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Haplotypes of the Estrogen receptor beta (*ESR2*) gene and breast cancer risk

From the Breast and Prostate Cancer Cohort Consortium*

Abstract

Exposure to exogenous (oral contraceptives, post-menopausal hormone therapy) and endogenous (number of ovulatory cycles, adiposity) steroid hormones is associated with breast cancer risk. Breast cancer risk associated with these exposures could hypothetically be modified by genes in the steroid hormone synthesis, metabolism, and signaling pathways. Estrogen receptors are the first step along the path of signaling cell growth and development upon stimulation with estrogens. The National Cancer Institute Breast and Prostate Cancer Cohort Consortium has systematically selected haplotype tagging SNPs in genes along the steroid hormone synthesis, metabolism, and binding pathways, including the estrogen receptor beta (*ESR2)* gene. Four htSNPs tag the six major (> 5% frequency) haplotypes of the *ESR2* gene. These polymorphisms have been genotyped in 5,789 breast cancer cases and 7,761 controls nested within the American Cancer Society Cancer Prevention Study II, European Prospective Investigation into Cancer and Nutrition, Multiethnic Cohort, Nurses' Health Study, and Women's Health Study cohorts. None of the SNPs were independently associated with breast cancer risk. One haplotype of the *ESR2* gene was associated with breast cancer risk before correction for multiple testing (OR 1.17, 95% CI 1.07–1.28, p=0.0007). This haplotype remained associated with breast cancer risk after adjustment for multiple testing using a permutation procedure. There was no statistically significant heterogeneity in SNP or haplotype odds ratios across cohorts. These data suggest that inherited variants in *ESR2*, while possibly conferring a small increased risk of breast cancer, are not associated with appreciable (OR > 1.2) changes in breast cancer risk among Caucasian women.

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Introduction

Exposures to estrogens from endogenous (lifetime ovulatory cycles, parity, adiposity) and exogenous (oral contraceptives, post-menopausal hormone therapy) sources are well established breast cancer risk factors. Estrogens act as growth factors in estrogen sensitive tissues, such as the breast, and this growth response to estrogens is mediated by estrogen receptors. Estrogen receptors are in the nuclear receptor superfamily of ligand-inducible transcription factors, and can interact directly with DNA, altering the expression of downstream genes.

Two estrogen receptor isoforms, ER-α and ER-β exist, and are coded by two separate genes, *ESR1* on chromosome 6 and *ESR2* on chromosome 14. Both proteins are expressed in normal breast luminal epithelial cells, the morphological cell type of most breast tumors [1]. Both isoforms can also be expressed in breast tumors. However somatic loss of expression is associated with tumors whose growth is no longer controlled by steroid hormones. Such tumors are more aggressive, and have poorer short-term prognosis.

Studies of associations between polymorphisms in *ESR2* and breast cancer risk have been inconclusive. In 2003, Försti et al [2] found no association between *ESR2* polymorphisms and breast cancer risk in a small case control study of 219 breast cancer cases and 248 healthy male controls. In 2004, Gold et al [3] reported on estrogen receptor genotypes and haplotypes, describing haplotypes of *ESR2* that may increase breast cancer risk among Ashkenazi Jewish women. In a larger case control study (723 cases and 480 controls), Maguire et al [4] described an *ESR2* haplotype which significantly increased breast cancer risk. In addition to the studies of associations between *ESR2* and breast cancer risk, the role of *ESR2* variants has also been explored in body weight extremes [5], ovulatory defects and menstrual disorders [6], anorexia nervosa [7] and Alzheimer's Disease [8]. *In vitro* studies also suggest that estrogen receptor beta variation may influence the susceptibility to and development of breast cancer. For example, variant *ESR2* mRNA transcripts have been isolated from human breast cancer cell lines [9] and tumors [10, 11]. *ESR2* coexpression with *ESR1* has been isolated in both normal and malignant breast tissue [12–14].

We hypothesized that inherited polymorphisms in genes related to sex steroid hormone synthesis, metabolism, and cell signaling could alter the function of these genes and the proteins they encode, therefore altering breast cancer risk; in this report, we present results for the estrogen receptor beta. We used a haplotype tagging approach, which aims to capture common variants in the *ESR2* gene. Here, we present these haplotypes and describe their association with breast cancer risk in a pooled analysis of nested case control studies from a large collaboration of prospective studies, the Breast and Prostate Cancer Cohort Consortium (BPC3) [15] which includes 5,789 cases of breast cancer and 7,761 controls.

Methods

Study Population

The BPC3 has been described in detail elsewhere [15]. Briefly, the consortium includes large well-established cohorts assembled in the United States and Europe that have both DNA samples and extensive questionnaire information (the American Cancer Society Cancer Prevention Study II (CPS-II) [16], the European Prospective Investigation into Cancer and Nutrition (EPIC) cohort [17], the Harvard Nurses' Health Study (NHS) [18] and Women's Health Study (WHS) [19], and the Hawaii-Los Angeles Multiethnic Cohort (MEC) [20]. With the exception of the MEC, most women in these cohorts are Caucasians of U.S. and European descent. Cases were identified in each cohort by self report with subsequent confirmation of the diagnosis from medical records or tumor registries, and/or linkage with population-based

tumor registries (method of confirmation varied by cohort). Controls were matched to cases by ethnicity and age, and in some cohorts, additional criteria, such as country of residence in EPIC.

Genotyping

Coding regions of *ESR2* were sequenced in a panel of 95 (15 from each of the five ethnic groups; African American, Latina, Japanese, Native Hawaiian, and Caucasian) advanced breast cancer cases from the MEC. All SNPs detected (8 total) in the sequencing scan existed previously in dbSNP or had been reported in the literature [5]. Forty SNPs with minor allele frequency >5% over all or > 1% in any one ethnic group were selected from this resequencing as well as those available in dbSNP from the nonsequenced areas to be used to select haplotype tagging SNPs. These SNPs were genotyped in a reference panel of 349 healthy women from the MEC populations (including 70 Caucasians) at the Broad Institute (Cambridge, MA) using the Sequenom and Illumina platforms, and five htSNPs were selected to ensure a minimum R^2_H (a measure of how well the SNPs selected describe the haplotypes observed in the screening population) among Caucasians of 0.7 or greater using the method of Stram et al [21]. Thellenberg-Karlsson et al. [22] described a polymorphism, rs2987983, in the 5′ region of *ESR2* which was associated with prostate cancer risk. This polymorphism failed to genotype in our initial screen, however using HapMap data (data release 21, July 2006 on NCBI build 35 and dbSNP build 124) we found that this polymorphism is in complete linkage disequilibrium (r^2 and D′ = 1.0) with rs3020450, one of the htSNPs we selected.

Genotyping of the five htSNPs (rs3020450, rs1256031, rs1256049, rs4986938 (ESR2_G1730A), and rs944459) in the breast cancer cases and controls was performed in 3 laboratories (University of Southern California, Los Angeles, CA USA, Harvard School of Public Health, Boston, MA USA, International Agency for Research on Cancer, Lyon, France) using a fluorescent 5′ endonuclease assay and the ABI-PRISM 7900 for sequence detection (Taqman). Initial quality control checks of the SNP assays were performed at the manufacturer (ABI); an additional 500 test reactions were run by the BPC3. Assay characteristics for the 5 htSNPs for *ESR2* are available on a public website (http://www.uscnorris.com/mecgenetics/CohortGCKView.aspx). Sequence validation for each SNP assay was performed and 100% concordance observed (http://snp500cancer.nci.nih.gov) [23]. To assess inter-laboratory variation, each genotyping center ran assays on a designated set of 94 samples from the Coriell Biorepository (Camden, NJ) [23]. The internal quality of genotype data at each genotyping center was assessed by typing 5–10% blinded samples in duplicate or greater (depending on study). One htSNP (rs944459) tagged a haplotype common only among African Americans, and as such was genotyped but not included in analyses. The four remaining htSNPs still tag the known variants of *ESR2* with an R^2_H of 0.70.

Statistical Analysis

We used conditional multivariate logistic regression to estimate odds ratios (ORs) for disease in subjects with a linear (log-odds additive) scoring for 0, 1 or 2 copies of the minor allele of each SNP. We also used conditional logistic regression with additive scoring and the most common haplotype as the referent to estimate haplotype-specific ORs using an expectation-substitution approach to assign expected haplotype counts based on the unphased genotype data and to account for uncertainty in assignment [24, 25]. Haplotype frequencies and subject-specific expected haplotype counts were calculated separately for each cohort (and country within EPIC or ethnicity in the MEC). We combined rare haplotypes (those with estimated individual frequencies less than 5% in all cohorts) into a single category with a combined frequency of less than 1.6% of the controls.

To test the global null hypothesis of no association between variation in *ESR2* haplotypes and htSNPs and risk of breast cancer (or subtypes defined by receptor status), we used a likelihood ratio test comparing a model with additive effects for each common haplotype (treating the most common haplotype as the referent) to the intercept-only model. In addition, we used permutation testing [26] to further evaluate the association between haplotypes and breast cancer risk. Ten thousand permuted data sets were generated by shuffling case-control status within each matched case-control set. Matching schemes and variables varied by cohort, ranging from 1:1 (WHS, CPS-II) to frequency matching (MEC). Associations between each SNP and haplotype were evaluated in each of the 10,000 permutations using the log-additive model. The minimum p-value across all the variants tested (4 SNPs, 6 haplotypes; each modeled independently for 10 tests per permutation) in each permuted data set was compared to the lowest p-value observed in the original data set. The multiple-comparisons-corrected p-value is the number of permutations where the minimum p-value was less than the smallest observed p-value divided by 10,000.

We considered conditional models adjusting for known breast cancer risk factors. The covariates included to account for breast cancer risk factors were age at menarche (≤ 12 years, 13–14 years, 15+ years), menopausal status (pre, post, unknown), parity (ever/never full term pregnancy), body mass index (BMI in kg/m^2 as a continuous variable), and use of postmenopausal hormones (ever/never). Other common risk factors, including family history of breast cancer, personal history of benign breast disease, and age at menopause were unavailable for large numbers of women, and therefore were not included in the models. We also evaluated these covariates (including those with large proportions of missing data) for possible interaction effects using likelihood ratio testing (LRT). Models with the main effect of genotype and the covariate of interest were compared to models with the main effects of genotype and the covariate of interest, plus a multiplicative interaction term of the two variables. Lastly, we tested whether the association between *ESR2* and breast cancer differed by receptor (ER and PR) status. Power calculations were carried out using the program Quanto [27]. The rmeta package in the R environment was used to create Figure 2 to examine heterogeneity across the cohorts.

Results

Figure 1 shows the genomic structure of the region around *ESR2*, which consists of a single haplotype block. The four haplotype tagging SNPs in Caucasians account for 96% of the haplotype diversity at this locus. Using all five htSNPs tags common haplotypes among Caucasians with minimum $R^2_H = 0.75$, African Americans $R^2_H = 0.58$, Japanese $R^2_H = 0.17$, Native Hawaiians $R^2_H = 0.23$, and Latinas $R^2_H = 0.12$. When restricting to the four htSNPs which tag the haplotypes among Caucasians, the R^2_H values are 0.75, 0.22, 0.17, 0.21, and 0.12, respectively. The haplotypes tagged by these four SNPs ranged in allelic prevalence from 5–46% among the MEC Caucasian samples used for tagSNP selection, and were similar in the case-control analyses (5–45%).

A total of 5,789 cases and 7,761 controls were available for genotyping among cases and controls from the participating cohorts. Samples not yielding a genotype were removed from individual SNP analyses, and samples not yielding a genotype for at least one SNP were removed from haplotype analyses. Genotyping concordance was above 99% among centers and was greater than 99% within centers for blinded QC samples. Genotype success rate among cases and controls in all cohorts was above 95%. One polymorphism (rs1256049) deviated from Hardy-Weinberg Equilibrium among the controls of the MEC Caucasians (p=0.016) and EPIC (p=0.003), however genotype distributions between all cohorts were similar.

None of the single nucleotide polymorphisms studied showed an association with breast cancer risk (Table 1). Tests of heterogeneity of risk estimates between participating cohorts ranged from 0.10 to 0.50 for each single nucleotide polymorphism. The global test for comparison of haplotype frequencies in cases and controls was not highly significant (d.f. = 6, p = 0.04). However, one haplotype showed an increase in breast cancer risk (p=0.0007; OR 1.17, 95% CI 1.07 – 1.28, Table 2). Heterogeneity tests of associations between haplotypes and breast cancer risk between cohorts ranged from 0.10 to 0.65. Figure 2 shows the risk associated with the CCAC haplotype in each cohort. We also used permutation testing to correct for multiple comparisons. Of the 10,000 permutations, only 20 yielded a minimum p-value less than that observed for the most significant haplotype. Therefore the multiple-comparisons corrected p-value for this haplotype is 0.002 (from 20/10,000).

Upon stratification by age at diagnosis (<63 or 63+, median age overall = 63 years), the risk associated with this haplotype was restricted to younger women (Table 3). No statistically significant interactions (p-interaction < 0.05) between haplotypes and breast cancer risk factors (recent hormone replacement therapy (HRT), ever HRT, age at first full term pregnancy (FTP), ever FTP, family history of breast cancer, age at menarche, age at menopause, personal history of benign breast disease, menopausal status, or body mass index (BMI in kg/m2 in three categories; <25, 25–29, ≥30)) were observed for this haplotype. No difference in risk was observed upon stratification by estrogen or progesterone receptor status (data not shown). Estrone and estradiol levels were available on postmenopausal cases and controls from EPIC and the NHS, and an interaction between the CCAC haplotype and estrone levels was observed (Table 4, p = 0.03), and similar, though not statistically significant results were observed with estradiol (data not shown).

Discussion

The estrogen receptor beta is an obvious candidate gene to harbor allelic variants which predispose to breast cancer risk along the sex steroid hormone synthesis, metabolism, and signaling pathway. However, it is not the only candidate along this pathway, and many other genes are currently under study to examine associations between common variants and breast cancer risk. At the present time, no clear consensus in the field has been reached with regards to studying the effect of variants in large numbers of genes simultaneously on disease risk. Therefore, we have chosen to present results from the *ESR2* gene independently of other genes.

Given that the global-test for association between *ESR2* haplotypes and breast cancer risk was of borderline significance (p=0.04), with only one (CCAC) of the six common haplotypes showing a statistically significant increase in risk (p=0.0007) we used permutation testing as an additional multiple comparisons correction procedure. After correction for multiple comparisons (at the gene level) using permutation testing, the CCAC haplotype remains nominally statistically significantly associated with breast cancer risk (corrected p-value = 0.002), though not at the stringent threshold (10^{-4}) that has been proposed for candidate gene studies.

The low magnitude of risk limits the power to detect interactions with non-genetic risk factors. Nevertheless, we did find some intriguing results upon stratification by age at diagnosis (Table 3) and estrone levels (Table 4). The stratified analyses by age suggest that the CCAC haplotype is a risk factor only in younger women. We have chosen to dichotomize at age 63, as this is the median age at diagnosis across all cohorts, and is similar to the median age at diagnosis in the SEER data (61 years)[22]. While breast cancer incidence rates increase dramatically after menopause, they continue to increase well into the seventh decade. In fact, risk factors for breast cancer, particularly body mass index, have been shown to vary in their effect on premenopausal or postmenopausal diagnosis of breast cancer. Therefore, the most likely

interpretation of the interaction between the CCAC haplotype and age at diagnosis on breast cancer risk is related to overall lifetime risk, as opposed to risk relative to some specific life event, such as menopause. Among women with lower estrone levels, women carrying the CCAC haplotype had a further reduction in breast cancer risk. This could imply that a variant on this haplotype reduces the ability of cells to respond to estrogen signaling by altering the function of the *ESR2* gene. These stratified analyses, particularly with respect to estrone levels where the number of samples available leads to very unstable risk estimates (as evidenced by the very wide confidence intervals) must be interpreted very cautiously however, and further replication is necessary before making definitive conclusions.

Examining the other polymorphisms genotyped in the screen for htSNPs does not yield any *a priori* candidate causal SNPs (ie non-synonymous or splice site SNPs) on this haplotype. However, a putatively causal polymorphism, either part of the screen or not, could be incompletely tagged by this haplotype, either due to incomplete linkage, different allele frequency, or both. Given that no obviously functional polymorphisms have been described on this haplotype, we can not rule out that the association we observe between the CCAC haplotype and breast cancer risk is due to chance.

The Breast and Prostate Cancer Cohort Consortium (BPC3) was established to overcome the sample size limitation of many studies which examine genetic variants for association with breast and prostate cancer. Given the sample size in this study (5,789 cases, 7,761 controls), we have >90% power with type I error rate of 10^{-4} to detect a 0.2 frequency allele with per-allele risk of 1.2. As such, the results we present here confidently exclude common variation of *ESR2* from being associated with moderate or greater breast cancer risk. However, one less common variant (the CCAC haplotype, 8% of control chromosomes) is found to be associated with a modest increase in breast cancer risk. Even with the large sample size of the current study, roughly 12,000 cases and controls would be needed for 80% power to detect a similar association (per-allele OR 1.17) at type I error rate of 10^{-4}. For this reason, we should be cautious when interpreting the association between the CCAC haplotype and breast cancer risk. Similarly, the population studied here is predominantly post-menopausal Caucasian women, and the htSNPs selected tag haplotypes most efficiently among Caucasians. Therefore, we can not make conclusions about the association between variants of *ESR2* and breast cancer risk in other populations, nor should these htSNPs be assumed to tag variants in non-Caucasian populations.

In conclusion, we have performed an exhaustive scan of SNPs in the *ESR2* gene, selected htSNPs based on this scan, and evaluated the association between these htSNPs and breast cancer risk. One haplotype of *ESR2* is significantly associated with a seventeen percent increase in breast cancer risk per copy of the haplotype carried among Caucasian women.

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Abreviations

BPC3	Breast and Prostate Cancer Cohort Consortium
htSNP	haplotype tagging single nucleotide polymorphism

ESR2	estrogen receptor beta
OR	odds ratio
SNP	single nucleotide polymorphism
ER	estrogen receptor
CPS-II	Cancer Prevention Study II
EPIC	European Prospective Investigation into Cancer and Nutrition
NHS	Nurses' Health Study
WHS	Women's Health Study
MEC	Multiethnic Cohort
BMI	Body Mass Index
PR	progesterone receptor
QC	quality control
HRT	hormone replacement therapy
FTP	full term pregnancy
LD	linkage disequilibrium
CI	confidence interval
BBD	benign breast disease

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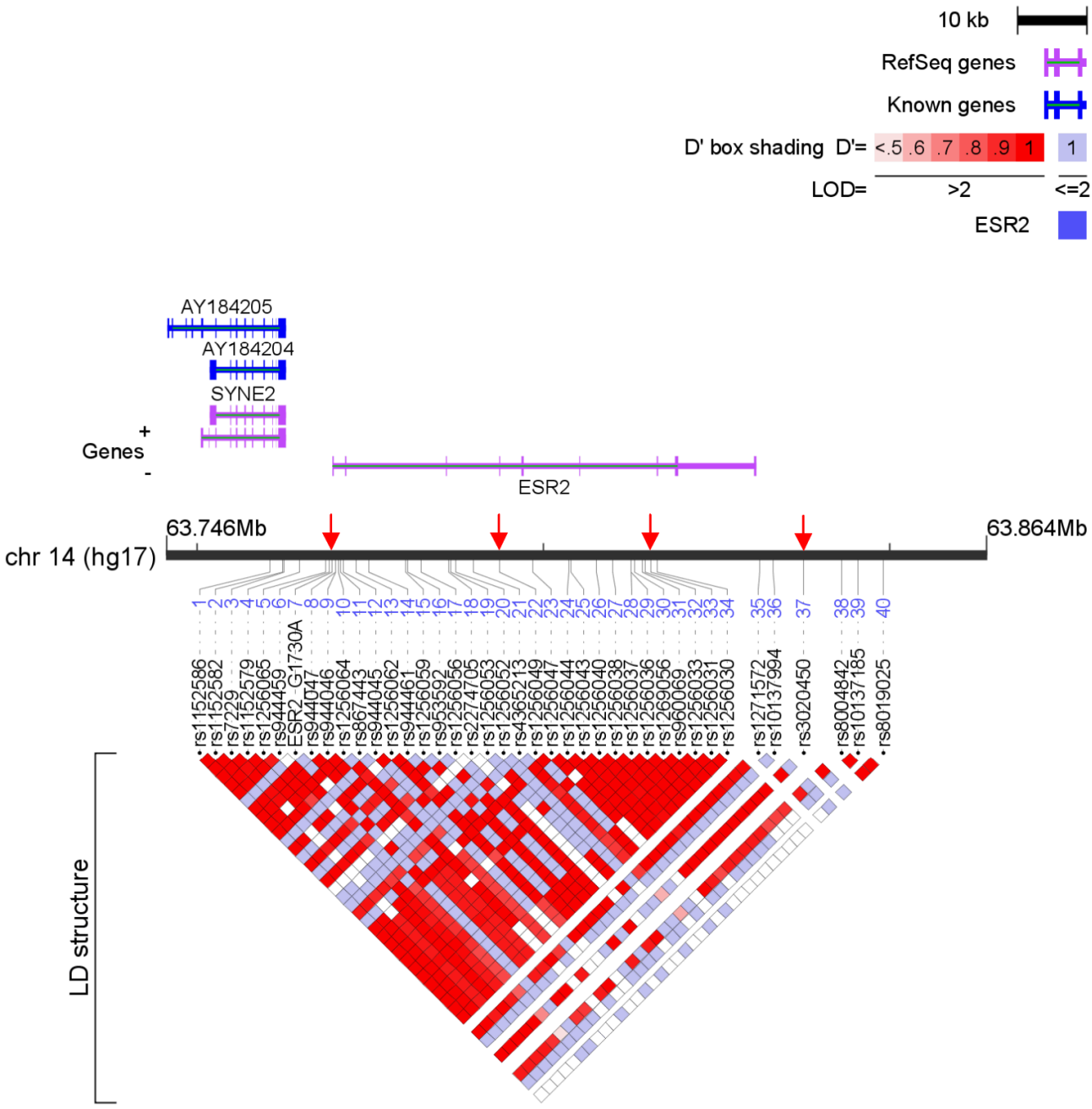

Figure 1.
Locusview [28] plot of *ESR2*. htSNPs among Caucasians are shown by red arrows.

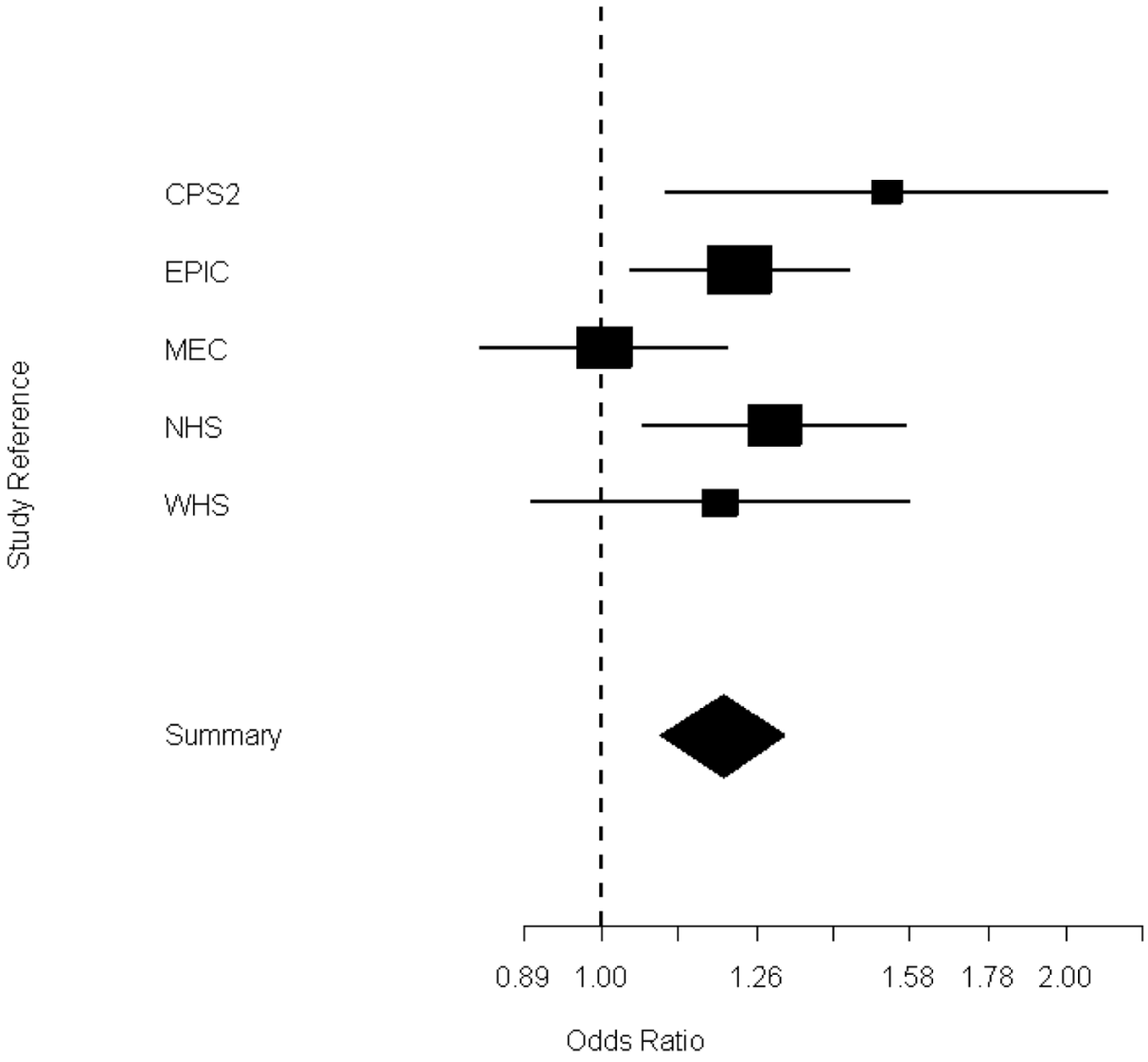

Figure 2.
Fixed Effects Mantel-Haenszel meta analyses of the dominant model for CCAC haplotype carriers. Significance level of 0.95 was used. Summary OR 1.20, 95% CI 1.09 –1.32, test for heterogeneity p-value = 0.183.

Table 1

Association between *ESR2* htSNPs and breast cancer risk in the Breast and Prostate Cancer Cohort

SNP	Genotype	Cases (%)*	Controls (%)*	OR (95% CI)**
ESR2_013	C/C	2513 (45)	3229 (43)	1.00 (Ref.)
rs4986938	C/T	2382 (42)	3304 (44)	0.95 (0.88 – 1.02)
	T/T	705 (13)	984 (13)	0.96 (0.86 – 1.06)
				p-trend = 0.19
ESR2_006	C/C	4987 (88)	6751 (89)	1.00 (Ref.)
rs1256049	C/T	610 (11)	734 (10)	1.09 (0.98 – 1.21)
	T/T	50 (1)	70 (1)	0.94 (0.70 – 1.28)
				p-trend = 0.27
ESR2_003	A/A	1644 (30)	2166 (29)	1.00 (Ref.)
rs1256031	A/G	2734 (50)	3634 (49)	0.99 (0.92 – 1.07)
	G/G	1135 (20)	1613 (22)	0.93 (0.85 – 1.02)
				p-trend = 0.15
ESR2_001	C/C	2640 (47)	3497 (46)	1.00 (Ref.)
rs3020450	C/T	2417 (43)	3208 (43)	1.01 (0.94 – 1.08)
	T/T	568 (10)	822 (11)	0.95 (0.85 – 1.06)
				p-trend = 0.54

* Cases and controls of invasive breast cancer from all participating studies

** Unadjusted logistic regression conditional on matched case-control sets

Table 2

Association between *ESR2* haplotypes and breast cancer risk in the Breast and Prostate Cancer Cohort

Haplotype	Cases (%) *	Controls (%) *	OR (95% CI)
hCCGC	2543 (44)	3454 (45)	1.00 (Ref.)
hCCAC	508 (9)	581 (8)	1.17 (1.07 – 1.28)
hTCAC	484 (8)	678 (9)	0.98 (0.89 – 1.07)
hCTAC	346 (6)	424 (5)	1.06 (0.97 – 1.17)
hCCAT	372 (7)	505 (7)	1.03 (0.93 – 1.13)
hTCAT	1418 (25)	1946 (25)	0.99 (0.90 – 1.09)
Freq<5%	91 (1)	121 (2)	1.04 (0.86 – 1.25)

* Cases and controls of invasive breast cancer from all participating studies, totals are the sum of haplotype scores

** Unadjusted logistic regression conditional on matched case-control sets. Global p-value for association of breast cancer risk with haplotypes = 0.04

Table 3

Association between *ESR2* haplotypes and breast cancer risk in the Breast and Prostate Cancer Cohort Consortium, stratified at age 63

< 63	**Haplotype**	**Cases (%)***	**Controls (%)***	**OR (95% CI)****
	hCCGC	1396 (44)	2004 (45)	1.00 (Ref.)
	hCCAC	287 (9)	323 (7)	1.23 (1.08 – 1.39)
	hTCAC	280 (9)	405 (9)	0.97 (0.86 – 1.09)
	hCTAC	180 (6)	239 (5)	1.07 (0.94 – 1.23)
	hCCAT	195 (6)	285 (6)	0.99 (0.87 – 1.14)
	hTCAT	789 (25)	1136 (25)	1.00 (0.92 – 1.09)
	Freq<5%	49 (1)	67 (1)	1.10 (0.84 – 1.44)
				global p=0.05
63 +	Haplotype	Cases (%)	Controls (%)	OR (95% CI)
	hCCGC	1159 (44)	1500 (45)	1.00 (Ref.)
	hCCAC	224 (8)	264 (8)	1.12 (0.98 – 1.27)
	hTCAC	205 (8)	278 (8)	0.98 (0.85 0 1.12)
	hCTAC	167 (7)	201 (6)	1.04 (0.90 – 1.19)
	hCCAT	178 (7)	233 (7)	0.99 (0.87 – 1.13)
	hTCAT	632 (24)	831 (25)	1.01 (0.93 – 1.10)
	Freq<5%	42 (2)	57 (2)	0.99 (0.76 – 1.30)
				global p=0.79

* Cases and controls of invasive breast cancer from all participating studies, totals are the sum of haplotype scores

** Unadjusted logistic regression conditional on matched case-control sets.

Table 4

Interaction between estrone levels with the CCAC haplotype and breast cancer risk in the BPC3

Estrone level*/CCAC Haplotype Copies			
Low/0	333 (36.6)	732 (42.4)	1.00 (Ref.)
Low/1	49 (5.4)	122 (7.1)	0.87 (0.59 – 1.27)
Low/2	1 (0.1)	10 (0.6)	0.23 (0.03 – 1.81)
High/0	448 (49.3)	766 (44.3)	1.37 (1.13 – 1.65)
High/1	74 (8.1)	93 (5.4)	1.96 (1.37 – 2.81)
High/2	4 (0.4)	6 (0.03)	1.53 (0.38 – 6.78)
			p-interaction = 0.03

*Estrone levels below (low) or above (high) the median. Median was determined separately by cohort (EPIC or NHS) among controls only

**Relative risk and 95% confidence interval from conditional logistic regression