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Center of Excellence for Individualization of Therapy for Breast Cancer

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The attached report contains report from subcontractors involved in the COE as well. These reports explain in detail the efforts of the COE during the past year.
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Center of Excellence for Therapeutic Individualization for Breast Cancer
Annual Report: General Overview

The report submitted herein includes reports from subcontractors involved in the COE, which explain in detail the efforts of the COE during the past year. This report will highlight the overall progress made by the Center of Excellence.

1. Creation of Research Infrastructure: During the past 12 months, the COE has established the research infrastructure necessary for carrying out the proposed research.
   a. Intra-program communications: We have performed regular monthly teleconferences linking the principal investigators, and have had our first face-to-face meeting (at the December San Antonio Breast Cancer Symposium), with a second planned face-to-face meeting at the May 2005 American Society of Clinical Oncology Meetings. Our Biostatistics Core has rigorously evaluated the internet-based communication system that will serve as the backbone for data communication has been rigorously evaluated by our biostatistics core.
   b. Clinical program development: We have identified clinical sites that will participate in the clinical trial, and have taken steps to insure that drugs will be available at our planned foreign site in Lima, Peru. We have developed our first clinical protocol (the master clinical protocol that represents the centerpiece of the program), and have submitted the protocol to the DOD IRB following initial approval by the Indiana University IRB and SRC. The DOD IRB approval currently represents the major barrier to initiation of the research plan of the COE; we are hopeful that DOD IRB approval will occur in the near future. We have developed a clinical procedures manual for the central protocol.
   c. Clinical specimen processing: The COE has developed procedures specific to the processing and shipping of clinical specimens from clinical sites to the Pathology Core laboratory at the University of Oklahoma, and the shipment of these specimens to the research laboratory cores. These procedures can be implemented within weeks of final protocol approval by the DOD IRB.
   d. Research Core Laboratories: A principal focus of the COE’s teleconference has involved the prioritization of clinical specimens for research evaluation. A prioritization process has been developed and is ready for use pending initiation of the clinical protocol. A laboratory procedure manual has been developed for use. The core research laboratories have developed standards for tissue processing, and (as outlined elsewhere) have hired personnel for specimen processing.

2. Consumer Advocacy core: The Consumer Core has been heavily involved with all of the above, participating regularly in teleconferences, reviewing clinical protocols and procedure manuals, and helping create information packets for patients potentially interested in the protocol. The first Research Advocates
Symposium will be held on Saturday, April 30th, and will bring together research advocates from the state of Indiana with clinical and laboratory researchers involved in the COE. It is hoped that this interaction will facilitate patient acceptance of the research program.

3. Current Development Plans: The Clinical Research Core (Kathy Miller, MD) is in the process of developing a Phase II trial focusing on the use of bevacizumab in metastatic breast cancer. This trial takes on additional importance given the recent demonstration (in Dr. Miller’s E2100 Phase III trial) that bevacizumab plays a clinically important role in the management of metastatic breast cancer.

Respectfully submitted,

George W. Sledge, Jr. MD
Center of Excellence for Individualization of Therapy for Breast Cancer: Proteomics Core Report
Statement of Work Task 1: Development of/Preparation for Metastatic Chemotherapy Parent Protocol

Participated in the monthly teleconferences related to the planning and IRB approvals needed for initiation of patient work and sample collection. Developed the logistics associated with data collection and data processing related to SELDI mass spectrometry of the patient serum and tissue specimens to be obtained during the course of the study. Meet with the biostatisticians to develop the precise tests and data transfer protocols to be used throughout the analysis of the mass spectrometry data. Attended the first biannual meeting of the working group in San Antonio during the annual San Antonio Breast Cancer Conference. Have made arrangements to meet in Orlando in early June for the 2nd biannual meeting of the working group.

Statement of Work Task 2: Performance of Metastatic Chemotherapy Trial/Tissue Collection/Patient Follow-up
Statement of Work Task 3: Analysis of Tissues by Laboratory Cores
Awaiting completion of Task 1.

Statement of Work Task 4: Performance of Prospective Validation Trial
Statement of Work Task 5: Performance of Investigational Agent Trials
Awaiting completion of Task 1-3
Center of Excellence for Individualization of Therapy for Breast Cancer: Clinical Core Report
Statement of Work Task 1: Development of/Preparation for Metastatic Chemotherapy Parent Protocol
Master protocol and informed consent completed; submitted to local IRB; and approval obtained from local IRB. Submitted to HSRRB initially on 14 OCT 2004. Reviewed at the 23 NOV 2004 HSRRB meeting. Conditional approval received with comments on 14 DEC 2004. Initial response sent 18 FEB 2005. Additional comments received from HSRRB 15 MAR 2005. Response sent 23 MAR 2005. Awaiting approval from HSRRB. eData submission is in development awaiting final approval of parent protocol before finalizing.

Assisted Patient Advocacy Core in creating patient recruitment information.

Site visits completed with each laboratory core. Infrastructure developed for the flow of specimens from clinical sites to laboratory cores. Monthly teleconferences conducted with George Sledge, M.D and Cores. First Face-to-Face meeting conducted 10 DEC 2004.

Statement of Work Task 2: Performance of Metastatic Chemotherapy Trial/Tissue Collection/Patient Follow-up
Statement of Work Task 3: Analysis of Tissues by Laboratory Cores
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Statement of Work Task 5: Performance of Investigational Agent Trials
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Center of Excellence for Individualization of Therapy for Breast Cancer: Advocate Core Report
Introduction

Advocate participation in this research study will allow them to better understand the science, the methodology and the results so they can take the lead in informing the advocacy community about this important project. The patient advocates will create and maintain a network of advocates and advocate organizations.

The Patient Advocates will work closely with the Clinical Trial Core to develop patient education materials and expand recruitment strategies. Plans are for: a brochure to provide general information to patients; Q & A for patients considering participating in the Center’s research; one-page summary explaining each study. All will be in English and Spanish.

An educational program for local advocates will increase their awareness and understanding of the objectives of the Center. One-day program offered at selected BCE sites will include researchers discussing their ongoing research, an overview of the Center and how advocates can be involved.
During this reporting period, advocates in the Advocate Core participated with researchers to provide the patient perspective to all discussion. The advocates participated in monthly conference calls and a face-to-face meeting at the San Antonio Breast Cancer Symposium. Several trips to Indianapolis enabled advocates to meet and interact with the PI and other associates. Meetings covered topics such as expectations of the advocates and researchers; development logistical arrangements for meeting objectives; and arrangements for the one-day Advocate Symposium.

Advocates worked closely with the co-PIs of the study Predicting Response and Toxicity in Patients Receiving Chemotherapy for Breast Cancer: A Multicenter Genomic, Proteomic, and Pharmacogenomic Correlative Study to develop a one-page summary. This summary contained the title, the sponsor, the rational, the purpose, the design and the eligibility requirements of the study. The summary received local IRB approval.

The development of a brochure to provide general information to patients about the Center Of Excellence For Individualization Of Therapy In Breast Cancer was completed by advocates with input from Center staff. A poster appropriate to be displayed in public spaces within the Center was also developed. Both educational pieces received IRB approval. The advocates recommended content for the public/advocate web page.

An Advocate Symposium was developed and will be offered, on April 30, 2005, to local advocates representing advocate organizations. The educational program will include researchers discussing their ongoing and proposed research; an overview of the Center and its research; and how advocates can be involved. Specific information about studies will be presented. The day will conclude with a tour of the laboratory providing advocates with information about how breast biopsies are conducted and how tissue is used.
Accomplishments

- Patient advocates participated in monthly core conference calls, face-to-face meeting in conjunction with the San Antonio Breast Cancer Symposium and meetings with Center staff.

- A brochure and a poster were developed to provide general information to patients about the Center. Both received approval from the local IRB.

- A one-page summary of *Predicting Response and Toxicity in Patients Receiving Chemotherapy for Breast Cancer: A Multicenter Genomic, Proteomic, and Pharmacogenomic Correlative Study* was developed.

- Content for the public/advocate web page was provided.

- An Advocate Symposium was developed and offered, on April 30, 2005, to local advocates representing advocate organizations to familiarize them with the research ongoing and proposed for the Center of Excellence.
Conclusions

- Advocates and researcher can work together to help ensure the success of research studies.

- The model of the development of patient educational material by advocates with input from researchers and staff proved to be successful.

- Developing a network of advocate, advocate organizations, and researchers can increase awareness and understanding of specific research projects.
Center of Excellence for Individualization of Therapy for Breast Cancer: Genomics Core Report
Introduction

Optimal systemic treatment after breast cancer is the most crucial factor in reducing mortality in women with breast cancer. Adjuvant chemotherapy and hormonal treatment both reduce the risk of death in breast cancer patients. However, while estrogen receptors status predicts for response to hormone treatments, there are no clinically useful predictive markers for chemotherapy responses. All eligible women are therefore treated in the same manner. Even denoval drug resistance will result in treatment failures in many breast cancer patients. Currently, there are no methods available to distinguish those patients who are likely to respond to specific chemotherapies, and given the accepted practice of prescribing adjuvant treatment to most parties, even if the average expected benefit is slow, the selection of appropriate patients represents a major advance in the clinical management of breast cancer today.

We therefore set out to identify gene expression patterns in breast cancer specimens that might predict response to taxenes. Chemotherapy allows for the sampling of the primary tumor for gene expression analysis and for direct assessment of response to chemotherapy by following changes in tumor size during the first few months of treatment. Hence, chemotherapy provides an idea platform to rapidly discover predictive markers of chemotherapy response.
In this present study, we hypothesize through high quantitation of gene expression, grade is possible to access thousands of genes simultaneously, and expression patterns in different breast cancers might correlate with and thereby predict response to treatment. The purpose of this study was to (1) demonstrate that sufficient RNA could be obtained from core biopsies to access gene expression, (2) to identify groups of genes that could be used to distinguish primary breast cancers to responsive or resistance to different chemotherapies and (3) to identify gene pathways that could be important in a mechanism of action of these agents.

**Body of Research**

We have been improving gene expression arrays obtained from small tissue samples, as technical development. We measured each core biopsy obtained from primary breast cancers as approximately 1 cm x 1 mm. As these core biopsies were too small for micro dissection, we ascertained the tumor cellularity of the pretreatment core biopsies. In general, the core biopsies showed good tumor cellularity with median tumor cellularity of 75% (range 40-100%). Each core biopsy yielded 3-6 mg of total RNA, which is more than sufficient to generate approximately 20 mg of label cRNA needed for hybridization with the Affymetrix U133A Genechip, using the manufacturer's standard protocols. We have also experimented with laser microdissection of tumors of lower tumor cellularity. There are no reportable outcomes as we are improving the technology for gene expression arrays for this study while samples are being collected in the clinical trial.
Center of Excellence for Individualization of Therapy for Breast Cancer: Pharmacodynamics/Pharmacogenomics Core Report
Introduction

The primary objective of the pharmacodynamics/pharmacogenomics core facility is to develop user-friendly techniques readily available to the clinician for measuring a specific aspect of response and/or toxicity, which will lead to the individualization of therapy. Critical determinants that govern individual responsiveness will be identified. These include markers, kinetic rate or metabolic outcome, which are often referred to as a pharmacokinetic or pharmacodynamic "signature". One critical advantage of this application is that these signatures will be directly compared to and contrasted with the genomic and proteomic analyses.

In order to identify such pharmacokinetic signatures, a number of techniques were established in our lab. For example, HPLC methods are used to quantify plasma and urinary levels of Doxorubicin, Cyclophosphamide and Diltiazem (Arm A) as well as some of their metabolites. A technique to evaluate tumoral protein levels of the P-glycoprotein (PgP) efflux pumps in these same patients has been set up as well. The plasma levels of Capecitabine and its metabolites, and levels of key enzymes involved in the metabolism of Capecitabine will be evaluated in patients of Arm B, using an HPLC method and an enzyme linked immunosorbent assay (ELISA) respectively. In the case of Vinorelbine (Arm C), we have established protocols enabling us to measure the concentrations of Vinorelbine and Diltiazem in both plasma and urine samples. Similarly, we were able to set up a technique to measure levels of Gemcitabine (Arm D) and its major metabolites in plasma and urine samples.

A correlation of the protein/enzyme activity profile with disease state, therapy and drug response would provide invaluable insight into monitoring inter-individual variations in efficacy and toxicity. Moreover, these observations could be used to help select appropriate drug and dosage regimens for each patient.
Protocol development:

Inventory system and miscellaneous:

In order to safely and promptly process all the incoming samples, we established an inventory system and database enabling us to manage and keep track of the samples under our responsibility. Among other features we planned the filing procedure, data entry procedure, backup system (backups offsite, CDROM and paper) as well as the storage of samples (split) in different storage spaces. The database will allow us to safely store all the relevant information relating to a particular sample or patient and make it possible to streamline the production of detailed reports. Although time-consuming, this initial step will permit a safe and effective processing of the samples.

Arm A: Doxorubicin, Cyclophosphamide and Diltiazem

Multi-drug associated P-glycoprotein (MDR/PgP) efflux pumps causes resistance to various drugs in a number of cancer patients and are the main pharmacogenomic parameters of interest for doxorubicin (DOX). DOX is metabolized in the liver to doxorubicinol (DOXol), which is then cleared more slowly. Metabolic phenotype can be determined by measuring the ratio of DOX and DOXol. It is also informative to determine the appearance of DOXol normalized to the concentration or volume distribution of diltiazem, a non cytotoxic MDR/PgP probe. The ratio of DOX to diltiazem will be measured in timed plasma and urine samples.

HPLC protocol development:

Doxorubicin: The chromatographic technique used for DOX and DOXol measurements is from de Bruijn et al (1999) with minor modifications. The HPLC system consists of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a series 200 Peltier oven. The stationary phase is composed of a ODS, Spheri-5, C18, 5 μm particle size, 250 x 4.6 mm i.d. Brownlee Column. The mobile phase is composed of water:acetonitrile:tetrahydrofuran (76:24:0.5, v/v/v)
with the pH adjusted to 2.0 with perchloric acid. The flow rate of the mobile phase is set at 1.25 ml/min under isocratic elution and the eluent was monitored fluorimetrically at an excitation wavelength of 560 nm, with a band width of 40 nm. The column temperature was set at 50°C. Detection and integration of chromatographic peaks are performed by the TotalChrom chromatography Data System.

Stock solutions of DOX (1.0 mg/ml), DOXol (0.5 mg/ml), and the internal standard daunorubicin (DNR) are prepared by dissolving the appropriate amount of drug in acetone: water (1:1, v/v) and stored in glass at -80°C. A 5.0 µg/ml working solution of the internal standard DNR is prepared by diluting the stock solution with acetone and stored in glass protected from light at -20°C.

Cyclophosphamide: The chromatographic technique used for cyclophosphamide measurements is from Griskevicus et al (2002) with minor modifications. The HPLC system consists of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a series 200 Peltier oven. The stationary phase is composed of a RP-18, Spheri-5, C18, 5 µm particle size, 150 x 4.6 mm i.d. Brownlee Column. The mobile phase is composed of phosphate buffer (10mM), pH 3.5 and acetonitrile (2/1 v/v). The flow rate of the mobile phase is set at 2 ml/min under isocratic elution and the eluent was monitored with the UV detector set at an excitation wavelength of 350 nm, and emission at 550 nm. The column temperature was set at 22°C. Detection and integration of chromatographic peaks are performed by the TotalChrom chromatography Data System.

Diltiazem: The chromatographic technique used for Diltiazem measurements is from Quaglia et al (2005) with minor modifications. The HPLC system consists of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a
series 200 Peltier oven. The stationary phase is composed of a RP-8, Spheri-5, C8, 5 μm particle size, 250 x 4.6 mm i.d. Brownlee Column. The mobile phase is composed of ammonium acetate buffer (10 mM, pH 6.58) containing 0.2% of dimethylamine and acetonitrile (60/40 v/v). The flow rate of the mobile phase is set at 150 μl/min under isocratic elution and the eluent was monitored fluorimetrically at an excitation wavelength of 237 nm, with a band width of 40 nm. The column temperature is set at 22°C. Detection and integration of chromatographic peaks are performed by the TotalChrom chromatography Data System.

Stock solutions: Diltiazem (1mg/ml) and the internal standard verapamil (1mg/ml) are prepared by dissolving the appropriate amount of drug in water and stored at 4°C.

**Western blot protocol development for MDR/PgP:**

For western blot analysis the primary antibody is a monoclonal mouse anti-P-glycoprotein (clone C-219; Catalog number BP1199) from ID Labs (London, ON, Canada) that recognizes both human MDR1 and MDR3 P-glycoprotein gene products. This anti-body reacts with a 170 kDa internal membrane epitope in humans. This primary anti-body was diluted (1/1000) in 3% BSA, and the secondary, a horseradish peroxidase conjugated anti-mouse, was diluted (1/10 000) in dry milk 5%.

To standardize for protein loading, the membranes are probed for PTP1D. In both cases immunoreactive proteins are visualized by enhanced chemiluminescence (ECL) according to the manufacturer's instructions. Densitometric measurements are done using a desktop scanner and arbitrary units, corrected for gel loading using PTP1D are determined with ImageJ software.
Arm B: Capecitabine

HPLC protocol development:

Capecitabine: The chromatographic technique used for Capecitabine measurements is from Zufia et al. (2004) with minor modifications. The HPLC system consists of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a series 200 Peltier oven. The stationary phase is composed of a ODS, Spheri-5, C18, 5 μm particle size, 150 x 4.6 mm i.d. Brownlee Column. For the mobile phase the method uses a gradient elution with a constant flow rate of 1.4ml/min. Initially the eluent was composed of, 10% solvent A (1% formic acid) and 90% solvent C (HPLC grade water) and a linear gradient is performed until there is 10% solvent A, 70% solvent B (Methanol) and 20 % solvent C. For the next 10 min the column is allowed to return to its initial conditions of 10% solvent A and 90% solvent C. The eluent was monitored at a wavelength of 266 nm for 5'-DFUR and 5-FU, 205 nm for FUH₂ and 310 nm for capecitabine. The column temperature was set at 30°C. Detection and integration of chromatographic peaks was performed by the TotalChrom chromatography Data System.

Stock solutions of Capecitabine, 5'-DFUR and the internal standard 5-bromouracil are prepared by dissolving the appropriate amount of compound in a known volume of methanol and stored at -20°C.

ELISA for TP and DPD

Photometric Enzyme-Linked ImmunoSorbent Assay (ELISA) kits for the quantitative determination of thymidine phosphorylase (TP) and dihydropyrimidine dehydrogenase (DPD) using antibody-coated microplates were obtained from Roche (catalog number 2158744 and 2207184 respectively). The tissue samples were homogenized in a tenfold excess volume of 10 mM Tris-HCl buffer (pH 7.4) containing 15 mM NaCl, 1.5 mM MgCl₂, and 50 μM potassium phosphate, and then centrifuged at 10,000 g for 15 min. The protein concentration of the supernatant was
determined using a DC protein assay kit (BioRad Laboratories, Hercules, Calif.). Samples and the serially diluted standard were dispensed into anti-TP or anti-DPD monoclonal antibody-coated microplate wells. The plates were incubated at 37°C for 1 h and then washed with 0.05% Tween 20 in 10 mM phosphate-buffered solution (pH 7.6). They were then incubated with a monoclonal anti-TP conjugated with peroxidase (HRP) or anti-DPD-HRP at 37°C for 1 h and washed. A substrate reaction was performed with the ABTS substrate solution under constant shaking. For up to 60 min. measurements were made every 10 min. at absorbance was measured at 450 nm, and the enzyme level was calibrated with that measured for the standard solution, and expressed as U/mg protein.

Arm C: Vinorelbine and Diltiazem

Efflux pumps and beta tubulin are recognized as key determinants of Vinorelbine efficacy. Vinorelbine is metabolized to active acetyl and inactive N-oxide with enterohepatic recycling. Metabolic phenotyping will include normalization for co-administered efflux pumps substrate, Diltiazem. The ratio of Vinorelbine to diltiazem will be measured in timed plasma and urine samples.

HPLC protocol development:

Vinorelbine: The chromatographic technique used for Vinorelbine measurements is from Gauvin et al. (2000) with minor modifications. The HPLC system consisted of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a series 200 Peltier oven. The stationary phase was composed of a ODS, Spheri-5, C18, 5 μm particle size, 250 x 4.6 mm i.d. Brownlee Column. The mobile phase consisted of a mixture of acetonitrile and 80 mM ammonium acetate (50:50, v/v); the pH of latter was adjusted to 2.5 with hydrochloric acid. The flow rate of the mobile phase was set at 1.00 ml/min under isocratic elution and the eluent was monitored fluorimetrically at an excitation wavelength of 560 nm, with a band width of 40 nm.
The column temperature was set at 20°C. Detection and integration of chromatographic peaks was performed by the TotalChrom chromatography Data System.

Stock solutions of VRB (1.0 mg/ml) and the internal standard vinblastine (VLB) (1.0 mg/ml) were prepared by dissolving the appropriate amount of drug in water and stored at -20°C. Working solutions at 10 and 100 ng/ml for VRB and at 0.5 µg/ml VLB were freshly prepared in water.

Diltiazem: The chromatographic technique used to measure Diltiazem is described in the doxorubicin section.

Arm D: Gemcitabine

Gemcitabine (difluorodeoxycytidine; dFdC) is a prodrug phosphorylated by deoxycytidine kinase (dCK) into a triphosphate metabolite that is incorporated into DNA resulting in cell death.

HPLC protocol development:

Gemcitabine: The chromatographic technique used for Gemcitabine measurements is from Sottani et al (2004) with minor modifications. The HPLC system consisted of a PerkinElmer TCCS QUAT diode array system (Sheldon, CT, USA), a PerkinElmer series 200 fluorescence detector, a PerkinElmer series 200 autosampler, and a series 200 Peltier oven. The stationary phase was composed of a ODS, Spheri-5, C18, 5 µm particle size, 250 x 4.6 mm i.d. Brownlee Column operated at 30°C. The mobile phase consists of a mixture of methanol-cyclohexane-1,2-dichloroethane (30:50:20, v/v/v) and the flow rate is set at 1.00 ml/min under isocratic elution. The detector is set to scan from 200 nm to 500 nm and has a discrete channel set at 272 nm which is the wavelength used for quantification. Detection and integration of
chromatographic peaks was performed by the TotalChrom chromatography Data System.

Stock solutions of gemcitabine (1.0 mg/ml) and the internal standards 2',-deoxyctydine and tetrahydrouridine (1.0 mg/ml) were prepared by dissolving the appropriate amount of drug in methanol. The internal standard stock solution is further diluted with methanol to a concentration of 10 \mu g/ml to obtain a working solution. All these solutions are stored at -20°C.

Conclusions

The facilities are prepared and the sample inventory system was set up. We have established the protocols described in the statement of work and are ready to process the incoming samples.
Reference List


Griskevicius L., Meurling L. and Hassan M. Simple method based on fluorescent detection for the determination of 4-hydroxycyclophosphamide in plasma. Ther Drug Monit. 2002; 24:405-9.


Center of Excellence for Individualization of Therapy for Breast Cancer: Pathology Core Report
Pathology Core

During the first year of this grant we have purchased a -80 freezer and storage racks for storage of specimens received from the various institutions. We have also purchased a computer capable of interfacing with the HOG computers. This will allow our research technician to enter data from cases into the main computer located at HOG. We have obtained a blanket IRB to cover the collection of specimens from the collaborating institutions and dissemination to the three core laboratories. We have reviewed various drafts of the laboratory manual for this project. We have also met with representatives from the central office in November 2004 in our laboratory and attended the semi-annual face to face meeting in conjunction with the San Antonio Breast Conference and Symposium in December 2004. Once the DOD IRB office approves the first of the protocols, we will expect a visit from the HOG information technology group to coordinate the final connections between the computers at OUHSC and HOG. We are in the process of establishing final laboratory protocols in conjunction with the HOG office.

Since the DOD IRB office has not yet officially approved the first protocol, we have not yet received any specimens from the collaborating institutions. Once the protocol has been approved, we anticipate we will begin to receive specimens from the outside institutions.