Mayo Clinic Cancer Center

MC1137 Breast Cancer Genome Guided Therapy Study (BEAUTY)

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√ Study contributor(s) not responsible for patient care

Document History	Effective Date
Activation	March 5, 2012
Addendum 1	April 13, 2012
Addendum 2	August 15, 2012
Addendum 3	October 3, 2012
Addendum 4	February 11, 2013
Addendum 5	May 6, 2013
Addendum 7	October 30, 2013
Addendum 8	March 14, 2014
Addendum 9	June 12, 2014
Addendum 10	August 14, 2014
Addendum 11	October 17, 2014
Addendum 12	January 16, 2015
Addendum 13	June 2, 2015
Addendum 14	July 14, 2016
Addendum 15	February 23, 2018
Addendum 16	September 3, 2019
Addendum 17	November 10, 2020
Addendum 18	September 8, 2021
Addendum 19	October 19, 2023

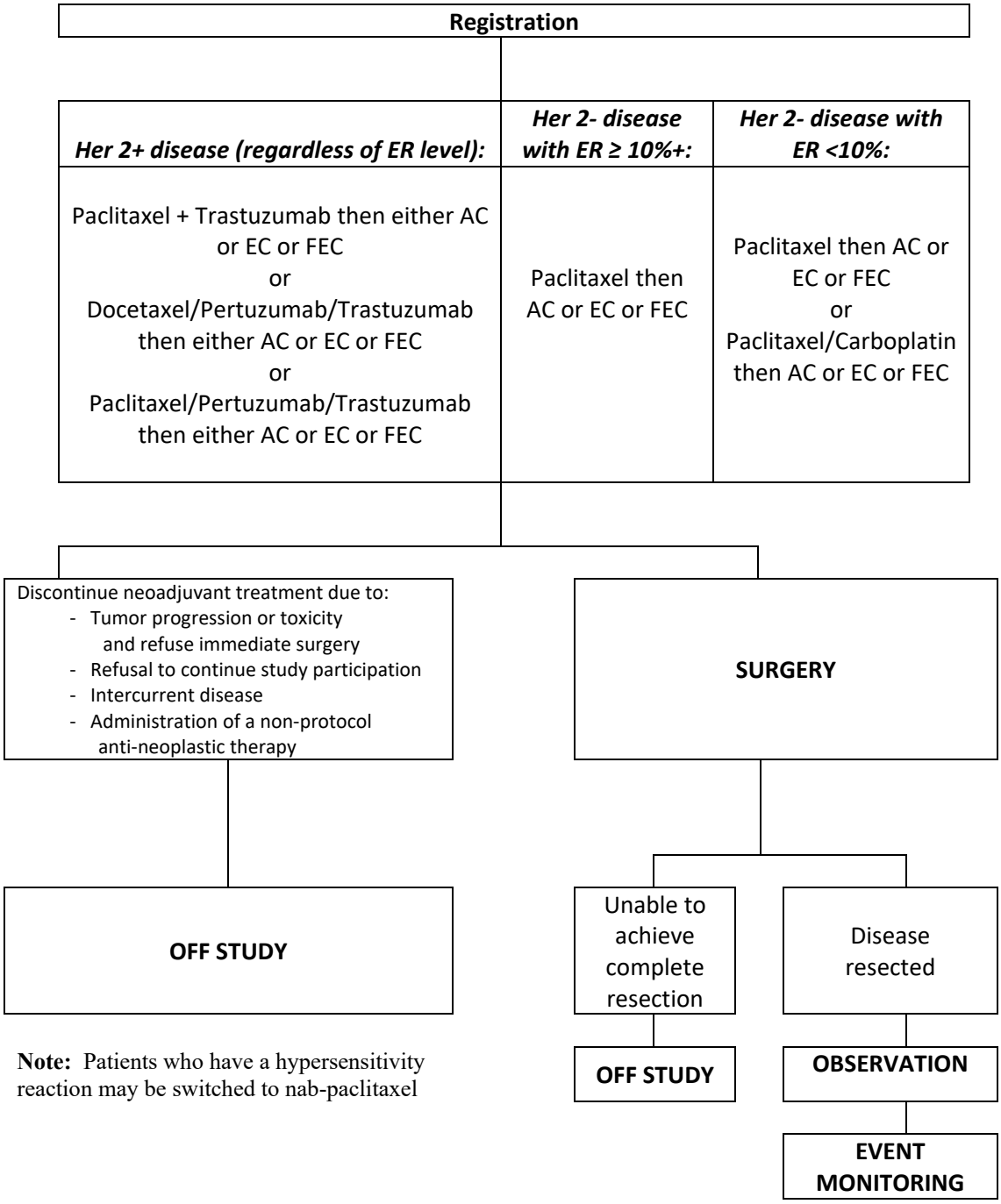
Protocol Resources

Questions:	Contact Name:
Patient eligibility*, test schedule, treatment delays/interruptions/adjustments, dose modifications, adverse events, forms completion and submission	[redacted]
Forms completion and submission	[redacted]
Research Study Nurse	[redacted]
Protocol document, consent form, regulatory issues	[redacted]
Non-paraffin biospecimens	[redacted]
Pathology Coordinator	[redacted]
CIM Genetics Counselor	[redacted]
Adverse Events (AdEERS, MedWatch, Non-AER, AML/MDS)	[redacted]

*No waivers of eligibility per NCI

Table of Contents

Schema



Note: Patients who have a hypersensitivity reaction may be switched to nab-paclitaxel

1.0 Background

1.1 Introduction

Breast cancer is diagnosed in 195,000 women annually in the United States alone; 45,000 women still die annually of this disease. Although many women now present with Stage I and II mammographically detected cancers and have excellent outcomes, 10–20% of newly diagnosed breast cancers present as locally advanced breast cancer (LABC) in which the risk of recurrence and death is significantly higher[1].

Development of multi-agent adjuvant chemotherapy regimens has substantially improved the clinical outcomes for women with breast cancer [2]. The most effective adjuvant combination regimens include anthracyclines, such as doxorubicin or epirubicin (topoisomerase II inhibitors), the alkylating agent cyclophosphamide, and taxanes (currently docetaxel or paclitaxel), which are microtubule stabilizers. These different mechanisms of action were designed to provide maximal cell kill within the range of tolerable toxicity for each drug, and broad coverage in resistant cell lines within a heterogeneous tumor population, The current standard of care for women with locally advanced breast cancer is neoadjuvant therapy prior to surgical resection.

While neoadjuvant chemotherapy does not lead to improved overall survival (compared to chemotherapy delivered after surgery), higher rates of breast conservation are achieved with neoadjuvant chemotherapy. Perhaps the most important neoadjuvant clinical trials performed to date were undertaken by the National Surgical Adjuvant Breast and Bowel Project (NSABP) and provide the largest randomized data to date comparing preoperative to standard adjuvant chemotherapy. The NSABP B18 trial randomized 1523 women to either preoperative or postoperative doxorubicin/cyclophosphamide for a total of four cycles [3]. Among those who were randomized to preoperative chemotherapy, 80% of patient had a reduction in breast tumor size; 49% had a clinical response (cCR: 36% and cPR: 13%); and 89% of the clinically node positive patients had a clinical nodal response.

The NSABP-B27 trial was designed to determine the impact on breast cancer response rates and overall survival (OS) of the addition of docetaxel to preoperative doxorubicin and cyclophosphamide [4]. Women with operable breast cancer (n = 2411) were randomly assigned to receive preoperative doxorubicin and cyclophosphamide followed by surgery, doxorubicin and cyclophosphamide followed by docetaxel and then surgery, or doxorubicin and cyclophosphamide followed by surgery and then docetaxel. Tamoxifen was initiated for estrogen receptor (ER) positive tumors. Adding docetaxel to doxorubicin and cyclophosphamide did not significantly impact disease free [1] survival (DFS) or OS but was found to significantly reduced the incidence of local recurrences as first events ($p = 0.0034$). Thus, the primary benefit of neoadjuvant chemotherapy is to downstage tumors, thereby improving optimal surgical resection and increasing the probability of breast conservation [5].

A meta-analysis was performed by the Early Breast Cancer Trialists Collaborative Group (EBCTCG) [6] that included eleven randomized clinical trials, performed between 1981–1993 encompassing 4675 women, demonstrated that preoperative therapy was associated with 18% fewer mastectomies and no significant differences in breast cancer recurrence, breast cancer mortality, or death within 10 years of follow-up comparing preoperative regimens with postoperative regimens.

An additional important finding in these studies was that the achievement of a pathologic complete response (pCR) (i.e., elimination of invasive tumor in breast and axillary lymph nodes, as assessed by pathology at surgery) is a useful surrogate for prognosis in breast cancer patients overall, suggesting that chemotherapy sensitivity, in and of itself, is an independent predictor of DFS and OS. Pathologic complete response, which occurred in 27% of patients in NSABP B-27 who received preoperative docetaxel, was a significant predictor of OS regardless of treatment (hazard ratio = 0.33; 95% CI, 0.23–0.47; $p < 0.0001$). Pathologic nodal status after chemotherapy was a significant predictor of OS ($p < 0.0001$). The pCR rates of doxorubicin-containing regimens are in the range of 12%; combining doxorubicin and taxanes leads to pCR rates in the 25–27% range. Conversely, the prognosis is quite poor for patients with significant residual disease in the breast, lymph nodes or both [7-10] following neoadjuvant chemotherapy.

Breast cancer is a heterogeneous disease, and there is a wealth of data demonstrating that the identification of pathways responsible for the malignant phenotype can lead to new drug development, leading to substantial gains in response and death. A growing understanding of the molecular, genetic, and biochemical changes that occur during the processes of carcinogenesis, progression, and metastasis has shifted drug development toward therapeutics that act on specific molecular targets responsible for the malignant phenotype. Recent advances in drug development have included drugs that target signal transduction (the HER receptor family, Ras, Raf, and MEK kinases); oncogenic proteins, such as BCR/ABL, cell-cycle regulating proteins, such as the cyclins, the cyclin-dependent kinases, and inhibitors of cyclin-dependent kinases; and proteins involved in the tumor angiogenesis, such as endothelial growth factor receptors.

While the most common drug target that drives the growth of breast cancer is the estrogen receptor, many women who present with locally advanced breast cancer have tumors that exhibit estrogen independent growth. An important pathway in breast cancer first described in the late 1980's by Slamon et al [11], is the discovery of the human epidermal growth factor receptor 2 (HER2) receptor tyrosine kinase. The HER2 protein (or ErbB2) is one of four structurally related receptor tyrosine kinases (HER1-4). The HER2 gene, is amplified in 20% of breast cancers and amplification leads to a substantial change in the malignant phenotype, including alterations in tumor size, lymph node status, angiogenesis, invasion, and metastasis. Blockade of the HER2 protein tyrosine kinase, either through the use of trastuzumab or small molecule inhibitors of the tyrosine kinase (lapatinib) has led to substantial reductions in recurrence, survival, and in the neoadjuvant setting substantial increases in pCR rates.

Molecular phenotyping has now been incorporated into the classification of tumors undergoing neoadjuvant therapy. These data have consistently demonstrated that response and pCR differs significantly by phenotype [12].

Patients with strongly ER positive tumors (those of the luminal A subtype) may have a low pCR rate, but subsequently have a favorable prognosis due to both the indolent natural history of the disease and the responsiveness of these tumors to anti-estrogen therapies. Conversely, many patients with triple negative breast cancer (i.e., ER/progesterone receptor (PgR)/HER2 negative by immunohistochemistry (IHC), or basal by molecular phenotyping) may have an excellent response to neoadjuvant chemotherapy, likely due to the high proliferative rate of these tumors. However, many of these patients subsequently relapse, and salvage strategies are lacking.

The optimal combination, sequencing, and schedule for neoadjuvant chemotherapy has not been established. Four cycles of doxorubicin/cyclophosphamide followed by paclitaxel is a common regimen for women with HER2/neu non-overexpressing LABC, and the addition of trastuzumab on a weekly schedule in combination with paclitaxel has been shown to be safe and effective for patients with HER2 positive disease [13]. However, several studies have examined variations to the standard regimen, with or without trastuzumab. The Eastern Cooperative Oncology Group (ECOG) 1193 trial, which compared doxorubicin followed by paclitaxel to the reverse sequence of paclitaxel followed by doxorubicin in the metastatic setting, demonstrated no difference in response rate [14]. Thus, clinical data to date suggests that the sequence of taxane and doxorubicin does not seem to impact efficacy, although randomized trials testing one sequence versus another have not been reported.

Breast cancer is a genetically and clinically heterogeneous disease. While multiple commercial gene expression profiles are available and can identify patients at higher risk for recurrence, this technology has not necessarily led to new therapeutics for breast cancer. Clinical progress has been limited by a poor understanding of the genetic events which drive the malignant phenotype and by limited preclinical models to study the disease. The rapid advancement of next-generation sequencing technologies allows comprehensive characterization of genomic changes. This technology is particularly useful for analysis of the entire tumor genome for the identification of novel genetic alterations in genes and gene pathways associated with resistance to standard chemotherapy.

Response to neoadjuvant chemotherapy is assessed by documenting whether residual invasive disease is present in the breast (complete pathologic response) as well as by residual cancer burden (RCB) classification (which takes into account amount invasive disease present in the breast and nodes).

Post-treatment proliferation (Ki67), has been shown to be an important prognostic factor among women receiving neoadjuvant chemotherapy[27] and neoadjuvant endocrine therapy [28]. The persistence of elevated Ki67 after treatment is associated with poor outcome. Moreover, patients with residual disease whose Ki67 levels are low after treatment have similar outcomes to patients with a complete pathological response[27].

Given the sequential nature of the regimens used in this study, we have the opportunity to interrogate the mechanisms of taxane resistance. For patients with elevated levels of Ki67 (Ki67 $\geq$ 15%) prior to paclitaxel, genetic differences between the tumors with elevated Ki67 levels after paclitaxel will be contrasted and compared to the tumors whose Ki67 levels are not elevated after paclitaxel.

Given that neoadjuvant chemotherapy followed by breast surgery is considered a standard for the initial treatment of locally advanced breast cancer, the neoadjuvant setting can be used for the development of new drugs in combination or following standard chemotherapy for women at high risk of breast cancer morbidity and mortality. Therefore, it is critical that the new drug development focus on the following:

1) The identification of patients at increased risk for treatment failure
2) Identification of novel mutations in genes and gene pathways that drive the malignant phenotype and those associated with resistance to standard therapy.

3) The ability to establish human tissue xenografts derived from the primary tumor tissue to directly test whether drugs which target the mutations and structural variations in genes and gene pathways will alter the growth and survival of these cells.
4) A personalized approach to introduce new drugs to women with high risk tumors whose mutation pattern would be expected to confer response to those novel drugs.

1.2 Current state of genomics in breast cancer

A core component in the development of personalized medicine is to identify specific gene mutations and their functional impact on signaling pathways involved in tumorigenesis. For example, the V600E mutation identified in *BRAF* through a sequencing effort of *BRAF* gene proved to be a unique drug target for BRAF inhibitors and the inhibitor is more efficacious in melanoma patients with this *BRAF* mutation. While first generation molecular signatures based on gene expression profiles are prognostic and appear to predict response to chemotherapy, the identification of genes and gene pathways that drive the malignant phenotype and are associated with resistance to standard therapy is a key step toward the realization of new therapies for breast cancer. High throughput assays including next generation sequencing, gene expression, and CpG methylation enable researchers to perform integrated analysis to identify molecular signatures associated with response to standard chemotherapy. Our group has extensive experience with these types of analyses. Under the NIH-Mayo Pharmacogenomics Research Network (PGRN), we have generated an *in vitro* model system consisting of 300 human lymphoblastoid cell lines obtained from 3 different ethnic groups. For each cell line, we have dense genomic information including 1.3 million SNPs, 54,000 gene expression probe sets, 47,000 CpG methylation sites and over 280 micro RNAs, plus over 20 different drug cytotoxicity phenotypes. With this information, we are able to perform integrated analysis of SNPs, expression, microRNA and CpG methylation with our drug cytotoxicity phenotypes to identify signatures associated with drug sensitivity. We also have experience with GWAS (genome wide association studies) and functional studies of GWAS signals using the lymphoblastoid cell lines in multiple disease settings including breast cancer.

In women with primary breast cancer who will be treated with neoadjuvant chemotherapy we will obtain tumor tissue prior to neoadjuvant treatment, post paclitaxel +/- trastuzumab, at surgery from those cases with residual tumor and at disease recurrence. These tissues will undergo whole exome sequencing and RNA seq of transcript levels and CpG methylation sites. Information gathered from these series of samples will enable us to identify signatures for Ki67 response, complete pathological response phenotype and the combination of both; identify mutations in genes of pathways that have known targets for therapy to individualize the patients with appropriate therapies, and identify additional novel mechanisms underlying different response phenotypes. Also, these tumor samples will be used to generate xenografts for future drug screening studies. Blood will be collected prior to the start of neoadjuvant therapy for isolation of germline DNA that will be compared to tumor DNA and will be used to assess the effect of polymorphisms on treatment response.

1.3 Molecular Breast Imaging **(MCR Participants Only)**

At Mayo Rochester we have developed Molecular Breast Imaging (MBI). This is a functional imaging tool that uses a small amount of injected radiotracer and a dual-head small field of view gamma camera system. Previous work has demonstrated a sensitivity of ~90% for the detection of lesions in the range 6-10 mm in diameter [15, 16] and shown that it can detect additional disease not seen on mammography.

We will examine tumor grade and cell proliferation index from the pre-treatment biopsy specimen and correlate these with the uptake in breast tumors of the radiopharmaceutical, 99mTc-sestamibi, prior to treatment. The images will be interpreted by breast imaging radiologists, and the type and distribution of radiotracer uptake will be documented according to the MBI lexicon [15].

99mTc-sestamibi MBI exams will be performed at 3 time points; the first after registration on the study (prior to any chemotherapy), the second after completion of taxane chemotherapy (prior to starting AC or FEC), and the third after completion of all neoadjuvant chemotherapy (prior to surgery). Changes in the pattern of tracer uptake will be assessed in terms of its association with pathologic tumor response and changes in Ki67 levels after NAC.

1.4 Summary of Background

In summary, patients with breast cancer who do not achieve a pathologic complete response (pCR) have a higher rate of recurrence and death. Current standard chemotherapy with taxanes (with or without trastuzumab for HER2+ disease) followed by anthracyclines achieves pCR rates of 30-50%. Additionally, elevated Ki67 after chemotherapy has also been shown to be associated with poor clinical outcome. Drug development in oncology is now focused on the identification of abnormalities in genes and gene pathways and the development of drugs designed to target those pathways. However, the identification of novel somatic changes that are "druggable" must be functionally validated before clinical utility can be evaluated. This requires human breast cancer tissue (xenografts) in order to perform functional studies Our long range goal is to individualize patient care by sequencing individual patient tumors, identifying mutations and alterations in tumors associated with the malignant phenotype, and using this information to personalize breast cancer therapy.

2.0 Goals

2.1 Primary Objectives

2.11a To ascertain the germline and somatic changes within gene and gene pathways that occur before and after completion of paclitaxel
and examine whether these changes differ between tumors that respond to paclitaxel and those that do not respond to paclitaxel

2.11b To ascertain the germline and somatic changes within gene and gene pathways that occur before and after completion of NAC and examine whether these changes differ between tumors that respond to treatment and those that do not respond to treatment

2.12 To determine the frequency of known tumor mutations for which current drug therapies already exist (e.g. BRAF, C-KIT, EGFR mutation, KRAS, PTEN, PI3K).

2.13 Using breast cancer tissue obtained prior to chemotherapy in all patients and following the completion of chemotherapy (in patients with residual disease), to develop tumor xenograft cell lines for mechanistic and functional studies to determine whether mutations identified are associated with the malignant phenotype and response to associated drugs which target the gene and/or pathways.

2.2 Secondary Objectives

2.21 Assess the association between changes in ^{99m}Tc-sestamibi uptake and response to treatment following neoadjuvant chemotherapy (**MCR participants only**).

2.22 To determine the association between T cell sequence and to treatment following neoadjuvant chemotherapy

2.23 To determine the ability of pre-treatment homologous recombination deficiency (HRD) scores to discriminate between those patients who will have a pCR after taxane and anthracycline-based neo-adjuvant chemotherapy regardless of tumor subtype and within tumor subtypes.

2.23a To determine if the pretreatment HRD score is associated with radiographic or Ki67 response to taxane therapy.

2.23b To determine if the pretreatment or post taxane HRD score is associated with MRI response after AC based therapy in patients with residual tumor on MRI after taxane therapy.

2.23c To determine if the pretreatment HRD score is associated with response to neoadjuvant chemotherapy as determined by pCR.

2.24 To examine the changes in HRD scores during the course of neoadjuvant chemotherapy and their association with surgical outcome.

2.25 To determine the association between baseline ER beta expression with

pCR after taxane and anthracycline-based neo-adjuvant chemotherapy in patients with triple negative or ER alpha (1-10%), HER2 negative breast cancer.

2.25a To determine if pretreatment ER beta expression is associated with radiographic or Ki67 response to taxane therapy in patients with triple negative or ER alpha (1-10%), HER2 negative breast cancer.

2.25b To determine if pretreatment ER beta expression is associated with MRI response after AC based therapy in patients with residual tumor on MRI after taxane therapy in in patients with triple negative or ER alpha (1-10%), HER2 negative breast cancer.

2.25c To determine if pretreatment ER beta expression is associated with PDX “take rate” as well as “growth rate” in patients with triple negative or ER alpha (1-10%), HER2 negative breast cancer.

2.26 An exploratory analysis of mutation status of additional genes known to be involved in DNA-damage response. Genes include AKT1, ATM, ATR, BAP1, BARD1, BRIP1, CDK12, CHEK1, CHEK2, CTNNB1, ERCC4, FAM175A,

FANCA, FANCD2, FANCE, FANCI, FANCL, KRAS, MLH1, MRE11A, MSH2, MSH6, MUTYH, NBN, PALB2, PIK3CA, PPP2R2A, PTEN, RAD50, RAD51, RAD51B, RAD51C, RAD51D, RAD52, RAD54B, RAD54L, RPA1, TP53, TP53BP1, XRCC2, and XRCC3

3.0 Patient Eligibility

3.1 Inclusion Criteria

3.11 Age ≥18 years.

3.12 Histological confirmation of invasive breast cancer.

3.13 Confirmation of breast cancer lesion ≥1.5 cm in size by any clinical (physical examination measurement) or radiographic criteria (mammogram, ultrasound or MRI) in the ipsilateral breast.

Note: Benign breast disease, LCIS or DCIS in the contralateral breast is allowed. Contralateral invasive breast cancer is allowed if disease is of clinically lower stage and the higher stage lesion will be the study lesion for all biopsies and tissue samples.

Note: Disease in axilla only is not eligible.

Note: Patients that have a contraindication or inability to have an MRI may still be enrolled on study and not participate in the MRI at any of the study specific time points.

Note: For patients with bilateral disease the higher clinical stage disease will be the study lesion that will undergo study biopsies and tissue samples from surgery and the contralateral lesion will NOT undergo research biopsies and tissue samples.

3.14 Men or women who are to begin neoadjuvant chemotherapy for treatment of Stage I-III Her 2 negative, ER10% or greater breast cancer with paclitaxel followed by either the combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) or the combination of epirubicin and cyclophosphamide (EC), or the combination of doxorubicin and cyclophosphamide (AC).

OR

Men or women who are to begin neoadjuvant chemotherapy for treatment of Stage I-III Her 2 negative, ER<10% breast cancer with paclitaxel with or without carboplatin followed by either the combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) or the combination of epirubicin and cyclophosphamide (EC) or the combination of doxorubicin and cyclophosphamide (AC).

OR

Men or women who are to begin neoadjuvant chemotherapy for treatment of Stage I-III Her 2 positive breast cancer with one of the following regimens:

1) Paclitaxel/trastuzumab followed by either the combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) or the combination of epirubicin and cyclophosphamide (EC) or the combination of doxorubicin and cyclophosphamide (AC).

2) Docetaxel/trastuzumab/pertuzumab followed by either the combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) or the combination of epirubicin and cyclophosphamide (EC) or the combination of doxorubicin and cyclophosphamide (AC).
3) Paclitaxel/trastuzumab/pertuzumab followed by either the combination of 5-fluorouracil, epirubicin and cyclophosphamide (FEC) or the combination of epirubicin and cyclophosphamide (EC) or the combination of doxorubicin and cyclophosphamide (AC).

Trastuzumab and/or pertuzumab will be given concurrently with the taxane portion. Pertuzumab should not be given with the anthracycline portion of chemotherapy. Trastuzumab can be given concurrently with FEC (but not AC at the discretion of the local medical oncologist (see section 7.0).

Note: Her2 positive disease is defined to be: HER2 score of 3+ by IHC or HER2 gene amplification by FISH.

3.15 Provide informed written consent.

3.16 Willing to return to Mayo Clinic in Rochester, MN, Mayo Clinic in Arizona, or Mayo Clinic in Florida for imaging correlative, surgery, and follow-up.

3.17 Willing to provide blood samples for correlative research purposes (see Sections 6.2 and 14.1).

3.18 Willing to provide tissue samples for correlative research purposes (see 6.2 and 17.1).

3.19 ECOG Performance Status ≤2.

3.2 Exclusion Criteria

3.21 Receiving any investigational agent which would be considered as a treatment for the primary neoplasm.

3.22 Other active malignancy ≤ 5 years prior to registration. EXCEPTIONS: Non-melanotic skin cancer or carcinoma-in-situ of the cervix.

Note: If there is a history or prior malignancy, they must not be receiving any other treatment for their cancer.

3.23 Patients who are not planning to receive neoadjuvant chemotherapy.

3.24 Biopsy proven Stage IV disease.

3.25 Patients who are pregnant or nursing.

4.0 Test Schedule

				Active Monitoring Phase					
Tests and procedures	≤60 days prior to registration	≤28 days prior to registration	After registration but prior to start of taxane	After the completion of taxane prior to anthracycline based chemotherapy (Cycle 1)	Suspicion of disease progression during neo-adjuvant treatment	After completion of anthracycline-based chemotherapy but prior to surgery (Cycle 2)	At the time of surgery (Cycle 3)	At first recurrence or post treatment if primary surgery is not to be performed	Annual Observation (Years 1-6 following surgery)
History and exam, Wt, PS*		X		X		X		X	X
Height		X							
Adverse Event Assessment[10]			X	X		X			
Pregnancy Test[8,13]		X							
Mammogram of the side of the study lesion	X[2,4]					X			
Breast ultrasound of the study lesion	X[4]					X			
Axillary ultrasound ipsilateral to study lesion	X[4]					X[16]			
Research Breast Biopsy[9,15,R]			X	X[11]				X[1,14,15]	
Tumor evaluation by Breast MRI[4]	X[3,4]			X[3,R]		X[3]			
^{99m}Tc-sestamibi MBI[13,R]			X	X		X			
Blood specimen[6,12,R]			X	X		X		X	

				Active Monitoring Phase					
Tests and procedures	≤60 days prior to registration	≤28 days prior to registration	After registration but prior to start of taxane	After the completion of taxane prior to anthracycline based chemotherapy (Cycle 1)	Suspicion of disease progression during neo-adjuvant treatment	After completion of anthracycline-based chemotherapy but prior to surgery (Cycle 2)	At the time of surgery (Cycle 3)	At first recurrence or post treatment if primary surgery is not to be performed	Annual Observation (Years 1-6 following surgery)
Diagnostic, surgical, & recurrent tumor specimen[15,R]			X[1]				X[17, 18]	X[5,9]	
Evaluation of disease status					X				X

1. Copies of the pathology report are to be submitted along with the tissue sample.
2. Unilateral mammogram (for unilateral breast cancer) on side of index cancer required ≤60 days prior to registration. Contralateral mammogram (if unilateral breast cancer) required to be done ≤3 months prior to registration.
3. Unilateral or bilateral after completion of paclitaxel ± trastuzumab but prior to the start of AC or FEC chemotherapy. * Patients with known bilateral disease must have a bilateral MRI performed at the follow-up time points
4. Imaging examinations performed at outside facilities prior to study registration will be accepted following review by Mayo Clinic radiologists. If deemed inadequate for interpretation or if further evaluation is needed, additional imaging will be performed at Mayo Clinic per standard practice. Outside examinations will not be accepted following enrollment into the study.
   Breast MRI from all 3 time points will undergo **central review** by two Mayo Clinic Radiologists. Submit MRI electronically via GEPACS.
5. At the time of recurrence, patients will return to Mayo Clinic for a fresh tumor sample from the locally recurrent or metastatic site. For patients who develop recurrence and are unable to return Mayo, tissue specimen and a copy of the pathology report will be requested from the treating institution.
6. For validation of biomarker studies.
7. Disease status evaluation by mammogram and physical examination if breast conserving surgery done or physical examination if breast mastectomy performed.
8. For women of childbearing potential only. Must be done ≤ 7 days prior to the initial sestamibi MBI.
9. Performing a tissue biopsy using imaging guidance is standard of care. The sample will be divided and some will go to pathology to establish a diagnosis (standard of care) and the other part will go to research. If a biopsy has been performed at an outside institution, then a repeat biopsy is required (if feasible).
10. Adverse event assessment for MBI only.
11. If no residual abnormality on MRI and no target lesion is seen at time of ultrasound guided biopsy, then no biopsy will be taken.
12. See section 14 for collection time and preparation of samples

13. MCR participants only.
14. If recurrence occurs in the study breast
15. See section 17 for collection time and preparation of samples.
16. Axillary ultrasound at this time point is required ONLY for cases that were node positive at diagnosis.
    Axillary ultrasound is not required after chemotherapy on cases that were clinically negative at initial presentation.
17. Tissue from any residual disease at surgery and from the lymph nodes if residual disease is confined to the lymph nodes must be submitted (see Section 17.243). Surgery and Pathology reports from all sites must be submitted within 30 days of date of surgery. ER, PR, Her2 and Ki67 should be performed clinically on the surgical specimen. In the event that ER, PR, Her2 and Ki67 testing is not performed clinically, banked tissue (up to 8 unstained tissue slides) from tumor registry will be accessed for ER, PR, Her2 and Ki67 testing and charged to the study.
18. For patients with bilateral disease the higher clinical stage disease will be the study lesion that will undergo study biopsies and tissue samples from surgery and the contralateral lesion will NOT undergo research biopsies and tissue samples.

R Research funded (see Section 19.0).

* Other standard of care tests done per treating physician discretion.

5.0 Stratification Factors

None

6.0 Registration/Randomization Procedures

6.1 To register a patient, access the Mayo Clinic Cancer Center (MCCC) web page and enter the registration/randomization application. The registration/randomization application is available 24 hours a day, 7 days a week. Back up and/or system support contact information is available on the Web site. If unable to access the Web site, call the MCCC Registration Office at [redacted] between the hours of 8 a.m. and 4:30 p.m. Central Time (Monday through Friday).

The instructions for the registration/randomization application are available on the [redacted] and detail the process for completing and confirming patient registration. Prior to initiation of protocol treatment, this process must be completed in its entirety and a MCCC subject ID number must be available as noted in the instructions. It is the responsibility of the individual registering the patient to confirm the process has been successfully completed prior to release of the study agent. Patient registration via the registration/randomization application can be confirmed in any of the following ways:

- Contact the MCCC Registration Office [redacted]. If the patient was fully registered, the MCCC Registration Office staff can access the information from the centralized database and confirm the registration.
- Refer to "Instructions for Remote Registration" in section "Finding/Displaying Information about A Registered Subject."

6.2 Correlative Research

A mandatory correlative research component is part of this study, the patient will be automatically registered onto this component (see Sections 3.1, 14.1 and 17.1).

6.3 Documentation of IRB approval must be on file in the Registration Office before an investigator may register any patients.

In addition to submitting initial IRB approval documents, ongoing IRB approval documentation must be on file (no less than annually) at the Registration Office (fax: 507-284-0885). If the necessary documentation is not submitted in advance of attempting patient registration, the registration will not be accepted and the patient may not be enrolled in the protocol until the situation is resolved.

When the study has been permanently closed to patient enrollment, submission of annual IRB approvals to the Registration Office is no longer necessary.

6.4 Prior to accepting the registration, registration application will verify the following:

- IRB approval
- Patient eligibility
- Existence of a signed consent form
- Existence of a signed authorization for use and disclosure of protected health information

6.5 At the time of registration, the following will be recorded:
- Patient has/has not given permission to store and use his/her sample(s) for future research of *breast cancer.*
- Patient has/has not given permission to store and use his/her sample(s) for future research to learn, prevent, or treat other health problems.
- Patient has/has not given permission for MCCC to give his/her sample(s) to researchers at other institutions.

6.6 Treatment cannot begin prior to registration and must begin ≤21 days after registration.

6.7 Pretreatment tests/procedures (see Section 4.0) must be completed within the guidelines specified on the test schedule.

7.0 Recommended Treatment Plan

7.1 Neoadjuvant taxane phase recommended treatment schedule for either HER2 negative or HER2+ breast cancer.

Note: The following recommended treatments are considered standard regimens for the neoadjuvant treatment of breast cancer. Taxane-based therapy can consist of paclitaxel (for HER2 negative breast cancer) or either paclitaxel or docetaxel for HER2+ breast cancer. For HER2+ breast cancer, either trastuzumab alone or the combination of trastuzumab + pertuzumab can be utilized. For ER≤10%, HER2 negative breast cancer, carboplatin may be combined with paclitaxel.

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with HER2-negative, ER≥10% breast cancer	paclitaxel	N/A	80 mg/m^2	IV	Day 1	Weeks 1-12

OR

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with HER2 – negative, ER≥10% or greater breast cancer	paclitaxel	N/A	175 mg/m^2	IV	Day 1	Weeks 1, 3, 5, 7

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with HER2-negative, ER<10% breast cancer	paclitaxel	N/A	80 mg/m^2	IV	Day 1	Weeks 1-12

OR

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with HER2 – negative, ER<10% breast cancer	paclitaxel	N/A	175 mg/m^2	IV	Day 1	Weeks 1, 3, 5, 7

OR

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with HER2 – negative, ER<10% breast cancer	paclitaxel	N/A	80 mg/m^2	IV	Day 1	Weeks 1-12
	carboplatin	after paclitaxel administered	AUC6	IV	Day 1	Weeks 1, 4, 7, 10

Reference: Sikov et al, CALGB 40603, SABCS December 2013

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with Her2 positive disease	paclitaxel	N/A	80 mg/m^2	IV	Day 1	Weeks 1-12
	trastuzumab	after paclitaxel administered	1st dose – 4 mg/kg	IV	Day 1	Week 1
			Subsequent doses – 2mg/kg		Day 1	Weeks 2-12

OR

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with Her2 positive disease	paclitaxel	N/A	175 mg/m^2	IV	Day 1	Weeks 1, 3, 5, 7
	trastuzumab	after paclitaxel administered	1st dose – 4 mg/kg	IV	Day 1	Week 1
			Subsequent doses – 2mg/kg		Day 1	Weeks 2-12

OR

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with Her2 positive disease	Trastuzumab Loading Dose [HERCEP LOAD]	N/A	8 mg/kg/day	IV	Day 1 only	
	Trastuzumab [HERCEP]	N/A	6 mg/kg/day	IV	Day 1	Weeks 4, 7, 10
	Pertuzumab (Perjeta) Loading Dose	After Trastuzumab	840 mg/day	IV	Day1.	
	Pertuzumab (Perjeta) [PERTUZU MAB]		420 mg/day	IV	Day 1	Weeks 4, 7, 10
	Paclitaxel	N/A	80 mg/m^2	IV	Day 1	Weeks 1-12

OR

	Agent	Time	Dose	Route	Rx Days	ReRx
Patients with Her2 positive disease	Trastuzumab Loading Dose [HERCEP LOAD]	N/A	8 mg/kg/day	IV	Day 1	
	Trastuzumab [HERCEP]	N/A	6 mg/kg/day	IV	Day 1	Weeks 4, 7, 10
	Pertuzumab (Perjeta) Loading Dose	After Trastuzumab	840 mg/day	IV	Day 1	
	Pertuzumab (Perjeta) [PERTUZUMAB]		420 mg/day	IV	Day 1	Weeks 4, 7, 10
	Docetaxel	N/A	75 mg/m^2	IV	Day 1	Weeks 1, 4, 7, 10

Any dose adjustments or use of growth factors will be at the discretion of the treating medical oncologist.

Patients may receive their chemotherapy at Mayo Clinic or at a local institution. Treating medical oncologist will be asked to submit a summary of chemotherapy.

For Mayo Clinic Rochester (MCR) patients only: Patients must return to Mayo Clinic Rochester after completion of paclitaxel ± trastuzumab taxane based chemotherapy for a physical examination, MRI, and MBI. Patients will also undergo an image guided biopsy of any residual tumor.

For Mayo Clinic Arizona (MCA) and Mayo Clinic Florida (MCF) patients: After completion of taxane based chemotherapy, patients must return to MCA or MCF for a physical examination and MRI. Patients will also undergo an image guided biopsy of any residual tumor.

For patients who develop an allergy to paclitaxel, nab-paclitaxel may be substituted, using a dose of 100 mg/m^2 once weekly, to complete a total of 12 weeks of taxane-based chemotherapy (total number of weeks for paclitaxel and nab-paclitaxel is 12 weeks).

7.2 Neoadjuvant AC or FEC or EC phase – recommended treatment schedule

Note: The following recommended treatments are considered standard regimens for the neoadjuvant treatment of breast cancer. Therefore, oncologists should use one of the following anthracycline containing regimens. Note, for patients with HER2+ breast cancer, trastuzumab (but not pertuzumab)may be combined with FEC as indicated below. Trastuzumab and pertuzumab should not be combined with

doxorubicin, as no safety data exist. The starting doses and schedule of each of these drugs is listed below and considered standard in clinical practice

	Agent	Dose	Route	Rx Days	ReRx
All patients	doxorubicin	60 mg/m^2	IV infusion	Day 1	every 2 weeks x 4 with growth factor support **or** every 3 weeks x 4 (with our without growth factor support
	cyclophosphamide	600 mg/m^2	IV infusion		

OR

	Agent	Dose	Route	Rx Days	ReRx
All patients	5 – fluorouracil	500 mg/m^2	IV push	Day 1	every 3 weeks x 4
	Epirubicin	75-90 mg/m^2	IV infusion		
	cyclophosphamide	500 mg/m^2	IV infusion		

OR

	Agent	Dose	Route	Rx Days	ReRx
All patients	Epirubicin	75-90 mg/m^2	IV infusion	Day 1	every 3 weeks x 4
	cyclophosphamide	500 mg/m^2	IV infusion		

For Her2 positive patients:

	Agent	Dose	Route	Rx Days	ReRx
Her 2 positive	doxorubicin	60 mg/m^2	IV infusion	Day 1	every 2 weeks x 4 with growth factor support **or** every 3 weeks x 4 (with our without growth factor support
	cyclophosphamide	600 mg/m^2	IV infusion		

OR

	Agent	Dose	Route	Rx Days	ReRx
Her 2 positive	5 – fluorouracil	500 mg/m^2	IV push	Day 1	every 3 weeks x 4
	Epirubicin	75-90 mg/m^2	IV infusion		
	Cyclophosphamide	500 mg/m^2	IV infusion		
Her 2 positive**	Trastuzumab	2 mg/kg	IV infusion	Days 1, 8 and 15	Weekly for 12 weeks

** = **OPTIONAL** - given data from ACOSOG Z1041 there is ample safety data to combine trastuzumab along with the FEC regimen for patients with Her2 positive breast cancer. Trastuzumab should not be combined with AC.

MCR patients only: After completion of all chemotherapy and prior to surgical resection, patients will undergo a physical examination, venipuncture, unilateral diagnostic mammogram, breast and axillary ultrasound (if pre-treatment axillary ultrasound was abnormal), MRI and MBI to evaluate the extent of residual disease.

MCF and MCA patients: After completion of all chemotherapy and prior to surgical resection, patients will undergo a physical examination, venipuncture, unilateral diagnostic mammogram, breast and axillary ultrasound (if pre-treatment axillary ultrasound was abnormal) and MRI to evaluate the extent of residual disease.

7.3 Surgical Approach

Surgery MUST be performed within 10 weeks of completion of neoadjuvant chemotherapy.

Surgical resection of the primary tumor can be by either lumpectomy or mastectomy at the discretion of the treating surgeon and the patient's preference. Resection of the primary tumor to negative margins is recommended. Management of the axillary lymph nodes is also per surgeon discretion. Resection of positive lymph nodes is recommended.

7.4 Pathology

Surgical specimens should be evaluated for residual disease in the breast and in the lymph nodes and reported using the elements for calculation of Residual Cancer Burden (RCB). Tissue from any residual disease in excess of diagnostic tissue at time of surgical resection will be flash frozen and submitted for research purposes (see section 17.243). Residual tissue from the surgical bed should be collected for research purposes, even if no residual cancer is present. In the event that ER, PR, Her2 and Ki67 testing is not performed clinically, banked tissue from tumor registry (up to 8 unstained tissue slides) will be accessed for ER, PR, Her2 and Ki67 testing.

7.41 If patient has bilateral disease, residual disease/tissue from the study lesion (the lesion that was biopsied and for which patient derived xenografts were generated) will be submitted. Any residual contralateral disease (non-index lesion) will have one representative section paraffin embedded and stored (see section 17.243). MCA and MCF will ship the flash frozen specimen and the paraffin embedded section (if indicated) to MCR (see section 17.2).

8..0 Dosage Modification Based on Adverse Events

Any dose adjustments or modifications will be at the discretion of the treating medical oncologist.

9.0 Ancillary Treatment/Supportive Care

Use of growth factors will be at the discretion of the treating medical oncologist.

10.0 Adverse Event (AE) Reporting and Monitoring

10.1 Adverse Event Characteristics

CTCAE term (AE description) and grade: The descriptions and grading scales found in the revised NCI Common Terminology Criteria for Adverse Events (CTCAE) version 4.0 will be utilized for AE reporting. A copy of the CTCAE version 4.0 can be downloaded from the CTEP web site [redacted]

10.11 Adverse event monitoring and reporting is limited to the research aspects of this study that are associated with the ^{99m}Tc-sestamibi MBI. The patient will be visually monitored by the nuclear medicine technologist from the time of injection of the radionuclide until image acquisitions are completed. Tc-99m sestamibi is an approved drug with an excellent safety profile. It has been used

routinely on a daily basis in over 40 patients a day in the Department of Radiology at Mayo Clinic for the last 15 years with no recorded adverse effects. Any side effects that occur during the MBI will be reported by the MBI staff in a note to file and sent to the clinical research coordinator for submission to the IRB following Mayo Clinic IRB policy.

10.111 First, identify and grade the severity of the event using a copy of the CTCAE v4.0. Next, determine if the adverse event is related to the medical treatment or procedure.

10.112 Assessment of Attribution

When assessing whether an adverse event is related to a research procedure, the following attribution categories are utilized:

Definite – The adverse event *is clearly related* to the investigational agent(s).
Probable – The adverse event *is likely related* to the investigational agent(s).
Possible – The adverse event *may be related* to the investigational agent(s).
Unlikely – The adverse event *is doubtfully related* to the investigational agent(s).
Unrelated – The adverse event *is clearly NOT related* to the investigational agent(s)

10.2 Expedited Reporting Requirements for Adverse Events that Occur in a Non-IND/IDE trial within 30 Days of the Last Administration of a Commercial Imaging Agent [1, 2]

FDA REPORTING REQUIREMENTS FOR SERIOUS ADVERSE EVENTS (21 CFR Part 312)
NOTE: Investigators **MUST** immediately report to the sponsor **ANY** Serious Adverse Events, whether or not they are considered related to the investigational agent(s)/intervention (21 CFR 312.64)
An adverse event is considered serious if it results in **ANY** of the following outcomes:

1) Death
2) A life-threatening adverse event
3) An adverse event that results in inpatient hospitalization or prolongation of existing hospitalization for ≥ 24 hours
4) A persistent or significant incapacity or substantial disruption of the ability to conduct normal life functions
5) A congenital anomaly/birth defect.
6) Important Medical Events (IME) that may not result in death, be life threatening, or require hospitalization may be considered serious when, based upon medical judgment, they may jeopardize the patient or subject and may require medical or surgical intervention to prevent one of the outcomes listed in this definition. (FDA, 21 CFR 312.32; ICH E2A and ICH E6).

ALL SERIOUS adverse events that meet the above criteria **MUST** be immediately reported to the sponsor within the timeframes detailed in the table below.

Hospitalization	Grade 1 Timeframes	Grade 2 Timeframes	Grade 3 Timeframes	Grade 4 & 5 Timeframes
Resulting in Hospitalization ≥ 24 hrs	7 Calendar Days			24-Hour 3 Calendar Days
Not resulting in Hospitalization ≥ 24 hrs	Not required		7 Calendar Days	

Expedited AE reporting timelines are defined as:

- "24-Hour; 3 Calendar Days" – The AE must initially be reported via MedWatch within 24 hours of learning of the AE, followed by a complete expedited report within 3 calendar days of the initial 24-hour report.
- "7 Calendar Days" – A complete expedited report on the AE must be submitted within 7 calendar days of learning of the AE.

[1]Serious adverse events that occur more than 30 days after the last administration of investigational agent/intervention and have an attribution of possible, probable, or definite require reporting as follows:

Expedited 24-hour notification followed by complete report within 3 calendar days for:

- All Grade 4, and Grade 5 Aes

Expedited 7 calendar day reports for:

- Grade 2 adverse events resulting in hospitalization or prolongation of hospitalization
- Grade 3 adverse events

For studies using PET or SPECT IND agents, the AE reporting period is limited to 10 radioactive half-lives, rounded UP to the nearest whole day, after the agent/intervention was last administered. Footnote "1" above applies after this reporting period.

Effective Date: May 5, 2011

Additional Instructions:

1. An increased incidence of an expected adverse event (AE) is based on the patients treated for this study at their site. A list of known/expected Aes is reported in the package insert or the literature, including Aes resulting from a drug overdose.
2. [redacted]

Mayo Clinic Cancer Center (MCCC) Institutions: Provide copies, along with the UPIRTSO cover sheet, by fax [redacted] to the MCCC Regulatory Affairs Unit (RAU) Risk Information Specialist who will determine and complete IRB reporting. The RAU will submit to the MCCC SAE Coordinator.

10.3 Adverse events to be graded after ^{99m}Tc-sestamibi MBI is performed (see Section 4.0) per Common Terminology Criteria for Adverse Events (CTCAE) CTEP Version 4.0 grading unless otherwise stated:

10.31 Submit via appropriate MCCC Case Report Forms (i.e., paper or electronic, as applicable) the following Aes experienced by a patient:

10.311 Grade 2 AEs deemed *possibly, probably, or definitely* related to ^{99m}Tc-sestamibi MBI.

10.312 Grade 3 and 4 AEs regardless of attribution to ^{99m}Tc-sestamibi MBI.

10.313 Grade 5 AEs (Deaths)

10.3131 Any death within 30 days of completion of chemotherapy or surgery

11.0 Disease Evaluation

11.1 Neoadjuvant chemotherapy phase

11.11 Ki67 response for those patients whose pre-treatment baseline Ki 67 level ≥ 15%.

Ki67 levels will be obtained from a breast biopsy samples taken prior to chemotherapy, at the completion of paclitaxel as well as at the completion of anthracycline therapy.
A Ki67 response is defined as a Ki67 value < 15% in the invasive component of the biopsy specimen or a biopsy specimen composed of neurotic and/or vacuolated cells (evidence of treatment effect) rendering Ki67 level undeterminable. OR a breast biopsy sample that contains no invasive disease will be considered to achieve a Ki-67 response.

For those patients where no area of disease is seen under ultrasound and, as such, the radiologist chooses not to biopsy the breast, these patients will be considered to achieve a Ki-67 response if central review of the 12 week MRI indicates a radiographic response (see 11.12)

11.12 Radiographic response (regardless of pretreatment Ki67 level)

Breast imaging will be performed at the completion of paclitaxel. Radiographic response is defined as a 30% or more decrease in the longest diameter of the lesion from pre-treatment size.

11.2 Surgery

11.21 Pathological response

A pathologic complete response is defined as no histologic evidence of invasive tumor cells in the surgical breast specimen and sentinel and/or axillary lymph nodes.

11.22 Residual Burden of Disease

RCB score is based upon four parameters of the residual tumor pathology review: the size of the residual tumor bed, the proportion of the tumor bed that contains invasive cancer, the number of axillary lymph nodes containing disease, and the diameter of the largest metastasis in an axillary lymph node.

Determination of Residual Cancer Burden (RCB) is described at [redacted]

11.23 Ki67 response for those patients whose pre-treatment baseline Ki67 level ≥ 15%.

A Ki67 response is defined as a Ki67 value < 15% in the invasive component of the pre-surgical biopsy specimen or a pre-surgical biopsy specimen composed of

neurotic and/or vacuolated cells (evidence of treatment effect) rendering Ki67 level undeterminable.

11.24 Radiographic response

Breast imaging will be performed at the completion of NAC. Radiographic response is defined as a 30% or more decrease in the longest diameter of the lesion from pre-treatment size.

11.3 Post Surgery

11.31 Local recurrence

Local recurrence is defined as histologic evidence of ductal carcinoma *in situ* or invasive breast cancer in the ipsilateral breast or ipsilateral chest wall.

11.32 Regional recurrence

Regional recurrence is defined as the cytologic or histologic evidence of disease in the ipsilateral internal mammary, ipsilateral supraclavicular, ipsilateral infraclavicular and/or ipsilateral axillary nodes or soft tissue of the ipsilateral axilla.

11.33 Distant recurrence

Distant recurrence is defined as the cytologic, histologic, and/or radiographic evidence of disease in the skin, subcutaneous tissue, lymph nodes (other than local or regional metastasis), lung, bone narrow, central nervous system or histologic and/or radiographic evidence of skeletal or liver metastasis.

11.34 Second primary breast cancer

Second primary breast cancer is defined as histologic evidence of ductal carcinoma *in situ* or invasive breast cancer in the contralateral breast or contralateral chest wall.

11.35 Second primary cancer (non-breast)

Any non-breast second primary cancer other than squamous or basal cell carcinoma of the skin, melanoma *in situ*, or carcinoma *in situ* of the cervix is to be reported and should be confirmed histologically whenever possible.

11.36 Death

Underlying cause of death is to be reported.

12.0 Molecular Breast Imaging (MCR Participants Only)

12.1 Molecular Breast Imaging procedure

All patients will undergo MBI with 99mTc-sestamibi prior to start of neoadjuvant chemotherapy, at time of switching from paclitaxel ± trastuzumab to AC or FEC chemotherapy and after completion of neoadjuvant chemotherapy prior to surgery.

For each scan, all patients will undergo cranio-caudal (CC) and medio-lateral oblique (MLO) views of the breast with the suspected lesion using a dual-detector MBI system at 5 minutes post-injection of 4-8 mCi ^{99m}Tc-sestamibi. A CC and MLO view of the contralateral breast will also be performed.

12.2 Evaluation of MBI

A radiologist will review the MBI study at each time point. Each exam will be interpreted in a blinded fashion using the established MBI lexicon [15]. The background parenchymal radiotracer uptake, number of lesions and their location, size of each lesion in three dimensions, intensity of radiotracer uptake, and associated findings will be evaluated and recorded. Final assessment codes, modeled after the Breast Imaging Reporting and Data System (BI-RADS) will be assigned on a per-breast basis. The interpreting radiologist will then be un-blinded and will correlate MBI findings with any available mammogram/ultrasound/MRI results.

Tumor uptake of ^{99m}Tc-sestamibi in each lesion will also be quantified using software developed in-house. Algorithms for calculation of tumor uptake from opposing views of the breast have been validated in phantom models and computer simulations [16]. This will allow calculation of true tumor / background ratio.

12.3 Aims of MBI studies

We will evaluate if tumor size can be satisfactorily assessed by MBI, if tumor size from MBI corresponds to tumor size evaluation by conventional imaging procedures (mammography, ultrasound, and MRI), and tumor size found at surgery, and if there are histopathologic features or biomarkers that correlate with tumor uptake of ^{99m}Tc-sestamibi.

The aims of this study are to a) determine the sensitivity of ^{99m}Tc-sestamibi for the evaluation of tumor size and function, b) to determine whether early changes in radiotracer uptake predicts eventual pathologic response, and c) to determine the correlation between tumor uptake and tumor grade and proliferation.

12.4 Participation in other MBI studies

If a patient meets the inclusion criteria for more than one MBI-related protocol, we will ask the patient if he/she is willing to provide authorization for use of their MBI images from this study in the other MBI-related protocols. A check box on the Patient Consent form is provided to document whether the patient has specifically agreed to this request.

If a patient agrees to participate in any additional research studies involving an MBI scan, the results from this study may be used in those protocols in lieu of performing an additional MBI scan provided that the MBI scan from this study meets the inclusion

criteria of the other study. This will eliminate unnecessary duplication of the MBI procedure in these patients.

13.0 Follow–up Decision at Evaluation of Patient

13.1 Prior to the drawing of any pre-treatment research bloods or MBI imaging (if applicable)

13.11 If a patient withdraws consent for any reason before any pre-treatment research bloods are drawn or MBI imaging is done, the patient will be considered a cancel. On-study material and the End of Active Treatment/Cancel Notification Form must be submitted. No further follow-up is to be done

13.2 Neoadjuvant chemotherapy phase

13.21 Patients who discontinue neoadjuvant chemotherapy due to toxicity or progression and choose to go directly to surgery will continue on study per protocol.

13.22 Patients who discontinue neoadjuvant chemotherapy due to:

- Tumor progression and/or toxicity on the taxane that do not go to anthracycline
- Tumor progression and/or toxicity on anthracycline that do not go to immediate surgery
- Refusal to continue study participation
- Intercurrent illness or pregnancy
- Administration of alternative anti-neoplastic therapy

will go off study. All data up until and including the point of discontinuation must be submitted. No further follow-up is required.

Future treatments are at the discretion of the patient's treating physician.

13.23 Patients who do not receive the recommended treatment sequence of taxane based chemotherapy followed by anthracycline based chemotherapy will go off study. All data up until and including the point of discontinuation must be submitted. No further follow-up is required.Future treatments are at the discretion of the patient's treating physician.

13.3 Surgery phase

13.31 Patients who are not considered surgical candidates or who refuse to undergo surgery will go off study. All data up until and including the point of determination the patient is not a surgical candidate or the patient refuses to undergo surgery must be submitted. No further follow-up is required. Future treatments are at the discretion of the patient's treating physician.

13.4 Post surgery phase

13.41 Patients whose disease could not be completely resected will go off study after post–operative visit. All data up until and including the post-operative visit must be submitted. No further follow-up is required. Future treatments are at the discretion of the patient's treating physician.

13.42 Patients who refuse to continue in the observation phase will go directly to event monitoring until death or a maximum of 6 years post-surgery.

13.5 Any point in the study

13.51 If a patient is found to be ineligible by the Data Center, that is, it is determined that at the time of registration, the patient did not satisfy each and every eligibility criteria for study entry. The patient will go off study and all data up until the point of confirmation of ineligibility must be submitted.

14.0 Body Fluid Biospecimens

14.1 Summary Table of Research Blood and Body Fluid Specimens to be Collected for this Protocol

Correlative Study (Section for more information)	Indicate if specimen is Manda-tory or Optional	Blood or Body Fluid being Collected; processed and submitted by participating site	Type of Collec-tion Tube (color of tube top)	Volume to collect per tube (# of tubes to be collected)	After registration but prior to start of taxane	After completion of taxane but prior to the start of anthracy-cline based chemo-therapy (cycle 1)	After comple-tion of anthracycline based chemotherapy, but prior to surgery (cycle 2)	At first recurrence or post treatment if primary surgery is not to be performed	Pro-cess at site? (Yes or No)	Additional processing required at site after blood draw?	Temper-ature Conditions for Storage /Shipping
Visit Alias – Lab Use Only					**V1B (Visit 1 Blood)**	**V2B (Visit 2 Blood)**	**V3B (Visit 3 Blood)**	**V4B (Visit 4 Blood)**			
Pharmaco-genetics	Mandatory	Blood	EDTA (purple)	10 mL (1)	X				No	Yes	Refrigerated or Wet ice
Circulating Tumor Cells	Mandatory	Blood	EDTA (purple)	10 ml (1)	X	X	X	X	No	No	Ambient
Circulating Tumor Cells	Mandatory	Blood	CellSave Tube (purple and yellow striped)	20 ml (2)	X	X	X	X	No	No	Ambient
Proteomics	Mandatory	Blood	EDTA (purple)	10 ml (1)	X	X	X	X	No	No	Refrigerated or Wet ice
Lymphocytes	Mandatory	Blood	Na Heparin (green)	10 mL (1)	X				No	No	Refrigerated or Wet ice
RNA	Mandatory	Blood	Paxgene (2)	2.5 ml	X	X	X	X	No	No	Ambient

14.2 Blood/Blood Products

14.21 **Mayo Clinic Rochester will use Special Study Cards in place of kits.**

All MCR samples must be collected **Monday-Friday.**

14.22 Kits will be used at Mayo Clinic in Arizona and Mayo Clinic in Florida for this study.

All specimens must be collected and shipped **Monday – Thursday ONLY. Sample processing must be completed by 3 pm on Thursday for overnight shipping to MN.**

14.23 Label specimen tube(s) with protocol number, study patient ID number, and time and date blood is drawn.

14.24 Collect and process all blood/blood products according to the table in 14.1.

14.241 Gently invert EDTA purple top and Na Heparin tubes 8 to 10 times and place on wet ice for transport to BAP Hilton CL-21. Gently invert the CellSave tubes 8 to 10 times (keep CellSave and 1 EDTA tubes ambient) and transport to BAP Hilton CL-21.

14.242 The CellSave tubes and (1) EDTA 10 mL purple top tube will be forwarded to Mike Campion at Hilton 10-66 for processing. The CellSave tubes will be processed per manufacture's protocol for capture and enumeration of CTCs. Briefly, a ferrofluid reagent conjugated with antibodies to the epithelial cell adhesion molecule (EpCAM) is used to immunomagnetically separate epithelial cells. These captured cells are then stained with DAPI, cytokeratin 8, 18,19, and CD45 to help distinguish true CTCs from leukocytes. Cells that are DAPI+/CD45-/CK+ are considered circulating tumor cells. The EDTA tube will be processed per manufacture's protocol for capture of epithelial cells. Briefly, this protocol is the same as the above protocol, however there is no staining of the captured cells. Any captured cells can be processed for molecular testing as seen appropriate. In this study, the captured cells will be frozen for future testing.

14.243 BAP will process specimens as follows:

Record receipt of specimens.

DNA will be isolated from 8 mLs EDTA whole blood. 2 runs of DNA will be done. DNA will be stored at 4°C until a box is full and then transferred to and stored in a −80°C freezer.

Extract Lymphocytes from **Na Heparin** whole blood using EBV slow freeze. Lymphocytes will be stored until the end of the study or upon request.

EDTA plasma will be collected from one EDTA tube. Centrifuge at lab standards and aliquot 1 mL into 4 aliquots, transfer WBC's into cryovial and freeze at -80° C.

CTC's, (2) CellSave 10 mL tubes, and (1) EDTA 10 mL purple top tube will be forwarded to Mike Campion at Hilton 10-66 for processing.

RNA (2) 2.5 mL PAXgene tubes will be collected. BAP will process RNA from (1) 2.5 mLPAXgene tube and will store the second 2.5mL PAXgene tube at -80°C.

14.244 Blood samples for MCA and MCF will be shipped to MCR for processing. Samples need to arrive within 24 hours of collection. The CellSave tubes and (1) EDTA tube must be kept ambient will be shipped to BAP Rochester and BAP will send to [redacted]. For CTC processing. Green tube and EDTA tube for lymphocytes and proteomics and pharmacogenomics will be shipped to BAP (refrigerated).

PAXgene tubes will be shipped to BAP ambient temperature.

14.3 Collection and Processing

14.31 Overview of pharmacogenomic study

Specimens (breast tumor and blood) will be obtained at initial time point prior to any chemotherapy. Additional tumor specimens will be obtained after completion of paclitaxel ± trastuzumab chemotherapy (prior to AC or FEC chemotherapy) and again at the time of definitive surgical resection (after completion of all chemotherapy) for sequencing. Additionally tissue from biopsy of any local or distant recurrence will undergo sequencing.

Blood will be used to isolate DNA for a series of high throughput assays, including genome wide SNP analysis using 2.5 M Illumina Omini chips and exome sequencing.

Blood will be collected and processed for DNA and lymphocyte isolation and proteomics at the [redacted]. DNA will be used for exome sequencing in the [redacted]b. DNA will also be sent for obtaining genome-wide SNPs at the [redacted] lab.

Lymphocytes will also be collected for future generation of lymphoblastoid cell lines that will be used in functional studies to assess the effect of germline polymorphisms on protein function and response to therapy.

Plasma will be collected for future proteomics.

Appendix V addresses the issue of reporting the results of germline DNA analyses to the patient.

14.32 Overview of Circulating Tumor Cells

Specimens will be obtained prior to initiation of chemotherapy, after completion of paclitaxel ± trastuzumab chemotherapy (prior to AC or FEC chemotherapy) and again after completion of all chemotherapy and at time of any disease recurrence. Blood will be used to measure the number of circulating tumor cells (CTCs) and isolate the intact CTCs for further testing. DNA isolated from the CTCs will be used for genotyping and comparison with the primary tumor.

14.33 Overview of RPPA

After RNA processing and collection in BAP lab, an aliquot of RNA from baseline (prior to chemotherapy), after completion of paclitaxel ± trastuzumab prior to AC or FEC chemotherapy) and from the time of surgery and from time of recurrence will be shipped to the Harris Lab at the University of Minnesota for quantitative expression profiling of APOBEC3B mRNA levels.

14.4 Shipping and Handling

14.41 Shipping Specimens

Verify ALL sections of the Blood Specimen Submission Form (see Forms Packet), BAP Requisition Form (provided in kit) and specimen collection labels are completed and filled in correctly.

Ship (1) EDTA and Na Heparin tubes with a properly prepared cold pack. CellSave and (1) EDTA tubes will be shipped ambient at the top of the refrigerated shipper. See kit instructions for specific details for cold pack preparation (i.e., frozen or refrigerated) and proper packing of blood and cold pack to avoid freezing of specimens.

Ship specimens, via Priority Overnight service, **Monday – Thursday ONLY,** to BAP Receiving according to the kit instructions. **Do not send samples on weekends or just prior to federal holidays.**

The BAP kits will include a smart shipper label (3x5 white barcoded label) affixed to the shipping boxes. The smart shipper label is a pre-addressed return label, which replaces the need for an airbill. Shipping costs will be covered by BEAUTY if the shipping box provided with the BAP kit is used for shipping specimens to BAP Freezer.

BAP Freezer will receive the samples and immediately forward specimens to the [redacted]

14.42 Handling Specimens

Samples will be stored in BAP.

14.5 Background and Methodology

14.51 Genome wide SNP analysis

2.5 M Illumina Omni chips will be used to obtain SNPs across the entire genome. These SNPs will serve as a quality control for both exome sequencing performed with the same DNA samples and that with tumor DNA. It will also serve as a base for further imputation to obtain SNPs outside exons, i.e. regulatory regions. The genome wide SNPs and imputed SNPs will then be examined in terms of its association with response phenotype.

Sequencing will be analyzed through the pipelines created in the Mayo bioinformatics Core. All the sequencing results and genome wide SNP results will be transferred to the statistical group for quality control and statistical analyses.

14.52 Exome sequencing

DNA isolated from blood will also be used to perform exome sequencing. The sequencing results will be compared with sequencing results of tumor DNA from the same patient. The comparison will allow us to identify tumor specific mutations in individual samples.

14.53 Future whole genome sequencing

If the price becomes affordable during the period of this project, we will apply whole genome sequencing instead of exome sequencing of DNA isolated from individual blood samples.

14.54 Ki 67 methodology and scoring

Immunohistochemical staining of the Ki67 antigen will be performed in formalin-fixed paraffin-embedded tissue sections using the MIB-1 clone on either a Leica Bond III or Ventana BenchMark-XT automated stainer. Image acquisition and analysis will be performed using the Dako ACIS® III or the NanoZoomer Digital Pathology (NDP) 2.0HT instrument (Hamamatsu Corporation) along with the Definiens Tissue Studio software package version 3.6. The percentage of positive staining tumor nuclei are quantitated by software after tumor selection by the technician. Results of tumor staining and appropriate controls will be reviewed by a pathologist.

14.55 Assessment of Clonality

[redacted] has developed a methodology to adapt genomic DNA isolated from flow cytometrically purified nuclei for use with genomic analyses including array comparative genomic hybridization (aCGH) and next-generation sequencing (NGS). Using this methodology, efficient high-resolution genomic analysis of clonal populations from patient biopsies is possible.

We plan to apply this approach to analyze distinct clonal tumor populations within clinically annotated BEAUTY breast cancer specimens in order to study their evolution under the selective pressure of therapy and to evaluate potential mechanisms of therapy resistance. In addition we will apply these same methods to study a set of tumor grafts derived from patients enrolled in BEAUTY.

Our initial focus will be comparing tissue obtained at the time of surgery from those patients with significant residual disease to those with partial and those

with complete response. Using samples from patients with varying degrees of response to standard chemotherapy, we will perform the following:

1) Utilize core biopsies obtained at baseline (prior to chemotherapy) and 12 weeks (after completion of paclitaxel), as well as any residual tumor tissue collected at the time of surgery. These tissues will be assessed by the study BEAUTY pathologist for determination of tumor cellularity, using an H&E cut from a cryosection (for baseline and 12 weeks). For the surgical specimen, a determination of tumor cellularity has already been conducted, and therefore the best surgical specimen will be utilized.

2) For those patients with established xenografts, we will additionally send tumor samples derived from the 2nd generation of tumor growth. If xenografts are available from both baseline and surgery, tissues from both time-points will be sent.

3) Following confirmation of tumor cellularity, a portion of each of these specimens from these 3 time points and the corresponding xenografts will be shipped to [redacted]

The remaining portion of the tumor will be retained here at Mayo Rochester for RNA seq.

4) For the portion of the tumor sent to [redacted], separation of the aneuploid and diploid populations will be performed using DAPI-based DNA content measures. Following this, [redacted] will profile each population with aCGH.

5) The diploid populations and any remaining tissue will be shipped to Mayo Rochester for further studies analyzing T cell receptors (collaboration with [redacted]. The DNA from sorted tumor populations will be submitted to [redacted] lab here at Mayo Clinic for sequencing.

14.56 T cell sequencing profile

Infiltration of immune cells including tumor-infiltrating T lymphocytes (TILs) in breast cancer tissues, and higher level of infiltration is considered to be a predictor of better clinical outcome of cancer patients (1). For example, the increased level of TILs in cancer tissues has been suggested as an independent predictive marker of response to neoadjuvant chemotherapy in breast cancer (2). However, most of studies are based on quantification of total TILs in tumors without examination of their functional characteristics that confer anti-tumor immune effects.

T cell receptor (TCR) is primarily a heterodimer of alpha (TCRA) and beta (TCRB) chains. These two genes cause rearrangement between VJ (alpha) and VDJ (beta) segments and determine functional diversity to recognize epitope of target cells. With the progress in next generation sequencing technologies, it has become possible to characterize millions of receptor clones representing the TCR repertoire of T cells from ~10 ml of blood or from cancer tissues (3). The TCR sequencing would be an effective way to examine the antigen specific expansion of T-cell subclones and in turn can help to characterize functional TILs of interest in response to cancer treatment.

The presence or absence of poly-(or oligo-) cloncal T cell population that may be associated with response to neoadjuvant chemotherapy will be investigated. The TCRA and TCRB cDNA of TILs in tumor biopsies that are collected before and after chemotherapy will be sequenced. To obtain cDNA samples of T lymphocytes, total RNAs will be isolated from peripheral blood mononuclear cells using Trizol reagent (Life Technologies, Carlsbad, CA) and then reversely-transcribed into cDNAs using random primers. The synthesized cDNA samples will be amplified by polymerase chain reaction (PCR) to amplify the TCRA and TCRB cDNA with specific primer sets. The obtained amplicons will be ligated to adaptors, and further amplified onto the proprietary Ion Sphere particles by emulsion PCR, and then sequenced on the Ion Torrent PGM sequencer (Life Technologies, Carlsbad, CA). These experiments will be conducted in the University of Chicago Medical Center ([redacted]

In this study, we plan to apply the next generation sequencing technology to profile TCR repertoire of T lymphocytes that could be related with response to neoadjuvant chemotherapy in breast cancer. Our findings will provide new insights for the establishment of immune markers predictive to neoadjuvant chemotherapy or can be applied as the surrogate biomarkers of monitoring patient's response during treatment.

To compare TCR profiled between two groups, 30 patients total, will be necessary, aiming for 15 each of responders and non-responders to neoadjuvant chemotherapy. The separated plasma for the proteomic samples and stored the frozen WBC aliquots (in storage), from both from baseline and surgery will be sent to [redacted] for sequencing.

Circulating T cells in blood will be examined as control TCR profiles of individual patients. To obtain cDNA samples of T lymphocytes, total RNAs will be isolated from peripheral blood mononuclear cells using Trizol reagent (Life Technologies, [redacted] and then reversely-transcribed into cDNAs using random primers. The synthesized cDNA samples will be amplified by polymerase chain reaction (PCR) to amplify the TCRA and TCRB cDNA with specific primer sets. The obtained amplicons will be ligated to adaptors, and further amplified onto the proprietary Ion Sphere particles by emulsion PCR, and then sequenced on the Ion Torrent PGM sequencer ([redacted] These experiments will be conducted in the University of Chicago Medical Center [redacted]):

[redacted]

Sequencing results will be analyzed by bioinformatics collaborator, [redacted]

From the sequencing data, the entire TCR profile will be compared between (1) blood and cancer tissue before treatment (clonality of TIL could be judged), (2)

blood and cancer tissue after treatment (clonality of TIL could be judged), and (3) between two tissues before and after the treatment (enrichment of certain T cell population(s) in cancer tissue and their relation with drug response can be examined). In addition, if the enrichment of specific type of TCR sequence(s) is identified, the information may be applied as immune markers to predict response of neoadjuvant chemotherapy.

DNA sequencing results will be abstracted onto a secure database which will be maintained by [redacted] lab. The whole system will be protected by password and a backup disk will be kept in a locked file cabinet.

14.57 APOBEC3B

The human DNA deaminase APOBEC3B is a newly discovered genetic marker for breast cancer, with expression studies demonstrating its over-expression in ~60% of breast cancers ([redacted] in process). APOBEC3B mutates the genomic DNA of breast cancer cells causing an overall increase in mutation frequency ([redacted]), in process). It is highly likely that this mutator activity contributes to the heterogeneity of breast cancer and in turn to negative outcomes (i.e. higher APOBEC3B = faster tumor evolution = more likely to evolve resistance to therapy and immune responses). Here, we will take an unbiased approach to testing this in the context of this protocol. Quantitative expression profiling of APOBEC3B mRNA levels will be performed in comparison to those of the endogenous housekeeping gene TBP [17]).

14.58 Proteomic studies

As part of ongoing research, we will collect plasma for proteomic studies, according to patient consent information. Samples will be frozen at −70ºC by BAP.

RPPA [redacted])
Reverse phase protein array (RPPA) is a high-throughput antibody-based technique developed for Functional Proteomics studies to evaluate protein activities in signaling networks. Protein extracted from tumor samples will be analyzed for multiple proteins important in drug response and resistance. A list of the proteins is included in Appendix III.

14.59a Homologous recombination assay ([redacted])

Homologous recombination (HR) is a critical pathway important in the repair of double and single stranded DNA. Both hereditary and somatic alterations in genes responsible for HR are implicated in predisposition to cancer, but additionally, genetic alterations in this pathway appear to be critically associated with response to chemotherapy drugs which

target genes in the HR pathway. For example, mutations in BRCA1 and BRCA2 have been demonstrated to have potential therapeutic relevance in the setting of DNA damaging agents and cells deficient in BRCA1 or BRCA2 are sensitive to PARP inhibitors.

Myriad developed a DNA-based HR score (HRD) based on genome-wide LOH analysis of ovarian tumors combined with comprehensive profiling of BRCA1 and BRCA2 defects. Recently, Telli et al assessed the ability of the HRD score to predict pathologic response to neoadjuvant platinum-based therapy in early-stage triple-negative and BRCA1/2 mutation-associated breast cancer and demonstrated that germline BRCA1/2 mutation status was known in all patients and 13/55 tumors were from carriers of deleterious BRCA1 (n = 9), BRCA2 (n = 3) or BRCA1&2 (n = 1) mutations. There was a statistical association between response to treatment and HRD score (p= 0.004).

Currently, a standard treatment employed in BEAUTY for high risk locally advanced breast cancer is taxane and anthracycline-based therapy. However, there are no data regarding the role of HRD and either taxane response or response to AC therapy. Therefore, a key goal of these translational studies will be to assess the response to individual chemotherapy regimens according to HRD score.

14.59b Analysis of estrogen receptor beta (ERβ) expression

ERβ is a member of the nuclear receptor superfamily of proteins that functions as a ligand mediated transcription factor and is classically thought of as a tumor suppressor in multiple cell and animal model systems. ERβ is known to be highly expressed in normal breast epithelial cells and tends to decrease in expression levels during progression from atypical hyperplasia to DCIS to breast cancer. However, substantial levels of ERβ protein are detectable in about 30-40% of all sub-types of breast cancer. For these reasons, ERβ is being considered as a novel therapeutic target.

Recent studies have also demonstrated that ERβ expression can sensitize breast cancer cells to the anti-cancer effects of multiple chemotherapy drugs. Ongoing studies from the Hawse laboratory have also shown that ERβ can suppress TGFβ expression in triple negative breast cancer cells, a pathway that is known to lead to the development of chemotherapy resistance. For these reasons, it is of interest to determine if ERβ positivity is associated with xenograft take rates and/or response to chemotherapy in triple negative breast cancer patients.

To determine ERβ protein levels in breast tumors, a 5 micron section will be stained with a highly specific and sensitive mouse monoclonal antibody (PPG5/10). IHC stains will be performed on a Leica Bond III stainer using a 1:75 dilution of this antibody purchased from Thermo Scientific. IHC stained slides will be analyzed by a dedicated breast cancer pathologised and scored based on nuclear extent and intensity of staining. A sum score will be calculated based on the estimated percentage of positive cells (less than 1% will be considered negative (0), 1%-25% (1), 26%-50% (2), 51%-75% (3) and 76%-

100% (4)) and the intensity of staining (none (0), weak (1), moderate (2) or strong (3)). Tumors will be grouped into ERβ negative/low (1-2), ERβ moderate (3-5) and ERβ high (6-7) using this sum score.

14.59c Human Leukocyte Antigen (HLA) Typing

Although the complex relationship between immune function and neoplastic cells is important in both carcinogenesis and cancer progression, at this time it is not fully understood. On average, a malignant cell will accumulate 10 mutations that alter the antigenic profile of the tumor and can be presented by class I major histocompatibility complex (MHC) molecules to CD8+ T cells. In addition, tumors often express proteins that aren't normally expressed by that cell type. Furthermore, tumor cells may undergo apoptosis, which can elicit an immune response, or can become senescent, resulting in the production of pro-inflammatory cytokines. Due to these changes, the host immune system can recognize and destroy cells that have diverged from normal development or are becoming malignant. It has been suggested that tumors develop or progress in part as a result of loss of immunostimulatory antigens or suppression of the immune response.

The Human Leukocyte Antigen (HLA) gene family is located on chromosome 6 and encodes for a group of related proteins that are utilized by the immune system to distinguish self proteins from foreign/non-self proteins. *HLA-A, HLA-B,* and *HLA-C* are class I genes and encode for proteins present on nearly all cells in humans, while *HLA-DPA1, HLA-DPB1, HLA-DQA1, HLA-DQBI, HLA-DRA,* and *HLA-DRB1* are class II genes and encode for proteins that are present primarily on the surface of immune system cells. Due to a high degree of sequence homology and the density of variable nucleotides, this region has been challenging to sequence. Therefore, we will use a software package to assign HLA types using the previously generated exome sequencing data. In order to validate that the software is working appropriately in the BEAUTY population, we will compare the results generated by the software package with HLA-typing performed in the Tissue Typing Laboratory at Mayo Clinic in a subset of BEAUTY patients. If there is good concordance, the HLA types generated by the software will be used to test for an association with breast cancer subtypes as well as response to chemotherapy using the various response phenotypes generated in the BEAUTY study.

14. 59d Evaluation for clonal hematopoiesis of indeterminate potential

Clonal hematopoiesis of indeterminate potential (CHIP) is defined as somatic mutations in hematopoietic cells in an individual with normal hematologic indices and without an underlying hematologic disorder. Whole exome sequencing in the normal population demonstrates that CHIP is an age-related phenomenon. CHIP is rarely identified in individuals younger than 40, but the incidence rises to more than 10% in persons above the age of 70.[18] Mutated genes are involved in regulating DNA methylation, hydroxyl-methylation, chromatin remodeling, splicing, tumor suppression and signal transduction, with frequently mutated genes including *DNMT3A*, *TET2,* and *ASXL1.* In large population studies, the presence of these premalignant clones was associated with an increased risk of developing a future hematologic malignancy, coronary heart

disease, and all-cause mortality.[18, 19] Further, in small retrospective studies of patients with solid organ or hematologic malignancy discovered that patients harboring CHIP at the time of primary malignancy diagnosis have an increased risk of developing a subsequent therapy-related myeloid malignancy (neither of these studies included patients with breast cancer).[20, 21] We further hypothesize that breast cancer patients with these abnormalities in hematopoietic cells may additionally experience more neutropenia and thrombocytopenia during chemotherapy. The correlative studies we propose aim to expand on the limited existing data on the incidence of CHIP in patients diagnosed with breast cancer, and explore the potential impact of CHIP on the development of cytopenias during treatment and subsequent therapy-related myeloid neoplasms.

Whole exome sequencing results will be analyzed by the Beauty team bioinformaticians in collaboration with [redacted]. To determine the incidence of CHIP, we will evaluate 74 genes identified in the Supplemental Table S3 of the Jaiswal et al. 2017 study in the peripheral blood specimens collected prior to neoadjuvant chemotherapy. We will utilize two variant detection programs, MuTect2 and VarDict to detect CHIP mutations. Variants will be annotated using the Ensembl Variant Effect Predictor program. Both callers have been reported to provide sensitivity in detecting low-allelic fraction variants while retaining respectable specificity in comparative evaluations. In patients where somatic mutations consistent with CHIP are identified, we will evaluate pre-treatment hematologic indices, and if within references ranges, confirm a diagnosis of CHIP. We will subsequently evaluate the entire cohort to correlate the presence of CHIP with delays or dose reductions in chemotherapy due to cytopenias, delayed count recovery after treatment, and developing therapy-related myeloid neoplasms.

15.0 Information concerning research procedures

None

16.0 Statistical Considerations and Methodology

16.1 Study Design and Specimen Collection

A prospective cohort study is being conducted to collect biospecimens before during and after completion of neoadjuvant chemotherapy in participants with Stage I-III breast cancer to identify novel somatic changes within gene and gene pathways that are potentially "druggable."

A minimum of 200 and a maximum of 230 participants will enroll onto this study to ensure at least 200 eligible patients with sufficient pre-neoadjuvant treatment tissue and a post-initiation neoadjuvant treatment tissue is available for analysis.

16.2 Analysis Plans

16.21 Pre-processing of genomic data

16.211 Exome sequencing data

Processing of the sequence reads will be done by mapping the short reads to the genome using Novoalign, followed by re-alignment and recalibration for greater efficiency in SNV calling. Gemline SNV calling for each sample will be completed using the union between GATK's Unified Genotyper and SNVMix. Somatic mutations will be identified using the union of SomaticSniper, JointSNVMix2, MuTect, and GATK' Somatic Indel detector – all of which simultaneously use the tumor and germline information to determine somatic status. We will then go back and use BEAGLE to refine and call missing genotypes using LD based imputation methods. All germline/somatic SNV and small insertions/deletions will be thoroughly annotated using BioR – which includes SIFT, PolyPhen2, and allele frequencies from numerous populations. Copy number variation will be assessed through PatternCNV – Mayo's in-house solution to derive copy number data from exome capture studies. Finally, using data from the 2.5 Million variant Illumina Omni chip, we will estimate variants outside of the exons using the 1000 Genomes Project Data and state of the art imputation methods that utilize linkage disequilibrium.

Variants will initially be prioritized based on whether they exist in genes relevant for breast cancer therapy (germline or somatic) or familial breast cancer risk (germline). These small lists of genes are reported in a user friendly HTML-based report that allows rapid, easy, and simplified access to genomic variants that have the most potential for clinical implications.

16.212 RNAseq data

For paired-end RNA-Sequencing data analysis, we will use MAP-RSeq - a comprehensive computational workflow developed at Mayo Clinic to obtain a variety of genomic features from an RNA seq experiment (http://bioinformaticstools.mayo.edu/research/maprseq/). MAP-RSeq uses a variety of freely available bioinformatics tools along with in-house developed methods. The main goal of the MAP-RSeq workflow is to align, assess and deliver multiple genomic features. These include gene expression, novel transcripts, alternative splice sites, expressed SNVs, fusion transcripts, long non-

coding RNAs, small insertions, small deletions, etc. Alignment of reads will be performed using Bowtie. Bowtie is a fast and memory efficient short sequence aligner. It aligns reads to the genome and maps them to a genome assembly. Unaligned reads from Bowtie are used by TopHat to map them to splice junctions. The aligned bam file from TopHat is used by HT-Seq software to calculate gene abundance per sample. Along with binary alignment (bam) and junctions bed file, TopHat also provides a list of expressed fusion transcripts using the TopHat-Fusion algorithm.

At [redacted], we have also developed a novel computational method (ESNV-Detect) to identify expressed single nucleotide variants from paired-end RNA-Seq data. We use a combination of open access bioinformatics tools along with our in-house developed method to identify expressed single nucleotide variants (eSNVs) from RNA-Seq data confidently. ESNV-Detect uses consensus data from multiple aligners to call eSNVs. Features like number of reads at a nucleotide position, reference supporting reads, alternate allele reads, forward strand supporting reads, reverse strand supporting reads, base qualities, mapping qualities etc. are used to call an expressed SNV. A variety of sources such as 1000 genome, dbSNP, and 5400 exome databases are used to annotate and identify known and novel variants from RNAseq datasets.

Detailed QC reports, gene expression counts, fusion transcripts data and eSNVs will be obtained from MAP-RSeq and ESNV-Detect for each patient before and after therapy for further analysis. As a QC method, the concordance/discordance between the eSNVs detected from the RNAseq data will be compared with the exome sequencing data.

16.213 Methylation data
Methylation analysis will be completed from tumor taken before, during, and after neoadjuvant therapy using Illumina's 450K methylation array to determine if any of the novel variants found from the sequencing of the tumor are contained or near known CpG sites (or islands) or if there is a change in CpG methylation pattern during and after neoadjuvant therapy.

16.22 Statistical/bioinformatic analyses of genomic data

The primary objectives of this study are to ascertain the germline and somatic changes within gene and gene pathways that occur before and after completion of paclitaxel and examine whether these changes differ between tumors that respond to paclitaxel and those that do not respond to paclitaxel as well as to ascertain the germline and somatic changes within gene and gene pathways that occur before and after completion of all NAC and examine whether these changes differ between tumors that respond to treatment and those that do not respond to treatment. These analyses will include a stratification by molecular subtype. We will take a discovery approach and will perform subset analyses throughout the course of this prospective study. Any interesting results will be further validated in subsequent cohort both within and outside of BEAUTY as well as through the use of laboratory functional studies as deemed relevant.

The response phenotypes are defined in section 11.0 and include the following: Ki-67 response, residual cancer burden, radiographic response. The response

phenotypes will be assessed post paclitaxel and at surgery (following completion of all chemotherapy)

Generalized linear models will be used to assess the association of the each somatic variant with the clinical endpoints described above. This will first be done using a univariate model and subsequently, we will adjust for known prognostic patient and tumor characteristics. Second, all somatic variants within a particular gene will be pooled into a single variable describing whether or not any somatic variant exists and we will evaluate the association between this pooled variable with the clinical endpoint under investigation. Third, rare variant collapsing methods [22, 23] will be used to determine the association of a "region" with the endpoint under investigation.

Fourth, pathway association analyses will be completed in which the number of variants a tumor has in a given pathway will be associated with the endpoint under investigation, using analysis approaches similar to that described by Morris and Zeggini [24]. If necessary, population stratification can be employed using an eigen analysis of the genome-wide SNP variants measured on the germline DNA [25]. Lastly, if a variant is significant, we can perform a eQTL analysis using the RNAseq data to evaluate function.

RNAseq data will be normalized using conditional quantile normalization (cqn). For each gene separately, a negative binomial model will be used to assess the association between the RNAseq gene counts and the clinical endpoints. The cqn parameters will be incorporated into the offset of the negative binomial models in order to normalize the data. Subsequently, we will redo analyzes adjusting for known prognostic variables. We will also evaluate if these differently expressed genes are in similar pathways as well as perform pathway analyses.

The fusion genes detected from the RNAseq data will summarized descriptively across the entire cohort, across molecular subtypes, as well as across the endpoints described above.

Methylation levels will be correlated with gene expression obtained by RNA seq and SNPs/mutations identified by sequencing. Generalized linear models will be used to examine the association between methylation status and each of the clinical endpoints.

As an exploratory approach, the correlation between methylation levels, gene expression, SNPs/mutations and/or CNV will be evaluated using canonical correlation analysis.

Adjustments for multiple testing of multiple variants will be made using both the simple (but conservative) Bonferroni adjustment along with permutation based adjustment [26]. False-discovery rates and q-values will be calculated [27].

16.221 HRD deficiency

An initial pilot of 5-10 samples will be sent to Myriad to assess quality of DNA in the specimens, without exchange of clinical data. The HRD results obtained from these samples as well as the HRD results obtained

from the remaining samples will be used in these analyses. It is anticipated that the failure rate of the HRD assay will be 10%. A sample size of approximately 120 patients is anticipated.

Based on prior research by Myriad, HR Deficiency is defined as either a high HRD score (≥42) or a BRCA1/BRCA2 mutation in the tumor.

Extended Fisher's exact test will be used to determine whether the proportion of patients with pretreatment HR deficiency differs among tumor subtypes (HER2- and ER+, HER2-and ER-, HER2+). In addition, for patients who are not BRCA1/BRCA2 mutational carriers, whether pretreatment HR scores differs among molecular subtypes will be assessed using either Kruskual Wallis test or general linear F-test using the results of fitting a one factor ANOVA model and whether the proportion or patients with high HRD scores differ among among molecular subtypes will be assessed using chi-square test.

Fisher's exact test will be used to determine whether

1. Radiographic or Ki67 response to taxane therapy rates differ with respect to pretreatment HR deficiency
2. MRI response after anthracycline based chemotherapy in patients with residual tumor seen on MRI after taxane based therapy differs with respect to either pretreatment or post-taxane HR deficiency
3. pCR to neoadjuvant therapy differs by pretreatment HR deficiency
4. pCR to neoadjuvant therapy differs by post-taxane HR deficiency
5. pCR to neoadjuvant therapy differs by post-anthracycline HR deficiency

Logistic regression modeling will be used to assess the impact of pretreatment HR deficiency on determining the likelihood of pCR after other patient and disease characteristics known to impact pCR rates are accounted for in the model. To maintain model stability, 1 dependent parameter can be added to the model for every 15-20 patients included in the model. If feasible, logistic regression modeling will also be used to assess the impact of pretreatment HR deficiency on determining the likelihood of pCR within molecular subgroups.

Time series plots will be used to examine the changes in HRD scores throughout the course of neo-adjuvant treatment to visually assess differences between those who had a pCR and those who did not, for all patients as well as by tumor subtype and BRCA1/BRCA2 status.

16.23 Molecular Breast Imaging

MCR patients only: Tumor uptake of ^{99m}Tc-sestamibi will be quantified using software developed in-house. Algorithms for calculation of tumor uptake from opposing views of the breast have been validated in phantom models and computer simulations [16]. This will allow calculation of true tumor / background ratio.

Spearman's rank correlation coefficient will be used to assess (1) the strength of the association between the uptake in pre-treatment breast tumors to ^{99m}Tc-sestamibi and tumor grade and cell proliferation index from the pre-treatment biopsy specimen.

The percent change in uptake in breast tumors to ^{99m}Tc-sestamibi during neoadjuvant chemotherapy from pretreatment levels will be measured. Spearman's rank correlation coefficients will be used to assess (1) the strength of the association between the percent change in uptake to ^{99m}Tc-sestamibi at completion of neoadjuvant chemotherapy and pathologic response and residual disease burden.

16.3 Sample Size justification:

Table 1: Detectable effect sizes (odds ratio) for various analysis with N = 200				
		Minor Allele Frequency or Variable Frequency for Region and Pathway Analyses		
Analysis	A	5%	10%	20%
Single Variant	0.00001	OR > 20	8.9	5.4
Region/Gene	0.0001	19.3	6.7	4.4
Pathway	0.001	10.1	5.0	3.6

With a sample of 200 randomly ascertained subjects, we will be able to detect 80% of the variants in a region (assuming 20 variants in the region) with minor allele frequency of 0.005 with probability 0.86[28].

The minimum detected odds ratio for pCR assuming that 40% of the 200 subjects enrolled will have a pCR is presented in Table 1 for a variety of minor allele frequencies/variable frequencies (5%, 10% and 20%) using a test of association with significant level α (0.00001, 0.0001 and 0.001). The significant levels used are to account for the testing of multiple hypotheses. Power was computed using the software package *Quanto* for a binary endpoint and assumed a dominant genetic model

Accrual Targets			
Ethnic Category	**Sex/Gender**		
	Females	**Males**	**Total**
Hispanic or Latino	12	0	12
Not Hispanic or Latino	216	2	218
Ethnic Category: Total of all subjects	228	2	230
Racial Category			
American Indian or Alaskan Native	1	0	1
Asian	15	0	15
Black or African American	13	0	13
Native Hawaiian or other Pacific Islander	1	0	1
White	198	2	200
Racial Category: Total of all subjects*	228	2	230

Ethnic Categories	**Hispanic or Latino –** a person of Cuban, Mexican, Puerto Rican, South or Central American, or other Spanish culture or origin, regardless of race. The term "Spanish origin" can also be used in addition to "Hispanic or Latino.
	Not Hispanic or Latino
Racial Categories	**American Indian or Alaskan Native –** a person having origins in any of the original peoples of North, Central, or South America, and who maintains tribal affiliations or community attachment.
	Asian – a person having origins in any of the original peoples of the Far East, Southeast Asia, or the Indian subcontinent including, for example, Cambodia, China, India, Japan, Korea, Malaysia, Pakistan, the Philippine Islands, Thailand, and Vietnam. (Note: Individuals from the Philippine Islands have been recorded as Pacific Islanders in previous data collection strategies.)
	Black or African American – a person having origins in any of the black racial groups of Africa. Terms such as "Haitian" or "Negro" can be used in addition to "Black or African American."
	Native Hawaiian or other Pacific Islander – a person having origins in any of the original peoples of Hawaii, Guam, Samoa, or other Pacific Islands.
	White – a person having origins in any of the original peoples of Europe, the Middle East, or North Africa.

17.0 Pathology Considerations/Tissue Biospecimens

17.1 Summary Table of Research Tissue Specimens to be Collected for this Protocol

Correlative Study (Section for more information)	Mandatory or Optional	Type of Tissue to Collect	After registration but prior to chemotherapy	After the completion of taxane prior to anthracycline based chemotherapy	At the time of surgery (cycle 3)	At first recurrence or post treatment if primary surgery is not to be performed	Process at site? (Yes or No)	Temperature Conditions for Storage /Shipping
Visit Alias – Lab Use Only			**V1T (Visit 1 Tissue)**	**V2T (Visit 2 Tissue)**	**V3T (Visit 3 Tissue)**	**V4T (Visit 4 Tissue)**		
Tumor sample (for genotyping)	Mandatory	Breast tumor	X	X	X+	X*	Yes	Frozen, Dry Ice
Tumor sample (for establishing xenograft)++	Mandatory	Breast tumor	X[1]		X[1+]	X[1]	Yes, Place in PBS	Refrigerated cold pack
Tumor sample (for correlative studies)	Mandatory	Breast tumor	X	X	X+	X*	Yes	Frozen, Dry Ice
Diagnostic slides and representative tumor block) from original and/or recurrent tissues (note, tumor cores are acceptable if an outside institution is unable to send blocks)	Mandatory		X		X	X		

* If patient develops disease recurrence – including local, regional, or distant disease site. Biopsy of locally recurrent (local or regional) or metastatic lesion for those patients with disease recurrence.

[1] Tissue samples for xenografts must be placed immediately into the 50 mL falcon conical tube containing 10 mL of PBS media in the procedure room. In MCR and MCF these are to be taken directly to the laboratory for xenografting. In MCA, place falcon tube containing tissue samples on wet ice until samples can be shipped refrigerate to Mayo Rochester.+ If preoperative imaging suggests complete response and complete response is confirmed by intra-operative frozen section then the lymph nodes should be evaluated for response as well. If the lymph nodes from axillary dissection demonstrate significant residual disease then fresh tissue samples can be obtained from these nodes for the purpose of xenografting, genomic sequencing and/or storage.++ Please email Dr. Liewei Wang's laboratory DL RST BEAUTY Xenograft Tissue Pickup Team, [redacted] nd [redacted] when you have a patient scheduled for biopsy and inform the team the date of biopsy. If biopsy is canceled for any reasons, please also send an email to inform [redacted]

17.2 Correlative Tissue Collection

17.21 Xenograft tissue shipping boxes will be provided for this protocol. Shipping boxes can be ordered from the fax supply order form.

Note: Tissue supplies to be ordered are:

Falcon tubes: 50 mL Conical 332600 and the 15 mL conical is 332590
PBS: GIBCO (catalogue number 10010-023)

17.22 Fresh Tissue for xenograft

Tumor for xenograft from MCA will be immediately stored in fresh PBS in a 50 ml falcon tube and shipped overnight to:

MCF will develop xenograft locally. Once the xenografts are established and frozen down, an aliquot of these cells will be shipped to Dr. Wang's laboratory for future sequencing and drug studies.

FFPE portions of the xenografts will go from [redacted] and undergo pathology review by [redacted]

17.221 Core biopsies from breast tumor biopsy prior to initiation of chemotherapy (obtained in radiology) and breast tumor tissue from time of surgery (obtained in the operating room and frozen section laboratory) will be placed into a labeled specimen container with protocol number, study patient ID number, and time and date sample is obtained. In MCR these specimens will be delivered by study staff to [redacted] From MCA the samples will be shipped overnight to [redacted] to pick up the samples. Samples will be injected into mice at the Barrier area on [redacted]

17.23 Frozen Tissue for sequencing

17.231 Tissues (Breast tumor) obtained at biopsy prior to chemotherapy, after completion of taxane prior to anthracycline based chemotherapy, and residual disease from time of surgery will be submitted. At time of surgery, tissue from frozen section lab after confirmation of residual viable disease will be submitted.

17.232 Tissue must be snap frozen in *liquid* nitrogen and remain frozen at temperatures between -60ºC and -80ºC until shipped and during shipping.

17.233 Label specimen container with protocol number, patient ID number, and patient initials. **Specimens must remain frozen at all times.**

17.24 Tissue for additional Correlative studies

Tissue for correlative studies will be collected at 4 time points: prior to initiation of chemotherapy, after completion of taxane prior to anthracycline based chemotherapy, at surgery and at time of recurrence. At each time point, the biopsy will be performed following the completion of the MBI (if applicable) and conventional imaging studies (mammogram, ultrasound, and MRI) to minimize post-biopsy changes on imaging.

17.241 Initial biopsy (prior to initiation of chemotherapy): Eight cores from a 14 gauge core needle biopsy device will be obtained using an image-guided biopsy device. Tissue will be preferentially utilized for sequencing (3 cores - frozen), establishing xenografts (3 cores – in PBS), FFPE in 15 mL conical tube (1 core) and the remaining 1core will be fresh frozen for future studies. The FFPE specimen will be utilized for ER, PR, HER2, and Ki67.

An aliquot of RNA derived from the RNA extraction will be shipped for quantitative expression profiling of APOBEC3B mRNA levels ([redacted].

An extract of protein from the frozen additional cores will be shipped to [redacted]

An aliquot of tumor DNA for HRD analysis will be shipped to [redacted]

17.242 2nd tumor biopsy (between taxane and AC or FEC chemotherapy): six cores from a 14 gauge core needle biopsy device will be obtained using an image-guided biopsy device. One core will be formalin fixed for FFPE for H&E and Ki67, ER/PR/HER2 and to quantitate and identify tumor cells. The additional cores (5) will be frozen in liquid nitrogen and sent to BAP for storage, one of which will be utilized for RPPA and two for DNA and RNA extraction. Cores from MCA and MCF will not be processed locally but will be shipped to MCR (5 cores shipped frozen, 1 as FFPE). Upon arrival they will be handled as outlined above.

An aliquot of RNA derived from the RNA extraction will be shipped for quantitative expression profiling of APOBEC3B mRNA levels [redacted]

An extract of protein from the frozen cores will be shipped to [redacted] for RPPA

An aliquot of tumor DNA for HRD analysis will be shipped to [redacted]

17.243 At the time of surgery the surgical specimen will be processed through the frozen section laboratory. Any residual invasive disease will be tested for estrogen receptor (ER), progesterone receptor (PR), Her2, and Ki67 per standard practice. If residual invasive disease is identified in the resected breast tissue any residual invasive tumor after tissue taken for clinical care will be collected and cut into three pieces. One piece will be flash frozen for DNA sequencing. A second piece will be flash frozen for RNA sequencing. The third piece will be placed in media for establishing xenografts. Any residual tissue from DNA/RNA specimens will be stored frozen in BAP for banking purposes.
In the event that ER, PR, Her2 and Ki67 testing is not performed clinically, banked tissue from tumor registry (up to 8 unstained tissue slides) will be accessed for ER, PR, Her2 and Ki67 testing.

Another piece will be flash frozen for future protein extraction for RPPA [redacted] – see Section 14).

An aliquot of RNA derived from the RNA extraction will be shipped for quantitative expression profiling of APOBEC3B mRNA levels ([redacted]

An aliquot of tumor DNA for HRD analysis will be shipped to Myriad Laboratories

In cases where there is no residual invasive disease in the breast a sample of the residual tumor bed should be submitted flash frozen for storage.

In cases where there is no residual invasive disease in the breast, but there is residual metastatic disease in the lymph nodes (sentinel node and/or axillary nodes), then tissue from the lymph nodes with disease will be flash frozen for DNA and RNA sequencing.

In the case where there is multifocal disease remaining in the breast at the time of surgery a representative portion from each of the tumors will be submitted for processing.

For patients with bilateral disease the tissue from the index side will be processed as outlined above and any residual disease from the contralateral side will be processed per usual pathology guidelines and a representative paraffin embedded block will be stored for research purposes for this study.

17.244 If patient develops disease recurrence – including local, regional, or distant disease site; a biopsy will be obtained (see section 17.3 below) and tissue will be distributed as follows:

Clinical sample for diagnostics (if not previously obtained) will be collected per routine clinical care for confirmation of disease recurrence.

Additional samples for correlative sciences will be divided into 3 specimens and prioritized as follows:

1) DNA and RNA sequencing (fresh frozen)
    a) An extract of protein will be shipped to [redacted]
    b) A portion of tumor specimen will be converted to RNA and sent to the [redacted] for quantitative expression profiling of APOBEC3B mRNA levels in comparison to those of the endogenous housekeeping gene TBP [17].
    c. If adequate tissue is available, a sample for HRD analysis will be sent to Myriad Laboratories .
2) If residual tissue – fresh tissue will be sent for establishment of xenograft.

17.245 Tissue from biopsy of recurrent disease – tissue will be utilized for sequencing, establishing xenografts. Any additional tissue will be paraffin embedded for future studies.

An aliquot of RNA derived from the RNA extraction will be shipped for quantitative expression profiling of APOBEC3B mRNA levels [redacted]

17.246 For MCA and MCR

1) Same collection information as above. One core will be placed in formalin and used for histologic processing to confirm the presence of tumor. The container will be shipped to [redacted] and immediately forwarded to [redacted] with the tissue submission paperwork. [redacted] will accession the formalin specimen and e-mail the pathology coordinator at [redacted] The pathology coordinator will pick up the formalin tissue container and take it to [redacted] for embedding. [redacted] will notify the pathology coordinator to pick up the tissue and store the sample with the [redacted].

17.25 Correlative Science – evaluation of tumor bed tissue from surgery

FFPE tumor blocks obtained from residual tumor and tumor bed samples collected at the time of surgery and banked in the [redacted] all 3 sites will be obtained and 3 slides each of 5 microns thickness will be cut – 1 will be stained for H&E and 2 unstained slides. The unstained slides will be stained for SNAIL IHC.

SNAIL can only be detected by IHC using FFPE tissue therefore stored samples from tissue registry will be used, rather than frozen tissue previously collected on this protocol.

17.3 Diagnostic slides from original tissue

ALL original diagnostic slides and a representative tissue block(s) containing invasive tumor from time of initial breast biopsy (note tissue cores are acceptable if the referring institution is unable to send blocks) will be obtained from the outside referring institution. Specimens should be clearly labeled and forwarded **as soon as possible after surgery** *OR* <30 days after registration for central review. ALL original diagnostic slides used to make the diagnosis of Breast cancer should be clearly labeled and forwarded < *30 days* of registration for QA audit according to shipping instructions below.

[redacted]

This Central Review is for research purposes only and not to verify diagnosis.
Central pathology review will not be placed into the patient's clinical record. In the unlikely event a significant change is noted between the local pathology findings and the central pathology review the central pathologist will communicate the variances with the treating physician.

17.31 Diagnostic slides from surgery

Diagnostic slides of the tumor bed and any residual disease (DCIS or invasive) and of the lymph nodes from time of surgery will be obtained from Mayo Clinic AZ, Mayo Clinic FL and Mayo Clinic Rochester locations. Specimens should be clearly labeled and forwarded as soon as possible after surgery for central review.

Additionally unstained slides from any residual invasive disease (breast or lymph node if no invasive disease in the breast) should be submitted from Mayo Clinic AZ and Mayo Clinic FL for tumor biomarker staining (ER, PR, Her2 and Ki67) as indicated below.

ALL diagnostic slides and 10 unstained slides (charged and cut at 5 microns) for tumor biomarkers should be clearly labeled and forwarded to:

[redacted]

This Central Review is for research purposes only (RCB, the primary endpoint) and not to verify diagnosis.

17.4 Recurrent disease tissue

17.41 Guidelines for tissue acquisition

17.411 Image guided biopsies are recommended where feasible. The amount of tissue collected will follow the guidelines listed below. If a patient has more than one site of disease, only one site needs to be biopsied.

17.412 Skin/chest wall: An incisional/excisional biopsy is preferred. If punch biopsy is performed, a minimum of 3 punch biopsies (≥ 5mm diameter).

17.413 Lymph node: A goal of 3-6 core biopsy specimens obtained using an 18-gauge needle or larger. Smaller or less samples will be obtained at the discretion of the physician performing the biopsy.

17.414 Liver: A goal of 2-3 core biopsy specimens obtained using an 18-gauge needle.

17.415 Lung: Because of the risk of pneumothorax associated with core needle biopsies of lung nodules, core biopsies are not required. If the tissue is to be obtained for clinical care; an additional 1-2 core biopsies are to be submitted for research purposes.

17.416 Bone: Because the yield of malignant tissue from bone biopsies tends to be relatively low, if a patient has another accessible site of disease (i.e., skin, lymph node, liver), that site should be biopsied preferentially. If bone is the only biopsy-accessible site, then a goal of 3-6 core biopsy specimens will be obtained using an 18-gauge needle.

17.5 Background and Methodology

17.51 Overview

A series of high throughput assays including exome sequencing, RNA seq and CpG methylation will be applied using tumor samples from a series well annotated samples obtained at the base line (biopsy), after initial chemotherapy and during the surgery (after finishing neoadjuvant chemotherapy). To control for possible batch effects, the exome sequencing of the tumor and germline DNA prior to treatment will be completed at same time. In addition, mRNA will be extracted from the tumor for RNA-seq at the same time as sequencing of the DNA.

Integrated analysis will then be performed to identify signatures that are associated with complete pathological response. With those non responders, sequencing analysis will be used to identify mutations in genes within the known signaling pathways that have available drug targets, such as PI3K, AKT, EGFR, mTOR, etc. These analyses will allow future individualized the therapy with selective targeted agents. Additional integrated analyses will be also performed with data obtained with exome sequencing, RNA seq and CpG methylation. The integrated analysis will help identify additional novel mutations and novel mechanisms underlying the different response phenotypes, all of which could

also help identify novel drug targets. Finally, samples obtained during the biopsy will be immediately injected into scid mice to generate human tissue xenografts. These tissues will be passaged in mice (see IACUC protocol A49111) and will be used to evaluate new drug therapies based on tumor sequencing data.

17.52 Exome sequencing of tumor DNA

Summary:--tumor DNA will be isolated from specimens obtained at three different time points, prior to the start of neoadjuvant treatment, at surgery and at recurrence. DNA will be processed and plated at Mayo BAP lab and then send to sequencing lab for exome sequencing using the Agilent SureSelect Human All Exon kit 50Mb and the Illumina HiSeq 2000. Typically, over 80% of the target region is covered at a depth of greater than 30-fold, allowing for high confidence identification of sequence variants across the known protein coding region of the genome.

Detailed Protocol--Paired-end indexed libraries are prepared following the manufacturer's protocol (Agilent). Briefly, 3 ug of target DNA in 120 ul TE buffer is fragmented using the Covaris E210 sonicator. The settings of duty cycle 10%, intensity 5, cycles 200, time 360 seconds generated double-stranded DNA fragments with blunt or sticky ends with a fragment size mode of between 150-200bp. The ends were repaired and phosphorylated using Klenow, T4 polymerase, and T4 polynucleotide kinase, after which an "A" base is added to the 3' ends of double-stranded DNA using Klenow exo- (3' to 5' exo minus). Paired end Index DNA adaptors (Agilent) with a single "T" base overhang at the 3' end are ligated and the resulting constructs are purified using AMPure SPRI beads from Agencourt. The adapter-modified DNA fragments are enriched by 4 cycles of PCR using InPE 1.0 forward and SureSelect Pre-Capture Indexing reverse (Agilent) primers. The concentration and size distribution of the libraries is determined on an Agilent Bioanalyzer DNA 1000 chip.

Whole exon capture is carried out using the protocol for Agilent's SureSelect Human All Exon kit 50MB. 500 ng of the prepped library is incubated with whole exon biotinylated RNA capture baits supplied in the kit for 24 hours at 65 °C. The captured DNA:RNA hybrids are recovered using Dynabeads MyOne Streptavidin T1 from Dynal. The DNA is eluted from the beads and purified using Ampure XP beads from Agencourt. The purified capture products are then amplified using the SureSelect Post-Capture Indexing forward and Index PCR reverse primers (Agilent) for 12 cycles. Libraries are validated and quantified on the Agilent Bioanalyzer.

Libraries are loaded onto paired end flow cells at concentrations of 4-5 pM to generate cluster densities of 300,000-500,000/mm^2 following Illumina's standard protocol using the Illumina cBot and HiSeq Paired end cluster kit version 1.

The flow cells are sequenced as 101 X 2 paired end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 1 and HiSeq data collection version 1.1.37.0 software. Base-calling is performed using Illumina's RTA version 1.7.45.0.

17.53 Sequence analysis of tumor RNA

Summary: RNA will be isolated from the tumor samples obtained at three different time points, prior to the start of neoadjuvant treatment, at surgery, and at recurrence. RNA will be first made into cDNA library and then subjected to paired-end 51 bp sequencing using Illumina HiSeq 2000 at a coverage of about 50 million reads per sample.

Detailed Protocol--Libraries for mRNA-seq are prepared using the TruSeq RNA sample prep kit v1 following the manufacturer's protocol (Illumina). PolyA containing mRNA is purified from total RNA using oligo-dT attached magnetic beads. The purified mRNA is fragmented using divalent cations at 95°C for 8 minutes, eluted from the beads and primed for first strand cDNA synthesis in a single step. The RNA fragments are copied into first strand cDNA using SuperScript (Invitrogen) reverse transcriptase and random primers. Second strand cDNA synthesis is performed using DNA polymerase I and RNase H. The double-stranded cDNA is purified using a single Ampure XP bead (Agencourt) clean-up step. The cDNA ends are repaired and phosphorylated using Klenow, T4 polymerase, and T4 polynucleotide kinase followed by a single Ampure XP bead clean-up. The blunt-ended cDNAs are modified to include a single 3' adenylate (A) residue using Klenow exo- (3' to 5' exo minus). Paired end DNA adaptors (Illumina) with a single "T" base overhang at the 3' end are immediately ligated to the 'A tailed' cDNA population. The resulting constructs are purified by 2 consecutive Ampure XP bead clean-up steps. The adapter-modified DNA fragments are enriched by 12 cycles of PCR using primers PE 1.0 and PE 2.0 (Illumina). The concentration and size distribution of the libraries is determined on an Agilent Bioanalyzer DNA 1000 chip. Twelve unique indexes are incorporated at the adaptor ligation phase if required for multiplex sample loading on the flow cells.
Libraries are loaded onto paired end flow cells at concentrations of 6 pM to generate cluster densities of 400,000/mm^2 following Illumina's standard protocol using the Illumina cBot and cBot Paired end cluster kit version 1.

The flow cells are sequenced as 51 X 2 paired end reads on an Illumina HiSeq 2000 using TruSeq SBS sequencing kit version 1 and HCS version 1.1.37.0 data collection software. Base-calling is performed using Illumina's RTA version 1.7.45.0.

17.54 RNA quantitative expression profiling of APOBEC3B

Summary: RNA will be isolate from the tumor samples obtained at three different time points, prior to the start of neoadjuvant treatment, at surgery, and at recurrence. RNA will be used for quantitative expression profiling of APOBEC3B mRNA levels in comparison to those of the endogenous housekeeping gene TBP [17].

Rationale: The human DNA deaminase APOBEC3B is a newly discovered genetic marker for breast cancer, with expression studies demonstrating its over-expression in ~60% of breast cancers ([redacted] APOBEC3B mutates the genomic DNA of breast cancer cells causing an overall increase in mutation frequency [redacted]). It is highly likely that this mutator activity contributes to the heterogeneity of breast cancer

and in turn to negative outcomes (i.e. higher APOBEC3B = faster tumor evolution = more likely to evolve resistance to therapy and immune responses).

17.55 CpG methylation analysis of tumor DNA

Understand epigenetic regulation in tumor and their effect on gene expression as well as response phenotypes. DNA isolated from tumor tissues obtained at three different time points will be used to perform CpG methylation assays using the Illumina 450K methylation chips that contain over 47,000 methylation sites.

17.56 Xenograft mice model

To generate individual tumor line for drug screening, drug cytotoxicity assay, specimen obtained during the initial biopsy will be immediately injected into scid mice for the creation of human tissue xenografts (See IACUC A49111). The xenograft tumor samples will be used to determine the functional implications tumor mutations identified from exome sequencing and for future drug screening and cytotoxicity assay. These lines will be also useful for future research purposes, such as testing novel compounds and different regimens. All the tumor tissues from xenografts will be stored on [redacted]

17.57 Stem Call Analysis

The cancer stem cell (CSC) hypothesis suggests that many cancers, including breast cancer, harbor a subpopulation of malignant cells with stem cell properties that may drive metastasis and resist chemotherapy, resulting in resistance and relapse. Two populations of CSCs have been identified: a mesenchymal-like population at the leading edge that stains CD24-/CD44+/ALDH- and a centrally-located epithelial-like population that stains ALDH+. Other groups have suggested that when tumors are treated with chemotherapy, often the CSCs are resistant. Thus, although the tumor size and cellularity may decrease, when the tumor is not eradicated the CSCs may be enriched in the remaining population—an interesting hypothesis that has not yet been tested to our knowledge. Interestingly, it has also previously been demonstrated that breast tumors containing increasing quantities of CSCs are capable of forming xenografts and that tumors expressing both CD24-/CD44+ and ALDH+ populations have the greatest tumor-initiating capacity.

CSCs will be analyzed to test the hypothesis that cancer stem cells are enriched in residual tumor and that they are related to response to therapy. Immunohistochemical stains (CD24, CD44, ALDH, and potentially other related CSC markers) using commercially available antibodies will be performed on human breast tumor tissue, as well as patient-derived xenografts. The quantity of cells that stain for each marker will be scored (% of tumor cells that stain positive) and the distribution of the positive-staining cells will also be noted (leading edge of tumor vs. center vs. uniformly distributed throughout). This CSC data will be correlated with other clinical, imaging, and genomic information to better understand the role of CSCs in breast cancer.

18.0 Records and Data Collection Procedures

Initial Material(s)

CRF	**Active-Monitoring Phase** (Compliance with Test Schedule Section 4.0)
On-Study Form	≤2 weeks after registration
Research Blood Submission Form	
Breast Biopsy Form	
MBI Related Adverse Event Form	
End of Active Treatment/Cancel Notification Form	
Research Tissue Submission Form	≤30 days after registration

Test Schedule Material(s)

	Active-Monitoring Phase (Compliance with Test Schedule Section 4.0)				
CRF	After the completion of taxane prior to anthracycline based chemotherapy (Cycle 1)	After completion of anthracycline based chemotherapy, but prior to surgery (Cycle 2)	After completion of surgery	Observation q 12 months after surgery until recurrence	At first recurrence or at completion of treatment if surgery is not to be performed
Evaluation/Treatment Form	X	X			
Active Monitoring Measurement Form	X	X			
Suspicion of Progression on Neoadjuvant Chemotherapy	X	X			
MBI Related Adverse Event Form	X	X			
Surgery Reporting Form			X		
Op & Path Reports			X		
Tumor Marker on surgical tissues (clinical report)			X		
Residual Cancer Burden Worksheet			X		
Evaluation/Observation Form				X	
Research Blood Submission Form	X (see Section 14.0)	X (see Section 14.0)			X (see Section 14.0)

CRF	Active-Monitoring Phase (Compliance with Test Schedule Section 4.0)				
	After the completion of taxane prior to anthracycline based chemotherapy (Cycle 1)	After completion of anthracycline based chemotherapy, but prior to surgery (Cycle 2)	After completion of surgery	Observation q 12 months after surgery until recurrence	At first recurrence or at completion of treatment if surgery is not to be performed
Research Tissue Submission Form	X (see Section 17.0)		X (see Section 17.0)		X (see Section 17.0)
Breast Biopsy Form	X (see Section 4.0)				X (see Section 4.0)
Start of Adjuvant Endocrine Therapy Form				X	
Changes in or Discontinuation of Adjuvant Endocrine Therapy Form				X	
End of Active Treatment/Cancel Notification Form			X		

Follow-up Material(s)

CRF	q. 12 months after recurrence[1, 2]	Death	New Primary
Event Monitoring Form	X	X	At each occurrence

1. If a patient is still alive 6 years after surgery, no further follow-up is required.
2. Submit copy of documentation recurrence to the MCCC Operations Office, Attention: QAS for MC1137.

19.0 Budget

19.1 Costs charged to patient: routine clinical care

19.2 Tests to be research funded: molecular breast imaging (MCR Participants only), research blood samples, research tumor biopsy, and MRI between paclitaxel ± trastuzumab and AC or FEC chemotherapy.

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Appendix I ECOG Performance Status

Grade

0	Fully active, able to carry on all pre-disease activities without restriction (Karnofsky 90-100).
1	Restricted in physically strenuous activity but ambulatory and able to carry out work of a light or sedentary nature, e.g., light housework, office work (Karnofsky 70-80).
2	Ambulatory and capable of all self-care, but unable to carry out any work activities. Up and about more than 50 percent of waking hours (Karnofsky 50-60).
3	Capable of only limited self-care, confined to bed or chair 50 percent or more of waking hours (Karnofsky 30-40).
4	Completely disabled. Cannot carry on any self-care. Totally confined to bed or chair (Karnofsky 10-20).
5	Dead

Appendix II Chemotherapy Guidelines

Weekly Paclitaxel in the Neoadjuvant Treatment of Breast Cancer				
Cycles: 3 Emetogenic Potential: 2 Time: 3 Hours				
DRUG	**DOSE**	**ROUTE**	**DAYS**	**COMMENTS**
Dexamethasone [DXM]	20 mg/dose	In 100 ml 0.9% NaCL IV infusion over 15 minutes immediately before Paclitaxel	1, 8, 15, 22	
Famotidine [PEPCID]	20 mg/dose	In 50 mL 0.9% NaCL IV infusion over 15 minutes immediately before Paclitaxel	1, 8, 15, 22	
Diphenhydramine [BEN]	50 mg/dose	In 100 mL 0.9% NaCl IV infusion over 15 minutes immediately before Paclitaxel.	1, 8, 15, 22	
Paclitaxel [TAXOL]	80 mg/m^2/week	In 250 mL 0.9% NaCL IV infusion over 1 hour (use non-pvc container and tubing)	1, 8, 15, 22	Dose rounding to the nearest 5 mg.

Retreatment: Every 28 days for 3 cycles (12 doses of Paclitaxel)
Reference: Sparano JA, Wang M et al. Weekly Paclitaxel in the Adjuvant Treatment of Breast Cancer. The New England Journal of Medicine 2008;358:1663-1671.

Weekly Paclitaxel/Carboplatin in the Neoadjuvant Treatment of Breast Cancer				
Cycles: 4 Emetogenic Potential: 3 Time: 5 Hours				
DRUG	**DOSE**	**ROUTE**	**DAYS**	**COMMENTS**
Dexamethasone [DXM]	20 mg	in 100 ml NS IV infusion over 15 minutes immediately before Taxol	1,8,15,	
Famotidine [PEPCID]	20 mg	IV infusion over 15 minutes immediately before Taxol	1,8,15,	
Diphenhydramine [BEN]	50 mg	IV infusion over 15 minutes immediately before Taxol	1,8,15,	

Paclitaxel [TAXOL]	80 mg/m^2	IV infusion in 250 ml 0.9% NaCL (non-PVC container) over 1 hour	1,8,15,	Dose rounding to the nearest 5 mg. If Inpatient, remain with the patient for the first 15 minutes of the infusion. Monitor vital signs prior to administration of paclitaxel, 15 and 30 minutes after initiation of paclitaxel, then hourly and at the completion of paclitaxel.
Carboplatin [CBDCA]	AUC = 6	in 250 ml 0.9% NaCl or D5W IV infusion over 30 minutes	1	Dosed using Calvert Formula with Cockroft & Gault Equation, actual body weight and maximum CrCL of 125 mL/min. Creatinine clearance should be estimated using a minimum serum creatinine value of 0.7 mg/dL. Dose rounding to the nearest 10 mg.

RETREATMENT: **Every 3 weeks**

Herceptin + Weekly Paclitaxel				
Emetogenic Potential: 1 Time: 3 Hours				
DRUG	**DOSE**	**ROUTE**	**DAYS**	**COMMENTS**
Dexamethasone [DXM]	20 mg/dose	In 100 ml 0.9% NaCL IV infusion over 15 minutes immediately before Paclitaxel	1, 8, 15, 22	May decrease Dexamethasone dose if patient does not experience reactions to Taxol.
Famotidine [PEPCID]	20 mg/dose	In 50 mL 0.9% NaCL IV infusion over 15 minutes immediately before Paclitaxel	1, 8, 15, 22	
Diphenhydramine [BEN]	50 mg/dose	In 100 mL 0.9% NaCl IV infusion over 15 minutes immediately before Paclitaxel.	1, 8, 15, 22	
Paclitaxel [TAXOL]	60 – 90 mg/m^2	In 250 mL 0.9% NaCL (non-pvc container and tubing) IV infusion over 1 hour	1, 8, 15, 22	Defaults to 80 mg/m^2/dose
Acetaminophen [APAP]	650 mg	PO prn fever	prn	
Meperidine [DEM]	25 mg/ dose	25 mg IV push prn rigors, may repeat once if first dose ineffective	prn	
Diphenhydramine [BEN]	50 mg	IV push prn fever & chills	prn	
Trastuzumab [HERCEPLOAD]	4 mg/kg loading	In 250 ml NS IV infusion over 90 minutes	1	Day 1 at 2 mg/kg on subsequent cycles. Left Ventricular Function should be evaluated in all patients prior to and during treatment with Herceptin.

Trastuzumab [HERCEP]	2 mg/kg	In 250 mL NS IV infusion over 30 minutes	8, 15, 22	Infusion rate to remain at 90 minutes until at least one dose is well tolerated. Then increase rate to 30 minutes.

Retreatment: Every 28 days x 3 cycles
Reference: N9831

Trastuzumab/Pertuzumab + Weekly Paclitaxel					
Agent	**Time**	**Dose**	**Route**	**Rx Days**	**ReRx**
Trastuzumab Loading Dose [HERCEP LOAD]	N/A	8 mg/kg/day	IV	Day 1 week 1	
Trastuzumab [HERCEP]	N/A	6 mg/kg/day	IV	day 1	Weeks 4, 7, 10
Pertuzumab (Perjeta) Loading Dose	After Trastuzumab	840 mg/day	IV	day 1 week 1	
Pertuzumab (Perjeta) [PERTUZUMAB]		420 mg/day	IV	Day 1	Weeks 4, 7, 10
Paclitaxel	N/A	80 mg/m^2	IV	Day 1	Weeks 1-12

Reference: Datko F, D'Andrea G, Theodoulou M et al. Phase II study of pertuzumab, trastuzumab, and weekly paclitaxel in patients with metastatic HER2-overexpressing metastatic breast cancer. Cancer Research 2012;72(24 Supplement):P5-18-20

Trastuzumab/Pertuzumab/Docetaxel					
Agent	**Time**	**Dose**	**Route**	**Rx Days**	**ReRx**
Trastuzumab Loading Dose [HERCEP LOAD]	N/A	8 mg/kg/day	IV	Day 1 Week 1	
Trastuzumab [HERCEP]	N/A	6 mg/kg/day	IV	Day 1	Weeks 4, 7, 10
Pertuzumab (Perjeta) Loading Dose	After Trastuzumab	840 mg/day	IV	Day 1 Week 1	
Pertuzumab (Perjeta) [PERTUZUMAB]		420 mg/day	IV	Day 1	Weeks 4, 7, 10
Docetaxel	N/A	75 mg/m^2	IV	Day 1	Weeks 1, 4, 7, 10

Reference: Gianni, L., T. Pienkowski, et al. (2012). "Efficacy and safety of neoadjuvant pertuzumab and trastuzumab in women with locally advanced, inflammatory, or early HER2-positive breast cancer (NeoSphere): a randomised multicentre, open-label, phase 2 trial." The lancet oncology 13(1): 25-32.
Schneeweiss, A., S. Chia, et al. (2013). "Pertuzumab plus trastuzumab in combination with standard neoadjuvant anthracycline-containing and anthracycline-free chemotherapy regimens in patients with HER2-positive early breast cancer: a randomized phase II cardiac safety study (TRYPHAENA)." Annals of oncology : official journal of the European Society for Medical Oncology / ESMO 24(9): 2278-2284.

Combination ADR + CTX Followed by Paclitaxel				
Cycles: 4 Emetogenic Potential: 4 Time: 2 Hours				
DRUG	**DOSE**	**ROUTE**	**DAYS**	**COMMENTS**
Doxorubicin [ADR]	60 mg/m^2	IV push into free flowing IV of NS	1	Maximum cumulative ADR dose is 240 mg/m^2.
Cyclophosphamide [CTX]	600 mg/m^2	IV infusion in 250 mL NS over 30 minutes	1	
Aprepitant [EMEND125]	125 mg/day	PO once pre-chemotherapy	1	Aprepitant 125 mg may be given on Gonda 10 or per Enterprise Orders prescription (Rx-EOP).
Aprepitant [EMEND80]	80 mg/day	PO once daily Days 2 and 3	2, 3	Aprepitant 80 mg once daily Days 2 and 3 should be given to the patient via Enterprise Orders prescription (Rx-EOP).
Filgrastim [GCSF]	5 mcg/kg	SQ	3 through 10	Suggest rounding dose to nearest vial size (300 mcg or 480 mcg). May substitute Neulasta (pegfilgrastim).
-- OR --				
Pegfilgrastim (NEULASTA)	6 mg	SQ	2	May substitute Neulasta (pegfilgrastim) for GCSF. Begin at least 24 hours post chemo.
Paclitaxel (Taxol)				
Cycles: 4 Emetogenic Potential: 2 Time: 4 Hours				
DRUG	**DOSE**	**ROUTE**	**DAYS**	**COMMENTS**
Dexamethasone [DXM]	20 mg	In 100 mL NS IV infusion over 15 minutes	1	
Famotidine [PEPCID]	20 mg	IV infusion over 15 minutes immediately before Taxol.	1	
Diphenhydramine [BEN]	50 mg	IV infusion over 15 minutes immediately before Taxol	1	

Paclitaxel [TAXOL]	175 mg/m^2	IV infusion in 500 mL 0.9% NaCL (non-pvc container and tubing) over 3 hours	1	Inpatient monitoring with the patient for the first 15 minutes of the infusion. Monitor vital signs prior to administration of paclitaxel, 15 and 30 minutes after initiation of paclitaxel, then hourly at the completion of paclitaxel.
Filgrastim [GCSF]	5 mcg/kg	SQ	3 through 10	Suggest rounding dose to nearest vial size (300 mcg or 480 mcg). May substitute Neulasta (pegfilgrastim).
-- OR --				
Pegfilgrastim (NEULASTA)	6 mg	SQ	2	May substitute Neulasta (pegfilgrastim) for GCSF. Begin at least 24 hours post chemo.

Retreatment: Every 2 weeks x 4 with growth factor support or every 3 weeks x 4 (with or without growth factor support)
Reference: C9741

FEC (Fluorouracil + Epirubicin + Cyclophosphamide)				
Emetogenic Potential: 3 Time: 3 Hours				
DRUG	**DOSE**	**ROUTE**	**DAYS**	**COMMENTS**
Epirubicin [EPIRUB]	75 mg/m2/day	IV push through a free-flowing IV line of 0.9% NaCL	1	
Fluorouracil [5FU]	500 mg/m2 /day	IV push	1	
Cyclophosphamide [CTX]	500 mg/m2/day	In 250 mL 0.9% NaCL IV infusion over 30 minutes	1	

Retreatment: Every 21 days
Reference: Buzdar, Ibrahim, Francis, et al. Significantly Higher Pathologic Complete Remission Rate After Neoadjuvant Therapy With Trastuzumab, Paclitaxel, and Epirubicin Chemotherapy: Results of a Randomized Trial in Human epidermal Growth Factor Receptor 2-Positive Operable Breast Cancer. JCO, Volume 23, Number 16, June 1, 2005. pp 3676-3685

Appendix III RPPA Standard Antibody List

No	Mills Lab Ab Name for Cataloging	Ab Name for Reports	Gene Name	Company	Catalog #	Ab ID	Species	Validation Status*	Recent Addition?
1	14-3-3_epsilon	14-3-3_epsilon	YWHAE	Santa Cruz	sc-2395	913.1	Mouse	Use with Caution	new
2	4E-BP1	4E-BP1	EIF4EBP1	CST	9452	2.8	Rabbit	Validated	
3	4E-BP1_pS65	4E-BP1_pS65	EIF4EBP1	CST	9456	3.1	Rabbit	Validated	
4	53BP1	53BP1	TP53BP1	CST	4937	985.1	Rabbit	Use with Caution	
5	ACC_pS79	ACC_pS79	ACACA ACACB	CST	3661	13.4	Rabbit	Validated	
6	ACC1	ACC1	ACACA	Epitomics	1768-1	14.1	Rabbit	Use with Caution	
7	AIB1	AIB1	NCOA3	BD Biosciences	611105	711.1	Mouse	Validated	
8	Akt	Akt	AKT1 AKT2 AKT3	CST	9272	21.13	Rabbit	Validated	
9	Akt_pS473	Akt_pS473	AKT1 AKT2 AKT3	CST	9271	23.10	Rabbit	Validated	
10	Akt_pT308	Akt_pT308	AKT1 AKT2 AKT3	CST	9275	25.11	Rabbit	Validated	
11	AMPK_alpha	AMPK_alpha	PRKAA1	CST	2532	39.4	Rabbit	Use with Caution	
12	AMPK_pT172	AMPK_pT172	PRKAA1	CST	2535	40.6	Rabbit	Validated	
13	Annexin_I	Annexin_I	ANXA1	Invitrogen	71-3400	795.1	Rabbit	Validated	
14	AR	AR	AR	Epitomics	1852-1	756.1	Rabbit	Validated	
15	Bad_pS112	Bad_pS112	BAD	CST	9291	63.7	Rabbit	Validated	new
16	Bak	Bak	BAK1	Epitomics	1542-1	71.1	Rabbit	Use with Caution	
17	Bax	Bax	BAX	CST	2772	73.3	Rabbit	Validated	
18	Bcl-2	Bcl-2	BCL2	Dako	Dako M0887	80.1	Mouse	Validated	
19	Bcl-X	Bcl-X	BCL2L1	Epitomics	1018-1	84.1	Rabbit	Use with Caution	
20	Bcl-xL	Bcl-xL	BCL2L1	CST	2762	85.5	Rabbit	Validated	
21	Beclin	Beclin	BECN1	Santa Cruz	sc-10086	87.1	Goat	Validated	
22	Bid	Bid	BID	Epitomics	1008-1	88.1	Rabbit	Use with Caution	
23	Bim	Bim	BCL2L11	Epitomics	1036-1	90.1	Rabbit	Validated	
24	Cadherin-E	E-Cadherin	CDH1	CST	4065	209.2	Rabbit	Validated	
25	Cadherin-N	N-Cadherin	CDH2	CST	4061	452.1	Rabbit	Validated	
26	Cadherin-P	P-Cadherin	CDH3	CST	2130	509.1	Rabbit	Use with Caution	
27	Caspase-3_active	Caspase-3_active	CASP3	Epitomics	1476-1	108.1	Rabbit	Use with Caution	
28	Caspase-7_cleavedD198	Caspase-7_cleavedD198	CASP7	CST	9491	109.6	Rabbit	Use with Caution	
29	Caspase-9_cleavedD330	Caspase-9_cleavedD330	CASP9	CST	9501	953.4	Rabbit	Use with Caution	new
30	Catenin-alpha	alpha-Catenin	CTNNA1	Calbiochem	CA1030	924.1	Mouse	Validated	new
31	Catenin-beta	beta-Catenin	CTNNB1	CST	9562	75.3	Rabbit	Validated	
32	Caveolin-1	Caveolin-1	CAV1	CST	3238	114.1	Rabbit	Validated	
33	CD31	CD31	PECAM1	Dako	M0823	127.1	Mouse	Validated	
34	CDK1	CDK1	CDC2	CST	9112	1007.5	Rabbit	Validated	
35	Chk1	Chk1	CHEK1	CST	2345	145.1	Rabbit	Use with Caution	
36	Chk1_pS345	Chk1_pS345	CHEK1	CST	2348	903.70	Rabbit	Use with Caution	
37	Chk2	Chk2	CHEK2	CST	3440	146.1	Mouse	Use with Caution	
38	Chk2_pT68	Chk2_pT68	CHEK2	CST	2197	147.2	Rabbit	Use with Caution	
39	cIAP	cIAP	BIRC2	Millipore	07-759	930.10	Rabbit	Validated	new
40	Claudin-7	Claudin-7	CLDN7	Novus	NB100-91714	852.1	Rabbit	Validated	
41	Collagen_VI	Collagen_VI	COL6A1	Santa Cruz	SC-20649	171.1	Rabbit	Validated	
42	COX-2	COX-2	PTGS2	Epitomics	2169-1	755.1	Rabbit	Use with Caution	
43	Cyclin_B1	Cyclin_B1	CCNB1	Epitomics	1495-1	192.1	Rabbit	Validated	
44	Cyclin_D1	Cyclin_D1	CCND1	Santa Cruz	SC-718	194.1	Rabbit	Validated	
45	Cyclin_E1	Cyclin_E1	CCNE1	Santa Cruz	SC-247	201.1	Mouse	Validated	
46	DJ-1	DJ-1	PARK7	Abcam	ab76008	891.10	Rabbit	Use with Caution	
47	Dvl3	Dvl3	DVL3	CST	3218	940.1	Rabbit	Validated	new
48	eEF2	eEF2	EEF2	CST	2332	1060.3	Rabbit	Validated	
49	eEF2K	eEF2K	EEF2K	CST	3692	1061.2	Rabbit	Validated	
50	EGFR	EGFR	EGFR	Santa Cruz	SC-03	215.2	Rabbit	Use with Caution	
51	EGFR_pY1068	EGFR_pY1068	EGFR	CST	2234	217.13	Rabbit	Validated	new
52	EGFR_pY1173	EGFR_pY1173	EGFR	Epitomics	1124	221.3	Rabbit	Use with Caution	
53	EGFR_pY992	EGFR_pY992	EGFR	CST	2235	222.4	Rabbit	Validated	
54	eIF4E	eIF4E	EIF4E	CST	9742	722.3	Rabbit	Validated	
55	ER-alpha	ER-alpha	ESR1	Lab Vision	RM-9101-S	238.6	Rabbit	Validated	new
56	ER-alpha_pS118	ER-alpha_pS118	ESR1	Epitomics	1091-1	241.1	Rabbit	Validated	
57	ERCC1	ERCC1	ERCC1	Lab Vision	MS-671-PO	247.1	Mouse	Use with Caution	
58	FAK	FAK	PTK2	Epitomics	1700-1	252.2	Rabbit	Use with Caution	
59	Fibronectin	Fibronectin	FN1	Epitomics	1574-1	262.10	Rabbit	Use with Caution	
60	FOXO3a	FOXO3a	FOXO3	CST	9467	269.4	Rabbit	Use with Caution	
61	FOXO3a_pS318_S321	FOXO3a_pS318_S321	FOXO3	CST	9465	270.1	Rabbit	Use with Caution	
62	GATA3	GATA3	GATA3	BD Biosciences	558686	764.1	Mouse	Validated	
63	GSK3_pS9	GSK3_pS9	GSK3A GSK3B	CST	9336	1082.12	Rabbit	Validated	new
64	GSK3-alpha-beta	GSK3-alpha-beta	GSK3A GSK3B	Santa Cruz	SC-7291	284.2	Mouse	Validated	
65	GSK3-alpha-beta_pS21_S9	GSK3-alpha-beta_pS21_S9	GSK3A GSK3B	CST	9331	285.12	Rabbit	Validated	
66	HER2	HER2	ERBB2	Lab Vision	MS-325-P1	1038.2	Mouse	Validated	new
67	HER2_pY1248	HER2_pY1248	ERBB2	Upstate (Millipore)	06-229	299.1	Rabbit	Validated	
68	HER3	HER3	ERBB3	Santa Cruz	sc-285	911.1	Rabbit	Validated	new
69	HER3_pY1298	HER3_pY1298	ERBB3	CST	4791	728.12	Rabbit	Use with Caution	new
70	IGF-1R-beta	IGF-1R-beta	IGF1R	CST	3027	336.1	Rabbit	Use with Caution	
71	IGFBP2	IGFBP2	IGFBP2	CST	3922	335.1	Rabbit	Validated	
72	INPP4B	INPP4B	INPP4B	Santa Cruz	SC-12318	912.1	Goat	Use with Caution	
73	IRS1	IRS1	IRS1	Upstate (Millipore)	06-248	802.1	Rabbit	Validated	
74	JNK_pT183_pT185	JNK_pT183_pT185	MAPK8	CST	4668	888.50	Rabbit	Validated	new
75	JNK2	JNK2	MAPK9	CST	4672	380.1	Rabbit	Use with Caution	
76	Jun-c_pS73	c-Jun_pS73	JUN	CST	9164	155.5	Rabbit	Use with Caution	
77	Kit-c	c-Kit	KIT	Epitomics	1522	157	Rabbit	Validated	
78	MAPK_pT202_Y204	MAPK_pT202_Y204	MAPK1 MAPK3	CST	4377	405.3	Rabbit	Validated	
79	MEK1	MEK1	MAP2K1	Epitomics	1235-1	417.1	Rabbit	Validated	

No	Mills Lab Ab Name for Cataloging	Ab Name for Reports	Gene Name	Company	Catalog #	Ab ID	Species	Validation Status*	Recent Addition?
80	MEK1_pS217_S221	MEK1_pS217_S221	MAP2K1	CST	9154	1076.3	Rabbit	Validated	new
81	Met-c	c-Met	MET	CST	3127	726.3	Mouse	Use with Caution	new
82	Met-c_pY1235	c-Met_pY1235	MET	CST	3129	727.50	Rabbit	Use with Caution	new
83	MIG-6	MIG-6	ERRFI1	Sigma	WH0054206M1	1062.1	Mouse	Validated	
84	MSH2	MSH2	MSH2	CST	2850	905.1	Mouse	Use with Caution	
85	MSH6	MSH6	MSH6	SDI	2203.00.02	1063.1	Rabbit	Use with Caution	
86	mTOR	mTOR	FRAP1	CST	2983	444.3	Rabbit	Validated	new
87	mTOR_pS2448	mTOR_pS2448	FRAP1	CST	2971	446.14	Rabbit	Use with Caution	new
88	Myc-c	c-Myc	MYC	CST	9402	161.20	Rabbit	Use with Caution	
89	NF2	NF2	NF2	SDI	2271.00.02	1046.1	Rabbit	Use with Caution	
90	NF-kB-p65_pS536	NF-kB-p65_pS536	NFKB1	CST	3033	457.4	Rabbit	Use with Caution	
91	Notch1	Notch1	NOTCH1	CST	3268	1064.1	Rabbit	Validated	
92	Notch3	Notch3	NOTCH3	Santa Cruz	sc-5593	767.1	Rabbit	Use with Caution	
93	p21	p21	CDKN1A	Santa Cruz	SC-397	470	Rabbit	Use with Caution	
94	p27	p27	CDKN1B	Epitomics	1591-1	897.1	Rabbit	Validated	
95	p27_pT157	p27_pT157	CDKN1B	R&D	AF1555	842.1	Rabbit	Use with Caution	
96	p27_pT198	p27_pT198	CDKN1B	Abcam	ab64949	878.1	Rabbit	Validated	
97	p38_MAPK	p38_MAPK	MAPK14	CST	9212	478.10	Rabbit	Use with Caution	
98	p38_pT180_Y182	p38_pT180_Y182	MAPK14	CST	9211	479.15	Rabbit	Validated	
99	p53	p53	TP53	CST	9282	481.3	Rabbit	Validated	
100	p70S6K	p70S6K	RPS6KB1	Epitomics	1494-1	493.1	Rabbit	Validated	
101	p70S6K_pT389	p70S6K_pT389	RPS6KB1	CST	9205	494.7	Rabbit	Validated	
102	p90RSK_pT359_S363	p90RSK_pT359_S363	RPS6KA1	CST	9344	770.2	Rabbit	Use with Caution	
103	PARP_cleaved	PARP_cleaved	PARP1	CST	9546	501.1	Mouse	Use with Caution	
104	Paxillin	Paxillin	PXN	Epitomics	1500-1	505.1	Rabbit	Validated	
105	PCNA	PCNA	PCNA	Abcam	ab29	511.1	Mouse	Validated	
106	PDK1_pS241	PDK1_pS241	PDK1	CST	3061	516.7	Rabbit	Validated	
107	PI3K-p110-alpha	PI3K-p110-alpha	PIK3CA	CST	4255	808.1	Rabbit	Use with Caution	
108	PI3K-p85	PI3K-p85	PIK3R1	Upstate (Millipore)	06-195	523.3 or 523.4	Rabbit	Validated	
109	PKC-alpha	PKC-alpha	PRKCA	Upstate (Millipore)	05-154	529.1	Mouse	Validated	
110	PKC-alpha_pS657	PKC-alpha_pS657	PRKCA	Upstate (Millipore)	06-822	530.2	Rabbit	Validated	
111	PR	PR	PGR	Epitomics	1483-1	549.1	Rabbit	Validated	
112	PRAS40_pT246	PRAS40_pT246	AKT1S1	Biosource	441100G	739.1	Rabbit	Validated	
113	PTCH	PTCH	PTCH1	SDI	2113.00.02	1051.1	Rabbit	Use with Caution	
114	PTEN	PTEN	PTEN	CST	9552	566.3	Rabbit	Validated	
115	Rab11	Rab11	RAB11A RAB11B	CST	3539	1083.3	Rabbit	Validated	new
116	Rab25	Rab25	RAB25	Covance Custom	Covance Custom	577.1	Rabbit	Use with Caution	
117	Rad50	Rad50	RAD50	Millipore	05-525	987.1	mouse	Use with Caution	
118	Rad51	Rad51	RAD51	Chem Biotech	na 71	579.3	Mouse	Use with Caution	
119	Raf-C	C-Raf	RAF1	Millipore	05-739	803	Rabbit	Validated	
120	Raf-C_pS338	C-Raf_pS338	RAF1	CST	9427	179.4	Rabbit	Use with Caution	
121	Ras-K	K-Ras	KRAS	Santa Cruz	sc-30 (F234)	1045.1	Mouse	Use with Caution	
122	Rb	Rb	RB1	CST	9309	552.7	Mouse	Validated	
123	Rb_pS807_S811	Rb_pS807_S811	RB1	CST	9308	557.9	Rabbit	Validated	
124	S6_pS235_S236	S6_pS235_S236	RPS6	CST	2211	600.8	Rabbit	Validated	
125	S6_pS240_S244	S6_pS240_S244	RPS6	CST		601.4	Rabbit	Validated	
126	Smac	Smac	DIABLO	CST	2954	610.4	Mouse	Validated	new
127	Smad1	Smad1	SMAD1	Epitomics	1649-1	922.2	Rabbit	Validated	new
128	Smad3	Smad3	SMAD3	Epitomics	1735-1	796.1	Rabbit	Validated	
129	Smad4	Smad4	SMAD4	Santa Cruz	sc-7866	920.1	Mouse	Validated	new
130	Snail	Snail	SNAI2	CST	3895	616.1	Mouse	Use with Caution	
131	Src	Src	SRC	Upstate (Millipore)	05-184	621.2	Mouse	Validated	
132	Src_pY416	Src_pY416	SRC	CST	2101	623.11	Rabbit	Use with Caution	
133	Src_pY527	Src_pY527	SRC	CST	2105	626.5	Rabbit	Validated	
134	STAT3_pY705	STAT3_pY705	STAT3	CST	9131	637.6	Rabbit	Validated	
135	STAT5-alpha	STAT5-alpha	STAT5A	Epitomics	1289-1	638.1	Rabbit	Validated	
136	Stathmin	Stathmin	STMN1	Epitomics	1972-1	718.1	Rabbit	Validated	
137	Syk	Syk	SYK	Santa Cruz	sc-1240	1033.1	Mouse	Validated	new
138	Tau	Tau	MAPT	Upstate (Millipore)	05-348	646.1	Mouse	Use with Caution	
139	TAZ_pS89	TAZ_pS89	WWTR1	Santa Cruz	sc-17610	779.0	Rabbit	Use with Caution	
140	TFF1	TFF1	TFF1	Epitomics	2044-1	1081.1	Rabbit	Validated	new
141	TIGAR	TIGAR	C12ORF5	Epitomics	S1711	1107.1	Rabbit	Validated	new
142	Tuberin	Tuberin	TSC2	Epitomics	1613-1	670.1	Rabbit	Use with Caution	
143	VASP	VASP	VASP	CST	3112	678.2	Rabbit	Use with Caution	
144	VEGFR2	VEGFR2	KDR	CST	2479	688.4	Rabbit	Validated	
145	XIAP	XIAP	XIAP	CST	2042	699.6	Rabbit	Use with Caution	
146	XRCC1	XRCC1	XRCC1	CST	2735	906.1	Rabbit	Use with Caution	
147	YAP	YAP	YAP1	Santa Cruz	sc-15407	780.0	Rabbit	Validated	
148	YAP_pS127	YAP_pS127	YAP1	CST	4911	782.1	Rabbit	Use with Caution	
149	YB-1	YB-1	YBX1	SDI	1725.00.02	700.1	Rabbit	Validated	
150	YB-1_pS102	YB-1_pS102	YBX1	CST	2900	835.1	Rabbit	Validated	

Appendix IV Molecular Breast Imaging (MCR Participants Only)

MBI Imaging Equipment

Cadmium-Zinc-Telluride (CZT) detectors are essentially equivalent to conventional gamma cameras, and differ only in that the material used to detect radiation is a semiconductor material rather than a scintillating crystal such as Sodium Iodide. Mayo currently has 3 dual-detector MBI systems. One MBI system is FDA approved, and the other 2 devices are prototypes and do not have 510k FDA approval. While we will attempt to utilize the FDA approved device in this study, this is unit may not be available at the appropriate time and patients will be imaged on an available system to expedite their imaging study. The 3 devices are essentially equivalent in terms of their imaging capabilities and performance. The 2 prototype detectors are mounted on modified mammographic gantries which were constructed in-house with assistance from the Department of Engineering, hence these 2 imaging systems are currently not FDA approved. However, under the FDA's Investigational Devices Exemptions Manual (HHH Publication FDA 96-4159), we believe that these 2 devices are exempt from the requirements of the IDE regulation, as they meet the requirements of a diagnostic device, i.e.,

a) it is non-invasive and does not require an invasive sampling procedure that presents significant risk

b) it does not by design or intention introduce energy into a subject

c) it is not used as a diagnostic procedure without confirmation by another medically established diagnostic product or procedure.

The Mayo-developed gantries comprise the support for mounting the detectors in a position for patient use, and controls to permit compression of the breast (15 lbs. force) between the two detectors. A safety stop was designed to limit the amount of compression that can be applied to the breast. These gantries have been safely used in over 2000 MBI patient studies to date.

In terms of patient safety, the 2 prototype CZT detectors are electrically isolated and are essentially equivalent to conventional gamma cameras. They utilize the same data acquisition/analysis computer that is used in some of our clinical gamma camera systems. Hence, we believe that use of these systems in this study is exempt from the requirements of the IDE regulations and only requires IRB approval.

Radiation Exposure

This protocol involves the administration of 4-8 mCi ^{99m}Tc-sestamibi. The target organs are the upper and lower large intestine, which receive approximately 1.44 and 1.0 rads / 8 mCi respectively. The radiation dose to the breasts is 0.02 rads. Full details of the absorbed radiation dose to various organs from ^{99m}Tc-sestamibi are given below.

The radiation dose from the combined injections of ~8 mCi ^{99m}Tc-sestamibi is significantly less than that from routine myocardial perfusion studies (60 mCi ^{99m}Tc-sestamibi) and the radiation dose to the breast is less than that from a mammogram.

Radiation Dosimetry

Absorbed radiation dose estimates for ^{99m}Tc-sestamibi, with an administered dose of 8 mCi and a 2 hour void *.

Target Organ	Dose (rads/8 mCi)
Breasts	0.05
Stomach	0.16
Small Intestine	0.80

Upper Large Intestine	1.44
Lower Large Intestine	1.04
Kidneys	0.54
Bladder Wall	0.54
Thyroid	0.19
Red Marrow	0.14
Ovaries	0.40
Testes	0.08
Whole Body	0.14

Cardiolite - Kit for the Preparation of Technetium ^{99m} Tc-Sestamibi for Injection, Bristol-Myers Squibb – Medical Imaging. T5-B001, May 2003.

Appendix V Return of Test Results

1. BACKGROUND

In MC1137 (the BEAUTY study), analysis of germline DNA is important for the identification of inherited mutations in cancer susceptibility genes, which can assist in patient counseling regarding treatment options.

Approximately 5-10% of breast cancer is caused by germline mutations in cancer susceptibility genes. A minimum of 200 and a maximum of 230 participants will enroll in BEAUTY. Therefore, it is reasonable to expect that germline mutations will be present in approximately 10-23 BEAUTY participants. Due to early-onset diagnosis and/or family history of cancer, we expect that many of these participants will have undergone clinical genetic testing previously as directed in their clinical care by the Mayo multidisciplinary team and will therefore already be aware of their genetic test results. However, there is the potential that the research germline DNA analysis will identify mutations in clinically relevant genes that the patient has not been tested for and therefore is not aware of. Thus far, in the analysis of the first 69 patients enrolled in MC1137, three BRCA1 and five BRCA2 gene mutations have been identified. Six of these participants have undergone clinical testing previously and are thus aware of their results, while two patients have not yet had clinical genetic testing to our knowledge.

We propose to offer the return of research germline DNA analysis results to MC1137 (BEAUTY) participants who are found to carry actionable germline mutations in cancer susceptibility genes and who are not already aware of their genetic test results through clinical testing. Please refer to Table 1 for a complete list of genes. The focus of our analysis will be on genes associated with hereditary breast cancer risk. We will also analyze other known cancer-predisposition genes, but we expect that identifying mutations in these other genes will be exceedingly rare. Mutations in all genes included in this proposal have associated medical management guidelines, such as National Comprehensive Cancer Network (NCCN) guidelines, and are thus actionable [redacted]). Mutations in genes that do not have medical management guidelines and are thus felt not to benefit the patient in terms of counseling will not be offered to be returned to the patient. Mutations in genes causing childhood-onset conditions will not be returned, given that women enrolling in BEAUTY are all beyond the age when these disorders would have typically manifested. Conditions with an autosomal recessive inheritance pattern will not be offered for return of results because if two mutations are detected, it is not possible to determine whether they occur in cis (on the same allele and thus not causative of the condition) or in trans (on opposite alleles and thus causative of the condition). Clinical testing in a CLIA-certified laboratory is available in the United States for all conditions in order to confirm these research results. Note, coverage of the exome sequencing may not be adequate to identify all disease associated mutations identified in Table 1. Results will be returned only for those mutations identified with the current technology and coverage.

Although there is no general consensus in the literature about whether individual research results should be returned to participants (1-3), ethical principles, studies of patient preferences, expert opinion in the literature, and national guidelines support the return of individual research results in certain situations. The ethical principles of beneficence and non-maleficence emphasize the need to act positively to maximize benefits and minimize harms for research participants. Studies of participants' attitudes toward return of research results indicate that the majority wish to receive study results (4, 5). Many experts believe that individual research results could or should be returned under specific conditions, often including: the finding has important health implications with substantial risks, the finding is actionable, e.g. strategies are available to reduce expected morbidity or mortality, the test is analytically valid, and the participant has consented/opted to receive the results (1, 6-8). Several national guidelines accept a similar stance that individual research results meeting certain criteria should be returned, including the

National Heart, Lung, and Blood Institute (NHLBI) and the National Cancer Institute Office of Biorepositories and Biospecimen Research (7, 11). Furthermore, the majority of researchers accept that there is a fundamental "duty to rescue," meaning that participants should be informed when an investigator discovers genetic information that clearly indicates a high probability of a serious condition for which an effective intervention is readily available (2, 3, 6, 9).

All BEAUTY participants have been consented to indicate whether they would like to be contacted and given the opportunity to receive germline test results in the future. Only those participants who checked "yes" will be contacted to offer return of research results if an actionable germline mutation is discovered.

The Clinical Laboratory Improvement Amendments (CLIA) of 1988 regulates testing on humans for health purposes and requires laboratories to establish analytical validity. Given that the germline testing in BEAUTY is not performed in a CLIA-certified laboratory, we will strongly recommend to all participants that their results be confirmed in a CLIA-certified laboratory before being used for clinical decision-making.

2. METHODS

2.1 ANALYTICAL METHOD

Results from the germline DNA analysis will be analyzed by a genetic counselor on the BEAUTY team. Germline variants in cancer-predisposition genes with existing medical management guidelines will be classified as benign, likely benign, variant of uncertain significance, likely deleterious, or deleterious using standard genetic variant software programs and databases including Alamut, Human Gene Mutation Database (HGMD), 1000 Genomes, Leiden Open Variation Database (LOVD), Breast Cancer Information Core (BIC), etc.

2.2 RETURN OF RESULTS METHOD

All women who previously opted-in to be contacted about germline test results, who are found to carry deleterious or likely deleterious germline mutations in a cancer-predisposition gene included in Table 1, and who have not had previous clinical testing that identified the germline mutation will be offered return of results.

1. A BEAUTY study coordinator will contact each eligible participant by telephone to inform her that the blood genetic test results are now available, and offer the participant an appointment with the BEAUTY genetic counselor at no charge for further information about these results. Please refer to the "Outline of Telephone Call for Study Coordinators" (section 1.2.3) for complete details regarding this phone call.
   The study coordinator will note that the participant does not need to make a decision about whether to accept or decline the appointment at this time, and offer to send a letter in the mail that summarizes the results disclosure procedure and includes information about genetic testing for hereditary cancer (see section 1.2.6, "Letter to Participants," below).

2. If the participant elects to proceed with an appointment with the genetic counselor, the participant will be given information about genetic testing for hereditary cancer, including risks, benefits, and limitations of test results, and an opportunity to ask questions (see section 2.4, "Outline of First Genetic Counselor Appointment," below).

   a. In an effort to make sure that participants are fully informed and have adequate time to think through this information before making a decision about whether or not to learn the genetic

test results, participants will not be given their results during the first appointment. Instead, it will be recommended that they consider their options and return for a second appointment during which they will learn their results. They will be asked to call the study coordinator to schedule a second appointment if they choose to receive their research results.

b. NOTE: the genetic counseling appointment can be performed in-person at Mayo Clinic or by telephone, depending on the participant's preference.

c. NOTE: the participant will not be charged for the genetic counseling appointment(s).

d. Note: if the patient desires to receive the results immediately after the first appointment and this is appropriate based on the judgment of the genetic counselor, the second appointment can occur immediately after the first appointment.

3. If the participant elects to receive their genetic test results, they will be given their research results at a second appointment (see section 2.5, "Outline of Second Genetic Counseling Appointment," below).

   a. We will clearly inform them that the research results must be confirmed in a CLIA-certified laboratory before any clinical decision is made based on these results.

      i. NOTE: the genetic counselor will assist with coordinating the testing at a CLIA-certified laboratory.

      ii. NOTE: the cost of CLIA-certified testing will be submitted to the participant's insurance company. In the event that the cost of this testing is not covered or only partially covered by the insurance company, funds from the BEAUTY study are available to cover the remainder.

   b. A summary letter and additional gene-specific hereditary cancer information will be mailed to the participant. A second copy will be included that the participant can share with their physician(s) if they chose to do so.

   c. A clinic note will be entered in the patient's electronic medical records documenting the visit discussion, the research results, and whether the patient elected to proceed with confirmatory clinical testing.

   d. Should the participant have any questions or concerns about their clinical management based on their results, they will be referred back to their oncologist for clinical management after CLIA testing.

4. If the participant chooses not to receive these research results:

   a. A letter will be sent summarizing the first appointment if the patient desires.

   b. A clinic note will be entered into the patient's electronic medical records documenting the first appointment and that the patient declined to receive the research results. The research results will not be entered into the patient's records.

2.3 OUTLINE OF TELEPHONE CALL FOR STUDY COORDINATORS

1. Introduce self/involvement in BEAUTY.
2. Explain reason for phone call:
   a. *You may recall filling out a consent form previously that asked if you were interested in learning more about your blood genetic test results. You had checked "yes" that you would like the opportunity to receive these results. We are contacting you as these research results are now available to give you the option to learn your results.*
3. Ask if the participant is still interested in receiving more information about her blood genetic test results and offer the participant an appointment with the genetic counselor (at no charge) for further information about these results.
   a. Are you still interested in receiving more information about your blood genetic test results? Would you like to make an appointment with the genetic counselor (at no charge) for more information about your results?
   b. The purpose of the initial appointment with the genetic counselor is to talk about the type of genetic testing that was performed, and to talk through the risks, benefits, and limitations of learning the test results.
   c. This appointment can either be in-person at Mayo Clinic or over the telephone.
   d. The genetic counselor will not give you the research results during this first appointment. If you decide after the first appointment with the genetic counselor that you want to receive your research results, you can call me [the study coordinator] back to set up a second appointment with the genetic counselor. Two appointments are planned to make sure that you are fully informed and have adequate time to think through the risks of benefits of receiving genetic test results before you make a decision. During the second appointment with the genetic counselor, you will have an opportunity to ask any questions or discuss any concerns that you may have thought of, and you will then be given your results if you wish.
4. The study coordinator will note that the participant does not need to make a decision about whether to accept or decline an appointment with the genetic counselor at this time, and will offer to send the participant a letter in the mail that summarizes the results disclosure procedure and includes information about genetic testing for hereditary cancer (see section 2.6, "Letter to Participants" below).
5. The study coordinator will note that these preliminary research results will need to be verified in a certified clinical laboratory before they are used to make any decisions about the patient's medical management. If their insurance does not pay for this testing, funds from the BEAUTY study are available to cover the cost. The participant will not be charged for the appointments with the genetic counselor.

2.4 OUTLINE OF FIRST APPOINTMENT

1. Genetic counselor introduction and description of role in BEAUTY
2. Ask participant's understanding of why this appointment is taking place
3. Discuss purpose of first appointment and plan for subsequent appointment if participant decides that they would like their results
4. Description of testing that is performed in BEAUTY - analysis of cancer susceptibility genes
5. Verify that participant has not already had genetic testing clinically/aware of their results
6. Discuss participant's personal/family history of cancer (formal pedigree not necessary)
7. Education about genetic testing for hereditary cancer
   a. Sporadic vs. familial vs. hereditary cancer
   b. Background information about genetics
   c. Various genes that can cause hereditary cancer
   d. Cancer risks associated with gene mutations

e. Medical management recommendations
f. Inheritance of gene mutations/ risks to relatives

8. Risks, benefits, and limitations of test results
    a. Potential risks:
        i. Psychological impact
            1. Fear of developing a second cancer
            2. Having to make additional decisions about management
            3. Guilt of passing mutation on to children
        ii. Family
            1. Burden of sharing information with family
            2. Impact on family members/family relationships
        iii. Genetic discrimination
            1. Genetic Information Non-discrimination Act (GINA)
            2. Protects against health insurance and employment discrimination
            3. Does not cover life, long-term care, or disability insurance
            4. State laws
    b. Potential benefits:
        i. Possible explanation for cause of breast cancer diagnosis (if a breast cancer susceptibility gene is identified)
        ii. Opportunity to prevent/reduce risks for other cancers
        iii. Opportunity to share information with at-risk relatives
        iv. Potential for enrollment in clinical trials/ selected therapies
    c. Limitations:
        i. Need to confirm research results in a CLIA-certified laboratory (will submit to insurance company; if test is not covered or partially covered, funds are available to pay remaining cost)
9. No cost for genetic counseling
10. Summary of research results will be in the medical records only if participant chooses to receive the results
11. Participant questions
12 Explain to participant that after thinking about this information, if they decide that they would like to receive their results, they can call the study coordinator to schedule a second appointment for results disclosure

2.5 OUTLINE OF SECOND APPOINTMENT

1. Answer any questions/concerns that participant has thought of since previous appointment
2. Confirm that the participant would like to receive their research results
    a. If so, disclose results
    b. If not, offer opportunity for results disclosure in the future if they change their mind
3. Discuss implication of results
    a. Cancer risks
    b. Medical management
    c. Inheritance/risks to relatives
4. Recommend CLIA-certified laboratory testing
    a. Facilitate testing if patient wants to proceed. After results are received from CLIA-certified laboratory, contact participant by telephone to review results.
5. Mail two copies of follow-up letter to participant (one for participant and one that they can give to their physician)

2.6 LETTER TO PARTICIPANTS

Participant Name
Address
City, State Zip

Dear *Participant* Name,

Thank you for your continued participation in the BEAUTY study here at Mayo Clinic. You may recall filling out a consent form previously that asked if you were interested in learning more about your blood genetic test results. You had checked "yes" that you would like the opportunity to receive these results. We are contacting you as these research results are now available to give you the option to learn your results. More information about genetic testing is included in the following pages.

If you are interested in learning more about your blood genetic test results or if you have questions:

Please call the study coordinator at 507-xxx-xxxx. The study coordinator will set up an appointment for you to talk with the genetic counselor ([redacted]

At the time of the initial appointment with the genetic counselor, you will be given more information about the genetic testing that was performed on the blood specimen that was collected at the time of your enrollment. The risks, benefits, and limitations of learning your results will be discussed. You will also have an opportunity to ask any questions you might have. The genetic counselor will not give you the research results during this first appointment.

If you decide after the first appointment with the genetic counselor that you want to receive your research results, you can call the study coordinator to set up a second appointment with the genetic counselor. Two appointments are planned to make sure that you are fully informed and have adequate time to think through this information before you make a decision about whether or not to learn the genetic test results. During the second appointment with the genetic counselor, you will have an opportunity to ask any questions or discuss any concerns that you may have thought of, and you will then be given your results if you wish.

These preliminary research results will need to be verified in a certified clinical laboratory before they are used to make any decisions about your medical management. If your insurance does not pay for this testing, funds from the BEAUTY study are available to cover the cost.

These appointments can be in person at Mayo Clinic or over the telephone if you prefer, depending on what is most convenient for you.

Results will not be available for up to 3 years from the date of this letter.

If you are NOT interested in learning your blood genetic test results from this study, check the box below and return this letter in the enclosed envelope.

☐ I do NOT want to receive my blood genetic test results from this study.

Please be assured that regardless of your response, your involvement in the BEAUTY study and your care at Mayo Clinic will not be impacted in any way. As always, we thank you for your participation in the BEAUTY study.

Sincerely,

Genetic Testing for Hereditary Cancer

Hereditary diseases are caused by gene mutations that are passed down through a family. A mutation is an alteration, or change, in a gene. Genetic tests can show whether you carry a gene mutation. Read this material to learn more about genetic testing. The material outlines things to think about related to genetic testing. It tells you what genetic test results may mean to you and your family.

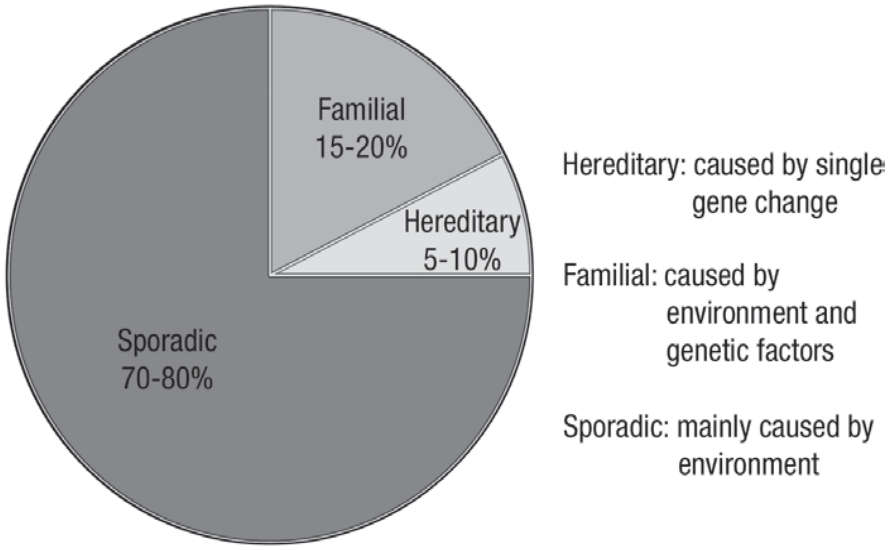
Causes of Cancer

Around 40 percent of people in the general population are diagnosed with cancer. Researchers do not know exactly what causes most cases of cancer. It happens when cells accumulate genetic changes and begin to divide and grow faster than healthy cells do. The cells then build up and form a tumor.

Most cancers are "sporadic". This means they happen by chance. Sporadic cancers are due mostly to aging, the environment, and lifestyle choices.

Some cancers appear to be "familial," meaning that there is more cancer in the family than would be expected by chance alone. Genetic factors may play a role in familial cancers, but families share many things besides their genes. For example, they may eat the same kinds of foods. They may live in the same kind of environment. They may have similar jobs. These things in combination might cause several cases of cancer in a family.

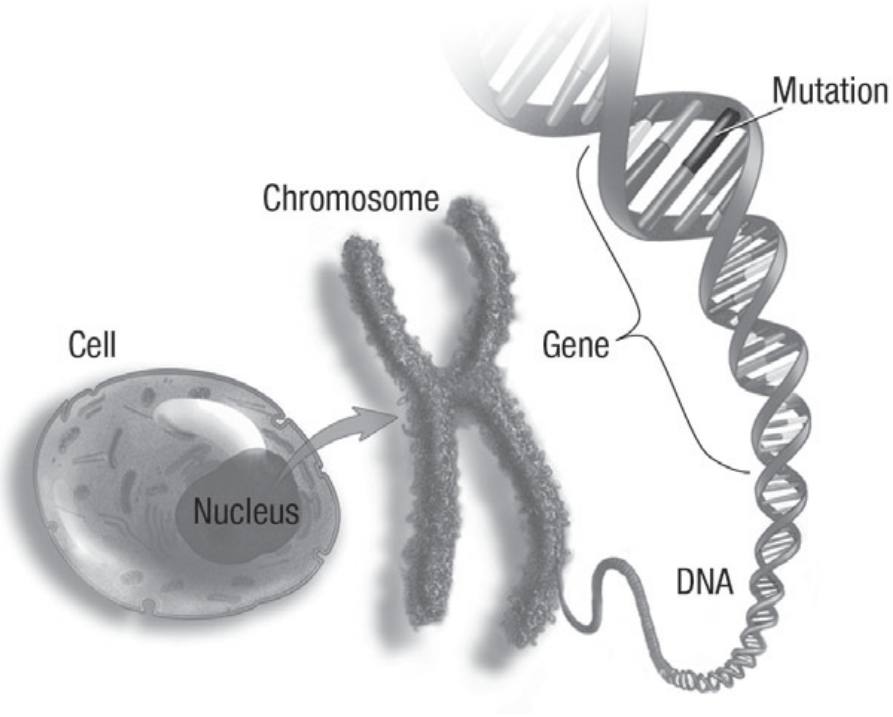
Only about 5 to 10 percent of breast cancers are "hereditary", or inherited. Certain gene changes, called mutations, that pass from parents to children can cause hereditary cancers. If you have one of these mutations your risk for certain cancers is higher. However, having a mutation does not guarantee that a person will develop cancer.



What are Genes and Mutations?

Some background information about genes can help explain how you may inherit a higher risk for cancer. Inside of each one of your cells are your chromosomes. Chromosomes are made of a long chain of a special chemical called DNA. Your DNA provides instructions that tell your cells how to work. DNA is divided into sections called genes. Each gene determines a specific trait, such as your eye color or blood type.

Mistakes or alterations in genes are called mutations. They are like words in a sentence that are spelled wrong or are in the wrong order. You can't read the sentence correctly. In the same way, mutations in genes can keep cells from doing their work correctly. That can lead to genetic diseases or other problems.



What Causes Hereditary Cancer?

Hereditary cancer is caused by mutations in certain genes. When these genes have no mutation and work normally, they stop tumor growth. But mutations in these genes increase the risk of certain cancers.

If a family has a gene mutation, cancer often affects several family members in more than one generation. Family members who have hereditary cancer often get it when they are very young, such as under age 50. They may have more than one type of cancer. They also may have rare cancers.

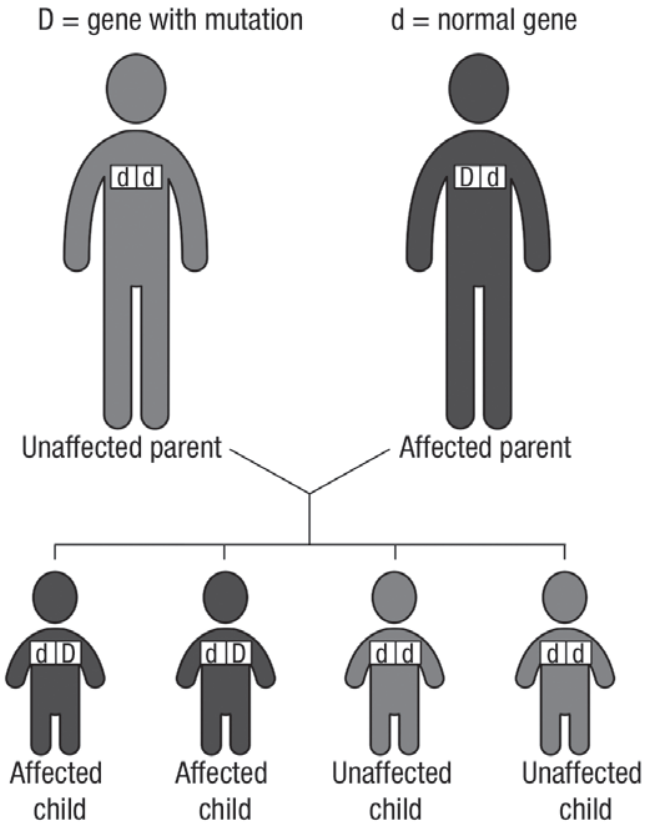
Please remember that having an inherited gene mutation does not guarantee that an individual will get cancer. Some people with gene mutations may live their entire life without developing cancer.

How are Gene Mutations Inherited?

Inherited mutations can pass from a parent to a child. You can inherit a gene mutation from either your mother or your father.

Gene mutations cannot skip generations. If your parent has a gene mutation that you do not inherit, you cannot pass the mutation on to your children.

Most gene mutations that cause hereditary cancer are inherited in an autosomal dominant pattern. This means that every child (both sons and daughters) of a parent who has a genetic mutation has one chance in two, or a 50-50 chance, to inherit the same mutation.



Possible Test Results

Positive test result

A positive result means that a mutation was found in one of your genes. This puts you at a higher risk to develop certain cancers. Your health care provider will talk with you about options for managing your cancer risks.

A positive test result also means that you could pass the mutation to your children. Your siblings and parents may carry the mutation as well. If your test result is positive, these family members may want to think about genetic testing.

Negative test result

A negative test result means that the test did not find a gene mutation. But the effect of this on your cancer risk is not always clear. Your genetic specialist looks at the test result in light of your family history. You may still be at increased risk of cancer if you have close family members with cancer.

Genetic testing can find most mutations in genes. However, you could have a gene mutation that the test was not able to find.

Deciding Whether to Receive Genetic Test Results

There are a number of things to think about before receiving genetic test results.

Benefits of genetic tests
Genetic testing for cancer may:

- Help show whether you are at higher risk for other cancers.
- Help you make medical choices, such as what type of surgery to have or how often to have screening procedures in the future.
- Explain the reason for your diagnosis of cancer, or for the cancer in your family.
- Let other family members learn about their risks so they can decide whether to have testing too.

Limitations of genetic tests
Be sure to talk with your genetic specialist about what genetic testing can and cannot tell you. Genetic test results may not give you a clear answer about your cancer risk. The tests may help to find people or families with a high risk of cancer. But the tests cannot tell for sure who will or will not get cancer.

Reactions to your test results

- *If your test result is positive:*
  - Knowing your risk for cancer may make you feel in control and able to take charge of your health. Being able to inform and educate your family members about their possible cancer risks may give you a positive feeling.
  - However, knowing that you carry a gene mutation can be hard. You may feel anxious, sad, afraid or angry. You may think that you surely will get cancer. You may feel guilty about the chance that you may pass a gene mutation on to your children.
  - You may need to make some hard choices about the best cancer prevention method for you. Talking about your choices with a genetic specialist or other cancer specialist can help guide you.
- *If your test result is negative:*
  - You may have a sense of relief to know that you do not have a gene mutation. If you don't have an increased cancer risk, you may not need to have screenings more often or make decisions about measures to prevent cancer.
  - However, don't let this relief lull you into a false sense of security. Even if your test result is negative, you can still get cancer. Women with other risk factors such as a family history of cancer or certain lifestyle choices may have an increased risk of cancer.
  - You may feel "survivor guilt" if other family members do carry a mutation and face a greater risk of cancer. In addition, because test results do not always give a clear answer, your health care provider may not be able to give you a definite conclusion about the meaning of your results. This can leave you feeling unsure about your cancer risk.

Other things to think about

- *Confirming the preliminary research results:*

  - Your preliminary research results will need to be verified in a certified clinical laboratory before they are used to make any decisions about your medical management.
- *Financial issues:*
  - Many insurance companies cover some or all of the cost of genetic testing. If your insurance company does not cover the cost of your confirmatory genetic testing, funds from the BEAUTY study are available to help you.
- *Talking with your family:*
  - Each person's situation is different. Some of your relatives may not want to know if a gene mutation is found within the family. Think about whether you will share test results with your family members. If you plan to share your results, think about how you will do this. Your health care provider or genetic specialist can help you find ways to share or ask for medical information within your family. For example, he or she may write a letter about your test results that you can give to family members.
- *Concerns over health insurance discrimination:*
  - There are federal and state laws to help keep your genetic information private. The federal Genetic Information Nondiscrimination Act, or GINA, helps protect you against discrimination in health insurance and employment. Laws to help protect you from discrimination in life insurance, long term care insurance and disability insurance vary from state to state.

Follow-up Care

If you have a positive test result, you may choose to take steps to lower your cancer risk. What you choose to do depends on your age and medical history. It depends on past treatments or surgeries you have had. Your personal preferences also will affect your choices.

Talk with your health care provider about your choices. Your provider may suggest that you have screening exams more often. Certain medicines may help lessen your risk for certain cancers. To help prevent cancer, some women have surgery to remove healthy tissue, such as the ovaries. Ask your health care provider to help you plan what is right for you.

A Final Word

Many things affect your decision about whether to receive genetic test results. Take time to think about what is important to you. Think about the benefits and limitations of genetic tests. If you wish, talk with your family and close friends about your concerns. Your health care provider and genetic specialist can give you more information to help you decide whether this is right for you.

2.7 EXAMPLE FOLLOW-UP LETTER TO PARTICIPANTS

MAYO CLINIC

CENTER FOR THE INDIVIDUALIZED MEDICINE

MONTHLY PROGRESS UPDATE PREPARED FOR MANAGEMENT TEAM AND BOARD OF GOVERNORS

Participant Name
Address
City, State Zip

Dear *Participant Name*,

It was a pleasure talking with you about your choice to learn your research genetic test results. As we discussed previously, your research results showed that you tested positive for a mutation in that causes . The specific mutation is in the ____ gene. Your result was confirmed by , a CLIA-certified clinical laboratory. The name of the specific mutation is: . A copy of your test result is included with this letter.

The discovery of this mutation may influence your medical management and provides some potentially useful information for your at-risk relatives. I encourage you to discuss these results with your doctor and with your family members. I have provided a second copy of this letter for you, which you may share with your family or your physician if you would like.

We hope this information is useful to you. If you have questions when you receive this, please do not hesitate to contact us.

As always, we want to express our appreciation for your continued participation in the BEAUTY study.

Sincerely,

[redacted]

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Appendix VI Return of Results Gene List

Gene name	Syndrome/ associated cancers	Listed in ACMG's recommendations for reporting incidental findings?	Medical management guidelines?	Inheritance Pattern	Return to participants?
APC	Familial Adenomatous Polyposis	Yes	NCCN	AD	Yes
BMPR1A	Juvenile polyposis	No	NCCN	AD	Yes
BRCA1	Hereditary breast/ovarian cancer syndrome	Yes	NCCN	AD	Yes
BRCA2	Hereditary breast/ovarian cancer syndrome	Yes	NCCN	AD	Yes
CDC73	Familial primary hyperparathyroidism/ Hyperparathyroidism-jaw tumor syndrome/ Parathyroid adenoma with cystic changes/ Parathyroid carcinoma	No	GeneReviews	AD	Yes
CDH1	Hereditary Diffuse Gastric Cancer	No	Fitzgerald et al., J Med Genet 2010;47:436-444	AD	Yes
CDK4	Hereditary Melanoma	No	Guidelines for CDKN2A but not CDK4; according to Puntervoll et al., J Med Genet 2013; 50(4):264-270, phenotype cannot be distinguished.	AD	Yes
CDKN2A	Hereditary Melanoma	No	Eckerle Mize et al., in Cancer Syndromes: NCBI; 2009	AD	Yes
DKC1	Dyskeratosis congenita	No	GeneReviews & Savage et al., Pediatr Blood Cancer 2009; 53:520-523.	XLR	Yes
EPCAM	Lynch syndrome	No	NCCN for other Lynch syndrome associated-genes, not specifically for EPCAM	AD	Yes

Gene name	Syndrome/ associated cancers	Listed in ACMG's recommendations for reporting incidental findings?	Medical management guidelines?	Inheritance Pattern	Return to participants?
FH	Hereditary Leiomyomatosis and Renal Cell Cancer	No	Provisional guidelines in GeneReviews; no consensus guidelines.	AD	Yes
FLCN	Birt-Hogg-Dube syndrome	No	GeneReviews	AD	Yes
KIT	Familial GIST	No	Provisional recommendations in Familial Cancer Suscetibility Syndromes handbook; no consensus guidelines.	AD	Yes
MAX	Hereditary Paraganglioma/Pheochromocytoma	No	GeneReviews; no consensus guidelines.	AD	Yes
MEN1	Multiple Endocrine Neoplasia type 1	Yes	GeneReviews & Thakker et al., J Clin Endocrin Metab 2012; 97(9): 2990-3011.	AD	Yes
MET	Papillary renal cell carcinoma	No	Provisional recommendations in Familial Cancer Suscetibility Syndromes handbook; no consensus guidelines.	AD	Yes
MLH1	Lynch syndrome	Yes	NCCN	AD	Yes
MSH2	Lynch syndrome	Yes	NCCN	AD	Yes
MSH6	Lynch syndrome	Yes	NCCN	AD	Yes
NF2	Neurofibromatosis type II	Yes	GeneReviews & Evans et al., Br J Neurosurg 2005a; 19: 5-12	AD	Yes
PDGFRA	Familial GIST	No	Provisional recommendations in Familial Cancer Suscetibility Syndromes handbook; no consensus guidelines.	AD	Yes
PMS2	Lynch syndrome	Yes	NCCN	AD	Yes
PRKAR1A	Carney complex	No	GeneReviews	AD	Yes
PTCH1	Gorlin syndrome	No	GeneReviews	AD	Yes

Gene name	Syndrome/ associated cancers	Listed in ACMG's recommendations for reporting incidental findings?	Medical management guidelines?	Inheritance Pattern	Return to participants?
PTEN	Cowden syndrome	Yes	NCCN	AD	Yes
RET	MEN2	Yes	GeneReviews & American Thyroid Association Guidelines Task Force, Thyroid 2009; 19(6): 565-612.	AD	Yes
SDHAF2	Hereditary Paraganglioma/Pheochromocytoma	Yes	GeneReviews; no consensus guidelines.	AD	Yes
SCG5	Hereditary Mixed Polyposis Syndrome	No	Proposed in various papers; see Calva and Howe, Surg Clin North Am 2008; 88(4):779.	AD	Yes
SDHB	Hereditary Paraganglioma/Pheochromocytoma	Yes	GeneReviews; no consensus guidelines.	AD	Yes
SDHC	Hereditary Paraganglioma/Pheochromocytoma	Yes	GeneReviews; no consensus guidelines.	AD	Yes
SDHD	Hereditary Paraganglioma/Pheochromocytoma	Yes	GeneReviews; no consensus guidelines.	AD	Yes
SMAD4	Juvenile polyposis	No	GeneReviews	AD	Yes
STK11	Peutz-Jeghers syndrome	Yes	NCCN	AD	Yes
TERC	Dyskeratosis congenita	No	GeneReviews & Savage et al., Pediatr Blood Cancer 2009; 53:520-523.	AD	Yes
TINF2	Dyskeratosis congenita	No	GeneReviews & Savage et al., Pediatr Blood Cancer 2009; 53:520-523.	AD	Yes
TP53	Li-Fraumeni syndrome	Yes	NCCN	AD	Yes
TSC1	Tuberous sclerosis	Yes	GeneReviews, de Vries et al., Eur Child Adolesc Psychiatry 2005; 14(4): 183-190, & Roach et al., J Child Neurol 1999; 14(6): 401-407.	AD	Yes

Gene name	Syndrome/ associated cancers	Listed in ACMG's recommendations for reporting incidental findings?	Medical management guidelines?	Inheritance Pattern	Return to participants?
TSC2	Tuberous sclerosis	Yes	GeneReviews, de Vries et al., Eur Child Adolesc Psychiatry 2005; 14(4): 183-190, & Roach et al., J Child Neurol 1999; 14(6): 401-407.	AD	Yes
VHL	Von Hippel-Lindau	Yes	GeneReviews & http://vhl.org/wordpress/library/Handbook/handbook40.pdf	AD	Yes

Appendix VII Digital Spatial Profiling of High Risk Hormone Receptor-Positive (HR+) Breast Cancer

Analytical Objective: In collaboration with the Mayo BEAUTY team, we will use the NanoString GeoMX Digital Spatial Profiling (DSP) platform to elucidate the immune architecture of high risk hormone-receptor positive HER2-negative (HR+) breast tumor samples from the BEAUTY study[1]. The initial experiments are designed to identify immune features of clinical high risk ER+/HER2 negative breast cancer in order to provide a new understanding of the immune landscape and biology of ER+/HER2- breast cancer both before and after chemotherapy. The overarching goal is to compare the immune landscape for those that develop a local/regional recurrence with those tumors with no recurrence. A secondary objective is to assess the immune landscape of tumors throughout the course of chemotherapy (baseline, after taxane, at surgery, and at the time of recurrence).

Clinical Rationale: The use of immune checkpoint inhibitors (ICIs) has revolutionized cancer treatment in the past decade. ICIs are believed to function primarily through inhibition of pre-existing host immune suppression. Consequently, an understanding of the baseline immune environment is critical for understanding and predicting the efficacy of immuno-therapy. Multiple clinical trials have demonstrated durable recurrence-free response with ICIs in "hot" or T-cell inflamed tumors such as melanoma or lung cancer. In contrast, the results of single agent ICIs in less immunogenic tumors such as breast cancer have been somewhat disappointing. This is particularly true in immunologically "cold" tumors like HR+/HER2-negative (i.e. luminal) breast cancer, that characteristically have lower amounts of tumor infiltrating lymphocytes (TILs)[2]. Clinical trials with a single agent ICI, anti-PD-L1 avelumab, showed that the objective response of single agent avelumab was only 2.8%[3] in such tumors.

There are several ongoing efforts to identify potential therapies to improve the efficacy of ICIs in luminal breast cancer. One such strategy is to combine conventional chemotherapy with ICIs. This strategy was evaluated in the recent I-SPY 2 trial in which patients with luminal breast cancer were randomized to receive standard neoadjuvant chemotherapy with or without pembrolizumab[4]. A 21% increase in complete pathological response (pCR) was observed when pembrolizumab was added to neoadjuvant chemotherapy (34% pCR with pembrolizumab plus chemotherapy vs. 13% with chemotherapy alone). However, the pCR rate in luminal breast cancer is still relatively low compared to more immunogenic tumors like triple negative breast cancer (TNBC), among which 60% of patients achieved pCR with pembrolizumab in combination with chemotherapy.

Recent studies suggest that pre-existing host immune features may play a critical role in therapeutic response of less immunogenic HR+ breast tumors, and additional studies are needed to evaluate the immune landscape of these tumors within the context of response to both conventional and Immune therapies. Limited data exist on the immune architecture of luminal breast cancer, and the significance of pre-existing host immune features and outcome of patients with luminal breast cancer remains to be determined. This is particularly true among patients with HR+ tumors with high risk of recurrence, generally defined as Grade 3 or Grade 2 with high proliferation.

There are also data that suggest that the significance of the baseline immune architecture may be quite different in HR+ breast cancer, compared to more immunogenic breast cancer like TNBC. Higher levels of TILs are associated with improved outcome in TNBC or HER2+ breast cancer; whereas high TILs in luminal HER2-negative breast cancer is associated with relatively poorer overall survival[2]. Such data reinforce the need for a more detailed understanding of the immune architecture of breast cancer subtypes.

Quantification of TILs and analysis of the prognostic and/or predictive potential of TILs is a subject of great interest in breast cancer research at this time. However, the type, activity, and function of specific immune cells cannot be determined by simple pathological assessment of TILs. For example, one of the genes that is associated with poor outcome in luminal breast cancer is CD68[5] which is highly expressed in

both circulating and tissue macrophages. This observation illustrates the need to begin to break down the relationship between specific types of TILs and clinical outcome as a function of the immune environment that prevails in different breast cancer subtypes. Elucidation of the immune architecture in luminal breast cancer is critical in moving the field of immune-oncology forward in breast cancer. DSP provides a new tool that makes it possible to examine features associated with tumor cell-immune cell interaction at a level of detail that was heretofore unavailable.

Samples: We will analyze tumor immune architecture as a function of clinical outcome following neoadjuvant therapy in a cohort of 52 high risk HR+/HER2- samples from the BEAUTY study. All women received weekly paclitaxel for 12 weeks, followed by AC chemotherapy, followed by surgery. Formalin-Fixed, paraffin embedded samples are available before treatment, after taxane, and after chemotherapy (residual disease). Additionally, a subset have an FFPE sample available from a loco-regional or distant recurrence.

For the primary aim, will assess in a co-primary endpoint of whether the immune landscape in the a) primary tumor biopsy (before chemotherapy) and b) surgical specimen (after chemotherapy) is associated with the risk of recurrence. A secondary analysis will be to assess whether the immune landscape using the baseline tumor is associated with chemotherapy response (RCB 0/1 vs 2/3). Finally, we will assess whether the immune landscape changes over time comparing the pre-treatment, b), after taxane c) surgical and d) recurrence. The latter will be an exploratory endpoint.

GeoMX Digital Spatial Profiling: DSP is a novel technology that allows simultaneous profiling of multiple proteins or RNAs while preserving the spatial information. The DSP technology is quite simple in concept and execution. FFPE sections (one 5-micron slide/sample) will be labeled with three fluorescent marker antibodies (pan-cytokeratin, anti-CD45, and anti-CD68) plus a DNA dye (SYTO13) to stain nuclei. These marker antibodies will be used to define regions of interest (ROIs). FFPE sections will also be incubated with the cocktail containing a panel of other "target" antibodies that are conjugated to synthetic oligonucleotides via a UV-cleavable cross linker. At present there are >40 well-validated target antibodies against immune function proteins (e.g. PD-L1, CD3, CD8, B2M, INO1, etc.), common tumor-related proteins (ERBB2, Ki67, BCL2, etc) or housekeeping proteins for normalization. The list of rigorously validated target antibodies grows almost daily. The current list of validated protein targets is shown below.

Figure 1A shows an ROI that has been segmented into tumor (pan-cytokeratin-green) and CD68-enriched (red) stroma. Stroma is segmented as SYTO13 positive/pan-cytokeratin negative.) Figure 1B shows a different ROI that has been segmented into tumor (green) and CD45-enriched (yellow) stroma. The individual segments within these ROIs are illuminated with UV light, releasing the oligonucleotides bound to the antibodies that were bound to their respective targets within each segment of each ROI. The oligonucleotides are "counted" using conventional nanoString technology, which provides a 5-log dynamic range for precise quantification of antibody binding to target proteins. Using this approach, we obtain an indirect measurement of the abundance of immune function proteins within the tumor in a spatially defined manner.

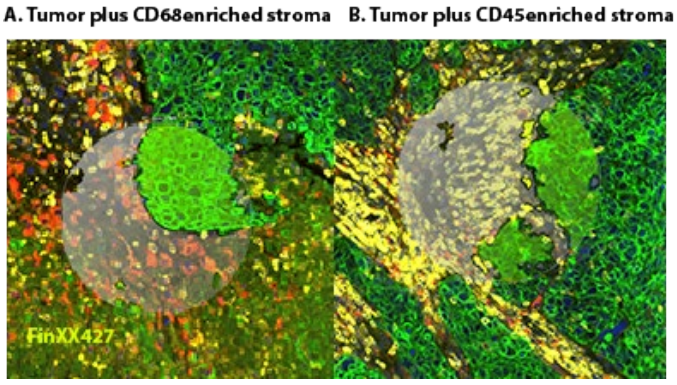
Draft Human IO†

Immune Cell Profiling Core*	IO Drug Target Module*	Immune Activation Status Module*	Immune Cell Typing Module	Pan-Tumor Module
Beta-2-microglobulin	4-1BB CD137	CD127	CD45RO	MART1
CD11c	LAG3	CD25	FOXP3	NY-ESO-1
CD20	OX40L	CD80	CD34	S100B
CD3	Tim-3	CD86	CD66b	Bcl-2
CD4	VISTA	ICOS	Gamma Delta TCR	EpCAM
CD45	ARG1	PD-L2	CD14	Her2/ErbB2
CD56	B7-H3	CD40	FAPalpha	PTEN
CD68	IDO1	CD40L	CD163	
CD8	STING	CD27		
CTLA4	GITR	CD44		
GZMB				
Ki-67				
PD1				
PD-L1				
Pan-cytokeratin				
HLA-DR				
SMA				
Fibronectin				
TGFB1				
+ Controls				

†Panel content subject to change prior to commercial launch
*Available for beta

We will use 3 fluorescent labeled antibodies to identify tumor cells (anti-pan-cytokeratin), leukocytes (anti-CD45), and macrophages (anti-CD68). Each ROI be "segmented" into two regions: a pan-cytokeratin+ segment that includes tumor, plus a STYO13+ (nuclear stain)/cytokeratin- segment that includes tumor-adjacent stroma. Twelve ROIs will be segmented for each sample: 6 ROIs that contain CD68-enriched stroma (Figure 1A), plus 6 ROIs that contain CD45-enriched stroma (Figure 1B). We will analyze 46 immune function proteins in tumor and stroma segments from each sample. Using this approach, we will test the hypothesis that macrophage (CD68)-associated vs. lymphocyte-associated (CD45+/CD68-) PD-L1 is prognostic for pCR in the neo-adjuvant setting. In addition, all other immune features will be tested for association with pCR.

Figure 1: DSP images of segmented regions of interest. Two 300 micron ROIs were segmented. The segments include pan-cytokeratin-positive tumor cells (green), CD68-enriched stroma (Panel A, red), or CD45-enriched stroma (Panel B, yellow).



Given the power of the technology, the uniqueness of the sample cohort, and the clinical significance of the question, we anticipate the results of these analyses will be of considerable clinical impact. The opportunity to use this powerful new technology to identify spatially-defined biomarkers is obvious. The wealth of genomic data that have been generated with these samples should inform subsequent studies and serve as preliminary data for future grant applications. We are also aware that some of these samples will be analyzed using the Fluidyne platform, and a cross-platform comparison would be of considerable interest, as well.

Appendix VII References:

[1] Goetz, M.P., et al., *Tumor Sequencing and Patient-Derived Xenografts in the Neoadjuvant Treatment of Breast Cancer*. Journal of the National Cancer Institute, 2017. 109(7).

[2] Denkert, C., et al., *Tumour-infiltrating lymphocytes and prognosis in different subtypes of breast cancer: a pooled analysis of 3771 patients treated with neoadjuvant therapy*. Lancet Oncology, 2018 Jan. 19(1): 40-50.

[3] Dirix, L.Y., et al., *Avelumab, an anti-PD-L1 antibody, in patients with locally advanced or metastatic breast cancer: a phase 1b JAVELIN Solid Tumor study*. Breast Cancer Research and Treatment, 2018 Feb. 167(3): 671-686.

[4] Nanda et al. Proc American Society of Clinical Oncology 2017

[5] Waks, A.G., et al., *The Immune Microenvironment in Hormone Receptor-Positive Breast Cancer Before and After Preoperative Chemotherapy.* Clinical Cancer Research, 2019 Aug 1. 15(15): 4644-4655.

Appendix VIII Gene Expression Analysis by the University of Bristol

Triple Negative Breast Cancer (TNBC) comprises around 20% of all breast cancer diagnoses, behaves more aggressively than other types of breast cancer and has targeted therapeutic options (Dent et al 2007). The majority of patients receive neoadjuvant chemotherapy in order to reduce tumour burden prior to their operation and this enables us to explore how tumours respond to chemotherapy drugs that are in use in routine clinical practice. In TNBC, there is a high-risk of early-relapse compared to other types of breast cancer, with most relapses having occurred by 3 years from diagnosis (Dent et al 2007 and Lietdke et al 2008). The relapse rate is significantly lower in those who obtain a complete pathological response to chemotherapy (Cortazar et al 2014 and Symanns et al 2017).

In trying to understand why TNBC behaves so aggressively attention has turned to cancer stem cells (CSCs) (Thiagarajan P et al 2018). Previous work has identified that CSCs (Al Hajj et al 2003), or stem-cell-like behaviour (Samanta et al 2014) could explain the aggressive characteristics of this type of breast cancer. TNBC has more CSCs as compared to other types of breast cancer (Dittmer et al 2018) and previous work has shown that CSCs increase in response to chemotherapy (Alamgeer et al 2014). In a previous study conducted at the Univsersity of Bristol, a set of 323 genes (whose expression was upregulated in purified murine normal mammary stem cells) was demonstrated to be highly prognostic of significantly shorter disease-free survival (DFS) in three human TNBC datasets (Soady et al 2015). This signature was particularly upregulated in the normal like breast molecular subtype as well as the BL1, M and MSL subtypes of TNBC but had prognostic ability for all subtypes of TNBC. Although this gene expression signature was highly prognostic of DFS in these retrospective analyses, chemotherapy response was not assessed. However, as is known, that response to chemotherapy is prognostic, a demonstration that the gene expression set (or a subset of these genes) could predict response to chemotherapy would provide a new tool for clinical management, enabling prediction of either de novo or acquired chemotherapy resistance and the possibility of changing treatment in patients as resistance emerges.

For this analysis, the University of Bristol will use gene expression data in combination with pathological response to be used as a part of the discovery cohort for their gene expression signature. The University of Bristol will investigate whether the previously identified CSC-signature is associated with chemosensitivity in this cohort and, in addition, they will perform explorative analyses in which we will look for genes which expression is associated with treatment response. Genes associated with Epithelial to Mesenchymal Transition (EMT), a process associated with cancer stem-cell like behaviour, were associated with resistance to chemotherapy. The University of Bristol has approximately 1300 patients in their discovery cohort and 700 in two separate validation cohorts.

Experimental approach:
The statistical analysis for this gene expression work will be undertaken by statisticians based at the Cancer Research UK Integrated Cancer Epidemology Programme (CRUK-ICEP) at the University of Bristol as well as having input from Dr Anita Grigoriadis at King's College London (KCL) who performed the statistical and bio-informatics analyses in the original stem cell gene signature study, and is leading in the cancer bioinformatics group at KCL [redacted] This will be based on the statistical plan as below.

Details of Statistical Plan:
Aim: The aim of our study is to predict neoadjuvant chemotherapy response in triple-negative breast cancer (TNBC) using gene expression.

Methods:

Integration of gene expression datasets

If the dataset arrays are all of the same version/type (for example Affymetrix arrays, Agilent arrays, RNA seq etc) then they will be processed concurrently. If the dataset arrays are different then they will be processed independently. Expression values will be summarized across whole genes independently. A list of common genes across all arrays will be built and the data will be converted to Z-scores independently. Afterwards, the data will be merged. There will likely be some batch effect so samples will be normalised using ComBat method and in downstream analyses, 'array type' will be included as a covariate.

Molecular subtypes of TNBC

Molecular subtypes of pre-processed and normalised raw data of TNBC will be determined by using the webtool [redacted] This is based upon the paper by Lehmann et al 2011. The data will also be run through the updated and condensed 4-type 2016 classification.

Classifier development and validation

The overall TNBC dataset will be divided into training (dataset A will have 2/3 of total samples) and validation subset (dataset B will have 1/3 of total samples). Exact numbers will be pre-determined before analysis dependent on cohorts included in the final dataset.

Linear models (univariate and multivariate (possible covariates will include age, T and N stage, grade as well as treatment received)) will be fit to dataset A and then an empirical Bayesian method will be used to compute the p-values corresponding to the t-statistics of differential expression. The genes will be ranked according to their most significant p-values, which will be adjusted for multiple testing by using Benjamini and Hochberg (BH) method. If any gene(s) are identified, the distribution of p value will be reviewed and used to empirically decide the cut-off to retain the genes for classification.

The classifier will be validated on dataset B using 1) blind validation and 2) leave one out cross validation (LOOCV). In all the classification approaches, a non-parametric k-nearest neighbour (KNN) classification method will be applied for responders and non-responders to chemotherapy.

TNBC is a heterogeneous disease. The above approach of classifier development and validation will be repeated for each of the molecular TNBC subtypes. Multi-class ROC analyses for all TNBC and each TNBC-subtype respectively will be performed to evaluation the classifier performance. The newly-identified genes will be compared with a previously published panel to establish the degree of overlap (Soady et al 2015).

There are pilot studies set up at the University of Bristol to examine whether they can capture this signature and look for dynamic changes throughout neoadjuvant chemo.

Appendix IX BCM/Mayo Clinic BEAUTY Collaboration Summary

Project Description
The [redacted] laboratories have a longstanding collaboration on two projects for which access to the BEAUTY quantitative MRI response and RNAseq data is requested. These two projects are:

1. Development of PDX-based molecular signatures of differential response to taxane vs. platinum-based chemotherapeutics.
2. Hypothesized requirement for the immune system for the growth of immunologically "hot" tumors, which have elevated expression of immune cell-associated gene expression.

For project 1, BEAUTY data will be used for clinical validation of the taxane response signature. Based on our ability to predict response in other PDX datasets, and one clinical dataset for cisplatin/paclitaxel response, we expect that our signature will predict paclitaxel response near-quantitatively and significantly better than chance.

For project 2, BEAUTY RNAseq data will be used to divide the clinical samples into "hot" and "cold" tumors using the [redacted] lab's computational cell type deconvolution algorithm. These classifiers will then be evaluated relative to the ability to grow PDX models from the clinical samples. Based on results from the BCM and HCI collections, we predict that the BEAUTY PDX will show similar results with only "cold" patient tumors yielding PDX models.

Proposed Logistics and Activities
During discussions in the meeting held August 5th, 2020, it was agreed that the BEAUTY trial RNAseq data would be made available to the [redacted] labs in a manner blinded to the quantitative MRI responses. However, we will need to know the ER/HER2 status for each sample as the PDX-based response predictor, and the "hot/cold" classification was developed exclusively using triple-negative models [That being said, we do have response data for four ER+ tumors so far, with additional models under study so we may be able to generate an ER+ chemotherapy response predictor at some point soon].

It is already known which clinical samples yielded PDX models.

RNAseq FASTQ files will be transferred either via the Globus file transfer system or by other means.
The [redacted] labs will process the RNAseq samples, conduct QC, and generate the Paclitaxel response predictions independently for triple-negative and ER+ clinical samples, and will determine "hot/cold" tumor classifications.

Ranked and quantitative paclitaxel response predictions will be sent to the Mayo Clinic BEAUTY Executive Committee for evaluation relative to quantitative MRI responses. The MAE and RMSE will be determined for the predictions vs the know outcomes. The ROC curve will also be determined for patients that did and did not have pathological complete response (pCR) to chemotherapy.

In addition, a set of genes ("multi-variate biomarker") used in the predictive model will also be sent to the Mayo Clinic BEAUTY Executive Committee for building of a model by Mayo on a subset of triple-negative "cold" tumors identified to be such by Baylor based on the analysis of RNA-seq data. Mayo team will determine predictive power of the model using Leave-one-out or similar validation method. This predictive power of the multi-variate biomarker will be compared to the predictive power of models built on random sets of genes. It is anticipated as a positive result that the model built on the multi-variate biomarker will outperform almost all the models built on random sets of genes. A negative result of this experiment will be highly informative, as it would suggest that the multi-variate biomarker is not informative.

Results of these analyses will be sent back to the [redacted] labs, and the BCM and Mayo Clinic groups will schedule a meeting to discuss.

Assuming response prediction is successful, these results will be included in a manuscript with Mayo Clinic personnel participating as authors.

If our initial response prediction is not successful, we would like the opportunity to have one more attempt to refine the signature and re-evaluate relative to response.

Depending on data availability for the TBCRC030 clinical trial completed at Dana Farber, the predictors may also be validated in this dataset for the publication. However, it may be advantageous to incorporate the paclitaxel (Mayo, RNAseq) and cisplatin (Dana Farber, Affy) data, as well as all available PDX data into a new set of predictors prior to use of TBCRC030 data.

Appendix X SimBioSys Collaboration

Synopsis

Data from the BEAUTY study will be shared with SimBioSys, Inc. for purpose of carrying out a validation of their TumorScope Algorithm for predicting response to NACT

Objectives
Primary Objective: To assess the accuracy of TumorScope™ in predicting pathological complete response (pCR) among women with Triple Negative & HER2+ breast cancer who receive **Neoadjuvant Chemotherapy (NACT)** therapy.

Hypothesis: TumorScope™ can accurately predict whether a patient will achieve a pathological complete response (pCR) using the AJCC staging system's definition of ypT0/Tis ypN0 as assessed by the local pathologist at the time of definitive surgery

Secondary & Exploratory Objectives:
Secondary & Exploratory objectives of the study are:

1. To assess accuracy of TumorScope™ in predicting pathological complete response(pCR) in women with HR+/HER2- Breast Cancer receiving NACT in combination with anti-Her2 therapy

   Hypothesis: TumorScope™ can accurately predict whether a patient will achieve a pathological complete response (pCR) using the AJCC staging system's definition of ypT0/Tis ypN0 as assessed by the local pathologist at the time of definitive surgery

2. To compare accuracy of volumetric response of TumorScope™ predictions to longitudinal imaging performed over the course of NACT

   Hypothesis: TumorScope™ can accurately predict the reduction of tumor volume (measured in cubic centimeters) over the course of NACT

3. To assess the relationship of pCR prediction from TumorScope™ to Event Free Survival (EFS)

   Hypothesis: TumorScope™ prediction of pCR is a prognostic marker for Early-Stage Breast Cancer as measured by Event Free Survival (EFS), independent of whether the pCR prediction is correct.

Study Design
A retrospective, independent, and blinded validation of the diagnostic accuracy of SimBioSys TumorScope™ prediction of pathological complete response (pCR) will be undertaken. The predictions

will be performed using only information available at the time of diagnosis and compared to ground truth for actual patient outcomes. The study design is depicted in the figure below.

The following variables will be shared with SimBioSys staff from the study database to perform predictions:

- **Demographic:** Race: Caucasian/African American/Asian/Hispanic/Other
- Age at start of NCT
- Cancer T Staging: T1, T2, T3, T4
- Cancer N Staging: N0, N1, N2, N3

- **Pathology Markers at time of Primary Diagnosis:**
- Estrogen Receptor Status
- Progesterone Receptor
- HER2+ Status: IHC (0 -3) or FISH Ratio
- Tumor Grade: 1-3
- Ki67: 0 – 100% (Optional, for analysis only)
- Histological Type: IDC/ILC/Mixed/Inflammatory

1. **Pre-Surgery Imaging**
   - DCE MRI Series: Minimum of 3 timepoint
   - Initial Size of Tumor on MRI imaging

2. **NCT regimen**
   - Regimen Given

3. **Dates for Volumetric Analysis (Optional):**
   - Lag between diagnostic MRI and start of NCT
   - Lag between diagnostic MRI and any additional MRIs to determine which time points to compare volumes at
   - Lag between the start of NCT and surgery

4. **Data regarding the Pathology Specimens**
   - weight of each breast pathology specimen for all specimens
   - dimensions (3 dimensions) of each breast and lymph node pathology specimen for all specimens

Blinding & Unblinding Procedures
This is a blinded study and any data indicative of potential response such assessment of pCR, Residual tumor size, post therapy imaging or any documented assessment of clinical response, recurrence of disease will not be utilized as an input or made available to SimBioSys staff at the onset of the study. Any subjects where data potentially indicative of response is accidently revealed will be excluded from the study and documented.

The Mayo team will provide blinded data and follow procedures with absolute integrity.

Submission of initial subject data will only include variables pre-identified as inputs into the model prediction. Upon delivery of initial data, SimBioSys will perform necessary validation and identify any subjects who should be excluded based on predefined criteria. Predictions of both primary, and secondary and exploratory endpoints will be performed and documented by SimBioSys staff. Simulation results will

be delivered to each site via email and confirmation of receipt will be required. The simulation prediction will be considered "locked" at this point and no changes to the results will be permitted.

Principal investigators alongside collaborating biostatisticians at the study site will internally unblind the entire cohort to perform initial analysis. The site-specific analysis will be shared with SimBioSys staff for discussion. At this point, the analysis and results will be considered "locked".

Once study site specific analysis is finalized, data used for validation and exploratory analysis will be unblinded and sent to SimBioSys staff. This data includes:

Output Variables
These variables may be collected at any time after the initiation of the study but will be **blinded to SimBioSys staff** until final predictions are submitted.

- **pCR:** Defined as ypT0/Tis & N0
- **Residual Size:** Measurement of residual tumor post-surgery (centimeters)
- **Recurrence:** Yes/No
- **Time to Recurrence:** Weeks
- **Last Follow-up Date:** Weeks

Statistical Methods
This study will be conducted in subjects who participated in Beauty Study (MC1137) at Mayo Clinic. Subjects will be selected based on inclusion criteria and availability of pre-treatment MRI.

Statistical Evaluation (pCR)
The investigational TumorScope™ test device has two possible predicted pCR outcomes, yes (+) or no (−). Each patient will have actual clinical outcomes (i.e., ground truth) with regards to the pCR, yes (+) or no (−). Clinical sensitivity and specificity will be determined by comparing the investigational TumorScope ™ predicted results to the ground truth at the subject level.

The following 2x2 table is constructed to illustrate the co-primary endpoints:

		Ground Truth		Total
		Yes (+)	No (-)	
Investigational Device	Yes (+)	a	b	a+b
	No (-)	c	d	c+d
Total		a+c	b+d	a+b+c+d

Sensitivity is the proportion of subjects who are pCR responders in the ground truth (i.e., yes) are also predicted responders in the investigational test (i.e., yes); calculated as 100% x a/(a+c).

Specificity is the proportion of subjects who are pCR non-responders in the ground truth (i.e., no) are also predicted non-responders in the investigational test (i.e., no); calculated as 100% x d/(b+d).

Positive Predictive Value (PPV) is the proportion of subjects who are predicted pCR responders in the investigational test are also responders in the ground truth; calculated as 100 x a/(a+b).

Negative Predictive Value (NPV) is the proportion of subjects who are predicted pCR non-responders in the investigational test are also non-responders in the ground truth; calculated as 100 x d/(c+d).

Diagnostic Accuracy is the proportion of correctly classified subjects among all patients; calculated as 100 x (a+d)/(a+b+c+d).

Exploratory Analysis
Predicted Tumor Volume
Using longitudinal MRIs, where available for each subject, segmented tumor volumes will be assessed by a practicing radiologist blinded to the prediction results.

The segmented volumes will be compared against predicted volumes previously submitted from TumorScope to assess performance. Performance will be reported as a Pearson Correlation between predicted volume and actual volumes. In addition, median absolute error, median absolute deviation, mean error, and standard error will be reported on predicted response (%) vs actual response (%) calculated as:

(Start Volume - End Volume)/Start Volume

Other analysis will be performed at the discretion of SimBioSys and Principal Investigators.

Event-Free Survival (EFS)
The EFS time distribution will be estimated by the Kaplan-Meier method A point and interval estimate of the 3 year EFS rate and the 5 year EFS rate will be constructed from these estimates. A log rank test will be used to assess whether the EFS differs with respect to whether the patient had a pCR after NCT as well as assess whether EFS differs with respect to whether TumorScope predicted the patient would have a pCR or not.

Target Subject Population
All patients enrolled on MC1137 (Beauty) who have previously received standard of care NACT and meet inclusion/exclusion criteria will be included in this study. The Primary End Point population will only include TNBC and HER2+ patients as, large, pooled analysis demonstrated strong association of pCR, when defined as no tumor in both breast and lymph nodes (ypT0 ypN0 or ypT0/Tis ypN0) following neoadjuvant therapy for breast cancer, with improved long-term benefit as measured by event-free survival and overall survival [4].

Introduction

Background
Breast cancer remains the most common malignancy in women in the United States. About 1 in 8 U.S. women will develop invasive breast cancer over the course of her lifetime [1]. Neoadjuvant chemotherapy (NACT) is considered a standard of care for some women with locally advanced breast cancers [2, 3]. Recently, FDA recognized a complete response from NACT referred to as a pathological complete response as a surrogate marker for accelerated drug approval. Large metanalysis confirms that patients who achieve pCR after NACT have excellent event-free and distant disease-free survival especially in the Triple Negative and HER2+ subtypes of the disease [4]. pCR rates differ among cancer subtypes that are defined by the tumor's ER, PR, and HER-2 receptor status. Specifically, pCR rates range from 10-15% in ER/PR+, HER2- cancers up to 70% in ERPR- HER2+ cancers [4]. Treatments are selected in clinical setting with the target to achieve pCR as this provides ability for physicians to prognosticate, escalate, or de-escalate therapy and develop treatment strategies post-surgery. Exploratory studies have shown factors that influence pCR rates include histology, morphologic features such as tumor size and enhancement pattern, and various imaging parameters [5-7]. Histological markers including higher Ki67 levels (greater

than 15% or greater than 35%, depending on the study) and greater percentages of tumor-infiltrating lymphocytes have been linked to higher pCR rates [10-12].

Current prediction methods involve comparing images taken before vs. after NACT and take into account information from dynamic contrast enhanced MRI (DCE-MRI) and histologic features. Many of these methods require data gathered after NACT initiation [8, 9]. While early response to NACT may predict attainment of a pCR, patients who have started receiving treatment will have delayed opportunities to pursue, or may be precluded from, other therapeutic options such as de-escalation or clinical trials.

Rationale for this Study
Even though a strong standard of care exists for early-stage breast cancer, there is still significant variation in decision making. Varying opinions among clinicians result from lack of tools to standardize how data is utilized in the planning process. Patient expectations are set using population-based statistics from clinical trials and references to literature, and the patient's role in the decision-making process is informed only by standard medical reports (MRI/Pathology), their interaction with their healthcare providers, and online forums/engines.

A reliable *a priori* prediction of pCR offers many benefits in clinical decision making oriented towards increased confidence of the decision for the clinician. Such a prediction would provide a patient-specific prognostication tool to supplement the commonly used clinic-pathological data points such as stage, grade and receptor status. However, none of the novel methods that have been recently developed have provided sufficient diagnostic accuracy to warrant use clinically. This justifies the urgent need for a comprehensive model, applicable to data obtained before NACT initiation, that can effectively differentiate between patients who are likely to achieve a pCR and those less likely to respond. Even though TNBC and HER2+ are chemosensitive compared to other breast cancer subtypes in the early-stage setting, residual disease rates post-NACT are still high and those with residual disease have an extremely poor outcome. Therefore, TNBC & HER2+ is a disease with high unmet medical need.

Selection of study population
Any patient previously indicated for Neoadjuvant Therapy in Early-Stage Breast Cancer who has completed therapy and whose surgical sample has been analyzed by a pathologist for attainment of pCR will be eligible for this study. The primary end-point analysis will be limited to Triple Negative and HER2+ subtypes. Exploratory analysis will be performed on all indicated patients.

Inclusion Criteria
For Inclusion in this study subjects must fulfill all the following criteria:

1. A diagnostic Dynamic Contrast Enhanced MRI was obtained prior to therapy
2. Patient is indicated for Neoadjuvant Therapy (HR+/HER2- subset only for secondary analysis)
3. Patients who completed treatment & surgery at the study site between 1/1/2001 – 12/31/2020
4. Treatment given was from the Standard of Care as defined by NCCN Guidelines listed below:

For HER2- Patients:

- Doxorubicin/Cyclophosphamide followed by Paclitaxel (AC-T)
- Paclitaxel followed by Doxorubicin/Cyclophosphamide (T-AC)
- Docetaxel/Cyclophosphamide (TC)
- Docetaxel/Carboplatin (TCb)
- Doxorubicin/Cyclophosphamide (AC)

For HER2+ Patients:

- Doxorubicin/Cyclophosphamide followed by Paclitaxel/Trastuzumab (AC-TH)
- Doxorubicin/Cyclophosphamide followed by Paclitaxel/Trastuzumab/Pertuzumab (AC-THP)

- Paclitaxel/Trastuzumab (TH)
- Paclitaxel/Trastuzumab/Pertuzumab (THP)
- Docetaxel/Carboplatin/Trastuzumab (TCbH)
- Docetaxel/Carboplatin/Trastuzumab/Pertuzumab (TCbHP)
- Docetaxel/Cyclophosphamide/Trastuzumab (TCH)
- Docetaxel/Cyclophosphamide/Trastuzumab/Pertuzumab (TCHP)

Exclusion Criteria
Any of the following are a criterion for exclusion from the study:

1. Confirmed distant metastasis prior to therapy
2. Patients with missing pathology or demographic input variables
3. Patients with no DCE MRI prior to initiation of therapy
4. Major MRI artifacts as defined by TumorScope including but not limited to poor fat suppression, significant coil flare, low signal to noise ratio, incomplete DICOM series and other non-correctable registration issues
5. Use of experimental or novel therapy alongside standard of care
6. Any patient with identifiable outcome information is shared prior to unblinding

Data Sources

Breast Magnetic Resonance Imaging (MRI)
Any Breast MRI performed as part of routine clinical management are acceptable for use as an input for the study if they were dated prior to the first dose of treatment.
Specifically, the T1-weighted DCE MRI images will be utilized both as inputs and validation (where available) for all subjects included in this study. Pre-Therapy (T0) Breast MRI images for all patients in the study will be sent to SimBioSys for quality review prior and then utilized for result predictions.

Upon unblinding and analysis of results, up to 3 additional MRIs will be used for validation of secondary endpoints:

- After the Neoadjuvant Treatment 1 Cycle 1
- Before Initiation of Neoadjuvant Treatment 2 Cycle 1 (any drug in regimen is changed, where applicable, Inter-Regimen)
- After completion of all Neoadjuvant Treatments but before Surgery (Pre-Surgery)

Changes from the baseline in terms of volume (cc) will be initial segmented by SimBioSys staff and then assessed by the investigator for validation.

Tumor Biopsy for Pathological Markers at Diagnosis
Patient tumor characteristics from the original core needle biopsy as assessed by the local pathologist's in the pathology report will be utilized for the subject inclusion, predictions, and result stratification.

Definitive Surgery Assessment
Assessment performed by the local pathologist after definitive surgery such as breast conservation surgery (BCS) or mastectomy with or without axillary lymph node dissection as part of the local standard of care approximately 10-35 weeks will be utilized as ground truth. If the assessment of any subjects is uncertain, specifically for the pCR outcome, they will be excluded from the analysis.

Invasive Disease-free Survival
Invasive disease-free survival is defined as the time from registration to any of the following events: progression of disease that precludes surgery, local or distant recurrence, second primary malignancy (breast or other cancers) or death due to any cause.

Publications
Mayo investigators will be included on any publications relating from this study or any future metanalysis performed using the corresponding results.

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