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Authors
Amir, Eitan
Cecchini, Reena S
Ganz, Patricia A
et al.

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CLINICAL TRIAL

25-Hydroxy vitamin-D, obesity, and associated variables as predictors of breast cancer risk and tamoxifen benefit in NSABP-P1

Eitan Amir · Reena S. Cecchini · Patricia A. Ganz · Joseph P. Costantino · Samantha Beddows · Nicola Hood · Pamela J. Goodwin

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Abstract Observational studies suggest that host factors are associated with breast cancer risk. The influence of obesity, vitamin-D status, insulin resistance, inflammation, and elevated adipocytokines in women at high risk of breast cancer is unknown. The NSABP-P1 trial population was used for a nested case–control study. Cases were drawn from those who developed invasive breast cancer and controls selected from unaffected participants (≤4 per case) matched for age, race, 5 year Gail score, and geographic location of clinical center as a surrogate for latitude. Fasting serum banked at trial enrolment was assayed for 25-hydroxy vitamin-D (25OHD), insulin, leptin (adipocytokine), and C-reactive protein (CRP, marker of inflammation). Logistic regression was used to test for associations between study variables and the risk of invasive breast cancer. Two hundred and thirty-one cases were matched with 856 controls. Mean age was 54, and 49% were premenopausal. There were negative correlations for 25OHD with body mass index (BMI), insulin, CRP, and leptin. BMI $\geq$ 25 kg/m^2 was associated with higher breast cancer risk (odds ratio [OR] 1.45, $p = 0.02$) and tamoxifen treatment was associated with lower risk (OR = 0.44, $p < 0.001$). Suboptimal 25OHD (<72 nmol/l) did not influence breast cancer risk (OR = 1.06, $p = 0.76$). When evaluated as continuous variables, 25OHD, insulin, CRP, and leptin levels were not associated with breast cancer risk (all $p > 0.34$). In this high risk population, higher BMI was associated with a greater breast cancer risk. Serum levels of 25OHD, insulin, CRP, and leptin were not independent predictors of either breast cancer risk or tamoxifen benefit.

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E. Amir · P. J. Goodwin
Division of Medical Oncology and Hematology, Princess Margaret Hospital and University of Toronto, Toronto, ON, Canada

R. S. Cecchini · J. P. Costantino
Biostatistical Center, National Surgical Adjuvant Breast and Bowel Project, Pittsburgh, PA, USA

P. A. Ganz
Los Angeles Schools of Medicine and Public Health, Jonsson Comprehensive Cancer Center, University of California, Los Angeles, CA, USA

J. P. Costantino
Department of Biostatistics, University of Pittsburgh Graduate School of Public Health, Pittsburgh, PA, USA

S. Beddows · N. Hood · P. J. Goodwin (✉)
Department of Medicine, Mount Sinai Hospital and University of Toronto, 1284-600 University Ave, Toronto, ON M5G 1X5, Canada
e-mail: pgoodwin@mtsinai.on.ca

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Introduction

Numerous risk factors for the development of breast cancer have been identified and quantified [4]. However, up to 60% of breast cancers arise in the absence of known risk factors [35]. Furthermore, established risk factors do not always account for all the attributable risk [4]. Therefore, there is a need to identify and validate new risk factors in women, regardless of the presence of recognized risk factors. Women could then be better advised on their individual risk and the need for risk-reducing strategies.

A considerable body of literature has examined the inverse association between blood levels of vitamin-D and overall cancer risk as well as the potential role of vitamin-D in cancer prevention [24, 28]. The greatest magnitude of association of breast cancer risk and vitamin-D comes from geographic studies which show higher incidence in patients residing at high latitudes [12]. Unfortunately, such studies do not provide direct evidence of an association of vitamin-D with breast cancer risk. Other data linking lower blood levels of vitamin-D to breast cancer risk are inconsistent [41] (Table 1).

A number of other host-related factors such as obesity and diet have also been postulated as breast cancer risks [26, 31]. The association of obesity and the development of breast cancer in post-menopausal women is relatively strong [27]; a weaker association of central obesity with the development of breast cancer exists in pre-menopausal women [27]. The mechanisms underlying this risk remain unclear. Possible explanations are elevated estrogen levels, insulin resistance with consequential hyperinsulinemia, and higher levels of insulin-like-growth-factor (IGF) [42]. Adipose tissue may directly influence tumor growth or differentiation by secretion of adipose tissue-derived hormones called adipocytokines, including adiponectin and leptin [30].

Many risk factors for breast cancer are inter-related. For instance, obesity and vitamin-D deficiency are associated

Table 1 Studies assessing blood levels of vitamin-D metabolites and breast cancer risk

Study	Study design	Number of cases/ controls	Comparison	OR or RR p value
Blood collected before diagnosis of breast cancer				
Bertone-Johnson [5]	Nested case–control (Nurses' health study)	701/724	Plasma 25-hydroxy vitamin-D Quintile 1 vs. 5	1.37 $p = 0.06$
Freedman [22]	Nested case–control (prostate, lung, colorectal and ovarian cancer screening trial)	1005/1005	Serum 25-hydroxy vitamin-D Quintile 1 vs. 5	0.96 $p = 0.81$
Chlebowski [8]	Nested case–control (Women's health initiative)	1067/1067	Plasma 25-hydroxy vitamin-D Quintile 1 vs. 5	1.22 $p = 0.20$*
McCullough [37]	Nested case–control (Cancer prevention study II)	516/516	Serum 25-hydroxy vitamin-D Quintile 1 vs. 5	0.92 $p = 0.60$*
Engel [17]	Nested case–control (French E3 N cohort)	636/1272	Serum 25-hydroxy vitamin-D <19.8 vs. >27 ng/ml	1.37 $p = 0.02$*
Almquist [3]	Nested case–control (Malmö diet and cancer study)	764/764	Serum 25-hydroxy vitamin-D Quartile 1 vs. 4	1.08 $p = 0.71$*
Veldhuis [48]	Cross-sectional study (Osteoporosis and fracture clinic)	56/829	Serum 25-hydroxy vitamin-D <50 vs. ≥50 nmol/l	1.43 $p = 0.18$
Eliassen [16]	Nested case–control (Nurses health study II)	613/1218	Serum 25-hydroxy vitamin-D Quartile 1 vs. 4	1.20 $p = 0.32$
Blood collected after diagnosis of breast cancer				
Lowe [34]	Hospital-based case–control (UK)	179/170	Plasma 25-hydroxy vitamin-D <50 vs. >150 nmol/l	5.83 $p < 0.001$
Abbas [1]	Population-based case–control (Pre-menopusal women, Germany)	289/595	Plasma 25-hydroxy vitamin-D <30 nmol/l vs. ≥60 nmol/l	2.22 $p < 0.001$*
Abbas [2]	Population-based case–control (Post-menopusal women, Germany)	1394/1365	Serum 25-hydroxy vitamin-D <30 vs. ≥75 nmol/l	3.23 $p < 0.001$*
Crew [11]	Population-based case–control (Long Island, NY)	1026/1075	Plasma 25-hydroxy vitamin-D <20 vs. >40 ng/ml	1.79 $p = 0.002$*
Rejnmark [40]	Nested case–control (Denmark)	142/420	Serum 25-hydroxy vitamin-D Tertile 1 vs. 3	1.92 $p < 0.05$
Yao [51]	Hospital-based case–control (USA)	220/156	Serum 25-hydroxy vitamin-D <20 vs. ≥30 ng/ml	2.70 $p < 0.001$*

OR odds ratio, *RR* risk ratio, *NS* not significant

* Statistical test for trend

with insulin resistance, inflammation, and elevated adipocytokines [25, 44, 47]. Cigarette smoking is associated with insulin resistance [21] as well as increased inflammation [23].

The primary aim of this study was to explore circulating 25-hydroxy vitamin-D, an indicator of vitamin-D status, as a predictor of breast cancer risk after adjustment for potential confounding baseline factors. Secondary aims included assessment of the relationships between obesity, 25-hydroxy vitamin-D levels, insulin resistance, C-reactive protein (CRP, a marker of inflammation), and leptin (an adipocytokine), and to evaluate whether these factors were associated with differential benefit from chemoprevention in a prospective cohort of patients enrolled in the National Surgical Adjuvant Breast and Bowel Project Protocol (NSABP) P1. We hypothesized that 25-hydroxy vitamin-D levels would be inversely related to breast cancer risk in women receiving both tamoxifen and placebo, that the effect of tamoxifen would be modified by 25-hydroxy vitamin-D levels, and that associations of 25-hydroxy vitamin-D levels with breast cancer risk would be independent of the effects of insulin, adipocytokines, and inflammatory markers.

Materials and methods

Study population

A case–control study design nested in the NSABP-P1 trial population was performed. Between 1992 and 1997, the NSABP-P1 trial randomized 13,388 women, age 35 years or older and at increased risk for breast cancer [Gail model 5 year score $\geq$ 1.66% or history of lobular carcinoma in situ (LCIS)] to 5 years of tamoxifen (20 mg daily) or matched placebo [19]. Prior to randomization, all participants provided fasting blood specimens which were processed into 1-ml aliquots and frozen at −80°C. The trial was stopped early after a median follow-up of 54.6 months as the data-monitoring committee determined that the 49% relative reduction in risk of invasive breast cancer had met pre-specified stopping rules. At that time, treatment allocation was unblinded and placebo participants were offered tamoxifen or the opportunity to participate in a subsequent chemoprevention trial. A total of 270 participants developed invasive breast cancer before the study was unblinded.

Cases included participants who developed invasive breast cancer before unblinding, had sufficient quantity and quality of stored blood samples, and provided additional consent for the use of these materials for further research. Controls included participants who did not develop invasive or non-invasive breast cancer during the course of follow-up before unblinding and were matched for age (±5 years), 5 year Gail score ($\leq$2.24%, 2.25–3.48%, $\geq$3.49%), race, and participant's clinical center as a surrogate for latitude of residence. Up to four matched controls for each invasive breast cancer were selected. The study was approved by the institutional ethics boards of both Mount Sinai Toronto and NSABP.

Laboratory assays

Frozen aliquots of serum measuring 1 ml were obtained from the NSABP biospecimen repository. Specimens were thawed and then analyzed for 25-hydroxy vitamin-D, insulin, and CRP on a single run. Testing for leptin was carried out on a separate run. 25-Hydroxy vitamin-D was assayed by the Liaison 25-hydroxy vitamin-D total assay chemiluminescent immunoassay (Diasorin Inc, Mississauga, ON, intra- and inter-assay co-efficient of variability (CV) were 6.7% and 11.7–18.4%, respectively). The range of detection for this platform was 10–375 nmol/l. Insulin was assayed by the Roche Diagnostics electrochemiluminescence immunoassay (Roche Diagnostics Canada Inc, Laval, QC, intra- and inter-assay CV were both <3.7%). This assay has an analyzable range of 1.39–6,945 pmol/l. CRP was assayed by the Roche Diagnostics particle-enhanced immunoturbidimetric assay (Roche Diagnostics Canada Inc, Laval, QC, intra and inter-assay CV were 1.8% and 5.2–5.7%, respectively) which can detect levels between 0.1 and 20 mg/l. Finally, leptin was measured by the Linco sandwich enzyme linked immunosorbent assay (Millipore Inc, Billerica, MA, intra- and inter-assay CV were 3.8 and 4.4%, respectively). This platform had a range of detection between 0.5 and 100 ng/ml.

Review and meta-analysis

In order to place the results of this study in context, our data were pooled together with other published data exploring 25-hydroxy vitamin-D and breast cancer risk. MEDLINE (Host: PubMed) was searched and a systematic review of the literature was carried out and trials reporting association between breast cancer and serum levels of 25-hydroxy vitamin-D were included. There were two pre-planned cohorts for this meta-analysis: (1) studies where blood was collected after the diagnosis of breast cancer and (2) studies where blood was collected before the diagnosis of breast cancer. Odds ratios (ORs) were extracted from individual studies, weighted using the generic inverse variance approach, and pooled using the DerSimonian and Laird random-effects method [15].

Statistical analysis

Spearman's rho was used to assess the correlation of 25-hydroxy vitamin-D levels with BMI, insulin, CRP, and leptin levels, and to assess the correlation between 25-hydroxy vitamin-D levels and the latitude of clinical center. The magnitude of correlation was assessed as described by Burnand et al. [6]. The distribution of cases by participant and tumor characteristics was determined and differences between the distributions for those with 25-hydroxy vitamin-D levels <72 nmol/l and those with levels ≥72 nmol/l were compared using the χ^2 test. This prior selected value for optimal blood levels of 25-hydroxy vitamin-D was based on the best available data at study initiation [13, 29]. It pre-dated the Institute of Medicine report suggesting a cut-off of 50 nmol/l [43]. In view of the inconsistent data regarding optimal cut-offs for optimal 25-hydroxy vitamin-D levels, in initial analyses, 25-hydroxy vitamin-D levels were evaluated as a dichotomized parameter cut at ≥72 nmol/l. Analyses were then repeated using log-transformed 25-hydroxy vitamin-D levels as a continuous variable. The association between serum 25-hydroxy vitamin-D levels and the risk of developing invasive breast cancer was evaluated using conditional logistic regression. The nature of the association was evaluated, initially in the univariable setting, and then in the multivariable setting with adjustment for potential confounding baseline factors including tamoxifen treatment, BMI, history of osteoporosis, cigarette smoking, and exogenous hormone use. Interaction between tamoxifen treatment and levels of serum markers was also assessed during multivariable modeling. The independent association with breast cancer risk was also evaluated for baseline serum levels of insulin, CRP, and leptin, all assessed as log-transformed continuous variables. When using continuous variables, ORs compared the midpoint of the upper quartile to the midpoint of the lower quartile. Statistical significance of parameters included in the regression models was assessed using the likelihood ratio test. Statistical significance of all testing was based on a two-sided test using an alpha level of 0.05.

Results

Data were available for 231 case participants (Fig. 1). Four matched controls were obtained for 196 of the cases (84.8%), three matched controls for 12 cases (5.2%), two matched controls for 13 cases (5.6%), and for ten cases (4.3%) only one matched control was obtained. Thus, the total study population comprised 1,087 participants (231 cases and 856 matched controls). The mean age was 53.6 years (standard deviation [SD] 8.7); mean BMI was 27.2 kg/m^2 (SD 5.7); and mean Gail model 5 year risk was 4.08% (SD 2.72). Forty-nine percent were pre-menopausal. Demographic factors for cases and controls and tumor characteristics for cases are shown in Table 2. Other than treatment, the only factor that showed a statistically significant difference between cases and controls was BMI. The percent of participants demonstrating sufficient levels (≥72 nmol/l) of 25-hydroxy vitamin-D levels was 24.2% among the cases and 27.8% among the controls.

Descriptive analysis of serum variables and correlations between variables are shown in Table 3 and correlations between BMI and serum variables are shown in Fig. 2. There was no correlation between 25-hydroxy vitamin-D levels and latitude of the participant's clinical center (rho = −0.02; $p = 0.45$). There were weak positive correlations between age and insulin, CRP and leptin levels, and a weak negative correlation with 25-hydroxy vitamin-D. The rho for age and insulin, CRP, leptin, and 25-hydroxy vitamin-D were 0.15, 0.20, 0.12, and −0.11, respectively (Supplementary Figure).

The results of univariable and multivariable conditional logistic regression modeling of the risk of developing invasive breast cancer when using 25-hydroxy vitamin-D as a dichotomous variable are shown in Table 4. The univariable OR for suboptimal vitamin-D status (serum 25-hydroxy vitamin-D <72 nmol/l) was 1.25, 95% confidence intervals (CI) were 0.88–1.77, ($p = 0.21$; Table 4-Model 1). When adjusting for tamoxifen treatment and BMI (Table 4-Model 3), the OR for the effect of suboptimal 25-hydroxy vitamin-D decreased to 1.06 (95% CI 0.73–1.53, $p = 0.76$). In this model, tamoxifen treatment showed a 56% reduction in the odds of invasive breast cancer (OR = 0.44, 95% CI 0.32–0.61, $p < 0.001$); and the OR for BMI ≥ 25 kg/m^2 was 1.45 (95% CI 1.06–2.00, $p = 0.02$). When BMI was assessed as three discrete categories (<25.0, 25.0–29.9, and ≥30.0 kg/m^2) in this multivariable model, there was little change to the effect of suboptimal 25-hydroxy vitamin-D (OR = 1.07, $p = 0.73$, Table 4-Model 4).

In univariable analysis as a continuous variable, 25-hydroxy vitamin-D again did not show a statistically significant association with invasive breast cancer (OR for upper versus lower quartile = 0.77, 95% CI 0.55–1.06, $p = 0.11$; Table 5, first row). When assessed as a continuous variable and adjusted for treatment and BMI (Table 5, fourth from bottom row), 25-hydroxy vitamin-D, did not show a statistically significant association with invasive breast cancer (OR for upper versus lower quartile = 0.86, 95% CI 0.62–1.21, $p = 0.40$).

There was no evidence of interaction between tamoxifen treatment and any serum markers. The p value for interaction between tamoxifen treatment and 25-hydroxy vitamin-D, insulin, CRP, and leptin were 0.52, 0.49, 0.83, and 0.68, respectively.

History of osteoporosis, cigarette smoking, and exogenous hormone use (oral contraceptive pill or hormone replacement therapy) were not associated with statistically

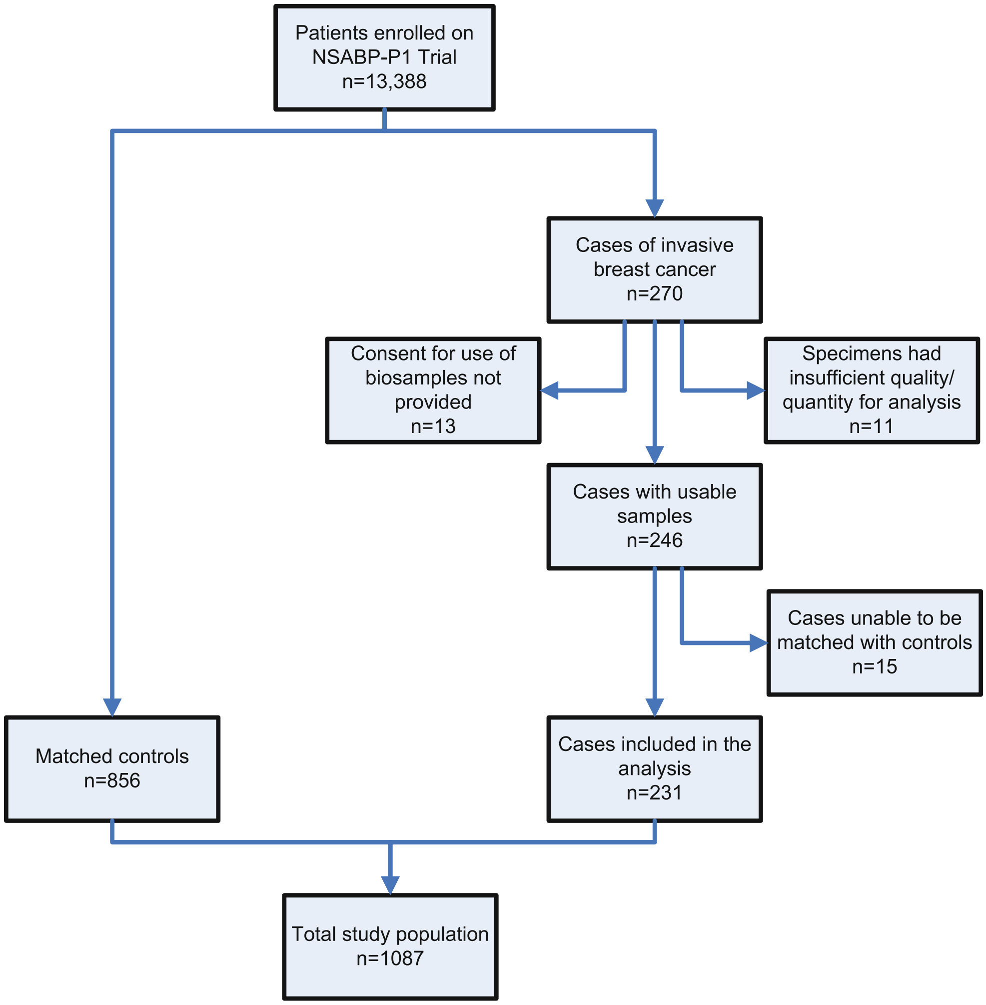

Fig. 1 CONSORT diagram

increased odds of invasive breast cancer. The univariable OR for history of osteoporosis was 1.42 (95% CI 0.74–2.76, $p = 0.30$). The univariable OR for smoking (upper quartile [≥28 years] versus never smoking) was 1.12 (95% CI 0.74–1.70, $p = 0.35$), and for exogenous hormone use (ever versus never) was 0.90 (95% CI 0.64–1.27, $p = 0.54$). History of osteoporosis, smoking, and hormone use remained non-significant when included in the multivariable model with suboptimal 25-hydroxy vitamin-D, tamoxifen treatment, and BMI (≥25.0 vs <25.0 kg/m^2). The OR for history of osteoporosis was 1.28 (95% CI 0.65–2.53, $p = 0.48$), the OR for smoking for at least 28 years compared with never smoking was 1.15 (95% CI 0.75–1.76, $p = 0.46$), and the OR for prior hormone use was 0.89 (95% CI 0.63–1.26, $p = 0.51$).

Menopausal status did not influence breast cancer risk in either univariable or multivariable analyses (Supplementary Table). The adjusted OR for post-menopause was 0.90 (95% CI 0.51–1.58, $p = 0.70$, Supplemental Table-Model 3). There was also no significant interaction between BMI and menopausal status (Supplemental Table-Model 4, $p = 0.32$).

In univariable analyses and after adjusting for tamoxifen treatment and BMI in multivariable modeling, plasma levels of insulin, CRP, and leptin did not show statistically significant effects on development of invasive breast cancer (Table 5, last three rows).

Meta-analysis of published studies assessing the association of blood levels of 25-hydroxy vitamin-D and breast cancer showed variability. In all six studies where blood levels of 25-hydroxy vitamin-D were measured after diagnosis of breast cancer, there was a significant inverse association between 25-hydroxy vitamin-D and breast cancer. Pooled data showed a highly significant OR of 2.49 (95% CI 1.93–3.21, $p < 0.001$). Among studies, where levels were measured before breast cancer diagnosis, only one out of nine studies showed a significant association between levels of vitamin-D metabolites and breast cancer and pooled data showed only limited association (OR = 1.10, 95% CI 1.00–1.20, $p = 0.04$, Table 1;

Table 2 Participant and tumor characteristics among cases and controls

Participant or tumor characteristic	Total	Cases		Controls		p*
		N	%	*N*	%	
Age (years)						
≤49	470	96	41.6	374	43.7	0.84
50–59	299	66	28.6	233	27.2	
≥60	318	69	29.9	249	29.1	
Treatment						
Placebo	605	164	71.0	441	51.5	<.0001
Tamoxifen	482	67	29.0	415	48.5	
5-year predicted breast cancer risk (%)						
≤2.24	300	64	27.7	236	27.6	0.99
2.25–3.48	293	63	27.3	230	26.9	
≥3.49	494	104	45.0	390	45.6	
Body mass index						
<25.0	444	79	34.2	365	42.6	0.02
≥25.0	643	152	65.8	491	57.4	
Smoking history (years)						
None	592	117	50.6	475	55.5	0.33**
<18	173	35	15.2	138	16.1	
18–27	143	39	16.9	104	12.1	
≥28	175	39	16.9	136	15.9	
Unknown	4	1	0.4	3	0.4	
History of hormone use						
No	273	60	26.0	213	24.9	0.73
Yes	814	171	74.0	643	75.1	
No. 1° relatives with breast cancer						
0	220	47	20.3	173	20.2	0.41
1	605	121	52.4	484	56.5	
≥2	262	63	27.3	199	23.2	
Menopausal status						
Premenopausal	530	113	48.9	417	48.7	0.73
Postmenopausal	493	102	44.2	391	45.7	
Unknown	64	16	6.9	48	5.6	
History of breast atypical hyperplasia						
No	986	204	88.3	782	91.4	0.16
Yes	101	27	11.7	74	8.6	
History of osteoporosis						
No	1034	216	93.5	818	95.6	0.20
Yes	53	15	6.5	38	4.4	
25-Hydroxy vitamin-D concentration						
<72 nmol/l	793	175	75.8	618	72.2	0.28
≥72 nmol/l	294	56	24.2	238	27.8	
Type of invasive cancer						
Infiltrating duct carcinoma	171	171	74.0	–	–	–
Other	60	60	26.0	–	–	
Estrogen receptor status						
Negative	56	56	24.2	–	–	–
Positive	158	158	68.4	–	–	
Unknown	17	17	7.4	–	–	

Table 2 continued

Participant or tumor characteristic	Total	Cases		Controls		p*
		N	%	N	%	
Progesterone receptor status						
Negative	85	85	36.8	–	–	–
Positive	123	123	53.2	–	–	
Unknown	23	23	10.0	–	–	
Presenting cancer stage						
I	131	131	56.7	–	–	–
II	76	76	32.9	–	–	
III–IV	14	14	6.1	–	–	
Unknown	10	10	4.3	–	–	

* p values are from χ^2 test unless otherwise specified

** Fisher's exact test

Table 3 Descriptive analysis of and correlations between serum variables

Variable	Mean	Standard deviation	Spearman's correlation rho*				
			25-Hydroxy vitamin-D	Insulin	CRP	Leptin	BMI
25-Hydroxy vitamin-D (nmol/l)	57.9	25.3	–	−0.23	−0.15	−0.22	−0.22
Insulin (pmol/l)	53.4	50.0	−0.23	–	0.44	0.66	0.58
CRP (mg/l)	3.5	9.7	−0.15	0.44	–	0.53	0.54
Leptin (μmol/l)	29.6	21.7	−0.22	0.66	0.53	–	0.79
BMI (kg/m^2)	27.2	5.7	−0.22	0.58	0.54	0.79	–

* All correlations statistically significant at $p < 0.001$

Fig. 3). There was a statistically significant difference between the two analysis subgroups ($p < 0.001$).

Discussion

Recent research has focused on the identification and validation of new risk factors predictive of breast cancer occurrence. There has been substantial interest in host factors such as obesity, vitamin-D status, adipocytokines, and inflammation and immune function. 25-Hydroxy vitamin-D concentrations have been related to factors associated with increased risk of cancer including obesity, circulating leptin levels [36], inflammatory markers [54], and insulin resistance [20]. It has been hypothesized that vitamin-D deficiency may explain increased cancer rates [33]. However, a recent report from the Institute of Medicine suggested that there are limited data supporting the association of vitamin-D status with cancer in general [43]. It remains unclear if any of the above-mentioned host factors are independent predictors of breast cancer risk.

This study, conducted in a population of women at high risk of breast cancer has shown that higher BMI was associated with increased breast cancer risk. There was a very weak, but statistically significant, negative correlation of BMI with 25-hydroxy vitamin-D and moderate to strong and significant positive correlations with insulin, CRP and leptin levels. However, when adjusting for baseline BMI, serum levels of 25-hydroxy vitamin-D, insulin, CRP and leptin were not significantly associated with breast cancer. It is unlikely that using different cut-offs for optimal 25-hydroxy vitamin-D would influence results as when assessed as a continuous variable, 25-hydroxy vitamin-D was also not seen to be associated with breast cancer risk. Of interest, there was no evidence of interaction between these factors and treatment. This suggests that these factors are not independent predictors of breast cancer risk or tamoxifen benefit in this high risk population.

The negative correlation between 25-hydroxy vitamin-D and BMI has been described previously [38], and may be explained by a volume-distribution effect with lower bioavailability of fat-soluble vitamin-D metabolites in overweight and obese individuals with excess adipose tissue [49].

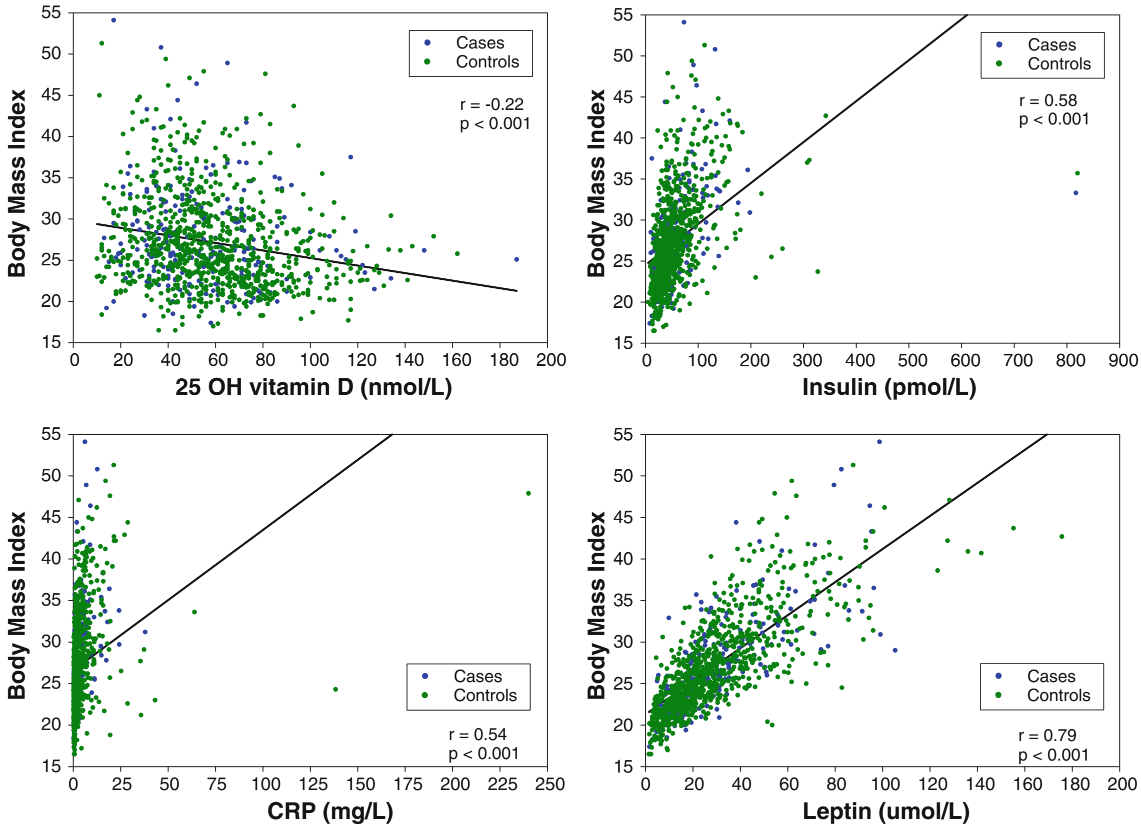

Fig. 2 Correlations between BMI and 25-hydroxy vitamin-D, insulin, CRP, and leptin

Positive correlations with insulin, leptin, and CRP have also been reported previously and may relate to the cytokine milieu associated with the metabolic syndrome [50]. No correlation was seen between 25-hydroxy vitamin-D levels and latitude of clinical center. This may be explained by the clinical center only being a rough surrogate for latitude of residence. Furthermore, no data were available on vitamin-D supplementation and this may confound this geographic analysis. However, a true absence of effect of latitude on 25-hydroxy vitamin-D levels in this population cannot be excluded.

A number of factors may explain the inconsistent association of blood levels of vitamin-D metabolites and breast cancer. First, it is possible that findings are compromised by reverse causation bias. Our meta-analysis has shown that significant association between vitamin-D metabolites and breast cancer were predominantly seen in studies where blood levels were collected after breast cancer diagnosis. This cohort was significantly different from the group where blood levels were collected before breast cancer diagnosis where only limited association between breast cancer and vitamin-D metabolite levels was seen. Similar results have been reported in other pooled analyses [7, 9, 52]. Analyses of blood drawn after breast cancer diagnosis may be prone to error as the blood parameter may be affected by the presence of breast cancer. Breast cancer cells have been shown to possess vitamin-D catalytic enzymes [18, 45] and these may interfere with standard 25-hydroxy vitamin-D assay techniques. Second, patients diagnosed with breast cancer may modify their lifestyle (such as diet, dietary supplementation, physical activity, and/or sun exposure), and this may lead to post-diagnosis changes in vitamin-D metabolite levels. Finally, the duration of follow-up for patients included in this analysis was less than 55 months. It is possible that this duration was not sufficiently long to observe the effects of blood levels of vitamin-D and cancer risk.

Prophylactic therapy with tamoxifen was protective of breast cancer with an OR of 0.44. This was consistent with the whole NSABP-P1 population suggesting balanced sampling of cases and controls. Consistent with prior data [31], higher BMI was shown to lead to increased breast cancer. Overweight and obese patients (BMI > 25 kg/m^2) had a statistically significant relative increase in the odds of breast cancer of 45%. A number of mechanisms have been suggested for this association. It has been proposed that obesity is associated with insulin resistance and hyperinsulinemia [42]. Our data suggest that the level of insulin does not influence cancer risk in this high risk population, and therefore implies that other, perhaps unknown

Table 4 Univariable and multivariable analysis of the association of 25-hydroxy vitamin-D with invasive breast cancer

Model number	Variables in model	Odds ratio	95% CI for odds ratio	*p* value
Model 1	25-Hydroxy vitamin-D			
	<72 nmol/l	1.25	0.88–1.77	0.21
	≥72 nmol/l	1.00		
Model 2	25-Hydroxy vitamin-D			
	<72 nmol/l	1.14	0.79–1.63	0.48
	≥72 nmol/l	1.00		
	Treatment			
	Placebo	1.00		
	Tamoxifen	0.45	0.33–0.61	<0.001
Model 3	25-Hydroxy vitamin-D			
	<72 nmol/l	1.06	0.73–1.53	0.76
	≥72 nmol/l	1.00		
	Treatment			
	Placebo	1.00		
	Tamoxifen	0.44	0.32–0.61	<0.001
	Body mass index			
	<25.0	1.00		
	≥25.0	1.45	1.06–2.00	0.02
Model 4	25-Hydroxy vitamin-D			
	<72 nmol/l	1.07	0.74–1.54	0.73
	≥72 nmol/l	1.00		
	Treatment			
	Placebo	1.00		
	Tamoxifen	0.44	0.32–0.61	<0.001
	Body mass index			
	<25.0	1.00		
	25.0–29.9	1.51	1.06–2.15	
	≥30.0	1.38	0.93–2.03	0.06

Table 5 Univariable and multivariable analyses of the association of log transformed plasma levels of 25-hydroxy vitamin-D, insulin, CRP, and leptin with invasive breast cancer

Adjustment variables	Variable assessed	Comparison*	Odds ratio	95% CI	*p*
None	25-Hydroxy vitamin-D (nmol/l)	86 vs. 31	0.77	0.55–1.06	0.11
	Insulin (pmol/l)	87 vs. 22	1.04	0.76–1.44	0.79
	CRP (mg/l)	6.5 vs. 0.4	1.05	0.85–1.30	0.64
	Leptin (μmol/l)	53.1 vs. 9.8	1.38	0.99–1.93	0.052
Treatment	25-Hydroxy vitamin-D (nmol/l)	86 vs. 31	0.81	0.58–1.13	0.23
	Insulin (pmol/l)	87 vs. 22	1.03	0.75–1.43	0.84
	CRP (mg/l)	6.5 vs. 0.4	1.07	0.86–1.33	0.53
	Leptin (μmol/l)	53.1 vs. 9.8	1.37	0.98–1.92	0.07
Treatment and BMI	25-Hydroxy vitamin-D (nmol/l)	86 vs. 31	0.86	0.62–1.21	0.40
	Insulin (pmol/l)	87 vs. 22	0.84	0.58–1.21	0.34
	CRP (mg/l)	6.5 vs. 0.4	0.95	0.75–1.21	0.68
	Leptin (μmol/l)	53.1 vs. 9.8	1.09	0.71–1.68	0.70

* Midpoint of the upper quartile to the midpoint of the lower quartile

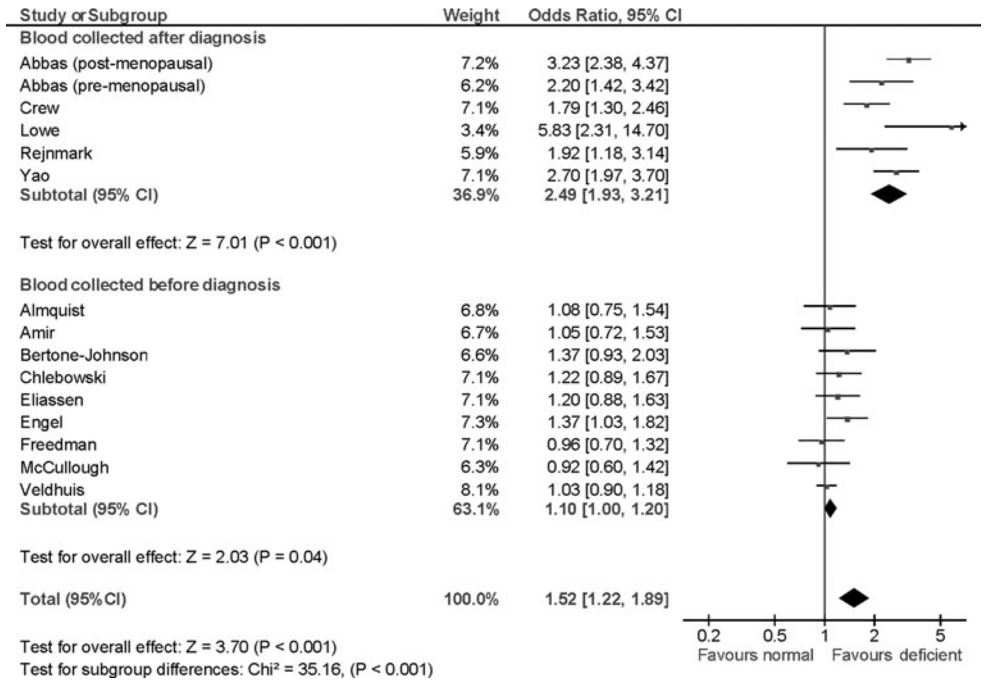

Fig. 3 Forest plot of odds ratios for breast cancer risk comparing low to high blood levels of vitamin-D metabolites. Odds ratios for each study are *represented by the squares*, the *size of the square* represents the weight of the trial in the meta-analysis, and the *horizontal line* crossing the square represents the 95% confidence interval. The *diamonds* represent the estimated pooled effect based on each cohort individually (labeled sub-total) and for all cohorts together (labeled total). Test of subgroup differences relates to the test of heterogeneity between the two subgroups as defined by Deeks et al. [14]. All p values are two-sided

mechanisms may explain the association of BMI and breast cancer risk. The association of BMI and breast cancer has been predominantly seen in post-menopausal women, and has also been observed in pre-menopausal women with central obesity [27]. In our study, 49% of patients were pre-menopausal. An analysis of the interaction between BMI and menopausal status was not significant. However, as cases and controls were matched for age and this variable is highly correlated with menopausal status, it is difficult to interpret these findings accurately.

Inflammation has been associated with increased risk of breast cancer [10, 39]. CRP is a marker of systemic inflammation, but data on its association with breast cancer risk remains sparse. Our data showed that baseline CRP did not predict for increased breast cancer risk. Similar data were reported in an analysis of the Women's Health Study [53]. Both these analyses were limited by single measurements of CRP which likely does not reflect long-term levels of inflammation. This limitation leads to uncertainty in the assessment of the association between inflammation and breast cancer risk.

These data have limitations. First, blood samples were collected from patients at only one time point. Therefore, these may not reflect levels of key mediators over time. Second, despite a large cohort population, the number of breast cancers was relatively small. This may have been influenced by our inclusion of only events occurring before unblinding of the study. Such case matching of a small subgroup of patients from a large randomized trial may be criticized. However, this was the only feasible methodology because after unblinding, patients on placebo were offered cross-over or enrolment on a randomized trial of tamoxifen versus raloxifene. It is hoped that the comprehensive background information and follow-up data derived from a randomized trial will negate these methodologic weaknesses. Third, due to matching by age, the differential effect of the tested blood levels in pre- and post-menopausal women could not be assessed robustly. Such analyses could also be confounded by pre-study use of hormone replacement therapy, although in our data, this variable did not appear to significantly affect breast cancer risk. Fourth, our choice of 25-hydroxy vitamin-D assay can be criticized. There can be substantial inter-assay differences in performance between different 25-hydroxy vitamin-D platforms [46], although these different methods have acceptable correlation [32]. Mass spectrometry-based assays likely results in the best calibration [46], but are not commonly used in clinical practice. Therefore, our use of electrochemiluminescence would likely have resulted in a balance between limited internal validity, but robust external validity. Finally, the population of women included in this study was at high risk for developing invasive breast cancer, and therefore may not be representative of all women. Despite these limitations, the meta-analysis shows that our findings are consistent with those from other studies where blood was collected before breast cancer diagnosis. These consistent findings which are not prone to reverse causation bias are likely to be a more accurate assessment of the association of vitamin-D metabolites and breast cancer risk.

In summary, when controlling for Gail score and adjusting for other factors independently associated with the risk of developing invasive breast cancer, suboptimal baseline levels of 25-hydroxy vitamin-D and increased baseline levels of insulin, CRP, and leptin levels do not show independent associations with the risk of breast cancer. BMI is a strong predictor of breast cancer, but the mechanisms underlying this association remain unclear. Further prospective data are required to further define how obesity influences breast cancer risk.

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