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Disturbed expression of phase I and II estrogen-metabolizing enzymes in endometrial cancer: Lower levels of CYP1B1 and increased expression of S-COMT

Neli Hevir[a], Jasna Šinkovec[b] and Tea Lanišnik Rižner[a]

[a]Institute of Biochemistry, Faculty of Medicine, University of Ljubljana, Ljubljana, Slovenia

[b]Department of Obstetrics and Gynaecology, University Medical Centre, Ljubljana, Slovenia

Short Title

Estrogen-metabolizing enzymes in endometrial cancer

Address all correspondence to: Dr. Tea Lanišnik Rižner

Institute of Biochemistry

Faculty of Medicine

University of Ljubljana

Vrazov trg 2

1000 Ljubljana

Slovenia

Tel: +386-1-5437657

Fax: +386-1-5437641

E-mail: tea.lanisnik-rizner@mf.uni-lj.si

ABSTRACT

Expression levels of genes encoding phase I and II estrogen-metabolizing enzymes: *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP3A5*, *CYP3A7*, *SULT1A1*, *SULT1E1*, *SULT2B1*, *COMT*, *UGT2B7*, and *GSTP1* were studied by real-time PCR in 38 samples of cancerous and adjacent control endometrium. We found significantly lower levels of *CYP1B1* and *CYP3A7*, higher levels of *SULT2B1*, *UGT2B7* and *GSTP1*, and no differences in expression of *COMT*, *CYP1A1*, *CYP3A5*, *SULT1E1* and *SULT2A1* in the endometrial cancers. The CYP1B1 and COMT proteins were also examined by Western blotting and immunohistochemical staining, supporting the real-time PCR analysis. Lower levels of CYP1B1 detected in cancerous endometrium suggest its important role in control, precancerous tissue. Additionally, we showed for the first time higher protein levels of soluble COMT in cancerous endometrium, and higher levels of membrane-bound COMT in control, precancerous endometrium. The importance of the changed ratio between soluble and membrane-bound COMT still needs to be evaluated in further studies.

Keywords:

Endometrial cancer; Estrogen-metabolizing enzymes; Cytochrome P450 1B1 (CYP1B1); Catechol-O-methyltransferase

1. Introduction

Endometrial cancer (EC) is the fourth most common cancer in women, and the most prevalent gynaecological malignancy in the developed World (Amant et al., 2005; Parkin et al., 2005). Based on clinicopathological and molecular characteristics, two different pathways have been described for tumourigenesis of sporadic endometrial carcinoma (Amant et al., 2005; Samarnthai et al., 2010). The first is type-I, or estrogen-dependent, endometrioid endometrial carcinoma, which is usually low-grade and represents the majority of cases (70% to 80%); this forms in a background of endometrial hyperplasias (Nieminen et al., 2009). The second is type II, or non-endometrioid endometrial carcinoma, which accounts for the remaining 10% to 20% of cases, and is believed to follow an estrogen-unrelated pathway. The type II cancer is typically high-grade, most frequently papillary serous, and less frequently clear-cell carcinoma.

Some tumours share pathological and molecular features of both types I and II. Therefore, many endometrial carcinomas are in the gray area between types I and II, and have overlapping clinical, morphological, immunohistochemical and molecular features (Liu 2007). There is, however, a clear association of excessive exposure to estrogens that is not opposed by progesterone, with the development of hyperplastic processes and the initiation and maintenance of malignant tumour growth in the endometrium (Henderson and Feigelson 2000).

Estrogens can induce cell proliferation and stimulate cell division, while they can also cause random errors during DNA replication that can result in a malignant phenotype (Zhu and Conney, 1998; Henderson and Feigelson, 2000; Hecht and Mutter, 2006; Bolton and Thatcher, 2008; Lanisnik Rizner 2009). The endogenous estrogens can undergo extensive oxidative metabolism at different positions, which is catalyzed by various cytochrome P450 (CYP) isoforms (Zhu and Lee, 2005) (Figure 1). These produce mostly A-ring metabolites by

2- and 4-hydroxylation, such as catecholestrogens (CEs), and to a lesser extent, D-ring metabolites by 16α-hydroxylation (Zhu and Conney, 1998). The metabolic activation of estrogens to form the CEs 2-hydroxy-estrone/estradiol and 4-hydroxy-estrone/estradiol (2-OH-E1/E2 and 4-OH-E1/E2, respectively) is catalyzed predominantly by CYP1A1/1A2 and CYP1B1, respectively (Yager and Davidson, 2006). These CEs can be oxidized to estrogen ortho-quinones (E1/E2-2,3-Q and E1/E2-3,4-Q, respectively), which can react with DNA, primarily forming unstable N3-adenine or N7-guanine DNA adducts. Greater carcinogenic potency is associated with 4-OH-E1/E2 than 2-OH-E1/E2, the latter of which are borderline carcinogens. Additionally, quinones undergo redox cycling via their reduction to semiquinones and subsequent oxidation back to quinones, forming the reactive oxygen species (ROS) superoxide (O_2^-) and then H_2O_2, which in the presence of Fe^{2+} forms hydroxyl radicals that can damage cellular proteins and DNA (Zhu and Conney, 1998; Lee et al., 2003; Cavalieri et al., 2006; Yager and Davidson, 2006; Bolton and Thatcher, 2008; Salama et al., 2008). In human extra-hepatic tissue, 16α-hydroxylation is mainly catalyzed by CYP3A5 (Zhu and Lee, 2005) and CYP3A7 (Lee et al., 2003). Like 4-OH-E2, 16α-OH-E1 and 16α-OH-E2 show specificity for the estrogen receptors ERα and ERβ (Zhu et al., 2006). Indeed, increased formation of 16α-OH-estrogens has been suggested to be associated with elevated risk of breast cancer (Zhu and Conney, 1998).

The metabolism of CEs and 16α-OH-estrogens is catalyzed by the phase II metabolic enzymes: catechol-O-methyltransferase (COMT) (Cavalieri et al., 2006; Salama et al., 2008), sulfotransferases (SULTs) (Raftogianis et al. 2000; Adjei and Weinshilboum, 2002; Lee et al. 2003), UDP glucuronosyl transferases (UGTs) (Lepine et al., 2004), and glutathione S-transferases (GSTs) (Lee et al., 2003). Quantitatively, methylation is the most important conjugative pathway for CEs (Raftogianis et al., 2000). COMT has an important role in estrogen-induced cancers because it inactivates CEs by converting them into non-

carcinogenic methoxyestrogens (MeO-estrogens). Furthermore, MeO-estrogens, and especially 2-MeO-E1/E2, have potent tumour-suppressing properties *in vitro* (Zhu and Liehr, 1996; Mannisto and Kaakkola, 1999). The COMT protein exists in two major forms: soluble (S-COMT) and membrane-bound (MB-COMT) (Jeffery and Roth, 1985). S-COMT and MB-COMT are products of the same gene and are regulated by the proximal *P1* and distal *P2* promoters, respectively (Tenhunen et al., 1994; Mannisto and Kaakkola, 1999). In most human tissues, the majority of COMT is present as S-COMT, with only a small fraction as MB-COMT (Tenhunen et al., 1994). Both COMT enzymes catalyze a wide variety of endogenous and exogenous catechol substrates. However, the kinetic properties and *in-vitro* regioselectivity of these enzymes are different (Pihlavisto and Reenila, 2002; Bai et al., 2007). Estrogens and CEs are also inactivated by sulfation, which is catalyzed by cytosolic SULTs (Raftogianis et al., 2000). Among these, SULT1A1 and SULT1E1 are the key enzymes of estrogen and CE inactivation (Hirata et al., 2008), while SULT2B1 might be important in CE conjugation (Adjei and Weinshilboum, 2002). Estrogens and their metabolites can also be eliminated by specific UGT proteins, forming inactive glucuronides. UGT2B7 is the major UGT expressed in the uterus, and it mainly catalyzes glucuronidation of 4-OH-estrogens. This can block the genotoxic oxidation of 4-OH-CEs to the quinone estrogens, and thus reduce their mutagenic potential (Zhu and Conney, 1998; Lepine et al., 2004). Also, conjugation of CEs with GSTs has an important role (Lo and Ali-Osman, 2007). Among the GSTs, the pi-class enzyme GSTP1 is the major GST isoform expressed consistently in a wide range of tissues, including endometrium (Terrier et al., 1990; Chan et al., 2005). Conjugation transforms estrogens into less active, and more polar and water-soluble metabolites, thus facilitating their excretion in the bile and urine (Neves et al., 2008). Clearly, the balance between the toxification and de-toxification metabolic pathways determines whether formation of the CE-quinones occurs (Joosten et al., 2004).

There are probably multiple overlapping mechanisms of estrogen carcinogenesis. Carcinogenesis is usually viewed as a step-wise process that begins with genotoxic effects (initiation), followed by enhanced cell proliferation (promotion). It is generally accepted that estrogens promote carcinogenesis by ER-mediated cell proliferation (Joosten et al., 2004), which results in an increased risk of genomic mutations during DNA replication. Another mechanism involves newly discovered membrane-associated ERs and appears to regulate extranuclear estrogen signaling pathways (Bolton and Thatcher, 2008). The third pathway of carcinogenesis involves the oxidative metabolism of estrogens to electrophilic/ redox-active estrogen ortho-quinones, with the concurrent formation of ROS, as a mechanism of tumour initiation by endogenous and exogenous estrogens (Cavalieri and Rogan, 2006; Bolton and Thatcher, 2008). Many studies have reported the carcinogenicity of estrogens and CEs through their mitogenic and mutagenic affects with regard to breast cancer. However, there are only a few studies of estrogen oxidative metabolism at the mRNA, protein and enzyme-activity levels in endometrial cancer (Sasaki et al., 2003a; Bochkareva et al., 2005; Chan et al., 2005; Lepine et al., 2010; Salama et al., 2008; Singh et al., 2008; Saini et al., 2009).

In the present study, we have characterized the local metabolism of estrogens in endometrial cancer and adjacent control endometrium at the mRNA as well as at the protein and cellular levels. We hypothesized that the alteration in estrogen phase I and II metabolism contributes to development of endometrial cancer.

2. Materials and methods

2.1. Tissue samples

Specimens of endometrial cancer and adjacent control endometrium were collected from 40 patients undergoing hysterectomies (mean age, 63 ±14 years) (Table 1). An additional nine control endometrium samples were obtained following hysterectomies from patients diagnosed with myoma uteri (mean age, 47 ±5 years) (Table 2). The study was approved by the National Medical Ethics Committee of the Republic of Slovenia. The specimens were immediately placed into RNA Later (Qiagene) RNA stabilization solution, and kept at –20 °C until RNA extraction. Diagnosis of carcinoma was confirmed histologically by an experienced gynaecological pathologist (J.Š.).

2.2. RNA isolation

Total RNA was isolated from 38 tissue samples using Tri Reagent (Sigma), according to the manufacturer instructions. The quality of the RNA samples was determined using Agilent 2100 Bioanalyser, and they showed an average RIN of 7.7. Total RNA was reverse transcribed using SuperScript® VILO™ cDNA Synthesis Kits (Invitrogen). One μg of total RNA was converted into cDNA (20 μl) according to the manufacturer instructions, and then stored at -20 °C.

2.3. Selection of normalization gene candidates

A cohort of 16 reference genes was tested on samples of endometrial cancer and adjacent control tissue (see Table 3). The TaqMan® Gene Signature Array Configuration (384) - Human Endogenous Control Array (Applied Biosystems, Foster City, CA, USA) was used. The most stable reference genes: *PPIA*, *HPRT1* and *POLR2A* were selected using both, GeNorm and NormFinder algorithms.

2.4. Quantitative real-time PCR

The expression of eleven genes of interest and three selected reference genes was examined by real-time TaqMan® PCR assay. Expression levels were determined with the exon-spanning hydrolysis probes (FAM or VIC dye labeled) that are commercially available as 'Assay on Demand' (Applied Biosystems, Foster City, CA, USA), with optimised primer and probe concentrations (Table 4). Quantification was accomplished with an Applied Biosystems 7500 real-time RT-PCR machine using TaqMan® Universal PCR Master Mix and universal thermocycling parameters recommended by Applied Biosystems. The RT-PCR samples were run in duplicates using 1 μL cDNA. The reactions were performed in 96-well plates (Applied Biosystems) in the reaction volume of 20 μL. The gene expression normalization factor for each sample was calculated based on the geometric mean of all three selected reference genes (Vandesompele et al., 2002). The Minimum Information for Publication of Quantitative Real-Time PCR Experiments (MIQE) guidelines were considered in the performance and interpretations of the qPCR reactions (Bustin et al., 2009).

2.5. Western blotting

Proteins were isolated from samples of cancerous endometrium and the adjacent control endometrium previously used for RNA isolation, following the Tri Reagent instructions. Protein aliquots of 60 μg were separated by SDS PAGE on 10% gels (NuPAGE® Novex 10% Bis-Tris Gel 1.0 mm, Invitrogen). The proteins were transferred from the gels to PVDF membranes (Millipore) and incubated with 5% non-fat milk for 1 h, to avoid non-specific binding. The membranes were then incubated overnight with the primary antibodies: rabbit anti-CYP1B1 (Abcam, ab33586, 1:20000), and rabbit anti-COMT (Millipore, ab5873, 1:7500) in TTBS with 1% non-fat milk powder. The next day, the secondary antibodies

(peroxidase-conjugated goat anti-rabbit IgG + IgM (H + L), Jackson ImmunoResearch Laboratories Inc., USA, 1:10000) were incubated with membranes for 2 h. The RapidStep ECL Reagent (Calbiochem) was used for detection of the bound antibodies, according to manufacturer instructions, using Fujifilm LAS4000 (Fujifilm, Japan). β-Actin (Sigma, A5441, 1:3000), cyclophilin A (Abcam, ab58144, 1:1000) and GAPDH (Sigma, G8795, 1:3000) were tested as normalisation controls. The mean values of the detected signals for cyclophilin A and GAPDH showed the best correlations to the amounts of proteins visualized on the gels after Coomassie Blue staining. Therefore, the protein levels of the investigated genes were normalised to these values. Quantification of Western blots was carried out with Multi-Gauge (Fujifilm software, Fujifilm, Japan).

2.6. Immunohistochemistry

Formalin-fixed, paraffin-embedded samples of the cancerous endometrium were de-waxed in xylene and rehydrated. Sections were incubated in H_2O_2 to block endogenous peroxidase. After antigen retrieval in sodium citrate buffer in a microwave oven, sections were incubated overnight with mouse anti-COMT (Abnova, H00001312-B01, 1:20) and rabbit anti-CYP1B1 antibodies (Abcam, ab33586, 1:200). The specificities of the antibodies were first checked by Western blotting. Control staining was performed by replacing the primary antibodies with serum of the same animal species. The peroxidase-antiperoxidase complex with the substrate 3,3'-diaminobenzidine (DAB Plus Substrate System, Thermo Fisher Scientific Inc.) were used to detect the bound antibodies.

Additionally, endometrial cancer/ adjacent paired tissue microarray (TMA; Pantomics, Inc, EMC241) was used to detect cellular CYP1B1 by immunohistochemical staining. TMA sections were first evaluated by a pathologist (J.Š.), to confirm the diagnoses determined by the manufacturer. Eleven pairs of cancerous and adjacent control sections were confirmed

(Table 5). In one pair, both of the sections were diagnosed as tumour sections, and so they were excluded from further evaluation. The TMA were processed as described above for full-section slides. A quick-score system was used for immunohistochemical scoring: the data represent the products of the percentages of positive cells and the intensities of the stain (scoring interval = 0-300).

2.7. Statistical analyses

Statistical calculations and tests were performed using the SPSS Software (SPSS Inc., USA). The Wilcoxon test was used for paired samples, and the Mann-Whitney test for unrelated samples. All of the statistical tests were two-tailed, and differences in p values of less than 0.05 were considered to be significant.

3. Results

3.1. *CYP1B1* codes for the most abundant phase I enzyme in human endometrium, and it is down-regulated in endometrial cancer

We initially studied the expression of five genes coding for estrogen phase I metabolizing enzymes by real-time PCR in 38 samples of cancerous and adjacent control endometrium: *CYP1A1*, *CYP1A2*, *CYP1B1*, *CYP3A5* and *CYP3A7* (Figure 2). There were significantly lower mRNA levels of *CYP1B1* (5.4-fold; $p < 0.0001$; 95% of samples) and *CYP3A7* (9.6-fold; $p = 0.0006$; 79% of samples) in the cancerous tissues. Among all of the genes coding for the phase I enzymes, *CYP1B1*, the prevalent estrogen 4-hydroxylase, was the most abundant CYP isoform in both control and cancerous tissues. The second most abundant was *CYP3A5*, which is mainly responsible for 16α-hydroxylation, but which was not aberrantly expressed in the tumors ($p = 0.4684$). The levels of *CYP1A1* were much lower (more than 10^4-fold) than the

levels of *CYP1B1*, and there was no significant difference in *CYP1A1* expression between cancerous and adjacent control endometrium (p = 0.7607). The expression of *CYP1A2* was hardly detectable. These more than 10^4-fold lower levels of *CYP1A1* and negligible mRNA levels of *CYP1A2* compared to *CYP1B1* suggested a marginal role for estrogen 2-hydroxylation in both control and cancerous endometrial tissue.

3.2. Protein levels of CYP1B1 are also decreased in endometrial cancer

Protein expression levels of CYP1B1 were examined in 15 samples of cancerous and adjacent control endometrium. The results were assessed by Western blotting and showed significantly lower protein levels of CYP1B1 in cancerous tissue (2.9-fold; p = 0.0002). Protein levels of CYP1B1 were decreased in 14 cases (93%) (Figure 3). CYP1B1 expression was also examined at the cellular level, using immunohistochemical staining (Figure 4). CYP1B1 was detected all over the tumour samples, predominantly in epithelial cells, but to a lesser extend also in stromal cells. The average scores using the quick-score system (see Methods) for 18 cancerous specimens was 175 ±35 for epithelial and 103 ±36 for stromal cells. In 14 specimens, normal and simple hyperplastic endometrial glands were also present, and were used as controls. The mean score for the control specimens was 197 ±31. The score was lower in 13 of 14 specimens and a small, and statistically significant, difference was seen for the cancerous gland staining, compared to control glands (p = 0.0143). As not all of the specimens contained control endometrial glands, we introduced an additional 8 control endometrial samples of myoma uteri patients. The mean score for these control specimens was 178 ±22 for epithelial and 160 ±23 for stromal cells. No difference was found between cancerous and control endometrial specimens of myoma patients for the staining of epithelial cells. A difference was, however, seen in stromal cells, where the staining was statistically

significantly weaker in tumour cells, compared to control endometrium of myoma patients ($p = 0.0009$).

TMA was also used to evaluate CYP1B1 cellular status (Figure 4). The TMA included 11 endometrial cancer samples paired with their corresponding non-involved tissue from the same patients. The mean score for cancerous and adjacent control sections was 146 ±43 and 162 ±33 for epithelial and 73 ±44 and 88 ±33 for stromal cells, respectively. Control sections showed slightly higher scores than cancer sections; this differences was, however, not statistically significant ($p = 0.1763$) (Figure 5).

3.3. Genes coding for phase II enzymes SULT2B1, UGT2B7 and GSTP1 are over expressed in cancerous tissue

The expression levels of genes coding for phase II enzymes, were also measured in 38 samples of cancerous and adjacent control endometrium: *SULT1A1, SULT1E1, SULT2B1, COMT, UGT2B7* and *GSTP1* (Figure 2). Significantly higher mRNA levels were seen in cancerous than control tissues for *SULT2B1* (10.3-fold; $p < 0.0001$; 84% of samples), *UGT2B7* (2.1-fold; $p = 0.0342$; 63% of samples) and *GSTP1* (1.4 fold; $p = 0.0001$; 75% of samples). *COMT* and *SULT1A1* expression were not altered ($p = 0.1315$; $p = 0.6322$; respectively). The levels of *SULT1E1* were decreased in 71% of cancerous samples, yet not with any statistical significance ($p = 0.1173$). The mRNA levels of *GSTP1* and *COMT* were the highest, suggesting that these represent the main detoxification force in endometrial tissue.

3.4. S-COMT is the prevalent COMT isoform in cancerous endometrium, and MB-COMT prevails in adjacent control tissue

COMT protein levels were examined in 16 samples of cancerous and adjacent control endometrium by Western blotting (Figure 3). Significantly lower protein levels of MB-COMT

(2.8-fold; p = 0.0015) were seen in cancerous tissue. Levels of S-COMT were elevated in the tumours of all 16 samples examined, and this increase was statistically significant (2.1-fold; p = 0.0005). The total COMT levels remained unchanged (p = 1.0000), which correlated well with our qPCR experiments. The comparisons of the cancerous samples and the control samples from the same individuals revealed that in control endometrium the levels of MB-COMT were 2.96-fold higher than the levels of S-COMT, whereas in the tumour the levels of MB-COMT were 2.71-fold lower. This MB-COMT/S-COMT ratio was thus increased 8.00-fold in adjacent control compared to cancerous endometrium (p = 0.0006), indicating that in cancerous endometrium, S-COMT is the most abundant COMT isoform, while in contrast, MB-COMT appears to be of greater importance in adjacent control tissue.

There are at present no specific antibodies available for MB-COMT or S-COMT, so we could only examine total COMT at the cellular level (Figure 4). Positive immunohistochemical staining was seen mainly in epithelial cells, while stromal staining was very weak. The mean score for 18 cancerous specimens was 141 ±24 for epithelial and 24 ±17 for stromal cells. Normal and hyperplastic endometrial glands were present in 14 of a total of 18 specimens, and the mean score for these control epithelial cell specimens was 147 ±24. As expected, there were no significant differences in these scores between cancerous and control epithelium.

We also stained 9 control endometrium specimens of myoma uteri patients. The mean score of these control specimens was 158 ±35 for epithelial and 84 ±40 for stromal cells. We saw no significant differences in epithelial cell staining between cancerous and control specimens of these myoma patients. Interestingly, the stromal cells scores for the control myoma samples were significantly higher than for the tumours (p = 0.0003).

3.5. The significantly lower CYP1B1/COMT ratio in endometrial cancer suggests decreased synthesis of 4-OH-E1/E2 and increased formation of MeO-estrogens

We compared the CYP1B1/COMT expression ratios to determine the contributions of these enzymes in cancerous and adjacent control tissues. We found a 4.84-fold lower CYP1B1/COMT ratio in tumours when comparing mRNA expression levels ($p < 0.0001$) (not shown), and a 3.50-fold decrease when analyzing protein levels ($p = 0.0001$) (Figure 3). A decreased ratio between the activating enzyme CYP1B1 and the detoxificating COMT suggests reduced synthesis of 4-OH-E1/E2 accompanied by increased formation of protective MeO-estrogens in the cancerous tissue.

Furthermore, we compared CYP1B1 protein levels with levels of the MB-COMT and S-COMT isoforms (Figure 3). The CYP1B1/MB-COMT ratio was surprisingly not altered in cancerous compared to adjacent control tissue (1.08-fold; $p = 0.3303$, statistically significantly effective pairing), suggesting that decreased CYP1B1 expression in tumours is associated with the same fold-lower MB-COMT expression. On the other hand, the CYP1B1/S-COMT ratio was statistically significantly reduced (11.47-fold; $p = 0.0001$) in the cancer tissue, showing that here a lower expression of CYP1B1 was accompanied by an increased expression of S-COMT.

4. Discussion

Estrogens have been shown to contribute to the growth and development of endometrial cancer. The most active estrogen, E2, stimulates cell proliferation and gene expression via the ERs, while by forming oxidative metabolites, it causes DNA damage (Zhu and Conney, 1998; Parl et al., 2009). In the present study, we assessed the mRNA expression profiles of the 10 most abundant estrogen phase I and phase II metabolizing enzymes. We also studied the

expression of the major phase I and II enzymes, CYP1B1 and COMT, at the protein and cellular levels.

The CYP1A1 and CYP1A2 enzymes are mainly responsible for 2-hydroxylation of estrogens (Zhu and Lee, 2005). 2-OH-E1/E2 can be oxidized to E1/E2-2,3-Q, which is believed to be less carcinogenic. The moderate expression of *CYP1A1* and the hardly detectable *CYP1A2* levels seen in our study suggest a marginal role for estrogen 2-hydroxylation in both control and cancerous endometrial tissues.

The major CYP isoform in human endometrium is CYP1B1, which mainly catalyzes 4-hydroxylation of estrogens. Previous studies have suggested that high levels of estrogen 4-hydroxylase activity in estrogen target tissues are important in estrogen-induced carcinogenesis (Zhu and Conney, 1998; Cavalieri and Rogan, 2006; Bolton and Thatcher, 2008). However, our data show significantly decreased levels of *CYP1B1* in cancerous endometrium compared to adjacent control tissue. This same expression pattern in endometrial cancer was reported by Singh et al. (2008), and recently by Lepine at al. (Singh et al., 2008; Lepine et al., 2010). Knowing that mRNA levels do not necessarily correlate with the amount of their respective proteins, we examined protein levels of CYP1B1 by Western blotting and immunohistochemical staining. The 2.9-fold decrease in CYP1B1 protein levels in cancerous tissue indicated reduced 4-hydroxylation in the tumours. The same pattern was seen at the cellular level. In contrast to our results Saini et al. reported up-regulation of CYP1B1 in endometrial cancer based on immunohistochemical staining of 48 cases, but their study was not supported by quantification and statistical analysis (Saini et al., 2009). As others have seen previously, we show that in human endometrium, the levels of the 4-hydroxylating enzymes exceed those of the 2-hydroxylating enzymes (Sasaki et al., 2003a; Lepine et al., 2010). Due to reduced expression of *CYP1B1* in the tumours, its possible role in precancerous tissue has also to be taken into consideration, especially knowing that our

adjacent control endometrium might have already undergone some hyperplastic changes, and endometrial hyperplasia is regarded as a preliminary stage of endometrioid carcinoma type 1.

It has been suggested that 16α-OH-E1 has an important role in mammary carcinogenesis (Nebert, 1993). CYP3A7 is the main 16α-hydroxylating enzyme, followed by CYP3A5 (Lee et al., 2003; Zhu and Lee, 2005). In the present study, both genes, *CYP3A7* and *CYP3A5* were expressed at high levels. No differences were seen for *CYP3A5*; however, *CYP3A7* was statistically significantly down-regulated in cancerous tissues, suggesting that, as with 4-hydroxylation, 16α-hydroxylation is also decreased in endometrial cancer.

Thus, two out of three reactions of estrogen oxidative metabolism appear to be deficient in endometrial cancer. Our data imply decreased production of potentially carcinogenic 4-OH-E1/E2 and 16α-OH-E1/E2 in the tumours, accompanied by unchanged, but low, 2-hydroxylation.

Oxidation of estrogens is followed by their methylation to the less harmful MeO-estrogens. COMT is expressed in various human tissues, but its precise role and the ratio between the S-COMT and MB-COMT isoforms remain unclear. A membrane-spanning extension in the amino-terminus of MB-COMT contributes to the higher binding affinity of its substrates (Lotta et al., 1995). And the catalytic efficiency of S-COMT for 4-O-methylation is approximately 50% lower than the efficiency for 2-O-methylation, whereas the efficiency of MB-COMT for both CEs is comparable (Bai et al., 2007). We observed no differences in the expression of total COMT at the mRNA and protein levels. In contrast to our results, Lepine at al. reported elevated mRNA levels of *COMT* in endometrial cancer compared to adjacent control tissue (Lepine et al., 2010). This difference in expression profiles may have arisen from different normalization procedures used in the qPCR data quantification. We carefully selected normalization controls among 16 genes examined, while

Lepine et al. used only one gene for normalization. Furthermore, Western blotting and immunohistochemical analyses also confirmed our qPCR data. Additionally, Western blotting revealed modified expression of the COMT isoforms: S-COMT was present in cancerous endometrium, while MB-COMT was the most abundant in the adjacent control endometrium. To the best of our knowledge, this is the first study showing increased levels of S-COMT in endometrial cancer suggesting lower capacity for 4-O-methylation in tumour. Our data showing decreased MB-COMT expression in endometrial cancer can be explained by the elevated CpG methylation of the MB-COMT promoter that was observed by Sasaki et al. in 78.3% of cancer cases, and was also confirmed by lower mRNA levels for *MB-COMT*. However, the same study showed that *S-COMT* was expressed and unmethylated (Sasaki et al., 2003b); therefore, the mechanisms behind the elevated levels of S-COMT in cancerous endometrium are not clear at the moment, and remain to be further defined.

We found no changes in the CYP1B1/MB-COMT ratio, which demonstrates that in the tumours, the decrease in CYP1B1 expression correlates well with the down-regulation of MB-COMT. Even more so, the fold-levels of the decreases here were almost identical (2.9 and 2.8 for CYP1B1 and COMT, respectively). However, the CYP1B1/S-COMT ratio was reduced, implying that the decreased CYP1B1 levels in the tumours were accompanied by elevated S-COMT expression. These data suggest that the expression of CYP1B1 and MB-COMT are linked at either the transcriptional or translational levels. The role of each COMT isoform in endometrial cancer therefore needs further investigation.

Also sulfate conjugation is an important pathway for estrogen biotransformation (Adjei and Weinshilboum, 2002; Adjei et al., 2003; Pasqualini and Chetrite, 2005). SULT1E1, SULT1A1 and SULT2B1 catalyze the sulfation of CEs and E2. In the present study, lower levels of *SULT1E1*, the most efficient of the SULT enzymes, were observed in 70% of cancer cases, thus suggesting decreased sulfation of CEs and estrogens. However, the

significant increase in *SUL2B1*, the most abundant SULT enzyme in the cancerous endometrium, indicated that lower levels of *SULT1E1* may be compensated for by much higher levels of *SULT2B1*.

Another important conjugative pathway of estrogens, CEs and MeO-E1/E2 is by glucuronidation. Several UGTs catalyze these reactions (Guillemette et al. 2004; Lepine et al., 2004; Lepine et al., 2010), but UGT2B7 has the highest catalytic efficiency towards the carcinogenic 4-OH-E1/E2 (Cheng et al., 1998; Raftogianis et al., 2000; Guillemette et al., 2004; Lepine et al., 2004). Significantly increased levels of *UGT2B7* were seen in the cancerous endometrium suggesting increased 4-OH-E1/E2 detoxification by glucuronidation. This is also in agreement with recently reported up-regulation of *UGT2B7* in endometrial cancer (Lepine et al., 2010).

GSTs catalyze the conjugation of CEs and CE-Qs, and thus have important roles in cell detoxification (Butterworth, et al. 1997; Bolton and Thatcher 2008). We observed statistically significantly elevated levels of *GSTP1* in cancerous compared to adjacent control endometrium. *GSTP1* was increased in 75% of specimens examined. This increase can be explained by the elevated local E2 levels in endometrial cancer (Berstein et al., 2003; Ito, 2007), as increased GST activities were observed in female rats treated with E2 (Sanchez et al., 2003). In contrast to our data, Chan et al. reported reduced *GSTP1* expression in 25 specimens of endometrial cancer (Chan et al., 2005). As GSTP1 is highly polymorphic and this study was performed on an Asian population, this may well be the reason for the observed differences.

Thus, among six genes coding for the most abundant conjugative enzymes, three were overexpressed (*SULT2B1, UGT2B7* and *GSTP1*), the levels of another two were unchanged (*COMT* and *SULT1A1*), and only one was down-regulated (*SULT1E1*), although not

statistically significantly, in cancerous endometrium. Our data suggest that the overall expression of estrogen phase II metabolising enzymes is increased in these tumours.

5. Conclusions

Our data show imbalance between the phase I and phase II enzymes in endometrial cancer. In cancerous endometrium we found lower levels of CYP1B1 suggesting decreased production of 4-hydroxycatechols. We also observed, for the first time, higher protein levels of S-COMT and lower levels of MB-COMT in cancerous endometrium. Although CYP1B1 and COMT enzymatic activities in cancerous and adjacent control endometrium still need to be evaluated, our data imply that the adjacent control endometrium may represent a preliminary stage of endometrioid carcinoma, where oxidative metabolism exceeds conjugation, thus favouring the production of the carcinogenic CEs and quinones.

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FIGURE LEGENDS

Figure 1. Phase I and II metabolism of the estrogens. Oxidative metabolism of estrogens to catecholestrogens (CEs): 2- and 4-hydroxy-estradiol/estrone (2-OH-E1/E2 and 4-OH-E1/E2), catalyzed predominantly by CYP1A1/1A2 and CYP1B1. CEs can be further oxidized to semiquinones and quinones, which can undergo redox cycling, thus producing reactive oxygen species that can further cause oxidative stress, lipid peroxidation, and DNA damage. Extrahepatic, 16α-hydroxylation is mainly catalyzed by CYP3A5 and CYP3A7. Metabolism of CEs and 16α-OH-E1/E2 is catalyzed by the phase II metabolic enzymes: COMT, SULT1A1, SULT2B1, SULT1E1, UDP2B7 and GSTP1. Enzymes of our interest are marked with assessed gene regulation. ↓ down-regulated genes, ↑ up-regulated genes, = not altered, and * statistically significant change in expression levels**.**

Figure 2. Gene expression of phase I and II estrogen-metabolic enzymes in paired samples of endometrial cancer.

Before-and-after graphs showing the normalized expression levels of the *CYP1A1, CYP1B1, CYP3A5, CYP3A7, COMT, SULT1A1, SULT2B, SULT1E1, UGT2B7* and *GSTP1* genes in their control (Control) and corresponding cancerous endometrium (Tumour). The levels of gene expression are presented on a logarithmic scale.

Figure 3. Western blotting for CYP1B1 and COMT.

A. ECL analysis of CYP1B1, MB-COMT and S-COMT in the 16 samples of cancerous - T and adjacent control endometrium - C. Placenta - P served as a reference sample on each membrane. B. Before-and-after graphs as for Figure 2, showing decreased levels of CYP1B1 and MB-COMT, increased levels of S-COMT and unchanged levels of total COMT, across control and tumour samples. Protein levels were normalized to cyclophilin A and GAPDH. C.

Box & whiskers graphs (whiskers: 5–95 percentile) demonstrate decreased ratios MB-COMT/S-COMT, CYP1B1/S-COMT and CYP1B1/Tot-COMT and unchanged ratios CYP1B1/MB-COMT in endometrial cancer. D. Representative ECL analysis of cyclophilin A and GAPDH expression used for normalization of CYP1B1 and COMT protein levels.

Figure 4. Immunohistochemical staining for CYP1B1 and COMT in cancerous and control endometrium

A. and B. Scores of control myoma endometrium (Myoma), cancerous endometrium (Tumour), and adjacent control endometrium (Control); shown on before-and-after graphs. Bars denote mean scores. C. Specificity of anti-COMT and anti-CYP1B1 antibodies determined by Western blotting of protein fractions of human placenta. D and E. Immunohistochemical staining for CYP1B1 and COMT in representative specimens of control myoma endometrium (M), cancerous endometrium (T) and adjacent control endometrium (C). F. Immunohistochemical stainig for CYP1B1 in tissue microarrays (TMAs) with 12 cores of endometrial cancer and corresponding uninvolved tissue cores. The whole TMA and one control tissue (C) and cancerous endometrium (T) core are shown. G.-I. The before-and-after graphs show CYP1B1 and COMT scores for cancerous tissue (Tumour) and adjacent control endometrium (Control), and the scores for CYP1B1 staining of TMA.

Table 1. Patient details for the cancer group.

No.	Sample	Age (years)	Pathology	Phase	Stage (TNM)
1	5	39	G2/G3	secretory	T1bN0M0
2	7	50	G1		T1bN0M0
3	8	83	G2/G3		T2aN0M0
4	10	53	G1	proliferative	T1aN0M0
5	11	60	G1		T1cN0M0
6	13	64	G2		T1cN0M0
7	14	73	G2		T1cN0M0
8	16	69	G1/G2		T1bN0M0
9	18	79	G1		T1cN0M0
10	19	74	G1/G2		T1bN0M0
11	20	76	G2/G3		T1bN0M0
12	21	53	G2	proliferative	T1aN0M0
13	22	36	G1/G2	NA	T1aN0M0
14	23	45	G1/G2	early s.	T1aN0M0
15	24	69	G2/G3		T1cN0M0
16	25	54	G3		T1aN0M0
17	26	72	G1/G2		T1bN0M0
18	30	54	G1		T1aN0M0
19	31	69	G2/G3		T1bN0M0
20	32	52	G1		T1aN0M0
21	33	77	G3		T1bN0M0
22	34	57	G1		T1bN0M0
23	35	61	G2		T1aN0M0
24	38	78	G2/G3		T1bN0M0
25	40	71	G1		T1aN0M0
26	42	81	G1		T1bN0M0
27	44	73	G1		T1bN0M0
28	46	50	G2/G3		T1aN0M0
29	47	27	G1/G2	late s.	T1aN0M0
30	49	70	G1		T1bN0M0
31	50	73	G1		T1aN0M0
32	51	75	G2/G3		T1bN0M0
33	52	75	G2/G3		T1bN0M0
34	53	50	G2/G3	proliferative	T1aN0M0
35	54	71	G1		T1bN0M0
36	55	75	G2		T1cN1M1
37	56	55	G1/G2		T1aN0M0
38	57	43	G1	late s.	T1aN0M0
39	39	63	G1		T2aN0M0
40	58	68	G2/G3		T1aN0M0

G1-G3, grading according to histological differentiation
NA: data not available

Table 2. Patient details for the further control group.

Sample	Age (years)	Diagnosis	Phase
M1	42	Uterus	non-proliferative
M2	47	Myoma uteri	late secretory
M3	43	Uterus	early secretory
M5	45	Uterus	proliferative
M6	50	Uterus	early secretory
M9	47	Uterus	proliferative
M10	48	Myoma uteri	proliferative
M11	43	Uterus	early secretory
M25	60	Myoma uteri	atrophic

Table 3. Reference gene details.

Gene symbol	Assay ID	Gene Name
18S	Hs99999901_s1	Eukaryotic 18S rRNA
ACTB	Hs99999903_m1	actin, beta
B2M	Hs99999907_m1	beta-2-microglobulin
GAPD	Hs99999905_m1	glyceraldehyde-3-phosphate dehydrogenase
GUSB	Hs99999908_m1	glucuronidase, beta
HMBS	Hs00609297_m1	hydroxymethylbilane synthase
HPRT1	Hs99999909_m1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan
IPO8	Hs00183533_m1	importin 8
PGK1	Hs99999906_m1	phosphoglycerate kinase 1
POLR2A	Hs00172187_m1	polymerase (RNA) II (DNA directed) polypeptide A,
PPIA	Hs99999904_m1	peptidylprolyl isomerase A (cyclophilin A)
RPLP0	Hs99999902_m1	ribosomal protein, large, P0
TBP	Hs99999910_m1	TATA box binding protein
TFRC	Hs99999911_m1	transferrin receptor (p90, CD71)
UBC	Hs00824723_m1	ubiquitin C
YWHAZ	Hs00237047_m1	tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein, zeta polypeptide

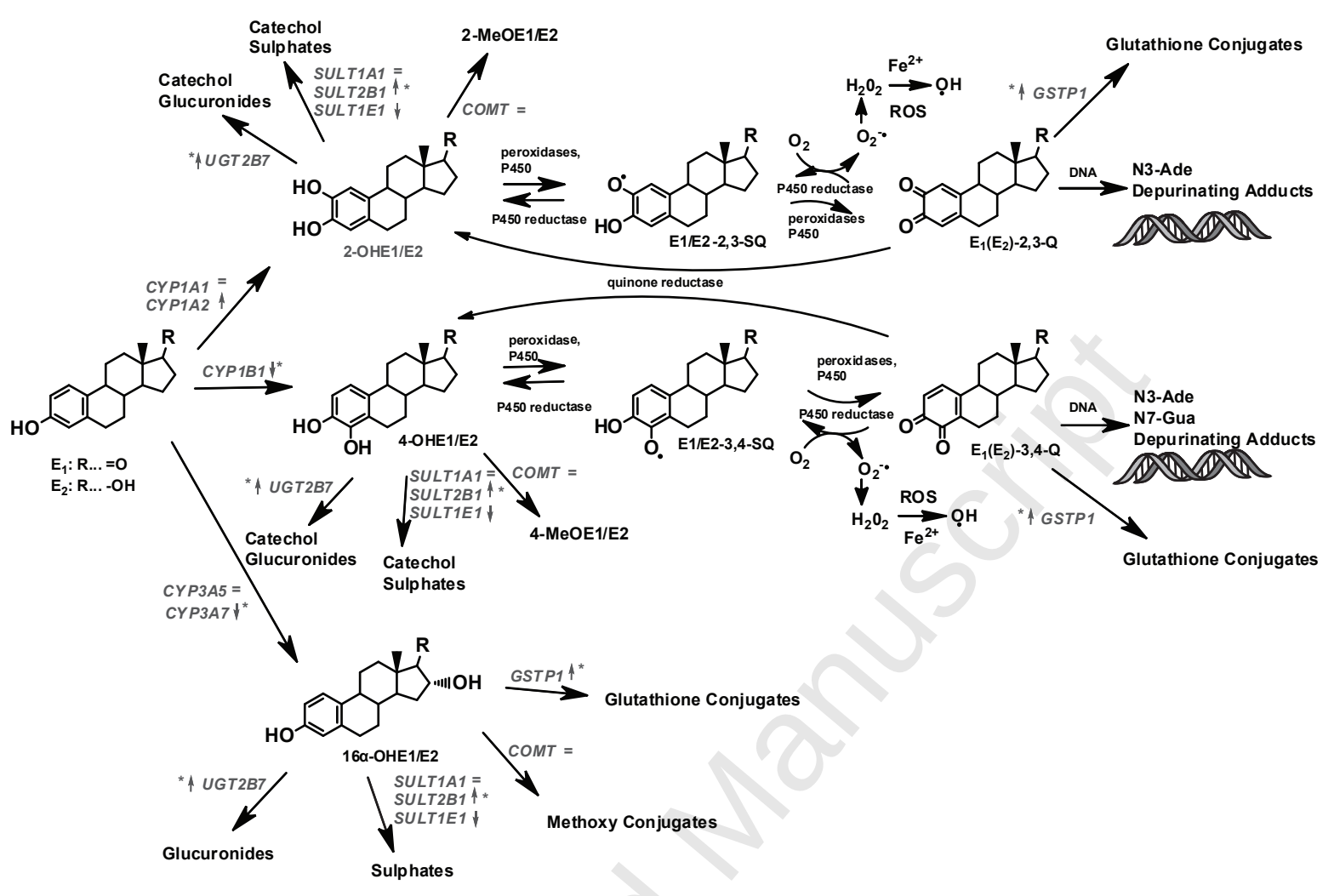
Table 4. Details of genes investigated.

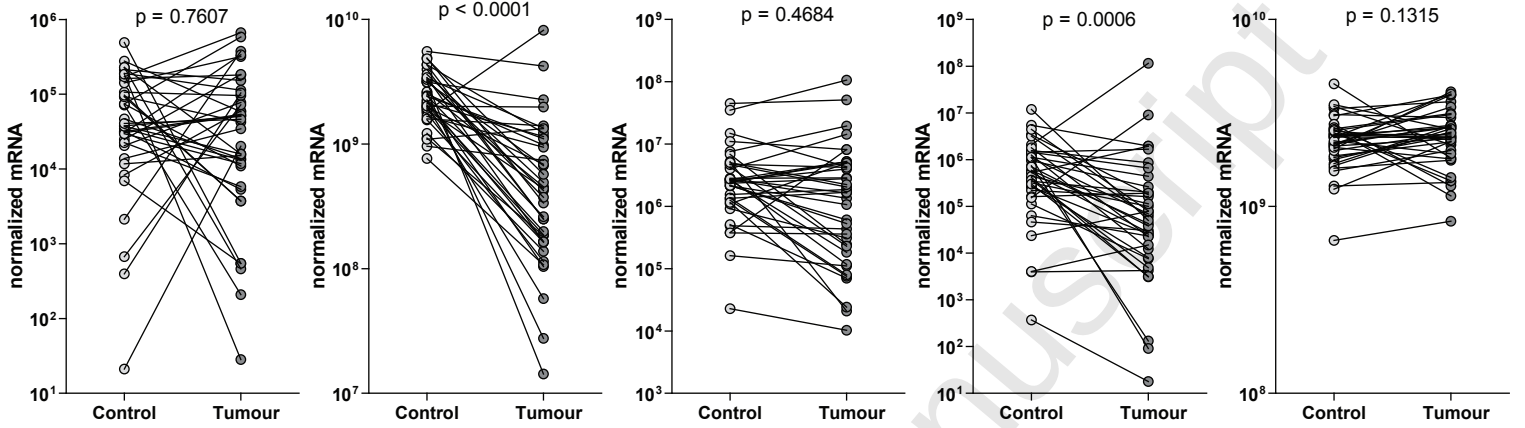
Gene Symbol	Assay ID	Gene Name
CYP1A1	Hs00153120_m1	cytochrome P450, family 1, subfamily A, polypeptide 1
CYP1A2	Hs00167927_m1	cytochrome P450, family 1, subfamily A, polypeptide 2
CYP1B1	Hs00164383_m1	cytochrome P450, family 1, subfamily B, polypeptide 1
CYP3A5	Hs00241417_m1	cytochrome P450, family 3, subfamily A, polypeptide 5
CYP3A7	Hs00426361_m1	cytochrome P450, family 3, subfamily A, polypeptide 7
COMT	Hs00984971_m1	catechol-O-methyltransferase
UGT2B7	Hs00426592_m1	UDP glucuronosyltransferase 2 family, polypeptide B7
SULT1A1	Hs00419411_m1	sulfotransferase family, cytosolic, 1A, phenol-preferring,
SULT2B1	Hs00190268_m1	sulfotransferase family, cytosolic, 2B, member 1
SULT1E1	Hs00193690_m1	sulfotransferase family 1E, estrogen-preferring, member 1
GSTP1	Hs00168310_m1	glutathione S-transferase pi 1
PPIA	Hs99999904_m1	peptidylprolyl isomerase A (cyclophilin A)
HPRT1	Hs99999909_m1	hypoxanthine phosphoribosyltransferase 1 (Lesch-Nyhan
POLR2A	Hs00172187_m1	polymerase (RNA) II (DNA directed) polypeptide A, 220

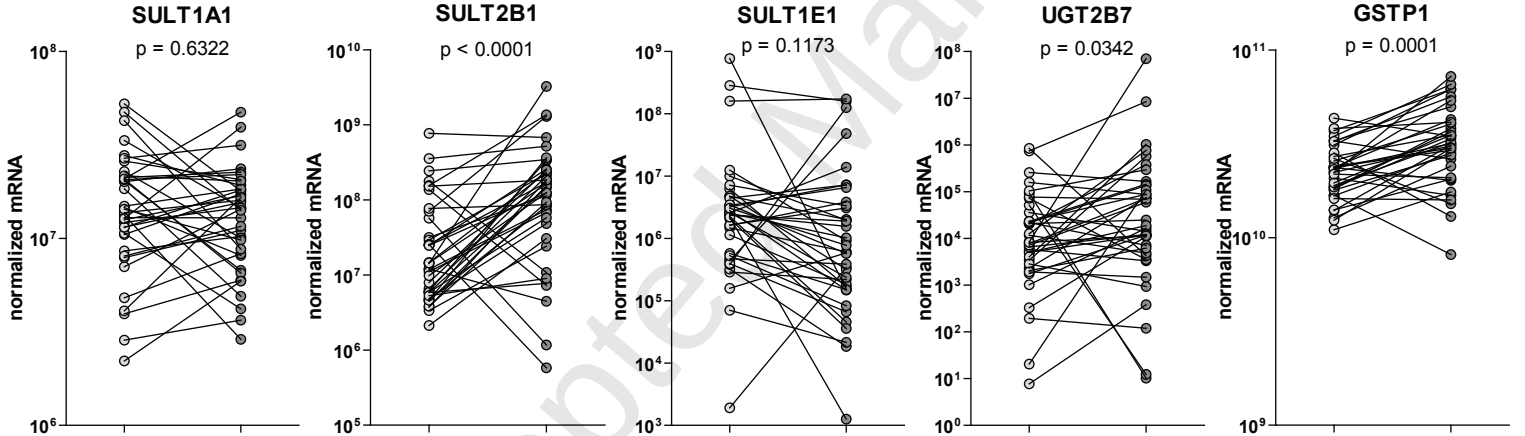
Table 5. Endometrial cancer/ adjacent paired tissue array details for scoring of CYP1B1 immunohistochemical staining.

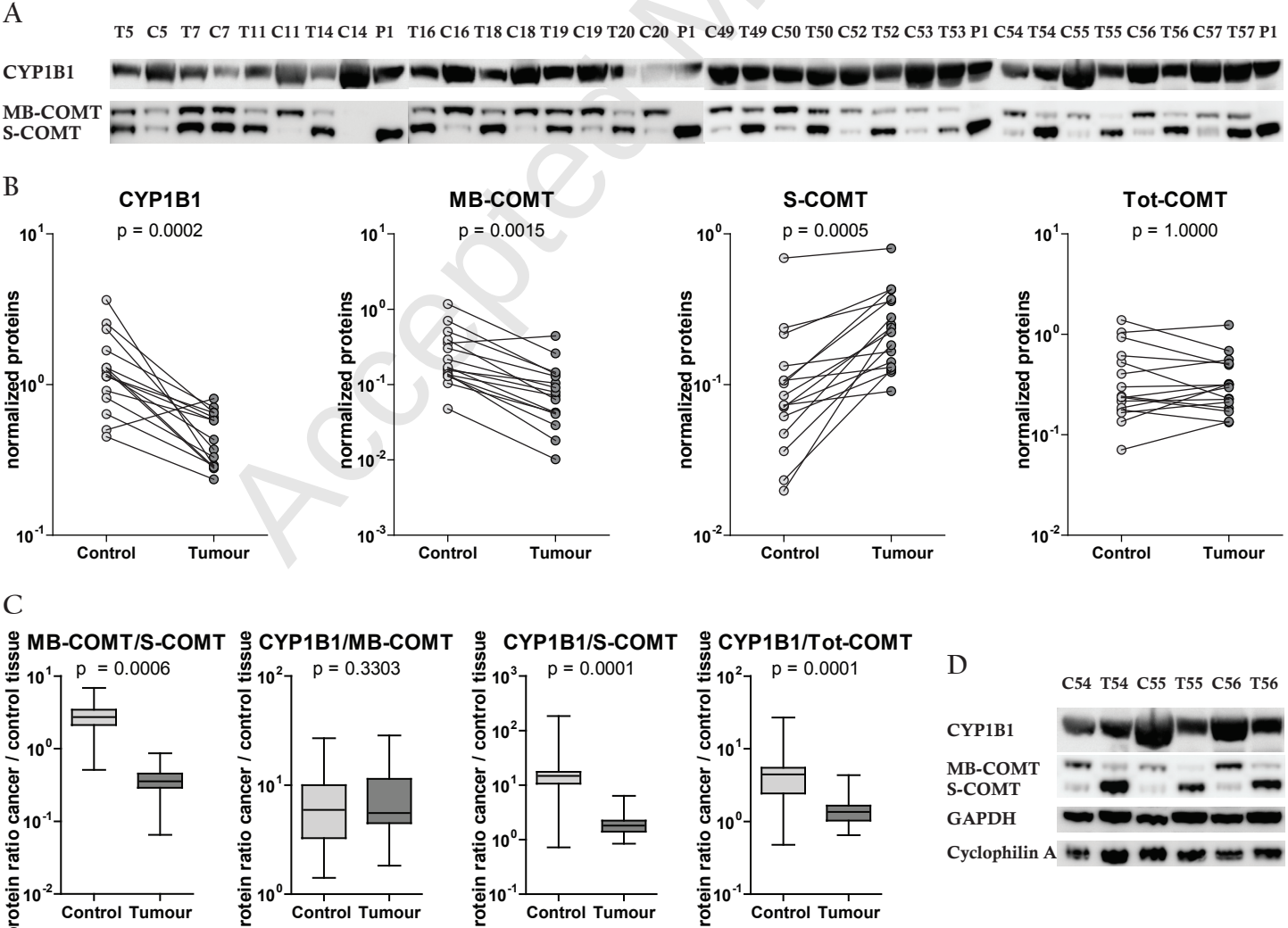
Sample	Age (years)	Pathology	Stage (TNM)	Score E	Score S
A1	44	Adenocarcinoma grade II	T1N0M0	210	115
B1	44	Uninvolved endometrial tissue	-	229	122
A2	56	Adenocarcinoma grade II	T1N0M0	176	133
B2	56	Uninvolved endometrial tissue	-	188	140
A3	51	Adenocarcinoma grade I-II	T1N0M0	151	40
B3	51	Uninvolved endometrial tissue	-	192	120
A4	36	Adenocarcinoma grade II-III	T1N0M0	130	120
B4	36	Uninvolved endometrial tissue	-	150	100
A5	40	Adenocarcinoma grade II-III	T2N1M0	154	100
B5	40	Uninvolved endometrial tissue	-	185	110
A6	60	Adenocarcinoma grade II-III	T1N0M0	170	110
B6	60	Uninvolved endometrial tissue	-	111	100
C1	49	Adenocarcinoma grade II-III	T1N1M0	180	40
D1	49	Uninvolved endometrial tissue	-	138	53
C2	45	Adenocarcinoma grade III	T1N0M0	160	60
D2	45	Uninvolved endometrial tissue	-	140	50
C4	51	Adenocarcinoma grade III	T3N1M0	110	50
D4	51	Uninvolved endometrial tissue	-	163	67
C5	53	Adenocarcinoma grade III	T2N0M0	50	0
D5	53	Uninvolved endometrial tissue	-	145	60
C6	56	Adenocarcinoma grade III	T1N0M0	120	33
D6	56	Uninvolved endometrial tissue	-	143	50

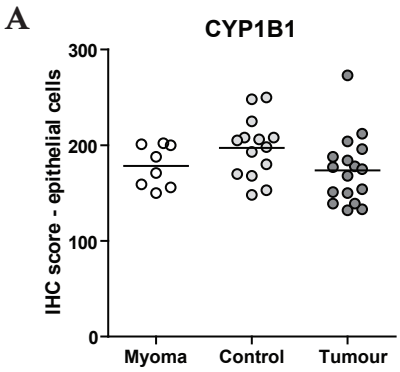
E: epithelial cells, S: stromal cells

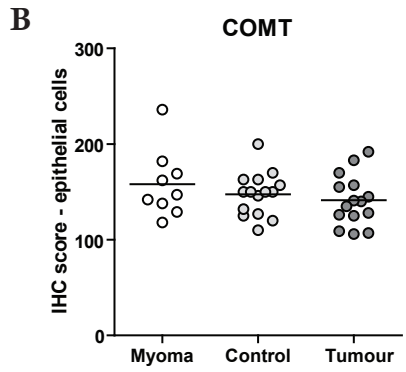

Catechol Sulphates
2-MeOE1/E2
Catechol Glucuronides
SULT1A1 =
SULT2B1 ↑*
SULT1E1 ↓
COMT =
*↑UGT2B7
peroxidases, P450
P450 reductase
2-OHE1/E2
E1/E2-2,3-SQ
O2
O2-•
Fe2+
H2O2
OH•
ROS
P450 reductase
peroxidases P450
*↑GSTP1
Glutathione Conjugates
DNA
N3-Ade Depurinating Adducts
E1(E2)-2,3-Q
quinone reductase
CYP1A1 =
CYP1A2 ↑
CYP1B1 ↓*
E1: R... =O
E2: R... -OH
peroxidase, P450
4-OHE1/E2
E1/E2-3,4-SQ
peroxidases, P450
P450 reductase
N3-Ade
N7-Gua
Depurinating Adducts
E1(E2)-3,4-Q
*↑UGT2B7
Catechol Glucuronides
SULT1A1 =
SULT2B1 ↑*
SULT1E1 ↓
Catechol Sulphates
COMT =
4-MeOE1/E2
CYP3A5 =
CYP3A7 ↓*
16α-OHE1/E2
GSTP1 ↑*
Glutathione Conjugates
COMT =
*↑UGT2B7
Glucuronides
SULT1A1 =
SULT2B1 ↑*
SULT1E1 ↓
Sulphates
Methoxy Conjugates

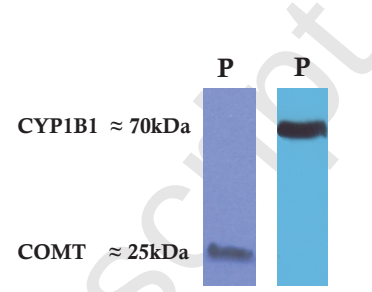


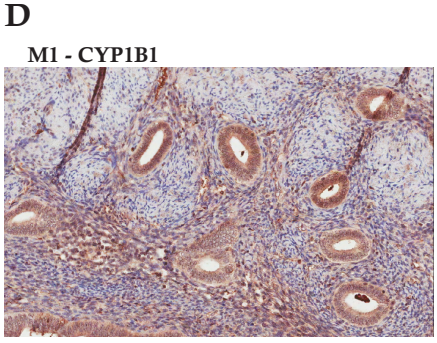


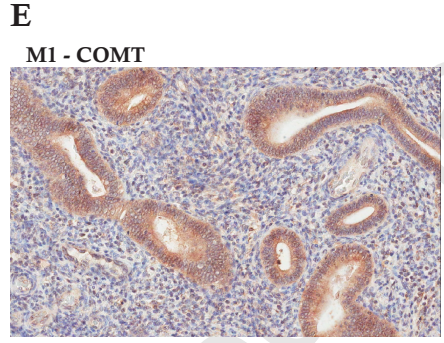



A
CYP1B1
IHC score - epithelial cells
300
200
100
0
Myoma
Control
Tumour

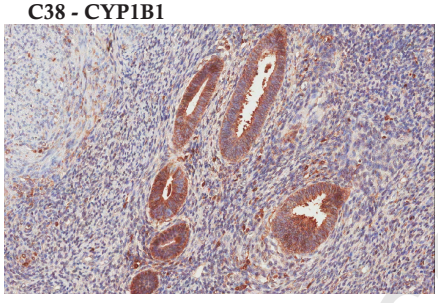

B
COMT
IHC score - epithelial cells
300
200
100
0
Myoma
Control
Tumour

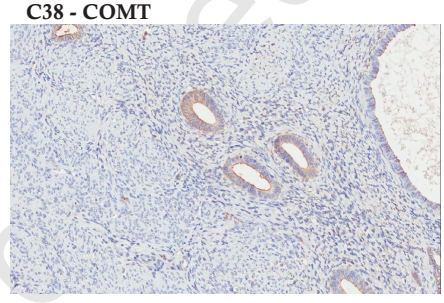

C
P
P
CYP1B1 ≈ 70kDa
COMT ≈ 25kDa

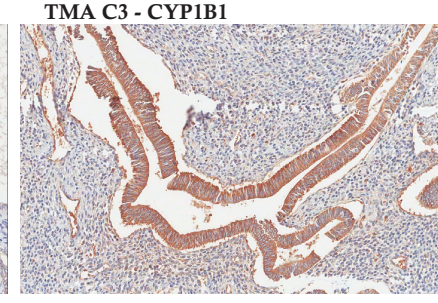

D
M1 - CYP1B1

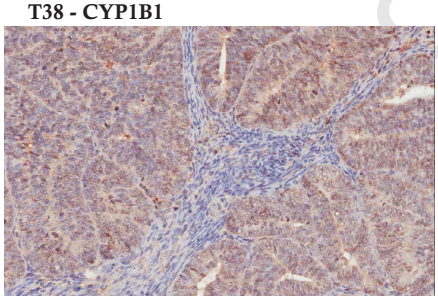

E
M1 - COMT

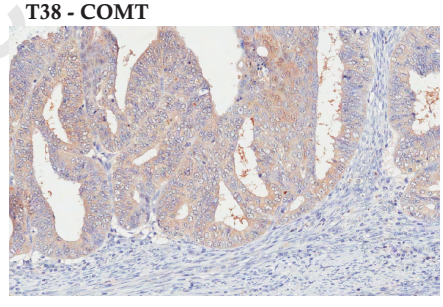

F
TMA - CYP1B1

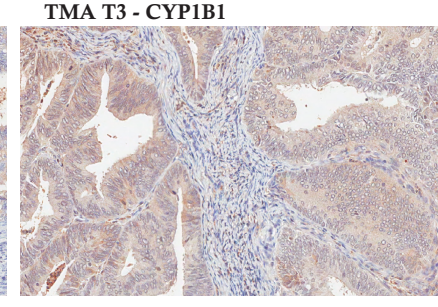

C38 - CYP1B1

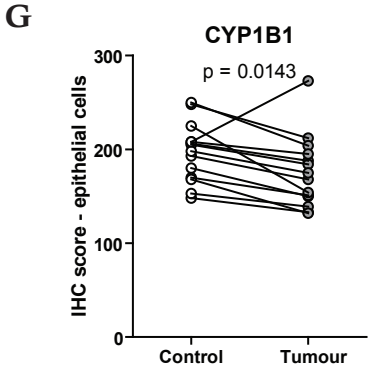

C38 - COMT

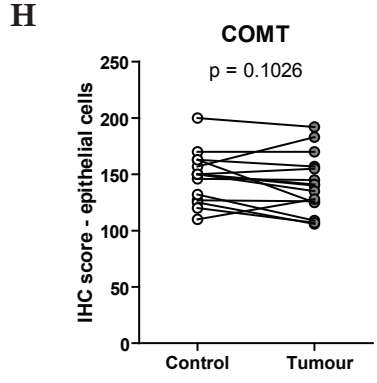

TMA C3 - CYP1B1

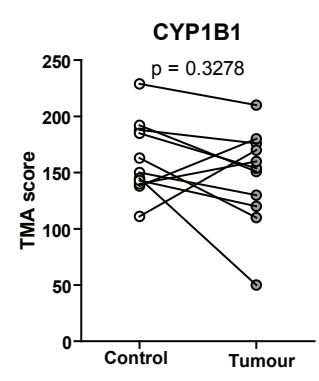

T38 - CYP1B1


T38 - COMT


TMA T3 - CYP1B1


G
CYP1B1
p = 0.0143
IHC score - epithelial cells
300
200
100
0
Control
Tumour


H
COMT
p = 0.1026
IHC score - epithelial cells
250
200
150
100
50
0
Control
Tumour


I
CYP1B1
p = 0.3278
TMA score
250
200
150
100
50
0
Control
Tumour