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13. ABSTRACT (Maximum 200) In Project 1(developing new biomarkers for hormones and growth factors), we have completed data analysis on sources of variability in EGF and TGF- α levels in breast fluid (BF) and prepared a manuscript. We have begun data collection for the next BFstudy, which will test 1) the relation of growth factor levels in BF to parenchymal density on screening mammograms, and 2) the relation of BF growth factor levels to reproductive and other risk factors for breast cancer. We have completed numerous assays of progesterone (PG) and estradiol (E2) in saliva, using new direct methods. The PG assay appears to be reliable and valid. Although the E2 assay is reliable and sensitive, correlations with plasma E2 levels were not as high as expected. We have undertaken several studies, including sharing samples with other labs, to resolve this issue, and are now modifying the assay to reduce background noise. We have completed a thorough review of the diet data from the Repeat Sample Study, and are now planning analyses that will relate diet, exercise and body size to hormone levels in these premenopausal women. In Project 2 (lobular differentiation in normal breast tissue), we have re-calibrated our scoring technique for lobule type in consultation with Dr. Jose Russo, and have evaluated the adequacy of mastectomy samples in preparation for a case-control study of lobular differentiation and breast cancer risk.				
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FOREWORD

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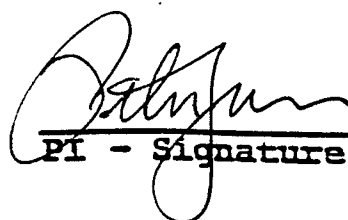
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Annual Report for Grant DAMD17 - 94 - J - 4203
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Annual Report: Grant DAMD17-94-J-4203
September 1, 1995 - August 31, 1996

Principal Investigator: Peter Gann, M.D., Sc.D.

Note: The previous annual report was submitted February 20, 1996 due to a change in the grant-type. The current report therefore primarily focuses on activity during the 7 months between February and October, 1996.

INTRODUCTION

The overall aim of this project is to develop new biological markers that can improve epidemiological investigations into the etiology of breast cancer. Our studies encompass three types of novel biomarkers: a) *breast fluid from nipple aspirates for measurement of growth factors and steroids*, b) *saliva for measurement of sex steroid concentrations*, and c) *normal breast tissue from biopsy samples for assessment of lobular differentiation*. These biomarkers will allow epidemiologists to study the development of breast cancer in greater biological detail than previously possible using conventional questionnaire-based research. **To allow consistency with our previous reports, we will refer to all activities related to hormone or growth factor levels as Project 1; activities related to lobular differentiation will be referred to as Project 2.**

In breast fluid, we have been studying concentrations of growth factors, including epidermal growth factor (EGF) and TGF- α , that are presumed to play a major role in controlling breast cell proliferation and differentiation¹. We seek to determine what extraneous factors influence these GF levels in breast fluid, whether GF levels are associated with breast cancer, and ultimately, whether GF levels are modulated by pro- or anti-carcinogenic exposures. We have completed an evaluation of assay sensitivity and precision for EGF and TGF- α , ascertained the relative amounts of intra- versus inter-woman variability, and evaluated the association between plasma hormones, menstrual cycle position and GF levels. A manuscript describing these studies is in the final stages of completion before being submitted for publication.

Our work with saliva samples focuses on development of new assay methods for estradiol and progesterone. These assays are direct assays - they avoid an extraction step that requires a large volume of sample and can introduce error. The new assays - which involve time-resolved fluoroimmunoassay for estradiol and a direct radioimmunoassay for progesterone - will provide ultrasensitive non-invasive methods for serial measurement of steroid hormone concentrations in premenopausal women.

As mentioned in our previous annual report, we decided, while collecting samples for development of the breast fluid and saliva assays, to extend our aims to include a cross-sectional analysis on the determinants of steroid hormone and growth factor levels in premenopausal women. We had observed that published data for premenopausal women on the relationship between hormone and GF levels and factors such as diet, physical activity and body size were quite sparse. We hypothesize that high fiber and low fat intake, high level

of physical activity, and low levels of abdominal fat (as measured by sagittal diameter) will be associated with reduced levels of bioavailable estradiol. The entire data collection effort, called the Repeat Sample Study (RSS) was completed in September, 1995. In the RSS, 62 women between ages 20 and 40, made four visits at weekly intervals to the Northwestern Clinical Research Center. At each visit, breast fluid, blood, saliva and urine were obtained. We expect the RSS to yield important information leading to several publications on GF assay variability in breast fluid, a new approach to measuring estradiol in saliva, and the association between diet, activity, body size and hormone profiles.

Project 2 deals with measurement of lobular differentiation in normal breast tissue. We postulate that it is feasible to use normal breast tissue from the margins of breast biopsies to obtain a histological index of the differentiation status of a woman's breast. This idea follows from the work of Russo, et al., which demonstrates the feasibility and usefulness of such a marker in a rodent model². Data on human lobular differentiation has heretofore been obtained from breast reduction specimens, and therefore is not abundant. Development of a histologic differentiation marker that can be used in readily available tissue would allow us to begin epidemiological studies aimed at identifying the major influences on human breast differentiation. It is presumed that extensive lobular differentiation will protect against breast cancer development. In this project thus far, we have worked with Dr. Jose Russo at Fox Chase Cancer Center to train pathologists and non-pathologists at Northwestern in scoring breast tissue under the microscope. We have also reviewed records at the Breast Center to determine the approximate number and suitability of biopsy samples available for analysis, and have initiated pilot studies using mastectomy specimens.

The progress of all these studies is diagrammed in Table 1. For Project 1, Phases 1 and 2 are completed and Phase 3 work has begun. For Project 2, Phase 1 work is ongoing, while Phase 2 work is underway.

BODY OF THE REPORT

A. Methods and Procedures: Project 1 (Hormones/Growth Factor Levels in Body Fluids, and Their Determinants)

[Note: Methods described below pertain to activities undertaken during the reporting period in question.]

1. Laboratory assays for breast fluid growth factors

EGF and TGF- α were measured in breast fluid at dilutions of 1:100 and 1:25 respectively, using a radioimmunoassay with reagents provided by Bio-Medical Technologies, Inc. (Stoughton, MA). Total protein was measured in each breast fluid sample by the Bradford method. We have initiated assays for TGF- β 1 in breast fluid using the Genzyme (Cambridge, MA) Predicta® ELISA kit.

2. Laboratory assays for breast fluid estrogens

We are adapting a time-resolved fluoroimmunoassay developed by Wallace, Inc. (Turku, Finland) to measure estradiol in breast fluid. This assay system (known as DELFIA) relies on

competition between steroids in the sample and europium-labelled steroid chelates for binding to an anti-steroid IgG. The europium label emits an intense, prolonged fluorescent signal³. Adaptation of the assay (which was developed for serum/plasma) involves constructing a standard curve and finding the appropriate sample dilution for testing breast fluid samples as well as determining the specific level of the background signal which we have detected in breast fluid samples, thereby allowing for proper adjustment of assay values. We are also adapting a competitive ELISA kit (Immunacare, Bethlehem, PA) for measuring 2-hydroxyestrone and 16 α -hydroxyestrone in breast fluid⁴.

3. Breast fluid growth factor data analysis

We have now completed our analysis of assay and biological variability in breast fluid EGF and TGF- α levels among the RSS participants. Completion of the analysis required: finding the optimal transformation (square root) of growth factor levels to provide normalization of the data, calculation of intra-class correlation coefficients between right and left breasts and between measurements taken in the same breast at different timepoints, developing random effects models to assess the associations between breast fluid GF levels and plasma hormones. We also used random effects models (PROC GLM in SAS) to assess the relation of EGF to TGF- α in the same breast fluid sample. The random effects models, which have both fixed and random coefficients, allow us to evaluate the correlation between two markers *within* each subject, as well as the correlation summarized across a group of subjects. The presence of multiple data points from each subject also required the application of a permutation method to compute the statistical significance of a correlation⁵. We believe that these data analytic methods, which were selected and evaluated in conjunction with Dr. Josee Dupuis, the project biostatistician, provide a valid and efficient template for future biomarker evaluation studies.

4. New study - Mammographic Density Study

Having demonstrated that EGF and TGF- α levels can be measured in breast fluid, and that these levels are consistent within a woman over time, we have designed and started our next breast fluid study. The aims of the Mammographic Density Study (MDS) are to 1) evaluate the association between breast fluid levels of EGF and TGF- α and breast parenchymal density as reflected in screening mammograms, and 2) evaluate the association between these breast fluid GF levels and reproductive risk factors for breast cancer. Aim 2 corresponds to Study 4 (Task 4 in SOW) in our original USAMRDC proposal. Mammographic density is increasingly recognized as a risk factor for breast cancer development. Moreover, tissue density appears to be controlled, at least partially, by ovarian hormonal influences⁶. We hypothesize that women with characteristically high levels of mitogenic growth factors in breast fluid will have increased mammographic density.

To measure mammographic density, we have decided to use the planimetry method employed by NCI investigators in their large epidemiologic study using BCDDP mammograms⁷. We have purchased and tested a Lasico (Los Angeles, CA) planimeter which is linked to a desktop PC. This planimeter allows accurate estimation of the surface area containing parenchymal density on a mammogram. The percentage of total breast area occupied by density was the measure that has given the highest risk gradient, in studies by both Saftlas, et al. and Byrne, et al^{7,8}. Use of the cranio-caudal view alone is preferable, because, although the lateral view adds information about the volume of a density, the total

breast area is not easy to measure on the lateral film. To validate our determination of percent density, we spent one whole day reviewing techniques with Martine Salane of Wolfe Radiological Associates in Detroit, who was the principal validated reader for the NCI study. Ms. Salane demonstrated her technique for tracing breast density on actual screening mammograms at the Northwestern Breast Center, and worked personally with Allison Ellman, who will be doing the tracings for our study. We will be exchanging mammograms with Ms. Salane by mail order to validate Ms. Ellman's readings.

Our MDS study protocol, which has been reviewed and approved by the Northwestern IRB, involves collection of breast fluid from women aged 35-60 who have come to the Breast Center for screening mammograms. We speculated that the compression from the mammogram could increase the yield of fluid; therefore, women are asked to participate in the study immediately after the mammogram, while they are waiting (in gowns) for the technologist to determine the adequacy of the film quality. Volunteers are then directed to a procedure room at the Center, where a research staff member completes informed consent, fills out a supplemental questionnaire concerning risk factors, and proceeds with breast fluid collection. Thus far, we have completed 30 aspiration attempts on mammography patients, and have obtained a measurable quantity of breast fluid from 15 women. Our sample size and power estimates call for a total of 120 breast fluid samples.

5. Assays for saliva progesterone and estradiol

To measure progesterone in saliva directly without extraction, we use an ultrasensitive competitive-binding radioimmunoassay. The antibodies for the assay were produced by Dr. Robert Chatterton, whose laboratory conducts the assay. In the assay, 200 μ l samples of saliva are mixed with radiolabelled progesterone and progesterone antibody. Bound progesterone is separated from free with the use of dextran coated charcoal. The bound is then counted in a liquid scintillation counter. The salivary estradiol assay uses time-resolved fluoroimmunoassay (tradename: DELFIA). In this assay, 100 μ l samples of saliva are mixed with antibodies to estradiol. The antigen-antibody complexes and any free antibodies are then captured to the solid phase in wells coated with anti-IgG antibodies. Estradiol chelated to europium is then added to the well and binds free anti-estradiol antibodies. After incubation and rinsing, the antibody-bound fluorescence signal over time is measured in a Wallac fluorometer designed for 96-well plates. Estradiol concentrations in the saliva are computed from comparison to standard curves.

6. Data analysis: saliva hormone assays

We computed the intra- and interassay coefficients of variation for both the progesterone and estradiol assays, based on blind quality control pool samples. We also conducted "stripping and add-back" studies in which male saliva was treated with charcoal to remove steroids, measured amounts of progesterone and estradiol were added, and assays performed. We analyzed the correlation of salivary hormone to contemporaneous plasma hormone levels using scatterplots and non-parametric correlation coefficients. We correlated salivary estradiol with total plasma estradiol, bioavailable estradiol and free estradiol. To estimate the association between saliva and blood estradiol levels both within subjects and in the group as a whole, we used random effects models (PROC GLM in SAS) containing dummy or indicator variables for each subject.

7. Refinement of the salivary estradiol assay

Concerns about the relatively low correlation between salivary estradiol and total estradiol (as measured in the Chatterton lab) led us to send a subset of saliva and plasma samples to reference laboratories. Sixty-two luteal phase saliva samples, plus unidentifiable quality control samples, were shipped while frozen to the lab of Dr. Peter Ellison and Dr. Susan Lipson at Harvard University. Plasma samples taken at the same time as each of these 62 salivas (plus QC samples) were sent to Dr. Christopher Longcope at the University of Massachusetts for analysis of total estradiol.

From our initial work with the DELFIA assay, we had noted that steroid-stripped saliva samples gave a "background" estradiol reading of approximately 2.8 pg/ml in our quality control pool samples. Our initial results for individual samples were therefore corrected by subtraction of 2.8 from each result. If the amount of background signal varied from one individual to the next, this assumption of a constant background would introduce error into the final assay results and error into the correlations with blood estradiol. We therefore conducted studies in which 300 μ l of saliva from individual subjects was stripped with charcoal and run in the DELFIA along with the unstripped sample. Tracer studies revealed that stripping was removing over 99% of the labelled estradiol.

To further explain the disappointing correlation we initially observed between saliva and plasma estradiol, we planned three additional studies. In one, which we have completed, free estradiol was measured in 31 plasma samples that had corresponding saliva samples assayed by both Chatterton and Ellison labs. To measure free estradiol we used a method described by Hammond employing centrifugal ultrafiltration dialysis to estimate the percent unbound estradiol in serum/plasma⁹. Second, we are planning to use matching blood-saliva stored from participants in the Two-Cycle Preliminary Study (another CRC-conducted biomarker development study) to determine whether the subtraction of individual assay background from each result improves the correlation with blood estradiol. This set of samples consists of 36 pairs of matching blood and serum, 2 sets from each of 18 women. In the third study, which we are calling the Repeat Measures Study, we will recruit 6 female volunteers between the ages of 20 and 40 to provide 12 matched saliva and blood samples - three per week over 4 weeks. These samples will be assayed for salivary estradiol and serum total and free estradiol. The aim of the Repeat Measures Study is to determine whether the saliva-blood estradiol correlation is significantly higher when correlations are made within individuals rather than with samples obtained from numerous women.

8. Refining of diet data

In the Repeat Sample Study, each participant provided four 24-hour diet recalls, which were obtained by a trained nutritionist from the Clinical Research Center at approximately weekly intervals. The data were initially analyzed using Nutritionist IV (Hearst Corp., San Bruno, CA), a software package designed to summarize macro- and micro-nutrient intake from diet reports. Since our Department normally uses the NDS program (Nutrition Coordinating Center, University of Minnesota) for estimating nutrient intake, we had a staff nutritionist review each 24-hour diet recall and re-input the data into NDS. This also served as a check on the initial data entry. We then compared the nutrient values obtained via Nutritionist IV and NDS. We found good agreement for all macro-nutrients (fat, carbohydrate, protein, calories), but poor agreement between the two databases for some micronutrients. For

example, the correlation for total fat was 0.85, but the correlation for alpha-tocopherol and beta-carotene were only 0.20 and 0.66 respectively.

B. Results/Discussion: Project 1

1. Breast fluid growth factors

The results of the analyses described above are presented and discussed in the manuscript submitted as Appendix A. This is a confidential manuscript.

2. Salivary progesterone and estradiol

Intra- and interassay reliability results for salivary progesterone (expressed in terms of the coefficient of variation) are shown in Table 2 below. The correlation between saliva and plasma progesterone, for luteal phase samples, is shown in Figure 1. As expected, given the assay reliability and correlation with blood, analysis of serial daily saliva samples, as shown in Figure 2, produces a profile across the menstrual cycle that is similar to that seen in blood.

The intra- and inter-assay CVs for salivary estradiol were excellent, see Table 2. However, we encountered a problem when we examined the correlation of salivary to plasma estradiol. This correlation was only 0.25 for 247 samples obtained from 62 women (see Figure 3). Similar correlation was seen with bioavailable estradiol rather than total. Our initial hypothesis was that a systematic error had occurred in either the saliva or plasma assay, since the intra- and interassay results indicated no problem with reliability of either assay. Figure 4 shows the correlation for salivary estradiol between the Chatterton and Ellison. Of the 62 samples sent to Ellison, results from one batch of 31 were discarded since the entire batch gave readings below the detection limit. For the remaining samples, the correlation between labs was reasonably high; however, the Chatterton values were generally higher than those recorded by Ellison. Figure 5 shows the plot and correlation of Ellison saliva versus Chatterton total plasma estradiol. This correlation was somewhat higher than that seen in Figure 3, therefore, we used the Ellison saliva data for subsequent analyses. Estradiol that is measurable in saliva is in the free form, therefore, comparison of saliva levels to free rather than total estradiol might improve correlation. Figure 6 shows the correlation of Ellison saliva with plasma free estradiol. There was essentially no difference in correlation, compared to that obtained with total estradiol.

Our recent results show that the amount of background noise in the salivary estradiol assay indeed varies between individuals, with a mean of 1.61 pg/ml and a standard deviation of 0.12. This constitutes a substantial portion of the total salivary estradiol measurement, and means that correction of each individual sample for its background could produce a real improvement in the saliva: blood correlation. Assays of the Two Cycle Preliminary Study samples (see above) are underway to answer this question. Published data on the estradiol correlation between saliva and blood are sparse. Dr. Ellison's lab, which is among the most experienced in salivary assays, has obtained unpublished data indicating saliva:serum correlations of about 0.80, when samples are obtained from the same woman. Our Repeat Measures Study will allow us to determine whether we see a higher correlation when samples from only one woman are analyzed. When we examined the Repeat Sample Study data (four samples per woman) with random

effects models, we did not find substantial correlations between salivary and plasma estradiol *within* women. However, the power of this analysis was restricted due to the small number of samples per woman.

A. Methods and Procedures: Project 2 (Lobular Differentiation in Normal Breast Tissue)

1. Reader validation

We are working with Dr. Jose Russo, Chairman of Pathology at the Fox Chase Cancer Research Center, to establish a "gold standard" for validating readers of lobular differentiation. Dr. Russo originated the system we are using for classifying lobules into 4 categories based on the complexity of the ductal branching. Our initial inter-reader results indicated that a pathologist at Northwestern (Dr. R. Goldschmidt) could obtain close agreement with Dr. Russo in classifying lobules from breast biopsy samples. However, we have begun working with Elizabeth Wiley, MD, the new chief of breast pathology at Northwestern, and hosted Dr. Russo in Chicago, so these pathologists could discuss classification criteria. Drs. Russo and Wiley agreed on stricter criteria for counting lobules (absence of hyperplasia, presence of visible central duct). We subsequently conducted blind review of breast biopsy samples by three readers: Dr. Wiley, Dr. Beth Bauer-Marsh (a pathology resident), and Allison Ellman (Dr. Gann's Project Coordinator). Ms. Ellman also scored 10 cases of benign biopsy previously read by Dr. Russo. These results, presented below, indicated that although correlations between readers were generally quite high, we were scoring a higher percentage of Type 2 lobules than previously. Dr. Gann and Ms. Ellman consequently travelled to Fox Chase in September, 1996 to meet with Dr. Russo and review previously-read as well as new cases. This meeting resulted in establishment of more explicit criteria for classifying lobules, and stricter criteria for including lobular structures for reading. The next step is for Ms. Ellman and Dr. Bauer-Marsh to read a validation set of cases that will be composed of 10 benign biopsy cases and 10 mastectomy cases. Dr. Russo will read the same cases, and we anticipate that we will see close agreement on lobule type, while overall correlation will remain high. We are also receiving bids for purchase of a used microscope with a teaching head that will be located in the Department of Preventive Medicine, and will be dedicated to this project.

2. Sample quality - mastectomy and biopsy specimens

We are continuing to examine various types of surgical specimens to determine their suitability for reading of lobule type in normal tissue. Representative slides from mastectomy cases, excision biopsies for palpable lumps and needle localization biopsies are being reviewed under 40X to determine if they contain a sufficient number of normal lobules. In the mastectomy cases, we have looked at samples from non-involved as well as involved breast quadrants. This activity can be conducted before completion of reader validation. In both mastectomy and benign biopsy cases we will, once reader validation is complete, score lobule type at various distances from the primary lesion (Task 2 in SOW).

3. Case-control study

We are designing a case-control study on the relation of lobular differentiation to the risk of breast cancer. This study will encompass, but extend beyond Task 3 in the original SOW. The cases will be patients diagnosed with breast cancer at Northwestern who received a mastectomy during 1995 or 1996. Preliminary sample size estimates call for 80 cases. We

will oversample women under age 50 in order to minimize the problem of lobule regression with aging. Since 1995, pathologists at Northwestern have routinely sampled normal breast tissue in all quadrants from the periareolar area on mastectomy specimens. The distribution of lobule type will be read from these non-involved areas. Control samples will be selected, matched for age, from excision biopsies performed for benign disease. We plan to have two controls per case. Lobule type will be read from the normal areas surrounding the benign lesion. Analysis will be based on the difference in lobule type distribution (types 1, 2 or 3) in cases versus controls. We hypothesize that cases will have a higher percentage of type 1 lobules and a lower percentage of type 3. The control series from this study will be used to analyze the relationship between breast cancer risk factors and lobule type (Task 3, SOW). The Breast Center database (developed under a separate USAMRDC grant to Dr. Monica Morrow) contains data on a wide range of breast cancer risk factors for all patients seen at the Lynn Sage Breast Center. We hypothesize that age at first parity, number of deliveries and exogenous hormone use will be associated with breast differentiation. Although the number of non-cancer samples in this analysis (n=160) is less than we originally proposed, it would represent by far the largest dataset on risk factors and breast differentiation in women, and would provide preliminary data to motivate further data collection.

B. Results/Discussion: Project 2

Figure 7 shows the correlations between readers (Ellman, Bauer-Marsh and Wiley) who were independently scoring lobule type in normal tissue from 10 cases of benign breast disease. We found that correlation between readers was high. Correlations for % type 1 are shown; results for % type 2 were similar. Ellman and Bauer-Marsh, in addition to having a high correlation, also had close agreement on the actual percent type 1 or type 2 (i.e., the regression line is close to a slope of 1). Dr. Wiley, on the other hand, tended to score more lobules as type 2. We also correlated the number of lobules scored by each reader and found relatively good agreement, although Dr. Wiley tended to score more structures. Comparison of Ellman to Russo, on 10 other benign biopsy cases, is shown in Figure 8. Again, although the correlation (0.80) is quite high, the slope is not equal to one because Ms. Ellman scored more as type 2 than Dr. Russo. We believe that the type 1 vs type 2 discrepancy will be greatly reduced as a result of the recent meeting in Philadelphia between Drs. Russo and Gann and Ms. Ellman. The new criteria for lobule type are: type 1 : 0-29 ductules, type 2: 30-79 ductules, and type 3: 80 or more ductules. We have also learned that prior to fall, 1995, most mastectomy cases at Northwestern Memorial Hospital had few slides containing breast lobules obtained from non-involved quadrants.

CONCLUSIONS

During the period from February to October, 1996, significant accomplishments of this project include:

- Completion of the first manuscript describing the assay for EGF and TGF- α in breast fluid, and documenting that individual women secrete concentrations of these mitogenic growth factors that are consistent between breasts and over time. The analysis also

reports, for the first time, an association between EGF and TGF- α levels in breast fluid within individual women, and an association, again within women, between breast fluid EGF and plasma total estradiol.

- Demonstration of a successful new method for direct assay of progesterone in saliva.
- Demonstration that a time-resolved fluoroimmunoassay for salivary estradiol is highly sensitive (can be performed on 100 μ l of saliva as opposed to 3 ml in standard methods) and reliable. We have also found fairly high correlation between two labs using different assay methods. However, the correlation of salivary estradiol to total or bioavailable estradiol in plasma was lower than expected. We have undertaken extensive studies to explain this, including sharing of samples with two reference labs and comparison of salivary estradiol to free estradiol in plasma. We are currently working on the hypothesis that correlation can be improved by running a charcoal-stripped sample beside each unstripped sample, and subtracting the background from each sample result. We are also examining the saliva:blood correlation when multiple pairs of samples are obtained from the same individual.
- Start of data collection for the Mammographic Density Study, which will test the hypothesis that elevated mitogenic growth factors in breast fluid are associated with increased parenchymal density on a screening mammogram. To conduct this study we have purchased a planimeter and begun the process of developing accurate measurements of mammographic density based on the percent of the total breast area occupied with radiodense tissue. This study will also allow us to analyze the relation of breast fluid EGF and TGF- α concentrations to reproductive and hormonal risk factors for breast cancer. Thus far, we have enrolled 30 women in the study.
- Re-calibration of our method for scoring breast lobule type. After testing three additional readers, we found that our previous criteria for scoring lobules was too lax, and may have over-counted type 1 lobules. We have established new, stricter criteria, and will test these using validation cases in the next two months. We have also reviewed mastectomy and biopsy samples, and have refined plans for a case-control study of lobular differentiation and breast cancer.

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Table 1. Developing New Epidemiologic Tools for Investigating Breast Cancer Risk (DAMD17-94-J-4203): Project Phases

	Phase 1 ^a	Phase 2	Phase 3
Project 1: Hormone/growth factor levels in body fluids, and their determinants	Repeat Sample Study (design, sample collection)	- assay breast fluid EGF, TGF-α, data analysis - assay bioavailable E2 - assay saliva PG, E2 - refine diet data	- begin sample collection for Mammographic Density Study - data analysis: diet, activity and body size - assay breast fluid E2, TGF-β - refine saliva E2 assay - measure free E2 - Repeat Measure Study
Project 2: Lobular differentiation in normal breast tissue	reader validation	- lobular homogeneity - sample quality	case-control study

Table 2. Intra and inter- assay coefficients of variance for salivary hormone assays

	Number of pairs	Intra -assay %CV	Inter-assay %CV
Salivary Progesterone	32	12.12	9.17
Salivary Estradiol	44	8.42	10.04

Figure 1. Salivary progesterone concentrations versus plasma progesterone concentrations from the luteal phase of the menstrual cycle

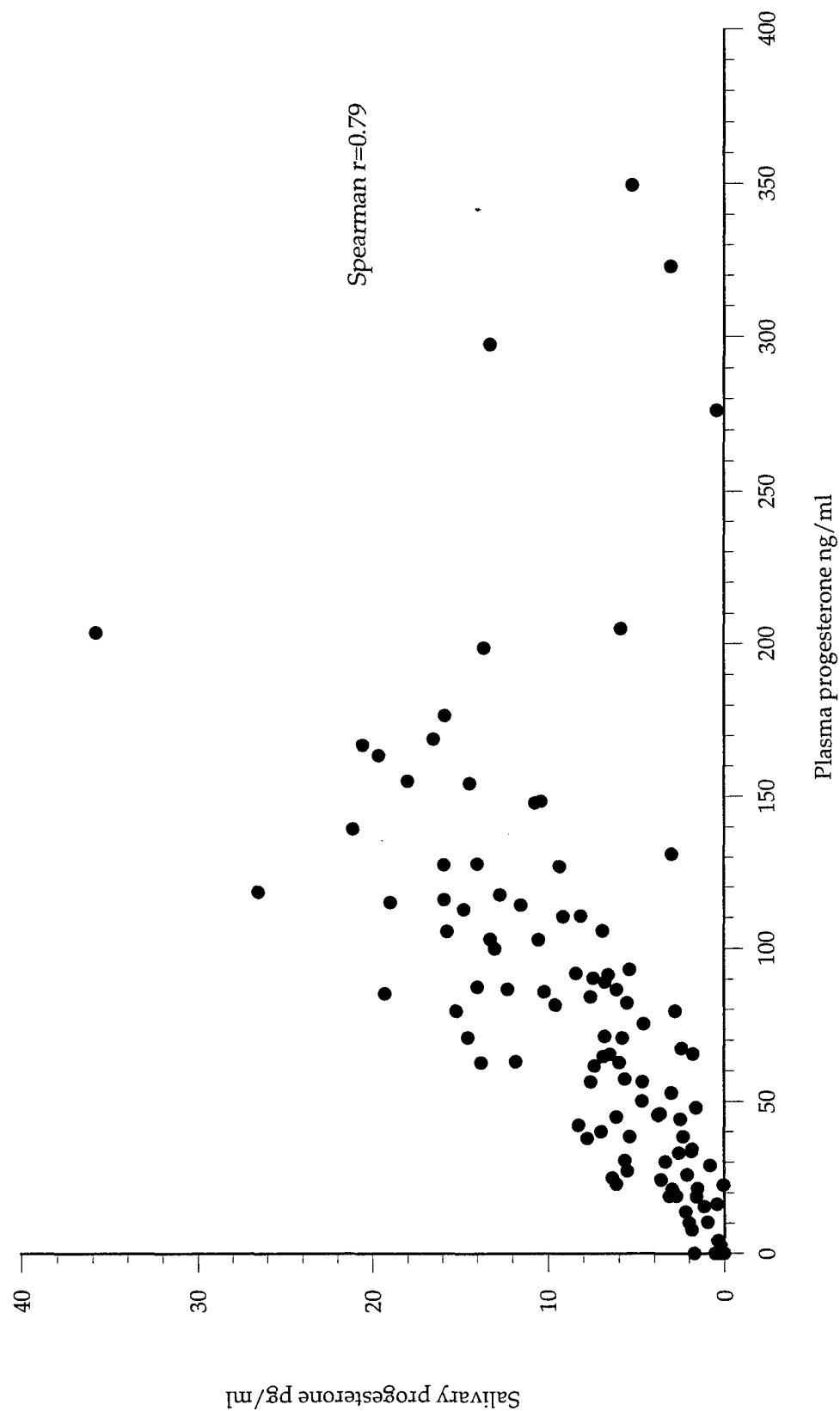


Figure 2. Salivary progesterone concentration by cycle day for RSS subjects - 188 samples obtained from 48 women

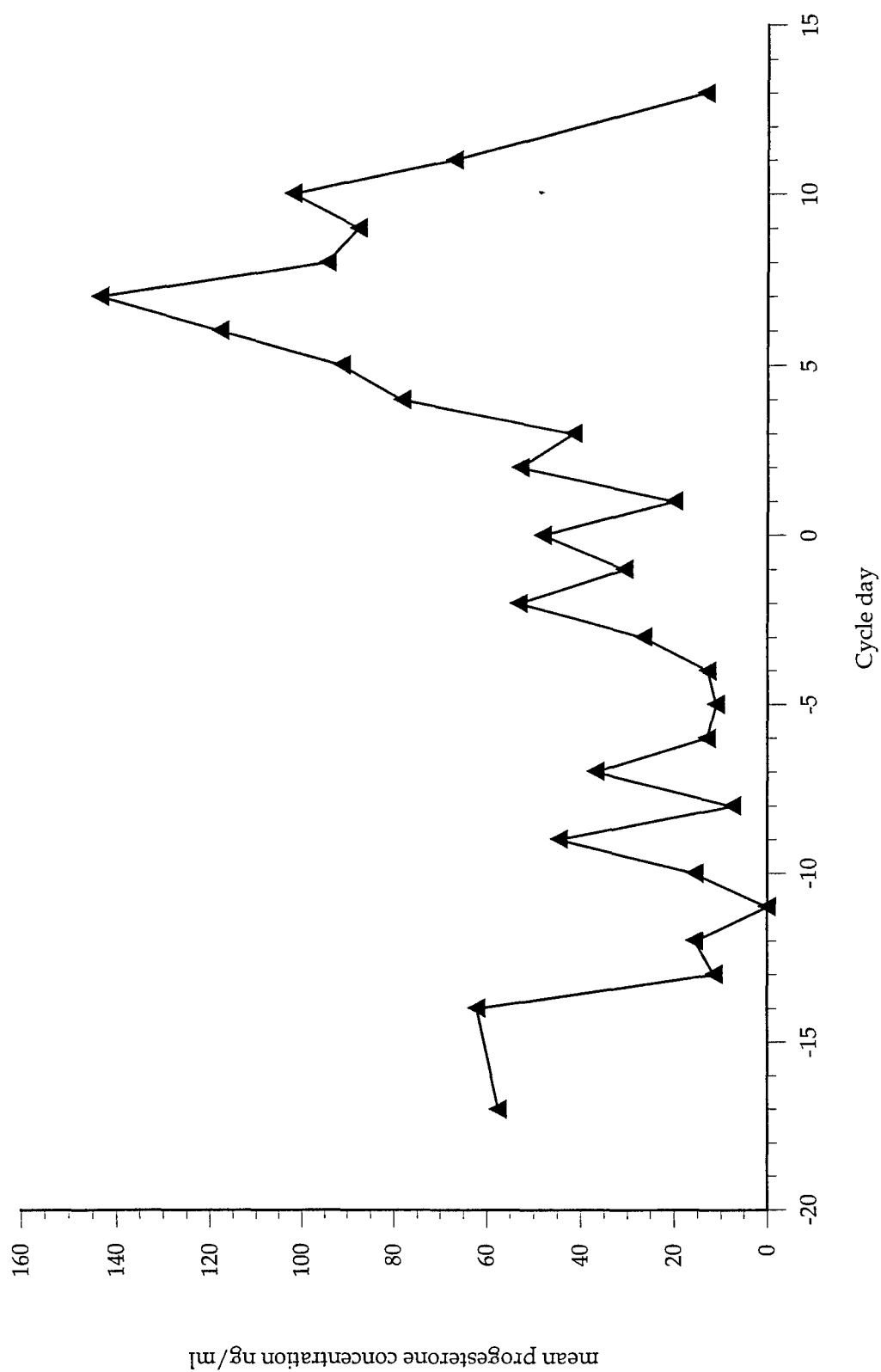


Figure 3. Chatterton laboratory salivary estradiol versus total plasma estradiol. A total of 247 samples obtained from 62 women

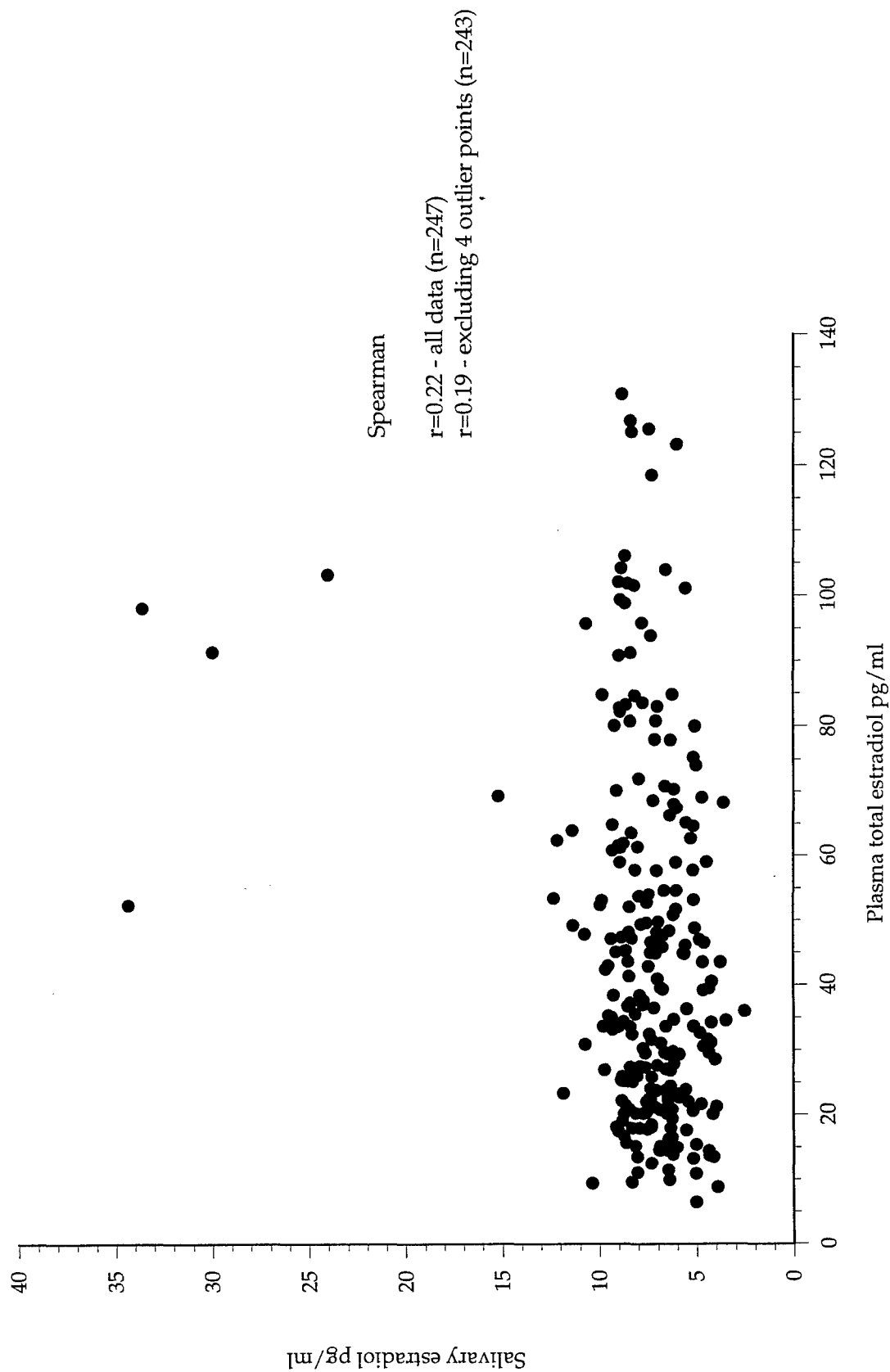


Figure 4. Salivary estradiol concentrations obtained from the Chatterton laboratory versus those obtained from the Ellison laboratory.

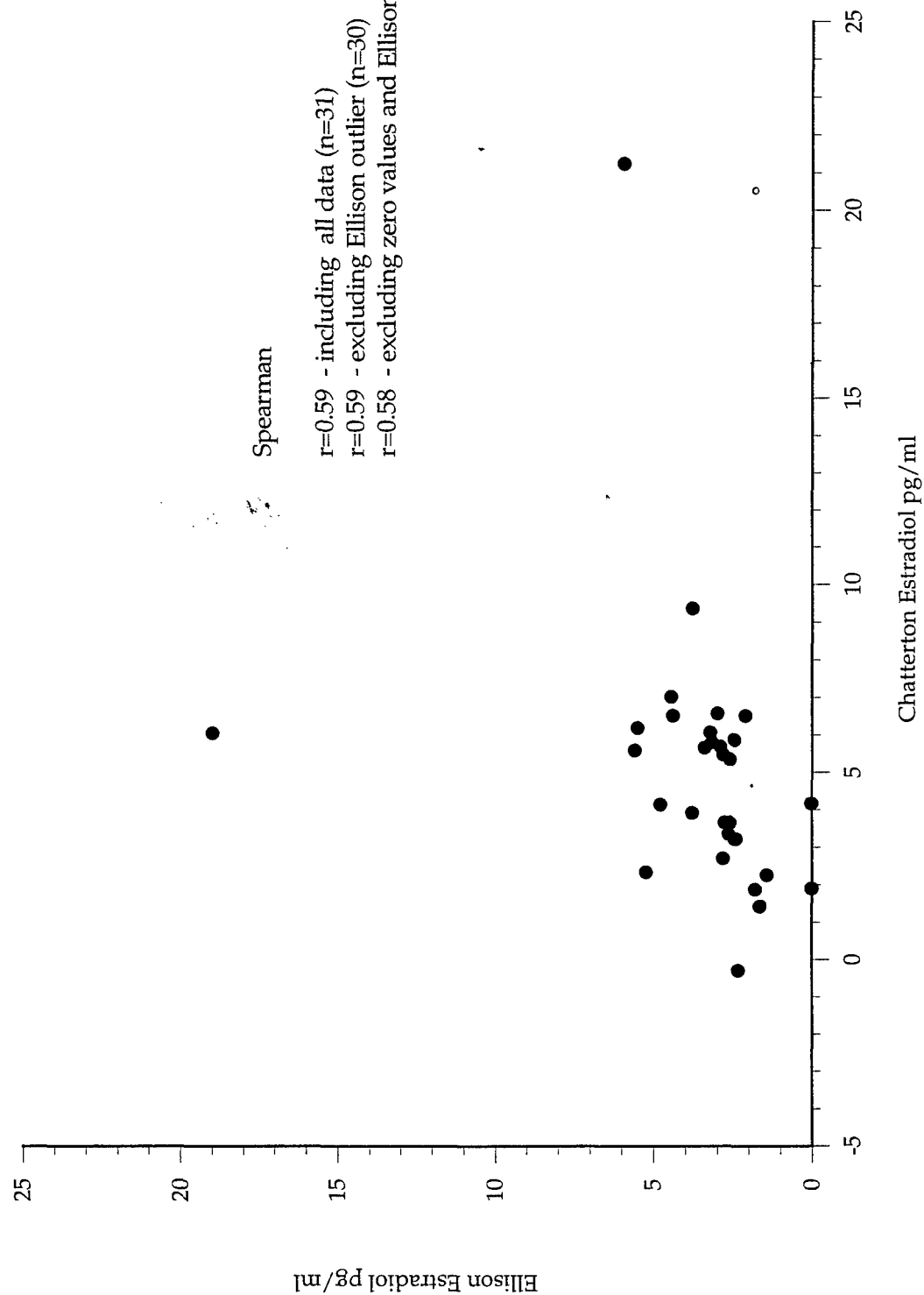


Figure 5. Salivary estradiol concentrations from the Ellison laboratory versus total plasma estradiol values from the Chatterton laboratory

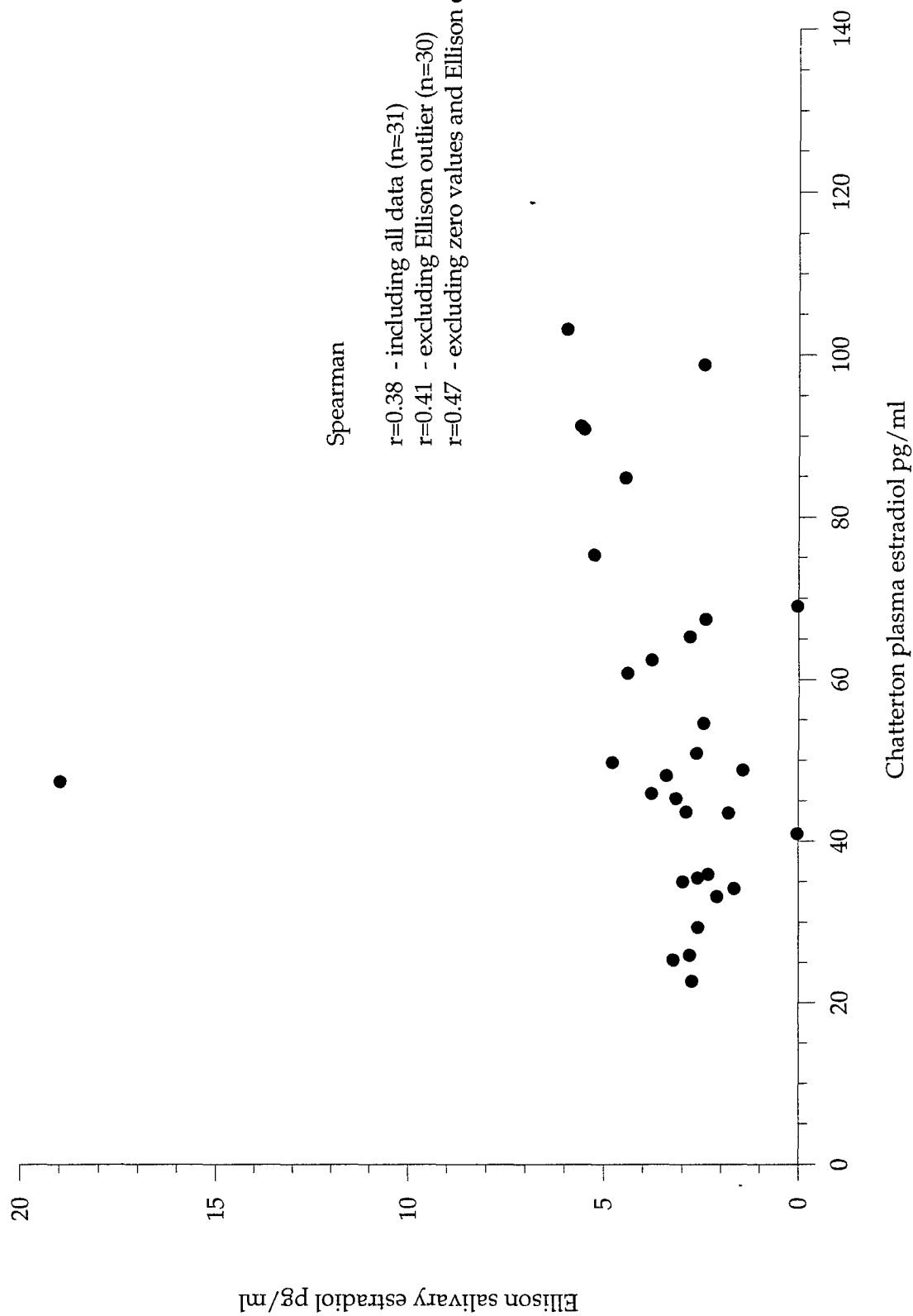
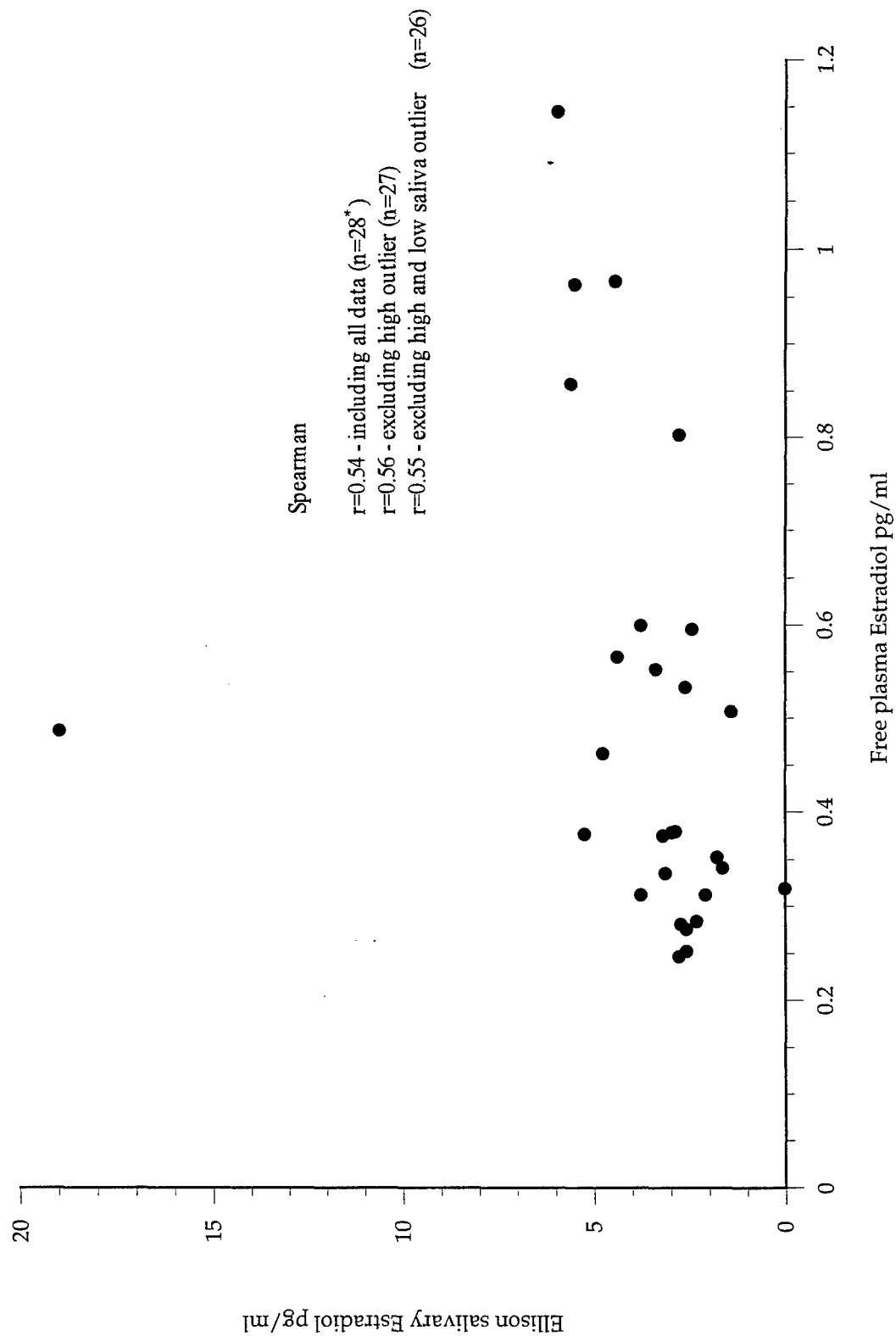


Figure 6. Ellison salivary estradiol versus Chatterton plasma free estradiol concentration



* 3 samples were inadequate for plasma free E2 analysis

Figure 7. Inter-reader correlation on lobular histology readings - Liz Wiley, Beth Bauer-Marsh and Allison Ellman

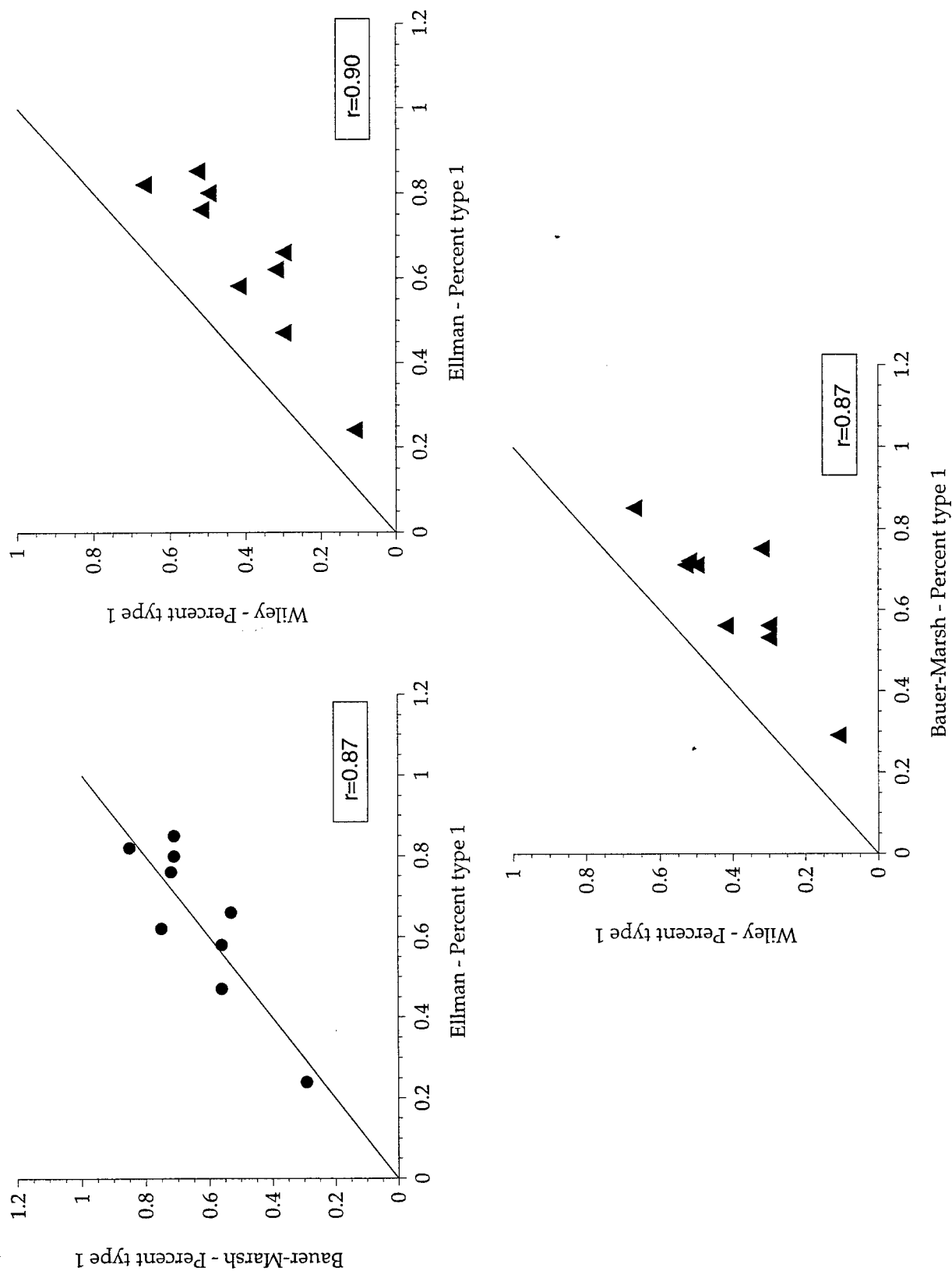
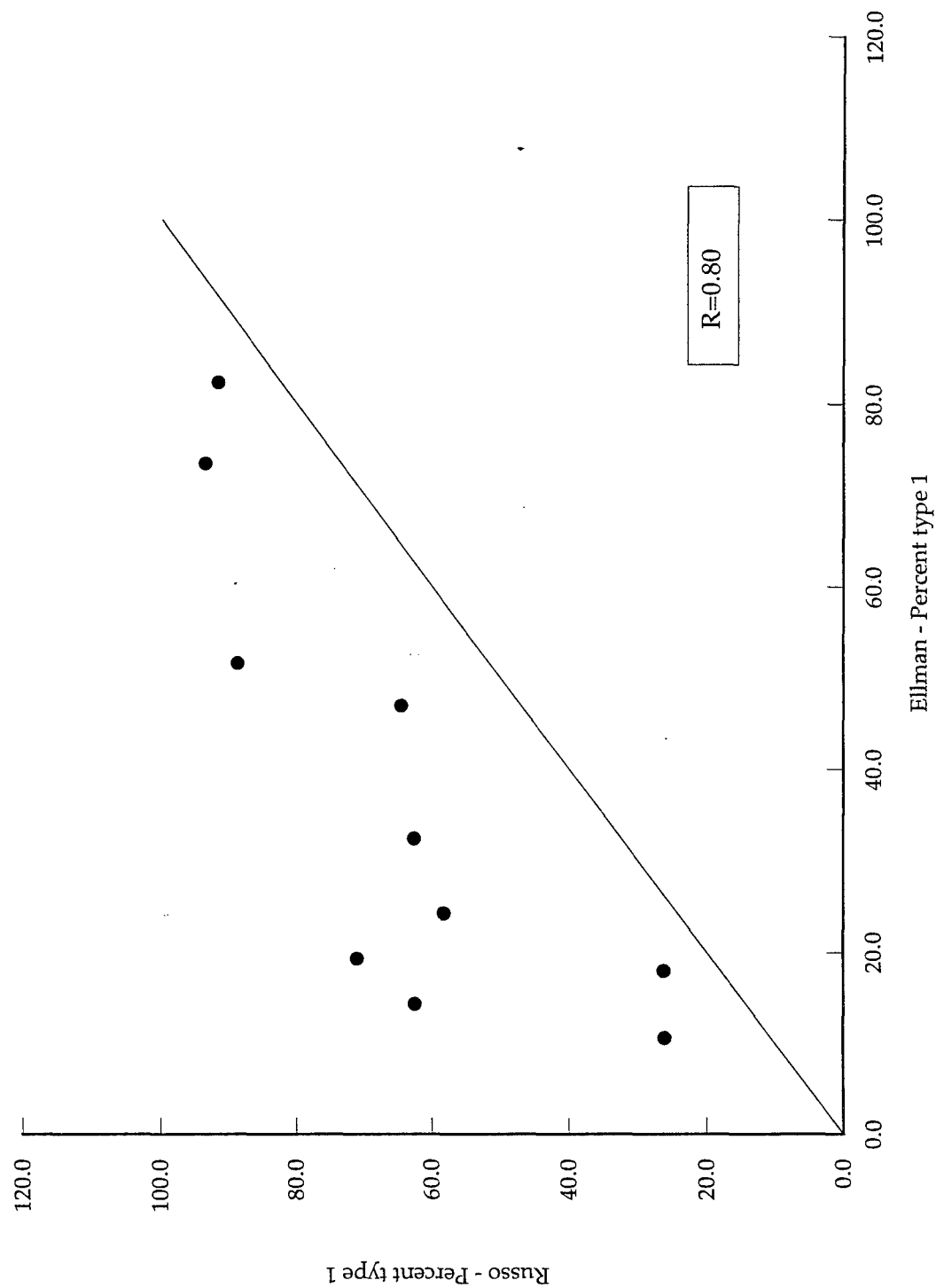


Figure 8. Inter-reader correlation: lobular differentiation of normal breast tissue from biopsy samples (percent type 1 lobules) - Russo and Ellman



APPENDIX A

DRAFT 10-15-96

**Mitogenic Growth Factors in Breast Fluid Obtained from Healthy Women:
Evaluation of Biological and Extraneous Sources of Variability**

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Funding sources: NCI KO7, USAMRMC DAMD17-94-J-4203, CRC grant, Sage Fund, Harris Memorial Fund

INTRODUCTION

The current model of breast cancer development assigns an important role to locally-acting autocrine/paracrine growth factors. Peptides such as epidermal growth factor (EGF) and transforming growth factor- α (TGF- α), which have a 30-40% amino acid homology, have a potent mitogenic effect on human breast cancer cells *in vitro*. In addition, the oncogene *erbB2* has been shown to encode a membrane receptor that binds both EGF and TGF- α . Amplification of this gene is observed in a significant proportion of breast cancers, and is an independent predictor of survival. Undoubtedly, these growth factors, whose structure is highly conserved across species, also play a role in control of normal breast cell proliferation. Current evidence indicates that estradiol, and anti-estrogens such as tamoxifen, have direct and opposite effects on production of these growth factors by epithelial or stromal cells in the breast. High levels of ovarian activity and of estrogen itself are related to increased breast cell proliferation, and indeed provide the most cogent explanation for the increased breast cancer risk attributable to diverse factors such as age at menarche, age at menopause and obesity. It is plausible, therefore, to hypothesize that healthy women with excessive production of mitogenic growth factors have an elevated risk of developing breast cancer.

Because EGF and TGF- α are produced locally and act locally, their concentrations in serum or urine are not necessarily relevant. On the other hand, nipple aspiration provides a non-invasive method for sampling fluid that is in close contact with ductal epithelial cells. Several groups of investigators have demonstrated that a small volume of breast fluid can be obtained from 40-70% of non-lactating women by using a simple pump-like device. Based on numerous biochemical analyses, this fluid appears to provide reasonable insight into the metabolic microenvironment of the breast. One published report describes EGF and TGF- α concentrations in breast fluid from 17 women. Two other studies report on these or similar growth factors in breast cyst fluid, although the comparability of cyst fluid to nipple aspirate fluid is questionable.

The studies described in this report were designed to address basic methodological questions concerning the assay of EGF and TGF- α in breast fluid. We evaluated the sensitivity and reproducibility of these assays, and the effect of specimen handling and storage. We then explored the variation in levels within women between breasts and within the same breast over time, comparing these within-woman variations to the amount of variation seen between women. For a biomarker to be useful in clinical or epidemiologic research, it is critical that there be a substantial amount of variation between individuals relative to the variation within individuals. Finally, we determined whether breast fluid EGF and TGF- α levels were related to the menstrual cycle phase, to plasma estradiol or progesterone levels, or to each other. By repeat sampling of individual women, we were able to assess these relationships within individual women, as well as in the group as a whole.

METHODS

Study population and sample collection

Following approval of the protocol and informed consent procedures by the Institutional

Review Board, we recruited women from the Chicago area to participate in the Repeat Sample Study. Criteria for eligibility included: age 25-45 years, no history of breast cancer, regular menstrual periods, no lactation within 6 months, no use of oral contraceptives or other exogenous hormones within 6 months, and no major concurrent illnesses. Sixty-five eligible women were scheduled for four outpatient appointments each, one week apart, to the Clinical Research Center (CRC) at Northwestern Memorial Hospital. Participants were allowed to start their visits during any day of the menstrual cycle, and arrived at the CRC in the morning after an overnight fast. The position of each visit day in the menstrual cycle was determined by recording the dates of onset of all menstrual bleeding immediately prior to and after the four visits. This allowed cycle position to be estimated by reverse-dating, in which the midcycle day is defined as the first day of bleeding minus 14 days, the average length of the luteal phase. At each visit, we collected plasma and breast fluid. In addition, we measured body size and fat composition, physical activity and dietary intake, for analyses not presented here.

Breast fluid collection

At each visit, a trained nurse attempted to aspirate breast fluid from both breasts of each participant. After lightly scrubbing the nipple with a water-moistened gauze pad, the nurse asked the participant to compress the breast at its base with both hands. A sterile suction device made from a 20 cc plastic syringe body was then applied over the nipple and vacuum pressure gradually applied (ref device). Suction was discontinued if fluid failed to appear at the nipple surface after 10 seconds. Droplets of breast fluid appearing at the duct openings were collected in 75mm plastic-coated capillary tubes that were then clay-sealed at both ends and kept on ice until storage at -70°C , no more than one hour after collection.

Assays for plasma estradiol, bioavailable estradiol and progesterone

Assays for breast fluid EGF, TGF- α and protein

Breast fluid was removed from the capillary tube while in a semi-frozen state, and, except when undiluted sample was needed for sensitivity studies, diluted with _____ prior to assay. We used competitive-binding radioimmunoassay kits purchased from BioMedical Technologies (Stoughton, MA) for both EGF and TGF- α . The EGF assay has a reported cross-reactivity of less than _____ with all related peptides tested, including _____ % cross-reactivity with TGF- α . The TGF- α assay has cross-reactivities of less than _____ %, and a cross-reactivity of _____ % for EGF. To evaluate assay sensitivity, dilutions ranging from undiluted to 1:200 were prepared. Intra-assay CV at each dilution was evaluated to determine the dilution level at which assay reliability became unacceptable. Inter-assay CV was evaluated by repeat testing of the same pooled sample in different assay runs. Total protein in breast fluid was measured by the Bradford method.

Data analysis

We calculated coefficients of variation (CV) and intraclass correlation coefficients (ICC) to assess intra- and inter-assay variability and the amount of variation within versus between individuals. The ICC is defined as the between-person variance divided by the total variance (between plus within). To determine which method minimized extraneous variation, we compared within-person CVs for GF concentrations expressed per unit breast fluid volume to

those expressed per weight of total protein. To compare right versus left breast results, and to compare EGF versus TGF- α or either growth factor versus plasma hormone levels, we computed the non-parametric Spearman correlation coefficients (r). These coefficients are unbiased, but because there were multiple measurements from the same person, the conventional variance of the coefficient estimates was too low. To obtain correct variance estimates and compute exact P values for r , we used a permutation method, which generates the actual distribution of r under the null hypothesis by evaluating all combinations in the dataset (ref Josee).

Growth factor and hormone concentrations were not normally distributed. To facilitate parametric analyses, we evaluated several data transformations and concluded that the square-root transformation provided the best normalization for the key variables as a group. Therefore, to compute mean GF concentration plus estimated 95% confidence intervals for each phase of the menstrual cycle, we obtained standard errors and confidence intervals from the transformed data and then converted back to the original units for reporting purposes. The menstrual cycle was divided into the following six phases, with 0 being the mid-cycle day: early (day -), mid- (days xx-xx), and late (days xx-xx) follicular; and early (days xx-xx), mid- (days xx-xx) and late (days xx-xx) luteal. P values for comparison of GF levels by cycle phase were obtained by random effects modelling using PROC GLM in SAS (ref). We also used random effects models, with EGF as the dependent variable, to determine the degree of linear association between EGF and TGF- α and plasma estradiol. An interaction term consisting of a binary dummy variable for each subject multiplied by the predictor level (TGF- α or plasma estradiol) allowed us to evaluate the biomarker associations within individuals. Similar models were developed with TGF- α as the dependent variable and EGF and plasma estradiol as the predictors. From these models we obtained estimates of the total variance and within-woman variance explained by each model term.

RESULTS

Table 1 shows results indicating the sensitivity and reliability of the EGF and TGF- α measurements in breast fluid. For EGF, analysis of samples diluted 1:100 with assay buffer gave acceptable intra-assay reliability. Thus, we were able to obtain reliable results using only 1 μ l of breast fluid, which contained concentrations in the range of 4-6 ng/ml in diluted samples from various quality control pools. For TGF- α , both intra-assay and inter-assay reliability were acceptable at dilutions of 1:25, but not at 1:50. We were therefore able to reliably measure TGF- α in only 4 μ l of breast fluid, with measured concentrations in pooled, diluted samples of about 0.1 ng/ml. EGF and TGF- α concentrations in pools prepared from women with abundant versus scant volumes of breast fluid were indistinguishable. In one experiment, the number of freeze-thaw cycles (ranging from two to six) was not associated with any trends in measured GF concentrations.

Mean growth factor levels and results on the variation between the right and left breast are shown in Table 2, part A. For EGF, the within-woman variation (between breasts) was considerable less than the variation in EGF levels between women. The intraclass correlation coefficient (ICC) implies that 48% of the total variance in EGF could be attributed to between-woman differences. For TGF- α , the within-woman variation between breasts was even lower,

and the ratio of between to within-woman variation was even higher. Eighty-eight percent of the total variance in TGF- α was attributable to between-woman differences. Table 2A also shows that expressing growth factor levels per weight of total protein rather than per unit volume did not improve, and in fact substantially reduced, the level of agreement between breasts. Total breast fluid protein levels were correlated between breasts (data not shown).

Figure 1 displays the agreement in EGF and TGF- α levels between breasts for individual women. Right and left breast levels were well-correlated; $r = 0.78$, $P = 0.003$ for EGF, and $r = 0.89$, $P = 0.001$ for TGF- α . Right versus left comparisons from our previous, larger study populations gave the same high level of correlation between breasts for both growth factors.

Table 2, part B shows the variation in growth factor levels between and within-women in the same breast over time. Once again, the variation between women was far greater than the variation within individual women over time, for both growth factors. The range of breast fluid EGF concentrations between women was extremely high (over 100-fold differences) and thus, the between-women variance for EGF was by far the dominant component of total variance ($ICC = 0.83$). Variances over time were not reduced by expressing results per weight of total protein rather than per volume. Figure 2 shows the EGF (part A) and TGF- α (part B) results for each woman over time. This graph provides visual evidence that women tend to have highly distinct levels of EGF that are relatively consistent over time. TGF- α levels for individual women (Figure 2B) also tend to remain stable over time, although the decreased variation between women, compared to EGF, is evident.

To more closely examine whether growth factor levels in breast fluid vary in conjunction with the menstrual cycle, we plotted the mean (square-root transformed) EGF and TGF- α concentrations for six cycle phases, as shown in Figures 3 and 4. We found no significant differences for either growth factor across cycle phases ($P = 0.23$ and 0.32 , respectively, based on a random effects model accounting for repeated measures). For EGF, there is the appearance of an increase during the luteal phase, but direct comparison of, for example, mid-luteal versus early or mid-follicular EGF indicated that the differences were highly compatible with chance ($P = 0.61$).

In Figure 5, EGF (part A) and TGF- α (part B) levels are plotted against concurrent total plasma estradiol levels. Neither growth factor was meaningfully correlated with plasma estradiol ($r = 0.15$ for EGF and $r = 0.02$ for TGF- α by the permutation method). We obtained similar results using plasma bioavailable estradiol (non-SHBG bound) instead of total estradiol.

The results shown in Figure 6 indicate that, when all samples from all women were considered, EGF and TGF- α concentrations in the same sample were not well-correlated ($r = 0.17$, $P = 0.50$). However, when we examined the EGF and TGF- α relationship for individual women, some striking co-variation was apparent. Table 3, part A, shows results from a random effects model that evaluates the EGF-TGF- α association within women. This model includes a universal coefficient reflecting the common relationship of EGF to TGF- α as well as a term reflecting the relationship for each individual woman. The universal coefficient was very small, consistent with the low r seen in Figure 6. However, the association within individual women was statistically significant ($P = 0.02$) and explained nearly 56% of the variance in EGF within women. The high percentage of variance explained (93.5%) by simply

specifying the individual subject corroborates the large amount of variation for EGF between women that was seen in the earlier analysis.

Table 3, part B shows a similar random effects analysis for the relationship of plasma estradiol to EGF. Again, although the overall correlation between EGF and estradiol was poor, the results indicate a significant correlation within individual women. Fifty-eight percent of the within-woman variance in EGF was explained by the plasma estradiol values. The within-woman association between breast fluid TGF- α and plasma estradiol was not substantial. Figure 8 includes graphs of selected participants showing strong co-variation between EGF and TGF- α levels (part A) and co-variation between EGF and plasma estradiol (part B).

DISCUSSION

Peptide growth factors such as EGF and TGF- α are potent signalling molecules for regulating the growth and perhaps differentiation of breast epithelial cells. Abnormal expression or activity of these factors could result from mutations of proto-oncogenes transcribing the growth factors themselves or their receptors. Alternatively, since these growth factors have a role in normal growth and therefore must be regulatable by endogenous signals, abnormal expression could occur as a result of up- or down-regulation of gene transcription by compounds such as steroid hormones. The data in this report, while preliminary to the study of any relationships *in vivo* between breast cancer and growth factor expression, indicate that immunoreactive EGF and TGF- α can both be detected reliably in breast fluid from healthy premenopausal women, and that individual women secrete distinctive amounts of these factors, amounts that are consistent both over time and between breasts. This study also provides evidence that levels of breast fluid EGF co-vary over time with TGF- α and plasma estradiol, within individual women.

One group of investigators has previously reported detection of EGF and TGF- α in breast fluid. The mean concentrations of both GFs were/not similar to ours xx They found EGF levels to be higher in women with benign breast disease than in controls xx Although the previously published results probably required higher volumes of breast fluid for analysis, we found no difference in GF concentrations between women with abundant versus scanty breast fluid volume. We utilized highly sensitive radioimmunoassays that require only 1 μ l and 4 μ l of breast fluid for EGF and TGF- α , respectively, and therefore make it possible to obtain measurements even on women with scanty breast fluid samples. In our hands, the average volume of breast fluid obtained is approximately 30 μ l, but the frequency distribution for sample volume is highly skewed, and many women have samples under 10 μ l. Highly sensitive assay methods are therefore important. We found no evidence for an effect of thaw-freeze cycles on GF concentrations, nor any evidence for a decay in measured growth factor during 18 months of storage at temperatures up to -20xx C.

Several aspects of our findings require further elaboration. In looking at variation over time, we found that between- versus within-woman variation was greater for EGF than TGF- α , largely because of a much greater difference between women for EGF. On the other hand,

TGF- α variation between the right and left breasts was very low ($r = 0.89$, $CV = 0.11$), so that the between-versus within-woman variation was greater for TGF- α than for EGF, even though the differences between women were once again greater for EGF. Taken together, these findings could suggest that breast fluid levels of TGF- α , the more potent of the two growth factors, are regulated within more narrow ranges than EGF in healthy women. More data are needed on this question.

The lack of a correlation between EGF and TGF- α or plasma estradiol concentrations when samples from many women are considered, and the presence of significant correlations *within* individual women can be puzzling at first. However, this type of result arises if the quantitative relationship of EGF to TGF- α and estradiol varies from one woman to another, while the levels of breast fluid EGF within an individual woman tend to change in proportion to changes in TGF- α and plasma estradiol. We conclude that these data provide preliminary evidence that breast fluid EGF and TGF- α are co-regulated *in vivo*, and that, in the case of EGF, regulation could involve circulating levels of estradiol. Other compounds, including other steroid hormones related to estradiol, could be involved in regulating TGF- α levels.

One limitation of these studies is that we were able to obtain breast fluid on only 60% of the women on whom nipple aspiration was attempted. Factors related to success in obtaining breast fluid have been studied extensively by Petrakis, et al., and appear to include age, parity, lactation history, Asian ethnicity, and cerumen type. Although we cannot rule out the possibility that our findings would not apply to the non-secretors had breast fluid been available from them, we find that argument to be implausible. The distinctions between secretors and non-secretors are more likely to involve differences in the volume of breast fluid secretion and/or the physical consistency of material that normally plugs the nipple ducts. Another limitation is that we have not yet confirmed the precise immunoreactive species in each radioimmunoassay. The kit manufacturer's testing indicates only minimal cross-reactivity for the antibodies used in the EGF and TGF- α assays; however, these cross-reactivities were determined in media other than breast fluid. We are currently conducting Western blot analyses to identify the immunoreactive species by molecular weight.

From these results, it appears that breast fluid EGF and TGF- α could eventually serve as useful biomarkers in studies of breast cancer etiology. Our next series of studies will examine whether growth factor concentrations are related to hyperproliferative states in the normal breast, to known or suspected breast cancer risk factors, and to the occurrence of breast cancer itself. In the long-term, establishment of altered local growth factor secretion as a link in the causal pathway of breast cancer development could allow breast fluid GF to play a role as an intermediate endpoint in the evaluation of interventions designed to reduce breast cancer risk.

Table 1. Intra-assay and inter-assay variability for replicate samples (including quality control pools and individual subject samples) assayed at various dilutions: EGF and TGF- α in breast fluid

	Dilution	Mean Intra-assay CV (# replicate pairs)	Mean Inter-assay CV (# replicate pairs)
EGF			
	1:50	12.2 % (26)	4.6% (6)
	1:75	4.3% (2)	-
	1:100	12.9% (36)	-
TGF- α			
	1:25	10.8% (59)	11.2% (4)
	1:50	24.9% (11)	-

Table 2. Breast fluid growth factor levels: variation between and within Repeat Sample Study participants

A. Left vs. right breast, same day						
	# subjects	# samples	mean	CV ^a between	CV within	ICC ^a
EGF	12	24	604 ng/ml	0.82	0.47	0.48
TGF	13	26	2.26 ng/ml	0.46	0.11	0.88
EGF/protein	12	24	7.11 ng/mg	0.74	0.65	0.08
TGF/protein	13	26	39.1 pg/mg	1.49	1.16	0.21
B. Same breast, different days						
EGF	15	57 ^b	494 ng/ml	1.2	0.26	0.83
TGF	15	58	2.68 ng/ml	0.78	0.42	0.37
EGF/protein	15	57	6.87 ng/mg	1.61	1.05	0.24
TGF/protein	15	58	73.2pg/mg	3.81	2.58	0.21

^a Abbreviations used: CV = coefficient of variation, ICC = intraclass correlation coefficient

^b One sample was not included in analysis of EGF levels due to a laboratory error.

Table 3. Random effects models evaluating the associations within individual women between breast fluid EGF and TGF- α (Model A) and breast fluid EGF and total plasma estradiol (Model B)

Model A: dependent variable, EGF

Independent variable	Sum of squares	% variance explained	% within-woman variance explained	P value
individual subject	3808.17	93.49	-	-
TGF- α	0.12	0.003	0.05	0.99
subject*TGF- α	148.0	3.63	55.82	0.02
error	116.87	2.87	-	-

Model B: dependent variable, EGF

Independent variable	Sum of squares	% variance explained	% within-woman variance explained	P value
individual subject	3808.17	93.49	-	-
plasma estradiol	2.39	0.06	0.92	0.65
subject*plasma estradiol	154.66	3.80	58.37	0.01
error	107.95	2.65	-	-

Figure 1. Scatterplots showing A. EGF, and B. TGF- α , concentrations in breast fluid from the right vs. left breast. Both breasts were sampled at the same time.

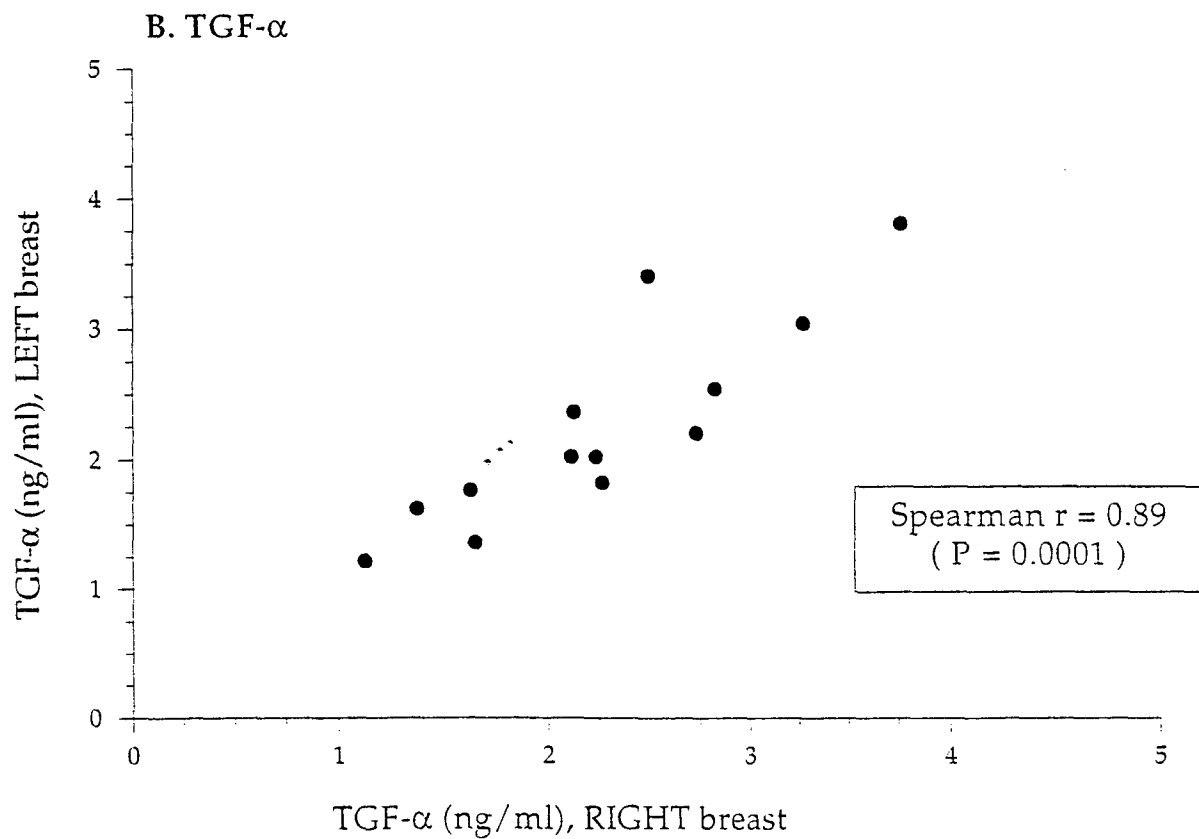
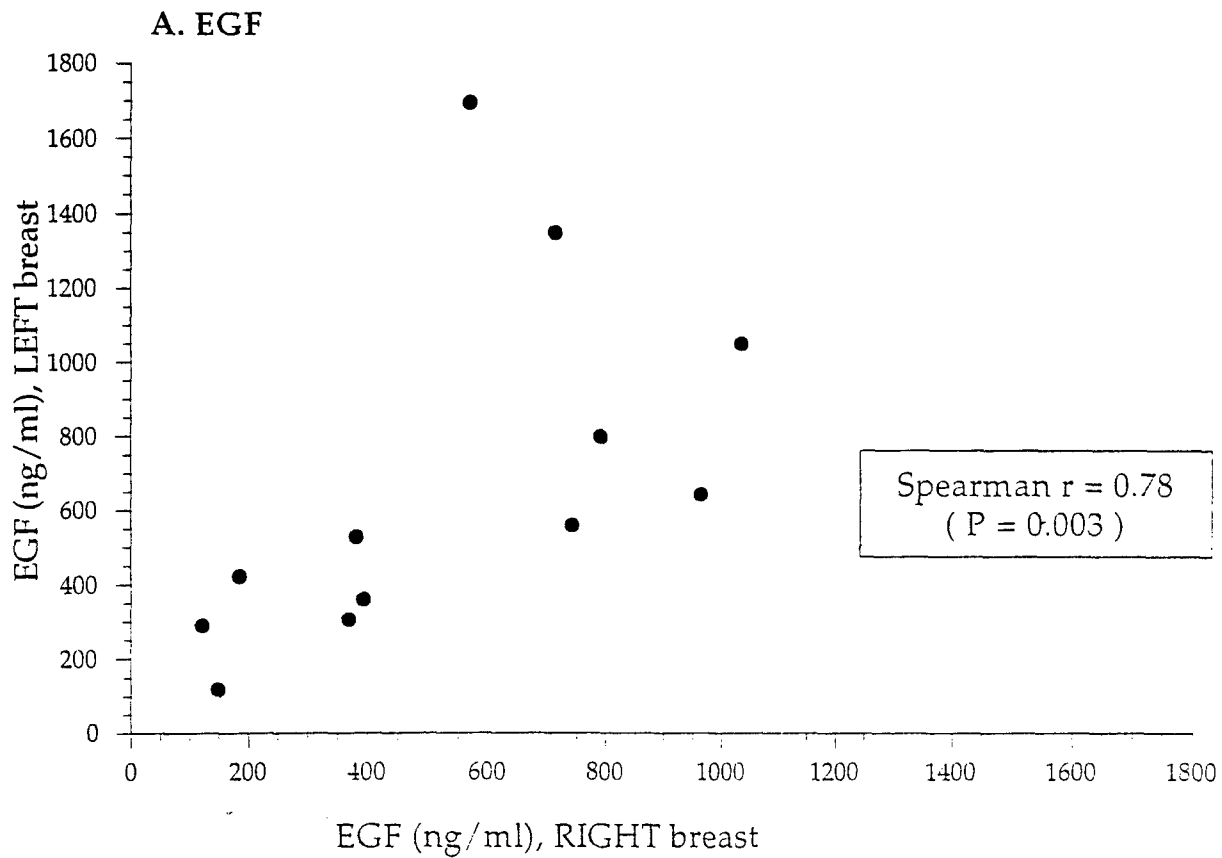


Figure 2. EGF (part A) and TGF- α (part B) concentrations in breast fluid across the menstrual cycle: repeated measures from 15 women.

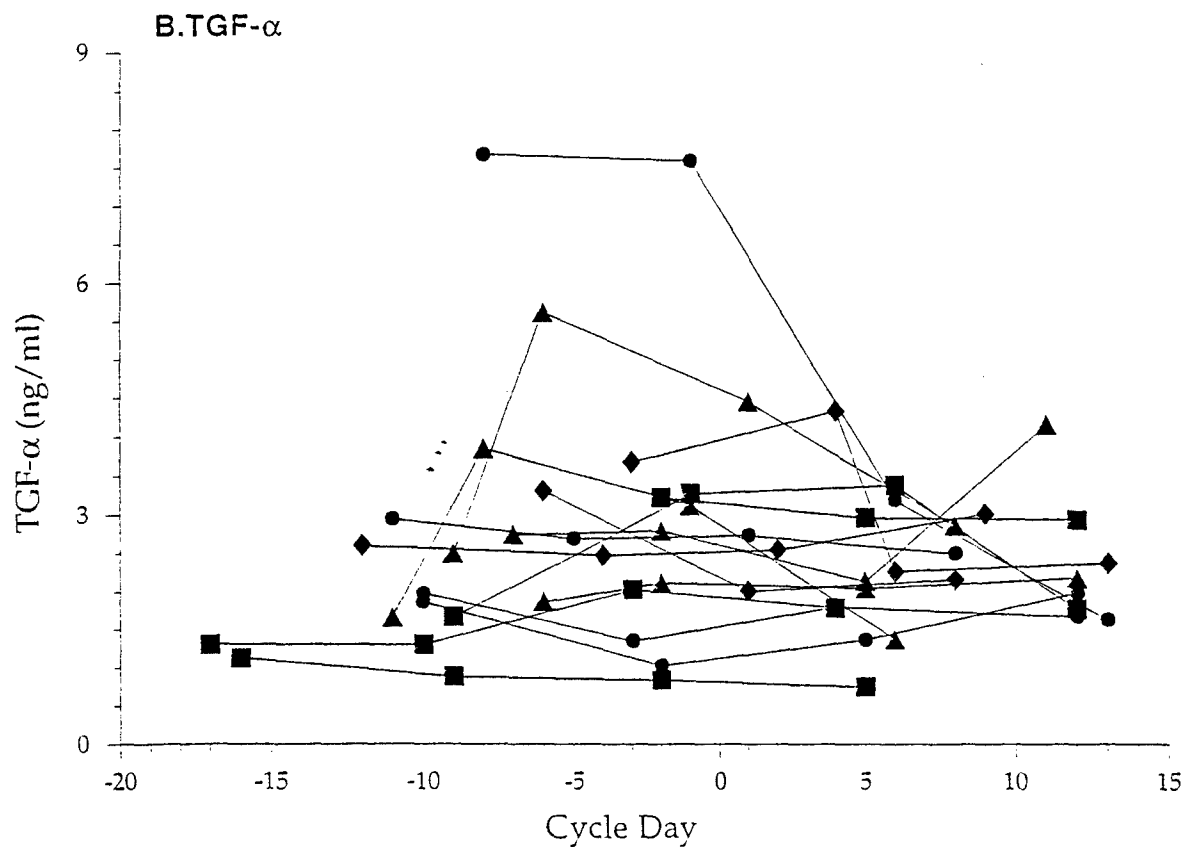
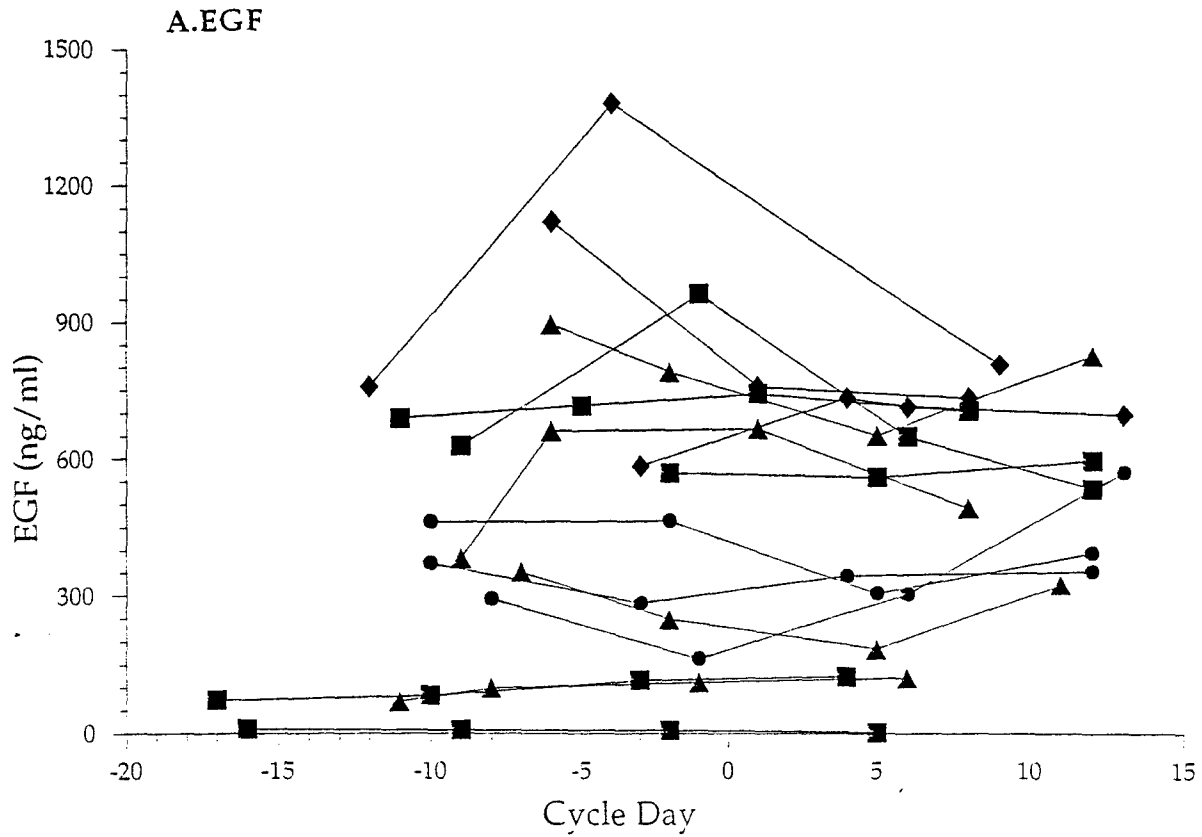


Figure 3. Mean concentration (plus 95% confidence intervals) of EGF in breast fluid obtained during six phases of the menstrual cycle.

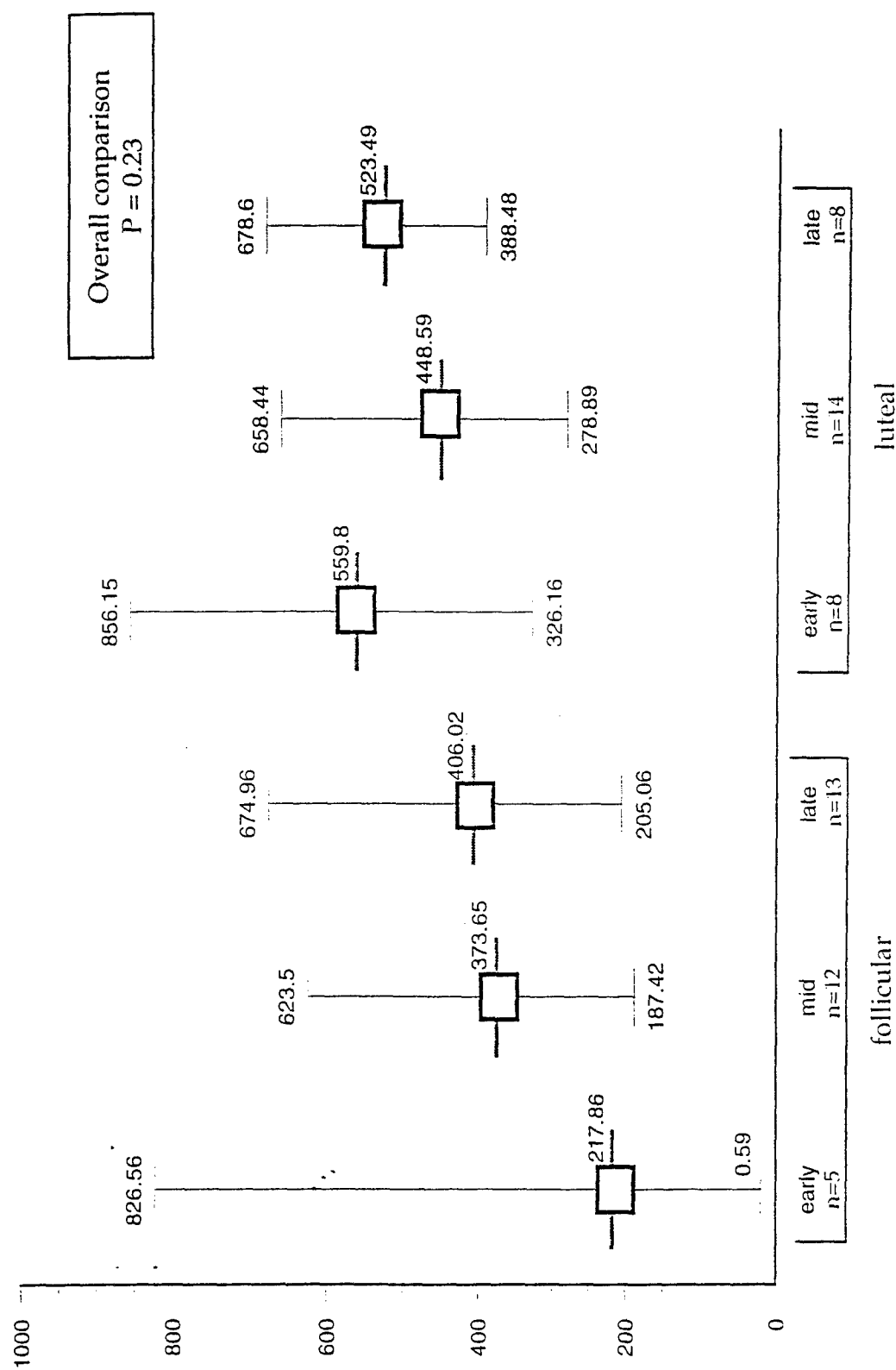


Figure 4. Mean concentration (plus 95% confidence intervals) of TGF- α in breast fluid obtained during six phases of the menstrual cycle.

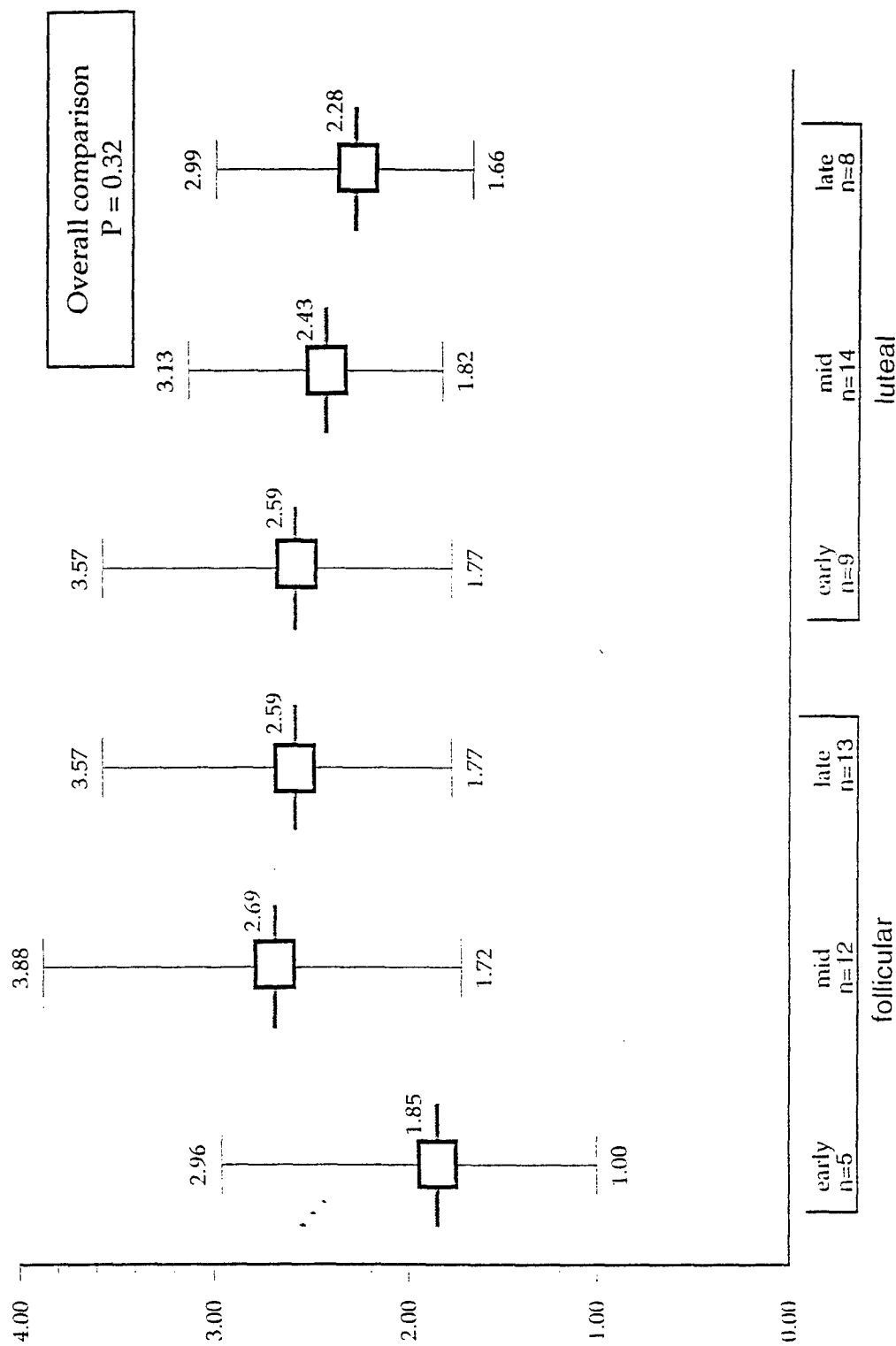


Figure 5. Plasma estradiol versus breast fluid EGF (A) and TGF- α (B), contemporaneous samples from 18 women. Correlation coefficient and P value determined by a permutation test to account for multiple samples per woman. Total samples=60 for EGF, 61 for TGF- α

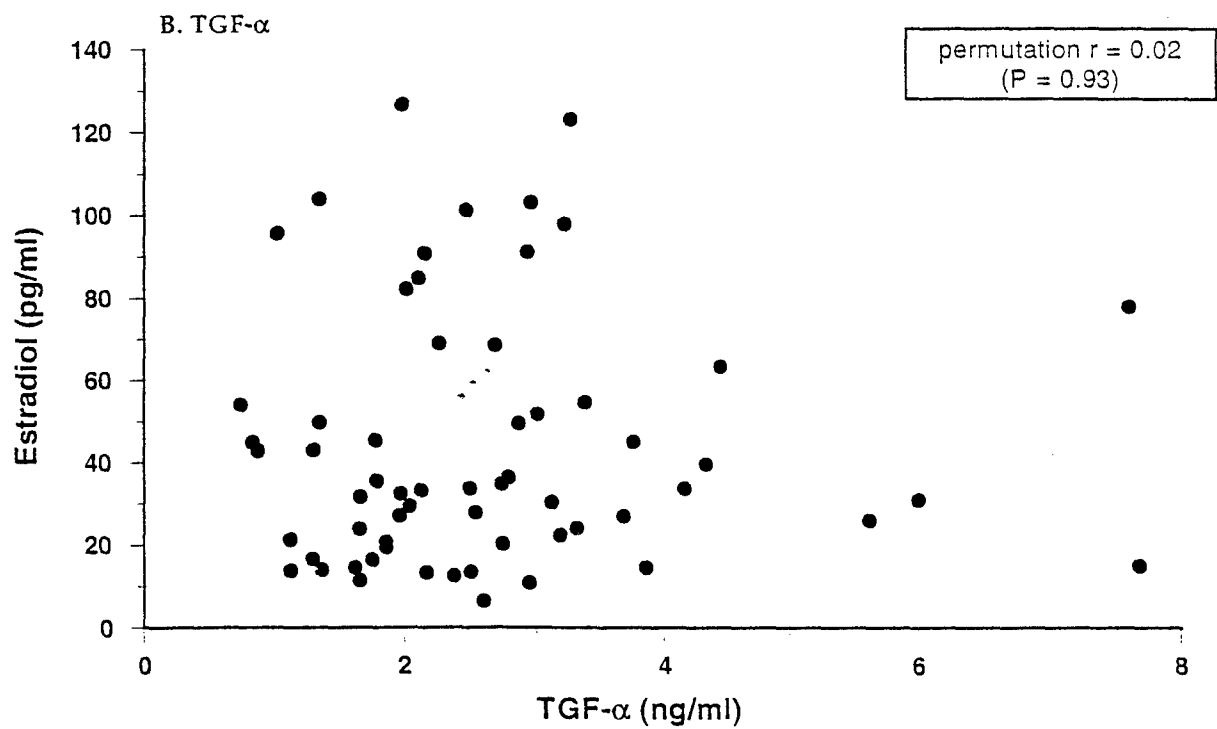
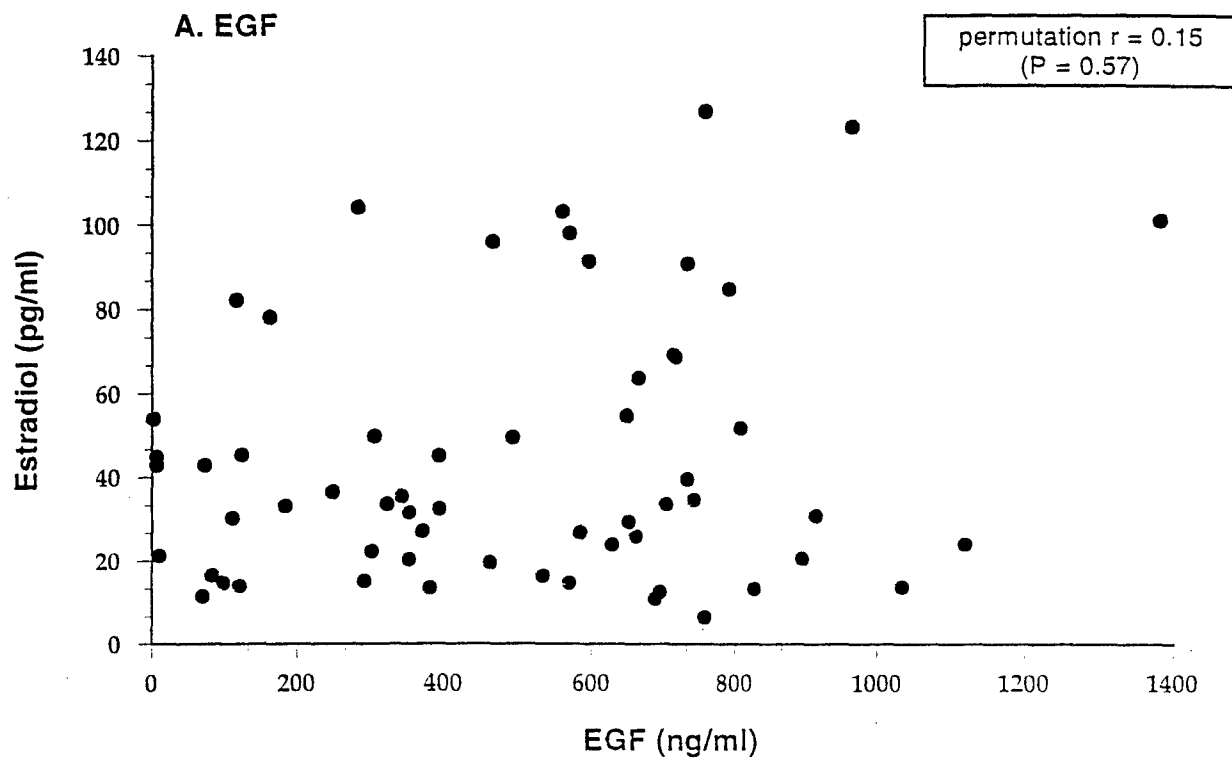


Figure 6. Scatterplot of EGF and TGF- α concentrations measured in the same breast fluid sample. The total number of samples is 60 obtained from 18 women

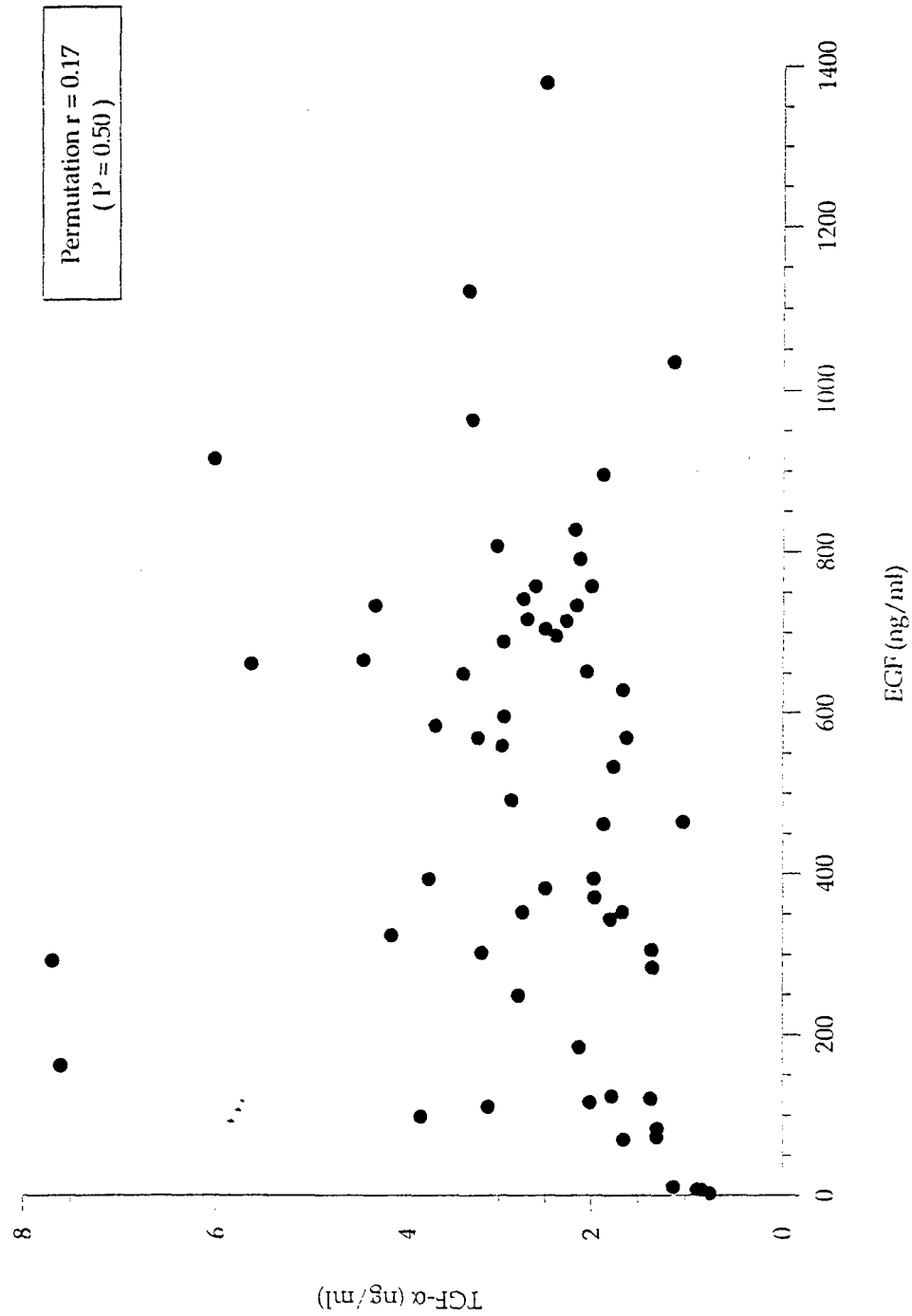
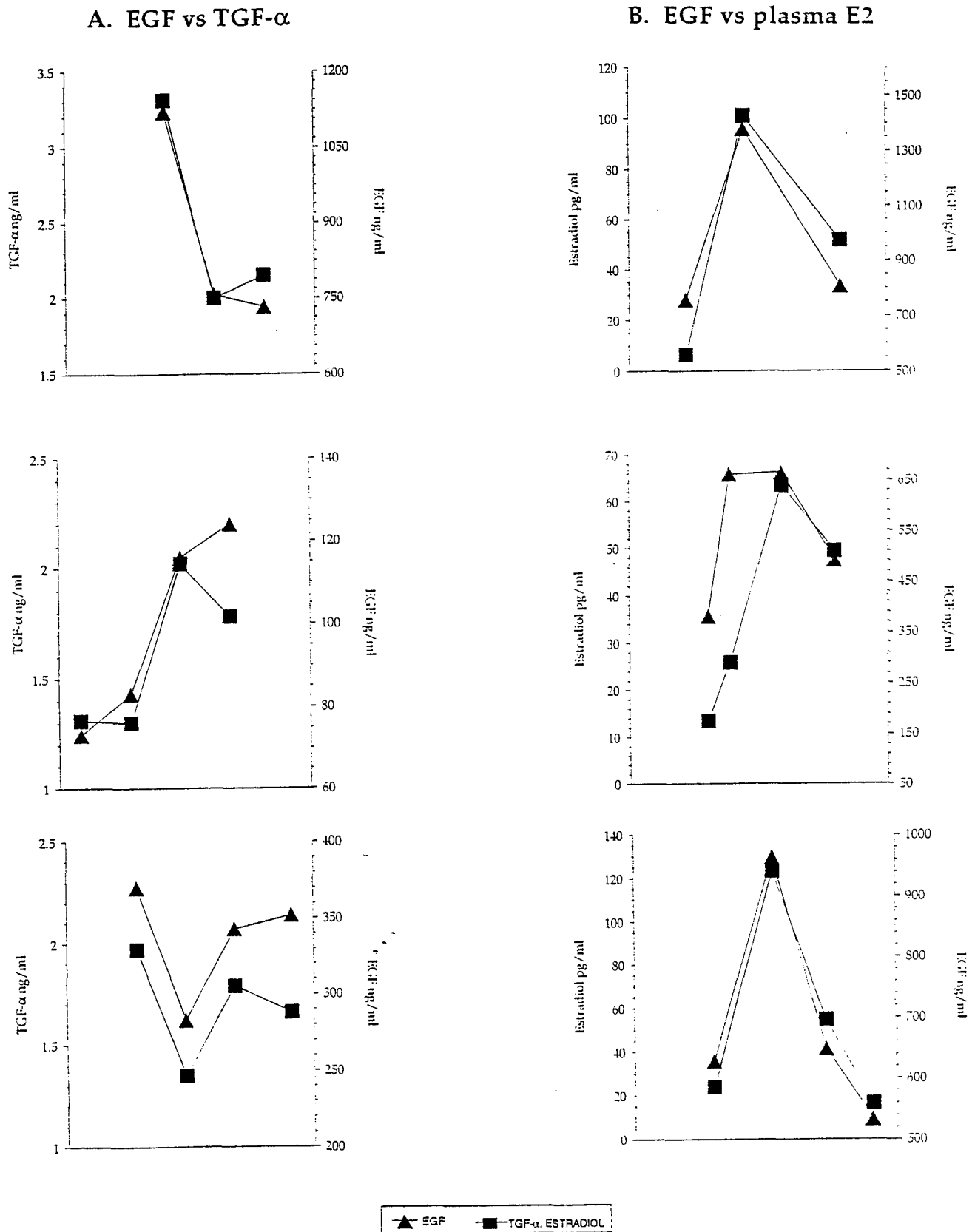


Figure 7. Scatterplots of repeat measure A. breast fluid EGF vs TGF- α and B. breast fluid EGF versus plasma estradiol across time for selected individuals





DEPARTMENT OF THE ARMY
US ARMY MEDICAL RESEARCH AND MATERIEL COMMAND
504 SCOTT STREET
FORT DETRICK, MARYLAND 21702-5012

REPLY TO
ATTENTION OF:

MCMR-RMI-S (70-1y)

1 JUN 2001

MEMORANDUM FOR Administrator, Defense Technical Information
Center (DTIC-OCA), 8725 John J. Kingman Road, Fort Belvoir,
VA 22060-6218

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1. The U.S. Army Medical Research and Materiel Command has reexamined the need for the limitation assigned to technical reports. Request the limited distribution statement for reports on the enclosed list be changed to "Approved for public release; distribution unlimited." These reports should be released to the National Technical Information Service.

2. Point of contact for this request is Ms. Judy Pawlus at DSN 343-7322 or by e-mail at judy.pawlus@det.amedd.army.mil.

FOR THE COMMANDER:

Encl

PHYLLIS M. RINEHART
Deputy Chief of Staff for
Information Management

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DAMD17-94-J-4499	ADB247447
DAMD17-94-J-4499	ADB258779
DAMD17-94-J-4437	ADB258772
DAMD17-94-J-4437	ADB249591
DAMD17-94-J-4437	ADB233377
DAMD17-94-J-4437	ADB221789
DAMD17-96-1-6092	ADB231798
DAMD17-96-1-6092	ADB239339
DAMD17-96-1-6092	ADB253632
DAMD17-96-1-6092	ADB261420
DAMD17-95-C-5078	ADB232058
DAMD17-95-C-5078	ADB232057
DAMD17-95-C-5078	ADB242387
DAMD17-95-C-5078	ADB253038
DAMD17-95-C-5078	ADB261561
DAMD17-94-J-4433	ADB221274
DAMD17-94-J-4433	ADB236087
DAMD17-94-J-4433	ADB254499
DAMD17-94-J-4413	ADB232293
DAMD17-94-J-4413	ADB240900