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TITLE: Evaluation of Feasibility for a Case-Control Study of Pituitary-Ovarian Function in Premenopausal Women with Breast Cancer

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CONTRACTING ORGANIZATION: Fox Chase Cancer Center
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14. ABSTRACT

Postmenopausal women with elevated serum estrogens are at an increased risk of breast cancer [1], but an association of serum estrogens with breast cancer in premenopausal women has not been clearly established. This may be partly due to methodological problems related to the dramatic variation in serum concentrations over the menstrual cycle. The purpose of this study was to evaluate variation in responsiveness of ovarian hormones to gonadotropin releasing hormone (GnRH) stimulation in healthy premenopausal women. GnRH stimulation was achieved by subcutaneous injection of a single bolus (1 •g/kg) of leuprolide acetate (Lupron) on days 1 to 5 after start of menses. This dose was chosen to keep serum estradiol levels within the normal range for premenopausal women. Luteinizing hormone (LH) and follicle stimulating hormone (FSH) increased following GnRH stimulation. Estradiol also generally increased following stimulation but there was some variation in the magnitude of the response. Further analyses are planned to identify determinants of the estradiol response to GnRH stimulation in premenopausal women.

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Introduction

Postmenopausal women with elevated serum estrogens are at an increased risk of breast cancer [1], but an association of serum estrogens with breast cancer in premenopausal women has not been clearly established. This may be partly due to methodological problems related to the dramatic variation in serum concentrations over the menstrual cycle. The original purpose of this study was to determine the safety and feasibility of conducting a case-control study that uses gonadotropin releasing hormone (GnRH) stimulation tests to evaluate the sensitivity of the hypothalamic pituitary ovarian (H-P-O) axis in premenopausal women with breast cancer compared to unaffected premenopausal women. However, the study as originally planned was not feasible because women with a history of breast cancer were unwilling to undergo a GnRH stimulation test strictly for research purposes. Therefore, in consultation with our Army Contracting Officer Representative, we modified the scope of work to evaluate variation in responsiveness of ovarian hormones to GnRH stimulation in healthy premenopausal women.

Body

Methods

The study took place at the Fox Chase Cancer Center and included 10 women living in the Philadelphia area who were identified via flyers and newspaper advertisements. To be eligible, women had to be less than 50 years old and healthy, have a regular menstrual cycle lasting 26-34 days, have no known breast pathology, be in the 90^{th} – 130^{th} percentile of ideal weight for height, have not been pregnant or lactating in the past 6 months, have no history of cancer other than non-melanoma skin cancer, agree to abstain from sexual intercourse for 3 days following GnRH stimulation, and not be taking any medications that could interfere with the study.

The study involved participation in three clinic visits. At the first clinic visit, which took place within 2 weeks before anticipated start of next menses, eligibility was determined and a serum pregnancy test was performed. At the second clinic visit, which took place in the afternoon on days 1-5 after start of menses, a second serum pregnancy was performed before GnRH stimulation. A single bolus (1 μg/kg) of leuprolide acetate (Lupron) was injected subcutaneously. This dose was chosen to keep serum estradiol levels within the normal range [2]. Blood was collected by venipuncture immediately before Lupron injection for baseline and at 60 minutes after Lupron injection to measure readily releasable gonadotropins and steroids [2]. At the third clinic visit, which took place 16 ±4 hours after Lupron administration, blood was collected to measure newly synthesized gonadotropins and steroids [2].

All hormones and cytokines were measured in serum. Blood was allowed to sit at room temperature for a minimum of 60 minutes before serum was separated, aliquoted into cryovials and stored at -80°.

All hormone assays were performed at the Reproductive Endocrinology Laboratory, University of Southern California under the direction of Dr. Frank Stanczyk. Estradiol and testosterone were quantified by validated, previously described radioimmunoassays (RIAs) [3-5]. Prior to RIA, steroids were extracted from serum with hexane:ethyl acetate (3:2). The steroids were then

separated by Celite column partition chromatography using either trimethylpentane, toluene in trimethylpentane, or ethyl acetate in trimethylpentane. Leutenizing hormone (LH), follicle stimulating hormone (FSH), insulin, insulin-like-growth-factor- 1 (IGF-1), IGF binding protein 3 (IGFBP-3), and sex hormone binding globulin (SHBG) were quantified by direct chemiluminescent immunoassays using the Immulite analyzer (Diagnostic Products Corporation, Los Angeles, CA). Leptin was measured by direct RIAs using kits obtained from Diagnostic Systems Laboratory (Webster, TX). Activin A and mullerian inhibitor substance (MIS) were measured by direct enzyme-linked immunosorbent assays (ELISAs) using kits obtained from Diagnostic Systems Laboratory. IL-6 and TNFα levels were determined by direct ELISAs using kits obtained from R&D (Minneapolis, MN).

All the immunoassay methods were shown to be reliable. Specificity was achieved by use of highly specific antisera and/or use of organic solvent extraction and chromatographic steps prior to quantification of the analytes. Assay accuracy was established by demonstrating parallelism between measured concentrations of a serially diluted analyte in serum with the corresponding standard curve. Intraassay and interassay coefficients of variation ranged from 4 to 8% and 8 to 13%, respectively.

Preliminary analyses presented in this report include graphical displays of data, estimation of means and standard deviations, and tests for differences in analyte levels at the various time points by repeated measures analysis of variance (ANOVA) implemented by SAS Proc Mixed. More detailed analyses are planned prior to publication.

Results

Basal and GnRH stimulated hormone concentrations for each participant are plotted in Figure 1. As expected, the gonadotropins, LH and FSH, rose significantly following GnRH stimulation. The overall test of differences in means concentrations at the three time points was significant for both (LH: $F_{2/9}$=25.86, p=.0002; and FSH: $F_{2/9}$=108.45, p<.0001). Although there were some differences in patterns of response, LH rose significantly from a mean of 5.4 mIU/ml at baseline to 27.26 mIU/ml at 60 minutes post GnRH stimulation (p=.003) and continued to rise to a mean of 46.69 mlU/ml at 16 hours (p=.0001 for change from baseline and p=.07 for change from 60 minutes post GnRH). FSH also rose significantly from 7.22 mIU/ml at baseline to 11.68 mIU/ml at 60 minutes (p=.01), and continued to rise to a mean of 21.77 mIU/ml at 16 hours (p=.0001 for change from baseline and p=.03 for change from 60 minutes). Thus, GnRH stimulation resulted in increased serum levels of both readily releasable and newly synthesized LH and FSH.

GnRH stimulation increased ovarian synthesis of the ovarian steroid hormones estradiol and testosterone. The mean serum estradiol level was unchanged from baseline at 60 minutes post GnRH stimulation but increased significantly to a mean of 184.19 pg/ml at 16 hours (p=.01 from baseline). There were some notable differences in the strength of the estradiol response to GnRH, with some women responding very little and others exhibiting a 2-to-3 fold increase. Similar to estradiol serum testosterone concentrations were unchanged from baseline at 60 minutes post GnRH stimulation, but were significantly elevated at 16 hours post GnRH reaching a mean of 37.24 ng/dl (p=.004).

Mean serum activin-A levels did not differ at the three time points ($F_{2/9}$=0.87, p=.45). However, patterns of activin-A response to GnRH varied considerably between women. Mean inhibin-B levels also were similar at the different time points ($F_{2/9}$=2.38, p=.15), but patterns of response to GnRH were variable.

MIS levels at the different time points were similar for each woman ($F_{2/9}$=0.54, p=.60), but there was considerable variation between women in MIS concentrations; the range extended from 0 to about 5 ng/ml.

Further analyses are planned to evaluate effects of age, weight, and menstrual cycle day of GnRH stimulation on response to GnRH stimulation as well as whether results varied by serum IGF-1, IGFBP-3, insulin, leptin, TNFα, IL-6, and SHBG levels (Figure 2).

Key Research Accomplishments:

- The goal of this pilot study was to determine the feasibility of conducting a case-control study to compare response of hypothalamic-pituitary-ovian (HPO) axis to GnRH stimulation in breast cancer patients vs. healthy controls. We demonstrated that such a study is not feasible at our institution because it was not possible to recruit breast cancer patients and it was very difficult to recruit healthy controls.

- The major reason women gave for not being interested in participating in the study was they did not want to take any drugs.

- The research team gained experience working together.

Reportable Outcomes

- We plan to write a manuscript reporting our results. Based on our current findings, it is likely that the focus of that manuscript will be on variation in serum estradiol responses to GnRH stimulation and factors that affect this variation such as age, menstrual cycle day of stimulation, weight, levels of insulin, IGF-1, IGFBP-3, SHBG, leptin, TNFα, IL6 and MIS.

- Based on what we learned about ovarian physiology while conducting this study and from other sources, we submitted a grant for a pilot study and obtained funding from the Fox Chase Cancer Center Ovarian Cancer SPORE to evaluate the long-term within person stability of mullerian inhibiting substance (MIS) to assess whether a study of basal MIS in relationship to cancer etiology is feasible. That study currently is underway.

- The knowledge that we gained through this project and other sources about hypothalamic-pituitary-ovarian function will be useful for developing new research projects related to breast and possibly ovarian cancer.

Conclusions

There is considerable between person variation in estradiol response to GnRH stimulation. Although the association of serum estradiol with breast cancer risk in premenopausal women has not been clearly established, most breast tumors are estrogen dependent and similar to postmenopausal women, premenopausal women with eleveated serum estradiol levels likely are at excess risk of breast cancer. Regrettably, we were unable to recruit breast cancer patients to compare HPO responsivity to GnRH as originally planned. However, identifying individual characteristics and serum biomarkers that influence estradiol responsivity to GnRH stimulation in healthy premenopausal women will increase our understanding of the regulation of HPO activity and could be informative about factors that potentially increase breast cancer risk via effects on the HPO axis.

References:

1. Endogenous sex hormones and breast cancer in postmenopausal women: reanalysis of nine prospective studies. J Natl Cancer Inst 2002;94:606-16.

2. Rosenfield RL, Perovic N, Ehrmann DA, Barnes RB. Acute hormonal responses to the gonadotropin releasing hormone agonist leuprolide: dose-response studies and comparison to nafarelin--a clinical research center study. J Clin Endocrinol Metab 1996;81:3408-11.

3. Goebelsmann U, Bernstein GS, Gale JA, Kletzky OA, Nakamura RM, Coulson AH, Korelitz JJ, al. e. Serum gonadotropin testosterone estradiol and estrone levels prior to and following bilateral vasectomy. In Lepow IH, Crozier R, editors. Vasectomy: Immunologic and pathophysiologic effects in animals and man. New York: Academic Press; 1979. p. 165.

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5. Scott JZ, Stanczyk FZ, Goebelsmann U, Mishell DR, Jr. A double-antibody radioimmunoassay for serum progesterone using progesterone-3-(O-carboxymethyl) oximino-[125I]-iodo-histamine as radioligand. Steroids 1978;31:393-405.

List of Personnel

- Dorgan, Joanne F. - Principal Investigator
- Weil, Susan – Co-Investigator
- Godwin, Andrew – Co-Investigator
- Moore, Dirk – Co-Investigator
- Stanczyk, Frank – Co-Investigator

Appendices

Email from Wendy Baker, USAMRAA approving request for change in work statement.

Supporting Data

Figure 1. Hormonal Responses to GnRH Stimulation in Premenopausal Women

Legend: Hormone concentrations 1 minute before GnRH stimulation (-1 min) and at 60 minutes (60 min) and 16 ±4 hours (16 hr) after GnRH stimulation in 10 healthy premenopausal women. Data for each woman is shown in a different color.

Figure 2. Serum IGF-1, IGFBP-3, Insulin, Leptin, TNF-α, IL-6 and SHBG Concentrations in Premenopausal Women

Legend: IGF-1, IGFBP-3, Insulin, Leptin, TNF-α, IL-6 and SHBG concentrations before GnRH stimulation. Data for each woman is shown in a different color.

From: Baker, Wendy A Ms USAMRAA [wendy.cockerham@us.army.mil]
Sent: Thursday, February 16, 2006 1:18 PM
To: Dorgan, Joanne F.
Cc: Sells, Mary Ann; Emgushov, Lisa; Moore, Katherine H Dr USAMRMC
Subject: RE: DAMD 170110236

Dr. Dorgan~

The GOR has now approved your request to utilize the remaining funds on this grant for the collection of pilot data on regulation of ovarian function in healthy premenopausal women by ?-endorphin, leptin, mullerian inhibiting substance, and possibly circulating cytokines. This approval is granted on the basis that it does not require additional sampling and the existing consent forms allow for additional analyses of the samples. This is now approved for implementation.

Regards,

Wendy Baker
Contract Specialist

-----Original Message-----
From: Dorgan, Joanne F. [mailto:JF_Dorgan@fccc.edu]
Sent: Wednesday, December 21, 2005 2:20 PM
To: 'kathy.moore@us.army.mil'
Cc: Baker, Wendy A Ms USAMRAA; Sells, Mary Ann; Emgushov, Lisa
Subject: FW: DAMD 170110236

Dear Dr. Moore,

This is to follow-up on our telephone conversation earlier today about my DOD Idea Award (DAMD 170110236). The proposed analysis of additional biomarkers would not require recruiting additional participants nor would it require collection of additional specimens. The analyses would be done using samples that we have already collected from participants and stored.
The informed consent document states that the samples may be used for other purposes and that the participant will not receive notice of any future use.
I hope that this clarifies the issues involved. Please inform me how I should proceed.

Thank you for your assistance,
Joanne Dorgan

-----Original Message-----
From: Baker, Wendy A Ms USAMRAA [mailto:wendy.cockerham@us.army.mil]
Sent: Thursday, December 15, 2005 1:00 PM
To: Dorgan, Joanne F.
Cc: Sells, Mary Ann; Emgushov, Lisa
Subject: RE: DAMD 170110236

Hi Joanne,

After conferring with the GOR, it has been determined that if you are able to do this new analysis on samples collected as part of your currently approved protocol, then this would be a good use of the funding. If a new protocol is needed, the GOR does not recommend approval of the revision to the project. Please advise.

Regards,

Wendy Baker
Contract Specialist

-----Original Message-----
From: Dorgan, Joanne F. [mailto:JF_Dorgan@fccc.edu]
Sent: Wednesday, December 07, 2005 4:55 PM
To: Baker, Wendy A Ms USAMRAA
Cc: Sells, Mary Ann; Emgushov, Lisa
Subject: DAMD 170110236

I received a DOD Idea Award in 2001 for a proposal entitled 'Evaluation of Feasibility for a Case-Control Study of Pituitary-Ovarian Function in Premenopausal Women with Breast Cancer' (DAMD 170110236). The objective of that study was to evaluate the feasibility of conducting a case-control study that uses gonadotropin releasing hormone (GnRH) agonist stimulation tests to compare pituitary-ovarian function in premenopausal women with
breast cancer with unaffected controls. The pilot study involved recruiting 10 premenopausal women with breast cancer and 10 healthy control women. Because of IRB concerns, the study was conducted in phases; the first phase included only healthy control women and the second phase included women with a history of breast cancer. We

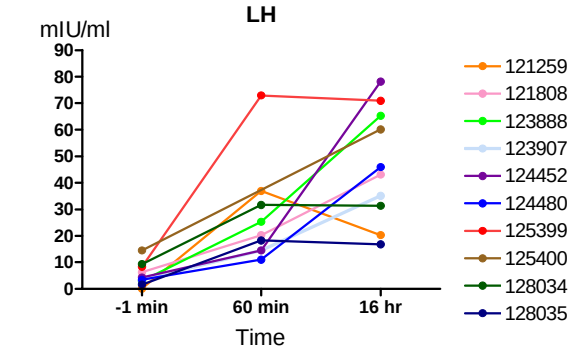
completed the first phase and the Data and Safety Monitoring Board at FCCC and the DOD IRB approved continuing to the second phase. However, although we have advertised widely, we have not had any success in recruiting for the second phase. Thus, we have concluded that a full-scale study is not feasible.

Some funds still remain in the budget and we propose that they be used for an analysis that is different from but related to our previous project.
Specifically, we propose that the remaining funds be used to collect pilot data on regulation of ovarian function in healthy premenopausal women by ?-endorphin, leptin, mullerian inhibiting substance, and possibly circulating cytokines. These data could be used to support a prospective study of serum levels of these biomarkers with breast cancer risk in premenopausal women and/or a genetic epidemiology study focused on polymorphisms in these genes in relationship to breast cancer risk.

Please let me know if you agree with this suggestion and how I should proceed.

Thank you for your assistance,
Joanne Dorgan

Figure 1. Hormonal Responses to GnRH Stimulation in Premenopausal Women



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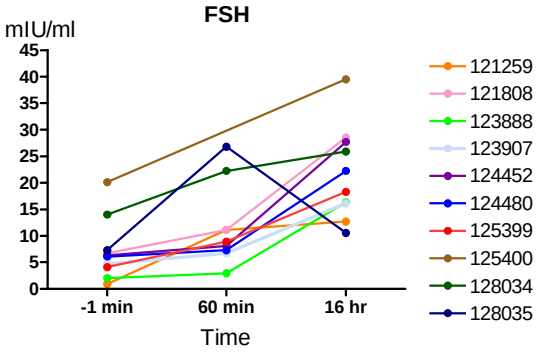
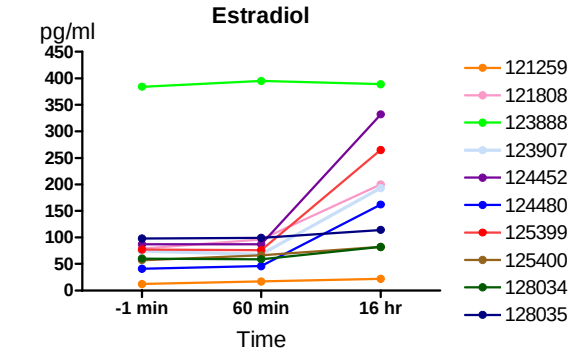
Std Dev:	4.28	19.10	21.27



	-1 min	60 min	16 hr
Mean:	7.22	11.68	21.77
Std Dev:	5.75	7.76	8.79



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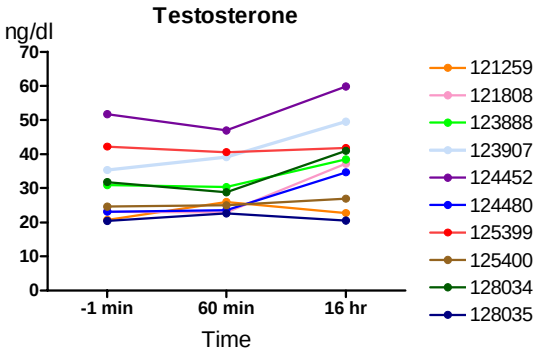
Mean:	96.87	100.82	184.19
Std Dev:	103.66	106.14	116.98



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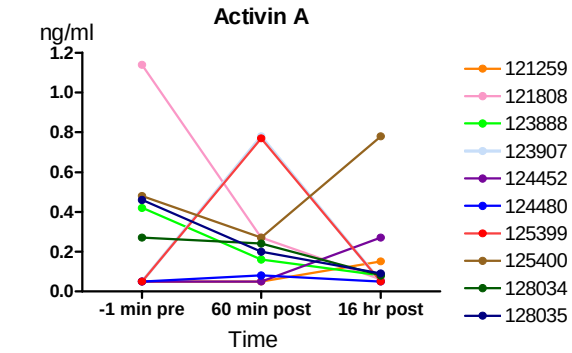
Std Dev:	10.30	8.60	12.02



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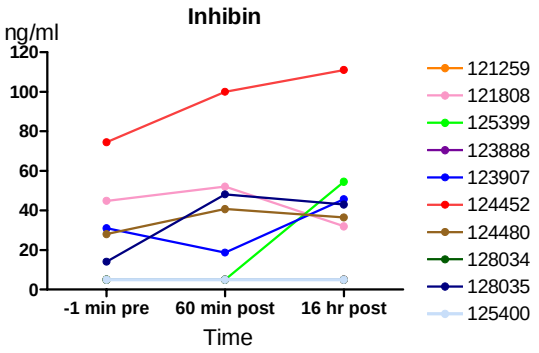
Mean:	0.30	0.29	0.16
Std Dev:	0.35	0.27	0.23



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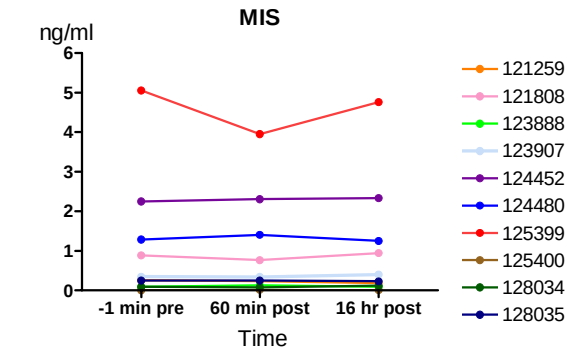
Mean:	21.75	28.49	34.34
Std Dev:	23.34	31.77	33.35



	-1 min pre	60 min post	16 hr post
Mean:	1.05	0.95	1.03
Std Dev:	1.57	1.28	1.50

Figure 2. Serum IGF-1, IGFBP-3, Insulin, Leptin, TNF-α, IL-6 and SHBG Concentrations in Premenopausal Women