Introduction
Breast cancer growth is regulated by estrogen, which acts by binding to its estrogen receptor alpha (ERα). The presence of ERα in breast tumors is used as a biological marker to identify patients who may respond to endocrine agents such as tamoxifen. However, one-half of the patients with ERα-positive tumors fail to respond favorably to antiestrogen treatment [1,2].

Methods: To test this hypothesis, we used real-time quantitative RT-PCR to quantify the mRNA expression of a large panel of genes coding for the major xenobiotic-metabolizing enzymes (12 phase I enzymes, 12 phase II enzymes and three members of the ABC transporter family) in a small series of normal breast (and liver) tissues, and in estrogen receptor alpha (ERα)-negative and ERα-positive breast tumors. Relevant genes were further investigated in a well-defined cohort of 97 ERα-positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone.

Results: Seven of the 27 genes showed very weak or undetectable expression in both normal and tumoral breast tissues. Among the 20 remaining genes, seven genes (CYP2A6, CYP2B6, FMO5, NAT1, SULT2B1, GSTM3 and ABCC11) showed significantly higher mRNA levels in ERα-positive breast tumors than in normal breast tissue, or showed higher mRNA levels in ERα-positive breast tumors than in ERα-negative breast tumors.

In the 97 ERα-positive breast tumor series, most alterations of these seven genes corresponded to upregulations as compared with normal breast tissue, with an incidence ranging from 25% (CYP2A6) to 79% (NAT1). Downregulation was rare. CYP2A6, CYP2B6, FMO5 and NAT1 emerged as new putative ERα-responsive genes in human breast cancer. Relapse-free survival was longer among patients with FMO5-overexpressing tumors or NAT1-overexpressing tumors ($P = 0.0066$ and $P = 0.000052$, respectively), but only NAT1 status retained prognostic significance in Cox multivariate regression analysis ($P = 0.0013$).

Conclusions: Taken together, these data point to a role of genes coding for xenobiotic-metabolizing enzymes in breast tumorigenesis, NAT1 being an attractive candidate molecular predictor of antiestrogen responsiveness.

Keywords: breast cancer, prognostic value, real-time RT-PCR quantification, tamoxifen xenobiotic-metabolizing enzyme expression
Several mechanisms have been forwarded to explain this lack of response in ERα-positive patients, one being based on altered tamoxifen metabolism or bioavailability [3–5].

Tamoxifen is metabolized by phase I enzymes such as cytochromes P450, lactoperoxidase, microsomal epoxide hydrolase and flavin-containing monoxygenase [6–9]. Tamoxifen metabolites may have not only antiestrogenic activity, but also estrogenic or genotoxic actions [10–13]. These tamoxifen metabolites are secondarily detoxified by phase II enzymes (conjugation enzymes) such as catechol-O-methyltransferase, UDP-glucuronosyltransferases, glutathione S-transferases, sulfotransferases, N-acetyltransferases and NAD(P):quinone oxidoreductase [14–18].

The three main tamoxifen metabolites are tamoxifen-N-oxide (catalyzed by flavin-containing monoxygenase, FMO1 and FMO5), 4-hydroxy-tamoxifen and N-desmethyl-tamoxifen (catalyzed by CYP2B6, CYP2C9, CYP2D6, CYP2E1, CYP3A4, etc. [7,8]). 4-Hydroxy-tamoxifen has the strongest antiestrogen activity (100-fold higher than tamoxifen itself) [6]. All three metabolites are secondarily detoxified by phase II enzymes [14–18].

Most xenobioc-metabolizing enzymes are expressed in the liver, but some are also expressed in breast tissue. Intratumoral tamoxifen or metabolites (generated by hepatic metabolism) could thus undergo further transformation in the breast in situ [19]. Altered intratumoral expression of genes coding for xenobioc-metabolizing enzymes is one potential mechanism of tamoxifen resistance.

Little is known of the function and clinical significance of the altered intratumoral expression of xenobioc-metabolizing enzymes with respect to tamoxifen resistance. Lower tumor tamoxifen concentrations have been observed in tamoxifen-resistant tumors from breast cancer patients [20]. CYP1A1 and CYP1B1 expression is increased in antiestrogen-resistant human breast cancer cell lines [21]. Fritz and colleagues [22] recently identified microsomal epoxide hydrolase as a predictor of the tamoxifen response in breast cancer.

To further investigate the possible relationship between altered intratumoral expression of xenobioc-metabolizing enzymes and both breast tumorigenesis and tamoxifen resistance, we used real-time quantitative RT-PCR assays to quantify mRNA expression of a large panel of genes coding for the major xenobioc-metabolizing enzymes (12 phase I enzymes, 12 phase II enzymes and three members of the ABC transporter family involved in multidrug resistance) in a small series of ERα-negative and ERα-positive breast tumors. Seven relevant genes thus identified were further investigated in a well-defined cohort of 97 ERα-positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone.

Materials and methods
Patients and samples
We analyzed tissue samples from primary breast tumors excised from 97 women at Centre René Huguenin from 1980 to 1994. The tumor samples were stored in liquid nitrogen immediately following surgery until RNA extraction. The patients (mean age, 71.1 years; range, 54–86 years) met the following criteria: primary unilateral nonmetastatic postmenopausal breast carcinoma; ERα-positive as determined at the protein level by biochemical methods (Dextran-coated charcoal method until 1988 and enzyme immunoassay thereafter) and at the mRNA level by ESR1/ERα real-time quantitative RT-PCR assay [23]; complete histological and biological information available from the primary tumors; no radiotherapy or chemotherapy before surgery; and clinical follow-up at Centre René Huguenin.

The standard prognostic factors are presented in Table 1. Thirty-one patients (32.0%) had modified radical mastectomy and 66 patients (68.0%) had breast-conserving surgery plus locoregional radiotherapy. Patients underwent physical examinations and routine chest radiography every 3 months for 2 years, and then annually. Mammograms were performed annually. The median follow-up was 6.5 years (range, 1.5–17.7 years). All the patients received postoperative adjuvant endocrine therapy (20 mg tamoxifen daily for 3–5 years), and no other treatment. Thirty-two patients relapsed (the distribution of first relapse events was 27 distant metastases, and five patients with both local and/or regional recurrences and metastases). Five ERα-negative tumors were also analyzed in order to investigate the relationship between target mRNA levels and ERα expression status.

Specimens of adjacent normal breast tissue from five breast cancer patients (patients who did not belong to the series of 97 patients analyzed in this study), and normal breast tissue from three women undergoing cosmetic breast surgery, were used as sources of normal breast RNA.

As xenobioc-metabolizing enzymes are mainly expressed in the liver, we also analyzed a pool of mRNA from three normal human livers (Clontech, Palo Alto, CA, USA) in order to compare mRNA levels between normal breast and liver tissues.

Real-time RT-PCR
Theoretical basis
Quantitative values are obtained from the cycle number (Ct value) at which the increase in fluorescent signal associated with an exponential growth of PCR products starts to be detected by the laser detector of the ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems, Foster City, CA, USA). This was done using the PE Biosystems analysis software according to the manufacturer’s manuals.
The precise amount of total RNA added to each reaction mix (based on optical density) and its quality (i.e., lack of extensive degradation) are both difficult to assess. We therefore also quantified transcripts of the gene coding for the TATA box-binding protein (TBP) (a component of the DNA-binding protein complex TFIID) as the endogenous RNA control, and each sample was normalized on the basis of its TBP content.

Results, expressed as n-fold differences in target gene expression relative to the TBP gene (termed ‘N\text{target}’), were determined by the formula: $N_{\text{target}} = 2^{\Delta Ct_{\text{sample}}}$, where the $\Delta Ct$ value of the sample was determined by subtracting the average Ct value of the target gene from the average Ct value of the TBP gene.

The $N_{\text{target}}$ values of the samples were subsequently normalized such that the mean of the $N_{\text{target}}$ values of the eight normal breast samples would equal a value of 1.

Target gene mRNA levels were confirmed using an additional endogenous RNA control for normalization; that is, the gene PPIA coding for the peptidylprolyl isomerase A (cyclophilin A).

### Primers

Primers for the 27 xenobiotic-metabolizing target genes, the $ESR1/ER\alpha$ gene (coding for the ER$\alpha$) and the $MKI67$ gene (coding for the proliferation-related Ki-67 antigen) were chosen with the assistance of the computer program Oligo 5.0 (National Biosciences, Plymouth, MN, USA). We conducted BLASTN searches against ‘dbEST’, ‘htgs’ and ‘nr’ (the nonredundant set of the GenBank, EMBL and DDBJ database sequences) to confirm the total gene specificity of the nucleotide sequences chosen for the primers, and to confirm the absence of DNA polymorphisms. In particular, the primer pairs were selected to be unique when compared with the sequences of the closely related family member genes or of corresponding retropseudogenes. To avoid amplification of contaminating genomic DNA, one of the two primers was placed, if possible, in a different exon. For example, the upper primer of TBP was placed at the junction between exons 5 and 6, whereas the lower primer was placed in exon 6. In general, amplicons were between 70 and 120 nucleotides. Agarose gel electrophoresis allowed us to verify the specificity of PCR amplicons.

The 27 target genes tested in this study are presented in Table 2. The nucleotide sequences of the primers are available on request.

### RNA extraction

Total RNA was extracted from breast specimens using the acid–phenol guanidinium method. The quality of the RNA samples was determined by electrophoresis through agarose gels and staining with ethidium bromide. The 18S and 28S RNA bands were visualized under ultraviolet light.

### cDNA synthesis

RNA was reverse transcribed in a final volume of 20 µl containing 1 x RT buffer (500 µM each dNTP, 3 mM MgCl$_2$, 75 mM KCl, 50 mM Tris–HCl; pH 8.3), 20 U RNasin Ribonuclease inhibitor (Promega, Madison, WI, USA), 10 mM dithiothreitol, 100 U Superscript II RNase H-reverse transcriptase (Gibco BRL, Gaithersburg, MD, USA), 3 µM random hexamers (Pharmacia, Uppsala, Sweden) and 1 µg total RNA. The samples were incubated at 20°C for 10 min and at 42°C for 30 min, and RT was inactivated by heating at 99°C for 5 min and cooling at 5°C for 5 min.

### PCR amplification

All PCR reactions were performed using an ABI Prism 7700 Sequence Detection System (Perkin-Elmer Applied Biosystems). PCR was performed using the SYBR® Green PCR Core Reagents kit (Perkin-Elmer Applied Biosystems). The thermal cycling conditions comprised an initial denaturation step at 95°C for 10 min and 50 cycles at 95°C for 15 s and at 65°C for 1 min.
Statistical analysis
The distribution of mRNA levels was analyzed on the basis of their median values and ranges. Relationships between mRNA levels of the different target genes, and comparisons between median target gene mRNA levels and clinical, histological and biological parameters were based on nonparametric tests – namely the Mann–Whitney test (link between one qualitative parameter and one quantitative parameter) and the Spearman rank correlation test (link between two quantitative parameters). Differences between two populations were judged significant at confidence levels greater than 95% ($P < 0.05$).

To visualize the capacity of target gene mRNA levels to discriminate between patients who relapsed and those who did not relapse (in the absence of an arbitrary cutoff value), we used the receiver–operating characteristic (available online).

Table 2

| Target genes tested |
|---------------------|
| Gene$^a$ | Genbank accession number | Chromosomal location | Description |
|---------|--------------------------|----------------------|-------------|
| **Phase I enzymes** |
| CYP1A1  | NM_000499 | 15q24.1 | Cytochrome P450, subfamily IA, polypeptide 1 |
| CYP1A2  | NM_000761 | 15q24.2 | Cytochrome P450, subfamily IA, polypeptide 2 |
| CYP1B1  | NM_000104 | 2p21 | Cytochrome P450, subfamily IB, polypeptide 1 |
| CYP2A6  | NM_000762 | 19q13.2 | Cytochrome P450, subfamily IIA, polypeptide 6 |
| CYP2B6  | NM_000767 | 19q13.2 | Cytochrome P450, subfamily IIB, polypeptide 6 |
| CYP2C9  | NM_000771 | 10q24.1 | Cytochrome P450, subfamily IIC, polypeptide 9 |
| CYP2D6  | NM_000106 | 2q13.1 | Cytochrome P450, subfamily IID, polypeptide 6 |
| CYP2E1  | NM_000773 | 10q24.3-qter | Cytochrome P450, subfamily IIE, polypeptide 1 |
| CYP3A4  | NM_017460 | 7q22.1 | Cytochrome P450, subfamily IIIA, polypeptide 4 |
| FMO1    | NM_002021 | 1q23-q25 | Flavin-containing monooxygenase 1 |
| FMO5    | NM_018578 | 1q21 | Flavin-containing monooxygenase 5 |
| LPO     | XM_042207 | 17q23.1 | Lactoperoxidase |
| **Phase II enzymes** |
| NQO1    | NM_000903 | 16q22.1 | NAD(P)H dehydrogenase, quinone 1 |
| NAT1    | NM_000662 | 8p23.1-p21.3 | N-acetyltransferase 1 |
| COMT    | NM_000754 | 2q21.1 | Catechol-O-methyltransferase |
| EPHX1   | NM_000120 | 1q42.1 | Epoxyde hydrolase 1, microsomal |
| SULT1A1 | NM_001055 | 16p12.1 | Sulfortransferase family, cytosolic, 1A, member 1 |
| SULT2A1 | NM_003167 | 19q13.3 | Sulfortransferase family, cytosolic, 2A, member 1 |
| SULT2B1 | NM_004605 | 19q13.3 | Sulfortransferase family, cytosolic, 2B, member 1 |
| UGT1A1  | NM_000463 | 2q37 | UDP-glucuronosyltransferase, 1 family, polypeptide A1 |
| GSTP1   | NM_000852 | 11q13 | Glutathion S-transferase pi 1 |
| GSTM1   | NM_000561 | 1p13.3 | Glutathion S-transferase mu 1 |
| GSTM3   | NM_000849 | 1p13.3 | Glutathion S-transferase mu 3 |
| GSTT1   | NM_000853 | 22q11.23 | Glutathion S-transferase theta 1 |
| **Phase III proteins** |
| ABCB1   | NM_000927 | 7q21.1 | ATP-binding cassette, subfamily B (MDR/TAP), member 1 (MDR1) |
| ABCC1   | NM_004996 | 16p13.1 | ATP-binding cassette, subfamily C (CFTR/MRP), member 1 (CFTR1) |
| ABCC11  | NM_033151 | 16q12.1 | ATP-binding cassette, subfamily C (CFTR/MRP), member 11 (MRP8) |

$^a$ LocusLink symbol.
(ROC)—area under the curve (AUC) method [24]. When a molecular marker has no discriminatory value, the ROC curve lies close to the diagonal and the AUC value is close to 0.5. When a marker has strong discriminatory value, the ROC curve moves to the upper left-hand corner (or to the lower right-hand corner) and the AUC value is close to 1.0 (or to 0).

Relapse-free survival was determined as the interval between diagnosis and detection of the first relapse. Survival distributions were estimated by the Kaplan–Meier method [25], and the significance of differences between survival rates was ascertained using the log-rank test. Cox’s proportional hazards regression model [26] was used to assess prognostic significance.

**Results**

**mRNA expression of the 27 target genes in normal breast and liver tissue, and in ERα-negative and ERα-positive breast tumors**

We quantified mRNA levels of the 27 xenobiotic-metabolizing enzyme genes, and the *MKI67* and *ESR1/ERα* genes, in a pool of normal liver tissue, in eight normal breast tissues, in five ERα-negative breast tumors and in 17 ERα-positive breast tumors.

Target gene mRNA levels were very low (detectable but not quantifiable by real-time quantitative RT-PCR assay, Ct > 35) in both normal and tumoral breast tissues for seven genes (*CYP1A1, CYP1A2, CYP2C9, CYP3A4, LPO, SULT2A1 and UGT1A1*) out of the 27 xenobiotic-metabolizing enzyme genes. *CYP1A2, LPO* and *UGT1A1* were very weakly expressed (Ct > 35) in the pooled liver tissues, while the other four genes (*CYP1A1, CYP2C9, CYP3A4 and SULT2A1*) showed significant expression (Ct < 30).

Means (± standard deviation) and ranges of mRNA levels for the 20 xenobiotic-metabolizing enzyme genes expressed in breast tissues, as well as for *ESR1/ERα* and for *MKI67*, are presented in Table 3. Target gene mRNA levels in the five ERα-negative breast tumors and in the 17 ERα-positive breast tumors (and in the pool of normal liver tissue) are expressed relative to the mean mRNA levels observed in the eight normal breast tissues.

From among the 20 xenobiotic-metabolizing enzyme genes, we selected seven genes of interest for further expression analysis in a large series of breast tumors (Table 3 and Fig. 1). These seven genes comprised four genes significantly upregulated in ERα-positive breast tumors as compared with normal breast tissue (i.e. *CYP2B6, NAT1, SULT2B1* and *ABCC11*), and three additional putative ER-responsive genes (*CYP2A6, FMO5* and *GSTM3*) that were significantly upregulated in ERα-positive tumors compared with ERα-negative tumors.

It is noteworthy (Table 3) that *CYP1B1* and *CYP2D6* were significantly upregulated in ERα-negative tumors compared with ERα-positive tumors, identifying them as candidate markers of tumor aggressiveness in ERα-negative human breast cancer.

Among the 20 xenobiotic-metabolizing enzyme genes, only *FMO5* showed markedly higher mRNA levels (>10-fold) in liver tissue than in breast tissue. *CYP1B1, NQO1* and *SULT2B1*, however, showed markedly lower mRNA levels (>10-fold) in liver tissue than in breast tissue. The other 16 genes showed close similar mRNA levels in the liver and the breast.

The mRNA levels of these 20 genes (except for *CYP2D6*; \(r = +0.453, \ P = 0.033\), Spearman rank correlation test) were not associated with the *MKI67* mRNA level (a proliferation-related marker), suggesting that they are not upregulated in rapidly proliferating cells in vivo (data not shown).

GSTM1 and/or GSTT1 mRNA was undetectable in some samples of both normal and tumoral breast tissue, probably owing to the particular polymorphism of these two genes (total absence of the two allele copies for these loci in ‘allele null’ patients).

The Ntarget values (calculated as described in Materials and methods) presented in Table 3 are based on the amount of target messenger relative to the TBP endogenous control, in order to normalize the amount and quality of total RNA; similar results were obtained with a second endogenous RNA control (*PPIA*) coding for cyclophilin A (data not shown).

**mRNA expression of seven selected genes in 97 ERα-positive breast tumors**

We quantified mRNA levels of the *CYP2A6, CYP2B6, FMO5, NAT1, SULT2B1, GSTM3* and *ABCC11* genes in a well-defined cohort of 97 ERα-positive breast tumors from postmenopausal patients treated by surgery who only received tamoxifen hormonotherapy thereafter.

The ranges, means and medians of the mRNA levels of the seven target genes in this series of 97 breast tumors are summarized in Table 4. Major interindividual differences in mRNA levels (at least two orders of magnitude) were observed for all seven genes. For example, N*CYP2B6* values ranged from 0.03 to 1053.1 (i.e. more than four orders of magnitude).

The cutoff points for altered gene expression in malignant breast tissues were determined using the Ntarget values (calculated as described in Materials and methods) obtained for the eight normal breast RNA samples. The mean values for the eight normal breast Ntarget plus five standard deviations were considered to represent the
cut-off point for overexpression. The mean values for the eight normal breast N\textsubscript{target} minus two standard deviations (or N\textsubscript{target} value = 0.1 when the latter calculation gave a negative value) were considered to represent the cut-off point for underexpression. The percentage of tumors overexpressing and underexpressing the seven genes is presented in Table 4. It is noteworthy that most alterations corresponded to overexpression (from 25\% of the tumors for CYP2A6 to 79\% for NAT1) and rarely to underexpression (from 0\% for NAT1 and SULT2B1 to 15\% for CYP2A6).

**Relationships between mRNA values of the seven selected genes in 97 ER\textalpha-positive breast tumors**

Using the Spearman rank correlation test (which compares continuous variables), we found a strong positive correlation between CYP2A6, CYP2B6, FMO5 and NAT1 mRNA levels (Table 5). We also quantified ESR1/ER\textalpha mRNA levels in this series of 97 ER\textalpha-positive breast tumors. We found a strong positive correlation with CYP2A6, CYP2B6, FMO5 and NAT1 mRNA levels and, to a lesser extent, with GSTM3 mRNA levels.

**Prognostic value of the seven selected genes in 97 ER\textalpha-positive breast tumors**

The comparison of median mRNA levels in tumors from patients without relapse \((n = 65)\) and in tumors from patients with relapse \((n = 32)\) identified significant differences in the expression of three genes (CYP2B6, FMO5 and NAT1) (Table 6). The three genes showed lower mRNA levels in the patients who relapsed than in those who did not relapse. The prognostic performance of each of the
seven selected genes for relapse was assessed using ROC curves. The overall prognostic value of these candidate molecular markers was compared using their AUC values, which identified \textit{NAT1} (AUC–ROC, 0.24) as the most discriminatory gene (Table 6).

Univariate and multivariate prognostic analyses were then applied to \textit{CYP2B6}, \textit{FMO5} and \textit{NAT1} status according to patient survival. As the percentage of patients with \textit{CYP2B6}-overexpressing and \textit{NAT1}-overexpressing tumors was high (76% and 79%, respectively; Table 4), the overexpressing tumors were subdivided into two equal subgroups with moderate and strong overexpression for univariate analysis (log-rank test). This analysis showed that longer relapse-free survival was linked to \textit{NAT1} overexpression (\(P=0.000052\); Fig. 2a) and to \textit{FMO5} overexpression (\(P=0.0066\); Fig. 2b). With regard to the two subgroups of \textit{NAT1}-overexpressing tumors, the higher the \textit{NAT1} mRNA level, the better the outcome (Fig. 2a).

Relapse-free survival was not significantly associated with \textit{CYP2B6} mRNA status (\(P=0.078\); Fig. 2c).

Multivariate analysis (Cox proportional hazards model) was used to assess the influence of \textit{NAT1} and \textit{FMO5} mRNA status on relapse-free survival, together with classical prognostic parameters identified by univariate analysis (histo-pathological grade, lymph node status and macroscopic tumor size) in this same series of patients (Table 1). Only \textit{NAT1} mRNA status and lymph node status retained their prognostic significance (Table 7; \(P=0.0013\) and \(P=0.016\), respectively).

\textbf{Discussion}

To test the hypothesis that altered tamoxifen metabolism and bioavailability could explain some cases of resistance, and to identify new candidate molecular markers to predict antiestrogen responsiveness in breast cancer, we used real-time quantitative RT-PCR to measure the
Table 4

mRNA levels of seven selected genes in 97 estrogen receptor alpha-positive breast tumors

| Gene   | mRNA levels | Expression status |
|--------|-------------|-------------------|
|        | Mean ± SD   | Median Range      | Underexpressed<sup>a</sup> | Normal     | Overexpressed<sup>b</sup> |
| CYP2A6 | 344.0<sup>c</sup> ± 1540.6 | 0.9 | 0.001–9741.1 | 15 (15.5)<sup>d</sup> | 58 (59.8)<sup>d</sup> | 24 (24.7)<sup>d</sup> |
| CYP2B6 | 103.3 ± 172.7 | 37.0 | 0.03–1053.1 | 1 (1.0) | 22 (22.7) | 74 (76.3) |
| FMO5  | 5.2 ± 6.3 | 2.6 | 0.10–30.1 | 1 (1.0) | 43 (44.3) | 53 (54.7) |
| GSTM3 | 3.1 ± 3.5 | 2.0 | 0.07–21.2 | 2 (2.1) | 60 (61.8) | 35 (36.1) |
| SULT2B1 | 7.5 ± 10.5 | 4.5 | 0.17–84.4 | 0 | 61 (62.9) | 36 (37.1) |
| NAT1  | 46.5 ± 55.4 | 21.5 | 1.1–295.6 | 0 | 20 (20.6) | 77 (79.4) |
| ABCC11 | 37.1 ± 60.4 | 15.9 | 0.04–461.7 | 3 (3.1) | 27 (27.8) | 67 (69.1) |

<sup>a</sup> Less than mean values for the normal breast N<sub>target</sub> minus two standard deviations (SDs) (or N<sub>target</sub> value = 0.1 when the latter calculation gave a negative value). <sup>b</sup> Greater than mean values for the normal breast N<sub>target</sub> plus five SDs. <sup>c</sup> The n-fold differences in target gene expression relative to the TATA box-binding protein (TBP) gene and the normal breast tissues. <sup>d</sup> Number of patients (percentage).

Table 5

Relationships between mRNA values of the seven selected genes and the ER<sub>α</sub> gene in the 97 estrogen receptor alpha (ER<sub>α</sub>)-positive breast tumor series

| CYP2B6 | FMO5 | NAT1 | SULT2B1 | GSTM3 | ABCC11 | ESR1/ERα |
|--------|------|------|---------|-------|--------|----------|
| CYP2A6 | +0.471 | +0.171 | +0.248 | +0.084 | −0.037 | −0.173 |
| CYP2B6 | +0.525 | +0.433 | +0.083 | +0.022 | +0.022 | +0.409 |
| FMO5  | +0.420 | −0.099 | −0.008 | +0.216 | +0.032 | +0.316 |
| NAT1  | +0.077 | +0.104 | +0.041 | +0.293 | +0.033 | +0.293 |
| SULT2B1 | +0.232 | +0.049 | +0.091 | +0.307 | +0.381 | +0.091 |
| GSTM3 | +0.198 | +0.049 | +0.239 | +0.018 | +0.049 | +0.239 |
| ABCC11 | +0.023 | +0.023 | +0.049 | +0.054 | +0.049 | +0.054 |

Data presented as Spearman rank correlation coefficient [P value (Spearman rank correlation test)]. NS, not significant.

Table 6

Relationships between the prognostic (± relapses) and the mRNA levels of the seven selected genes in 97 estrogen receptor alpha-positive breast tumors

| Gene   | Tumors without relapses (n = 65) | Tumors with relapses (n = 32) | <sup>P</sup> | ROC–AUC<sup>b</sup> |
|--------|---------------------------------|-------------------------------|------------|-------------------|
| CYP2A6 | 1.2 (0.001–9741.1)<sup>c</sup> | 0.8 (0.01–7228.8) | NS (0.17) | 0.41 (0.29–0.54)<sup>d</sup> |
| CYP2B6 | 56.1 (0.3–1053.1) | 14.7 (0.03–249.8) | 0.011 | 0.34 (0.23–0.45) |
| FMO5  | 3.9 (0.2–30.1) | 1.4 (0.1–23.9) | 0.0016 | 0.30 (0.19–0.41) |
| GSTM3 | 2.1 (0.07–12.2) | 1.7 (0.09–21.2) | NS (0.80) | 0.48 (0.35–0.62) |
| SULT2B1 | 4.9 (0.2–84.4) | 3.5 (0.4–21.0) | NS (0.65) | 0.47 (0.35–0.59) |
| NAT1  | 35.9 (1.6–295.6) | 10.0 (1.1–134.1) | 0.000047 | 0.24 (0.14–0.35) |
| ABCC11 | 15.9 (0.06–195.5) | 16.6 (0.04–461.7) | NS (0.60) | 0.46 (0.33–0.60) |

<sup>a</sup> P value, Mann–Whitney test; NS, not significant. <sup>b</sup> Receiver–operating characteristics (ROC)–area under curve (AUC) analysis. <sup>c</sup> Median (range) of gene mRNA levels. <sup>d</sup> AUC value (95% confidence interval).
expression of a large panel of genes \( (n = 27) \) coding for major xenobiotic-metabolizing enzymes. These 27 genes encode 12 phase I enzymes (including CYP2C9, CYP2D6, CYP3A4 and FMO1, known to be involved in the hepatic metabolism of tamoxifen \([6,8]\)), 12 phase II enzymes and three members of the ABC transporter family involved in multidrug resistance in a series of human breast tumors.

Real-time quantitative RT-PCR has a major advantage over cDNA microarrays in the present setting in that it can distinguish closely related family member genes. Indeed, some xenobiotic-metabolizing enzyme genes are clustered in the same chromosomal region, and their nucleotide sequences show considerable homology. This is the case for the genes coding for certain cytochrome P450s, UDP-glucuronosyltransferases, glutation S-transferases and sulfotransferases. Real-time RT-PCR can use primer pairs that are unique relative to closely related family member genes. It is important to study these highly homologous genes individually, as they frequently code for enzymes with very different substrates.

Although we did not study all existing xenobiotic-metabolizing enzyme genes, our results nevertheless demonstrate the usefulness of real-time RT-PCR and identify several candidate marker genes of potential clinical value.

Xenobiotic-metabolizing enzyme genes in breast cancer have mainly been studied by investigating the relationship between genetic polymorphisms and cancer susceptibility \([19]\). This DNA-level approach was not suited to our aims, as it does not distinguish between hepatic gene expression and/or mammary gene expression.

We first quantified the mRNA expression of 27 genes coding for major xenobiotic-metabolizing enzymes in a small series of ER\(\alpha\)-negative \((n = 5)\) and ER\(\alpha\)-positive \((n = 17)\) breast tumors. Seven genes of interest were then further investigated in a well-defined cohort of 97 ER\(\alpha\)-
positive postmenopausal breast cancer patients treated with primary surgery followed by adjuvant tamoxifen alone. This two-step strategy significantly limited the required number of PCR experiments.

The results of the first step yielded the following information about the involvement of xenobiotic-metabolizing enzymes in breast tumorigenesis. Among the 27 genes we identified seven genes (CYP1A1, CYP1A2, CYP2C9, CYP3A4, LPO, SULT2A1 and UGT1A) whose expression is very weak or undetectable in breast tissue, in partial agreement with published results [27–29]. In particular, the recent study of Iscan and colleagues [30] observed marked expression of CYP1B1, CYP2B6, CYP2D6 and CYP2E1 in breast tumors, but low expression or no expression of CYP1A1 and CYP3A4. The only discrepancy with our study concerns CYP2A6 expression, which was not observed in breast tissue by Iscan and colleagues.

The 20 remaining genes, except CYP1B1, FMO5, NQO1 and SULT2B1, showed mRNA expression variation <10-fold between normal breast and liver. Another result was that CYP1B1 and CYP2D6 were significantly upregulated in ERα-negative (poorly differentiated) tumors relative to ERα-positive tumors. These two genes would thus correspond to markers of tumor aggressiveness.

Two genes (GSTM1 and GSTT1) had undetectable mRNA expression in a number of breast tumors, and in normal breast tissues, probably owing to their particular polymorphism (a total absence of the two allele copies of these two loci in ‘allele null’ patients), although this needs to be confirmed at the DNA level. It is noteworthy that GSTM1 and GSTT1 polymorphisms are associated with the risk of breast cancer and that inherited metabolic variability may also influence breast cancer treatment outcome [31–33].

Finally, we identified seven genes of interest (CYP2A6, CYP2B6, FMO5, NAT1, SULT2B1, GSTM3 and ABCC11) and further investigated their expression in a larger series of ERα-positive breast tumors. These genes either showed strong upregulation in ERα-breast tumors compared with normal breast tissue, suggesting a role in breast tumorigenesis, and/or showed upregulation in the ERα-positive tumors compared with the ERα-negative tumors, making them putative ERα-responsive genes.

It is noteworthy that due to lack of expression in breast tissue (CYP2C9 and CYP3A4), due to no expression differences between normal and tumoral breast tissue (FMO1) and due to expression upregulation in ERα-negative compared with ERα-positive breast tumors (CYP2D6), these genes classically described to metabolize the tamoxifen in the liver were not further investigated in the 97 ERα-positive breast tumor series.

In the second part of this study we examined relationships between the expression status of these seven genes and the risk of disease recurrence and the response to tamoxifen therapy. The results point to CYP2A6, CYP2B6, FMO5 and NAT1 as new ERα-responsive genes, and point to NAT1 as an independent predictor of response to tamoxifen. Indeed, expression levels of the CYP2A6, CYP2B6, FMO5 and NAT1 genes were strongly linked to ERα mRNA levels in our ERα-positive breast tumor series. Total validation of these four genes as effective ERα-responsive genes will require the use of classical in vitro or in vivo expression models, and the identification of estrogen-responsive elements within the promoters of the four genes.

The most important result of this study is that both univariate and multivariate prognostic analysis identified NAT1 as both an independent prognostic factor of breast cancer relapse and as a putative predictor of the response to tamoxifen. The predictive value of NAT1 in the response to endocrine therapy of breast cancer must now be confirmed in a prospective randomized study designed to show that this parameter influences outcome only in patients who receive adjuvant tamoxifen as compared with untreated patients. Indeed, previous epidemiological studies of the potential link between the NAT1 genotype, breast cancer risk and lifestyle factors (including cooked meat and cigarettes) showed an increased risk among individuals with certain NAT1 alleles who eat well-cooked meat [34]. We thus cannot rule out the possibility that the prognostic value of NAT1 in our breast cancer series was due to individual variations in the metabolism of xenobiotics other than tamoxifen, influencing outcome independently of endocrine treatment.

Human aryl N-acetyltransferases are encoded by two genes (NAT1 and NAT2) physically linked in chromosomal region 8p21.3-23.1. Despite their strong homology at the amino acid level (81%), NAT1 and NAT2 enzymes have distinct substrate specificity, although they do share certain substrates such as aromatic and heterocyclic amine carcinogens [35]. These enzymes also have distinct tissue expression profiles: NAT2 is principally expressed in human liver and intestine, while NAT1 is expressed more ubiquitously [36]. In normal breast tissue, NAT1 enzyme levels are high, while NAT2 enzyme levels are very low [37]. It is noteworthy that NAT1 gene expression may reliably be studied at the mRNA level because NAT1 mRNA expression detected by RT-PCR analysis seems to be highly associated with positive NAT1 immuno-histochemistry staining [37]. Immunochemistry-based studies show that NAT1 expression is strictly limited to epithelial cells, stromal tissues showing no NAT1 staining [37]. Few data are available on NAT1 expression in breast tumors. A recent study showed increased NAT1 enzyme activity in a series of 12 breast tumors as compared with normal breast tissue [38].
NAT1 overexpression was associated with good outcome in our cohort of ERα-positive postmenopausal breast cancer patients treated with adjuvant tamoxifen alone. We hypothesize that strong intratumoral NAT1 expression could lead to increased detoxification of genotoxic and/or estrogenic tamoxifen metabolites, while having no action on the major antiestrogenic tamoxifen metabolites such as 4-hydroxy-tamoxifen, which is again metabolized by phase I enzymes (i.e. cytochromes P450).

Conclusions
In conclusion, this study points to a role of altered intratumoral expression of xenobiotic-metabolizing enzyme genes in breast tumorigenesis, identifies four putative ERα-responsive genes (CYP2A6, CYP2B6, FMO5 and NAT1) and points to NAT1 as an attractive candidate molecular marker predictive of antiestrogen responsiveness in breast cancer. This latter hypothesis is currently being tested in a large, prospective and homogeneous patient cohort.

Competing interests
None declared.

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