progression) to be reported to the DSMC within 24 hours for medical monitor ad hoc review between meetings to determine if immediate action is required. Reports may be emailed to the DSMC Admin at GroupSOCCICCTODSMCAdmin@cshs.org.

7.6.2.2 Reporting to the Institutional Review Board (IRB)

The IRB must be notified within 10 business days of "any unanticipated problems involving risk to subjects or others."

1. Any serious event (injuries, side effects, deaths or other problems), which in the opinion of the Principal Investigator was unanticipated, involved risk to subjects or others, and was possibly related to the research procedures.
2. Any serious accidental or unintentional change to the IRB-approved protocol that alters the level of risk.
3. Any deviation from the protocol taken without prior IRB review to eliminate apparent immediate hazard to a research subject.
4. Any new information (e.g., publication, safety monitoring report, updated sponsor safety report), interim result or other finding that indicates an unexpected change to the risk/benefit ratio for the research.
5. Any breach in confidentiality that may involve risk to the subject or others.
6. Any complaint of a subject that indicates an unanticipated risk or that cannot be resolved by the Principal Investigator.

7.6.3 Routine Reporting

All other adverse events- such as those that are expected, or are unlikely or definitely not related to the study participation are to be reported annually as part of regular continuing reviews or data submissions.

7.6.4 Reporting to the Food and Drug Administration

The investigator or his designee must submit SAEs on FDA Form 3500A (MedWatch) according to the following reporting criteria:

- Reporting any unexpected fatal or life-threatening suspected adverse reactions no later than 7 calendar days after initial receipt of the information
- Reporting any (1) serious, unexpected suspected adverse reactions (SUSAR), (2) findings from other clinical, animal, or in-vitro studies that suggest significant human risk, and (3) a clinically important increase in the rate of a serious suspected adverse reaction no later than 15 calendar days after determining that the information qualifies for reporting

7.7 Reporting to US Army Medical Research and Material Command Office (USARMC) of Research Protections (ORP) Human Research Protection Office (HRPO)

The protocol will not be initiated until written notification of approval of the research project is issued by the USARMC ORP HRPO.

(1) Substantive modifications to the research protocol and any modifications that could potentially increase risk to subjects must be submitted to the HRPO for approval **prior to implementation**. The USAMRMC ORP HRPO defines a substantive modification as a change in Principal Investigator, change or addition of an institution, elimination or alteration of the consent process, change to the study population that has regulatory implications (e.g. adding children, adding active duty population, etc), significant change in study design (i.e. would prompt additional scientific review) or a change that could potentially increase risks to subjects.

(2) Any changes of the IRB used to review and approve the research will be promptly reported to the USAMRMC ORP HRPO.

(3) All unanticipated problems involving risk to subjects or others must be promptly reported:

by telephone (301-619-2165),
by email (usarmy.detrick.medcom-usamrmc.other.hrpo@mail.mil), or
by facsimile (301-619-7803) to the HRPO.

A complete written report will follow the initial notification. In addition to the methods above, the complete report can be sent to the US Army Medical Research and Materiel Command,

ATTN: MCMR-RP,
810 Schreider Street,
Fort Detrick, Maryland 21702-5000.

(4) Suspensions, clinical holds (voluntary or involuntary), or terminations of this research by the Institutional Review Board (IRB), the institution, the Sponsor, or regulatory agencies will be promptly reported to the USAMRMC ORP HRPO.

(5) A copy of the continuing review approval notification by the IRB of Record must be submitted to the HRPO as soon as possible after receipt. For greater than minimal risk research, a copy of the continuing review report approved by the IRB must also be provided. Please note that the HRPO also conducts random audits at the time of continuing review. Additional information and documentation may be requested at that time.

(6) The final study report, including any acknowledgement documentation and supporting documents, must be submitted to the HRPO when available.

(7) The knowledge of any pending compliance inspection/visit by the FDA, DHHS Office of Human Research Protections (OHRP), or other government agency concerning this research, the issuance of Inspection Reports, FDA Form 483, warning letters or actions taken by any regulatory agencies including legal or medical actions and any instances of serious or continuing noncompliance with the regulations or requirements, must be promptly reported to the HRPO

8.0 CORRELATIVES/SPECIAL STUDIES

8.1 Assay Methodology

H&E TIL Score: A dedicated breast pathologist will score TILs on fresh frozen paraffin embedded (FFPE) tumor specimens.[56] Guidelines for TIL score measurement will be according to the Salgado criteria, and can be found in Appendix B.[56] TILs score will be rounded to the nearest tenth percentile. TIL scores will then be averaged, and mean/SD fold-changes will be reported for each cohort. Residual cancer burden (RCB) values will also be obtained for post-neoadjuvant pathologic tumor staging to assess pCR at the time of curative-intent surgery.

Multispectral IHC (mIHC): In FFPE tumor samples, we will use mIHC to quantify TIL subsets including regulatory T-cells, effector T-cells, macrophages, and PD-L1+ subsets. Using the immediately adjacent H&E as reference, the study pathologist will identify and circle areas of viable tumor tissue, and within this area, intratumoral versus stromal areas will be gated on the basis of cytokeratin-positivity. Densities of various cell types will be quantified in both intratumoral and stromal compartments, and spatial relationships between effectors and suppressors will be analyzed. Staining will include CD3 (T-cell), CD8 (effector T-cell), FOXP3 (regulatory T-cell), CD163 (macrophage), PD-L1, and Ki-67 (activated T-cell).

Flow Cytometry: Frozen blood and tumor samples will undergo fluorescent immunophenotyping to assess baseline and changes in composition/activation status of lymphocyte subsets including CD8+ regulatory T-cells and FOXP3+ effector T-cells through the course of treatment. Expression of markers of activation and proliferation will also be evaluated.

Circulating Cytokines: Frozen blood will also be evaluated for the prevalence of circulating cytokines.

T-Cell Receptor Sequencing: Frozen blood and FFPE tumor samples will be submitted for DNA extraction and T-cell receptor deep sequencing to assess clonality/diversity of the T-cell population.

Whole Genome/Exome Sequencing: Frozen or FFPE tumor samples will be submitted for DNA extraction and whole genome/exome sequencing to assess the expression level of different genes within the tumor.

MerFISH: In conjunction with Dr. Simon Knott (Biomedical Sciences, Cedars-Sinai), frozen tumor samples will be submitted for copy number and spatial assessment of thousands of different RNA species in single cells using fluorescence in situ hybridization (FISH).[44] The eventual goal of this technology is to build a 3-D model of the tumor microenvironment with data on expression levels of various RNAs in tumor, stroma, and immune infiltrate. Changes in gene expression levels through the course of therapy will allow for the identification of drivers of safety and response.

Single-cell RNA Sequencing: In collaboration with Dr. Simon Knott, correlative endpoints have been designed to establish the immunogenic contribution of RT to checkpoint blockade therapy

and define the molecular signatures that are associated with amplified tumor immunogenicity. Single cell gene expression profiling will be applied to research biopsies performed prior to pembrolizumab (in the TNBC cohort only) and in all cohorts, sequentially following administration of pembrolizumab and pembrolizumab + RT to identify tumors with an immunogenic response. For each biopsy, 10,000 live single cells will be isolated from the mixture using Fluorescence Activated Cell Sorting (FACS), disregarding cell type. These cells will be profiled individually using the 10X genomics single cell RNA-sequencing platform. The result of this processing will be a set of 10,000 gene expression signatures for each biopsy, where each profile informs on the type and molecular state of a single tumor cell.

Optional MR Breast Imaging:
Imaging will be performed as an optional sub-study after consent at baseline, within 14 days prior to C1D1, prior to C2D1 (+/- 5 days), and prior to Week 7(+/- 5 days) at the time of initiation of standard of care treatment. Timing to coincide with research biopsies**.**

If the patient agrees to participate in the study, he/she will undergo the following procedures:

- The patient will be set up in the prone position. A breast MR coil will be placed on the patient body.
- The patient will be asked to breathe freely. Standard breast MR sequences, including T2 weighted sequences (anatomy and analysis), T1 gradient-echo sequences will be performed and used as a comparison.
- Research MR sequence including free-breathing dynamic contrast-enhanced (DCE) MR, diffusion MR, multi-tasking 4D-MR with T1/T2 map and Chemical exchange saturation transfer **(**CEST) will be performed on the patient.
- The patient will be given contrast for the DCE sequence.
- The MR scan will take 1-1.5hrs. A patient arrives at the research imaging core 45min before the scan for consent and IV placement.

Data will be collected by the imaging core and analyzed by the Shiao Lab in conjunction with his collaborators in the Cedars-Sinai Biomedical Imaging Research Institute (BIRI).

Optional Fecal Samples for Microbiome Analysis:
In conjunction with Dr. Stephen Shiao (Radiation Oncology, Cedars-Sinai), optional fecal samples will undergo microbiome analysis. Kits for sample collection will be provided to all participants who opt to participate in this optional part of the trial, upon registration to the trial. Each kit will be provided with a pre-labeled box to be shipped back by the patient for analysis to the laboratory of the CSMC PI who will be conducting this analysis:

Dr. Stephen Shiao
Cedars-Sinai Medical Center
110 N. George Burns Rd #D4094D
Los Angeles, CA 90048

All specimens will contain the subject's name and date of collection. The shipping and receipt of specimens will be tracked in a database and by emails sent to Dr. Shiao and the PI. Alternatively, samples may be delivered to Dr. Shiao's lab via the study coordinator.

8.2 Sample Collection / Specimen Banking

Research bloods to be used for flow cytometry analysis, measurement of circulating cytokines, and TCR sequencing will be collected prior to C1D1, on C2D1, and at the time

of initiation of standard of care treatment. Samples will be frozen and stored for subsequent analysis in the laboratory of Dr. Stephen Shiao.

Research biopsies will be conducted prior to C1D1 (standard of care diagnostic biopsy can be used if sufficient archived tissue available for the ER+ cohort), on C2D1, and at the time of initiation of standard of care treatment. If subsequent standard of care treatment is breast-conserving surgery, excision of tumor tissue+/- surrounding normal tissue can be collected in lieu of biopsy. At each time point, at least 2 cores (1cm each) will be collected. One will be frozen for flow cytometry analysis and MerFISH and the other will be paraffin embedded for TIL score, mIHC, and TCR and exome sequencing. For the ER+ cohort, if archived tissue is used in lieu of the first research biopsy, flow cytometry and MerFISH will not be conducted on this sample as fresh or frozen tissue is required for these studies.

If subjects receive neoadjuvant systemic therapy prior to proceeding to surgery as part of SOC treatment, fresh specimens may be collected at the time of surgery.

9.0 STATISTICAL CONSIDERATIONS

9.1 Sample Size

Phase Ib:
It is imperative that any experimental pre-operative therapeutic innovation not undermine the timing of curative-intent treatment in localized breast cancer, the primary endpoint for the Phase Ib study is safety/tolerability, as defined by the number of patients who do not necessitate a delay in standard of care treatment (breast surgery or neoadjuvant systemic therapy followed by breast surgery). A small number of patients of TNBC will be included in this cohort to establish safety of the regimen as described below for consideration for further study. Along with safety, assessment of TILs through the course of treatment in this cohort will establish the immunogenicity of this combination. If at least 80% of the first 10 subjects in the TNBC cohort proceed with standard of care therapy without delay, the combination will be deemed safe in the cohort, and enrollment in the HR+ cohort (10 subjects) will occur at that time.

Phase II:
Once the protocol has been determined to be safe and the protocol modified accordingly, we will employ Simon's two stage trial design[56]. For this cohort, the null hypothesis that the true response rate is 10% will be tested against a one-sided alternative. In the first stage, 22 patients will be accrued. If there are 2 or fewer responses in these 22 patients, the study will be stopped. Otherwise, 18 additional patients will be accrued for a total of 40 patients. The null hypothesis will be rejected if 8 or more responses are observed in 40 patients. This design yields a type I error rate of 0.04 and power of 0.80 when the true response rate is 25%.

9.2 Data Sets Analyzed

Data Analyses/Study Endpoints

9.2.1 Efficacy Analysis

The primary analysis will be based on an intention-to-treat approach and will include all subjects who receive a dose of study drug.

Phase Ib:

The primary efficacy endpoint will be feasibility as defined by the number of patients who do not necessitate a delay in standard of care treatment. Let P_d be the true probability that a patient experiences a delay. The trial will stop if there is statistical evidence that P_d exceeds 20%. We will use a Bayesian sequential design by checking whether P_d exceeds this threshold value after 3, 6, 9, … patients are evaluable for feasibility. The decision rule is to stop the trial if the posterior probability that P_d exceeds 0.20 is 0.95 or more; $P(P_d > 0.2 \mid \text{data}) > 0.95$. A non-informative prior distribution for P_d will be used. Table 9.2.1 gives the stopping rules for the design at each look and column 2 gives the maximum number of patients with delay in order for the trial to proceed. Stopping rules will also apply for any patients with clinical progression of disease during study treatment, although this is expected to be uncommon. For example, if 4 or more delays in treatment are observed after enrolling 6 patients, the trial stops. The third column gives the probability of stopping the trial when in fact, the true $P_d = 0.20$. This is the equivalent of the Bayesian type I error probability. The target type I error probability was set at 0.05.

Table 9.2.1 Stopping rules based on four interim looks

Number of Patients	Number to Continue	Probability to Stop	Cumulative Probability to Stop
3	2	0.008	0.008
6	3	0.013	0.021
9	4	0.010	0.031
10	4	0.011	0.042

Number to continue is the maximum number of delays in standard of care treatment for not stopping the trial.

Table 9.2.2 gives the design operating characteristics under selected values of the true probability P_d for the HR+ cohort. It gives the probability of stopping the trial under the alternative hypothesis, the expected sample size, and the average sample size given that the trial stopped. For example, if the true value of P_d is 0.5, then there is an 64% chance that the trial is stopped early and the average sample size is about 8. On the other hand, there is a small chance of stopping the trial if P_d is small; 0.3% chance of stopping the trial when in fact $P_d = 0.1$. Tables 9.2.3 and 9.2.4 list the same operating characteristics for the TNBC cohort.

Table 9.2.2 Design operating characteristics under different scenarios for the true probability of P_d

True Value of P_d	Probability to Stop	Expected N	Expected N given that we Stopped
0.10	0.003	9.99	6.32
0.30	0.17	9.55	7.38
0.50	0.64	8.01	6.90
0.60	0.84	6.92	6.35

Table 9.2.3 Stopping rules based on seven interim looks

Number of Patients	Number to Continue	Probability to Stop	Cumulative Probability to Stop
3	2	0.008	0.008
6	3	0.013	0.021
9	4	0.010	0.031
12	5	0.007	0.038
15	6	0.005	0.044
18	7	0.004	0.047
20	8	0.009	0.048

Table 9.2.4 Design operating characteristics under different scenarios for the true probability of P_d for TNBC cohort

True Value of P_d	Probability to Stop	Expected N	Expected N given that we Stopped
0.10	0.003	19.96	6.48
0.30	0.233	17.76	10.38
0.50	0.834	11.01	9.28
0.60	0.967	8.11	7.70

The co-primary efficacy endpoint with be change in TILs. TILs will be assessed in paraffin embedded samples after the study is completed and will have no bearing on impact on continued enrollment for the trial. For each cohort, the mean percent change in TIL score before treatment and after pembrolizumab alone μ_1 will be compared to the mean change in TIL score before treatment and after pembrolizumab plus radiation μ_2 using the paired t-test or Wilcoxon sign test depending on the distribution of the data. A significance of 0.05 will be used to declare statistical significance. Let SD(diff) be the standard deviation of the difference in percent change in TIL score before treatment and after pembrolizumab and before and after treatment with pembrolizumab and radiation. Data from 8 evaluable patients in the HR+ cohort achieve 80% power to detect an effect size of 1.16 using a two-sided paired t-test at the 0.05 level of significance. The effect size is defined as the difference between the two means divided by SD(diff), $(\mu_1 - \mu_2) / SD(diff)$. This is equivalent to an absolute difference of 20% between the mean change in TIL scores assuming an SD(diff) of 17%. For the TNBC cohort, assuming 16 patients are evaluable, the effect size that is detectable with the same power and type I error is 0.75.

Comparisons of clinical characteristics among cohorts will be conducted using descriptive summary statistics using median and range. Changes in TIL score will be compared between cohorts using Fishers exact test.

Phase II:

The efficacy endpoint with be pathologic complete response rate (pCR) as determined at the time of surgery assuming that the trial continues through both stages. For this cohort, the pCR rate at the time of surgery which is after RT, pembrolizumab and, in most cases, chemotherapy will be determined. A significance of 0.05 will be used to declare statistical significance. Data from 50 evaluable patients achieve 80% power to detect an effect size

of 1.20 using a two-sided paired t-test at the 0.05 level of significance compared to the historical pCR rate for triple negative breast cancer90. The effect size is defined as the difference between the mean pCR rate for this trial compared to historical data for triple-negative breast cancer. This is equivalent to an absolute difference of 20% between the mean pCR rate between the two groups assuming a standard deviation (SD) of 15%.

Comparisons of clinical characteristics for this cohort will be conducted using descriptive summary statistics using median and range. Changes in TIL score, immune populations, gene expression, microbiome populations and cytokine expression will be compared between different time points and responder/non-responder groups if sufficient numbers for analysis are available using the paired t-test or Wilcoxon sign test depending on the distribution of the data.

9.2.2 Safety Analysis

All subjects entered into the study on C1D1 will be included in the safety analysis. The frequencies of AEs that occur through week 15 after the second dose of pembrolizumab by type, body system, severity and relationship to study drug will be summarized. SAEs (if any) will be described in detail. Potential association with study treatment will be assessed.

9.2.3 Invasive Disease-Free Survival (IDFS)

Secondary objective of IDFS will be evaluated by the Kaplan-Meier method. IDFS is defined as the time from completion of surgery to the first occurrence of the following events: invasive ipsilateral, local, regional, or distant recurrence, or death due to breast cancer[60].

10.0 STUDY MANAGEMENT

10.1 Conflict of Interest

Any reportable conflict of interest by study investigators will be disclosed to the local IRB and will be outlined in the Informed Consent Form. Conflict of interest will be defined by a situation in which a study investigator is involved in an interest, financial or otherwise, which could affect the decision-making of that individual as it relates to this study.

10.2 Institutional Review Board (IRB) Approval and Consent

It is expected that the IRB will have the proper representation and function in accordance with federally mandated regulations. The IRB should approve the consent form and protocol.

In obtaining and documenting informed consent, the investigator should comply with the applicable regulatory requirement(s), and should adhere to Good Clinical Practice (GCP) and to ethical principles that have their origin in the Declaration of Helsinki.

Before recruitment and enrollment onto this study, the patient will be given a full explanation of the study and will be given the opportunity to review the consent form. Each consent form must include all the relevant elements currently required by the FDA Regulations and local or state regulations. Once this essential information has been provided to the patient and the investigator is assured that the patient understands the implications of participating in the study, the patient will be asked to give consent to participate in the study by signing an IRB-approved consent form.

Prior to a patient's participation in the trial, the written informed consent form should be signed and personally dated by the patient and by the person who conducted the informed consent discussion.

10.3 Registration Procedures

All subjects that sign informed consent will be assigned a subject number sequentially by their date of consent. Those subjects that do not pass the screening phase will be listed as screen failures on the master list of consented subjects. Eligible subjects, as determined by screening procedures and verified by a treating investigator, will be registered on study at Cedars Sinai Medical Center (CSMC) by the Study Coordinator.

Issues that would cause treatment delays after registration should be discussed with the Principal Investigator (PI). If a patient does not receive protocol therapy following registration, the patient's registration on the study may be canceled. The Study Coordinator should be notified of cancellations as soon as possible.

Assignment of Subject ID: The study team will track all subjects who sign consent on a subject screening/enrollment log using a unique screening ID (S001, S002, etc.). Subjects found to be ineligible will be recorded as screen failures. Subjects found to be eligible will be registered using only the three-digit numeric ID assigned at screening that follows the standard SOCCI format (001, 002, etc.).

A) Eligibility Verification

Prior to registration, all subjects must undergo eligibility verification by the SOCCI Cancer Clinical Trial Office (CCTO). The following documents will be completed and provided for review:

- Registration form (or equivalent)
- Copy of applicable source documents
- Eligibility checklist (signed by investigator)
- Signed patient consent form and Subject's Bill of Rights
- HIPAA authorization form

B) Registration

After eligibility is verified, registration is completed as follows:

- Assign a patient study number
- Entry of the patient in OnCore by the Study Coordinator
- Notify the investigational pharmacy and treating physicians that a subject has gone on study and anticipated treatment start date

Oversight by the principal investigator is required throughout the entire registration process.

10.4 Data Management and Quality Control and Reporting

The data will be entered into a HIPAA-compliant database. The Study Staff will be responsible for data processing, in accordance with procedural documentation. Database lock will occur once quality assurance procedures have been completed.

All procedures for the handling and analysis of data will be conducted using good computing practices meeting FDA guidelines for the handling and analysis of data for clinical trials.

10.5 Data and Safety Monitoring

10.5.1 Data Monitoring and Quality Assurance

High Risk Monitoring

Adherence to the protocol, Good Clinical Practices (GCP) and institutional policy will be monitored by the PI during the course of the study through routine Disease Research Group (DRG) meetings (or equivalent). In addition, the SOCCI Cancer Clinical Trials Office (CCTO) Quality Management Core (QMC) will conduct internal monitoring visits and audits for data quality and protocol adherence. QMC reports will then be forwarded to the SOCCI Data and Safety Monitoring (DSMC).
QMC will also conduct the following:

1. Central eligibility verification for all subjects enrolled as described in protocol Section 4.
2. Central review of all eligibility waivers by a SOCCI Medical reviewer to assess appropriateness and risk to ensure quality data and ensure subject safety protections for investigator-initiated research

10.5.2 Safety Monitoring

High Risk Monitoring

Oversight of the progress and safety of the study will be provided by the PI. The PI will maintain continuous safety monitoring for the duration of the study by reviewing subject/study data. Adverse events and unanticipated problems are not expected, but if they occur they will be documented and reported according to CSMC IRB policies and procedures. If the PI becomes aware of any new safety information that may place subjects at increased risk than what was previously known the IRB will be promptly notified and if warranted, enrollment may be held until the PI determines whether a modification to the study is necessary and/or the informed consent documents are updated accordingly.

In addition, this protocol will utilize the SOCCI Data and Safety Monitoring Committee will provide another layer of data and safety oversight. Committee membership includes experts in the field of oncology, nursing, pharmacy, and biostatistics in reviewing the over data, safety, quality, and study integrity of SOCCI interventional IITs. DSMC membership and responsibilities are governed by the committee charter. The DSMC findings and recommendations will be reported in writing to the Principal Investigator. A summary letter will be forwarded by the Principal Investigator or his/her designee to the Cedars-Sinai Medical Center IRB. .

DSMC Medical Monitor: Ronald L. Paquette, MD
The DSMC Medical Monitor appointed to this study is independent of the research team listed in this protocol. The DSMC Medical Monitor also assumes the specific duties and responsibilities of the Department of Defense (DoD) Research monitor as listed below.

DSMC Monitors responsibilities include:
• Attend the DSMC meeting at which the submitted protocol is to be reviewed in order to present a brief synopsis of the reported study outcomes.
• Assessment of DSM risk category and the proposed DSMP. Provide recommendation for PRMC's final determination. The Data and Safety Monitoring (DSM) Risk Category Worksheet may be used. The DSMP Reviewer Worksheet may be used.
• Submit a written review summary to the DSMC Coordinator prior to the meeting. The DSMC medical/biostatistician monitor reviewer worksheet may be used. In the rare event

the medical monitor or biostatistician are unable to attend and a substitute appointed DSMC Chair or Vice Chair, the monitor's written review summary may be read during the room. Proxy voting is not allowed.
• Review of data and safety monitoring reports for each study is complete and accurate.

DoD Research Monitor Duties, Authorities, and Responsibilities:

- Observe recruitment and enrollment procedures and the consent process for individuals, groups or units
- Oversee study interventions and interactions
- Review monitoring plans and UPIRSO reports
- Oversee data matching , data collection, and analysis
- At a minimum, the research monitor: may discuss the research protocol with the investigators, interview human subjects, and consult with others outside of the study about the research; shall have authority to stop a research protocol in progress, remove individual human subjects from a research protocol, and take whatever steps are necessary to protect the safety and well-being of human subjects until the IRB can assess the monitor's report; shall have the responsibility to promptly report their observations and findings to the IRB or other designated official and the HRPO.

10.6 Record Retention

Study documentation includes all Case Report Forms, data correction forms or queries, source documents, monitoring/auditing logs/letters, and regulatory documents (e.g., protocol and amendments, IRB correspondence and approval, signed patient consent forms).

Source documents include all recordings of observations or notations of clinical activities and all reports and records necessary for the evaluation and reconstruction of the clinical research study. Government agency regulations and directives require that the study investigator must retain all study documentation pertaining to the conduct of a clinical trial. Study documents should be kept on file per local guidelines.

10.7 Adherence to Protocol

Except for an emergency situation in which proper care for the protection, safety, and well-being of the study patient requires alternative treatment, or a protocol exception request approved by the SOCCI Medical Director and CSMC IRB, the study shall be conducted exactly as described in the approved protocol.

10.7.1 Emergency Modifications

Investigators may implement a deviation from, or a change of, the protocol to eliminate an immediate hazard(s) to trial subjects without prior IRB approval. For any such emergency modification implemented, the IRB must be notified as soon as possible, but no more than 10 days from the investigator's awareness of the event.

10.7.2 Protocol Exceptions and Eligibility Waivers

An exception is an anticipated or planned deviation from the IRB-approved research protocol, as described in the IRB Policy*, Reporting Possible Unanticipated Problems Involving Risks to Subject or Others (UPIRSO) Policy: Institutional Review Board/Research Compliance and Quality Improvement.*

A protocol exception most often involves a single subject and is not a permanent revision to the research protocol. Protocol exceptions that extend beyond a single subject should result in a protocol amendment to avoid serial violations.

All exception requests must be reviewed by the SOCCI CCTO Medical Director and the Institutional Review Board prior to implementation. The PI or her/his designee is responsible for submitting a protocol exception and its supporting documents to the SOCCI Medical Director for review. Planned exceptions to the protocol that are more than logistical and/or have the potential to affect the subject's safety and/or study integrity may not be implemented without prior approval from the SOCCI Medical Director and IRB.

Special considerations for Eligibility Waivers (EW)
In general, subjects who do not meet the eligibility requirements should not be enrolled. In the rare event that it is appropriate for subject inclusion, the rationale/justification and subject case history should be forwarded to the SOCCI CCTO Medical Director for assessment **prior** to submission to the IRB for approval.

The CCTO Medical Director will review the case and contact the PI if additional information is needed or further discussion is warranted. The CCTO Medical Director will provide a written assessment/recommended course of action. The CCTO Medical Director's assessment must be uploaded into CS-IRB with the waiver request for IRB review and consideration. The CCTO Medical Director may recommend future protocol changes.

Eligibility Waiver Submission Process
The PI and/or treating physician should provide written request for a waiver which includes case history and justification for prospective deviation from the study design to the SOCCI CCTO Medical Director.

10.7.3 Other Protocol Deviations

Logistical deviations from the protocol (e.g., minor changes to the study schedule for an individual subject) do not require prior IRB approval unless the deviation has the potential to affect the subject's safety. Such planned deviations that do meet this definition and do not affect the subject's safety should be noted in the subject's research record or deviation log as described in the SOCCI CCTO's Standard Operating Procedure 12: Deviation and Noncompliance Reporting.

Unintentional deviations from the protocol that might affect subject safety or study integrity should be reported to the IRB within 10 days from when the investigator becomes aware that such a deviation has occurred, as outlined in the SOCCI CCTO's Standard Operating Procedure 12: *Deviation and Noncompliance Reporting*. In this case, a Protocol Deviation report must be submitted in CS-IRB, per IRB policy, *Reporting Possible Unanticipated Problems Involving Risks to Subject or Others (UPIRSO) Policy: Institutional Review Board/Research Compliance and Quality Improvement*. All submissions should include a description of the plan to avoid similar deviations or exceptions in the future.

10.7.4 Amendments to the Protocol

Should amendments to the protocol be required, the amendments will be originated and documented by the PI. It should also be noted that when an amendment to the protocol substantially alters the study design or the potential risk to the patient, a revised consent form might be required.

The written amendment, and if required the amended consent form, must be sent to the IRB for approval prior to implementation. Repeat exceptions or deviations to the protocol may suggest a protocol amendment is needed.

10.8 Obligations of Investigators

The PI is responsible for the conduct of the clinical trial at the site in accordance with Title 21 of the Code of Federal Regulations and/or the Declaration of Helsinki. The PI is responsible for personally overseeing the treatment of all study patients. The PI must assure that all study site personnel, including co-investigators and other study staff members, adhere to the study protocol and all FDA/GCP/NCI regulations and guidelines regarding clinical trials both during and after study completion.

The PI will be responsible for assuring that all the required data will be collected and entered onto the Case Report Forms and/or into a HIPAA-compliant study database. Periodically, monitoring visits will be conducted and the PI will provide access to his/her original records to permit verification of proper entry of data. At the completion of the study, all case report forms will be reviewed by the PI and will require her final signature to verify the accuracy of the data.

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APPENDIX A: PROTOCOL SUMMARY OF CHANGES

Amendment 1(Changes made in Protocol Version 2 dated 13MAR2018)

1. PI change from Alice Ho, MD to Stephen Shiao, MD, PhD
2. Revisions to study schema for clarification
3. Throughout document: research MRI has been removed
4. Formatting throughout document
5. Addition of Table 3: Dose Modification and Toxicity Management Guidelines for Immune-related AEs Associated with Pembrolizumab
6. Section 6.1.8: Research-Related Laboratory Evaluation
   Clarification that *"Labs must be drawn, but do not require results to determine eligibility or treatment continuation."*
7. Section 6.4: Time and Events Table
   - Clarification of Pathology review
   - Clarification of safety bloodwork and thyroid function panel (to be obtained at screening; additional specimens not required C1D1)
   - Clarification of follow up schedule
8. Section 7.6: Reporting Requirements for Adverse Events
   - Change AE collection from within 30 days of last dose of pembrolizumab to 90 days
   - Updated expedited reporting contact and phone number

- 10. Section 11.0: Removal of reference 55

Amendment 2 (Changes made in Protocol Version 3 dated 09APR2018)

1. List of abbreviations updated to include YR and SUSAR
2. Radiation treatment timing and dose clarified throughout document
3. Section 2.3: added new exploratory objective 2.3.5 to assess changes in breast cosmesis
4. Section 2.4: Safety evaluations clarified to include those related to both pembrolizumab and radiation therapy
5. Section 3.0: updated to clarify cohort number as 2 cohorts only
6. Section 4.1.17: WOCBP definition added to eligibility criteria regarding negative serum pregnancy test
7. Section 5.1.2: revisions to radiation treatment parameters that clarify total treatment dose
8. Section 5.1.2.3 – 5.1.2.9: radiation dosing constraints clarified
9. Section 5.4: Table 2 removed and replaced with table 3 (Table 3 relabeled as table 2).
10. Section 6.1, 6.2 & 6.3: added directions and timing of cosmesis study procedures
11. Section 6.1: clarification of screening procedure timing within 14 days prior to C1D1
12. Section 6.2: clarification of adverse event collection for both pembrolizumab and radiation therapy
13. Section 6.4: Time and Events Table
    - Cosmesis study added to table
    - Clarification of adverse event collection
    - Clarification of pregnancy evaluations
    - Clarification of follow up scheduled, Q6Mo's post treatment
14. Section 7.3: clarification of adverse event collection timepoints
15. Section 7.6.4: addition of IND reporting guidelines
16. Appendix C: Harvard Four Tier Cosmesis Scale added to accompany breast cosmesis study
17. Formatting throughout document

Amendment 3 (Changes made in Protocol Version 4 dated 16AUG2018)

1. Formatting throughout document
2. Section 4.1.6: Clarified eligibility criteria to match study schema and current practices. TNBC patients (defined as ER<10%, PR<10%, HER2-neu 0-1+ by IHC or FISH-negative; or as per MD discretion).

Amendment 4 (changes made in Protocol Version 5 dated 10SEP2018)

1. Formatting changes throughout document
2. Addition of 10 TNBC subjects throughout document:
    a. Study Schema and Study Summary
    b. Section 3.0 Study Design
    c. Section 9.1 Sample Size
3. Radiation Therapy window updated to 5 business days throughout document
4. Section 5.8 Subject Replacement: Clarified subjects who fail to complete the study intervention or are not evaluable for the primary endpoint will be replaced.
5. Section 6.0 Study Procedures: Screening window updated to 28 calendar days
6. Section 9.0 Statistics: Update to statistics to reflect enlarged sample size.

Amendment 5 Changes made in Protocol Version 6 dated 04DEC2018

1. Formatting changes throughout document
2. Addition of optional research breast MRI to the following sections:
    a. Study schema and study summary updated
    b. 1.6 Correlative Studies
    c. Secondary Objective 2.3.6 added MRI to assess disease status
    d. Section 4.2.8: exclusion criteria added: For subjects who agree to the research breast MRI sub-study: Four or more previous gadolinium contrast scans in the past 12 months due to the risk of brain deposits following repeated use of gadolinium-based contrast agents.
    e. Study procedures 6.0 updated with correct MRI timepoints
    f. Section 6.4 Schedule of events updated to include MRI
    g. Optional MR Breast Imaging section added to 8.0 Correlatives/Special Studies

Amendment 6 Changes made in Protocol Version 7 dated 30MAY2019

1. Formatting changes throughout document
2. Study objective 2.2.3 clarified safety/tolerability to feasibility throughout document
3. Addition of new study objective: To assess the pathological complete response (pCR) rates.
    a. Study summary updated
    b. Secondary Objective 2.3.7 added
    c. New study endpoint added to section 2.4
4. Clarification to section 3.0 to state that:
    - *If at least 80% of the **first** 10 subjects in the TNBC cohort proceed with standard of care therapy without delay, the combination will be deemed safe in the cohort, and enrollment in the HR+ cohort, as well as continued enrollment in the TNBC cohort (from 10 to 20 subjects), will occur at that time.*
5. Eligibility updates for inclusion criteria, section 4.1:
    a. Addition of coagulation parameters
    b. Standard of care imaging criterion clarified
6. Eligibility updates for exclusion criteria, Section 4.2:
    a. Addition of evidence of metastatic disease
    b. Addition of exclusion of immunodeficiencies

c. Addition of exclusion of additional known malignancies progressing or receiving active treatment in past 3 years.
d. Revised exclusion of concurrent diseases
e. Revised exclusion of prohibited treatments and/or therapies
f. Exclusion of WOCBP with positive pregnancy test within 72 hours prior to study start.

7. Toxicity information updates from Merck added to section 5.4 Dosing
    a. Inclusion of Table 3 Pembrolizumab Infusion Reaction Dose modification and Treatment Guidelines; provided by Merck
8. Changed Section 10.5 to reflect the addition of the Data Safety and Monitoring Committee and revised risk categories and safety monitoring.

Amendment 7 Changes made in Protocol Version 8 dated 15AUG2019

1. Revised study title
2. Clarification of Principal Investigators
    a. Stephen Shiao, MD, PhD as overall study PI
    b. Reva Basho, MD as PI for ER+ cohort
3. Revisions to Study Schema for clarification of cohorts
4. Study Summary
    a. Number of subjects changed from 30 to 60
5. Background and Rationale
    a. Section 1.3.2 Clinical Data updated with recent observations to proceed with the phase II portion of this trial
6. Study Objectives
    a. Section 2.1 Primary Objectives revised
7. Study Design Section 3.0
    a. Updated description of study design to Phase Ib/II study
    b. Expansion of cohorts to 50 TNBC and 10 HR+ women
    c. Clarified safety assessment
8. Exclusion Criteria 4.2.8 minor correction to incomplete sentence
9. Exclusion Criteria
    a. Removed pregnancy testing within 72 hours prior to study start and moved as part of Study Procedures
10. Time and Events Table
    a. Added footnote regarding pregnancy testing for clarification on timepoints
11. Added Section 7.7 for Reporting to US Army Medical Research and Material Command Office (USARMC) of Research Protections (ORP) Human Research Protection Office (HRPO)
12. Statistical Considerations
    a. Updated Section 9.1 Sample Size with description of Phase 1b and Phase II
    b. Minor revision to Section 9.2.1 Efficacy Analysis for clarification and addition of Phase II
13. Registration Procedures Section 10.3 revised for new CCTO name
14. Safety Monitoring Section 10.5.2
    a. Added details and reference to the DSMC Charter and appointed DSMC Medical Monitor for this study

Amendment 8 Changes made in Protocol Version 9 dated 06NOV2019

1. Formatting changes throughout document
2. Inserted Protocol Signature Page
3. Study Schema: added procurement of fresh surgical tissue collection post neoadjuvant systemic therapy
4. Inclusion Criteria Clarification
    a. 4.1.7: minor revision to include acceptable assessment by clinical exam
    b. 4.1.12: minor revision to clarify that eligibility includes candidacy for breast-conserving therapy versus planned

5. Section 6.4: Time & Events Table
    a. Revised footnote #10 to add option to procure fresh tissue specimen if surgery is after completion of SOC neoadjuvant systemic therapy
6. Section 6.5: minor corrections to subheading typos
7. Section 7.6.2: Expedited Reporting
    a. Added DSMC required language regarding expedited reporting for interventional cancer IITs
8. Section 8.1: Assay Methodology
    a. Correction of Appendix label in reference to the Salgado criteria
    b. Added description RCB value as a supplemental methodology for pCR assessment
9. Section 9.1 Sample Size: revised description to align with Section 3.0.
10. Addition of language to allow for fresh specimen collection at the time of SOC surgery if subject receives neoadjuvant systemic therapy after study treatment. The following sections incorporate this change:
    a. Section 6.2.2: Prior to standard of care treatment or at the time of surgery if that is the planned standard of care treatment
    b. Section 6.4: Time and Events Table; Footnote #10
    c. Section 8.2: Sample Collection / Specimen Banking
11. Sections 10.5.2 and 10.7.3 updated with standard institutional language and required language by the Department of Defense. Removed references to the DSMC Charter as this is an internal document not to be released.

Amendment 9 Changes made in Protocol Version 10 dated 08JAN2020

1. Section 6.3: Follow-up Procedures
    a. Added 6.3.5 description of endocrine toxicity assessment, including collection of full thyroid function panel and cortisol results at one year +/- 2 months post-treatment follow-up.
    b. Section 6.3.2: AE Assessment revised to clarify that AE collection may be completed via phone call for subjects who are receiving standard treatment at their local treating physician's office.
2. Section 6.4: Time and Events Table
    a. Minor revision for additional timepoint for full thyroid function panel at one year post-treatment follow-up
    b. Added cortisol results collection at one year post-treatment follow-up as part of standard of care endocrine toxicity assessment
    c. Added Footnote #16 for description of cortisol lab results along with full thyroid function panel at one year post-treatment follow-up (+/- 2 months).
3. Section 7.0: Adverse Events
    a. Added note to clarify that AE collection may be done via phone call, usually around Weeks 7 – 25, for subjects receiving standard chemotherapy at their treating physician's office.

Amendment 10 Changes made in Protocol Version 10.1 dated 25MAR2020

1. Section 4.1 Inclusion Criteria
    a. Inclusion criteria 4.1.17: Added note to clarify that in the setting of egg harvesting, patients are not required to take a pregnancy test within 72 hours of C1D1 due to the high likelihood of false positive results.

2. Section 6.1 Study Procedures
    a. Study Procedures 6.1.10: Added note to clarify that in the setting of egg harvesting, patients are exempt from taking a pregnancy test within 72 hours of C1D1 due to the high likelihood of false positive results.

3. Section 6.4 Time & Events Table
    a. Footnote #8 clarifies pregnancy testing requirements in the setting of egg harvesting.

Amendment 11 Changes made in Protocol Version 11 dated 22JUL2020

1. Study Summary
    a. For addition of survival status assessment for up to 5 years, study duration changed from “Approx. 1 year” to “Approx. 5 years”

2. Section 4.1 Inclusion Criteria
    a. Inclusion criterion 4.1.17: Added note to incorporate language from Protocol Clarification Memo dated 30JUN2020 regarding pregnancy testing exemption for egg harvesting patients where due to false positive results lasting for at least two weeks (and therefore are exempt from pregnancy testing within 14 days of C1D1), these subjects may enroll only per investigator discretion.

3. Section 6.1 Study Procedures
    a. Study Procedures 6.1.10: Added note to incorporate language from Protocol Clarification Memo dated 30JUN2020 regarding pregnancy testing exemption for egg harvesting patients where due to false positive results lasting for at least two weeks (and therefore are exempt from pregnancy testing within 14 days of C1D1), these subjects may enroll only per investigator discretion.
    b. Section 6.3.6: Added survival status assessment and any evidence of recurrence to be conducted every 6 months for up to 5 years
    c. Research blood tubes corrected to 3 CPT, 1 red-top tube, and 1 green-top tube of blood.

4. Section 6.4 Time and Events Table
    a. Added timepoint for survival status assessment and documentation of recurrence for 5 years
    b. Research blood tubes corrected to 3 CPT, 1 red-top tube, and 1 green-top tube of blood.
5. Section 8.0 Correlatives/Special Studies
    a. Added description of whole genome/exome sequencing from frozen or FFPE tumor samples.

APPENDIX B: SALGADO CRITERIA FOR TIL SCORE ASSESSMENT[56]

Recommendations for assessing tumor-infiltrating lymphocytes (TILs) in breast cancer

1. TILs should be reported for the stromal compartment (=% stromal TILs). The denominator used to determine the % stromal TILs is the area of stromal tissue (i.e. area occupied by mononuclear inflammatory cells over total intratumoral stromal area), not the number of stromal cells (i.e. fraction of total stromal nuclei that represent mononuclear inflammatory cell nuclei).
2. TILs should be evaluated within the borders of the invasive tumor.
3. Exclude TILs outside of the tumor border and around DCIS and normal lobules.
4. Exclude TILs in tumor zones with crush artifacts, necrosis, regressive hyalinization as well as in the previous core biopsy site.
5. All mononuclear cells (including lymphocytes and plasma cells) should be scored, but polymorphonuclear leukocytes are excluded.
6. One section (4–5 μm, magnification ×200–400) per patient is currently considered to be sufficient.
7. Full sections are preferred over biopsies whenever possible. Cores can be used in the pretherapeutic neoadjuvant setting; currently no validated methodology has been developed to score TILs after neoadjuvant treatment.
8. A full assessment of average TILs in the tumor area by the pathologist should be used. Do not focus on hotspots.
9. The working group's consensus is that TILs may provide more biological relevant information when scored as a continuous variable, since this will allow more accurate statistical analyses, which can later be categorized around different thresholds. However, in daily practice, most pathologists will rarely report for example 13.5% and will round up to the nearest 5%–10%, in this example thus 15%. Pathologist should report their scores in as much detail as the pathologist feels comfortable with.
10. TILs should be assessed as a continuous parameter. The percentage of stromal TILs is a semi quantitative parameter for this assessment, for example, 80% stromal TILs means that 80% of the stromal area shows a dense mononuclear infiltrate. For assessment of percentage values, the dissociated growth pattern of lymphocytes needs to be taken into account.

Lymphocytes typically do not form solid cellular aggregates; therefore, the designation ‘100% stromal TILs would still allow some empty tissue space between the individual lymphocytes.

11. No formal recommendation for a clinically relevant TIL threshold(s) can be given at this stage. The consensus was that a valid methodology is currently more important than issues of thresholds for clinical use, which will be determined once a solid methodology is in place. lymphocyte-predominant breast cancer can be used as a descriptive term for tumors that contain more lymphocytes than tumor cells. However, the thresholds vary between 50% and 60% stromal lymphocytes.

APPENDIX C: HARVARD FOUR-TIER COSMESIS SCALE

Score	
Excellent	Treated breast nearly identical to untreated breast
Good	Treated breast slightly different from untreated breast
Fair	Treated breast clearly different from untreated breast but not seriously distorted
Poor	Treated breast seriously distorted