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TITLE: Biologic and Computational Modeling of Mammographic Density and Stromal Patterning

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(2) Section I - A brief introduction covering the purpose and scope of the research effort.

INTRODUCTION:

Mammographic density serves as independent marker of short term breast cancer risk and a surrogate marker of response to a variety of prevention agents[1-3]. Although a majority of breast cancers are epithelial in origin, there is evidence that stromal content of the breast is an important predictor or mammographic density. There is increasing evidence that the stroma plays a role in breast cancer initiation[4]. However, currently we lack an understanding of how mammographic density is affected by the individual contribution of epithelial and stromal components and the biological potential of stromal and/or epithelial cells. The goals of this synergistic grant proposal are to develop computational and biological tools to investigate the relationship between mammographic density, stromal content of the breast, and the role of stromal/epithelial interactions in regulating proliferation, and ultimately, short-term breast cancer risk. To achieve these goals we bring together investigators with expertise in mathematical fractal pattern assessment, 3-D models of stromal/epithelial interactions, and clinical breast cancer risk assessment. Together we propose to correlate computational models of mammographic and stromal patterning with biological assays of stromal/epithelial proliferation, and clinical outcome leading to the construction of multi-disciplinary tools for the classification of breast cancer risk and response to prevention strategies.

Random Periareolar Fine Needle Aspiration (RPFNA) is a research technique that has been prospectively validated to assess *1)* short-term breast cancer risk and *2)* response to chemoprevention in high-risk women. While RPFNA was originally developed to evaluate early epithelial changes, RPFNA also provides a representative sampling of stromal cells in high-risk women. prevention agents, and therefore, mammographic In this Synergy Proposal, we are currently testing the ***hypothesis*** that in women with epithelial atypia, 1) mammographic and stromal patterning does not consistently predict the degree of epithelial atypia (measured by Masood Cytology Index) and 2) mammographic density may not be a reliable measure of epithelial response to prevention agents.

(3) Section II - A brief description of overall progress to date plus a separate description for each task or other logical segment of work on which effort was expended during the report period. Description shall include pertinent data and graphs in sufficient detail to explain any significant results achieved. If this award includes the recruitment of human subjects for clinical research or a clinical trial, report progress on subject recruitment (i.e., number of subjects enrolled versus total number proposed).

Objective 1: To investigate the relationships between mammographic density, mammary stromal patterns and computational image analysis of the breast. The goals of this aim are to 1) Quantitate the stromal-epithelial cell ratios obtained from RPFNA and quantitate imaged breast density computer modeling; 2) Perform comparisons and correlations between RPFNA stromal-epithelial cell ratios, and mammographic density; 3) Statistically examine the relationship between mammographic density, MRI fibroglandular volume, and RPFNA stromal to epithelial composition and stromal patterning.

Task 1: RPFNA, Digitizing, Annotation, and Posting - *COMPLETE*

TIMELINE: *Years 1-2:* 50 RPFNA will be performed in high-risk women, slides will be prepared, cytology assessed, slides will be digitized, annotated and posted.

MILESTONES: *Year 1:* 50 RPFNA performed, tested, and posted.

Task 1 Progress: We performed serial RPFNA on 25 high-risk women and 25 high-risk women taking tamoxifen chemoprevention. Women not taking tamoxifen were risk-matched to the 25 women who took tamoxifen. Each woman underwent an average of 4.4 RPFNA. A total of 228 RPFNA were analyzed. Subject demographics are presented in **Table 1**. All 228 RPFNA slides have been digitized, annotated, on a password protected server.

The average time of total observation for women was 33 months (range 18 to 54 months) and the average time on tamoxifen prevention was 22 months (range 12 to 48 months). The average age of women was 46 (range 40 to 52). Eighty percent of women were premenopausal and 20% were either perimenopaual or postmenopausal. Twenty percent of women were African American and 80% were Caucasian. See **Table 1** for subject demorgraphics.

We previously used RPFNA to test for cytological response to tamoxifen chemoprevention in high-risk women with atypia. We observed that disappearance of atypia occurs within the first 12 months of initiating tamoxifen. After 12 months, women do not have disappearance of atypia. In the 25 women taking tamoxifen chemoprevention in this study, we observe that 14/25 women have disappearance of atypia after 12 months tamoxifen prevention and 11 women have persistent atypia. This is consistent with the Breast Cancer Prevention Trial (P1) which demonstrated a 50% reduction in estrogen receptor-positive breast cancer.

Task 2: Analysis of Epithelial/Stromal Counts.

TIMELINE: *Years 1-2: Cytological Quantization:* Using a standard volume of suspended RPFNA cells, four cytology slides will be generated. Epithelial cell and stromal cell counts will be quantitated by a blinded cytologist in triplicate.

***Computational Pattern Analysis*:** Fractal pattern analysis of epithelial and stromal cells will be performed on digitized images of fixed cell slides from the RPFNA.

MILESTONES: *Year 1:* Stromal and Epithelial Cell Counts will be tested from 50 subjects using cytological quantitation and computational pattern analysis.

Task 2 Progress:

Complete: Stromal and epithelial cell counts from 50 RPFNA have been counted and quantitated.

Ongoing: Fractal analysis is ongoing by Dr. Lo's team.

Epithelial/Stromal Counts: Epithelial cell and stromal cell counts were analyzed in from the serial 50 subjects (228) described above in **Task 1**. We observed a correlation between an overall decrease in cell counts and stromal cell counts after 12 months tamoxifen chemoprevention ($p<0.001$). There was however, no correlation between a decrease in stromal cell counts and epithelial cell counts. Of the 14 women who had

disappearance of atypia all had >75% decrease in RPFNA cell counts. There was no additional response to Tam after 12 months in the 11 women who had persistent atypia after 12 months tamoxifen prevention.

Task 3: Analysis of Mammographic Density - *COMPLETE*

TIMELINE: *Year 1-2:* Mammographic density will be assessed quantitatively using 1) visual assessment of mammographic density and 2) a novel automated computer method

MILESTONES: *Year 2:* 100 Mammograms analyzed by visual assessment and computer automated methods. A total of 250 (150 old; 100 new) will be completed.

Progress Task 3: Over 475 serial screen-film mammograms have been digitized from the 50 women described in **Task 1**: 25 high-risk women taking tamoxifen prevention and 25 high-risk women who elected not to take tamoxifen. Woman had an average of 4.3 mammographic determinations. Mammograms from both breasts were digitized, including cranial caudal and medial lateral views.

Mammograms were digitized from 25 women taking tamoxifen prevention and 25 controls using a new Howtek MultiRad 860 digitizer. The anonymized mammographic images were stored on our private computer network and referenced in the database. Mammographic density was assessed quantitatively using established computer modeling techniques. We are using the public Digital Database for Screening Mammography. To verify the reproducibility and robustness with respect to imaging technique, we compared the medio-lateral oblique and craniocaudal views of the same digitized breast.

We found in the course of this analysis that assessment of mammographic breast density by analysis of films suffers from variability. We are now analyzing 127 MRI from the 50 women described in **Task 1**.

Task 4: Analysis of MRI

TIMELINE: *Year 1-2:* MRI slices will be segmented manually and total voxel volumes for the fibroglandular tissue will be computed over the whole breast. Patterns of suspicious MRI signal enhancement will be preliminarily evaluated.

MILESTONES: *Year 2:* Analysis of 100 MRIs will be completed. A total of 250 (150 old; 100 new) will be completed.

Progress Task 4: MRI Image Analysis is ongoing to evaluate breast density. We performed and collected an average of 3.1 breast MRI on each of our 50 subjects that are described in **Task 1** and have a bank of 427 additional MRI for analysis.

All MRI were performed with a commercial system using a dedicated breast coil. The digital files were obtained from the Picture Archiving and Communication System (PACS) and placed on our private computer network for the specified analysis. We are currently performing a preliminary semi-automatic analysis of the 3-D MRI images. MRI slices are segmented manually and total voxel volumes for the fibroglandular tissue are computed over the whole breast.

We observed 2 breast cancers in the 50 subjects in Task 1; there were also patterns of suspicious MRI signal enhancement present in 7/50 subjects that are being prospectively analyzed. Unfortunately an additional 28 women in our cohort developed breast cancer over the past 12 months. A majority of these breast cancers were not focal in origin.

Task 5: Statistical analysis

TIMELINE: *Years 1-2:* Statistical analysis will be performed to correlate mammographic density with, MRI patterning, stromal cell counts, and stromal patterning.

MILESTONES: *Year 2:* Statistical analysis will be completed.

Statistical Analysis: Statistical comparison are on-going and methods include, *1)* Pearson's correlation coefficient, *2)* Spearman rank correlation coefficient, and *3)* mutual information. *Pearson's correlation coefficient* measures linear dependence between random variables. *Spearman rank correlation coefficient* can show correlation between rank-ordered data. Since the data is ranked, *1)* the values are not used directly; *2)* the measure of correlation is independent of scales; *3)* no assumptions are made about the distribution of the underlying data. *Mutual information* is a method for measuring the general statistical dependence between random variables. Mutual information will be computed to test whether a more general statistical dependence

exists between mammographic density, fractal patterning, and stromal/epithelial counts. Questions that we are currently testing include:

a) Do stromal and/or epithelial counts predict mammographic density? - COMPLETED
Neither stromal or epithelial counts predicted density.

Observation to date: We have completed this analysis for 50 subjects and observe that epithelial counts do not predict mammographic density. We observe a direct correlation between epithelial cell counts and Masood Cytology abnormalities ($p<0.001$). Stromal cell counts are in process.

b) Is there a correlation between the presence or absence of atypia after tamoxifen chemoprevention and changes in mammographic density? - COMPLETED
No correlation.

Observation to date: We tested for a correlation in the 25 subjects described in **Task 1** who took tamoxifen chemoprevention. There was no correlation ($p>0.5$) when the data analysis was performed for individual women or individual breasts. Two women developed breast cancer while taking tamoxifen chemoprevention. Both women had a decline in mammographic density. In contrast, a minority of women had correlation between mammographic density and disappearance of atypia in RPFNA.

c) Is there a correlation between mammographic density, mammographic and stromal fractal patterning, and RPFNA Masood epithelial cytology?

Observation to date: In subjects with atypia there is not a direct correlation. These studies will provide rational for developing MRI based measures of short-term breast cancer risk and response to prevention strategies.

Based on our preliminary studies, we hypothesize that MRI patterning (density of high and moderate intensity small patterning) is predictive of subsequent breast cancer, especially when the density increases on subsequent MRI. These studies are on-going. We have identified that the density of small moderate enhancing non-specific lesions correlate with atypia and coalescence of these small foci correlate with progression to DCIS.

OBJECTIVE 2: **To test whether increased mammographic density correlates with increased stromal proliferation.** To accomplish this aim we are using combinations of 1) defined epithelial cell and 2) patient-derived epithelial cells obtained RPFNA will be co-cultured with stroma isolated from subjects with high- and normal-mammographic density. Co-culture methods will include 3-D culture and 3-D rotary bioreactor culture, stromal and epithelial cells will be tested for proliferation and transcriptional activation.

Task 1: Isolation of Mammary Stromal and Epithelial Cells from RPFNA - *COMPLETED*

TIMELINE: *Years 1-2:* Obtain matched HMECs and stromal cells from high-risk patients with high and low-medium mammographic density.

MILESTONES: *Year 2:* Obtain 10 matched sets of stroma and epithelial cells from RPFNA.

Progress to date: We have collected matched HMECs and stroma from 10 high-risk patients with high mammographic density.

Task 2: Epithelial/Stromal Co-Culture.

TIMELINE: *Years 1-2:* Perform 3-D culture with combinations of stroma and epithelial cells obtained from women with high and low-medium mammographic density.

MILESTONES: *Year 2:* 3-D culture performed on 30 sample combinations.

Progress to date: We initiated co-culture of defined and patient-derived epithelial/stroma cells. Cells have been isolated from high-risk women with *1)* atypia who have *2)* high or normal mammographic density. Co-culture methods include 3-D bioreactor culture. We are currently testing for dominance of stroma versus epithelium using the strategy outlined below. We experience technical problems. The bioreactor culture resulted in unacceptable rates of contamination. We observed decreased stromal proliferation in 3-D culture. Currently we are adjusting cell culture conditions and may attempt a dual chamber co-culture but this would be suboptimal since this would not provide cell-cell contact.

Task 3: Statistical analysis correlating stromal proliferation with mammographic density and breast stromal composition.

TIMELINE: ***Year 2:*** Statistical analysis will be performed.

MILESTONES: ***Year 2:*** Statistical analysis will be completed

Results to date: We have initiated these studies in 3-D culture. However, we experience decreased proliferation of stromal cells. We are working to optimize cell culture conditions to improve stromal proliferation.

Table 1:

	High-risk subjects NOT on tamoxifen prevention	High-risk subjects on tamoxifen prevention	Total subjects
Number	25	25	50
Average Age	41 (41-48)	40 (40-52)	46 (40-52)
Menopausal status Premenopausal Perimenopausal	 19/25 6/25	 21/25 4/25	 40/50 (80%) 10/50 (20%)
Race Caucasian African American	 21/25 4/25	 19/25 6/25	 40/50 (80%) 10/50 (20%)
Risk Atypia/LCIS DCIS	 19/25 6/26	 19/25 6/25	 38/50 (76%) 12/50 (24%)
Time of observation	30 mos (18 to 50 mos)	31 mos (18 to 54 mos)	31 mos (18 to 54 mos)
Duration of tamoxifen	n/a	22 mos (12 to 48 mos)	n/a
Average density			
Average mammographic density	57% (range 31% to 55%)	54% (range 34% to 67%)	55% (range 31% to 67%)
Average number of RPFNA	4.5	4.6	4.4 (3-6 RPFNA)
RPFNA change Disappearance of atypia Persistence of atypia	 1/25 24/25	 14/25 11/25	 15/50 35/50
Development of breast cancer	2	2	4

(4) Section III - Problem Areas

(a) A description of current problems that may impede performance along with proposed corrective action.

We had delayed IRB approval. This delayed the initiation of our studies. This problem was corrected prior to the last review.

We initiated 3-D co-culture and experienced difficult with maintaining stromal proliferation. The rotary culture resulted in a high rate of contamination. We will continue with the 3-D culture and work to optimize culture conditions with modulation of EGR and insulin.

(b) A description of anticipated problems that have a potential to impede progress and what corrective action is planned should the problem materialize.

None.

(5) Section IV - A description of work to be performed during the next reporting period.

Studies for Objective 1 for next period of reporting:
Test for the correlation between MRI patterning and mammary atypia.

Studies for Objective 2 for next period of reporting:
We will continue with 3-D culture co-culture, stromal and epithelial cells will be tested for proliferation and transcriptional activation.

(6) Section V - Administrative Comments (Optional) - Description of proposed site visits and participation in technical meetings, journal manuscripts in preparation, coordination with other organizations conducting related work, etc.

1) Manuscripts
Lo, J, Barron, A, and Seewaldt, VL. Presence or absence of atypia in RPFNA does not correlate with mammographic density changes after 12 months tamoxifen prevention. Submitted Cancer Epi Biomarkers Prevention, 2009.

2) Presentations
Lo, J, Barron, A, and Seewaldt, VL. Presence or absence of atypia in RPFNA does not correlate with mammographic density changes after 12 months tamoxifen prevention. Presented *SPORE*, December, 2008.

Seewaldt, VL. Multidisciplinary assessment of atypia in RPFNA, MRI patterning, and early mammary carcinogenesis. Presented *NIEHS*, January, 2009.

Seewaldt, VL. Multidisciplinary assessment of early mammary carcinogenesis. Presented *Wayne State*, May, 2009.

Seewaldt, VL. Multidisciplinary assessment of atypia in RPFNA, MRI patterning, and early mammary carcinogenesis. Presented *U. Minnesota*, May, 2009.

Seewaldt, VL. MRI patterning and breast cancer risk. Presented *FORCE Plenary Session*, May, 2009.

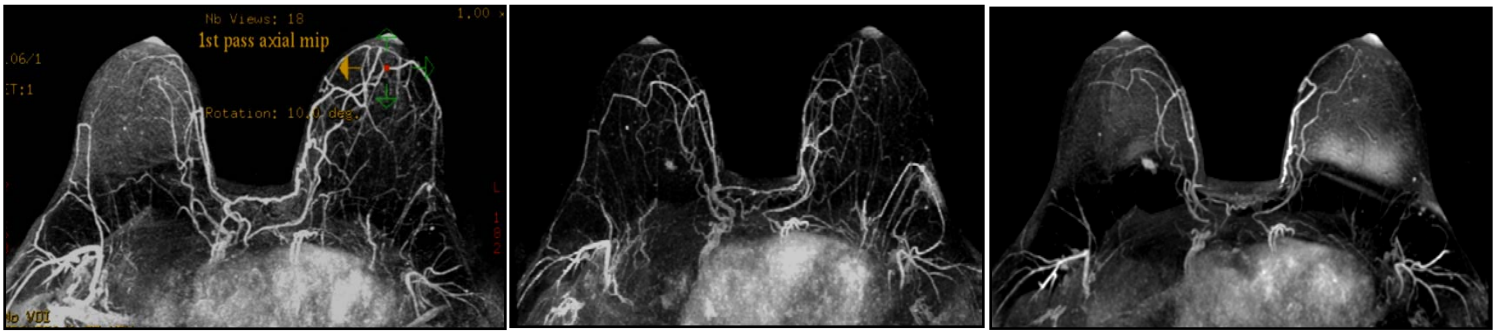
3) Funding

CALGB multi-institutional trial to test for correlation between mammographic density, MRI determined breast density, and RPFNA cytology. Concept submitted and approved Prevention Committee, now being submitted to full CALGB review (Seewaldt)

DoD SIDA not funded (Seewaldt)
DoD IDEA pending (Seewaldt/Weaver)
Susan G. Komen Disparities, not funded (Seewaldt)
K01 Career Development Award, re-submitted (Ibarra PI, Seewaldt Mentor)
Environmental Cancer Core Grant, pending (DeGuilio PI, Seewaldt Cancer Core Leader)

4) Training
Graduate student Nicholas D'Amato, DoD Predoctoral Award - Awarded.

Graduate student Matthew Sweede, DoD Predoctoral Award - Pending
Junior Faculty, Julie Ostrander, DoD Multi-Disciplinary Award – Awarded
K07- pending.
Junior Faculty, Catherine Ibarra, Komen Career Catalyst – Awarded.
K01 award. – resubmitted (A2)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