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ACTIVITIES OF METABOLIZING ENZYMES IN HUMAN PLACENTA

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Highlights

- CYP19A1, UGT, GST and CAT activities were confirmed in human placenta.
- High interindividual variation was found in the enzyme activities.
- Several chemicals affected the enzyme activities in human placental perfusion
- Antipyrine in placental perfusion may alter the activities of the analyzed enzymes.

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Abstract

In addition to the transfer across the placenta, placenta displays hormonal and xenobiotic metabolism, as well as enzymatic defense against oxidative stress. We analyzed aromatase (CYP19A1), uridine 5'-diphospho-glucuronyltransferase (UGT), glutathione-S-transferase (GST) and catalase (CAT) activitiesin over 70 placentas from nonsmokers stored at -80ºC from former perfusion studies. A wide interindividual variation in all activities was found. Longterm storage at -80ºC did not affect the activities. Ethoxyresorufin-O-deethylase (EROD, CYP1A1) was not detected in any of the studied placentas perfused with chemicals. Several compounds in placental perfusion changed statistically significantly the enzyme activities in placental tissue. Melamine and nicotine increased CYP19A1, melamine increased UGT and GST, PhIP with ethanol decreased CYP19A1 and increased GST, and PhIP with buprenorphine decreased CAT. Antipyrine in 100µg/ml also changed the studied enzyme activities, but not statistically significantly. Because antipyrine is a reference compound in placental perfusions, its potential effects must be taken into account in human placental perfusion. Enzyme activities deserve further studies as biomarkers of placental toxicity.

Finally, enzyme activities deserve further studies as biomarkers of placental toxicity.

Keywords:

Aromatase (CYP19A1); CYP1A1; Uridine 5'-diphospho-glucuronyltransferase (UGT); glutathione-S-transferase (GST); catalase (CAT); antipyrine; placental perfusion

1. Introduction

Human placenta is metabolically a very active organ with hormone metabolism, an active antioxidative system, with antioxidant enzymes, as well as xenobiotic metabolizing enzymes (Myllynen et al. 2009). This metabolic capacity is essential for the development and growth of the placenta and fetus during gestation. Placenta is responsible for the production of many hormones (e.g. estrogen and progesterone) that are important for the maintenance of pregnancy (Costa, 2016). Aromatase (CYP19A1), an essential enzyme that catalyzes the synthesis of estrogen from androgen, is expressed in human placental syncytiotrophoblast (For recent reviews, see Blakemore and Naftolin, 2016; Kao et al., 2019), Several antioxidant enzymes,

such as superoxide dismutase (SOD), catalase (CAT), and glutathione peroxidase (GPx) (Qanungo and Mukherjea, 2000) are found in human placenta (Myllynen et al., 2009). Many functionalization and conjugation enzymes of xenobiotic metabolism are expressed in human placental syncytiotrophoblast (For reviews, see Myllynen et al., 2009; Syme et al., 2004).

Enzymatic xenobiotic metabolism in human placenta is a potentially important determinant of transplacental transfer of xenobiotics and toxicity in fetus. Xenobiotics may exert their toxicity directly on the placental cells or alter expression and production of hormones and functions of proteins, such as enzymes or transporters, in the placenta (Karttunen et al., 2017). The activity of CYP1A1, important in functionalization reactions in xenobiotic metabolism, has long been known to be induced in placentas of women exposed to tobacco smoke (Vähäkangas et al., 1989). Induction of CYP1A1 may lead to activation of toxic chemicals such as benzo(a)pyrene (BP) which is metabolized by CYP1A1 into reactive metabolites capable of binding to DNA. In our earlier study, we found BP-DNA adducts in a human placenta perfused with BP, which implicates CYP1A1 catalyzed formation of reactive toxic metabolites (Karttunen et al., 2010). GST and UGT are important conjugation enzymes and they are known to be functionally active during pregnancy in human placenta (Myllynen et al., 2009). GST is known to be important in the metabolism of endogenous substance and hormones (Syme et al., 2004) but there is little information about its role in metabolism of xenobiotics in human placenta. UGT is the most important conjugating enzyme in human placenta (Myllynen et al., 2009). Its activity in human placenta has also been established earlier (Collier et al., 2002).

It is known that xenobiotics can induce or inhibit placental enzymes. The best-known case is the induction of CYP1A1 by maternal smoking (Vähäkangas et al. 1989). A correlation between DNA adducts and enhanced ethoxyresorufin-O-deethylase (EROD, CYP1A1) activity with organochlorine exposure has been found in placentas of women exposed to food contaminated by organochlorine compounds (Lagueux et al., 1999). In choriocarcinoma derived cells JEG-3 and placental explants the anti-HIV drug AZT (3´azido-3´-deoxythymidine) has been reported to increase the activity of GST, CYP1A1, and β-glucuronidase, and reduce the activity of UGT (Collier et al., 2003). Decrease in conjugating enzymes may result in accumulation of reactive toxic metabolites, and because they take part in hormone metabolism also, disruption of hormonal balance in human placenta may occur (for a review, see Myllynen et al., 2009). Also aromatase in hormone metabolism may be affected. In JEG-3 cells, non-cytotoxic doses of bisphenol A have been found to downregulate the genes

of CYP19A1 and to reduce the final levels of steroidogenesis related hormones such as progesterone and estradiol (Chu et al., 2018).

Alterations in placental enzyme activities have been suggested as predictors of placental toxicity (for reviews, see Kodydkova et al., 2014; Myllynen et al., 2007; Pereira and Martel, 2014). Aromatase may reflect on the wellbeing of fetus with potentially longterm effects. Effects on aromatase activity may serve as a biomarker for toxicity in placenta and fetus (Vähäkangas et al 2019). Catalase is an important enzyme in counteracting the effects of oxidative stress. Changes in the level of catalase have been used to reflect induction of oxidative stress as in the case of preeclampsia and diabetes (Aouache et al., 2018; D'Souza et al., 2016; Wdowiak et al., 2015). Chiapella et al. (2014) suggested that CAT activity can be used as a biomarker for exposure to pesticides.

Due to the variation in the morphology, physiology and structure of placentas among species (Benisrschke, 1998), it is difficult to extrapolate data from non-human placenta for human placental toxicity. Further studies with better models simulating the actual situation of placental physiology *in vivo* would help to predict such effects. Naturally, the *in vivo* human studies are restricted to life-style exposures, such as smoking, accidental cases of exposure or very low levels of environmental exposures, with great challenges in determining the actual level of exposure.

Ex vivo human placental perfusion maintains the integrity of placental tissue. In our studies (e.g. Annola et al. 2008; Partanen et al. 2010; Karttunen et al. 2010; Veid et al. 2011; Mohammed et al. 2018), as well as in those by other groups (e.g. Conings et al., 2019; Mandelbrot et al., 2019) placental perfusion is usually used to pursue transplacental transfer of xenobiotics. Over the years, we have collected placental tissue from placentas used for perfusions, both before and after the perfusions (Karttunen et al. 2015). This gives a unique opportunity to study placental enzyme activities and their inter-individual variation in a large sample set. Furthermore, it is possible to pursue a preliminary view on the effects of a wide range of perfused chemicals, including medicinal drugs, food borne toxicants, hazardous compounds in tobacco smoke, pesticides and food adulterants, as well as antipyrine, the reference compound used in all of our placental perfusions. The selected enzyme activities for this study represent placental hormone metabolism (CYP19A1), defense against oxidative damage (CAT), and activating (CYP1A1) and detoxicating (UGT, GST) capacity within xenobiotic metabolism .

2. Materials and Methods

2.1.Collection of new placentas

Four placentas from healthy nonsmokers were collected during the study to compare the enzyme activities of newly collected placentas with previously preserved placentas. Also, the enzyme activities of the newly collected placentas were compared before and after being frozen for one year at -80^{0}C. Two of the collected placentas were also used to investigate the intra-individual difference in enzyme activities between central and peripheral parts of the placenta.

2.2.Stored placental samples

All placentas collected for perfusions carried out between 2008 and 2018, with placental tissue available for analysis, were used in this study (Supplementary table 1). All placentas (both the new and formerly collected) were full term placentas collected in Kuopio University Hospital (KUH) from healthy non-smoking mothers after either elective cesarean section or normal delivery. It is important to mention that not all the available placentas were analyzed for all studied enzyme activities due to limited amount of available tissue.

All the donating mothers reported not having smoked or abused drugs and none of them were on drug therapy. The samples from placental tissue had been taken prior to and after the end of perfusions. The samples had been snap-frozen in liquid nitrogen and stored at -80°C.

2.3.Ethical aspects

An approval for placental perfusion studies from the Ethics Committee of the University Hospital District of North Savo had been granted in 2005 (29/2005) and in 2007 (54/2007). Placentas were always collected anonymously after a written informed consent was signed by the donors. Information on gender, date of birth, gestational weeks and weight of the placenta, but no personal information were recorded. Collection of placentas for perfusion studies has been shown to produce no harm or risk to donating mothers (Halkoaho et al., 2011).

2.4.Placental perfusions

All samples from perfusions are from earlier studies by our group. No new placental perfusions were carried out for this paper and the perfusion method had been similar in all of these studies. The used dual re-circulating perfusion method of a single placental lobule is explained in detail by Karttunen et al. (2015). Shortly, the collected placenta is flushed with heparinized KRB solution and the fetal vein and artery cannulated. The trimmed lobule is then fixed into the

perfusion chamber. The perfusion in maternal side is started after inserting two blunt cannulas into the intervillous space. The perfusion medium contains RMPI cell culture medium, L-glutamine, penicillin-streptomycin, sodium pyruvate, non-essential amino acids, dextran, heparin and albumin. Antipyrine (either 25µg/ml or 100µg/100) is used as a reference compound in all the perfusions. Details of the duration of perfusions, concentrations of chemicals and antipyrine used, and number of perfusions in each study are listed in the Supplementary Table 1.

2.5.Preparation of microsomal and cytosolic tissue fractions

Placental tissues were cut into small pieces and homogenized into buffer solution (0.1 M Tris-HCL and 1 mM K2-EDTA, pH 7.4). The homogenate was centrifuged for 30 minutes at 10 000 X g at 4^0C. The supernatant was collected and further centrifuged at 100 000 X g for 1 hour at 4^0C. The supernatant which represents the cytosolic fraction was collected and stored at -80^0C. The pellet which represents the microsomal fraction was homogenized into the buffer (see above) and the samples were collected and stored at -80 ^{0}C, as well. Protein concentration was determined in tissue fractions with the Bradford method and using bovine serum albumin (BSA) as a standard (Kruger, 1994).

2.6.Enzyme assays

EROD activity catalyzed by CYP1A1 was analyzed in microsomal fractions of placenta using spectrofluorometry (Shimadzu RF 5000 spectrofluorometer, Japan) following the method by Burke et al. (1985). Initially, about 0.1 mg protein was added to the mixture of 1 M Tris-HCl pH 7.4, 100 mM $MgCl_2$, 0.1 M Tris-HCl pH 7.4, 1mM K_2-EDTA and 0.1 mM 7-ethoxyresorufin (Sigma-Aldrich, Israel) as a substrate, and the reaction was initiated after addition of 0.5 mM NADPH. After 10 minutes of incubation at 37°C, the reaction was terminated by addition of methanol. Subsequently, the sample was centrifuged for 10 minutes at 3000 g at room temperature and the supernant was transferred into 1ml cuvette for the measurement.

The assay for catalase follows the continuous decline in absorbance of H_2O_2 attributed to the decomposition of H_2O_2 into oxygen molecules and water (Aebi 1984). Briefly, about 0.25 – 0.5 mg protein of cytosolic fraction was treated with 0.1 mM phosphate buffer containing 10 mM H_2O_2 in 1 ml cuvette, and measured at 240 nm wavelength at room temperature using Hitachi U-2000 (Japan) or Cary 50 Bio, Varian® (Australia) spectrophotometer.

The activity of GST was analyzed in placental cytosolic fractions following the spectrophotometric method by Habig et al. (1974) utilizing 1-chloro-2,4- dinitrobenzene (CDNB) as a substrate. About 0.25 mg cytosolic protein was added to the reaction mixture of 0.1 potassium phosphate buffer, 1 mM reduced glutathione (GSH; Sigma-Aldrich, Japan), and 1 mM CDNB. The speed of the reaction was measured at 340 nm wavelength using Hitachi U-2000 (Japan) or Cary 50 Bio, Varian® (Australia) spectrophotometer.

CYP19A1 activity was analyzed in microsomal fractions of placentas according to the method by Pasanen (1985) using radioactive [^{3}H]-androstenedione as a substrate. The activity was measured using 2540 Micro Beta®, microplate scintillation counter (Perkin Elmer, USA). Briefly, about 1 mg of placental microsomes was incubated for 40 minutes at 37°C with 1 M Tris HCL pH 7.4 containing the cold substrate, 0.2 mM androstenedione, and the hot substrate [^{3}H]-androstenedione, and a NADPH regenerating system. The reaction was terminated by 33% trichloroacetic acid and the sample was centrifuged at 10000 g for 5 minutes. The supernant was filtered via Sep-Pak filters and mixed with an equivalent volume of scintillation liquid for the measurement.

UDP-glucuronyl transferase (UGT) assay was analyzed in human placental microsomes using the spectrofluorometric method by Collier et al. (2002). Briefly, the reaction mixture consisted of 0.1 M Tris-HCL pH 7.4, 5 mM $MgCl_2$, 10 mg/ml BSA, 50 µM 4-methylumbelliferone (4MU) as a substrate, 2 mM uridine diphosphate glucuronic acid trisodium salt (UDPGA, Sigma) and minimum of 0.1 mg microsomal proteins. The UGT activity assay follows the drop in the fluorescence that results from the conversion of 4MU into 4-MU-glucuronide. The samples were measured by the Envision spectrofluorometer (Perkin Elmer, USA).

2.7.Statistical analysis:

One-way analysis of variance (ANOVA) was used to compare groups of placentas over preservation time (Figure 1).

For statistical presentation of interindividual variation in the studied enzyme activities, data are presented as a mean±SD, showing also the range. The ratios of the highest to the lowest enzyme activity was also calculated (Table 1).

Paired t-test was used to compare the studied enzyme activities in human placentas before and after perfusions, and in fresh non-perfused placentas before and after being stored at -80^{0}C. The *P*-values <0.05 were considered statistically significant.

Percentage of change in enzyme activities in placentas before and after perfusions was calculated using the formula: $(E_a-E_b)/E_b$ x 100. Where E_b is the enzyme activity before perfusion and E_a is the enzyme activity after perfusion.

3. Results

3.1.Enzyme stability in storage.

Because the samples in this study were stored at -80°C for up to 12 years, it was important to consider the stability of the enzymes. For this, we compared the mean activities of enzymes in relation to time (Figure 1). Placental tissues were divided into groups according to the year the paper was published. This gives an idea of the storage duration of the tissues at -80^0C. There was no statistically significant systematic change in enzyme activities over the course of preservation time among the different groups of placentas (Figure 1).

We also compared the enzyme activities of UGT, GST and CAT in the four newly collected placentas before and after being stored for one year at -80^0C. There was no statistically significant difference in the studied enzyme activities between the two groups.

3.2.Enzyme activities in non-perfused placental tissues.

Activities of CYP19A1, uridine 5'-diphospho-glucuronyltransferase (UGT), glutathione-S-transferase (GST) and catalase (CAT) were detected in all the studied placentas (Table 1, Supplementary Table 2). Wide inter-individual variation in the activities of CYP19A1, UGT, GST and CAT in placental tissues was demonstrated (Table 2, Figure 1). EROD activity, as expected, because these placentas are from non-smokers, were very low or non-existent in non-perfused placental tissue. We found no clear differences in these enzyme activities between central and peripheral parts of the same placenta in the two studied placentas (data not shown).

3.3.Effects of antipyrine in placental perfusion on enzyme activities.

Antipyrine is an important chemical in human placental perfusion. In our group, we have used either 25µg/ml or 100µg/ml, of antipyrine as a reference compound in placental perfusions. The series of perfusion used in this study included a group with no other chemicals than antipyrine in the perfusion. The effects of antipyrine on the studied enzyme activities were studied and the two different concentrations compared. There was not sufficient data to calculate the statistics for the effects of 25µg/ml antipyrine (AP25) on the studied metabolizing enzymes. During perfusions, the higher concentration of antipyrine, 100µg/ml (AP100), did

not statistically significantly change the activities of any of the studied placental enzymes. However, the percent change in CYP19A1, UGT and GST activities was high in some individual cases (Figure 3, Supplementary Table 2).

3.4.Effects of the perfused chemicals on the enzyme activities in human placenta.

Several, but not all of the chemicals used in human placental perfusion affected the studied placental enzyme activities statistically significantly when the placental tissue before and after perfusion were compared (Table 2; Supplementary table 2). It was more common for the chemicals to increase the enzyme activities than to decrease (Table 2 and Supplementary Table 2). Melamine statistically significantly increased the activity of UGT (Table 2). As to GST, melamine and a combination of nicotine and ethanol statistically significantly increased GST activity. The combination of ethanol and nicotine in perfusion also increased GST.

A statistically significant decrease in CAT activity was only seen in perfusions with a combination of PhIP+buprenorphine. However, CAT activity tended to decrease in most studied perfusions (Supplementary Table 2).

EROD, representing the functional activity of CYP1A1/2, was below detection also after perfusion in the 33 placentas perfused with PhIP, PhIP+buprenorphine, diuron, doxorubicin, nicotine+ethanol, NDMA, ochratoxin A or ethanol.

4. Discussion

4.1.Enzyme activities in non-perfused placental tissues

Two lines of evidence confirms the the activities of the studied enzymes are preserved at -80^{0}C: 1) There was no statistically significant systematic change in the enzyme activities in the placentas stored for a different number of years at -80^{0}C and 2) no statistically significant difference was found between the enzyme activities in samples from newly collected placentas before and after storage for one year at -80^{0}C. In contrast, a decline of enzyme activities has been reported when stored at -20^{0}C (Cryer and Bartley, 1974). This study, where a large number of placentas was used, confirms the activities of CYP19A1 (Pasanen, 1995), GST (Radulovic and Kulkarni, 1986), UGT (Collier et al., 2002) and CAT (Misra and Mukherja, 1985) in human placenta. The high inter-individual variation in the activities of placental enzymes supports our earlier findings (Vähäkangas et al., 1989; Partanen et al., 2010). It is thus important to consider the inter-individual variation in the activities of placental enzymes when incorporating data into risk assessment of chemicals for placental toxicity.

The activities of CYP19A1 in healthy human placentas analysed by the Pasanen 1985 method (Huuskonen et al., 2016; Maliqueo et al., 2013; Paakki et al., 2000; Pasanen, 1985) have been in average higher, with less inter-individual variation compared to this study. In a study (Johnston et al., 2016) with a sample size of 57, the inter-individual variation in placental CAT activity was as high as in this study, with, however, much lower average activity. Similarly to our study, UGT activity, using the same method as in this study, was found in all placentas by Collier et al. (2002) and Huuskonen et al. (2016). The variation in UGT activity in the study by Huuskonen and coworkers was much lower compared to this study (mean ± SD, 5+/1.38 vs. 9.2 ± 9 pmol/min/mg, respectively). However, also the number of placentas in their study was much lower (n=7) compared to this study (n=>70).

In the literature, there are somewhat discrepant data about the studied enzymes in different parts of the placenta with most data showing no difference (Johnston et al., 2016; Maliqueo et al., 2013; Sahay et al., 2015). We could not find such differences, either.

4.2.Effects of antipyrine on studied human placental enzymes

Our study is the first that examines the impact of perfusion conditions including the reference compound, antipyrine, on enzyme activities in human placenta. Lack of evident modifications of CYP19A1, UGT and CAT activities by AP25 alone suggests that the potential effects of perfusion conditions such as temperature, oxygenation, perfusion duration and culture media on these placental enzymes are insignificant under the given circumstances.

Although not statistically significant at group level, the significant increase in the activity of CYP19A1 and UGT in individual cases by the higher concentration of antipyrine implicates and effect and dose dependence. More studies are required to conclude whether antipyrine in placental perfusion has an effect on the UGT and CYP19A1. Data on transfer kinetics of antipyrine in human placental perfusion have demonstrated that regardless of the concentration of antipyrine and the duration of perfusion the transplacental transfer of antipyrine in perfusion is always consistent (Karttunen et al., 2015). This implicates that antipyrine with either of the indicated doses can be used as a reference for transplacental transfer in human placental perfusion. However, in terms of toxicity, this may not be the case. Especially, if the perfused placenta is to be analyzed for enzyme activities, effects of antipyrine may confound the actual effects of the studied compounds.

It is unknown how exactly antipyrine exerts its effects on the analysed enzymes. There is an old hypothesis that the antipyretic effect of antipyrine is partly mediated by increasing the level of hydrogen peroxide in the body (Williamson and Rudge, 1948). Since hydrogen peroxide has direct inhibitory effects on CAT (for comprehensive reviews, see Goyal and Basak, 2010; Kodydkova et al., 2014), the reduction in CAT observed in our study may be related to this mechanism.

4.3.Effects of perfused chemicals on the studied enzymes

This study provides the first set of data on the effect of xenobiotics in human placental perfusion on placental metabolizing enzyme activities. It is important to mention that the concentrations of chemicals used in these perfusions usually reflect the actual background levels of exposure (e.g., Partanen et al., 2010; Veid et al 2011). Thus, placental perfusion can serve as an important model to study the effects of chemicals on placental enzyme activities. However, long enough perfusions to allow for metabolic changes are important. We have previously shown that a perfusion of 6 hours is sufficient to show metabolism and DNA-binding of benzo(a)pyrene (BP) in a perfusion of a placenta from a non-smoker with no adducts before perfusion (Karttunen et al., 2010). EROD activity was increased in the same placenta after 6 hours in perfusion with BP (Karttunen et al., 2010). Also diuron was metabolized in 6-hour perfusions (Mohammed et al., 2018), as well as aflatoxin B1 (AFB1;Partanen et al., 2010) and acrylamide (Annola et al., 2008) in 4-hour perfusions.

Benzo(a)pyrene diolepoxide (BPDE)-DNA adducts have been described in placentas from smokers (Manchester et al., 1988), and placental microsomes from smokers, but not from non-smokers, can activate BP (Vähäkangas et al., 1989) confirming inducible CYP1A1 activity in human placenta. Induction of CYP1A1 occurs through aryl hydrocarbon receptors which are known to be expressed and active in human placenta (for a comprehensive review, see Wakx et a., 2018). In this study no EROD (CYP1A1) activity was found in any of the studied placentas with any of the used chemicals, of which PhIP (Sekimoto et al., 2016) and diuron (Rudzok et al., 2009) have been reported to induce CYP1A1/2 in human hepatocellular carcinoma HepG2 cells, and nicotine in rat lung (Iba et al., 1999). Inducibility is genetically as variant as the basal enzymes activities (Hakkola et al., 1998; Nebert et al., 2004), and thus even with long perfusion time, not every placenta can be expected to show similar changes in enzyme activities with chemicals in placental perfusion.

Potential modifications of CYP19A1 activity by xenobiotics in human placenta have been seldom explored. We found a statistically significant increase in CYP19A1 activity during placental perfusion also by nicotine. Similarly, nicotine and its metabolite, cotinine, inhibited the metabolism of androgen in human placental microsomes and human placental trophoblastic JAr cells (Barbieri et al., 1986). Based on their results, nicotine and cotinine exert direct inhibitory effects on aromatase by competing with the substrate, androgen. On the other hand, Pasanen (1985) did not find a significant difference in CYP19A1 activity between placentas from smokers and non-smokers.

In this study, melamine increased CYP19A1 statistically significantly. In the literature, no data is available on the effect of melamine on human CYP19A1. Consistent, although statistically not significant alteration in CYP19A1 by diuron is of high interest. This is because in our previous work, diuron was found to cross human placenta easily suggesting fetal exposure (Mohammed et al., 2018). With its potential effects on placental CYP19A1 combined with fetal exposure, diuron is potentially harmful to the development of the fetus.

Also, melamine increased UGT and GST activities statistically significantly. No former information is available of the effects of melamine on UGT and GST activities. We have previously shown that melamine is cytotoxic to human placental trophoblastic BeWo cells, and crosses human placenta in perfusion indicating fetal exposure (Partanen et al., 2012). Our new data further stresses the possibility that melamine poses a risk to the function of human placenta and possibly to the development of fetus.

In addition to melamine, GST activity was clearly increased in perfusion by the combination of nicotine and ethanol. Thus, overall our findings show that placental GST, as well as aromatase, can respond to external stimuli by chemicals, and could be studied as a biomarkers of chemical exposure in the placenta. Interestingly, alteration in GST activity has been proposed as a potential biomarker of exposure to chemicals in cancer tissues regardless of cancer type (for a review, see Noguti et al., 2012).

In conclusion, with a large number of placentas, our results provide confirmation of the activities of CYP19A1, UGT, GST and CAT in human placenta with a wide inter-individual variation. In perfusions long enough it seems to be possible to detect enzyme induction in placental tissue making placental perfusion an attractive model to study effects of chemicals on placental enzymes. However, the series of perfusions in this paper with each of the chemicals are too small for any conclusions of their use as biomarkers, but implicate the

importance of further studies. Our study for the first time implicates in perfused human placentas effects of antipyrine on enzymes catalyzing xenobiotic metabolism, hormone synthesis or protecting from oxidative stress. Because of potential effects of the higher dose of antipyrine on the studied enzymes, it is justifiable to use as low a concentration of antipyrine as possible as a reference compound in placental perfusions.

Contributions of authors

Ideas and planning	Ali Mohammed, Kirsi Vähäkangas
Enzyme analysis	Ali Mohammed
Human placental perfusion	Ali Mohammed, Heidi Sahlman, Jenni Repo, Kirsi Myöhänen, Jackson Woo, Vesa Karttunen, Päivi Myllynen, Kirsi Vähäkangas
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Accepting the final paper	All authors
Supervision of the work	Kirsi Vähäkangas

Declaration of interests

The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

Conflict of Interest

None of the authors have conflicts of interest.

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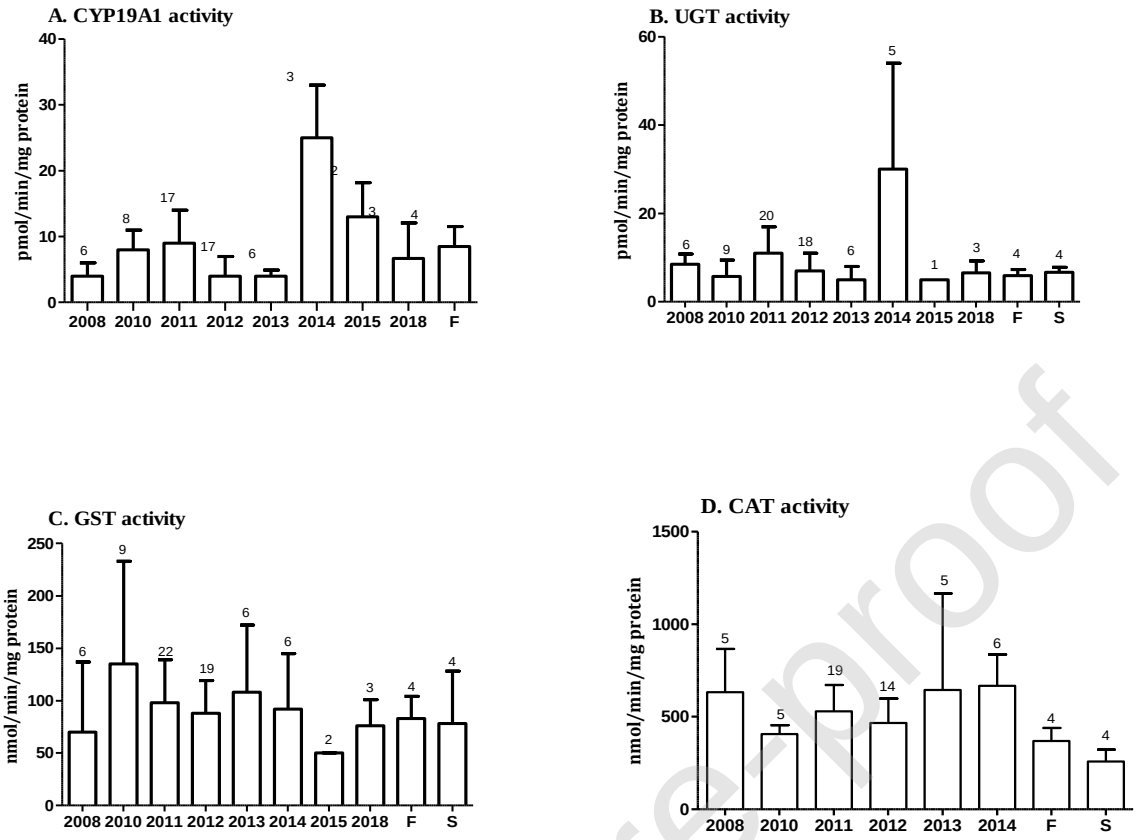

Figure (1): Enzyme activities in human placenta. The number on top of each bar represents the number of placentas. Non-perfused tissues stored at -80^{0}C from earlier placental perfusion studies, published in the year indicated below the bar, were used. Four new placentas were collected and analyzed before (F=fresh) and after (S=stored) for one year at -80^{0}C. Using One-way of analysis of variance (ANOVA), no statistically significant, systematic change was found in the studied enzymes over time. Using the paired t-test, no statistically significant difference was observed in the newly collected placenta before and after storage. CYP19A1= aromatase (A), UGT= UDP-glucuronyltransferase (B), GST= glutathione-S-transferase (C), CAT= catalase (D).

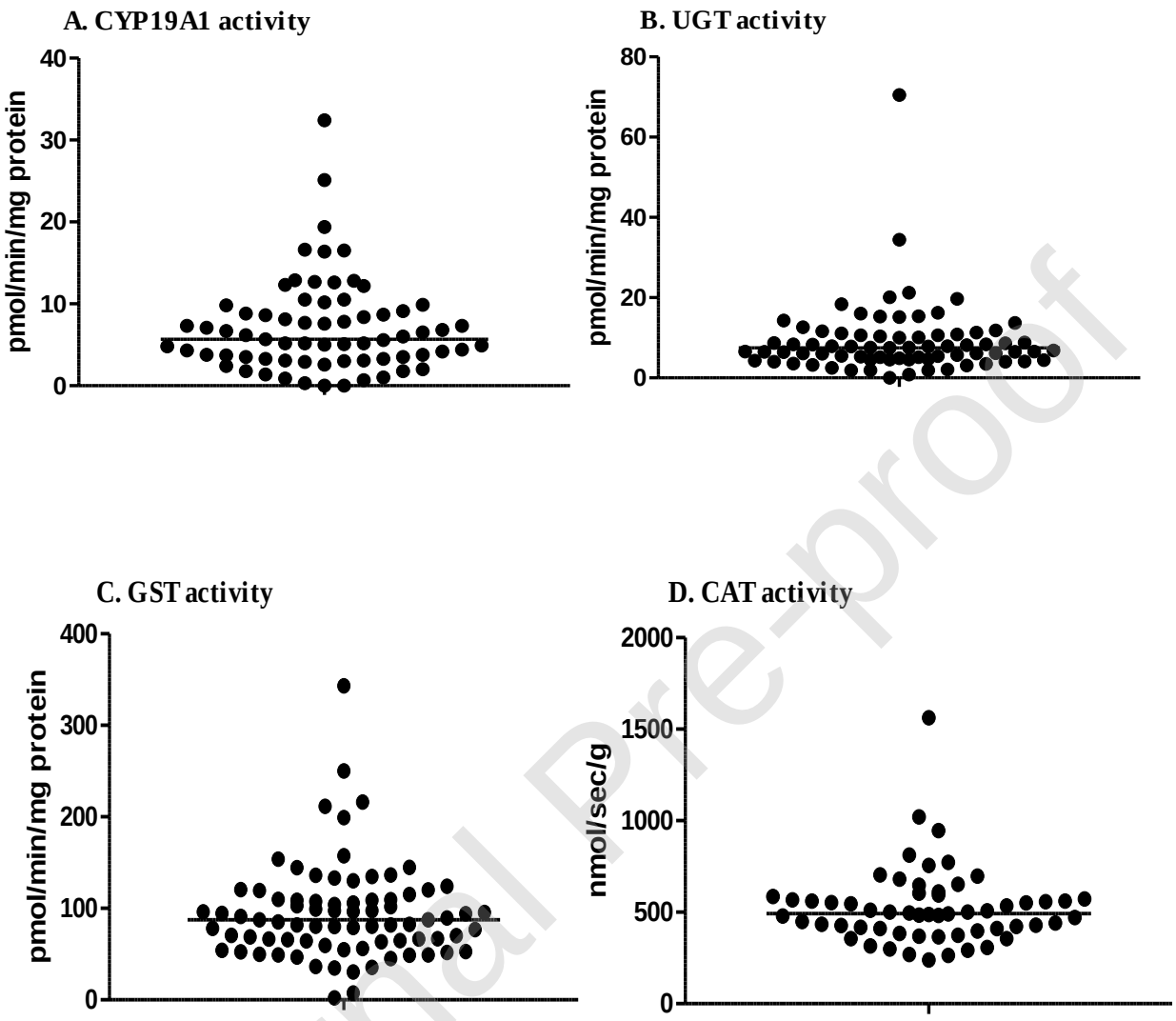

Figure (2): Interindividual variation in the activity of the studied enzymes in human placenta from healthy nonsmokers. Median of activities is indicated by a line. CYP19A1= aromatase (A) (*n*=66), UGT= UDP-glucuronyltransferase (B) (*n*=72), GST= glutathione-S-transferase (C) (*n*=77), CAT= catalase (D) (*n*=57).

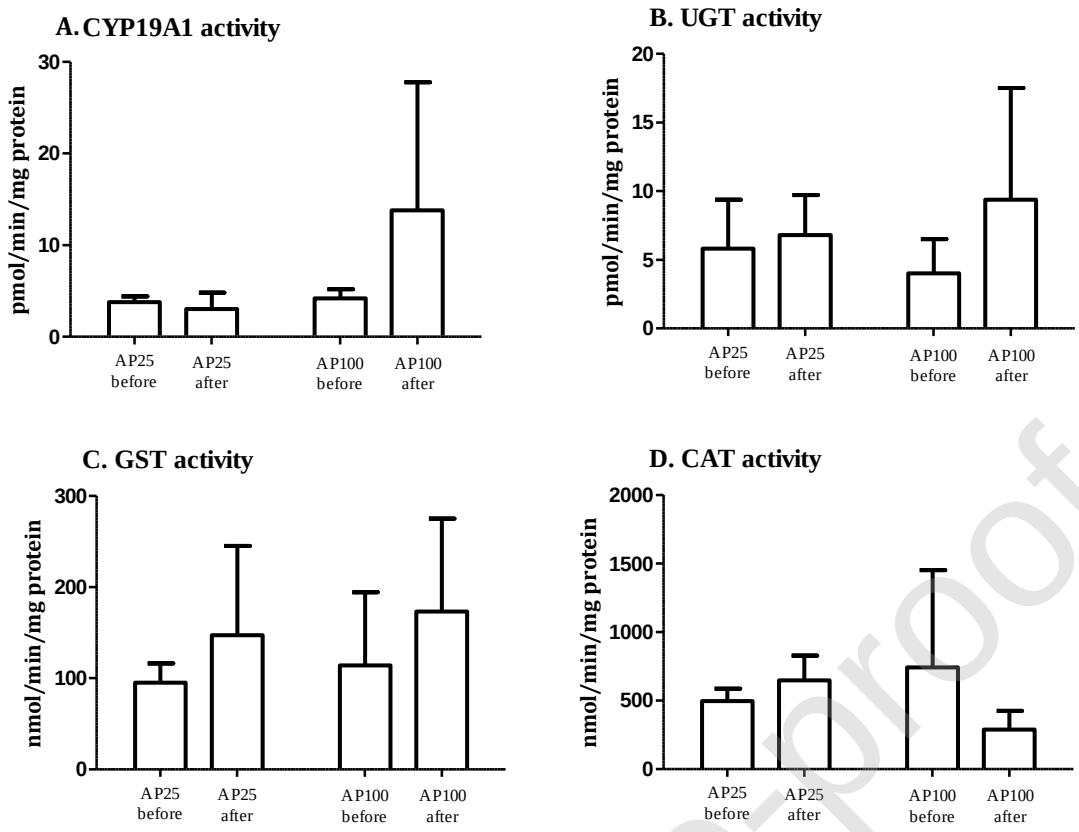

Figure (3). Enzyme activities in placental tissues before and after 4-hour antipyrine perfusions. Each bar represents a mean ± SD of the enzyme activities of human placentas from perfusion studies (n=2 for AP25; n=4 for AP100, except for CAT n=3). Due to the low number of AP25, statistical significance could only be calculated with AP100 and there were no statistically significantly differences between before and after perfusions (paired t-test). CYP19A1= aromatase (A), UGT= UDP-glucuronyltransferase (B), GST= glutathione-S-transferase (C), CAT= catalase (D). AP25= antipyrine 25µg/ml. AP100= antipyrine 100µg/ml.

Table 1. Activities of xenobiotic metabolizing enzymes in non-perfused human placental tissues.

	CYP19A1 (pmol/min/mg)	UGT (pmol/min/mg)	GST (nmol/min/mg)	CAT (nmol/min/mg)

n (number of placentas)	66[a]	72[a]	77[a]	57[a]
Mean ± SD	7.5 ± 5.8	9.2 ± 9	94 ± 52.4	526 ± 211
Range	0.25 - 32	0.8 - 71	2.2 - 343	239 - 1563
Highest/lowest[b]	128	89	156	6.5

[a] includes newly collected as well as placentas from earlier perfusions. Insufficient material in some cases reduced the number of placentas used. [b] Highest/lowest refers to a ratio of the highest measured enzyme activity to the lowest enzyme activity (shown in the range) in the studied placentas.

Table 2. Effects of chemicals on the activities of enzymes in placentas from human placental perfusions. Activities were analyzed in tissues stored from the placenta before and after perfusion with the indicated chemical.

Perfused chemical	**CYP19A1** pmol/mg/min			**UGT** pmol/mg/min			**GST** **n**mol/mg/min			**CAT** **n**mol/mg/min		
	Before	**After**	***n***	**Before**	**After**	***n***	**Before**	**After**	***n***	**Before**	**After**	***n***
Melamine	**3.0±2.4**	**6.3±4.1**[c]	5	**6.7±2.9***	**12±4**[c]	6	**93±27**	**202±84**[c]	7	560±109	677±211	6
Nicotine	**2.6±1.1**	**3.6±1.5**[c]	3	15±4	15±5	3	88±36	196±90	4	495±53	387±148	4
Nicotine+Ethanol	9.4±5.7	9.7±3.8	5	10±6	14±3	**7**	**79±42**	**186±83**[c]	6	475±96	491±196	4
PhIP[a]+ethanol	**12±4**[b]	**7±5**[c]	5	14±4	12±4	5	124±55	256±133	5	635±116	1032±322	4
PhIP+Buprenorphine.	648±184	495±159	5	29±28	15±7	4	79±46	168±107	5	**648±184**	**495±159**[c]	**5**

[a] 2-Amino-1-methyl-6-phenylimidazo(4,5-b)pyridine

[b] mean ± SD.

[c] Statistically significant difference (P <0.05; paired t-test) between "before" and "after".