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Effect of *CYP19* rs6493497 and rs7176005 haplotype status on *in vivo* aromatase transcription, plasma and tissue estrogen levels in postmenopausal women

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ABSTRACT

High plasma levels of estradiol (E_2) are correlated to increased breast cancer risk in postmenopausal women. Recently, a study reported two single nucleotide polymorphisms (SNPs) (rs6493497; C → T and rs7176005; G → A) in the aromatase (*CYP19*) promoter 1.1 to be associated with elevated plasma E_2 levels, most likely due to enhanced transcription. *In silico* predictions suggested increased transcription factor binding for the rs7176005_A allele. We genotyped 46 breast cancer patients for rs6493497 and rs7176005 status and assessed the potential association between *CYP19* SNP status and (i) CYP19 mRNA levels in tumour and normal breast tissue, and (ii) estrogen levels in plasma, tumour and normal breast tissue. In addition, we measured *CYP19* SNP status and correlated it to plasma estrogen levels in a confirmatory dataset of 108 healthy postmenopausal women. We found no correlation between either of the two SNPs and CYP19 mRNA level. In the breast cancer patients, the rs6493497_T/rs7176005_A variant haplotype was associated with low plasma estrone (E_1) ($p = 0.038$) and low E_2 ($p = 0.050$) levels; however, no correlation was recorded between SNP status and plasma estrogen levels in the cohort of 108 healthy postmenopausal women. Our findings indicate rs6493497 and rs7176005 status not to enhance *CYP19* transcription or increase estrogen levels in postmenopausal women.

1. Introduction

Estrogens play a key role in breast cancer development, and early loss of ovarian function substantially reduces breast cancer risk in premenopausal women [1]. More recent evidence has linked high plasma estradiol (E_2) concentrations to an increased risk of breast cancer in postmenopausal women [2]. The pivotal role of estrogens promoting growth of estrogen receptor positive (ER+) breast cancer is further underlined by the profound antitumor effects of endocrine therapy [3].

After menopause, estrogens are produced by peripheral aromatisation. The main pathway is aromatisation of androstenedione into estrone (E_1), with a smaller contribution from aromatisation of testosterone into E_2 [4]. Both reactions are catalyzed by the aromatase enzyme (CYP19). Currently, aromatase inhibitors (AIs) represent first line endocrine treatment for postmenopausal women (reviewed in Ref. [5]).

The aromatase (CYP19) enzyme is a member of the cytochrome P450 family. The protein is encoded by the *CYP19* gene, localized on chromosome15q21.2 [6,7]. *CYP19* consists of nine coding exons (II–X), and several alternative, untranslated first exons that are expressed under the control of tissue-specific promoters [8,9]. Thus, while *CYP19* transcription is regulated by different promoters in different tissues, the gene codes for a single CYP19 protein only.

In healthy breast adipose tissue *CYP19* is expressed at low, but distinct levels [10], primarily expressed from promoter 1.4 [8]. In breast cancer tissue, there is a switch in promoter usage, and *CYP19* is expressed via promoter II, 1.3 and 1.7, in addition to promoter 1.4 [8]. Thus, alternative promoter use in malignant breast tissue probably explains elevated total CYP19 mRNA level recorded in breast cancer as compared with normal breast tissue (reviewed in Ref. [11]).

Genetic variations in *CYP19* have been extensively studied, and several single nucleotide polymorphisms (SNPs) have been identified [12]. While conflicting evidence has linked various *CYP19* SNPs to elevated enzyme activity [12,13], plasma estrogen levels [14–17] and breast cancer risk [18–20], there is no general consensus whether any of these SNPs affect CYP19 enzyme activity. Ma and co-workers [12] identified 88 *CYP19* SNPs, including two

Abbreviations: E_2, estradiol; E_1, estrone; E_1S, estrone sulphate; CYP19, aromatase; AI, aromatase inhibitor; SNP, single nucleotide polymorphism; ER, estrogen receptor.

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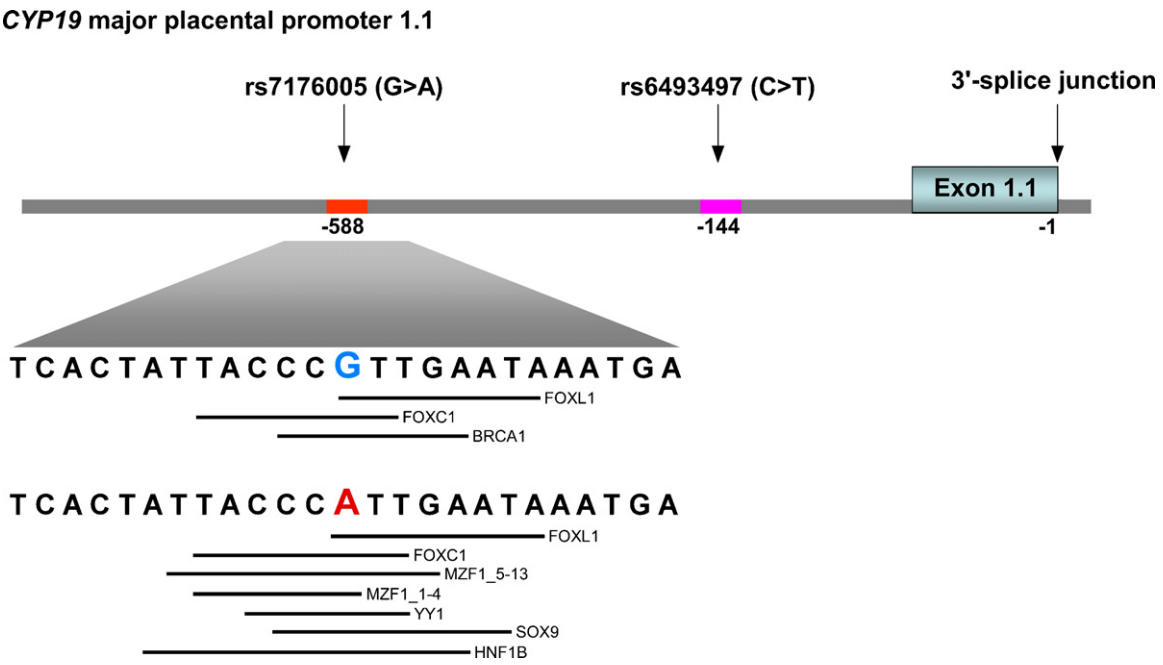

Fig. 1. Schematic presentation of the *CYP19* promoter 1.1. The 3′-end of exon 1.1 is spliced onto a common splice junction in exon II, immediately upstream of the coding region, giving rise to a 5′-UTR contiguous with the first coding exon (exon II) [10]. SNPs rs6493497 and rs7176005 are located 144 and 588 nucleotides upstream of the 3′-splice junction in exon 1.1. The transcription factor binding sites predicted by JASPAR for the rs7176005 wild-type sequence (G), and the variant haplotype (A) are indicated by lines under the sequences. The predicted binding-scores for the various binding sites are summarized in Table 2.

SNPs affecting promoter 1.1, the major promoter regulating *CYP19* expression in placental tissue [8,9]. The two SNPs (rs6493497; C → T and rs6493497; G → A) locates 144 bp and 588 bp upstream of the 3′-splice junction in exon 1.1, respectively (depicted in Fig. 1). Recently, the rs6493497_T and rs7176005_A variants were reported to be associated with elevated total CYP19 enzyme activity in breast tumour tissue, elevated plasma E_2 levels and a greater inhibitory effect of AIs [21]. Based on their findings, the authors suggested these SNPs to possibly increase *CYP19* transcription *in vivo*.

An effect of rs6493497 and rs7176005 status on plasma and tissue estrogen levels may have significant implications to breast cancer therapy and, potentially, endocrine prevention. The aim of this study was to confirm the impact of the *CYP19* rs6493497_T/rs7176005_A haplotype on estrogen levels in postmenopausal women. To do so, we evaluated the potential impact of these two SNPs on transcription factor binding by *in silico* analyses. Subsequently, we determined rs6493497/rs7176005 haplotype status and correlated it to (i) CYP19 mRNA levels in tumour and normal tissue from 46 breast cancer patients and (ii) estrogen levels in plasma, normal breast and breast cancer tissue previously determined in the subcohort of 33 postmenopausal patients from the same series [22]. Finally, we compared the rs6493497/rs7176005 haplotype status with plasma estrogen levels in a confirmatory cohort of 108 healthy postmenopausal women.

2. Materials and methods

2.1. Subjects

The breast cancer patients included in this study have been described previously (see [22] for details). In brief, normal breast and breast cancer tissue specimens were collected from 46 breast cancer patients (13 pre- and 33 postmenopausal women) undergoing mastectomy at Haukeland University Hospital, Bergen, Norway. Women using oral hormone replacement therapy or contraceptives were excluded. The samples were snap-frozen in liquid nitrogen immediately upon removal in the operating theatre, and stored in liquid nitrogen until processing. Plasma and tissue estrogen levels in these patients have been reported elsewhere [22]. Here, we determined rs6493497 and rs7176005 status and CYP19 mRNA levels in benign breast and breast cancer tissue in these patients.

To confirm a potential effect of rs6493497 and rs7176005 status on plasma estrogen levels, we determined both SNPs in white blood cells from a cohort of 108 healthy postmenopausal woman previously characterized for plasma estrogen values [23].

2.2. Estrogen measurements

Plasma and tissue estrogen levels in the breast cancer patients and plasma levels in the cohort of healthy women have previously been reported [22,23]. The samples were analyzed by highly sensitive and specific radioimmunoassays involving sample prepurification steps described in detail elsewhere [24,25].

2.3. DNA extraction

Genomic DNA was extracted using QIAamp DNA Mini Kit (Qiagen) according to the manufacturer's protocol.

2.4. PCR amplification and sequencing

The genomic region covering the *CYP19* SNPs rs6493497 and rs7176005 was amplified and sequenced using the primers listed in Table 3. PCR amplification was performed using the DyNazyme EXT polymerase system (FINNZYMES) in a 50 μL reaction mix containing 1 × PCR buffer, 1.5 mM $MgCl_2$, 0.5 mM of each deoxynucleotide triphosphate, 5% DMSO, 0.2 μM of each primer, 0.5 U DyNazyme polymerase and ~5–50 ng genomic DNA. The thermocycling conditions used were an initial denaturation step of 5 min at 94 °C, followed by 30 cycles of denaturation (94 °C for 1 min), annealing (59.1 °C for 30 s) and elongation (72 °C for 1 min), and a final elongation step of 7 min at 72 °C. Following amplification, the PCR product was treated with ExoSAP-IT® (USB® Products, Affymetrix, Inc.) at 37 °C for 30 min and 80 °C for 15 min according to the

Table 1
Distribution of *CYP19* SNPs rs6493497 and rs7176005, and correlations to tissue and plasma estrogen levels.

	CYP19 exon 1.1 SNPs: genotypes		Correlation to plasma estrogens[a]			Correlation to tumour breast tissue estrogens[a]			Correlation to normal breast tissue estrogens[a]		
	rs6493497: C>T	rs7176005: G>A	E_2	E_1	E_1S	E_2	E_1	E_1S	E_2	E_1	E_1S
Breast cancer patients ($n=33$)	CC: $n=27$ CT: $n=6$	GG: $n=27$ GA: $n=6$	$p=0.050$	$p=0.038$	$p=0.119$	$p=0.107$	$p>0.500$	$p>0.500$	$p>0.500$	$p>0.500$	$p>0.500$
Healthy women ($n=108$)	CC: $n=89$ CT: $n=19$	GG: $n=89$ GA: $n=19$	$p>0.500$	$p=0.289$	$p>0.500$	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]	NA[b]

[a] Mann–Whitney rank-test.
[b] Not available.

manufacturer's protocol. DNA sequencing was performed in a 10 μL reaction mix containing 1 × sequencing buffer, 1 μM primer and 1 × BigDye v.1.1 (Applied Biosystems). Capillary electrophoresis was performed on an automated DNA sequencer (ABI 3730), and the sequences were analyzed using the Sequence Scanner v. 1.0 software (Applied Biosystems).

2.5. RNA extraction and cDNA synthesis

Total RNA was extracted from snap-frozen biopsies using Trizol reagent (Life technologies) according to the manufacturer's protocol, and dissolved in DEPC-treated deionised water as described by Knappskog et al. [26]. The RNA concentration in all samples was determined using a Nanodrop ND1000 spectrophotometer and adjusted to 25 ng/μL. Single strand cDNA was synthesised from 125 ng total RNA, using the Transcriptor reverse transcriptase system (Roche) according to the manufacturer's protocol. Both oligoT (16-mers) and random hexamers were used as primers in the cDNA synthesis reaction mix.

2.6. Quantitative PCR (qPCR)

CYP19 mRNA levels were quantified in duplex reactions together with the ribosomal protein P2 (RPLP2) mRNA, using the Lightcycler 480 instrument (Roche). The amplification primers and BlackBerry-quenched hydrolysis probes (TIB MOLBIOL) that were used are listed in Table 3. Amplification was performed using the LC480 Probes Master (Roche) reaction mix, 0.5 μM of each primer, 0.125 μM of each hydrolysis probe and 0.5 μL cDNA synthesised from 125 ng total RNA. The following thermocycling conditions were used: Initial denaturation at 95 °C for 5 min, 50 cycles of denaturation at 95 °C for 10 s, annealing/elongation at 55 °C for 30 s, and a final cooling step at 40 °C for 10 s. Water was used as negative control in each run. For each analysis, the results were converted into relative concentrations using an *in run* standard curve, and the observed relative concentrations for CYP19 mRNA were normalised by RPLP2 mRNA levels. Each analysis was performed in triplicate runs.

2.7. In silico predictions

Putative transcription factor binding sites and binding strength in the area ±12 nucleotides of SNPs rs6493497 and rs7176005 in the promoter area of *CYP19* exon 1.1 were predicted both for the wild-type and the variant haplotype, using the JASPAR database (http://jaspar.genereg.net) [27] at 80% profile score threshold (default settings).

2.8. Statistical analysis

The statistical analyses were performed using the PASW Statistics 18.0 software package (Chicago, IL, USA). All *p*-values are given as two-sided.

3. Results

3.1. Distribution of SNPs rs6493497 and rs7176005

Normal and tumour breast tissue samples from 46 patients (13 pre- and 33 postmenopausal) were available for *CYP19* genotyping. Each of the 13 premenopausal individuals examined harboured the wild-type sequence for rs6493497 and rs7176005. Among the postmenopausal women 6 of 33 individuals (18.2%) revealed heterozygosity for the rs6493497_T-allele and rs7176005_A-allele (Table 1). The two SNPs occurred in concert in all 6 individuals, suggesting they may constitute a distinct haplotype. While Wang and co-workers [21] reported these SNPs to be tightly linked, they did not provide figures for individual haplotypes. We found no individual homozygous for either the rs6493497_T or rs7176005_A genotype. Thus, the allele distribution of the SNPs was in accordance with Hardy-Weinberg equilibrium.

3.2. rs7176005 alters CYP19 transcription factor binding in silico

Using the JASPAR database (http://jaspar.genereg.net) [27], we compared the predicted transcription factor binding sites and binding strengths for the *CYP19* variant haplotype rs6493497_T/rs7176005_A versus the wild-type sequence (rs6493497_C/rs7176005_G). Our predictions revealed no significant differences in the transcription factor binding scores between the C- and T-allele of rs6493497. However, the analysis predicted five additional transcription factor binding sites for the rs7176005_A-allele compared to the wild-type G-allele, in addition to increased binding strength for two of the binding sites identified in the wild-type allele (see details depicted in Fig. 1, and binding scores listed in Table 2). These findings indicate that individuals harbouring the rs7176005_A-allele could have increased *CYP19* transcription directed from this locus.

Table 2
In silico predictions for transcription factor binding to the rs7176005 G- and A-alleles.

Transcription factor bindings sites (predicted by JASPAR)	rs7176005_G	Rs7176005_A
	Score	Score
FOXC1	4.086	4.349
FOXL1	4.478	5.135
BRCA1	5.642	–
HNF1B	–	9.381
MZF_5–13	–	8.038
MZF_1–4	–	4.846
YY1	–	5.236
SOX9	–	8.311

Table 3
Primers and probes used in this study.

Primers/probes	Sequences	PCR/sequencing
CYP19 exon1.1_Forward1	CTCTCAGCAATACCCACCATTAACC	1. Round PCR
CYP19 exon1.1_Reverse2	GAGTGTCTGATCCCACAGGTTG	1. Round PCR
CYP19 exon1.1_Forward1	CTCTCAGCAATACCCACCATTAACC	2. Round PCR
CYP19 exon1.1_Reverse3	AGATCCCGAGCACAGGAC	2. Round PCR
CYP19 exon1.1_Forward2	TCACCCCCAACACATAGCAC	Sequencing
CYP19 forward primer	ATCCTCAATACCAGGTCCTGGC	qPCR
CYP19 reverse primer	AGAGATCCAGACTCGCATGAATTCT	qPCR
CYP19 FAM-labelled probe	6FAM-ACCCGGTTGTAGTAGTTGCAGGCACT-BBQ	qPCR
RPLP2 forward primer	GACCGGCTCAACAAGGTTAT	qPCR
RPLP2 reverse primer	CCCCACCAGCAGGTACAC	qPCR
RPLP2 Cy5-labelled probe	Cy5-AGCTGAATGGAAAAAACATTGAAGACGTC-BBQ	qPCR

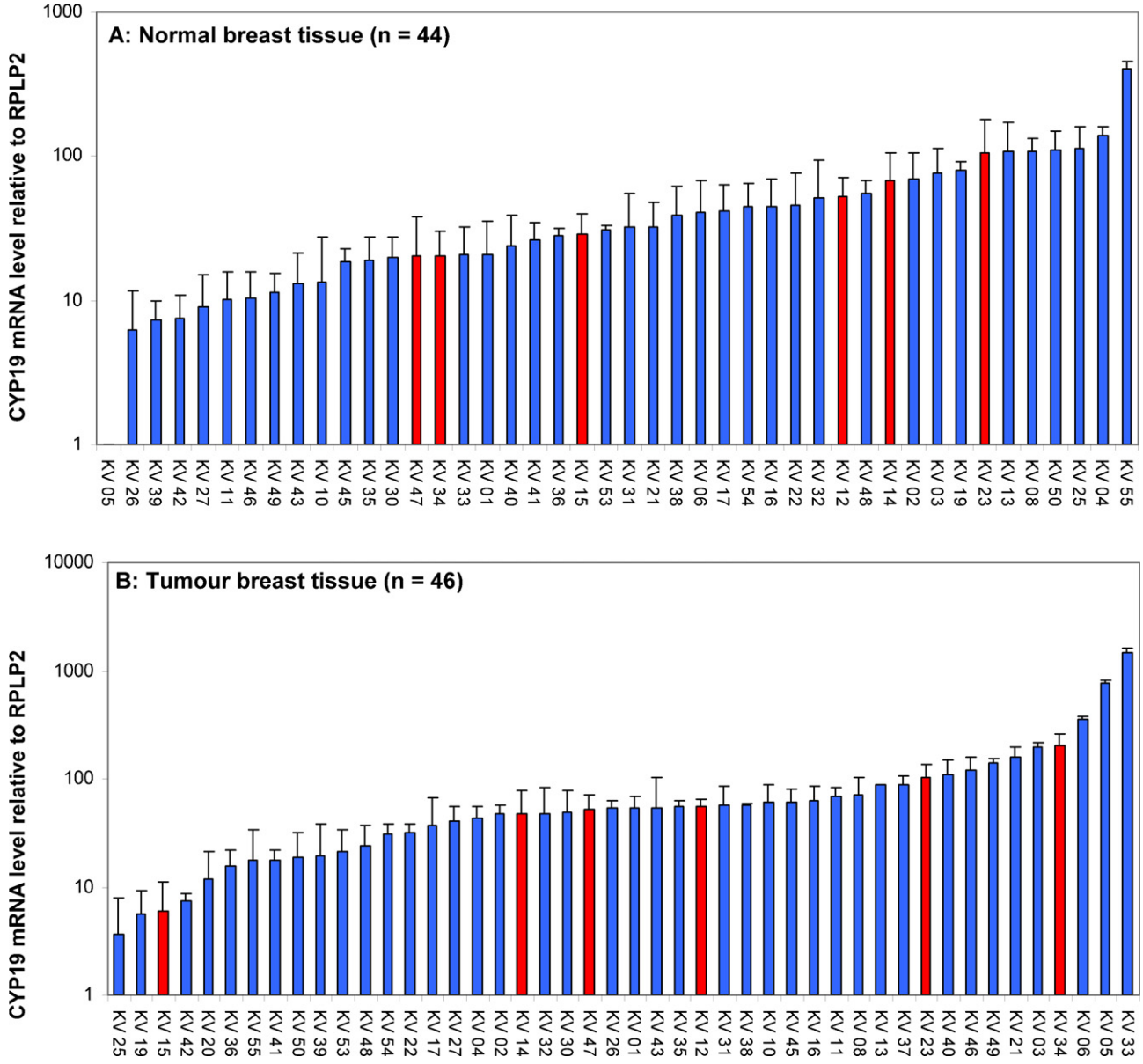

Fig. 2. *CYP19* expression related to rs6493497 and rs7176005 SNP-status in breast cancer patients. (A) Normal breast tissue and (B) tumour breast tissue. The samples are sorted according to increasing CYP19 mRNA level. Patients harbouring the rs6493497_T/rs7176005_A – haplotype variant are indicated by red bars. No association between the haplotype variant and CYP19 mRNA level in either normal breast or tumour breast tissue was observed ($p > 0.5$).

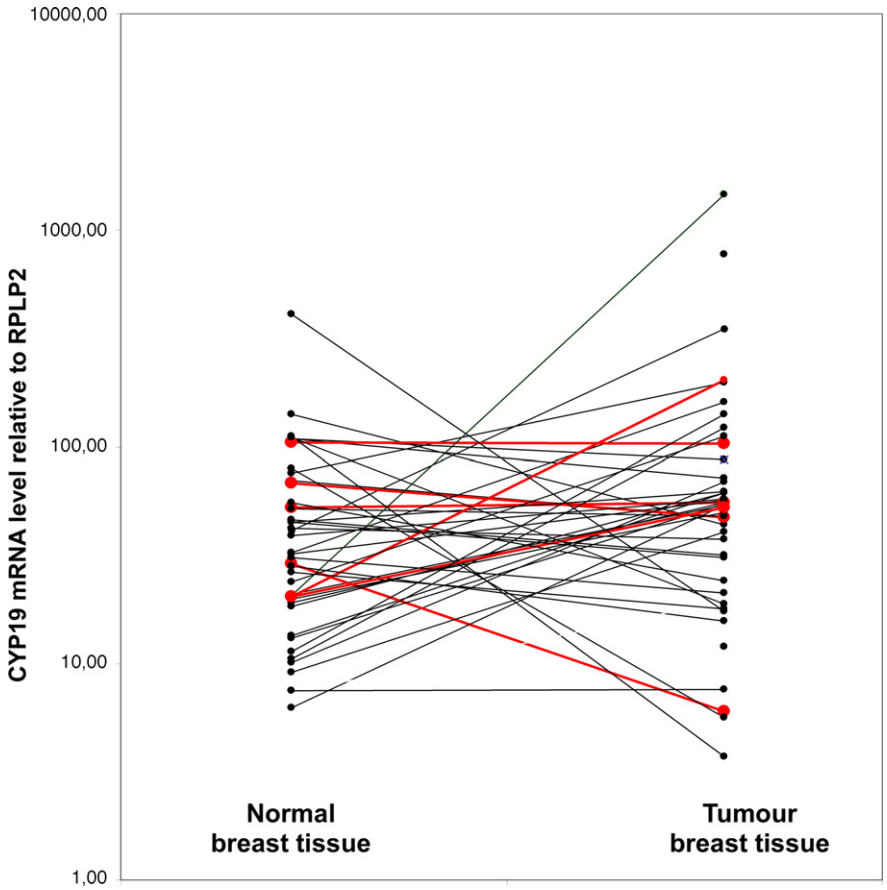

Fig. 3. *CYP19* expression in normal and tumour breast tissue. Patients harbouring the rs6493497_T/rs7176005_A – haplotype variant are indicated by red lines, and those harbouring the wild-type sequence with black lines. As may be seen, no consistent trend for a higher or lower *CYP19* expression level in tumour as compared to normal breast tissue was observed among patients harbouring the rs6493497_T/rs7176005_A – haplotype variant or wild-type *CYP19*.

3.3. CYP19 expression level in relation to SNPs rs6493497 and rs7176005 SNP in breast cancer patients

CYP19 mRNA levels in normal and tumour breast tissue were measured by qPCR. The CYP19 mRNA data for 34 of these breast cancer patients were included in a previous study [10] and correlated to tissue estrogen levels but not to *CYP19* haplotype status. Here, we present the CYP19 mRNA levels for the entire patient cohort ($n = 46$) and correlate expression levels to haplotype status.

CYP19 mRNA levels were not different between individuals harbouring the rs6493497_T/rs7176005_A – haplotype or those harbouring the *CYP19* wild-type sequence in either normal or tumour breast tissue (See Fig. 2; $p > 0.5$ for both, and Fig. 3). Similar findings were recorded when limiting the analysis to the subgroup of 33 postmenopausal patients (data not shown). Due to the low number of premenopausal patients ($n = 13$), no separate statistical analysis was performed within this subgroup.

3.4. SNPs rs6493497 and rs7176005 in relation to plasma and tissue estrogen levels

Plasma and tissue estrogen levels were available from 29 and 31 out of the total group of 33 postmenopausal women. Contrasting our expectations, we observed low levels of both plasma E_1 ($p = 0.038$) and E_2 ($p = 0.050$) among individuals harbouring the rs6493497_T/rs7176005_A – haplotype. In contrast, no correlations were observed between the variant haplotype and plasma estrone sulphate (E_1S) or tissue E_2, E_1 and E_1S levels ($p > 0.10$ for all; Table 1).

In order to validate the results obtained from the breast cancer patients, we genotyped a cohort of 108 healthy postmenopausal women for whom plasma estrogen levels were available [23]. In this cohort, 19 out of 108 (21.3%) women were heterozygous for the rs6493497_T/rs7176005_A – haplotype (Table 1), mirroring an incidence of 18.2% in our cohort of breast cancer patients. Similar to what was recorded among our breast cancer patients, the SNPs occurred in concert in all the 19 healthy individuals. None of them were homozygous for the variant haplotype, and the distribution was in accordance with the Hardy-Weinberg equilibrium. No difference in plasma levels of either E_2, E_1 or E_1S ($p > 0.20$ for all) was observed between individuals harbouring the rs6493497_T/rs7176005_A – haplotype and those harbouring the wild-type sequence (Table 1).

4. Discussion

Peripheral tissue aromatisation is the sole pathway of estrogen synthesis in postmenopausal women [4]. While there has been much interest in *CYP19* SNPs as possible explanations to variation in postmenopausal estrogen levels, so far no major effects have been recorded [14–17]. Recently, Wang et al. [21] reported two tightly linked *CYP19* SNPs (rs6493497 and rs7176005) to be associated with elevated plasma E_2 level. The aim of this study was to confirm this finding, and to explore the possible impact of these SNPs on *CYP19* transcription.

Conducting *in silico* predictions, we detected several transcription factor binding sites for the rs7176005_A-allele; five additional binding sites were generated by the variant haplotype compared

to the wild-type G-allele. In addition, the A-allele was predicted to have a higher affinity than the G-allele for two transcription factors (summarized in Fig. 1 and Table 2), suggesting that the variant haplotype might enhance *CYP19* transcription, which in turn could be responsible for increased plasma and tissue estrogen levels.

Analyzing CYP19 mRNA with highly sensitive q-RT-PCR assays based on hydrolysis probes, we were able to quantify CYP19 in the entire patient cohort; the levels were undetectable in two individuals only. We found no difference in expression levels in either normal breast or breast cancer tissue between individuals harbouring the rs6493497_T/rs7176005_A – haplotype and those harbouring the *CYP19* wild-type sequence. Secondly, we compared plasma and tissue estrogen levels to *CYP19* rs6493497/rs7176005 haplotype status. In contrast to our expectations, we detected lower plasma E_2 and E_1 levels among postmenopausal breast cancer patients carrying the rs6493497_T/rs7176005_A – haplotype variant. As this result was not reproduced in the larger cohort of healthy women, we believe this finding in the breast cancer cohort to have occurred by chance only. Taken together, our results contrast the findings of Wang and co-workers, reporting the SNPs to be associated with elevated plasma E_2 [21].

A limitation of our dataset relates to the number of patients enrolled. Based on the number of healthy individuals examined, assuming a one-sided alpha power of 5%, we estimated the chance of detecting a 30% or 40% average increase in plasma E_1 levels among individuals carrying the SNPs rs6493497/rs7176005 variants of 75% and 92%, respectively (beta value). While we may not exclude a minor (10–20%) increase in estrogen levels related to the SNPs, our data argues against a major effect on plasma and tissue estrogen levels. In addition, our finding that breast cancer patients carrying the SNPs rs6493497/rs7176005 variants actually expressed lower plasma levels of both E_2 and E_1 compared to individuals carrying the wild type variant add further support to this conclusion.

As described previously, the expression of *CYP19* is controlled by a number of tissue-specific promoters. The *CYP19* SNPs rs6493497 and rs7176005 are located in the promoter element immediately upstream of exon 1.1; known as the major placenta specific promoter [8,9]. However, even though promoter 1.1 is the major promoter regulating *CYP19* expression in the placenta, it has been shown to be active in several other tissues as well [28]. *CYP19* is expressed both in normal breast and breast cancer tissue [10]. However, due to a rapid plasma to tissue transfer rate, the key factor regulating normal breast as well as breast cancer tissue estrogen levels is the plasma hormone concentration [29–30]. Thus, any factor influencing estrogen synthesis in any body compartment subsequently leading to alterations in plasma estrogen levels should influence normal breast and breast cancer tissue estrogen levels as well.

The possibility exist that SNPs rs6493497 and rs7176005 could be markers for a general haplotype, involving other SNPs that may influence *CYP19* activity. However, data from Ma and co-workers who carefully sequenced the coding as well as non-coding parts, including the promoter areas of the *CYP19* gene in 240 patients (including 60 Caucasians) [12], does not support this hypothesis.

In conclusion, our findings are in contrast with Wang et al. [21], suggesting that SNPs rs6493497 and rs7176005 do not affect *CYP19* transcription levels in normal breast or breast cancer tissue. Consistent with that, we found no correlation between *CYP19* rs6493497 and rs7176005 haplotype status and either plasma or tissue estrogen levels in postmenopausal women.

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