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Given breast cancer, is fat better than thin? Impact of the estrogen receptor beta gene polymorphisms

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Abstract

The role of estrogen receptor beta (ERβ) in breast cancer has been investigated since its identification in 1996. Studies based on protein expression have indicated that ERβ is a favorable prognostic marker. Further, ERβ expression is lower in obese breast cancer patients. Fewer studies have focused on the prognostic impact of ERβ polymorphisms. Therefore, we analyzed the associations between four previously identified haplotype tagging single nucleotide polymorphisms (htSNPs), associated haplo- and diplotypes, and breast cancer-free survival according to body constitution.

The patient cohort included 634 women from the prospective breast cancer and blood study (BC-Blood study, Sweden) with a median follow-up of 4.92 years. Four htSNPs (i.e., rs4986938, rs1256049, rs1256031, rs3020450) in the *ESR2* gene and the correlating haplo- and diplotypes were analyzed and correlated to selected patient and tumor characteristics and to disease-free survival, including stratification for BMI. Based on the four htSNPs, seven haplotypes and eight diplotypes were identified. The patient and tumor characteristics were well-balanced across all geno- and haplotypes. Disease-free survival differed according to rs4986938 and rs1256031 (Log-Rank p=0.045 and p=0.041, respectively) and the number of haplotype copies of the wildtype CCGC and TCAC (Log-Rank p=0.027 and p=0.038, respectively). In the survival analyses stratified for BMI, significant survival differences between alleles were observed among overweight women (rs4986938 and rs1256031 with Log-Rank p=0.001 and p=0.001, respectively). The BMI-stratified survival analyses based on haplotypes showed shorter disease-free survival for overweight women with null copies of CCGC (Log-Rank p=0.001) and for overweight women with any TCAC copy (Log-Rank p<0.0001). Markedly impaired disease-free survival was found for genotypes in two out of four *ESR2* htSNPs and for two haplotypes.

ESR2 polymorphisms seem to divide patients into good and poor survivors based on BMI, stressing the need of taking host factors into consideration in the evaluation of prognostic markers.

Key words: ESR2, estrogen receptor beta, breast cancer, prognostic, polymorphisms, BMI

Introduction

Estrogen signaling that is mediated by the estrogen receptors (ERs) is essential for breast cancer development and endocrine treatment response [33]. The classical estrogen receptor, i.e., estrogen receptor alpha (ERα), was discovered in the 1960s and is encoded by the *ESR1* gene located on chromosome 6, whereas the more recently identified estrogen receptor beta (ERβ) is encoded by the *ESR2* gene located on chromosome 14 [21]. Many studies have investigated the prognostic and predictive role of the immunohistochemical expression of ERβ and thus observed a survival benefit and an enhanced response to tamoxifen treatment in women with high ERβ expression [12, 13, 28]. In contrast, genetic studies on *ESR2* polymorphism have focused on overall breast cancer risk, and the results have been inconsistent to date [6, 8, 9, 31, 34]. A recent meta-analysis on *ESR2* polymorphisms and breast cancer risk by Yu et al. suggested an association between SNP rs4986938 and the haplotypes in the *ESR2* gene and increased breast cancer risk [34]. The National Cancer Institute's Breast and Prostate Cancer Cohort Consortium has selected four haplotype tagging single nucleotide polymorphisms (htSNPs) that tag the six major haplotypes of the *ESR2* gene, and genotyping was performed in almost 6,000 breast cancer cases and the corresponding controls without finding any associations between these htSNPs and breast cancer risk [6]. Studies on the *ESR2* gene in prostate cancer patients have noted the influence of body mass index (BMI) on the prognostic value of different alleles [4]. Generally, breast cancer studies agree on the substantial prognostic impact of anthropometric measures with an impaired survival among overweight women [7, 17, 22, 24]. Further, the relevance of anthropometrics to the efficacy of the endocrine treatment was addressed by Sestak et al. [26] and highlighted in the comments by Goodwin and Pritchard, who refer to obesity and hormone therapy as an "unfinished puzzle" [10]. However, no studies to date have investigated the prognostic and predictive relevance of gene polymorphism in the *ESR2*

gene in breast cancer patients while considering anthropometrics. In this study, we analyzed the associations between four previously identified htSNPs, associated haplo- and diplotypes, and breast cancer-free survival according to body constitution. The Breast Cancer and Blood cohort (BC Blood) was used for the sample group in this study (i.e., 634 female breast cancer patients enrolled between October of 2002 and October of 2008 and followed until January 1st, 2012, providing a median follow-up time of 4.92 years).

Material and Methods

Patients and Blood sampling

The BC Blood study is an ongoing study at Lund University Hospital in Sweden and has recruited women with primary breast cancer in order to study genetic factors and their association with prognosis and treatment response. Enrollment takes place at the preoperative visit where participants fill out an extensive questionnaire on lifestyle factors and medical history. Follow-up questionnaires at 3 to 6 months and one, two, three, five, and seven years postoperatively track clinical information and changes in lifestyle factors. A vast majority of study patients attended follow-up visits as reported previously [15].

Anthropometric measures, such as weight, height, waist and hip circumference, and breast volume, were assessed at the preoperative visit [23]. Tumor size, histological type and grade, axillary node involvement, signs of distant metastases, and ERα and progesterone receptor (PR) statuses were obtained from each patient's pathology report. Tumors with >10% positive nuclear ERα and/or PR staining were considered receptor positive [14]. Blood samples were collected at the pre-operative visit and stored at −70°C as serum, EDTA-plasma, and blood cells; samples were labeled with serial codes to ensure blind analyses.

This study incorporated a total of 634 women between October of 2002 and October of 2008. The study adheres to the REMARK criteria [20]. A written informed

consent was obtained from all participants. The study has been approved by The Ethics Committee at Lund University (Dnr 72-02 and Dnr 37-08).

SNP genotyping

The Wizard Genomic DNA Purification Kit (Promega, Madison, WI, USA) was used to extract genomic DNA from peripheral blood, and genotyping of the *ESR2* htSNPs was accomplished via the manufacturer's protocol in laboratories at the Region Skåne Competence Center in Skåne University Hospital at Malmö, Sweden. The analyses of *ESR2* rs4986938, rs1256031, rs1256049, and rs3020450 were performed using a matrix-assisted laser desorption-ionization time-of-flight mass spectrometer with a Sequenom MassARRAY platform (Sequenom, San Diego, CA, USA). Around 10% of the samples were run in duplicate for the purpose of quality control with a concordance of 100% for all validated samples.

***ESR2* haplo- and diplotype construction**

The four htSNPs were successfully analyzed in most cases, i.e., with missing information for only two cases, in each of three htSNPs (i.e., rs4986938 [htSNP1], rs1256049 [htSNP2], and rs3020450 [htSNP4]) and in eight cases for htSNP rs1256031 (htSNP3). The relevant htSNP could be imputed for two patients based on the remaining three SNPs. Cross-tabulations were performed for each SNP against the other three SNPs. Based on the most likely combinations, haplotypes and diplotypes were constructed. Seven different haplotypes (i.e., CCGC, CCAC, CCAT, CTAC, TCAC, TCGC, and TCAT) and eight diplotypes (i.e., CCGC/CCGC, CCGC/TCAT, CCGC/TCAC, CGCC/CCAC, CCAC/TCAT, TCAT/TCAC, TCAT/TCAT, and CCGC/CCAT) were constructed. Due to a lack of information on genotypes, diplotype construction could not be performed for ten patients. The diplotype

variants that were present in less than 5% of patients were classified as rare and seen in 97 patients.

Statistical analysis

All analyses were performed using SPSS Statistics 19 (IBM, Chicago, IL, USA). The anthropometric variables were not normally distributed and thus transformed using the natural logarithm (ln). BMI was dichotomized in order to stratify patients into normal- and overweight. A cut-off of 25 kg/m^2 was used. The association between genotypes and axillary lymph node involvement was assessed using the Chi-square test. Breast cancer events were defined as local or regional recurrences, contra-lateral cancers, or distant metastasis. Breast cancer free survival was based on the time from the date of inclusion to the date of any breast cancer event, death from a non-breast cancer related cause, or last study follow-up (i.e., prior to January 1st, 2012). In the survival analyses, we excluded all patients with non-invasive breast cancer (i.e., *in situ* carcinoma, n=14), patients who had received neo-adjuvant treatment (n=30) or pre-operative interstitial laser thermotherapy (n=11 + 1 uncertain), and patients with a breast cancer event within three months after inclusion (n=2). Survival analyses were performed using Kaplan-Meier curves, including the log-rank test, and Cox-regression was used for generating hazard ratios. Additionally, adjusted analyses included potential confounders, such as linear age, tumor size (i.e., ≥21 mm or pT4, yes/no), and lymph node metastasis (i.e., yes/no). Interaction terms between haplotypes and BMI were constructed to test for interactions. For htSNP1, htSNP3, and htCCGC, a dose-dependent association was observed, and these were analyzed in a dose-dependent manner. Yet, for htTCAC, any copy (yes/no) was used since there were few patients with two copies. Prior power calculations assuming 600 patients with an accrual interval of 6 years and additional follow-up time of 2 years and a SNP frequency of 20% showed that the study was able to

detect true HRs between 0.679 and 1.565 with 80% power and α of 5% (Power and Sample size calculation programme, PS, version 2.1, developed by Dupont and Plummer; http://biostat.mc.vanderbilt.edu/wiki/Main/PowerSampleSize). Furthermore, simulations with different frequencies of failure rates (20% to 30%) and genotypes were also performed and showed that the study was powered to detect increased HRs between 1.8 and 2.3 with a genotype frequency of 20%.

Nominal p-values without correction for multiple testing are presented herein [1]. All statistical tests were two-sided, and p-values less than 0.05 were interpreted as significant.

Results

Patient characteristics

This study is based on a sample of 634 women with primary breast cancer. The median age of the participants was 59.6 years (range 25 to 99 years). Further patient characteristics, such as anthropometric factors (e.g., BMI and waist-hip-ratio), parity, and use of hormone replacement treatment in relation to htSNPs and haplotypes, are described in Tables 1 and 2. In general, the anthropometric factors showed a balanced distribution among different geno- and haplotypes with the exception of women carrying the htSNP4 T/T and women with the TCAT haplotype who were slimmer according to all measured anthropometric factors. The percentage of nulliparous women in different geno- and haplotype groups varied from 81% to 95% for different genotypes and from 81% to 100% for different haplotypes (i.e., in subgroups with more than ten individuals).

Tumor characteristics

Table 3 presents the distributions of standard pathological parameters (e.g., tumor size, histological grade, hormone receptor status, and lymph node metastasis) among

women with different genotypes. In patients with htSNP4 T/T, tumors were often larger than 20 mm, while the tumors in women with htSNP3 G/G were smaller in relation to the average distribution among all patients. Regarding tumor grade, the lowest number of grade III tumors was found in patients with htSNP3 G/G, whereas patients with htSNP1 T/T displayed the highest number of high-grade tumors. Hormone receptor status differed to a lesser extent across genotypes with exception to a few outliers. Patients with htSNP3 G/G were more prone to develop ERα+ and/or PR+ tumors; in contrast, patients with htSNP4 T/T more often developed ERα- and PR- tumors. There was an increasing trend towards any axillary lymph node involvement with increasing number of htSNP1 T-alleles (P_{trend}=0.003) and htSNP3 A-alleles (P_{trend}=0.044).

Table 4 displays pathological factors in relation to different haplotypes. Only three haplotypes, namely, CCGC, TCAC, and TCAC, were associated with tumor characteristics. Diversion from the average tumor size was predominantly seen in patients with two copies of the wild type CCGC haplotype allele and had smaller tumors, whereas one or two alleles of the TCAT haplotype corresponded with larger tumors. As for grade, the most prominent deviation was seen in patients with one allele of the TCAC haplotype who developed few grade II tumors and relatively more grade I and grade III tumors. Comparatively, most low-grade tumors were found in patients with two alleles of the CCGC haplotype. ERα+ tumors were seen most infrequently in patients with two alleles of the TCAT haplotype and most frequently in patients carrying two alleles of the CCGC haplotype. A corresponding pattern was seen for the distribution of tumors according to PR status. Lymph node negative disease was most often seen in homozygous CCGC carriers (P=0.012), whereas patients with the TCAT haplotype in duplicates were somewhat more prone to develop node positive disease (P=0.077)

Prognostic impact of *ESR2* polymorphisms

Breast cancer-free survival was used as an endpoint in the survival analyses, and a total of 73 breast cancer events were detected during a median follow-up time of 4.92 (IQR 3.01-6.36) years. Among all breast cancer events, 46 events were classified as distant metastasis.

In the analyses of the four different htSNPs, htSNP1 and htSNP3 demonstrated a significant prognostic impact on their respective genotypes. As for htSNP1, a significant trend towards shorter disease-free survival for each T-allele was observed (LogRank 1 df P=0.045, Fig. 2A). Cox regression crude analyses provided hazard ratios (HR) of 1.57 (95% CI 0.92-2.66); (P=0.098) for C/T and 1.90 (95% CI 0.95-3.79); (P=0.070) for T/T. The multivariate analyses adjusted for age showed that the HRs for tumor size and lymph node metastasis were materially the same. Likewise, shorter disease-free survival was found for women with the htSNP3 A/A genotype as compared to the A/G and particularly the G/G genotypes (LogRank 1 df P=0.041 per A-allele, Fig. 2B). In the Cox regression analyses using the G/G as reference, the HR was 2.43 for A/G (95% CI 1.03-5.74); (P=0.044) and 3.00 for A/A (95% CI 1.24-7.26); (P=0.013) with a significant Cox-trend value (P=0.040). Multivariate analyses showed a slight decrease in adjusted HR to 2.24 (95% CI 0.93-5.25); (P=0.073) for the A/G genotype and adjusted HR 2.64 (95% CI 1.08-6.45); (P=0.030) for the A/A genotype. The remaining two htSNPs (i.e., htSNP2 and htSNP4) showed no survival differences between genotypes (data not shown).

Regarding the different haplotypes, the number of copies showed a significant prognostic value for the wildtype CCGC haplotype (LogRank 1 df P=0.027 per copy, Fig. 2C). Patients with null copies displayed a significantly increased risk for early events (crude HR 2.80 with 95% CI 1.16-6.78; P=0.023), while patients with one copy had a non-significantly increased risk (HR 2.34 with 95% CI 0.99- 5.54; P=0.053) as compared to

patients homozygous for the wildtype CCGC haplotype. Adjusted analyses showed slightly weaker HRs. Conversely, for the TCAC haplotype, patients with null copies had longer disease-free survival as compared to patients with any copy (LogRank P=0.038, Fig. 2D). Only a few patients had two copies of the TCAC haplotypes. The remaining haplotypes showed no prognostic value (data not shown). The results were similar for patients with ERα positive versus ERα negative tumors.

Diplotypes showed less prognostic value with the exception to patients who were homozygous carriers of the wildtype CCGC who had somewhat longer disease-free survival (LogRank P=0.025), but this was no longer significant in the adjusted analyses.

Anthropometric factors and *ESR2* polymorphism

In the disease-free-survival analyses of htSNP1 stratified for BMI, a notable survival difference was observed between alleles among overweight patients (LogRank 1 df P=0.001), whereas normal-weight patients displayed similar survival curves independent of the htSNP1 alleles (p=0.65, Fig. 3A/B). A significant interaction was observed between htSNP1 and BMI on disease-free survival ($P_{interaction}$ =0.015).

Similar results were found in the analyses of htSNP3 in overweight and normal-weight patients (LogRank 1df P=0.001 and LogRank 1 df P=0.68, respectively, Fig. 3C/D). The interaction between htSNP3 and BMI was of borderline significance (P=0.066). Notably, overweight patients who were homozygous for the major allele G/G had no breast cancer events at all. Again, these results were independent of ERα status. Using distant metastasis as an endpoint, interaction analyses of BMI and htSNP1 respective to htSNP3 revealed similar results as the analyses of disease-free survival (data not shown). Meanwhile, htSNP2 and

htSNP4 did not show any prognostic relevance in crude analyses or analyses stratified for BMI.

The disease-free survival analyses based on haplotypes highlighted the distinct impact of anthropometric factors. For the CCGC haplotype, the number of copies was not associated with disease-free survival among normal-weight women (LogRank 1 df P=0.78) as opposed to overweight patients, who had an impaired survival rate in the case of null copies as compared to patients with one copy and especially patients with two copies (Fig. 3E/F, LogRank 1 df P=0.001), and the interaction was of borderline significance ($P_{interaction}$=0.062). Very few patients had two copies of TCAC; therefore, we analyzed any TCAC. The survival curves of normal and overweight patients differed. Shorter disease-free survival was seen for patients with null copies compared to patients with any copy of TCAC (LogRank P=0.037); conversely, significantly shorter disease-free survival was observed in overweight patients with at least one TCAC copy (LogRank P<0.0001). The interaction between TCAC and BMI was significant ($P_{interaction}$=0.004, Fig. 3G/H).

Since htSNP1, htSNP3 and CCGC were associated with axillary lymph node involvement, further stratification according to node status was performed. Among normal weight patients, the lack of association between the different genotypes and risk for early events remained. Among women with a BMI of 25 kg/m^2 or higher and axillary lymph node involvement, there was an increased risk for early events with increasing number of htSNP1 T-alleles (LogRank 1 df; P=0.013), increasing number of htSNP3 A-alleles (LogRank 1 df; P=0.023) as well as decreasing number of CCGC copies (LogRank 1 df; P=0.033). There was only a non-significant association between these genotypes and risk for early events in overweight patients without axillary lymph node involvement.

Predictive relevance of *ESR2* polymorphisms

Survival analyses, including different regimens of endocrine treatment (i.e., tamoxifen and aromatase inhibitors), showed no evident predictive impact of ESR2 polymorphisms, neither in crude nor in BMI-stratified analyses.

Discussion

Herein, we present data indicating that *ESR2* polymorphisms affect breast cancer outcome for two of the four investigated htSNPs that harbor prognostic information just as two common haplotypes, including the wild type haplotype, do. Moreover, body constitution greatly impacted the prognostic information obtained from *ESR2* polymorphisms as survival in overweight patients differed extensively depending on *ESR2* polymorphisms in contrast to that of normal-weight patients.

For this study, certain methodological aspects need further explanation. The findings are based on a Southern Swedish cohort consisting of 634 patients who represent 58% of all incident breast cancer cases within the study period. In previous analyses, the participants were representative of all operated breast cancer patients regarding age and hormone receptor status (ERα and PR), and we consider the study population adequately representative for Southern Sweden. Regarding the frequencies of different haplotypes within this cohort, we found a distribution similar to the distribution seen for the cohort from the Breast and Prostate Cancer Cohort Consortium, i.e., CCGC being the most common, followed by TCAT, CCAC, TCAC, CCAT, and CTAC in descending order [5]. The evident similarity in haplotype distribution between the smaller Swedish cohort and a large international cohort supports our findings in this study. The genotypes were directly constructed in almost all cases; i.e., only two ht3SNPs were imputed.

The study was powered to detect increased HRs of 1.8 to 2.3 with simulated failure rates of 20% to 30%. The estimated failure rates exceeded the observed failure rates, which may have influenced the power in this study. In addition, this study assessed numerous variables, and some of the findings may be due to chance. The study was an exploratory study and correction for multiple testing was not performed in accordance with Benjamini *et al* [1]. The results need confirmation in independent cohorts,

Anthropometric studies might include a risk for recall bias. However, anthropometric measures were gathered by a research nurse in this case; thus, BMI is considered a valid variable in this study. This study is based on a predominantly Caucasian population in Sweden, and since the BMI range differs widely internationally and according to ethnicity [5], the results may not be representative for all breast cancer populations. Nevertheless, *ESR2* polymorphisms are important in overweight women and may thus be even more important in more obese populations. Previously, we have shown that overweight patients were more adherent to endocrine therapy; moreover, non-adherence was associated with a significantly increased risk of early breast cancer events [18]. In this study, the duration of endocrine treatment was not associated with the number of variant alleles of htSNPs1-4, number of htCCGC copies, or the presence of any htTCAC variant, indicating that adherence did not affect our findings.

Furthermore, nodal involvement may be a potential confounder in this study as geno-and haplotypes were associated with nodal involvement. In the multivariate analyses, nodal involvement was adjusted for and the results remained significant. Additionally, the main findings of significant associations between *ESR2* genotypes and risk for early events remained significant in the group of overweight patients with axillary lymph node involvement in spite of the lower number of patients in each strata. This finding suggests that the association was not simply driven by the node status.

This study with an average follow-up time of approximately five years may identify recurrences from more aggressive and less ERα+ breast cancer types. An extended follow-up time may influence the impact of *ESR2* polymorphisms on survival in the cases of correlations between ERα expression and *ESR2* polymorphisms. To date, no data for extended follow-up times are available. Previously, we published results from another Swedish cohort on the tumoral expression of ERα and ERβ and showed a co-expression of the two ER receptors in approximately half the cases [2]; thus, ERα status may influence *ESR2* polymorphisms' impact on survival. This study demonstrated prognostic information based on *ESR2* polymorphisms irrespective of ERα status, indicating that although interrelated, the two ERs probably play independent roles in tumor progression. Herein, ERα status was defined using the current Swedish cut-off of 10%. The novel international guidelines for ERα positivity (defined as more than 1% positive cells) could not be incorporated into this study. As most tumors are homogeneous in ERα expression, few tumors show an ERα fraction between 2 and 10%, thus the results are believed to be applicable in populations where the cut-off of 1% is applied..

The risk of classification bias in pathological tumor data is considered inappreciable as all tumor tissues were evaluated in the same pathological department by two senior breast pathologists. Information on HER2 status and Ki67 might have been of interest as covariates in the survival analyses and should be incorporated into future studies.

In this study, we have evaluated four different htSNPs based on the data from the National Cancer Institute's Breast and Prostate Cancer Cohort Consortium; from their extensive scan of SNPs in the *ESR2* gene, they identified the four htSNPs tagging the six major haplotypes of the *ESR2* gene [6]. More than these four htSNPs could have possibly been evaluated, but limiting the analyses to four htSNPs was reasonable based on the profound work generated from the Breast and Prostate Cancer Cohort Consortium [6].

Yu et al. recently addressed *ESR2* polymorphisms in relation to overall breast cancer risk and indicated a decreased risk among women with a variant allele in htSNP1 and an association between different haplotypes and breast cancer risk [34]. However, less is known about the field of *ESR2* polymorphisms and prognosis following breast cancer, and to the best of our knowledge, this study is a pioneer in the field. *ESR2* polymorphisms do seem to contribute prognostic information in addition to standard pathological parameters, such as tumor size and axillary lymph node involvement. However, the striking findings among overweight women, for whom *ESR2* polymorphisms had a profound impact on prognosis, point towards the field of gene-environment interaction. The findings demonstrated in this study are in line with other studies on *ESR2* polymorphisms and anthropometrics, i.e., an inverse relation between one *ESR2* haplotype and overweight [11, 16, 25]. In the field of prostate cancer, Chae et al. found an interactive association between BMI and genotypes of *ESR2* and prostate cancer risk [2]. This and previous studies indicate that the association between *ESR2* polymorphisms and cancer risk might be clarified by incorporating host factors, such as anthropometrics, into the risk stratification of the population for preventive efforts. Similarly, in calculations of prognosis for breast cancer patients based on *ESR2* polymorphisms, anthropometrics should be considered. By doing so, a more specific calculated recurrence risk for overweight breast cancer patients tested for *ESR2* polymorphisms, could be obtained and support the decision for adjuvant treatment.

A tentative explanation for the association between *ESR2* polymorphisms and anthropometrics might be found in the methylation of the ERβ promoter. ERβ is considered to play an important role as a tumor-suppressor gene [19], and in 2005, Rody et al. showed that the methylation of ERβ promoter correlates with the loss of ERβ expression in breast cancer and was an early indicator of premalignant lesions [24]. Additionally, methylation has been associated with BMI in melanin-concentrating-hormone-receptor1 (MCHR1) [29]. In breast

cancer, a greater waist-hip ratio was associated with the increased likelihood of methylation of E-cadherin, p16, and RARβ-2 and might contribute to support for an interaction between body constitution and methylation [30]. In summary, body constitution may be associated with the methylation of the ERβ promoter, thereby possibly leading to decreased ERβ expression in epithelial cells, which is an early event in carcinogenesis and the loss of ERβ in breast cancer cells that corresponds with reduced anti-proliferative effects [27, 32]. The interaction between overweight and ERβ is supported by previous findings in another Southern Swedish cohort, in which a significant association between overweight and less tumoral ERβ expression was demonstrated [3].

Conclusion

Herein, we present a shorter breast cancer-free survival in patients with the minor allele of rs4986938 (htSNP1) or the major allele of rs1256031 (htSNP3) and for two of seven haplotypes. Interestingly, the prognostic impact of *ESR2* polymorphisms was highly dependent on the patient's BMI. Genotyping of breast cancer patients may serve as a future prognostic tool in terms of identifying a selected set of patients with a less favorable prognosis and the need for further treatment; conversely, this approach can also identify patients who may need less adjuvant treatment.

Figure captions

Fig. 1

Schematic overview of the htSNPs in *ESR2* based on NM_001437

Fig. 2

Kaplan-Meier plots showing breast-cancer-free survival analyses with respect to two different *ESR2* genotypes (i.e., htSNP1 (a) and htSNP3 (b)) and two haplotypes (i.e., CCGC (c) and

TCAC (d)) in the ongoing BC Blood study. Since the BC blood study is an ongoing study, the number of patients in the life tables decreases as the follow-up increases.

Fig. 3

Kaplan-Meier analyses stratified for BMI (< 25/≥ 25), demonstrating breast-cancer-free survival with respect to two different *ESR2* genotypes (i.e., htSNP1 (a, b) and htSNP3 (c,d)) and two haplotypes (i.e., CCGC (e,f) and TCAC (g,h)) in the ongoing BC Blood study. Since the BC blood study is an ongoing study, the number of patients in the life tables decreases as the follow-up time increases.

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Disclosure of Potential Conflicts of Interest

The authors declare that they have no conflict of interest.

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Schematic overview over the htSNPs in ESR2 based on NM_001437

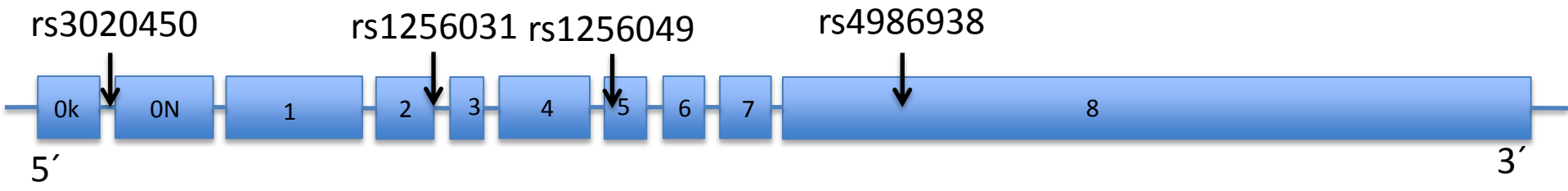
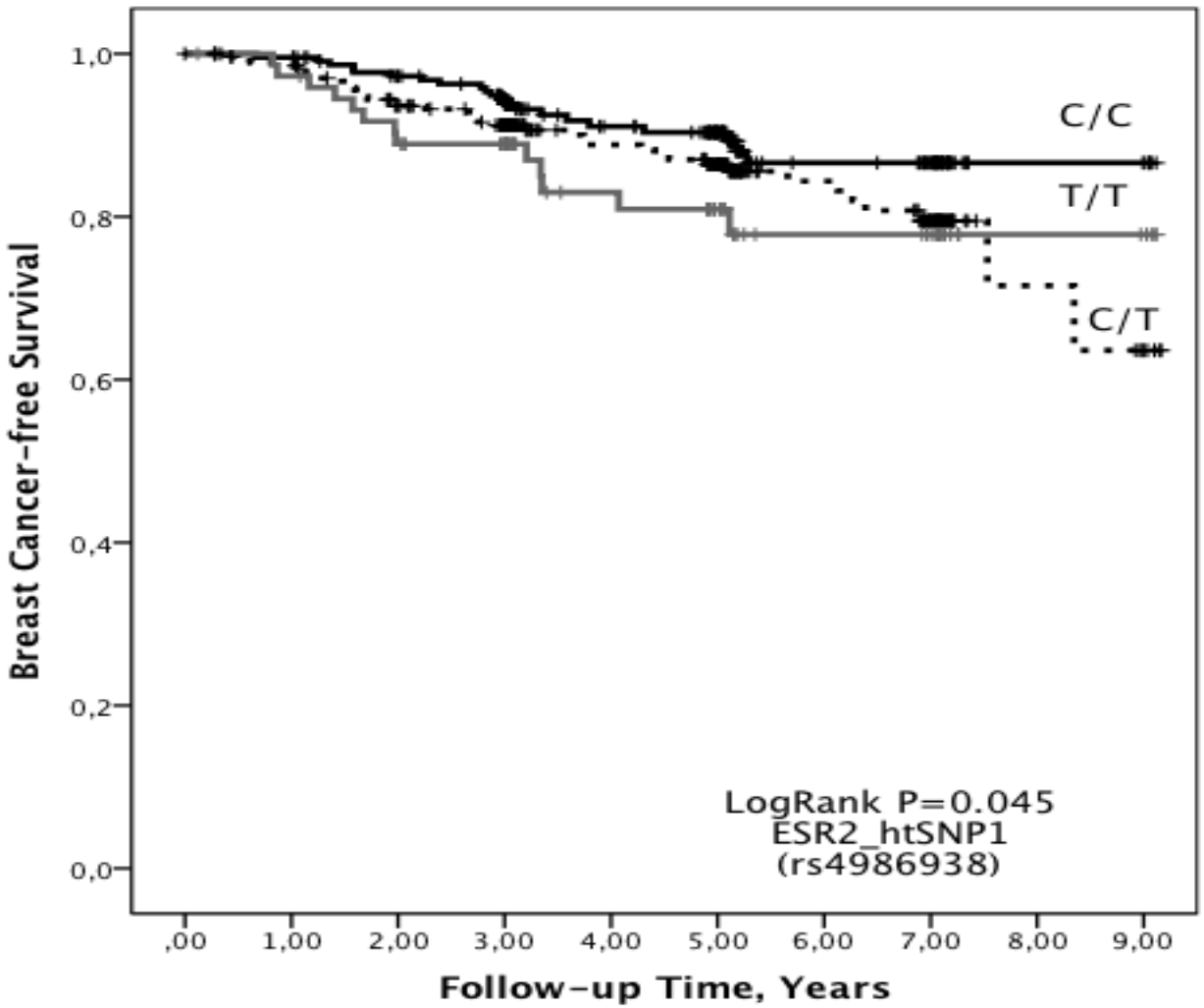
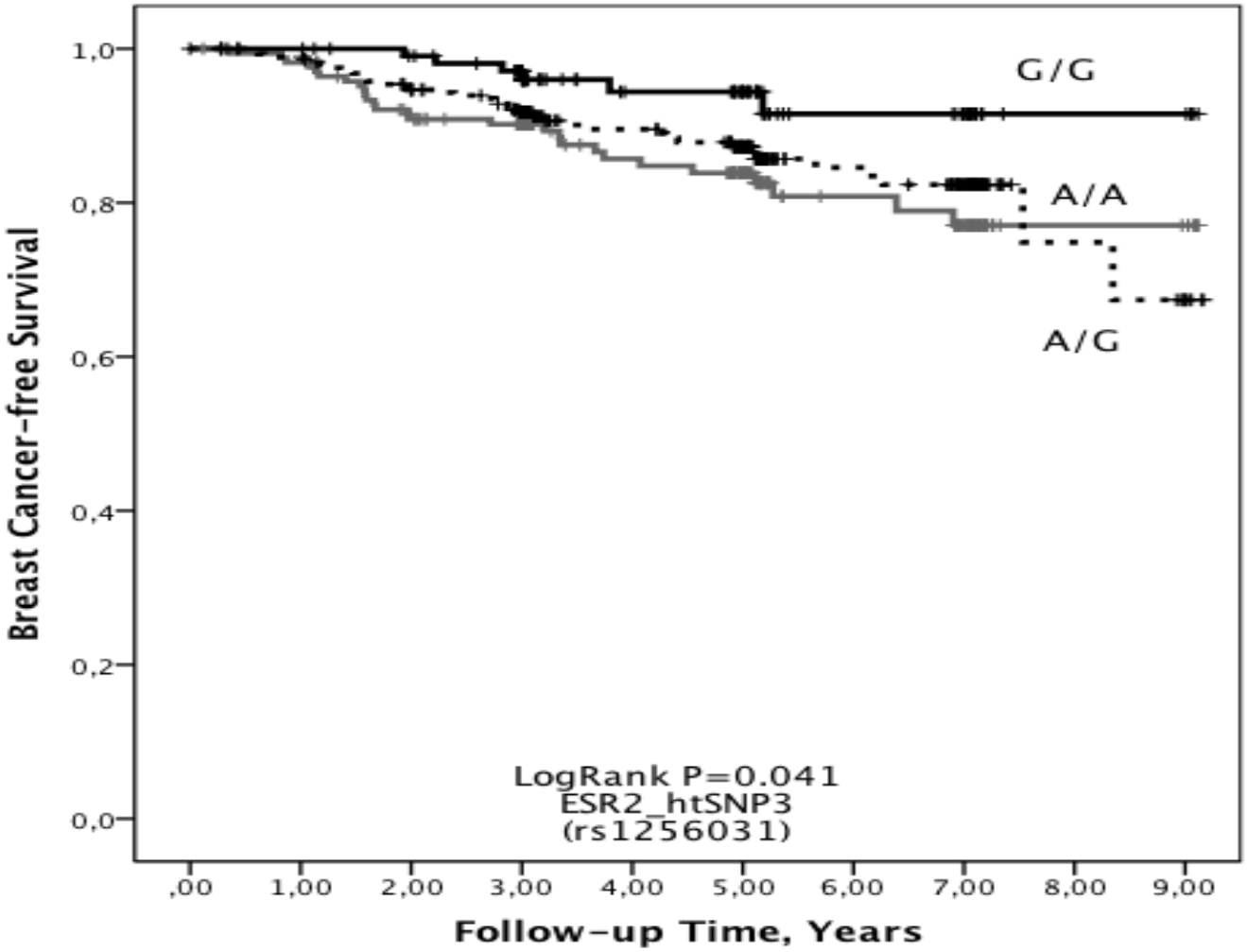


Fig 2A



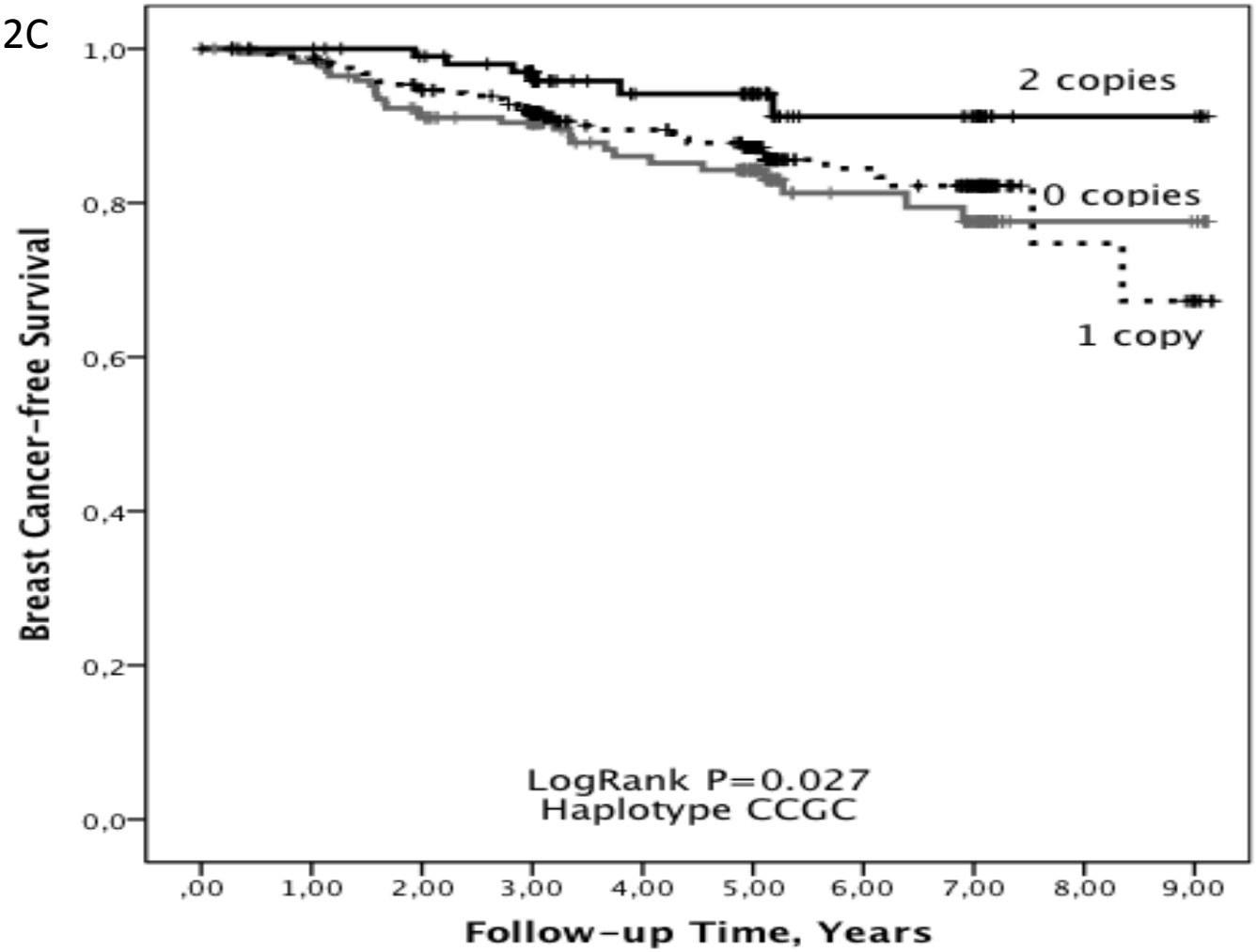
C/C n=	226	222	209	178	127	100	55	44	7	6
C/T n=	274	266	242	215	147	124	70	49	9	5
T/T n=	74	71	64	57	40	31	20	18	5	4

Fig 2B



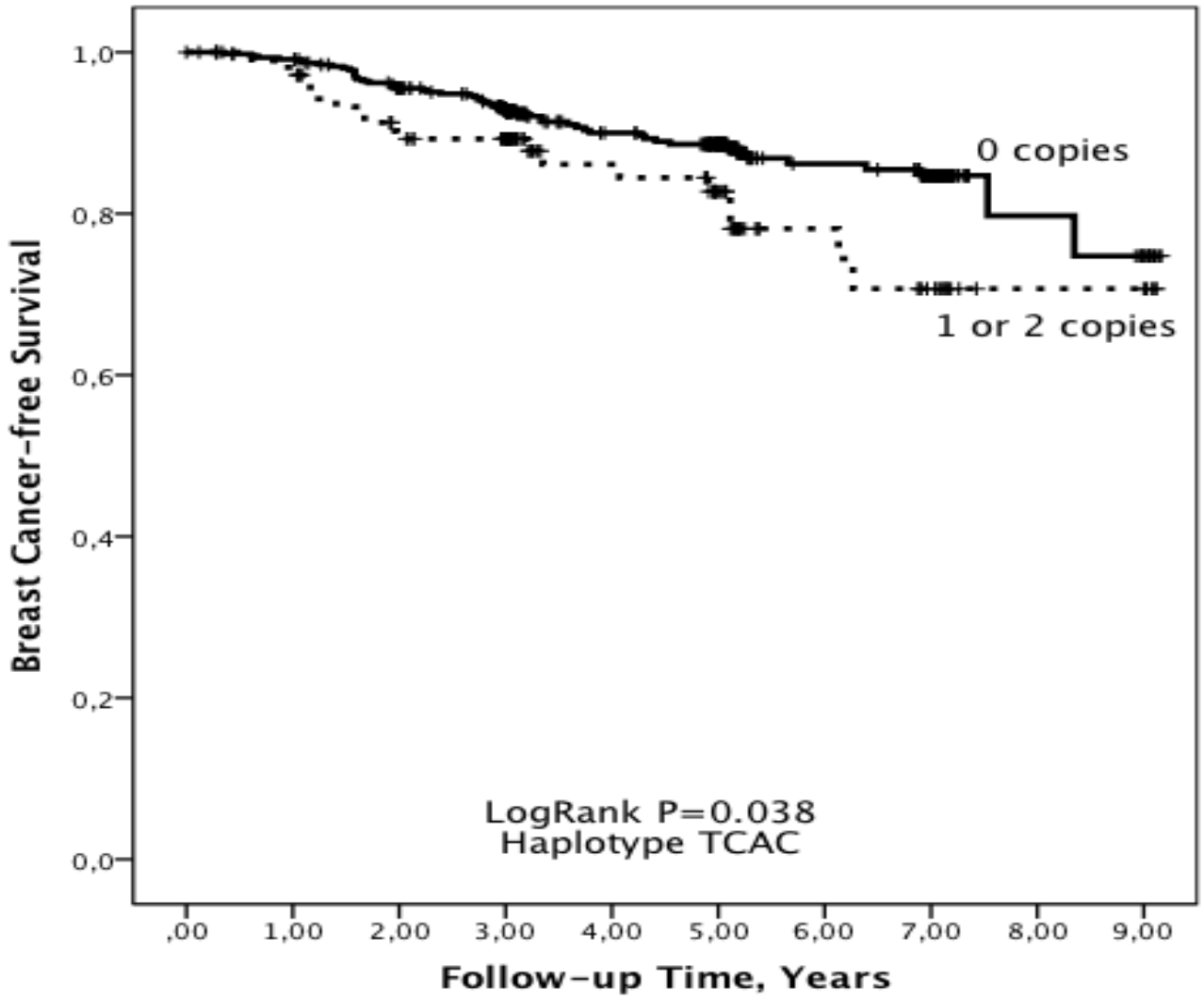
G/G n=	112	109	104	88	57	46	25	20	5	5
A/G n=	288	281	262	231	161	131	76	56	10	5
A/A n=	170	165	145	129	94	77	43	34	6	5

Fig 2C



2 copies n=	108	105	100	84	54	43	24	20	5	5
1 copy n=	286	279	260	230	160	130	75	54	10	5
0 copies n=	174	169	149	132	97	80	44	35	6	5

Fig 2D



0 copies n =	461	449	421	365	259	213	122	94	16	10
1 or 2 copies n=	107	104	88	81	52	40	21	15	5	5

Fig 3A

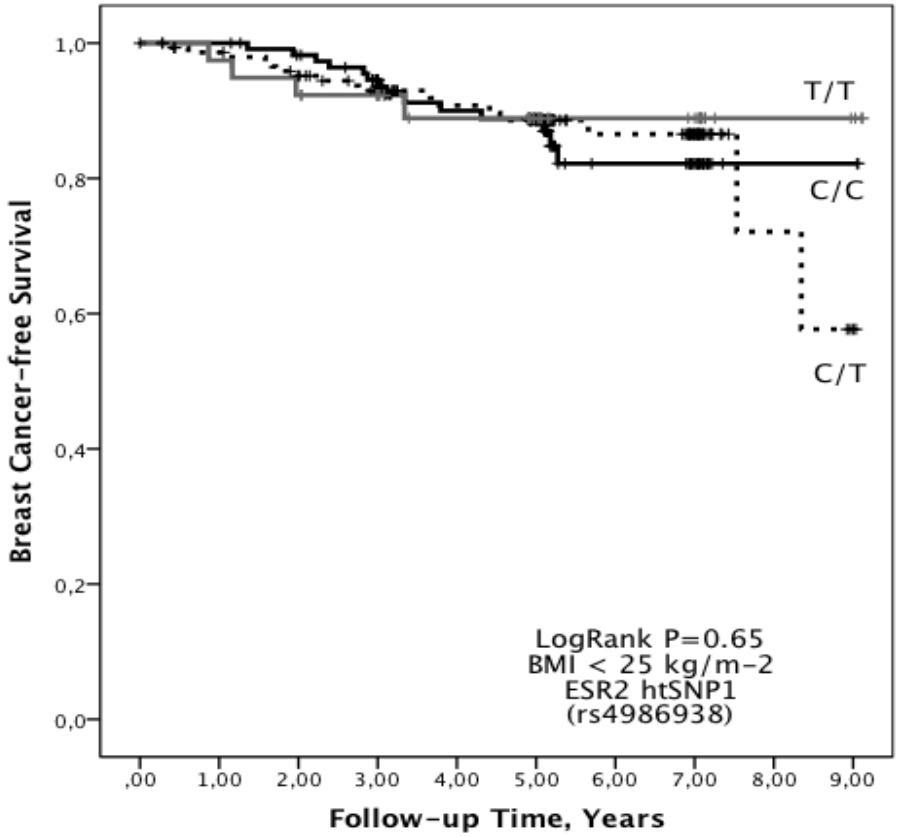
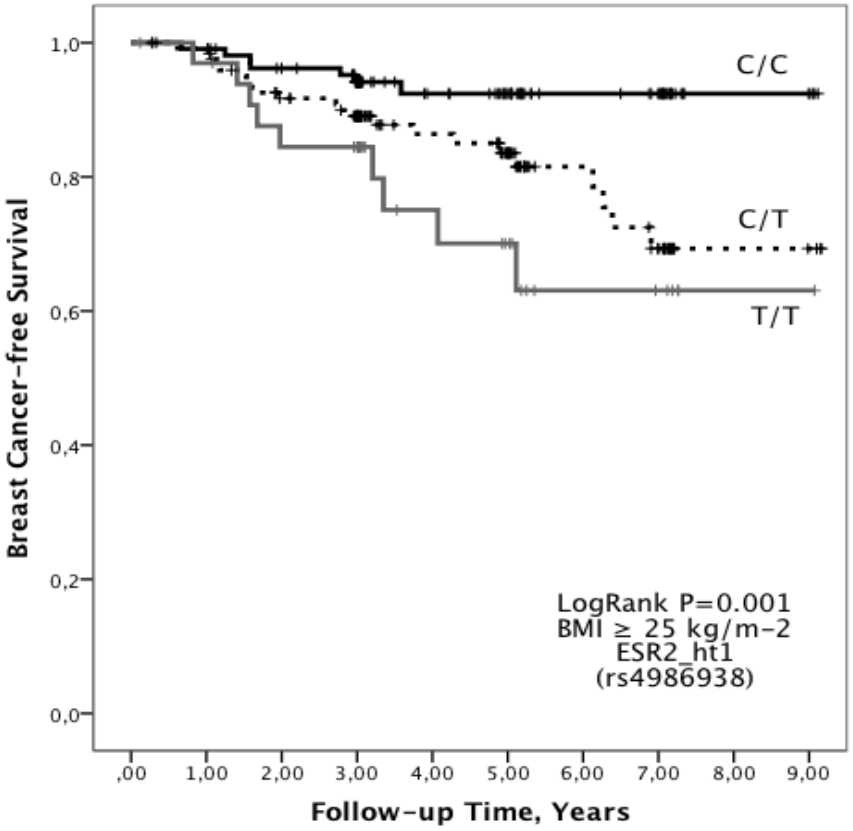


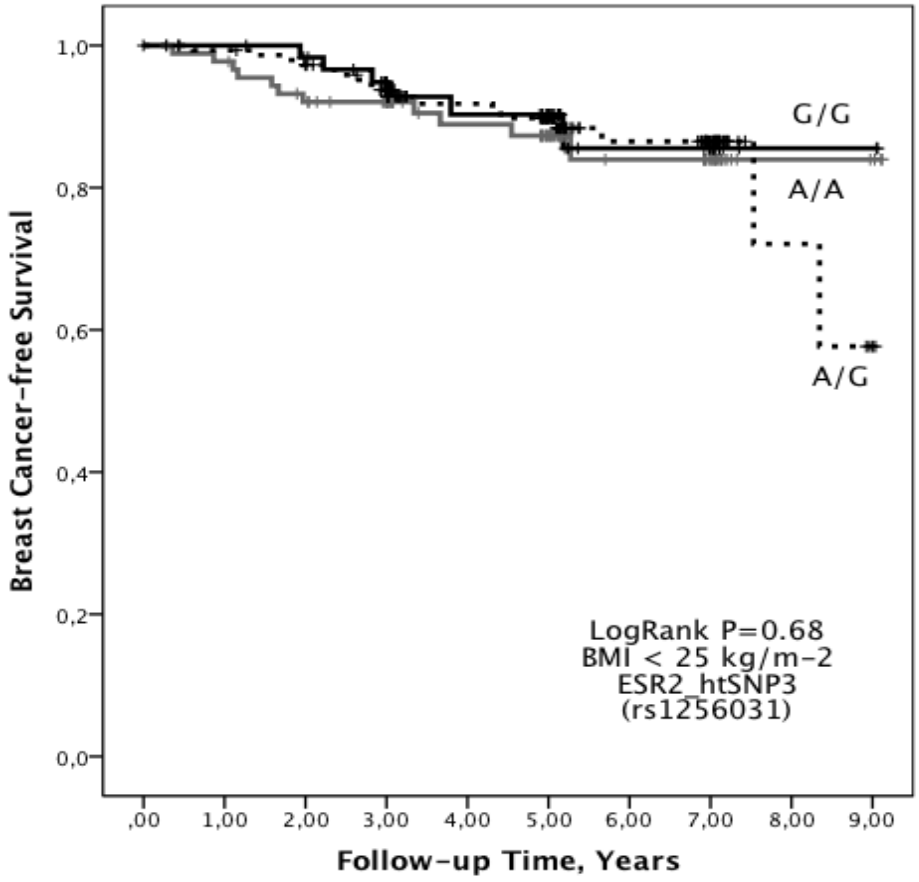
Fig 3B



C/C n=	115	114	109	93	75	59	29	22	2	2
C/T n =	149	143	135	121	83	72	42	28	5	2
T/T n=	39	38	36	32	25	19	14	13	4	3

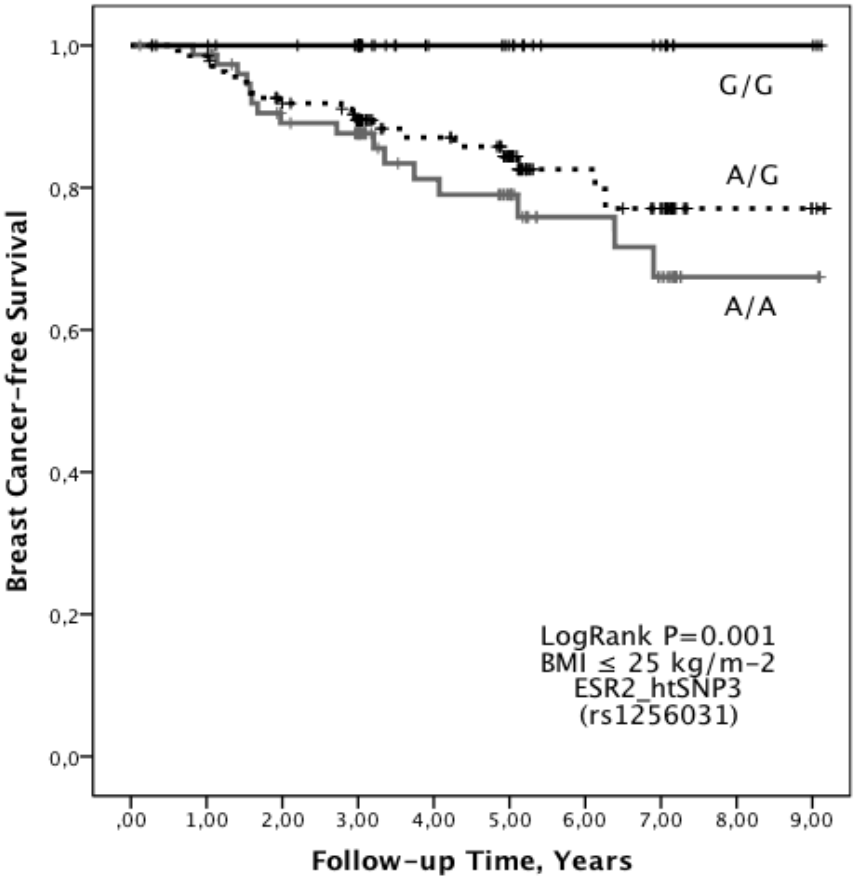
C/C n=	110	107	99	84	51	41	26	22	5	4
C/T n =	124	122	106	93	63	51	27	20	4	3
T/T n=	34	32	27	25	15	12	6	5	1	1

Fig 3C



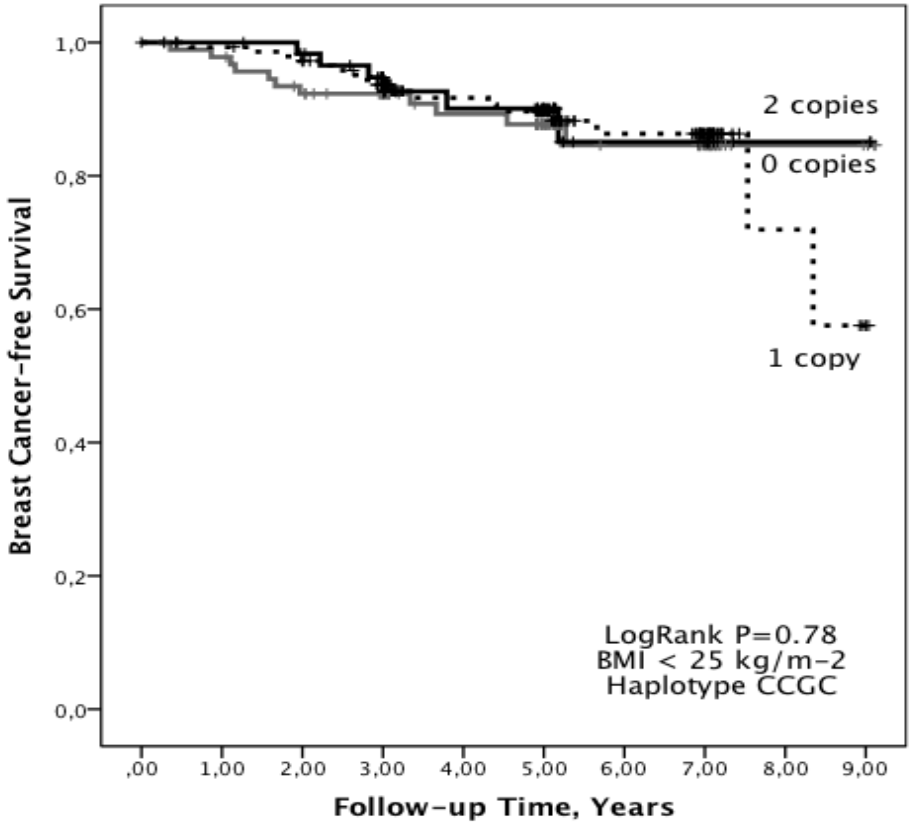
G/G n=	62	61	58	46	36	29	15	12	2	2
A/G n =	150	146	141	127	91	75	46	33	5	2
A/An=	90	87	80	72	56	46	24	18	4	3

Fig 3D



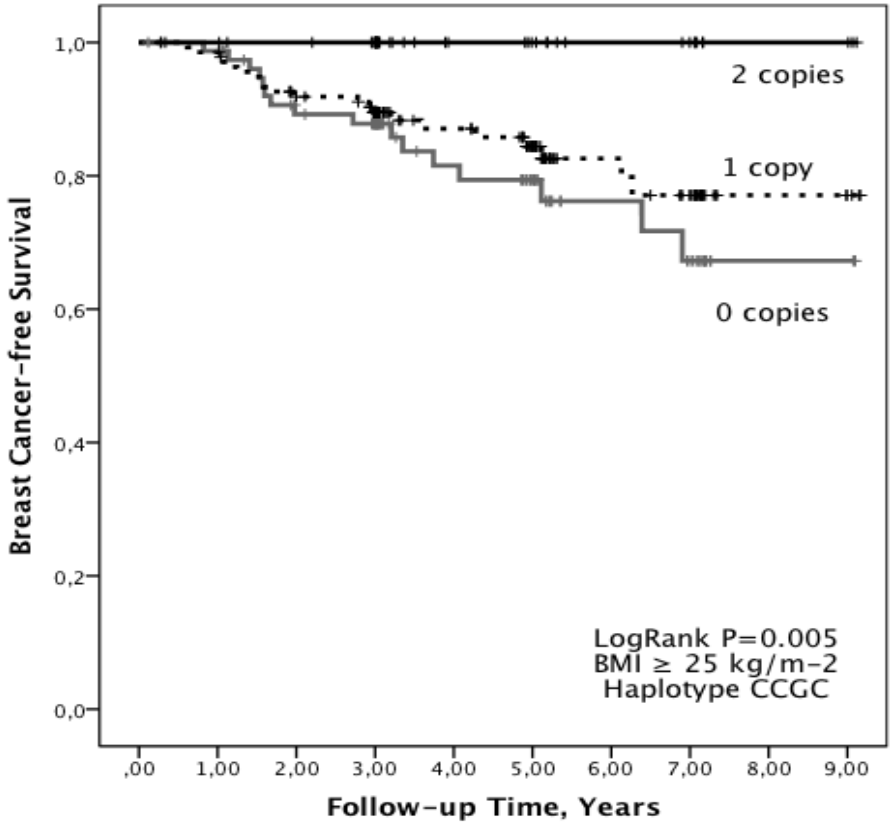
G/G n=	49	47	45	41	20	17	10	8	3	3
A/G n =	138	135	121	104	70	56	30	23	5	3
A/An=	78	76	63	56	37	30	18	15	2	2

Fig 3E



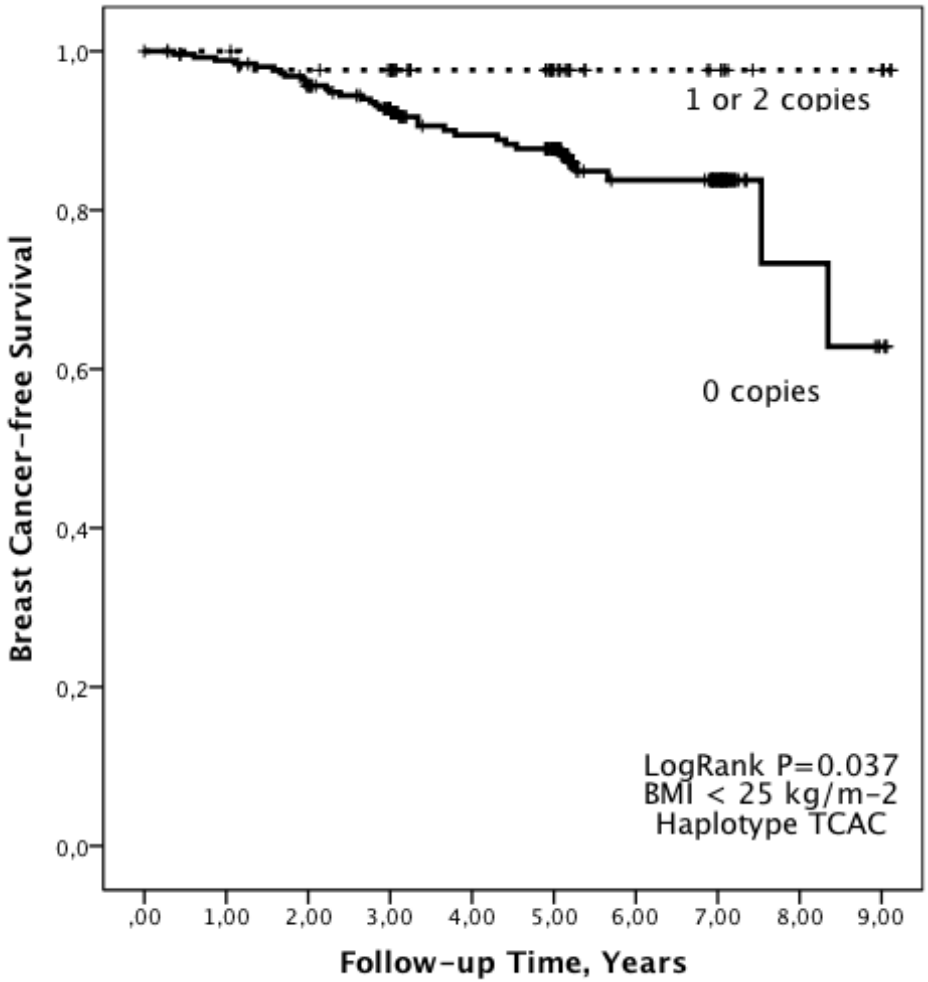
2 copies n= 61	60	57	45	35	28	14	12	2	2
1 copy n = 148	144	139	126	90	74	45	31	5	2
0 copies n= 93	90	83	74	58	48	26	20	4	3

Fig 3F



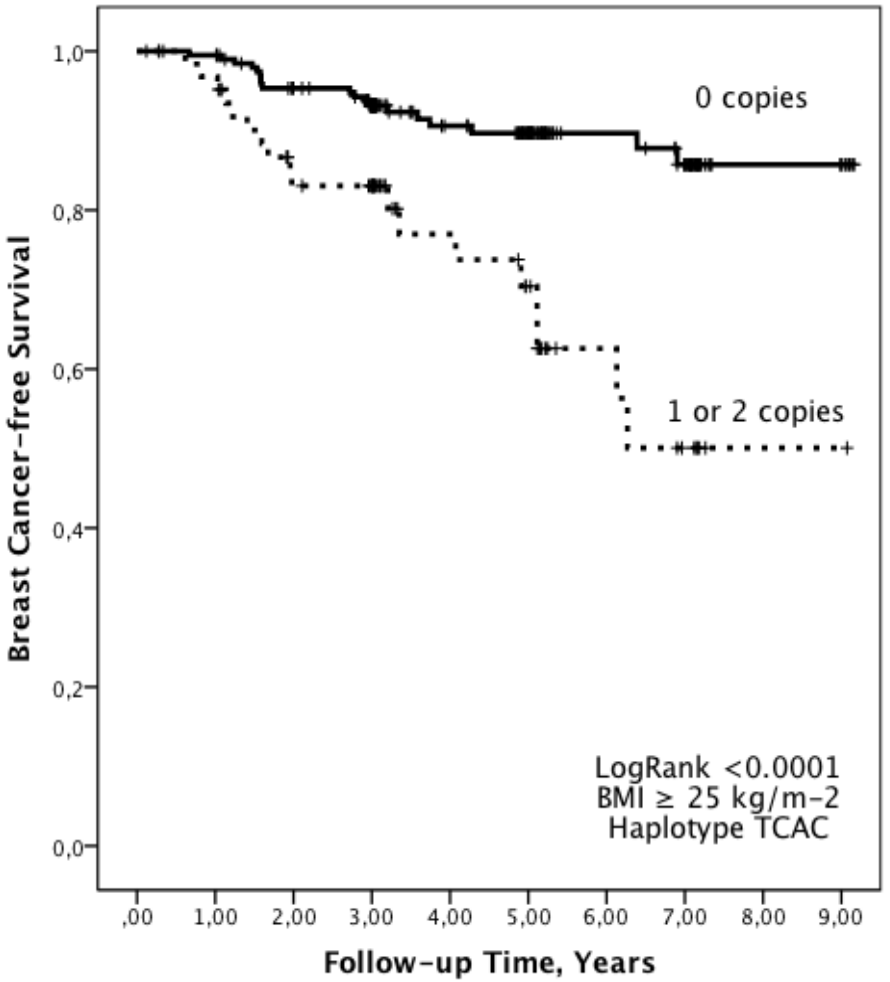
2 copies n= 46	44	42	38	18	15	10	8	3	3
1 copy n = 138	135	121	104	70	56	30	23	5	3
0 copies n= 79	77	64	57	38	31	17	14	2	2

Fig 3G



0 copies n=258	251	238	206	155	129	74	54	7	3
1 or 2 copies n=	44	43	41	39	28	21	11	9	4	4

Fig 3H



0 copies n=201	196	181	157	102	83	47	39	9	7
1 or 2 copies n=	62	60	46	42	24	19	10	6	1	1

Table 1 Patient characteristics and ERβ genotypes

	Age at diagnosis,	BMI, kgs/m2	Waist-Hip ratio	Parous%	HRT*%
All	634	631	630	634	633
median or %	59.6	24.6	0.84	84.7	45.3
IQR	51.1-66.1	22.3-27.8	0.78-0.89		
rs4986938a					
C/C (n)	251	250	250	251	251
median/%	59.1	24.8	0.84	84	47
IQR	52.0-66.0	22.5-28.2	0.80-0.89		
C/T (n)	300	299	298	300	300
median/%	60.9	24.4	0.84	85	46
IQR	51.4-67.6	22.2-27.7	0.79-0.89		
T/T	81	80	80	81	80
median/%	57.4	24.5	0.82	88	38
IQR	48.0-63.3	22.2-27.5	0.77-0.88		
rs1256049a					
C/C (n)	589	586	585	589	589
median/%	59.6	24.6	0.84	84	45
IQR	51.2-65.9	22.3-27.7	0.79-0.89		
C/T (n)	41	41	41	41	41
median/%	60,8	23,6	0,84	95	44
IQR	51.1-67.2	22.1-28.3	0.77-0.90		
T/T (n)	2	2	2	2	2
median/%	62,1	22,6	0,83	100	50
IQR	57.8-62.1	18.7-22.6	0.80-0.83		
rs1256031a					
A/A	186	184	185	186	186
median/%	59.9	24.5	0.83	87	42
IQR	49.7-67.9	22.3-28.0	0.78-0.88		
A/G	316	316	314	316	316
median/%	59.7	24.5	0.84	85	46
IQR	52.4-65.8	22.3-27.9	0.79-0.89		
G/G	126	125	125	126	125
median/%	58,7	24,6	0,83	81	49
IQR	50.5-65.0	22.2-27.7	0.78-0.89		
rs3020450a					
C/C (n)	297	296	296	297	296
median/%	60,1	24.8	0.84	84	49
IQR	52.2-66.6	22.6-28.2	0.79-0.89		
C/T (n)	282	281	279	282	282
median/%	59	24.4	0.84	87	42
IQR	50.0-65.3	22.1-27.5	0.79-0.89		
T/T (n)	53	52	53	53	53
median/%	57.4	23.8	0.81	83	40
IQR	48.4-68.6	21.9-26.4	0.76-0.89		

Abbreviations: ERβ= estrogen receptor beta; IQR= interquartile range.*Ever use of hormone replaceme

Table 2 Patient characteristics and ERβ haplotypes

	Age at diagnosis, years	BMI, kgs/m^2	Waist-Hip ratio	Parous%	HRT*%
Ht _CCGC					
0 (n)	190	188	189	190	189
median (IQR)	59.6 (48.8-67.4)	24.4 (22.3-27.8)	0.83 (0.78-0.88)	87	42
1 (n)	314	314	312	314	314
median (IQR)	59.7 (52.4-65.9)	24.7 (22.3-27.9)	0.84 (0.79-0.89)	84	47
2 (n)	122	121	121	122	122
median (IQR)	59.0 (51.1-65.0)	24.5 (22.0-27.6)	0.83 (0.78-0.89)	81	48
Ht_CCAC					
0 (n)	501	498	497	501	500
median (IQR)	59.6 (50.6-65.4)	24.5 (22.2-27.7)	0.84 (0.78-0.89)	85	44
1 (n)	118	118	118	118	118
median (IQR)	60.1 (53.1-68.8)	24.7 (22.7-28.7)	0.84 (0.79-0.88)	84	52
2 (n)	7	7	7	7	7
median (IQR)	53.6 (45.2-58.0)	26.4 (23.7-29.8)	0.82 (0.78-0.99)	71	29
Ht_CCAT					
(missing=8)					
0 (n)	569	567	565	569	568
median (IQR)	59.6 (51.1-65.7)	24.4 (22.3-27.7)	0.84 (0.78-0.88)	84	46
1 (n)	55	54	55	55	5
median (IQR)	59.9 (50.1-69.7)	25.5 (22.1-27.8)	0.86 (0.81-0.92)	85	42
2 (n)	2	2	2	2	2
median (IQR)	65.2 (62.8-65.2)	27.0 (26.2-27.0)	0.84 (0.83-0.84)	100	50
Ht_CTAC					
0 (n)	583	580	579	583	582
median (IQR)	59.6 (51.2-65.9)	24.6 (22.3-27.7)	0.84 (0.78-0.89)	84	46
1 (n)	41	41	41	41	41
median (IQR)	60.8 (51.1-67.2)	23.6 (22.1-28.3)	0.84 (0.77-0.90)	95	44
2 (n)	2	2	2	2	2
median (IQR)	62.1 (57.8-62.1)	22.6 (18.7-22.6)	0.83 (0.80-0.83)	100	50
Ht_TCAC					
0 (n)	510	508	507	510	509
median (IQR)	58.7 (50.8-65.8)	24.4 (22.2-27.7)	0.83 (0.78-0.89)	84	45
1 (n)	110	109	109	110	110
median (IQR)	61.7 (53.5-68.0)	25.8 (22.9-28.3)	0.84 (0.78-0.90)	85	47
2 (n)	6	6	6	6	6
median (IQR)	59.9 (55.1-66.5)	25.8 (22.4-28.8)	0.85 (0.81-0.89)	100	33
Ht_TCGC					
0 (n)	618	615	614	618	618
median (IQR)	59.6 (51.3-66.0)	24.5 (22.3-27.7)	0.84 (0.78-0.88)	84	45
1 (n)	6	6	6	6	6
median (IQR)	52.2 (47.4-68.9)	24.0 (23.2-26.7)	0.88 (0.83-0.91)	83	33
2 (n)	2	2	2	2	2
median (IQR)	46.4 (43.9-46.4)	28.3 (26.0-28.3)	0.81 (0.81-0.81)	100	100
Ht_TCAT					
0 (n)	336	335	335	336	335
median (IQR)	59.9 (52.0-66.4)	25.0 (22.6-28.0)	0.84 (0.79-0.89)	100	48
1 (n)	255	253	252	255	255
median (IQR)	59.8 (50.6-65.9)	24.4 (22.2-27.5)	0.84 (0.79-0.88)	86	43
2 (n)	35	35	35	35	35
median (IQR)	53.3 (47.7-59.5)	22.7 (21.8-25.9)	0.78 (0.75-0.88)	83	34

Abbreviations: ERβ= estrogen receptor beta; IQR= interquartile range.*Ever use of hormone replaceme

Table 3, htSNPs	All	rs4986938 (CC)	rs4986938 (CT)	rs4986938 (TT)	rs1256049 (CC)	rs1256049 (CT)	rs1256049 (TT)	rs1256031 (AA)	rs1256031 (AG)	rs1256031 (GG)	rs3020450 (CC)	rs3020450 (CT)	rs3020450 (TT)
No pre-operative treatment, n	**592**	**234**	**281**	**75**	**550**	**38**	**2**	**173**	**299**	**114**	**277**	**263**	**50**
Tumor size, n (%)													
In situ	14 (2.4)	7 (3.0)	6 (2.1)	1 (1.3)	14 (2.5)	0	0	3 (1.7)	9 (3.0)	2 (1.8)	4 (1.4)	9 (3.4)	1 (2.0)
≤ 20 mm	424 (71.6)	179 (76.5)	191 (68.0)	53 (70.7)	392 (71.3)	29 (76.3)	2 (100)	122 (70.5)	209 (69.9)	89 (78.1)	213 (76.9)	179 (68.1)	31 (62.0)
21-50 mm	144 (24.3)	47 (20.1)	79 (28.1)	18 (24.0)	135 (24.5)	9 (23.7)	0	42 (24.3)	77 (25.8)	23 (20.2)	59 (21.3)	71 (27.0)	14 (28.0)
> 50 mm	9 (1.5)	0	5 (1.8)	3 (4.0)	8 (1.5)	0	0	5 (2.9)	4 (1.3)	0	1 (0.4)	4 (1.5)	3 (6.0)
T4	1 (0.2)	1 (0.4)	0	0	1 (0.2)	0	0	1 (0.6)	0	0	0	0	1 (2.0)
Missing	0	0	0	0	0	0	0	0	0	0	0	0	0
Histological grade													
I	157 (26.6)	71 (30.3)	70 (25.0)	16 (21.3)	146 (26.6)	11 (28.9)	0	41 (23.7)	76 (25.5)	38 (33.3)	88 (31.8)	60 (22.9)	9 (18.0)
II	308 (52.1)	118 (50.4)	153 (54.6)	35 (46.7)	282 (51.4)	22 (57.9)	2 (100)	92 (53.2)	156 (52.3)	58 (50.9)	135 (48.7)	144 (55.0)	27 (54.0)
III	126 (21.3)	45 (19.2)	57 (20.4)	24 (32.0)	121 (22.0)	5 (13.2)	0	40 (23.1)	66 (22.1)	18 (15.8)	54 (19.5)	58 (22.1)	14 (28.0)
Missing	1	0	1	0	1	0	0	0	1	0	0	1	0
Hormone receptor status													
ER+	502 (86.7)	199 (86.9)	237 (86.5)	64 (86.5)	465 (86.6)	34 (89.5)	1 (50)	144 (85.2)	250 (85.6)	102 (91.1)	239 (87.2)	220 (87.0)	41 (82.0)
ER-	77 (13.3)	30 (13.1)	37 (13.5)	10 (13.5)	72 (13.4)	4 (10.5)	1 (50)	25 (14.8)	42 (14.4)	10 (8.9)	35 (12.8)	33 (13.0)	9 (18.0)
PR+	402 (69.4)	158 (69.0)	190 (69.3)	52 (70.3)	369 (68.7)	31 (81.6)	0	117 (69.2)	196 (67.1)	84 (75.0)	193 (70.4)	174 (68.8)	33 (66.0)
PR-	177 (30.6)	71 (31.0)	84 (30.7)	22 (29.7)	168 (31.3)	7 (18.4)	2 (100)	52 (30.8)	96 (32.9)	28 (25)	81 (29.6)	79 (30.0)	17 (34.0)
Missing	13	5	7	1	13	0	0	4	7	2	3	10	0
Axillary node involvement													
0	368 (62.4)	159 (68.2)	171 (61.1)	36 (48.0)	339 (61.7)	25 (67.6)	2 (100)	102 (59.3)	181 (60.7)	82 (71.9)	184 (66.7)	155 (59.2)	27 (54.0)
1-3	167 (28.3)	54 (23.2)	83 (29.6)	30 (40.0)	159 (29.0)	8 (21.6)	0	54 (31.4)	89 (29.9)	21 (18.4)	66 (23.9)	84 (32.1)	17 (34.0)
4+	55 (9.3)	20 (8.6)	26 (9.3)	9 (12.0)	51 (9.3)	4 (10.8)	0	16 (9.2)	28 (9.4)	11 (9.6)	26 (23.9)	23 (8.8)	6 (12.0)
Missing	2	1	1	0	1	1	0	1	1	0	1	1	0

Table 4	Tumor size				Histological grade			Hormone receptor				Axillary lymph nodes		
Haplotype Copy Number	*In situ*	*≤ 20 mm*	*21-50 mm*	*> 50 mm*	*I*	*II*	*III*	*ER+*	*ER-*	*PR+*	*PR-*	*0*	*1-3*	*4+*
All Patients*														
n=592 #	14 (2.4)	424 (71.6)	144 (24.3)	9 (1.5)	157 (26.6)	308 (52.1)	126 (21.3)	502 (86.7)	77 (13.3)	402 (69.4)	177 (30.6)	368 (62.4)	167 (28.3)	55 (9.3)
HtCCGC														
0 (n=177)	3 (1.7)	125 (70.6)	44 (24.9)	4 (2.3)	41 (23.2)	95 (53.7)	41 (23.2)	147 (85.0)	26 (15.0)	119 (68.8)	54 (31.2)	105 (59.7)	54 (30.7)	17 (9.7)
1 (n=297)	9 (3.0)	207 (69.7)	77 (25.9)	4 (1.3)	77 (26.0)	153 (51.7)	66 (22.3)	249 (85.9)	41 (14.1)	195 (67.2)	95 (32.8)	178 (60.1)	89 (30.1)	29 (9.8)
2 (n=110)	2 (1.8)	87 (79.1)	21 (19.1)	0	37 (33.6)	56 (50.9)	17 (15.5)	98 (90.7)	10 (9.3)	81 (75.0)	27 (25.0)	80 (72.7)	21 (19.1)	9 (8.2)
HtCCAC														
0 (n=464)	11 (2.4)	331 (71.3)	113 (24.4)	8 (1.7)	121 (26.1)	241 (52.1)	101 (21.8)	396 (85.3)	59 (13.0)	321 (70.5)	134 (29.5)	284 (61.5)	131 (28.4)	47 (10.2)
1 (n=114)	3 (2.6)	82 (71.9)	29 (25.4)	0	31 (27.2)	60 (52.6)	23 (20.2)	93 (84.5)	17 (15.5)	71 (64.5)	39 (35.5)	76 (66.7)	30 (26.3)	8 (7.0)
2 (n=6)	0	6 (100.0)	0	0	3 (50.0)	3 (50.0)	0	5 (83.3)	1 (16.7)	3 (50.0)	3 (50.0)	3 (50.0)	3 (50.0)	0
HtCCAT														
0 (n=530)	11 (2.1)	384 (72.5)	128 (24.2)	7 (1.3)	145 (27.4)	276 (52.2)	108 (20.4)	452 (87.1)	67 (12.9)	362 (68.3)	157 (29.6)	329 (62.2)	152 (28.7)	48 (9.1)
1 (n=52)	2 (3.8)	35 (67.3)	14 (26.9)	1 (1.9)	10 (19.2)	27 (51.9)	15 (28.8)	41 (82.0)	9 (18.0)	32 (64.0)	18 (36.0)	32 (62.7)	12 (23.5)	7 (13.7)
2 (n=2)	1 (50.0)	0	0	1 (50.0)	0	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	1 (50.0)	2 (100.0)	0	0
HtCTAC														
0 (n=544)	14 (2.6)	388 (71.3)	133 (24.4)	8 (1.5)	144 (26.5)	280 (51.6)	119 (21.9)	459 (86.4)	72 (13.6)	364 (68.5)	167 (31.5)	336 (61.9)	156 (28.7	51 (9.4)
1 (n=38)	0	29 (76.3)	9 (23.7)	0	11 (28.9)	22 (57.9)	5 (13.2)	34 (89.5)	4 (10.5)	31 (81.6)	7 (18.4)	25 (67.6)	8 (21.6)	4 (10.8)
2 (n=2)	1	2 (100.0)	0	0	0	2 (100.0)	0	1 (50%)	1 (50%)	0	2 (100.0)	2 (100.0)	0	0
HtTCAC														
0 (n=476)	13 (2.7)	339 (71.2)	117 (24.6)	6 (1.3)	123 (25.8)	260 (54.6)	92 (19.3)	403 (86.9)	61 (13.1)	318 (68.5)	146 (31.5)	302 (63.6)	129 (27.2)	44 (9.3)
1 (n=103)	1 (1.0)	75 (72.8)	25 (24.3)	2 (1.9)	31 (30.1)	41 (39.8)	31 (30.1)	86 (84.3)	16 (15.7)	73 (71.6)	29 (28.4)	59 (57.8)	34 (33.3)	9 (8.8)
2 (n=5)	0	5 (100.0)	0	0	1 (20.0)	3 (60.0)	1 (20.0)	5 (100.0)	0	4 (80%)	1 (20.0)	2 (40.0)	1 (20.0)	2 (40.0)
HtTCGC														
0 (n=576)	14 (2.4)	414 (71.9)	139 (24.1)	9 (1.6)	154 (26.8)	299 (52.0)	122 (21.2)	487 (86.5)	76 (13.5)	390 (69.3)	173 (30.7)	357 (62.2)	164 (28.6)	53 (9.2)
1 (n=6)	0	4 (66.7)	2 (33.3)	0	1 (16.7)	3 (50.0)	2 (33.3)	5 (83.3)	1 (16.7)	3 (50.0)	3 (50.0)	5 (83.3)	0	1 (16.7)
2 (n=2)	0	1 (50.0)	1 (50.0)	0	0	2 (100.0)	0	2 (100.0)	0	2 (100.0)	0	1 (50.0)	0	1 (50.0)
HtTCAT														
0 (n=313)	7 (2.2)	237 (75.7)	67 (21.4)	2 (0.6)	94 (30.0)	155 (49.5)	64 (20.4)	265 (86.0)	43 (14.0)	213 (69.2)	95 (30.8)	207 (66.6)	74 (23.8)	30 (9.6)
1 (n=239)	7 (2.9)	161 (67.4)	66 (27.6)	5 (2.1)	55 (23.0)	131 (55.0)	52 (21.8)	203 (87.9)	28 (12.1)	162 (70.1)	69 (29.9)	141 (59.0)	76 (31.8)	22 (9.2)
2 (n=32)	0	21 (65.6)	9 (28.1)	2 (6.3)	6 (18.8)	18 (56.3)	8 (25.0)	26 (81.3)	6 (18.8)	20 (62.5)	12 (37.5)	15 (46.9)	14 (43.8)	3 (9.4)

**Only patients without pre-operative treatment*

#Histological grade was missing for 1 patient, Hormone receptor status was missing for 13 patients, axillary lymph node status was missing for 2 patients.