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<b>13. ABSTRACT (Maximum 200)</b> In Project 1 (developing biomarkers for growth factors and key steroids in breast fluid) we have published an initial paper characterizing the levels of EGF and TGF- $\alpha$ in breast fluid within and between individuals. We have also begun analysis of samples in the Mammographic Density Study, which focuses on the relation of breast fluid growth factor levels to breast tissue density, an independent risk factor for breast cancer. Results thus far indicate that mammographic density might be associated with EGF levels. We have halted development of the fluoroimmunoassay for salivary estradiol in favor of a new ultrasensitive radioimmunoassay. The new assay appears promising. In Project 2 (lobular differentiation in normal breast tissue), we have finalized our protocol for reading lobule type, resulting in improved inter-reader agreement. In addition, we have established homogeneity of lobule type across breast regions within an individual, and have completed preliminary analysis of our case-control study. This study thus far shows a shift towards immature lobule type in women with breast cancer, particularly among those who are parous. The data also suggest that nulliparity is associated with immature lobule type. We are accruing more data; this will become the largest analysis of these hypotheses to date in humans.			
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FOREWORD

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Annual Report: Grant DAMD17-94-J-4203  
September 1, 1996 - August 31, 1997

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

## 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), TGF- $\alpha$  and TGF- $\beta$ , that are presumed to play a major role in controlling breast cell proliferation and differentiation<sup>1</sup>. 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- $\alpha$ , 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 was published in *Cancer Epidemiology, Biomarkers & Prevention*<sup>2</sup>. Having established that the assay was sensitive and reliable, and that women secrete consistent yet individually distinct amounts of EGF and TGF- $\alpha$  in breast fluid, we next addressed the hypothesis that levels of these mitogenic growth factors are correlated with breast tissue density as determined by mammograms. Mammographic density is presently accepted as an independent risk factor for breast cancer<sup>3</sup>. This report includes initial results of our Mammographic Density Study.

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 will provide ultrasensitive non-invasive methods for serial measurement of steroid hormone concentrations in premenopausal women. This report describes our problems with the fluorimetric assay for salivary estradiol, and our recent conversion to an ultrasensitive radioimmunoassay, which we are currently testing. We have recently completed the Repeat Measures Study, which is designed to permit measurement of the correlation between salivary and serum concentrations of estradiol *within* individual women.

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 model<sup>4</sup>. Data on human lobular differentiation previously has been obtained from breast reduction or autopsy specimens, and therefore are 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 both pathologists and non-pathologists at Northwestern in scoring breast tissue under the microscope using a standardized protocol. In addition to validating readers, we have reviewed records at the Breast Center to determine the approximate number and suitability of biopsy samples available for analysis, and completed studies on the heterogeneity of lobule type within individual breasts. We have also completed a substantial amount of data collection on a case-control study comparing lobular differentiation in patients with breast cancer compared to controls with no evidence of malignant disease.

The progress of all these studies is diagrammed in Table 1. For Project 1, Phases 1 and 2 are completed and Phase 3 work is partially complete. For Project 2, Phases 1 and 2 have been completed, and Phase 3 work is well underway.

## **BODY OF THE REPORT**

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

#### **1. Laboratory assays for breast fluid growth factors**

Having completed development of the assays for EGF and TGF- $\alpha$ , we have recently focused attention on measurement of TGF- $\beta$ 1 in breast fluid using the Genzyme (Cambridge, MA) Predicta® ELISA kit. Repeated measurement of total TGF- $\beta$ 1 in pooled samples at various dilutions indicates that immunoreactive growth factor will be detectable using as little as 0.5-1.0  $\mu$ l of breast fluid. TGF- $\beta$ 1 is released from binding proteins by acidification prior to assay, but extraction does not appear to be necessary. Once the sources of laboratory variability are quantified and judged to be acceptable, we will use previously collected samples to determine the correlation of TGF- $\beta$ 1 levels between breasts and over time, in the same woman.

#### **2. Laboratory assays for breast fluid estrogens**

We are adapting a time-resolved fluoroimmunoassay developed by Wallac, Inc. (Turku, Finland) to measure estradiol in breast fluid. This assay system (known as DELFIA) utilizes a Europium label that gives antigen-antibody complexes an intense, prolonged fluorometric signal. 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. To achieve the required sensitivity, we have found it necessary to add an ether extraction step. Extraction efficiency measured using labeled E2 is approximately 80%. We are now using our

standard sequence of steps to characterize sensitivity and sources of variability of this assay. These steps include measurement of CVs at serial dilutions, observed vs. expected levels with dilution, and recovery of added growth factor. Once these steps are completed, we will use samples from the Repeat Sample Study to evaluate the correlation of E2 levels in breast fluid between breasts and over time, particularly in relation to menstrual cycle phase and concomitant serum E2 level.

### 3. **Mammographic Density Study**

The Mammographic Density Study (MDS) was begun in 1996 with the following aims: 1) to evaluate the association between breast fluid levels of EGF and TGF- $\alpha$  and breast parenchymal density as reflected in screening mammograms, and 2) to 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 and appears to be controlled, at least partially, by ovarian hormonal influences. We hypothesize that women with characteristically high levels of estradiol and mitogenic growth factors in breast fluid will have increased mammographic density.

Following IRB approval, we began to collect breast fluid from women receiving mammograms at the Lynn Sage Breast Center at Northwestern. Women were eligible if they were between 35-60 years old, had no history of breast cancer, were at least 2 years post-lactation, were scheduled for a screening mammogram as opposed to a mammogram for follow-up of an abnormal finding, and had no nipple soreness or lesions that would preclude breast fluid sampling. After the films were taken, radiology technicians gave each eligible woman a cover letter and consent form to review and sign while waiting (still in examination gown) for the film quality to be checked. Thus, all nipple aspirations were performed soon after breast compression. Following nipple aspiration, an interviewer administered a brief questionnaire to obtain supplemental information on reproductive history, exogenous hormone use and family history of breast cancer. A staff member validated according to procedures described earlier in the project traced the whole breast area and areas of density on the cranio-caudal view mammogram. These tracings were then re-traced with a computerized planimeter to measure total breast area and total area occupied by radiographic densities.

### 4. **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  $\mu$ l samples of saliva are mixed with radiolabelled progesterone and progesterone antibody. The resulting bound progesterone is separated using dextran-coated charcoal, counted in a scintillation counter, and counts are compared to a standard curve. The first salivary estradiol assay we evaluated uses time-resolved fluoroimmunoassay (tradename: DELFIA). In this assay, 100  $\mu$ l samples of saliva are mixed with antibody to estradiol, and then pipetted into plastic wells coated with anti-IgG. Estradiol bound to chelated Europium is added to the well. After incubation and rinsing, the solid phase 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. Due to problems with the DELFIA assay described under Results, we have begun adapting a new ultrasensitive

radioimmunoassay (Diagnostic Systems Laboratory, Webster, TX) for estradiol. In this assay,  $I^{125}$ -labeled estradiol competes with salivary estradiol for binding to antibody in buffer solution. Addition of anti-IgG results in the formation of antigen-antibody complexes, which are then precipitated and separated from unbound hormone by centrifugation. This assay thus avoids potential problems caused by interference between substances in saliva and binding to the solid-phase secondary antibody used in DELFIA.

#### 5. **The Two-Cycle Preliminary Study (TCPS) and the Repeat Measures Study (RMS)**

Initially, we evaluated the correlation between salivary and serum hormone levels in the Repeat Sample Study, a population of 62 premenopausal women who each provided 4 saliva samples over a single menstrual cycle. We found unacceptably low correlations in these samples between estradiol in saliva and serum. As discussed in the previous report, we hypothesized that this poor correlation could be due to a variable amount of background signal produced in the DELFIA assay by unknown substances in saliva. We therefore assayed individual saliva samples that had been stripped of estradiol by charcoal (99% extraction efficiency) together with the unstripped sample. These studies did reveal presence of background interference that varied between individuals. We then selected 36 saliva-serum concurrent pairs, collected from 18 premenopausal women in the Two-Cycle Preliminary Study, and assayed both stripped and unstripped saliva to determine if subtraction of individual background levels could improve the correlation with serum estradiol.

Attacking a separate hypothesis for explaining the low serum-saliva correlation, we started a new study - the Repeat Measures Study (RMS) - to determine if correlations would be improved by looking at multiple serum-saliva pairs obtained from the same individual. Informed consent was obtained from 9 premenopausal volunteers, who then provided fasting blood samples and both morning and evening saliva samples 2-3 days per week throughout an entire menstrual cycle. Salivary estradiol was measured in unstripped samples by DELFIA, and in serum by radioimmunoassay. Assays have been completed for 6 individuals, with each individual's samples assayed in a single run to avoid interassay variability. Scatterplots and correlations were produced for each individual.

### **B. Results/Discussion: Project 1**

#### 1. **Breast fluid growth factors**

Development of the assays for EGF and TGF- $\alpha$  was reported in the journal *Cancer Epidemiology, Biomarkers & Prevention* (Appendix A). Our results for TGF- $\beta$ 1 thus far indicate that we can easily detect TGF- $\beta$ 1 in pooled breast fluid at concentrations of approximately 2 ng/ml. Intra-assay reliability is good (CV = 8.7-9.5%) over a 5-fold range of TGF- $\beta$  concentrations. However, interassay variability remains unacceptably high (>15%) and requires further investigation.

#### 2. **Breast fluid estradiol**

Results for estradiol measured in a pool of breast fluid at various dilutions without an extraction step are shown in Table 2. "Count" CVs - those shown below as "duplicate CVs" - represent the agreement in fluorometric counts among replicates. These values indicate

excellent reproducibility at each dilution. However, the "dose CV" includes variability introduced by reading a final result off the standard curve. When the count corresponds to a standard at a very low, non-linear part of the standard curve, dose CV can be much higher than count CV. The dose CV at 1:5 is acceptable; however, the interassay dose CV is too high (45.8%). Results on another breast fluid pool after ether extraction are shown in Table 3. The intraassay dose CV at 1:5 is similar to the results without extraction and the interassay dose CV is reduced to 18.5%. A direct comparison of extracted and unextracted samples is shown in Table 4. We infer that the assay for estradiol in breast fluid is sufficiently reliable when run at dilutions of 1:5 following ether extraction. We believe it will be possible to measure estradiol in samples with volumes as low as 2.5  $\mu$ l. It is important to note that the amount of estradiol we are detecting in these pooled samples is approximately 140 pg/ml. This is comparable to estradiol levels in serum during the luteal phase of the menstrual cycle, but is about 3-fold lower than the levels reported earlier by Petrakis, et al.

### 3. Mammographic Density Study

Through August, 1997, we enrolled 93 MDS women for nipple aspiration; we obtained breast fluid from 47 of these women. We have obtained EGF concentrations in breast fluid and mammographic density readings from the same breast for 23 women. The relationship between EGF and mammographic density in the corresponding breast for these women is shown in Figure 1. There is a low-moderate correlation ( $r=0.32$ ,  $P = 0.13$ ), with several notable outliers. Removal of a single influential outlier (with density=62% and EGF=300 ng/ml) gave a considerably stronger correlation ( $r=0.45$ ,  $P=0.04$ ). We obtained EGF measurements from both right and left breasts for 7 women - the correlation was 0.74, similar to the correlation observed in our previous studies. In conclusion, we do not feel confident that the current dataset is large enough to permit firm inferences about the relationship of breast fluid EGF to mammographic density. We intend to collect more data on EGF, as well as to analyze the MDS samples for TGF- $\alpha$ , TGF- $\beta$ 1 and estradiol.

### 4. Salivary progesterone and estradiol

Results with the salivary progesterone assay continue to be excellent. Profiles of salivary progesterone levels from daily serial samples from 11 women are shown in Figure 2. These show a distinct, smooth luteal peak. We have just completed progesterone assays on saliva obtained from women in the TCPS. These samples represent days 10 to the final day of two consecutive cycles from 18 women. Within the next month, we will analyze these results to determine how consistent the progesterone peak in saliva is *between* cycles in these normal young women. Not surprisingly, data on intra-individual variability in progesterone levels is scarce since serum methods would require a large number of serial daily venipunctures.

Previous work with charcoal-stripped samples had shown that the amount of background noise in the salivary estradiol assay indeed varies between individuals, ranging from 1 to 3 pg/ml. Measurement of this background in each individual sample and correction for it did not, however, improve the correlation between serum and saliva in the samples we analyzed from the TCPS (18 women, 2 serum-saliva pairs from each). This leaves open the possibility that there are interfering substances in saliva that are not removable by charcoal treatment.

As stated in the previous report, 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. Serum-saliva correlations for 6 women from the more recent Repeat Measures Study are shown in Figure 3. These are evening saliva results - similar results were obtained with the morning saliva. Samples from three subjects (1, 2, and 4) were assayed in the first run and those from 3 more (subjects 5,6,7) in a second run. Subject 1 most likely had an anovulatory cycle, so that estradiol levels never rose above 50 pg/ml in serum. Discounting results from that subject, the correlations for Subjects 2 and 4 were encouraging. However, the correlations from subjects 5-7 are actually inverse in 2 cases. Thus, the overall correlation from all 59 RMS samples (Figure 4) is essentially zero.

We have adopted the premise that something in saliva, probably not a charcoal-extractable steroid, interferes with the DELFIA system, perhaps by affecting the binding properties of the solid phase-fixed second antibody. The effect of this interference is quite reproducible in replicate samples, so that we consistently observe excellent "sensitivity" and reliability in the salivary estradiol assay - but unfortunately, inadequate validity. Our preliminary results with the DSL solubilization assay, which does not involve solid phase antibodies, are encouraging. Daily estradiol profiles during normal menstrual cycles from 11 women are shown in Figure 5. A distinct preovulatory estradiol peak can be seen, as well as a typical luteal plateau. We are currently repeating the RMS serum-saliva correlations using the new assay and expect to have final results within the next month.

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

##### **1. Final development of standardized reading protocol and reader validation**

Dr. Gann and Allison Ellman visited Dr. Jose Russo at Fox Chase Cancer Center in Philadelphia in September, 1996. The purpose was to revise the standard criteria for scoring lobule type in histologic specimens, and review discrepancies between Dr. Russo and Ms. Ellman. After review of many cases, we concluded that inter-reader agreement would be improved by: 1. adopting strict criteria for presence of a central duct in order to count a lobule, and 2) excluding lobules with diffuse hyperplasia defined as greater than two epithelial cell layers of thickness.

##### Final protocol for scoring lobules

The reader's goal is to provide an estimate of the distribution of lobule type (types 1, 2 and 3) in normal breast tissue obtained by excision biopsy or mastectomy. Type 1, 2 and 3 lobules are distinguished solely on the basis of the number of acini contained within a lobular unit, as follows:

Type 1	2 - 29 acini
Type 2	30-79
Type 3	≥ 80

Figures 6 and 7 illustrate the appearance of typical lobules types 1 and 2 (Figure 6) and type 3 (Figure 7).

Slides stained with H&E are examined first at 40X to establish the suitability of the slide for reading (presence of lobular structures, absence of diffuse pathology or artifact), and the approximate number of slides that will be needed to complete the reading. Multiple slides are used for each patient. The reader then proceeds to type lobules at 100X, moving through adjacent fields in an organized manner. 200X magnification is used to resolve questions about specific lobules. Results for each patient are recorded on tally sheets that allow us to reconstruct the order in which lobules were read, by slide.

The following criteria must be met in order to count a lobule:

- Central duct must be visible (step sections can be used to identify a central duct), unless there are 80 or more acini already visible.
- There must not be artifact or atrophy that could distort the number of acini counted.
- There must be no more than 2 cell layers of epithelium in the acini, which would indicate hyperplasia.
- The lobule must be free of other definable pathology (fibrocystic change, adenosis, LCIS, etc.)

Analysis of data from scoring an unlimited number of lobules led us to conclude that improvement in inter-reader agreement is negligible after 100 lobules have been counted using the above procedures. Therefore, no more than 100 lobules are counted per case.

Following establishment of these criteria and the review session in Philadelphia, Ms. Ellman read 20 new cases, which were also read independently by Dr. Russo. Scatterplots and correlation coefficients were used to assess inter-reader agreement on the percentage of lobules in each category. A second reader, Dr. Elizabeth Bauer-Marsh, subsequently reviewed training cases with Ms. Ellman and read the same 20 "validation cases" read by Russo and Ellman. Dr. Bauer-Marsh is a senior resident in Pathology at Northwestern Memorial Hospital. Scatterplots and inter-reader correlations were also produced.

## **2. Homogeneity across breast regions**

Whole mount studies had indicated no significant systematic variation in lobule type across breast regions. To determine if the same was true for the histologic index, and to determine if distance from a tumor would influence lobule type, we examined slides from 6 mastectomy patients in detail (4 from previously stained sections and 2 from new sections made from formalin-fixed mastectomy tissue). We had a pathology technician make new slides from all quadrants of 6 recently-obtained mastectomy specimens - however, 4 of these had too few lobules present on the slides to permit scoring. For each patient, we read 10 slides from each breast quadrant, and recorded the distance of each section from the breast cancer.

## **3. Case-control study**

We are currently collecting data in a case-control study on the relation of lobular differentiation to the risk of breast cancer. This study encompasses, and extends beyond Task 3 in the

original SOW. The specific aims of this study are to apply the index to conduct pilot studies exploring the relationship between lobular differentiation and a.) breast cancer risk, and b.) reproductive and hormonal variables hypothesized to be determinants of breast tissue maturation.

Thus far, we have randomly selected 23 patients under age 55 diagnosed with invasive breast cancer since 1995 at Northwestern Memorial Hospital. We also randomly selected 23 patients from the same age stratum who had recent biopsies that did not reveal malignant disease. Age was restricted to younger women to reduce distortion of lobular typing by age-related lobular regression. Slides from each patient were scored for lobule type by validated readers. Dr. Wiley initially reviews all cases and all slides containing breast cancer are not sent to the reader to avoid bias.

Analysis is 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.

## **B. Results/Discussion: Project 2**

Figure 8 shows the final correlations between validated readers (Ellman and Bauer-Marsh), and the reference expert, Dr. Russo.

These plots indicate a high level of agreement between readers, and that our standardized protocol allows us to readily validate new readers. Note also that the slopes of the line for inter-reader agreement are close to 1.0. Ms. Ellman left Northwestern in June, 1997 to return to medical school. Therefore, Dr. Bauer-Marsh is currently our sole validated reader. We have begun training two additional senior residents in pathology.

In our homogeneity analyses, lobule type was essentially the same in all breast quadrants (including the involved quadrant) in patients with breast cancer. Lobule type was also the same for regions located either 2-4 cm or > 4 cm from the primary tumor (the number of lobules < 2 cm from tumors was insufficient for analysis). We counted 152-454 lobules per case in these analyses.

Table 5 shows the comparison of lobule type distribution in breast cancer cases versus benign controls, thus far. Patients with breast cancer were slightly older than the benign group. Among all samples, the cancer group had a slightly higher percentage of type 1 and lower percentages of types 2 and 3 than the benign group. Four patients from the benign group were exceptionally young (ages 24, 26, 27, 27). With exclusion of these patients, which made the mean ages more comparable, the differences between the cancer and benign group were greater. Differences were greatest for parous women - the cancer group had 14% higher type 1 and 38 % lower type 2 than those with benign diagnoses (10.8% and 7.7% absolute

differences, respectively). DCIS/LCIS patients appeared to have intermediate lobular type, although only 3 such cases were available so far for analysis.

It is worthy of note that the highest % of type 3 lobules was observed in a patient who was diagnosed with breast cancer while pregnant. Pregnancy is expected to involve a largely transient shift towards higher lobule type.

Table 6 shows the comparison of lobule type by parity on 37 women for whom parity has been verified. There was no difference in lobule type for parous vs. nulliparous women among breast cancer cases. However, among women with benign breast tissue, nulliparity was associated with a higher percentage of immature lobules.

## CONCLUSIONS

During the period from October, 1996 to October, 1997, significant accomplishments of this project include:

- Publication of the first paper describing a comprehensive evaluation of assays for EGF and TGF- $\alpha$  in breast fluid. (This research also contributed to the publication of a second paper, dealing with characterization of EGF and TGF- $\alpha$  in expressed prostatic fluid, in September, 1997<sup>5</sup>.)
- We have detected assayable quantities of TGF- $\beta$ 1 and estradiol in breast fluid, and are refining immunoassays to permit detection of these important growth regulatory compounds in minimal volumes of sample.
- Demonstration that the time-resolved fluoroimmunoassay for salivary estradiol is perturbed by non-specific binding, even though it is highly sensitive and reliable. We believe this non-specific binding causes a relatively low correlation between saliva and synchronous serum samples. A new ultrasensitive RIA is currently providing profiles across the menstrual cycle that correspond to the expected serum values. Further testing of this new assay is underway.
- We have collected a substantial number of samples in 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. Breast fluid samples and mammographic density readings have been obtained from 47 women. Initial analyses suggest a possible moderate degree of correlation between EGF concentrations in breast fluid and mammographic density.
- We have re-calibrated 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 have confirmed that these criteria produce much better inter-reader agreement. We have obtained data indicating that the distribution of lobule type is relatively homogeneous across breast regions and at various distances from a localized breast cancer. Finally, we

have now obtained pilot data comparing breast cancer patients to patients with benign breast histology, and have found small differences in lobule type. Thus far, nulliparous women do appear to have less developed lobular patterns, and the association between immature lobule type and breast cancer appears to be strongest among parous women. We are in the process of obtaining more lobular typing data from breast cancer cases and controls.

## REFERENCES

1. Dickson RB, Lippman ME. Growth factors in breast cancer. *Endocr Rev* 1995; 16: 559-589.
2. Gann P, Chatterton R, Vogelsong K, Dupuis J, Ellman A. Mitogenic growth factors in breast fluid obtained from healthy women: evaluation of biological and extraneous sources of variability. *Cancer Epidemiol Biomarkers Prev* 1997; 6:421-428.
3. Byrne C, Schairer C, Wolfe J, Parekh N, Salane M, Brinton LA, et al. Mammographic features and breast cancer risk: effects with time, age, and menopause status. *J Natl Cancer Inst* 1995; 87:1622-1629.
4. Russo J, Russo IH. Toward a physiological approach to breast cancer prevention. [Review]. *Cancer Epidemiol Biomarkers Prev* 1994; 3:353-364.
5. Gann PH, Chatterton R, Vogelsong K, Grayhack JT, Lee C. Epidermal growth factor-related peptides in human prostatic fluid: sources of variability in assay results. *Prostate* 1997; 32:234-240.

### **Publications / Abstracts resulting from this grant:**

1. Gann P, Chatterton R, Vogelsong K, Dupuis J, Ellman A. Mitogenic growth factors in breast fluid obtained from healthy women: evaluation of biological and extraneous sources of variability. *Cancer Epidemiol Biomarkers Prev* 1997; 6:421-428.
2. Gann PH, Chatterton R, Vogelsong K, Grayhack JT, Lee C. Epidermal growth factor-related peptides in human prostatic fluid: sources of variability in assay results. *Prostate* 1997; 32:234-240.
3. Gann PH, Ellman A, Wiley EL, Russo J. A histologic index of lobular differentiation in breast cancer cases versus controls with benign breast disease, the 13<sup>th</sup> Annual San Antonio Breast Cancer Symposium, December, 1997.

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

	Phase 1 <sup>a</sup>	Phase 2	Phase 3
<b>Project 1: Hormone/growth factor levels in body fluids, and their determinants</b>	<ul style="list-style-type: none"> <li>■ Repeat Sample Study (design, sample collection)</li> </ul>	<ul style="list-style-type: none"> <li>■ assay breast fluid EGF, TGF-<math>\alpha</math>, data analysis</li> <li>■ assay bioavailable E2</li> <li>■ assay saliva PG, E2</li> <li>■ refine diet data</li> </ul>	<ul style="list-style-type: none"> <li>■ begin sample collection for Mammographic Density Study</li> <li>■ data analysis: diet, activity and body size</li> <li>■ assay breast fluid E2, TGF-<math>\beta</math></li> <li>■ refine saliva E2 assay</li> <li>■ measure free E2</li> <li>■ Repeat Measure Study</li> </ul>
<b>Project 2: Lobular differentiation in normal breast tissue</b>	<ul style="list-style-type: none"> <li>■ reader validation</li> </ul>	<ul style="list-style-type: none"> <li>■ lobular homogeneity</li> <li>■ sample quality</li> </ul>	<ul style="list-style-type: none"> <li>■ case-control study</li> </ul>

Table 2. Detection of E<sub>2</sub> using DELFIA in unextracted pooled breast fluid samples

Sample	Dilution	Avg. E <sub>2</sub> ±S.D. (pg/ml) <sup>n</sup>	Avg. intraassay duplicate CV(%) <sup>a</sup>	Avg. intraassay dose CV(%) <sup>b</sup>	Interassay dose CV(%)
BF Pool	1:5	95.07±43.58	3.38	13.39	45.8
	1:10	178.15±93.7	4.19	35.79	52.6
	1:20	321.43±53.9	2.11	28.26	16.8

<sup>a</sup> Represents the average difference between fluorescence values for duplicate wells containing the same sample in the same assay plate.

<sup>b</sup> Represents the average difference between values for the dose determined from a standard curve for duplicate wells containing the same sample in the same plate.

Table 3. Detection of E<sub>2</sub> using DELFIA in pooled breast fluid samples extracted with ether

Sample	Dilution	E <sub>2</sub> (pg/ml) ± S.D.	Avg. intra-assay dose CV (%) <sup>a</sup>	Interassay dose CV (%)
Ether extracted BF pool	1:2	141.6 ± 1.1	9.3	0.8
	1:5	147.1 ± 27.2	15.9	18.5
	1:10	316.0	39.6	-

<sup>a</sup> Represents the average difference between fluorescence values for duplicate wells containing the same sample in the same assay plate.

Table 4. Direct comparisons of extracted and unextracted BF pools in the same run:

Run	Dilution	Unextracted BF pool			Ether-extracted BF pool		
		nmol/L	pg/ml	Dose CV	nmol/L	pg/ml	Dose CV
1	1:5				0.525	143.01	6.02
	1:10	1.56	424.9	4.6	0.47	128.03	11.7
	1:50				0.55	149.82	24.6
2	1:100	1.40	381.36	20.9	1.30	354.12	29.0
	1:5				0.69	187.96	14.7
	1:10				1.0	272.4	4.9
2	1:20	1.18	321.43	28.7	1.16	315.98	39.5

**Table 5. Preliminary results comparing lobule type in normal tissue from breast cancer cases and benign biopsy controls**

	N	Mean age	Mean lobule type, %			P, type 1 a vs. c*	P, type 2 a vs. c*
			1	2	3		
<u>All samples</u>							
a. Breast ca.	19**	42.2	83.4	13.2	3.4		
b. DCIS/LCIS	3	44.3	77.7	19.0	3.3		
c. Benign	22	36.2	80.8	15.1	4.1	0.30	0.30
<u>Age &gt; 30 only</u>							
a. Breast ca.	19	42.2	83.4	13.2	3.4		
b. DCIS/LCIS	3	44.3	77.7	19.0	3.3		
c. Benign	18	38.5	77.8	17.4	4.8	0.15	0.14
<u>Parous, age &gt;30</u>							
a. Breast ca.	13	43.4	85.5	12.4	2.1		
b. DCIS/LCIS	3	44.3	77.7	19.0	3.3		
c. Benign	10	39.4	74.7	20.1	5.2	0.05	0.07

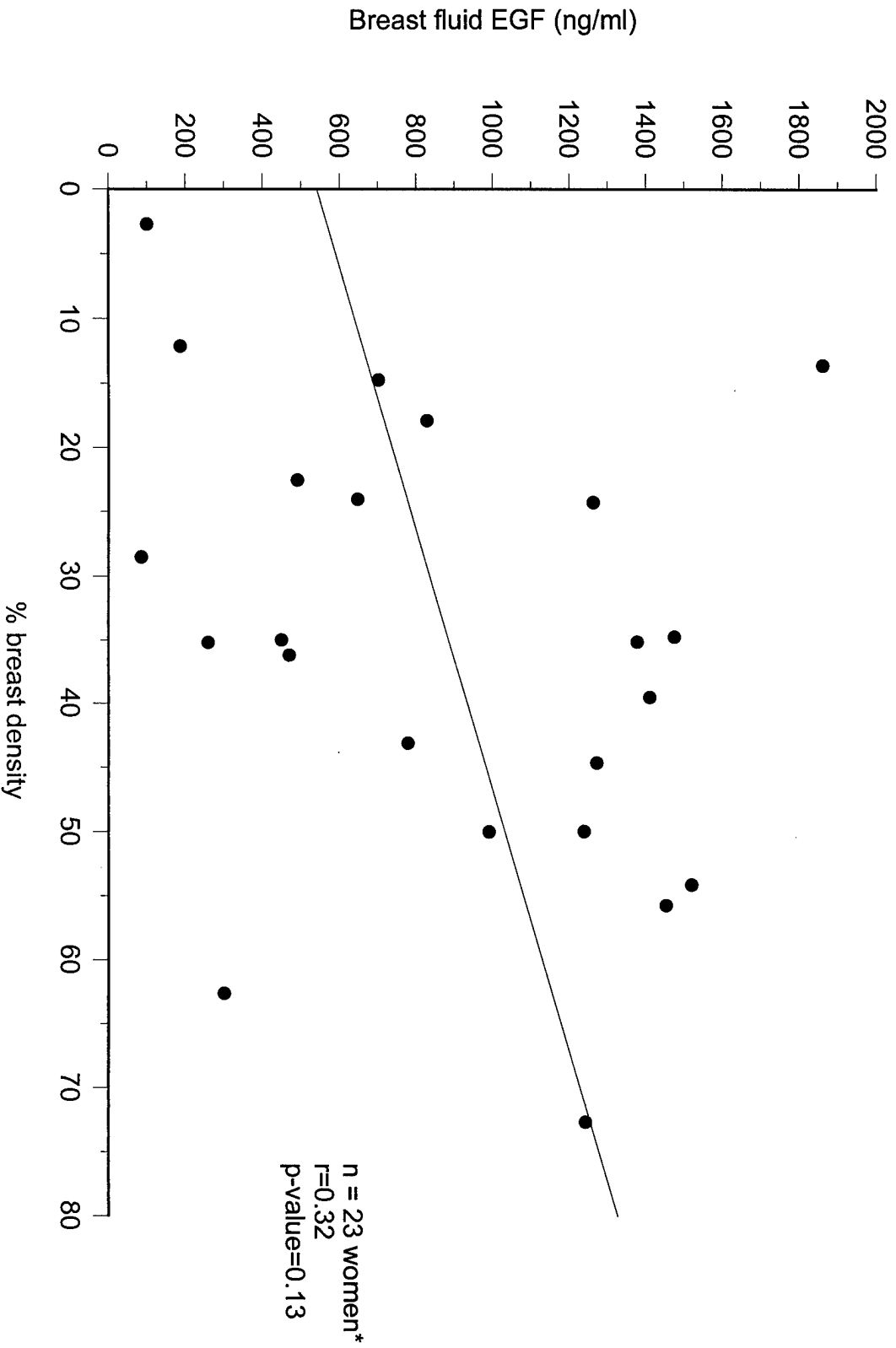
\* One-tailed t-test.

\*\* Excludes 1 patient with invasive breast ca. who was pregnant at diagnosis and had 36% type 3, and a second patient with invasive breast ca. < 1 year postpartum.

**Table 6. Distribution of lobule type in normal tissue by parity, stratified by breast diagnosis**

	N	Mean lobule type, %			P, type 1 a vs. b	P, type 2 a vs. b
		1	2	3		
<b>Breast cancer</b>						
a.Nulliparous	4	85.0	12.2	2.8		
b.Parous	13	85.6	12.4	2.1	0.48	0.49
<b>DCIS/LCIS</b>						
a.Nulliparous	1	76.0	19.0	5.0		
b.Parous	2	78.5	19.0	2.5	-	-
<b>Benign</b>						
a.Nulliparous	8	89.7	8.2	2.1		
b.Parous	10	74.7	20.1	5.1	0.02	0.01
<b>Benign, &gt;age 30</b>						
a.Nulliparous	5	86.9	10.7	2.5		
b.Parous	10	74.7	20.1	5.1	0.08	0.08

Figure 1. Scatterplot of correlation between EGF concentration in breast fluid and mammographic density in the corresponding breast



\*Right breast excluded for women with results from both breasts.

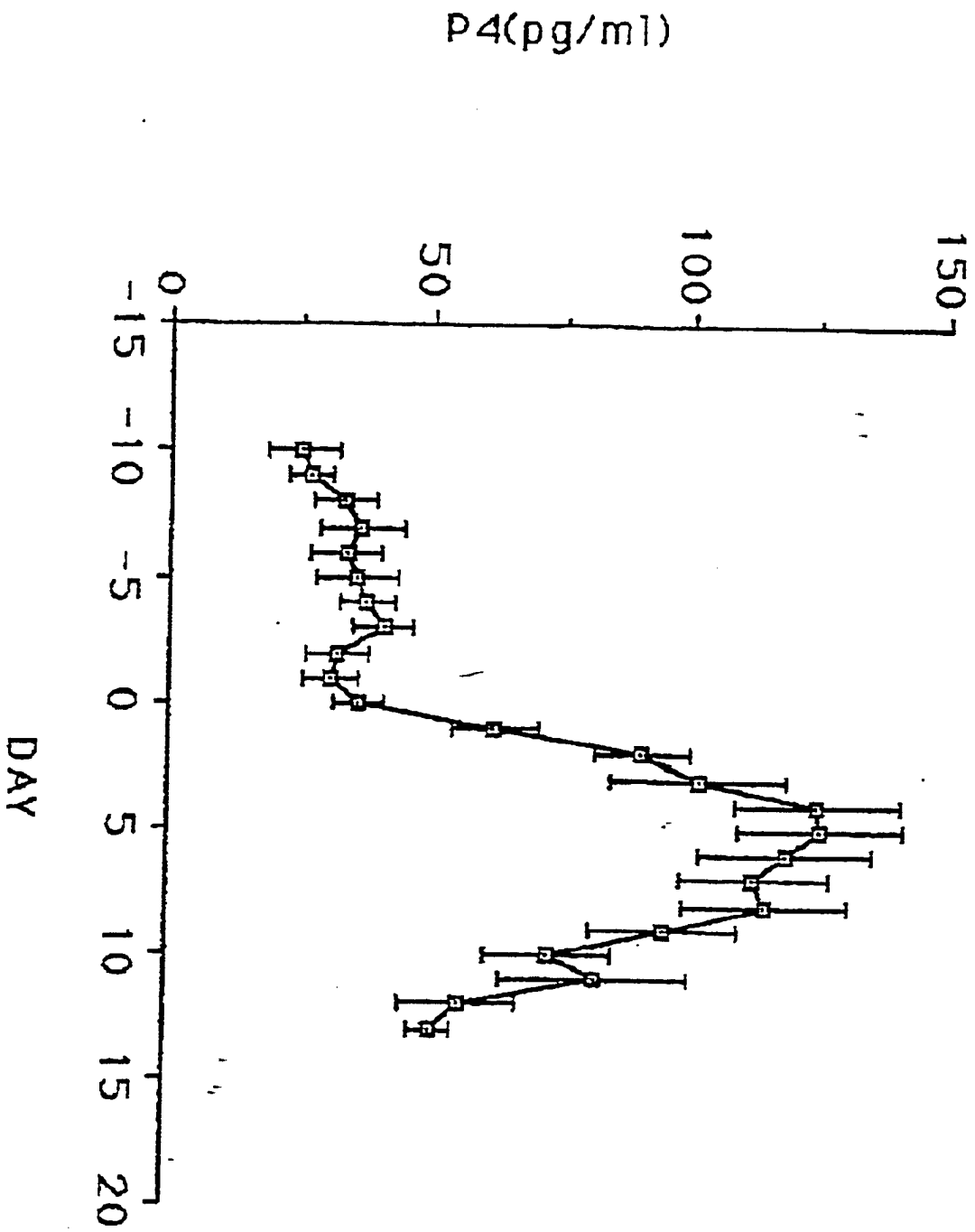


Figure 2. Salivary progesterone (P4) during the menstrual cycle: 11 healthy women, DSL ultrasensitive radioimmunoassay

22 Figure 3. Correlation between evening salivary (by DELFIA) and morning serum (by RIA) levels of estradiol: six women from the Repeat Measures Study

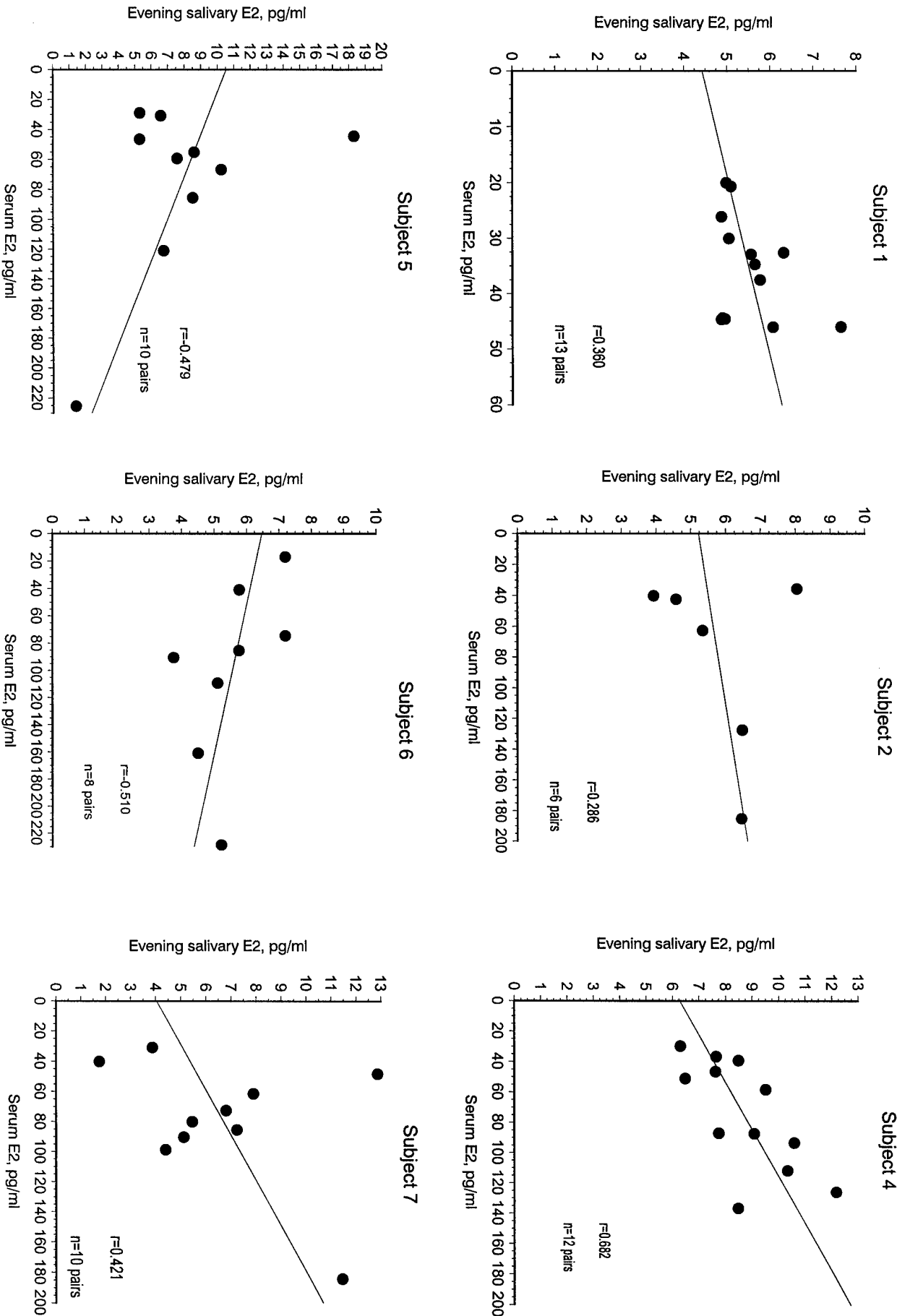
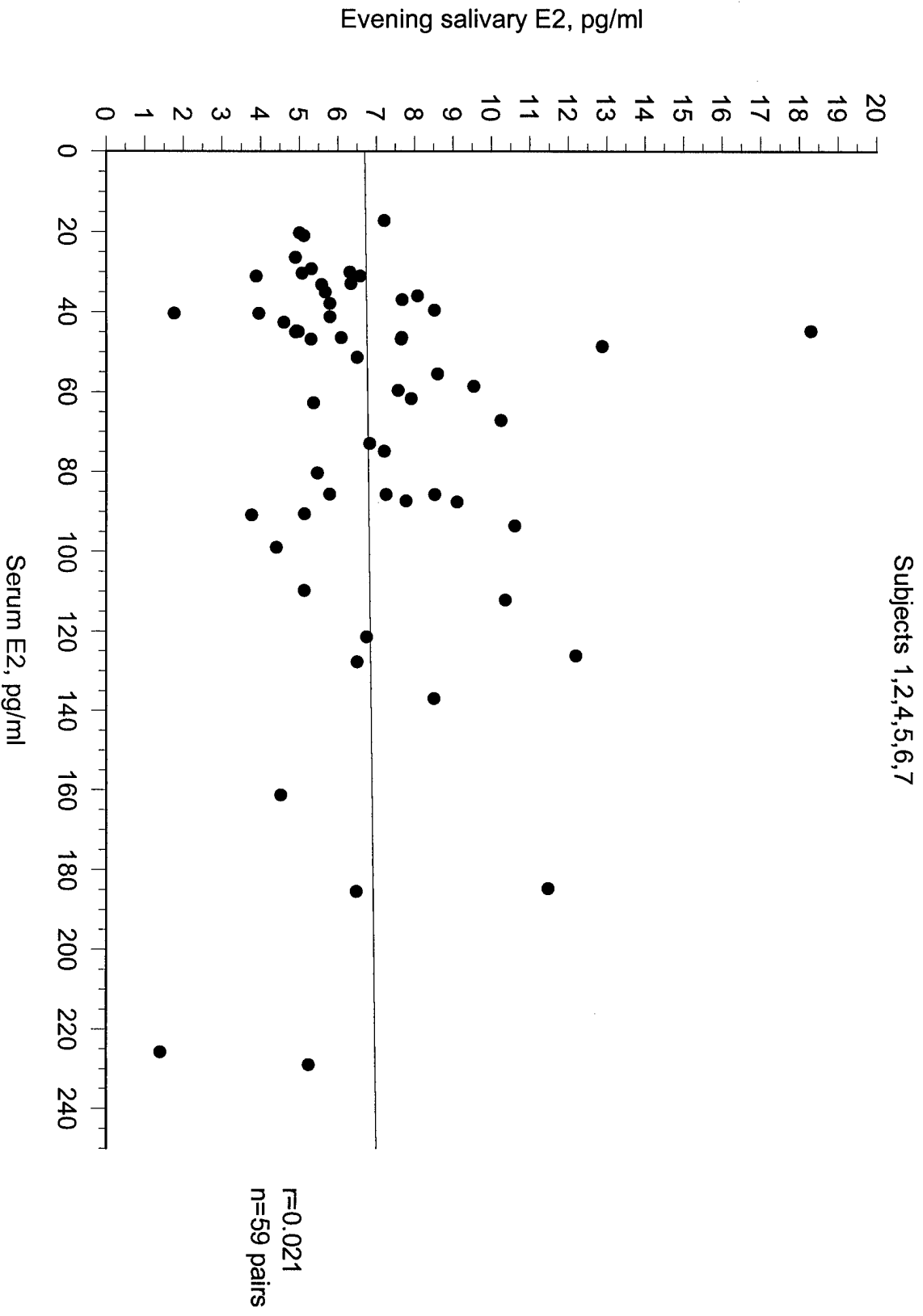


Figure 4. Correlation between evening salivary (by DELFIA) and morning serum (by RIA) levels of estradiol: six women from the Repeat Measures Study



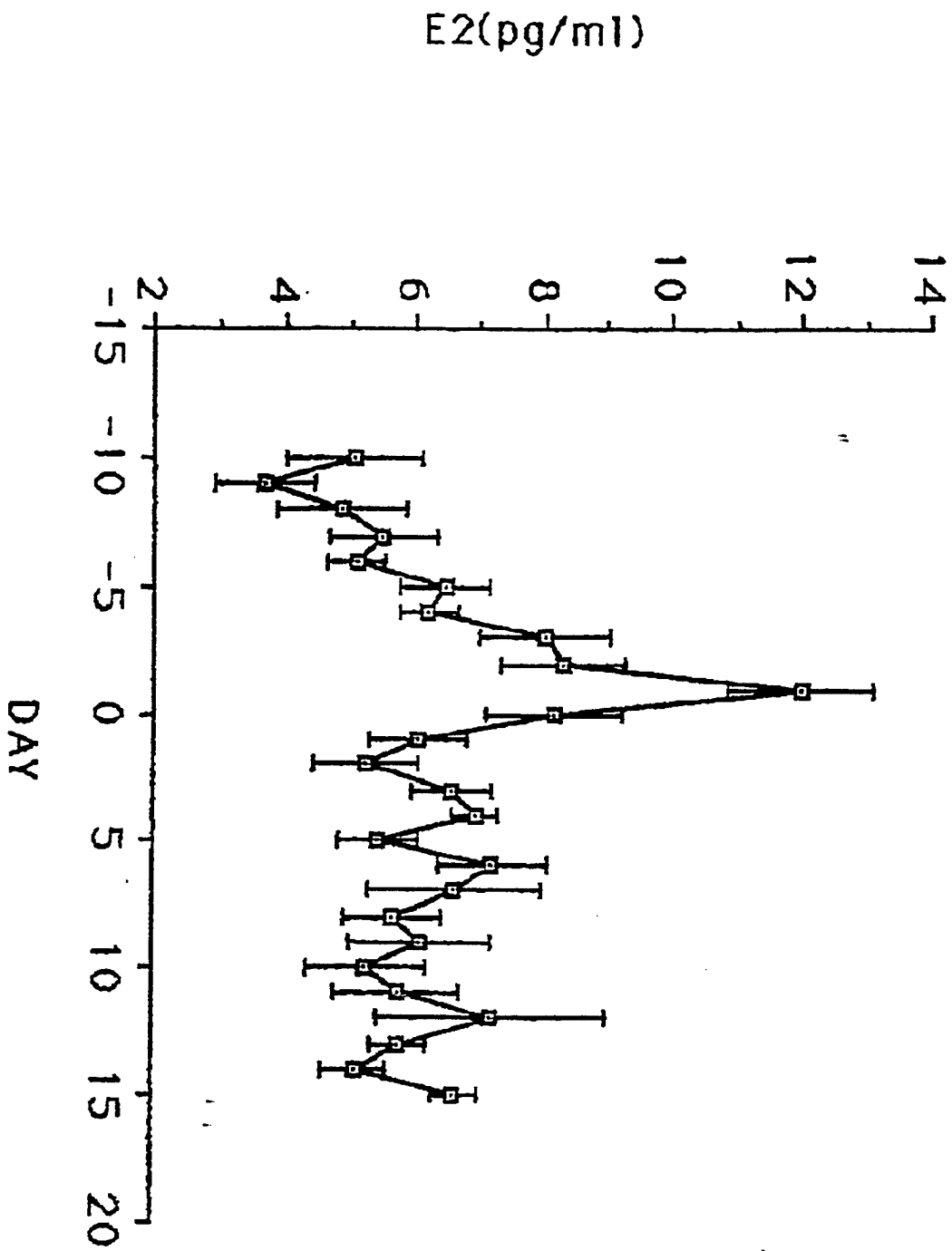


Figure 5. Salivary estradiol (E2) during the menstrual cycle: 11 healthy women

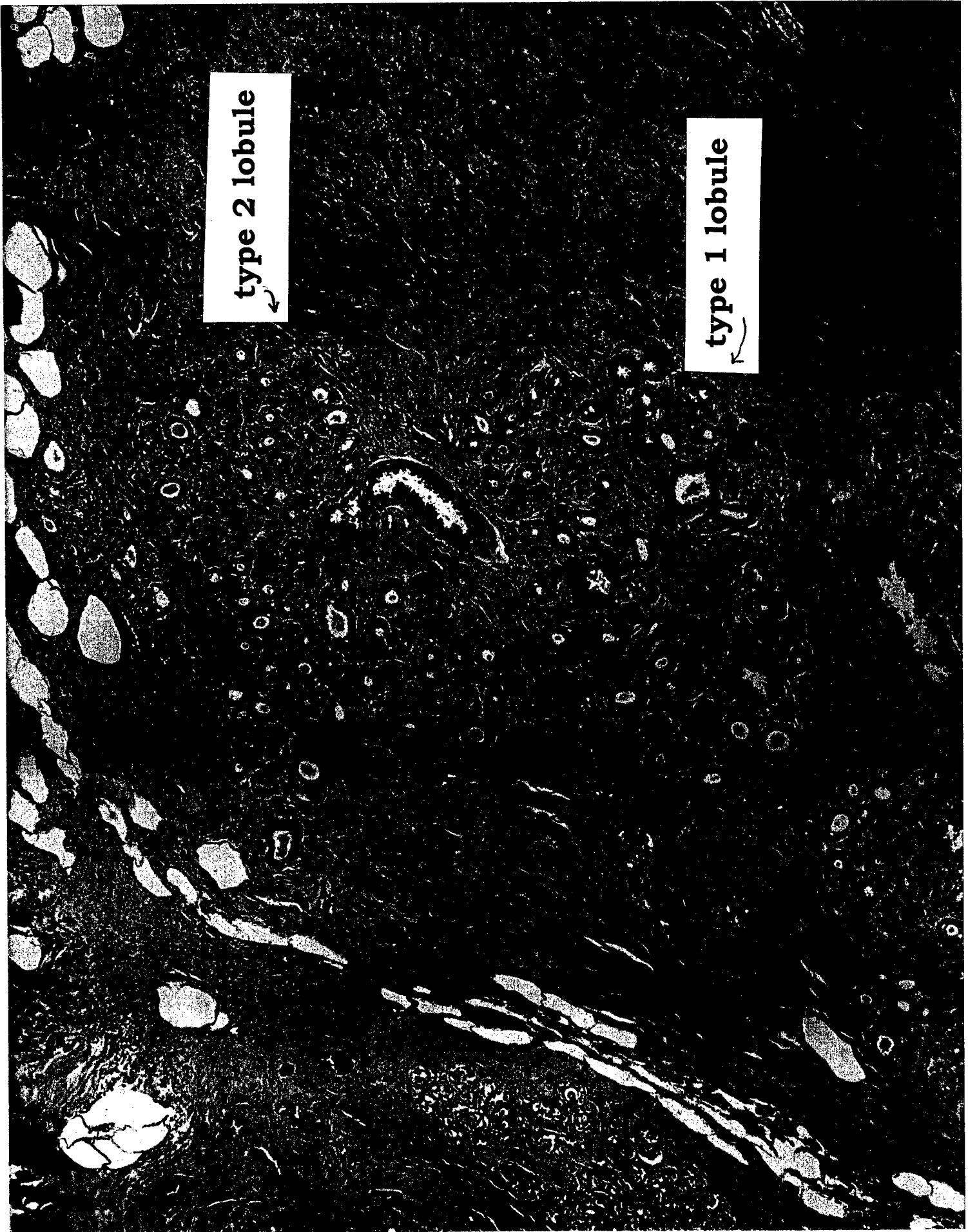


Figure 6. Appearance of typical lobule types 1 and 2 in normal breast tissue from a biopsy specimen

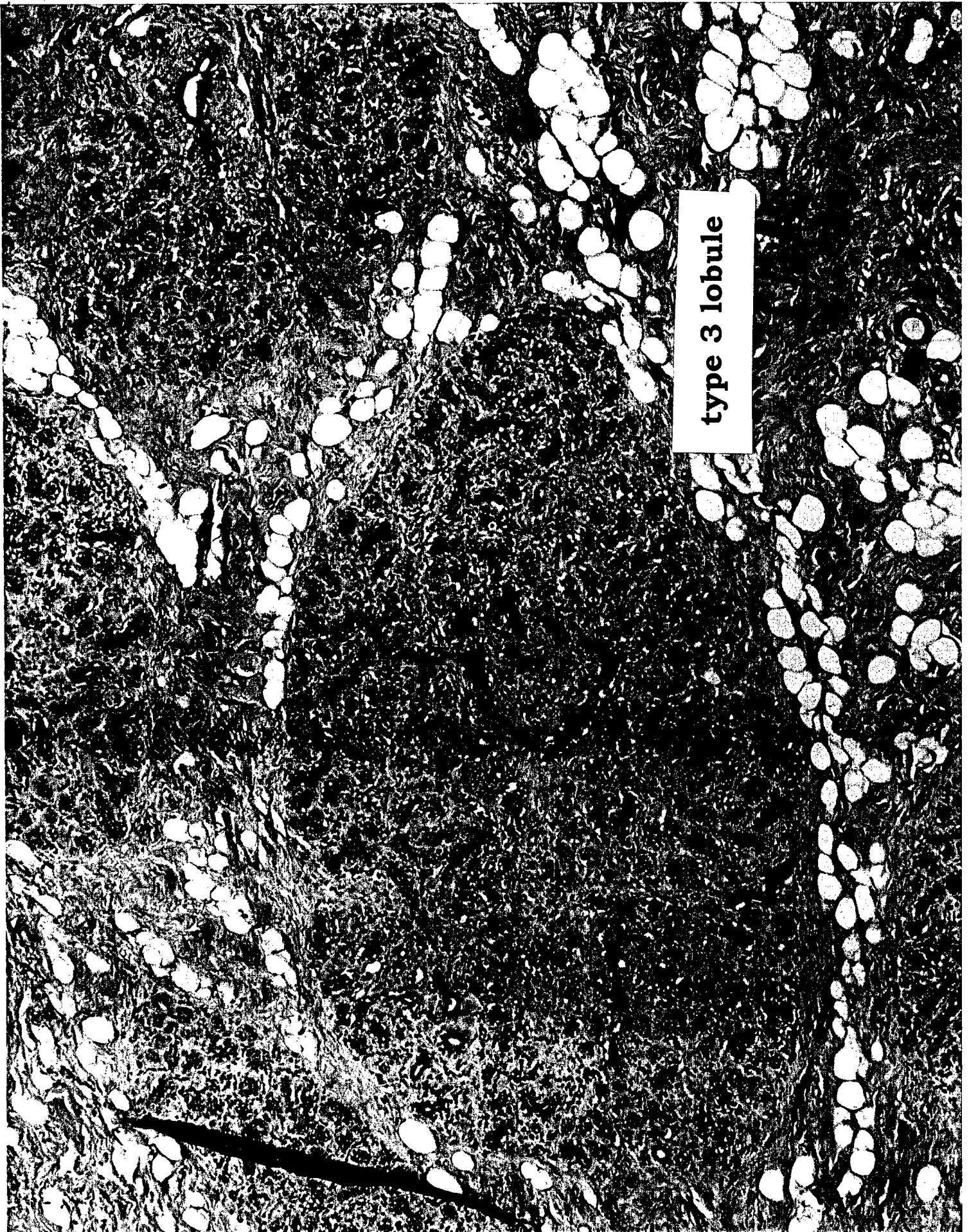
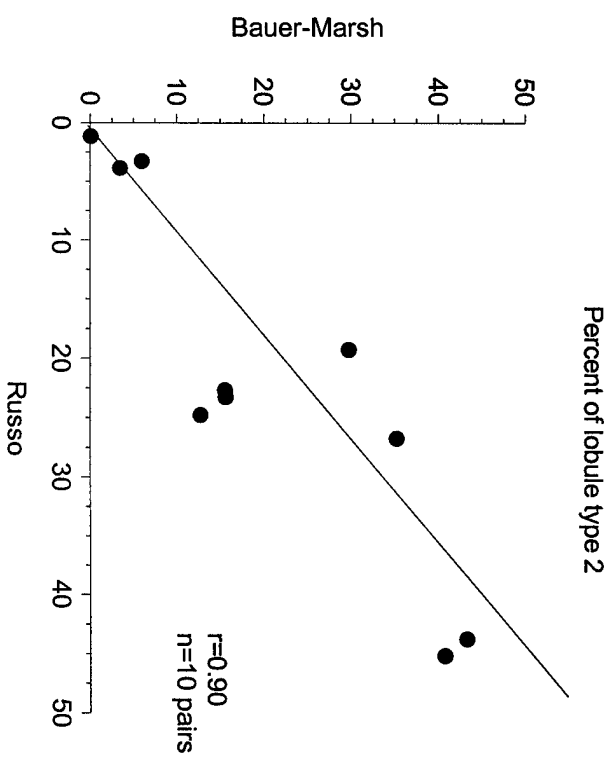
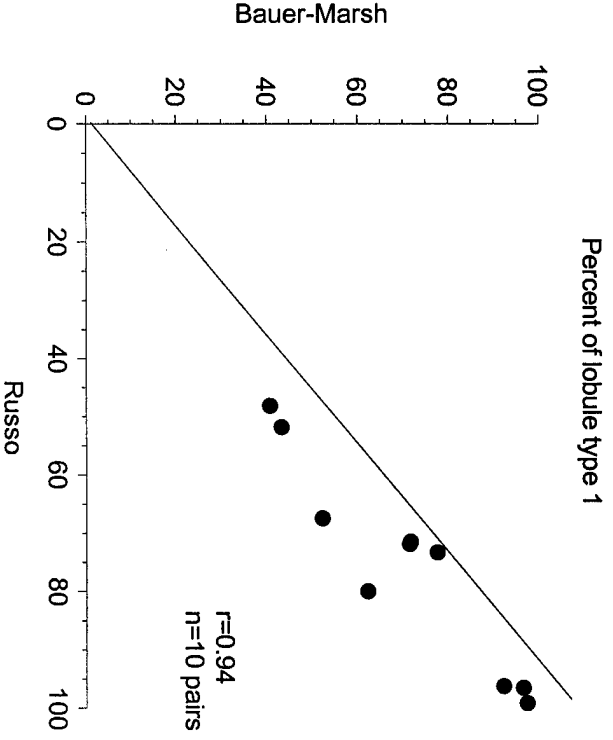
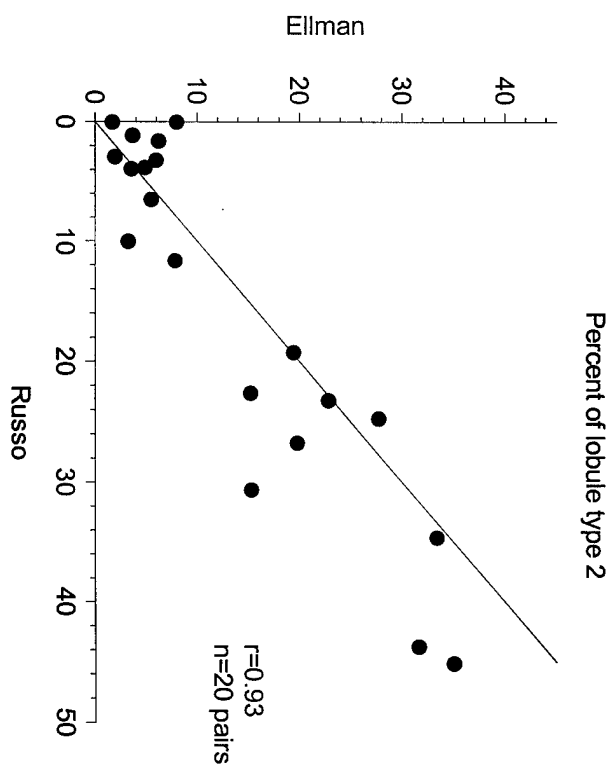
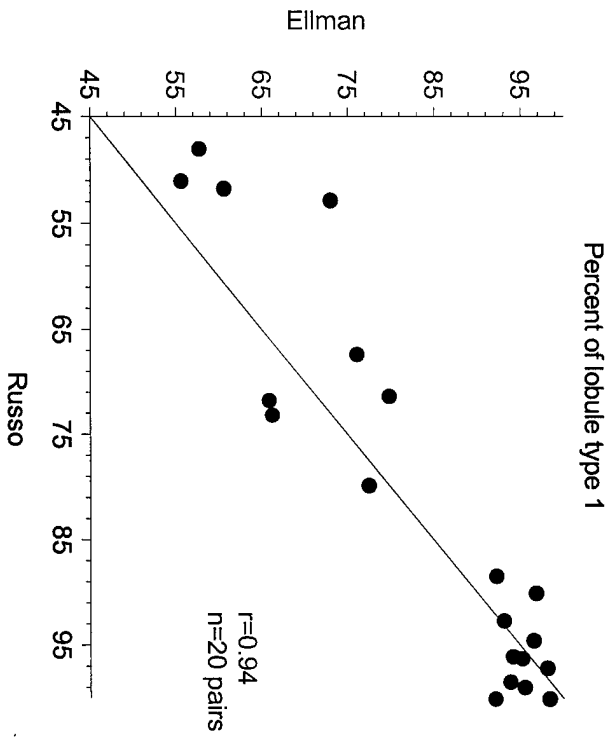


Figure 7. Appearance of typical lobule type 3 in normal breast tissue from a biopsy specimen

Figure 8. Scatterplots of percent of lobule type: correlation between readings of non-professional (Ellman and Bauer-Marsh) readers vs. an expert reader (Russo)



## APPENDIX A

### Reprints of papers resulting from this grant:

1. Gann P, Chatterton C, Vogelsong K, et al. Mitogenic growth factors in breast fluid obtained from healthy women: evaluation of biological and extraneous sources of variability. *Cancer Epidemiol Biomarkers Prev* 1997; 6:421-428.
2. Gann PH, Chatterton R, Vogelsong K, et al. Epidermal growth factor-related peptides in human prostatic fluid: sources of variability in assay results. *Prostate* 1997; 32:234-240.

## Mitogenic Growth Factors in Breast Fluid Obtained from Healthy Women: Evaluation of Biological and Extraneous Sources of Variability<sup>1</sup>

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Departments of Preventive Medicine [P. G., J. D., A. E.], Obstetrics and Gynecology [R. C., K. V.], and Physiology [R. C.], Northwestern University Medical School, Chicago, Illinois 60611

### Abstract

Peptide growth factors (GFs), including epidermal GF (EGF) and transforming GF- $\alpha$  (TGF- $\alpha$ ), are presumed to play an important role in the local regulation of breast cell proliferation. Breast fluid collected by nipple aspiration provides a potential means to assess the concentration of these factors in contact with the ductal epithelium. Although identification of immunoreactive EGF-like GFs in breast fluid has been reported previously, we performed this study to evaluate the sensitivity and reliability of newer RIA methods and to characterize the sources and amounts of both intra- and intersubject variability. We also evaluated the relationship of breast fluid EGF and TGF- $\alpha$  levels to each other and to plasma levels of estradiol and progesterone. Breast fluid and plasma samples were obtained two to four times at weekly intervals from 18 healthy, premenopausal women. EGF and TGF- $\alpha$  were measured by competitive binding RIA. Both GFs were detected with good precision in all breast fluid samples analyzed, using dilutions as low as 1:100 for EGF (1  $\mu$ l) and 1:25 for TGF- $\alpha$  (4  $\mu$ l). The correlations between the right and left breasts, sampled concurrently, were  $r = 0.78$  ( $P = 0.003$ ) for EGF and  $r = 0.89$  ( $P = 0.0001$ ) for TGF- $\alpha$ . For both GFs, the variation between women was substantially greater than the variation between breasts or over time in an individual woman, particularly for EGF, for which there were 100-fold differences between women in mean levels. When samples from multiple women were analyzed together, we found no apparent relationships between EGF and TGF- $\alpha$  levels or between either GF level and menstrual cycle phase or plasma hormone concentrations. However, in random effects

analyses, EGF levels within an individual were significantly associated overall with both TGF- $\alpha$  ( $P = 0.02$ ) and plasma estradiol levels ( $P = 0.01$ ). These data, which are the first comprehensive results on the feasibility of measuring mitogenic GFs in breast fluid, support the conclusion that women secrete consistent and individually distinct levels of EGF and TGF- $\alpha$  and that, in at least some women, EGF secretion *in vivo* covaries with both TGF- $\alpha$  in breast fluid and circulating estradiol.

### Introduction

The prevailing model of breast cancer development assigns an important role to locally acting autocrine/paracrine GFs<sup>3</sup> (1). Peptides such as EGF and TGF- $\alpha$ , which have a 30–40% amino acid homology, demonstrate potent mitogenic effects on human breast cancer cells *in vitro* (2). In addition, the EGF receptor and the homologous product of the oncogene *c-erbB2*, which bind both EGF and TGF- $\alpha$ , are overexpressed in a significant proportion of breast cancers, particularly those with a poor prognosis (3, 4). Undoubtedly, these GFs, the structures of which are highly conserved across species, also play a role in control of normal breast cell proliferation. Current evidence indicates that estradiol and antiestrogens such as tamoxifen have direct and opposite effects on production of these GFs by epithelial or stromal cells in the breast (5). 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. Overexpression of EGF-type GFs is strongly associated with mammary cancer in transgenic mice and the early stages of spontaneous mammary tumor development in normal mice (6, 7). It is plausible, therefore, to hypothesize that healthy women with excessive production of these mitogenic GFs have an elevated risk of developing breast cancer.

Because EGF and TGF- $\alpha$  are produced locally and act locally, their concentrations in serum or urine are not necessarily relevant. On the other hand, nipple aspiration provides a noninvasive 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 nonlactating women by using a simple pump-like device (8). On the basis of numerous biochemical analyses, this fluid appears to provide reasonable insight into the hormonal and metabolic microenvironment of the breast. One group of investigators has reported immunoreactive EGF and TGF- $\alpha$  concentrations in breast fluid (9, 10). Other studies

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<sup>3</sup> The abbreviations used are: GF, growth factor; EGF, epidermal GF; TGF- $\alpha$ , transforming GF- $\alpha$ ; CV, coefficient of variation; QC, quality control; ICC, intraclass correlation coefficient.

**Table 1** Intra-assay and interassay variability for replicate samples (including QC pools and individual subject samples) assayed at various dilutions: EGF and TGF- $\alpha$  in breast fluid

	Dilution	Mean intra-assay CV (no. of replicate pairs)	Mean interassay CV (no. of replicate pairs)
EGF	1:50	0.12 (26)	0.05 (6)
	1:75	0.04 (2)	
	1:100	0.13 (36)	
TGF- $\alpha$	1:25	0.11 (59)	0.11 (4)
	1:50	0.25 (11)	

**Table 2** Breast fluid GF levels: variation between and within Repeat Sample Study participants (left vs. right breast, same day)

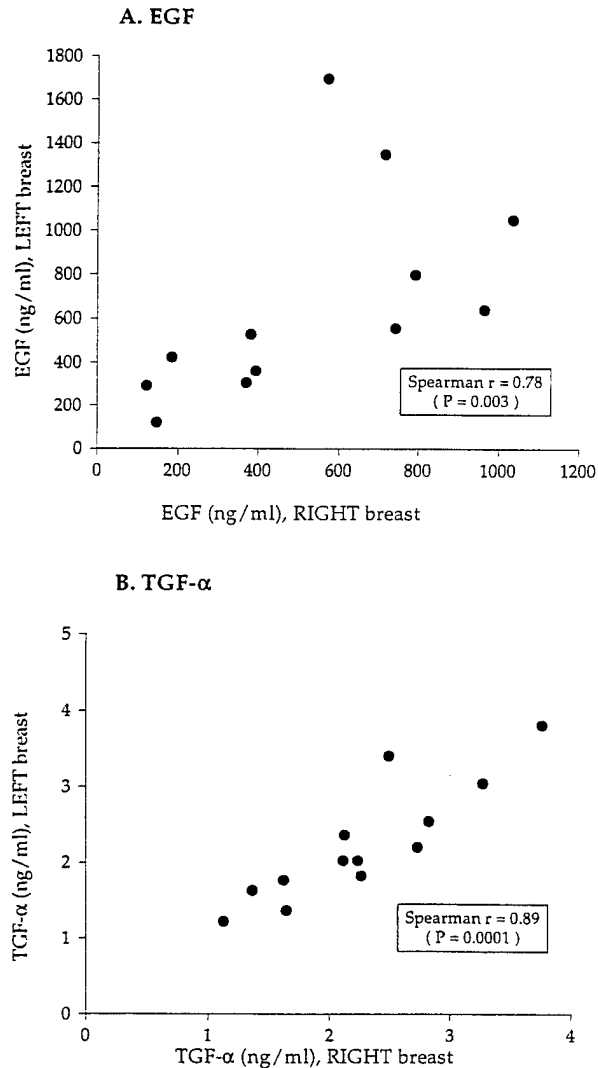
	No. of subjects	No. of samples	Mean	CV between	CV within	ICC
EGF	12	24	604 ng/ml	0.82	0.47	0.48
TGF- $\alpha$	13	26	2.26 ng/ml	0.46	0.11	0.88
EGF/protein	12	24	7.11 ng/mg	0.74	0.65	0.08
TGF- $\alpha$ /protein	13	26	39.1 pg/mg	1.49	1.16	0.21

have reported on these or similar GFs in human milk or breast cyst fluid, although the comparability of milk or cyst fluid to nipple aspirate fluid is questionable (11–13).

The studies described in this report were designed to address basic methodological questions concerning the assay of EGF and TGF- $\alpha$  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 epidemiological research, it is critical that there be a substantial amount of variation between individuals relative to the variation within individuals (14). Finally, we determined whether breast fluid EGF and TGF- $\alpha$  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.

## Subjects and 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: ages 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, 1 week apart, at the Clinical Research Center at Northwestern Memorial Hospital. Participants were allowed to start their visits during any day of the menstrual cycle, and they arrived at the Clinical Research Center 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. The mean



**Fig. 1.** Scatterplots showing concentrations of EGF (A) and TGF- $\alpha$  (B) in breast fluid obtained at the same time from the right versus left breast. EGF,  $n = 12$ ; TGF- $\alpha$ ,  $n = 13$ .

age of the participants was 33.7 years; 76% were white, 11% were African-American, and 4% were Asian.

**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 was gradually applied (15). Suction was discontinued if fluid failed to appear at the nipple surface after 10 s. Droplets of breast fluid appearing at the duct openings were collected in 75-mm plastic-coated capillary tubes that were then clay-sealed at both ends and kept on ice until storage at  $-20^{\circ}\text{C}$ , no more than 1 h after collection. We obtained at least 2  $\mu\text{l}$  of breast fluid from 39 (60%) of the 65 women who attended at least one visit. For the assays in this report, we selected samples from 18 women, 15 of whom gave samples at three or four visits and 13 of whom gave samples from both breasts on at least one visit.

Table 3 Breast fluid GF levels: variation between and within Repeat Sample Study participants (same breast, different days)

	No. of subjects	No. of samples	Mean	CV between	CV within	ICC
EGF	15	57 <sup>a</sup>	494 ng/ml	1.20	0.26	0.83
TGF- $\alpha$	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- $\alpha$ /protein	15	58	73.2 pg/mg	3.81	2.58	0.21

<sup>a</sup> This sample was not included in analysis of EGF levels due to a laboratory error.

**Assays for Plasma Estradiol, Bioavailable Estradiol, and Progesterone.** Plasma estradiol was measured using a RIA kit obtained from Diagnostic Systems Laboratories (Webster, TX). This double antibody assay has a sensitivity of 29 pmol/liter and low cross-reactivity with forms of estrogen other than estradiol. The mean intra- and interassay CVs based on blinded QC samples were 0.08 and 0.04, respectively. Sex hormone binding globulin-bound estradiol was measured using the concanavalin A-Sepharose method described by Bonfrer *et al.* (16). This assay gave intra- and interassay CVs of 0.04 and 0.09, respectively, in our QC samples. Plasma progesterone concentrations were measured by RIA using antibodies prepared by Dr. R. Chatterton (17). The intra- and interassay CVs were 0.09 and 0.10, respectively, in our QC samples.

**Assays for Breast Fluid EGF, TGF- $\alpha$ , and Protein.** Breast fluid was removed from the capillary tube while it was in a semi-frozen state, and, except when undiluted sample was needed for sensitivity studies, it was diluted with a Tris-saline buffer (pH 8.0) prior to assay. The initial dilution was made without BSA in the buffer, an aliquot was taken for protein assay, and the final dilutions were made with BSA to a concentration of 0.15%. We used competitive binding RIA kits purchased from BioMedical Technologies (Stoughton, MA) for both EGF and TGF- $\alpha$ . The EGF assay has an estimated cross-reactivity of less than 0.1% with human TGF- $\alpha$  and undetectable binding with other human peptides tested. The TGF- $\alpha$  assay has cross-reactivities of less than 0.1% with human EGF and undetectable binding with other human peptides tested. To evaluate assay sensitivity, dilutions of breast fluid ranging from undiluted to 1:200 were prepared. The intra-assay CV at each dilution was evaluated by analyzing replicates in the same assay run to determine the dilution level at which assay reliability became unacceptable. Total protein in breast fluid was measured by the Bradford method.

**Data Analysis.** We calculated CVs and ICCs to assess intra- and interassay variability and the amount of variation within *versus* between individuals. Interassay CV was calculated from the variance between assays with the intra-assay variance removed. The ICC is defined as the between-person variance divided by the total variance (between plus within; Ref. 18). 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- $\alpha$  or either GF *versus* plasma hormone levels, we computed the nonparametric Spearman correlation coefficients ( $r$ ). These coefficients are unbiased, but because there were multiple measurements from the same person, the conventional variance estimate of the coefficient estimates was too low. To obtain correct variance estimates and compute  $P$ s for  $r$ , we used a permutation method, which generates an approximate distribution of  $r$  under the null hypothesis (19).

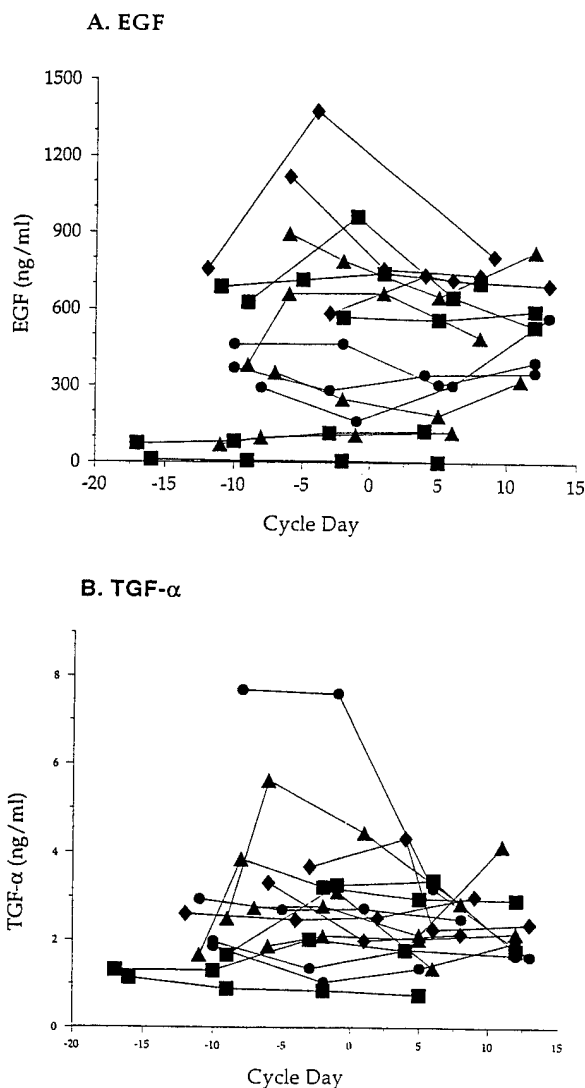


Fig. 2. Concentrations of EGF (A) and TGF- $\alpha$  (B) in breast fluid across the menstrual cycle: repeated measures from 15 women. Midcycle day (day 0) was determined by reverse dating from subsequent menses.

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 SEs 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 designated as the midcycle day: early (days < -10), mid- (days -10 to -6), and late (days -5 to -1) follicular and early (days 0-4), mid- (days 5-9), and late (days 10-13) luteal.  $P$ s for comparison of GF levels by cycle phase were obtained by random effects modeling using PROC GLM in SAS (SAS Institute, Inc., Cary, NC). We also used random effects models, with EGF as the dependent variable, to determine the degree of linear association between EGF and TGF- $\alpha$  and plasma estradiol (20). An inter-

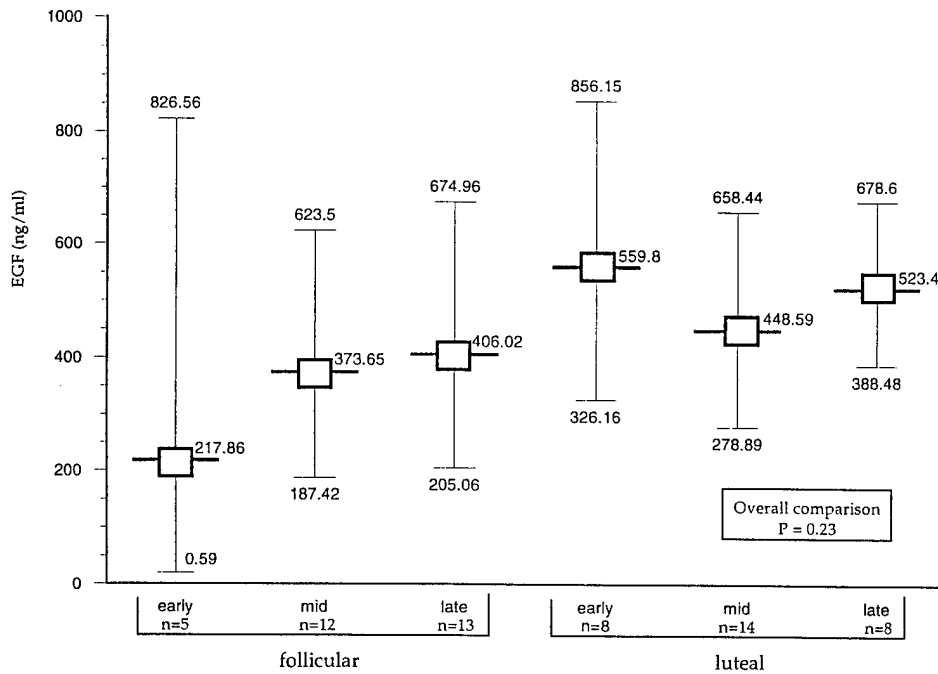


Fig. 3. Mean concentration (plus 95% confidence intervals) of EGF in breast fluid obtained during six phases of the menstrual cycle.

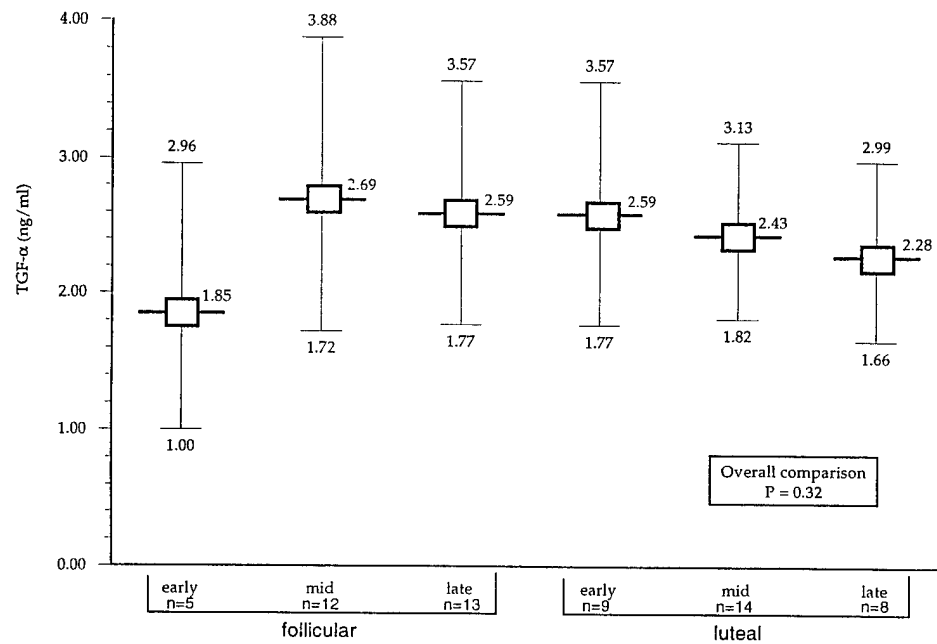


Fig. 4. Mean concentration (plus 95% confidence intervals) of TGF-α in breast fluid obtained during six phases of the menstrual cycle.

action 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. Although we had enough data to estimate the contribution of within-woman correlation to overall model fit, the small number of samples available per woman precluded testing hypotheses about correlation in specific individuals.

### 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 μl of breast fluid, which contained concentrations in the range of 4–6 ng/ml in diluted samples from various QC 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 μl 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 GF levels and results on the variation between the right and left breast are shown in Table 2. EGF levels were unobtainable from both breasts in one woman due to a laboratory error. For EGF, the within-woman variation (between breasts) was considerably less than the variation in EGF levels between women. The ICC implies that 48% of the total variance in EGF could be attributed to between-woman differences. For TGF- $\alpha$ , the within-woman variation between breasts was even lower, and the ratio of between-woman to within-woman variation was even higher. Eighty-eight % of the total variance in TGF- $\alpha$  was attributable to between-woman differences. Table 2 also shows that expressing GF 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).

Fig. 1 displays the agreement in EGF and TGF- $\alpha$  levels between breasts for individual women sampled in both breasts on the same date. Right and left breast levels were well correlated: EGF,  $r = 0.78$  and  $P = 0.003$ ; and TGF- $\alpha$ ,  $r = 0.89$  and  $P = 0.001$ .

Table 3 shows the variation in GF 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 GFs. 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. Fig. 2 shows the EGF (Fig. 2A) and TGF- $\alpha$  (Fig. 2B) 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- $\alpha$  levels for individual women (Fig. 2B) also tend to remain stable over time, although the decreased variation between women, compared to EGF, is evident.

To more closely examine whether GF levels in breast fluid vary in conjunction with the menstrual cycle, we plotted the mean EGF and TGF- $\alpha$  concentrations for six cycle phases, as shown in Figs. 3 and 4. We found no significant differences for either GF 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 midfollicular EGF indicated that the differences were highly compatible with chance ( $P = 0.61$ ).

In Fig. 5, EGF (Fig. 5A) and TGF- $\alpha$  (Fig. 5B) levels are plotted against concurrent total plasma estradiol levels. Neither GF was meaningfully correlated with plasma estradiol ( $r = 0.15$  for EGF and  $r = 0.02$  for TGF- $\alpha$  by the permutation method). We obtained similar results using plasma bioavailable (*i.e.*, non-sex hormone binding globulin bound) estradiol or progesterone instead of total estradiol.

The results shown in Fig. 6 indicate that, when all samples from all women were considered, EGF and TGF- $\alpha$  concentrations in the same sample were not well correlated ( $r = 0.17$ ,  $P = 0.50$ ). However, when we examined the EGF and TGF- $\alpha$  relationship for individual women, some striking covariation was apparent. Table 4 shows results from a random effects

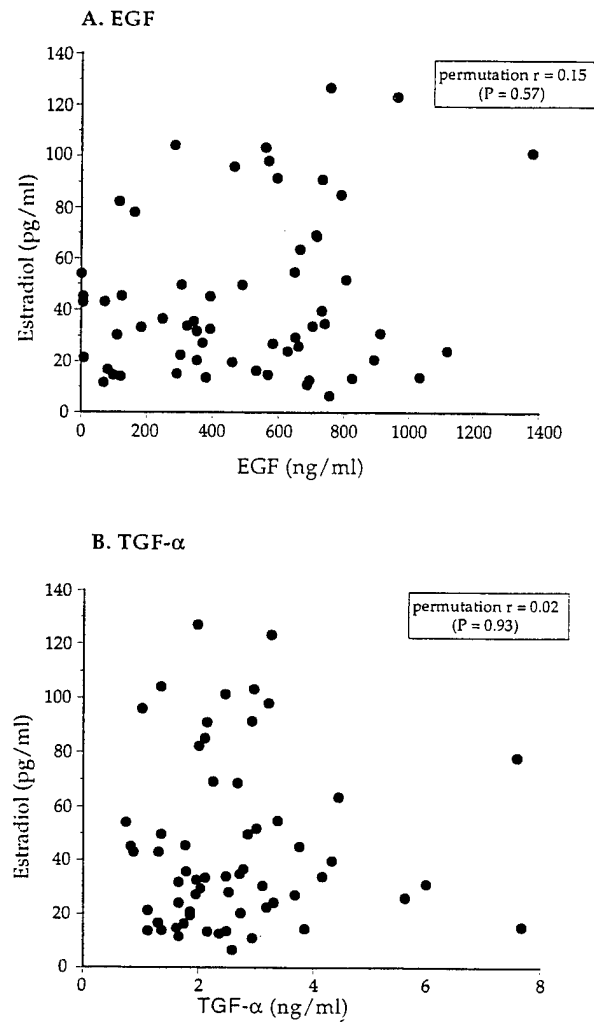


Fig. 5. Plasma estradiol *versus* breast fluid EGF (A) and TGF- $\alpha$  (B): contemporaneous samples from 18 women. Correlation coefficients and  $P$ s determined by a permutation test to account for multiple samples per woman. EGF,  $n = 60$ ; TGF- $\alpha$ ,  $n = 61$ .

model that evaluates the EGF-TGF- $\alpha$  association within women. This model includes a universal coefficient reflecting the common relationship of EGF to TGF- $\alpha$ , 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 Fig. 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 (93.5%) of variance explained by simply specifying the individual subject corroborates the large amount of variation for EGF between women that was seen in the earlier analysis.

Table 5 shows a similar random effects analysis for the relationship of EGF to plasma estradiol. Again, although the overall correlation between EGF and estradiol was poor, the results indicate a significant correlation within individual women ( $P = 0.01$ ). Fifty-eight % of the within-woman variance in EGF was explained by the plasma estradiol values. The within-woman association between breast fluid TGF- $\alpha$  and plasma estradiol was not substantial. Fig. 7 includes graphs of selected participants showing strong covariation between EGF

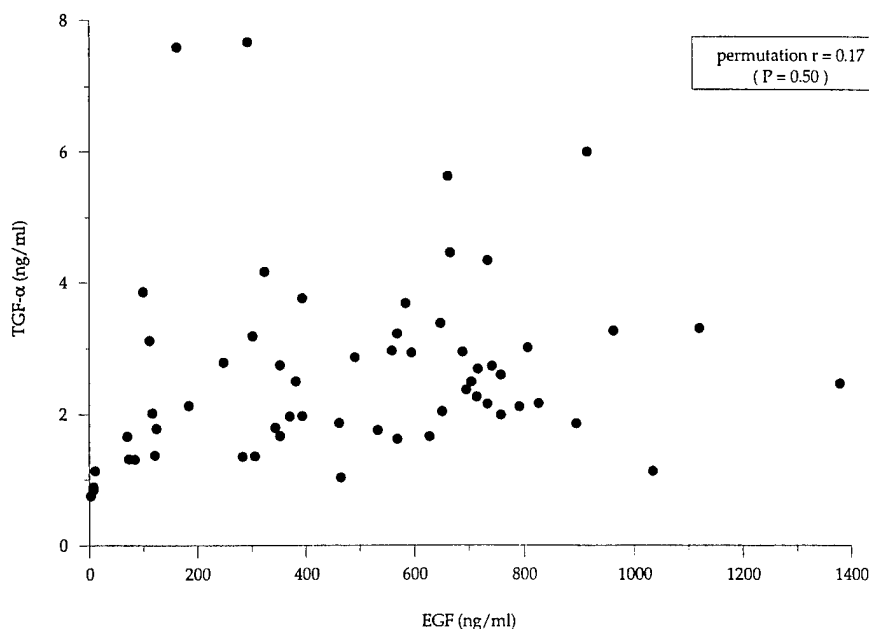


Fig. 6. Scatterplot of EGF and TGF- $\alpha$  concentrations measured in the same breast fluid sample.  $n = 60$ , obtained from 18 women.

Table 4 Random effects model evaluating the associations within individual women between breast fluid EGF and TGF- $\alpha$ <sup>a</sup>

Independent variable	Sum of squares	% variance explained	% within-woman variance explained	P
Individual subject	3808.17	93.49		
TGF- $\alpha$	0.12	0.003	0.05	0.99
Subject $\times$ TGF- $\alpha$	148.0	3.63	55.82	0.02
Error	116.87	2.87		

<sup>a</sup> Model: dependent variable, EGF;  $n = 60$  samples from 18 women.

Table 5 Random effects model evaluating the associations within individual women between breast fluid EGF and total plasma estradiol<sup>a</sup>

Independent variable	Sum of squares	% variance explained	% within-woman variance explained	P
Individual subject	3808.17	93.49		
Plasma estradiol	2.39	0.06	0.92	0.65
Subject $\times$ plasma estradiol	154.66	3.80	58.37	0.01
Error	107.95	2.65		

<sup>a</sup> Model: dependent variable, EGF;  $n = 61$  samples from 18 women.

and TGF- $\alpha$  levels (Fig. 7A) and covariation between EGF and plasma estradiol (Fig. 7B). These participants were not atypical, although the extent of covariation in some individuals appeared to be lower. The small number of replicates per woman, however, prevents us from drawing reliable conclusions about correlation in specific individuals.

## Discussion

Peptide GFs such as EGF and TGF- $\alpha$  are potent signaling 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 GFs themselves or their receptors. Alternatively, because these GFs have a role in normal growth and therefore must be regulated 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, although preliminary to the study of any relationships *in vivo* between breast cancer and GF expression, indicate that immunoreactive EGF and TGF- $\alpha$  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 tend to covary over time with TGF- $\alpha$  and plasma estradiol, within individual women.

The presence of EGF in human milk has been demonstrated previously. In fact, Carpenter (11) reported that 90% of the mitogenic activity of human milk in cell culture systems was negated by a neutralizing antibody for human EGF. Connolly and Rose (9, 10) have reported previously the detection of EGF and TGF- $\alpha$  in breast fluid from healthy premenopausal women. They found concentrations of TGF- $\alpha$  in single samples from 21 women ranging from 0 to 50 ng/ml, with a median of 5.1 ng/ml, levels somewhat higher than we found. They also found EGF levels in samples from 17 women similar to ours; intriguingly, however, 9 of these women with unspecified biopsy-confirmed benign breast disease appeared to have higher EGF levels than the 8 women designated as controls (9). Although the results published previously 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 used highly sensitive RIAs that require only 1 and 4  $\mu$ l of breast fluid for EGF and TGF- $\alpha$ , respectively, and therefore make it possible to obtain measurements even on women with scanty breast fluid samples. The median volume of breast fluid we obtain is approximately 25  $\mu$ l, but the frequency distribution for sample volume is highly skewed, and many women have samples under 10  $\mu$ l. Highly sensitive assay methods are therefore important. We found no evidence for an effect of thaw-refreeze cycles on GF concentrations, nor did we find any evidence for a decay in measured GF during 18 months of storage at temperatures of  $-15$  to  $-20^\circ\text{C}$ .

Several aspects of our findings require further elaboration. In looking at variation over time, we found that be-

**A. EGF vs TGF- $\alpha$**

**B. EGF vs plasma E2**

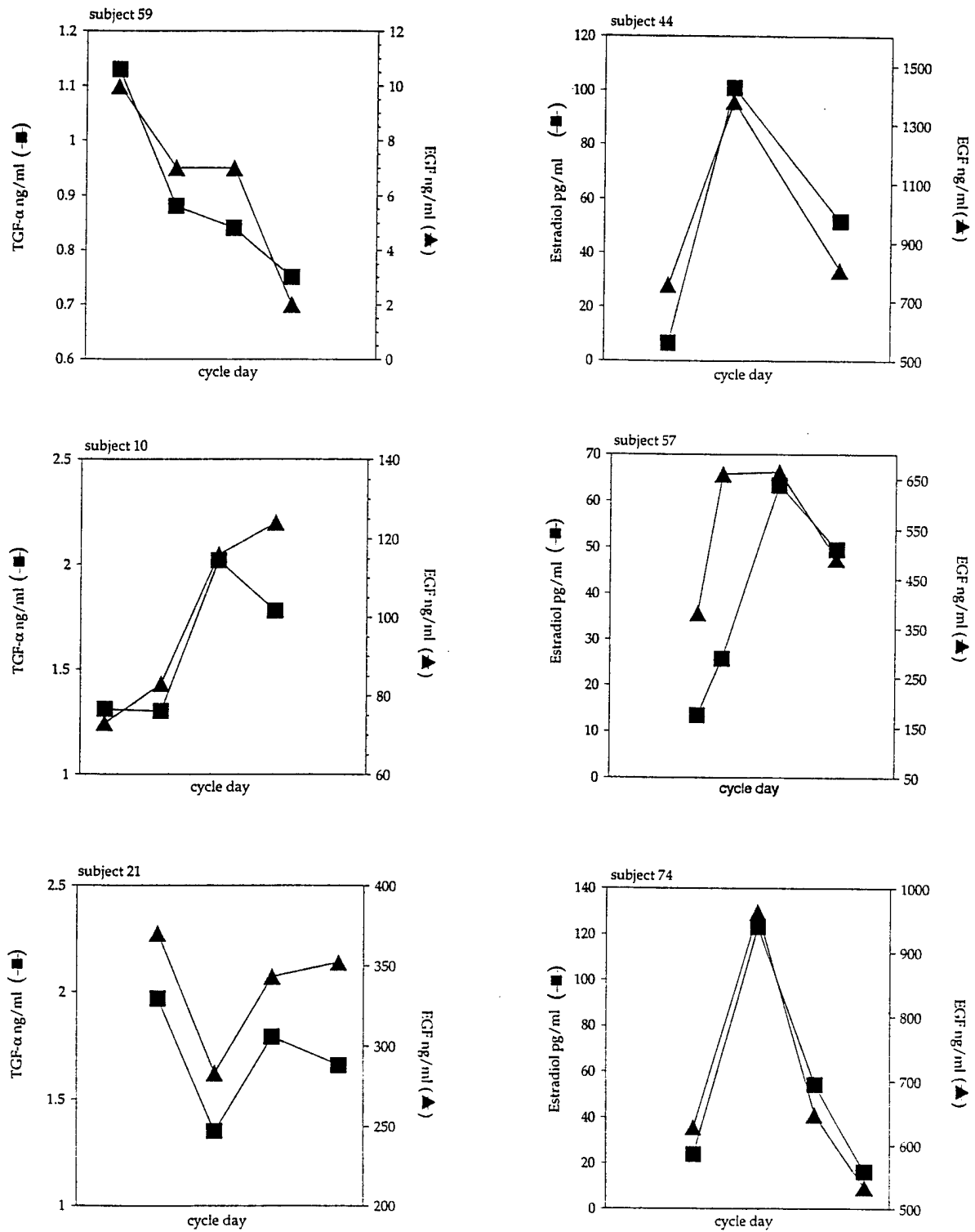


Fig. 7. Breast fluid EGF versus TGF- $\alpha$  (A) and breast fluid EGF versus plasma estradiol (B) across time for selected individuals.

tween- versus within-woman variation was greater for EGF than TGF- $\alpha$ , largely because of a much greater difference between women for EGF. However, TGF- $\alpha$  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- $\alpha$  than for EGF, although the differences between women were once again greater for EGF. Taken together, these findings imply that breast fluid levels of TGF- $\alpha$ , perhaps the more potent of the two GFs, 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- $\alpha$  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- $\alpha$  and estradiol varies from one woman to another, whereas the levels of breast fluid EGF within an individual woman tend to change over time in proportion to changes in TGF- $\alpha$  and plasma estradiol. Correlation analyses composed of samples from multiple women, such as those shown in Figs. 3–6, can fail to reveal these relationships. We conclude that these data provide preliminary evidence that breast fluid EGF and TGF- $\alpha$  are coregulated *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- $\alpha$  levels, or alternatively, estradiol and TGF- $\alpha$  levels could be related but less well synchronized than estradiol and EGF.

One limitation of these studies is that we were able to obtain breast fluid from only 60% of the women on whom nipple aspiration was attempted. Factors related to success in obtaining breast fluid have been studied extensively and appear to include age, parity, lactation history, Asian ethnicity, and cerumen type (21). In this study, such factors did not strongly differentiate those who provided fluid and those who did not; however, the study population was small and relatively homogeneous. Although we cannot rule out the possibility that our findings would not apply to the nonsecretors, had breast fluid been available from them, we find that argument to be implausible. The distinctions between secretors and nonsecretors 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, which is in part genetically determined (22). We found no evidence for a relationship between GF concentration and the amount of breast fluid obtained. Another limitation is that we have not yet confirmed the precise immunoreactive species in each RIA. The kit manufacturer's testing indicates only minimal cross-reactivity with other peptides for the antibodies used in the EGF and TGF- $\alpha$  assays; however, these cross-reactivities were determined for selected peptides that might or might not be present in breast fluid. We are currently conducting Western blot analyses to identify the immunoreactive species by molecular weight and ionic charge.

From these results, it appears that breast fluid EGF and TGF- $\alpha$  could eventually serve as useful biomarkers in studies of breast cancer etiology. Our next series of studies will examine whether GF 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. If altered local GF secretion can be established as a link in the

causal pathway of breast cancer development, breast fluid GF levels could provide novel intermediate end points for the evaluation of suspected risk factors or of interventions designed to reduce breast cancer risk.

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### References

- Dickson, R. B., and Lippman, M. E. Growth factors in breast cancer. *Endocr. Rev.*, 16: 559–589, 1995.
- Lippman, M. E., Dickson, R. B., Bates, S., Knabbe, C., Huff, K., Swain, S., McManaway, M., Bronzert, D., Kasid, A., and Gelmann, E. P. Autocrine and paracrine growth regulation of human breast cancer. *Breast Cancer Res. Treat.*, 7: 59–70, 1986.
- Paik, S., Hazan, R., Fisher, E. R., Sass, R. E., Fisher, B., Redmond, C., Schlessinger, J., Lippman, M. E., and King, C. R. Pathologic findings from the National Surgical Adjuvant Breast and Bowel Project: prognostic significance of erbB-2 protein overexpression in primary breast cancer. *J. Clin. Oncol.*, 8: 103–112, 1990.
- Fox, S. B., Smith, K., Hollyer, J., Greenal, M., Hastrich, D., and Harris, A. L. The epidermal growth factor receptor as a prognostic marker: results of 370 patients and a review of 3009 patients. *Breast Cancer Res. Treat.*, 29: 41–50, 1994.
- Clarke, R., Dickson, R. B., and Lippman, M. E. Hormonal aspects of breast cancer: growth factors, drugs and stromal interactions. *Crit. Rev. Oncol. Hematol.*, 12: 1–23, 1992.
- Dickson, R. B., Gottardis, M. M., and Merlino, G. T. Molecular insights into breast cancer from transgenic mouse models. *Bioessays*, 13: 591–596, 1991.
- Medina, D., Kittrell, F. S., Oborn, C. J., and Schwartz, M. Growth factor dependency and gene expression in preneoplastic mouse mammary epithelial cells. *Cancer Res.*, 53: 668–674, 1993.
- Petrakis, N. L. Nipple aspirate fluid in epidemiologic studies of breast disease. *Epidemiol. Rev.*, 15: 188–195, 1993.
- Rose, D. P. Hormones and growth factors in nipple aspirates from normal women and benign breast disease patients. *Cancer Detect. Prev.*, 16: 43–51, 1992.
- Connolly, J. M., and Rose, D. P. Epidermal growth factor-like proteins in breast fluid and human milk. *Life Sci.*, 42: 1751–1756, 1988.
- Carpenter, G. Epidermal growth factor is a major growth-promoting agent in human milk. *Science (Washington DC)*, 210: 198–199, 1980.
- Ness, J. C., Sedghinasab, M., Moe, R. E., and Tapper, D. Identification of multiple proliferative growth factors in breast cyst fluid. *Am. J. Surg.*, 166: 237–243, 1993.
- Valenti, G., Zanardi, S., Cerruti, G., Fassio, T., Bruzzi, P., DeFranchis, V., Barreca, A., Del Monte, P., and Minuto, F. Epidermal growth factor in breast cyst fluid: relationship with intracyclic cation and androgen conjugate content. *Cancer Res.*, 48: 5860–5863, 1988.
- Wilcosky, T. C. Criteria for selecting and evaluating markers. In: B. S. Hulka, T. C. Wilcosky, and J. D. Griffith (eds.), *Biochemical Markers in Epidemiology*, pp. 28–55. New York: Oxford University Press, 1990.
- Kesaree, N., Banapurmath, C. R., Nanapurmath, S., and Shamanur, K. Treatment of inverted nipples using a disposable syringe. *J. Hum. Lact.*, 9: 27–29, 1993.
- Bonfrer, J. M. G., Bruning, P. F., and Nooljen, W. J. A simple method for the measurement of the steroid fraction bound to sex hormone binding globulin. *J. Steroid Biochem.*, 33: 227–231, 1989.
- Chatterton, R. T., Haan, J. N., Jenco, J. M., and Cheesman, K. L. Radioimmunoassay of pregnanediol concentrations in early morning urine specimens for assessment of luteal function in women. *Fertil. Steril.*, 37: 361–366, 1982.
- Fleiss, J. L. Reliability of measurement. In: *The Design and Analysis of Clinical Experiments*, pp. 1–32. New York: John Wiley & Sons, 1986.
- Good, P. *Permutation Tests: A Practical Guide to Resampling Methods for Testing Hypotheses*, pp. 96–97. New York: Springer-Verlag New York, Inc., 1994.
- Diggle, P. J., Liang, K., and Zeger, S. L. In: *Analysis of Longitudinal Data*, p. 169. Oxford: Oxford University Press, 1994.
- Wrensch, M. R., Petrakis, N. L., Gruenke, L. D., Ernster, V. L., Miike, R., King, E. B., and Hauck, W. W. Factors associated with obtaining nipple aspirate fluid: analysis of 1428 women and literature review. *Breast Cancer Res. Treat.*, 15: 39–51, 1990.
- Petrakis, N. L. Cerumen phenotype and epithelial dysplasia in nipple aspirates of breast fluid. *Am. J. Phys. Anthropol.*, 62: 115–118, 1983.

## Epidermal Growth Factor-Related Peptides in Human Prostatic Fluid: Sources of Variability in Assay Results

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**BACKGROUND.** Prostatic fluid (PF) provides a unique medium for noninvasive evaluation of critical growth and differentiation signals in the prostatic microenvironment. The purpose of this study was to establish the feasibility of measuring two prostatic mitogens, epidermal growth factor (EGF) and transforming growth factor- $\alpha$  (TGF- $\alpha$ ) in PF, and specifically to quantify extraneous variability attributable to the assay itself, sample handling, or biological variation within an individual over time.

**METHODS.** PF was collected by transrectal massage from consecutive patients attending a urology clinic. Pooled PF and individual samples from 25 men with stable benign prostatic hyperplasia (BPH) were analyzed for EGF and TGF- $\alpha$  by radioimmunoassay and for total protein.

**RESULTS.** Reproducibility was adequate at dilutions as low as 1:50 (2- $\mu$ l pooled sample) and 1:5 (20  $\mu$ l) for EGF and TGF- $\alpha$ , respectively. Results were not affected by freeze-thaw cycles, time in storage, or protease inhibition in fresh PF. EGF and TGF- $\alpha$  were detectable in 100% and 92% of individual men, with respective means of 152 and 0.2 ng/ml. Correlations between two samples obtained from the same man within 12 months were highly significant (EGF  $r = 0.89$ , TGF- $\alpha$   $r = 0.71$ ). Protein concentrations were consistent over time; expression of either peptide per weight of protein rather than per volume did not improve within-man correlation. Between-man variability far exceeded within-man variability for both peptides, and was estimated to account for 84% and 61% of the total variability in EGF and TGF- $\alpha$ , respectively. There was no correlation between EGF and TGF- $\alpha$  in the same samples.

**CONCLUSIONS.** We conclude that men with BPH secrete consistent and distinct levels of EGF-related peptides in PF, and that these levels can be detected with acceptable sensitivity

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and precision by radioimmunoassay (RIA). Measurement of TGF- $\alpha$ , which has not been reported previously, requires a relatively larger sample. *Prostate* 32:234–240, 1997.  
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**KEY WORDS:** prostate; epidermal growth factor-urogastrone; transforming growth factor-alpha

## INTRODUCTION

A substantial body of evidence suggests that epidermal growth factor (EGF) and transforming growth factor-alpha (TGF- $\alpha$ ) play a role in controlling the replication of prostatic epithelial cells. These peptides, which both interact with the EGF receptor, are potent mitogenic stimuli for human prostate cells in vitro [1], are overexpressed in cancerous compared to benign prostate [2], and are potentially important in the local mediation of androgen effects in the prostate [3]. Development of a noninvasive tool for assessing growth factor levels would be of considerable benefit to clinical or epidemiological research aimed at identifying exposures that enhance or inhibit prostate carcinogenesis. However, since these growth factors act primarily through autocrine or paracrine processes, systemic levels (as can be measured in serum or urine) are not likely to serve as useful biomarkers. Prostatic fluid (PF) produced by prostatic epithelium provides a reflection of the metabolic status of the prostate, and can be obtained repeatedly from most men by transrectal massage [4].

Although EGF-related peptides have previously been identified in PF, no systematic study of assay variability has been conducted. In this study, we used pooled and individual samples of PF to evaluate the assay sensitivity and degree of variability in immunoreactive EGF and TGF- $\alpha$  measurements attributable to sample handling, variation in total protein, biological variation over time, and the assay procedure itself. Assessment of these sources of variation is critical before we go on to examine the role played by alterations in levels of EGF-related peptides in the development and progression of prostate pathology.

## MATERIALS AND METHODS

### Prostatic Fluid Samples

Prostatic fluid samples collected from consecutive patients seen in our Urology Clinic were examined microscopically for cellular elements, sperm, and seminal vesicle globules. Uncontaminated PF samples were immediately placed in a refrigerator freezer and transported on ice to a  $-20^{\circ}\text{C}$  freezer within 4 hr. The mean sample volume was approximately 75  $\mu\text{l}$ . Samples were entered into a database that includes information on the principal diagnoses at time of col-

lection. For analyses of assay sensitivity and precision, we made several pools of PF, each containing fluid from at least 10 men. For analyses relating to variation over time, we selected samples from 25 men in the sample bank who met the following criteria: 1) no history of prostate cancer or prostatic-specific antigen (PSA) greater than 6 ng/ml, 2) two PF samples of at least 30  $\mu\text{l}$  each obtained on separate visits within 12 months, 3) stable benign prostatic hyperplasia (BPH) with no evidence of changes in symptoms, physical examination, PSA, or therapy during the interval between samples, and 4) no medications that would possibly influence androgen levels. For 2 men we selected a third sample obtained within the 12-month period, thus yielding 23 pairs of samples and two triplets. All but five sets of samples were obtained within 6 months of each other. At sampling, subjects' ages ranged from 54–78 years, with a mean of 68 years.

### Assays for EGF and TGF- $\alpha$

Immunoreactive EGF and TGF- $\alpha$  were measured in prostatic fluid by competitive binding radioimmunoassay (RIA) using commercially available reagents (Biomedical Technologies, Stoughton, MA). Hereafter, the terms "EGF" and "TGF- $\alpha$ " refer to their immunoreactive identities. Prostatic fluid was diluted in a saline-Tris-BSA buffer as provided in the kits for EGF and TGF- $\alpha$  assays. Growth factors were measured in a double-antibody RIA with  $^{125}\text{I}$ -labeled ligands. For each assay run, we included a wide range of purified growth factor concentrations in order to construct a standard curve. The range of standard concentrations was selected to provide at least one standard level below the lowest measurable sample. Occasional samples with concentrations above the highest standard were diluted to bring them within the standard curve range. Total protein assays were done on 18 sets of samples using the standard Coomassie blue method of Bradford, which requires only 4  $\mu\text{l}$  of PF.

To determine assay sensitivity, we made progressive dilutions of pooled PF (from 1:2–1:200), assayed identical aliquots at each dilution level, and calculated the within-assay coefficient of variation (CV) at each dilution. The lowest dilution yielding a CV of less than 15% was used as one indication of assay sensitivity, and was used as the standard dilution for assaying individual samples. Assay precision was determined

by calculating the mean intraassay CV for replicate samples from several assay batches. Interassay CV was determined by comparing results for identical samples of pooled PF inserted into each assay batch. These quality-control (QC) pools were also used to monitor for interassay drift, indicating possible degradation of samples in storage.

#### Effects of Freeze-Thaw Cycles and Protease Activity

To evaluate the effect of freeze-thaw cycles on measured growth factor levels in PF, we thawed and refroze pooled samples 1, 2, 4, and 6 times and then assayed them together. To assess the possible effect of proteases in PF on EGF and TGF- $\alpha$ , we collected fresh PF from 3 men, and immediately divided the PF into a regular tube and a tube containing 100  $\mu$ l of glycine-HCl buffer (0.21% glycine in 0.13 M HCl) to obtain a sample pH of 2.0. This level of acidification is known to inhibit nearly all proteases [5]. Acidified samples were neutralized, and both these and nonacidified aliquots were then assayed together for EGF and TGF- $\alpha$ , as well as PSA.

#### Data Analysis

Intra- vs. interindividual variability was assessed primarily by calculation of intraclass correlation coefficients (ICC) based on pairs of samples from 25 men. For the 2 men with triplet samples, we used the two samples collected closest together in time, unless one of these samples had a missing protein value. The ICC is the proportion of total variance (including between- and within-subject components) contributed by between-subject variability. We calculated the exact lower bound of the 95% confidence interval for each ICC using the method described by Fleiss [6]. We also calculated CVs within and between men and F statistics from a one-way analysis of variance (ANOVA) comparing variance within and between men. We plotted EGF and TGF- $\alpha$  measurements at two time-points and calculated both Pearson and Spearman correlation coefficients. The two types of coefficient were virtually identical; we chose to report the nonparametric Spearman coefficients. For each growth factor, calculations were performed with concentrations expressed per unit volume and per weight of total protein. We used a scatterplot and correlation analysis to compare EGF and TGF- $\alpha$  for the same sample (same date).

## RESULTS

Data shown in Table I indicate that TGF- $\alpha$  in pooled prostatic fluid could be reliably measured with RIA at

**TABLE I. Intraassay and Interassay Coefficients of Variation (CVs) at Various Dilutions of Pooled Prostatic Fluid: TGF- $\alpha$  and EGF**

Dilution	Concentration (ng/ml)	Mean intraassay CV (%)	Mean interassay CV (%)
TGF- $\alpha$			
Undiluted	0.21		5.8%
1:5	0.04	10.3%	16.1%
1:10	0.02	36.9%	
EGF			
1:25	5.82	3.6%	
1:50	3.03	2.7%	7.3%
1:100	1.54	22.6%	29.8%

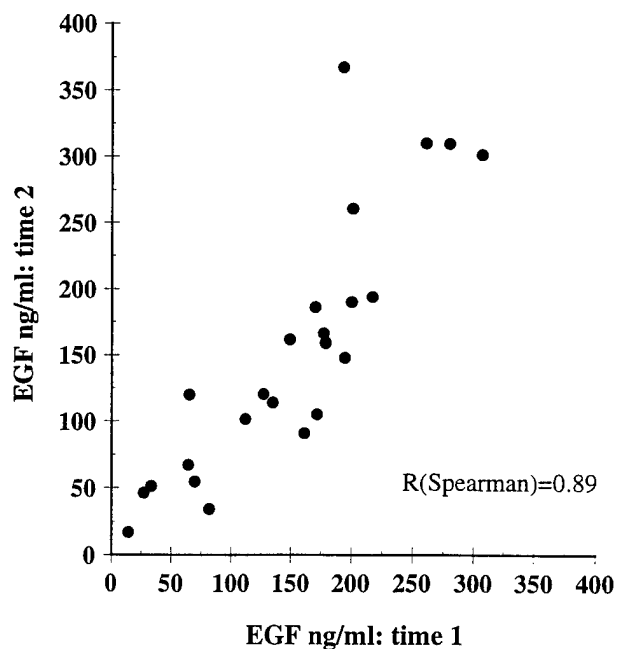
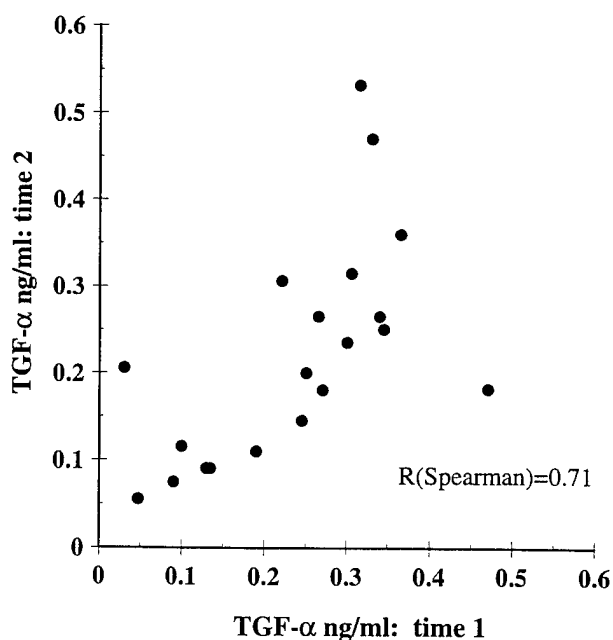
a dilution of 1:5, requiring 20  $\mu$ l of fluid. This dilution corresponded to a concentration of approximately 0.04 ng/ml. Purified TGF- $\alpha$  standards were precisely measured with linear results across a range from 0.015–2.5 ng/ml. EGF could be reliably measured with a 1:50 dilution, which required 2  $\mu$ l of sample and corresponded to a concentration of approximately 3 ng/ml. The assay provided precise and linear results for purified EGF standards at concentrations between 0.25–50 ng/ml. We did not observe a trend towards lower values for either growth factor with progressive freeze-thaw cycles, or a downward trend in values for QC pools assayed up to 11 months apart. Growth factor and PSA levels in fresh PF, acidified immediately after collection, were similar to those in the unacidified aliquots.

Table II shows the comparison of variability within men over time vs. variability between men. TGF- $\alpha$  was not detectable in any sample from 2 men. Three other men had one sample that was considered non-detectable, yielding a total of 20 TGF- $\alpha$  pairs for analysis. Fourteen pairs of samples had both detectable levels of TGF- $\alpha$  and protein levels available. Results are shown both with and without adjustment for total protein concentration. Total protein averaged 15 mg/ml, and was highly correlated when measured at separate time points ( $r = 0.83$ ). EGF was easily detectable in every sample assayed, and at a higher concentration than TGF- $\alpha$  in each sample. Between-man variability was far greater than within-man variability for both growth factors, by several measures. *P* values for the *F* statistics, which test the hypothesis that samples from the same man represent less variability than samples drawn from the entire data set, were all extremely low. The intraclass correlation coefficients indicated that most of the total variance (e.g., 84% for EGF and 61% for TGF- $\alpha$ ) was contributed by variability between men. Expressing results relative to total protein

**TABLE II. Variability Within Individuals Over Time Compared to Variability Between Individuals: Prostatic Fluid TGF- $\alpha$  and EGF**

Reproducibility measure	TGF- $\alpha$	TGF- $\alpha$ /protein	EGF	EGF/protein
No. of sample pairs	20	14	25	18
Mean level	0.23	0.017	152.02	10.56
	ng/ml	ng/mg	ng/ml	ng/mg
SD	0.11	0.014	83.35	4.84
Standard error of measurement	0.07	0.005	33.88	2.27
$CV_{\text{between}}$	0.65	1.12	0.78	0.65
$CV_{\text{within}}$	0.31	0.30	0.22	0.22
$CV_{\text{b}}/CV_{\text{w}}$	2.10	3.73	3.55	2.95
F statistic	4.41	13.97	12.11	9.07
F statistic <i>P</i> value	0.0009	>0.0001	>0.0001	>0.0001
Intraclass correlation coefficient (ICC)	0.61 <sup>a</sup>	0.86	0.84	0.80
ICC 95% confidence interval, lower bound	0.35	0.70	0.72	0.61

<sup>a</sup>ICC = 0.82 (95% CI lower bound = 0.63) when restricted to same pairs used in TGF- $\alpha$ /protein analysis.

**A. EGF****B. TGF- $\alpha$** 

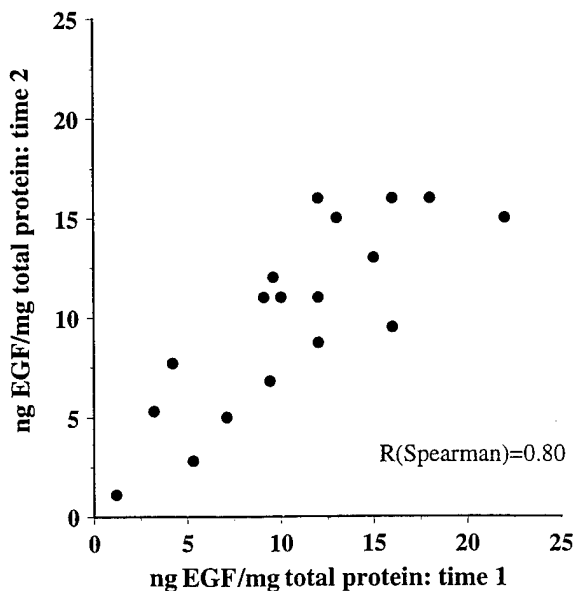
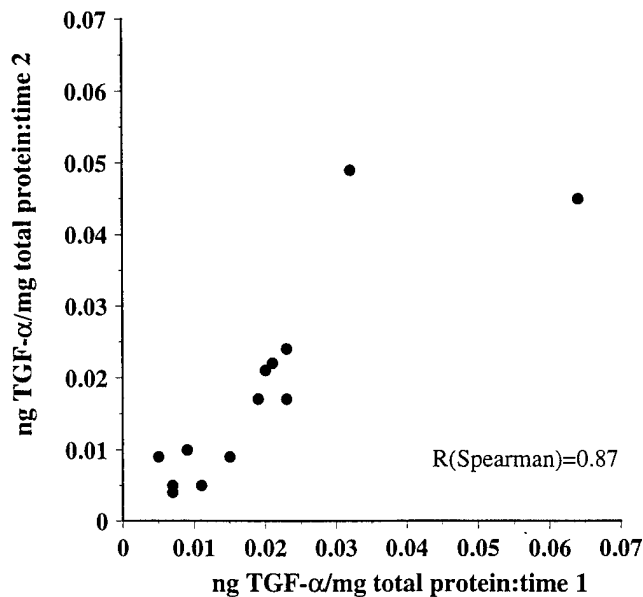
**Fig. 1.** Correlation of **(A)** EGF ( $n = 25$ ) and **(B)** TGF- $\alpha$  ( $n = 20$ ) concentrations in prostatic fluid obtained at two time-points from the same individual.

rather than volume had no discernible effect on the ICC for EGF. The increase in ICC for TGF- $\alpha$  expressed per weight protein occurred because, by chance, several sample pairs that had a missing protein value were less highly correlated. The ICC for TGF- $\alpha$  alone,

using the same pairs as in the TGF- $\alpha$ /protein analysis, was 0.82.

Figure 1 shows scatterplots for EGF (Fig. 1A) and TGF- $\alpha$  (Fig. 1B) measured from the same individual at two time points. Figure 2 shows similar plots for EGF/

## A. EGF / total protein

B. TGF- $\alpha$  / total protein

**Fig. 2.** Correlation of (A) EGF ( $n = 18$ ) and (B) TGF- $\alpha$  ( $n = 14$ ) in prostatic fluid obtained at two time-points from the same individual, expressed per weight of total protein.

total protein (Fig. 1A) and TGF- $\alpha$ /total protein (Fig. 1B). For EGF expressed per unit volume, the nonparametric correlation coefficient was 0.89, and 0.80 for EGF expressed per weight of protein. For TGF- $\alpha$ , the correlation coefficient was 0.71 per unit volume, and 0.87 per weight of protein. The TGF- $\alpha$  per unit volume correlation, with analysis restricted to sample pairs with protein levels available, was 0.86. The growth factor concentrations detected ranged widely, from 14.5–367 ng/ml for EGF and from 0.04–0.47 ng/ml for TGF- $\alpha$ .

Figure 3 shows the scatterplot for EGF vs. TGF- $\alpha$  measured in the same samples from 23 men. These results were not correlated ( $r = -0.16$ ).

### DISCUSSION

Peptide growth factors such as EGF and TGF- $\alpha$  appear to be potent signalling molecules for regulating the growth and differentiation of prostate cells, and, in all likelihood, their abnormal expression plays a role in the carcinogenic process [7]. Abnormal expression could result from mutation of protooncogenes transcribing the growth factors themselves or their receptors. However, research to date has not identified any strong associations between such protooncogenes and prostate cancer [8]. Alternatively, since these growth factors have a function in normal growth and development and therefore must be regulatable by endog-

enous signals, abnormal expression could occur as a result of up- or downregulation of the normal mechanisms for controlling growth factor gene transcription. This view allows that growth factor expression could be altered diffusely in prostatic tissue during the early stages of cancer development. An imbalance of stimulatory and inhibitory signals, for example, could create a "field effect" in which hyperproliferation leads to somatic mutation and clonal selection. Our motivation for studying growth factors in prostatic fluid therefore stems more from an interest in the influence of etiologic factors in the environment (including diet) on growth factor expression than it does from an interest in detecting prostate cancer earlier due to specific patterns of growth factor expression confined to nests of neoplastic cells.

Our results indicate that EGF and TGF- $\alpha$  can be reliably measured in prostatic fluid by radioimmunoassay. TGF- $\alpha$ - and EGF-like material were detectable in nearly all samples, with levels of EGF 700–800 times higher on average than TGF- $\alpha$ . Required sample volumes are small enough to permit analysis of both growth factors in most individual specimens. However, TGF- $\alpha$  will be difficult or impossible to measure in some samples with low concentrations and low sample volume. Men with low sample volumes were excluded from this study.

Levels of TGF- $\alpha$  and EGF in individual men re-

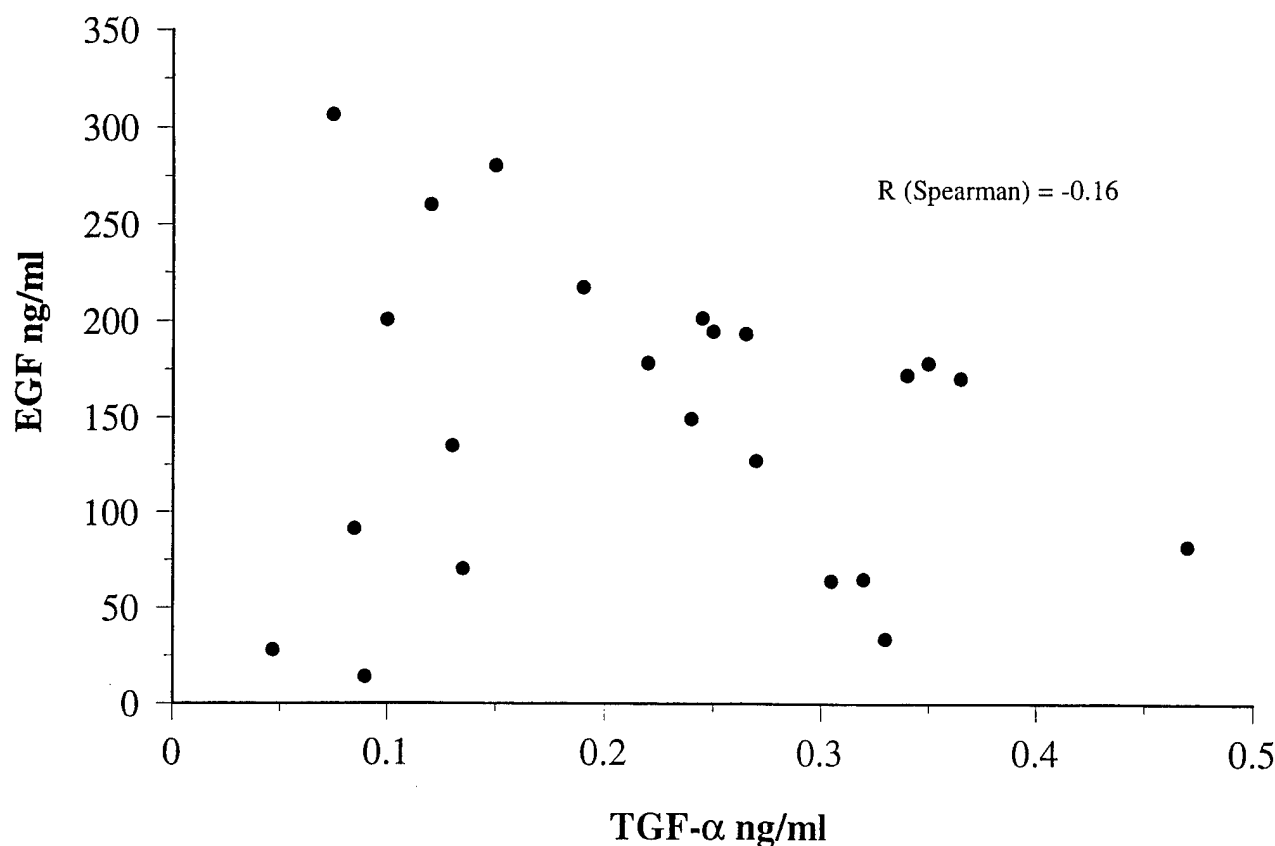


Fig. 3. Correlation of EGF and TGF- $\alpha$  concentrations in the same prostatic fluid sample ( $n = 23$ ).

mained fairly constant when sampled twice within 12 months. Greater variability between vs. within men suggests that individuals can be correctly ranked within study populations, which is vital for epidemiologic analyses. The within-man variability for TGF- $\alpha$  appeared to be reduced by computing the ratio of growth factor to total protein rather than sample volume. However, most of the apparent improvement in correlation, e.g., in comparing Figures 1B and 2B, was due to chance dropout of poorly-correlated observations, because both protein and TGF- $\alpha$  measurements were not available for some samples. The correlation coefficient for TGF- $\alpha$  per unit volume for the same 14 pairs of samples plotted in Figure 2B was 0.86. Measurement of total protein is easily performed with as little as 4  $\mu$ l of sample; however, it does not appear to be helpful.

Levels of TGF- $\alpha$  and EGF in prostatic fluid are not correlated. We previously found them to be highly correlated ( $r = 0.88$ ) in breast fluid. The reasons for this difference need to be explored, because they suggest that in PF, expression of these growth factors might have different regulatory mechanisms. We are currently determining the relationship between growth factor and steroid hormone levels in PF.

Only two previous studies provide data on growth factors in PF. Tackett et al. [9] identified a 30-kD pep-

ptide in PF that was mitogenic to cultured fibroblasts as well as a smaller, unidentified inhibitory peptide. Gregory et al. [10] measured EGF-like material by RIA in PF from men with BPH and clinically normal prostate glands. Among the "normal" men with a mean age of 67.3, the mean EGF concentration was 272 ng/ml, whereas among similarly aged men with BPH the mean EGF was 155 ng/ml, a statistically significant difference. EGF levels in PF did not appear to vary by age among the normals. This study failed to detect EGF-like material in tissue sections, which led the authors to speculate that the prostate does not produce EGF itself, but instead just concentrates and secretes EGF obtained from the blood. BPH could thus involve an impairment in the ability to package and secrete EGF. These intriguing observations on EGF, to our knowledge, have not been subjected to further study. Both EGF- and a TGF- $\alpha$ -like material have been identified in seminal fluid [11]. We were unable to find any data published prior to this report on TGF- $\alpha$  in PF.

The available data on EGF and TGF- $\alpha$  in human prostatic tissue are sparse and difficult to interpret. In one study, EGF was detected by immunohistochemistry in both BPH and carcinoma; TGF- $\alpha$  was detected in carcinoma only [2]. An earlier study reported less frequent detection of EGF in BPH tissue compared to cancer (6% vs. 68%) [12]. Yang et al. [13] measured

EGF/TGF- $\alpha$  in homogenized tissue by RIA and found equivalent amounts of both GFs in BPH and cancer tissue. Since the distribution of these GFs within tissue sections is not homogeneous, assays of total GF per weight of tissue might not reflect the biologically relevant concentrations. Saturation analysis of EGF binding sites on resected human prostate tissue revealed lower levels of EGF binding in samples of containing BPH compared to cancer or histologically normal tissue [14]. Considered together, these results suggest a role for the EGF family in human prostatic disease; however, we do not have enough data yet to formulate detailed hypotheses regarding mechanisms.

This is the first study to report on sources of variability in growth factor measurements in prostatic fluid and the first to report detection of TGF- $\alpha$ . However, we note several limitations to our data. All samples assayed were obtained from men with BPH, because these were the most numerous in the sample bank, and it is possible that assay characteristics are different in men without clinical prostatic disease or those in other age groups. Furthermore, if growth factor levels in PF are associated with the condition of the gland, then interindividual variation in a more general, less highly-selected population would be even greater than our estimates indicate. We limited patients selected to those with stable BPH and a minimal interval between samples. Failure to identify changes in growth factor levels related to disease progression would have led to conservative overestimation of within-man variability. We refer to the measured growth factors as EGF and TGF- $\alpha$ , but do not presume that the immunoreactive species necessarily correspond to pure EGF and TGF- $\alpha$ . Although the antibodies display minimal crossreactivity in other biological media, crossreactivity in PF has not been specifically tested. Furthermore, it is possible that the detectable growth factors include higher molecular weight forms. Earlier investigators identified a 6-kD form consistent with pure EGF in addition to a higher molecular species in PF [10], and only a 6-kD form of TGF- $\alpha$  similar to pure TGF- $\alpha$  in seminal fluid [11]. Our preliminary analyses indicate that the immunoreactive EGF we are measuring includes some higher molecular weight forms. The biological activity of the forms in PF is also yet to be determined.

We are currently pursuing more detailed characterization of the EGF-related species present in PF and are investigating assays for additional growth factors. In the meantime, we conclude that these data support the feasibility of using assays for EGF-like peptides in prostatic fluid as biomarkers in clinical or epidemiological research. We plan to further explore the associations between EGF-like peptides in prostatic fluid and prostate cancer, as well as the identification of

factors influencing growth factor levels, such as local steroid concentrations.

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### REFERENCES

1. Sherwood ER, Lee C: Epidermal growth factor-related peptides and the epidermal growth factor receptor in normal and malignant prostate. *World J Urol* 1995;13:290-296.
2. Myers RB, Kudlow JE, Grizzle WE: Expression of transforming growth factor- $\alpha$ , epidermal growth factor and the epidermal growth factor receptor in adenocarcinoma of the prostate and benign prostatic hyperplasia. *Mod Pathol* 1993;6:733-737.
3. Liu XH, Wiley HS, Meikle AW: Androgens regulate proliferation of human prostate cancer cells in culture by increasing transforming growth factor- $\alpha$  (TGF- $\alpha$ ) and epidermal growth factor (EGF)/TGF- $\alpha$  receptor. *J Clin Endocrinol Metab* 1993;77:1472-1478.
4. Grayhack JT, Lee C, Oliver L, Schaeffer AJ, Wendel EF: Biochemical profiles of prostatic fluid from normal and diseased prostate glands. *Prostate* 1980;1:227-237.
5. Clement-Jones V, Lowry PJ, Rees LH, Besser GM: Development of a specific extracted radioimmunoassay for methionine enkephalin in human plasma and cerebrospinal fluid. *J Endocrinol* 1980;86:231-243.
6. Fleiss JL: "The Design and Analysis of Clinical Experiments," New York: Wiley, 1995:12.
7. Habib FK, Chisholm GD: The role of growth factors in the human prostate. *Scand J Urol Nephrol [Suppl]* 1991;138:53-58.
8. Peehl DM: Oncogenes in prostate cancer: An update. *Cancer* 1993;71:1159-1164.
9. Tackett RE, Heston WDW, Parrish RF, Pletscher LS, Fair WR: Mitogenic factors in prostatic tissue and expressed prostatic secretion. *J Urol* 1985;133:45-48.
10. Gregory H, Willshire IR, Kavanagh JP, Blacklock NJ, Chowdhury S, Richards RC: Urogastrone—Epidermal growth factor concentrations in prostatic fluid of normal individuals and patients with benign prostatic hypertrophy. *Clin Sci* 1986;70:359-363.
11. Yie S, Lobb DK, Clark DA, Younglai EV: Identification of a transforming growth factor alpha-like molecule in human seminal plasma. *Fertil Steril* 1994;61:129-135.
12. Fowler JE Jr, Lau JLT, Ghosh L, Mills SE, Mounzer A: Epidermal growth factor and prostatic carcinoma: An immunohistochemical study. *J Urol* 1988;139:857-861.
13. Yang Y, Chisholm GD, Habib FK: Epidermal growth factor and transforming growth factor  $\alpha$  concentrations in BPH and cancer of the prostate: Their relationships with tissue androgen levels. *Br J Cancer* 1993;67:152-155.
14. Davies P, Eaton CL: Binding of epidermal growth factor by human normal, hypertrophic, and carcinomatous prostate. *Prostate* 1989;14:123-132.