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FOREWORD

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Esther C. J. Snowley Sept 8, 1997
PI - Signature Date

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Introduction

Recent work has defined a previously unsuspected involvement of vitamin D in cellular growth and differentiation^{1,2}. This recognition has fostered an interest in the investigation of a possible role for vitamin D in carcinogenesis³. Several cancers have been evaluated for a relationship between tumor occurrence and low levels of both the active and precursor metabolites of vitamin D. There is a suggestion in the literature that decreased intake of vitamin D may be associated with cancer of the colon⁴; blood levels of the active vitamin D metabolite, 1,25-dihydroxyvitamin D (1,25-D), are lower in African-American and white men with cancer of the prostate than in similar men without prostate cancer⁵.

Evidence for the possible relationship between vitamin D and breast cancer is based on several lines of investigation. Ecologic studies generally support a relationship between low levels of sunlight exposure and breast cancer^{6,7,8}. A single case-control study in Canada, however, failed to demonstrate an association between low levels of vitamin D as determined by dietary history, and breast cancer⁹. Studies in rats show an increased incidence of mammary tumors under conditions of low dietary vitamin D (0.05 IU/kcal) and calcium (0.25 mg/kcal)¹⁰. 1,25-D, the hormonally active metabolite, displays a growth inhibitory effect on human breast adenocarcinoma cells irrespective of their sex-steroid dependence¹¹. Cultures of the human breast cancer cell line BT-20 demonstrate increased differentiation when exposed to 1,25-D daily for 8 days¹².

The purpose of our current work was to determine whether there are differences in blood levels of 1,25-D between women with breast cancer and two control groups of women without breast cancer. We used archived samples of blood obtained from women in a recently-completed case-control study of genetic risk factors for breast cancer. Control subjects were frequency matched to cases on age (within three years) and race; in addition, control group 1 was matched on clinic and month of blood drawing. Control group 2 subjects came from a general medical clinic and had non-breast-related complaints. The study was restricted to females aged 21 or older; women were excluded from the study if

study was restricted to females aged 21 or older; women were excluded from the study if there was a current or past diagnosis of epithelial hyperplasia, with or without atypia.

We also attempted to modify the immunohistochemical technique for detection of vitamin D receptors (VDR) described for frozen tissue for use with paraffin embedded tissue. This would have enabled us to evaluate the VDR status of tumor tissue from our subjects with breast cancer. Our results with immunohistochemical staining have been variable and to date, we have been unable to successfully analyze breast tumor tissue for VDRs.

We were, however, able to analyze our study population's blood for polymorphisms in the gene for the VDR and to investigate the effect of genotype on the vitamin D metabolite blood level/breast cancer relationship. This investigation was made possible through a collaboration with Drs. Jack Taylor and Doug Bell at the National Institute of Environmental Health Sciences. We did not request money from the DOD for this task and did not use DOD money for the polymorphism determinations.

The specific aims of our study were as follows: 1) To determine if blood levels of 25-hydroxyvitamin D (25-D) and 1,25-D are lower in women at the time of first diagnosis of breast cancer than in comparable women who do not have breast cancer; 2) To describe the relationship between levels of 25-D and 1,25-D and previously described risk factors for breast cancer. We were able to successfully complete these aims. However, we were unable to accomplish our two specific aims related to identification of VDRs in breast tumor tissue because of difficulties in developing a specific immunoassay: 3) To identify and quantify the distribution of vitamin D receptors in tumor samples from the women diagnosed with breast cancer; and 4) To identify the relationship between previously described risk factors for breast cancer and blood level of 25-D and 1,25-D with VDR (+) and VDR (-) breast cancers.

Body

Task 1: Analysis of Vitamin D metabolites.

We have completed analysis of the vitamin D metabolites for the entire group of 511 samples. Our vitamin D assays were done in 13 batches of 40 samples each with an admixture of randomly selected samples consisting of approximately one-third cases and one-third controls from each of the two control groups. We used the calf-thymus radioreceptor assay from INCSTAR (Stillwater, Minnesota) for determination of 1,25-D and the radioimmunoassay from INCSTAR (Stillwater, Minnesota) for determination of 25-D. Fourteen percent of the samples had to be reassayed because of technical difficulties such as problems with recovery.

A summary of the mean levels of the precursor metabolite, 25-D, and the active metabolite, 1,25-D, as well as median values and ranges by race are presented in Table 1. Eight Asian-American, one Native-American, and five Hispanic women were excluded from the analyses because their numbers were too small to provide meaningful results. This brings the number of subjects evaluated in Table 1 to 497.

There was a significant difference in 1,25-D pg/ml blood levels between white cases and each control group; there were no differences in mean values of 25-D ng/ml. There were no statistically significant differences in blood levels of either vitamin D metabolite between black cases and either black control group. The data are consistent with previously reported racial differences in vitamin D metabolite levels, with blacks subjects often having lower levels of 25-D and higher levels of 1,25-D than white subjects.

The analyses of the vitamin D metabolites support the research hypothesis in white women: subjects with breast cancer have lower blood levels of 1,25-D at the time of first diagnosis of their disease than comparable women without breast cancer. We did not see the same relationship between 1,25-D level and disease risk in the small number of black women in this study.

Task 2: Development of Assay for VDR.

During the past three years we have been trying to establish methods in our laboratory for the specific and sensitive immunolocalization of the vitamin D receptor (VDR) in both frozen and fixed tissue specimens. Although our goal is to identify VDRs in breast tumor tissue, our efforts have been focused on establishment of the assay with tissue specimens from normal duodenum. This tissue contains VDRs and is a "positive control". We have met with numerous difficulties during this process, most of which have been attributed to the lack of specificity of the available antibodies. To date, we have had mixed success with obtaining specific immunostaining. We have eliminated the background problem that was initially encountered but now have non-specific staining in the cell cytoplasm of the sections. The changes we have made in the protocols are described below. Dr. H.F. DeLuca provided us with other, more specific antibodies against the VDR: V-D2F12 and IV-G8C11. These are mouse monoclonal antibodies that have been shown to be specific for and have high affinity against the VDR. These have been used extensively in Western blot analysis and ELISA by Dr. DeLuca's group for quantification of extracted VDR. Our initial results were very positive with these antibodies and both antibodies appeared to give specific nuclear staining in the sections. These results, however, have been variable and to date, no breast tissues have been successfully analyzed. Current efforts focus on the competition of the binding with purified VDR to show specificity and optimization of assay conditions to reduce non-specific binding. Although there is no further funding from the DOD for this project, we plan to continue these analyses and bring them to completion with the addition of funds from other sources. Results from the continuation of these studies will be forwarded to the DOD at their completion.

The following methods for immunohistochemical localization of VDR in frozen sections of human gut have been used during the past year. Frozen specimens of normal human intestine have been obtained from Dr. K. Lund, Department of Pathology, UNC-CH. The specimens were divided in two, with one piece frozen in embedding medium and

the other fixed in 10% neutral buffered formalin (the slides have been stored at 4⁰ C for later use). 5 micron serial sections were cut from the frozen specimens on a cryostat and mounted onto superfrost plus slides (Fisher Scientific). Slides were then stored at -80⁰ until staining. All incubations were carried out in a humidified chamber. At the time of immunostaining, slides were fixed for 10 minutes in a neutral buffered formalin according to the method of Milde, *et al.*¹³. Following washes with Tris buffered saline (TBS) (pH=7.4), slides were treated with ice cold methanol and acetone for 3 and 1 minutes, respectively. After rinsing with TBS, sections were blocked by incubation for 15 minutes each with the following agents: 5% normal goat serum, avidin (5mg/ml) and biotin (5 mg/ml). Endogenous peroxidase activity was inhibited by incubating sections with 0.3% hydrogen peroxide for 20 minutes. Slides were then incubated with primary antibody, either Mab 9A7g (rat monoclonal) or one of the two mouse monoclonal antibodies against the VDR supplied by Dr. DeLuca, V-D2F12 and IV-G8C11, at dilutions of 1:50 and 1:100 overnight at 4⁰ C. After incubation, slides were washed with TBS and incubated for 30 minutes with biotin-labeled secondary antibody (goat anti-rat or goat anti-mouse) at room temperature. After two rinses with TBS, slides were treated with streptavidin-peroxidase for 30 minutes at room temperature. After four rinses with TBS, staining was visualized with 0.05% diaminobenzidine with 0.04% hydrogen peroxide solution (DAB) for 15 minutes. Slides were viewed to determine adequate color development and then rinsed, dehydrated and cleared prior to placement of coverslips. In all cases, controls were run in which primary antibody was omitted.

As mentioned above we were able to obtain specific nuclear staining with this method and are now in the process of showing the specificity of this staining by competition with purified VDR. Once these experiments are complete, we will begin to apply these methods to breast tissue. We have a limited number of frozen specimens for initial application and then will begin the analysis of the fixed specimens. At this point, we anticipate doing antigen retrieval with microwave pre-treatment prior to immunostaining

with antibodies. The methods to be applied will be identical to those for the estrogen and progesterone receptors in our SPORE Breast Cancer Immunohistochemistry Laboratory. Lynn Dressler, Director of this facility, will act as a consultant to us on this aspect of the project.

Task 3: Detection of VDRs in paraffin-embedded tumor specimens.

Formalin-fixed, paraffin-embedded (FFPE) breast tumor tissue blocks were acquired from the three participating hospitals. Tumor tissue for a total of 96 incident breast cancer cases, 83 white and 13 black subjects, was successfully acquired; 19 of the cases were diagnosed with intraductal carcinoma, 79 with invasive ductal carcinoma, and one with lobular carcinoma. Our results with immunohistochemical staining have been variable and to date, we have been unable to successfully analyze breast tumor tissue for VDRs.

We were, however, able to analyze our study population's blood for polymorphisms in the gene for the VDR. Genomic DNA was extracted from the peripheral blood at the time of subject interview using the salting out method of Miller and Bulbrook¹⁴. We had samples for 138 of our subjects with incident breast cancer (18 black, 120 white) and 163 women (24 black, 139 white) from control group 1, frequency matched to cases on age (within three years), race, clinic, and month of blood drawing. We included 143 women (23 black, 140 white) from control group 2, frequency matched to cases on age (within three years) and race, in the genetic analyses, but not in the blood level analyses for reasons of comparability.

VDR *TaqI* genotype was determined by a PCR-based method described by Riggs, et al¹⁵. Briefly, a 740-bp fragment was amplified using PCR primers (5' -cag agc atg gac agg caa and 5' gca act cct cat ggc tga ggt etc) located within intron 8 and exon 9. The PCR fragment was subjected to *TaqI* digestion and then separated on 3% Nusieve 3:1 agarose gels (FMC Bioproducts, Rockland, ME), stained with ethidium bromide and visualized using ultraviolet light. *TaqI* digestion results in three distinct banding patterns signifying three genotypes: (a) homozygous absence of the restriction site (TT) with two fragments of

495 bp and 245 bp; (b) homozygous presence of the *TaqI* polymorphism (tt) with three fragments of 290 bp, 245 bp, and 205 bp; and (c) heterozygosity (Tt) with all four fragments, 495 bp, 290 bp, 245 bp, and 205 bp present. The 245-bp fragment is constant among all genotypes, having been created by a nonpolymorphic *TaqI* site within the amplification fragment, and acts as an internal control for the digestion¹⁶.

Associations between disease and genotype were assessed by calculating the OR and 95% CI¹⁷. Because genotypes can differ by race, estimates were computed separately for blacks and whites. Since our work indicates that the races differ in the effect of low 1,25-D on breast cancer risk, races were analyzed separately for potential effect modification of genotype on the 1,25-D/breast cancer relationship.

Task 4: Data analysis and report writing.

A. Blood level analyses. We have completed analysis of the data related to Specific Aim 1 of our study to determine if blood levels of 25-D and 1,25-D are lower in women at the time of initial diagnosis of breast cancer than in comparable women who do not have breast cancer. Table 2 describes the crude odds ratios (ORs) associated with being in successive quartiles of 1,25-D with the highest quartile as the reference. Quartiles were based on the distribution of 1,25-D among the respective control groups. Among all study subjects, the OR (95% CI) for breast cancer risk of lowest versus highest quartile of 1,25-D was 2.7(1.6, 4.8); the same comparison among white subjects only was 3.0 (1.6, 5.5). Tests for trend using the Mantel extension test¹⁸ were significant at <0.05 level for the entire group and the white subgroups. Black subjects did not show the pattern of increased risk associated with lowest quartile of 1,25-D compared to highest quartile seen in white subjects (OR [95% CI]: 0.7[0.1, 3.7]). Table 3 presents the adjusted risk estimates for breast cancer according to quartile of 1,25-D among cases and control group 1 subjects. Control group 2 was excluded from this analysis because it was not comparable to either the case group or control group 1 in month of blood drawing; this may affect the vitamin D metabolite levels. Adjustment for age, sample storage time, assay batch, month of blood

draw, and clinic increased the OR(95% CI) for white women in the lowest quartile relative to the highest quartile to 5.2(2.1, 12.8). Because of the possibility that more advanced disease could affect the blood level of 1,25-D, a subset analysis was carried out, omitting those women with lymph node metastases at the time of diagnosis (n=26) from the multivariate logistic regression (Table 3). The estimated OR (95%CI) for the lowest quartile of 1,25-D relative of the highest quartile, adjusted for the matching variables, assay batch and sample storage time, was 4.0 (1.5, 10.8).

There were no significant associations between either lower or higher levels of 25-D and previously described risk factors for breast cancer, such as older age, postmenopausal status, higher body mass index (BMI), younger age at menarche, and nulliparity.

We performed a stratified analysis with white cases and control 1 subjects to investigate possible interactions between 1,25-D and selected covariates. We considered an interaction present if the Breslow-Day statistic for homogeneity of the ORs was significant at the 0.05 level. We compared the risk of breast cancer associated with levels of 1,25-D below the median value of 22.24 pg/ml to those above the median value by age, menopausal status, value of BMI, level of 25-D, or use of hormone replacement therapy. The magnitude of risk was greater for women above the median age of 54 (adj. OR 4.7, 95% CI: 2.1, 10.2) than at or below age 54 (adj. OR 1.5, 95% CI: 0.7, 3.0), $X^2=4.7$, $p=0.03$. There were no differences in breast cancer risk by level of any of the other covariates.

We conducted a case-case comparison among white subjects to examine the relationship between stage of disease and the 1,25-D breast cancer relationship. There were no differences in risks between those subjects with ductal carcinoma *in situ* (n=31) and subjects with invasive ductal carcinoma (n=95). Similarly, among women with invasive ductal carcinoma the risk associated with low 1,25-D levels did not differ significantly between women with and without lymph node metastases at time of diagnosis.

We also evaluated the relationship between estrogen receptor (ER) and progesterone (PR) status (ER and/or PR present, or both absent) and vitamin D-related breast cancer risk (Table 4). The adjusted risk estimate associated with low versus high 1,25-D was higher for receptor positive than receptor negative breast cancer, 5.0 (95% CI: 2.3, 11.0) versus 1.1 (95% CI: 0.5, 3.0). The Breslow Day X^2_{1df} was 5.9, $p=0.02$, indicating that estrogen receptor status may modify the breast cancer risk related to low 1,25-D blood levels.

B. Polymorphism/blood level analyses. There were significant differences between the races in distribution of the *TaqI* VDR genotypes ($X^2_{2df}=10.39$, $p=0.006$); black subjects had more TT genotypes and fewer tt genotypes than white subjects (Table 5). There were no differences in breast cancer risk by *TaqI* VDR genotypes in either race (Table 6). Among white women, breast cancer cases had significantly lower mean blood levels of 1,25-D than controls for genotypes TT and Tt (Table 7); the magnitude of the difference was less and not statistically significant for tt, although there were fewer subjects in this group (Table 7). Table 8 demonstrates the effect of *TaqI* VDR genotype on the relationship between low blood levels of 1,25-D (at or below the median of 22.24 pg/ml) and breast cancer risk. For women with low levels of 1,25-D compared to women with levels above the median, the adjusted ORs (95% CI) for breast cancer risk were 3.0(1.3, 6.9) and 5.0(1.9, 13.1), respectively for the TT and Tt genotypes. For women with the tt genotype, the corresponding OR estimate for breast cancer risk was 0.8(0.3, 2.5). Among women with the TT and Tt genotypes combined, the breast cancer risk associated with 1,25-D at or below the median compared to levels above the median was 3.6 (1.9, 6.4); this was significantly different from the OR(95% CI) of 0.8(0.3, 2.5) for a similar comparison among women with the tt genotype, $X^2_{1df}=4.5$, $p=0.03$.

Table 6 indicates that, among cases, white women with tt were more likely than TT and Tt to have *in situ* disease, 2.3(0.9, 6.0), and were less likely to have lymph node metastases, 0.5(0.1, 1.7).

We have prepared and submitted one manuscript describing the methods used to assess assay validity and reliability for 25-D and 1,25-D in whole blood. We have prepared and submitted a manuscript on the results of the blood level analyses and we are preparing a manuscript on the results of the polymorphism/blood level relationships.

Conclusion

Our data indicate that white women in the lowest quartile of 1,25-D have an adjusted OR (95% CI) of 5.2 (2.1, 12.8) for risk of breast cancer compared to similar women in the highest quartile. The risk, OR (95% CI), was greater among women over the age of 54 than among those at or below age 54, 4.7 (2.1, 10.2) versus 1.5 (0.7, 3.0) when 1,25-D levels below the median were compared to those above the median.

Future work needs to address the temporal relationship between low levels of vitamin D metabolites and the development of breast cancer. A case-control study design cannot evaluate the temporal relationship; a prospective study would provide certainty on this question. A replication of this study using the preferred substrate of plasma or serum would allow a more accurate assessment of the 1,25-D levels which confer protection; it would also allow a more precise evaluation of 25-D levels. Further, we had no data on dietary intake of vitamin D, phosphorus, calcium, vitamin supplementation, or sunlight exposure. The lack of difference in the blood level of 25-D between cases and controls supports the probability that both groups had adequate physiologic amounts of the precursor hormone. However, selective decreases in dietary calcium and/or phosphorus among both groups of controls or selective increases among the cases relative to both control groups, while unlikely, could have affected the 1,25-D levels.

The small number of black women in our study did not demonstrate the same relationship between low levels of 1,25-D and breast cancer risk. It would be important to study a larger group of black women and other minority women to clarify the vitamin D/breast cancer relationship in this population.

The information on VDRs is important; differences in quantity and/or function may be associated with breast cancer risk or prognosis. Two recent papers describe an association between *TaqI* VDR genotype and prostate cancer risk^{16,19}. Our data do not demonstrate a relationship between *TaqI* VDR genotype and breast cancer risk. However, *TaqI* VDR genotype may modify the relationship between 1,25-D blood level and breast

cancer risk. Among women with the TT and Tt genotypes combined, the breast cancer risk associated with 1,25-D at or below the median compared to levels above the median was 3.6 (1.9, 6.4); this was significantly different from the OR(95% CI) of 0.8(0.3, 2.5) for a similar comparison among women with the tt genotype, $X^2_{1df}=4.5$, $p=0.03$.

The significance of this work relates to the potential for prevention of breast cancer. Recent work has demonstrated the antineoplastic effect of a novel vitamin D analogue, 1 α -hydroxyvitamin D₅²⁰. Vitamin D analogues are potential chemotherapeutic as well as chemopreventive agents for breast cancer. Finally, there is the possibility of better understanding the mechanisms of carcinogenesis as we define the role of vitamin D in this process.

References

1. DeLuca HF and Ostrem V. The relationship between the vitamin D system and cancer. *Adv Exp Med Biol*, 206:413-429, 1987.
2. Reichel H, Koeffler HP, Norman AW. The role of the vitamin D endocrine system in health and disease. *NEJM*, 320:980-991, 1989.
3. Manoglas SC. Vitamin D and its relevance to cancer. *AntiCancer Research*, 7:625-638, 1987.
4. Garland CF, Garland FC, Shaw KO, Comstock GW, Helsing KJ, Gorham ED. Serum 25-hydroxyvitamin D and colon cancer: eight-year prospective study. *Lancet* 1176-1178, Nov. 18, 1989.
5. Corder EH, Guess HA, Hulka BS, Friedman GD, Sadler M, Vollmer RT, Lobaugh B, Drezner MK, Vogelman JH, Orentreich N. Vitamin D and prostate cancer: a prediagnostic study with stored sera. *Cancer Epidemiology, Biomarkers & Prevention*, 2:467-472, 1993.
6. Blot WJ, Fraumeni JF, Stone BJ. Geographic patterns of breast cancer in the United States. *J Natl Cancer Inst*, 59:1407-1411, 1977.
7. Gorham ED, Garland FC, Garland CF. Sunlight and breast cancer incidence in the USSR. *Int J Epidemiol*, 19:820-824, 1990.
8. Garland FC, Garland CF, Gorham ED, Young JF. Geographic variation in breast cancer mortality in the United States: a hypothesis involving exposure to solar radiation. *Preventive Med*, 19:614-622, 1990.
9. Simard A, Vobecky J, Vobecky JS. Vitamin D deficiency and cancer of the breast: an unprovocative ecological hypothesis. *Can J Public Health*, 82:300-303, 1991.
10. Jacobson EA, James K, Newmark HL, Carroll KK. Effects of dietary fat, calcium, and vitamin D on growth and mammary tumorigenesis induced by 7,12-dimethylbenz(a)anthracene in female Sprague-Dawley rats. *Cancer Res*, 49:6300-6303, 1989.
11. Chouvet C, Vicard E, Devonec M, Saez S. 1,25-dihydroxyvitamin D₃ inhibitory effect on the growth of two human breast cancer cell lines (MCF-7, BT-20). *J Steroid Biochem*, 24:373-376, 1986.
12. Frappart L, Falette N, Lefebvre MF, Bremond A, Vauzelle JL, Saez S. In vitro studies of effects of 1,25-dihydroxyvitamin D₃ on the morphology of human breast cancer cell line BT-20. *Differentiation*, 40:63-69, 1989.
13. Milde P, Merke J, Ritz E, Haussler MR, Rauterberg EW. Immunohistochemical detection of 1,25-dihydroxyvitamin D receptors and estrogen receptors by monoclonal antibodies: comparison of four immunoperoxidase methods. *J Histochem Cytochem*, 37:1609-1617, 1989.
14. Miller AB, Bulbrook RD. UICC multidisciplinary project on breast cancer: the epidemiology, aetiology and prevention of breast cancer. *Int J Cancer*, 37:173-177, 1986.

15. Riggs BL, Nguyen TB, Melton LJ, Morrison NA, O'Fallon WM, Kelly PJ, Egan KS, Sambrook PN, Muhs JM, Eisman JA. The contribution of vitamin D receptor alleles to the determination of bone mineral density in normal and osteoporotic women. *J Bone Miner Res*, 10:991-995, 1995.
16. Taylor, JA, Hirvonen A, Watson M, Pittman G, Mohler JL, Bell DA. Association of prostate cancer with vitamin D receptor gene polymorphism. *Cancer Res*, 56:4108-10, 1996.
17. Kleinbaum DG, Kupper LL, Morgenstern H. Epidemiologic Research Principles and Quantitative Methods. Van Nostrand Reinhold, New York, 1982.
18. Mantel N. Chi-square tests with one degree of freedom: extensions of the Mantel-Haenszel procedure. *J Am Stat Assoc*, 58:690-700, 1963
19. Ingles SA, Ross RK, Yu MC, Irvine RA, La Pera G, Haile RW, Coetzee GA. Association of prostate cancer risk with genetic polymorphisms in vitamin D receptor and androgen receptor. *J Natl Cancer Inst*, 89:166-170, 1997.
20. Mehta RG, Moriarty RM, Mehta RR, Penmasta R, Lazzaro G, Constantinou A, Guo L. Prevention of preneoplastic mammary lesion development by a novel vitamin D analogue, 1α -hydroxyvitamin D₅. *J Natl Cancer Inst*, 89:212-218, 1997.

Table 1. Comparison of vitamin D metabolite blood levels in cases and controls by race

	Black		White	
	case n=21	control 1 n=30	control 2 n=23	control 1 n=149
1,25-D, pg/ml				
* mean (SD)	24.17 (10.15)	22.28 (10.99)	26.56 (11.22)	23.21 (9.09)
median	21.01	19.06	24.15	22.24
range	6.78 - 49.22	4.91 - 46.13	6.92 - 48.90	1.79 - 46.96
25-D, ng/ml				
* mean (SD)	8.91 (6.50)	9.94 (7.40)	11.06 (6.42)	14.71 (7.41)
median	6.80	8.13	7.99	12.98
range	3.52 - 28.47	1.87 - 40.33	3.81 - 26.73	2.14 - 48.40

*P-value for t test of difference in means:

Black

1,25-D case versus control 1 0.54 case versus control 1 0.0001
 case versus control 2 0.46 case versus control 2 0.0009

25-D

case versus control 1 0.61 case versus control 1 0.72
 case versus control 2 0.28 case versus control 2 0.24

White

Table 2. Crude Odds ratios (95% CI) for the risk of breast cancer associated with quartile of 1,25-D

	n	Quartile of 1,25-D pg/ml*				p trend
		1	2	3	4 (ref)	
All subjects	511	2.7 (1.6, 4.8)	1.9 (1.0, 3.4)	1.4 (0.7, 2.6)	1	<0.0001
Black subjects	74	0.7 (0.1, 3.7)	2.0 (0.5, 8.3)	1.5 (0.3, 6.6)	1	0.85
White subjects	423	3.0 (1.6, 5.5)	1.7 (0.9, 3.3)	1.2 (0.6, 2.3)	1	<0.0001
cases/control 1	280	3.2 (1.6, 6.3)	1.4 (0.7, 2.9)	0.9 (0.5, 2.6)	1	<0.0001
cases/control 2	274	3.1 (1.5, 6.2)	1.4 (0.7, 3.0)	1.6 (0.8, 3.4)	1	0.002
quartiles*:		1	2	3	4	
all	≤15.96	15.97 - 21.46	21.47 - 28.95	> 28.95		
black	≤16.11	16.12 - 21.41	21.42 - 35.10	>35.10		
white	≤16.05	16.06 - 21.74	21.75 - 28.49	>28.49		
control 1	≤17.43	17.44 - 22.24	22.25 - 27.36	>27.36		
control 2	≤15.65	15.66 - 20.34	20.35 - 28.73	>28.73		

*quartile ranges in picograms/ml are based on the distribution of metabolite in the respective control group; values for control groups 1 and 2 are based on white subjects

Table 3. Odds ratios (95% CI) for the risk of breast cancer associated with quartile of 1,25-D, white subjects: cases and control group 1

	n	Quartile of 1,25-D pg/ml			
		1 ≤ 17.43	2 17.44 - 22.24	3 22.25 - 27.36	4 (ref) > 27.36
Adjusted OR* (95% CI)	280	5.2 (2.1, 12.8)	1.9 (0.8, 4.8)	0.8 (0.3, 2.1)	1
Adjusted OR** (95% CI)	279	5.1 (2.1, 12.6)	2.0 (0.8, 4.9)	0.8 (0.3, 2.1)	1
Adjusted OR*** (95% CI)	279	5.8 (2.3, 14.9)	1.7 (0.6, 4.4)	0.9 (0.3, 2.5)	1
Subjects with lymph node metastases at diagnosis removed from analysis					
Adjusted OR* (95% CI)	254	4.0 (1.5, 10.8)	1.5 (0.5, 4.0)	0.5 (0.2, 1.5)	1
Adjusted OR** (95% CI)	254	4.0 (1.5, 10.4)	1.7 (0.7, 4.5)	0.5 (0.2, 1.4)	1
Adjusted OR*** (95% CI)	254	4.6 (1.6, 13.1)	1.4 (0.5, 3.9)	0.6 (0.2, 1.9)	1

*adjusted for age, assay batch, month of blood draw, clinic, sample storage time

**adjusted for BMI, 25(OH)D, age, assay batch, clinic, sample storage time

***adjusted for BMI, BMI², age, assay batch, month of blood draw, clinic, sample storage time

Table 4. Evaluation of breast cancer risk associated with levels of 1,25-D below the median value by ER and PR* status: white cases and control group 1

Group	1,25-D, pg/ml, n		OR (95% CI) [†]	Adj OR (95% CI)#
	≤ 22.24	>22.24		
Receptor positive**				
case	44	9	4.8 (2.2, 10.6)	5.0 (2.3, 11.0)
control 1	75	74	1	1
Receptor negative***				
case	11	10	1.1 (0.5, 2.7)	1.1 (0.5, 3.0)
control 1	75	74	1	1

*ER=estrogen receptor, PR=progesterone receptor

**ER and/or PR present

***neither receptor present

[†]Breslow Day $\chi^2_{1df} = 5.9, p = 0.02$

#adjusted for age, clinic, sample storage time

Table 5. VDR *TaqI* genotype frequencies among black and white controls

Ethnic group	Genotype frequency*(%)			Total
	TT	Tt	tt	
White	99 (35)	121 (43)	59 (21)	279
Black	21 (45)	24 (51)	2 (4)	47

* $X^2_{2df} = 10.39, p = 0.006$

Table 6. ORs and 95% CIs for the association between breast cancer and *TaqI* VDR genotype for all cases and by disease stage

Group	TT	Tt	tt	OR tt vs. (TT + Tt)	95% CI
Blacks					
controls	21	24	2	.*	-
cases	11	6	1	-	-
Whites					
controls	99	121	59	1.0	
cases	47	49	24	0.9	0.5, 1.6
Cases by stage					
DCIS**	12	8	9	2.3	0.9, 6.0
LN mets***	12#	11+	3	0.5	0.1, 1.7

The OR estimates for risk of DCIS and LN mets are comparisons among white cases

*Strata too sparse for a stable estimate

**Ductal carcinoma *in situ*

***Lymph node metastases

#does not include 5 black cases

+does not include 3 black cases

Table 7. Blood levels of 1,25(OH)₂D by genotype among white breast cancer patients and controls

Group	TT		Tt		tt				
	n	1,25(OH) ₂ D pg/ml, mean (SD)	p*	n	1,25(OH) ₂ D pg/ml, mean (SD)	p*	n	1,25(OH) ₂ D pg/ml, mean (SD)	p*
Controls	50	24.15 (8.15)		54	22.51 (9.68)		35	22.41(9.35)	
Cases	47	18.79 (9.20)	0.003	49	17.13 (7.65)	0.002	24	20.51 (10.76)	0.5

*p value for t-test of differences in means between cases and controls; there were no statistically significant differences among cases or controls by genotype

Table 8. Effect of low versus high 1,25(OH)₂D levels on risk of breast cancer according to VDR *TaqI* genotypes: white subjects

Group	1,25(OH) ₂ D* pg/ml		OR (95%CI)	Adj OR** (95% CI)
	≤ 22.24	> 22.24		
TT				
case	32	15	2.9 (1.3, 6.8)	3.0 (1.3, 6.9)
control	21	29	1	1
Tt				
case	42	7	4.8 (1.8, 12.6)	5.0 (1.9, 13.1)
control	30	24	1	1
tt				
case	14	10	0.9 (0.3, 2.6)	0.8 (0.3, 2.5)
control	21	14	1	1

*median value in white control group = 22.24 pg/ml

**adjusted for age

Bibliography

1. Janowsky EC, Lester GE, Hulka BS. Vitamin D and breast cancer. *Am J Epid*, 143:S37, 1996.
2. Janowsky EC, Lester GE, Hulka BS. Measurement of 1,25-dihydroxyvitamin D and 25-hydroxyvitamin D in archived samples of whole blood. Submitted.
3. Janowsky EC, Lester GE, Weinberg CR, Millikan RC, Schildkraut JM, Garrett PA, Hulka BS. The association between low levels of 1,25-dihydroxyvitamin D and breast cancer risk. Submitted.
4. Janowsky EC, Lester GE, Taylor JA, Bell DA, Hulka BS. *TaqI* VDR genotype may modify the breast cancer risk associated with low levels of 1,25(OH)₂D. In preparation.

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