The gut microbiome and gastrointestinal toxicities as determinants of the response to neoadjuvant chemotherapies for advanced breast cancer

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ABSTRACT

Many chemotherapeutic agents compromise the integrity of the mucosal barrier in the gut, allowing translocation of gram-positive bacteria in secondary lymphoid organs. While this has, until recently, been considered an undesirable side-effect, it may also represent one mechanism by which chemotherapy stimulates an effective anti-cancer immune response. It has been proposed that the priming of Th17 T-cell responses against commensal microbes following disturbance of the gut mucosal barrier facilitates the accumulation of Th1 helper T cells at the site of the tumor, and thus contributes to tumor regression. Gut microbes may also contribute to the efficacy of chemotherapeutic agents through metabolism of the chemotherapeutic agent to its active form. Our hypothesis is that gut microbial composition can influence immune response to the tumor, and therefore may account for variations among patients in the response to anti-cancer therapies. In preparation for epidemiologic studies of this hypothesis, we propose a pilot study of as many as 50 women diagnosed with breast cancer and referred for neoadjuvant chemotherapies at the University of Arkansas for Medical Sciences. Participants will provide blood and fecal samples prior to, and immediately following completion of neoadjuvant chemotherapies, and will authorize retrieval of archived breast tumor tissues collected prior to initiation of chemotherapy. Microbial content of fecal samples will be evaluated based upon 454-pyrosequencing of 16s-rRNA. Host immune response will be assessed by measuring relative abundances of immune cells in blood and in tumor tissue. Complete pathologic response will be assessed based upon findings at the time of cancer surgery. The aims of the proposed study include demonstration of the feasibility of study measures, and estimation of key parameters including the occurrence of complete pathologic response in all participants and in specific subgroups, and the magnitude of associations between microbial and clinical measures.

BACKGROUND

The presence of immune cells in breast tumors was noted as early as the 1970's and described using immunohistochemical staining of fixed tissues [1]. In the 1980's, scientists at the National Institutes of Health observed, in patients whose disseminated breast cancer was treated using interleukin-2, that marked infiltration of breast tumors by T lymphocytes occurred following treatment and predicted tumor regression [2].

An effective immune response to precancerous or malignant cells culminates in the destruction of abnormal cells by Natural Killer cells and cytotoxic T-lymphocytes [3]. In contrast, maladaptive immune responses may contribute to cancer development when chronic local inflammation stimulates intrinsic programs of tissue remodeling and angiogenesis [4]. Both chronic inflammation and impaired immune surveillance may facilitate processes of carcinogenesis, invasion and metastasis [5]. Current research suggests that immune factors may play important roles in cancer risk and cancer outcomes, by contributing to or preventing the pathogenesis of solid tumors [3] and by mediating the effects of diverse cancer therapies [6]. Immune markers also represent potentially useful biomarkers of treatment response and/or prognosis [7].

Recent research has highlighted interactions of commensal microbes with the developing mucosal immune system as an important modulator of systemic immunity with implications for

growth, development and health [8]. In two recent studies [9, 10] differences were observed in treatment efficacies of various therapies, including an immunotherapy and two commonly used therapeutic agents, platinum and cyclophosphamide, when they were employed in germ-free mice compared to counterparts with an intact gut microbiome. Based upon this work, investigators have suggested that translocation of gram-positive bacteria following disruption of the intestinal barrier may elicit systemic a Th17-type response, indirectly facilitating accumulation of effector Th1 cells at the site of the tumor and contributing to tumor regression [9-11]. This has been demonstrated only for a few specific chemotherapeutic agents but many chemotherapeutic regimens have gastrointestinal side effects, suggesting that this mechanism of action may be generalizable across drug classes [12]. Thus, while GI toxicities have been considered as entirely undesirable, they may also represent one mechanism by which chemotherapy treats cancer. In addition, gut microbes are also known to play important roles in the metabolism of many chemotherapeutic agents to their active forms, which may also influence internal dose, tolerability, and outcomes of chemotherapeutic regimens [13, 14].

Marked alterations of the gut microbiome have been observed in several studies of patients when the composition, diversity, and metabolic activities were compared in fecal samples collected prior to, and following completion of chemotherapy [15-17]. The changes in gut microbial composition may be influenced by the chemotherapeutic regimen, host factors, or the stability of the gut microbial population at baseline. Changes in composition occurring over this interval may shed some light on which bacteria translocate and evoke immune responses and lasting changes in gut mucosal tolerance.

Neoadjuvant chemotherapy, in the setting of breast cancer, is defined as a chemotherapeutic regimen provided prior to breast surgery. The specific regimen may differ depending on prognostic and predictive factors that include tumor stage and histologic grade; pathologic characteristics of the primary tumor including hormone receptor status, HER2 status, and multi-gene test results; and patient characteristics including age, comorbidities, and menopausal status. Neoadjuvant chemotherapy has generally been indicated for women with locally advanced invasive breast cancer (2-3 positive axillary nodes or involvement of skin or chest wall). metastatic breast cancer, or triple negative breast cancers (ER-/PR- and HER2-).

More recently, neoadjuvant therapies have been applied in a wider range of breast cancer patients since randomized trials have demonstrated that pre- and post-surgical chemotherapy have comparable rates of both recurrence-free and overall survival [18]. The major benefit of neoadjuvant chemotherapy to patients is increased rates of breast conserving surgery [19]. In addition, tumor response to neoadjuvant chemotherapy, measured as achievement of pathologic complete response (pCR), provides a surrogate end-point for long-term survival [20-22]. A recent single-arm trial of a specific neo-adjuvant chemotherapy regimen included 40 breast cancer patients enrolled at UAMS and demonstrated heterogeneous outcomes by both tumor and host characteristics [23].

Among women with breast cancer who undergo neoadjuvant chemotherapy, complete pathologic response is a predictor of long-term outcomes across and within many subgroups variously defined by intrinsic subtypes, stage, grade, and nodal status [24-26]. In a meta-analysis, Gluck and colleagues pointed out an exception; among women with early-stage luminal A breast cancers, prognosis was not predicted by pathological complete response [27].

A recent meta-analysis gathered results from studies of immune infiltrates in breast tumors from patients who subsequently underwent neoadjuvant cancer therapies, and its findings confirm that

immune infiltrates in pre-treatment breast tissue, and CD8+ infiltrates in particular, are predictive of pathologic complete response in many, but not all of the distinct breast cancer subtypes, including luminal A breast cancers ; *her*2+ breast cancers [28]; and basal breast cancers [24]. CD8+ immune infiltrates have been also observed to predict recurrence-free survival, breast cancer specific survival, and overall survival in breast cancer patients treated surgically prior to hormonal or chemotherapies [24]. Similar associations have been observed in studies looking at early stage breast cancers [25] and advanced breast cancers ; in patients receiving different anti-cancer therapies, including first-line chemotherapies [26], rescue chemotherapies, immune therapies and endocrine therapies; and in studies with different endpoints, including recurrence-free survival, overall survival and breast cancer specific-mortality.

We hypothesize that some of the heterogeneity in treatment outcomes may be explained by variations in systemic immune response and its determinants, including the gut microbiome. Therefore we are proposing a pilot study to evaluate feasibility of the proposed measures and to estimate parameters that will aid in planning a larger study designed to test the hypotheses that the gut microbiome can modulate the efficacy of neoadjuvant chemotherapies to treat breast cancer.

SPECIFIC AIMS

Many chemotherapeutic agents compromise the integrity of the mucosal barrier in the gut, allowing translocation of gram-positive bacteria in secondary lymphoid organs. While this has, until recently, been considered an undesirable side-effect, it may also represent one mechanism by which chemotherapy stimulates an effective anti-cancer immune response. It has been proposed that the priming of Th17 T-cell responses against commensal microbes following disturbance of the gut mucosal barrier facilitates the accumulation of Th1 helper T cells at the site of the tumor, and thus contributes to tumor regression. Gut microbes may also contribute to the efficacy of chemotherapeutic agents through metabolism of the chemotherapeutic agent to its active form. We will test the hypothesis that gut microbial composition can influence immune response to the tumor, resulting in inter-individual differences in the response to anti-cancer therapies. We will also explore how host factors and microenvironmental factors influence the local immune response to the tumor.

We propose the following specific aims:

1. To demonstrate feasibility and acceptability of protocol procedures and measures, including in-home collection of a fecal sample, collection of a blood sample by venipuncture, and collection of breast tissue from newly diagnosed breast cancer patients prior to initiation of neoadjuvant breast cancer therapy.
2. To estimate rates of gastrointestinal side effects and pathological complete response in women with breast cancer treated with neoadjuvant chemotherapies, by breast cancer subtypes, tumor characteristics, cancer treatment, and by categorical measures of gut microbial composition, diversity, and metabolic activity.
3. To estimate the associations of pre-treatment gut microbial composition, diversity, and metabolic activity with anticancer immune responses, measured as relative abundance of immune infiltrates in pre-treatment FFPE breast tumor tissues, and as relative abundance of circulating lymphocytic subtypes (Th1, Th2, Th17, and T_{reg}) in blood samples collected prior to initiation and following completion of neoadjuvant

chemotherapy.

4. To estimate associations of local and systemic immune responses with host factors such as age, BMI, and smoking status, and with measures of the breast microenvironment including mammographic density and benign parenchymal enhancement on breast MRI.

Study Population

We anticipate identification of 60 eligible women among patients diagnosed with breast cancer at UAMS and recommended for neoadjuvant therapy over the course of a year and estimate that, of these, 40 will consent to participate and complete study assessments.

Potentially eligible participants will be identified by their treating oncologist in the Medical Oncology Clinic. The study will be presented to potential participants prior to initiation of chemotherapy. We will allow the patient as much time as she needs to think about participation, and discuss her concerns with friends, relatives, and professional staff before making a decision.

Written consent will be requested from patients who meet eligibility criteria. The informed consent process will be conducted by study staff. We will not advertise for study participants. No compensation will be provided to study participants.

Inclusion Criteria

Eligible participants are women aged 18 years or older, who are:

- Diagnosed with invasive breast cancer and prescribed a regimen that includes neo-adjuvant therapy prior to breast surgery.
- Able to provide informed consent.

Exclusion Criteria

- History of any previous malignancy, other than non-melanoma skin cancers
- Inability to tolerate phlebotomy
- Immunosuppressive therapy for any other condition
- Fever or active uncontrolled infection in the last 4 weeks
- Inflammatory bowel disease
- Surgery of the stomach, small or large intestines, appendectomy, gastric bypass or gastric banding in the past 6 months.
- Active autoimmune disease, including, but not limited to, SLE, MS, ankylosing spondylitis

Study Design

We propose a small longitudinal study to demonstrate the feasibility of measuring the gut microbiome and immune infiltrates in pre-treatment tumor tissue from newly diagnosed breast cancer patients who will subsequently undergo neoadjuvant therapies. This study will provide preliminary data in support of a proposal for an outcomes study of the roles of the gut microbiome in response to cancer therapies.

This protocol involves the collection of three types of biospecimens from each participant. These collections would occur as follows: fecal samples will be collected prior to, and following completion of neoadjuvant chemotherapy by the participant, in her home, using a standard collection kit; venipuncture-collected blood samples will be collected prior to, and following completion of neoadjuvant chemotherapy. In addition, we will retrieve archival breast tissue samples, if available, from a clinically-indicated pre-treatment biopsy. Participants will be asked to complete a baseline questionnaire which includes items querying demographic and lifestyle factors and medical history which may be related to breast health and disease prognosis. Participants will be asked to complete FACT-G7 (Quality of life) questionnaire and a questionnaire on gastrointestinal side effects while at routine chemotherapy-related visits.

All study procedures are considered to be of minimal risk.

Schedule of Study Events

Assessment	**Screening**	**Baseline (prior to chemo)**	**Interim** (during chemo at the time of routine visits)	**Prior to Surgery**	**After Surgery**
Informed consent	X				
Eligibility	X				
Archival tumor tissue from pre-treatment biopsy for IHC		If available			
Data from clinically indicated CBC with diff		If available			
In home collection of fecal sample		X		X	
Study blood sample (<30mL)		X		X	
General questionnaire (demographic information, health history and lifestyle)		X			
Pretreatment mammographic evaluations and breast MRI		If available			
FACT-G7 (Health-related quality of life)		X	Every 2 weeks		
Gastrointestinal symptom checklist			Every 2 weeks		
Assessment of pathologic complete response during routine clinical care					X

Outcome definition

The study outcome would be assessed at the time of surgery. Complete pathologic response will be defined as the absence of any residual invasive cancer on hematoxylin and eosin (H&E) evaluation of the resected breast specimen and all sampled ipsilateral lymph nodes following completion of neoadjuvant systemic therapy (ypT0 ypN0 in the current AJCC staging system).

Microbial DNA assays

Fecal collection: The first fecal sample should be collected prior to initiation of neoadjuvant therapy; a second fecal collection will be collected after chemotherapy and prior to surgery. In each case, the sample will be collected by the participant at home using an OMNIgene GUT sample collection kit. The samples, labeled with a code and no other patient identifiers, can be brought to the clinic or can be mailed by participants to University of Arkansas for Medical Sciences, Cancer Clinical Trials Office, Re: IRB # 204897, 4301 West Markham Street, Slot 724, Little Rock, AR 72205. If mailed, the postage date must be on or before the first day of chemotherapy treatment to be considered eligible to continue. It is vital that the first fecal sample be collected prior to the first dose of chemotherapy. Following receipt, samples will be aliquoted into cryovials and stored in a -80 C freezer in the Cannon lab, until the complete batch can be sent to Dr. Jacques Ravel, c/o Mike Humphrys / Melissa Nandy, at the Insitute for Genome Sciences, University of Maryland School of Medicine, BioPark - 6th floor, 801 W. Baltimore Street, Baltimore, MD 21201. Samples will be labeled with a unique code and no other identifiers prior to sending to the University of Maryland.

DNA extraction and sequencing: DNA will be extracted from fecal samples at the Ravel lab. Microbial 16S rRNA genes will be amplified using indexed primers and sequenced using 454-pyrosequencing, as described in the paper by Fuhrman et al 2013 [29]. The DNA analysis that will be performed will focus on the identification and study of microbes in the sample rather than characterization of human genes.

Bioinformatics: Reads will be filtered through the Institute of Genome Sciences bioinformatics pipeline [15]. Operational taxonomic units (OTUs) will be defined using the Quantitative Insights Into Microbial Ecology (QIIME) pipeline as sequences with at least 97% identity, rarified (randomly sampled with replacement) to the minimum number of observed OTUs. Using the Ribosomal Data Project Bayesian classifier in QIIME, OTUs will be assigned to phylum, class, order, family, and genus level-taxa, and their relative abundances will be calculated [16]. Additional microbial measures of interest include alpha diversity (Shannon index, Inverse Shannon), beta diversity (UNIFRAC distances), and phylogenetic diversity (whole tree phylogenetic diversity), as measured using QIIME after using rarefaction to account for differences in amplification across fecal samples [16]. In addition, we would like to consider as exposures phylogenetic composition, assessed down to the levels of phylum, family, and genus. Finally, we will use the programs PICRUSt and Lefse to generate metagenomic data and measures of KEGG pathway abundances from phylogenetic profiles [17].

Characterization of immune response

Breast tissue collection: Tissue-based measures of immune response will require formalin-fixed paraffin-embedded tissues from primary tumor samples. After informed consent is

obtained, archival tissue from a pre-treatment biopsy of primary breast tumor, in the form of a FFPE tissue block or pre-cut slides will be requested either from the diagnosing center or from the Tissue Biorepository. *Four s*lides will be received or cut from formalin-fixed paraffin-embedded tissues from primary tumor samples collected prior to chemotherapy. Slides will be prepared from an FFPE tumor tissue block of at least 5-µm thickness. An H&E slide will be scanned using an Aperio slide scanner and will be used to characterize the sample in terms of tissue composition (cell density, relative abundance of stromal, epithelial, and adipose cells). We will stain slides for T-lymphocytes and microenvironmental cues using antibodies for CD8, CD4, FoxP3, and PDLM1 using standard methods for cell surface markers and intracellular markers as described in the following reference [30].

Blood sample collection: A sample of not more than 30 mL or approximately 6 teaspoons (three 10-mL yellow ACD tubes) will be taken by venipuncture in order to obtain peripheral blood leukocytes. This will be done twice, prior to the initiation, and after completion, of chemotherapy. A trained phlebotomist or registered nurse will draw the blood required for clinical and research purposes. This blood sample will be collected in coordination with clinical care to minimize the number of blood draws experienced by participants. It is important that the first sample be obtained prior to the first dose of chemotherapy. For logistical reasons, blood draws should be completed before 2:30 pm Monday-Friday so that the sample can be processed the same day. After collection, the sample should be kept and transported at room temperature.

Delivery to the laboratory: Research staff will pick up specimens from the Cancer Institute Blood Draw or Infusion Centers. Research staff will re-label specimens with a patient identifier, date of the specimen, and name of visit. When a research specimen is collected, designated research staff will be notified to coordinate delivery and processing of the sample at the Cannon Laboratory, in Room B532 of Biomedical Building 1, in the Department of Microbiology and Immunology at the University of Arkansas for Medical Sciences.

Processing: Samples will be processed within 24 hours of the blood draw. Peripheral blood mononuclear cells (PBMCs) will be freshly isolated (within 8 h after the blood draw) from EDTA-treated samples using Ficoll-Hypaque gradient separation (Lymphoprep, Axis Shield, Oslo Norway) and washed, following a standard protocol.

Storage: One of the three aliquots will be stored in the Tissue Biorepository at -80ºC or lower. Samples will be kept indefinitely. If a sample is deemed unviable at the time of use, the sample will be mixed with bleach and disposed in a sink. All specimen containers (tubes and containers) will be disposed into biohazard red plastic bags according to UAMS Occupational Health & Safety policy. Red bag waste will be picked up by Occupational Health & Safety for final disposal.

Flow cytometry: Leukocytes will be isolated and tagged using monoclonal antibodies directed against CD4, CD8. Following permeabilization, cells will be incubated with anti-FOXP3 mAb (eBioscience, USA). Samples will be analyzed using a FACS Canto flow cytometer (BD Biosciences, USA). Number of events will be acquired in list mode. The FACS Diva software package (BD Biosciences) will be used.

Measures of the breast microenvironment: mammographic density and benign parenchymal enhancement

Available mammographic and MRI-based evaluations carried out at UAMS as a part of the diagnostic process will be reviewed by study investigators. Collected mammographic images would be used for measurement of volumetric mammographic breast density using Volpara software [6]. For each woman, standard views (CC and MLO views) will be reviewed. MRI images will be reviewed by Dr. Malak in order to measure benign parenchymal enhancement. In the final dataset, mammographic and MRI-based measures will be stored with the participant's study code.

Withdrawal from the protocol

Subjects may discontinue participation in the research at any time without penalty or loss of benefits to which the subject is otherwise entitled. In the event that the participant is unable to attend study visits or tolerate study assessments, the Principal Investigator reserves the right to terminate the subject's participation without the subject's consent. Data from samples collected prior to withdrawal may be used in data analysis unless the subject provides a written request to the Principal Investigator requesting that data and/or any stored samples be destroyed.

Reporting of protocol modifications, deviations or violations

This study will be reviewed by the UAMS Institutional Review Board (IRB).

Any modifications to the protocol will be submitted to the UAMS IRB for review and approval before any such changes are put into effect. If modifications of the consent form are required, these will also be reviewed and approved by the IRB before the study proceeds. In exceptional circumstances, involving major changes in the protocol, patients already entered into the study may be asked to sign a revised consent form (following IRB approval). However, as this study does not involve patient treatment, and is no more than minimal risk, this last circumstance is not likely.

Study activities will be reported to the UAMS IRB according to the guidelines for reporting outlined in IRB Policy 10.2.

In addition, this study will undergo scientific review by the Cancer Institute's Protocol Review and Monitoring Committee (PRMC). Approval by both the IRB and PRMC is required before the clinical trial can be activated.

Disposition of data

Data will be collected and recorded according to protocol requirements for all subjects enrolled to this trial. Data obtained during the study will be entered into the clinical trial management suite. Subjects will be registered in C3PR, a cancer Biomedical Informatics Grid (caBIG®, NCI) application of the clinical trial management suite. Data will be entered into OpenClinica through electronic web-based case report forms (CRFs). OpenClinica is a secure open source system for electronic data capture and clinical data management. Data from OpenClinica can be easily extracted for analysis throughout the study**.**

Future research

In the course of the present study, data is collected on many factors, including tumor characteristics, host characteristics, lifestyle factors, medical history, cancer therapies, gastrointestinal symptoms, comorbidities and clinical course for use in meeting stated study aims.

In addition to data, blood and fecal specimens are collected for analysis. For participants who agree to allow it, data collected in the course of the present study may be stored for future studies. Unused portions of blood and fecal samples may, with the participants' permission, be securely stored for use in future research studies. Any future research would also be about factors that influence breast cancer risk and outcomes. The informed consent has a separate section in which the participant is given the option to allow or decline use of their data and/or their biospecimens in future research. If the subject declines to have data and/or biospecimens stored for future research, data and biospecimens will be used only for the research described in this protocol. If the participant chooses, he or she can contact Dr. Fuhrman at any time with a written request to have his or her data be destroyed.

In accordance with federal policy for NIH-funded investigators, the study shall send data to an NIH data repository. This data will be coded and will not contain any direct identifiers. The study is required to submit documentation that describes how the confidentiality of research subjects is protected, project protocols, and data variable descriptions. Access to the repository would be granted by an NIH-appointed Data Access Committee, which would review all applicant projects and put data-use agreements in place and only be available for the purposes of biomedical research.

In addition, the informed consent has a section in which the participant is given the option to allow the research staff to contact them in the future regarding new clinical trials of which they would be eligible to participate. If the subject declines, they will not be contacted for future clinical trials.

Benefits and Risks

There will be no direct benefits to the study participants; however, knowledge gained from the study could potentially benefit patients in the future.

Physical risks of blood withdrawal are limited to possible slight bruising at the site of needle puncture, the slight chance of fainting at the time of blood withdrawal, infection, bleeding and pain.

There is a risk that the genetic information could be linked to the identity of the participant. This could result in genetic discrimination. Genetic discrimination occurs if people are treated unfairly because of differences in their genes that increase their chances of getting a certain disease. In the past, this could have resulted in the loss of health insurance or employment. Because of this, The Genetic Information Nondiscrimination Act of 2008, also referred to as GINA, was passed by Congress to protect Americans from such discrimination. The new law prevents discrimination from health insurers and employers. This act was signed into federal law on May 21, 2008, and went into effect May 2009.

An unlikely risk to study participants is the potential for loss of confidentiality. Measures to protect the confidentiality of study participants will be implemented as described in the Data Handling and Recordkeeping section below.

Data Handling and Recordkeeping

The Principal Investigator will carefully monitor study procedures to protect the safety of research subjects, the quality of the data and the integrity of the study. All study subject material will be assigned a unique identifying code or number. Data obtained during the study will be entered into C3PR and OpenClinica located in the UAMS clinical trial management suite. C3PR is a cancer Biomedical Informatics Grid (caBIG®, NCI) application that will keep a list of subjects

enrolled and specific information regarding their enrollment. Data will be entered into OpenClinica through electronic web-based case report forms (CRFs). OpenClinica is a secure open source system for electronic data capture and clinical data management. Data from OpenClinica can be easily extracted for analysis throughout the study.

After informed consent is obtained, the data will be collected, coded with a study ID number, and stored indefinitely.

Data Analysis

Analyses will be conducted according to study aims. As the aims are considered exploratory, P values < 0.05 will be considered statistically significant and no adjustments will be made for multiple comparisons.

Aim 1. To assess the acceptability and feasibility of study procedures.

We will describe numbers and characteristics of women who are 1) screened, 2) eligible, 3) consented, and 4) complete collections for each type of biospecimen. In a brief form which will accompany the fecal sample, we will ask enrolled women specifically about the acceptability of, and logistical difficulties with, the procedures for collection and packaging of the fecal specimen.

Aim 2. To estimate proportions of patients with pathological complete response by host factors, breast cancer subtypes, tumor characteristics, reported gastrointestinal symptoms, and by categorical measures of gut microbial composition, diversity, and metabolic activity.

We will estimate and compare rates of pCR by each factor using chi-squared tests for crude comparisons and multivariable logistic regression for adjusted comparisons. Factors of interest will include host factors such as age and menopausal status; and tumor characteristics including: intrinsic subtype assessed using clinically indicated measures (ER and PR status, Her2/neu status, and Ki67 positivity); standard clinical prognostic indicators (TNM stage and histologic grade), and subgroups defined using tertiles of all continuous gut microbial measures.

Aim 3. To estimate the associations of pre-treatment gut microbial composition, diversity, and metabolic activity with anticancer immune responses, measured as relative abundance of immune infiltrates in pre- treatment FFPE breast tumor tissues, and as relative abundance of circulating immune cell subtypes (Th1, Th2, Th17, and T_{reg}) in baseline and longitudinal blood samples. Bivariate and covariate-adjusted associations among measures will be evaluated using Spearman's rank test and General Linear Models, respectively.

Aim 4. To estimate associations of the measures of local and systemic immune responses with host factors such as age, BMI, and smoking status, and with measures of the breast microenvironment including mammographic density and benign parenchymal enhancement on breast MRI. Bivariate and covariate-adjusted associations among measures will be evaluated using Spearman's rank test and General Linear Models, respectively.

Power and Sample Size:

Aim 1: If we screen 60 potential participants, we should be able to estimate, with 95%

confidence, the true proportion of this patient population willing to participate in the study with precision of +/- 20%.

Aim 2: If 40 participants are enrolled and complete study measures, and the expected pCR in the category with the greatest risk of mortality is assumed to be 20%, then we will have 47% power to detect a statistically significant trend in rates of pCR across tertiles of microbiome measures if the odds ratio for the highest tertile is 5.0.

Aim 3 and 4: If we have 20 participants with both tumor infiltrate measures and measures of gut microbial composition, we will have adequate power (ß=80%) to detect relationships between the dependent variable (relative abundances of tumor infiltrating lymphocytes and circulating lymphocytes) and the independent variables gut microbial composition, diversity and metabolic activity; host factors, and measures of the tumor microenvironment) at a two-sided α=0.05 significance level, when the true change in the dependent variables is 0.67 standard deviations per one standard deviation change in the independent variables.

Ethical Considerations

This study will be conducted in accordance with all applicable government regulations and University of Arkansas for Medical Sciences research policies and procedures. This protocol and any amendments will be submitted and approved by the UAMS Institutional Review Board (IRB) to conduct the study. In addition, all study personnel must have completed human subject protection training.

The formal consent of each subject, using the IRB-approved consent form, will be obtained before that subject is submitted to any study procedure. All subjects for this study will be provided a consent form describing this study so they can make an informed decision about their participation in this study. The person obtaining consent will thoroughly explain each element of the document and outline the risks and benefits, alternate treatment(s), and requirements of the study.

The consent process will take place in a quiet and private room, and women may take as much time as needed to make a decision about their participation. The privacy of all subjects will be maintained and questions regarding participation will be answered. No coercion or undue influence will be used in the consent process. This consent form must be signed by the subject and the individual obtaining the consent. A copy of the signed consent will be given to the participant, and the informed consent process will be documented in each subject's medical record.

Dissemination of Data

Results of this study may be used for presentations, posters, or publications. The publications will not contain any identifiable information that could be linked to a participant.

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