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PRINCIPAL INVESTIGATOR: Giske Ursin, M.D., Ph.D.

CONTRACTING ORGANIZATION: University of Southern California
School of Medicine
Los Angeles, California 90033

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13. Abstract consists of 3 separate projects; The first aims at assessing the association between lifestyle factors (hormone use) and breast cancer; the second aims at elucidating the role of estrogen metabolism in the development of breast cancer; the third aims at understanding the relation of female hormone levels to changes in mammographic densities, a possible intermediate endpoint in cancer prevention studies. The first project uses data from a case-control study of Asian-American immigrants where the relative risks associated with oral contraceptive use will be assessed. The second project consists of two studies; a case-control study of postmenopausal breast cancer patients and a case-control study of premenopausal women with "high" and "normal" risk of breast cancer. Urinary estrogen metabolites will be compared to test the hypothesis that the 16-alpha pathway of estrone metabolism is associated with breast cancer risk. Project 3 is 2 studies of hormone induced changes in mammographic densities. These studies will test the hypothesis that reducing serum estrogen and progesterone levels reduces mammographic densities.				
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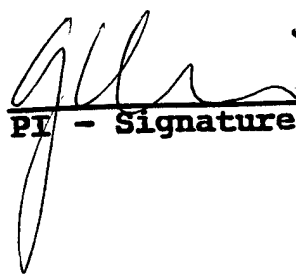
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(5) Introduction:

Project 1: Case-control study of breast cancer in Asian-American

The incidence rates of breast cancer vary substantially between countries. Rates in Asia have consistently been much lower than those in the U.S. and Western-Europe (1-3). Around 1985, breast cancer incidence rates were 2.5-4 times higher in the U.S. than in the Philippines, Japan and China (1). There is substantial evidence that when Chinese, Japanese and Filipina women migrate to the U.S., their risk of breast cancer increases over several generations and approaches those of white U.S. women (4-8). Ziegler et al. have previously reported that risk of breast cancer increases rapidly in a population of Asian immigrants the first decade after they arrive in the US (9). This increase in breast cancer risk may be due to changes in the prevalence of known risk factors for breast cancer. Known or suspected risk factors for breast cancer include early age at menarche, nulliparity, late age at first birth, late age at menopause, postmenopausal weight, hormone use and dietary factors (10).

By studying immigrants at various stages of acculturation in a case-control study of breast cancer, it is possible to determine how much of the increase in rates is due to differences in known risk factors. In the same population of Asian-American immigrants as studied by Dr. Ziegler, Wu et al. (11), studies have found that although reproductive factors are associated with risk of breast cancer in this population, changes in reproductive factors cannot explain the increased risk of breast cancer occurring the first decade after migration to the US. The question remains whether this risk increase can be explained by lifestyle factors.

Although initially reassuring, a number of recent studies have found an association between oral contraceptive use and breast cancer, either overall or in subgroups of women. Some studies have suggested an increased risk of breast cancer in young women associated with long-term use or use starting at an early age (12-17). Because oral contraceptive (OC) use is very uncommon in Asia, and very common in the US, we hypothesized that OC use could explain the increase in breast cancer risk observed in these Asian immigrants the first decade after they arrive in the US.

The research question for this project is to quantify the relative risk of breast cancer associated with oral contraceptive use in this population of Asian immigrants (13,14) and to examine how much of the difference in breast cancer risk observed in recent versus long-term immigrants can be explained by differences in patterns of oral contraceptive use.

Project 2a. Estrogen metabolism in breast cancer cases and controls

There is considerable evidence that increased serum estrogen levels are associated with an increased risk of breast cancer in postmenopausal women (18-28); and increased urinary excretion rates of estrogen (estrone, estradiol and estriol conjugates) in postmenopausal breast cancer cases compared to controls have also been consistently found (29-36). In addition to increased serum estrogen levels, it has also been suggested that the pathway by which estrone (E1) is metabolized may be important in determining breast cancer risk (37-38). Of the two main metabolic pathways for E1, the 16-hydroxy pathway yields biologically active products, while the 2-hydroxy pathway yields metabolites which are essentially devoid of estrogen activity (39-41). The suggestion is

that an elevated rate of 16-hydroxylation as demonstrated by an increased ratio of urinary 16-alpha-hydroxyestrone (16 α OHE1) to urinary 2-hydroxyestrone (2OHE1) is associated with an increased risk of breast cancer (37-38, 42-43). However, the epidemiologic data to support this hypothesis are sparse, consisting of a solitary case-control study of 9 perimenopausal and 24 postmenopausal breast cancer cases and 10 postmenopausal controls (42); where the ratio of 16 α OHE1 to 2OHE1 was 31.3% in the breast cancer cases and 23.0% in the controls. There are no data evaluating whether the ratio 16 α OHE1/2OHE1 is a risk factor independent of total urinary estrogens. Demonstrating a difference in 16 α OHE1/2OHE1 between breast cancer cases and controls, especially if independent of total urinary estrogens, would be important for the understanding of breast cancer pathogenesis. It could also be very important as regards future prevention strategies because there is evidence that the pathway of E1 metabolism may be altered by dietary (in particular, cruciferous vegetables) and other factors (44-48).

The research question for this project is to examine whether the ratio of urinary 16 α OHE1 to 2OHE1 is higher in postmenopausal breast cancer cases than in controls; and whether the ratio is higher after adjusting for other breast cancer risk factors including total urinary estrogens.

Project 2b. Estrogen metabolism in women at high and low risk of breast cancer.

In a study by Osborne et al. (43), 17 women at 'high risk' of breast cancer (family history of breast cancer or epithelial atypia in previous biopsy) were compared with women without high risk lesions or a family history ('low-risk' controls). The comparison of urinary 16 α OHE1 to 2OHE1 was very similar to that found in the case-control study of Schneider et al. (42). No further details regarding the study subjects were provided, and no other studies have been reported attempting to confirm or refute this finding. In this project we plan to investigate the ratio of urinary 16 α OHE1 to 2OHE1 in women who have a strong family history of breast cancer, and to compare the results to those from control women. As 'cases' we use premenopausal daughters or sisters of women diagnosed with either premenopausal bilateral breast cancer before the age of 50, or unilateral breast cancer before the age of 40. These 'cases' are at increased risk of breast cancer (relatives of premenopausal bilateral breast cancer cases have been estimated to be at as much as a 5-fold increased risk of breast cancer compared to women with no such family history (52-53)).

The research question for this project is to examine whether the ratio of urinary 16 α OHE1 to 2OHE1 is higher in premenopausal women at high risk of breast cancer than in 'normal risk' women; and whether the ratio is higher after adjusting for other breast cancer risk factors including total urinary estrogens.

Project 3a: Changes in mammographic densities associated with surgical menopause

This project has been changed to: "Changes in mammographic densities in women on a gonadotropin-releasing hormone agonist contraceptive regimen". The reason for this change was: 1) problems with the originally proposed project and 2) very interesting

preliminary mammographic findings in the pilot-study of this contraceptive regimen that suggested that these mammographic data would warrant further investigation.

The problems associated with the originally proposed project were as follows: First, the project was never funded. Second, a number of problems arose while we were conducting a pilot-study for this project. We had proposed to conduct the study at the Los Angeles County Women's Hospital. At the time when this project was developed, the gynecologists there frequently performed bilateral oophorectomies on premenopausal women undergoing hysterectomies. However, this policy has now changed; bilateral oophorectomies are no longer routinely done on premenopausal women undergoing hysterectomies. This obviously made it almost impossible to find eligible women. It took 6 months to recruit 4 women for our pilot study. Further, in November 1994, California voters passed Proposition 187 which requires hospitals to report individuals who are illegally in the United States to the Immigration and Naturalization Services. The patients base for Women's Hospital is mainly Hispanic (>65%). Although Proposition 187 has not yet gone into effect, it has caused fear and resentment in the Hispanic population, making recruitment to, and compliance with, study protocols at the Women's Hospital even more difficult. Finally, the fiscal problems of the Los Angeles County Medical Center this year has complicated matters substantially for projects at the Women's Hospital.

The project that replaced the oophorectomy project arose from a study we completed last year (51). In this project we found a decrease in mammographic densities associated with use of a gonadotropin releasing hormone agonist (GnRHA). This project examines changes in mammographic densities with artificially induced menopause. The background for this new project is described below.

New Project 3a: Changes in mammographic densities in women on a gonadotropin-releasing hormone agonist contraceptive regimen

There is substantial epidemiological and experimental evidence that ovarian hormones (in particular, estrogens, and possibly progesterone) increase the risk of breast cancer (52-54). Although certain serum levels of ovarian steroid hormones are necessary for optimal health, premenopausal women who are not trying to conceive appear to require considerably less of these hormones than is produced by the ovary (55-56). Spicer and Pike have developed a gonadotropin releasing hormone (GnRH) agonist plus ultra-low-dose estrogen and ultra low-dose progestogen hormonal contraceptive regimen, that attempts to reduce the levels of estrogen and ultra-low-dose progestogen to a minimum, while still preserving the essential beneficial effects of estrogen (56-57). With the use of ultra-low-dose estrogen and progestogen alone ovarian function is not blocked by these sex steroids as with standard hormonal contraceptives; blocking of ovarian function is achieved through the use of the GnRH agonist (GnRHA) which results in the suppression of pituitary follicle stimulating hormone (FSH) and luteinizing hormone (LH) release. Sufficient estrogen is given to prevent hypo-estrogenic symptoms (such as hot flashes); and intermittent progestogen is given to prevent any estrogen-induced endometrial hyperplasia. Since the GnRH agonist also blocks testosterone production from the ovary, sufficient testosterone is also included in the regimen to just replace that no longer produced by the ovary.

The regimen substantially lowers the levels of female sex hormones. Such a regimen may provide long-term reduction in breast cancer risk by reducing breast cell division (55,56). The effects of this regimen on densities in the mammographic image of the breast taken after being on the regimen for 1 year have previously been reported (51). A large reduction of parenchymal densities in the mammograms was observed in the women who have been on the regimen for 1 year.

Mammographic densities have been demonstrated to be significantly associated with breast cancer risk, independent of other breast cancer risk factors, with higher densities being associated with up to a 5-fold increase in risk (58-61). Mammographic densities appear to decline with menopause (60) and increase in women who take hormonal replacement therapy.

We hypothesize that mammographic densities reflect the endogenous hormone milieu in the breast and that these densities are reduced (as compared to baseline) as long as women are on the GnRHA regimen (and the serum levels of female sex hormones are lowered), and increase again once the regimen is stopped (and the serum levels of sex hormones return to normal). In other words, mammographic densities after 2 years on the regimen should also be reduced compared to baseline, and the densities 1 year after end of treatment should be higher than during the time of treatment. If the regimen does have the hypothesized effects on mammographic densities, then this adds to the evidence that mammographic densities reflect the hormonal milieu of the breast, and may be useable as intermediate endpoints for breast cancer risk in chemoprevention studies.

The research question for this project is to examine whether women receiving a regimen consisting of a gonadotropin-releasing hormone agonist (GnRHA) to completely suppress ovarian function, conjugated estrogens at a daily dose of 0.625 mg and intermittent (14 days every fourth 28-day cycle) and medroxy-progesterone acetate for 2 years have lower mammographic densities than at baseline, and whether the mammographic densities increase again once these women come off the regimen.

Project 3b: Changes in mammographic densities during the normal menstrual cycle

The results from cross-sectional studies of the effect of menopause and from the pilot GnRHA-based prevention trial suggest that mammographic parenchymal patterns change with endogenous hormone levels. The observed changes in the GnRHA trial were in women who had been on the regimen for 1 year. However, the changes may be discernible much sooner. In this study I will investigate whether mammographic changes occur with the changing hormone levels occurring during the menstrual cycle.

There appears to have been no previous systematic study of the effects of menstrual cycle on mammographic patterns, but a single case demonstrating a significantly greater amount of densities in the breast in the late luteal phase was reported by De Paredes (62); other mammographers have reported to us that they have knowledge of such cases, but we have been unable to find any further documentation. If there are changes in mammographic densities during the natural menstrual cycle, then this would imply that studies of mammographic densities in premenopausal women should obtain mammograms for comparison at the same day in the cycle. This would also imply that

studies that examine changes in densities over time should take into account recent exposures, and that intervention studies aimed at reducing mammographic densities should expect to see changes after a relative short period. A rapid change in mammographic densities will also make it possible to realistically study over a short period of time with cross-over type of designs the effects of different HRT regimens on breast mammographic patterns.

The research question for this project is to examine whether there are observable changes in mammographic densities during the menstrual cycle.

(6) Body

Project 1: Case-control study of breast cancer in Asian-Americans

METHODS

This project is based on data from a completed case-control study where cases were all women of Chinese, Japanese or Filipina ethnicity diagnosed with histologically confirmed breast cancer between age 20 and 55 in San Francisco, Los Angeles and Hawaii between 1983 and 1987 (NIH grant: N01 CP95659). Controls were selected by random digit dialing methods in Los Angeles and San Francisco, and from the annual household sample survey in Hawaii. The data collection is complete, and the data set contains information on 597 cases and 966 controls. An in-person interview was conducted. The standardized questionnaires included questions on residence history, menstrual, reproductive history, anthropometric variables and diet at three different time periods. Details can be found in Ziegler et al., (9). Odds ratios of breast cancer associated with oral contraceptive use were estimated using logistic regression, adjusting for potential confounders in the model (70). Statistical analyses were performed using SAS (SAS Institute Inc., Cary, NC) and EPILOG (Epicenter Software, Pasadena, CA).

RESULTS

Prevalence of OC use was lowest among women who have been in the US for less than 7 years; only 20% had ever used OCs, and less than 10 % had used OCs for more than one year. Asian-Americans born in the US had the highest prevalence of OC use: 46% had ever used OCs. There was, however, no increased risk of breast cancer associated with duration of OC use, nor with an early age at start of OC use, or with recent use of OCs. Dr. Ziegler reported that the risk of breast cancer essentially doubled the first decade after migration to the US (9). Adjusting these relative risk estimates for oral contraceptive use had no effect. This suggests that oral contraceptive use cannot explain the rapid increase in risk that occurs the first decade after Asian women migrate to the US. These data were presented at the Society for Epidemiologic Research earlier this year (63), and a manuscript will be prepared shortly.

Project 2a. Estrogen metabolism in breast cancer cases and controls

METHODS

I was awarded a small grant to perform this study (DAMD17-94-J-4289). Postmenopausal subjects, who are participants in an ongoing case-control study of breast cancer at our institution (NIH grant: 5 P01 CA17054) are eligible for inclusion in this study. Exclusion criteria are: having: ever - been treated with chemotherapy, or been diagnosed with systemic lupus erythematosus or liver cirrhosis; over the previous 3 years - smoked; over the past 6 months - used medications that may interfere with estrogen metabolism (estrogen, progesterone, tamoxifen, cimetidine, carbamazepin, phenytoin, barbiturates, thyroxin, corticosteroids or omega-3 fatty acid supplements); over the past 3 months - had general anesthesia; and currently - weighing more than 200 lbs. The cases in the ongoing case-control study are incident cases of histologically confirmed breast cancer, and were ascertained through the population based cancer registry for Los Angeles County (Los Angeles County/University of Southern California Cancer Surveillance Program, LACCSP, a NCI SEER registry). Controls were matched to cases by age and neighborhood of residence. My role will be to conduct the E1 metabolism study in this population.

Early morning urine samples are collected from 100 breast cancer cases and 100 controls. The following urinary metabolites are determined: 16 α OHE1, 2OHE1, estrone (E1), estradiol (E2) and estriol (E3). The 16 α OHE1 and 2OHE1 are determined by enzyme immunoassay by Dr. Leon Bradlow at the Strang-Cornell Cancer Research Laboratory in New York. E1, E2 and E3 conjugates are determined by radioimmunoassay in the laboratory of Dr. Frank Stanczyk at Los Angeles County/USC Women's Hospital. Data on current body weight, recent diet and alcohol consumption are also being collected in order to be able to study possible effects of these factors. Dietary intake is assessed with a semi-quantitative food frequency questionnaire developed by Dr. Walter Willett at Harvard University (64-65).

Results will be analyzed statistically using t-tests and standard analyses of covariance techniques, as well as logistic regression. The values of some of the hormone measurements will need to be log transformed before analyses to achieve approximate normality of results. In the logistic regression, the odds ratio per unit increase in 16 α OHE1, 2OHE1 and 16 α OHE1/2OHE1 (with and without adjustment for urinary E1, E2, E3 and other known risk factors (in particular, weight and menstrual and reproductive history)) will be calculated.

RESULTS

We have so far contacted 407 cases and 445 controls. Responses have been obtained from approximately 700 women so far. Approximately 170 subjects (20%) have been found to be eligible so far. Major reasons for ineligibility are tamoxifen use (35% of cases) and estrogen use (38% of controls). Currently, urine samples have been collected from 157 subjects (71 cases and 86 controls).

The first batches of urine samples have been sent to Dr. Bradlow in New York and Dr. Stanczyk at USC for analysis. Preliminary results of urine samples from the first 55 subjects did not yield significant differences between cases and controls on 16 α -2-OHE1. (The differences between cases and controls on E1, E2, E3 or the combination of the three were not quite statistically significant). Our study coincides with a reproducibility/validity study of the EIA

assays of 16 α - and 2-OHE1 conducted by Dr. Regina Ziegler, NCI. Based on Dr. Ziegler's results, the 16 α - and 2-OHE1 assays have during the past few months undergone adjustments to account for the lower levels of estrogens in urine of postmenopausal women (Leon Bradlow, personal communication). We are currently waiting for the final results from Dr. Ziegler's reproducibility/validity study before we submit further urinary samples for analysis. Because of these laboratory problems, we requested (and obtained) a 1 year no-cost extension for this grant; the current grant ends September 30, 1996; the project should be completed by that time.

Project 2b. Estrogen metabolism in women at high and low risk of breast cancer.

METHODS

I was awarded a small grant to perform this study (DAMD17-94-J-4231). 'Cases' in this study are premenopausal sisters and daughters of patients with a) premenopausal bilateral breast cancer who participated in a genetic-epidemiologic study (66), or b) unilateral breast cancer before age 40 who participated in a breast cancer case-control study. Eligible women have never themselves been diagnosed with breast cancer. 'Controls' are daughters or sisters of women participating as controls in the breast cancer case-control study (67-68) or in the Woman's CARE study (P.I. Leslie Bernstein). As in project 2a, exclusion criteria are: having: ever - been treated with chemotherapy, or been diagnosed with systemic lupus erythematosus or liver cirrhosis; over the previous 3 years - smoked; over the past 6 months - used medications that may interfere with estrogen metabolism (estrogen, progesterone, tamoxifen, cimetidine, carbamazepin, phenytoin, barbiturates, thyroxin, corticosteroids or omega-3 fatty acid supplements); over the past 3 months - had general anesthesia; and currently - weighing more than 200 lbs.

RESULTS

We have so far contacted 461 women with premenopausal uni- or bilateral breast cancer and 413 controls. One hundred and eighty-one of these cancer cases and 124 controls had at least one daughter or sister between the ages of 20 and 50 living in California (total of 262 case daughters or sisters and 205 control daughters or sisters). We have contacted all of these case and control daughters and sisters. If there is more than one daughter/sister in each family, we will include the youngest one above age 20 if eligible. This means that we have to await a response from the younger potential eligible member before we decide who should be included in the study. To date we have obtained responses from approximately 180 case daughters or sisters and 140 control daughters or sisters. Since only one member from each family is eligible, this means that we have a total of 78 eligible case daughters or sisters and 28 eligible control daughters or sisters. Major reasons for ineligibility include current oral contraceptive use (30%), current smokers (10%), other medications (5-10%), irregular periods (10% of controls), currently pregnant/breastfeeding (10% of controls). We have so far collected urines on 70 case daughters or sisters and 20 control daughters or sisters. We are contacting eligible women on a regular basis to remind them of contacting us when their menstrual period begins. However, we are now revising our procedure, and will in the next couple of months, make a major effort to increase the number of control participants.

We have not started shipping urines to the laboratories for two reasons: first because of problems obtaining controls, and second because as a result of Dr. Ziegler's validation study (see 2a, above) the 16 α - and 2-OHE1 assays are undergoing adjustments in order to also be used in

postmenopausal women. This means that the current assay may need further adjustments before it can be used in premenopausal women. The new, adjusted assays are currently being validated (both for pre- and post-menopausal women) by Ziegler and colleagues at NCI. We have therefore decided to wait with sending further samples until the results of Dr. Ziegler's study are available. Because of the problems in identifying controls, and because of these problems with the laboratory assays, we have requested (and have obtained) a 1-year no-cost extension of this grant; the current grant ends September 30, 1996; the project should be completed by that time.

Project 3a: Changes in mammographic densities in women on a gonadotropin-releasing hormone agonist contraceptive regimen

METHODS

Scanning of Mammograms: The mammograms are scanned with an Omnimedia XRS scanner and the software Adobe Photoshop, version 3.0 with a specially designed plug-in program for the scanner. The mammograms are scanned at 150 dpi. The scanning procedure takes about 3-4 minutes per mammogram. Using Adobe photoshop all personal identifying information is deleted from the image. The mammographic image is subsequently saved on Bernoulli disks identified only by the study ID.

Mammographic Density Determinations: The density assessments have so far been performed by myself as well as two radiologists at our institution (Drs. John Pearce and Yuri Parisyk). We use a computer-assisted quantitative measure of the amount of radiologic densities from digitized cranio-caudal mammographic images. Byng and his colleagues have published such a method, an interactive thresholding technique (69), and we have developed and validated a measure which closely follows their approach. The software program we have developed works as follows: In the digitized mammographic image, the user colors yellow all pixels she (or he) considers represent mammographic densities using a mouse to drag a specific coloring key. We use a system based on 256 different gray levels, with 0 being the darkest value and 255 the whitest. The user colors yellow the pixels between 255 and X (where X is any color value selected by the user between 0 and 255) using the coloring key. The pixel count (corresponding to the area colored) can then be recorded from the screen. The area of the breast is later determined using standard outlining tools in Adobe Photoshop.

Statistical analysis of this study will be done using standard methods (70,71). Statistical analyses will be performed using the SAS (SAS Institute Inc., Cary, NC) and EPILOG (Epicenter Software, Pasadena, CA). We will treat mammographic densities as a continuous and as a categorical value. We will consider both absolute amount and relative amount (% of breast) of mammographic densities.

RESULTS

We have developed and validated a computer-assisted quantitative measure of mammographic densities (see above). Our method closely follow the approach of Byng and his colleagues (69). The Canadians have demonstrated that their method yields results very similar to that of a traditional six category subjective classification (61). We have also compared our method to the amount of mammographic densities determined using a subjective classification. We invited Ms. Salane, Wolfe's Radiological Services, Detroit to USC to read a set of our mammograms. Ms. Salane has extensive experience in determining the amount of mammographic

densities from mammograms (72,73). Our method was found to be highly correlated with Ms. Salane's density determinations (Salane vs. Ursin $r = 0.8$), and I have obtained high within observer correlations ($r > 0.9$). We are still in the process of improving the technique in order to obtain even better correlations with Ms. Salane. We are also testing the effects of using different types of scanners, different exposure settings at the time of scanning, and different computer hardware (monitor and videocard) at the time of density assessment.

The GnRHA pilot study has now been completed. Currently mammograms at year 2 are available for all but 2 women in the trial, and 1 year post-treatment mammograms are available for more than 2/3 of the participants. We are currently attempting to localize the missing mammograms. All available mammograms have now been scanned. I have read the baseline and 1-year mammograms for these women using our new computerized system twice, and obtain the same results as those in the original (51) publication (unpublished data). The remainder of the mammograms will be read shortly. This project should be completed within the next year.

Project 3b: Changes in mammographic densities during the normal menstrual cycle
METHODS/RESULTS:

This study has not been funded yet. However, I have conducted a pilot-study where I examined mammographic changes in 10 volunteer premenopausal women with regular menstrual cycles. The mammograms were obtained in the follicular (day 7-10) and late luteal phase (days 24-27) of the same cycle. Blood samples were drawn at the time of the second (luteal phase) mammogram. I scanned the mammograms on the XRS Omnimedia scanner (as described in methods for 3a). I deleted all personal identifiers from the mammograms. The mammograms were saved with a code number. Although I knew which mammograms belonged together, I coded the mammograms so that, when I read them, I was blinded for the time at which each mammogram was obtained. I determined the densities of these mammograms twice using the method described in 3a.

There was a non-significant increase in mammographic densities from the follicular to the luteal phase of the cycle. This increase was on average approximately 10%. These results will be used in a NIH grant application to obtain funds to conduct the larger study.

(7) Conclusion (of all five projects)

One change has been made in the original project plan (project 3a); instead of examining mammographic changes with surgical menopause, the current project deals with mammographic changes associated with artificial menopause or chemoprevention. Overall, the work so far is in concordance with the original proposed timeplan. Note that instead of waiting with planning/executing projects 3a and 3b until the first projects were completed, all projects were started this first year. As a result, more time was spent on these projects (3a and 3b) than anticipated this year. Therefore project 1 has not yet been completed. However, the data analysis for project 1 is almost complete, and the manuscript will be written up shortly. Projects 2a and b are currently running according to the timeplan.

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