VITAMIN D, TISSUE RESISTANCE, BONE MINERAL DENSITY AND BREAST CANCER RISK

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Etiologic factors such as vitamin D and estrogen are potentially related to breast cancer development, although details of their mechanisms are not completely understood. We prospectively investigated correlates of breast cancer risk among postmenopausal women in the Study of Osteoporotic Fractures (SOF). First, we undertook a case-cohort study to test the hypothesis that low serum 25-hydroxyvitamin D [25(OH)D] will be associated with an increased risk of ER+ breast cancer (N=502). Low 25(OH)D levels were not associated with an increased risk of breast cancer and do not support an association between 25(OH)D and ER+ breast cancer development. Second, we utilized fractional calcium absorption (FCA) as a marker of tissue resistance to vitamin D to test the hypothesis that low FCA will be associated with an increased risk of breast cancer (N=5035). To the contrary, over a mean 9.6 years, increasing rates of FCA were associated with a higher risk of invasive breast cancer. A stronger positive relationship was noted among women with low dietary calcium intake. The findings support a modestly increased risk of breast cancer with higher FCA rates particularly among those who have low calcium intake. Finally, we examined the long-term association of an initial bone mineral density (BMD) measure and change in BMD (annual percent change assessed 3.5 years later) on breast cancer risk (N=5385). Furthermore, we tested the hypothesis that the risk associated with an initial BMD measure would be strengthened by the addition of the change variable. Over a mean 9.5 years, there was no association between increasing levels of BMD, change in BMD, or a combined model and breast cancer. The effect of BMD was found to be dependent upon family history of breast cancer. Among women with a positive family history, high BMD was associated with a 3-fold higher risk of breast cancer compared to low BMD. Through our investigations of two etiologic factors and their association with breast cancer development, we have enhanced our knowledge regarding the interdependence of vitamin D, calcium, and estrogen. These findings may lead to improved opportunities for prevention and early detection and are of significant public health relevance.

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1.0 INTRODUCTION

Breast cancer is the most commonly diagnosed cancer, and the second leading cause of cancer mortality among women in the United States. Although the rising incidence has recently waned, our ability to assess a woman's risk of developing breast cancer and implement preventive measures is limited. The focus of this dissertation is on two potentially etiologic factors for breast cancer.

First, the association of vitamin D in tumor development was investigated. In addition to its role in building bone, vitamin D has a separate function in cancer prevention. Specifically, 1,25-dihydroxyvitamin D [1,25$(OH)_2$D], the biologically active form of vitamin D, is responsible for genetic regulation of cellular processes such as controlling proliferation, inhibiting angiogenesis, and inducing differentiation and apoptosis. These actions are carried out locally in tissues containing the vitamin D receptor (VDR) such as the breast where circulating 25-hydroxyvitamin D [25(OH)D] is converted to 1,25$(OH)_2$D. Therefore, the serum concentration of 25(OH)D may be a predictor of breast cancer risk. There is the potential, however, for tissue to lose its sensitivity to 1,25$(OH)_2$D with age. This is demonstrated in the gut where fractional calcium absorption (FCA), a measure of the rate of calcium uptake, is maintained despite increased levels of 1,25$(OH)_2$D. It is not known whether other tissues might also develop resistance to 1,25$(OH)_2$D over time. FCA could potentially be used as an indicator of tissue sensitivity to 1,25$(OH)_2$D beyond the small intestine and help to clarify the role of vitamin D in breast cancer development.

Second, we investigate estrogen. Estrogen plays an important role in the development of breast cancer. Estrogen, along with growth factors, vitamin D, parathyroid hormone (PTH), and calcium are important factors contributing to bone health. Bone mineral density (BMD), a measure of bone strength, is hypothesized to be a surrogate marker for cumulative estrogen exposure because bone contains estrogen receptors making it sensitive to levels of circulating estrogens. BMD may predict future breast cancer occurrence.

In light of the number of women affected with breast cancer, it becomes increasingly important to understand this disease and to identify potentially modifiable risk factors along with the women who might benefit from targeted prevention strategies. Established risk factors explain little of the variability in breast cancer and therefore there is a need to identify additional risk factors. This becomes an increasingly difficult task given the heterogeneity of breast cancer risk factors that exists between premenopausal and postmenopausal women, as well as the differences in pathologic features of breast tumors in older women. The following literature review presents a brief overview of the epidemiology of breast cancer and known risk factors. A more detailed background on vitamin D, fractional calcium absorption, and bone mineral density, as they relate to breast cancer is also provided.

2.0 LITERATURE REVIEW

2.1 EPIDEMIOLOGY OF BREAST CANCER

2.1.1 Incidence, Survival and Mortality

Breast cancer is the most commonly diagnosed cancer, and the second leading cause of cancer mortality among women in the United States. The incidence of breast cancer from 2000 to 2004 in the US was 125.3/100,000 and the mortality rate was 25.5/100,000.[1, 2] The number of women affected by breast cancer annually is not insignificant, with a projected 182,460 new cases of invasive breast cancer and 40,480 deaths expected to occur in the United States in 2008.[2] After increasing for several decades, female breast cancer incidence rates decreased between 2001 and 2004.[2] Two distinct patterns in recent breast cancer trends have emerged. The subtle downturn in incidence rates for all women over age 45 is reflective of the saturation of screening mammography utilization, where as the sharp decrease in incidences among women aged 50 to 69 years is more likely to be attributed to the reduction in the use of hormone therapies (HT), as tumors in women this age are predominantly estrogen receptor positive (ER+) and hence sensitive to levels of circulating hormones.[3] Overall, mortality due to breast cancer has been steadily declining since the early 1990's.[2] The 5-year relative survival from breast cancer is 89%, but varies greatly depending upon stage at diagnosis with a range of 98% for localized tumors to 27% for metastatic disease.[2] Survival is greatest for women diagnosed

at age 40 or older with a 5-year survival of 89% compared to 82% for those under age 40.[4] Long-term survival is greater among individuals with a ER+ tumor and a stronger degree of positivity for tamoxifen therapy. In quantitative terms, women with estrogen receptor negative (ER-) tumors have an 8% to 35% lower 5-year survival rate compared to those with ER+ tumors.[5]

2.1.2 Age

The majority of breast cancer cases occur among women over age 50, with a peak in incidence at 75-79 years of age.[6] Women over the age of 50 have an incidence rate of 375 / 100,000 compared to 42.5 / 100,000 among women less than 50 years of age.[6] For women, the lifetime probability of developing invasive breast cancer is 1 in 8; age specific probabilities are 1 in 26 (40-59 years), 1 in 28 (60-69 years), and 1 in 15 for those over age 70.[2] The proportion of women diagnosed with distant-stage disease increases with age,[7] as does the proportion of tumors expressing hormone receptors.[5, 8] **Figure 1** shows the age-specific invasive breast cancer incidence rates overall and by estrogen receptor status. Approximately 75% of breast cancers in older women are ER+. Such pathologic differences may reflect unique biologic influences on breast cancer occurrence in older women.[9]

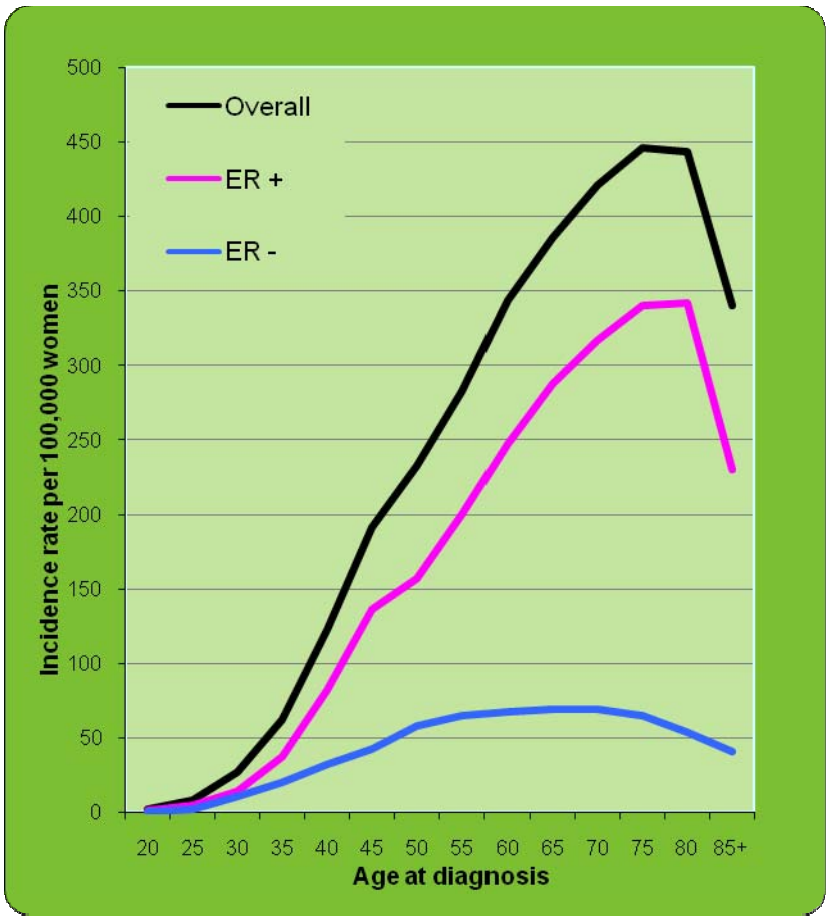

Figure 1. Age-specific invasive breast cancer incidence rates, 2004-2005
National Cancer Institute Surveillance, Epidemiology, and End Results Program (SEER)

2.1.3 Race and Ethnicity

Breast cancer is the most common cancer among women of every major ethnic group. However, there are differences by race. Age-adjusted incidence rates are highest among white women (133/100,000) and lower among black women (118/100,000), Asian American and Pacific Islanders (89/100,000), Hispanic/Latinas (89/100,000), and America Indian and Alaskan Natives (70/100,000) for the time period of 2000-2004.[2] The distribution of age at onset differs by race; black women have earlier age at onset with less frequent postmenopausal breast cancer occurrence. With the exception of black women, differences in incidence rates among

racial and ethnic groups were shown to be explained by differences in other risk factors.[10] There are also differences in survival and mortality among racial groups. The death rate is 36% higher in black women compared to white women in the United States, and the relative 5-year survival is 77% and 90% for black and white women respectively.[2]

2.2 RISK FACTORS FOR POSTMENOPAUSAL BREAST CANCER

2.2.1 Reproductive Aspects

Reproductive factors influencing the risk of breast cancer include age at menarche, age at first live birth, age at menopause, parity, and breastfeeding, as these factors are key determinants of hormone exposure. Prolonged exposure and higher concentrations of endogenous estrogen increases the risk of breast cancer in postmenopausal women.[11] Estrogen production is controlled by ovarian function, however, after menopause, the main sources of estrogen are from peripheral conversion of androtestosterone, an adrenal hormone, to estrone which primarily occurs in fat tissue.[12] Older age at menarche[13] and younger age at menopause[11, 13, 14] are both associated with a lower risk of breast cancer possibly due to a decreased lifetime exposure to hormones. Indeed, induced menopause through surgical means (i.e. bilateral oophorectomy) decreases the risk of breast cancer.[15]

Parous women have a decreased risk of breast cancer compared to nulliparous women,[11, 13, 16, 17] and the younger a woman is at her first full-term pregnancy, the lower her risk of breast cancer.[11, 13, 17] The Nurses' Health Study showed a 20% decreased risk of breast cancer in parous versus nulliparous women with a first birth at age 20.[17] The benefit is reduced to 10% with a first birth is at age 25. The risk however for a first birth at age 35 is actually

greater than that of nulliparous women. The protection associated with parity, is proposed to be due to pregnancy induced cellular differentiation of breast tissue which guards against exposures to cancer initiating events. The extent of the protective effect is greater at younger ages of first birth as the tissue maturation process is completed earlier in a woman's lifespan.[18] The greater the number of subsequent live births incrementally decreases the risk of breast cancer, there is however a short-term increased risk of breast cancer following each pregnancy.[16]

Breastfeeding is weakly protective against breast cancer depending on both the duration (4.3% per 12 months) and number of births (7% per child).[19, 20] Prolonged lactation offers a small amount of decreased risk, although this may be limited to premenopausal breast cancer, likely due to the inhibitory effect of breastfeeding on ovulation and reduced estrogen production.[21]

2.2.2 Hormonal Factors

Sex hormones play a central role in the etiology of breast cancer as evidenced by the rapid increase in breast cancer rates in the premenopausal years followed by a sudden slowing in the increasing rates at menopause when endogenous hormone levels decline rapidly. Among postmenopausal women, there is a clear increased risk of breast cancer with increasing levels of circulating endogenous hormones, including estradiol and testosterone.[22-28] Results are even stronger for ER+ breast cancers.[26] Studies of the relationship in premenopausal women have found conflicting results,[28-32] and again, differences are seen by estrogen receptor status.[29, 32]

Exogenous hormone exposures are typically through oral contraceptives among premenopausal women and hormone therapy (HT) among postmenopausal women. Little to no increased risk of breast cancer is associated with oral contraceptive use.[33, 34] Conversely, HT is

related to an increased risk of breast cancer. As demonstrated in the randomized controlled trial of the Women's Health Initiative (WHI), combined estrogen plus progesterone is clearly related to an increase in the risk of breast cancer.[35, 36] Risk is greater for ER+ cancers,[37] and with longer duration of use.[23, 25] As recently reviewed by Santen et al., the evidence is less clear concerning hormone therapies where estrogen is unopposed, typically prescribed for women who have undergone a hysterectomy.[38] The estrogen-alone arm of the WHI randomized trial found no association.[39] Other studies have found no association,[39, 40] a decreased risk,[41-43] or an increased risk.[41, 44, 45]

2.2.3 Anthropometry

Obesity is positively related to postmenopausal breast cancer, however, there is an opposite, inverse association among premenopausal women. The higher levels of circulating estrogens found in heavier women have been attributed to the greater amount of adipose tissue.[46, 47] In postmenopausal women, adipose tissue is the major source of estrogen, and obese postmenopausal women have both higher levels of endogenous estrogen and a higher risk of breast cancer.[48, 49] Among postmenopausal women, higher body mass index (BMI) posed a greater risk of breast cancer (RR 1.3 for ≥ 25 vs. < 21 kg/m^2, 95%CI 1.1-1.5).[50] In addition, the increased risk among heavier women appears to be greater for never users of HT.[51, 52] A study of women enrolled in the WHI who never used HT found increased BMI to be a strong predictor of breast cancer risk among younger postmenopausal women (50-59 years) but not associated among older women (70-79 years).[51] This is in contrast to an earlier study reporting a greater risk among older postmenopausal women.[50] Being obese (BMI ≥ 30 kg/m^2) is also associated with an increased risk of dying from breast cancer.[53] Recently, the effect of BMI on breast cancer mortality was shown to be age dependent with an increased risk of death with higher

BMI at age 65 yet a decreased risk of death with higher BMI at age 85.[54] The relationship among premenopausal women on the other hand, is contradictory; high BMI (> 31 kg/m^2) is protective against breast cancer compared to low BMI (< 21 kg/m^2).[50] The reduction in breast cancer risk among obese premenopausal women may be due in part to suppressed ovulation.[55]

Height is positively associated with breast cancer risk. Taller women (≥ 175 cm) have been found to be 20% more likely to develop breast cancer than shorter women (< 160 cm).[50]

Higher levels of dense breast tissue are associated with increased breast cancer risk.[56, 57] Several factors suggest that the relationship maybe independent of estrogen. Firstly, mammographic density is largely an inherited trait,[58] secondly, ER+ and ER- tumors are equally represented,[59] and thirdly, breast density measures have not been found to correlate highly with sex hormone levels.[60]

2.2.4 Family History / Genetics

Family history is an important risk factor for breast cancer despite the fact that only 15-20% of women with breast cancer report a positive family history. Risk of breast cancer increases with a greater number of affected first degree relatives.[61] Specific genes such as BRCAI/II, p53, and AT convey an increased risk of breast cancer. While the risk to individuals with such a mutation is high (approximately 90% for BRCA), the prevalence is low and hence they account for only about 5% of breast cancer cases in the general population.

2.2.5 Benign Breast Disease

Of the major histologic categories of benign breast disease, atypical hyperplasia, proliferative lesions, are most highly associated with increased breast cancer risk (RR 3.9, 95%CI 2.6-5.9)

as observed in the Nurses' Health Study.[62] The risk, however, appears to be greater among premenopausal (RR 5.3, 95%CI 2.6-10.7) compared to postmenopausal women (RR 3.7, 95%CI 2.1-6.6).

2.2.6 Lifestyle

Physical activity is modestly protective against developing breast cancer, with an estimated average risk reduction of 30 to 40%.[63-66] This evidence is more consistent for postmenopausal women.[63, 67-69] The Women's Health Initiative reported a reduced risk of 37% for normal weight women engaging in at least 10 hours of brisk walking per week.[68] This may be mediated through weight control, or the associated reduction in circulating estrogen concentrations with increasing levels of physical activity.[47, 70]

Alcohol consumption has been consistently associated with an increased risk of breast cancer. Compared with nondrinkers, daily alcohol consumption has been associated with as much as a 40% increased risk (RR 1.41 for 2+ drinks/day, 95%CI 1.18-1.69) by a pooled analysis of 6 cohort studies,[71] and as little as a 6% increased risk (RR 1.06 for 1 drink/day, 95%CI 1.00-1.11) by a meta-analysis of 5 cohort studies.[72] No differences were noted by menopausal status.[72] The effect is more pronounced for ER+ breast cancer[73-75] and when combined with HT.[73, 74, 76] The combination of 1.5 to 2 drinks daily with current HT use for at least five years was associated with a doubling of the risk of breast cancer compared to nondrinking, nonusers of HT (RR 1.99, 95%CI 1.42-2.79).[76]

The relationship between smoking and breast cancer risk is complex due to interactions with other risk factors including alcohol, obesity, and endogenous hormones.[77, 78] Despite this, most studies have shown a modestly increased risk of breast cancer with smoking (RR 1.17, 95%CI 1.02-1.34).[79] In addition, the risk may differ my menopausal status with a protective

effect among postmenopausal women (OR 0.5, 95%CI 0.3-0.9) who experienced increasing BMI throughout life and initiated smoking later.[78]

2.2.7 Risk Factors among Older Women

Reports of differences in traditional breast cancer risk factors by both menopausal status and older age continue to mount. A study of women 75 years and older, found women with a high BMI (HR 1.44 for > 29.5 vs. < 23.5 kg/m^2, 95%CI 1.12-1.84), a family history of breast cancer in a first degree relative (HR 1.54, 95%CI 1.24-1.93), and an older age at menopause (p_{trend}=0.07) to have an increased risk of breast cancer, while having had five or more children compared to one or two was protective (HR 0.67, 95%CI 0.51-0.88).[9] Traditional breast cancer risk factors not associated with increased risk among the those over 75 years were nulliparity, age at first live birth, and age at menarche. A pooled analysis of reproductive risk factors by age at diagnosis (premenopausal or < 50 years vs. post-menopausal or > 50 years) found that while breast cancer risk decreased with increasing age at menarche for both groups, the decreased risk was approximately 9% per year of later menses onset for the younger women compared to 4% per year among the older women.[80] Similar results were found for age at first birth; risk of breast cancer increased 5% per year vs. 3% per year for younger vs. older women. In contrast, the risk of breast cancer was decreased by 12% for each live birth among the older women, but only 3% among the younger women.[81] It has been suggested that risk factors representing hormonal exposures of the distant past, will show attenuated risk ratios with rising breast cancer incidence with age, where as more recent indicators remain relevant to the risk of breast cancer among the elderly.[9]

2.2.8 Risk Factors by Estrogen Receptor Status

Breast cancer risk factors vary by hormone receptor status. The protective effects of reproductive risk factors such as delayed menarche,[82] higher parity,[82-85] younger age at first birth,[82-86] and early menopause[82, 85] tend to be stronger for ER+ breast cancer. However such findings are not entirely consistent as others have reported similar risks for ER+ and ER- tumors for older age at menarche,[84, 85] and parity.[86, 87] A meta-analysis by Ma et al. reported an 11% reduction in ER+/PR+ breast cancer risk with each additional birth and a 27% increased risk for women in the oldest versus the youngest category of age at first birth.[84]

Other breast cancer risk factors that show a greater association with ER+ breast cancer include height,[88] hormone use,[37, 86, 88] body mass index,[82, 85, 86, 89] physical activity,[67] and alcohol intake.[73-75] The Women's Health Study reported a modest relative risk per 1 drink/day of alcohol intake of 1.11 (95%CI 1.03-1.20) for ER+/PR+ tumors, 1.00 (95%CI 0.81-1.24) for ER+/PR- tumors, and 0.99 (95%CI 0.82-1.20) for ER-/PR- tumors.[74] Similar results were reported by a case-control study among a group of women aged 65-79 years who had ever used alcohol.[75] The Swedish Mammography Cohort however, reports an elevated risk of postmenopausal breast cancer with ≥ 1 drink/day versus none for both ER+/PR+ (RR 1.35, 95%CI 1.02-1.80) and ER+/PR- (RR 2.36, 95%CI 1.56-3.56) but not ER- subtypes.[73]

Family history, on the other hand, is one breast cancer risk factor found to be more strongly related to ER- breast cancer.[82]

2.2.9 Summary of Risk Factors

Table 1. Summary of breast cancer risk factors by menopausal status

Risk Factor	Premenopausal	Postmenopausal
Family history of breast cancer	+++	+++
Benign breast disease	++++	++++
Late age at menarche	+	+
Late age at menopause	n/a	++
Late age at first birth	+	+
Higher parity	--	--
Breastfeeding	--	--
Exogenous hormone use	+	++
Height	++	++
Weight	--	++
Obesity	--	++
Physical activity	--	--
Alcohol	+	+
Smoking	++	+

Scale: RR < 1.0, --; 1.0-1.25, +; 1.25-1.50, ++; 1.50-2.00, +++; > 2.00, ++++

2.3 HORMONE RECEPTOR STATUS DETERMINATION

Breast cancer is dependent upon estrogen or progesterone for growth. The stimulatory effect is mediated through estrogen receptors (ER_α and ER_β) and progesterone receptors (PR_a and PR_b), which are over-expressed in most breast tumors.[90] Estrogen receptors belong to a superfamily of nuclear hormone receptors, including other steroid hormone receptors such as vitamin D (VDR), that function as transcription factors when they are bound to their respective ligands.[90] The majority of breast tumors co-express ER_α and ER_β. A study of ER expression found 62% of breast tumors to be $ER_\alpha+/ER_\beta+$, 14% $ER_\alpha+/ER_\beta^-$, 15% $ER_\alpha^-/ER_\beta+$, and 9% ER_α^-/ER_β^-.[91]

ER and PR detection and quantification is done by either dextran-coated charcoal (DCC), which utilizes competitive binding of radiolabeled steroid ligand, or immunohistochemistry (IHC) and enzyme immunoassay (EIA) which are based on recognition of the receptor protein by specific antibodies.[92] The two ER subtypes (α and β) have similar estrogen binding affinity, however, ER_{α} is the predominant isoform used to determine ER status. Although ER status is often considered to be a dichotomous factor, i.e. positive or negative, ER concentration is actually measured on a continuous scale from 0 to 1,000 femtomoles per milligram (fmol/mg) with a positive range between 3 and 20 fmol/mg.[92]

While DCC is highly reproducible, variation is greatest between 3 and 10 fmol/mg and therefore 10 fmol/mg is the typical cutpoint used to determine ER+ status.[92] DCC is also known to give false-negative results in instances of high levels of circulating hormones such as estrogens due to receptor site saturation unlike IHC which is not affected by steroid hormone levels.[92] IHC's simplicity and relatively low cost make it the predominant method utilized in clinical practice. One drawback to IHC is that, unlike EIA it is not objectively quantitative. Recent studies have reported a bimodal ER status with 90% of tumors being either completely negative or very strongly positive with the IHC technology.[93] Despite this, comparative studies have reported high correlations in ER status reporting for DCC to IHC (80-90%), EIA to DCC (80%), and EIA to IHC (90%).[92]

In many observational studies, ER status is mainly determined through review of patient medical records. A recent comparison of ER status abstracted from pathology reports (where ER status was determined from many different labs, over a long period of time, and by several different methods) to corresponding measures by a single method (IHC) at a central laboratory found agreement for 87% of specimens (kappa=0.64, $p<0.01$), indicating that pathology reports are a reliable source for determining ER status.[94] In addition, the rate of tumors with

unspecified ER/PR status has decreased from more than 80/100,000 cases in 1990 to approximately 40/100,000 cases in 2003.[3]

2.4 VITAMIN D AND BREAST CANCER RISK

2.4.1 Biologic Plausibility

Vitamin D is available through exposure to sunlight, supplements, and dietary intake. Vitamin D produced in the skin and consumed in fortified foods (vitamin D_3, cholecalciferol, technically a prosteroid hormone) or consumed in the form of plant sterols (vitamin D_2, ergocalciferol) is biologically inert and must undergo two enzymatic hydroxylations to become biologically active.[95] **Figure 2** details the synthesis and metabolism of vitamin D. Following absorption, vitamin D is first metabolized by the liver into its principal circulating metabolites, 25-hydroxyvitamin D_3 [25(OH)D_3], and 25-hydroxyvitamin D_2 [25(OH)D_2]. 25(OH)D refers to both 25(OH)D_2 and 25(OH)D_3 and will be used throughout the text. Because liver production of 25(OH)D is not highly regulated, measured levels of these metabolites directly reflect cutaneous production and dietary intake and therefore is used as a marker to determine vitamin D sufficiency status.[96, 97] 25(OH)D_3 is far more abundant in circulation than 25(OH)D_2, with a normal concentration of 20-100 ng/ml but a preferred range of 30-60 ng/ml.[98] Circulating concentrations below 20 ng/ml are considered deficient, 21-29 ng/ml insufficient, and above 30 ng/ml sufficient.[98-100] As reviewed by Holick, a high prevalence of inadequate 25(OH)D levels has been documented for many different populations: young and old, healthy and ill, white and non-white, in the United States and abroad.[101] The prevalence of inadequate 25(OH)D levels in the United States is estimated to be more than 35% in healthy young adults (18 to 29 years)

and as high as 60% in hospitalized patients. Studies of osteoporotic postmenopausal women have reported values ranging from 50% to 75%. Among those over age 50 hospitalized with non-traumatic fractures, 97% had 25(OH)D levels below 30 ng/ml. Inadequate 25(OH)D is even more prevalent among non-white populations where 42% of black women aged 15 to 49 years had levels below 15 ng/ml and 84% of elderly black individuals had levels below 20 ng/ml.[101]

25(OH)D is subsequently hydroxylized in the kidney, as well as other tissues, into its most biologically active form, $1,25(OH)_2D$.[95] Circulating concentrations of $1,25(OH)_2D$ are 1000 times lower than that of 25(OH)D.[102] Renal production of $1,25(OH)_2D$ is tightly regulated by PTH through end product inhibition (i.e. a negative feedback loop) by $1,25(OH)_2D$. PTH increase with higher 25(OH)D levels and stabilizes at a 25(OH)D concentration of 30-40 ng/ml.[98, 103] Other regulators include calcium, phosphate, growth hormone, and prolactin.[95, 96]

Biological activities of $1,25(OH)_2D$ are mediated by vitamin D receptors in the target tissues.[104-106] Animal models have shown that normal and cancerous mammary cells have the ability to convert 25(OH)D into $1,25(OH)_2D$.[107, 108] Breast cells contain the VDR which becomes activated through interaction with $1,25(OH)_2D$ and can inhibit cellular proliferation and induce differentiation and apoptosis in normal mammary gland and breast cancer cells.[105, 109] A possible mechanism for the malignant transformation of breast cells is through insufficient 25(OH)D levels which limits the synthesis of $1,25(OH)_2D$, thus preventing activation of the VDR to regulate the cell cycle.[98] Furthermore, $1,25(OH)_2D$ down regulates inflammatory markers which has an anti-proliferative effect.[103] Hence, vitamin D has the potential to influence the development of breast cancer.

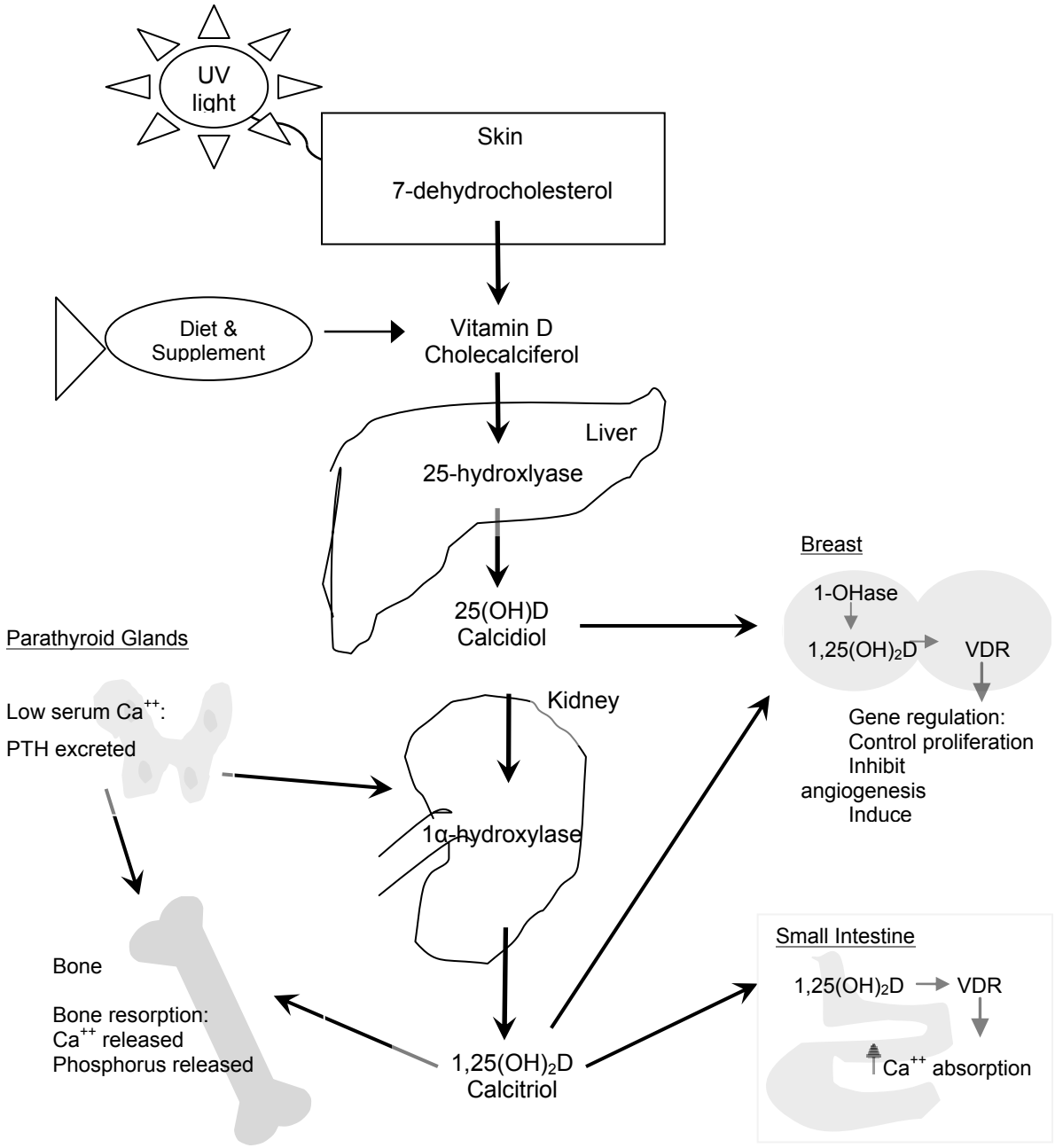

Figure 2. Vitamin D metabolism and action in bone, intestine, and breast tissue
In addition to the kidney, active vitamin D ($1,25(OH)_2D$) is produced locally in the breast where it interacts with the VDR to regulating genes that control cellular proliferation, inhibit angiogenesis, and induce differentiation and apoptosis. Subsequently, $1,25(OH)_2D$ is rendered biologically inert through catabolism. In the small intestine, $1,25(OH)_2D$ enhances intestinal calcium absorption through interaction with the VDR which aids in the expression of the calcium binding channel and the calcium-binding protein. In bone, along with PTH, $1,25(OH)_2D$ interacts with the VDR on osteoblasts, causing expression of RANKL which binds to its receptor RANK on preosteoclasts, and initiates the transition to mature osteoclasts. Osteoclasts promote bone mineralization by removing Ca++ and phosphorus from bone in order to maintain circulating levels of these minerals.[98, 110]

2.4.2 Ecologic Evidence

Indeed, ecologic data have shown that vitamin D from UV-B radiation is associated with a reduction in breast cancer incidence and mortality. Populations living at sunny lower latitudes (regions with higher levels of solar UV-B radiation) have higher circulating levels of 25(OH)D,[111] have decreased breast cancer risk,[112, 113] and lower mortality rates[114-118] compared with populations living at higher latitudes (regions with lower levels of UV-B radiation). These finding suggest that part of the relation between sun exposure and breast cancer risk could be explained by the vitamin D metabolic pathway. The major limitation of such studies however is the potential for ecologic fallacy in which the observed statistical association at the population level does not necessarily represent the true association present at the individual level.

2.4.3 Studies of Dietary Intake

The potentially protective effects of vitamin D from dietary sources on breast cancer risk were investigated by case control[119, 120] and cohort studies,[121-125] and most recently randomized controlled trials.[126, 127] Evidence of a role for vitamin D in reducing breast cancer risk is mixed. The randomized trial by Lappe et al. found a statistically significant reduced risk of overall cancer with combined daily calcium (1500 mg) and vitamin D (1100 IU) supplementation among postmenopausal women over age 55.[127] The findings of the Women's Health Initiative randomized trial showed no association with breast cancer (HR 0.96, 95%CI 0.85-1.09), however the dose of vitamin D (400 IU/day) is not thought to have been high enough.[126] The Nurses' Health Study found vitamin D intake to be inversely associated with breast cancer risk, but this effect was seen in premenopausal women only (RR 0.66, 95%CI 0.43-1.00).[125] Other prospective observational studies of dietary intake found no association or a weak association

that lacked statistical significance. Such studies are limited in their ability to quantify dietary intake and may not translate into physiologic levels.

2.4.4 Biomarker Studies

Given the difficulty in accurately estimating vitamin D intake from diet, and the tight physiologic control of $1,25(OH)_2D$, studies measuring circulating levels of 25(OH)D to determine vitamin D status are preferred. There has been much debate, however, surrounding the proper method of measurement for 25(OH)D, which is complicated further due to its two circulating forms, D_2 and D_3. While HPLC is considered to be the gold standard as it is able to quantify both metabolites, it is expensive, time consuming, requires a large sample, and is not readily available. Therefore alternatives such as the radio-immune assay (RIA) and enzyme immunoassay (EIA) are widely used. Reports from DEQAS (Vitamin D External Quality Assessment Scheme), an international laboratory quality control initiative, indicates that some assays routinely over estimate 25(OH)D levels.[128] Furthermore, while various assay manufactures claim to measure total circulating 25(OH)D (i.e. 100% cross-reactivity with both $25(OH)D_2$ and D_3) only one assay, the Diasorin RIA, was found to accurately measure 25(OH)D by detecting both metabolites in human serum.[129] Secondary hyperparathyroidism in individuals with low levels of 25(OH)D, leads to normal or elevated $1,25(OH)_2D$ levels and hence $1,25(OH)_2D$ should not be used as a measure of vitamin D status.[101] Because breast tissue acquires 25(OH)D from blood and converts it to $1,25(OH)_2D$ locally, circulating levels of 25(OH)D is the appropriate measure to study the effect of vitamin D on the risk of breast cancer.

Six studies, including one pooled analysis, have investigated the association of 25(OH)D concentration to breast cancer risk and are summarized in **Table 2**. Important covariates of vitamin D status including age, BMI, race, and season of blood draw were adjusted for as

appropriate. Most recently, a case-cohort study from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial found no association overall among women 55-74 years (RR 1.04 for < 18.3 vs. ≥ 33.7 ng/ml, 95%CI 0.75-1.45,) or when limited to women aged 60 and older (results not reported).[130] The only other prospective study, a matched nested case-control from the Nurses' Health Study, found a modest reduction in breast cancer risk with high levels of 25(OH)D (RR 0.73 for ≥ 42 vs. ≤ 22 ng/ml, 95%CI 0.49-1.07, p_{trend}=0.06). The association was stronger among women 60 and older (283 cases) in the highest (≥ 42 ng/ml) versus the lowest (≤ 22 ng/ml) quintile although the results were not statistically significant (RR 0.57, 95%CI 0.31-1.04, p_{trend}=0.03).[131]

Two case-control studies have reported a positive association between low levels of measured 25(OH)D and increased breast cancer risk. A population-based matched case-control study among postmenopausal women found an odds ratio (OR) of 0.31 (95%CI 0.24-0.42) for the highest (≥ 30 ng/ml) versus the lowest (< 12 ng/ml) category of 25(OH)D.[132] A matched case-control study with 179 cases found an overall odds ratio of 3.54 (95%CI 1.86-6.61) for breast cancer risk among individuals with < 20 ng/ml 25(OH)D compared to > 20 ng/ml.[133] The study also found a five-fold risk of breast cancer among women in the lowest (< 20 ng/ml) versus the highest (> 60 ng/ml) quartile of vitamin D levels (OR 5.83, 95%CI 2.31-14.7). One smaller matched case-control study (156 cases) found no difference in 25(OH)D levels between cases and controls (μ difference 0.87, 95%CI -0.47-2.21).[134] The findings of case-control studies are of limited relevance to the development of breast cancer due to the fact that blood samples were collected after the cancer was diagnosed. The preventive effect of vitamin D may only be applicable during the early stages of carcinogenesis.[135]

The only study to investigate estrogen receptor status and breast cancer risk for 25(OH)D reported a borderline significant inverse association (p_{trend}=0.08) with ER-/PR-, however they had limited power and used a combined population of premenopausal and

postmenopausal women.[131] This finding is inconsistent with the reported stronger association among older women who are far more likely to have ER+ opposed to ER- breast cancer. Furthermore, ER+ and ER- tumors likely have different etiologies.[8]

Table 2. Results and characteristics of studies investigating the association between endogenous 25(OH)D levels and incident breast cancer

Author	Study Design	Case / Control / Population	Comparisons	Results (95%CI)	Matched on (M) / Adjusted for (A)
Freedman 2008 PLCO Cancer Screening Trial, US	Frequency matched, case-cohort	1005/1005 Multi-racial Mixed-menopausal	Quintiles <18 vs. ≥34 ng/ml Aged 60+	RR=1.04 (0.7-1.4) No association	M: age, year of study entry, season; A: BMI, menarche age, menopause age, HT, BBD, FH, parity/age first birth, smoking, alcohol, calcium intake
Abbas 2008 Population-based, Germany	Matched case-control post-diagnosis samples	1394/1365 Post-menopausal	Categories = 5 ≥30 vs. <12 ng/ml	OR=0.31 (0.2-0.4)	M: season, age; A: menopause age, FH, BBD, parity, menarche age, breastfed, number of mammograms, HT, BMI, education, smoking
Garland 2007*	Pooled analysis		Quintiles Median ng/ml 48 vs. 6	OR=0.50 (p_{trend}<0.001)	
Bertone-Johnson 2005 Nurses' Health Study, US	Nested matched case-control	701/724 Multi-racial Mixed-menopausal	Quintiles ≥42 vs. ≤22 ng/ml Aged 60+	RR=0.73 (0.5-1.1) RR=0.57 (0.3-1.0)	M: age, menopause status, HT, season, fasting; A: BMI, parity, age first birth, FH, BBD, menarche age, menopause age, alcohol, α-carotene, estradiol, HT duration
Lowe 2005 Hospital-based, UK	Matched case-control	179/179 Caucasian Mixed-menopausal	Quartiles <20 vs. >60 ng/ml	OR=5.83 (2.3-14.7)	M: season, age, menopause status
Janowsky 1999 Hospital based, US	Case-control post-diagnosis samples	131/149 Caucasian Mixed-menopausal	Cases vs. Controls	Mean difference = 0.87 (-0.47-2.21)	M: age, race, clinic, season

*Pooled analysis included two studies; Berton-Johnson et.al and Lowe et.al.

Abbreviations used: BBD, benign breast disease; BMI, body mass index; CI, confidence interval; FH, family history; HT, hormone therapy; OR, odds ratio; RR, relative risk

2.4.5 Summary

Vitamin D has been shown to be inversely related to breast cancer through studies of ultraviolet radiation,[112, 113] dietary intake,[119, 121, 125, 127] and circulating concentrations.[131-133] In addition, postmenopausal women are thought to be at increased risk of vitamin D deficiency and related health consequences.[136] Insufficient vitamin D intake coupled with low sunlight exposure (the source of more than 90% of our vitamin D requirements), and the reduced ability of aged skin to synthesize cholecalciferol contributes significantly to vitamin D deficiency in this age group.[137-139] In light of the apparently contradictory findings from the Nurses' Health Study, and given the heterogeneity of risk factors by estrogen receptor status, it becomes increasingly important to disentangle these effects. However, the relationship between serum 25(OH)D and breast cancer has never been studied solely in postmenopausal women by histological subtype.

2.5 FRACTIONAL CALCIUM ABSORPTION AND BREAST CANCER RISK

2.5.1 Determinants of FCA

Fractional calcium absorption, a measure of intestinal calcium absorption, varies widely from person to person. Among postmenopausal women, FCA values have been reported to range from 0.07 to 0.68 with an average of 0.27 ± 0.10 (SD).[140] Given the high reproducibility of calcium absorption measures (r=0.8 over 8 weeks, r=0.5 over 5 years), the variation is thought to be largely due to biological need.[141] Indeed, several factors have been shown to effect intestinal calcium absorption, including calcium intake, age, estrogen, and serum levels of the biologically active $1,25(OH)_2D$.

By far, the most significant factor affecting intestinal calcium absorption is its hormonal regulator, $1,25(OH)_2D$.[142-145] See **Figure 2**. Approximately 20% of the variation in calcium absorption between individuals can be explained by circulating $1,25(OH)_2D$.[142] Some studies,[142, 145, 146] but not all,[147-153] have reported decreased levels of serum $1,25(OH)_2D$ with age. Alternatively, some investigators have reported 25(OH)D, but not $1,25(OH)_2D$, to be highly and positively correlated with calcium absorption.[152, 154-156] Serum 25(OH)D concentration was estimated to be responsible for 25% of the variation in calcium absorption,[154] and has been proposed to be more biologically active in intestinal calcium absorption than $1,25(OH)_2D$.[152] Studies of treatment with 25(OH)D have been shown to increase both $1,25(OH)_2D$ levels and calcium absorption.[156-158]

FCA is also affected by other factors. There is overwhelmingly consistent evidence that FCA varies inversely with calcium intake (i.e. low absorption values are associated with high

calcium intake and high absorption values are associated with low calcium intake).[140, 145, 150, 159, 160] In quantitative terms, with a daily intake of 200 mg of calcium, the mean absorption fraction was 0.45 compared to 0.15 at 2000 mg/day among non-estrogen deprived women.[160] As much as 26% of the variation in calcium absorption among individuals is attributed to calcium intake (both dietary and supplemental).[160]

It is well established that intestinal calcium absorption declines with increasing age.[137, 143, 145, 151, 160, 161] There is some debate however, as to whether there is a dual phase decrease which first occurs at menopause, and then subsequently with older age,[160] or if there is a single menopause related decrease in calcium absorption that can be counteracted with hormone therapy.[145] One study reported a decrease in calcium absorption of about 0.21% per year after age 40, with menopause itself responsible for a drop of 2.2%.[160] A more recent and larger study reported a 30% decrease in intestinal calcium absorption among women greater than 75 years of age, in addition to the decline that occurs at menopause.[143] Not all studies however, have reported an age related decrease in intestinal calcium absorption.[150, 153]

Studies have not only shown that calcium absorption is lower in postmenopausal compared to premenopausal women, but that it can be reversed by estrogen replacement.[162, 163] Researchers are uncertain however, if the exogenous estrogen directly increases calcium absorption, or if it works through other mechanisms such as increasing the circulating levels of $1,25(OH)_2D$.

Two additional factors that have been associated with calcium absorption are body size and smoking. Height and weight, but not BMI were positively associated with FCA in a study of middle aged women even after adjustment for estrogen status.[164] In a separate study of elderly adults (mean age 70), FCA was significantly ($p<0.05$) lower among smokers regardless of gender, age, and calcium and vitamin D intakes, and among the heaviest smokers (≥ 20 cigarettes/day, $p_{trend}<0.02$).[165] There is conflicting evidence regarding the effect of smoking on

serum sex hormone levels, with reports of both increased and decreased levels among smokers.[165] Given that estrogen depletion among postmenopausal women is known to decrease calcium absorption, the lower FCA among smokers may be caused by a similar mechanism, i.e. altered sex hormone levels.[165] Other identified correlates of FCA include weight loss and bone mineral density.[166]

2.5.2 Evidence of Tissue Resistance

Intestinal calcium absorption has been considered a marker of tissue responsiveness to vitamin D and aging has been associated with reduced sensitivity to $1,25(OH)_2D$.[167] The evidence supporting this claim is of varying strength. One study demonstrated unchanging calcium absorption with increasing age despite higher levels of $1,25(OH)_2D$, suggestive of intestinal resistance to $1,25(OH)_2D$ with aging.[150] Similarly, reduced calcium absorption was reported among non-estrogen users over age 75, despite unchanging $1,25(OH)_2D$ or 25(OH)D levels.[143] Perhaps the most compelling findings are from a study by Pattanaungkul et al. which clearly shows that in young women (mean 29 years) FCA increases with increasing serum concentrations of $1,25(OH)_2D$, while the increase in FCA among elderly women (mean 73 years) is significantly diminished ($p=0.03$) with increasing serum concentrations of $1,25(OH)_2D$.[168]

The actions of $1,25(OH)_2D$ on the small intestine and other target tissues are mediated by the VDR, an intracellular protein, and are ultimately involved with calcium transport.[169, 170] Cellular responsiveness to $1,25(OH)_2D$ is dependent on the concentrations of both the VDR and $1,25(OH)_2D$.[169] Consequently, changes in the intestinal VDR protein concentration may contribute to the decline in calcium absorption with age.[151] While there has been a report of lower concentrations of intestinal VDR proteins with age, fractional calcium absorption remained

unchanged with increasing age among elderly women, thus providing further evidence of an impaired intestinal response to 1,25$(OH)_2$D with increasing age.[153] Another study did not find lower VDR concentrations with age, but did find a decrease in calcium absorption that was only partially explained by changes in 1,25$(OH)_2$D serum concentrations indicating some level of intestinal resistance to 1,25$(OH)_2$D.[151] Indeed, animal models have supported the theory of age-related resistance to 1,25$(OH)_2$D. Old rats were not found to have lower VDR counts compared to young rats, however they did have significantly lower calcium absorption (46%) despite receiving supplemental 1,25$(OH)_2$D to raise plasma levels.[171]

Evidence from recent dietary studies of calcium and vitamin D provides additional support to the theory of intestinal resistance to vitamin D with age. The Nurses' Health Study, a large prospective cohort study of over 88,000 women found an inverse association between breast cancer risk and vitamin D intake among premenopausal but not postmenopausal women.[125] These findings were confirmed in the Women's Health Study, which showed a lower risk of premenopausal but not postmenopausal breast cancer with higher vitamin D intake.[122] Similarly, the WHI randomized trial of calcium plus vitamin D in postmenopausal women did not report any significant influence of supplementation on breast cancer risk.[126]

Other investigators have hypothesized that the age-related decline in calcium absorption is due to an estrogen deficiency induced decrease in intestinal response to 1,25$(OH)_2$D.[151] In support of this theory, a placebo controlled clinical trial found that both FCA and serum 1,25$(OH)_2$D concentration were increased after treatment with estrogen in postmenopausal osteoporotic women.[172]

2.5.3 Summary

Based upon the current evidence suggesting intestinal resistance to vitamin D with age, it is conceivable that other vitamin D sensitive tissues, such as breast tissue, may also have diminished response to vitamin D with aging. In fact, the decreased ability to absorb calcium with age, indicative of reduced gut tissue responsiveness to vitamin D, may be representative of other tissue's impaired responsiveness. This relationship however, has never been studied.

2.6 BONE MINERAL DENSITY AND BREAST CANCER RISK

2.6.1 Biologic Plausibility

Bone remodeling is the process by which the two types of bone tissue (cancellous or trabecular bone and cortical or compact bone) renew themselves. Normal bone turnover involves the metabolism of bone-resorbing cells (osteoclasts) followed by bone-forming cells (osteoblasts).[173] Estrogen is integral to bone health, exerting a multitude of actions on bone tissues, with estrogen deprivation leading to accelerated bone loss.[173] Bone strength is a reflection of both density and quality and is measured by several different methods. The assessment of bone strength can be either radiologic (i.e. bone mineral density), biochemical (i.e. markers of bone turnover), or histologic (i.e. bone biopsy for histomorphometry).[173] Measures of bone mineral density are the least invasive and the most widely used in clinical practice. Dual-energy x-ray absorptiometry (DXA), the standard device used to measure bone mineral density, is highly precise with coefficients of variation ranging from 1-3% depending on the skeletal site.[174]

Estrogen is thought to play a central role in the development of breast cancer due to its ability to stimulate proliferation of breast tissue.[175] Factors that increase exposure of breast tissue to estrogens, such as early menarche, older age at first birth, or late menopause, are associated with breast cancer risk.[176] Indeed, prolonged exposure to high levels of endogenous estrogens may increase breast cancer risk in postmenopausal women.[177] However, it is difficult to classify a woman's long-term exposure to endogenous estrogen by a single measurement because serum estrogen levels are highly variable over time.[178]

Bone mineral density, on the other-hand, is hypothesized to be a surrogate measure of lifetime estrogen exposure.[179] Bone contains estrogen receptors and is sensitive to circulating estrogen levels.[180] BMD is positively correlated with endogenous estrogen levels,[181] early menarche, parity, and the length of a woman's reproductive lifecycle.[182] In addition to the underlying age related decrease, BMD also decreases in postmenopausal women, mostly due to loss of ovarian estrogen beginning around the time of menopause. Even among postmenopausal women, however, the rate of bone loss is variable. Factors affecting postmenopausal bone loss include sustained estrogen exposure due to exogenous estrogen use and/or endogenous estrogen released from fat, age, BMI, calcium and vitamin D intake from dietary and supplemental sources, and level of physical activity.[183]

2.6.2 Etiologic Studies

As first proposed by Cauley and colleagues in the Study of Osteoporotic Fractures (SOF), increased BMD, reflecting high estrogen exposure throughout life, may be a predictor of future breast cancer occurrence.[184] Bone mineral density and other related surrogate markers of long-term estrogen exposure including height loss and history of fractures have been shown to be associated with the risk of breast cancer. Lower BMD (RR per 1 SD increase = 1.50, 95%CI 1.16-1.95), greater height loss (OR 0.67 for -2.5cm vs. 0, 95%CI 0.47-0.96), and more recent fracture experience (OR 0.79 for <5yr vs. none, 95%CI 0.65-0.95), are each associated with a reduced risk of breast cancer.[184, 185] Other studies of women who have experienced bone fractures late in life, an indicator of low BMD, have been noted to have a reduced risk of breast cancer.[185-189] Several prospective studies have sought to confirm the association between high BMD and increased breast cancer risk, and found a similar or slightly weaker relationship.[190-194]

Additionally, the risk of breast cancer related to BMD appears to differ by family history of breast cancer,[190, 195] stage at diagnosis,[196] and estrogen receptor status.[197] The more recent studies have focused on the effect of circulating sex hormones, particularly estradiol and testosterone, on the association between BMD and breast cancer risk. Two studies found that the relationship between BMD and breast cancer was attenuated when endogenous hormone levels were controlled for.[197, 198] However, in one study, when stratified by estradiol level, high BMD (> 0.62 g/cm^2) was a significant predictor of breast cancer risk in individuals with low estradiol concentrations (HR 2.6 for ≤ 10pmol/l, 95%CI 1.2-5.7), but not associated in individuals with high estradiol concentrations (HR 0.9 or > 10pmol/l, 95%CI 0.4-1.8).[199] **Table 3** provides an overview of the important characteristics of the published studies that have investigated the association between BMD and breast cancer risk along with details of the major findings.

Table 3. Population characteristics and results of studies assessing the association of bone mineral density and incident breast cancer

Author Population	Study Type / Length	Study Population	BMD Measure / Results*	Adjustment Factors
Cauley 1996 Study of Osteoporotic Fractures (SOF)	Prospective cohort 3.2 years mean follow-up	97 cases / 6854 cohort Postmenopausal, 65+ years, Caucasian, no HBC, excluded cases within first year, not currently on ERT	<u>Proximal radius (SPA):</u> RR=1.3 (1.1-1.6) Q1 RR=1.00 P_{trend} =.01 Q2 RR=1.9 (1.0-3.7) Q3 RR=2.4 (1.3-4.7) Q4 RR=2.3 (1.2-4.5) <u>Distal radius (SPA):</u> RR=1.4 (1.1-1.7) Q1 RR=1.00 P_{trend} =.004 Q2 RR=1.9 (1.0-3.7) Q3 RR=2.1 (1.1-3.9) Q4 RR=2.7 (1.4-5.1) <u>Calcaneus (SPA):</u> RR=1.2 (1.0-1.5) Q1 RR=1.00 P_{trend} =.01 Q2 RR=2.1 (1.1-4.2) Q3 RR=2.4 (1.2-4.8) Q4 RR=2.5 (1.3-5.0) <u>Femoral neck (DXA):</u> RR=1.5 (1.2-1.9) Q1 RR=1.00 P_{trend} =.001 Q2 RR=2.1 (0.8-5.6) Q3 RR=4.1 (1.6-10.0) Q4 RR=4.0 (1.6-9.7) <u>Total spine (DXA):</u> RR=1.4 (1.1-1.7) Q1 RR=1.00 P_{trend} =.01 Q2 RR=1.8 (0.8-4.1) Q3 RR=1.5 (0.6-3.5) Q4 RR=3.3 (1.6-7.1)	Multivariable adjustment did not affect study results.
Zhang 1997 Framingham Study	Prospective cohort 22.1 years median follow-up	91 cases / 1373 cohort Postmenopausal, 47-80 years, no HBC, race unknown	<u>Second metacarpal (Radiograph):</u> Q1 RR=1.0 P_{trend} =.001 Q2 RR=1.3 (0.6-2.8) Q3 RR=1.3 (0.6-2.7) Q4 RR=3.5 (1.8-6.8)	Multivariable adjusted rate ratios did not affect results.

Table 3. continued

Author Population	Study Type / Length	Study Population	BMD Site / Results*	Adjustment Factors
Kuller 1997 Study of Osteoporotic Fractures (SOF)	Prospective cohort 3.2 years mean follow-up	121 cases / 8065 cohort Postmenopausal, 65+ years, Caucasian, no HBC, excluded cases within first year	Proximal radius (SPA): Q1 IR=2.5 (1.5-4.3) Q2 IR=4.1 (2.1-6.2) Q3 IR=5.1 (3.5-7.4) Q4 IR=5.5 (3.9-7.8) Other sites were not presented	Multivariable adjustment did not affect study results.
Lucas 1998 Study of Osteoporotic Fractures (SOF)	Prospective cohort 3.2 years mean follow-up	104 cases / 7250 cohort Postmenopausal, 65+ years, Caucasian, no HBC, excluded cases within first year	Proximal radius (SPA): T1 RR=1.0 T2 RR=1.5 (0.9-2.6) T3 RR=1.8 (1.0-3.1) Family history (+/-): RR=4.2 (2.0-9.0) T3+ vs. T1- Distal radius (SPA): T1 RR=1.0 T2 RR=1.6 (0.9-2.8) T3 RR=2.4 (1.4-4.2) Calcaneus (SPA): T1 RR=1.0 T2 RR=1.5 (0.8-2.5) T3 RR=1.5 (0.9-2.7)	35% increase in breast cancer relative risk per 1 SD increase in radial BMD based on multivariable adjusted models.
Nguyen 2000 Dubbo Osteoporosis Epi Study (DOES)	Nested case-control Cases self-reported at baseline	30 cases / 120 controls Postmenopausal, 60+ years	Femoral neck (DXA): RR=1.4 (1.0-2.3) Lumbar spine (DXA): RR=2.0 (1.3-3.0)	Matched on age and weight (±3kg) Years of ovulation, BMI, age at menarche, parity, HRT use in past 5 years
Buist$_a$ 2001 Fracture Intervention Trial (FIT)	Prospective cohort 3.7 years mean follow-up	131 cases / 8203 cohort Postmenopausal, 54-80 years, majority Caucasian, no HBC, excluded cases within first 6 months	Proximal femur (DXA): Q1 RR=1.0 Q2 RR=1.9 (1.1-3.2) Q3 RR=1.5 (0.8-2.6) Q4 RR=1.5 (0.8-2.7) Family history (+/-): Q1+ RR=1.8 (0.6-4.8) Q2-4+ RR=2.3 (1.1-4.5) Q1- RR=1.0 Q2-4- RR=1.7 (1.0-2.9)	Multivariable adjustment

Table 3. continued

Author Population	Study Type / Length	Study Population	BMD Measure / Results*	Adjustment Factors
Buist$_b$ 2001 Fracture Intervention Trial (FIT)	Case-cohort study 3.7 years mean follow-up	109 cases / 173 controls Postmenopausal, 54-80 years, majority Caucasian, no HBC, excluded cases within first 6 months	Proximal femur (DXA): Q1 RR=1.0 Q2 RR=2.1 (1.0-4.8) Q3 RR=1.5 (0.6-3.6) Q4 RR=1.4 (0.5-4.0)	The relationship between BMD and breast cancer risk is truncated when measured hormone levels and other covariates are controlled for.
Zmuda 2001 Study of Osteoporotic Fractures (SOF)	Prospective cohort 6.5 years mean follow-up	315 cases / 8905 cohort Postmenopausal women aged 65+, Caucasian, no HBC, excluded cases within first year	Proximal radius (SPA): Q1 RR=1.0 Q2 RR=1.6 (1.1-2.3) Q3 RR=1.7 (1.2-2.4) Q4 RR=2.0 (1.4-2.9) Distal radius (SPA): Q1 RR=1.0 Q2 RR=1.4 (1.0-2.1) Q3 RR=1.4 (0.9-1.9) Q4 RR=2.0 (1.4-2.9) Calcaneus (SPA): Q1 RR=1.0 Q2 RR=1.1 (0.8-1.7) Q3 RR=1.8 (1.3-2.6) Q4 RR=1.8 (1.3-2.6)	Multivariable adjustment did not affect study results.
Ganry 2001	Case-control	126 cases / 126 controls Postmenopausal, age unknown	Lumbar spine: significant Femoral neck: NS Trochanter: significant Ward's Triangle: significant Q4 vs. Q1 RR range (2.5-4.8)	Limited information based on abstract only

Table 3. continued

Author Population	Study Type / Length	Study Population	BMD Measure / Results*	Adjustment Factors
Nelson 2002 NHANES I Epidemiologic Followup Study	Prospective cohort 19 years follow-up	41 cases / 1091 cohort Postmenopausal, 55+ years, majority Caucasian, no HBC	Wrist: Q1 RR=1.0 P_{trend} =.04 Q2 RR=0.2 (p=0.03) Q3 RR=1.6 (p=0.23) Q4 RR=1.7 (p=0.26)	Age, race, and BMI
Van der Klift 2003 Rotterdam Study	Prospective cohort 6.5 years mean follow-up	74 cases / 3107 cohort Postmenopausal women 55+ years, no HBC, race not addressed	Lumbar spine (DXA): T1 RR=1.5 (0.8-2.9) T2 RR=1.0 T3 RR=2.1 (1.1-3.7) Femoral neck (DXA): T1 RR 0.8 (0.4-1.4) T2 RR=1.0 T3 RR 1.0 (0.6-1.7) Intertrochanteric (DXA): T1 RR 0.7 (0.4-1.3) T2 RR=1.0 T3 RR 1.1 (0.6-1.9)	Multivariable adjustment did not affect study results.
Nelson 2004	Hospital based matched case-control	221 cases / 197 controls Caucasian, mixed menopausal status, aged 40-85 years, no HBC, no steroids/bisphosphonates >1month	Proximal radius (DXA): OR=2.0 (1.1-3.6) Z-score >0 vs. <0	Matched on ethnicity and age BMI, menopausal status, age, HRT use

Table 3. continued

Author Population	Study Type / Length	Study Population	BMD Measure / Results*	Adjustment Factors
Ganry 2004 Epidemiologic Study of Osteoporosis (EPIDOS)	Prospective cohort 7 years mean follow-up	45 cases / 1504 cohort Postmenopausal, 75+ years, Caucasian, no HBC, excluded cases within first 6 months, no metabolic bone disease	Femoral neck (DXA): T1 RR=1.0 T2 RR=2.6 (1.1-6.8) T3 RR=3.1 (1.2-7.8) Trochanter (DXA): T1 RR=1.0 T2 RR=1.4 (0.6-3.3) T3 RR=2.2 (1.1-4.8) Ward's Triangle (DXA): T1 RR=1.0 T2 RR=1.6 (0.7-3.5) T3 RR=2.2 (1.0-4.8)	Multivariable adjustment
Kerlikowske 2005 San Francisco Mammography Registry	Nested case-control 2 years mean follow-up	208 cases / 436 controls Aged 28+ years, no HBC, excluded breast augmentation/mastectomy	Total hip (DXA): Q1 OR=1.0 Q2 OR=1.1 (0.7-1.9) Q3 OR=1.3 (0.8-2.1) Q4 OR=1.2 (0.7-2.1)	Age, family history, age at first live birth/nulliparous, breast density, race, BMI
Stewart 2005	Prospective cohort 9.7 years mean follow-up	87 cases / 3,013 cohort Peri/early-menopausal, 45-54 years, no HBC	Lumbar spine (DXA): RR=1.2 (1.0-1.5) / 1 SD decrease Femoral neck (DXA): RR=1.2 (0.9-1.5) / 1 SD decrease	Age, height, weight, menopausal status, HRT use
Cummings 2005 Study of Osteoporotic Fractures (SOF)	Case-cohort 10.5 years follow-up	196 cases / 378 controls Postmenopausal, 65+ years, no ERT,	Distal radius (SPA): Estrogen Receptor Positive RR=1.2 (0.9-1.5)/1SD increase	Age, weight, education, testosterone, estradiol

Table 3. continued

Author Population	Study Type / Length	Study Population	BMD Measure / Results*	Adjustment Factors
Cauley 2007 Multiple Outcomes of Raloxifene Evaluation (MORE) & Continuing Outcomes Relevant to Evista (CORE)	Clinical Trial – Placebo arm 13,698 py follow-up	65 cases / 2,511 controls Postmenopausal, ≤80 years, >2 years past menopause, osteoporotic, no HBC/endometrial cancer/stroke/venous thromboembolism in past 10 years	<u>Femoral neck (DXA):</u> Low estradiol (≤10pmol/l) RR=2.6 (1.2-5.7) >0.62/≤0.62 (g/cm^2) High estradiol (>10pmol/l) RR=0.9 (0.4-1.8) >0.62/≤0.62 (g/cm^2) <u>Lumbar spine (DXA):</u> not associated	Age, family history, estradiol, estradiol-BMD interaction term
Hadji 2007 Marburg Breast Cancer & Osteoporosis Trial (MABOT)	Case-control	242 cases / 2250 controls (matched analysis = 242 controls) Untreated cases, no family history, aged 22-88 years	<u>Calcaneus (Ultrasonometry):</u> Q1 OR=1.0 Q2 OR=1.9 (1.1-3.2) Q3 OR=2.3 (1.3-3.9) Q4 OR=2.9 (1.7-5.0) T-score p_{trend} = <0.001	Post-matched (1:1) on age, body weight, height, BMI, duration of estrogen exposure (endogenous and exogenous), estrogen use, age at menarche, age at menopause, parity, and breast feeding

* Includes *carcinoma in situ* along with invasive breast cancer cases
Abbreviations used: ERT, estrogen replacement therapy; DXA, dual energy x-ray absorptiometry; HBC, history of breast cancer; IR, incident rate = cases/1000 person years; OR, odds ratio; RR, relative risk; SPA, single photon absorptiometry

2.6.3 Variation in BMD Measure

BMD has been investigated at a number of skeletal sites covering six body regions. These regions are the forearm (sites include the proximal radius),[184, 195, 196, 200, 201] wrist (distal radius),[184, 192, 195-197] hand (second metacarpal),[194] spine (total spine and lumbar spine),[184, 193, 199, 202-204] hip (total hip, femoral neck, proximal femur, intertrochanteric, trochanter, and Ward's triangle),[184, 190, 191, 193, 198, 199, 202-205] and the heel (calcaneus).[184, 195, 196, 206] Results of prospective studies reporting relative risks for individual skeletal sites are highlighted in **Figure 3**.

The literature is fairly consistent in its assessment of the increasing risk of breast cancer with higher BMD measurements and these results hold for a variety of skeletal sites. The magnitude of the results, however vary by study and by skeletal site and are at best moderate to weak in their association. Peripheral skeletal sites and the lumbar spine (which have more trabecular bone and may therefore be more sensitive to estrogen) have been proposed to be better predictors of breast cancer risk than measurements of the hip, since it is a major load-bearing site and potentially more affected by lifestyle differences.[201] The two studies utilizing the Fracture Intervention Trial cohort reported a distinct threshold effect, while the other studies showed a more gradual increase with increasing level of BMD.[190, 198] The strongest results are found when several skeletal sites are assessed in conjunction with one another. Because BMD can vary from one skeletal site to another on a single woman, Kuller et al. investigated the effect of low BMD at all sites versus high BMD at one or more sites and found that women with low BMD at several skeletal locations were highly protected against breast cancer compared to a woman with any measure of high BMD (RR=0.23, 95%CI 0.07-0.72).[200] Some studies have found no association.[204, 205] There are study specific issues that may have contributed to the inconsistencies in the findings, such as differences in skeletal region measured, bone mass

measurement methods, and characteristics of the populations studied, including inclusion criteria, sample size, and length of follow-up.

Although the relationship between higher BMD and an increased risk of breast cancer in postmenopausal women has been well studied, the association is moderate at best, and critics still question the validity and/or biologic plausibility of these findings.[207] One reason that a stronger association has not been documented, may be that even among women with low BMD, their exposure to estrogen has been sustained in a manner that negates the protective effect of having a lower BMD. Or conversely, among women with a higher BMD, their estrogen exposure has been diminished so that their increased risk of breast cancer has been attenuated. By looking at a women's change in postmenopausal BMD level, it may be possible to relate BMD to risk of breast cancer in a way that accounts for sustained estrogen exposure. The one study to have looked at change in BMD over 6.9 years and the risk of breast cancer did so only among peri-postmenopausal women aged 45-54.[204] They found no relationship with a mean follow-up of 9.7 years. The HR (95%CI) for 1 SD change in BMD at the spine was 1.17 (0.80-1.71) and 1.15 (0.79-1.68) at the femoral neck. Given there were only 34 incident breast cancer cases, power was low and likely inadequate to detect a difference.

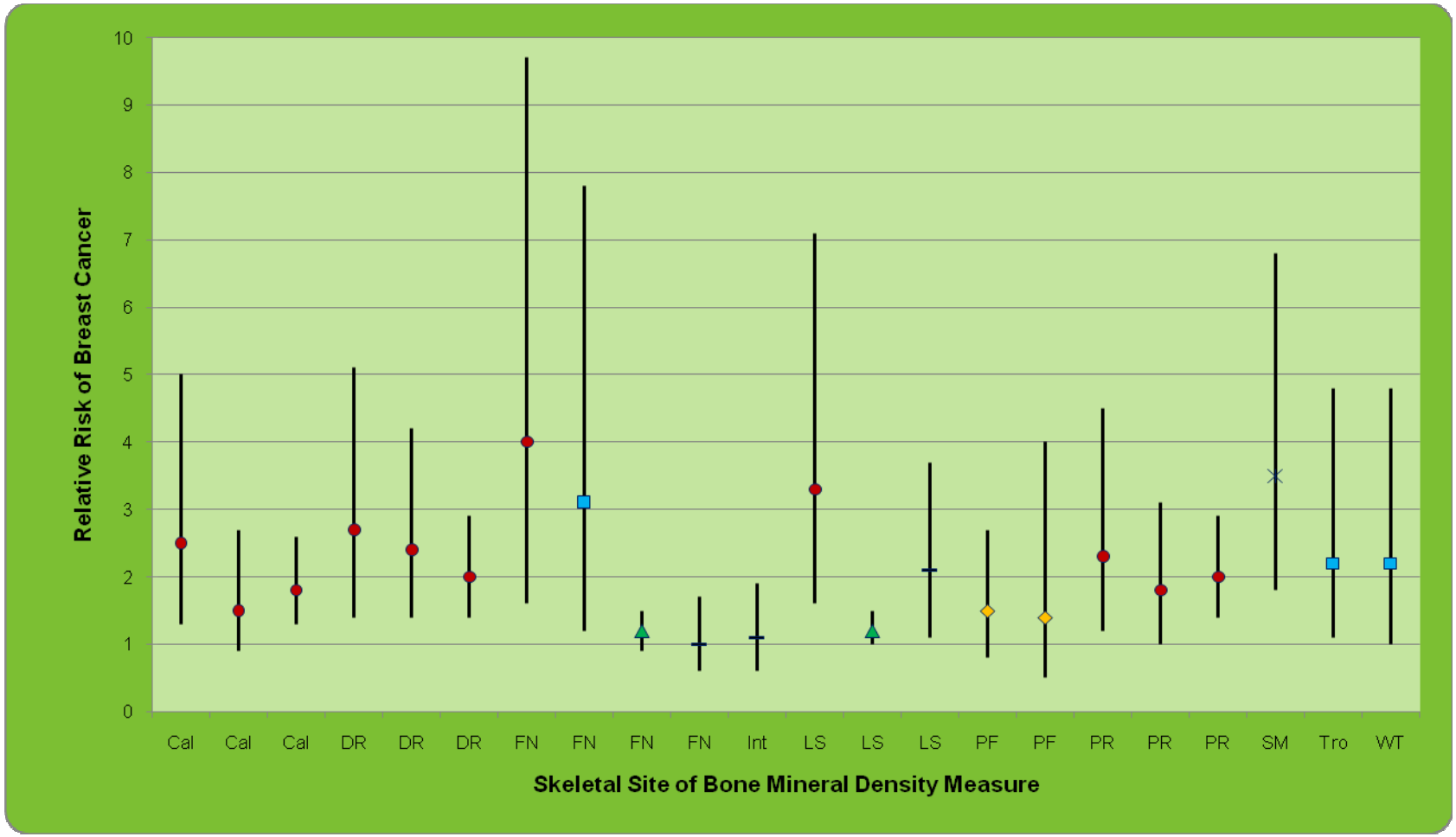

Figure 3. Relative risk of breast cancer for measures of bone mineral density from prospective cohort studies
Comparisons groups are study specific and between the highest and lowest BMD category. Markers indicate point estimates; symbols designate cohorts:●=SOF, ■=EPIDOS, ▲=population based, ▬=Rotterdam, ◊=FIT, x=Framingham. Vertical lines represent 95% confidence intervals. BMD skeletal sites: Cal=calcaneus, DR=distal radius, FN=femoral neck, Int=Intertrochanteric, LS=lumbar spine, PF=proximal femur, PR=proximal radius, SM=second metacarpal, Tro=trochanter, WT=Ward's triangle.

2.6.4 Summary

While the proportion of elderly women with high bone density in the general population is less than 5%, the risk of breast cancer attributable to BMD is approximated to be 21%.[203] Moreover, a recent analysis from the Women's Health Study found that BMD predicted breast cancer risk independently of Gail score.[208] There are many risk factors for breast cancer, and bone mineral density is just one of them. Because of its close ties to estrogen exposure, BMD may help to elucidate the underlying biology linking estrogen and breast cancer. BMD also has the potential to be an important and easily measured marker of breast cancer risk, if it is found to be a reliable predictor. There is much to be clarified concerning the strength of the relationship between BMD and breast cancer including the short-term versus the long-term risk associated with elevated BMD and if the rate of bone loss might be a better surrogate of estrogen exposure.

2.7 SUMMARY

As highlighted in **Figure 2**, the role of vitamin D in the body varies widely from building bone to regulating gene expression. Aside from its function in calcium homeostasis, the action of vitamin D in the breast is completely separate. Maintaining adequate levels of vitamin D has the potential to prevent breast cancer through control of cellular processes including differentiation, proliferation, and apoptosis. The few existing studies of circulating 25(OH)D and risk of breast cancer have produced inconsistent results which are likely due to their inadequate size and/or design. Alternatively, lack of findings regarding risk of breast cancer in older women could be a result of lost sensitivity to $1,25(OH)_2D$ with age. FCA may be an easily measured surrogate for general tissue responsiveness to $1,25(OH)_2D$.

Bone mineral density, a marker of lifetime estrogen exposure, has been shown to be a moderately weak predictor of breast cancer among postmenopausal women over a relatively short period of time. However, the association is not as strong as expected. Additional studies are needed to determine if the relationship is maintained/strengthened/weakened with longer follow-up, and if other measures of BMD might be better at predicting breast cancer risk.

3.0 SPECIFIC AIMS AND HYPOTHESES

The following specific aims and hypotheses are proposed to address the areas requiring additional research identified above.

1) **To examine the association between serum concentrations of 25(OH)D and the risk of ER+ breast cancer among postmenopausal women.** *It is hypothesized that postmenopausal women with a low serum concentration of 25(OH)D will have an increased risk of breast cancer.*

    a. Secondary aims are to test the hypotheses that this association differs by age and obesity. *Further hypotheses are that the inverse association between low serum levels of 25(OH)D and higher breast cancer risk will be stronger among older and obese women.*

2) **To examine the association between fractional calcium absorption and the risk of breast cancer among postmenopausal women.** *It is hypothesized that postmenopausal women with lower fractional calcium absorption are at increased risk of breast cancer.*

    a. Secondary aims are to test the hypotheses that this association differs for ER+ cancers and by calcium intake. *Further hypotheses are that the inverse association between low FCA and higher breast cancer risk will not differ for ER+ cancers, and that by category of calcium intake, women with low FCA*

will have an increased risk of breast cancer compared to women with high FCA.

3) **To assess whether the positive association between higher bone mineral density and increased breast cancer risk is maintained over a longer follow-up period, and if a measure of BMD change strengthens the association.** Specifically, does a repeated bone mineral density measure (i.e. annualized percent change) enhance the prediction of breast cancer over that of a single measure and what is the association between change in BMD and breast cancer risk? *It is hypothesized that postmenopausal women with a high vs. low BMD will have an increased risk of breast cancer. Furthermore, it is hypothesized that a lower rate of bone loss will be associated with an increased risk of breast cancer.*

a. Secondary aims are to test the hypotheses that this association differs for ER+ cancers and by family history of breast cancer. *Further hypotheses are that the positive association between high BMD and increased breast cancer risk will be stronger for ER+ cancers and those with a positive family history of breast cancer.*

4.0 ARTICLE 1: SERUM 25-HYDROXYVITAMIN D AND RISK OF ER+ BREAST CANCER IN POSTMENOPAUSAL WOMEN

Manuscript in Preparation

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4.1 ABSTRACT

Evidence suggests that vitamin D may reduce the incidence of breast cancer. The few epidemiologic studies that have investigated the relationship between circulating levels of 25-hydroxyvitamin D [25(OH)D] and breast cancer risk have produced inconsistent results. We examined the subsequent risk of estrogen receptor positive (ER+) breast cancer related to serum levels of 25(OH)D in a case-cohort study within the Study of Osteoporotic Fractures (SOF), a prospective cohort of 9,704 postmenopausal, Caucasian women aged 65 and over. Serum 25(OH)D level was measured for 170 incident ER+ breast cancer cases and a random subcohort (n=332) of SOF participants using serum specimens collected at baseline (1988-1989). A case-cohort analysis was performed to compute relative risks of breast cancer and 95% confidence intervals. Mean time between blood draw and diagnosis was 6.0 years. The median 25(OH)D level was 27.5 ng/ml and did not differ between cases and non-cases (p=0.5). Low levels of 25(OH)D were not associated with an increased risk of breast cancer. Compared to women with sufficient levels of 25(OH)D (≥ 30 ng/ml), women with lower levels (20-30 and < 20 ng/ml) had relative risks (95%CI) of 0.94 (0.61-1.46) and 1.15 (0.63-2.12), respectively (p_{trend}=0.8) in multivariable models adjusted for age (as timescale), clinic site, season of blood draw, BMI, smoking history, and estrogen therapy (ET). The findings of this prospective study of postmenopausal women are not supportive of an overall association between serum 25(OH)D concentration and the development of ER+ breast cancer.

4.2 INTRODUCTION

Evidence of the inverse association between vitamin D and breast cancer incidence and mortality has been shown through ecological studies of ultraviolet radiation. Populations living at sunny lower latitudes (regions with higher levels of solar UV-B radiation) have higher circulating levels of 25(OH)D,[111] have decreased breast cancer risk,[112, 113] and lower mortality rates[114-118] compared with populations living at higher latitudes (regions with lower levels of UV-B radiation). Studies of vitamin D intake from dietary and supplemental sources have reported mixed findings.[119, 121-125] A recent randomized controlled trial (RCT) of calcium and vitamin D supplementation from the Women's Health Initiative (WHI) reported no association with breast cancer, but the low dose of vitamin D used (400 IU) was inadequate to raise vitamin D concentrations to a sufficient level.[126]

25-hydroxyvitamin D is the principal circulating vitamin D metabolite, directly reflecting both cutaneous production and dietary intake and is therefore the measure used to determine vitamin D sufficiency status.[96] 1,25-dihydroxyvitamin D [$1,25(OH)_2D$] is the biologically active form of vitamin D[95] and its activities are mediated by vitamin D receptors (VDR) in target tissues.[104-106] Both normal and cancerous mammary cells have the ability to convert 25(OH)D into $1,25(OH)_2D$.[107] Breast cells contain the VDR which becomes activated through interaction with $1,25(OH)_2D$ and can inhibit cellular proliferation and induce differentiation and apoptosis in normal mammary gland and breast cancer cells.[105, 109] A possible mechanism for the malignant transformation of breast cells is through insufficient 25(OH)D levels which limits the synthesis of $1,25(OH)_2D$, thus preventing activation of the VDR to regulate the cell cycle.[98] Therefore, vitamin D has the potential to influence the development of breast cancer.

More than 50% of women, aged 60+ years, who were surveyed in the Third National Health and Nutrition Examination Survey were found to have inadequate serum 25(OH)D levels (i.e. < 62.5 nmol/L or 25.0 ng/ml) during the summer months at northern latitudes in the United States.[209] Despite the high prevalence of low vitamin D status among older women, the association between circulating 25(OH)D and breast cancer risk is under-studied. The few prior studies have produced inconsistent results and, have been limited by combined premenopausal and postmenopausal populations[130, 131, 133, 134] and cross-sectional case-control design.[132-134] In fact, the association has never been investigated prospectively among postmenopausal women by histological subtype. This is an important distinction given that the majority of postmenopausal cancers are ER+, and that ER+ and ER- tumors likely have different etiologies.[8] We undertook a case-cohort study to investigate to the risk of ER+ breast cancer associated with serum 25(OH)D concentrations among postmenopausal women. Specifically we tested the hypothesis that low levels of 25(OH)D will be associated with an increased risk of ER+ breast cancer. We also tested whether this association differed by age or obesity as secondary aims.

4.3 METHODS

4.3.1 Study Population

The Study of Osteoporotic Fractures is a longitudinal cohort of 9,704 community-dwelling, Caucasian, postmenopausal women aged 65 and over who were recruited at 4 US clinical centers between 1986 and 1988. Women with a history of bilateral hip replacements and those who were unable to walk unassisted were excluded.[210] At the baseline examination, women

provided informed consent and risk factor and health measures were collected through physical measurements, questionnaires, and functional assessments. Serum samples were also obtained and immediately frozen at -20^{o}C for no more than two weeks before being stored in liquid nitrogen at -190^{o}C.

4.3.2 Study Design

The study is a case-cohort design,[211] and is a secondary analysis of an existing case-cohort within SOF. The original study investigated the relationship of serum sex hormone levels and ER+ breast cancer risk.[197] The original subcohort was a random sample of the entire SOF cohort. The original case group included all incident ER+ breast cancer cases validated through December 2000 when the case-cohort study was formed.

4.3.3 Study Subjects

Participants in this study were part of a previous case-cohort study. Incident breast cancer cases were defined by the diagnosis of estrogen receptor positive breast cancer occurring after the baseline examination. All incident cases with sufficient stored serum were included (n=160). A random subcohort of 363 women, with available serum, was chosen and formed the comparison group (subcohort), which included 15 cases. Twenty individuals reporting a prior history of breast cancer at baseline were excluded from this analysis. A single case subject was excluded due to a missing 25(OH)D measure. The final study was therefore comprised of 332 subcohort non-cases, 14 subcohort cases, and 156 cases from outside the subcohort.

4.3.4 Serum 25(OH)D Laboratory Measurement

Serum 25(OH)D was measured by the designated Reproductive Endocrine Research Laboratory of the University of Southern California under the direction of Frank Stanczyk, PhD. Specimens were shipped via overnight courier in a Styrofoam box packed with dry ice, and arrived frozen and undamaged. The laboratory stored the samples at -70°C until they were assayed using 25(OH)D RIA kits and reagents from Diasorin (Stillwater, MN). This kit effectively detects both the D2 and D3 forms of endogenous 25(OH)D in human serum and has been shown to produce more reliable results than other commercially available 25(OH)D kits.[129] In order to minimize interassay variability, the same kit lot number was used to analyze all the samples. In addition, a single highly trained technician was used to carry out all the assays and was masked to subject identity and all participant characteristics including case-control status. Samples were labeled by number only. Assay methods were identical for cases and the subcohort and were completed in a single batch. The performance of the 25(OH)D RIA kit was first checked by completing an assay using the manufacturer provided quality control (QC) samples and confirming that the obtained values were within the expected range reported in the instruction manual. The samples for the assay were thawed by standing at room temperature before being mixed thoroughly by inversion. 25(OH)D was extracted from the calibrators, high and low level controls and study samples by first aliquoting 50 µl of each into separate tubes containing 500 µl of acetonitrile and then vortexing each tube. Following a centrifugation step, 25 µl aliquots were taken from each of the supernatants and transferred into a new set of labeled tubes. RIA was carried out in the usual manner by addition of ^{125}I-25(OH)D and 25(OH)D antiserum, incubation, and addition of second antibody to separate the antibody-bound and unbound 25(OH)D. The tubes were then counted in a gamma counter, and results were calculated using an RIA program. The assay sensitivity was 1.5 ng/ml, and the interassay

CVs 11.7%, 10.5%, 8.6% and 12.5% at 8.6, 22.7, 33.0 and 49.0 ng/ml, respectively. Comparison of the present RIA method with the liquid chromatography mass-spectroscopy (LC-MS/MS) assay carried out at the Mayo Clinic Endocrine Laboratory (Rochester, MN) showed a high correlation (Spearman correlation r=0.92; p=<0.001) between 25(OH)D values measured by the two assay methods.[212] Furthermore, there was no significant difference in the mean 25(OH)D levels between the two methods (paired t-test, p=0.73). Approximately 5% of our study samples (n=25) were tested in a blinded duplicate fashion. The correlations of assay values determined in the duplicate samples were high (R^2=0.76) (**Appendix A**).

4.3.5 Covariate Information

At the baseline examination (V1), participants completed a questionnaire and were interviewed. Demographic (age, education), reproductive history (menarche age, parity, age at first birth, number of live births, breastfeeding), height at age 25, menopausal status (menopause age, surgical vs. natural menopause), and breast cancer risk factor (benign breast disease, family history of breast cancer) data were collected. Women were asked about smoking status, alcohol use (average number of alcoholic drinks/week), and physical activity. Body weight was measured using a balance-beam scale. BMI was calculated by dividing the V1 weight (kg) by the square of height (m) at age 25 years. Bone mineral density (g/cm^2) of the proximal femur was measured using dual-energy x-ray absorptiometry (QDR 1000, Hologic, Waltham, Massachusetts).[210, 213] Exogenous estrogen use (i.e. ET) was defined as currently taking estrogen pills. Current supplemental vitamin D use was defined as taking vitamin D or a multivitamin containing vitamin D at least once per week. Calcium supplementation was determined by asking about current use at least once per week. Dietary calcium intake was estimated by using a validated 20-item Block semi-quantitative food-frequency questionnaire

developed from the Second National Health and Nutrition Survey (NHANES II).[214, 215] Total calcium intake was calculated by summing dietary calcium intake (mg/d) and daily dose of calcium supplements (mg/d). Month of blood draw was grouped into seasons of winter (December-February), spring (March-May), summer (June-August), and fall (September-November). Variables were categorized based on common cutpoints (e.g. BMI) or the original response categories collapsed to prevent small cell counts (e.g. age at first birth). Continuous variables were additionally dichotomized at their median and/or divided into quartiles for analytic purposes.

4.3.6 Incident Breast Cancer Ascertainment

Follow-up occurred every four months by either postcard or telephone (98% complete) in addition to clinic visits approximately every 2 years. Breast cancer outcomes were ascertained through self-report or death certificate review and were adjudicated by physicians locally and centrally at the San Francisco Coordinating Center. Medical records and pathology reports were used to record information on date of breast cancer diagnosis, stage at diagnosis, and estrogen- and progesterone-receptor status.[184]

4.3.7 Statistical Analysis

Smoothed density plots and kernel density plots of serum 25(OH)D by age at baseline for the cases, subcohort cases, and the non-case subcohort were investigated (**Appendix A**). No meaningful differences in the distribution of the subcohort cases and non-subcohort cases were found. All subsequent analyses were conducted with a combined case group (n=170).

As part of the preliminary data analysis, the distribution of baseline characteristics among the cases and the non-cases were compared by t-test for continuous variables and the chi-square test for categorical data. The median values of 25(OH)D were also compared by age, season of blood draw, vitamin D supplement use, and BMI using either the Wilcoxon rank sum test or the Kruskal-Wallis test. Supplemental vitamin D use, defined as taking vitamin D or a multivitamin containing vitamin D at least once per week, was assessed at the baseline examination and categorized as current/past/never user. The median difference in 25(OH)D level was evaluated by category of vitamin D supplementation. The log transformation of 25(OH)D was investigated, however, it did not significantly improve normality, and therefore modeling was pursued with the non-transformed values (**Appendix A**).

The main analysis estimated hazard ratios (HR) and 95% confidence intervals (95%CI) for the association of serum 25(OH)D level and with the risk of incident ER+ breast cancer using multivariable Cox proportional hazard regression models that include robust standard error estimates to account for the case-cohort design,[211, 216] with age as the underlying time scale.[217-219] The analysis of a case-cohort study differs from that of a traditional cohort in that the denominator sums over subjects at risk in the subcohort rather than subjects at risk in the entire cohort. Subcohort members (cases and non-cases) contributed to the analysis over their entire time in the study whereas the cases outside the subcohort contributed only at their event time. At each event (failure), a risk set was formed which consisted of only the subcohort members (cases and non-cases) and any non-subcohort cases that failed at that time. The addition of non-subcohort cases at their respective event times results in non-nested risk sets.

The level of the 25(OH)D serum marker was entered as a continuous variable to estimate the relative risk (RR) of breast cancer. Levels of vitamin D were also assessed as categories of deficient (< 20 ng/ml), insufficient (≥ 20 to < 30 ng/ml), and sufficient (≥ 30 ng/ml).[98] The relative risk of breast cancer, estimated as hazard ratios, was estimated for each

category using the highest category as the reference and adjusting for covariates as necessary. Because laboratory measured vitamin D is a continuous measure with clinically important threshold values, cubic splines were fit to investigate non-linear effects. Restricted cubic splines allow continuous data to fit within the Cox model without assuming linearity.[220] Cubic splines are piecewise polynomial functions that are constrained to join at control points (knots) in the data. Forcing the first and second derivatives of the functions to agree at the knots results in smooth splines. RCS (restricted cubic splines), a SAS macro, was used to create the cubic splines.[221] Knots were placed at typical clinical cut-points used to assess vitamin D status (15, 20, and 30 ng/ml).[98-100] To ensure that the location of the knots did not influence the results, a spline with 4 knots placed at standard percentiles (5, 25, 75, 95) was also investigated.[221]

All models were adjusted for SOF clinic site and season of blood draw. Preliminary multivariable models were fit separately and included potential confounders described above based on their significance (p=0.1) in the univariate analyses as well as a priori established breast cancer risk factors. In situations where variables are correlated, e.g. various measures of body size, variable choice was based on statistical association, scientific knowledge, and/or variable distribution. The potential correlation of continuous modeling covariates was investigated using a covariance matrix (**Appendix A**). Dummy variables were created for categorical variables as appropriate. Overall model significance was assessed by partial likelihood ratio tests comparing each of the fitted models to the univariable model. The final model was selected by entering all the covariates from earlier models and using a backward elimination strategy.[222, 223] Variables were selected for elimination one at a time based on univariate Wald tests. After each variable was removed from the model, the partial likelihood ratio test was calculated comparing the nested models. Removed variables remained out of the models if they made no significant contribution to the model. Likelihood ratio tests were used to evaluate the significance of potential interactions by comparing the model including the

interaction term to the main effects model. Analyses were repeated among subgroups defined by age (<75 and ≥75 years) and BMI (<30 and ≥30 kg/m^2). Due to small numbers, 25(OH)D was investigated as a continuous variable in subgroup analyses. For the final model, probability values < 0.05 were considered statistically significant. All tests were two-tailed.

Power was calculated a priori using previously reported SOF data (serum 25(OH)D mean (SD) = 25.8 (10.7) ng/ml)[224] with PASS 2005 software (NCSS, Kaysville, Utah). A two-sided two-sample t-test with equal variance, an alpha level of 0.05, 175 cases, and a 350 member subcohort, provides 80% power to detect a mean difference of 2.78 ng/ml (Cohen's *d* = 0.26) in the cases compared to the subcohort. Data descriptions, including graphical presentations, were carried out in STATA version 10. Cox proportional hazards modeling was performed using SAS software release 9.1.3 (SAS Institute Inc., Cary, NC).

4.4 RESULTS

This case-cohort study of postmenopausal women within the Study of Osteoporotic Fractures was comprised of 170 incident ER+ breast cancer cases and 332 non-cases. The mean time between baseline blood draw and breast cancer diagnosis was 6.0 years. **Table 4** presents the characteristics, including socio-demographic variables and established breast cancer risk factors of the study population at baseline. The mean age of cases and non-cases at baseline are 70.4 and 71.2 years, respectively. Compared to non-cases, cases were heavier (weight p<0.01; BMI p=0.02), and taller (p=0.03). Cases also had slightly higher bone mineral density (p=0.06), and were less likely to have ever smoked (p=0.06), although these differences were of borderline statistical significance.

Median serum concentrations of 25(OH)D were 27.3 ng/ml and 27.5 ng/ml among cases and non-cases, respectively (p=0.51). **Table 5** gives details of the median 25(OH)D levels by important study characteristics. Median 25(OH)D levels were highest among current vitamin D supplement users (31.1 ng/ml) compared to past (23.2 ng/ml) and never (24.5 ng/ml) users (p<0.01) and among women with a normal BMI (28.6 ng/ml) compared to those who were overweight (25.9 ng/ml) or obese (27.9 ng/ml) (p=0.03). Measurements were highest when taken during the summer months (28.9 ng/ml), and lowest during the spring (25.7 ng/ml); this difference was of borderline statistical significance (p=0.06). There was no significant difference in median 25(OH)D concentration by age group (p=0.69).

Table 6 shows the multivariable adjusted regression results for the association between serum level of 25(OH)D and incident breast cancer. In the simple analysis (model 1) of serum 25(OH)D categorized at clinically relevant cutpoints, which adjusted for age (as timescale), clinic site, and season of blood draw, there was no association with breast cancer (HR 1.13 for < 20 ng/ml, 95%CI 0.65-1.96; reference level ≥ 30 ng/ml). In model 2, the addition of BMD, weight, and smoking status slightly increased the risk estimate from 13% to 22% although it was not significant (HR 1.22 for < 20 ng/ml, 95%CI 0.66-2.24; reference level ≥ 30 ng/ml). Adjustment for a priori breast cancer risk factors in model 3 attenuated the risk estimate (HR 1.06 for < 20 ng/ml, 95%CI 0.54-2.10; reference level ≥ 30 ng/ml). In the main analysis (model 4), the lack of association between serum concentration of 25(OH)D and risk of postmenopausal ER+ breast cancer persisted. Compared with the highest category (≥ 30 ng/ml), the HR's (95%CI) for lower serum concentrations (20-30 and < 20 ng/ml) were 0.94 (0.61-1.46) and 1.15 (0.63-2.12), respectively (p=0.68). A test of linear trend was not significant (p_{trend}=0.69). No significant association was found with serum 25(OH)D modeled as a continuous variable (RR 1.01 per 1 SD decrease, 95%CI 0.80-1.26).

An examination of the shape of the risk function using cubic splines, displayed in **Figure 4**, showed no significant non-linearity (p=0.65).

Results of the subgroup analyses addressing the secondary aims are presented in **Table 7**. In multivariable models with 25(OH)D modeled as a continuous variable, no association between 25(OH)D and breast cancer was observed among women less than 75 years of age (RR 1.09 per 1 SD decrease, 95%CI 0.84-1.41). However, among women 75 years and older, a significant decrease in the risk of breast cancer was found with lower levels of 25(OH)D (RR 0.50 per 1 SD decrease, 95%CI 0.27-0.92). The interaction between age and 25(OH)D was not significant ($p_{interaction}$=0.43). There was no difference in the relationship between 25(OHD) and breast cancer risk by obesity, RR (95%CI) of breast cancer per 1 SD decrease in 25(OH)D was 1.00 (0.79-1.27) and 0.79 (0.27-2.29) for non-obese (BMI < 30 kg/m^2) and obese (BMI ≥ 30 kg/m^2) women, respectively ($p_{interaction}$=0.27).

4.5 DISCUSSION

This prospective, case cohort study of 502 postmenopausal women from the Study of Osteoporotic Fractures, showed little evidence of an association between serum 25(OH)D and ER+ breast cancer. A positive relationship was noted among women greater than 75 years of age. The association between serum 25(OH)D and ER+ breast cancer did not seem to differ by obesity.

Previous studies reporting on the association between 25(OH)D concentration and postmenopausal breast cancer show disparate results; two have found an inverse association, while the third shows no association.[130-132] Most recently, a case-cohort study from the Prostate, Lung, Colorectal, and Ovarian Cancer Screening Trial found no association overall

among women 55-74 years (RR 1.04 for < 18.3 vs. ≥ 33.7 ng/ml, 95%CI 0.75-1.45, p_{trend}=0.81) or when limited to women aged 60 and older (results not reported).[130] The Nurse's Health Study reported a modest but non-significant decreased risk of breast cancer in women 60 years and older (RR 0.57 for ≥ 41.7 vs. ≤ 22.0 ng/ml, 95%CI 0.31-1.04, p_{trend}=0.03).[131] In addition, they evaluated the relationship by estrogen and progesterone receptor status, and while they reported an inverse association for ER-/PR- (p_{trend}=0.08) but not for other subtypes (ER+/PR+ p_{trend}=0.30; ER+/PR- p_{trend}=0.33), statistical significance was not reached. Moreover, these later findings were for a combined pre- and post-menopausal population. These two findings appear to be contradictory given the observed associations were greater among older women who are more likely to have ER+ breast cancer, and for ER- cancers which are more likely to occur among younger women. In contrast, a single case-control study, reporting a strong inverse association among 1394 postmenopausal cases (OR=0.31 for ≥ 30 vs. < 12 ng/ml, 95%CI=0.2-0.4), reported no effect modification by ER status.[132] These findings are of limited relevance to the development of breast cancer as blood samples were collected after the diagnosis of breast cancer. Furthermore, as noted by Abbas et al., dietary and behavioral changes, as well as cancer therapy have the potential to affect circulating levels of 25(OH)D following a diagnosis.[132]

Our findings are noteworthy as they add to the small body of epidemiologic data concerning circulating 25(OH)D and breast cancer. The overall lack of association of serum 25(OH)D and ER+ breast cancer is consistent with the limited information currently known regarding the association with different tumor subtypes. One potential explanation is that circulating 25(OH)D is not an adequate measure of localized levels in the breast. It is also possible, due to the estrogen sensitivity of ER+ tumors, that estrogen plays a greater role in the development of breast cancer than the capacity of vitamin D to act as a preventive factor. More than 18% of the women in this study reported current estrogen use at baseline. However, adding exogenous estrogen use (current/past/never) to our multivariable models had little effect

on our 25(OH)D estimates. The body's reaction to vitamin D also may deteriorate with old age. The lack of an association may reflect either a decline in the interaction between $1,25(OH)_2D$ and VDRs in breast tissue, or diminished VDR expression. A study of muscle tissue found a significant decrease in VDR expression with increasing age ($r=0.5$, $p=0.004$) that was not correlated to either circulating 25(OH)D or $1,25(OH)_2D$ levels.[225] Furthermore, reports of tumors developing mechanisms to negate the anti-proliferative effects of $1,25(OH)_2D$ at the cellular level, such as enhancing expression of 24-OHase, the enzyme responsible for destroying $1,25(OH)_2D$, have been made.[226]

Body fat has been shown to negatively impact vitamin D levels; obese individuals have lower vitamin D bioavailability,[227] and 25(OH)D concentration is inversely associated with adiposity.[228] While we did see a significant difference in median 25(OH)D level by BMI category, the risk of ER+ breast cancer according to 25(OH)D level was not different for obese and non-obese women in this study. It has been hypothesized that because older women have a lower lean muscle to fat ratio for a given weight compared to younger women, BMI may not be an accurate measure of obesity among these women.[51] Nevertheless, obesity may be an important mediating factor in the association between vitamin D and breast cancer risk.

Our positive finding of a 50% reduced risk with lower 25(OH)D concentration among women 75 years of age and greater has never been reported. We do not interpret this to mean that higher levels of 25(OH)D lead to the development of breast cancer, but rather that there may be age related changes in the interaction of vitamin D and breast tissue. This result may have occurred by chance and further confirmation of this finding is needed.

While there is no established optimal serum 25(OH)D concentration, it is accepted to be at least 30 ng/ml which causes a plateau in serum parathyroid hormone level.[229] Recommended levels for cancer prevention are even higher (36-48 ng/ml).[100] There has only been one randomized controlled trial (RCT) of vitamin D supplementation and breast cancer

risk. The findings of the WHI showed no association with incident breast cancer risk.[126] However the dose of vitamin D (400 IU / day) administered in the WHI is not thought to have been high enough to adequately raise vitamin D levels. A RCT of overall incident cancer found a statistically significant reduced risk of cancer with combined daily calcium (1500 mg) and vitamin D (1100 IU) supplementation among postmenopausal women over age 55.[127]

Strengths of this study include its prospective case-cohort design from the large and long-standing population-based SOF cohort. We utilized pre-diagnostic serum samples and quantitatively measured 25(OH)D with the reliable Diasorin RIA assay. The 25(OH)D metabolite integrates all sources of vitamin D from diet, supplement, and sunlight making it a clinically relevant indicator of vitamin D status.

Limitations of this study include our reliance upon a single measure of 25(OH)D, which may not reflect long-term status. While our measure of total 25(OH)D was not able to distinguish between $25(OH)D_2$ and $25(OH)D_3$, this is not of particular concern as both metabolites have been shown to have a similar extremely high affinity in our assay (100% cross-reactivity).[129] Furthermore, we lacked a measure of the biologically active vitamin D metabolite $1,25(OH)_2D$. Because our study is comprised of older Caucasian women, our results may not be generalizable to other populations.

In conclusion, the findings of this study do not support a protective effect of 25(OH)D on ER+ breast cancer risk in postmenopausal women 65 years of age and older. However, the positive association among women 75 years and greater is suggestive of a change in the interaction of vitamin D in the breast. Further investigations of the role of vitamin D on breast cancer development among elderly women are warranted. In particular, it will be important to better define the factors influencing 25(OH)D levels in this vulnerable age group. Future studies should also include adequate numbers of women with ER- breast cancer.

4.6 TABLES AND FIGURES

Table 4. Baseline characteristics of study population, Study of Osteoporotic Fractures

	Breast Cancer Cases (n=170)		Subcohort Non-Cases (n=332)		
Characteristic	N	%	N	%	**p-value***
Age, y, mean(SD)	70.44 (4.54)		71.21 (4.70)		0.08†
Clinic Site					0.98#
A	42	24.71	83	25.00	
B	49	28.82	93	28.01	
C	40	23.53	83	25.00	
D	39	22.94	73	21.99	
Education					0.48#
< High School	31	18.24	73	21.99	
High School	70	41.18	140	42.17	
> High School	69	40.59	119	35.84	
Distal Radius BMD, g/cm^2, mean(SD)	0.38 (0.09)		0.37 (0.09)		0.06
Body Weight, kg, median(IQR)	69.9 (61.1-78.2)		65.5 (59.3-73.6)		<0.01‡
BMI, kg/m^2, median(IQR)	25.8 (23.1-29.2)		24.9 (22.7-27.6)		0.02‡
BMI					0.08#
< 25	78	44.24	164	51.57	
25-29	58	35.15	112	35.22	
≥ 30	34	20.61	42	13.21	
Waist/Hip Ratio, mean(SD)	0.81 (0.07)		0.81 (0.06)		0.70†
Height at age 25 y, cm, mean(SD)	163.52 (5.46)		162.39 (5.66)		0.03†
Age at Menarche, y					0.87#
≤ 11	24	15.19	43	14.01	
12-13	85	53.80	162	52.77	
≥ 14	49	31.01	102	33.22	
Nulliparous	25	14.71	66	19.88	0.15#
Number of Live Births					0.29#
Never pregnant	25	14.71	66	19.88	
0	4	2.35	6	1.81	
1-2	75	44.12	121	36.45	
3-4	45	26.47	105	31.63	
5+	21	12.35	34	10.24	
Age at First Birth, y					0.76#
≤ 20	30	17.65	52	15.66	
> 20	104	61.18	198	59.64	
Never gave birth	27	15.88	65	19.58	
Unknown	9	5.29	17	5.12	

Table 4. continued

Characteristic	Breast Cancer Cases (n=170) N	%	Subcohort Non-Cases (n=332) N	%	p-value*
Ever Breastfed	99	68.28	185	69.55	0.79#
Age at Menopause, y					0.32#
≤ 40	10	5.88	27	8.13	
41-45	30	17.65	46	13.86	
46-50	45	26.47	110	33.13	
≥ 51	55	32.35	89	26.81	
Unknown	30	17.65	60	18.07	
Surgical Menopause	28	16.77	40	12.54	0.20#
Walks for Exercise	84	49.41	180	54.22	0.31#
Alcohol, drinks/week					0.59#
None	74	43.53	141	42. 47	
≤1	63	37.06	129	38.86	
2-7	19	11.18	44	13.25	
8+	14	8.24	18	5.42	
Smoking					0.06#
Never	119	70.00	202	61.03	
Past	36	21.18	103	31.12	
Current	15	8.82	26	7.85	
Dietary Calcium Intake, mg/d, mean(SD)	726.72 (428.62)		765.81 (458.71)		0.36†
Supplemental Calcium Intake, mg/d, mean(SD)	388.58 (555.43)		360.67 (524.62)		0.59†
Total Calcium Intake, mg/d, mean(SD)	1130.30 (708.80)		1118.60 (683.87)		0.86†
Current Calcium Supplement Use	71	41.76	137	41.64	0.98#
History of Osteoporosis	23	13.86	43	13.03	0.80#
Oral Estrogen Use					0.78#
Never	86	51.05	171	52.62	
Past	53	31.74	94	28.92	
Current	28	16.77	60	18.46	
Any Current Estrogen Use	34	20.61	75	23.36	0.49#
Benign Breast Disease	35	22.01	51	16.24	0.12#
Family History of Breast Cancer	22	13.41	42	13.13	0.93#

*Reported p-values are from tests of significance comparing the combined case-groups (n=170) and the non-case subcohort (n=332)
†T-test
‡Wilcoxon two sample rank-sum test
#Chi-square test
Abbreviations used: BMI, body mass index; IQR, interquartile range; SD, standard deviation

Table 5. Median 25-hydroxyvitamin D level by disease status and important study characteristics, Study of Osteoporotic Fractures

	25(OH)D, ng/ml		
	Median	**(IQR)**	**p-value**
Group			
Cases, n=170	27.26	21.6-32.4	0.51†
Non-Cases, n=332	27.49	22.7-33.2	
Age*			
65-69, n=147	28.04	23.4-33.6	
70-74, n=105	27.13	22.0-32.5	0.69‡
75-79, n=58	26.46	21.6-34.2	
80+, n=22	29.67	20.7-31.6	
Season*			
Winter, n=64	26.50	20.9-35.9	
Spring, n=79	25.74	21.5-31.3	0.06‡
Summer, n=106	28.92	24.5-33.4	
Fall, n=83	27.56	22.8-34.6	
Vitamin D Supplement Use*			
Current, n=144	31.10	26.3-35.9	<0.01‡
Past, n=35	23.21	20.2-31.5	
Never, n=147	24.51	20.7-29.7	
BMI*, kg/m^2			
< 25, n=164	28.59	23.6-35.3	
25-29, n=112	25.94	21.7-32.2	0.03‡
$\geq$ 30, n=42	27.91	21.5-31.4	

*Comparison is among non-cases only
†Wilcoxon two-sample rank-sum test
‡Kruskal-Wallis test
Abbreviations used: IQR, interquartile range

Table 6. Association of serum 25(OH)D level and ER+ breast cancer, Study of Osteoporotic Fractures

	Model 1 N=502	**Model 2** N=487	**Model 3** N=437	**Model 4** N=479
	HR (95%CI)	HR (95%CI)	HR (95%CI)	HR (95%CI)
Categorical 25(OH)D ng/ml				
<20	1.13 (0.65-1.96)	1.22 (0.66-2.24)	1.06 (0.54-2.10)	1.15 (0.63-2.12)
≥20 to <30	0.94 (0.62-1.43)	0.90 (0.59-1.38)	0.95 (0.59-1.51)	0.94 (0.61-1.46)
≥30	1.00	1.00	1.00	1.00
p-value	0.73	0.42	0.88	0.68
Continuous 25(OH)D				
RR*	1.01 (0.83-1.23)	1.01 (0.81-1.27)	0.96 (0.75-1.23)	1.01 (0.80-1.26)
p-value	0.96	0.91	0.75	0.95
Clinic Site				
A	1.00	1.00	1.00	1.00
B	1.16 (0.69-1.96)	1.10 (0.63-1.93)	1.09 (0.59-2.02)	1.02 (0.58-1.80)
C	1.04 (0.61-1.79)	1.05 (0.60-1.83)	1.02 (0.53-1.94)	1.03 (0.59-1.80)
D	1.14 (0.67-1.95)	1.09 (0.62-1.90)	0.96 (0.50-1.83)	1.07 (0.61-1.89)
Season				
Winter	0.86 (0.51-1.46)	0.72 (0.41-1.26)	0.76 (0.38-1.53)	0.76 (0.43-1.35)
Spring	0.99 (0.60-1.63)	0.98 (0.58-1.65)	1.16 (0.62-2.15)	1.01 (0.60-1.70)
Summer	0.54 (0.33-0.90)	0.51 (0.30-0.85)	0.58 (0.33-1.04)	0.50 (0.29-0.84)
Fall	1.00	1.00	1.00	1.00
Distal Radius BMD, g/cm^2	-----	1.44 (0.13-15.45)	-----	-----
Body Weight, kg	-----	1.03 (1.01-1.05)	1.03 (1.01-1.05)	1.03 (1.01-1.04)
Smoking				
Never	-----	1.00	-----	1.00
Past	-----	0.59 (0.37-0.93)	-----	0.59 (0.37-0.93)
Current	-----	0.99 (0.49-1.99)	-----	1.06 (0.52-2.18)
Oral ET Use				
Never	-----	-----	1.00	1.00
Past	-----	-----	1.16 (0.70-1.92)	1.06 (0.66-1.70)
Current	-----	-----	0.96 (0.48-1.93)	1.13 (0.64-2.00)
Benign Breast Disease	-----	-----	1.45 (0.76-2.74)	-----
Family History of Breast Cancer	-----	-----	0.97 (0.50-1.85)	-----
Age at Menarche, y				
≤ 11	-----	-----	1.00	-----
12-13	-----	-----	1.18 (0.64-2.18)	-----
≥ 14	-----	-----	1.03 (0.50-2.10)	-----
Number of Live Births				
No term pregnancy	-----	-----	0.83 (0.16-4.13)	-----
1-2	-----	-----	1.00 (0.47-2.14)	-----
3-4	-----	-----	0.66 (0.40-1.09)	-----
5+	-----	-----	1.00	-----

Table 6. continued

	Model 1 N=502	Model 2 N=487	Model 3 N=437	Model 4 N=473
	HR (95%CI)	HR (95%CI)	HR (95%CI)	HR (95%CI)
Age at Menopause, y				
≤40	-----	-----	1.00	-----
41-50	-----	-----	1.29 (0.73-2.29)	-----
≥51	-----	-----	1.39 (0.77-2.49)	-----
Walks for Exercise	-----	-----	0.83 (0.53-1.29)	-----
Current Alcohol Use	-----	-----	0.76 (0.48-1.21)	-----

*Relative risk for a 1 SD (9.2 ng/ml) decrease in serum 25(OH)D; continuous 25(OH)D modeled separately from categorical 25(OH)D
Models 1-4: All models are adjusted for age (as timescale) season of blood draw, and clinic site
Model 2: Adjusted for baseline characteristics found to be significantly different between cases and the subcohort in univariate analyses at p<0.1
Model 3: Adjusted for a priori established breast cancer risk factors
Model 4: Adjusted for ET use in addition to covariates in Models 2 and 3 that remained statistically significant at p<0.1 via manual backwards elimination strategy
Abbreviations used: CI, confidence interval; HR, hazard ratio; RR, relative risk

Table 7. Estimated relative risk of serum 25(OH)D level and ER+ breast cancer according to risk subgroup, Study of Osteoporotic Fractures

Risk Subgroup	Cases / Non-cases	Relative Risk of Breast Cancer RR (95%CI)*	P value
Age			0.43†
< 75 years	135 / 234	1.09 (0.84-1.41)	0.52
≥75 years	27 / 76	0.50 (0.27-0.92)	0.03
BMI			0.27†
< 30 kg/m^2	128 / 268	1.00 (0.79-1.27)	0.97
≥ 30 kg/m^2	34 / 42	0.79 (0.27-2.29)	0.66

*Relative risk for a 1 SD (9.2 ng/ml) decrease in serum 25(OH)D; adjusted for age (as timescale), clinic site, season of blood draw, BMI, smoking history, and oral ET use
†P-value for interaction between subgroup variable and 25(OH)D
Abbreviations used: BMI, body mass index; CI, confidence interval; RR, relative risk

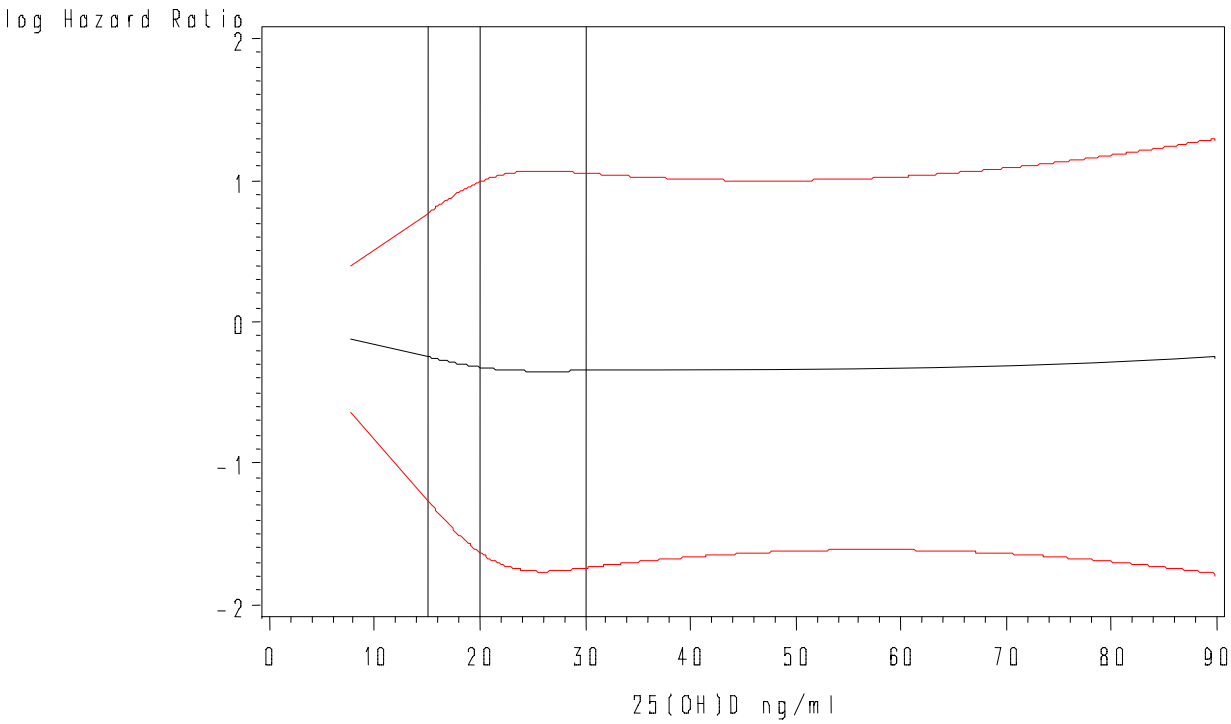

Figure 4. Cubic spline transformation of serum 25(OH)D concentration
Knot placement indicated by horizontal lines at 15, 20 and 30 ng/ml. Outer bands represent 95% confidence intervals. $P_{linearity}$=0.65.

5.0 ARTICLE 2: RISK OF BREAST CANCER USING FRACTIONAL CALCIUM ABSORPTION AS A MARKER OF VITAMIN D RESISTANCE

Manuscript in Preparation

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5.1 ABSTRACT

Although epidemiologic evidence suggests that vitamin D may reduce the incidence of breast cancer, reports in the literature have been inconsistent, particularly in regard to postmenopausal women. The possibility that age related tissue resistance to the active vitamin D metabolite (1,25-dihydroxyvitamin D) may interrupt the protective mechanism by which vitamin D is thought to prevent breast cancer has not been investigated. In this prospective study, we examined the association between fractional calcium absorption (FCA), utilized as a marker of tissue resistance, and breast cancer risk in the Study of Osteoporotic Fractures (SOF), a cohort of 9,704 Caucasian, postmenopausal women aged 65 and older. Of these, the rate of FCA was obtained by single isotope method for 5035 women, 257 of whom later developed an incident case of breast cancer. A Cox proportional hazards analysis was performed to compute hazard ratios (HRs) and 95% confidence intervals (95%CIs). Subgroup analyses by calcium intake, vitamin D supplementation, age, and estrogen therapy (ET) were also performed. Mean time between FCA measure and breast cancer diagnosis was 9.6 years. The mean FCA rate was higher among cases compared to non-cases (38.7% vs. 37.6%), although of borderline significance (p=0.05). In multivariable models, increasing rates of FCA were associated with a slightly higher risk of invasive breast cancer (HR 1.15 per 1 SD increase, 95%CI 1.00-1.32, p=0.05). Compared with the lowest quartile of FCA (≤ 0.314), women with higher FCA (0.315-0.372, 0.373-0.434, ≥ 0.435) had relative risks (HR, 95%CI) of 1.54 (1.01-2.34), 1.50 (0.99-2.29), and 1.47 (0.96-2.26), respectively (p_{trend}=0.14). In a subgroup analyses with FCA dichotomized at the lowest quartile (0.314), a stronger positive relationship was noted among women with low (HR 2.34 ≤ 525 mg/d, 95%CI 1.21-4.52) but not high (HR 1.12 > 525 mg/d, 95%CI 0.71-1.76) dietary calcium intake ($p_{interaction}$=0.06). The findings of this prospective study

of postmenopausal women are supportive of a modestly increased risk of breast cancer with higher FCA rates particularly among those who have low calcium intake.

5.2 INTRODUCTION

The role of vitamin D in breast cancer etiology has been proposed through ecologic,[112, 113] dietary intake,[119, 121-125, 127] and analytic studies.[130-134] Both normal and cancerous mammary cells have the ability to convert circulating 25-hydroxyvitamin D [25(OH)D] into the biologically active form of vitamin D, 1,25-dihydroxyvitamin D [$1,25(OH)_2D$].[95, 107] Breast cells also contain the vitamin D receptor (VDR) which is activated through interaction with $1,25(OH)_2D$ to play a direct role in growth regulation of both normal mammary gland and breast cancer cells by inducing cell differentiation and apoptosis and inhibiting cellular proliferation.[105, 109] A possible mechanism for the malignant transformation of breast cells is through insufficient 25(OH)D levels, which limits the synthesis of $1,25(OH)_2D$ and prevents activation of the VDR to regulate the cell cycle.[98] Alternatively, decreased sensitivity of breast tissue to $1,25(OH)_2D$ may also serve to limit VDR mediated cell cycle regulation.

Fractional calcium absorption, a measure of intestinal calcium absorption, varies widely from person to person. Among postmenopausal women, FCA values have been reported to range from 0.07 to 0.68 with an average of 0.27 ± 0.10 (SD).[140] It is well established that intestinal calcium absorption declines with increasing age.[137, 143, 145, 151, 160, 161] By far, the most significant factor affecting intestinal calcium absorption is its hormonal regulator, $1,25(OH)_2D$.[142-145] Approximately 20% of the variation in calcium absorption between individuals can be explained by circulating $1,25(OH)_2D$.[142] Some studies,[142, 145, 146] but not all,[147-153] have reported decreased levels of serum $1,25(OH)_2D$ with age.

Intestinal calcium absorption has been touted as a marker of tissue responsiveness to vitamin D, and aging associated with reduced sensitivity to $1,25(OH)_2D$.[167] The evidence

supporting this claim is of varying strength. One study demonstrated unchanging calcium absorption with increasing age despite higher levels of $1,25(OH)_2D$, suggestive of intestinal resistance to $1,25(OH)_2D$ with aging.[150] Similarly, reduced calcium absorption was reported among non-estrogen users over age 75, despite unchanging $1,25(OH)_2D$ or 25(OH)D levels.[143] Perhaps the most compelling findings are from a study by Pattanaungkul et al. which clearly shows that in young women (mean 29 years) FCA increases with increasing serum $1,25(OH)_2D$ concentration, while the increase in FCA among elderly women (mean 73 years) is significantly diminished with increasing serum $1,25(OH)_2D$ concentration ($p=0.03$).[168]

Based upon the current knowledge, it is conceivable that other vitamin D sensitive tissues, such as breast tissue, may also have diminished response to vitamin D with aging. Decreased ability to absorb calcium with age, indicative of reduced gut tissue responsiveness to vitamin D, may be representative of other tissue's responsiveness to vitamin D. In this prospective cohort study, we investigated the relationship between FCA, utilized as a marker of tissue responsiveness to vitamin D, and breast cancer among postmenopausal women. Specifically, we tested the hypothesis that low FCA will be associated with an increased risk of invasive and estrogen receptor positive (ER+) breast cancer. We also tested whether this relationship differed by calcium intake, vitamin D supplementation, and hormone therapy as secondary aims. The association between FCA and breast cancer has never been studied.

5.3 METHODS

5.3.1 Study Population

The Study of Osteoporotic Fractures is a longitudinal cohort of 9,704 community-dwelling, Caucasian, postmenopausal women aged 65 and over who were recruited at 4 US clinical centers between 1986 and 1988. Women with a history of bilateral hip replacements and those who were unable to walk unassisted were excluded.[210] At the baseline examination, women provided informed consent and risk factor and health measures were collected through physical measurements, questionnaires, and functional assessments.

Fractional calcium absorption was assessed at the fourth clinic visit (V4) between 1992 and 1994. All SOF participants with a V4 fractional calcium absorption measure (n=5452) were eligible. Individuals reporting a history of breast cancer at enrollment (n=240) and those with an incident breast cancer diagnosed prior to V4 (n=116) were excluded from this analysis. Additionally, women with missing outcome data (n=71) were excluded. **Figure 5** details the selection process for the analysis population. Follow-up continued through December 2006 at which time the women were censored who did not develop breast cancer, experience death, or were not lost to follow-up. This analysis includes 257 cases and 4778 non-cases.

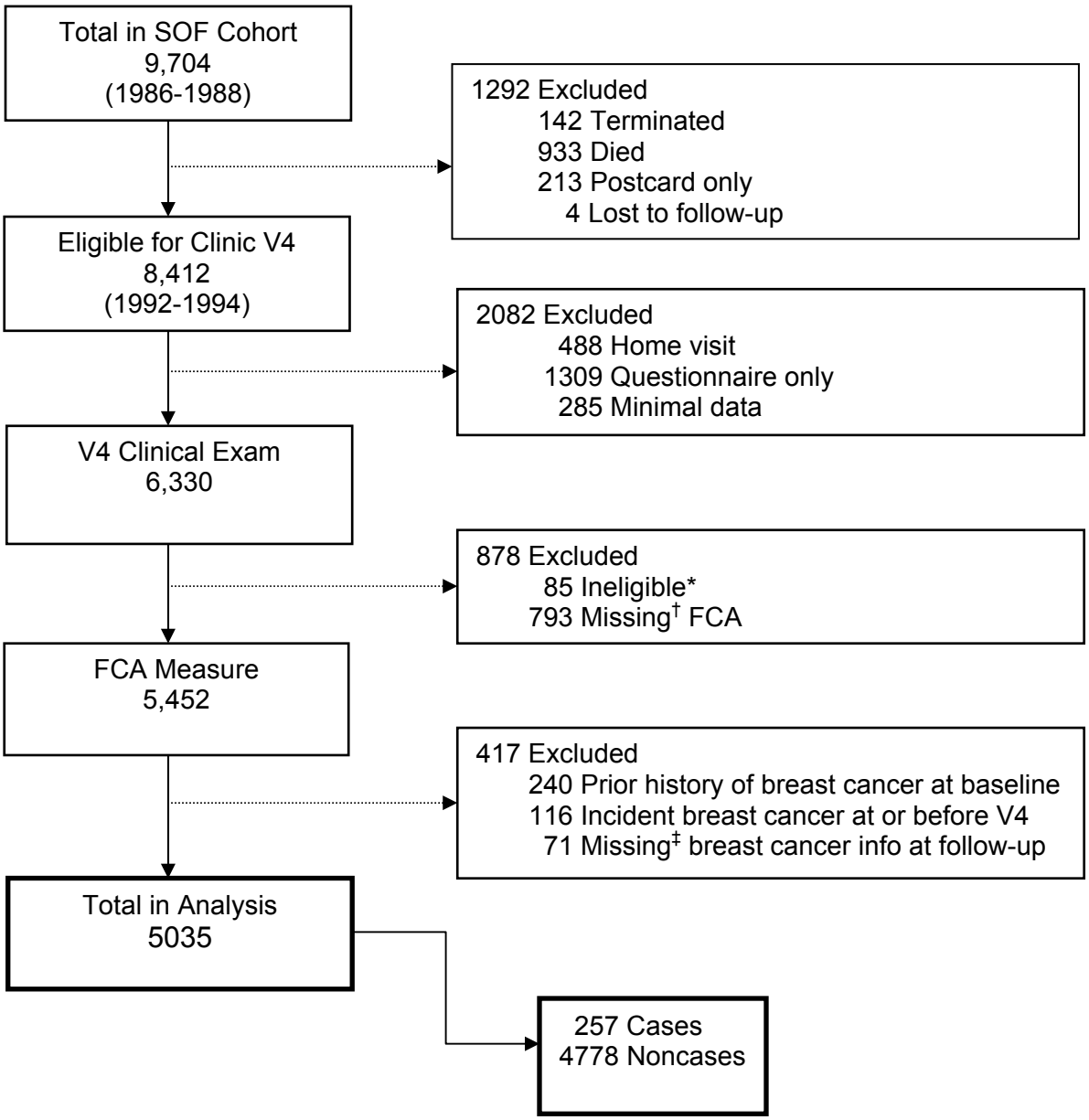

Figure 5. Cascade of analysis population determination, Study of Osteoporotic Fractures
*Women were deemed ineligible for the FCA test if they reported experiencing nausea, vomiting or diarrhea in the 48 hours preceding the exam. †Missing FCA includes those who refused testing or had incomplete tests. ‡Missing values coded as refused to answer (n=54), unable to answer (n=6), never had a period (n=11).

5.3.2 Fractional Calcium Absorption Measurement

Details of the FCA assessment have been published previously.[166] FCA testing was completed in the morning after a 5-hour fast. Participants were instructed not to take any calcium supplements for 12 hours before testing. Midway through consumption of a standardized light test meal, participants ingested a mixture of 50 grams of radio-labeled apple juice (containing 63 mg of ^{45}Ca) and 120 grams of unlabeled apple juice (Speas Farm, Sundor Brands, Mt. Dora, Florida) for a total calcium load of 215 mg. The mixture was prepared on site at each of the four clinics with labeled ^{45}Ca from the Osteoporosis Research Center at Creighton University in Omaha Nebraska. Fractional ^{45}Ca absorption was estimated from the appearance of ^{45}Ca in the blood. Blood was drawn into a serum separator tube exactly 3 hours after ingestion of the tracer and was allowed to clot at room temperature. Within 2 hours of collection, serum was separated and frozen at $-70^{0}C$ until analysis. Frozen serum samples were later shipped on dry ice by overnight delivery to Creighton University, where fractional calcium absorption was estimated by a single isotope method.[230, 231]

5.3.3 Covariate Information

At the baseline (V1) and fourth clinic visits (V4), participants completed a questionnaire and were interviewed. Demographic (age, education), reproductive history (menarche age, parity, age at first birth, number of live births, breastfeeding), height at age 25, menopausal status (menopause age, surgical vs. natural menopause), and breast cancer risk factor (benign breast disease, family history of breast cancer) data were collected at the baseline examination. At the V4 examination, updated smoking status, alcohol use, physical activity, and ET data were collected. Body weight was measured using a balance-beam scale. Weight change was

calculated by subtracting weight at V1 from V4. Body mass index (BMI) was calculated by dividing the V4 weight by the square of height at age 25 years. Bone mineral density (g/cm^2) of the proximal femur was measured using dual-energy x-ray absorptiometry (QDR 1000, Hologic, Waltham, Massachusetts).[210, 213] In addition, they were asked to bring current medications including vitamins and supplements and the total daily dose of calcium and vitamin D were recorded. Current supplemental vitamin D use was defined as taking vitamin D or a multivitamin containing vitamin D at least once per week. Calcium supplementation was determined by asking questions about dose and frequency of multivitamin use, specific vitamin and mineral supplements, and antacids containing calcium. Dietary calcium intake was estimated by using a validated 60-item block semi-quantitative food-frequency questionnaire developed from the Second National Health and Nutrition Survey (NHANES II).[214, 215] Total calcium intake was calculated by summing dietary calcium intake (mg/d) and daily dose of calcium supplements (mg/d). Month of FCA measure was grouped into seasons of winter (December-February), spring (March-May), summer (June-August), and fall (September-November). Variables were categorized based on common cutpoints (e.g. BMI) or the original response categories collapsed to prevent small cell counts (e.g. age at first birth). Continuous variables were additionally dichotomized at their median and/or divided into quartiles for analytic purposes.

5.3.4 Incident Breast Cancer Ascertainment

Follow-up occurred every four months by either postcard or telephone (98% complete) in addition to clinic visits approximately every 2 years. Breast cancer outcomes were originally ascertained through self-report or death certificate review and were adjudicated by physicians locally and centrally at the San Francisco Coordinating Center. Medical records and pathology reports were used to record information on date of breast cancer diagnosis, stage at diagnosis, and estrogen- and progesterone-receptor status.[184]

5.3.5 Statistical Analysis

As part of the preliminary data analysis the distribution of baseline characteristics (measured at V1 or V4) were compared by disease status (cases vs. non-cases) using t-tests for continuous measures and chi-square tests for categorical data. Mean FCA also was compared by disease status (case/non-case), age (<75/≥75 years), season of blood draw (winter/spring/summer/fall), vitamin D supplement use (current/not current), total calcium intake (<775/≥775 mg/d), oral estrogen use (current/not current), height at age 25 (<163/≥163 cm), body weight (<65/≥65 kg), BMI (<30/≥30 kg/m^2), alcohol use (<1/≥1 drink/week), and smoking status (current/not current) using either t-tests or ANOVA.

The Kaplan-Meier method for survival analysis was used to compare time to breast cancer diagnosis by quartile of FCA. A log-rank test was used to assess differences by FCA quartile. Follow-up time for each woman was calculated in days from V4 to breast cancer diagnosis, death, loss to follow-up, or censoring.

The main analysis estimated hazard ratios and 95% confidence intervals for the association between FCA and the risk of breast cancer using Cox proportional hazard regression models with age as the underlying time scale.[217-219] Age at entry in days, was calculated by multiplying age at V4 (time of the FCA measure) by 365.25. Age at exit from the study was calculated by adding follow-up time in days from V4 to study end (i.e. time to breast cancer diagnosis, death, loss to follow-up or censoring) to entry age. Levels of FCA were entered as a continuous variable to estimate the HR of breast cancer per one standard deviation (SD) increase in FCA. FCA levels were also assessed as quartiles based on the distribution of FCA in the entire cohort and using the lowest category as the reference. All models were adjusted for SOF clinic site. The potential confounders described above were evaluated for inclusion in the multivariable adjusted model based on their significance ($p \leq 0.1$) in the univariate and bivariate analyses. In situations where variables are correlated, e.g. various

measures of body size, covariates were chosen based on statistical association, scientific knowledge, and/or variable distribution. Dummy variables were created for categorical variables as appropriate. Variables were selected for elimination one at a time using a backward elimination strategy based on individual Wald tests.[222, 223] After each variable was removed from the model, the partial likelihood ratio test was calculated comparing the nested models. Removed variables remained out of the models if no significant contribution to the model was determined ($p \geq 0.1$). Interactions were evaluated using likelihood ratio tests that compared the model with the interaction term to the main effects model ($p \geq 0.05$).

The potential for non-linear effects was investigated using cubic splines. RCS (restricted cubic splines), a SAS macro, was used to fit the cubic splines.[221] Knot placement was set at the FCA quartile cut-points. All breast cancers including in situ and invasive only breast cancers were analyzed separately. Although the inclusion of in situ did not substantially impact the results, regression results for invasive only and ER+ cancers are presented. The final multivariable model was assessed for a linear contrast with FCA quartile 1 vs. quartiles 2-4. Analyses were also repeated among subgroups defined by age, dietary calcium intake, use of calcium supplements, total calcium intake, vitamin D supplementation (current/not current), and oral estrogen use (current/not current). FCA was split at quartile 1 vs. quartiles 2-4 for subgroup analyses; continuous variables were dichotomized at the median value. Additionally, in response to the recent WHI report of an interaction between calcium and vitamin D supplementation and hormone therapy (HT),[232] FCA was investigated by total calcium intake and oral estrogen use simultaneously. Likelihood ratio tests were used to evaluate possible interactions between the subgroup variable and FCA by comparing a multivariable model with and without the interaction term expressed as the product of the variable and dichotomous FCA. For the final models, probability values <0.05 were considered statistically significant. All tests were two-tailed. Schoenfeld residuals were used to test the proportional hazards assumption.

Power was calculated a priori using previously reported SOF data and PASS 2005 software (NCSS, Kaysville, Utah). With a mean fractional calcium absorption level of 0.38 and a standard deviation of 0.09,[166] we have a minimum of 80% power to detect a mean difference of 0.02. A two-sided two-sample t-test was conducted using an alpha level of 0.05. Calculations were based on 250 cases and 4775 controls. Data descriptions, including graphical presentations, were carried out in STATA version 10. Kaplan-Meier survival analysis and Cox proportional hazards modeling was performed using SAS software release 9.1.3 (SAS Institute Inc., Cary, NC).

5.4 RESULTS

This prospective cohort study investigated the relationship between fractional calcium absorption, utilized as a marker of tissue responsiveness to vitamin D, and breast cancer among 5035 postmenopausal women in the Study of Osteoporotic Fractures. **Table 8** presents the characteristics, including socio-demographic variables and established breast cancer risk factors of the analysis population at baseline or V4.

The mean age of cases and non-cases at V4 are 75.5 and 76.5 years, respectively. Compared to non-cases, cases weighed more (p<0.001), had higher BMI (p=0.004), had higher hip BMD (p=<0.0001), were older at menopause (p=0.007), took more supplemental calcium (p=0.03), had a greater total daily calcium intake (p=0.02), took ET more frequently (25.7% vs. 18.6%, p=0.005), and were more likely to have a positive family history of breast cancer (19.4% vs. 12.8%, p=0.002).

Mean FCA rates were 38.7% and 37.6% for cases and non-cases, respectively (p=0.05). **Table 9** gives details of the mean FCA rate by important study characteristics. Mean FCA was highest among women under 75 years of age (39.5% vs. 36.3%, p=<0.0001), those consuming

less than 775 mg of calcium per day (38.6% vs. 36.8%, p<0.0001), and those not currently taking a vitamin D supplement (38.2% vs. 37%, p<0.0001). Mean FCA did not differ according to the season the measure was taken (p=0.16), or current ET use (p=0.51).

Over a mean 9.6 years of follow-up, 257 women were diagnosed with incident breast cancer. Invasive breast cancers accounted for 222 cases while 35 were in situ. Estrogen receptor status was obtained for 206 cases and was positive in 175 (85%). Results of the Kaplan-Meier survival analysis did not show a significant difference by quartile of FCA, $p_{logrank}$=0.30 (**Appendix B**).

In the Cox PH regression analyses, there was a significant positive association between FCA and risk of invasive breast cancer in the age adjusted models that persisted in the multivariable adjusted models, albeit borderline significant (**Table 10**). Adjusted for age (as timescale), clinic site, weight, menopause age, average total daily calcium intake, ET, and family history of breast cancer, FCA was significantly positively related to breast cancer (HR 1.15 per 1 SD increase in FCA, 95%CI 1.01-1.32, p=0.05). Compared with the lowest quartile of FCA, the HR (95%CI) for Q2-Q4 were 1.54 (1.01-2.34), 1.50 (0.99-2.29), and 1.47 (0.96-2.26), respectively (p_{trend}=0.14). A linear contrast for FCA quartile 1 vs. quartiles 2-4 was significant (HR 1.50, 95%CI 1.04-2.17, p=0.03).

An examination of the shape of the risk function using cubic splines did not indicate a deviation from a linear relationship (p=0.67) (**Appendix B**).

Results of the subgroup analyses are presented in **Table 10**. In multivariable models comparing dichotomous FCA (quartile 1 vs. quartiles 2-4), there was an increased risk of breast cancer associated with higher FCA among women with low (HR 2.34 ≤ 525 mg/d, 95%CI 1.21-4.52) but not high (HR 1.12 > 525 mg/d, 95%CI 0.71-1.76) dietary calcium intake, although of borderline statistical significance ($p_{interaction}$=0.06). Investigated individually using likelihood ratio tests, there was no interaction between dichotomous FCA and the other subgroup variables (i.e., vitamin D supplementation, calcium supplementation, estrogen therapy, total calcium

intake, age) in a multivariable model. In the analysis of FCA by both total calcium intake and estrogen therapy, there was no interaction between FCA, calcium and ET in the multivariable model of FCA dichotomized at the median ($p_{interaction}$=0.23; results not shown).

Results were similar in multivariable regressions including estrogen receptor positive cancer only (HR 1.12 per 1 SD increase in FCA, 95%CI 0.96-1.31) (**Table 11**). Compared with the lowest quartile of FCA, the HR (95%CI) for Q2-Q4 were 1.43 (0.88-2.30), 1.57 (0.99-2.51), and 1.39 (0.85-2.25), respectively (p_{trend}=0.20). A linear contrast for FCA quartile 1 vs. quartiles 2-4 was borderline significant (HR 1.46, 95%CI 0.97-2.21, p=0.07).

5.5 DISCUSSION

This prospective cohort study of 5035 postmenopausal women from the Study of Osteoporotic Fractures showed a modestly significant increased risk of invasive breast cancer with higher fractional calcium absorption. A stronger positive association was found among women with low dietary calcium intake. However, there was no difference in the magnitude of the association between current and not current users of vitamin D supplements, calcium supplements, estrogen therapy, by total calcium intake or age. Results were similar for estrogen receptor positive tumors.

Indeed, the hypothesized relationship between calcium and breast cancer prevention is not new. Direct effects on cell proliferation and differentiation have been reported in vitro.[233, 234] Furthermore, calcium has been shown to reduce fat-induced cell proliferation in a rodent model by maintaining intracellular calcium concentrations.[235] The literature is limited regarding calcium intake and breast cancer among postmenopausal women and the findings are inconsistent. The prospective Cancer Prevention Study II Nutrition Cohort reported a lower risk of breast cancer among women with the highest dietary calcium intake, > 1,250 mg/d compared to ≤ 500 mg/d

(p=0.02).[123] Two other prospective cohorts, the Women's Health Study and the Nurses' Health Study, failed to find an association between total calcium intake and postmenopausal breast cancer.[122, 125]

Likewise, the relationship between vitamin D intake and breast cancer has been investigated recently. Vitamin D is hypothesized to reduce breast cancer risk via mechanisms similar to, but independent of, calcium. Vitamin D regulates the cell cycle through antiproliferative actions and by promoting differentiation.[105, 233, 236] Breast tissue can directly convert 25(OH)D to $1,25(OH)_2D$,[109] and has also been shown to inhibit mammary tumorigenesis.[237] Prospective cohort studies of vitamin D intake have not shown an association with postmenopausal breast cancer risk.[122-125, 238]

It is well documented that intestinal calcium absorption is most efficient when calcium intakes are low and decreases with increasing amounts of calcium intake.[160] The mechanism by which low calcium intake raises fractional calcium absorption is complex. In response to low levels of circulating extracellular calcium, the parathyroid gland releases parathyroid hormone (PTH). In-turn, PTH up-regulates 25-hydroxy 1-alpha hydroxylase in the kidney, an enzyme responsible for converting 25(OH)D to its active form, $1,25(OH)_2D$.[239] In the intestine, $1,25(OH)_2D$ serves as the hormonal regulator of calcium absorption mediated by the vitamin D receptor. The VDR is an intracellular protein, which regulates the expression of vitamin D-dependent genes, such as calbindin D, a cytosolic protein believed to be the rate-limiting molecule in vitamin D-induced intestinal calcium transport.[240] Therefore, lower calcium intake, resulting in elevated calcium transport, may be the link between higher FCA and increased breast cancer risk.

Recently, the Women's Health Initiative (WHI) published results of a re-analysis of their randomized trial of calcium and vitamin D supplementation on colorectal cancer risk in postmenopausal women in which they found a previously unreported interaction with hormone therapy. Among women assigned to the placebo HT arms, calcium and vitamin D

supplementation are protective (HR 0.71, 95%CI 0.46-1.09), whereas the opposite was found among women assigned to the active HT arms (HR 1.50, 95%CI 0.96-2.33) ($p_{interaction}$=0.018).[232] The WHI proposed several mechanisms by which estrogen may interfere with the effect of calcium and vitamin D on cancer prevention including increasing intestinal calbindin expression independent of vitamin D which would lead to decreased circulating calcium levels and inhibit calcium and vitamin D dependent apoptosis, and activating osteoblast formation resulting in increased calcium mineralization in bone and reduced bioavailability of systemic calcium.[232] The WHI randomized trial of calcium plus vitamin D on breast cancer risk in postmenopausal women did not report any significant effect of supplementation.[126] However, these results may also be influenced by an unrecognized interaction with hormone therapy and should be re-analyzed.

We found a statistically significant difference in mean FCA by age in a bivariate analysis, but no difference in the association between FCA and breast cancer risk according to age in a multivariable regression. Studies have shown that calcium absorption is lower in postmenopausal compared to premenopausal women,[162] and that it can be reversed by estrogen replacement.[162, 163] However, we did not find a difference in mean FCA by ET use in a bivariate analysis, a multivariable regression, or in an analysis of FCA by both total calcium intake and ET. These findings may have lacked significance due to reduced power for subgroup analyses.

Strengths of this study include its prospective cohort design from the large and long-standing population-based SOF cohort. We utilized FCA rates measured using a widely accepted single isotope method and were able control for a number of factors related to breast cancer and FCA. Limitations of this study include our reliance upon a single measure of FCA, which may not reflect long-term status. Measurements of potentially mediating factors such as level of 25(OH)D, and endogenous estrogen levels were also not available. Most notably, our

study is comprised of elderly, community-dwelling white volunteers and therefore our results may not be generalizable to other populations.

In conclusion, our results suggest that high FCA, particularly among those with a low dietary calcium intake, is associated with an increased risk of postmenopausal breast cancer. Correlates of FCA, including calcium, vitamin D, and estrogen have been investigated individually as etiologic factors for breast cancer. However, with the exception of estrogen, the reported associations have been inconsistent. Further investigations into the role of estrogen in conjunction with calcium and vitamin D levels on FCA may help to clarify their interdependence. Confirmation of the association between FCA and postmenopausal breast cancer is necessary.

5.6 TABLES

Table 8. Descriptive characteristics and risk factors for breast cancer among cases and non-cases, fourth examination, Study of Osteoporotic Fractures

	Cases (n=257)		Non-Cases (n=4778)		
Characteristic	**N**	**%**	**N**	**%**	**p-value***
Clinic Site					0.10
A	85	33.1	1452	30.4	
B	67	26.1	1017	21.3	
C	39	15.2	924	19.3	
D	66	25.7	1385	29.0	
Age, y, mean(SD)	75.52 (3.85)		76.51 (4.70)		<0.0001
Education					0.29
< High School	41	16.0	951	19.9	
High School	107	41.6	1924	40.3	
> High School	109	42.4	1899	39.8	
Total Hip BMD, g/cm^2, mean(SD)	0.77 (0.12)		0.73 (0.13)		<0.0001
Body Weight, kg, mean(SD)	68.68 (11.78)		66.21 (11.84)		0.001
Weight Change†	-0.50 (4.67)		-0.86 (4.97)		0.26
BMI, kg/m^2, mean(SD)‡	25.83 (4.34)		25.03 (4.27)		0.004
BMI					0.02
< 18	1	0.4	81	1.8	
18-24	112	45.0	2421	52.6	
25-29	94	37.8	1505	32.7	
$\geq$ 30	42	16.9	596	13.0	
Height at age 25 y, cm, mean(SD)	163.12 (5.69)		162.64 (5.82)		0.20
Age at Menarche, y					0.74
$\leq$ 11	27	11.2	569	12.5	
12-13	130	53.7	2464	54.3	
$\geq$ 14	85	35.1	1508	33.2	
Nulliparous	40	15.6	741	15.5	0.98
Number of Live Births					0.97
Never pregnant	40	15.7	741	15.6	
0	8	3.1	117	2.5	
1-2	100	39.2	1914	40.2	
3-4	82	32.2	1533	32.2	
5+	25	9.8	459	9.6	

Table 8. continued

Characteristic	Cases (n=257) N	Cases (n=257) %	Non-Cases (n=4778) N	Non-Cases (n=4778) %	p-value*
Age at Menopause, y, mean(SD)	49.06 (4.86)		48.11 (5.71)		0.007
Age at First Birth, y					0.88
Never gave birth	31	12.5	555	12.0	
≤ 20	171	68.7	3261	70.2	
> 20	47	18.9	830	17.9	
Ever Breastfed	64	29.5	1255	31.1	0.62
Age at Menopause, y					0.15
≤ 40	15	7.3	404	10.3	
41-50	113	54.6	2245	57.1	
≥ 51	79	38.2	1282	32.6	
Surgical Menopause	34	13.8	544	11.8	0.36
Walks for Exercise	141	54.9	2436	51.1	0.24
Alcohol, drinks/week, mean(SD)	1.24 (2.50)		1.29 (3.04)		0.76
Current Alcohol Use	128	49.8	2160	45.3	0.15
Smoking					0.54
Never	167	65.0	2931	61.6	
Past	77	30.0	1576	33.1	
Current	13	5.1	252	5.3	
Calcium Intake, mg/d, mean(SD)					
Dietary Calcium	615.08 (363.00)		597.91 (359.98)		0.46
Supplement Calcium	501.43 (773.80)		392.64 (677.0)		0.03
Total Calcium	1116.51 (853.18)		990.55 (778.57)		0.02
Current Calcium Supplement Use	125	48.6	2149	45.0	0.25
Vitamin D Supplement Use	111	43.2	2010	42.1	0.72
Current Oral Estrogen Use	66	25.7	887	18.6	0.005
Benign Breast Disease	40	16.1	678	14.6	0.51
Family History of Breast Cancer	49	19.4	596	12.8	0.002

*P-values from t-tests for continuous variables, and chi-square tests for categorical variables
†Weight change since baseline
Abbreviations used: BMI, body mass index; SD, standard deviation

Table 9. Mean fractional calcium absorption by disease status, personal and behavioral characteristics, Study of Osteoporotic Fractures

Characteristic*	Fractional Calcium Absorption Mean	SD	p-value
Group			
Cases, n=257	0.387	0.092	0.05†
Non-Cases, n=4778	0.376	0.088	
Age, y			
< 75, n=2128	0.395	0.088	<0.0001†
≥ 75, n=2907	0.363	0.086	
Total Calcium Intake, mg/d			
≤ 775, n=2512	0.386	0.089	<0.0001†
> 775, n=2523	0.368	0.086	
Dietary Calcium Intake, mg/d			
≤ 525, n=2503	0.382	0.089	<0.0001†
> 525, n=2532	0.371	0.087	
Season			
Winter, n=1138	0.372	0.088	0.16‡
Spring, n=1269	0.377	0.087	
Summer, n=1068	0.379	0.089	
Fall, n=1560	0.378	0.088	
Vitamin D Supplement Use			
Current, n=2121	0.370	0.086	<0.0001†
Not Current, n=2913	0.382	0.089	
Oral Estrogen Use			
Current, n=953	0.370	0.083	0.51†
Not Current, n=4081	0.377	0.089	
Height at age 25 y, cm			
< 163, n=2958	0.379	0.090	0.05†
≥ 163, n=2077	0.374	0.090	
Body Weight, kg			
< 65, n=2549	0.363	0.087	<0.0001†
≥ 65, n=2486	0.391	0.087	
BMI, kg/m^2			
<30, n=4395	0.373	0.087	<0.001†
≥30, n=640	0.403	0.088	
Alcohol, drinks/week			
< 1, n=3881	0.377	0.089	0.54†
≥ 1, n=1154	0.375	0.085	
Smoking			
Current, n=265	0.377	0.093	0.97†
Not Current, n=4751	0.377	0.088	

*Comparisons are among the entire analysis population
†T-test
‡ANOVA
Abbreviations used: BMI, body mass index; SD, standard deviation

Table 10. Results of multivariable Cox proportional hazards regressions for association between fractional calcium absorption and invasive breast cancer, Study of Osteoporotic Fractures

	Invasive Breast Cancer					
	Age Adjusted			Multivariable* Adjusted		
FCA	**N**	**HR (95%CI)**	**P value**	**N**	**HR (95%CI)**	**P value**
Continuous[†]	5000	1.14 (1.00-1.30)	0.048	4759	1.15 (1.00-1.32)	0.050
Categorical[‡]	5000			4759		
≤ 0.314		1.00			1.00	
0.315-0.372		1.53 (1.01-2.30)	0.043		1.54 (1.01-2.34)	0.046
0.373-0.434		1.51 (1.01-2.28)	0.046		1.50 (0.99-2.29)	0.057
≥ 0.435		1.46 (0.97-2.20)	0.073		1.47 (0.96-2.26)	0.079
p_{trend}			0.124			0.138
Dichotomous[§]	Cases / Non-cases			Cases / Non-cases		
Age						0.86**
<75 years	112/1997	1.01 (0.61-1.65)	0.97	107/1904	1.15 (0.68-1.95)	0.60
≥75 years	110/2781	2.09 (1.26-3.47)	0.004	107/2641	1.86 (1.12-3.11)	0.02
Dietary Calcium Intake						0.06**
≤ 525 mg/d	108/2380	2.30 (1.23-4.30)	0.009	103/2269	2.34 (1.21-4.52)	0.01
> 525 mg/d	114/2398	1.15 (0.74-1.79)	0.53	111/2276	1.12 (0.71-1.76)	0.64
Calcium Supplement						0.44**
Not Current	121/2628	1.33 (0.80-2.20)	0.27	117/2500	1.31 (0.78-2.21)	0.30
Current	101/2149	1.67 (1.01-2.76)	0.04	97/2045	1.63 (0.97-2.75)	0.06
Total Calcium Intake						0.66**
≤ 775 mg/d	102/2402	1.63 (1.91-2.92)	0.10	100/2284	1.70 (0.92-3.12)	0.09
> 775 mg/d	120/2376	1.46 (0.93-2.30)	0.10	114/2261	1.38 (1.86-2.19)	0.18
Vitamin D Supplement						0.56**
Not Current	127/2767	1.43 (0.88-2.31)	0.15	123/2629	1.37 (0.83-2.26)	0.21
Current	95/2010	1.57 (0.93-2.66)	0.09	91/1916	1.61 (0.93-2.79)	0.09
Oral Estrogen Use						0.23**
Not Current	166/3890	1.52 (1.01-2.31)	0.05	159/3701	1.50 (0.98-2.30)	0.06
Current	56/887	1.42 (0.71-2.82)	0.32	55/844	1.54 (0.74-3.17)	0.24

*Adjusted for age (as timescale), clinic site, weight, menopause age, calcium intake, vitamin D supplementation, estrogen therapy, and family history of breast cancer
[†]Hazards ratios in this row are for a 1 SD (8.8%) increase in fractional calcium absorption
[‡]Quartile distribution among entire cohort; range 0.11-0.74
[§]Hazard ratios comparing FCA dichotomized at quartile 1 (0.314)
**P-value for interaction between subgroup variable and FCA
Abbreviations used: CI, confidence interval; FCA, fractional calcium absorption; HR, hazard ratio; SD, standard deviation

Table 11. Results of multivariable Cox proportional hazards regressions for association between fractional calcium absorption and estrogen receptor positive breast cancer, Study of Osteoporotic Fractures

	Estrogen Receptor Positive					
	Age Adjusted			Multivariable* Adjusted		
FCA	N	HR (95%CI)	P value	N	HR (95%CI)	P value
Continuous†	4953	1.12 (0.97-1.30)	0.14	4713	1.12 (0.96-1.31)	0.15
Categorical‡	4953			4713		
≤ 0.314		1.00			1.00	
0.315-0.372		1.38 (0.87-2.20)	0.17		1.43 (0.88-2.30)	0.15
0.373-0.434		1.56 (0.99-2.44)	0.05		1.57 (0.99-2.51)	0.06
≥ 0.435		1.37 (0.87-2.17)	0.18		1.39 (0.85-2.25)	0.19
p_{trend}			0.18			0.20

*Adjusted for age (as timescale), clinic site, weight, menopause age, average total daily calcium intake, oral estrogen use, and family history of breast cancer

†Hazards ratios in this row are for a 1 SD (8.8%) increase in fractional calcium absorption

‡ Quartile distribution among entire cohort; range 0.11-0.74

Abbreviations used: CI, confidence interval; FCA, fractional calcium absorption; HR, hazard ratio; SD, standard deviation

6.0 ARTICLE 3: LONG-TERM PREDICTION OF BREAST CANCER RISK IN POSTMENOPAUSAL WOMEN BY BONE MINERAL DENSITY

Manuscript in Preparation

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6.1 ABSTRACT

Breast cancer occurrence is positively related to bone mineral density (BMD), but previous studies have been limited to short follow-up durations and have not accounted for recurrent BMD measures. In this prospective study, we examined the association of an initial BMD measure and change in BMD on breast cancer risk in the Study of Osteoporotic Fractures (SOF), a cohort of 9,704 Caucasian, postmenopausal women. Total hip BMD was measured twice, a mean 3.5 years apart in 5383 women, 263 of whom later developed an incident case of breast cancer. A Cox proportional hazards analysis was performed to compute hazard ratios (HRs) and 95% confidence intervals (95%CIs). Mean time between the repeat BMD measure and breast cancer diagnosis was 9.5 years. Mean BMD was significantly higher among cases compared to non-cases for both the initial and repeat measures ($p<0.0001$). In multivariable models, there was no association between increasing levels of BMD and invasive breast cancer (HR 1.06 per 1 SD increase, 95%CI 0.88-1.20). Compared with the lowest category of BMD T-score (≤ -2.5), women with higher T-Scores (-2.5 to -1.0, and ≥ -1.0) had relative risks (HR, 95%CI) of 1.99 (1.03-3.85), and 2.01 (1.00-4.01), respectively ($p=0.07$). Change in BMD (annualized % change between initial and repeat measure) was not associated with a significant increased risk of breast cancer (HR 1.09 per 1 SD increase, 95%CI 0.93-1.26). Similar results were obtained in a combined model with initial BMD and change in BMD. In a subgroup analysis of initial BMD dichotomized at the median, the effect of BMD was dependent upon family history ($p_{interaction}=0.01$). Women with a positive family history and high BMD had a 3 times higher risk of breast cancer (95%CI 1.25-7.12) compared to women with low BMD. In this prospective study of postmenopausal women, the effect of higher BMD on breast cancer risk varied by family

history of breast cancer. These results support an increased risk of breast cancer over a long follow-up period among women with low to normal T-scores compared to osteoporotic women.

6.2 INTRODUCTION

Estrogen is thought to play a central role in the development of breast cancer due to its ability to stimulate proliferation of breast tissue.[175] Factors that increase exposure of breast tissue to estrogens, such as early menarche, older age at first birth, or late menopause, are associated with breast cancer risk.[176] Indeed, prolonged exposure to high levels of endogenous estrogens may increase breast cancer risk in postmenopausal women.[177] However, it is difficult to classify a woman's long-term exposure to endogenous estrogen by a single measurement because serum estrogen levels are highly variable over time.[178]

Bone mineral density, on the other-hand, is thought to be a surrogate measure of lifetime estrogen exposure.[179] Bone contains estrogen receptors and is sensitive to circulating estrogen levels.[180] BMD is positively correlated with endogenous estrogen levels,[181] early menarche, parity, and the length of a woman's reproductive lifecycle.[182] In addition to the underlying age related decrease, BMD also decreases in postmenopausal women, mostly due to estrogen deprivation beginning at the time of menopause. Even among postmenopausal women, however, the rate of bone loss is variable. Factors affecting postmenopausal bone loss include sustained estrogen exposure due to exogenous estrogen use and/or endogenous estrogen released from fat, calcium and vitamin D intake from dietary and supplemental sources, and level of physical activity.

Higher BMD, reflecting higher estrogen exposure throughout life, has been shown to predict future breast cancer in older women.[184] Data supporting the BMD-breast cancer link were initially published by SOF investigators more than a decade ago. To update the early SOF reports which had fewer than 4 years of follow-up,[184, 195] we investigated the relationship between BMD and breast cancer among postmenopausal women after 13 years of follow-up

(mean 9.5 years). Specifically we studied the long-term association of BMD and risk of invasive and estrogen receptor (ER+) positive breast cancer. Furthermore, we tested the hypothesis that the risk associated with an initial BMD measure would be strengthened by the addition of a repeat BMD measure (annual percent change) assessed 3.5 years later. We also tested whether this association differed by body mass index (BMI), hormone therapy, and family history of breast cancer as secondary aims.

6.3 METHODS

6.3.1 Study Population

The Study of Osteoporotic Fractures (SOF) is a longitudinal cohort of 9,704 Caucasian, community-dwelling postmenopausal women aged 65 and older who were recruited at 4 US clinical centers between 1986 and 1988. At the baseline (V1) examination, women provided informed consent and risk factor and health measures were collected through physical measurements, questionnaires, and functional assessments. Women with a history of bilateral hip replacements and those who were unable to walk unassisted were excluded.[210]

Total hip bone mineral density was measured at the second (V2) and fourth (V4) clinic visits, approximately 2 and 6 years after enrollment. All SOF participants with matching V2 and V4 hip BMD measures were eligible for the current analysis. Individuals reporting a history of breast cancer at enrollment (n=269) and those with missing outcome data (n=336) were excluded. In order to compare the same breast cancer outcomes, only incident cases diagnosed after the V4 repeat measure are included. The 136 incident cases diagnosed prior to V4 were excluded. Follow-up for incident breast cancer cases begins after the V4 repeated hip BMD measure and continued through December 2006 at which time the women were censored who

did not develop breast cancer, experience death, or were not lost to follow-up. The analysis population was therefore comprised of 263 cases and 5120 non-cases. The study timeline is detailed in **Figure 6** and the selection process in **Figure 7**.

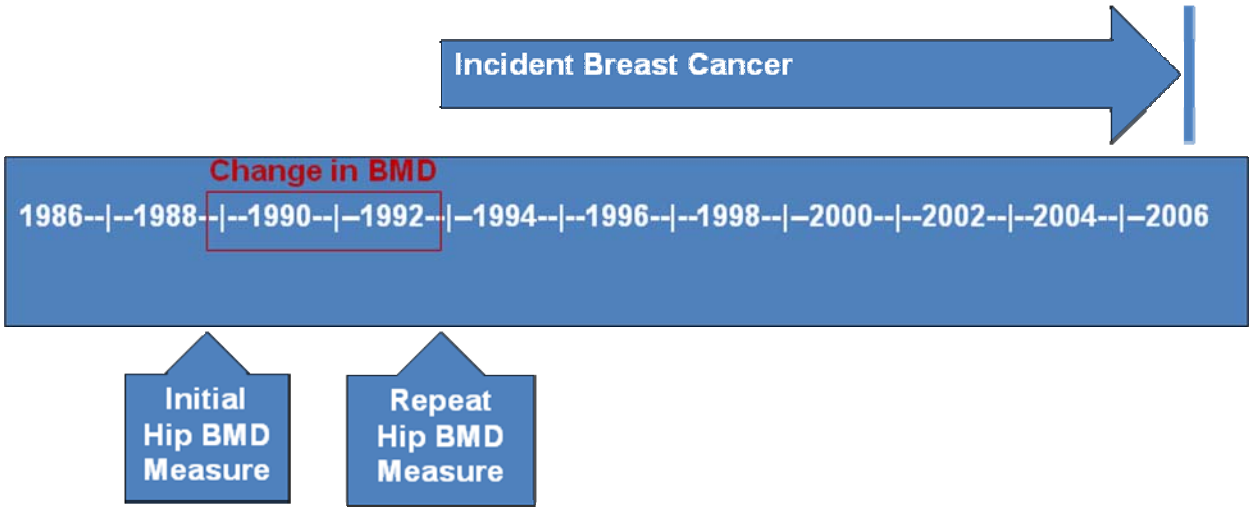

Figure 6. Timeline of total hip bone mineral density measurements and follow-up period, Study of Osteoporotic Fractures

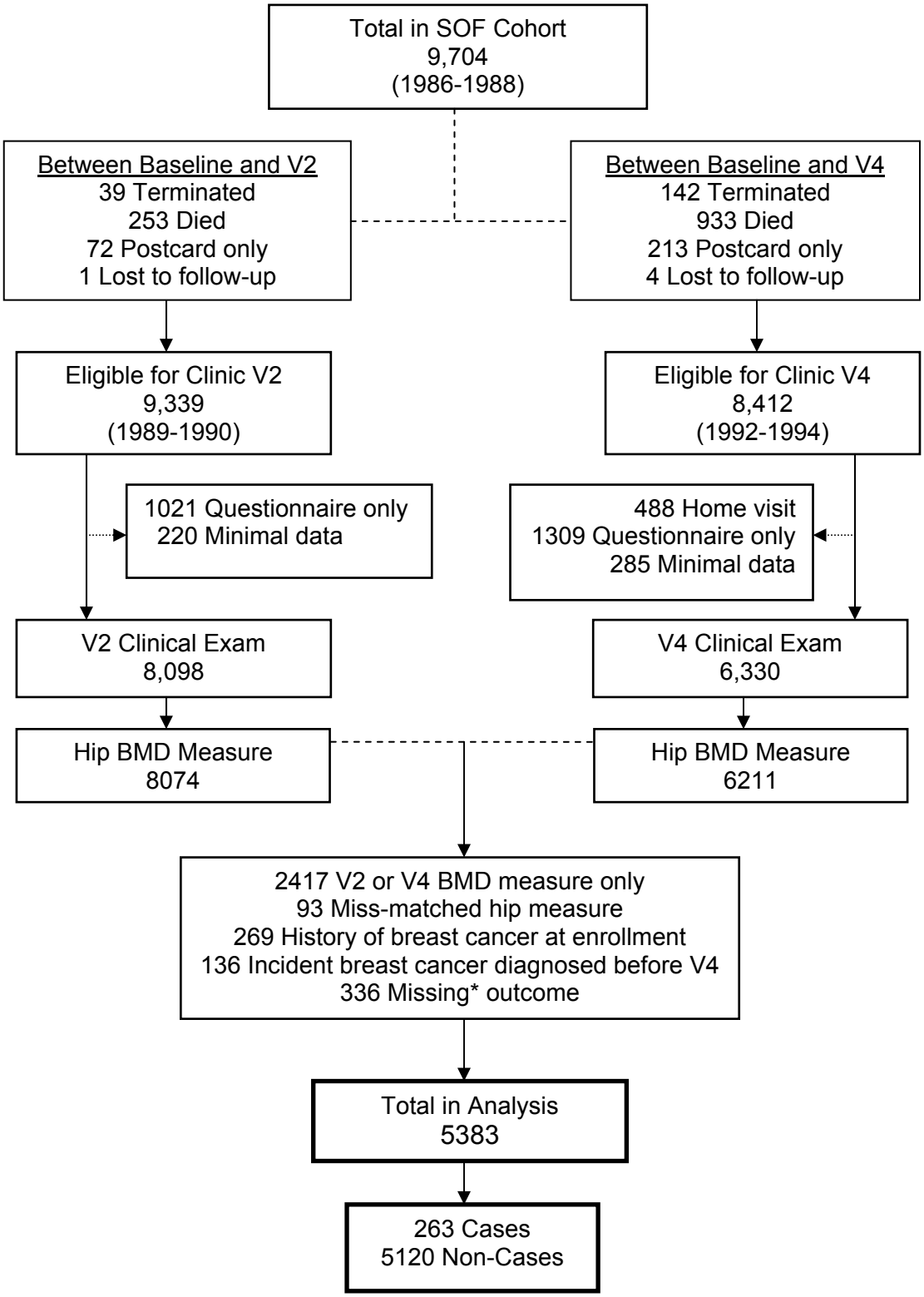

Figure 7. Cascade of analysis population determination, Study of Osteoporotic Fractures
*Missing values coded as refused to answer (n=63), unable to answer (n=8), never had a period (n=265).

6.3.2 Bone Mineral Density Measurements

Details of the BMD measures have been published previously.[210, 213] Briefly, total hip BMD (bone mineral density, g/cm^2, of the proximal femur) was measured at V2 and V4 using dual-energy x-ray absorptiometry (DXA) (QDR 1000, Hologic Inc., Bedford, Massachusetts). The mean coefficient of variation was 1.2% for the femoral neck. BMD was categorized into quintiles based in on the distribution in the SOF cohort with cutpoints at 0.648, 0.721, 0.782, and 0.86. T-scores were calculated using the National Health and Nutrition Examination Survey reference values,[241] and categorized based on the World Health Organization Criteria for the Diagnosis of Osteoporosis in Caucasian Women; normal bone mass is less than or equal to 1 SD below the young-adult mean, low bone mass is between 1 and 2.5 SD below the young-adult mean, osteoporosis is greater than or equal to 2.5 SD below the young-adult mean.[242] The rate of change in BMD was calculated using the total hip BMD measures taken at V2 and V4, and expressed as the annualized percent change over a mean 3.5 years.

6.3.3 Covariate Information

At the baseline and V2 examinations, participants completed a questionnaire and were interviewed. Demographic (age, education), reproductive history (menarche age, parity, age at first birth, number of live births, breastfeeding), height at age 25, menopausal status (menopause age, surgical vs. natural menopause), and breast cancer risk factor (benign breast disease, family history of breast cancer) data were collected at the baseline examination. At the V2 examination, updated data concerning smoking status, alcohol use, and physical activity were collected. Body weight was measured using a balance-beam scale. BMI was calculated by dividing the V2 weight by the square of height at age 25 years. Estrogen therapy (ET) was defined as currently taking estrogen pills. Current supplemental vitamin D use was defined as

taking vitamin D or a multivitamin containing vitamin D at least once per week. Calcium supplementation was determined by asking about current use at least once per week. Variables were categorized based on common cutpoints (e.g. BMI) or the original response categories collapsed to prevent small cell counts (e.g. age at first birth).

6.3.4 Incident Breast Cancer Ascertainment

Follow-up occurred every four months by either postcard or telephone (98% complete) in addition to clinic visits approximately every 2 years. Breast cancer outcomes were ascertained through self-report or death certificate review and were adjudicated by physicians locally and centrally at the San Francisco Coordinating Center. Medical records and pathology reports were used to record information on date of breast cancer diagnosis, stage at diagnosis, and estrogen- and progesterone-receptor status.[184]

6.3.5 Statistical Analyses

As part of the preliminary data analysis, baseline characteristics of the analysis population and the remainder of the SOF cohort were compared using t-tests for continuous measures and chi-square tests for categorical data. Additionally, change in important characteristics between V2 and V4 were assessed for the analysis population. Mean T-score per quintile of initial BMD also was calculated. Finally, the distribution of baseline characteristics (measured at V1 or V2) of the analysis population were compared for cases and non-cases.

The main analysis used Cox proportional hazard regression models with age as the underlying time scale to estimate hazard ratios and 95% confidence intervals for the association between bone mineral density and the risk of breast cancer.[217-219] Entry time was defined as age at V4 in days (calculated by multiplying V4 age by 365.25). Exit time was defined as age at

breast cancer diagnosis, death, loss to follow-up, or censoring (calculated by adding follow-up time in days from V4 to entry age). BMD was assessed 3 ways: 1) initial V2 BMD alone, 2) change in BMD between the initial and repeated measure, and 3) a combined model of initial BMD plus the change in BMD. Continuous BMD measures were expressed per one standard deviation (SD) increase in BMD. Initial BMD levels also were assessed as quintiles based on the distribution of BMD in the entire SOF cohort; the lowest category served as the reference. BMD was further assessed by T-score category with the osteoporotic group as the reference. Potential confounders, described above, were evaluated for inclusion in the multivariable adjusted models based on their significance ($p<0.25$) in the bivariate analyses. Correlated variables, e.g. multiple measures of body size, were chosen as covariates based upon statistical association, scientific knowledge, and/or variable distribution. Dummy variables were created for categorical variables as appropriate. Variables were selected for elimination one at a time using a backward elimination strategy based on individual Wald tests.[222, 223] After each variable was removed from the model, the partial likelihood ratio test was calculated comparing the nested models. Removed variables remained out of the models if no significant contribution to the model was determined ($p\geq0.1$). Interactions were evaluated using likelihood ratio tests that compared the model with the interaction term to the main effects model ($p\leq0.05$).

The potential for non-linear effects was investigated using cubic splines. RCS (restricted cubic splines), a SAS macro, was used to fit the cubic splines.[221] Knot placement was set at the BMD quintile cut-points. All breast cancers including in situ and invasive only breast cancers were analyzed separately. While the inclusion of in situ did not affect the results, regression results for invasive only cancers are presented. Analyses are also repeated among subgroups defined by age (split at median < 75 vs. ≥ 75 years), BMI (< 30 and ≥ 30 kg/m^2), ET (current/not current), and family history of breast cancer (negative/positive). Likelihood ratio tests were used to evaluate possible interactions between the subgroup variable and BMD by comparing a multivariable model with and without the interaction term expressed as the product of the

variable and dichotomous BMD. For the final models, probability values <0.05 were considered statistically significant. All tests were two-tailed. Schoenfeld residuals were used to test the proportional hazards assumption. SAS software release 9.2 (SAS Institute Inc., Cary, NC) was used for all analyses.

6.4 RESULTS

This prospective cohort study investigated the relationship between bone mineral density and breast cancer among 5383 postmenopausal women enrolled in the study of osteoporotic fractures. Baseline characteristics of the analysis population and the remainder of the SOF cohort (i.e. non-participants) are provided in **Table 12**. Meaningful differences were that the analysis population tended to be younger at enrollment and experience menopause later, were less likely to be current smokers or have a positive family history of breast cancer, and were more likely to be current users of calcium supplements, vitamin D supplements, and estrogen therapy and to walk for exercise.

The mean age of women at V2 was 72.7 years. The mean total hip BMD was 0.76 g/cm^2 (T-score of -1.34). Changes in important participant characteristics between V2 and V4 for the analysis population are provided in **Table 13.** At V4 a smaller proportion of women were current smokers, and a greater proportion of women were current users of calcium supplements, vitamin D supplements, and estrogen therapy. Mean T-scores by quintile of BMD at V2 are displayed in **Table 14**.

After a mean 9.52 years of follow-up after V4, 263 incident breast cancer cases were identified. Invasive breast cancers accounted for 224 cases while 39 were in situ. Estrogen receptor status was obtained for 206 cases and was positive in 178 (86%).

Table 15 presents the characteristics of the analysis population at baseline or V2. Women with breast cancer were slightly younger and more likely to be heavier, taller, and walk for exercise. As expected, breast cancer risk factors including older age at menopause, estrogen therapy use, and family history of breast cancer were found to be more prevalent in the incident cases than the non-case group.

Results of the Cox proportional hazards regression analyses are presented in **Table 16**. In Model 1, the initial BMD measure was a significant predictor of incident breast cancer risk in the age adjusted models. The positive association persisted in multivariable models, but was attenuated with the addition of weight as a covariate. Adjusted for age (as time scale), clinic site, weight, vitamin D supplement use, ET, and family history of breast cancer, initial BMD was not associated with and increased risk of invasive breast cancer (HR 1.06 per 1 SD increase in BMD, 95%CI 0.88-1.20). By quintile, with the lowest BMD level serving as the reference, the HR (95%CI) for increasing categories were 1.09 (0.64-1.85), 1.39 (0.84-2.31), 1.44 (0.87-2.39), and 1.13 (0.66-1.95), respectively (p=0.43). Compared to the lowest T-score category (≤ 2.5 corresponding to the WHO designation of osteoporosis), the HR (95%CI) for increasing categories were 1.99 (1.03-3.85) and 2.01 (1.00-4.01), respectively (p=0.07). In Model 2, change in BMD was not a significant predictor of incident breast cancer risk in the age adjusted, or either of the multivariable adjusted models (HR 1.09 per 1 SD increase, 95%CI 0.93-1.27). In Model 3, the risk estimates were similar to those in the earlier models with initial BMD or change in BMD alone.

Results were similar in multivariable regressions including estrogen receptor positive cancer only (**Table 17**). In Model 1, initial BMD was not associated with and increased risk of ER+ breast cancer (HR 1.04 per 1 SD increase, 95%CI 0.878-1.24). Compared to the lowest T-score category, the HR (95%CI) for increasing categories were 2.75 (1.19-6.33) and 2.45 (1.02-5.86), respectively (p=0.02). In Model 2, change in BMD was not a significant predictor (HR 1.06

per 1 SD increase, 95%CI 0.90-1.26). In Model 3, containing both initial BMD and BMD change, the risk estimates were similar.

Results of the subgroup analyses are presented in **Table 18**. In multivariable models with initial BMD dichotomized at the median, higher BMD was associated with an increased risk of invasive breast cancer among women with a positive (HR 2.99, 95%CI 1.25-7.12) but not a negative (HR 1.07, 95%CI 0.77-1.50) family history of breast cancer ($p_{interaction}$=0.01). There was no difference in the magnitude of the association by age, ET, or BMI. Investigated individually using likelihood ratio tests, no other interactions between dichotomous BMD and the subgroup variable in a multivariable model were found.

An examination of the shape of the risk function using cubic splines did not indicate a deviation from a linear relationship for BMD overall (p=0.11), or by family history of breast cancer (negative family history p=0.20, positive family history p=0.17) (**Appendix C**).

6.5 DISCUSSION

In this large, prospective, cohort study of 5383 postmenopausal women from the Study of Osteoporotic Fractures, we examined the association between BMD and the development of breast cancer over an average of 9.5 years. Overall a continuous BMD measure was not associated with breast cancer risk after adjusting for known breast cancer risk factors including weight, ET, and family history. Furthermore, there was no improvement in the overall predictive value with a second measure of BMD, obtained over a mean 3.5 years later, in the determination of breast cancer risk. Results were similar for estrogen receptor positive breast cancer. We found a significantly increased risk of invasive breast cancer with total hip T-score measures in the normal (≥ -1.0) and low (-1.0 to -2.5) ranges compared to osteoporotic (≥ -2.5)

based on WHO criteria. Having a positive family history of breast cancer was associated with a significant 3-fold increase in breast cancer risk among women with high compared to low BMD.

Data supporting the BMD-breast cancer link were initially published by SOF investigators between 1996 and 2001 with follow-up ranging from 3 to 6 years.[184, 195, 196, 200] Several other prospective studies have sought to confirm this association, and found a similar or slightly weaker relationship,[190-194] while other still found no association.[204, 205] Furthermore, a recent analysis from the Women's Health Study found that BMD predicted breast cancer risk independent of Gail score.[208] The fact that we did not find an overall association between BMD and breast cancer risk, indicates that BMD may be a stronger predictor of early opposed to later breast cancer risk as we did not include incident cancers that occurred within approximately 4 years following the initial hip BMD scan. Nevertheless, we are using BMD as a measure of long-term exposure.

While the relationship between higher BMD and an increased risk of breast cancer in postmenopausal women has been well studied, the association is moderate at best. One reason that a stronger association has not been documented, may be that among women with higher BMD, their estrogen exposure has diminished resulting in an attenuated increased risk of breast cancer. Or conversely, among women with low BMD, their exposure to estrogen has been sustained essentially negating the protective effect of having a lower BMD.

To our knowledge, this is the first study to investigate the predictive value of a repeat BMD measurement compared with the initial BMD among postmenopausal women. We found change in BMD not to be a risk factor for breast cancer. The one study to have looked at change in BMD over 6.9 years and the risk of breast cancer did so only among peri-menopausal women aged 45-54.[204] With only 34 incident breast cancer cases, they too found no relationship with a mean follow-up of 9.7 years. The HR (95%CI) for 1 SD change in BMD at the femoral neck was 1.15 (0.79-1.68). Our results also suggest that a repeat BMD measure does not add value in determining future breast cancer risk.

The risk of breast cancer related to BMD has been reported to differ by estrogen receptor status[197] and family history of breast cancer. Results regarding family history come from a separate cohort study[190] and from an early SOF report which utilized cases that were diagnosed within the first 3.5 years of follow-up and that do not overlap with the cases in this analysis.[195] While our results were similar for invasive and estrogen receptor positive breast cancers, we did find a significant dependence on family history in the relationship between BMD and breast cancer. Indeed, these women may be more sensitive to cumulative estrogen exposure as reflected by their BMD measurements. Genetic differences in estrogen synthesis, metabolism, and the enzymes involved could account for the association due to altered bioavailability and/or biologic activity of steroid hormones such as estrogen.[243]

Our study has several important strengths in that it is a large prospective study of postmenopausal women, with repeated and rigorously controlled BMD measurements. The 3.5 year interval present in this analysis reflects the time between repeat BMD measurements common in clinical practice. Additionally, incident breast cancer diagnoses were confirmed by pathology records and adjudicated by a physician and detailed covariate information was collected.

Limitations of our study include the fact that women who were able to attend the follow-up examination to have their BMD measurement were healthier than those who did not attend the follow-up examination. Those who did not attend, were likely to have been older, weaker, and possibly may have had greater BMD loss. Thus, our results are relevant to healthy postmenopausal white women aged 65 and greater, and may not be generalizable to other populations. Furthermore, we lacked data regarding disease history in more distant relatives, aunts and grandmothers for instance, as well as paternal family history.

In conclusion, BMD was not found to be a significant long-term predictor of breast cancer risk after multivariable adjustment. Furthermore, a repeat BMD measure does not appreciably enhance the predictive value. However, our results do indicate that the association

between bone mineral density and invasive breast cancer is modified by family history of breast cancer. Women with a positive family history coupled with high BMD are at increased risk. Identifying women at elevated risk is a critical step towards breast cancer risk reduction. Because preventive interventions are limited, continued vigilance in breast cancer screening is recommended for these women into old age. Routine screening can result in earlier detection of breast carcinomas, less invasive treatment options, better outcomes, and increased survival.

6.6 TABLES

Table 12. Baseline characteristics of the analysis population and non-participants, Study of Osteoporotic Fractures

Characteristic	Analysis Population (n=5383)		Non-Participants (n=4321)		p-value
	Mean	**SD**	**Mean**	**SD**	
Age, y	70.69	4.64	72.80	5.66	<0.0001
Education, y	12.81	2.73	12.30	2.79	<0.001
Body Weight, kg	67.15	11.83	66.82	12.26	0.19
BMI, kg/m^2	26.39	4.38	26.42	4.59	0.72
Height at Age 25 y, cm	162.60	15.82	162.50	5.65	0.75
Age at Menopause, y	48.11	45.75	47.71	5.79	<0.01
Age at Menarche, y	13.02	1.44	13.06	1.50	0.23
Number of Live Births	2.72	1.53	2.54	1.48	<0.001
Alcohol Use, drinks/week	1.90	3.90	1.93	4.3	0.72
	N	**%**	**N**	**%**	
Clinic Site					
A	1524	28.31	934	21.62	0.42
B	1211	22.50	1210	28.00	
C	1171	21.75	1253	29.00	
D	1477	27.44	924	21.38	
Nulliparous	843	15.67	731	16.93	0.10
Age at First Birth, y					
≤ 20	633	14.63	562	19.00	<0.001
21-30	3040	70.24	1973	66.70	
31-40	633	14.63	408	13.79	
> 40	22	0.51	15	0.51	
Ever Breastfed	3108	68.52	2468	68.88	0.73
Surgical Menopause	645	12.44	512	12.40	0.95
Walks for Exercise	2951	54.83	1915	44.33	<0.0001
Current Smoker	450	8.38	517	12.01	<0.0001
Current Calcium Supplement Use	2356	43.85	1776	41.20	<0.01
Current Vitamin D Supplement Use	2441	46.14	1832	43.25	<0.01
Current Oral Estrogen Use	818	15.37	513	12.08	<0.0001
Family History of Breast Cancer	689	13.17	605	15.14	<0.01

Abbreviations used: BMD, bone mineral density; BMI, body mass index; SD, standard deviation

Table 13. Change in important participant characteristics between the second and fourth clinic visits, analysis population (N=5383), Study of Osteoporotic Fractures

	V2		V4		
Characteristic	**Mean**	**SD**	**Mean**	**SD**	**p-value***
Age, y	72.71	4.62	76.45	4.63	<0.01
Body Weight, kg	66.60	11.77	66.26	11.97	0.14
BMI, kg/m^2, mean(SD)	26.21	4.37	26.44	4.52	0.01
Total Hip BMD, g/cm^2	0.76	0.13	0.74	0.13	<0.01
	N	**%**	**N**	**%**	
Current Smoker	372	6.95	296	5.50	<0.001
Current Calcium Supplement Use	2034	38.65	2401	44.61	<0.001
Current Vitamin D Supplement Use	2137	40.53	2247	41.75	<0.001
Current Oral Estrogen Use	8584	16.24	1046	19.44	<0.001

*P-values from paired t-tests for continuous variables, and two-dependent proportion chi-square tests for categorical variables
Abbreviations used: BMD, bone mineral density; BMI, body mass index; SD, standard deviation

Table 14. Mean T-score by quintile of BMD at V2, analysis population, Study of Osteoporotic Fractures

	T-score		
BMD Quintile, g/cm^2	**N**	**Mean**	**SD**
≤ 0.648	975	-2.72	0.41
0.649-0.721	1065	-1.95	0.16
0.722-0.782	1094	-1.44	0.13
0.783-0.860	1110	-0.91	0.17
≥ 0.861	1139	0.08	0.62

Abbreviations used: BMD, bone mineral density; SD, standard deviation

Table 15. Descriptive characteristics and risk factors for breast cancer among cases and non-cases, analysis population, Study of Osteoporotic Fractures

Characteristic	Cases (N=263)		Non-cases (N=5120)		p-value*
	N	%	N	%	
Clinic Site					
A	82	31.18	1442	28.16	0.31
B	66	25.10	1145	22.36	
C	48	18.25	1123	21.93	
D	67	25.48	1410	27.54	
Age, y, mean(SD)	71.79	3.77	72.75	4.66	<0.0001
Education					
< High School	39	14.83	1001	19.57	0.16
High School	113	42.97	2067	40.42	
> High School	111	42.21	2049	40.01	
Body Weight, kg, mean(SD)	68.98	11.63	66.48	11.76	0.001
BMI, kg/m^2, mean(SD)	26.83	4.54	26.18	4.36	0.02
Height at age 25 y, cm, mean(SD)	163.26	5.62	162.54	5.83	0.05
Age at Menarche, y					
≤ 11	24	9.72	602	12.29	0.47
12-13	138	55.87	2691	54.95	
≥ 14	85	34.41	1604	32.75	
Nulliparous	42	15.97	801	15.65	0.89
Age at First Birth, y					
Never gave birth	30	11.76	603	12.06	0.98
≤ 20	178	69.80	3495	69.89	
> 20	47	18.43	903	18.06	
Number of Live Births, mean(SD)					
Never pregnant	42	16.09	801	15.69	0.79
0	6	2.30	130	2.55	
1-2	99	37.93	2079	40.73	
3-4	91	34.87	1603	31.41	
5+	23	8.81	491	9.62	
Ever Breastfed	64	28.96	1364	31.61	0.41
Age at Menopause, y, mean(SD)	48.88	4.93	48.07	5.78	0.02
Surgical Menopause	38	14.90	607	12.31	0.22
Walks for Exercise	158	60.08	2793	54.56	0.08
Alcohol, drinks/week, mean(SD)	1.89	3.65	1.90	3.91	0.97
Current Smoker	15	5.75	357	7.01	0.43
Current Calcium Supplement Use	103	40.55	1931	38.56	0.52
Current Vitamin D Supplement Use	93	36.47	2044	40.74	0.17
Current Oral Estrogen Use	56	21.88	802	15.96	0.01
Benign Breast Disease	45	17.72	743	14.90	0.23
Family History of Breast Cancer†	49	18.92	640	12.87	0.005

Table 15. continued

Characteristic	Cases (N=263) N	Cases (N=263) %	Non-cases (N=5120) N	Non-cases (N=5120) %	p-value*
Initial BMD at V2					
Total Hip, g/cm^2, mean(SD)	0.79	0.12	0.76	0.13	<0.0001
T-score					
≤ -2.5	13	4.94	622	12.15	
> -2.5 to -1.0	135	51.33	2755	53.81	<0.0001
≥ -1.0	115	43.73	1743	34.04	
Repeat BMD at V4					
Total Hip, g/cm^2, mean(SD)	0.77	0.12	0.73	0.13	<0.0001
T-score					
≤ -2.5	21	7.98	904	17.66	
> -2.5 to -1.0	146	55.51	2835	55.37	<0.0001
≥ -1.0	96	36.50	1381	26.97	
Annual % BMD change, mean(SD)[‡]	-0.43	1.29	-0.58	1.43	0.07

*P-values from t-tests for continuous variables, and chi-square tests for categorical variables
[†]Self-reported breast cancer diagnosis in a first degree female relative (i.e. mother or sister)
[‡]Over a mean (SD) 3.54 (0.30) years between measurements
Abbreviations used: BMD, bone mineral density; BMI, body mass index; SD, standard deviation

Table 16. Results of multivariable Cox proportional hazards regression to predict risk of invasive breast cancer with initial and change in total hip BMD measures, Study of Osteoporotic Fractures

	Invasive Breast Cancer					
	Age Adjusted		Multivariable Adjusted‡		Multivariable Adjusted‡	
Cases / Non-Cases	**224 / 5119**		**214 / 4872**		**210 / 4769**	
Model	**HR (95%CI)**	**P value**	**HR (95%CI)**	**P value**	**HR (95%CI)**	**P value**
1: Initial BMD						
Continuous*	1.19 (1.04-1.35)	0.01	1.14 (0.99-1.30)	0.06	1.06 (0.88-1.20)	0.75
Quintiles						
≤ 0.648	1.00		1.00		1.00	
0.649-0.721	1.18 (0.70-1.97)		1.19 (0.70-2.01)		1.09 (0.64-1.85)	
0.722-0.782	1.62 (1.00-2.63)	0.01§ 0.06**	1.56 (0.95-2.57)	0.04§ 0.15**	1.39 (0.84-2.31)	0.49§ 0.43**
0.783-0.860	1.75 (1.09-2.81)		1.72 (1.06-2.80)		1.44 (0.87-2.39)	
≥ 0.861	1.68 (1.04-2.70)		1.52 (0.93-2.49)		1.13 (0.66-1.95)	
T-score						
≤ -2.5	1.00		1.00		1.00	
> -2.5 to -1.0	2.09 (1.12-3.88)	<0.01§ <0.01**	2.21 (1.15-4.24)	0.01§ <0.01**	1.99 (1.03-3.85)	0.19§ 0.07**
≥ -1.0	2.56 (1.36-4.81)		2.54 (1.31-4.93)		2.01 (1.00-4.01)	
2: Change in BMD†	1.09 (0.94-1.25)	0.70	1.10 (0.95-1.28)	0.19	1.09 (0.93-1.26)	0.28
3: Initial BMD	1.18 (1.04-1.35)	0.01	1.13 (0.99-1.30)	0.07	1.03 (0.88-1.20)	0.75
Change in BMD	1.08 (0.93-1.25)	0.33	1.10 (0.94-1.28)	0.23	1.09 (0.93-1.27)	0.28

*Per 1 SD (0.13 g/cm^2) increase in BMD
†Per 1 SD (1.42%) increase in BMD
‡Adjusted for age (as time scale), clinic site, vitamin D supplement use, estrogen therapy use, and family history of breast cancer; the final multivariable model additionally includes an adjustment for weight
§P value for trend
**Overall p value
Abbreviations used: BMD, bone mineral density; CI, confidence interval; HR, hazard ratio; SD, standard deviation

Table 17. Results of multivariable Cox proportional hazards regression to predict risk of ER+ breast cancer with initial and change in total hip BMD measures, Study of Osteoporotic Fractures

	Estrogen Receptor Positive					
	Age Adjusted		Multivariable Adjusted‡		Multivariable Adjusted‡	
Cases / Non-Cases	**178 / 5119**		**172 / 4872**		**168 / 4769**	
Model	**HR (95%CI)**	**P value**	**HR (95%CI)**	**P value**	**HR (95%CI)**	**P value**
1: Initial BMD						
Continuous*	1.22 (1.05-1.40)	0.008	1.16 (1.00-1.34)	0.07	1.04 (0.87-1.24)	0.66
Quintiles						
≤ 0.648	1.00		1.00		1.00	
0.649-0.721	1.09 (0.60-1.98)		1.06 (0.58-1.93)		0.97 (0.53-1.78)	
0.722-0.782	1.93 (1.13-3.31)	0.02§ 0.04**	1.84 (1.07-3.17)	0.06§ 0.07**	1.63 (0.94-2.84)	0.59§ 0.16**
0.783-0.860	1.70 (0.98-2.93)		1.65 (0.96-2.86)		1.36 (0.77-2.41)	
≥ 0.861	1.72 (1.00-2.96)		1.52 (0.87-2.63)		1.09 (0.59-2.01)	
T-score						
≤-2.5	1.00		1.00		1.00	
>-2.5 to -1.0	3.15 (1.38-7.21)	0.01§ <0.01**	3.08 (1.34-7.05)	0.03§ <0.01**	2.75 (1.19-6.33)	0.37§ 0.02**
≥-1.0	3.50 (1.52-8.08)		3.21 (1.38-7.44)		2.45 (1.02-5.86)	
2: Change in BMD†	1.07 (0.91-1.25)	0.42	1.08 (0.91-1.28)	0.36	1.06 (0.90-1.26)	0.47
3: Initial BMD	1.21 (1.05-1.40)	0.01	1.15 (0.99-1.34)	0.06	1.04 (0.87-1.24)	0.66
Change in BMD	1.06 (0.90-1.24)	0.52	1.07 (0.90-1.27)	0.42	1.06 (0.90-1.26)	0.48

*Per 1 SD (0.13 g/cm^2) increase in BMD
†Per 1 SD (1.42%) increase in BMD
‡Adjusted for age (as time scale), clinic site, weight, vitamin D supplement use, estrogen therapy use, and family history of breast cancer
§P value for trend
**Overall p value
Abbreviations used: BMD, bone mineral density; HR, hazard ratio; SD, standard deviation

Table 18. Results of subgroup analyses to assess the association between BMD and risk of invasive breast cancer, Study of Osteoporotic Fractures

	Invasive Breast Cancer					
	Age Adjusted			Multivariable Adjusted[†]		
Initial BMD*	Cases / Non-cases	HR (95%CI)	P value	Cases / Non-cases	HR (95%CI)	P value
Age						0.76[‡]
<75 years	111/2139	1.59 (1.05-2.39)	0.03	103/1972	1.49 (0.94-2.36)	0.09
≥75 years	113/2980	1.51 (1.04-2.19)	0.03	107/2797	1.05 (0.69-1.59)	0.83
BMI						0.60[‡]
<30	171/4204	1.53 (1.13-2.08)	0.007	160/3891	1.28 (0.91-1.79)	0.15
≥30	53/915	1.13 (0.58-2.21)	0.73	50/878	0.97 (0.48-1.96)	0.93
ET						0.97[‡]
Not current	172/4223	1.51 (1.11-2.06)	0.009	165/4012	1.30 (0.92-1.82)	0.14
Current	46/802	1.50 (0.76-2.96)	0.24	45/757	1.13 (0.55-2.32)	0.73
Family History						0.01[‡]
Negative	183/4330			172/4153		
Low BMD		0.77 (0.57-1.04)	0.09		0.94 (0.67-1.31)	0.69
High BMD		1.30 (0.96-1.75)	0.09		1.07 (0.77-1.50)	0.69
Positive	38/640			38/616		
Low BMD		0.28 (0.12-0.65)	0.003		0.34 (0.14-0.80)	0.01
High BMD		3.52 (1.53-8.11)	0.003		2.99 (1.25-7.12)	0.01

*Hazard ratios comparing BMD dichotomized at median (0.751)
[†]Adjusted for age (as time scale), clinic site, weight, vitamin D supplement use, estrogen therapy use, and family history of breast cancer
[‡]P value for interaction between subgroup variable and BMD
Abbreviations used: BMD, bone mineral density; HR, hazard ratio; SD, standard deviation

7.0 GENERAL DISCUSSION

Osteoporosis and breast cancer are two diseases that predominantly affect postmenopausal women. Osteoporosis, characterized by low bone mass, is the most common bone disease and a leading cause of fractures among the aging.[14] Among women over age 50 in the United States, the prevalence of osteoporosis is between 13 and 18 percent.[241] Breast cancer is the most commonly diagnosed cancer, and the second leading cause of cancer mortality among women in the United States.[1] During 2008 alone, more than 182,000 new cases of invasive breast cancer and approximately 40,500 deaths were expected to occur.[2]

Notwithstanding their different pathophysiologies, breast cancer and osteoporosis share several etiologic factors including estrogen. Estrogen is thought to play a central role in the development of breast cancer, due to its ability to stimulate proliferation of breast tissue,[175] and osteoporosis, as evidenced by the rapid decrease in bone density following menopause.[244] As the number of women with low bone mass increases with age (approximately 30% over age 50 and more than 50% over age 80),[245] so does the incidence of osteoporosis. However, the incidence of breast cancer also continues to increase with age, peaking at 75-79 years,[6] despite the dramatic drop in endogenous estrogen levels at menopause.

The mechanisms by which breast cancer develops are not well understood, thus limiting opportunities for disease prevention. Therefore, it is extremely important to pursue research focused on breast cancer etiology. Only through the identification of modifiable factors associated with this disease can effective breast cancer prevention be realized. We undertook investigations of two factors of potential etiologic importance to breast cancer: vitamin D level

and endogenous estrogen exposure. We sought to examine the association between three physiologic measures related to bone metabolism and calcium homeostasis, vitamin D, fractional calcium absorption, and bone mineral density, and breast cancer risk.

7.1 ARTICLE 1: SERUM 25-HYDROXYVITAMIN D AND RISK OF ER+ BREAST CANCER IN POSTMENOPAUSAL WOMEN

We used a case-cohort design to study the association between serum 25(OH)D and ER+ breast cancer in 502 women within the Study of Osteoporotic Fractures (SOF). 25(OH)D was measured using serum specimens collected at baseline. Low 25(OH)D was not associated with an increased risk of ER+ breast cancer overall. However, a protective effect was noted with lower 25(OH)D levels among women over 75 years of age.

The results of our prospective analysis are in agreement with a previous study which demonstrated no association among postmenopausal women.[130] Two other studies reported an inverse association.[131, 132] However, the comparability of our results with these later studies are limited due to methodological differences. A reduced tissue response to vitamin D with greater age is possible. Our observed association may be reflective of either a decline in the interaction between $1,25(OH)_2D$ and VDRs in breast tissue, or diminished VDR expression with old age. Alternatively, tumors may develop mechanisms to negate anti-proliferative effects of $1,25(OH)_2D$ at the cellular level.[226] The positive association of a 50% reduction in risk among women 75 years and older has not been reported previously and may indicate a change in the interaction between vitamin D and breast tissue.

7.2 ARTICLE 2: RISK OF BREAST CANCER USING FRACTIONAL CALCIUM ABSORPTION AS A MARKER OF VITAMIN D RESISTANCE

In this prospective study, we examined the association of between fractional calcium absorption, utilized as a marker tissue resistance to vitamin D, and breast cancer risk in SOF. Epidemiologic evidence suggests that vitamin D may reduce the incidence of breast cancer. However, reports in the literature have been inconsistent, particularly in regard to postmenopausal women. The possibility that age related tissue resistance to $1,25(OH)_2D$, the active vitamin D metabolite, may interrupt the protective mechanism by which vitamin D is thought to prevent breast cancer has not been investigated previously.

Contrary to our hypothesis, we found a modestly significant increased risk of invasive breast cancer with increasing FCA. A stronger positive association was obtained among women over age 75 and those with low dietary calcium intakes (≤ 525 mg/d). Both calcium and vitamin D are thought to prevent breast cancer development through similar but independent effects on cell proliferation and differentiation.[105, 233, 234, 236] The rate of FCA is inversely proportional to calcium intake and is also regulated by $1,25(OH)_2D$ and potentially estrogen.[160, 162, 163, 239]

Estrogen may interfere with the effect of calcium and vitamin D on cancer prevention by increasing intestinal calbindin D expression independent of vitamin D.[232] Calbindin D is a cytosolic protein and the rate-limiting molecule in vitamin D-induced intestinal calcium transport.[240] Increased calbindin D expression would lead to decreased circulating calcium levels, limiting calcium and vitamin D dependent apoptosis, and activate osteoblast formation resulting in increased calcium mineralization in bone and reduced bioavailability of systemic calcium.[232] We did not find an interaction with estrogen therapy use, however small numbers may have been prohibitive.

7.3 ARTICLE 3: LONG-TERM PREDICTION OF BREAST CANCER RISK IN POSTMENOPAUSAL WOMEN BY BONE MINERAL DENSITY

In this prospective study, we investigated the association between BMD and the development of breast cancer over an average of 9.5 years in SOF. Overall a continuous BMD measure was not associated with breast cancer risk after adjusting for known breast cancer risk factors. We found a significantly increased risk of invasive breast cancer with total hip T-score measures in the normal (≥ -1.0) and low (-1.0 to -2.5) ranges compared to osteoporotic (≤ -2.5) based on WHO criteria. Furthermore, there was no improvement in the overall predictive value in a second measure of BMD, obtained over a mean 3.5 years later, in the determination of breast cancer risk. However, having a positive family history of breast cancer was associated with a significant 3-fold increase in breast cancer risk among women with high compared to low BMD.

The lack of an overall association, indicates that BMD may be a stronger predictor of early opposed to later breast cancer risk. Our results are consistent with a small study of peri-menopausal women which also found no association between change in BMD and breast cancer risk.[204] The significant interaction with family history of breast cancer is in-line with previous reports.[190, 195] These women may be more sensitive to cumulative estrogen exposure as reflected in their BMD measurements. Genetic differences in estrogen synthesis, metabolism, and enzymes involved could enhance the bioavailability and/or biologic activity of steroid hormones such as estrogen.[243]

7.4 SUMMARY & FUTURE DIRECTIONS

These research studies explored the association between vitamin D level and estrogen, two potentially etiologic factors for breast cancer. Our findings do not support a protective effect of 25(OH)D level on ER+ breast cancer risk in postmenopausal women 65 years of age and older. The positive association among women 75 years and greater is suggestive of a change in the interaction of vitamin D in the breast; confirmation of this association in other populations is needed. Further investigations of the role of vitamin D on breast cancer development among elderly women are warranted. In particular, it will be important to better define the factors influencing 25(OH)D levels in this vulnerable age group. An important next step will be to measure localized levels of $1,25(OH)_2D$ and 25(OH)D in both healthy and malignant breast tissues and to compare them as the correlations between these two metabolites are currently unknown. Future studies should also include adequate numbers of women with ER- breast cancer.

We are the first to investigate the association between FCA and breast cancer risk. Our results suggest that high FCA, particularly among those with a low dietary calcium intake, is associated with an increased risk of postmenopausal breast cancer. More research is needed to fully understand the association between FCA and breast cancer risk. Accounting for other physiological measures such as sex steroid hormone levels, calcium, $1,25(OH)_2D$, 25(OH)D, and PTH in multivariable models may be useful. Correlates of FCA, including calcium, vitamin D, and estrogen have been investigated individually as etiologic factors for breast cancer. However, with the exception of estrogen, the reported associations have been inconsistent. Further investigations into the role of estrogen in conjunction with calcium and vitamin D levels

on FCA may help to clarify their interdependence. Additionally, replication of these findings in other populations will be important.

BMD was not found to be a significant long-term predictor of breast cancer risk after multivariable adjustment. Furthermore, a repeat BMD measure does not appreciably enhance the predictive value. However, our results do indicate that the association between bone mineral density and invasive breast cancer is modified by family history of breast cancer. Women with a positive family history coupled with high BMD are at increased risk. The extent to which BMD can be used to further elucidate the risk of postmenopausal breast cancer among women already at higher risk due to a positive family history should be studied. Identifying women at elevated risk is a critical step towards breast cancer risk reduction. Moreover, the research in these areas has not addressed the association among other racial groups, in particular those with darker skin pigmentation for whom inadequate circulating vitamin D levels are more prevalent and baseline BMD measurements are higher on average.

8.0 PUBLIC HEALTH SIGNIFICANCE

The public health burden of breast cancer and osteoporosis is substantial. These two diseases predominantly affect postmenopausal women, and due to our rapidly aging population, their impact is expected to worsen. While primary prevention of both these diseases is a desirable and sought-after goal, shared risk factors in opposing directions complicates such efforts. For instance, estrogen is thought to play a central role in the development of breast cancer, due to its ability to stimulate proliferation of breast tissue,[175] and osteoporosis, as evidenced by the rapid decrease in bone density following menopause.[244] Increased understanding of the etiologic factors and their underlying mechanisms are needed in order to identify potentially modifiable risk factors along with the women who might benefit from targeted prevention strategies. Established risk factors explain little of the variability in breast cancer and therefore there is a need to identify additional risk factors. This becomes an increasingly difficult task given the heterogeneity of breast cancer risk factors that exists between premenopausal and postmenopausal women, as well as the differences in pathologic features of breast tumors in older women. We therefore focused on how vitamin D, fractional calcium absorption, and bone mineral density relate to breast cancer risk.

More than 50% of women over age 60 are reported to have inadequate summer serum 25(OH)D levels,[209] however the association with breast cancer risk has been understudied. Our investigation of the association between 25(OH)D and ER+ breast cancer adds to the small body of epidemiologic evidence. It is not known yet whether FCA rates will be useful in distinguishing between women with an increased breast cancer risk, however, our findings may

help to clarify the inconsistent associations seen with studies of calcium and vitamin D to date. Finally, we have demonstrated a significant interaction in the relationship between bone mineral density and breast cancer by family history. Approximately 30% of women over age 50 have low bone mass as do more than 50% of women over age 80,[245] potentially indicating that they have a reduced breast cancer risk. While routine screening can result in earlier detection of breast carcinomas, less invasive treatment options, better outcomes, and increased survival, screening recommendations for older women are severely lacking. The ability to better pinpoint a woman's risk of postmenopausal breast cancer will allow for the establishment of solid screening guidelines.

Through our investigations of these potentially etiologic factors and their association with breast cancer development, we have enhanced our knowledge regarding the interdependence of vitamin D, calcium, and estrogen. These results, along with those of future studies expanding upon our findings, may lead to improved opportunities for prevention and early detection of breast cancer.

APPENDIX A

SERUM 25-HYDROXYVITAMIN D AND RISK OF ER+ BREAST CANCER IN POSTMENOPAUSAL WOMEN

A.1 CASE-COHORT SELECTION

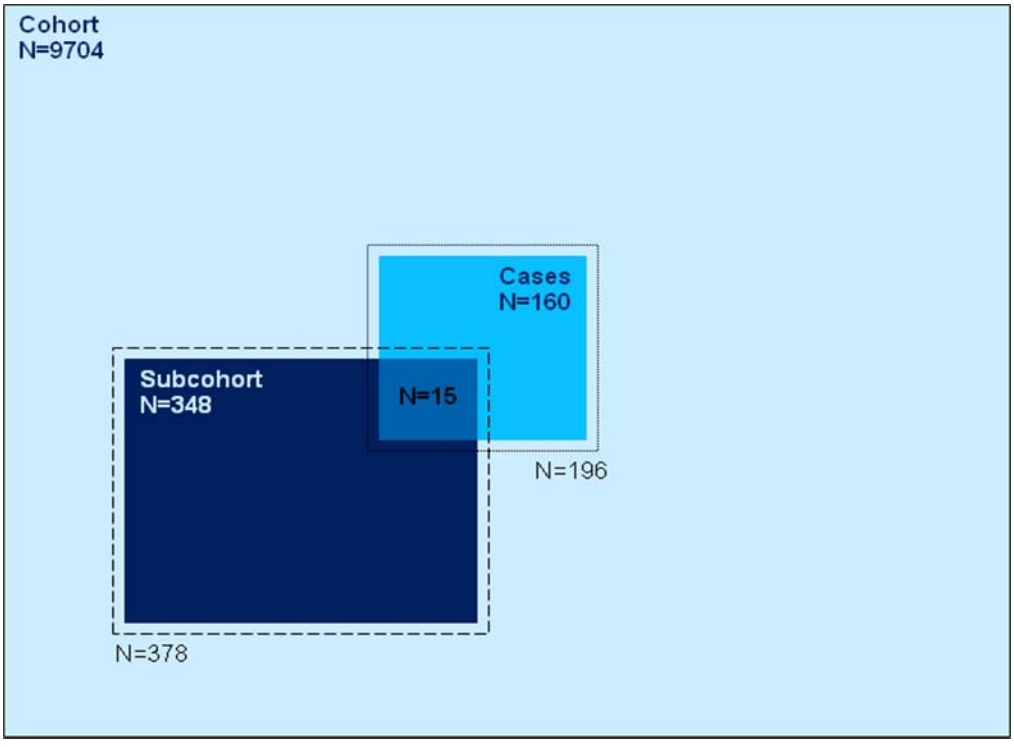

Figure 8. Case-cohort participant selection, Study of Osteoporotic Fractures
The subcohort includes all individuals from the original subcohort (a random sample of the entire cohort, represented by a dashed - - - outline) with available serum. Fifteen cases were selected into the subcohort. The case group includes all incident ER+ breast cancer cases from the original case group (all ER+ cases diagnosed through 2000, represented by a dotted . . . outline) with available serum.

A.2 ASSAY RELIABILTY SUBSTUDY

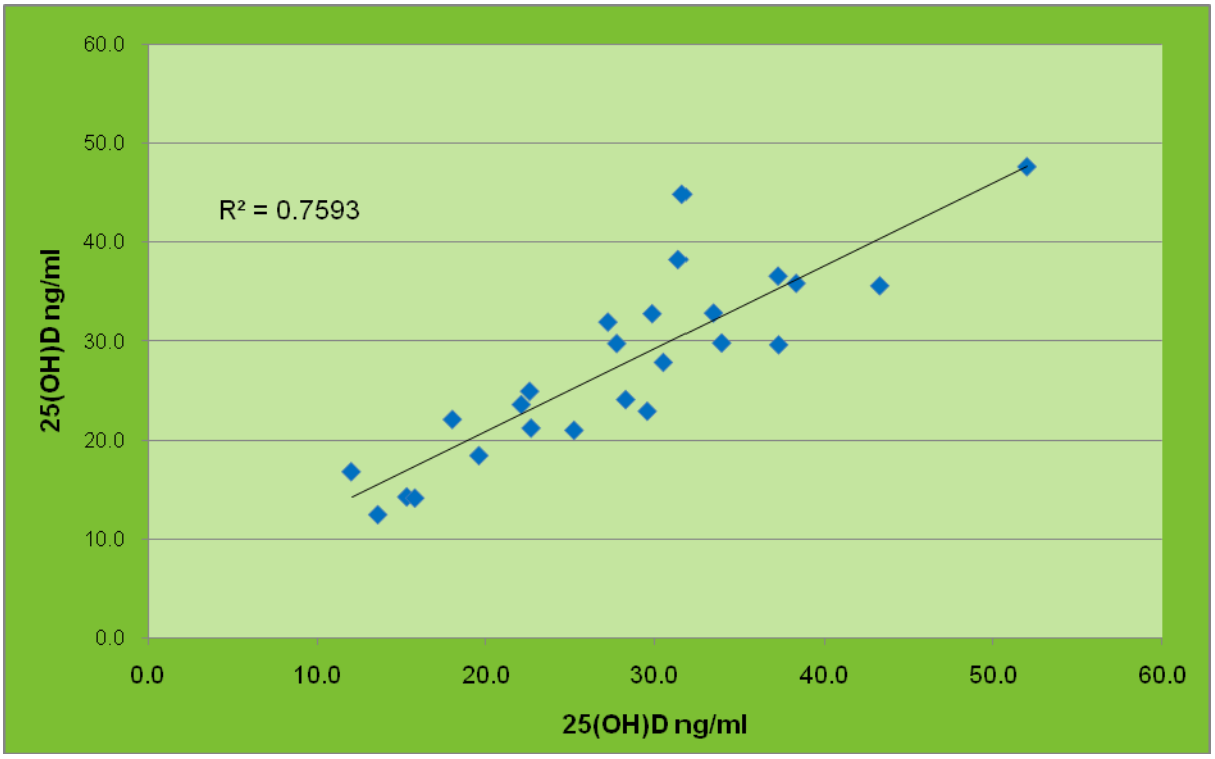

Figure 9. Diasorin RIA 25(OH)D assay reliability, Study of Osteoporotic Fractures
Masked duplicate serum 25(OH)D samples (n=25).

A.3 KERNEL DENSITY PLOT OF 25(OH)D BY CASE COHORT STATUS

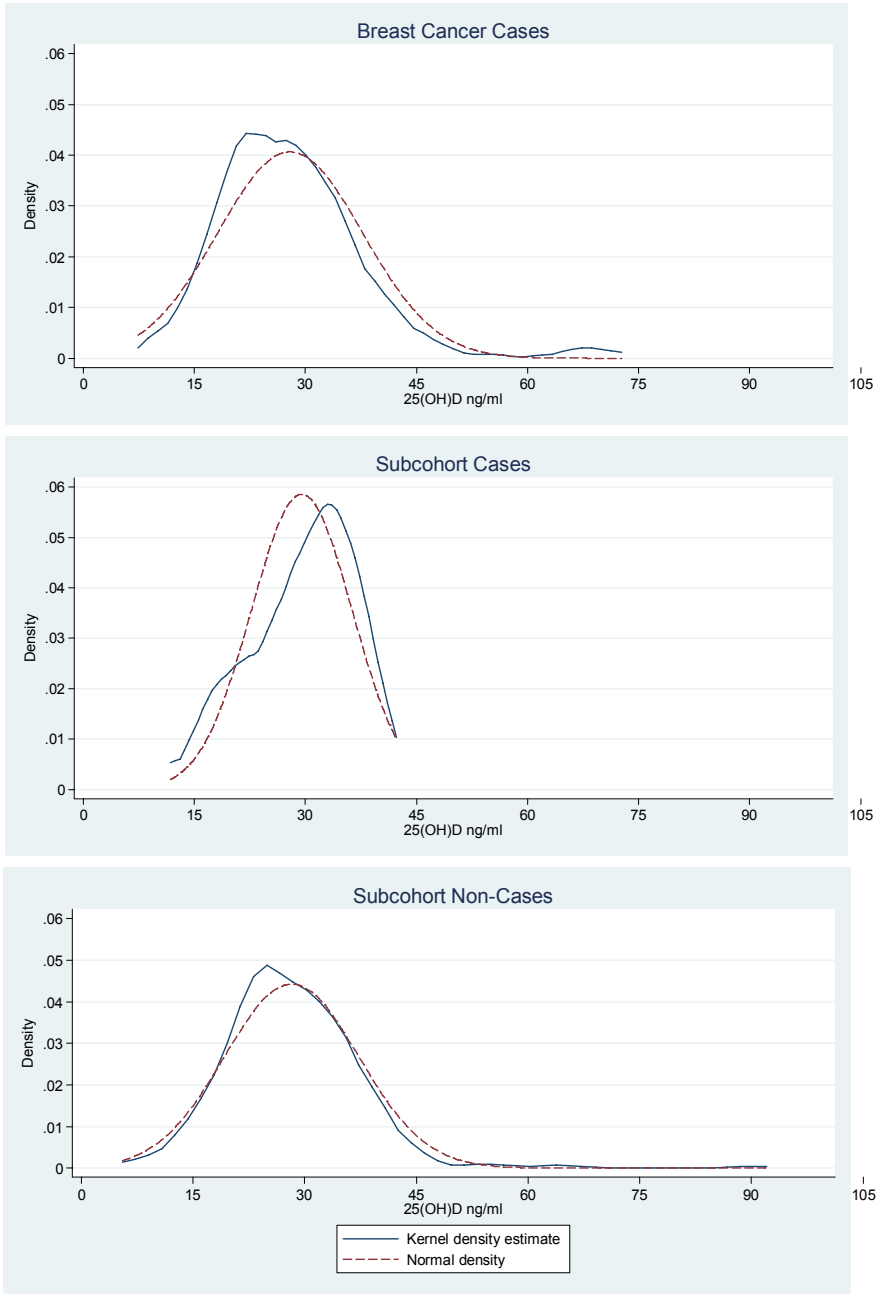

Figure 10. Kernel density plots of serum 25(OH)D by case-cohort status
Breast cancer cases (n=156), subcohort cases (n=14), and subcohort non-cases (n=332).

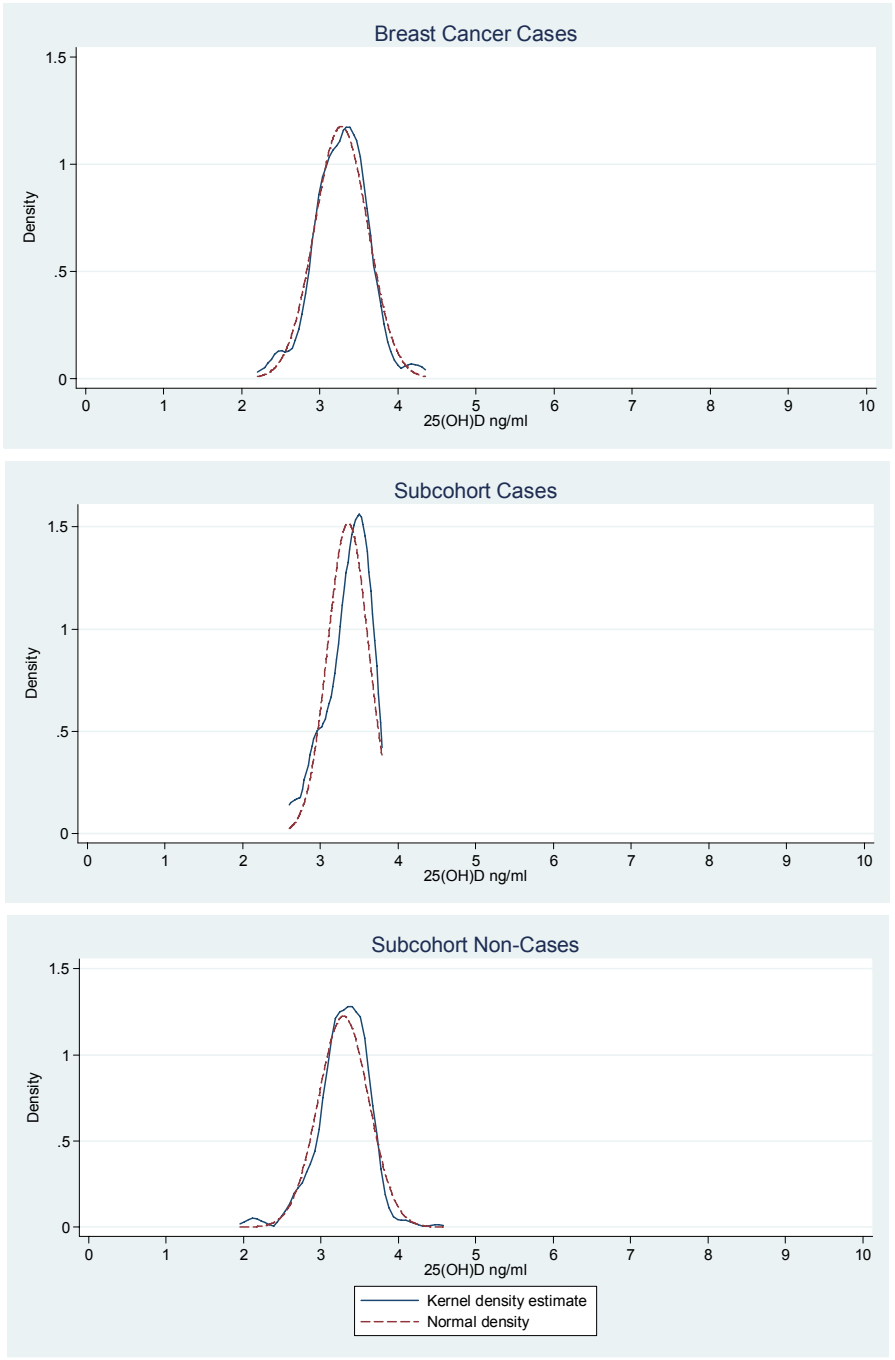

Figure 11. Kernel density plots of log transformed serum 25(OH)D by case-cohort status
Breast cancer cases (n=156), subcohort cases (n=14), and subcohort non-cases (n=332).

A.4 LOWESS SMOOTHER OF 25(OH)D BY AGE AND CASE COHORT STATUS

Figure 12. Serum 25(OH)D distribution by age at baseline and case-cohort status
Breast cancer cases (n=156), subcohort cases (n=14), and subcohort non-cases (n=332).

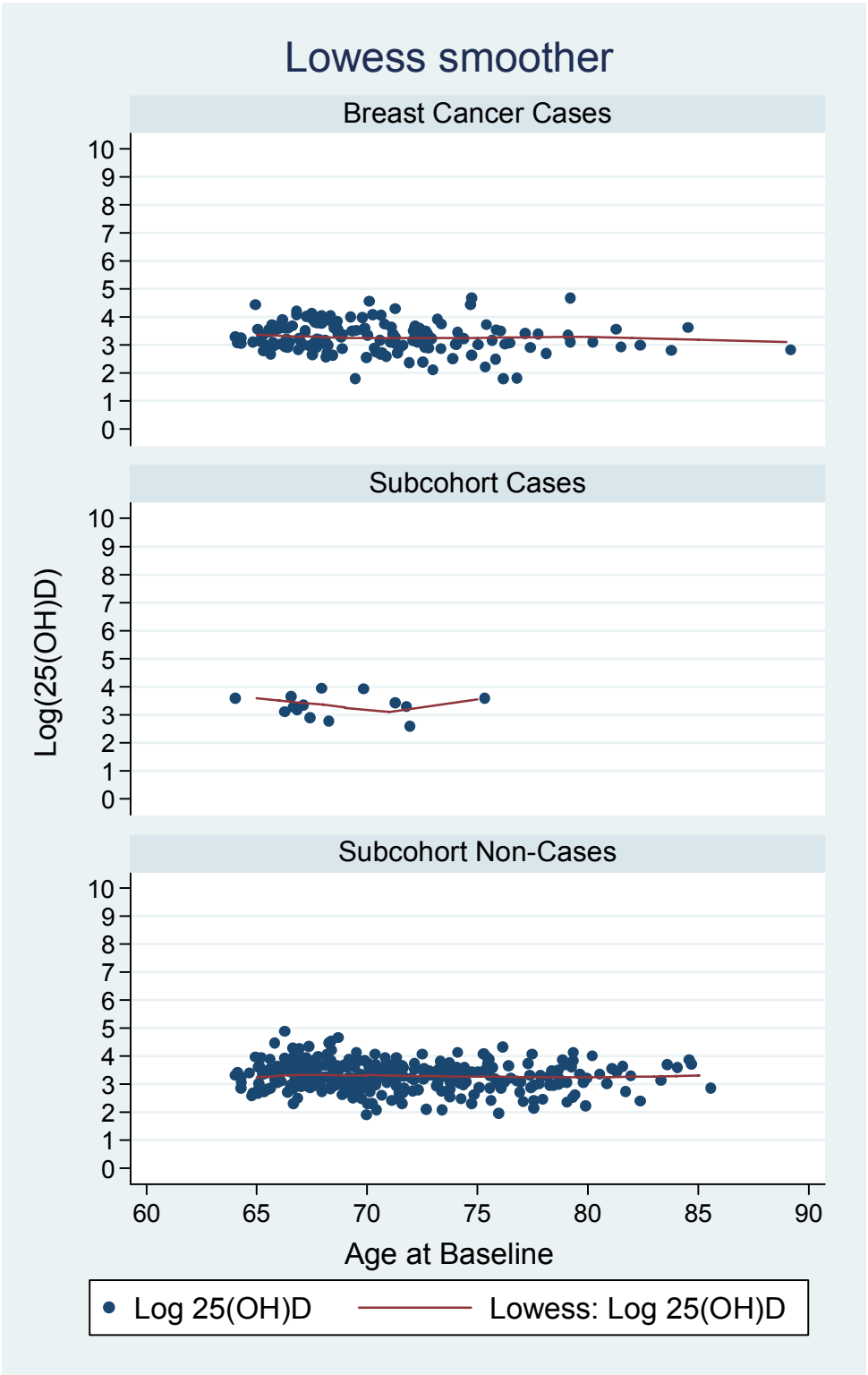

Figure 13. Serum log(25(OH)D) distribution by age at baseline and case-cohort status
Breast cancer cases (n=156), subcohort cases (n=14), subcohort non-cases (n=332).

A.5 LOG 25(OH)D BY AGE, SEASON, SUPPLEMENT USE, BMI AND DISEASE STATUS

Table 19. Median serum log (25(OH)D) by disease status and important study characteristics, Study of Osteoporotic Fractures

	Log Transformed 25(OH)D		
	Median	(IQR)	p-value
Group			
Cases, n=170	3.31	3.1-3.5	0.51†
Subcohort Non-Cases, n=332	3.31	3.1-3.5	
Age*			
65-69, n=147	3.33	3.2-3.5	
70-74, n=105	3.30	3.1-3.5	0.69‡
75-79, n=58	3.28	3.1-3.5	
80+, n=22	3.39	3.0-3.5	
Season*			
Winter, n=64	3.28	3.0-3.6	
Spring, n=79	3.25	3.1-3.4	0.06‡
Summer, n=106	3.36	3.2-3.5	
Fall, n=83	3.32	3.1-3.5	
Vitamin D Supplement Use*			
Current, n=144	3.44	3.3-3.6	<0.01‡
Past, n=35	3.14	3.0-3.5	
Never, n=147	3.20	3.0-3.4	
BMI*, kg/m^2			
< 25, n=164	3.35	3.2-3.6	
25-29, n=112	3.26	3.1-3.5	0.03‡
$\geq$ 30, n=42	3.33	3.1-3.5	

*Comparison is among non-cases only
†Wilcoxon two-sample rank-sum test
‡Kruskal-Wallis test
Abbreviations used: IQR, interquartile range

A.6 COVARIANCE MATRIX OF POTENTIAL MODEL COVARIATES

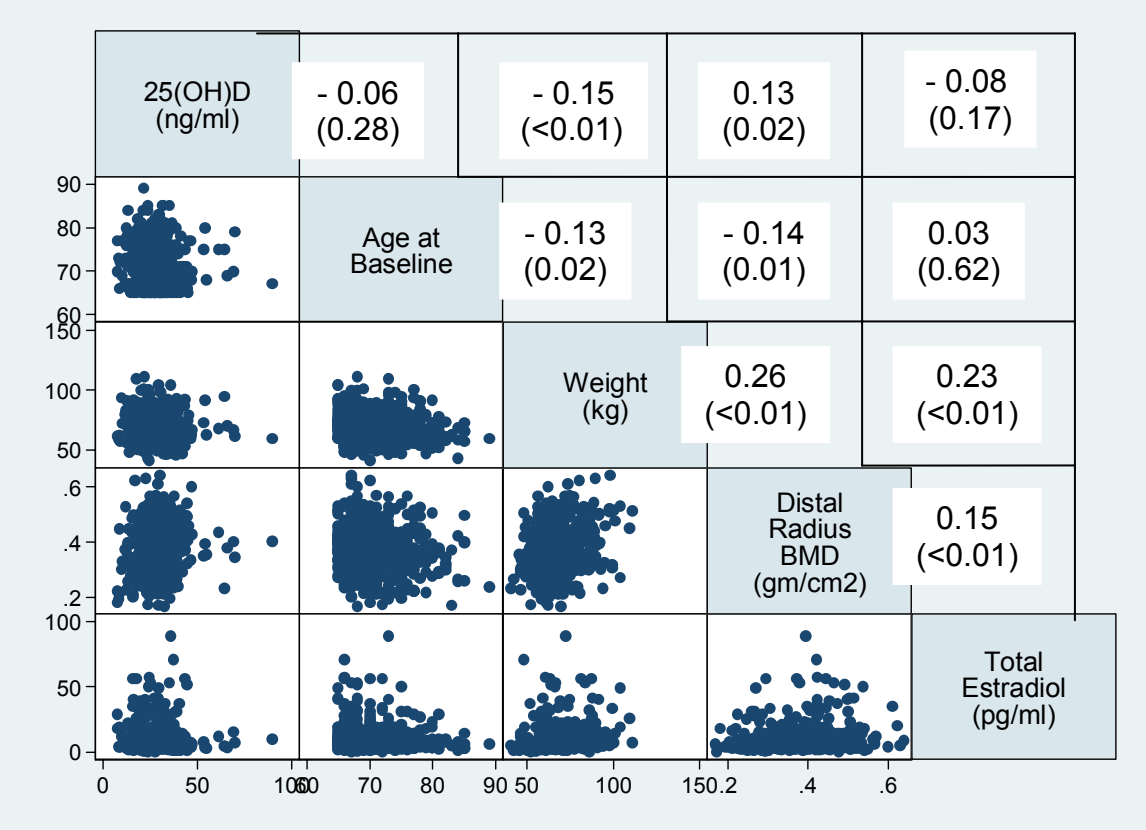

Figure 14. Covariance matrix of continuous model covariates
Spearman rank correlation: rho (p-value).

APPENDIX B

RISK OF BREAST CANCER USING FRACTIONAL CALCIUM ABSORPTION AS A MARKER OF VITAMIN D RESISTANCE

B.1 LOWESS SMOOTHER OF FCA AND AGE BY DISEASE STATUS

Figure 15. Fractional calcium absorption distribution by age and disease status
FCA measure at study baseline (SOF clinic visit 4). Breast cancer cases (n=257), non-cases (n=4778).

B.2 KERNEL DENSITY OF FCA BY CALCIUM INTAKE AND DISEASE STATUS

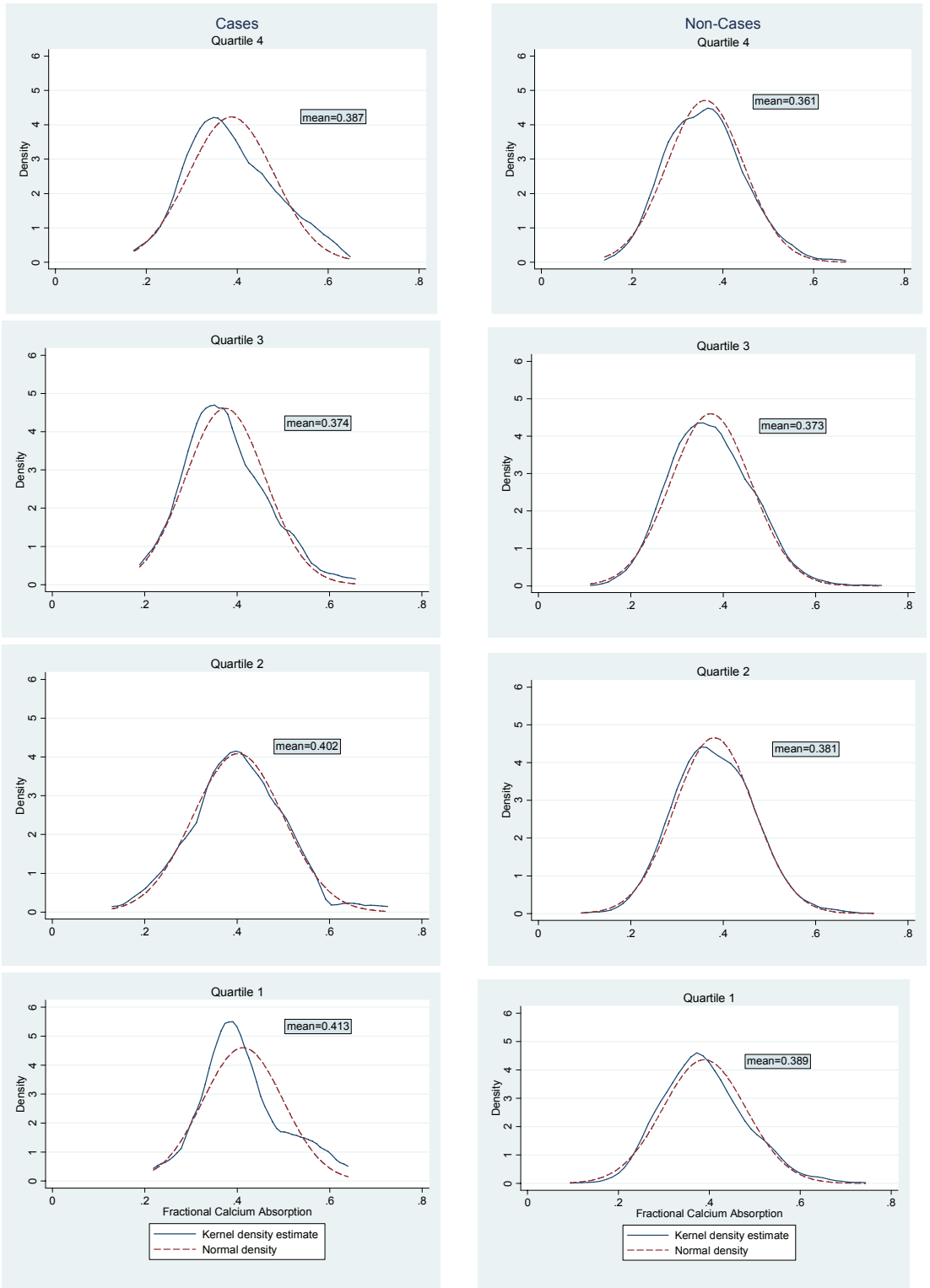

Figure 16. Kernel density plots of FCA distribution for cases and non-cases by quartile of calcium intake

Calcium quartile cutpoints: 25%=455, 50%=775, 75%=1321.

B.3 KAPLAN-MEIER SURVIVAL CURVE

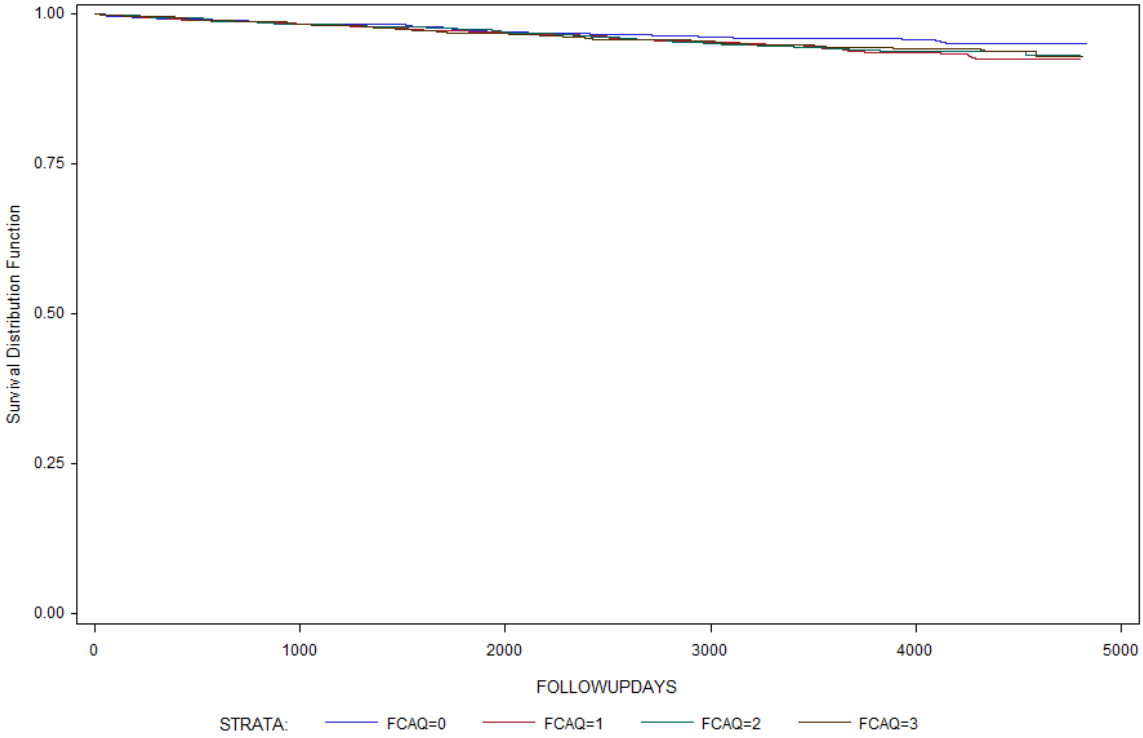

Figure 17. Kaplan-Meier survival curve of time to breast cancer diagnosis by FCA quartile
$P_{logrank}$ = 0.30. Follow-up in days from study baseline (i.e. clinic visit 4).

B.4 CUBIC SPLINE OF FCA

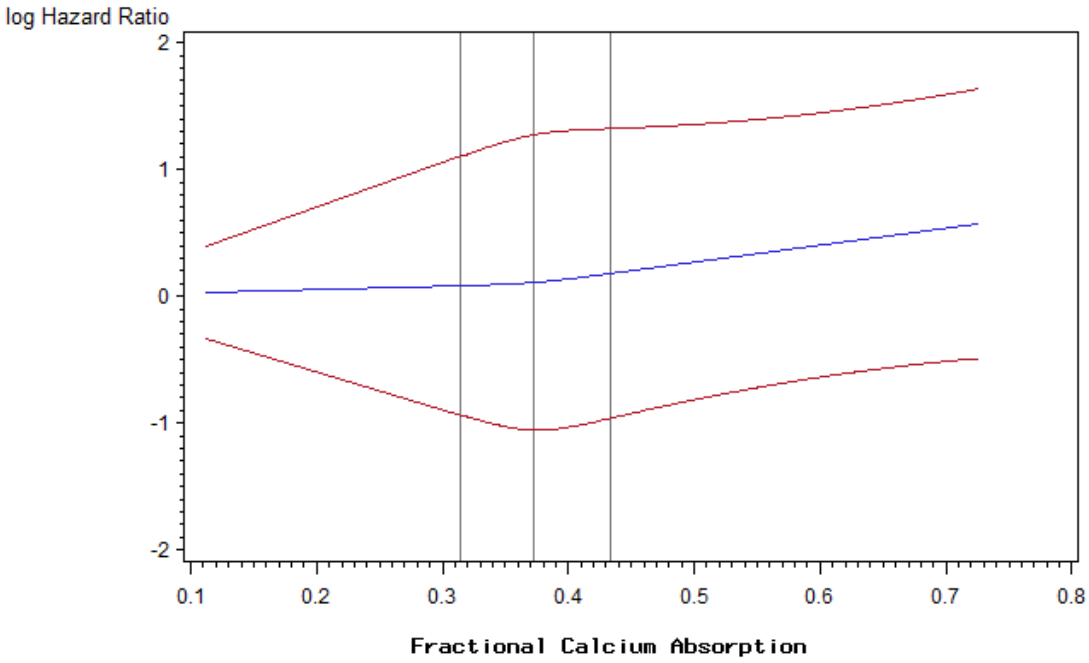

Figure 18. Cubic spline transformation of fractional calcium absorption
Knot placement indicated by horizontal lines at quartile cutpoints (0.31420, 0.37175, 0.43370). Outer bands represent 95% confidence intervals. $P_{linearity}$=0.67.

APPENDIX C

LONG-TERM PREDICTION OF BREAST CANCER RISK IN POSTMENOPAUSAL WOMEN BY BONE MINERAL DENSITY

C.1 CUBIC SPLINES OF BMD

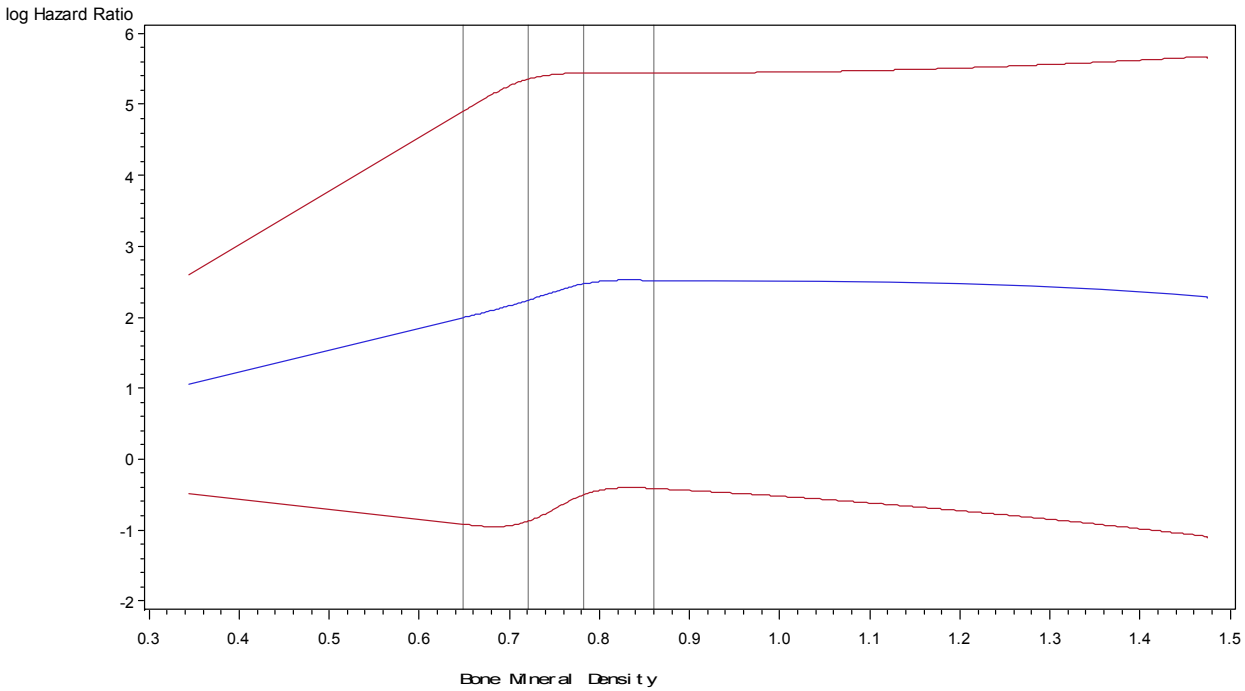

Figure 19. Cubic spline transformation of bone mineral density
Knot placement indicated by horizontal lines at quintile cutpoints (0.648, 0.721, 0.782, 0.860). Outer bands represent 95% confidence intervals. $P_{linearity}$=0.11.

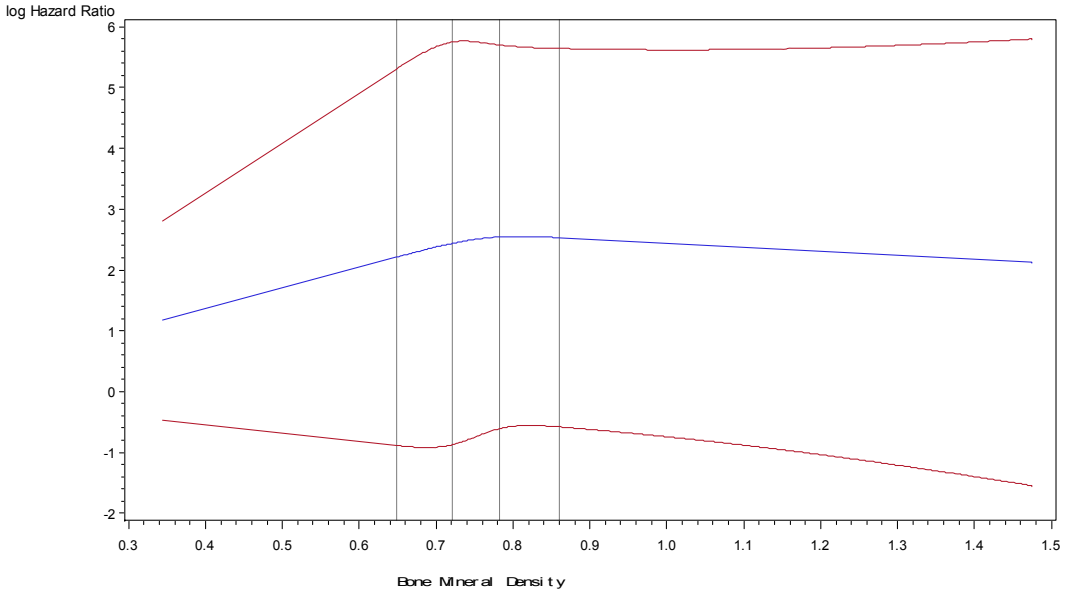

Figure 20. Cubic spline transformation of bone mineral density among women with a negative family history of breast cancer
Knot placement indicated by horizontal lines at quintile cutpoints (0.648, 0.721, 0.782, 0.860). Outer bands represent 95% confidence intervals. $P_{linearity}$=0.20.

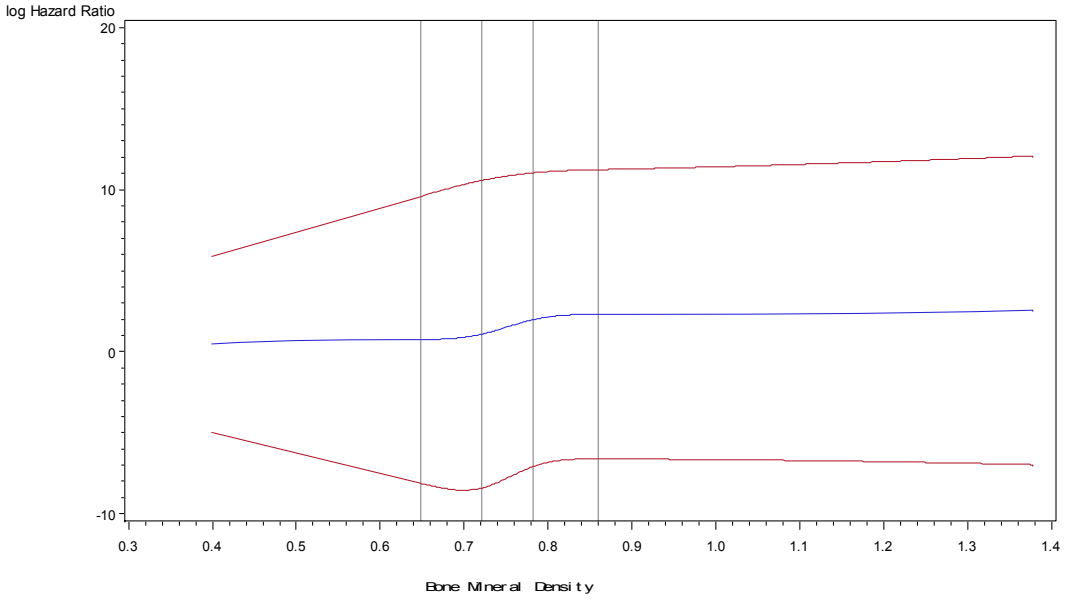

Figure 21. Cubic spline transformation of bone mineral density among women with a positive family history of breast cancer
Knot placement indicated by horizontal lines at quintile cutpoints (0.648, 0.721, 0.782, 0.860). Outer bands represent 95% confidence intervals. $P_{linearity}$=0.17.

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