Ultrasound and Near Infrared Imaging for Predicting and Monitoring Neoadjuvant Treatment

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1. STUDY OVERVIEW

1.1. Monitoring Neoadjuvant Treatment Response

Twenty percent (20%) of all newly diagnosed breast cancers are locally advanced. Neoadjuvant treatment, including both neoadjuvant chemotherapy (NAC) and neoadjuvant endocrine therapy (NAE) is being used more frequently in treating these patients. When used prior to surgery, chemotherapy may allow for breast conservation by reducing tumor size. An additional benefit of neoadjuvant treatment is the opportunity to assess the treatment response of the tumor *in vivo*. Moreover, important prognostic information is obtained. In the setting of NAC, when a pathologic complete response (pCR) is achieved, patients have increased disease-free (DFS) and overall survival (OS). Since there are many chemotherapeutic agents available, it is important to assess tumor response early, so the optimal agent or regimen can be adopted as quickly as possible. The expedience of the assessment can both minimize morbidity of ineffective treatment and maximize the success of effective treatment.

Conventional methods to monitor response to NAC include physical examination, and imaging with ultrasound (US), and mammography. However, conventional imaging has been shown to be suboptimal in their assessment of tumor response [1, 21(yeh et al.)]. Contrast-enhanced magnetic resonant imaging (MRI) and positron emission tomography (PET) have been increasingly used to evaluate locally advanced breast cancers and may potentially depict changes associated with the regression of tumor angiogenesis and metabolic changes after chemotherapy [2-4,22,23]. However, MRI and PET require intravenous contrast agents and are also costly, which may limit their use for repeated monitoring throughout the treatment. Contrast enhanced mammography (CEM) is an FDA approved emerging technology, which has shown promise in assessing neoadjuvant treatment response [Patel BK, Lobbes MBI, Lewin J. Semin US CT MRI 2018; 39:70-79, ElSaid NAE,.EgyptJRadio NuclMed2017;48(2):519-527]. Like MRI, CEM demonstrates enhancement at the site of viable malignancy, but is less expensive and more accessible than MRI.

Similar to NAC, NAE therapy results in comparable rates of clinical response, pathologic complete response, and breast conservation surgery (26, 27). However, clinical or conventional imaging monitoring of response (i.e. by mammography and ultrasound) in patients receiving NAE therapy does not consistently predict adjuvant therapy effectiveness and long term outcome (28, 29). In contrast, biological endpoints, such as Preoperative Endocrine Prognostic Index (PEPI) score, which combines the cell proliferation index Ki67, ER, and pathology stage at surgery and on-treatment Ki67 of tumor biopsies have been shown to be predictive of long term outcomes in retrospective studies (30). Monitoring with Ki67 during neoadjuvant treatment has identified up to 20% of patients who are resistant to NAE therapy (31). However, Ki67 monitoring requires tumor biopsy which is invasive and the clinical use of PEPI score and Ki67 is still being investigated in prospective clinical trials (32). Non-invasive measures of NAE response are unmet clinical need. NAE appears to reduce angiogenesis (33) and may affect neovascularization (33-36), properties that lend themselves to evaluation with NIR optical imaging, which measures hemoglobin content. We therefore propose to include an exploratory cohort of patients with ER+HER2- breast cancer undergoing neoadjuvant endocrine therapy in this study.

1.2. Optical Tomography Using Near Infrared Light Guided or Assisted With Ultrasound (NIR/US)

Near infrared (NIR) light provides a unique approach for functional and molecularly based diagnostic imaging and for monitoring neoadjuvant treatment response [5-16,24]. The primary limitation of diffuse optical tomography is related to the fact that scattering dominates NIR light propagation in tissues, making three-dimensional localization of lesions and accurate quantification of lesion optical properties difficult. Recently, optical tomography guided by co-registered MRI, x-ray, and US has demonstrated a great potential to overcome lesion location uncertainty and to improve light quantification accuracy [7,9,10,13].

We have introduced the Optical Tomography Using Near Infrared Diffused Light Assisted With Ultrasound (NIR/US), which utilizes co-registered US to localize breast lesions and optical tomography to assess how tumor vasculature reacts to neoadjuvant treatment and to determine how well the vascular response correlates with the tumor pathologic response. This unique hybrid technique is implemented by simultaneously deploying NIR optical sensors and a commercial ultrasound transducer mounted on a hand-held probe, and utilizing co-registered lesion structure information provided by ultrasound to improve the inverse optical tomography reconstruction. As a result, NIR/US has overcome problems associated with intense light scattering and has provided reliable tumor angiogenesis distribution and quantification via total hemoglobin contrast. Initial results obtained from a group of 32 patients who underwent NAC have shown that the tumor angiogenesis content and changes imaged at the early treatment cycles can be used to predict and assess pathological response of breast cancer patients to NAC [17-18].

The technical aspects of the NIR optical imaging system have been described in detail [7,8,13,18]. Briefly, NIR light is delivered from multiple source positions embedded within the ultrasound transducer and the reflected light (from normal and pathologic breast tissue) is collected by multiple detection fibers within the transducer that are coupled to photomultiplier tubes (PMTs). The transmitted NIR light is delivered to each transducer source position sequentially and reflected NIR light is detected in parallel from all PMT detectors. The entire acquisition from all source detector pairs takes approximately 3 to 4 seconds.

1.3. Assessing Response Using NIR/US

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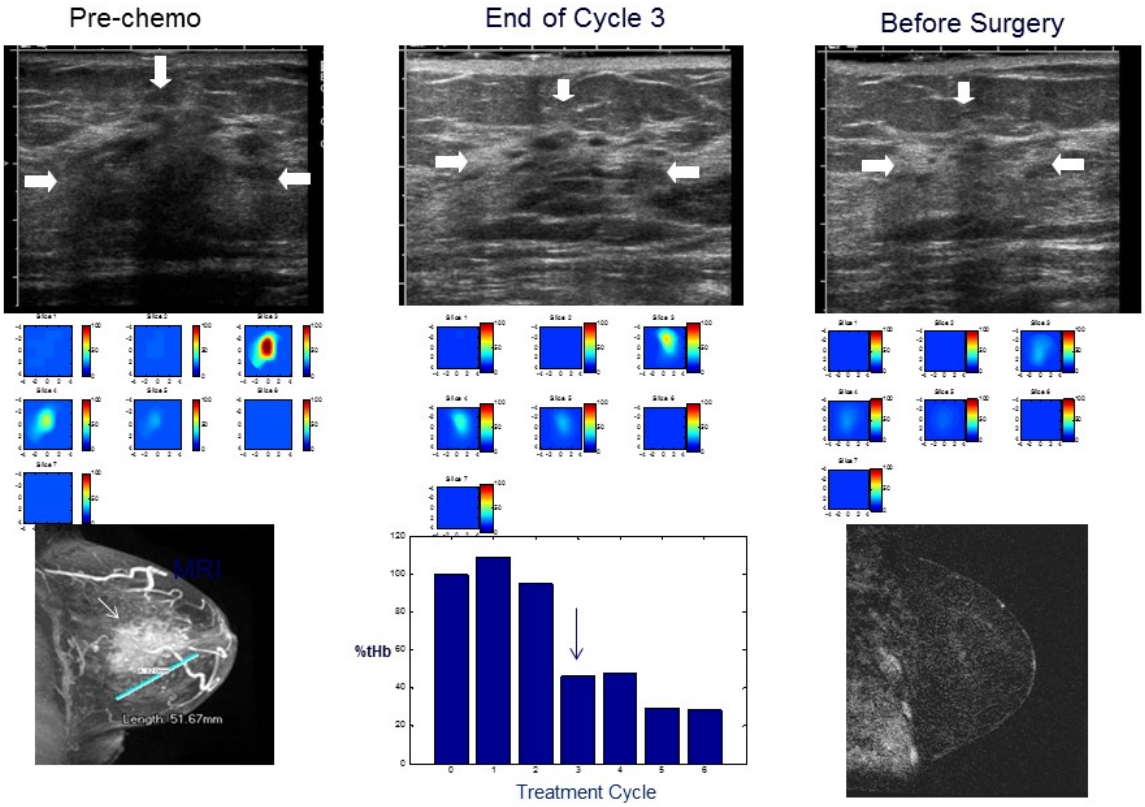

Figure 1. An example of pCR who had a HER2 positive tumor. 55-year-old woman with an intermediate grade invasive ductal carcinoma treated with Herceptin-based regimen. Top panel: US images at three time points. The tumor was ill-defined and heterogeneous seen by US, and was palpable at the beginning of the treatment. Middle panel: total hemoglobin concentration maps obtained at the three time points. Each map shows 7 sub-images marked as slice 1 to 7 and each sub-image shows spatial x and y distribution of tHb concentration reconstructed from 0.5 cm to 3.5 cm depth range from the skin surface. The spacing between the sub-images in depth is 0.5 cm. The tHb reduced from 113 µmol/L measured at pretreatment or baseline to 33 µmol/L measured before surgery (71% reduction). Dramatic reduction occurred at the end of cycle 3 (55% reduction) (middle column of Bottom panel). Bottom panel: MRI images before (left) and after treatment before surgery (right). The MRI measurements were 3.2 x 4.8 x 5.2 cm before treatment with no visible residual tumor at the end of treatment. The percentage ratio is 0% or reduction in largest dimension was 100%. Middle column: total hemoglobin level normalized to baseline (%tHb) vs. treatment cycles. This patient had a complete pathologic response with no residual tumor; her tumor was Miller-Payne grade 5.

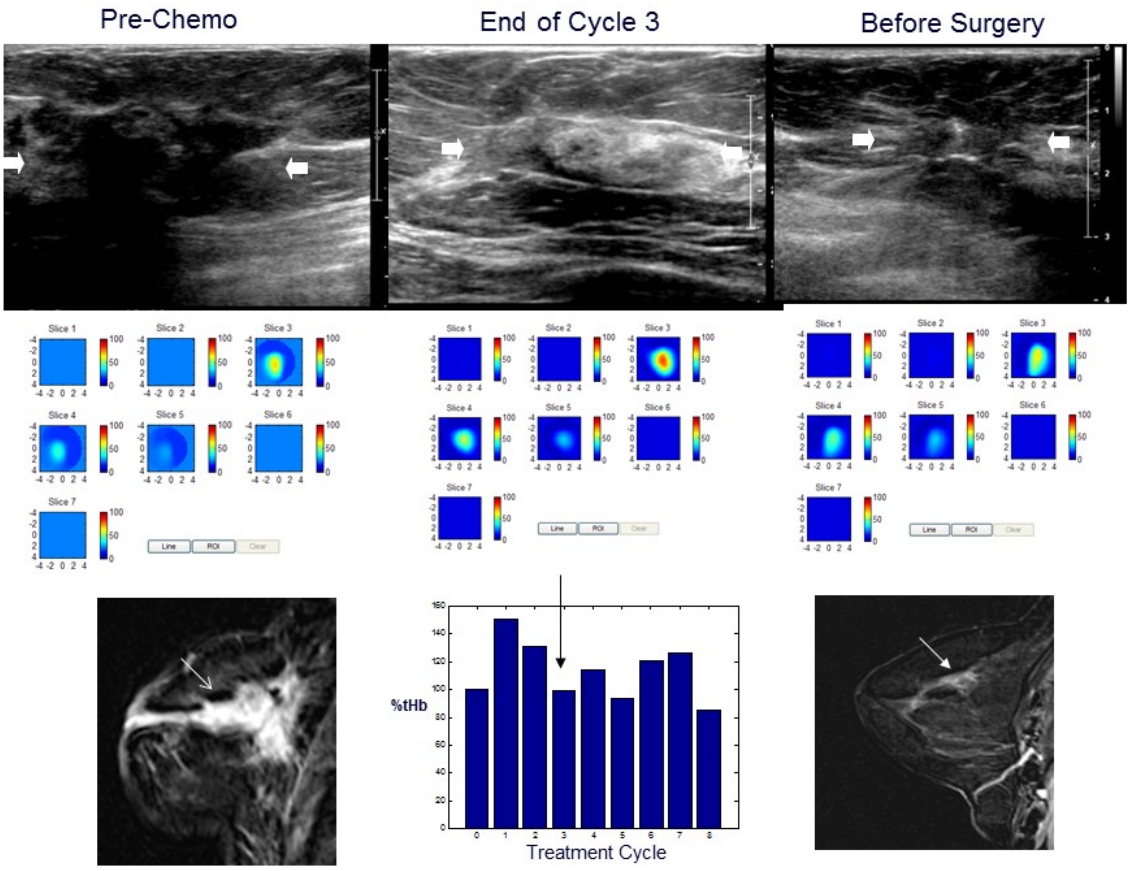

Figure 2. An example of a non-responder. 42-year-old woman with an intermediate grade invasive lobular carcinoma treated with ACT+Bev with capecitabine. Top panel: US images at the three time points. The tumor was irregular seen by US and was palpable at the beginning of the treatment. Middle panel: The tHb level fluctuated but was never reduced during the entire treatment period (middle column of Bottom panel). All image dimensions are the same as Fig.1. Bottom panel: MRI images before (left) and after treatment showed reduced enhancement and size. The tumor size measurements were 3.7x4.4x4.0 before treatment and 1.1 x 1.0x 1.9 after treatment with significantly reduced contrast enhancement. The percentage ratio was 42.2% or reduction of largest dimension was 56.8%. Middle column: %tHb concentration vs. treatment cycles. Pathologically a 6.5 cm by 3.5 cm residual tumor was present; an additional 2.3 cm residual tumor was present at re-excision. Her tumor was Miller-Payne grade 2.

2. OBJECTIVE

Primary Objective: To evaluate the use of NIR/US to predict and assess pathological response of breast cancer patients undergoing NAC.

Exploratory Objective: To evaluate the use of NIR/US to predict treatment response, measured by PEPI score (0 vs not), in breast cancer patients undergoing NAE.

Additional Objective: To compare the results of NIR/US to predict and assess pathological response in patients undergoing neoadjuvant treatment with CEM in the subset of patients who have undergone CEM as part of their standard of care evaluation.

3. ELIGIBILITY CRITERIA

1. Participants who are candidates for neoadjuvant chemotherapy or neoadjuvant endocrine therapy for the treatment of newly diagnosed, invasive breast cancer.
2. At least 18 years of age.
3. Female.
4. Not pregnant and/or breastfeeding.
5. No prior history of breast cancer.
6. No prior history of chest wall radiation.
7. No prior history of breast reconstruction, reduction, or augmentation.
8. Able to understand and willing to sign an IRB-approved written informed consent document.

3.1. Inclusion of Women and Minorities

Women and members of all races and ethnic groups are eligible for the study.

4. REGISTRATION PROCEDURES

Patients must not start any protocol intervention prior to registration through the Siteman Cancer Center.

The following steps must be taken in order to register patients to this study:

1. Confirmation of patient eligibility
2. Registration of patient in the Siteman Cancer Center OnCore database
3. Assignment of unique patient number (UPN)

4.1. Confirmation of Patient Eligibility

Confirm patient eligibility by collecting the information listed below:

1. Registering MD's name
2. Patient's race, sex, and DOB
3. Three letters (or two letters and a dash) for the patient's initials
4. Copy of signed consent form
5. Completed eligibility checklist, signed and dated by a member of the study team
6. Copy of appropriate source documentation confirming patient eligibility

4.2. Patient Registration in the Siteman Cancer Center OnCore Database

All patients must be registered through the Siteman Cancer Center OnCore database.

4.3. Assignment of UPN

Each patient will be identified with a unique patient number (UPN) for this study. All data will be recorded with this identification number on the appropriate CRFs.

5. STUDY PROCEDURES

Patients will have the NIR/US baseline scan performed before their first treatment. The desirable schedule will be 7 to 10 days after initial biopsy to avoid confounding effects from the biopsy related acute inflammatory response. We will attempt to perform pretreatment imaging as far out from the time of biopsy as possible, understanding that there is urgency to begin neoadjuvant treatment due the patient's clinical circumstances, i.e. locally advanced or regionally metastatic cancer. Time of NIR-US relative to biopsy will be tracked.

All patients that undergo core needle biopsy at the Joanne Knight Breast Center at SCC have metallic biopsy markers placed at the time of biopsy. This is particularly important for patients who subsequently undergo neoadjuvant treatment but is part of our standard operating procedure for all patients. Only patients refusing marker placement will not have a marker placed. Given the importance of marker placement for patients undergoing neoadjuvant treatment this occurrence, i.e. refusal of marker, is felt to be extremely unlikely.

Patients who will be consented to the study will have NIR/US performed according the schedule in the table below. The number of NIR/US study visits for these subjects will vary from 5 to 6 depending on the subject's treatment regimen. End of cycle 5 is optional and only intended for those patients whose regimen was changed. For patients undergoing NAE, "At change of Treatment" visit is only intended for those patients who have had a change in their NAE regimen. All end of cycle NIR/US exams may be obtained within a 4 day window from the proposed time. The prior to surgery exam will be performed after completion of the neoadjuvant treatment regimen and prior to surgery and usually coinciding with the SOC breast surgeon preoperative visit. Timing is variable, but this typically occurs within one month of treatment surgery. The expected duration of study participation for these patients is approximately 6 months.

5.1. Procedure Table (Neoadjuvant Chemotherapy Cohort)

	Baseline[1]	**End of cycle 1**	**End of Cycle 2**	**End of Cycle 3**	**End of Cycle 5[2]**	**Prior to surgery**
consent	X					
NIR/US	X	X	X	X	X	X

1. Prior to initiation of chemotherapy and ≥ 7 days after initial core needle biopsy
2. NIR/US exam will only be obtained at this time point if the treatment regimen has changed and there is a need to continue assessing response based on imaging results of first three cycles.

5.2. Procedure Table (Neoadjuvant Endocrine Cohort)

	Baseline[1]	**End of cycle 1**	**End of Cycle 2**	**End of Cycle 3**	**At Change of Treatment[2]**	**Prior to surgery**
consent	X					
NIR/US	X	X	X	X	X	X

1. Prior to initiation of endocrine therapy and ≥ 7 days after initial core needle biopsy
2. "At change of Treatment" visit is only intended for those patients who have had a change in their NAE regimen.

6. IMAGING

NIR/US will be performed in the Department of Radiology. Dr. Poplack, Dr. Appleton, Dr. Young and Dr. Covington will assist Dr. Zhu's team in performing all NIR/US studies. They will not be blinded to any clinical data. At a minimum they will be aware of the imaging presentation leading up to diagnosis, the pathology result of the large core needle biopsy, and imaging documenting post biopsy marker clip position. They may or may not be aware of the neoadjuvant treatment regimen employed. The function of the imaging specialist in this study is to ensure that the correct anatomic location of the index malignancy is being imaged. If this technology is accepted for clinical use, the sonographer/sonologist would be expected to have this level of awareness about the patient undergoing neoadjuvant treatment that they were imaging.

A hand-held hybrid probe, consisting of a commercially available US transducer located in the middle and near-infrared source and detector optical fibers distributed at the periphery (see Figure 1), will be used for scans. For each patient, co-registered digital US images from the commercial ultrasound unit and optical measurements from our NIR optical imaging device are acquired simultaneously at multiple locations including the lesion of interest region and the same region in the mirror-image contralateral breast. The contralateral location is chosen as the reference site. The difference between measurements obtained from the lesion and the reference site is used for optical imaging reconstruction. Optical absorption distributions at four different wavelengths are reconstructed and lesion total hemoglobin concentration (tHb), oxygenated and deoxygenated hemoglobin concentrations (oxyHb and deoxyHb) are computed from absorption maps. The total data acquisition time is about 10 to 15 minutes once the lesion is identified by US.

Two examples obtained from a complete pathological responder (pCR) and a non-responder are given in Section 1.3 to demonstrate the utility of the technique in assessing neoadjuvant treatment.

Following each patient study, the near infrared images will be reconstructed by Dr. Zhu's group who will not know the final pathological response. At the end of this two-year trial, the near infrared data will be pooled for final statistical analysis. In the subset of patients who have had CEM as part of their clinical care, to compare results of NIR-US with CEM. In particular to correlate the presence and extent of enhancement at the malignant site with total hemoglobin concentration (tHb).

6.1. Safety and Setup for Imaging

The US-guided NIR device has been used safely by Prof. Zhu's group in the past 14 years for ~500 patients [7,9,13,17,18,20]. There is no known risk associated with the use of the device. The light sources are low-power laser diodes with wavelengths in the NIR range and are fiberoptic coupled to the probe. The laser light sources can only be activated when the surface of the probe, i.e., the tips of the fibers, is in contact with the skin.

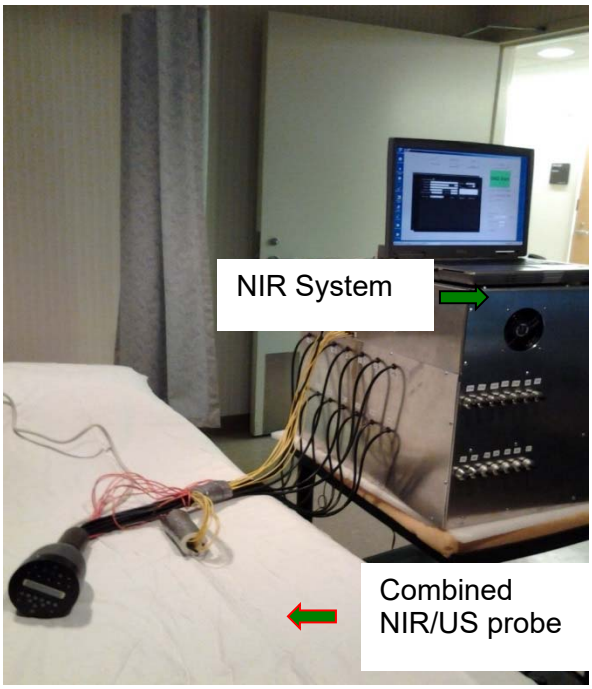

Figure 3: Hand-held US-guided Near Infrared system

7. DATA COLLECTION AND ANALYSIS

Study specific data will be collected from subjects after informed consent is obtained. The key data elements will likely include:

- Pathology results from initial diagnosis, during neoadjuvant treatment and final surgical management with specific attention to Miller Payne pathologic response (see below)
- Neoadjuvant Treatment information, including: medication regimen, frequency, relevant basic lab results and complications
- Imaging results from the serial US exams conducted during the NIR visits
- NIR results
- Comparison of CEM with NIR in subset with CEM exam

In some cases, the pathologist may review existing biopsy specimens with special staining to look for signs of increased blood supply and vessel density. This will not require additional slicing of standard of care biopsy specimens; thus, samples will not be labeled or stored for the research study. The results of these tests will not be placed in the patient medical record. The results of these tests may be given to the investigators to correlate to the results of the NIR/US procedures.

The biopsy grading was based on Miller-Payne system [19]. In the Miller-Payne system, the pathologic response is divided into 5 grades based on comparison of tumor cellularity between pre-neoadjuvant core biopsy and definitive surgical specimen as:

- grade 1: no change or some alteration to individual malignant cells but no reduction in overall cellularity (pNR)

- grade 2: a minor loss of tumor cells but overall cellularity still high; up to 30% (pPR)
- grade 3: between an estimated 30% and 90% reduction in tumor cells (pPR)
- grade 4: a marked disappearance of tumor cells such that only small clusters or widely dispersed individual cells remain (almost pCR); more than 90% loss of tumor cells
- grade 5: no malignant cells identifiable in sections from the site of the tumor; only vascular fibroelastonic stroma remains often containing macrophages (pCR) (however, ductal carcinoma *in situ* (DCIS) may be present)

Dr. Souzan Sanati, specialist in breast pathology, will assess patient treatment responses and provide Miller-Payne scores as well as other pathological findings from patients' standard of care assessments.

7.1. Data Analysis and Validation

In our early study, among the 32 patients (recruited from 2007 to 2011) from whom data was reported in Ref. 18, 24 HER2 negative patients (75%) were treated with a Taxol-based regimen and five HER2 positive patients (16%) were treated with Herceptin-based regimens. The rest three were treated with a Taxol-based regimen in combination with bevacizumab. This anti-angiogenesis regimen was stopped by the FDA later due to its side effects.

The primary goal of this study is to assess the predictive value of NIR/US in monitoring early tumor vasculature and oxygen changes and to correlate these changes with patients' pathological response. We observed that the Her2+ patients, triple receptor negative patients, and ER+ patients treated with different regiments respond differently. Based on small sample size of Her2+ patients (n=5), triple receptor negative patients (n=6), and ER+ and Her2- patients (n=22), the early changes in total hemoglobin level were observed at different treatment cycles. Our goal in this study is to extend the pilot study into a larger patient pool for Her2+, triple receptor negative and ER+,Her2- patients and benchmark the optical signature of neoadjuvant treatment response of subsets of breast cancer based on molecular phenotype.

A secondary goal of this study is to assess the correlation of optical imaging parameters with the presence and intensity of contrast enhancement of the index malignancy in patients who have undergone CEM.

8. ADVERSE EVENT MONITORING AND REPORTING

The entities providing oversight of safety and compliance with the protocol require reporting as outline below.

The Washington University Human Research Protection Office (HRPO) requires that all events meeting the definition of unanticipated problem or serious noncompliance be reported as outlined in Section 8.2.

There are minimal side effects associated with the NIR/US, therefore we will not be tracking AEs. All SAEs that occur within 24 hours of an NIR/US exam will be brought to the attention of the PI and a determination will be made if they are related or unrelated to study participation. Any SAE that is related to the NIR/US intervention, will be reported to the IRB.

8.1. Definitions

8.1.1. Adverse Events (AEs)

Definition: any unfavorable medical occurrence in a human subject including any abnormal sign, symptom, or disease.

Attribution (Relatedness), Expectedness, and Seriousness: the definitions for the terms listed that should be used are those provided by the Department of Health and Human Services' Office for Human Research Protections (OHRP). A copy of this guidance can be found on OHRP's website at: http://www.hhs.gov/ohrp/policy/advevntguid.html.

8.1.2. Serious Adverse Event (SAE)

Definition: any adverse drug experience occurring at any dose that results in any of the following outcomes:

- Death
- A life-threatening adverse drug experience
- Inpatient hospitalization or prolongation of existing hospitalization
- A persistent or significant disability/incapacity (i.e., a substantial disruption of a person's ability to conduct normal life functions)
- A congenital anomaly/birth defect
- Any other experience which, based upon appropriate medical judgment, may jeopardize the subject and may require medical or surgical intervention to prevent one of the outcomes listed above

8.1.3. Unexpected Adverse Experience

Definition: any adverse drug experience, the specificity or severity of which is not consistent with the current investigator brochure (or risk information, if an IB is not required or available).

8.1.4. Life-Threatening Adverse Experience

Definition: any adverse drug experience that places the subject (in the view of the investigator) at immediate risk of death from the reaction as it occurred, i.e., it does not include a reaction that, had it occurred in a more severe form, might have caused death.

8.1.5. Unanticipated Problems

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

- related or possibly related to participation in the research (in this guidance document, possibly related means there is a reasonable possibility that the incident, experience, or outcome may have been caused by the procedures involved in the research); and
- suggests that the research places subjects or others at a greater risk of harm (including physical, psychological, economic, or social harm) than was previously known or recognized.

8.1.6. Noncompliance

Definition: failure to follow any applicable regulation or institutional policies that govern human subject research or failure to follow the determinations of the IRB. Noncompliance may occur due to lack of knowledge or due to deliberate choice to ignore regulations, institutional policies, or determinations of the IRB.

8.1.7. Serious Noncompliance

Definition: noncompliance that materially increases risks, and results in substantial harm to subjects or others, or that materially compromises the rights or welfare of participants.

8.1.8. Protocol Exceptions

Definition: A planned deviation from the approved protocol that are under the research team's control. Exceptions apply only to a single participant or a singular situation. Pre-approval of all protocol exceptions must be obtained prior to the event.

8.2. Reporting to the Human Research Protection Office (HRPO)

The PI is required to promptly notify the IRB of the following events:

- Any unanticipated problems involving risks to participants or others which occur at WU, any BJH institution, or that impacts participants or the conduct of the study.
- Noncompliance with federal regulations or the requirements or determinations of the IRB.
- Receipt of new information that may impact the willingness of participants to participate or continue participation in the research study.

These events must be reported to the IRB within **10 working days** of the occurrence of the event or notification to the PI of the event. The death of a research participant that qualifies as a reportable event should be reported within **1 working day** of the occurrence of the event or notification to the PI of the event.

8.3. Reporting to the Quality Assurance and Safety Monitoring Committee (QASMC) at Washington University

The PI is required to notify the QASMC of any unanticipated problem occurring at WU or any BJH or SLCH institution that has been reported to and acknowledged by HRPO as reportable. (Unanticipated problems reported to HRPO and withdrawn during the review process need not be reported to QASMC.)

QASMC must be notified within **10 days** of receipt of IRB acknowledgment via email to a QASMC auditor.

8.4. Time Frame for Reporting Required Events

8.4. As noted above minimal adverse events are associated with NIR/US. If we are made aware of an AE/SAE then we will report this to the HRPO in the timeline noted in section 8.2.

9. DATA AND SAFETY MONITORING

In compliance with the Washington University Institutional Data and Safety Monitoring Plan, the Principal Investigator will provide a Data and Safety Monitoring (DSM) report to the Washington University Quality Assurance and Safety Monitoring Committee (QASMC) semi-annually beginning six months after accrual has opened (if at least five patients have been enrolled) or one year after accrual has opened (if fewer than five patients have been enrolled at the six-month mark). This report will include:

- HRPO protocol number, protocol title, Principal Investigator name, data coordinator name, regulatory coordinator name, and statistician
- Date of initial HRPO approval, date of most recent consent HRPO approval/revision, date of HRPO expiration, date of most recent QA audit, study status, and phase of study
- History of study including summary of substantive amendments; summary of accrual suspensions including start/stop dates and reason; and summary of protocol exceptions, error, or breach of confidentiality including start/stop dates and reason
- Study-wide target accrual and study-wide actual accrual
- Protocol activation date
- Average rate of accrual observed in month 1, month 2, and subsequent months
- Expected accrual end date
- Objectives of protocol with supporting data and list the number of participants who have met each objective
- Measures of efficacy
- Early stopping rules with supporting data and list the number of participants who have met the early stopping rules
- Abstract submissions/publications
- Summary of any recent literature that may affect the safety or ethics of the study

The study principal investigator and Research Patient Coordinator will monitor any potential issues on an ongoing basis. Once the principal investigator or Research Patient Coordinator

becomes aware of an adverse event, the AE will be reported to the HRPO and QASMC according to institutional guidelines.

10. STATISTICAL CONSIDERATIONS

It is known that tumor pathologic variables, such as invasive ductal carcinoma, high tumor grade and high proliferative activity, are associated with a better response to neoadjuvant treatment. It is also known that triple-receptor negative and HER2 positive tumors respond best to cytotoxic and Herceptin-based regimens, respectively. To estimate the predictive value of these parameters as well as newly acquired hemoglobin parameters from US-guided NIR system, we have developed a logistic regression model which can be used to describe the relationship of several predictor variables X1, X2, … Xk to a dichotomous response variable Y, where Y is coded as 1 (responder) or 0 (non-responder) for its two possible categories. We also assess the overall quality of the prediction models through the ROC curves and the area under ROC (AUC). For this purpose, all parameters will be inputted to the prediction model to estimate the predict power on final pathological response. Once the model is validated from the new patient data, it can serve as a guideline for predicting patient response to neoadjuvant treatment.

10.1. Sample Size Calculations

For triple receptor negative breast cancers, recent studies have shown that 40% of the patients achieved pathologic complete response [25]. For Her2 positive breast cancers, recent studies have demonstrated that new anti-cancer regimens trastuzumab (Herceptin) plus lapatinib (Tykerb), or with trastuzumab plus pertuzumab (Perjeta) have resulted in a statistically significantly larger number of patients achieving pathologic complete response [25]. From our pilot study, we have observed a 25% change in total hemoglobin level when compared to pre-treatment baseline (%tHb) between the responder (Miller-Payne grades 4-5) and non-responders (Miller-Payne grades 1-3) at the end of cycle one. However, no statistical test was performed because of the small sample size (n=5) for this subgroup. Based on our earlier pilot data of response at end of cycle 1, n=5, the estimated sample size needed at significance level of $\alpha = 0.01$ (power 0.99) using difference in mean values and pooled standard deviations of two responder groups obtained from the pilot data of five patients at the end of cycle 1 suggests that 18 patients are required for the Her2 positive group in the new study. We observed an even greater difference of 34.1% in total hemoglobin level when compared to baseline between the two responder groups for triple receptor negative patients at the end of cycle 3. Again, no statistical test was performed because of the small sample size (n=6). The estimated sample size needed at significance level of $\alpha = 0.01$ (power 0.99) using difference in mean values and pooled standard deviations of two responder groups obtained from these 6 patients at the end of cycle 3, suggests that 19 patients are required for the triple receptor negative group in the new study. MINITAB two sample t-test was used for calculations.

For ER+ and Her2- patients, recent studies have shown that a pathological complete response is rarely achieved by neoadjuvant chemotherapy. At current practice, most of the patients were treated by hormone therapy which is a much more prolonged treatment than neoadjuvant chemotherapy. We do not plan to immediately recruit patients who will be treated by hormone therapy. In our pilot study, we had a total of 22 patients with ER+ and HER2- cancers and have obtained statistical significance based on tHb difference between responder and non-responder groups at the pre-treatment. We also obtained statistical significance based on %tHb at the end of cycle 1. The estimated sample size needed at

significance level of α =0.01 (power 0.99) using difference in mean values and pooled standard deviations of two responder groups at pre-treatment and end of the cycle 1, suggests that 12 patients are required for this group of patient in the new study. Again, MINITAB two sample t-test was used for calculations.

Based on our past experience, about 20% of the patients may either go to early surgery or drop the study due to side effects of treatment. With the 60 planned patients, we expect to have approximately 50 patients who will complete the study. Based on the patient pool at Washington University, we expect ~18 HER2 positive patients, ~19 triple receptor negative patients in the next two years of the award. The rest will be ER+ and HER- patients.

The adequate sample size of HER2 positive patients and triple receptor negative patients will allow us to evaluate the observation that the best time windows for predicting early response based on %tHb changes is within the first cycle for HER+ patients, and the best window for predicting early response is within the first three cycles for triple receptor negative patients. The new data that will be obtained at the end of day 7 may offer new insight into early tumor responses of these two major treatment groups.

Although the Taxol-based and Herceptin-based regimens have an important place in the treatment of breast cancer, many alternatives or new anti-cancer regimens are equally interesting and acceptable. We anticipate that a small population of patients of the proposed large patient cohort will be treated with novel regimens as these new treatments become available for clinical trials, and the results for these regimens can be used as pilot data for the efficacy of the new regimens.

For the exploratory cohort, we plan to enroll 10 patients.

In summary, sixty eligible patients will be enrolled and ~50 patients are expected to complete the study. Based on the patient pool at Washington University, we expect to recruit ~18 HER2-positive patients, ~19 triple negative patients, and the rest may be ER-positive and HER2-negative patients.

We will thereby begin to benchmark the optical signature of neoadjuvant treatment response of subsets of breast cancer based on molecular phenotype.

10.2. Primary Endpoint

The primary endpoint is pathologic response based on the Miller-Payne grading system (See Section 7 for description on grade 1~5). Patients will be grouped into responder (grade 4/5) and non-responder (grade 1~3) for statistical analysis.

10.3. Data Analysis

The multivariate logistic regression models will be used to model the binary primary endpoint. The receiver operating characteristics (ROC) curve analysis will be applied based on the fitted multivariate logistic model to evaluate the predictive ability of tumor vascularity parameters measured by NIR/US at baseline and at different treatment cycles including total, oxygenated and deoxygenated hemoglobin concentrations (abbreviated as tHb, oxyHb, deoxyHb, respectively) without and with inclusion of classic pathological variables including tumor histology, estrogen and progesterone receptor status, HER2

status, Ki67 proliferation index, Nottingham score, mitotic index, intrinsic subtype. To summarize the resulting predictive ability, the area under ROC (AUC) with Delong's method for confidence interval calculation and the sensitivity, specificity, positive and negative predictive value corresponding to an optimal cutoff will be derived. Two models will be compared based on their AUCs based on the DeLong's method and the likelihood ratio test. To robustly assess the predictive ability, the cross validation (CV) procedure will be applied: at each CV, 2/3 of the observations will be used to train the model while the remaining will be used to validate the model.

For the exploratory neoadjuvant endocrine cohort, the analysis will also include correlation with PEPI scores.

For the additional aim, correlate tHb with enhancement on CEM.

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