



**COLUMBIA UNIVERSITY
MEDICAL CENTER**

**Herbert Irving Comprehensive Cancer Center
Protocol**

**Effects of MK-3475 (Pembrolizumab) on the Breast
Tumor Microenvironment in Triple Negative Breast
Cancer With and Without Intra-operative RT: a
Window of Opportunity Study**

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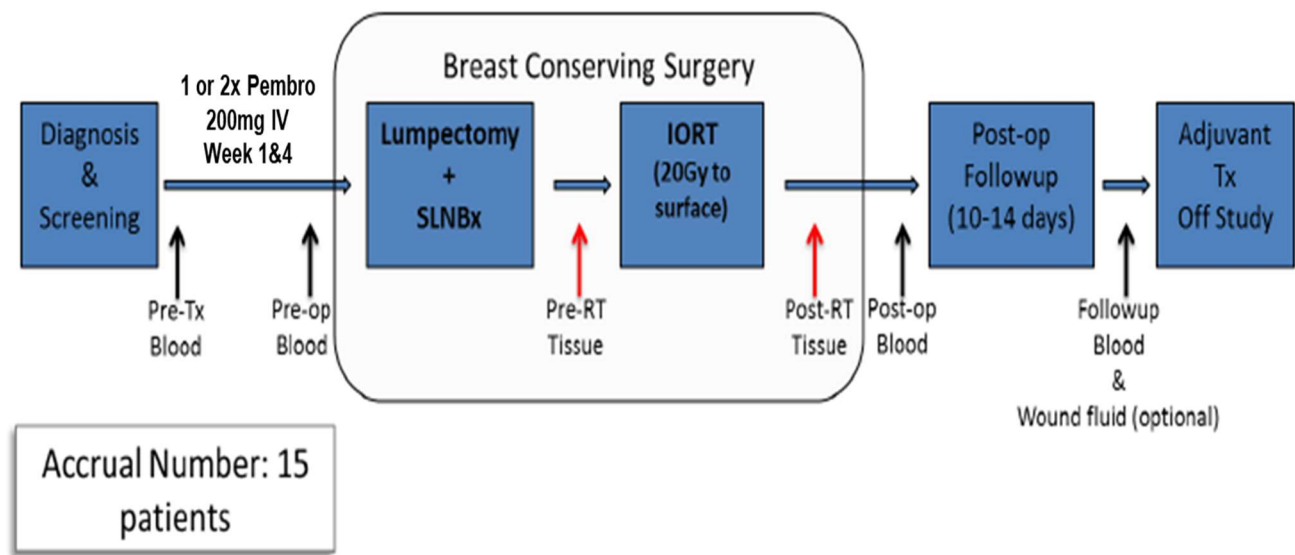
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Protocol Synopsis

Title	Effects of MK-3475 (Pembrolizumab) on the breast tumor microenvironment in triple negative breast cancer with and without Intra-operative RT: a window of opportunity study.
Short Title	Window of Opportunity Pembrolizumab Trial
Phase	Phase I
Methodology	Single arm open label- Preoperative window of opportunity study
Study Duration	Estimated duration for the main protocol : 12 months
Study Center(s)	Multi-Center; 2 additional sub-sites
Objectives	Assess response to MK-3475 in both primary tumor, normal breast stroma, circulating lymphocytes and serum exosomes in treatment naive TNBC patients.
Number of Subjects	15 subjects
Diagnosis and Main Inclusion Criteria	Newly diagnosed triple negative breast cancer, ≤ 3 cm, Node negative
Study Product, Dose, Route, Regimen	Pembrolizumab 200 mg IV q3 weeks x 1-2 doses
Duration of administration	30 minutes
Statistical Methodology	The study is designed to provide an initial assessment of the mean percent change in TILS comparing tumor tissue from initial core biopsy samples to pathology samples from definitive surgery after two cycles of MK-3475. A sample size of 15 subjects will produce a two-sided 95% confidence interval with a margin of error equal to 0.06, given an estimated standard deviation of 0.10.
Duration of Participation	90 days

Protocol Schema:



Aims: Immunologic biomarkers, Cancer genetics, and proteomics

Blood-based test	Method(s)	Tumor-based test	Method
Quantification of T-cell subset	Flow cytometry	PD-L1	IHC/Flow
Myeloid-derived suppressor cells	Flow cytometry	Other co-stimulatory markers	Flow cytometry
Tumor antigen-specific T-cell quantification and function	ICS/Flow/Sequencing	Quantification and localization of TILs	IHC/Flow
Quantification of RNA levels for immune related genes and biomarker genes of interest	Microarray/Nanostring	Sequencing of tumor and TILs	Next-gen sequencing
Serum Exosome proteomic analysis	LC MS/MS analysis	Shave margin tissue	Method
Tumor antigen-specific antibodies	ELISA	PD-L1/PD-L2	IHC/Flow
		T-cell/other immune subsets	IHC/Flow
		Proteomic studies	LC MS/MS analysis
			MALDI-MSI

1 INTRODUCTION

This document is a protocol for a human research study. This study is to be conducted according to US and international standards of Good Clinical Practice (FDA Title 21 part 312 and International Conference on Harmonization guidelines), applicable government regulations and Columbia University Medical Center institutional research policies and procedures.

2 STUDY OBJECTIVES

2.1 Primary Objective

Objective: Assess for change in tumor-infiltrating lymphocytes (TILs) both stromal (sTILs) and intraepithelial (iTILs) in newly diagnosed early stage triple negative breast cancer (TNBC) patients treated with MK-3475 prior to lumpectomy. We will also compare to specimens collected from TNBC patients without MK-3475 exposure (samples from a separate prospective study).

Hypothesis: Immune modulation with PD1 antibody MK-3475 will increase TILs in newly diagnoses TNBC at resection in comparison with the initial core biopsy pre-treatment specimen.

2.2 Secondary Objectives

- (1) **Objective:** Measure patient response with histopathologic features of the tumor microenvironment as assessed by PD-1, PD-L1, and PD-L2 expression, quantification of infiltrating CD8+ T cells at the invasive margin and within the center of the tumor, and assessment of CD2 expression. Initial diagnostic biopsy and lumpectomy resection specimen will be analyzed and compared.

Hypothesis: MK-3475 treatment will lead to an alteration in histopathologic biomarkers such as PD-1, PD-L1, and PD-L2 expression as well as infiltrating T-cells within TNBC tumors.

- (2) **Objective:** Measure patient response using genomic features of the tumor microenvironment as assessed by NanoString with measurement of immune related genes (53 gene panel). Expression levels will be compared pre-treatment and post-treatment.

Hypothesis: Expression of immune pathway genes within a TNBC tumor and the tumor microenvironment will be altered after exposure to MK-3475

- (3) **Objective:** Evaluate the total phosphoproteome profile by LC-MS/MS analysis of adjacent “normal” breast tissue compared to immune markers/gene expression above. We will also compare to specimens collected from TNBC patients without MK-3475 exposure (samples from a separate prospective study).

Hypothesis: We will be able to detect difference in global protein expression and the phosphoproteome in the tumor microenvironment between TNBC patients exposed to

MK-3475 and those unexposed (previously prospectively collected IORT only cohort).

- (4) **Objective:** Determine if significant alteration in immune pathways occur following single fraction IORT in breast microenvironment. Compare phospho-protein and cytokine expression profiles by LC-MS/MS analysis of prospectively collected tissue obtained pre and post RT from the same individual to identify radiation specific changes. Compare to alterations observed in TNBC patients without MK-3475 exposure (samples and data from a separate prospective IORT only study).
- (5) **Hypothesis:** The phospho-expression profiles of the tumor microenvironment following IORT in TNBCs will be altered when patients are treated with MK-3475 compared to untreated (IORT only cohort)

2.3 Exploratory Objectives

- (1) **Objective:** Compare protein profile from tissue specimens to serum exosomes isolated from serial blood specimens collected from the same patient. Determine if response to therapy can be observed and if serum exosomes may be used as a surrogate biomarker for response to MK-3475 and IORT.

Hypothesis: Serum exosome profiles will demonstrate similar patterns of change as observed in tissue specimens.

- (2) **Objective:** Determine if serum exosome profile can be used to monitor drug efficacy as well as potential toxicity by comparing IORT with and without PD1 Ab. These markers can be adapted in clinics to monitor patient response in the future.

Hypothesis: Serum exosome profiles can be used as a surrogate biomarker for response to MK-3475 and IORT.

- (3) **Objective:** Utilize MALDI-MSI imaging for localization of targeted proteins

Hypothesis: MALDI-MSI can be utilized to localize proteomic signatures such as immune specific signatures in heterogeneous tissue specimens following MK-3475 treatment and IORT.

- (4) **Objective:** Explore effects on circulating lymphocytes of preoperative exposure to MK-3475. Serial blood samples will be collected to monitor response over time and changes due to both drug treatment and IORT.

Hypothesis: Circulating lymphocyte profiles will be altered by both MK-3475 and IORT treatment.

3 INVESTIGATIONAL AGENT

3.1 Background

Pembrolizumab is a potent humanized immunoglobulin G4 (IgG4) monoclonal antibody (mAb) with high specificity of binding to the programmed cell death 1 (PD-1) receptor, thus inhibiting its interaction with programmed cell death ligand 1 (PD-L1) and programmed cell death ligand 2 (PD-L2).

3.2 Pharmaceutical and Therapeutic Background

The importance of intact immune surveillance function in controlling outgrowth of neoplastic transformations has been known for decades [Disis, 2010]. Accumulating evidence shows a correlation between tumor-infiltrating lymphocytes in cancer tissue and favorable prognosis in various malignancies. In particular, the presence of CD8⁺ T-cells and the ratio of CD8⁺ effector T-cells/FoxP3⁺ regulatory T-cells (T-regs) correlates with improved prognosis and long-term survival in solid malignancies, such as ovarian, colorectal, and pancreatic cancer; hepatocellular carcinoma; malignant melanoma; and renal cell carcinoma. Tumor-infiltrating lymphocytes can be expanded ex vivo and reinfused, inducing durable objective tumor responses in cancers such as melanoma [Dudley et al., 2005; Hunder et al., 2008].

The PD-1 receptor-ligand interaction is a major pathway hijacked by tumors to suppress immune control. The normal function of PD-1, expressed on the cell surface of activated T-cells under healthy conditions, is to down-modulate unwanted or excessive immune responses, including autoimmune reactions. PD-1 (encoded by the gene *Pdcd1*) is an immunoglobulin (Ig) superfamily member related to cluster of differentiation 28 (CD28) and cytotoxic T-lymphocyte-associated protein 4 (CTLA-4) that has been shown to negatively regulate antigen receptor signaling upon engagement of its ligands (PD-L1 and/or PD-L2) [Greenwald et al., 2005; Okazaki et al., 2001].

The structure of murine PD-1 has been resolved [Zhang et al., 2004]. PD-1 and its family members are type I transmembrane glycoproteins containing an Ig-variable-type (IgV-type) domain responsible for ligand binding and a cytoplasmic tail responsible for the binding of signaling molecules. The cytoplasmic tail of PD-1 contains 2 tyrosine-based signaling motifs, an immunoreceptor tyrosine-based inhibition motif, and an immunoreceptor tyrosine-based switch motif. Following T-cell stimulation, PD-1 recruits the tyrosine phosphatases, SHP-1 and SHP-2, to the immunoreceptor tyrosine-based switch motif within its cytoplasmic tail, leading to the dephosphorylation of effector molecules such as CD3 zeta (CD3 ζ), protein kinase C-theta

(PKC θ), and zeta-chain-associated protein kinase (ZAP70), which are involved in the CD3 T-cell signaling cascade [Okazaki et al., 2001; Chemnitz et al., 2004; Sheppard et al., 2004; and Riley, 2009]. The mechanism by which PD-1 down-modulates T-cell responses is similar to, but distinct from, that of CTLA-4, because both molecules regulate an overlapping set of signaling proteins [Parry et al., 2005; Francisco, 2010]. As a consequence, the PD-1/PD-L1 pathway is an attractive target for therapeutic intervention in triple negative breast cancer.

3.3 Rationale for Dose Selection/Regimen/Modification

This is not a dose finding study as the dose of pembrolizumab has been established and only a single dose of pembrolizumab will be given. Patients will be monitored closely for treatment related adverse events. The use of corticosteroids will be considered for management of immune-related adverse events (irAEs).

3.4 Preclinical Data

3.5 Rationale

3.5.1 Rationale for the Trial and Selected Population

The goal of this project is to determine if immune modulation through treatment with MK-3475 (Pembrolizumab) in triple negative breast cancer (TNBC) patients will alter expression of immune tolerant markers [including PD-L1], within the primary tumor. We hope to determine if MK-3475 exposure will change the molecular signature of breast stroma from “normal” adjacent breast tissue (non-tumor tissue) obtained prospectively at the time of breast conserving surgery (BCS) in TNBC patients. Finally, we will investigate how MK-3475 alters breast stromal response to high dose radiation delivered by intraoperative radiation therapy (IORT). Our primary aim is to determine if there MK-3475 exposure will cause a change in tumor-infiltrating lymphocytes (TILs) both stromal (sTILs) and intraepithelial (iTILs) in newly diagnosed TNBC.

While breast tissue adjacent to tumor tissue might be considered “normal,” recent evidence demonstrates that there is significant cross talk between the tumor, adjacent breast stroma and infiltrating non-tumor cells, which plays an important role in tumor behavior including growth, invasion, response to therapy, local recurrence and metastasis (Finak, Bertos et al. 2008). In breast cancer following breast-conserving surgery, postoperative radiation (RT) is the standard of care to reduce the risk of local recurrence (Vaidya, Wenz et al. ; Veronesi, Orecchia et al.). Clinical experience has shown us that the risk of local recurrence varies depending on breast cancer subtype, from ~ 5% at 10 years for luminal A to >15% for triple negative breast cancer (TNBC) (Voduc, Cheang et al.). One implication of this finding is that the post-lumpectomy bed is different between breast cancer subtypes, and this leads to differences in recurrence risk.

RT itself causes significant alteration in the host tissue stroma, leading to induction of cytokines, chemokines, and growth factors through signaling pathways such as PI3K pathway and TGF- β ,

as well as a host immune response which may be either protective of the tumor cells or targeted against them(Kuonen, Secondini et al.). All of these changes have been shown to be highly dependent on the dose and fractionation schedule of RT delivered(Hellevik and Martinez-Zubiaurre). In RT resistant tumors, the alterations in host stroma induced by RT has been linked to increased tumor recurrence both locally as well as distantly (Kuonen, Secondini et al.). It is suggested that in RT resistant cancers such as TNBCs, changes induced in host stroma by RT might promote migration and invasion abilities of surviving tumor cells as well as a protective immune response(Moncharmont, Levy et al.).

Our understanding of how cancers induce immune tolerance, and our ability to intervene, has improved greatly over the past several years. MK-3475 [Pembrolizumab] is an antibody to the programmed death-1 (PD-1) receptor.(2013; Hamid, Robert et al. 2013) An anti-PD1 agent, MK-3475 interrupts the tolerance signal imparted on regulatory T-cells by cancer cells and TAMs through the interaction of PD-1 on T-cells with its ligand, PD-L1, on cancer cells and TAMs. Adams et al recently confirmed the important prognostic value of TILs in TNBC.(Adams, Gray et al.) Interestingly, the presence of PD-1+ TILs were found to be associated with poor prognosis in TNBC arguing that there may be phenotypically separate populations of TILs.(Muenst, Soysal et al. 2013)

Anti-PD-1 antibody MK-3475 has shown clinical efficacy with durable responses in multiple tumor types(Hamid, Robert et al. 2013); additionally PD-L1 expression has emerged as a potential biomarker of sensitivity to anti-PD-1 antibodies.(Topalian, Hodi et al. 2012) In a recent phase I study of MK-3475 in pretreated PD-L1 positive recurrent/metastatic TNBC patients, 16.1% tumors responded, and 9.7% had stable disease. This study demonstrated MK-3475 to be well tolerated and have significant therapeutic activity in a subset of heavily pretreated TNBC patients. [Nanda et. al. SABCS 2014]. PD-L1 is not expressed in normal breast tissue but shows significant expression in breast cancers and is highly enriched within TNBC cases (20%).(Mittendorf, Philips et al. 2014) The relationship between PD-L1 expression and TILs remains uncertain with one report in breast cancers showing a positive correlation between CD8+ T-cell infiltrates and PD-L1 expression, while inverse correlations observed in NSCLC and oral cancers.(Konishi, Yamazaki et al. 2004; Cho, Yoon et al. 2011; Mittendorf, Philips et al. 2014)

In summary, there is a clearly identifiable and important subset of TNBCs with significant co-expression of immune tolerant markers [including PD-L1], that will likely benefit from MK-3475 treatment, all of which have a still unclear relationship to potentially heterogeneous TILs populations. Additionally MK-3475 may inhibit induction of an immune tolerant phenotype in the host stroma of TNBC patients following local RT. In this application, we propose to test our hypothesis through a prospective window of opportunity study of MK-3475 given preoperatively to 15 women with early stage TNBC undergoing BCS with IORT from whom we will collect breast tissue samples from the lumpectomy cavity both pre- and post-RT as well as compare diagnostic biopsy and lumpectomy resection specimens. Serial blood samples will also be collected to evaluate serum exosomes and circulating lymphocyte populations during the course of treatment.

4 METHODOLOGY

4.1 General Design

This is an open-label, single-arm, single institution pre-operative, Phase I “window of opportunity study” of pembrolizumab in subjects with newly diagnosed triple-negative, ≤ 3 cm, node negative breast cancer. Subjects may not have received any prior neo/adjuvant chemotherapy, definitive surgery or radiation treatment prior to enrollment.

15 patients with histologically confirmed triple negative subtype (negative for ER, PR and HER2) invasive breast carcinoma will be enrolled on this preoperative trial. Patients must have clinically or radiographically tumors ≤ 3 cm and be node negative. Following enrollment, a pretreatment blood sample will be collected. Patients will receive 1 or 2 doses of pembrolizumab 200 mg IV prior to their scheduled surgery. The amount of doses of pembrolizumab will be determined by each investigator. In those subjects receiving one dose of pembrolizumab, a second set of blood samples will be collected at any time between at the Day 22 visit and the scheduled surgery. In those subjects receiving two doses of pembrolizumab, a second set of blood samples will be collected before second dose of pembrolizumab. Breast conserving surgery with sentinel lymph node biopsy and intraoperative radiation will be scheduled 10 days (-2/ + 8 days) following the second dose of pembrolizumab. A preoperative blood sample will be collected. Lumpectomy will be according to practice standards; complete macroscopic excision of the tumor with the widest margin of excision while maintaining a good cosmetic outcome.

Following tumor resection a shave margin measuring up to H=7 mm, Ø=33 mm should be obtained if accessible and immediately sectioned into 3 parts: one for paraffin fixation to assess final margin status, one frozen and one part heat stabilized using the Stabilizer™ system, per manufacturers guidelines. Patients will then undergo planned IORT using the Intrabeam® Photon Radiosurgery System receiving a single fraction of 20Gy prescribed to the lumpectomy cavity surface delivered over 15-40 minutes depending on applicator size. Immediately after completion of IORT, a second shave margin should be obtained and sectioned into 3 parts and processed as above. The surgical wound will then be closed per normal technique and a postoperative blood sample will be collected. Patients will be seen in routine follow-up 10 to 30 days postoperatively, at which time another blood sample will be obtained. Should removal of excess seroma fluid from the wound cavity be required clinically, a sample will also be saved for research purposes. Patients will also be consented to allow analysis of previously obtained core biopsy as well primary tumor resection. Patients are then off study and will continue with appropriate standard adjuvant therapy, including whole breast irradiation and systemic therapy, per standard of care.

The aim of this window of opportunity study is to collect blood, tumor tissue and “normal” breast tissue from patients with newly diagnosed TNBC tumors, following one or two cycles of pembrolizumab and assess the immune response in both primary tumors, normal breast tissue and serum from these patients. Using these prospectively collected biospecimens we propose a series of immunologic profiling assays, which will be performed, as appropriate, to evaluate our exploratory aims listed in section 2.3 above.

4.2 Dose Limiting Toxicities

There will be no dose reductions for the study drug.

5 SUBJECT SELECTION AND WITHDRAWAL

5.1 Number of Patients

15 subjects total will be accrued across all sites

45 will be enrolled to account for screen fails, drop outs and withdrawals

5.2 Participant Inclusion Criteria

A total of 15 adult female subjects newly diagnosed with early stage triple negative breast cancer will be enrolled.

The subject must meet the following criteria in order to be eligible for participation in this trial:

1. Ability to understand and the willingness to sign a written informed consent document
2. Be ≥ 21 years of age on day of signing informed consent.
3. Histologically proven invasive breast carcinoma with triple negative receptor status (Estrogen receptor, Progesterone receptor and HER2 negative by IHC and/or FISH). Patients who are weakly positive for the estrogen or progesterone receptor (i.e. $\leq 10\%$) are eligible.
4. Clinically ≤ 3 cm unifocal lesion by imaging or physical examination.
5. Clinically node negative, no evidence of metastatic disease.
6. No prior systemic anti-cancer therapy including investigational agents, radiation therapy, or breast resection within 6 months of study entry.

7. Breast size B cup or larger, to allow for IORT procedure.
8. Have a performance status of 0 or 1 on the ECOG Performance Scale.
9. Demonstrate adequate organ function as defined in Table 1.

Table 1: Adequate Organ Function Laboratory Values

System	Laboratory Value
Hematological	
Absolute neutrophil count (ANC)	$\geq 1500/\mu\text{L}$
Platelets	$\geq 100\,000/\mu\text{L}$
Hemoglobin	$\geq 9.0\text{ g/dL}$ or $\geq 5.6\text{ mmol/L}$ Criteria must be met without erythropoietin dependency and without packed red blood cell (pRBC) transfusion within last 2 weeks.
Renal	
Creatinine <u>OR</u> Measured or calculated ^a creatinine clearance (GFR can also be used in place of creatinine or CrCl)	$\leq 1.5 \times \text{ULN}$ <u>OR</u> $\geq 30\text{ mL/min}$ for participant with creatinine levels $> 1.5 \times \text{institutional ULN}$
Hepatic	
Total bilirubin	$\leq 1.5 \times \text{ULN}$ OR direct bilirubin $\leq \text{ULN}$ for participants with total bilirubin levels $> 1.5 \times \text{ULN}$
AST (SGOT) and ALT (SGPT)	$\leq 2.5 \times \text{ULN}$
Coagulation	
International normalized ratio (INR) OR prothrombin time (PT)	$\leq 1.5 \times \text{ULN}$ unless participant is receiving anticoagulant therapy as long as PT or aPTT is within therapeutic range of intended use of anticoagulants
Activated partial thromboplastin time (aPTT)	$\leq 1.5 \times \text{ULN}$ unless participant is receiving anticoagulant therapy as long as PT or aPTT is within therapeutic range of intended use of anticoagulants
^a Creatinine clearance (CrCl) should be calculated per institutional standard.	

10. Female subject of childbearing potential should have a negative urine or serum pregnancy within 72 hours prior to receiving the first dose of study medication. If the urine test is positive or cannot be confirmed as negative, a serum pregnancy test will be required.

Note: in the event that 72 hours have elapsed between the screening pregnancy test and the first dose of study treatment, another pregnancy test (urine or serum) must be performed and must be negative in order for subject to start receiving study medication.

11. Female subjects of childbearing potential must be willing to use an adequate method of contraception for the course of the study through 120 days after the last dose of study medication.

Note: Abstinence is acceptable if this is the usual lifestyle and preferred contraception for the subject.

5.3 Exclusion Criteria

The subject must be excluded from participating in the trial if the subject:

1. Multifocal disease within the breast.
2. Has primary lesion >3cm in size radiographically or by physical examination. Pathologically proven nodal disease at diagnosis is not allowed.
3. Has metastatic disease.
4. Has a known additional malignancy that is progressing or requires active treatment in the last 5 years. Exceptions include basal cell carcinoma of the skin, squamous cell carcinoma of the skin, or in situ ductal carcinoma or cervical cancer that have undergone potentially curative therapy.
5. Patients for whom radiotherapy for breast cancer is contraindicated because of medical reasons (e.g., connective tissue disorder or prior ipsilateral breast radiation).
6. Has a diagnosis of immunodeficiency or is receiving chronic systemic steroid therapy (in dosing exceeding 10mg daily of prednisone equivalent) or any other form of immunosuppressive therapy within 7 days prior to the first dose of study drug.
7. Has a known history of active TB (Bacillus Tuberculosis)
8. Has severe hypersensitivity ($\geq$ Grade 3) to pembrolizumab and/or any of its excipients.
9. Has 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 and is allowed.
10. Has a history of (non-infectious) pneumonitis/interstitial lung disease that required steroids or has current pneumonitis/interstitial lung disease
11. Has an active infection requiring systemic therapy.

12. Has a history or current evidence of any condition, therapy, or laboratory abnormality that might confound the results of the study, interfere with the subject's participation for the full duration of the study, or is not in the best interest of the subject to participate, in the opinion of the treating investigator.
13. Has known psychiatric or substance abuse disorders that would interfere with cooperation with the requirements of the trial.
14. Is pregnant or breastfeeding, or expected to conceive children within the projected duration of the study, starting with the screening visit through 120 days after the last dose of trial treatment.

The effects of MK-3745 on the developing human fetus are unknown. For this reason and because monoclonal antibody neoplastic agents are known to be teratogenic, women of child-bearing potential must agree to use adequate contraception (hormonal or barrier method of birth control; abstinence) prior to study entry and for the duration of study participation. Should a woman become pregnant or suspect she is pregnant while she is participating in this study, she should inform her treating physician immediately.

15. Has received prior therapy 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).
16. Has known history Hepatitis B (defined as HBsAg reactive) or known active Hepatitis C virus (defined as HCV RNA [qualitative] is detected) infection.

Note: no testing for Hepatitis B and Hepatitis C is required unless mandated by local health authority.

17. Has received a live vaccine or live-attenuated vaccine within 30 days prior to the first dose of study therapy. Administration of killed vaccines is allowed.
18. Has a medical history of allogenic stem cell transplant
19. Has received a solid organ transplant
20. Has a known history of Human Immunodeficiency Virus (HIV) infection.
21. Note: No HIV testing is required unless mandated by local health authority.

5.4 Lifestyle Considerations

5.4.1 Meals and Dietary Restrictions

Participants should maintain a normal diet unless modifications are required to manage an AE such as diarrhea, nausea or vomiting.

5.4.2 Contraception

Pembrolizumab may have adverse effects on a fetus in utero. Refer to Section 5.3.14 for

approved methods of contraception.

For this study, male participants will be considered to be of non-reproductive potential if they have azoospermia (whether due to having had a vasectomy or due to an underlying medical condition).

5.4.3 Pregnancy

If a participant inadvertently becomes pregnant while on treatment with pembrolizumab, the participant will be immediately discontinued from study intervention(s). The site will contact the participant at least monthly and document the participant's status until the pregnancy has been completed or terminated.

5.5 Inclusion of Women and Minorities

Women of all races and ethnic groups are eligible for this trial.

5.6 Subject Recruitment

Subjects will be recruited from Columbia University Medical Center, Emory University and from an additional sub-site that may be added in the future.

At each institution, potential subjects will be discussed at Interdepartmental meetings. Each participating institution may utilize advertisement in accordance to their local policies and procedures.

5.7 Early Withdrawal of Subjects

5.7.1 When and How to Withdraw Subjects

Patients will be removed from study when any of the criteria listed below applies. The reason for study removal and the date the patient was removed will be documented in the Case Report Form.

Subjects may withdraw consent at any time for any reason or be dropped from the trial at the discretion of the investigator should any untoward effect occur. In addition, a subject may be withdrawn by the investigator or the Sponsor if enrollment into the trial is inappropriate, the trial plan is violated, or for administrative and/or other safety reasons.

A subject must be discontinued from the study treatment but continue to be monitored in the

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study for any of the following reasons:

- The subject or legally acceptable representative requests to discontinue study intervention or withdraws consent.
- After prolonged study intervention interruption that prohibits restarting study intervention if agreed upon by the Sponsor
- Any progression or recurrence of any malignancy, or any occurrence of another malignancy that requires active treatment
- Any study intervention-related toxicity specified as a reason for permanent discontinuation as defined in the guidelines for dose modification due to AEs in Section 8.
- 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.
- Unacceptable adverse experiences as described in Section 9
- Intercurrent illness that prevents further administration of treatment
- Investigator's decision to withdraw the subject
- The subject has a confirmed positive serum pregnancy test
- Noncompliance with trial treatment or procedure requirements
- The subject is lost to follow-up
- Administrative reasons

Patients will be withdrawn from the study if one of the following is met:

- Confirmed positive pregnancy test
- The patient fails to comply with the defined treatment plan and follow-up evaluations,
- If the patient withdraws consent for continued participation, he/she will be removed from study.
- Subject requires the use of prohibited concomitant medications
- If at any time the patient develops unacceptable toxicity, he/she will be removed from study.

- If at any time the patient is found to be ineligible for the protocol as designated in the section on Criteria for Patient/Subject Eligibility (i.e., a change in diagnosis), the patient will be removed from study.

5.7.2 Data Collection and Follow-up for Withdrawn Subjects

For any subject that is withdrawn prematurely:

- Data collected will be utilized
- Medical record will be reviewed for survival and adverse events
- Referring and treating physicians will be contacted for information on the subject
- If the subject withdraws without receiving all or any pembrolizumab or IORT, pathology biopsy specimens (pre and post surgery) will be requested.
- If the subject withdraws without receiving all or any pembrolizumab but receives IORT, preoperative and post op shave margins, tissue and blood at the time points listed in the study calendar will be collected.

Even though subjects may be withdrawn prematurely from the study, it is imperative to collect at least survival data on such subjects throughout the protocol defined follow-up period for that subject. Such data is important to the integrity of the final study analysis since early withdrawal could be related to the safety profile of the study drug. It must be a high priority to try to obtain at least survival data on all subjects lost to follow-up and to note what methods should be used before one can state the subject is truly lost to follow-up (e.g. number of phone calls to subject, phone calls to next-of-kin if possible, certified letters, etc.). Subjects withdrawn because of unacceptable adverse events will be followed-until resolution or stabilization of the adverse event.

5.8 Clinical Criteria for Early Trial Termination

Early trial termination will be the result of the criteria specified below:

1. Quality or quantity of data recording is inaccurate or incomplete
2. Poor adherence to protocol and regulatory requirements
3. Incidence or severity of adverse drug reaction in this or other studies indicates a potential health hazard to participants
4. Plans to modify or discontinue the development of the study drug

7 TREATMENT PLAN

7.1 Trial Intervention

Treatment will be administered on an outpatient basis. Reported adverse events and potential risks for Investigational Agent are described in Section 10. Appropriate dose modifications for Investigational Agent are described in Section 8. No investigational or commercial agents or therapies other than those described below and in section 8 may be administered with the intent to treat the patient's malignancy.

The intervention to be used in this trial is outlined below in Table 2.

Table 2 Trial Intervention

Drug	Dose/Potency	Dose Frequency	Route of Administration	Regimen/Treatment Period	Use
Pembrolizumab	200 mg	Q3W	IV infusion	Day 1 of each 3 week cycle; 1-2 cycles	Experimental

Trial intervention(s) should begin on the day of randomization or as close as possible to the date on which intervention is allocated/assigned.

STATISTICAL CONSIDERATIONS

Study Design/Endpoints

The primary objective of this window of opportunity study is to determine if immune modulation therapy with MK-3475 will increase TILs in newly diagnosed TNBC tumors. To accomplish this, the study is designed to provide an initial assessment of the mean percent change in TILs comparing tumor tissue from initial core biopsy samples to pathology samples from definitive surgery after two cycles of MK-3475. A sample size of 15 subjects will produce a two-sided 95% confidence interval with a margin of error equal to 0.06, given an estimated standard deviation of 0.10.

Secondarily we hope to determine if a measurable difference in the total proteome and immune pathway markers occurs within the tumor bed microenvironment in TNBC patients treated with MK-3475 compared to untreated TNBC patients (prospectively accrued historical control). Based on existing proteomic profiling data from in vitro breast cancer cell lines we assume: 1180 proteins /samples per group. The mean/sd of each protein is calculated within each group, and the average of them is taken across all proteins. We expect ~12.5% proteins to be differentially expressed; with at least a 2-fold difference is expected between groups. Using a power calculation based on $p=0.05$, to detect a fold change of 1.5, 2, 4 and 10, the sample size (in each group) needed to ensure 80% power is 28, 11, 4, 3 respectively. Therefore we plan 30 patients in two cohorts (15 patients each: IORT alone from prior prospective study and MK-3475 + IORT) to detect a 2-fold difference with at least an 80% power.

Finally, we will explore whether there are significant changes in the phospho-proteome within individuals following treatment with IORT plus MK-3475.

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