Effect of breed upon cytochrome P450s and phase II enzymes expression in cattle liver.

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Abbreviations:

16βOH-T, 6βOH-T and 2βOH-T, 16β-, 6β- and 2β-testosterone hydroxylase, respectively; Aniline-OH, aniline hydroxylase; BA, Blonde d'Aquitaine; BENZDEM, benzphetamine *N*-demethylase; BPOH, benzo[a]pyrene hydroxylase; CDNB, 1-chloro-2,4-dinitrobenzene; CH, Charolais; CYP, cytochrome P450; DCNB, 3,4-dichloronitrobenzene; DMEs, drug metabolising enzymes; ERDEM, erythromycin *N*-demethylase; EROD, ethoxyresorufin *O*-deethylase; ETDEM, ethylmorphine *N*-demethylase; ETR, ethoxyresorufin; GSH, glutathione; GST, glutathione *S*-transferase; HPLC, high performance liquid chromatography; NAD(P)H-R, NAD(P)H cytochrome *c* reductase; PBS, phosphate buffer solution; PM, Piedmontese; pNP-OH, *p*-nitrophenol hydroxylase; pNP-UGT, *p*-nitrophenol UGT; Q RT-PCR, quantitative *Real Time* polymerase chain reaction; RT, reverse transcription; SDS-PAGE, sodium dodecyl sulphate-polyacrylamide gel electrophoresis; SNP, single nucleotide polymorphism. TAO, triacetyloleandomycin; TAODEM, TAO *N*-demethylase; TBT, tolbutamide; TBT4-OH, tolbutamide 4-hydroxylase; TST, testosterone; UGT, uridinediphosphoglucuronosyltransferase; αN-UGT, α-naphthol UGT.

Abstract

Cattle represent an important source of animal-derived food-products; nonetheless, our knowledge about the expression of drug metabolizing enzymes (DMEs) in present and other food-producing animals still remains superficial, despite the obvious toxicological consequences. Breed represents an internal factor which modulates DMEs expression and catalytic activity. In the present work, the effect of breed upon relevant phase I and II DMEs was investigated at the pre-transcriptional and post-translational levels in male Charolais (CH), Piedmontese (PM) and Blonde d'Aquitaine (BA) cattle. As specific substrates for cattle have not yet been identified, the breed effect upon specific cytochrome P450s (CYPs), glucuronyl or glutathione transferase (UGT and GST, respectively) DMEs, in terms of catalytic activity, was determined by using human marker substrates. Among CYPs, benzphetamine N-demethylase, 16β-, 6β- and 2β-testosterone hydroxylase, aniline and p-nitrophenol hydroxylase, α-naphthol and p-nitrophenol UGT activities were significantly higher in CH; by contrast, lower levels of CYP1A1- and CYP1A2-like, CYP2B6-like, CYP2C9- and CYP2C18-like, CYP3A4-like, and UGT1A1-like mRNAs were noticed, with CH<PM≤BA as a trend. Cytochrome P450 2B and CYP3A mRNA results were confirmed with immunoblotting, too. As regards conjugative DMEs, UGT1A6-like mRNA levels were consistent with respective catalytic activities. Both 1-chloro-2,4-dinitrobenzene and 3,4-dichloronitrobenzene GST activities were higher in BA, and these results agreed with GSTA1-, GSTM1- and GSTP1-like mRNA amounts. Correlation analysis between catalytic activities and mRNAs showed either significant or uneven results, depending on the substrate. These findings confirm previous data obtained in laboratory species; however, further studies are required to ascribe this behaviour to pre-transcriptional or post-translational phenomena.

Introduction

Historically, studies concerning drug metabolising enzymes (DMEs) expression and regulation in veterinary food-producing species (e.g. cattle, swine, poultry) have always been considered of lesser interest, if compared to those done in man and laboratory animals. This is rather peculiar. Basically, the DMEs comparative knowledge is useful either to extrapolate pharmaco-toxicological data from one species to another or to extend veterinary drug licences of use from major to minor or exotic species. On the other hand, farm animals are often exposed to pesticides, pollutants or drugs (sometimes used illicitly, to increase growth performances), which are potentially harmful for the animal itself and also for humans, if the consumption of animal edible tissues containing relevant amounts of residues occurs. Consequently drug metabolism studies, performed in these animals, are of value for the evaluation of the consumer's risk (Nebbia et al., 2003; Sivapathasundaram et al., 2001, 2003a). Despite these obvious toxicological implications, the knowledge about cytochrome P450 (CYP) and phase II conjugation systems in veterinary species is still elusive or superficial, even after five decades of intensive worldwide research efforts dedicated to drug metabolism.

Cattle represents one of the major economically important veterinary species worldwide. A few studies, mostly addressed to investigate the post-translational effects of species, age and gender (some of constitutional factors influencing the overall biotransformation capability), upon the cattle liver DMEs have been published in the past decade (Nebbia et al., 2003; Machala et al., 2003; Sivapathasundaram et al., 2003b; Szotáková et al., 2004; Dacasto et al., 2005; Gusson et al., 2006). By contrast, only recently DMEs expression and regulation have begun to be studied at the gene expression level (Greger et al., 2006).

In one of fore-mentioned studies, breed-differences in the hepatic CYP3A apoprotein and CYP3A-dependent catalytic activities have been reported among Limousine and

Piedmontese (PM) cattle (Dacasto et al., 2005). In the present one, the effect of breed upon the most relevant phase I and II DMEs has been investigated both at the pre-transcriptional and/or post-translational level, in two French (Charolais, CH, and Blonde d'Aquitaine, BA) and one Italian (PM) meat cattle breeds.

Since in cattle, as well as in most veterinary species, the definition of substrates which are specifically metabolised by a P450 isoform or a conjugative DME is still debatable, catalytic activities were measured by using probes whose selectivity toward each DMEs had been previously established in rat and human species. Likewise, commercially available antibodies, raised toward human and rat antigens, were used for CYP1A, 2B, 2C, 2E and 3A immunoblotting; in fact, to the best of Authors' knowledge, mono- or polyclonal antibodies directed to cattle antigens have not yet produced so far. In effect, such an approach has been already adopted, at least partly, in prior studies where cattle liver drug metabolism was compared with that of other Ruminants, other farm animals or reference species (i.e., the rat) as well (Dacasto et al., 2005; Gusson et al., 2006; Ioannides, 2006; Machala et al., 2003; Nebbia et al., 2003; Nebbia et al., 2004; Sivapathasundaram et al., 2001; Sivapathasundaram et al., 2003a; Sivapathasundaram et al., 2003b; Szotáková et al., 2004). On the contrary, bovine specific primers pairs were designed for CYPs and phase II enzymes isoforms mRNA, to be used in the relative quantification by means of quantitative *Real time* polymerase chain reaction (Q RT-PCR). In fact, the entire bovine genome has been recently sequenced but a definitive nomenclature for bovine CYPs as well as phase II DMEs is still actually lacking. For this reason, in the present study bovine DMEs sequences were identified with the name of the human sequence sharing the highest percentage of identity, based on protein sequence alignments, followed by the suffix -like. Finally, a correlation analysis between each gene expression profile and the relative supposed catalytic activity was performed, in an attempt to

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define the bovine CYP or phase II enzyme mostly involved in the metabolism of chosen
model substrates.

Material and Methods

Chemicals and reagents. Bovine serum albumin, glucose 6-phosphate, glucose 6-
phosphate dehydrogenase, $NADP^+$ and 4-aminophenol were from Boehringer Ingelheim
(Ingelheim, Germany). All other reagents used for the estimation of catalytic activities were
obtained from Sigma-Aldrich (Milano, Italy), except for methanol, acetonitrile, NADPH and
glacial (HPLC grade) acetic acid (from Mallinkrodt – Baker, Milano, Italy), ammonium
acetate and hydrogen chloride (from Fluka, Milano, Italy). The MilliQ Synthesis water
purification system (Milano, Italy) provided purified deionised water. The goat anti-rabbit
CYP1A1/2 polyclonal antibody was from Oxford Biomedical Research (Milano, Italy), the
goat anti-rat CYP2B1/2 polyclonal antibody from Daiichi Pure Chemicals Co. (Tokyo,
Japan), the anti-rat CYP3A1/2 monoclonal antibody was a kind gift of H.V. Gelboin, National
Institute of Health and Cancer (Bethesda, Maryland, USA), whereas rabbit anti-human
CYP2C8/9/19 and rat CYP2C12 as well as sheep anti-human and rat CYP2E1 polyclonal
antibodies were from Chemicon International (Milano, Italy). Rabbit anti-goat and donkey
anti-sheep peroxidase-conjugated IgGs were purchased from Sigma Aldrich (Milano, Italy);
donkey anti-rabbit and sheep anti-mouse peroxidase-conjugated IgGs were from Amersham
Biosciences (GE-Healthcare, Milano, Italy). All the other immunoblotting reagents were of
molecular biology grade. Chloroform, isopropyl alcohol and ethyl alcohol were obtained from
Carlo Erba Reagents (Val de Reuil, France), while TRIzol® reagent and agarose were from
Invitrogen (Paris, France). High Capacity cDNA Archive Kit and Power SYBR® Green PCR
Master Mix were from Applied Biosystems (Courtaboeuf, France). Oligonucleotide primers
were synthesized by Invitrogen (Paris, France).

Animals. Certified male CH (*n*=10, about 700 kg bw), PM (*n*=8, about 650 kg bw) and BA (*n*=7, about 600 kg bw) beef cattle from private breeding farms located in the Piedmonte Region (Italy) were used. All animals were about 18 - 20 months old and were selected in the course of a research project whose major role was to define basic immunological, histological, zootechnical and pharmaco-toxicological parameters to be used in the screening of illicit drug treatments in meat cattle farming. At the slaughterhouse, after the exsanguination step, the liver lobe was removed and small aliquots for the total RNA extraction (about 100 mg each) were collected in sterility, immediately snap-frozen in liquid nitrogen and stored at -80°C. The remaining part of the lobe was cut in small pieces, washed in chilled isotonic 1,15% KCl and brought to the laboratory, within two hours after the animal death, wrapped in aluminium foils and kept in ice.

Preparation of subcellular fractions. Once in the laboratory, 20-30 g aliquots free from pathological lesions were blotted dry, chopped and homogenized in a Potter homogenizer. Therefore, cytosolic and microsomal subcellular fractions were isolated by differential ultracentrifugation as reported elsewhere (Nebbia et al., 2003). Aliquots of supernatants (cytosolic fraction) were frozen in liquid nitrogen and stored at -80°C until use. Pellets (microsomal fraction) were resuspended with 0.1 M phosphate buffer solution (PBS), containing 0.1 M EDTA and glycerol 20% (v/v) and stored as above. Protein concentration was determined according to Lowry et al. (1951), using bovine serum albumin as standard.

Enzyme assays. With regards to phase I DMEs catalytic activities, the rate of *N*-demethylation of benzphetamine (1mM), erythromycin (1 mM), ethylmorphine (6 mM), triacetyloleandomycin (TAO, 0.3 mM) as well as the hydroxylation of aniline (5 mM), 4-nitrophenol (0.2 mM) and benzo[a]pyrene (0.08 mM), were measured with an Uvikon 941 spectrophotometer (Kontron, Milano, Italy) or, alternatively, a LS30 fluorescence spectrometer (PerkinElmer, Monza, Italy), after an aerobic incubation step at 37°C by using

conditions previously reported by Nebbia et al. (2003). The testosterone (TST: 250 µM) hydroxylation in 2β-, 6β- and 16β positions (2βOH-T, 6βOH-T and 16βOH-T, respectively) was measured by a high performance liquid chromatography (HPLC) method (Capolongo et al., 2003). The ethoxyresorufin-*O*-deethylase (EROD) activity was measured by a modified HPLC method with fluorimetric detection, according to Hanioka et al. (2000). Briefly, 0.2 mg microsomal protein were diluted in PBS (50 mM, pH 7.4) and incubated (37°C, 3 min) in presence of 0.1 M $MgCl_2$, 10 mM NADPH and 0.5 µM ethoxyresorufin (ETR) as substrate. The final assay volume was 400 µL. The reaction was stopped by adding 400 µL of ice–cold methanol. Samples were vortexed, cooled on ice for 15 min and centrifuged (3500xg, for 20 min at +4°C). Surnatant (10 µl) was finally injected onto a Luna C18 column (100 Å, 150 mm x 4.6 mm, 5 µm; Phenomenex, Anzola Dell'Emilia, Bologna. Italy). The produced analyte (resorufin) was eluted isocratically with a mobile phase consisting of PBS (25 mM, pH 7.0) – methanol (58:42, v/v), at a 0.8 ml/min flow rate and detected fluorimetrically at 530 nm (excitation) and 580 nm (emission) wavelengths. The HPLC analysis was run by using a Jasco HPLC apparatus consisting of a PU 980 pump, an AS-1555 autoinjector, a DG1580-53 degasser and a FP-920 fluorescence detector (Jasco Europe, Cremella, Lecco, Italy). The rate of tolbutamide (TBT) hydroxylation was measured by HPLC as reported by Palamanda et al. (2000), with minor modifications. In short, 1.25 mg/ml microsomal protein diluted in PBS (0.1 M, pH 7.4) were incubated with 1.5 mM TBT (final concentration), as above mentioned for EROD. The final assay volume was 400 µL. The reaction was stopped with 2 mL ice-cold acetonitrile. After shaking (15 min), a sample aliquot (2 mL) was collected, evaporated under vacuum (40°C) using a Stepbio EZ2 Plus evaporator (Bologna, Italy), dissolved in 400 µL PBS (0,1 M, pH 7.4) and acidified with 2 M HCl (pH 2). Finally, 50 µl were injected onto a Luna C8 column (100 Å, 250 mm x 3 mm, 5 µm; Phenomenex, Anzola Dell'Emilia, Bologna. Italy), at a flow rate of 0.5 ml/min with 10 mM ammonium acetate (pH 4.3: A) and

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acetonitrile (B) as mobile phase. The hydroxylated metabolite was separated by using a linear gradient, consisting of 20% B for 14 min which increased up to 50% in 2 min, remained constant for 7 min, decreased to 20% in 2 min and then kept constant until the end of runtime (30 min). the HPLC analysis was performed using a JASCO HPLC system consisting of a PU-2089 PLUS pump, a PU 2089 PLUS degasser, a AS 2055 PLUS autoinjector and a UV-2070 PLUS detector (Jasco Europe, Cremella, Lecco, Italy).

Among phase II DMEs, UDP-glucuronosyltransferase (UGT) and glutathione-S-transferase (GST) catalytic activities were measured. The activity of the former toward substrates 1-naphthol and p-nitrophenol (αN-UGT and pNP-UGT, respectively) was measured on microsomal proteins, according to Gusson et al. (2006). With regards to cytosolic GSTs, the conjugation rate of either the substrate 1-chloro-2,4-dinitrobenzene (CDNB 1 mM; α-, μ-, π-GST) or 3,4-dichloronitrobenzene (DCNB 1 mM; μ-GST) were measured as previously described by Gusson et al. (2006).

Sodium dodecyl sulphate-polyacrilamide gel electrophoresis (SDS-PAGE) and immunoblotting. Microsomal proteins (30 µg) were separated by 10% SDS-PAGE in a Bio-Rad Miniprotean cell (Milano, Italy) and transferred to Protran® nitrocellulose membranes (Whatman Schleicher & Schuell, Dassel, Germany), as previously reported by Laemmli (1970) and Towbin et al. (1979), respectively. Membranes were then firstly probed with goat anti-rabbit CYP1A1/2 (1:100 final dilution), goat anti-rat CYP2B1/2 (1:1000), rabbit anti-human CYP2C8/9/19 and rat CYP2C12 (1:1000), sheep anti-human and rat CYP2E1 (1:2000) and anti-rat CYP3A1/2 (1:500) poly- or monoclonal antibodies. Therefore, they were incubated with peroxidase-conjugated rabbit anti-goat (final dilution 1:5000 for CYP1A and 1:4000 for CYP2B), donkey anti-rabbit (for CYP2C: 1:2000), donkey anti-sheep (for CYP2E1: 1:1000) and sheep anti-mouse (for CYP3A: 1:5000) IgGs, respectively. Band detection was performed by using a chemiluminescence kit (SuperSignal® West Pico

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Chemiluminescent Substrate, Pierce, Milano, Italy), according to the manufacturer's instructions. Immunopositive bands were captured by the Agfa ScanWise™2.0. software for the Agfa Snapscan 1212U scanner and their optical density analysed by the ImageJ 1.34s image analysis software. Two further samples, consisting of liver microsomal proteins from control or induced (β-naphtoflavone for CYP1A, phenobarbital for CYP2B and CYP2C, dexamethasone for CYP3A) rats were run on each minigel to allow the densitometric analysis of results as well as a molecular marker, respectively.

Total RNA isolation from bovine liver. Total RNA was isolated from frozen samples using the TRIzol® reagent according to the manufacturer's instructions. Briefly, 1mL of TRIzol® was added to a small aliquot (80 mg) of liver tissue in a Qbiogene Lysing Matrice Tube (MP Biomedicals, Illkirch, France) and immediately homogenized using Qbiogene Fast-Prep® FP120 (MP Biomedicals, Illkirch, France) twice for 20 seconds. Then, samples were put on ice and purified with a classical phenol-chloroform extraction step. Total RNA concentration and quality were checked by using a Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France). The RNA quality was estimated by the 260/280 and 260/230 nm absorbance ratios and confirmed by denaturing agarose gel electrophoresis.

Reverse-transcription (RT). The RT was performed using the High Capacity cDNA Archive Kit, following the manufacturer's procedure. Two µg of total RNA were added to the reaction mixture to reach a final volume of 20 µl. The reaction was performed in a 96-Well GeneAmp® PCR System 9700 (Applied Biosystems, Courtaboeuf, France) under the following conditions: 10 min at 25°C and 2 h at 37°C. Complementary DNA was then stored at -20°C until use.

Quantitative Real time polymerase chain reaction. *Bos taurus* mRNA sequences of target genes were obtained from GenBank and Ensembl Genome Browser web sites (http://ncbi.nlm.nih.gov/ and http://www.ensembl.org/, respectively). Primers sequences for Q

RT-PCR were designed using Primer Express[TM] Software (version 2.0, Applied Biosystems, Courtaboeuf, France) and are reported in Table 1. Primers concentrations were optimized in the 300-900 nM range. Melting curve analysis and agarose gel electrophoresis confirmed the amplification of a single amplicon of the expected size as well as the absence of primer dimers and genomic DNA amplification. Calibration curves, using a 10-fold serial dilution of a cDNAs pool, revealed PCR efficiencies close to 100%; thus, the $\Delta\Delta Ct$ method (Livak et al, 2001) was used to analyze data, expressed as fold-change compared to CH, arbitrarily chosen as the control. The β-actin was considered as the reference gene. The absence of polymorphisms on cDNA sequences complementary to primers used for Q RT-PCR measurements was checked on databases and the PCR efficiency evaluation was executed on cDNAs from all breeds. The Q RT-PCR was performed on 5 µL, out of 25 µL final volume, of 20-fold diluted cDNA by using an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Courtaboeuf, France) using standard PCR conditions.

Other methods. The total cytochrome P450 content was measured as the carbon monoxide difference spectrum (450 – 490 nm) of sodium dithionite-reduced microsomal suspensions, according to Nebbia et al. (2003). Both NADPH and NADH-P450 cytochrome *c* reductases activities were estimated following respectively the NADPH or NADH-dependent reduction of cytochrome *c* at 550 nm, according to Thomford and Dziuk (1986). The cytosolic glutathione (GSH) content was measured by using the Ellman's procedure (1959).

Statistical and data analysis. Data were expressed as mean values ± SD. Statistical analysis (Graph Pad Instat 2.01, San Diego, California, USA) was performed by the one-way analysis of variance (ANOVA) followed, if appropriate, by the Tukey's post-test. The Spearman nonparametric test was used for the correlation analysis between the gene expression and catalytic activities. In both circumstances, the presence of a value of $p < 0.05$ was considered as significant.

Results

Total cytochrome P450 content, GSH content and NAD(P)H cytochrome c reductase activity. No statistically significant differences were ever noticed in the amount of microsomal and cytosolic proteins (data not shown), total CYP content and NADH cytochrome *c* reductase activity (NADH-R: see Table 2) between breeds. The NADPH cytochrome *c* reductase activity (NADPH-R) showed a lower enzymatic activity in PM compared to the CH one ($p<0.01$). No statistically significant differences in GSH content were ever observed among the three cattle breeds.

Effect of breed upon phase I and II DMEs. Effects of breed upon phase I and II DMEs at the catalytic activity, protein and mRNA level are summarised in tables 3-4, 5 and 6, respectively. The nonparametric Spearman correlation analysis results are reported in Table 7.

CYP1A activity, protein and mRNA. Benzo[a]pyrene and ETR were chosen as CYP1A-dependent substrates. Neither catalytic activity or CYP1A apoprotein showed significant differences between breeds, with the mere exception of a higher benzo[a]pyrene hydroxylation (BPOH) rate in BA *vs* PM ($p<0.05$). By contrast, CH showed lower amounts, in terms of relative abundance, of both CYP1A1- and CYP1A2-like mRNAs when compared to PM ($p<0.001$ and $p<0.05$, respectively) and BA (only for CYP1A1: $p<0.001$) ones. No statistically significant correlation was ever found between selected substrates and CYP1A1-like mRNA. In CH, both CYP1A-dependent catalytic activities were significantly correlated with CYP1A2-like mRNA levels, with a nonparametric Spearman correlation coefficient (r_S) equal to 0.83 and 0.79 for BPOH and EROD, respectively ($p<0.05$).

CYP2B activity, protein and mRNA. Charolais cattle disclosed the highest capability to *N*-demethylate benzphetamine ($p<0.01$ *vs* PM) and hydroxylate TST in the 16β-position ($p<0.01$, *vs* PM and $p<0.01$ *vs* BA), which had been selected as CYP2B-dependent model substrates. On the contrary, it was BA that showed the highest CYP2B apoprotein content

($p<0.05$ *vs* CH). In figure 1, a representative CYP2B immunoblotting is reported. Such a result was confirmed also at the CYP2B6-like gene expression level; in both cases the trend was BA>PM>CH. The linear correlation analysis between 16βOH-T and CYP2B6-like gene expression pointed out a positive correlation for PM and BA ($p<0.05$) and a negative one for CH (r_S = 0.71, 0.79 and -0.38, respectively). Besides, a good correlation coefficient was noticed in PM if we took into account benzphetamine *N*-demethylase (BENZDEM: r_S = 0.74, $p<0.05$). In fig. 2, the overall effect of breed upon CYP2B catalytic activities, apoprotein content and mRNA together with the linear correlation analysis between 16βOH-T and CYP2B6-like mRNA is reported.

CYP2C activity, protein and mRNA. Differences in tolbutamide 4-hydroxylase (TBT4-OH) and CYP2C protein amount were never observed among cattle breeds. Likewise, no statistically significant differences were noticed in CYP2C8- and CYP2C19-like mRNAs (despite the higher amounts found in PM). By contrast, significant lower amounts of CYP2C9- and CYP2C18-like mRNAs were found in CH ($p<0.01$ *vs* PM and BA and $p<0.001$ *vs* BA, respectively); in the case of CYP2C18-like transcript, significant differences were noticed between BA and PM, too ($p<0.05$). The TBT4-OH catalytic activity was significantly correlated to liver CYP2C19-like mRNA expression in CH group (r_S = 0.82, $p<0.05$).

CYP2E activity, protein and mRNA. Aniline and *p*-nitrophenol were chosen as CYP2E1 model substrates. Liver CH microsomes showed the greater catalytic activity toward the fore-mentioned substrates. In particular, the aniline hydroxylase (aniline-OH) activity was about 2- and 1.5-fold higher than that reported in PM and BA microsomes ($p<0.001$ and $p<0.05$, respectively). With regards to *p*-nitrophenol hydroxylase (pNP-OH), a significantly higher catalytic activity was pointed out in CH, if compared to the BA one ($p<0.05$). Immunoblotting was performed by using a human and rat anti-CYP2E1 polyclonal antibody raised in sheep; faint cross-reacting bands, which hampered their consequent densitometric analysis, were

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noticed (data not shown). No statistically significant differences were ever observed in CYP2E1-like mRNA even if, and quite unexpected, BA showed a higher CYP2E1-like mRNA abundance (BA>CH>PM). As a whole, these results were partially confirmed by the correlation analysis: in fact, in BA group the aniline-OH catalytic activity was negatively correlated to CYP2E1-like gene expression (r_S = -0.94, $p<0.05$).

CYP3A activity, protein and mRNA. No differences were found between CH, PM and BA in the *N*-demethylation of CYP3A-dependent substrates ethylmorphine, erythromycin or TAO (ETDEM, ERDEM and TAODEM, respectively). On the contrary TST, another CYP3A substrate, was metabolized to a greater extent in CH: the rate of TST hydroxylations in 6β- and 2β positions was about 2-fold higher than in PM and BA ($p<0.01$). By contrast, CYP3A protein was expressed to a greater extent in BA group ($p<0.05$, *vs* CH). The Q RT-PCR results were in line with those obtained with immunoblotting, as BA was the cattle breed gifted of the higher CYP3A4-like transcript ($p<0.05$, BA *vs* CH). No significant correlations were noticed between used substrates and the CYP3A4-like gene expression profile.

UGT activity and mRNA. In our experimental conditions, CH was proved as the cattle breed which metabolised to a greater extent either α-naphthol or *p*-nitrophenol, two UGT1 specific substrates. As regards the former, the catalytic activity was about 1.5- and 2-fold higher than in PM ($p<0.05$) and BA ($p<0.01$), respectively. At the mRNA level, UGT1A6-like expression was perfectly in line with pNP-UGT activity results: in fact, CH showed a mRNA relative abundance 3- and 8-fold greater than that measured in PM ($p<0.01$) and BA ($p<0.01$), respectively. By contrast, UGT1A1-like transcript was found highly expressed in BA ($p<0.01$), with an opposite trend: BA>PM>CH. No significant correlation was ever observed between the two UGT1-dependent conjugation activities and UGT1A1- and UGT1A6-like gene expression profiles.

GST activity and mRNA. With regards to GST-dependent conjugation reactions, in figure 3 the activity of GSTs accepting two different substrates, namely CDNB for total GST and DCNB for GST μ1, are reported. The pattern of CDNB metabolism was lowest in CH ($p<0.01$ *vs* PM and $p<0.001$ *vs* BA), whereas the DCNB one was of the same order of magnitude in CH and PM, but more elevated in BA ($p<0.001$). At the pre-transcriptional level, likewise to enzymatic activities, CH showed an overall lesser amount of considered GSTs transcripts, reaching in certain instances the level of statistical significance ($p<0.05$ *vs* PM for GSTA1-like and $p<0.05$ *vs* BA for GSTP1-like). As far as GSTM1-like mRNA is concerned, CH showed about 3- and 6-fold lower amounts than PM and BA ($p<0.05$ and $p<0.001$, respectively: see figure 3). The correlation analysis between the two selected DMEs activities and GSTM1-like gene expression was statistically significant ($p<0.05$) in PM cattle, with r_S values of 0.82 and 0.89 for CDNB and DCNB, respectively.

Discussion

In the present study, the effect of breed upon most DMEs was investigated in cattle liver both at pre-transcriptional and post-translational levels. In fact, previously published were mostly addressed to the evaluation of catalytic activities and apoprotein content, and only recently DMEs gene expression has gained increasing interest in this veterinary species (Greger et al., 2006; Cantiello et al., 2005).

Substrates mostly used to monitor CYP1A activity are benzo[a]pyrene and ETR, being this latter metabolised to a greater extent in cattle than rats (Nebbia et al., 2003; Ioannides, 2006). In the present study both catalytic activities and apoprotein levels were in line with previously published ones (Nebbia et al., 2003; Szotáková et al., 2004; Sivapathasundaram et al., 2001). In the rat EROD reflects CYP1A1 activity, but it has been hypothesised that CYP1A2 might participate, albeit much less efficiently than CYP1A1, to ETR metabolism. In the cattle liver, the higher EROD activity might be due to a more relevant expression of

CYP1A1, an extra-hepatic isoform only inducible in the liver (Sivapathasundaram et al., 2001); in this respect, the presence of liver constitutive CYP1A1-like mRNA was confirmed by the Q RT-PCR technique, but correlation indexes between catalytic activities and CYP1A1- or CYP1A2-like mRNA were higher for this latter. The observed significant breed-differences in CYP1A1/2 expression were consistent with previous data reported in mouse strains (Casley et al., 1997).

Benzphetamine and 16α-hydroxylated TST are considered CYP2B-dependent substrates in man and rat, respectively (Wang et al., 2006; Ioannides, 2006). In cattle this isoform was believed to be poorly involved in TST hydroxylation (Ioannides, 2006; Sivapathasundaram et al., 2001) but, recently, veal calf liver microsomes were proved able to produce, out of 6β- and 2β-OH TST, detectable amounts of 16β-OH TST, too (Capolongo et al., 2003); besides, a 3-fold increase in 16β-OH TST production was noticed in liver microsomes from beef cattle induced with phenobarbital (Cantiello et al., 2006), which up-regulates CYP2B10 expression in the mouse (Peffer et al., 2007). In our study, CYP2B-dependent DMEs activities were clearly and significantly higher in CH. The immunoblotting, where we used the same anti-rat CYP2B1/2 polyclonal antibody reported by Nebbia et al. (2004) and Sivapathasundaram et al. (2001), gave significant results which agreed with CYP2B6-like expression profile, but not with catalytic activities. A good correlation was obtained between 16β-OHT and CYP2B6-like gene expression (especially in BA), thereby confirming data reported in the mouse by Peffer et al. (2007).

Tolbutamide hydroxylation was chosen as a CYP2C-dependent DME because it reflects human 2C9 and it has been already measured in cattle, too (Ioannides, 2006). Breed did not affect either TBT4-OH or CYP2C apoprotein level, which were not correlated each other (data not shown); similar results were obtained by Löfgren et al. (2004) in the mouse, where the same polyclonal antibody which recognises several CYP2C isoforms as a single

immunoreacting band was successfully used. On the contrary significant breed-differences were noticed at the mRNA level except for CYP2C19-like, whose expression was significantly correlated with TBT4-OH in CH, however. In veterinary species, CYP substrates are usually classified according to their specificity toward human or rat isoforms; unfortunately, a substrate specifically metabolised by a certain CYP in fore-mentioned species might undergoes different biotransformation pathways in other ones, with consequent changes in CYP selectivity (Szotáková et al, 2004). Therefore, further studies are required to identify bovine CYP2C isoform sequences and clarify their involvement in xenobiotics metabolism.

Several rodent CYP2E-dependent DME activities, including pNP-OH and Aniline-OH, have been measured in cattle (Ioannides, 2006). Present results are consistent with those reported by Nebbia et al. (2003) and suggestive of differences in catalytic activities among cattle breeds. A poor cross-reactivity was noticed at the immunoblotting level, which did not allow a correlation between enzymatic activity and CYP2E1 protein; on the other hand, contrasting results in terms of cross-reactivity, number of cross-reacting bands as well as accordance with corresponding catalytic activities have already been reported in veterinary species, following the use of anti-human or anti-rat CYP2E1 antibodies (Machala et al., 2003; Nebbia et al.,; Szotáková et al., 2004; Ioannides, 2006). No differences were noticed at the mRNA level, consequent to the high inter-individual variability. Noteworthy was the correlation coefficient found for Aniline-OH (significant for BA), which would indirectly confirm this latter as a CYP2E substrate in cattle, too; this isoform represents one of the mostly conserved subfamilies, and corresponding orthologous proteins often show the same substrate specificity (Ioannides, 2006).

Concerning CYP3A, both 6βOH-T and 2βOH-T activities pointed out significant differences among breeds, whereas ERDEM, ETDEM and TAODEM ones did not. These contrasting results partially disagreed with those reported by Dacasto et al. (2005), where

significant differences, between PM and LIM cattle, were found for ERDEM, ETDEM and 2βOH-T. Out of ERDEM, whose protocol and the resulting catalytic activity in PM (the only used in both studies) were similar, methodological differences might be probably offered as a justification for such a discrepancy; for ERDEM, in terms of substrate concentration or instrument sensitivity; about 6βOH-T, of more sensitivity and precision of the used method (HPLC). In this respect, the correlation analysis among catalytic activities and CYP3A4-like mRNA gave better coefficients with HPLC results, which indirectly suggest TST as a more specific and useful substrate to study CYP3A expression in cattle. The immunoblotting, where the same anti-rat CYP3A1/2 cited by Nebbia et al. (2003) was used, gave contradictory results compared with catalytic activities (according to Dacasto et al., 2005) but consistent with CYP3A4-like mRNA.

With regards to phase II DMEs, we choose human microsomal UGT1A substrates α-naphthol and *p*-nitrophenol, which had already been used in cattle (Gusson et al, 2006). Both were metabolised to a greater extent in CH. These data were in line with UGT1A6-like mRNA levels, but disagreed with UGT1A1-like ones. Moreover, no correlation was found between catalytic activities and gene expression. Interestingly a UGT2B17-like isoform, involved in human steroid conjugation (Xu et al., 2004), was highly expressed in BA, likewise to UGT1A1-like (data not shown). Present results might be justified by the fact that many UGTs (including those here selected, and whose cattle sequences are available in databases), show broad overlapping substrates specificities (Nagar and Remmel, 2006) even in the mouse animal model (Katoh et al., 2005).

Nowadays, seven GSTs classes are recognized in mammals (Frova, 2006); among their substrates, CDNB is considered as a general one whereas DCNB is specific for the Mu isoform in rat, mouse, man and rabbit (Gusson et al., 2006). In our study BA elicited the most efficient pattern of biotrasformation, in contrast with CYPs and UGTs data; besides, such a

behaviour was confirmed pre-transcriptionally, too. Furthermore, in PM the GSTM1-like mRNA was significantly correlated with both substrates. On a knowledge basis this is the first report concerning GSTs mRNA expression in cattle, suggestive of breed-differences as previously noticed in rats (Jang et al., 2001).

As a whole our data are suggestive of breed-differences in DMEs expression in cattle, likewise to laboratory species (Casley et al.,1997; Löfgren et al., 2004; Jang et al., 2001; Saito et al., 2004; Stott et al., 2004). Differences in DMEs expression are likely to influence both the bioavailability and clinical efficacy of xenobiotics (Sallovitz et al., 2002) and these same might be of particular concern in farm animals, for the hypothetical presence of potentially harmful residues in foodstuffs of animal origin. Moreover, the presence of uneven results suggest that such an effect cannot be clearly set at the level of enzyme translation rather than transcription *a priori*.

Microsomal and cytosolic proteins as well as CYP and GSH contents were entirely similar among breeds; nonetheless CH comparatively showed, for the great majority of considered DMEs, the lower protein and gene expression levels but the more elevated catalytic activity. A first possible explanation of such a behaviour might be a more efficient catalytic cycle, a hypothesis which might be justified by the higher NADPH-R activity measured in CH itself; in this respect, a similar behaviour has been recently recorded, for CYP3A, in the horse (Tydén et al., 2007). A second one might be traced back in the presence either of post-translational phenomena (like phosphorylation) or, alternatively, post-transcriptional effects (i.e., microRNA); in fact, the former ones have been proved able to modulate CYP activity and substrate specificity as well (Oesch-Bartlomowicz and Oesch, 2005), whereas the second ones, instead, might play a role in the regulation of DMEs expression (as recently reported in the case of CYP1B1: Tsuchiya et al., 2006). Finally, the hypothetical presence of single nucleotide polymorphisms (SNP) in DMEs sequence cannot

be altogether excluded; recently, an increased metabolic activity found in CH compared to German Holstein cattle has been attributed to SNPs in some functional liver and intestine genes (Schwerin et al., 2006). Ongoing studies, aiming to sequence genes coding for major DMEs in these cattle breeds are envisaged in our laboratory.

Acknowledgements

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Footnotes

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Legend for figures

Fig. 1: Representative (CYP2B) immunoblotting of liver microsomes (30 µg) from CH, PM and BA cattle. Membranes were probed with an anti-rat CYP2B1/2 polyclonal antibody raised in goat. A control sample, consisting of microsomal proteins obtained from the liver of untreated rats (R) was run to allow the densitometric analysis as well as the normalization of results, particularly for any developing and fixing discrepancies among different blots.

Fig. 2: Effect of breed upon (A) liver CYP2B-dependent 16β-OHT, CYP2B apoprotein and CYP2B6-like mRNA in CH (*n*=10), PM (*n*=8) and BA (*n*=7) cattle and (B) correlation analysis between the enzymatic activity and the gene expression data. Catalytic activity, immunoblotting and Q RT-PCR data were obtained as described in Material and Methods. All data are expressed as means ± S.D. In particular, immunoblotting densitometric values represent the mean ± SD of integrated density data for every group, expressed as arbitrary units (a.u.) of the density recorded in the control (R, liver microsomes from untreated rats). Catalytic activity data are expressed as nmol/min/mg protein and Q RT-PCR ones as fold-change (arbitrary units, a.u.) compared to the CH group which was arbitrarily chosen as the control group. [a,b,c]Statistically significant differences between CH and PM, CH and BA, PM and BA, respectively (ANOVA + Tukey's post-test; b: $p<0.05$; aa,bb,cc: $p<0.01$). r_S corresponds to the nonparametric Spearman correlation coefficient (range from -1 to +1; considered significant for $p<0.05$).

Fig. 3: Effect of breed upon liver GST catalytic activities (CDNB and DCNB) and GSTM1-like mRNA in CH (*n*=10), PM (*n*=8) and BA (*n*=7) cattle (A) and correlation analysis between the enzymatic activity (CDNB) and the gene expression data. Catalytic activity, immunoblotting and Q RT-PCR data were obtained as described in Material and Methods. All

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data are expressed as means ± S.D. Catalytic activity data are expressed as nmol/min/mg protein and Q RT-PCR ones as fold-change (arbitrary units, a.u.) compared to the CH group which was arbitrarily chosen as the control group. [a,b,c]Statistically significant differences between CH and PM, CH and BA, PM and BA, respectively (ANOVA + Tukey's post-test; a: $p<0.05$; aa: $p<0.01$; bbb, ccc: $p<0.001$). r_S corresponds to the nonparametric Spearman correlation coefficient (range from -1 to +1; considered significant for $p<0.05$).

Table 1: Target and housekeeping genes selected for Q RT-PCR: NCBI mRNA reference sequences and primers

DMEs	GenBank accession no.	5' → 3' primer sequence	Primer length (bp)	Amplicon size (bp)
CYP1A1-like	XM_588298	F : GACCTGAATCAGAGGTTCTACGTCT	25	81
		R : CCGGATGTGACCCTTCTCAA	20	
CYP1A2-like	XM_591450	F : ACCATGACCCGAAGCTGTG	19	78
		R : CAATGGTGGTGCCATCAGAC	20	
CYP2B6-like	NM_001075173	F : GCGGACCTCATCCCCATT	18	80
		R : GTGCCCTTGGGAAGGATGT	19	
CYP2C8-like	XM_868633	F : CCCAGAGGTCATAGCTAAGGC	21	56
		R : CGGTGTCTGCCAAGCTCAT	19	
CYP2C9-like	XM_612374	F : TCCCTGGACATGAACAACCC	20	71
		R : TTGTGCTTTTCCTGTTCCATCTT	23	
CYP2C18-like	NM_001076051	F : ATGTTAAGAACATTGGCAAATCCTT	25	51
		R : GGCCATAGGTGTTTGAGAGATTG	23	
CYP2C19-like	XM_600421	F : TCCCAAGGGCACAACCATA	19	56

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		R : CCTTGCCATCGTGCAGG	17	
CYP2E1-like	NM_174530	F : ACCCGGAGGTTGAAGAGAAAC	21	51
		R : GCCCAATCACCCTGTCAATTT	21	
CYP3A4-like	NM_174531	F : GCCAGAGCCCGAGGAGTT	18	77
		R : GCAGGTAGACGTAAGGATTTATGCT	25	
UGT1A1-like	DQ115935	F : ACCATCCTACGTGCCCAGG	19	71
		R : TGTTCTTCACCCGCTGCAG	20	
UGT1A6-like	NM_174762	F : CAACACGGTCCTCATCGGA	19	71
		R : CATAGGCTTCAAATTCCTGAGACAG	25	
GSTA1-like	NM_001078149	F : TTCCCTCTGCTAAAGGCCCTA	21	84
		R : CTTCCTCTGGCTGCCAGG	18	
GSTM1-like	NM_175825	F : TCCTTGACATGCACCGCATA	20	99
		R : CAGAGATCTTCTTCAGGCCCTCA	23	
GSTP1-like	NM_177516	F : CCTCATTTACACCAACTACGAGGC	24	72
		R : AAAGGCTTCAGGTGCTGGG	19	
β-Actin	NM_173979	F : GTCGACACCGCAACCAGTT	19	85

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R : AAGCCGGCCTTGCACAT   **17**

Table 2: Cytochrome P450 (CYP) and GSH content, NAD(P)H-R activities[¤] in cattle breeds.

	Cattle breeds		
	CH	**PM**	**BA**
CYP content	0.63 ± 0.09	0.66 ± 0.14	0.67 ± 0.06
GSH content	3.5 ± 0.3	3.7 ± 0.3	3.8 ± 0.4
NADPH-R	90.3 ± 10.7^{aa}	61.1 ± 11.0	74.4 ± 15.8
NADH-R	939 ± 224	925 ± 192	978 ± 191

Note. Data are expressed as means $\pm$ SD (CH, $n= 10$; PM, $n=8$; BA, $n=7$).

[¤]: nmoles/mg protein (CYP content); µmoles GSH/g liver weight (GSH content); nmoles/min/mg protein (NADPH-R and NADH-R).

[a]Statistically significant differences between CH and PM (ANOVA + Tukey's post test; aa: $p<0.01$).

Table 3: Phase I DMEs activities* in cattle breeds.

Phase I DMEs	Catalytic Activities	Cattle breeds		
		CH	PM	BA
CYP1A	BPOH	0.75 ± 0.13	0.59 ± 0.23^c	0.90 ± 0.15
	EROD	430.1 ± 59.3	323.5 ± 43.0	383.4 ± 94.5
CYP2B	BENZDEM	2.06 ± 0.39^{aa}	1.36 ± 0.28	1.77 ± 0.46
	16βOH-T	$0.32 \pm 0.11^{aa,bb}$	0.19 ± 0.06	0.18 ± 0.08
CYP2C	TBT4-OH	0.04 ± 0.02	0.04 ± 0.01	0.05 ± 0.02
CYP2E	Aniline-OH	$0.56 \pm 0.18^{aaa,b}$	0.26 ± 0.06	0.36 ± 0.07
	pNP-OH	0.37 ± 0.11^b	0.35 ± 0.19^c	0.13 ± 0.10
CYP3A	6βOH-T	$0.88 \pm 0.25^{aa,bb}$	0.53 ± 0.15	0.49 ± 0.20
	2βOH-T	$0.30 \pm 0.10^{aaa,bb}$	0.15 ± 0.05	0.17 ± 0.07
	ERDEM	0.56 ± 0.12	0.60 ± 0.28	0.52 ± 0.15
	ETDEM	1.66 ± 0.35	1.46 ± 0.31	1.38 ± 0.18
	TAODEM	0.42 ± 0.11	0.42 ± 0.31	0.49 ± 0.14

Note. Data are expressed as means $\pm$ SD (CH, n=10; PM, n=8; BA, n=7).

*: μg quinine sulphate/min/mg protein (BPOH); pmoles/min/mg protein (EROD); nmoles/min/mg protein (BENZDEM, 16βOH-T, TBT4-OH, Aniline-OH, pNP-OH, 6βOH-T, 2βOH-T, ERDEM, ETDEM, TAODEM).

[a,b,c]Statistically significant differences between CH and PM, CH and BA, PM and BA, respectively (ANOVA + Tukey's post test; b, c: p<0.05; aa, bb: p<0.01; aaa: p<0.001).

Table 4: Phase II DMEs activities[#] in cattle breeds.

Phase II DMEs	Catalytic Activities	Cattle breeds		
		CH	PM	BA
UGT	αN-UGT	13.5 ± 5.2^a	8.5 ± 2.5	9.2 ± 2.4
	pNP-UGT	$15.1 \pm 5.4^{a,bb}$	9.3 ± 2.5	7.5 ± 2.5
GST	CDNB	$295.5 \pm 43.7^{aa,bbb}$	367.4 ± 29.4	417.0 ± 56.9
	DCNB	0.34 ± 0.06^{bbb}	0.33 ± 0.08^{ccc}	0.56 ± 0.11

Note. Data are expressed as means $\pm$ SD (CH, $n=10$; PM, $n=8$; BA, $n=7$).

[#]: nmoles/min/mg protein

[a,b,c]Statistically significant differences between CH and PM, CH and BA, PM and BA, respectively (ANOVA + Tukey's post test; a: $p<0.05$; bb: $p<0.01$; aaa, bbb, ccc: $p<0.001$).

Table 5: Effect of breed upon liver P450 apoprotein[†] in CH, PM and BA cattle.

Apoprotein	Immunoblotting		
	CH	**PM**	**BA**
CYP1A1/2	2.92 ± 0.37	2.89 ± 0.76	2.70 ± 0.47
CYP2B1/2	0.96 ± 0.23^{b}	1.21 ± 0.33	1.34 ± 0.33
CYP2C8/9/19-2C12	0.81 ± 0.17	0.87 ± 0.16	0.95 ± 0.27
CYP3A1/2	0.99 ± 0.43^{b}	1.16 ± 0.37	1.63 ± 0.46

[†]: arbitrary units (a.u.). Immunoblotting densitometric values represent the mean $\pm$ SD of integrated density data for every group (CH, $n=10$; PM, $n=8$; BA, $n=7$), expressed as arbitrary units of the density recorded in the control (R, liver microsomes from untreated rats).

[b,c]Statistically significant differences between CH and BA, PM and BA, respectively (ANOVA + Tukey's post test; b: $p<0.05$).

Table 6: Liver P450 and Phase II DMEs mRNA relative abundance° in CH, PM and BA cattle breeds.

Genes	Fold-change		
	CH	**PM**	**BA**
CYP1A1-like	$1.00 \pm 0.35^{aaa,bbb}$	2.05 ± 0.50	2.05 ± 0.40
CYP1A2-like	1.00 ± 0.54^{a}	1.73 ± 0.38	1.63 ± 0.61
CYP2B-like	1.00 ± 2.34^{b}	3.82 ± 3.28	4.77 ± 2.46
CYP2C8-like	1.00 ± 1.89	4.28 ± 3.34	2.41 ± 1.92
CYP2C9-like	$1.00 \pm 0.52^{aa,bb}$	3.34 ± 1.43	3.29 ± 1.35
CYP2C18-like	1.00 ± 0.29^{bbb}	1.33 ± 0.27^{c}	1.93 ± 0.5
CYP2C19-like	1.00 ± 0.84	1.56 ± 1.01	1.00 ± 0.49
CYP2E1-like	1.00 ± 1.52	0.57 ± 0.70	1.47 ± 1.05
CYP3A4-like	1.00 ± 0.37^{b}	1.32 ± 0.45	1.72 ± 0.46
UGT1A6-like	$1.00 \pm 0.68^{a,bb}$	0.36 ± 0.18	0.12 ± 0.10
UGT1A1-like	1.00 ± 0.42^{bb}	1.02 ± 0.28^{cc}	1.75 ± 0.37
GSTA1-like	1.00 ± 0.41^{a}	1.75 ± 0.77	1.56 ± 0.48
GSTM1-like	$1.00 \pm 0.60^{a,bbb}$	3.85 ± 1.65	6.64 ± 3.61
GSTP1-like	1.00 ± 0.23^{b}	1.13 ± 0.25	1.41 ± 0.31

Note. Data are expressed as means ± SD (CH, $n=10$; PM, $n=8$; BA, $n=7$).

°: arbitrary units (A.U.).

[a,b,c]Statistically significant differences between CH and PM, CH and BA, PM and BA, respectively (ANOVA + Tukey's post test; a, b: $p<0.05$; aa, bb, cc: $p<0.01$; aaa, bbb: $p<0.001$).

Table 7: Nonparametric Spearman correlation analysis between catalytic activities and gene expression in CH, PM and BA cattle breeds.

Catalytic Activity	Gene	r_S [§]		
		CH	PM	BA
BPOH	CYP1A1-like	0.50	0.71	0.43
	CYP1A2-like	0.83*	0.02	0.43
EROD	CYP1A1-like	0.36	0.46	0.03
	CYP1A2-like	0.79*	0.21	-0.25
16βOH-T	CYP2B6-like	-0.38	0.71	0.79*
BENZDEM		0.05	0.74*	0.61
TBT4-OH	CYP2C8-like	0.57	-0.2	-0.43
	CYP2C9-like	0.18	-0.07	-0.36
	CYP2C18-like	0.37	0.54	-0.46
	CYP2C19-like	0.82*	0.25	0.20
Aniline-OH	CYP2E1-like	0.43	0.51	-0.94*
pNP-OH		0.29	0.14	-0.54
6βOH-T		0.57	0.32	0.29
2βOH-T		0.55	0.32	0.29
ERDEM	CYP3A4-like	0.18	-0.10	0.52
ETDEM		0.22	-0.29	-0.25
TAODEM		-0.05	-0.05	0.64
αN-UGT	UGT1A1-like	-0.22	-0.68	-0.50
pNP-UGT		-0.17	-0.43	-0.39
αN-UGT	UGT1A6-like-like	0.07	-0.25	0.29

pNP-UGT		0.50	-0.43	0.46
CDNB	**GSTA1-like**	0.49	0.12	0.21
CDNB	**GSTM1-like**	-0.35	0.82*	0.83
DCNB		0.17	0.89*	0.38
CDNB	**GSTP1-like**	0.32	-0.02	0.36

§: r_S corresponds to the nonparametric Spearman correlation coefficient (range from -1 to +1; considered significant for $p < 0.05$).
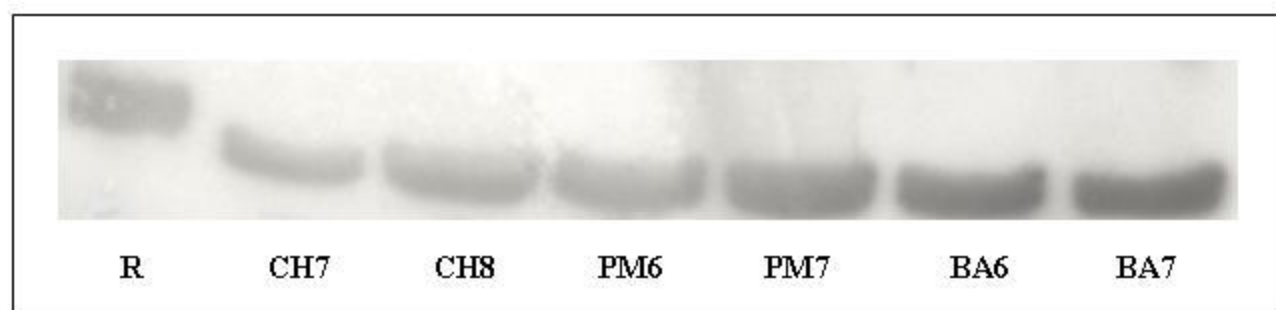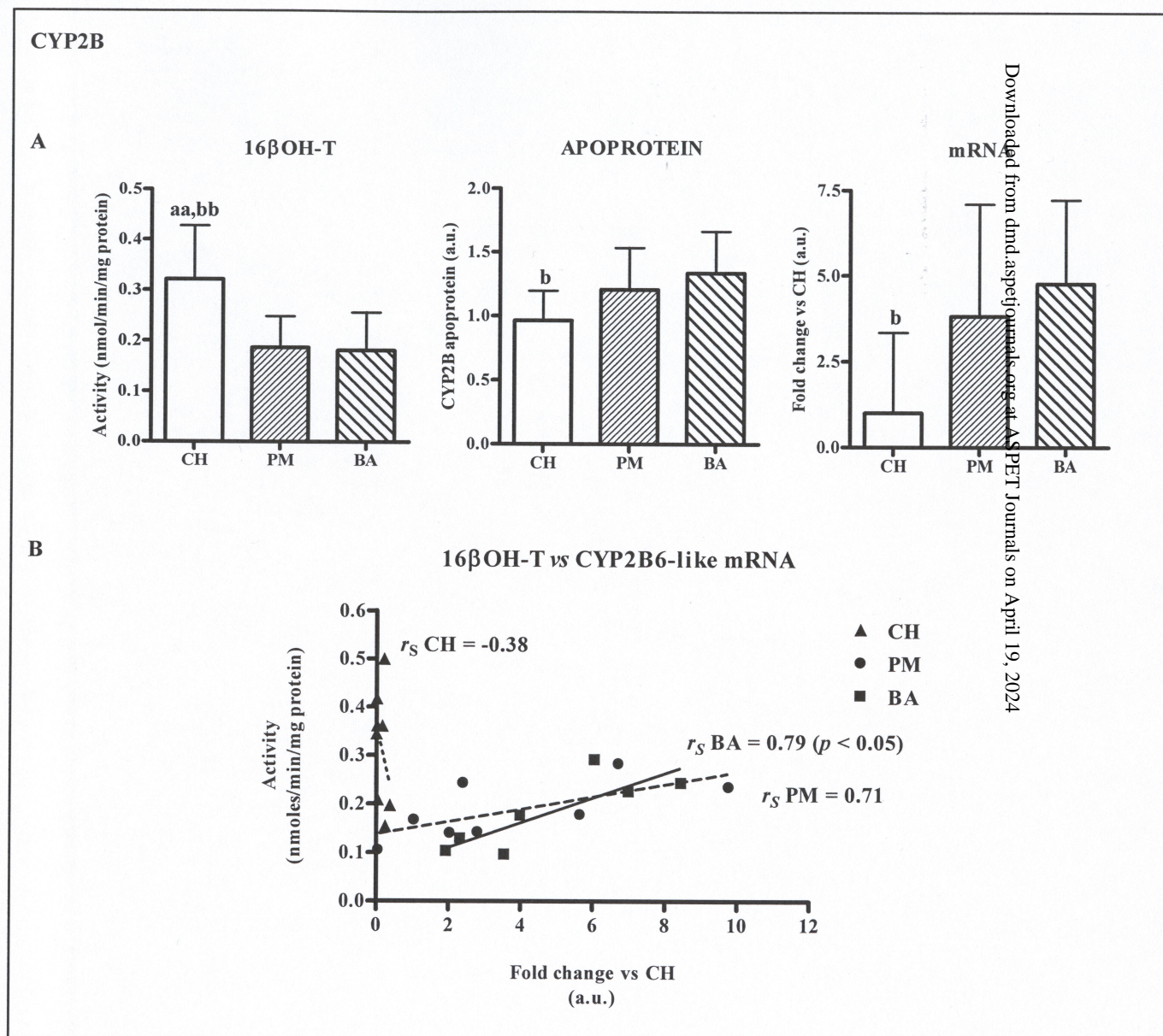
*: $p < 0.05$

Figure 1

| R | CH7 | CH8 | PM6 | PM7 | BA6 | BA7 |

Figure 2

CYP2B

A

16βOH-T

APOPROTEIN

mRNA

B

16βOH-T *vs* CYP2B6-like mRNA

Figure 3

GSTM

A

CDNB

DCNB

mRNA

B

DCNB *vs* GSTM1-like mRNA

r_S BA = 0.83

r_S PM = 0.82 (*p* < 0.05)

r_S CH = - 0.35

▲ CH
● PM
■ BA

Activity (nmoles/min/mg protein)

Fold change vs CH (a.u.)