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DRUG METABOLIZING ENZYMES AND TRANSPORTERS IN *BOS TAURUS* AND CRYOPRESERVED PIG HEPATOCYTES: CONSTITUTIVE EXPRESSION AND TRANSCRIPTIONAL MODULATION

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“The secret of getting things done is to act!”

Dante Alighieri.

ABSTRACT

The exposure to xenobiotics may alter the transcription of a broad array of genes expressed in multiple tissues and vital organs such as the liver, kidney, intestine, lungs, brain, placenta, and pancreas, in order to modulate their own metabolism and excretion (Tolson & Wang, 2010). The mammalian nuclear receptor superfamily of transcription factors is involved in the first step of detoxification mechanism, regulating the gene expression of phase I and II metabolizing enzymes and drug transporters (di Masi et al., 2009). Over the past ten years, remarkable advances have been made about the nuclear receptor-dependent activation of drug metabolizing enzymes and their consequences. Despite this, such a knowledge is still restricted to human and rodent species and much less is known in veterinary species. Aim of the present PhD thesis, which is presented in form of a collection paper, was first to propose a new nomenclature for bovine cytochrome P450 sequences involved in xenobiotic metabolism through a phylogenetic analysis and, thereafter, to improve knowledge on distribution and regulation of genes involved in xenobiotic metabolism of cattle and pig, two of the most important food-producing species. For this reason the transcriptional effects of phenobarbital were investigated upon bovine hepatic and extra-hepatic tissues nuclear receptors, drug metabolizing enzymes and major uptake and efflux transporters. Furthermore, transcriptional and post-translational effects of culturing time and prototypical cytochrome 3A inducers were investigated on principal transcription factor and metabolizing enzyme coding genes in long-term stored cryopreserved pig hepatocytes.

The phylogenetic analysis pointed out several inconsistencies between currently used cattle cythochromes P450 names and true evolutionary relationship among cytochrome P450 isoforms. Name changes mostly mirrored the orthologous counterparts established for other species, and new names were created when no clear orthologous sequences were identified. With regard to drug metabolizing enzymes and drug transporter expression in cattle tissues, the obtained information confirm for the most part data obtained in humans and rodent model species. Therefore, even if present results are not suggestive of a clear bovine-specific pattern of gene expression, the existence of a tissue-specific mechanism of cythochromes P450 induction cannot be excluded. Phenobarbital, orally administered at inducing dosages, up-regulated cattle hepatic cytochrome 2B, 2C and 3A, as well as the major conjugative enzymes UDP glucuronosyltransferase 1A1-*like* and glutathione S-transferase α 1-*like*. In extrahepatic tissues, results showed a different responsiveness to phenobarbital when compared to liver. A general trend to inhibition was observed in small intestine, and the sulfotransferase 1A1-*like* gene expression was significantly

inhibited. About drug transporters, liver, intestine and testis were proved to be tissues mostly involved in transport activity and, ATP-binding cassette C2 mRNA was shown to be significantly up-regulated by phenobarbital in the liver. By contrast, renal ATP-binding cassette G2 mRNA was inhibited. Likewise to former studies made in primary or cryopreserved hepatocytes obtained from different species (including the pig, too), a time-dependent down-regulation of cytochrome mRNAs and proteins was observed, whereas nuclear receptor gene expression data showed some differences in terms of response (crash and rise effect, up-regulation, no effect). When exposed to prototypical cytochrome 3A inducers, both nuclear receptor (essentially following the incubation with dexamethasone) and cytochromes gene expression profiles were up-regulated; furthermore, phase I enzymes modulation was confirmed at the post-translational level, although to a lower extent.

In conclusion, the gene expression profiling represents nowadays a helpful and sensitive method, compared to protein and catalytic activity assays, to detect a modulation of drug metabolizing enzymes expression and function following a xenobiotic exposure. Our findings on cattle nuclear receptors, drug metabolizing enzymes and transporters gene expression and possible modulation contributed to improve the knowledge on comparative drug metabolism.. Likewise, cryopreserved pig hepatocytes have been shown to be, even if stored for longer times than usual, an useful tool to study the expression and regulation of genes requiring a functional transcription factor machinery.

RIASSUNTO

L'esposizione a xenobiotici può modulare la trascrizione di un ampio spettro di geni espressi in tessuti ed organi diversi tra cui fegato, rene, intestino, polmone, cervello, placenta e pancreas al fine di favorirne il metabolismo ed il processo di escrezione (Tolson e Wang, 2010). La superfamiglia genica dei fattori di trascrizione codificante per i recettori nucleari è responsabile dell'attivazione del meccanismo di detossificazione dello xenobiotico regolando, a livello trascrizionale, l'espressione degli enzimi del metabolismo di fase I e II, nonché di fase III (trasportatori di membrana; di Masi *et al.*, 2009). Nel corso degli ultimi dieci anni, nell'uomo e nelle specie da laboratorio è stato chiarito il meccanismo di attivazione dei geni regolati dai recettori nucleari; nonostante ciò, poche informazioni sono tutt'ora disponibili per quanto riguarda le specie di interesse veterinario. Scopo di questa Tesi di Dottorato, presentata in forma di "*collection papers"* è stato *in primis* di proporre una nuova nomenclatura tramite un'accurata analisi filogenetica, per le sequenze di bovino del citocromo P450 coinvolte nel metabolismo degli xenobiotici. In seguito, sono state implementate le conoscenze riguardanti la distribuzione tissutale e la regolazione degli enzimi coinvolti nel metabolismo degli xenobiotici nel bovino e nel suino, due delle specie veterinarie da reddito più importanti. A questo proposito, è stato condotto uno studio volto a delucidare gli effetti *pre*-trascrizionali del fenobarbitale, nel fegato e in tessuti extra-epatici di bovino, sui principali recettori nucleari, enzimi biotrasformativi e trasportatori di membrana,. Oltre a ciò, in epatociti crioconservati di suino, sono stati valutati gli effetti a livello trascrizionale e *post*-traduzionale del tempo in coltura e di classiche molecole induttrici del citocromo 3A sui principali recettori nucleari ed enzimi di fase I.

L'analisi filogenetica ha rivelato numerose imprecisioni nell'attuale nomenclatura utilizzata per le sequenze di citocromo P450 nel bovino. I cambiamenti proposti riprendono prevalentemente la nomenclatura in uso per la specie la cui sequenza è risultata ortologa a quella di bovino, mentre, per le sequenze alle quali non è stato possibile assegnare con certezza una relazione di ortologia, è stato creato un nuovo acronimo. La distribuzione tissutale nel bovino osservata per i fattori di trascrizione, enzimi del metabolismo e trasportatori presenta alcune caratteristiche comuni con l'uomo e specie da laboratorio. I risultati ottenuti non sarebbero pertanto indicativi di un profilo di espressione bovino-specifico di tali geni, anche non si può escludere la presenza di un meccanismo di induzione tessuto-specifico. Il fenobarbitale ha indotto a livello epatico la trascrizione dei citocromi appartenenti alle famiglie 2B, 2C e 3A nonché quella di enzimi coniugativi quali l'UDP glucuronosiltransferasi 1A1-*like* e la glutatione S-transferasi α 1-

like. Per quanto riguarda i tessuti extra-epatici, nell'intestino è stato osservato un generale effetto inibitorio (significativo per sulfotransferasi 1A1-*like*). Anche nel bovino, il fegato, l'intestino e il testicolo si sono rivelati essere gli organi che esprimono geni codificanti per proteine di afflusso ed efflusso e l'espressione del trasportatore *ATP-binding cassette* C2 è stata significativamente aumentata a livello epatico dal fenobarbitale. Diversamente, nel rene il livello di mRNA dell' *ATP-binding cassette* G2 è stato significativamente ridotto. Similmente a quanto riportato in studi effettuati in precedenza su epatociti primari di diverse specie (inclusa quella suina) preparati a fresco e crioconservati, è stata osservata una diminuzione in funzione del tempo nel contenuto di citocromo P450 (sia livello di trascritto sia di proteina), mentre per i recettori nucleari non è stata evidenziata nessuna differenza in termini di risposta. L'esposizione a molecole induttrici del citocromo 3A (soprattutto a desametasone) ha significativamente indotto l'espressione dei geni codificanti per i recettori nucleari e gli enzimi di fase I, e per quest'ultimi tale modulazione è stata confermata anche a livello *post*-traduzionale.

Concludendo, lo studio dei profili di espressione genica si è rivelato un metodo precoce e sensibile per determinare la modulazione degli enzimi del metabolismo da parte degli xenobiotici rispetto agli studi effettuati a livello proteico e di attività catalitica. I risultati ottenuti dallo studio della distribuzione tissutale e di induzione dei recettori nucleari, enzimi deputati al metabolismo e dei trasportatori nel bovino contribuiscono ad implementare la conoscenza del metabolismo non solo nel fegato ma anche nei tessuti extra-epatici. Infine, è stato dimostrato che gli epatociti crioconservati di suino, anche se stoccati per lungo tempo, possono considerarsi uno strumento valido per lo studio dei meccanismi di regolazione di quei geni che richiedono un inalterato apparato trascrizionale.

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ABBREVIATIONS

17βE	17β-estradiol
βNF	β-naphthoflavone
ABC	ATP-binding cassette
ACTB	β-actin
ADME	absorption, distribution, metabolism and excretion
AhR	aryl hydrocarbon receptor
B2M	β_2-microglobulin
BCRP	breast cancer resistance protein
BLAST	basic local alignment search tool algorithm
bp	base pairs
BSEP	bile salt export pump
CAR	constitutive androstane receptor
CCRP	cytoplasmic retention protein
CITCO	2,3,3',4',5',6-hexachlorobiphenyl
COUP-TF	chicken ovalbumin upstream promoter transcription factor
CpG	cytosine-guanine
CPH	cryopreserved pig hepatocyte
C_t	cycle threshold
CTRL	control group
CYP	cytochrome P450
DBD	DNA binding domain
DEX	dexamethasone
DME	drug metabolizing enzyme
DMSO	dimethyl sulfoxide
DR	direct repeat
DT	drug transporter
ER	everted repeat
ERα	estrogen receptor alpha
E_x	PCR efficiency
FCS	fetal calf serum
FXR	farnesoid X receptor
G6PDH	glucose-6-phosphate dehydrogenase
GAPDH	glyceraldehydes-3-phosphate dehydrogenase
GR	glucocorticoid receptor alpha
GRE	glucocorticoid response element

GRIP1	p160 transcription factor GR-interacting protein 1
GST	glutathione S-transferase
HNF	hepatocytes nuclear factor
hr	hour
Hsp	heat shock protein
HTS	high throughput screening
ICG	internal control gene
LBD	ligand binding domain
LDH	lactate dehydrogenase
LXR	liver X receptor
MDR1	multidrug resistance protein 1
ML	maximum likelihood
MRP	multidrug resistance-associated protein
NCoR	nuclear co-repressor
NR	nuclear receptor
Nrf-2	nuclear factor-E2 related factor-2
NTCP	sodium taurocholate co-trasporting polypeptide
OAT	organic anion transporter
OATP	organic anion transporting polypeptide
OCT	organic cation transporter 1
OCTN	organic cation/carnitine transporter
OHTST	hydroxyl TST
PB	phenobarbital
PBREM	PB-responsive enhancer module
PBRU	PB-responsive unit
PCN	pregnenolone-16alpha-carbonitrile
PD	pharmacodynamics
PGC1	peroxisome proliferative activated receptor, PPAR gamma, coactivator 1
P-gp	*p*-glycoprotein
PHEN	phenobarbital treated gruop
PK	pharmacokinetics
PPIA	cyclophilin A
PXR	pregnane X receptor
qPCR	quantitative Real Time RT-PCR
RIF	rifampicin
RPLP0	ribosomal protein, large P0

RR	reticolo-rumen
RXR	retinoid X receptor
SA	salicylic acid
SHP	small heterodimer partner
SLC	solute carrier
SLCO	solute carrier organic anion transporter
SMRT	silencing mediator for retinoid and thyroid hormone receptor
SRC1	nuclear receptor coactivator 1
SULT	sulfotransferase
TBP	TATA-box binding protein
TBT	tolbutamide
TCDD	2,3,7,8-tetrachlorodibenzo-p-dioxin
TCPOBOP	3,5-dichloro-2-[4-(3,5-dichloropyridin-2-yl)oxyphenoxy]pyridine
TPD	2,4,6-triohenyldioxane-1,3
TST	testosterone
UGT	uridine-5'-diphosphate glucuronosyl transferase
UTR	untraslated region
VDR	vitamin D receptor
WEM	Williams' E medium
XREM	xenobiotic responsive enhancer module

1. Introduction

The cell membrane represents an efficient barrier that protects the cell from toxic, water-soluble xenobiotics. However, lipophilic substances can cross this boundary much more easily and subsequentially may accumulate within the cell until toxic levels are reached. Biological organisms protect themselves through two general mechanisms: biotransformation and transport (Handschin & Meyer, 2003). Biotransformation are required to convert lipophilic substances into more water-soluble metabolites that can be excreted from the body. Cytochromes P450 (CYP), together with dehydrogenases, reductases and other oxidases represent the main enzymatic system responsible for primary modifications of lipophilic compounds (phase I reactions). Functional groups, resulting from CYPs catalyzed monooxygenase reactions, can be used by other enzymes,such as phase II enzymes, for further modifications. Phase II reactions consist mainly of glucuronidation, sulfation, attachment of glutathione, methylation, *N*-acetylation, or conjugation with amino acids (Handschin & Meyer, 2003). Cells transport the resulting polar conjugates through export transporters which have been classified as the phase III of metabolism. Likewise, conjugates can also be taken up into cells, i.e. into hepatocytes for biliary excretion, and these uptake processes have been termed phase 0 (Köhle & Bock, 2009).

Liver represents the organ mostly endowed with CYPs and, the most important forms involved in human metabolism of therapeutic agents are CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/3A5 (Graham & Lake, 2008). Extrahepatic CYPs contribute to bioavailability and systemic effects of drugs; in this respect, a well known example is represented by the role played by drug-metabolizing enzymes (DMEs) and efflux proteins in the small intestine, where they contribute to the first-pass metabolism (Caldwell et al., 1995). In addition to liver and intestine, CYPs are found in many other tissues such kidney, lung and upper airways, brain, adrenals, gonads, heart and skin (Pelkonen et al., 2008). Sometimes, an enzymatic induction may occur following a CYP-dependent reactions; such a phenomenon is well known, and first examples date up to 40 years ago (Conney 1967). The ability of CYPs to increase their expression from a low basal level in the presence of a xenobiotic represents a cellular defense mechanism promoting the conversion of a compound to a more polar (sometimes more active) metabolite (Handschin & Meyer, 2003).

In veterinary medicine, one of the driving forces to study drug metabolism across different animal species, is the need to identify and describe depletion of residues in tissues of farms animals intended for human consumption (Fink-Gremmels, 2008). Until

now, limited information about DMEs expression, regulation and catalytic activity are available for veterinary species, compared to human and rodent model species. In order to safely develop therapeutic agents for use in food-producing animals and limits the presence of harmful residues for consumers, it is therefore essential to know the nature of enzymes involved in xenobiotic metabolism pathways (Ioannides, 2006).

2 Background

2.1 Transcriptional regulation of genes involved in drug metabolism

Pharmacogenomics is the study of the impact of genetic variation on drug effects, with the ultimate goal of achieving a "personalized medicine". Since the completion of the Human Genome Project, great strides have been made towards the goal of personalized dosing of drugs in people, as exemplified by the development of gene-guided dosing of the anticoagulant drug warfarin. Although the pharmacogenomics of domestic animals is still at an early stage of development, there is great potential for advances in the coming years as the direct result of complete genome sequences currently being derived for many of the species of significance to veterinary and comparative medicine. This sequence information is being used to discover sequence variants in candidate genes associated with altered drug response, as well as to develop whole genome high density single nucleotide polymorphism arrays for genotype–phenotype linkage analysis (Mosher & Court, 2010).

Transcriptional regulation has a pivotal role in the overall control of gene expression and down-stream processes in every organism (Georgitsi et al., 2011). Differential gene expression of phase I and II enzymes and drug transporters (DTs, involved in phase 0 and III of drug metabolism) can be related to variabilityin drug responses noticed in human population (Glubb & Innocenti, 2010). Transcriptional expression, leading to protein abundance, is in part determined by genetic elements and by the complex control involving the action of transcription factors (see Figure 1.; Köhle & Bock, 2009). Nuclear receptors belong to a large family of structural related, ligand-activated transcriptional regulators that includes vitamin D, thyroid, retinoid, steroid, and orphan receptors. They play a fundamental role not only in the recognition of xenobiotics and endogenous molecules, but also on development process and adult physiology. Protein organization of NR family members presents common features: the highly conserved DNA binding domain (DBD) which contains two zinc finger motifs, involved in receptor dimerization and in the binding of specific DNA sequences, the carboxyterminal region of nuclear receptors includes the ligand binding domain (LBD). The LBD serves as a docking site for ligands, co-regulator recognition, dimerization and contains a AF-2 transactivation region that is necessary for inducing transcription of target genes. The N-terminal region of some nuclear receptors also contains an activation domain called AF-1 that is ligand independent and can interact with the AF-2 domain (Akiyama & Gonzalez, 2003). The general activation pathway for NRs activation involves an interaction between the receptor and a specific ligand, the ligand binding induces a conformational change on the

NR allowing it to dimerize, producing both homodimers and heterodimers, and to traslocate into nucleus where specific hormone response elements present in DNA are recognized (di Masi et al., 2009).

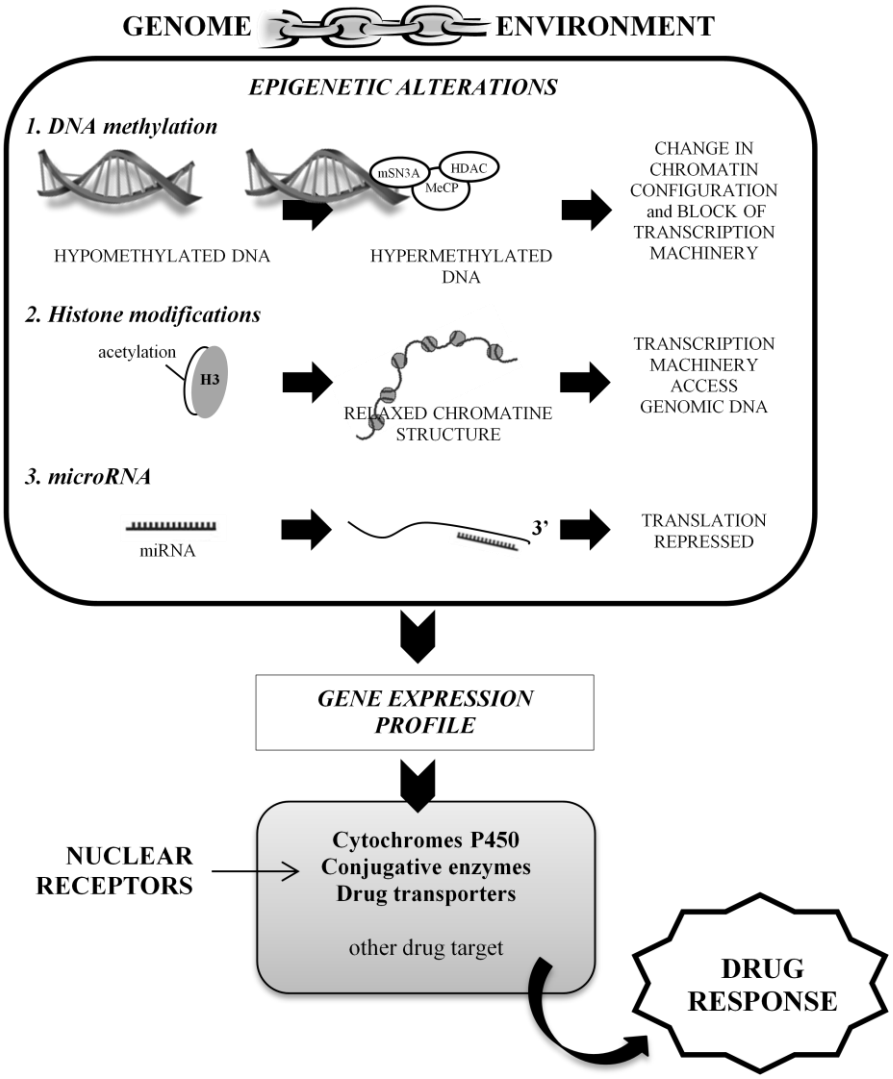

Figure 1: Transcriptional regulation of genes involved on drug response. Modified from Baer-Dubowska et al. (2011).

2.2 Pharmacokinetics: an overview on species differences

Basically, pharmacokinetics (PK) comprehends the following steps:

- **A**bsorption, leading the xenobiotic entry into the body systemic circulation;
- **D**istribution into the various tissues of the body;
- **M**etabolism, in which lipophilic substances are converted into more water-soluble metabolites that can be excreted from the body;
- **E**xcretion through renal or biliary route.

Commonly, we refer to these steps by using the ADME acronym. Humans and other animals are daily exposed to many xenobiotics. To achieve its effect, whether therapeutic

or toxic, a xenobiotic and/or its metabolites must be present in appropriate concentrations at sites of action. In addition to classical processes influencing xenobiotic concentration, namely dose, formulation and route of administration, rate and extent of absorption, distribution and binding to tissues, biotransformation and excretion, nowadays growing importance is actually attributed to DTs (Caldwell et al., 1995; Scherrmann, 2009). Transporters are ubiquitous within cells of all mammalian organs, including intestine, liver, and kidney. In these organs they play a key role in the absorption, facilitating the movement of solutes into cells, i.e. influx (import) transporters, and in the elimination of substances from the cytosol of cells through efflux (export) transporters (Scherrmann, 2009).

Species-differences in drug response can be explained either by variation in PK or pharmacodynamics (PD, essentially the xenobiotic mechanism of action), the magnitude of which varies from drug to drug. In light of this, the main challenge for veterinarians is not to select a drug but determinea rational dosage regimen; in fact, a drug dosage regimen in a given species may depend upon its anatomy, biochemistry, physiology and behavior as well as on the nature and causes of the condition requiring treatment. Causes and examples of interspecies differences in PK are abundant (see some examples in Table 1) and reflect species differences in physiological processes involved in the handling of drugs. These differences are most common and of greatest magnitude whenever functions phylogenetically divergent between species, such as digestive functions (ruminant *vs* non-ruminant), are involved in drug absorption and metabolism. On the other hand, species-differences in drug action and effect (PD), reflecting differences in target organ functions (anatomy, physiology, pathology) and/or target receptors are less known and not so well documented (Toutain et al., 2010).

As a whole, the presence of such numerous and often unpredictable species-differences don’t allow generalizations and valuable interspecies extrapolations (i.e. prediction of biotransformation in sheep from cattle data or for avian species from poultry). A more comprehensive view of interspecies differences, also between breeds with existing polymorphism, is needed. Thus, recent and future advances in molecular biology and pharmacogenetics will allow the investigation of each candidate drug in a species-specific manner, to guarantee its effective and safe use, ensuring the well-being of animals and safeguarding of the environment and human consumption of animal products (Toutain et al., 2010).

Process	Organ	Interspecies differences
Absorption	Stomach and intestine	<u>Monogastric species</u> Dogs (carnivorous monogastric) possess a relatively simple colon but well developed small intestine (long villi; Martinez et al., In horse and rabbit antibiotics administered by an oral route, having a poor bioavilability and extensively excreted in the bile or by enterocyte efflux after parenteral administration should not be used. The microbial digestion of these species, occurring mainly in ceacum and colon, can be compromised (Toutain et al., 2010). <u>Poligastric species (ruminants)</u> The reticolo-rumen (RR) due to its large capacity (100 - 225L in cattle and 10 24L in sheep and goat) leads to dilution and affects the residence time of orally administered drug (Toutain et al., 2010). The microflora of the rumen can inactivate drugs by metabolic or chemical reactions and a potential site for ion trapping due to its strict pH range (Martinez et al., 2002). For drugs not absorbed in the rumen and having short half-life the passage through the reticulo-omasal orifice is a problem: from 6 to 15 hours (hrs) are needed for a drug in solution in the liquid phase of the ruminal content and even 50-60 hours if a drug is strongly bound to cellulose (Toutain et al., 2010).
Metabolism	Liver	Horse had the lowest catalytic ability to demethylate (and hence detoxify) monensin among pigs, broiler chickens, cattle and rats (Nebbia et al., 2001). Faster clearance of drugs in herbivores, i.e. salicylic acid (SA): less than one hr in herbivores (cattle and horse); from 3 to 6 hrs in omnivores (man and pigs); up to 9 hrs in dogs; from 22 to 48 hrs in cat due to a deficit in glucurono-conjugation (Toutain et al., 2010). Rabbits pigs and horses show the maximal glucuronidation capacity toward phenolic substrates, while broilers and cattle display a relatively low conjugation rate (Toutain et al., 2010).
Excretion	Kidney	Differences on urinary pH determined mainly by the composition of the diet (alkaline in herbivorous species and acidic urinary pH in carnivores) can be relevant to overall drug elimination via renal clearance. As an example in the alkaline urine of herbivores, the SA conjugate is wholly in the ionized form and cannot be re-absorbed by the nephron (Toutain et al., 2010).

Table 1: Examples of interspecies differences affecting the absorption, metabolism and excretion of drugs.

2.3 From phase 0 to phase III of human metabolism

Classical drug metabolism was earlier divided into phase I and phase II. Nowadays it has been realized that two additional steps, called phase 0 and phase III of drug disposition, are just as important as the previously known processes. Both phases involve the modulation of the cellular entry and exit, respectively, of either the unmodified or metabolized compounds. In phase 0, a large number of more-or-less selective transporters can increase or decrease the cellular entry of various compounds before they would reach the intracellular compartments or cross tissue barriers. In fact, this mechanism results in a significant modulation of pharmacological effects, by not allowing, or greatly augmenting drug interactions with intracellular targets. The term phase III was introduced for the process of efficient elimination of the already detoxified molecules, also carried out by multiple transporters (Szakács et al., 2008).

2.3.1 Phase 0: uptake transporters

The first group of DTs includes the so-called 'uptake transporters', which are multispecific solute carrier (SLC) transporters, facilitating the cellular entry or exit of a wide range of compounds, without a direct ATP hydrolysis. The current classification of these uptake transporters distinguishes transporters for organic anions and organic cations. The SLCO gene family encodes for the organic anion transporting polypeptides (OATPs). The SLC22 gene family encodes for the organic cation transporters (OCTs), the organic cation/carnitine transporters (OCTNs), and the organic anion transporters (OATs). Some of these transporters perform obligatory exchange of organic compounds (i.e. OAT3), while in others transport is modulated and/or driven by monovalent ions and the membrane potential (i.e. OCTN, Szakács et al., 2008).

The substrates of OATPs include bile salts, steroid conjugates, thyroid hormones, anionic oligopeptides and several therapeutic drugs and other xenobiotics. Through the selective expression in the apical and basolateral membranes of polarized cells in tissues such as liver, kidneys, the intestinal wall and the blood–brain barrier, OATP transporters may significantly affect the PK and the effects of their substrate drugs (Niemi, 2007). Likewise to DMEs, uptake DTs have been shown to be regulated by NRs in healthy and diseased tissues. Induction of OATP1A2, which contains a pregnane X receptor (PXR, coding gene NR1I2) response element in its promoter, was shown in human breast cancer cell lines after the exposure to the PXR agonist rifampicin (Svoboda et al., 2011). Following the activation of liver X receptor (LXR, NR1H3) and farnesoid X receptor (FXR, NR1H4) but neither of PXR nor the constitutive androstane receptor (CAR, NR1I3) the OATP1B1 gene is induced in cancer cell lines and isolated primary human

hepatocytes (Svoboda et al., 2011). In precision-cut slices from human liver, a down-regulation of OATP1B3 was noticed after the exposure to RIF, while phenobarbital (PB) increased its expression. Furthermore, the hepatocyte nuclear factor 1 alpha (HNF1α) has been shown to be of importance for the hepatic expression of OATP1B1. Further studies made in liver slices showed that a high bile acid concentrations down-regulated HNF4α, the HNF4-dependent HNF1α and OATP1B1 genes (Svoboda et al., 2011). In human hepatocytes, OATP1B3 and OATP2B1 gene expression can be inhibited by some activators of CAR and aryl hydrocarbon receptor (AhR) such as PB and 2,3,7,8-tetrachlorodibenzo-p-dioxin (TCDD), respectively; in contrast, OATP1B1 is induced by RIF and TCDD (Svoboda et al., 2011). The activation of drug-sensing receptors AhR, CAR, PXR, and the nuclear factor-E2 related factor-2 (Nrf-2) by their respective ligands changed the expression of a number of drug transporters (DTs) in the hepatocyte. In particular, PB (acting through CAR) effectively decreased OAT2 expression whereas the activation of other receptors had smaller effects (Rizwan & Burckhardt, 2007).

2.3.2 Phase I and II: cytochromes P450 and conjugative enzymes

Cytochromes P450 are a gene superfamily of heme proteinsencoding for the principal enzymatic system involved in the metabolism of lipophilic xenobiotics. In August 2009, the CYP superfamily consisted of more of 11.000 named sequences in animals, plants, fungi, protists, bacteria and viruses (http://drnelson.uthsc.edu/P450.statsfile.html). These DMEs are also important in the oxidative, peroxidative, and reductive metabolism of numerous endogenous compounds including steroids, bile acids, fatty acids, prostaglandins, leukotrienes, biogenic amines, and retinoids. Together with dehydrogenases, reductases, and oxidases, CYPs catalyze phase I reactions (Handschin & Meyer, 2003). Cytochrome P450 enzymes are found in practically all tissues, with highest abundance and largest number of individual CYP forms present in the liver but also in the intestine, lung, kidney, brain, adrenals, gonads, heart, nasal and tracheal mucosa, and skin. The human genome has 57 CYP genes and ifteen individual CYPs, belonging to families 1-3 metabolize xenobiotics, including the majority of drugs currently in use (Pelkonen et al., 2008). Functional groups deriving from phase I reactions are considered as reactive groups by phase II DMEs for further modifications. Phase II reactions consist mainly of glucuronidation, sulfation, attachment of glutathione, methylation, *N*-acetylation, or conjugation with amino acids. In addition, esterases, amidases, imidases, epoxide hydrolases, or other hydrolytic processes are likely to increase the xenobiotic hydrophilicity (Handschin & Meyer, 2003). The complex network of NRs shown to be involved in the regulation of DTs, contribute to the expression and regulation phenomena

of the biotransformation process, ultimately to adjust the organism to the requirements of the chemical environment (Pelkonen et al., 2008).

2.3.2.1 Pregnane X receptor

The pregnane X receptor is a key transcription factor for endobiotic and xenobiotic metabolism in humans, mice and rats. It is expressed primarily in liver, intestine and kidney but also in other tissues such as lung, stomach, uterus, ovary, placenta, breast, heart, adrenal gland, bone marrow and specific regions of the brain (di Masi et al., 2009). The pregnane X receptor resides in the cytosol in a multi-protein complex containing CAR cytoplasmic retention protein (CCRP) and the heat shock protein (Hsp) 90. After the ligand recognition, PXR dissociates from the multi-protein complex and traslocates into the nucleus, where dimerization with the retinoid X receptor α (RXRα, NR2B1) and co-activators recruitment activates target gene transcription (see Figure 2). Co-activators recruit a group of histone acetyltransferase or methyltransferase, which may be involved in chromatin relaxation and association with the basic transcriptional machinery; by contrast, co-repressors such as the nuclear co-repressor (NCoR) and the silencing mediator for retinoid and thyroid hormone receptor (SMRT), bind to inactivated PXR receptor and recruit various forms of histone de-acetylases leading to chromatin condensation and repression of gene expression (Lim and Huang, 2008). Pregnane X receptor was at first demonstrated to regulate CYP3A gene, and then it was shown to contribute to regulation of other inducible CYPs such as CYP2Bs, 2Cs, 2A6, 4F12 and 7A1 (Tolson and Wang, 2010). Among conjugative enzymes transcriptionally regulated by PXR, the uridine-5'-diphosphate glucuronosyl transferase (UGT) 1A1 contain a PXR responsive element and UGT1A3, 1A4, 1A6 and 1A9 were increased by PXR activation, even if exact DNA responsive elements has not been yet identified. The bile acid sulfotransferase SULT2A1 was the first SULT enzyme being identified as regulatory tarhet of PXR (Tolson and Wang, 2010). Finally, other outstanding conjugation enzymes, such as glutathione S-transferases (GSTs) α, π and μ classes are under positive control of PXR in a tissue- and sex-specific manner (Tolson and Wang, 2010).

2.3.2.2 Constitutive androstane receptor

CAR is highly expressed in the liver and in the epithelial cells of the small intestine, but also, at low levels, in mouse and human heart, skeletal muscle, brain, kidney and in human lung (di Masi et al., 2009). The two closely related nuclear receptors, PXR and CAR, are implicated in mediating the effects of xenobiotics on CYP2B and CYP3A gene expression. In particular, CAR and PXR share a significant cross-talk in target gene recognition by binding to similar xenobiotic responsive elements and in their xenobiotic

activators. However, a discrepancy still exists and needs some discrimination. For example, PB and 5b-pregnane-3,20-dione (a biologically active metabolite of plasma progesterone) are activators of both CAR and PXR. On the other hand, clotrimazole activates human PXR, but it is an efficacious deactivator of CAR (Lim & Huang, 2008). Furthermore, CAR displays unique activation mechanisms compared to PXR and other orphan receptors, involving both direct ligand binding and indirect ligand-independent pathways (Tolson & Wang, 2010). As regards to CAR activation mechanism, similarly to what previously reported for PXR, this NR in absence of a stimulation is retained in the cytoplasm by a multi-component complex including Hsp90, CCRP, protein phosphatase 1, regulatory subunit 16A and phosphatase 2A (see Figure 2). Consequently to the direct binding of a ligand, such as 3,5-dichloro-2-[4-(3,5-dichloropyridin-2-yl)oxyphenoxy]pyridine (TCPOBOP) for mouse CAR, or a ligand-independent activation operated by PB or bilirubin, CAR translocates and accumulates in the nucleus assisted by the p160 transcription factor and the glucocorticoid receptor (GR) -interacting protein 1 (GRIP1). Translocation of CAR into the nucleus is followed by CAR/RXRα heterodimerization, CAR-DBD binding to the response element present in the target gene promoters and the recruitment of co-activators (GRIP1, peroxisome proliferative activated receptor, PPAR γ, coactivator 1 (PGC1) α, and nuclear receptor coactivator 1, SRC1; di Masi et al., 2009).

It has been understood that CAR governs not only CYP2Bs through PB-responsive enhancer module (PBREM; Honkakoski et al., 1998), but also other DMEs including human CYP1A1 and 1A2 (showing a CAR binding site located in the 5'-flanking region: (Yoshinari et al., 2010), CYP2A6, 3A4, 2C9 and 2H (Itoh et al., 2006; Goodwin et al., 2001; Sueyoshi et al., 1999; Gerbal-Chaloin et al., 2002; Handschin & Meyer, 2000). Among glucuronidation enzymes reported to be potential CAR target genes, UGT1A1 was the first in which a distal PBREM was identified (Sugatani et al., 2001) and, in general, CAR activation increases the major pathway of bilirubin clearance by inducing UGT1A1, MRP2, OATP2, GSTA1 and A2 gene expression (Qatanani et al., 2005).

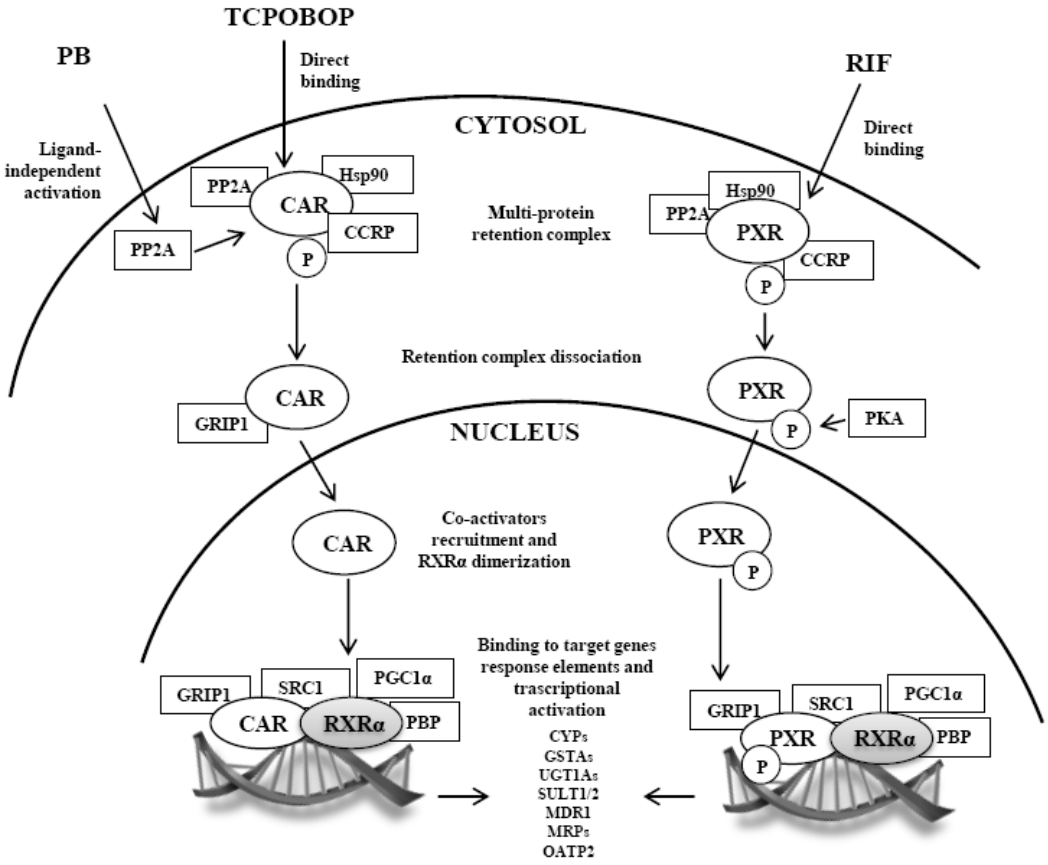

Figure 2: Proposed activation mechanism for the mammalian constitutive androstane receptor (CAR) and pregnane X receptor (PXR) arose from in vitro studies. Normally, both nuclear receptors are retained in the cytoplasm compartment through a multi-protein complex; after ligand binding or activation, releasing from the retention complex and intermediate events lead to the nuclear translocation. Subsequentially, CAR and PXR heterodimerize with retinoid X receptor α (RXRα), bind to their respective response elements and increase transcription of target genes. Modified from di Masi et al., (2009).

2.3.2.3 Hepatocyte nuclear factor 4α

Hepatocyte nuclear factor 4α is mostly expressed in the liver, gut and kidney. It is localized in the nucleus of hepatocytes and binds as a homodimer to its DNA recognition site, a direct repeat element (AGGTCA) with either a one- or two nucleotide spacer (DR-1 or DR-2, respectively; Jover et al, 2009). This NR is fundamental for the constitutive and inducible expression of many hepatic CYP genes. Cytochrome P450 2C9 was the first human CYP whose expression was found to be regulated by HNF4α; today, many other CYP genes, including CYP2A6, 2B6, 2C8, 2C9, 2C19, 2D6, 3A4 and 3A5, have been identified as HNF4α targets. In particular, HNF4a is required for both PXR- and CAR-mediated transcriptional activation of CYP3A4. Furthermore, when an HNF4a binding site in the CYP3A4 enhancer region is mutated, both PXR and CAR fail to

activate the CYP3A4 promoter. Such a NR plays an important role also in regulating the basal expression of some phase II enzymes, such as SULT2A1. The expression of the UGT1A6 and 1A9 genes were found to be positively correlated with HNF4α and HNF1α expression in human liver; moreover, functional HNF4α and HNF1α binding sites were identified in the promoter regions of both UGT genes.

2.3.2.4 Glucocorticoid receptor α

The actions of glucocorticoids (cortisol is the major glucocorticoid in humans) are mediated through a 94 kDa intracellular receptor, the GR, which is a member of the nuclear receptor superfamily. In the absence of a ligand, the GR forms an inactive multiprotein complex in the cytoplasm, containing chaperone Hsp 90, 70 and 50, immunophilins, as well as other proteins. Once glucocorticoid binds to GR, the activated receptor undergoes a conformational change that results in dissociation from this multi-protein complex and rapid translocation to the nucleus. Within the nucleus, the receptor binds as a homodimer to glucocorticoid response elements (GREs) in the promoter region of target genes, and regulates positively or negatively their expression depending both on the GRE sequence and the promoter context (Nicolaides et al., 2010; Taniguchi-Yanai et al., 2010). Following the transcriptional activation or inhibition of glucocorticoid-responsive genes, GR dissociates from the ligand and shows a lower affinity for binding to GREs. It remains within the nucleus for a considerable length of time and is then exported to the cytoplasm. Either within the nucleus than in the cytoplasm, GR may be recycled and/or degraded bythe proteasome (Nicolaides et al., 2010).

An indirect role of human GR in CYP2B6 induction was hypothesized for the ability of DEX-activated GR to increase expression of PXR and CAR in human hepatocytes. Further potential mechanisms underlying this interaction might include a GR-mediated recruitment of specific co-activator proteins or as enhancer of PXR and CAR binding to the CYP2B6 PBREM and xenobiotic responsive enhancer module (XREM) via protein-protein interactions (Wang & Tompkins, 2008). This NR induce human CYP2C subfamily through homologous GREs identified in the 5'-flanking region of CYP2C8, 2C9 and 2C19. With regard to CYP3A, dexamethasone (DEX) induces CYP3A by a biphasic mechanism: at low DEX concentration (nanomolar) GR indirectly controls the induction by increasing the expression of PXR, CAR and RXRα, while, at supramicromolar DEX concentrations only PXR is involved in CYP3A4 induction (Pascussi et al., 2002). Within conjugation enzymes UGT1A1, SULT1A3 are considered glucocorticoid-responsive genes. In this respect, at submicromolar DEX concentrations

GR directly binds to GREs in their regulatory region (Sugatani et al., 2005; Bian et al., 2007).

2.3.3 Phase III: efflux transporters

Drug transporters are constitutively expressed in several organs with an important barrier function, and efflux pumps are determinant for the absorption, distribution, and excretion of xenobiotics and their metabolites. In intestinal cells, the action of two ABC-transporters, namely P-glycoprotein (P-gp; coding gene ABCB1) and breast cancer resistance protein (BCRP; ABCG2) leads to a decreased drug concentration in the liver. In addition, drug distribution to the brain is hampered by P-gp, multidrug resistance associated protein 1 and 2 (MRP1/2; ABCC1/2), MRP4 (ABCC4), and BCRP, expressed at the blood-brain barrier level (Huls et al., 2009). Excretion of drugs or their derivatives into the bile is also affected by P-gp, bile salt export pump (BSEP; ABCB11) and MRP2; on the other hand, their passage in the systemic circulation depends on MRP1/3 expression and function. More water-soluble drugs can be excreted into urine by the kidney, where ABC transporters (P-gp, MRP2/4 and BCRP) are localized along the apical membrane of renal proximal convoluted tubule cells (Huls et al., 2009).

Although most of their substrates are hydrophobic, MDR–ABC pumps are also capable to extrude also a variety of amphipathic anions and cations. Whereas P-gp substrates are essentially unmodified neutral or positively charged hydrophobic compounds, those of ABCC subfamily members (the MRPs) and ABCG2 comprehend also organic anions and phase II metabolic products. This synergism between the efflux systems and the metabolizing/conjugating enzymes provides a very efficient network for drug elimination (Szakács et al., 2008).

The expression of these canalicular DTs are subject to transcriptional regulation, and NRs are master regulators of ABC-transporters expression, while post-transcriptional regulation is usually dependent from endogenous and exogenous compounds and different pathologic situations. The constitutive androstane receptor and PXR regulates several DTs: the former participates in regulation of MRP2, 3, and 4 transcription, while PXR influence MDR1, MRP2 and 3 gene expression(Faber et al., 2003; Mottino & Catania, 2008). Moreover, a down-regulation of RXRα can affect the expression of a number of DTs, owing to its heterodimerizing role for most of NR proteins. Other transcription factors, such as FXR and HNF1α are regulators of several uptake transporter gene. Farnesoid X receptor acts as a bile salt sensor in the liver and its activation leads to enhanced ABCB11, SLCO1B3 and SHP-1 genes expression (this latter one in turn

represses the transcription of sodium/bile acid cotransporter: NTCP, SLC10A1 gene), while human SLCO1B1 and 1B3 are responsive to HNF1α (Faber et al., 2003).

2.4 Species-differences in drug metabolism: focus on veterinary species

As mentioned above, CYPs are considered the most important DMEs. Despite the common function, significant species-differences exist in the level of individual CYP expression and substrate specificity, even in phylogenetically related animal species (Ioannides et al., 2006). Recently, the NR-dependent activation has been recognized to be the molecular basis of species-differences in the induction of hepatic CYP forms. For example, differences between both CAR and PXR activators are attributable to species-differences in the LBD of these two NRs (Graham & Lake, 2008). In human and laboratory animals extensive data are available on CYP expression, regulation and function, while in veterinary species similar information are still incomplete. As an example, enzyme substrates were selected in the basis of the available knowledge from rodent or human studies, but enzyme velocity has not been characterized in the individual target animals, and enzyme kinetic data have been determined only in very few studies (Fink-Gremmels, 2008). Despite the presence of phase II conjugative enzymes has been reported in several veterinary species such as horse, pig, goat and cattle, even less data compared to phase I enzymes are available on their characterization. A similar situation is pointed out for DTs for which a lack of systematic data regarding the physiological levels in animal tissues and their transcriptional regulation is recorded (Fink-Gremmels, 2008; Fink-Gremmels, 2010; Mosher & Court, 2010). In the following paragraphs, species-differences in DMEs and DTs expression and transcriptional regulation recorded among most important veterinary species, with particular interest toward cattle and pig, are reviewed, ones.

2.4.1 Species differences in CYPs expression

Cattle liver constitutively express multiple CYP mRNAs (Giantin et al., 2010). In extrahepatic tissues, CYPs expression is associated with a specific physiological role; nevertheless, their modulation may affect the local disposition of xenobiotic or endogenous compounds, thus altering their effects (Pavek & Dvorak, 2008; Seliskar & Rozman, 2007). In gastrointestinal tract of neonatal calves, CYP2A6, 2C8 and CYP2E1 are not expressed in jejunum and colon (Krüger et al., 2005). Cattle coronary tissue express CYP1A, 2C, 2E and CYP2J mRNAs, with a potential role in vasoregulation (Grasso et al., 2005). In testis, in which steroidogenesis occurs CYPs can be clustered in two subsets: steroidogenic CYPs and CYPs involved in xenobiotic metabolism. The presence of mRNA coding for DMEs has been recently demonstrated, although to a lower

extent if compared to liver (Lopparelli et al., 2010). With regard to NRs, measurable mRNA amounts of some of them (peroxisome preliferator-activated receptor α, PPARα; PXR, CAR, RXRα) have been measured in cattle testis (Lopparelli et al., 2010), thereby confirming the significant role played by CYPs and NRs in testicular toxicity. On the other hand, PPARα mRNA was not detected in jejunum and colon of neonatal calves (Krüger et al., 2005). Apart from the liver, few data about CYPs expression in extrahepatic tissues have been published in cattle so far.

Breed-differences in CYP gene expression profiles have been only recently become subject of interest. Differences in CYP1A1, 1A2, 2B22, 2C87, 2C88 and CYP3A28 mRNAs were noticed between Charloais, Piedmontese and Blonde d'Aquitaine cattle; furthermore, CYP2B22 and CYP3A28 mRNA results were confirmed at the protein level, too (Giantin et al., 2008). In a more recent study, Angus pregnant cows appeared to have higher CYP2B22 and 2E1 mRNA amounts compared to Simmental ones (Ashwell et al., 2011). Thus, breed might effectively affect CYP gene expression in cattle, albeit further confirmatory molecular studies are needed.

Post-translational changes in DMEs occur during development in humans (Hines et al., 2007), but knowledge about CYPs expression and regulation phenomena occurring during maturation remains incomplete. In cattle, hepatic CYP2C8-, 3A4-*like*, CAR and PXR mRNAs were increased with age in pre-term, full-term calves immediately after birth, full-term calves 5-days old, and veal calves at the age of 159 days; in contrast, waving results were obtained for RXRα (Greger et al., 2006).

Sex-based differences in hepatic drug metabolism have been demonstrated in humans (Waxman & Halloway, 2009) and cattle (only at the protein level; Dacasto et al., 2005). Only one transcriptional study has been published so far, in which Angus heifer calved showed higher amounts of CYP3A28 mRNA than Angus steers (Ashwell et al., 2011).

Over the last decade, an increasing amount of mechanistic information has emerged on the altered regulation and function of CYP genes by dietary constituents (Murray, 2006). Such an argument has gained a recent increasing interest in cattle husbandry, too. Retinods are essential nutrients and contribute to important physiological processes; differences in hepatic relative abundance of CYP2E1 and 2C8 have been pointed out in male calved fed either with a formula deficient in vitamin A, a formula supplemented with vitamin A or colostrums. Furthermore, a different pattern of CYP2B22 and RXRα gene expression was observed in the colon (Krüger et al., 2005). The nutritional status has been shown to affect lactating dairy cows reproductive performances; cows either provided a gluconeogenic feedstuff or treatment with insulin showed lower amounts of CYP3A28 and 2C31 mRNAs (Lemley et al., 2008). Similarly, CYP2C31 gene expression

was decreased in cows fed a high cornstarch diet compared with cows fed a higher fiber diet (Lemley et al., 2010).

Cythochromes P450 can be either induced or inhibited by xenobiotics, in most cases through NR-mediated mechanisms (Graham & Lake, 2008). Cattle primary cultures exposed for 24 hrs to dioxin, dioxin-*like* compounds and extracts of fish oil used as feed ingredients for animal feed showed a dose dependent up-regulation of both CYP1A1 and 1B1 mRNAs (Guruge et al., 2009). Veal calves administered with DEX showed a reduction of hepatic CAR and PXR mRNA levels (Greger & Blum, 2007). Steroids and corticosteroids are often illicitly used in cattle husbandry, and their effects upon CYP genes has recently become subject of investigation. In the veal calf, CYP3A28 gene expression was inhibited to a varying extent following the administration of DEX illicit schedules (Cantiello et al., 2009). Likewise, in beef cattle liver, a common CYP2B22 and 2E1 down-regulation was noticed following the use of two illicit protocols containing DEX or DEX plus 17β-estradiol (17βE). In contrast, CYP3A28, CAR and RXRα mRNAs were up-regulated with DEX alone (Giantin et al., 2010), and an increase of CYP1A1, 2E1 and PPARα mRNA abundances was observed in testes of DEX plus 17βE-treated cattle (Lopparelli et al., 2010). Altogether, these results would confirm that species-differences exist in CYPs expression and regulation as well as in xenobiotic response.

The interest in biotransformation processes of pig species is not only related to veterinary medicine. Pig is in fact considered a good model in biomedical research because of its anatomical, physiological, and biochemical similarity to humans (Bode et al., 2010). Recently, Puccinelli and colleagues (2011) exhaustively reviewed not only the tissue distribution, substrates and inhibitors, inducibiliy and regulation of CYPs 1-4 families and related key NRs (AhR, CAR, PXR, PPARα and HNF4α). Liver express constitutively up to 15 CYP forms and abovementioned transcription factors. As regards to extra-hepatic tissues, kidney was shown to express the greater number of CYP and NR mRNAs, although to lower extent compared to liver; furthermore, CYP genes were found in lung, intestine, respiratory and olfactory nasal mucosa, brain and heart. Thanking to cloning and expression in a recombinant system, actually the following recombinant porcine CYPs are available: CYP1A1, 1A2, 2A19, 2C33, 2C49, 2D21, 2D35, 2E1, 3A29, 4A21 and 4A24/25).

Noteworthy, when comparing data from pigs with human ones, it's important to discriminate between conventional (farm) animals and special breeds such as the Göttinger, Bama and Yucatan minipigs. Minipigs contain the highest total CYP450 activity (0.81 nmol/mg microsomal protein), whereas different strains of conventional pigs has an activity of 0.46-0.57 nmol/mg protein, values more close to the total human

liver CYP content of 0.43 nmol/mg protein (Fink-Gremmels, 2008). However, the primary structure of CYP enzymes in conventional pig and minipigs is not expected to differ significantly. Porcine CYP3A29, likewise to CYP3A4 in humans, gives the greatest contribution of CYP3A activity in hepatic microsomes (Puccinelli et al., 2011). Sex-based differences were observed for CYP1A2 and 2A activity that were particularly high in female minipigs, additionally CYP2E activity was higher in female of both, conventional and minipigs (Fink-Gremmels, 2008). Generally investigation on pig CYPs inducibility were conducted with liver or intestine microsomes, which were used to measure the conversion of known model substrates for individual CYP-mediated reactions; less studies are available for extra-hepatic pig tissues which required an *in vivo* approach. Table 2 summarizes findings obtained at the transcriptional level in pigs

Very little is known about the regulation of porcine CYP genes, and there is a lack of knowledge about regulatory response elements in CYPs promoters. The constitutive androstane receptor has been demonstratedto be involved *in vitro* in pig CYP2As regulation, showing similarities with human CAR-regulated CYPs. In the olfactory and respiratory porcine nasal mucosa, the lack of induction of CYP2B22 following the *in vivo* treatment with RIF, β-naphthoflavone (βNF) and PB has been hypothesized to be due to the scarce expression of CAR and PXR NRs nor to a more complex tissue-specific regulation. With regard to the regulation mechanisms of swine CYP2Cs, very little is known. It has been suggested that the PB induction of pig CYP2Cs is primarily due to a tissue-specific activation of CAR, PXR, and HNF4α, likewise to humans. Phenobarbital has been demonstrated to suppress CYP2D25 activity via a direct enzyme inhibition and transcriptionally down-regulating the gene promoter via a mechanism involving PXR or CAR. Finally, few information are available on CYP3As regulation; pig liver express NRs involved in human CYP3A genes induction (PXR, CAR and HNF4α) and mechanisms involved in CYP3A induction seem to be similar to human ones (Puccinelli et al., 2011).

In sheep liver and intestinal mucosa the expression of CYP3A28-*like* as well as of GRα, CAR, PXR and RXRα transcription factors has been recently measured following DEX administration; the glucocorticoid induced hepatic CYP3A28-*like* gene, apoprotein and related catalytic activity. By contrast, in jejunum and colon DEX significantly increased CYP3A28-*like* only at the posttranslational level (Maté et al., 2011).

In horses CYP3A mRNA was present in the duodenum and constantly decreased in jejunum, ileum, cecum, and colon. The amount of CYP3A mRNA in the liver was about the same noticed in the anterior part of the jejunum, but about 4.5 times lower than in the

anterior part of the duodenum. All of this is in contrast with humans, in which CYP3A shows higher mRNA amounts in the liver compared to the intestine (Tydén et al., 2004).
In 2008, 2009, and 2010 several equine CYP enzyme isoforms, named CYP2D50, 2C92, 3A89, 396 and 3A97 were, for the first time, sequenced and their activities compared to human CYP2C, 2D and 3A isoforms. There are some reports of sexual dimorphism of drug metabolism in horses and there is also evidence for an age-dependent increase in catalytic activity of many phase I and II DMEs (Scarth et al., 2010).
Recently, the tissue distribution of feline CYP2D6 and two novel hepatic CYP3A homologues, CYP3A131 and CYP3A132, were characterized. The former gene was found predominantly in liver and scarce amounts were detected in testis and brain, heart, kidney and ovary (Komatsu et al., 2010). Messenger RNA expression of both feline CYP3As was found in several tissues, including brain (cerebrum), heart, lung, liver, small intestine, and kidney. Among the six tissues addressed, feline CYP3A131 was expressed predominantly in liver and small intestine, while much lower amounts were detected in brain. It was poorly expressed in other tissues. A low expression of CYP3A132 was observed in liver; moreover, CYP3A132 expression in male liver did not differ from female one (Honda et al., 2011). Cytochromes P4501A1 and 1A2 cDNAs were cloned and CYP1A1 protein sequence homology was estimated to be 71, 72, 68, 66 and 67% with dog, pig, human, rat and mouse sequences, respectively; even CYP2E1 was cloned and apparently three distinct isoenzymes have been identified, of which two (CYP2E-b and CYP2E-c) mostly in the liver, whereas CYP2E-a was found in various tissue including liver, kidney, lung, stomach and intestine (Fink-Gremmels, 2008).
Dogs possess several unique CYP isoenzymes such as a canine form of CYP1/2, CYP2B11, CYP2C31 and 2C41, 2D15, 3A12 and 3A26 (Fink-Gremmels, 2008). The most prominent differences between dogs and other species exist in the CYP2B family; in fact, CYP2B11 is unique in dogs and comprise approximately 20% of total hepatic CYPs. As regards to CYP3A expression in dog, higher mRNA levels are usually found in the liver than in the duodenum. The hepatic expression of CYP3A26 is greater than CYP3A12, with CYP3A26 comprising 75.2% of the total hepatic CYP3A mRNAs. Conversely, duodenal expression of CYP3A12 accounts for comprising 99.8% of duodenal CYP3A mRNAs (Mealey et al., 2008). No sex-differences in the expression or protein amount of CYP3A12, CYP2B11 and CYP2C21 were found in dog liver (Graham et al., 2006).

CYP subfamily	Inducers	Tissue	Study features
CYP1A1	β-naphtoflavone	liver lung, heart, kidney	primary hepatocytes *in vivo*
CYP1B1	β-naphtoflavone	liver midbrain, capillaires	primary hepatocytes *in vivo*
CYP2B22	phenobarbital rifampicin	liver, small intestine liver, kidney	primary hepatocytes, *in vivo* *in vivo*
CYP2C33	phenobarbital	liver, small intestine	*in vivo*
CYP2C42	phenobarbital	liver, small intestine, kidney	*in vivo*
CYP2C49	phenobarbital	liver, small intestine, kidney	*in vivo*
CYP2E1	phenobarbital	liver	*in vivo*
CYP3A22	rifampicin	liver, intestine	*in vivo*, primary hepatocytes and enterocytes
	dexamethasone	liver, intestine	primary hepatocytes and enterocytes
	phenobarbital	liver, intestine	*in vivo*, primary hepatocytes and enterocytes
CYP3A29	rifampicin	liver, intestine, cerebellum, midbrain, blood-brain interfaces	*in vivo*, primary hepatocytes and enterocytes
	dexamethasone	liver, intestine	primary hepatocytes and enterocytes
	phenobarbital	liver, intestine	*in vivo*, primary hepatocytes and enterocytes
CYP3A46	rifampicin	liver, intestine	*in vivo*, primary hepatocytes and enterocytes
	dexamethasone	liver, intestine	primary hepatocytes and enterocytes
	phenobarbital	liver, intestine	*in vivo*, primary hepatocytes and enterocytes

Table 2: Inducibility at transcriptional level of porcine CYPs involved in drug metabolism (Puccinelli et al., 2011).

2.4.2 Species-differences in conjugative enzyme expression

Glucuronidation represents one of the major phase II reactions in the metabolism of drugs. Among food-producing animals, rabbits, pigs, and horses show the maximal glucuronidation capacity towards phenolic substrates, while broilers and cattle display a relatively low conjugation rate. Differences on UGT and GST activity and mRNA levels were observed in liver among three different cattle breeds by Giantin and colleagues (2008). At the mRNA level, UGT1A6-*like* expression was statistically significant higher in Charolais comprared to Piedmontese and Blonde d'Aquitaine; this result was also confirmed at the catalytic activity level by using two UGT1 marker substrates.

Conversely, UGT1A1-*like* expression showed an opposite behavior: highly expressed in Blonde d'Aquitaine, followed by Piedmontese and Charolais. As regards GSTs, Charolais showed the lowest expression of GSTA1-, M1- and P1-*like* genes and measurement of general GST catalytic ativity would support transcriptional results. In pig GSTM2 mRNA expression analysis shown an high transcript level in liver and testis, a medium level in longissimus dorsi muscle, adipose tissue, spleen and lung and a low level in kidney and heart (Huang et al., 2007). Recently porcine GSTA1-1 and A2-2 has been characterized and, the expression profiles were respectively similar to those of human GSTA1-1/A2-2 and GSTA1-1/A3-3. The highest mRNA expression for GSTA1-1 was found in the adipose tissue, followed by liver and pituitary gland; also in the adrenal gland, kidney, lung, prostate, skin, testis and ovary has been detected (Fedulova et al., 2011). While for GSTA2-1 high mRNA amounts were found in testis, ovary and liver and to a lower extent in the pituitary gland and skin (Fedulova et al., 2010). The porcine SULT1A1 shown, as in human, a wide tissue distribution; a difference in SULT1A1 expression was observed for the small intestine, in which such transcript was not detected in pig (Lin et al., 2003).

Mainly post-transcriptional data on phase II DMEs are available for the other major veterinary species such as sheep (Maté et al., 2010), goat (Martinez et al., 2002) and horse (Scarth et al., 2010) and no data at mRNA level are actually reported.

Finally, in cats liver only UGT1A1 and 1A02 transcripts were detected while UGT1A6 resulted a pseudogene; therefore, the lower capacity to glucuronidate planar phenolic xenobiotics compared with most other mammalian species appear to be related with a less diverse pattern of UGT1A isoform expression (Court & Greenblatt, 2000). More recently, Shresta and colleagues (2011) shown *Felidae*, classified together with Phocidae hypercarnivores species, display a reduced UGT1A6 amino acid fixation rates leading to multiple inactivating mutations. In fact, UGT1A6 is likely representative of a set of mammalian genes that are essential for effective utilization of plants as a nutritional source, but dispensable during adaption to a primarily animal-based diet.

2.4.3 Species-differences in the expression of drug transporters

Species differences in the expression of DTs should be considered in interspecies scaling, as these ones can have implications on the kinetics of xenobiotics. Therefore, data referring to species-specific expression of DTs transporters in different tissues are needed. Only four members of the ABC transporter family are known to be responsible for transporting drugs that are commonly used in veterinary patients: ABCB1, ABCG2, ABCC1 and ABCC2. Among these ones, the most well characterized in terms of

clinically relevant polymorphisms in both human and veterinary medicine are ABCB1 and ABCG2 (Mealey et al., 2011).

In dairy cattle mammary gland, expression of ABCG2 is particularly important due to its involvement in the excretion of xenobiotic into the milk (Schrickx & Fink-Gremmels). This could be potentially harmful, due to undesiderable presence of residues in milk obtained from animals administered/exposed with drugs/xenobiotics which are BCRP substrates. A differential expression of ABCG2 gene in the mammary gland was observed between lactating and nonlactating cows with a decreased expression during the "dry period" (Farke et al., 2008) and fluoroquinolones such as enrofloxacin and danofloxacin-mesylate, two licensed veterinary drugs used in cases of acute and severe inflammatory processes in the mammary gland, are substrated of BCRP (Schrickx & Fink-Gremmels, 2008).

In the brain capillary vessels of pigs and cattle, P-gp was detected by using Western blotting and its expression in chorioid plexus of sheep by means of flow cytometry (Zahner et al., 2010). In pigs, the presence of P-gp and BCRP mRNAs have been demonstrated in the liver and kidney. The organ distribution of MRP2 in apparently similar in pigs, dogs and other species as well, except for the rat in which its high constitutive expression leads to an high capacity in transport in sulphate and glucuronide conjugates (Fink-Gremmels, 2008).

In horses, MDR1 mRNA was shown to be expressed in liver, kidney, intestine and peripheral blood lymphocytes. Highest mRNA level was found in the gastrointestinal tract, major amounts were found in duodenum and proximal jejunum. Gene expression declined rapidly in the distal jejunum, ileum, caecum and colon (Tydén et al., 2009). Additionally, the presence of BCRP, MRP1 and MRP2 was demonstrated in the horse liver, intestine and kidney; moreover, it appeared that in most tissues the expression of MRP1 was much lower than for BCRP and MRP2 (Tydén et al., 2010).

In sheep, P-gp is actually the most studied DT due to its crucial role in the multidrug resistance of pathogenic helminths as well as in antiparasitic drug pharmacokinetics in the host. The ovine P-gp was cloned and expressed and characterized in Madin Darby canine kidney cells (MDCK: Zahner et al., 2010).

In dogs, a 4-bp deletion in the Mdr1 gene was shown to be responsible of ivermectin neurotoxicity; , additionally, both P-gp mRNA and protein amounts measured in dog liver, kidney, intestine and brain are similar to human ones (Schrickx & Fink-Gremmels, 2008; Zahner et al., 2010).

Finally, chicken and turkey ABCB1 mRNA levels found have been shown to be comparable to humans, although in this latter the renal expression was higher that the

intestinal one; a further remarkable finding is the relatively low expression of P-gp in the adrenal gland, differently from humans in which this tissue showed the highest expression compared to all other organs (Schrickx & Fink-Gremmels, 2008).

3. Aims

The molecular mechanisms involved in DMEs and DTs regulation and the role of putative NRs have been mainly subject of investigation in human and laboratory species; on the contrary, in veterinary species there is still a lack of knowledge about DMEs regulation. Objective of the present PhD thesis was to provide preliminary information on the expression and regulation of foremost cattle and porcine NRs and CYPs as well as of bovine DTs. More in detail, the specific goals were:

1. perform a phylogenetic analysis to solve the uncertainly of cattle CYPs families 1-4, known to be those mostly involved in xenobiotic drug metabolism;

2. provide a first overview on cattle hepatic and extrahepatic tissue distribution of most important oxidative, conjugative DMEs and related transcription factors, through quantitative Real Time RT-PCR (qPCR) approach; then, to investigate transcriptional effects of an oral PB inducing protocol upon the abovementioned set of target genes;

3. measure, by using a qPCR approach, mRNA levels of leading SLC- and ABC-transporter in liver, intestine, kidney, testis, adrenal gland and skeletal muscle tissues of cattle orally administered *in vivo* with PB at inducing dosages;

4. investigate the transcriptional effects of time and prototypical CYP3A inducers upon major target NRs and CYPs in cryopreserved pig hepatocytes (CPHs) stored up to ten years in liquid nitrogen.

4. Proposed new nomenclature for Bos taurus cytochromes P450 involved in xenobiotic drug metabolism[1]

Abstract. The CYP superfamily of drug metabolizing enzymes plays a central role in the oxidative metabolism of xenobiotics to which living organisms are exposed. In *Bos taurus* (cattle), a definitive nomenclature for CYP proteins is still lacking, and to unambiguously settle cattle nomenclature a phylogenetic analysis of proteins belonging to CYP 1-4 families was performed. Sequences obtained from GenBank and Dr. Nelson's P450 homepage databases were analyzed according to the maximum likelihood method. Phylogenetic outputs showed that CYPs sharing the same name and obtained from different species did not form, in several instances, monophyletic groups. Some cattle CYPs did not group with their supposed human orthologous counterparts, thus requiring a new nomenclature. Name changes mostly mirrored the orthologous counterparts established for other species, and new names were created when no clear orthologous sequences were identified. The new nomenclature will allow a more appropriate investigation of biochemical and molecular mechanisms involved in the expression and regulation of these drug metabolizing enzymes.

[1] Adapteed with the permission of Wiley-Blackwell from: **Zancanella, V., Giantin, M., Lopparelli, R.M., Patarnello, T., Dacasto, M. & Negrisolo, E., (2010) Proposed new nomenclature for *Bos taurus* cytochromes P450 involved in xenobiotic drug metabolism. J vet Pharmacol Therap, 33, 528-536.**

4.1 INTRODUCTION

Bos taurus (cattle), like humans, during lifetime is exposed to xenobiotics of natural (i.e., mycotoxins) or anthropogenic origin (i.e., drugs, pesticides and environmental pollutants).

Some of these chemicals possess sufficient lipophilicity to cross cellular membranes, enter the blood stream and reach substantial concentrations in target tissues. As most xenobiotics are potentially detrimental for the animal's survival, during their evolution living organisms developed a number of hepatic and extra-hepatic enzyme reactions catalysing the production of hydrophilic metabolites, which can be readily eliminated. The most important enzyme system involved in xenobiotic drug metabolism is undoubtedly represented by the CYP superfamily of DMEs.

This multigene membrane-bound enzyme system catalyses the oxidation of xenobiotics and relevant endogenous compounds (i.e., steroids, bile acids). Most tissues and organs are endowed of CYPs, but these ones are more abundant in the liver, which is therefore considered to be the major site of drug metabolism (Xu, 2005; Ioannides, 2006).

In past fifty years CYPs have been extensively studied in human and rodent model species such as *Mus musculus* (mouse) or *Rattus norvegicus* (rat), leading to a significant improvement of the knowledge about their expression and regulation. Advances in bioinformatics facilitated the development of large data banks, like GenBank (http://www.ncbi.nlm.nih.gov/Genbank/), the Directory of P450-Containing Systems (http://www.icgeb.org/~p450srv/), the Cytochrome P450 Database (Dr. Nelson's Homepage at http://drnelson.utmem.edu/CytochromeP450.html), the Human CYP Allele Nomenclature Committee database (http://www.cypalleles.ki.se/), and SWISS-PROT (http://www.ebi.ac.uk/uniprot/). In these databases expressed sequence tags, complete genes, translated proteins, three-dimensional structures, as well as information about CYPs clinical relevance and genetic polymorphisms are reported (Lisitsa et al., 2001). Actually, the characterisation of cattle CYPs is still incomplete. A fully defined systemic approach to CYPS composition and function is needed for this economically relevant food-producing species. Moreover, the molecular mechanisms involved in the pre-transcriptional regulation of cattle CYPs have only recently become subject of investigation (Greger et al., 2006; Ioannides, 2006; Greger & Blum, 2007; Fink-Gremmels, 2008; Giantin et al., 2008; Giantin et al., 2010).

In 1996, individual members of CYP superfamily were classified for the first time into families and subfamilies based on their amino acid sequences or genes coding for the enzymes: those sharing more than 40% sequence identity were assigned to the same family (i.e., CYP1), whereas those with more than 55% identity were assigned to the

same subfamily (i.e., CYP1A: Ioannides, 2006). So far, more than thirty-six gene families have been described and twelve of them (which comprise twenty-two subfamilies) are expressed in mammals. Members of CYPs families 1-4 are primarily involved in drug metabolism (Xu et al., 2005; Fink-Gremmels, 2008). Previous phylogenetic studies made in humans and rodent model species proved that some CYP isoforms were well conserved within the phylogenetic tree, while other ones diverged each other about 2 billion of years ago (Fink-Gremmels, 2008). This process of diversification was mirrored by marked species-differences in the expression and substrate specificity (Sivapathasundaram et al., 2001; Xu et al., 2005; Ioannides, 2006; Fink-Gremmels, 2008). The general evolutionary mechanism named birth and death process, implying multiple rounds of genes multiplication and selective losses, was indicated as the responsible of the extreme isoforms diversity observed in CYP superfamily (Thomas, 2007). Consequently, the true relationships of orthology among different polypeptides was difficult to assess.

Current cattle CYPs nomenclature is based upon the human nomenclature. The correspondence between each human and cattle CYP isoform is usually established through the basic local alignment search tool algorithm (BLAST), taking into account the percentage of identity (Altschul et al. 1990). Alternatively, the suffix *–like* was used whenever cattle CYP isoform could not be equated without uncertainty with the CYP isoform resulting the best hit in a BLAST search (Greger & Blum, 2007; Giantin et al., 2008). The identification of true orthologous CYPs (i.e. protein products of the same gene in different species) is not a trivial task and requires a phylogenetic approach (Thomas, 2007). In the present study, a phylogenetic analysis was performed to solve the uncertainty of cattle CYPs. Cytochromes P450 sequences from primates and laboratory species (considered as reference sequences in CYP nomenclature), most of veterinary species, fishes, birds as well as species belonging to Insecta, Crustacea, and Amphibia were obtained through multiple BLAST searches (Altschul et al., 1990) against GenBank (http://www.ncbi.nlm.nih.gov/) and Ensembl (http://www.ensembl.org/index.html) databases. In particular, sequence data sets included CYPs proteins belonging to families 1-4, which are known to be those mostly involved in xenobiotic drug metabolism (http://drnelson.utmem.edu/cytochromeP450.html/).

4.2 MATERIALS AND METHODS

The full lists of amino acid sequences accession numbers ,used to built CYP 1-4 families data sets, are provided in the supporting material online of this work on published version (Zancanella et al., 2010).

Multiple CYP proteins alignments were done with the MUSCLE program (Edgar, 2004).

Phylogenetic trees were inferred through the maximum likelihood (ML) approach (Felsenstein, 2004). Models which best fitted the data sets were selected using the ProtTest program and according to the Akaike criterion (Abascal et al., 2005). The JTT+I+G resulted the best model for all alignments. This model uses the amino acid substitution matrix JTT (Jones, Taylor and Thornton) developed by Jones et al. (1992), considers a fraction of positions in the alignment as invariable (I) and models the heterogeneity of the substitution process among different positions through the gamma distribution (G) approximate with four categories (Felsenstein, 2004). The ML phylogenetic analyses were performed by using PHYML 2.44 (Guindon & Gascuel, 2003). The non parametric bootstrap (Felsenstein, 1985) was performed to test the robustness of ML tree topologies (1000 replicates). The new nomenclature for cattle CYPs was created following the criteria explained below. The original name was maintained for cattle CYP under scrutiny if a relationship of orthology between this latter and the reference sequence for that isoform was identified in the phylogenetic analysis (i.e., CYP1A1). If a marked incongruence resulted between the assigned cattle nomenclature and the phylogenetic output, two different approaches were used to rename the cattle isoforms. When the investigated cattle CYP resulted to be orthologous (bootstrap support ≥80%) to sequences that already possess an unambiguous established name, cattle isoform was renamed using the same acronym (i.e., CYP2C31). Conversely, if no clear orthologous counterparts were identified, a new name was created for the considered cattle isoform (i.e., CP2C88). Complete phylogenetic trees of CYPs families 1-4 are provided in Figures 3-6.

4.3 RESULTS

Analysis of tree topologies confirmed that the diversity observed among various CYPs isoforms was produced through a process of successive duplications/multiplications and selective losses. Phylogenetic output of the CYP 1 family (see Figure 3) showed that cattle sequences were placed among their orthologous counterparts; therefore, no nomenclatorial changes were necessary. The CYP 2 analysis (see Figure 4) proved that cattle *Bos taurus* CYP2B, CYP2C18 and CYP2C19 did not group with their supposed human orthologous counterparts. Consequently, former *Bos taurus* CYP2B and 2C19 were renamed respectively CYP2B22 and CYP2C31 taking into account their close relationships, supported by high bootstrap values (≥80%), respectively with *Sus scrofa* (swine) CYP2B22 and *Capra hircus* (goat) CYP2C31. Conversely the new name CYP2C88 was introduced for former *Bos taurus* CP2C18 that has no clear orthologous counterpart among currently available sequences. The cytochrome P450 3 family tree

(see Figure 5) provided a similar result, with *Bos taurus* CYP3A4 isoforms and CYP3A5 located in a totally different position with respect to the supposed human orthologous sequences. The name CYP3A28 was assigned to *Bos taurus* CYP3A4, following the nomenclature proposed in SwissProt database, while new acronyms CYP3A38 and CYP3A48 were selected to rename former *Bos taurus* CYP3A5 and *Bos taurus* CYP3A4 (niphedipine oxidase), respectively. Following the phylogenetic analysis of CYP4 family (see Figure 6), a new nomenclature was assigned to former *Bos taurus* CYP4A11, CYP4A22 and CYP4F3 (*Bos taurus* CYP4A10, CYP4A41, and CYP4F46, respectively). Moreover, the former CYP4F3 was renamed in agreement with the orthologous *Ovis aries* (sheep) sequence. Changes in the nomenclature of cattle CYP proteins are summarized in Table 3.

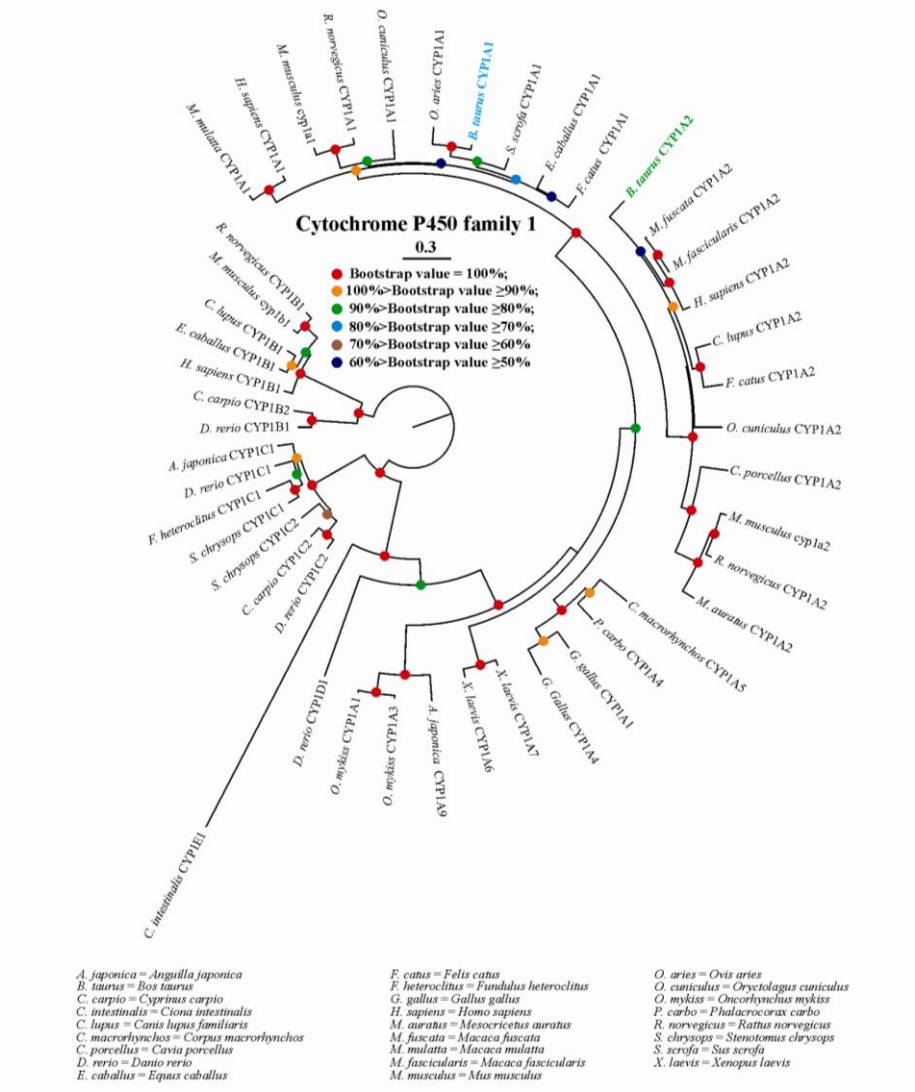

Figure 3: Phylogenetic analysis of the CYP1 family of drug metabolizing enzymes, with particular reference to CYP1A1 and CYP1A2 subfamilies. Maximum likelihood tree (-InL = 20624.87342) of CYP1. Bar represents 0.3 substitution per site.

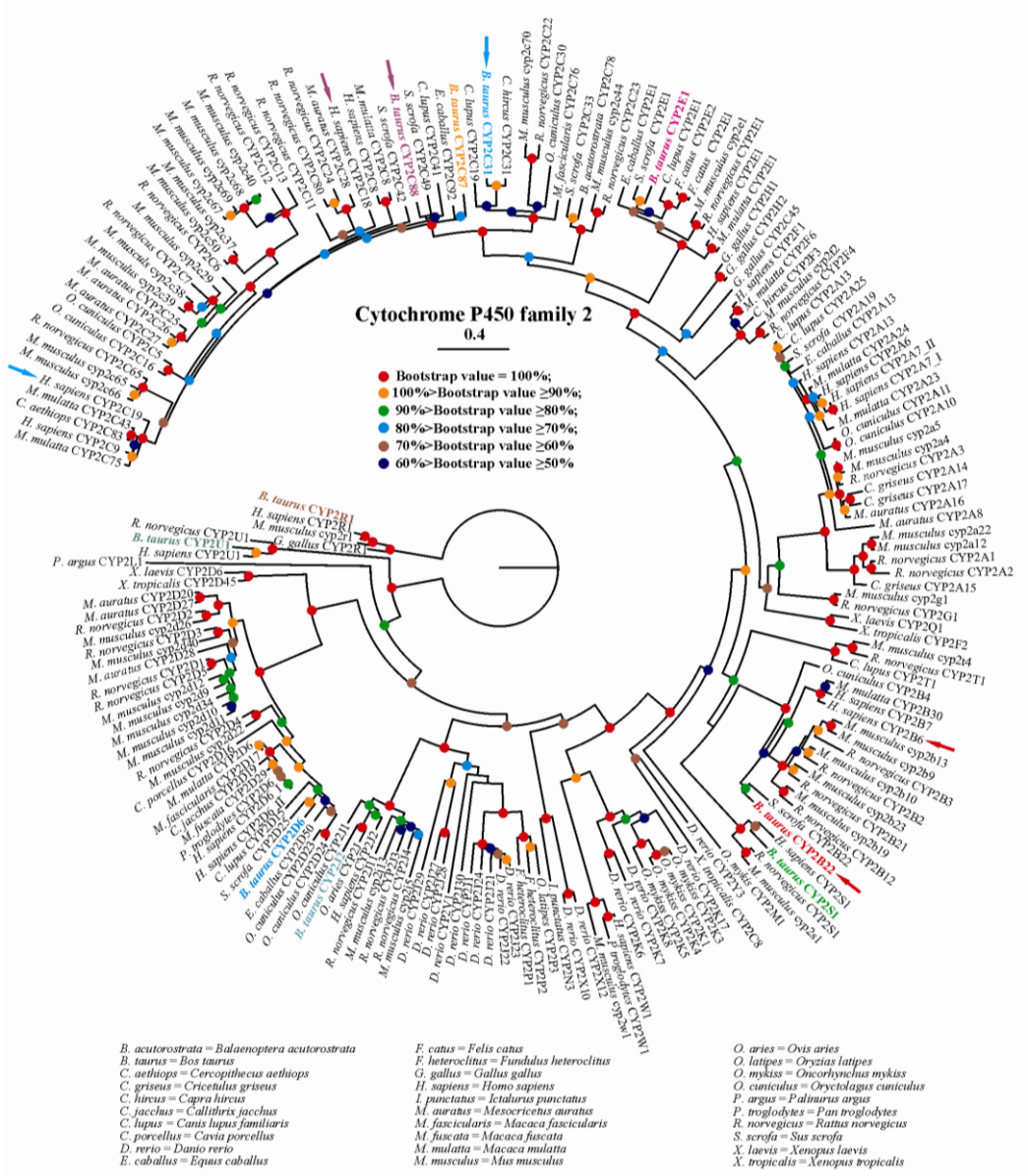

Figure 4: Phylogenetic analysis of the CYP2 family of drug metabolizing enzymes, with particular reference to CYP2C and CYP2B subfamily members. Maximum likelihood tree (-InL = 75029.66193) of CYP2. Bar represents 0.4 substitution per site. Names of cattle isoforms are provided in different colours to allow their immediate identification in the tree. Coloured arrows are used to pinpoint previously considered orthologous isoforms.

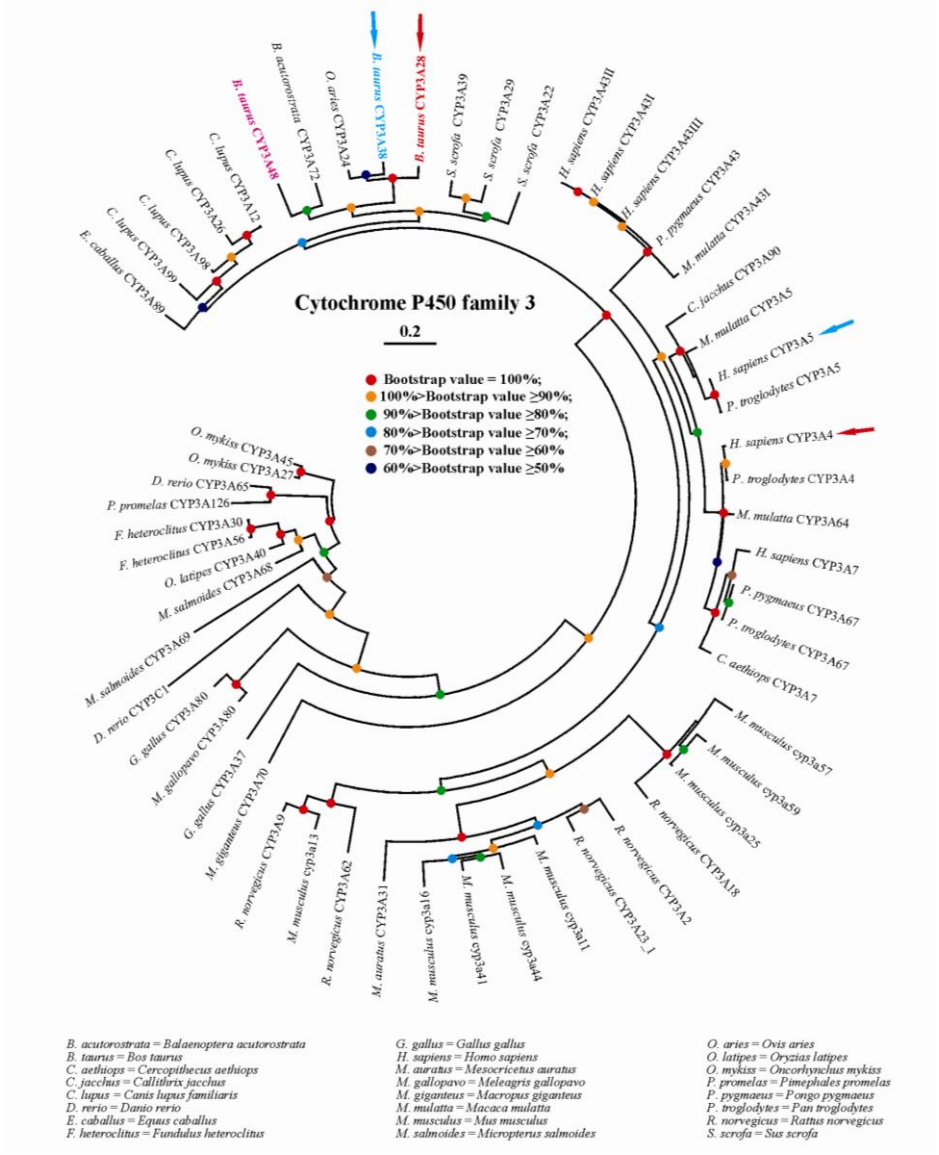

Figure 5: Phylogenetic analysis of the CYP3 family of drug metabolizing enzymes. Maximum likelihood tree (-InL = 17734.77393) of CYP3. Bar represents 0.2 substitution per site. Names of cattle isoforms are provided in different colours to allow their immediate identification in the tree. Coloured arrows are used to pinpoint previously considered orthologous isoforms.

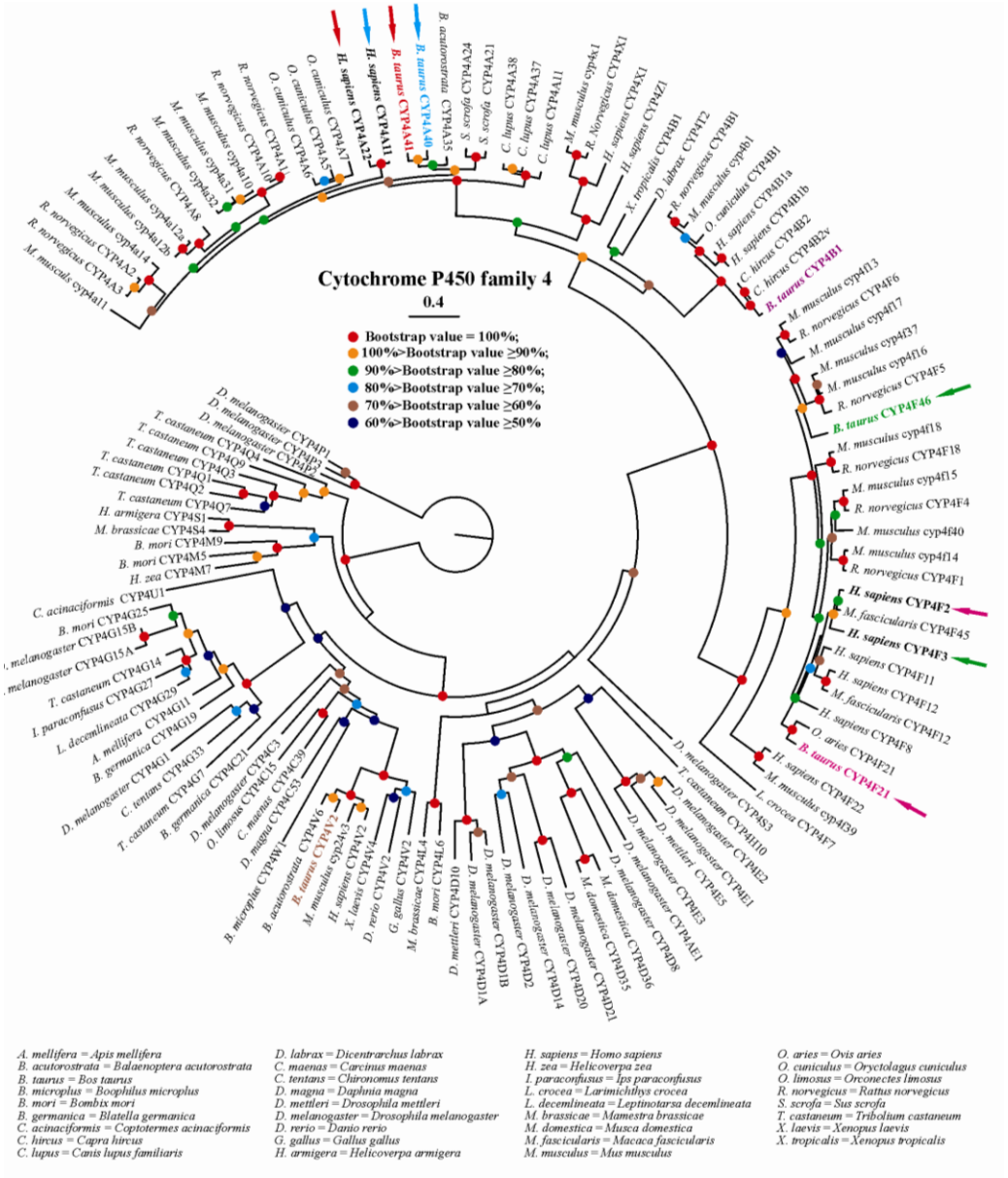

Figure 6: Phylogenetic analysis of the CYP4 family of drug metabolizing enzymes, with particular reference to CYP4A and CYP4F subfamily members. Maximum likelihood tree (-InL = 67780.90976) of CYP4. Bar represents 0.4 substitution per site. Names of cattle isoforms are provided in different colours to allow their immediate identification in the tree. Coloured arrows are used to pinpoint previously considered orthologous isoforms.

Previous nomenclature	**Result of phylogenetic analysis and nomenclature implication**	**Proposed nomenclature**
CYP1A1	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP1A1
CYP1A2	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP1A2
CYP2B	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned *Sus scrofa* orthologous sequence nomenclature	CYP2B22
CYP2C87	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2C87
CYP2C18	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned a new nomenclature	CYP2C88
CYP2C19	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned *Capra hircus* orthologous sequence nomenclature	CYP2C31
CYP2D6	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2D6
CYP2E1	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2E1
CYP2J2	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2J2
CYP2R1	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2R1
CYP2S1	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2S1
CYP2U1	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP2U1
CYP3A4	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned the pre-existing SwissProt nomenclature identifying *Bos taurus* CYP3A4	CYP3A28
CYP3A4 *(niphedipine oxidase)*	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned a new nomenclature	CYP3A48
CYP3A5	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned a new nomenclature	CYP3A38
CYP4A11	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned a new nomenclature	CYP4A40
CYP4A22	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned a new nomenclature	CYP4A41
CYP4B1	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP4B1
CYP4F2	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned *Ovis aries* orthologous sequence nomenclature	CYP4F21
CYP4F3	No orthology between *Bos taurus* and *Homo sapiens* sequence; assigned a new nomenclature	CYP4F46
CYP4V2	*Bos taurus* sequence orthologous to *Homo sapiens* one; confirmed previous nomenclature	CYP4V2

Table 3: Results of phylogenetic analysis and proposed changes in cattle CYPs nomenclature.

4.4 DISCUSSION

In the present work our phylogenetic analysis was based on the maximum likelihood method that is considered statistically very sound and capable to deals with the complex evolutionary histories (Felsenstein, 2004) like that experienced by the P450 superfamily (Thomas, 2007).

Discrepancies among the current nomenclature and phylogenetic analysis results were quite expected, considering the mechanism of gene multiplication and loss that often proved to be restricted to a single species or phyletic lineage (e.g. Thomas, 2007). Current CYPs nomenclature problems are probably further exacerbated by the naming strategy adopted for different species. In several cases naming strategy was very probably based on BLAST comparisons rather than established through rigorous phylogenetic analysis (e.g. see the placement of *Xenopus tropicalis* CYP2C8 and CYP2F2 on Figure 2).

In previously published studies, species-differences in CYPs-dependent catalytic activities were noticed, even among animal species presumed phylogenetically related (i.e., Ruminants: Ioannides, 2006; Fink-Gremmels, 2008). Among reasons offered as a justification for such a behaviour there was the substrate selection strategy (to use substrates considered specific for a particular human CYP isoform or, alternatively, for a rodent model species). Such an approach is nowadays questionable; it's known that a chosen substrate might be oxidized in cattle (but also in other veterinary species) either by a different CYP (change in selectivity) or simultaneously by several CYP isoforms (loss of selectivity: Szotáková et al., 2004). Moreover, there are very few instances where the substrate specificity for that CYP isoform has been investigated by measuring classical kinetic constants such as K_m and V_{max} (Fink-Gremmels, 2008). All of this might result in a low correlation between the measured catalytic activity and CYP protein amount (Szotáková et al., 2004).

Nebbia and colleagues in their comparative study on liver CYPs expression in farm animals (2003) affirmed that phylogeny did not affect the *in vitro* metabolism of marker substrates in food-producing species; on the contrary, there is a number of papers where even orthologous CYP proteins were shown to exhibit substantial species-differences in substrate specificity (Ioannides, 2006). Our phylogenetic results, outlining several discrepancies among the true orthologous relationships and traditional nomenclature for cattle CYPs, offer also an explanatory key to deal with several inconsistencies observed in the function of various CYP isoforms (e.g. differences in the substrate specificity and catalytic activity) expressed in cattle and other animal species. Some examples are considered below.

Ethoxyresorufin represents the substrate most commonly used in man and other animal species to monitor catalytic activity of P450 1A1. In cattle, the Eadie-Hofstee plot (a kinetic model used to determine if one or more enzymes are involved in the substrate biotransformation) confirmed CYP1A1 as the unique enzyme involved in the CYP-dependent *O*-deethylation of ethoxyresorufin. Moreover, a single immunoreacting band was observed following the incubation of microsomal proteins with goat anti-rabbit CYP1A1/2 or sheep anti-rat CYP1A1 antibodies (Sivapathasundaram et al., 2001; Pegolo et al., 2008). In the present nomenclature (Figure 3), *Bos taurus* CYP1A1 sequence was placed among their orthologous counterparts, confirming *Bos taurus* CYP1A1 orthology. The cytochrome P450 2E1 is another supported monophyletic CYP subfamily, and aniline, *p*-nitrophenol, chlorzoxazone and dimethylnitrosamine have been used as substrates to measure its catalytic activity. The immunoblotting of cattle microsomal proteins with human or rat anti-CYP2E1 antibodies showed a single band (Ioannides, 2006). To the best of Authors' knowledge, no kinetic data about some of aforementioned substrates in cattle have never been published. On the other hand, the good correlation found between the rate of aniline hydroxylation and cattle liver CYP2E1-*like* mRNA levels should indirectly confirm its usefulness as a CYP2E1 model substrate (Giantin et al., 2008). Likewise to CYP1A1, in the present phylogenetic analysis *Bos taurus* CYP2E1 grouped itself with orthologous counterparts (Figure 4), whereas other members of the CYP 2 family did not. In humans, four CYP2C isoforms (namely, CYP2C8, CYP2C9, CYP2C18, CYP2C19), sharing a >82% amino acid identity, have been described. The oral hypoglicemic agent tolbutamide (TBT) is a suitable and sensitive marker to measure CYP2C9 activity, the most abundant CYP2C isoform found in the human liver. In cattle, TBT is metabolized to a lower extent, with about 3-7 and 4-5 fold differences in K_m and V_{max} values, respectively. Moreover, the Eadie-Hofstee plot suggests that a single, but different enzyme, with a similar K_m value, might be responsible for TBT methyl hydroxylation (Pegolo et al., 2010). A single immunoreacting band was noticed following the incubation of cattle liver microsomes with antibodies raised against either human CYP2C9 or human CYP2C8/CYP2C9/CYP2C19 and rat CYP2C12 (Ioannides, 2006; Giantin et al., 2008), but this CYP2C9-*like*-dependent catalytic activity did not correlate with the respective apoprotein amount (Giantin et al., 2008). Thus, the hypothetical involvement of other CYP2C isoforms in the TBT methyl-hydroxylation cannot be excluded. Interestingly, in the present nomenclature, *Homo sapiens* CYP2C9 did not group either with *Bos taurus* CYP2C31, CYP2C88 (now identifying former CYP2C19 and CYP2C18 isoforms) and CYP2C87 or other CYP2C isoforms from other food-producing species (horse, goat, and swine: Figure 4).

In humans, testosterone (TST) is mainly hydroxylated at the 6β-position by CYP3A4. In cattle, TST is hydroxylated at different positions, but the 6β-hydroxy TST (OHTST) represents the major TST metabolite, followed by 2β-OHTST (Capolongo et al., 2003). Several antibodies raised against sheep CYP3A or rat CYP3A1/2 cross-reacted with a single protein (Sivapathasundaram et al., 2001; Dacasto et al. 2005; Ioannides, 2006). Among other hydroxylated metabolites, 16α-OHTST and 16β-OHTST are considered CYP2B-dependent substrates in human and rat (the former), and in mouse (the latter). Veal calf microsomes were shown to produce detectable amounts of 16β-OHTST; moreover, a 3-fold increase in 16β-OHTST production was noticed in phenobarbital-treated cattle (Cantiello et al., 2006). Therefore, 16β-OHTST was considered as a CYP2B-*like* (now CYP2B22) substrate (Giantin et al., 2008). It should be here inferred, however, that in a recent study where K_m and V_{max} values of TST hydroxylated metabolites were determined in beef cattle microsomes by means of chemical and immunoinhibition protocols, the active participation of CYP2B22 in 16β-OHTST production was not confirmed. Rather, results indicate that a CYP3A isoform might be actively involved in the production of such a TST metabolite (Pegolo et al., 2010). These contradictory results might be mirrored by the phylogenetic analysis; in fact, *Bos taurus* CYP3A28, CYP3A38 and CYP3A48 were far away from the supposed orthologous human counterparts, although they grouped together with other farm animals (see Figure 5).

4.5 CONCLUSIONS

In conclusion, such a new nomenclature, that mirrors the true evolutionary relationships in bovine CYP isoforms, will contribute to a more appropriate investigation about cattle CYPs biochemical properties and molecular mechanisms. Results aid in choosing suitable marker substrates to measure the catalytic activity, obtaining species-specific antibodies to measure cattle CYP protein amounts, and increasing knowledge about the pre-transcriptional regulation of CYP expression. These advances are not only essential for understanding metabolism in this relevant food-producing species, but will also contribute to veterinary comparative drug metabolism studies. Clearly, more investigations are needed and it will be necessary to constantly improve present results about CYP 1-4 subfamilies, taking into account new sequence annotations or revisions in B. taurus itself as well as in other species; a further step might be represented by the extension of evolutionary analysis to other DMEs, such as conjugative enzymes (i.e., GSTs, UGTs).

5. Tissue distribution and phenobarbital induction of SLC- and ABC- transporters in cattle tissues[2]

Abstract. Uptake and efflux drug transporters modulate the kinetic behavior of endogenous compounds and xenobiotics. In veterinary pharmaco-toxicological sciences, data about drug transporter expression and regulation phenomena are still lacking. In the present study, the constitutive expression of major SLC- and ABC- drug transporters, as well as their possible transcriptional modulation following PB administration, were investigated in cattle tissues through a qPCR approach.

Only two out of the seven target drug transporters (SLCO1B3: solute carrier organic anion transporter 1B3 and SLC10A1) were not constitutively expressed in target tissues. The greatest number of drug transporters (SLCO2B1, ABCB1, ABCC2, ABCG2) were expressed in the liver, followed by testis (SLCO2B1, ABCB1, ABCG2), adrenal gland (SLCO2B1, ABCB1, ABCG2), lung (ABCB1, ABCG2), kidney and skeletal muscle (ABCG2). Phenobarbital administration never altered drug transporter mRNA levels, except for an increase of hepatic ABCC2 mRNA and a down-regulation of renal ABCG2.

Altogether, these results confirm data obtained in humans and laboratory species, with few exceptions; clearly, they should be considered a preliminary step for further molecular investigations about species-differences in drug transporter gene expression and regulation as well as in drug transporter protein expression and function.

[2] From: **Zancanella, V., Giantin, M., Lopparelli, R.M., Nebbia, C. & Dacasto, M. Tissue distribution and phenobarbital induction of SLC- and ABC- transporters in cattle tissues.** Submitted.

5.1 INTRODUCTION

Besides the classical phase I and phase II reactions of drug metabolism, two additional steps (phase 0 and phase III of drug disposition) have nowadays assumed an increasing importance in pharmaco-toxicology. In phase 0, the xenobiotic uptake occurs through the action of multi-specific SLC transporters, for which an ATP hydrolysis is not required. In phase III, the xenobiotic efflux is basically carried out by transporters of the MDR superfamily of ABC transporters, requiring an ATP binding and hydrolysis (Zaïr et al., 2008; Hagenbuch 2010).

Drug metabolizing enzymes and transporters show coordinated mechanisms of regulation and expression; thus, they cooperate in detoxification and excretion of xenobiotics. A clear example is represented by PB: its administration has been shown to modulate the expression of drug transporters like the MRP2 and OATPs (Johnson et al., 2002; Patel et al., 2003), thereby altering the glucuronidation and the following biliary excretion of endogenous substrates or, to a wider extent, of xenobiotics (Ritter et al., 1999).

In human and rodent model species an extensive literature about the expression of aforementioned drug transporters in organs involved in xenobiotic metabolism or blood-tissues barriers as well their substrates/inhibitors has been published (Faber et al., 2003; Augustine et al., 2005; Zaïr et al., 2008; Vähäkangas & Myllynen, 2009; Hagenbuch, 2010; Kim et al., 2007; Mruk et al., 2011). In veterinary pharmaco-toxicology, species-differences in the expression and substrate specificity of ABC-transporters have been recently reviewed (Martinez et al., 2008; Schrickx & Fink-Gremmels, 2008; Mealey, in press); furthermore, information about the constitutive expression of some efflux transporters in equine, trout, turkey, poultry and cattle have been published, too (Taguchi et al., 2002 ; Haritova et al., 2008; Warren et al., 2009; Haritova et al., 2010; Lončar et al., 2010; Mani et al., 2010; Tydén et al., 2010). Nevertheless, in veterinary species there is still a lack of knowledge about drug transporters tissue distribution and molecular mechanisms involved in their transcriptional regulation.

In the present study, the mRNA levels of chief SLC- and ABC-transporters were measured, for the first time and by using a qPCR approach, in liver, small intestine, kidney, testis, adrenal gland and skeletal muscle tissues of cattle administered *in vivo* with PB at inducing dosages. Target genes were chosen on a literature basis, and were essentially represented by hepatic drug transporters known to be specifically modulated by PB in human and laboratory species. In particular, the SLCO1B3 and 2B1; the SLC10A1 as well as ABCB1, ABCB11, ABCC2 and, finally, ABCG2 (Fardel et al., 2001; Courtois et al., 2002; Patel et al., 2003; Cheng et al., 2005; Jigorel et al., 2006;

Martin et al., 2008; Olinga et al., 2008; van de Kerkhof et al., 2008; Lambert et al., 2009; Le Vee et al., 2009; Martin et al., 2010).

5.2 MATERIALS AND METHODS

5.2.1 Chemicals

Phenobarbital was obtained from Sigma-Aldrich (St. Louis, MO). Chloroform, isopropyl and ethyl alcohol were obtained from Thermo Electron Corporation (Waltham, MA), whereas TRIzol® reagent and agarose from Invitrogen (Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit, RNAlater® solution and Power SYBR® Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany).

5.2.2 Animals, treatments and tissue collection

The experiment was run in an authorized facility located nearby the Faculty of Veterinary Medicine of Turin, according to the European Community Directive 86/609, recognized and adopted by the Italian Government (DLgs 116/92). The experimental plan was approved by the Italian Ministry of Health.

Seven healthy male Friesian cattle (about 300 kg bw and 10 months old) were divided, on a weight basis, into two groups of three and four animals. The former one served as control (CTRL), while individuals of the second group received PB (PHEN; 18 $mg \cdot kg^{-1} \cdot body\ weight^{-1} \cdot day^{-1}$ and for 7 days) by oral gavage. Cattle were slaughtered the day after the suspension of PB administration. After the bleeding step, aliquots (about 200 mg each) of liver, small intestine, kidney, lung, adrenal gland, testis and skeletal muscle were collected, immediately frozen in liquid nitrogen and, then, stored at -80°C until use.

5.2.3 Total RNA extraction and reverse-transcription

Total RNA was isolated by using the TRIzol® reagent, according to the manufacturer's instruction. Ribonucleic acid concentration and quality were determined by using the Nanodrop ND-1000 spectrophotometer (Labtech France, Paris, France). Its integrity was confirmed by denaturing gel electrophoresis and visualization of 18S and 28S rRNA bands. The reverse transcription of 2 μg of RNA was performed by using the High Capacity cDNA Reverse Transcription Kit (Foster City, CA) and random primers (final assay volume of 20 μL) following the purchaser's procedure. Furthermore, the SPUD assay was carried out to detect the presence of inhibitors in cDNA generated from RNAs extracted from target tissues (Nolan et al., 2006).

5.2.4 Quantitative Real Time RT-PCR

Primer oligonucleotide sequences of candidate internal control genes and target drug transporters used for qPCR are listed in Table 4 and 5, respectively. Primers were designed *ex novo* except for β-actin (ACTB), glyceraldehydes-3-phosphate dehydrogenase (GAPDH) and glucose-6-phosphate dehydrogenase (G6PDH) (Toffolatti et al., 2006), ribosomal protein, large P0 (RPLP0) and 18S rRNA (Robinson et al., 2007) as well as the TATA-box-binding protein (TBP) (Lisowski et al., 2008).

Bos taurus coding sequences were obtained from GenBank website [http://ncbi.nlm.nih.gov/], and best primer pairs for amplification were chosen by using the Primer Express™ Software 3.0 (Applied Biosystems, Foster City, CA, USA). This latter software as well as OligoAnalyzer 3.1 [http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx] were used to confirm the absence of primer dimers and hairpin formation. The SYBR Green chemistry was used and a careful validation of primer pairs was undertaken to be sure that only the target gene sequence was amplified. Primers specificity was checked *in silico* by the using BLAST and Primer-BLAST NCBI tools [http://blast.ncbi.nlm.nih.gov/Blast.cgi]; furthermore, to avoid amplification of potentially contaminating genomic DNA, one of two primers was designed spanning an exon-exon junction. Finally, the presence of specific amplification products was confirmed by agarose gel electrophoresis and dissociation curve after qPCR reaction. Each primers pair was optimized to 300-900 nM range to indentify primers concentration providing the highest sensitivity.

Calibration curves were obtained following the amplification of decreasing amounts of different cDNA pools. Two cDNA pools were prepared according to preliminary information about gene amplification in target tissues (data not shown). The former included liver, small intestine, adrenal gland and testis, while the second one comprised kidney, lung and skeletal muscle. Just to avoid inaccurate results, tissues in which target genes were not constitutively expressed or, alternatively, showed mean cycle threshold (C_t) values over 30 or very close to the limit of quantification of the thermal cycler were not taken into consideration. Pooled cDNA diluted at 3- or 4-fold intervals were used to evaluate qPCR performances, such as PCR efficiency (E_x) determined by using the equation $E_x = 10(-1/\text{slope}) - 1$), sensitivity assay and test linearity correlation. Only E_x values comprised between 1.9 (90% of efficiency) and 2.1 (110% of efficiency) as well as high correlation coefficients ($0.9846 \leq r \leq 0.9998$) were accepted.

The qPCR was performed on 5µL of cDNA in a final volume of 20 µL, by using Power SYBR®Green PCR Master Mix and an ABI-Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA) under standard qPCR conditions. The $\Delta\Delta C_t$ method (Livak

& Schmittgen, 2001) was then used to analyze data. Target genes constitutive expression in extra-hepatic tissues and their modulation following PB administration, were expressed as n-fold change, that is normalized to the $\Delta\Delta C_t$ mean values of liver and CTRL group, respectively, to whom an arbitrary value of 1 were assigned.

5.2.5 Statistical analysis

Open source geNorm^{PLUS} (Vandesompele et al., 2002) and NormFinder version 0.953 (Andersen et al., 2004) algorithms were used to identify the two most stably expressed internal control genes. Differences in drug transporters mRNA levels between CTRL and PHEN were analyzed by using the Student's *t*-test (GraphPad InStat 3, San Diego, California, USA). A p value < 0.05 was considered as statistically significant. Data were expressed as mean arbitrary units (a.u.) ± standard deviation (SD).

Gene acronym	GenBank ID	Primer sequence 5' → 3'	Reference	Amplicon size (bp)
ACTB	NM173979	F: GTCATCACCATCGGCAATGAG R: AATGCCGCAGGATTCCATG	Toffolatti et al., 2006	84
B2M	NM173893	F: TCGTGGCCTTGGTCCTTCT R: AATCTTTGGAGGACGCTGGAT	designed *ex novo*	71
GAPDH	U85042	F: ACACCCTCAAGATTGTCAGCAA R: TCATAAGTCCCTCCACGATGC	Toffolatti et al., 2006	102
G6PDH	XM583628	F: GCAAAGAGATGGTCCAGAACC R: TGTCCCGGTTCCAAATGG	Toffolatti et al., 2006	75
PPIA	NM178320	F: CTCTTTTGAGCTGTTTGCAGACA R: CCAAATCCTTTCTCTCCAGTGCT	designed *ex novo*	81
RPLP0	NM001012682	F: CAACCCTGAAGTGCTTGACAT R: AGGCAGATGGATCAGCCA	Robinson et al., 2007	227
TBP	NM001075742	F: ACAACAGCCTCCCACCCTATGC R: GTGGAGTCAGTCCTGTGCCGTAA	Lisowski et al., 2008	111
18S	DQ222453	F: GTAACCCGTTGAACCCCATT R: CCATCCAATCGGTAGTAGCG	Robinson et al., 2007	152

Table 4: Candidate internal control genes: GenBank accession numbers, oligonucleotide sequences, references and amplicon sizes (base pairs, bp).

Gene acronym	GenBank ID	Primer sequence 5’ → 3’	Reference	Amplicon size (bp)
SLCO1B3	NM205804	F: CACTAACTATTCGAACGCTAGGAGG R: AGTTGTTGATGGACCACTTCATACA	designed *ex novo*	93
SLCO2B1	NM174843	F: GTGTGGAATACATCACGCCCT R: TTGGTGTAGAAGACCTGGCTTTT	designed *ex novo*	88
SLC10A1	NM001046339	F: GCTTCTCCTTGTTGCCATCTTTAG R: AGGTCATTTTTGTGTCATCTCTGG	designed *ex novo*	71
ABCB1	XM590317	F: GGAAGAGCACAGTCGTCCA R: CCTTGCCATCGATTAACACTG	designed *ex novo*	75
ABCB11	NM001192703	F: CAGCCATCATTGGTCTGAGTGT R: GACTTCATCAGCAACTGAGCCA	designed *ex novo*	86
ABCC2	XM599177	F: TCTGTCCAATGCACTTAATATCACA R: TCGCTCAACAGCCACAATGT	designed *ex novo*	91
ABCG2	NM001037478	F: CCCCATGAGGATGTTACCAAGTA R: CCTTTGGCTTCAGTCCTAACAGA	designed *ex novo*	71

Table 5: Target drug transporters: GenBank accession numbers, oligonucleotide sequences, references and amplicon sizes.

5.3 RESULTS

5.3.1 Validation of qPCR assays and selection of the best internal control genes

A relatively "pure" RNA (260 and 280 nm absorbance ratios were about 1.9 - 2.0) without co-purified contaminants (absorbance ratios at 260 and 230 nm comprised between 1.8 - 2.2) was obtained from each tissue aliquot. All qPCR primers allowed the obtainment of specific amplicons, distinct dissociation peaks and a single band of the expected size in agarose gel electrophoresis.

To identify the best internal control gene to be used for data normalization, mRNA levels of eight candidate genes (ACTB, B2M: β_2-microglobulin, GAPDH, G6PDH, PPIA: cyclophilin A, RPLP0, TBP and 18S) were measured in target tissues. Thereafter, a pre-selection was made by using the mean C_t value obtained from pooled cDNA samples as a criterion. The 18S gene was excluded as a result of its high constitutive expression in target tissues (C_t~18). On the contrary, GAPDH as well as G6PDH and TBP were not taken into account as they were shown to be poorly expressed (C_t > 29) in the lung (the former one) and kidney, lung and skeletal muscle (the two other ones). Once verified the absence of significant differences between CTRL and PHEN, the amplification data of the remaining candidate internal control genes (ACTB, B2M, PPIA and RPLP0) were submitted to geNorm^{PLUS} [http://www.biogazelle.com/mybiogazelle/] and NormFinder [http://www.mdl.dk/Files/NormFinder-HowTo%20v18.pdf] algorithms to identify the most stable couple of internal control genes. In both analyses, ACTB was the best internal control gene, with the lowest NormFinder stability and geNorm^{PLUS} M values (0.078 and 0.99, respectively). The NormFinder software identified ACTB and B2M as the best combination of two internal control genes, with a stability value of 0.058. On the other hand, geNorm^{PLUS} recognized ACTB, B2M and RPLP0 as the three reference genes with the lowest M value. However, the variability value among sequential normalization factors (based on the n and n+1 least variable targets) was considered greater than expected (the threshold value is set at 0.15) by the software. In this case, the use of n-1 reference targets showing the lowest M value is recommended; therefore, ACTB, B2M and RPLP0 were used as internal control genes in the present study.

The main features of each qPCR assay (4 internal control genes, 7 drug transporters), namely slope, E_x, y-intercept, correlation coefficients (R^2) and linear dynamic range are reported in Tables 6 and 7.

Gene acronym	cDNA pool	Slope	E_x (%)	*y*-intercept	R^2	Dynamic range (C_t)
ACTB	Lv. I. T. A.	-3.39	97.2	27.63	0.9934	18.20 - 26.17
	Ln. K. M.	-3.51	92.7	30.53	0.9949	23.24 - 28.45
B2M	Lv. I. T. A.	-3.32	100.1	26.55	0.9935	18.61 - 25.07
	Ln. K. M.	-3.32	100.1	29.26	0.9850	23.36 - 29.43
PPIA	Lv. I. T. A.	-3.46	94.5	29.12	0.9956	20.73 - 27.44
	Ln. K. M.	-3.31	100.5	31.31	0.9943	24.50 - 30.41
RPLP0	Lv. I. T. A.	-3.35	98.8	26.42	0.9898	19.61 - 26.76
	Ln. K. M.	-3.28	101.8	28.08	0.9872	21.53 - 28.46

Table 6: Main features (slope, E_x, y-intercept, R^2 and linear dynamic range) of best internal control gene qPCR assays. Lv, liver; I, small intestine; T, testis; A, adrenal; Ln, lung; K, kidney; M, muscle.

Gene acronym	cDNA pool	Slope	E_x (%)	*y*-intercept	R^2	Dynamic range (C_t)
SLCO1B3	Lv.	-3.36	98.4	32.17	0.9998	20.05 - 26.13
SLCO2B1	Lv. I. T. A.	-3.27	102.2	36.51	0.9846	24.60 - 30.39
SLC10A1	Lv.	-3.33	99.7	32.08	0.9986	20.13 - 26.13
ABCB1	Lv. I. T. A.	-3.49	93.4	33.50	0.9860	21.12 - 29.24
	Ln.	-3.25	103.1	37.81	0.9901	28.20 - 34.05
ABCB11	Lv.	-3.36	98.4	35.65	0.9911	23.40 - 31.81
ABCC2	Lv. I. T.	-3.49	93.4	36.76	0.9944	24.20 - 32.79
ABCG2	Lv. I. T. A.	-3.48	93.8	34.74	0.9947	24.26 - 28.45
	Ln. K. M.	-3.34	99.3	38.38	0.9922	28.32 - 34.38

Table 7: Main features (slope, E_x, y-intercept, R^2 and linear dynamic range) of target drug transporters qPCR assays. Lv, liver; I, small intestine; T, testis; A, adrenal; Ln, lung; K, kidney; M, muscle.

5.3.2. Drug transporter constitutive expression in target tissues

The constitutive expression of SLC- and ABC-transporters in target tissues is reported in Figures 7 and 8. To help the reader's understanding, gene expression profiles were compared with liver ones, in which all drug transporters were shown to be constitutively expressed. On the whole, lesser amounts of drug transporter mRNAs were found in extra-hepatic tissues, except for ABCB1. Sometimes, target genes were expressed at negligible levels (i.e., SLCO1B1, SLC10A1, ABCB11). Only three out of seven drug transporters (ABCG2, ABCB1 and, to a lower extent, SLCO2B1) were expressed in almost all target tissues. Apart from the liver, the small intestine was the tissue in which the larger amount of drug transporters (SLCO2B1, ABCB1, ABCC2, ABCG2) were confirmed to be constitutively expressed, followed by testis and adrenal gland (SLCO2B1, ABCB1, ABCG2 and SLCO2B1, ABCB1, ABCG2, respectively), the lung (ABCB1 and ABCG2) and, finally, kidney and skeletal muscle (only ABCG2 for both).

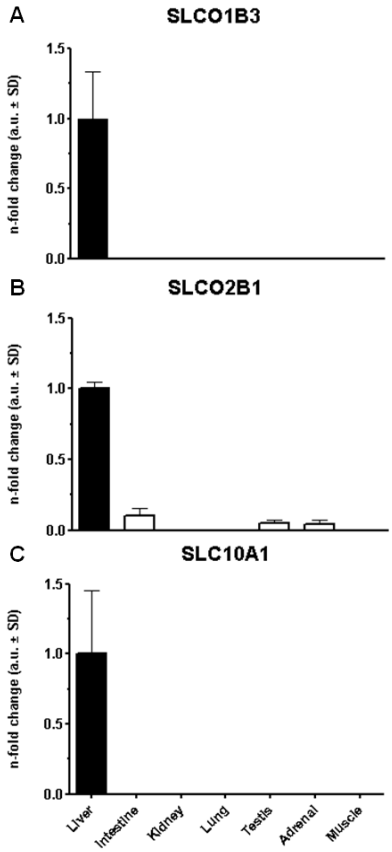

Figure 7: Constitutive expression of SLC- drug transporters in cattle tissues. Messenger RNA was extracted from cattle tissue aliquots and drug transporter mRNA levels were measured by a qPCR approach. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of liver tissue, to whom an arbitrary value of 1 was assigned.

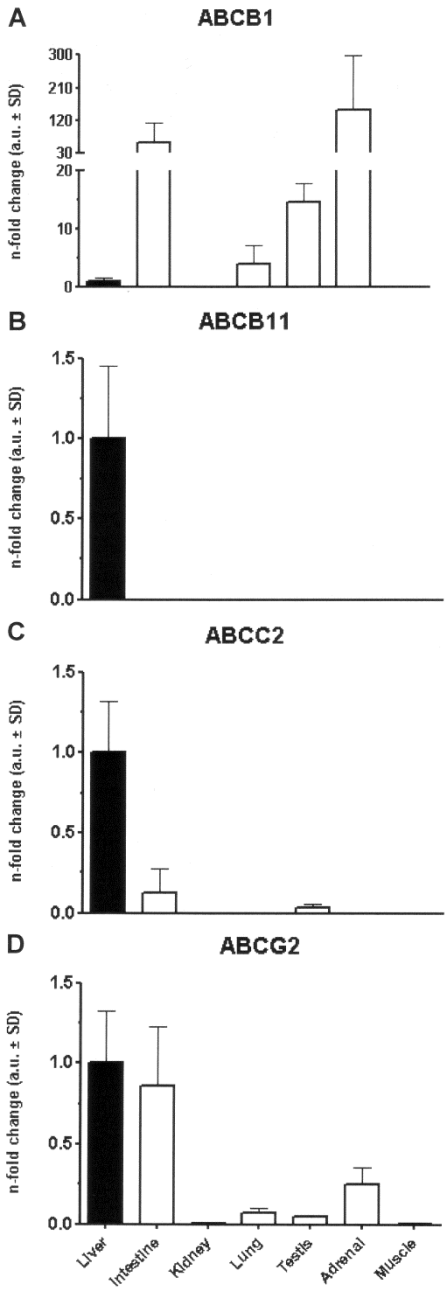

Figure 8: Constitutive expression of ABC- drug transporters in cattle tissues. Messenger RNA was extracted from cattle tissue aliquots and drug transporter mRNA levels were measured by a qPCR approach. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of liver tissue, to whom an arbitrary value of 1 was assigned.

5.3.3. Modulation of drug transporter gene expression by PB

Whole results on the effects of PB upon drug transporter gene expression in cattle target tissues are reported in Tables 8 and 9. No transcriptional modulation of target drug transporters was ever noticed, except for an increase ($p < 0.05$) of ABCC2 mRNA in the liver (see Figure 9A) and a down-regulation ($p < 0.05$) of ABCG2 gene in the kidney (see Figure 9B).

	n-fold change (a.u.) ± SD					
	SLCO1B3		**SLCO2B1**		**SLC10A1**	
Tissue	**CTRL**	**PHEN**	**CTRL**	**PHEN**	**CTRL**	**PHEN**
Liver	1.00 ± 0.34	1.49 ± 0.43	1.00 ± 0.05	1.31 ± 0.36	1.00 ± 0.45	0.92 ± 0.32
Small intestine	N.D.[a]		1.00 ± 0.45	1.46 ± 0.19	N.D.	
Kidney	D.N.Q.[b]		D.N.Q.		N.D.	
Lung	N.D.		D.N.Q.		D.N.Q.	
Testis	D.N.Q.		1.00 ± 0.54	1.45 ± 0.95	D.N.Q.	
Adrenal	D.N.Q.		1.00 ± 0.74	0.97 ± 0.59	N.D.	
Muscle	D.N.Q.		D.N.Q.		D.N.Q.	

Table 8: Relative abundances of SLC-transporters mRNA in cattle orally administered with PB. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

[a]*: not detected.*

[b]: *detected but not quantifiable*.

	n-fold change (a.u.) ± SD							
	ABCB1		**ABCB11**		**ABCC2**		**ABCG2**	
Tissue	**CTRL**	**PHEN**	**CTRL**	**PHEN**	**CTRL**	**PHEN**	**CTRL**	**PHEN**
Liver	1.00 ± 0.54	2.29 ± 0.86	1.00 ± 0.45	1.11 ± 0.40	1.00 ± 0.31	2.00 ± 0.55*	1.00 ± 0.32	1.36 ± 0.13
Small intestine	1.00 ± 0.90	0.62 ± 0.27	D.N.Q.[b]		1.00 ± 1.17	0.67 ± 0.96	1.00 ± 0.75	0.43 ± 0.26
Kidney	D.N.Q.		D.N.Q.		D.N.Q.		1.00 ± 0.32	0.29 ± 0.23*
Lung	1.00 ± 0.77	1.86 ± 1.71	D.N.Q.		D.N.Q.		1.00 ± 0.61	1.24 ± 0.47
Testis	1.00 ± 1.55	0.22 ± 0.18	D.N.Q.		1.00 ± 0.71	1.07 ± 1.14	1.00 ± 0.65	0.97 ± 0.71
Adrenal	1.00 ± 1.01	0.63 ± 0.83	D.N.Q.		D.N.Q.		1.00 ± 0.69	0.55 ± 0.20
Muscle	D.N.Q.		D.N.Q.		D.N.Q.		1.00 ± 0.35	0.64 ± 0.28

Table 9: Relative abundances of ABC-transporters mRNA in cattle orally administered with PB. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

[a]*: not detected.*

[b]: *detected but not quantifiable*.

[*]*: significant differences ($p<0.05$) vs CTRL*

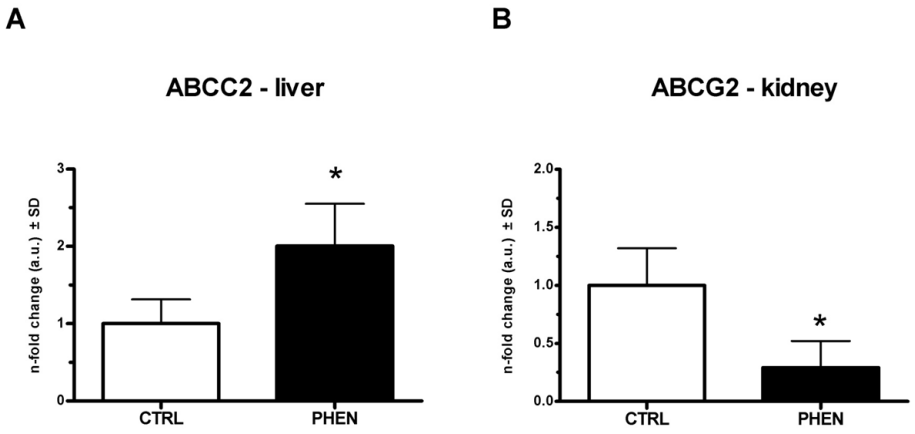

Figure 9: Effect of PB on hepatic ABCC2 and renal ABCG2 gene expression. Messenger RNA was extracted from tissue aliquots obtained from CTRL and PHEN animals, and target drug transporter mRNA levels were measured by a qPCR approach. Data (arithmetic means ± SD) are expressed as n-fold change (a.u.) normalized to $\Delta\Delta C_t$ mean value of CTRL, to whom an arbitrary value of 1 was assigned.

**: significant differences ($p < 0.05$) vs CTRL*

5.4 DISCUSSION

In the past two decades the characterization of drug transporters constitutive expression, their substrate specificities and modulation by prototypical drug metabolizing enzymes inducers provided outstanding information about molecular mechanisms governing the disposition of endogenous compounds and xenobiotics in humans (Jigorel et al., 2006; Martin et al., 2008; Zolk & Fromm, 2011).

Likewise to humans and laboratory species, drug transporters are of extremely importance also in veterinary medicine, as many drugs commonly used in veterinary chemotherapy are substrates for one or more drug transporters (Schrickx & Fink-Gremmels, 2008). Despite this, few data are actually available about their gene expression in veterinary species. This study was undertaken to provide a first overview about tissue distribution of SLC- and ABC-transporters in cattle and their transcriptional response following the oral administration of PB at doses presumably known to induce drug metabolizing enzymes.

5.4.1 Identification of the best internal control genes

A reliable normalization is a fundamental prerequisite for gene expression data analysis, and such an approach includes the measurement of an internal control gene. In the present study, geNorm^{PLUS} was chosen among the different free algorithms used to identify the best internal control gene. Such a choice was justified by the fact that both algorithms

indicated ACTB and B2M as the two most stable genes, but geNorm^{PLUS} provided an additional information about the optimal number of internal control genes to be used in the present experiment. A third internal control gene was suggested to be used besides ACTB and B2M for a more accurate data normalization, and RPLP0 was identified by geNorm^{PLUS}.

5.4.2 Solute carrier transporters

Among SLC-transporters, the SLCO1B3 gene encodes for OATP1B3, a protein mostly expressed in the hepatocyte basolateral membrane (Hagenbuch, 2010). Apart from the liver, SLCO1B3 has been shown to be constitutively expressed in extra-hepatic tissues like placenta (Briz et al., 2003), prostate (Hamada et al., 2008), colon (Ballestero et al., 2006) and gastrointestinal tumors (Hagenbuch & Meier, 2004). Similarly, SLCO1B3 was predominantly expressed in cattle liver, and poor mRNA amounts were detected in kidney, testis, adrenal gland and skeletal muscle, too. Phenobarbital did not affect SLCO1B3 gene expression in cattle, but in humans this influx transporter did not respond to PB in an unequivocal manner: a down-regulation was noticed in primary human hepatocytes (Jigorel et al., 2006), while in liver slices the SLCO1B3 gene expression was unchanged or induced (Olinga et al., 2008).

Compared to SLCO1B3, the SLCO2B1 gene presents a wider tissue distribution, as it is constitutively expressed in cells of excretory organs and in a number of blood/organ barriers (Hagenbuch, 2010; Meyer zu Schwabedissen & Kim, 2009). In cattle, the SLCO2B1 gene was expressed in all the analyzed tissues and comparable mRNA amounts were found in liver and small intestine as well as in testis and adrenal gland. In human primary hepatocytes, the SLCO2B1 gene transcription was suppressed after 72 hrs of incubation with PB (Jigorel et al., 2006); what's more, PB significantly decreased murine OATP2B1 expression *in vivo* (Cheng et al., 2005). By contrast, cattle SLCO2B1 mRNA levels were never affected by PB in liver and in extra-hepatic tissues as well.

Among SLC-transporters a key role is played by SLC10A1, encoding for the NCTP protein; this latter one is exclusively expressed at the hepatocyte basolateral membrane and represents the foremost uptake system for conjugated bile salts (Hagenbuch, 2010). Likewise to humans, measurable amounts of SLC10A1 mRNA were just found in cattle liver. With regard to PB effects on SLC10A1 gene expression, conflicting results were obtained in previous *in vitro*/*in vivo* studies. A reduction of SLC10A1 mRNA was observed in PB-exposed human hepatocyte primary cultures (Jigorel et al., 2006); Olinga and colleagues (2008) pointed out a NTCP up-regulation following the exposure of human liver slices to PB (25 μM and 50 μM), but a higher PB concentration (3.2 mM)

down-regulated SLC10A1 mRNA; lastly, SLC10A1 expression was not significantly affected at both transcriptional and post-translational levels in rats and mice intraperitoneally injected with PB (Hagenbuch et al., 2001, Wagner et al., 2005). In a more recent study made in HepaRG cells, drug metabolizing enzyme and drug transporter gene expression profiles were shown to be modulated in a dose- and time-dependent way by PB; in particular, SLC10A1 mRNA was significantly down-regulated only with PB concentrations close to 3.2 mM (Lambert et al., 2009). Therefore, definitive conclusions cannot still be drawn, although the presence of low PB concentrations in cattle liver might hypothetically explain, in addition to species-differences in drug transporter molecular regulatory mechanisms, the absence of a transcriptional effect on SLC10A1 here observed.

5.4.3 ATP-binding cassette transporters

Only four genes of the ABC-transporter family are believed as responsible for the efflux of drugs commonly used in veterinary species: ABCB1, ABCG2, ABCC1 and ABCC2. In the present study, ABCB1 mRNA was expressed in most of tissues subject of investigation (adrenal gland>small intestine>testis>lung>liver). This finding confirms previous studies made in humans and model species (monkey and rat), in which highest amounts of mRNAs coding for MDR1 and mdr1a/1b proteins were noticed in gastrointestinal tract and adrenal gland, followed by liver, lung, kidney, testis and skeletal muscle (Brady et al., 2002; Nishimura & Naito, 2005; Nishimura et al., 2009). Phenobarbital has been recognized as a *p*-glycoprotein (P-gp) substrate in humans (Luna-Tortós et al., 2008; Zhang et al., 2010; Chan et al., 2011). This barbiturate increases P-gp protein expression and transport activity either in mouse brain capillaries (Abbott et al., 2010; Wang et al., 2010) than in other *in vitro* models such as human primary hepatocytes (Jigorel et al., 2006), liver and intestinal slices (Olinga et al., 2008; van de Kerkhof et al., 2008), HepG2, LS180 and Caco-2 cell lines (Martin et al., 2008; Schuetz et al., 1996). Furthermore, it has been recently shown how both PXR and CAR are actively involved in the regulation of P-gp expression (Chan et al., 2011). In the present study, PB increased (250% *vs* CTRL) liver ABCB1 mRNA, and the lack of a statistical significance might be essentially attributed to the high biological variability noticed within PHEN group.

In cattle, quantifiable amounts of ABCB11 mRNA were found exclusively in the liver, thereby confirming its predominant hepatic localization. Based on raw C_t values information, ABCB11 was also constitutively expressed, but not at quantifiable levels, in cattle testis. These data agree with those reported for humans (Nishimura & Naito, 2005). Phenobarbital did not affect ABCB11 mRNA in cattle, but in humans distinct

transcriptional effects like an up- or down-regulation, a biphasic increase and following decrease as well as a dose- and time-dependent modulation, have been observed *in vitro* (Jigorel et al., 2006; Olinga et al., 2008; Lambert et al., 2009). A CAR-independent mechanism of regulation of ABCB11 gene expression has been hypothesized and a post-translational effect of the barbiturate has been signaled *in vitro* in the mouse (Wagner et al., 2005).

In human and laboratory species, the ABCC2 gene is predominantly expressed in liver, kidney, small intestine and, to a lower extent, in testis, lung and adrenal gland (Nishimura and Naito, 2005; Kim et al., 2007; Nishimura et al., 2009). Except for the kidney, a similar pattern of gene expression was noticed in bovine tissues. This would suggest that ABCB1 and ABCC2, as well as P-gp and MDR2 proteins, might play a fundamental role in intestinal drug efflux mechanisms not only in humans but also in cattle. In cattle liver, PB significantly up-regulated ABCC2 gene expression, as previously reported *in vitro* by using either hepatocyte primary cultures or established cell lines (Jigorel et al., 2006; Martin et al., 2008; Olinga et al., 2008; Lambert et al., 2009). Moreover, it has been recently shown how P-gp, Mrp2 and breast cancer resistance protein (BCRP) transport activity and protein expression are enhanced, through CAR activation, in brain capillaries and liver of PB treated rats (Wang et al., 2010).

The gene coding for BCRP is expressed in a number of mammalian tissues, mostly in those having important barrier function: intestine, liver, brain, placenta, kidney and mammary gland (Jonker et al., 2005; Han and Sugiyama, 2006; Huls et al., 2009). Present results would confirm the ubiquity of ABCG2 gene, as it was the drug transporter more expressed in bovine target tissues (liver≈intestine>adrenal gland>lung>kidney≈skeletal muscle). Phenobarbital is considered neither a BCRP substrate or an inhibitor (Cerveny et al., 2006); on the other hand, the ABCG2 gene has been shown to be up-regulated by PB in HepaRG cell line, hepatocyte primary cultures and rodent brain capillaries (Jigorel et al., 2006; Lambert et al., 2009; Wang et al., 2010). In the present experiment a significant down-regulation of ABCG2 gene was noticed in bovine kidney, a result actually with no possible explanation.

5.5 CONCLUSIONS

To date, drug transporters are believed to play a significant role not only in multi-drug resistance phenomena, but also in kinetic behavior (i.e., absorption and distribution) of endogenous compounds as well as of xenobiotics. The knowledge on the impact of drug transporters in veterinary pharmaco-toxicology is still limited, though some progress has recently been done (Martinez et al., 2008; Schrickx & Fink-Gremmels, 2008; Haritova et

al., 2008; Haritova et al., 2010; Mealey, in press). The major drawback is the lack of systematic data about drug transporter physiological levels in animal tissues and information about molecular mechanisms involved in their transcriptional regulation. In fact, and similar to drug metabolizing enzymes, the presence of significant species-differences in drug transporter expression and regulation phenomena have been hypothesized. Hence, a closer molecular approach on localization and function of drug transporters in various species and, within a species, in target tissues is nowadays needed. This will allow to predict the clinical relevance of drug-drug interactions and the development of new drugs tailored to reach faster and effectively the target tissue (Schrickx & Fink-Gremmels, 2008). To the best of our knowledge, this is the first study providing new information about the mRNA tissue distribution of foremost uptake and efflux drug transporters in cattle. As a whole, the pattern of drug transporter gene expression has been shown to be, with few exceptions, similar to that observed in humans and rodent model species; in particular, liver, small intestine and testis were proved to be tissues mostly involved in transport activity. Phenobarbital is considered as a general substrate of many drug transporters; in the present study PB, orally administered at inducing dosages, prompted on a significant modulation of hepatic ABCC2 and renal ABCG2, while variation of ABCB1 gene expression in liver was very close to significance. Present results should be considered as a starting point for further molecular studies in cattle, designed to investigate more in depth the presence of species-differences in drug transporter gene expression and regulation as well as in drug transporter protein expression and function, which might ultimately result in a different kinetic behavior and/or in dangerous drug-drug interactions.

6. Constitutive expression and phenobarbital modulation of drug metabolizing enzymes and related nuclear receptors in cattle liver and extra-hepatic tissues[3]

Abstract. In humans and laboratory species, PB and PB-*like* compounds induce hepatic and extra-hepatic DMEs through the activation of specific NRs, and basic molecular mechanisms governing such a phenomenon have been clearly understood in aforementioned species. On the contrary, few data about the transcriptional effects PB in veterinary species are available. In the present work the constitutive expression and modulation, after an oral PB challenge, of a number of PB-responsive NR and DME genes was investigated in cattle liver and extrahepatic tissues (small intestine, kidney, lung, testis, adrenal and muscle).

As reported in humans and rodents, target genes were always expressed to a lower extent compared to the liver with few exceptions, i.e. NR2A1 in kidney, NR2B1, cytochrome CYP2B22, SULT1A1-*like* in lung, and NR2B1 in skeletal muscle. Following PB oral administration, hepatic CYP2B22, 2C31, 2C87, 3A and UGT1A1-*like*, GSTA1-*like* and SULT1A1-*like* mRNAs and protein amounts were significantly modulated; among extrahepatic tissues, only the small intestine showed a significant down-regulation of both SULT1A1-*like* mRNA and corresponding apoprotein.

As a whole, present results represent the first evidence about the constitutive expression of foremost DME and NR genes in different extra-hepatic tissues of cattle; furthermore, data obtained following the PB exposure will improve knowledge about the PB-dependent modulation of DMEs expression and function in liver and extrahepatic tissues of this worldwide relevant food-producing species.

[3] From: Zancanella, V., Giantin, M., Lopparelli, R.M., Nebbia, C. & Dacasto, M. **Constitutive expression and phenobarbital modulation of drug metabolizing enzymes and related nuclear receptors in cattle liver and extra-hepatic tissues.** Submitted

6.1 INTRODUCTION

In mammals, the CYP superfamily of DMEs play an outstanding role in the oxidative metabolism of drugs, xenobiotics as well as of relevant endogenous compounds (i.e., steroids, bile acids). Liver represents the organ with the highest abundance of CYP isoforms, and CYP1A2, CYP2B6, CYP2C9, CYP2C19, CYP2D6 and CYP3A4/3A5 represent those mostly involved in human drug metabolism (Graham & Lake, 2008). Extrahepatic CYPs play a part in drug bioavailability and systemic effects, and a clear example in this respect is represented by the interaction among DMEs and efflux proteins at the small intestine level, contributing to the first-pass drug metabolism (Martignoni et al., 2004). Besides liver and gastrointestinal tract, CYPs are expressed in many other extrahepatic tissues, such as kidney, lung and upper airways, brain, adrenal gland, gonads, heart and skin (Pelkonen et al., 2008). On a comparative basis, limited information about DMEs constitutive expression, regulation and function in tissues other than liver have been published hitherto in veterinary species such as in cattle, which represent a relevant food-producing species worldwide. In particular, the few available data refer to gastrointestinal mucosa (Kawalek & el Said, 1994; Virkel et al., 2010), kidney (Darwish et al., 2010; Merlanti et al., 2007), lung (Darwish et al., 2010) and olfactory tract (Longo et al., 1997), testis (Lopparelli et al., 2010), tongue (Yang et al., 2003) and coronary arteries (Grasso et al., 2005).

Cytochromes P450 induction represent a well known phenomenon, with first reports dating from 50 years ago (Conney, 1967). Such an event has a major impact on CYP-dependent drug metabolism, pharmacokinetics and drug-drug interactions, the carcinogenicity of xenobiotics as well as on the activity and the disposition of endogenous hormones. Among substances known to up-regulate CYP gene expression and function there are barbiturates, dexamethasone, rifampicin, polycyclic aromatic hydrocarbons and ethanol. What's more, a specific group of xenobiotic-sensor receptors (the nuclear receptors, NRs) have been shown to be involved in the transcriptional CYP activation and the resulting protein induction (Handschin & Meyer, 2003; Xu et al., 2005; Tolson & Wang, 2010).

Phenobarbital is a hypnotic and antiepileptic drug that causes numerous effects in the liver including hypertrophy, proliferation of the smooth endoplasmatic reticulum and tumor promotion. Within this pleiotropic response PB induces CYP2A, 2B, 2C and 3A subfamilies and various transferases, too (Honkakoski & Negishi, 1997). A certain number of compounds have been shown to elicit this same behavior, although most of them have no evident structural relationship either with PB or each other (Audet-Walsh et al., 2009).

In human and rodent model species much is known about the molecular mechanism of CYPs induction by PB and PB-*like* compounds: it's a transcriptional activation of target genes for the most part mediated by CAR and also possibly PXR (Handschin &Meyer, 2003; Timsit & Negishi, 2007).

Beyond human and lab species, a transcriptional up-regulation of hepatic CYPs has been documented in other animal species administered with PB, such as pig (Puccinelli et al., 2010), rabbit (Marini et al., 2007), chicken (Goriya et al., 2005) and dog (Graham et al., 2006; Makino et al., 2009). On the contrary, no data are actually available for cattle. The aim of the present study was to provide a first overview about the constitutive expression in cattle extrahepatic tissues of foremost PB-responsive (in human and lab species) oxidative and conjugative DMEs and related xenosensors (NRs), by using a qPCR approach; target tissues were represented by known classical sites of absorption/metabolism/excretion (small intestine, lung, liver and kidney) as well as by testis, adrenal and muscle. The transcriptional effects of PB upon the abovementioned set of target genes were then investigated and, whenever a significant outcome observed, confirmed at the post-translational level through immunoblotting investigations. This study is an integral part of a wider project addressed to characterize the global transcriptional and post-translational effects of PB upon cattle DMEs, NRs and drug transporters, whose just preliminary liver post-translational data have been published thus far (Cantiello et al., 2006).

6.2 MATERIALS AND METHODS

6.2.1 Chemicals and reagents

Phenobarbital was from Sigma-Aldrich (St. Louis, MO). Chloroform, isopropyl and ethyl alcohol were obtained from Thermo Electron Corporation (Waltham, MA), whereas TRIzol® reagent and agarose from Invitrogen (Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit, RNAlater® solution and Power SYBR® Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). The Power SYBR®Green PCR Master Mix was obtained from Applied Biosystems (Foster City,CA). The Quant-iT® Protein Assay Kit ws from Invitrogen (Eugene, OR). NuPAGE® Novex® 4-12% Bis-Tris Gels and iBlot™ Gel Transfer Stack, Nitrocellulose, Mini were obtained from Invitrogen (Eugene, OR). The rabbit anti-human CY2B6 was purchased from LifeSpan BioScences Inc. (Seattle, WA); the rabbit anti-human CYP2C8/9/19 and anti-human UGT1A1 were from Millipore (Temecula, CA); the rabbit anti-human CYP3A4, anti-human GSTA1 and anti-human SULT1A1 were purchased from GeneTex Inc. (Irvine,

CA). The peroxidase conjugated goat anti-rabbit IgG and the molecular weight marker ChemiBlot™ were obtained from Millipore (Temecula, CA). The chemiluminescence kit SuperSignal West Pico Chemiluminescent Substrate was from Pierce Chemical (Rockford, IL).

6.2.2 Animal phase and tissues sampling

Complete protocols adopted during the animal phase and tissue sampling has been previously described on chapter 5.2.2 of the present thesis (page 39).

6.2.3 Total RNA extraction and reverse-transcription

The experimental procedures are reviewed on chapter 5.2.3 (page 39).

6.2.4 Quantitative Real Time RT-PCR

Oligonucleotide primers of internal control genes and target DMEs and NRs were taken from previously published studies, except for those of HNF4α, that were designed *ex novo*. Briefly, *Bos taurus* coding sequences were obtained from GenBank website [http://ncbi.nlm.nih.gov/] and best primer pairs for amplification were chosen by using the Primer Express™ Software 3.0 (Applied Biosystems, Foster City, CA, USA). This same software as well as OligoAnalyzer 3.1 [http://eu.idtdna.com/analyzer/applications/oligoanalyzer/default.aspx] were used to confirm the absence of primer dimers and hairpin formation. A careful validation of primer pairs was undertaken to be sure that only the target gene sequence was amplified. Primers specificity was checked *in silico* by using the NCBI Nucleotide Basic Local Alignment Search Tool and Primer- Nucleotide Basic Local Alignment Search Tool [http://blast.ncbi.nlm.nih.gov/Blast.cgi]; furthermore, to avoid amplification of potentially contaminating genomic DNA, one of the two primers was designed spanning an exon-exon junction. Finally, the presence of specific amplification products was confirmed by agarose gel electrophoresis and dissociation curve after qPCR reaction. Primers pair was optimized in the 300-900 nM range to identify primers concentration providing the highest sensitivity. Calibration curves were obtained following the amplification of decreasing amounts of different cDNA pools. In particular, two pools were prepared according to preliminary information about gene amplification in target tissues (data not shown). The former included liver, small intestine, adrenal gland and testis, the second one kidney, lung and skeletal muscle. To avoid inaccurate results, tissues in which target genes were not constitutively expressed or, alternatively, showed mean cycle threshold (C_t) values over 30 or very close to the limit of quantification of the thermal cycler were not taken into consideration. Pooled cDNA diluted at 3- or 4-fold intervals were used to

evaluate qPCR performances, such as the PCR efficiency (E_x) determined by using the equation $E_x = 10^{-1/slope}$, the sensitivity assay as well as the test of linearity correlation. Only E_x values comprised between 1.9 (90% of efficiency) and 2.1 (110% of efficiency) as well as high correlation coefficients ($0.970 \leq R^2 \leq 1.000$) were accepted. Primers references or oligonucleotide sequences of internal control genes (ICGs), target DMEs and NRs are listed in Table 10. The geometric mean of β-actin (ACTB), β-2-microglobulin (B2M) and ribosomal protein, large P0 (RPLP0) Ct values was used for data normalization. These ICGs were chosen within a set of candidates genes that were processed by using the geNorm^{PLUS} algorithm [http://www.biogazelle.com/mybiogazelle/].

The qPCR was performed on 5 µL of 100-fold diluted cDNA in a final volume of 20 µL, by using the Power SYBR®Green PCR Master Mix and an ABI-Prism 7000 thermal cycler (Applied Biosystems, Foster City, CA) under standard qPCR conditions. The $\Delta\Delta C_t$ method (Livak & Schmittgen, 2001) was used to analyze data.

Gene acronym	GenBank ID	Primer reference or sequence (5’ → 3’)	Amplicon size (bp)
NR1I2	NM_001103226	Cantiello et al. 2009	68
NR1I3	NM_001079768	Cantiello et al. 2009	63
NR2A1	NM_001015557	*forward*: 5’-TTGACGATGGGCAATGACACA-3’ *reverse*: 5’-GCACTGACACCCAGGCTGTT-3’	74
NR2B1	XM_881943	Cantiello et al. 2009	120
CYP2B22	NM_001075173	Giantin et al. 2008	80
CYP2C31	XM_600421	Giantin et al. 2008	56
CYP2C87	XM_612374	Giantin et al. 2008	71
CYP2C88	NM_001076051	Giantin et al. 2008	51
CYP3A	NM_174531	Cantiello et al. 2009	77
UGT1A1-*like*	NM_001105636	Giantin et al. 2008	71
GSTA1-*like*	NM_001078149	Giantin et al. 2008	84
SULT1A1-*like*	NM_177521	Lopparelli et al. 2010	84
SULT2A1-*like*	NM 001046353	Lopparelli et al. 2011	81
ACTB	NM_173979	Toffolatti et al. 2006	84
RPLP0	NM_001012682	Robinson et al. 2007	227
B2M	NM_173893	*forward*: 5’-TCGTGGCCTTGGTCCTTCT-3’ *reverse*: 5’-AATCTTTGGAGGACGCTGGAT-3’	71

Table 10: GenBank accession numbers, oligonucleotide sequences or references of primers used for qPCR, and amplicon size.

6.2.5 Immunoblotting

Subcellular fractions were isolated from CTRL and PHEN tissue aliquots by differential centrifugation according to Pegolo et al. (2010) and then stored at -80°C until analysis. Cytosolic and microsomal protein amounts were determined by using the Qubit®Fluorometer and the Quant-iT® Protein Assay Kit. Proteins (50 μg) were initially separated on NuPAGE® Novex® 4-12% Bis-Tris Gels by using the XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen, Eugene, Oregon) and, then, transferred onto nitrocellulose filters through the iBlot™ Dry Blotting System, consisting in the iBlot™ Gel Transfer Stack, Nitrocellulose, Mini and the iBlot™ Gel Transfer Device (Invitrogen™, Eugene, Oregon). Next, membranes were firstly incubated with rabbit anti-human CYP2B6 (1:1000 final dilution), CYP2C8/9/19 (1:7500), CYP3A4 (1:1500), UGT1A1 (1:3000), GSTA1 (1:1000) and SULT1A1 (1:1000) polyclonal antibodies and, subsequently, with peroxidase-conjugated donkey anti-rabbit IgGs (1:6000 final dilution for membranes probed with antibodies raised against CYP2B6, CYP3A4, UGT1A1, GSTA1 and SULT1A1, and 1:10000 for the one probed with anti-CYP2C8/9/19). Peroxide conjugates were detected by means of a chemiluminescence kit according to the manufacturer's instructions. On each minigel, a positive control sample (liver microsomal proteins obtained from a PB-induced rat) as well as a molecular marker were blotted, too. Immunopositive bands were captured by the Photo Studio 5 version 5.0.0.53 software for the Canon CanoScan Lide 20 (LED indirect exposure) scanner, and their optical density was analyzed by the ImageJ 1.44p image analysis software.

6.2.6 Statistical analysis

Data concerning the constitutive expression of target genes in extrahepatic tissues as well as their possible modulation after PB administration (relative quantification, arithmetic means ± standard deviation, SD) were expressed as the fold change normalized to the $\Delta\Delta C_t$ mean values respectively of liver and CTRL group, to whom an arbitrary value of 1 was assigned.

Immunoblotting densitometric data (mean arbitrary units, a.u. ± SD) were normalized to the integrated density mean value of CTRL, to whom an arbitrary value of 1.00 was assigned. Results (a.u. ± S.D.) were finally analyzed by using the Student's *t*-test (GraphPad InStat 3, San Diego, California, USA). A p value < 0.05 was considered as statistically significant.

6.3 RESULTS

6.3.1 Validation of qPCR assay

Quantitative real-time RT-PCR primers gave rise to specific amplicons; furthermore, a distinct dissociation peak and single bands of the expected size in agarose gel electrophoresis were obtained. Efficiencies values comprised between 1.931 and 2.092 and high linear regression coefficients ($0.972 \leq R^2 \leq 0.999$) were recorded for each qPCR assay (see Table 11).

6.3.2 Constitutive expression of DMEs and NRs in cattle extrahepatic tissues

To assist the reader's understanding, extrahepatic gene expression profiles of target NRs, CYPs and conjugative enzymes were compared with liver ones. Both NR1I2 and the retinoid X receptor alpha (RXRα, gene symbol NR2B1) were constitutively expressed, at analyzable levels, in all target tissues; in addition, high amounts of NR2B1 mRNA were noticed in the lung. As regards NR1I3 and NR2A1, their mRNA was detected only in liver, small intestine, kidney and, only for NR2A1, in testis (see Figure 10.A).

As expected, target DMEs were mostly expressed in the liver except for CYP2B22, for which higher mRNA levels were found in the lung. The greatest number of CYP mRNAs were detected in the small intestine (CYP2B22, 2C87, 2C88, 3A), followed by lung (CYP2B22, 2C31 and 2C88), testis (CYP2B22, 2C31 and 3A), kidney (CYP2B22 and 2C87), adrenal (CYP2B22 and 2C31) and muscle (CYP2B22 only: see Figure 10.B). Among SULT1A1-, SULT2A1- and GSTA1-*like* genes, the former was by far the gene more constitutively expressed in extrahepatic tissues and particularly in the lung, where its mRNA levels were comparable to the hepatic ones. On the contrary, the UGTA1-*like* gene was detected only in small intestine, testis and adrenal (see Figure 10.C).

Assay	cDNA pool	Slope	E_x	R^2	Linearity range (C_t)
NR1I2	Lv. I. T. A.	-3.25	2.031	0.998	24.35 – 34.29
	Ln. K. M.	-3.12	2.092	0.972	29.29 – 33.04
NR1I3	Lv. I.	-3.30	2.009	0.996	25.43 – 33.31
	K.	-3.27	2.022	0.998	27.68 – 33.66
NR2B1	Lv. I. T. A.	-3.30	2.009	0.994	23.90 – 36.00
	Ln. K. M.	-3.29	2.013	0.986	24.61 – 30.47
NR2A1	Lv. I. T. A.	-3.33	1.997	0.988	23.97 – 30.1
	Ln. K. M.	-3.48	1.938	0.990	25.84 – 34.15
CYP2B22	Lv. I. T. A.	-3.40	1.968	0.999	19.98 – 32.21
	Ln. K. M.	-3.35	1.988	0.996	23.50 – 33.30
CYP2C31	Lv. I. T. A.	-3.25	2.031	0.992	23.26 – 31.04
	Ln.	-3.32	2.001	0.995	28.53 – 32.52
CYP2C87	Lv. I. T.	-3.35	1.988	0.982	23.36 – 31.68
CYP2C88	Lv. I. A.	-3.42	1.961	0.995	23.85 – 32.17
	Ln.	-3.16	2.072	0.987	28.36 – 36.19
CYP3A	Lv. I. T.	-3.50	1.931	0.988	20.08 – 30.35
UGT1A1-*like*	Lv. I. T. A.	-3.30	2.009	0.990	24.10 – 34.11
GSTA1-*like*	Lv. I. T. A.	-3.33	1.997	0.993	19.70 – 29.82
	Ln. K. M.	-3.28	2.018	0.996	24.09 – 32.04
SULT1A1-*like*	Lv. I. T. A.	-3.35	1.988	0.998	21.45 – 33.68
	Ln. K. M.	-3.21	2.049	0.993	24.42 – 30.16
SULT2A1-*like*	Lv. I. T. A.	-3.21	2.049	0.998	23.34 – 32.96
	Ln. K. M.	-3.15	2.077	0.993	28.15 – 31.95
ACTB	Lv. I. T. A.	-3.39	1.972	0.993	18.2 – 26.17
	Ln. K. M.	-3.51	92.7	0.995	23.24 – 28.45
RPLP0	Lv. I. T. A.	-3.35	98.8	0.990	19.61 – 26.76
	Ln. K. M.	-3.28	101.8	0.987	21.53 – 28.46
B2M	Lv. I. T. A.	-3.32	100.1	0.993	18.61 – 25.07
	Ln. K. M.	-3.32	100.1	0.985	23.36 – 29.43

Table 11: Main features, namely slope, efficiency (Ex), linear regression coefficients (R2) and linear dynamic range (cycle threshold, Ct), of each qPCR assays, measured by using two different tissue pools (liver, intestine, adrenal gland and testis; kidney, lung and skeletal muscle). Tissues in which target genes were not constitutively expressed or showed Ct values over 30 (or close to thermal cycler limit of quantification) were not taken into consideration. Lv, liver; I, small intestine; T, testis; A, adrenal; Ln, lung; K, kidney; M, muscle.

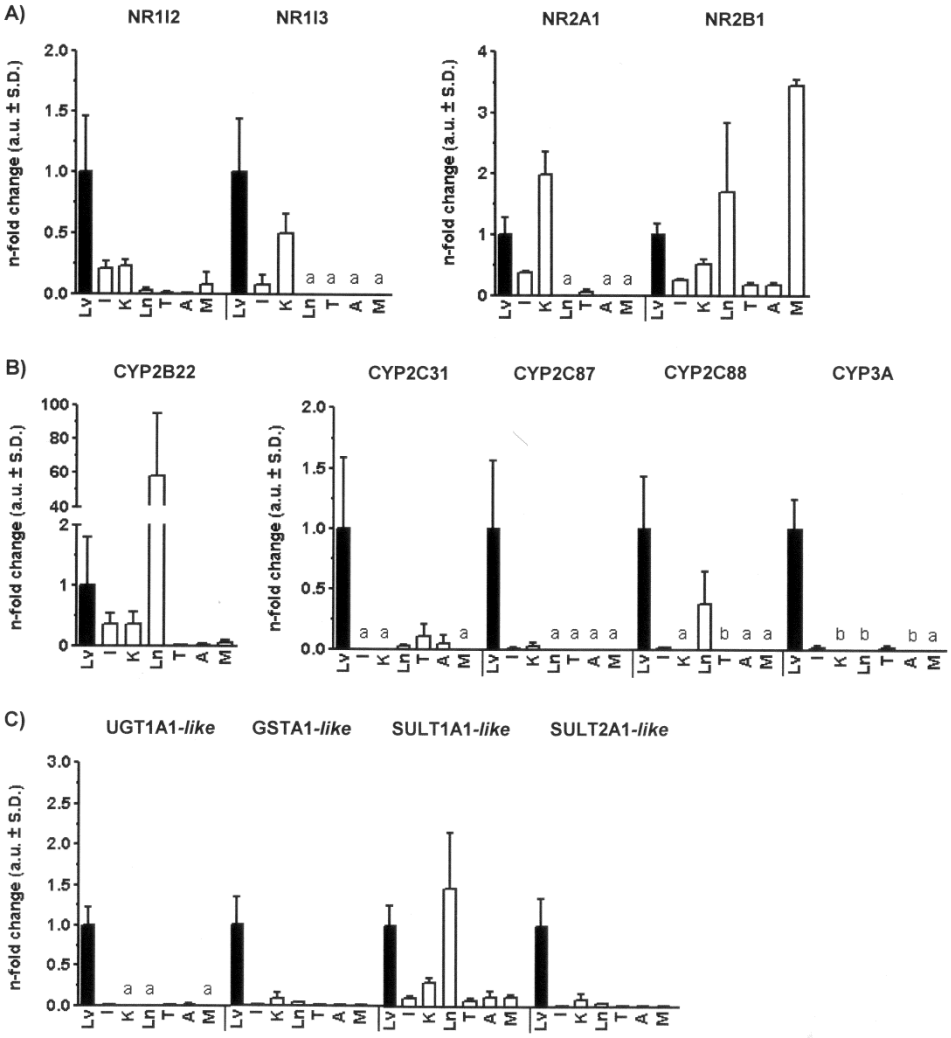

Figure 10: Constitutive expression of target genes, namely NRs (A), CYPs (B) and conjugative DMEs (C) in small intestine, kidney, lung, testis, adrenal and muscle of control cattle (n = 3). Data of relative quantification (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to ΔΔCt mean value of liver tissue, to whom an arbitrary value of 1 was assigned.

6.3.3 Effects of PB upon target gene mRNAs

Phenobarbital did not cause a transcriptional effect upon target NRs; by contrast, a modulation of a number of genes coding for oxidative and conjugative DMEs was observed in liver and, merely for SULT1A1-*like*, in the small intestine (see Table 12). No differences in candidate gene expression profiles were ever noticed between CTRL and PHEN in the other extrahepatic tissues here considered (see Tables 13 and 14).

Hepatic CYP2B22, 2C31, 2C87 and 3A mRNA levels were significantly up-regulated by PB ($p<0.001$, $p<0.001$, $p<0.01$ and $p<0.001$, respectively: see Table 12). As a result, CYP2B, 2C and 3A apoprotein amounts were increased in the same way ($p<0.01$, $p<0.001$ and $p<0.01$, respectively: see Figure 11). With regard to conjugative DMEs, GSTA1- and UGT1A1-*like* gene expression was induced in the liver ($p<0.05$ and $p<0.01$, respectively); on the contrary, SULT1A1-*like* transcript was down-regulated ($p<0.05$) at the small intestine level (Table 12). Immunoblotting investigations confirmed the hepatic UGT1A1-*like* gene up-regulation ($p<0.01$) as well as the intestinal SULT1A1-*like* mRNA decrease ($p<0.05$; see Figure 12).

Target gene	n-fold change (a.u.) ± S.D.			
	Liver		Small intestine	
	CTRL	PHEN	CTRL	PHEN
NR1I2	1.00 ± 0.46	0.99 ± 0.09	1.00 ± 0.24	0.73 ± 0.29
NR1I3	1.00 ± 0.44	0.90 ± 0.14	1.00 ± 1.05	0.37 ± 0.26
NR2A1	1.00 ± 0.28	1.19 ± 0.11	1.00 ± 0.03	0.84 ± 0.35
NR2B1	1.00 ± 0.17	1.02 ± 0.16	1.00 ± 0.10	0.76 ± 0.20
CYP2B22	1.00 ± 0.78	90.51 ± 19.66***	1.00 ± 0.51	0.53 ± 0.36
CYP2C31	1.00 ± 0.58	6.03 ± 0.99***	D.N.Q.	
CYP2C87	1.00 ± 0.57	3.73 ± 0.59**	1.00 ± 0.51	0.60 ± 0.20
CYP2C88	1.00 ± 0.43	1.29 ± 0.40	1.00 ± 0.43	1.17 ± 0.82
CYP3A	1.00 ± 0.25	2.67 ± 0.29***	1.00 ± 0.68	0.29 ± 0.25
UGT1A1-*like*	1.00 ± 0.22	1.62 ± 0.16**	1.00 ± 0.27	1.12 ± 0.71
GSTA1-*like*	1.00 ± 0.42	2.75 ± 0.94*	1.00 ± 0.83	1.05 ± 1.24
SULT1A1-*like*	1.00 ± 0.25	0.71 ± 0.21	1.00 ± 0.17	0.65 ± 0.12*
SULT2A1-*like*	1.00 ± 0.34	1.59 ± 0.55	1.00 ± 1.10	0.61 ± 0.34

*Table 12: Nuclear receptors, CYPs and conjugative DMEs mRNA relative abundances in liver and small intestine of cattle orally administered with PB. Data (arithmetic means ± S.D.) are expressed as n–fold change (normalized to $\Delta\Delta C_t$ mean value of the respective control group to whom an arbitrary value of 1 was assigned). *,**,***: significant differences (p<0.05, 0.01 and 0.001, respectively) vs CTRL (Student's t-test). D.N.Q., detected but not quantifiable.*

Target gene	n-fold change (a.u.) ± S.D.			
	Kidney		Lung	
	CTRL	PHEN	CTRL	PHEN
NR1I2	1.00 ± 0.26	2.76 ± 2.86	1.00 ± 0.64	1.68 ± 0.92
NR1I3	1.00 ± 0.33	2.26 ± 1.99	D.N.Q.	
NR2A1	1.00 ± 0.20	1.36 ± 1.03	D.N.Q.	
NR2B1	1.00 ± 0.16	1.36 ± 0.88	1.00 ± 0.68	0.59 ± 0.05
CYP2B22	1.00 ± 0.54	0.68 ± 0.44	1.00 ± 0.63	0.73 ± 0.73
CYP2C31	D.N.Q.		1.00 ± 0.30	13.91 ± 14.86
CYP2C87	D.N.Q.		D.N.Q.	
CYP2C88	D.N.Q.		1.00 ± 0.74	0.55 ± 0.62
CYP3A	N.D.		N.D.	
UGT1A1-*like*	D.N.Q.		D.N.Q.	
GSTA1-*like*	1.00 ± 0.22	1.16 ± 0.53	1.00 ± 0.56	3.41 ± 1.99
SULT1A1-*like*	1.00 ± 0.19	1.25 ± 0.45	1.00 ± 0.46	0.76 ± 0.58
SULT2A1-*like*	1.00 ± 0.98	0.54 ± 0.57	1.00 ± 0.21	1.63 ± 1.79

Table 13: Nuclear receptors, CYPs and conjugative DMEs mRNA relative abundances in kidney and lung of cattle orally administered with PB. Data (arithmetic means ± S.D.) are expressed as n–fold change (normalized to $\Delta\Delta C_t$ mean value of the respective control group to whom an arbitrary value of 1 was assigned). D.N.Q., detected but not quantifiable; D.N., not detected.

Target gene	n-fold change (a.u.) ± S.D.					
	Testis		Adrenal		Muscle	
	CTRL	PHEN	CTRL	PHEN	CTRL	PHEN
NR1I2	1.00 ± 0.85	1.45 ± 1.01	1.00 ± 0.35	0.61 ± 0.45	1.00 ± 1.12	0.30 ± 0.18
NR1I3	D.N.Q.		D.N.Q.		D.N.Q.	
NR2A1	1.00 ± 0.80	1.06 ± 1.00	D.N.Q.		D.N.Q.	
NR2B1	1.00 ± 0.29	0.96 ± 0.44	1.00 ± 0.29	0.67 ± 0.02	1.00 ± 0.03	1.16 ± 0.52
CYP2B22	1.00 ± 0.57	0.96 ± 0.32	1.00 ± 1.03	0.36 ± 0.33	1.00 ± 0.86	2.93 ± 2.04
CYP2C31	1.00 ± 0.87	1.18 ± 1.0	1.00 ± 1.38	0.02 ± 0.02	D.N.Q.	
CYP2C87	1.00 ± 1.48	0.25 ± 0.18	D.N.Q.		D.N.Q.	
CYP2C88	N.D.		D.N.Q.		D.N.Q.	
CYP3A	1.00 ± 0.83	0.98 ± 0.87	N.D.		D.N.Q.	
UGT1A1-*like*	D.N.Q.		1.00 ± 0.81	0.09 ± 0.02	D.N.Q.	
GSTA1-*like*	1.00 ± 0.83	0.51 ± 0.22	1.00 ± 0.49	0.54 ± 0.63	1.00 ± 0.85	0.47 ± 0.14
SULT1A1-*like*	1.00 ± 0.56	0.41 ± 0.10	1.00 ± 0.66	1.23 ± 1.25	1.00 ± 0.26	0.90 ± 0.14
SULT2A1-*like*	1.00 ± 0.73	0.59 ± 0.22	1.00 ± 1.05	0.07 ± 0.01	1.00 ± 0.39	3.96 ± 1.94

Table 14: Nuclear receptors, CYPs and conjugative DMEs mRNA relative abundances in kidney and lung of cattle orally administered with PB. Data (arithmetic means ± S.D.) are expressed as n–fold change (normalized to $\Delta\Delta C_t$ mean value of the respective control group to whom an arbitrary value of 1 was assigned). D.N.Q., detected but not quantifiable; D.N., not detected.

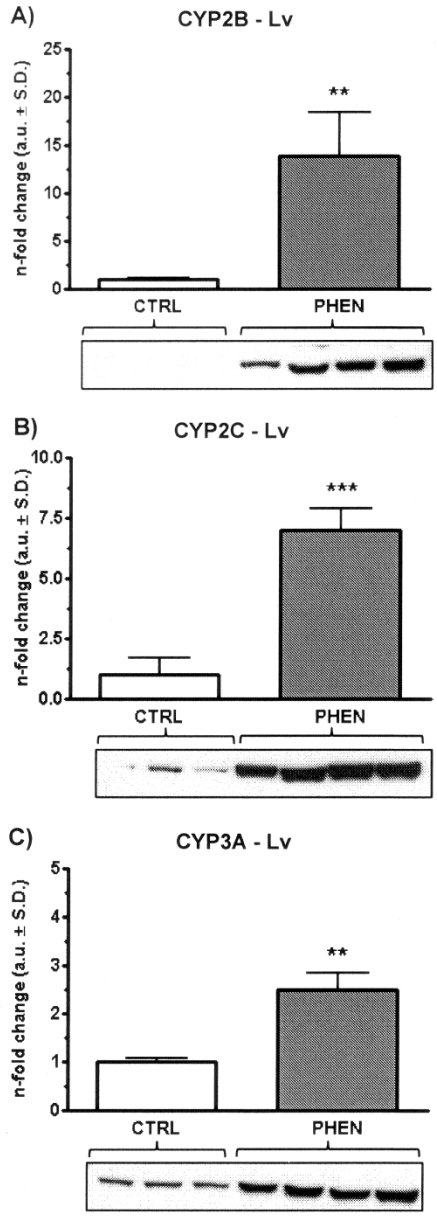

*Figure 11: Hepatic CYP2B (A), 2C (B) and 3A (C) immunoblotting. Densitometric data (mean arbitrary units, a.u. ± SD) from PHEN group microsomal proteins were normalized to the integrated density mean value of CTRL, to whom an arbitrary value of 1.00 was assigned. Results are expressed as mean a.u. ± S.D. **: significant differences ($p<0.01$) vs CTRL (Student's t-test); ***: significant differences ($p<0.001$) vs CTRL (Student's t-test). Lv, liver.*

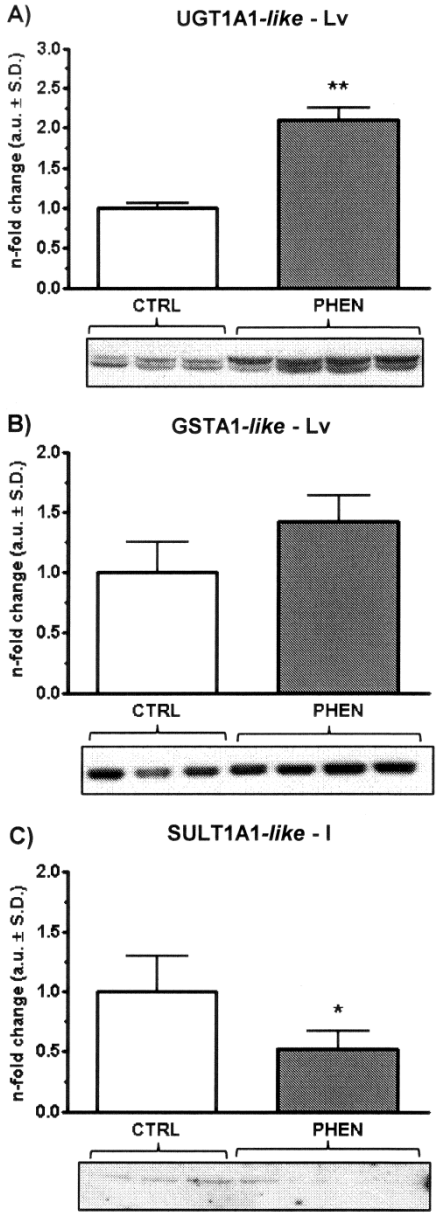

Figure 12: Hepatic GSTA1-like (A), UGT1A1-like (B) and small intestinal SULT1A1-like (C) immunoblotting. Densitometric data (mean arbitrary units, a.u. ± SD) from PHEN group microsomal proteins were normalized to the integrated density mean value of CTRL, to whom an arbitrary value of 1.00 was assigned. Results are expressed as mean a.u. ± S.D. **: significant differences ($p<0.05$) vs CTRL (Student's t-test);* ***: significant differences ($p<0.011$) vs CTRL (Student's t-test). Lv, liver; I, small intestine.*

6.4 DISCUSSION

Drug metabolizing enzymes constitute a complex network of enzymes whose prevailing roles are detoxifying xenobiotics to whom the living organisms are exposed during lifetime as well as to participate in the biosynthesis and catabolism of endogenous substrates with relevant physiological functions. The organ mostly endowed of DMEs is the liver, which represent the hinge of drug metabolism; however, a number of extrahepatic tissues constitutively express DMEs; among these ones, gastrointestinal tract, kidney, lung, adrenal, gonads, and brain (Pelkonen et al., 2008). In the past decade, DMEs have been extensively studied in human and model species, thereby improving knowledge about their expression and regulation phenomena. In this respect, a milestone in the research on regulation of DMEs was the discovery of NRs gene superfamily, a number of key ligand-activated transcriptional regulators of DMEs involved also in several physiological functions (di Masi et al., 2009).

Cattle represent a relevant food-producing species worldwide, but the knowledge about DMEs expression and their regulatory mechanisms, likewise to other species of veterinary interest, is still incomplete and largely based on post-transcriptional approaches (Ioannides, 2006; Fink-Gremmels, 2008; Antonovic and Martinez, 2011). Nevertheless, the release of cattle genome and methodological advances in the field of bioinformatics and transcriptomics have now provided novel tools for studying more in depth the molecular biology of cattle DMEs and NRs. As a consequence, few papers about the constitutive expression of DMEs and NRs in cattle liver and extrahepatic tissues as well as their transcriptional modulation either caused by some constitutional (i.e., breed, gender, age) and dietary factors or following the exposure to xenobiotics (induction/inhibition phenomena) have been recently published or shortly reviewed (Darwish et al., 2010; Dacasto et al., 2011). In the present study, the constitutive expression of PB-responsive DMEs and NRs was initially characterized in foremost cattle extrahepatic tissues, thereby enforcing the knowledge about the comparative drug metabolism; on a second instance, the effects of PB orally administered at inducing dosage regimens were explored in these same tissues and target genes, to unveil a potential noxious transcriptional effect upon cattle overall biotransformation capacity.

As mentioned above, NRs are ubiquitous transcription factors involved in the regulation of DMEs; mechanistically speaking, they make possible the regulation of gene expression at the transcriptional level, following the exposure to a xenobiotic/endogenous ligand (Tolson and Wang, 2010). In general, few data about NRs expression in extrahepatic tissues have been published in cattle so far (Krüger et al., 2005; Lopparelli et al., 2010). In the present study, major interest was addressed to NRs that transcriptionally activate

CYP2B, 2C and 3A promoters following the exposure to PB-*like* compounds (Xu, 2005; Bell and Michalopoulos, 2006; Chen and Goldstein, 2009). Apart from the liver (showing highest mRNAs amounts), the NR1I2 gene was mostly expressed in the gastrointestinal tract and kidney, while lower mRNA levels were found in other extrahepatic tissues (I~K>M>Ln>A~T). These results agree with previously published data referring to humans, rat, pig and cattle as well (Zhang et al., 1999; Nishimura et al., 2004; Nannelli et al., 2008; Xu et al., 2009; Lopparelli et al., 2010). A similar behavior was noticed for NR1I3, another key NR involved in the PB-dependent transcriptional activation of many DMEs; this gene was highly expressed in cattle kidney and gastrointestinal tract (K>I), while it was not quantifiable in the other tissues here examined. This pattern of expression would confirm preceding human and pig data (Baes et al., 1994; Nishimura et al., 2004; Nannelli et al., 2008), albeit species-differences have been noticed in the extrahepatic constitutive expression of NR1I3; as an example, the NR1I3 mRNA was noticed in the rat lung but not in that of mouse and rabbit (Chirulli et al., 2005). Interestingly, also NR2A1 has a role in the regulation of DMEs that have NR2A1-binding sites (Bell and Michalopoulos, 2006; Kamiyama et al., 2007). Likewise to humans, in which this xenosensor is expressed in liver, kidney, intestine and pancreas (Drewes et al., 1996; Jiang et al., 2003; Nishimura et al., 2004; Bell and Michalopoulos, 2006), evident NR2A1 mRNA amounts were noticed in cattle gastrointestinal tract and kidney; moreover, renal NR2A1 gene expression was higher than the liver one (K>Lv>I). The expression of this xenosensor in extrahepatic tissues is still largely unknown, but the presence of tissue-specific variation in NR2A1 gene expression has recently been hypothesized in rodents (Dean et al., 2010). Finally, the heterodimerizing partner NR2B1 was the NR undoubtedly more expressed in cattle extrahepatic tissues; moreover, higher mRNA amounts, when compared to liver ones, were noticed in skeletal muscle and lung (M>Ln>Lv>K>I~T~A). While a large interindividual variation was observed in the lung, thereby preventing further consideration, skeletal muscle transcriptional data are consistent with human ones (Nishimura et al., 2004).

Mammals express multiple CYPs in liver as well as in a variety of other tissues, including kidney, lung, adrenal, gonads, brain and many other ones. In extrahepatic tissues, the expression of CYPs appears to be very low if compared with the predominant expression in adult liver, and they are usually associated with a specific physiological role; nevertheless, it is believed that their modulation may affect the local disposition of xenobiotic or endogenous compounds, thus altering their effects (Seliskar and Rozman, 2007; Pavek and Dvorak, 2008). Cattle liver constitutively express multiple CYP mRNAs (Giantin et al., 2008; Giantin et al., 2010); in contrast (and similarly to NRs), a small

number of transcriptional data about CYPs gene expression in cattle extrahepatic tissues have been published so far (Grasso et al., 2005; Krüger et al., 2005; Lopparelli et al., 2010). In human liver and extrahepatic tissues, CYP2B genes are expressed constitutively and inducible (Nishimura et al., 2003; Bièche et al., 2007). In cattle, CYP2B22 was mostly expressed in lung and liver, followed by gastrointestinal tract and kidney; finally, barely detectable mRNA levels were noticed in muscle, adrenal and testis (Ln>Lv>I~K>M~T~A). Remarkable was the CYP2B22 constitutive expression in the lung, where it reached a value up to 20-fold the liver one. This finding is consistent with previous comparative data indicating CYP2B as the most abundant CYP isoform in pulmonary tissue of mammals, whose amount might be equal to the liver or even higher (Hukkanen et al., 2001; Chirulli et al., 2005; Bièche et al., 2007; Nannelli et al., 2008; Darwish et al., 2010). Such a result was not astonishing; in fact, the respiratory tract is exposed either to inhaled or systemic xenobiotics, and a number of pneumotoxic chemicals undergo metabolic activation through pulmonary CYPs (Lee et al., 1998; Hynes et al., 1999; Ding and Kaminsky, 2002). Data about CYP2B22 mRNA levels in the other extrahepatic tissues subject of investigation agree with those previously obtained either in cattle (limited to kidney and testis: Darwish et al., 2010; Lopparelli et al., 2010) than in the same human extrahepatic tissues (Nishimura et al., 2003; Bièche et al., 2007). In humans, four CYP2C isoforms (namely, CYP2C8, 2C9, 2C18, and 2C19) have been described. Altogether, CYP2Cs are primarily distributed in the liver, where they account for approximately 20% of total CYPs (2C9>2C8>2C19). Among human extrahepatic tissues, CYP2Cs are expressed to a variable extent in gut, kidney, testis, muscle, brain, and cardiovascular tissues, while contradictory results have been published for the lung (Klose et al., 1999; Ferguson et al., 2002; Nishimura et al., 2003; Bergheim et al., 2005; Bièche et al., 2007; Chen and Goldstein, 2009). Likewise to humans, cattle 2C31, 2C87 and 2C88 (corresponding to human CYP2C19, 2C9, and 2C18, respectively: Zancanella et al., 2010) were basically found in the liver. Furthermore, CYP2C87 mRNA was detected only in gastrointestinal tract and kidney (K>I), a finding that only partially match with human data, indicating CYP2C9 as a gene mostly expressed in intestine and kidney (I>K), followed by skeletal muscle, adrenal, and testis (Klose et al., 1999; Nishimura et al., 2003; Bièche et al., 2007). As regards CYP2C31 and 2C88, the former was for the most part expressed in testis, lung and adrenal (T>Ln>A), the second one only in the lung and gastrointestinal tract (Ln>I). Present results look inconsistent on a comparative basis: in fact, CYP2C18 and C19 are large distributed in gut and, to a lower extent, in kidney, lung, muscle, adrenal and testis (Nishimura et al., 2003; Bièche et al., 2007). Nevertheless, it should be emphasized that CYP2Cs exhibit species-differences in

their extrahepatic pattern of gene expression. What's more, different results about the extrahepatic distribution of human CYP2C have been published and finally, it has been recently shown that human CYP2Cs are not orthologous to corresponding cattle sequences (Klose et al., 1999; Tsao et al., 2001; Nishimura et al., 2003; Bièche et al., 2007; Chen and Goldstein, 2009; Zancanella et al., 2010). Cytochrome P450 3A represents the most abundant CYP subfamily in human liver and small intestine; in addition, it is expressed in a number of other extrahepatic tissues, such as kidney, lung, testis, adrenal and skeletal muscle (Nishimura et al., 2003, Burk and Wojnowski, 2004; Thelen and Dreissman, 2009). Four human CYP3A genes have been described so far: CYP3A4, 3A5, 3A7 and 3A43; among these ones, CYP3A4 represents the most consistently expressed and most abundant isoenzyme in adult liver and small intestine (Burk and Wojnowski, 2004; Thelen and Dressman, 2009); in contrast, CYP3A5 and 3A43 may be expressed at higher levels than CYP3A4 in some extrahepatic tissues (Daly, 2006). In the present study, the CYP3A gene was mostly distributed in liver, gastrointestinal tract and testis, according to human and cattle previously published data (limited to the gonad in cattle: Burk and Wojnowski, 2004; Thelen and Dressman, 2009; Lopparelli et al., 2010). By contrast, conflicting results (no expression) were obtained for kidney, lung and adrenal. There is an evidence for the expression of CYP3As in some cell types of human and equine lung (Larsson et al., 2003; Raunio et al., 2005) and kidney (Wojnowski, 2004; Bièche et al., 2007; DiMaio Knych et al., 2010); moreover, CYP3As measurable amounts were detected in the adrenal of human, non-human primate and miniature pig (Nishimura et al., 2003; Bièche et al., 2007; Nishimura et al., 2009; Shang et al., 2009; Uno et al., 2009). In general, there are still controversial results about the expression of CYP3As in tissues different from liver and gut; furthermore, in extrahepatic tissues CYP3A isoforms (i.e., CYP3A5) are prone to interindividual variation in gene expression (Burk and Wojnowski 2004; Daly, 2006). Altogether, these evidences might partly justify present cattle results.

Glucuronidation represents the primary phase II reaction and an essential detoxification pathway in humans. Liver is the organ in which most UGTs are expressed; however, some isoforms (namely, UGT1A7, 1A8 and 1A10) are distributed exclusively at the extrahepatic level, for the most part in the gut (Mohamed and Frye, 2011). In addition, human and rat organs mostly contributing either to xenobiotic first pass metabolism or excretion (liver, intestine and kidney, respectively) greatly express the UGT1A1 gene (Fisher et al., 2000; Shelby et al., 2003; Gregory et al., 2004; Nishimura and Naito, 2006; Nakamura et al., 2008; Bellemare et al., 2011). In the current study, the UGT1A1-*like* gene was mostly expressed in the liver and, to a lower extent, in gastrointestinal tract,

testis and adrenal. In contrast, measurable amounts of UGT1A1-*like* mRNA were never found in kidney. However, another UGT1A isoform (UGT1A6) has been shown to be strongly expressed in cattle kidney (Iwano et al., 2001); therefore, it should be infer that cattle UGTs tissue distribution support, to some extent, aforementioned comparative data.

Glutathione transferases play an essential role in detoxification processes both in animals and plants, catalyzing the conjugation of glutathione to electrophilic metabolites and usually yielding a product with a decreased reactivity (Forkert et al., 1999). According to previously published data (Rowe et al., 1997; Nishimura and Naito, 2006), the GSTA1-*like* gene was expressed to a greater extent in cattle liver and, at lower amounts, in extrahepatic tissues (K>Ln>I~T~A~M). By contrast, highest GSTA1-*like* mRNA levels (compared to liver ones) have been recently noticed in cattle kidney and testis (Darwish et al., 2010; Lopparelli et al., 2011). In this respect, GSTA has been shown to be essentially found in bovine steroidogenic cells (Rabahi et al., 1999); moreover, constitutional factors such as breed have been shown to affect GSTA1-*like* gene expression (Giantin et al., 2008).

The sulphotransferase superfamily contains five families of phase II DMEs (SULT1-5), in charge of the conjugation of a wide range of compounds; among these ones, members of SULT1 and 2 families recognized as substrates phenolic and hydroxysteroid compounds, respectively (Strott, 2002). Sulfotransferases tissue distribution has been extensively investigated in human and rodent laboratory species, whereas few data are actually available for veterinary species including cattle (Tsoi et al., 2001; Tsoi et al., 2002; Lin et al., 2004; Giantin et al., 2010; Lopparelli et al., 2010). The sulfotransferase 1A1 gene is primarily expressed in the liver, while species-differences in extrahepatic tissue distribution have been noticed. As a whole, the gastrointestinal tract (small intestine or colon), lung and kidney have been shown to express to a greater extent SULT1A1 (Dunn and Klaassen, 1998; Tsoi et al., 2002; Alnouti and Klaassen, 2006; Riches et al., 2009). In cattle, the SULT1A1-*like* gene was distributed, besides liver, in all the considered extrahepatic tissues (Ln>Lv>K>A~I~M>T); additionally, highest SULT1A1-*like* mRNA levels were found in lung and liver. Therefore, lung and kidney appear to be the principal extrahepatic tissues contributing to sulfation pathway, likewise to other species; by contrast, the small intestine would not play a relevant role. However, further studies about SULT1A1-*like* gene expression in gut, useful to clarify this contrasting result, are needed. Similar to SULT1A1-*like*, the SULT2A1-*like* gene was constitutively expressed in liver and extrahepatic tissues ((Lv>K>Ln> I~T~A~M). This pattern of gene expression basically reflect that previously reported in human and rodent model species (Alnouti and Klaassen, 2006; Riches et al., 2009). This gene codes for an

enzyme playing a key role in sex steroids biosynthesis, bile acid metabolism and detoxication (Aldred et al., 2000), and a similar role might be hypothesized for cattle, too. Altogether, in the present study the constitutive expression of known PB-responsive DMEs and NRs was demonstrated, for the first time and to the best of our knowledge, in cattle extrahepatic tissues. Albeit the individual variability in gene expression was not taken into account, it was quite evident that gene abundances were, with few exceptions, lower when compared to liver ones.

The term induction denotes a dose-dependent increase in DMEs expression (gene-protein) and function (catalytic activity). The magnitude of induction strongly depends on constitutional (i.e., genetic and physio-pathological), dietary and environmental factors. Such a phenomenon influence the xenobiotic drug metabolism, kinetics, drug-drug interactions, toxicity and carcinogenicity as well as the activity and disposition of endogenous compounds (i.e., hormones). Inducible drug metabolism was discovered more than 35 years ago, and five classes or prototypical inducers have been identified. One of them is PB, a prototype of a large group of structurally unrelated chemicals (PB-*like* compounds) that induce a number of hepatic DMEs (mostly CYP2B, 2C, 3A and transferases). Phenobarbital induction, whose main feature is its pleiotropicity, is observed in a wide number of species ranging from bacteria to humans. In vertebrates, the induction occurs predominantly in the liver, intestine and, to a lower extent, in other extrahepatic tissues, i.e. kidney and lung (Kakizaki et al., 2003; Denison and Whitlock, 1995; Goriya et al., 2005). Actually, much is known about the molecular mechanisms by which PB and PB-*like* compounds induce DMEs; following the exposure to the barbiturate, the up-regulation of target gene transcription is mostly driven by NR1I3 and by NR1I2, but many questions regarding this NR activation by xenobiotics in different animal species is still subject of debate (Pustylnyak et al., 2005; Kodama and Negishi, 2006; Yamada et al., 2006; Audet-Walsh et al., 2009; Tamasi et al., 2009). Few papers about the *in vivo* PB-dependent transcriptional up-regulation of DMEs in veterinary species have been published, and none in cattle (van't Klooster et al., 1993; Chirulli et al., 2005; Goriya et al., 2005; Marini et al., 2007; Makino et al., 2009).

In the present study, PB never affected target NRs gene expression in liver and extrahepatic tissues. Basically, the transcriptional activation of PB-responsive genes is usually mediated by NR1I3 and NR1I2 (Tamasi et al., 2009); however, PB can modestly up-regulate also NR2A1 by means of similar, albeit independent of NR1I3 and NR1I2, molecular mechanisms (Bell and Michalopoulos, 2006). The cytochrome P450 2B induction is strictly related to the corresponding mRNA levels of NR1I3 in liver and extrahepatic tissues (Pustylnyak et al., 2009). Nonetheless, present cattle results were not

astonishing. Significant species-differences in the induction of hepatic DMEs have been reported, and NRs are considered as the molecular basis of such an event (Graham and Lake, 2008). Furthermore, the presence of tissue-differences in DMEs regulation has been hypothesized (Marini et al., 2007). The aforementioned specie-differences in CYP2B response to PB and some PB-*like* compounds may occur through variations in NR ligand binding domain sequences as well as in NR1I3/NR1I2-dependent transcriptional activation of target genes (Kiyosawa et al., 2008; Pustylnyak et al., 2009; Kojima et al., 2011). On a second instance, dose- and time-dependent effects of PB upon NR1I3 and NR1I2 genes have been observed (Lambert et al., 2009; Zheng et al., 2011); in particular, NR1I3 mRNA levels increased with PB concentration, while NR1I2 gene expression was unchanged even at higher barbiturate concentrations (Lambert et al., 2009). What's more, a similar behavior (no effect upon NR1I2, 1I3, 2A1 and 2B1 genes) was noticed in veal calves administered at growth promoting purposes with low doses of dexamethasone (a prototypical CYP3A inducer: Cantiello et al., 2009). In this respect, marked species- and dose-dependent differences in NR1I2/NR1I3 transactivation and consequent CYP3A induction have been observed in the presence of different CYP3A inducers, such as dexamethasone, rifampicin and pregnenolone-16alpha-carbonitrile (Burk and Wojnowski, 2004; Luo et al., 2004; Kojima et al., 2011). Collectively, previous and present results suggest that basic regulatory molecular mechanisms by which NR ligands transactivate cattle NR1I3/NR1I2/NR2A1/NR2B1 genes network and increase the transcription of target genes need further basic and applied clarifying molecular studies.

Since early 60s, it has become evident that PB and PB-*like* compounds elicit pleiotropic effects on liver function, but PB is best known for the induction of genes coding for oxidative and conjugative DMEs, such as CYP2B, 2C, 3A, UGTs and GSTs (Jones et al., 1998; Kakizaki et al., 2003; Kodama and Negishi, 2006). In particular, PB has been shown to induce hepatic CYP gene expression profiles in human and rodent model species (Joannard et al., 2000; Wagner et al., 2005; Pelkonen et al., 2008) as well as in rabbit (Chirulli et al., 2005), goat (van't Klooster et al., 1993), pig (Puccinelli et al., 2010), chicken (Goriya et al., 2005) and dog (Makino et al., 2009). Just preliminary post-translational data have been published in cattle (Cantiello et al., 2006). In the current study, PB significantly increased primarily cattle hepatic CYP2B22 mRNA levels (about 90-fold the CTRL value), followed by 2C31, 2C87 and 3A, likewise to humans and rodents (Waxman and Azaroff, 1992; Handschin and Meyer, 2003). Noteworthy, the PB transcriptional effect upon CYP mRNAs was reflected at the protein level, as

demonstrated by the significant augmentation of CYP2B, 2C and 3A apoprotein amounts. Moreover, catalytic activity assays confirmed gene/protein results (Cantiello et al., 2006).

Besides CYPs, some phase II DMEs have been shown to be modulated by PB, and UGT1A1 is perhaps the most important among these ones. Phenobarbital up-regulates the UGT1A1 gene in humans and rodents (Smith et al., 2005; Olinga et al., 2008; Buckley and Klaassen 2009), and such an effect is regulated by the same transcriptional pathway (through NR1I3: Sugatani et al., 2001). Likewise to humans (Smith et al., 2005; Olinga et al., 2008), PB significantly increased cattle UGT1A1-*like* mRNA and apoprotein amounts. A similar behavior was noticed for GSTA1-*like*, for which a significant increase of its mRNA (two-fold the CTRL value) was noticed and confirmed, albeit not in a statistically significant way, at the protein level. These findings agree with data previously published for rodents (Langouet et al., 1996; Bulera et al., 2001; Ejiri et al., 2005). Furthermore, present UGT1A1-*like* and GSTA1-*like* data did not find confirmation at the catalytic activity level (Cantiello et al., 2006). In contrast, hepatic SULTs were never modulated by PB. In general, murine SULT1A1 and 2A1/2 mRNA expression is not affected by PB or PB-*like* compounds (Alnouti and Klaassen, 2008), while diverse PB dosages (35, 80 and 100 mg/kg for 3 days) elicited an dose-dependent inhibitory effect upon SULT1/2 transcription in the rat (Runge-Morris et al., 1998). Thus, present transcriptional results, together with the inhibitory trend noticed by using an anti-rat SULT1A1 antibody (data not shown) would be consistent with abovementioned rodent data.

Besides liver, PB has been shown to increase transcription of DMEs in the intestine and, to a lower extent, in other extrahepatic tissues (Denison and Whitlock, 1995; Kakizaki et al., 2003; Goriya et al., 2005; van de Kerkhof et al., 2008). In gastrointestinal tract, a general trend to inhibition was observed, reaching the level of statistical significance for SULT1A1-*like* mRNA; in addition, such a finding was confirmed at the protein level. Therefore, present SULT1A1-*like* results match with those obtained in the liver. On a comparative basis, most of published reports about the *in vivo/in vitro* transcriptional effects of PB upon this tissue are evidence of a common CYP genes up-regulation, albeit with some variations/exceptions (Zhang et al., 2003; Martignoni et al., 2004; Martin et al., 2008; van de Kerkhof et al., 2008). As an example, PB (0.5 mM) up-regulates CYP3A4 expression and catalytic activity as well as CYP2B6 mRNA levels in human intestinal slices (van de Kerkhof et al., 2008); in rat intestinal slices, the barbiturate (1 mM) increased CYP2B1 gene transcription but no effect was noticed on CYP3A1 mRNA (Martignoni et al., 2004). Finally, increasing amounts of intestinal CYP2B and 2C mRNAs and proteins have been noticed following a challenge with PB in the mouse

(Zhang et al., 2003). On the other hand, some conflicting results have been published for UGTs (Shelby and Klaassen, 2006; van de Kerkhof et al., 2007; van de Kerkhof et al., 2008), while to the best of our knowledge few data (if any) are actually available for GSTs and SULTs; as far as GST1A1 gene is concerned, species-differences in the transcriptional regulation .of GSTA1 by chemicals that normally induce GSTs in rodents have been signaled; in particular, human GSTA1 gene expression was down-regulated after the in *vitro* exposure (24 hours with 5mM PB) to PB (Romero et al., 2006).

In the other cattle extrahepatic tissues, the PB oral administration wasn't successful. Few comparative data on the effects of PB upon target gene mRNA levels in kidney and lung have been published, while no information are actually available for testis, adrenal and skeletal muscle. What's more, these information usually refer to CYPs. As a whole, present results are consistent with at hand data. In fact, PB did not elicit a transcriptional effect on CYP2B in human and mouse kidney (Jarukamjorn et al., 2001; Kakizaki et al., 2003). As regard the lung, rat and rabbit pulmonary CYP2Bs did not respond to PB, even though NR1I2 and NR1I3 were expressed in both species (Lee et al., 1998; Skarin et al., 1999; Chirulli et al., 2005); likewise, mouse pulmonary CYP2B gene and protein were not modulated by PB, but in this species measurable amounts of NR1I3 mRNA were not detected (Pustylnyak et al., 2009). Finally, PB has been shown to induce CYP3A5 in the human A549 cell line (Hukkanen et al., 2000).

Overall, present data suggest that PB, orally administered in cattle by using a prototypical induction protocol, increases the transcription of six out of nine genes coding for DMEs (CYP2B22, 2C31, 2C87 and 3A, UGT1A1-*like* and GSTA1-*like*) in the liver; what's more, such an effect was confirmed at the post-translational level. On the other hand, the small intestine was the only extrahepatic tissue in which PB elicited an effect, consisting merely in a down-regulation of SULT1A1-*like* mRNA and protein amounts. Finally, no PB-transcriptional effects were ever noticed in NRs gene expression. In the overwhelming majority of events, present transcriptional data corroborate previously published comparative ones, but some contradictory results need further molecular investigations.

6.5 CONCLUSIONS

In the present study, the constitutive expression of a number of PB and PB-like compounds responsive genes, namely those NRs involved in DMEs regulation (NR1I2, 1I3, 2A1, 2B1), CYP2B22, 2C31, 2C87, 2C88, 3A and conjugative enzymes (UGT1A1-*like*, SULT1A1-*like*, SULT2A1-*like* and GSTA1-*like*) was demonstrated in cattle liver and extrahepatic tissues. As reported in humans and rodent model species, these genes

were expressed to a lower extent compared to the liver with few exceptions, i.e. NR2A1 in kidney, NR2B1, CYP2B22 and SULT1A1-*like* in lung and NR2B1 in skeletal muscle. Phenobarbital is a prototypical DME inducer, whose transcriptional and ensuing post-translational effects in humans and rodents are mediated by NRs. Following cattle exposure to an oral PB inducing protocol, hepatic CYP2B22, 2C31, 2C87, 3A and UGT1A1-, GSTA1- and SULT1A1-*like* mRNAs and protein amounts were significantly modulated; among extrahepatic tissues, only the small intestine showed a significant down-regulation of both SULT1A1-*like* mRNA and corresponding apoprotein. Data here obtained improved knowledge about the xenobiotic-dependent modulation of DMEs expression and function in cattle liver and extrahepatic tissues. Overall, these findings are suggestive of possible species-differences in DMEs expression, regulation and function between cattle, humans and laboratory species, whose relevance needs to be better characterized and comprehended by using basic molecular investigations, particularly addressed to define the molecular nature of PB-induction response and its tissue-specific control in this outstanding veterinary species.

7. Effects of time culture and prototypical cytochrome P450 3A (CYP3A) inducers on CYP2B22, CYP2C, CYP3A and nuclear receptors (NRs) mRNAs in long-term cryopreserved pig hepatocytes (CPHs)[4]

Abstract. At present, no data about NRs gene expression and CYP NR-dependent regulation are available on cryopreserved pig hepatocytes (CPHs.) Therefore, transcriptional and post-translational effects of culturing time and prototypical CYP3A inducers on principal NRs, CYP2B22, 2C and 3A were investigated in long-term stored (~10 years) CPHs. In the time-course study, a crush and rise effect was observed for NR1I2 and NR1I3 mRNAs, while a time-dependent increase of NR2B1 was noticed. Cytochrome P450 gene expression profiles were down-regulated as a function of time. In the induction study, an increase of NR1I2, NR1I3 and NR2B1 mRNAs was observed in dexamethasone-exposed CPHs. About CYPs, an overall up-regulation was seen in CPHs exposed to PB, while DEX and RIF up-regulated only CYP3A. Transcriptional CYP results, obtained from both studies, were confirmed through post-translational investigations: CYP2B and 3A enzyme activities results are consistent with mRNA data while, immunoblotting analysis shown no effect of PB on CYP2B protein level. Present data demonstrate that long-term stored CPHs may be used to investigate mechanisms involved in CYPs regulation, expression and function; provide further info about NR regulation of CYPs, and confirm species-differences in these mechanisms of regulation; finally, suggest the usefulness and relevance of gene expression profiling to early detect any modulation of CYPs expression and bioactivity.

[4] From: Giantin, M., Zancanella, V., Lopparelli, R.M., Granato, A., Carletti, M., Vilei, M.T., Muraca, M., Baratto, C. & Dacasto, M. **Effects of time culture and prototypical cytochrome P450 3A (CYP3A) inducers on CYP2B22, CYP2C, CYP3A and nuclear receptors (NRs) mRNAs in long-term cryopreserved pig hepatocytes (CPHs)**. Submitted.

7.1 INTRODUCTION

Cryopreservation represents the best method for the long-term storage, in high amounts, of functional primary cells (Madan et al., 1999). The rat represents the species most commonly used for cryopreservation experiments, but human and monkey have been used, too (Chesné et al., 1991; Loven et al., 2005). Nonetheless, the best donor of liver cells for cryopreservation and bioreactors is the pig, owing to the large amount of cells achievable (if compared to rodent species) and the poor availability of human liver tissue (Behnia et al., 2000).

Oxidative and conjugative DMEs activities have been characterized in fresh and CPHs. If referred to rodent and dog hepatocytes, CPHs better reproduce the human CYP-dependent oxidative drug metabolism (Donato et al., 1999; Langsh et al., 2009), although it has been recently demonstrated that porcine hepatocytes may not be suitable in clinical liver support or xenotransplantation, for significant species-differences in CYP gene regulation and bioactivity (Schrem et al., 2006; Kleine et al., 2008) Despite this, porcine hepatocytes are still considered a valuable non rodent, non primate nor dog bioassay for pharmaco- and toxicological studies, useful to avoid or decrease the number of experimental animals (Langsh et al., 2009; Hansen et al., 2000; Schneider et al., 2006).

Previous studies showed that isolated hepatocytes, successfully cryopreserved and carefully stored in liquid nitrogen, maintain their liver specific functions up to four years (Chesné et al., 1993; Lu et al., 2011); on the other hand, a reduced metabolic capacity, essentially attributable to the use of energy to restore membrane and intracellular environment, was observed (Loven et al., 2005).

At present, few data about the effect of cryopreservation, time of culture and prototypical inducers upon porcine hepatocytes DMEs have been published; moreover, most of them refer to post-translational changes (Madan et al., 1999; Baldini et al., 2009) or to mRNA levels measured by using semiquantitative methods, i.e., northen and/or dot blot, PCR (Monshower et al., 1998; Desille et al., 1999; Gillberg et al., 2006). Furthermore, no data about NR constitutive gene expression and modulation have been reported so far. Aim of the present work was to investigate, in CPHs stored up to ten years in liquid nitrogen, the transcriptional effects of time and prototypical CYP3A inducers upon CYP2B22, 2C and 3A as well as on NRs mostly involved in DMEs regulation; in particular, NR1I2, NR1I3, NR2B1 and GRα. Thus, mRNA levels of abovementioned genes were measured, by using qPCR approach, 24, 48, 72 and 96 hours (hrs) after seeding as well as following the exposure (96 hrs) to PB, PCN, DEX and RIF. Post-translational investigations (immunoblotting and catalytic activity) were also executed to confirm CYPs transcriptional results.

7.2 MATERIALS AND METHODS

7.2.1 Chemicals and reagents

Cryopreserved pig hepatocytes were provided by Circe Biomedical Inc. (Lexington, MA). Collagen type I from rat tail, RPMI-1640 and Williams' E medium (WEM), fetal calf serum (FCS), NaH_2PO_4, Na_2HPO_4, $NaHCO_3$, $CaCl_2$, $MgCl_2$, penicillin G potassium salt, streptomycin sulfate salt, L-glutamine, glucagon, DEX, PB sodium salt, PCN, dimethyl sulfoxide (DMSO), trypan blue, pyruvic acid and β-nicotinamide adenine dinucleotide phosphate reduced form, dipotassium salt, were obtained from Sigma-Aldrich (St. Louis, MO). Humulin®I insulin was purchased from Eli Lilly Italia (Sesto Fiorentino, Italy) and Rifadin from Sanofi-Aventis (Milano, Italy). The Cytotoxicity Detection Kit (LDH) and L-LDH standards (from hog muscle) were obtained from Roche (Mannheim, Germany). Chloroform, isopropyl and ethyl alcohol were obtained from Thermo Electron Corporation (Waltham, MA), whereas glycerol, TRIzol® reagent and agarose from Invitrogen (Carlsbad, CA). High Capacity cDNA Reverse Transcription Kit and Power SYBR® Green PCR Master Mix were from Applied Biosystems (Foster City, CA). Oligonucleotide primers were synthesized by Eurofins MWG Operon (Ebersberg, Germany). Quant-iT® Protein Assay Kit, NuPAGE® Novex® 4-12% Bis-Tris Gels and iBlot™ Gel Transfer Device and iBlot™ Gel Transfer Stack, Nitrocellulose, Mini were from Invitrogen™ (Eugene, Oregon). The rabbit anti-human CY2B6 was purchased from LifeSpan BioScences Inc. (Seattle, WA), the rabbit anti-human CYP2C8/9/19 from Millipore (Temecula, CA) and the anti-human CYP3A4 from GeneTex Inc. (Irvine, CA). The peroxidase conjugated goat anti-rabbit IgG and the molecular weight marker ChemiBlot™ were from Millipore (Temecula, CA), while the chemiluminescence kit SuperSignal West Pico Chemiluminescent Substrate was from Pierce Chemical (Rockford, IL).

7.2.2 Thawing, viability and seeding of CPHs

Cryopreserved pig hepatocytes, stored in liquid nitrogen for about 10 years, were thawed in a 37°C water bath. The content of each bag was placed in tubes and mixed (1:5, v/v) with WEM supplemented with 10% FCS, 8.4% $NaHCO_3$, $CaCl_2$ and $MgCl_2$ (0.5 M), penicillin/streptomycin solution (6.329%/1%), L-glutamine (2 mM), glucagon (140 μg/ml) and insulin (100 U/ml). Cells were centrifuged at low-speed to remove DMSO and suspended in supplemented WEM. The post-thaw viability was determined by trypan blue exclusion test. Hepatocytes were seeded at a density of 1.5×10^6 cells/ml (6 ml/dish) onto collagen-coated (150 μl) plastic culture dishes (100-mm diameter), which had been previously re-hydrated (twice) with RPMI medium. Therefore, cells were placed in a

humidified incubator at 37°C with a 95%/5% mixture of air/CO_2; the medium was changed for the first time after 4 hrs of incubation and, then, every 24 hrs. Cell morphology was observed (light microscope) pending all the experiments.

7.2.3 Cell culture treatment and harvesting

Three freezing bags were used to perform three independent cell cultures.

Time-dependent changes in NRs and CYPs gene expression and bioactivity were investigated after 24, 48, 72 and 96 hrs of incubation. With regards to CPHs responsiveness to prototypical CYP3A inducers, 24 hrs after plating the medium was replaced by supplemented WEM containing PB (2 mM final concentration), PCN (10 μM), RIF (10 μM) or DEX (10 μM). Inducers were dissolved in DMSO, except for PB (in water). The final DMSO concentration was 0.1% (v/v). Control CPHs were incubated with medium containing only DMSO and supplemented WEM.

The cytotoxicity was measured by using a lactate dehydrogenase (LDH) leakage colorimetric enzymatic assay as an end point; the standard curve was prepared by using L-LDH standards from the hog muscle, and the released LDH activity was calculated as Units/liter/10^6cells (U/l/10^6cells).

Hepatocytes were harvested either at 24, 48, 72 and 96 hrs (time-course study) or 96 hrs (induction study) after seeding. The medium was aspirated, the monolayer was washed twice with 2 ml of ice-cold phosphate-buffered saline (PBS 0.1 M, pH 7.4) and scraped off. Cells were pelleted and suspended either in 1ml of TRIzol® for RNA extraction or 4 mL of PBS for the obtainment of subcellular fractions, and finally stored at -80°C until use. For each cell culture, four biological replicates were considered for gene expression analysis, and two for immunoblotting and catalytic activity investigations.

7.2.4 Total RNA extraction, cDNA synthesis and qPCR

Total RNA was isolated by using the TRIzol® reagent according to the manufacturer's instruction. The nucleic acid concentration and quality were determined as reported by Lopparelli et al. (2010). The reverse transcription (2 μg of total RNA in a final volume of 20 μL) was performed by using the High Capacity cDNA Reverse Transcription Kit (Foster City, CA), random primers and following the purchaser's procedure.

Sus scrofa coding sequences of target genes were obtained from GenBank web site [http://ncbi.nlm.nih.gov/]. Primer oligonucleotide sequences are listed in Table 15. ACTB, GAPDH, CYP2B22, CYP2C and CYP3A primer pairs were designed *ex novo* by using Primer 3 Software [http://frodo.wi.mit.edu/primer3/]. As regards CYP2C and CYP3A, highly conserved sequence regions were taken into account to obtain a qPCR assays not isoform-specific. To help in the selection of the most stable internal control

gene (ICG), three additional reference target genes, namely the PPIA, RPLP0 (Robinson et al., 2007) and the 18S were included among the candidate genes. These ones had already been used in cattle (Zancanella et al., paper submitted) geNorm^{PLUS} [http://www.biogazelle.com/mybiogazelle/] and NormFinder version 0.953 [http://www.mdl.dk/publicationsnormfinder.htm] algorithms were chosen to identify the best ICGs. For each qPCR assay, a strict validation process was performed as previously reported in Lopparelli et al. (Lopparelli et al., 2011). The qPCR was performed on 2.5 μL of 100-fold diluted cDNA (in a final volume of 10 μL) by using, under standard qPCR conditions, the Power SYBR®Green PCR Master Mix and Stratagene Mx3000P thermal cycler (Agilent technologies, Santa Clara, CA). The $\Delta\Delta C_t$ method was used to analyze data (Livak & Schmittgen, 2001). Results were expressed as n–fold change, that is normalized to the $\Delta\Delta C_t$ mean value of controls. These latter ones were represented by cells cultivated for 24 hrs (time-course) and DMSO-exposed cells (induction), to whom arbitrary values of 100.00 and 1.00 were assigned, respectively.

Gene Symbol	Primer	Sequence 5' → 3'	Tm (°C)	%GC	Amplicon Length (bp)	GenBank Accession No.
ACTB	*Forward*	GGATGCAGAAGGAGATCACG	60	55.0	84	DQ845171
	Reverse	ACACGGAGTACTTGCGCTCT	60	55.0		
GAPDH	*Forward*	CTTTTAACTCTGGCAAAGTGGAC	59	43.5	78	AF017079
	Reverse	GGAACATGTAGACCATGTAGTGGA	60	45.8		
PPIA	*Forward*	CTCTTTTGAGCTGTTTGCAGACA	58.9	43.5	81	NM_214353
	Reverse	CCAAATCCTTTCTCTCCAGTGCT	60.6	47.8		
RPLP0	*Forward*	CAACCCTGAAGTGCTTGACAT	57.9	55.6	227	NM_001098598
	Reverse	AGGCAGATGGATCAGCCA	56	59.1		
NR1I2	*Forward*	AAGGCCTACATCGAGTGCAA	60	50.0	66	NM_001038005
	Reverse	AGCCATGATCTTCAGGAACAG	59	47.6		
NR1I3	*Forward*	CAAGTCTCCGGGACAGGTT	60	57.9	61	NM_001037996
	Reverse	CTTCGGAGCTCAGCCAATA	59	52.6		
NR2B1	*Forward*	GTCCTCTTCAACCCGGACT	58.8	57.9	79	XM_001927453
	Reverse	GGGACGCGTAGACCTTCTC	61.0	63.2		
NR3C1	*Forward*	CAGTGGAAGGACAGCACAATTA	60	45.5	113	NM_001008481
	Reverse	TCCAGCTTGAAGACACTTTCTG	59	45.5		
CYP2B22	*Forward*	TTCCAGACCTTTCTGCTCATC	59	47.6	67	NM_214413
	Reverse	TCAAGAAGGCGGAGTAGAGC	59	55.0		
CYP2C	*Forward*	AACGCGTTCAAGAGGAA	60	52.6	127	NM_214420
	Reverse	CGATTGTGGAAAATGATGGA	60	40.0		
CYP3A	*Forward*	CAAGGACTCCATAAACCCTTACA	59	43.5	69	NM_001195509
	Reverse	CTCATGCCGATGCAGTTG	59	55.6		

Table 15: Reference genes for qPCR assays. Primers pair, melting temperature (Tm), percentage of GC (%GC), amplicon length and GenBank accession number for qPCR assays.

7.2.5 Preparation of microsomal subcellular fractions

Microsomes were isolated by differential centrifugation (Pegolo et al., 2010) and stored at -80°C until analysis. The microsomal protein content was determined by using Qubit®Fluorometer and Quant-iT® Protein Assay Kit.

7.2.6 Cytochromes P450 2B, 2C and 3A immunoblotting

Microsomal proteins (30 μg) were separated on NuPAGE® Novex® 4-12% Bis-Tris Gels by using the XCell SureLock™ Mini-Cell electrophoresis system (Invitrogen™, Eugene, Oregon). Proteins were transferred onto nitrocellulose filters through the iBlot™ Dry Blotting System (consisting in the iBlot™ Gel Transfer Device and iBlot™ Gel Transfer Stack, Nitrocellulose, Mini). Membranes were firstly incubated with rabbit anti-human CYP2B6 (1:1000 final dilution), CYP2C8/9/19 (1:7500) and CYP3A4 (1:1500) polyclonal antibodies and, then, with peroxidase-conjugated donkey anti-rabbit IgGs (1:6000 for CYP2B6 and CYP3A4, 1:10000 for CYP2C8/9/19). Peroxide conjugates were detected by using a chemiluminescence kit and according to the manufacturer's instructions. A positive control sample (liver microsomal proteins from a rat induced with PB) and a molecular marker were present on each minigel. Immunopositive bands were captured by the Photo Studio 5 version 5.0.0.53 software for the Canon CanoScan Lide 20 scanner (LED indirect exposure), and their optical density analyzed by the ImageJ 1.44p image analysis software.

7.2.7 Cytochrome P450 2B- and 3A-dependent catalytic activities

Pooled microsomal samples obtained from the three cultures were used to measure CYP2B- and CYP3A-dependent catalytic activities, according to Nebbia et al. (2003) and by using model substrates, namely the *O*-dealkylation of benzyloxyresorufin (BROD, CYP2B) and the *N*-demethylation of ethylmorphine (ETDEM, CYP3A). The cytochrome P450 2C catalytic activity was not determined, owing to the low amount of available proteins.

7.2.8 Statistical analysis

Data for each target gene were expressed as the arithmetic mean ± SD of $\Delta\Delta C_t$ values expressed as n–fold changes. The presence of statistically significant differences in gene and protein expression were checked through the analysis of variance (ANOVA), followed by the Tukey's post test (GraphPad InStat 3, San Diego, California, USA). A p value < 0.05 was considered statistically significant.

7.3 RESULTS

7.3.1 Cryopreserved hepatocytes viability and morphology

The mean number of CPHs was 1.5 x 10^9 cells/bag, and the mean viability of cell suspensions after thawing (trypan blue dye exclusion test) was 77.3% ± 3.8%. Four hrs after seeding most of hepatocytes showed a polygonal shape, while not adherent and/or damaged cells maintained a spherical figure and were removed with medium renewal. Between 24 and 96 hrs, monolayers displayed the "typical" hepatocyte phenotype: polygonal feature, presence of distinct cell-cell borders and bile canaliculus-*like* structure. Cells covered all dish surface, with minimal morphological abnormalities.

In the time-course study, LDH leakage values at 24 and 48 hrs after seeding (111.21 ± 5.85 and 130.93 ± 13.59 U/l/10^6cells, respectively) were higher than those measured at 72 and 96 hrs (101.20 ± 10.48 and 81.84 ± 4.05 U/l/10^6cells). On the other hand, in the induction study no differences in LDH-leakage values were noticed among control (111.44 ± 6.97 U/l/10^6cells), DMSO- (118.17 ± 17.86 U/l/10^6cells) and PB-, PCN-, DEX- or RIF-exposed cells (80.35 ± 6.81, 112.97 ± 16.81, 90.24 ± 8.28 and 132.78 ± 10.56 U/l/10^6cells, respectively).

7.3.2 Quantitative Real Time RT-PCR assays validation and selection of ICGs

Chosen primers gave rise to specific amplification products. For each qPCR assay, primers concentration were optimized and PCR efficiency (*E*) and linearity were tested. Only PCR with *E* (%) comprised between 90% and 110% and high linearity (linear regression coefficient $0.98 \leq r \leq 1$) were considered as acceptable (see Table 16).

Five reference genes (ACTB, GAPDH, PPIA, RPLP0 and 18S) were screened to identify the best ICG for qPCR data normalization. One of them (18S) was excluded from further analysis as a consequence of its high constitutive expression (raw C_t value of about 12), while the other ones (C_t values comprised between 17 and 23) were considered good enough, also because they did not show time- or treatment-dependent significant variations in their expression profile. Their amplification data were then submitted to geNorm^{PLUS} and NormFinder algorithms. In the time-course study, NormFinder indicated RPLP0 as the best ICG, but recommended the use of a combination of two genes (RPLP0 and PPIA: see Table 3). The results of geNorm^{PLUS} analysis revealed PPIA as the most stable gene and the candidate ICG with the lowest average expression stability value (M value); moreover, the use of a combination of three ICGs (PPIA, RPLP0 and GAPDH) was suggested (see Table 17). As far as the induction study is concerned, NormFinder identified ACTB as the most stable gene, and its association with RPLP0 the best combination to normalize qPCR data (Table 18). By contrast, the best reference gene

identified by geNorm^{PLUS} was RPLP0; moreover, geNorm^{PLUS} itself was unable to determine the optimal number of ICGs to be used, for the high pairwise variation values between two sequential normalization factors containing one more gene. Whenever such an evidence occurs, it is recommended to use the n-1 reference targets with the lowest M value (in this case, RPLP0, PPIA and ACTB: see Table 17). Taking these results as a whole, the geometric mean of PPIA, RPLP0 and GAPDH was used to normalize data from the time-course study, while that obtained from RPLP0, PPIA and ACTB for the normalization of data obtained with the induction study.

Target gene	**Slope**	**Efficiency (%)**	***y*-intercept (C_t)**	**Linear dynamic range (C_t)**	**Linear regression coefficients (R^2)**
ACTB	-3.347	99.0	29.04	17.42 – 26.76	0.990
GAPDH	-3.396	97.0	32.46	20.59 – 32.45	1.000
PPIA	-3.388	97.3%	37.29	20.66 – 34.70	0.999
RPLP0	-3.468	94.2%	35.28	18.57 – 32.95	0.998
NR1I2	-3.314	100.3	33.51	22.00 – 31.31	0.996
NR1I3	-3.385	97.4	40.75	26.58 – 33.69	0.996
NR2B1	-3.327	99.8	37.25	23.32 – 34.95	0.999
NR3C1	-3.333	99.5	36.07	24.51 – 33.75	0.997
CYP2B22	-3.363	98.3	35.47	23.86 – 35.00	0.996
CYP2C	-3.449	95.0	32.36	20.33 – 33.18	1.000
CYP3A	-3.370	98.0	33.76	19.80 - 31.56	0.998

Table 16: qPCR assays validation. Parameters obtained from qPCR assays validation: slope, efficiency (%), y-intercept, linear dynamic ranges and linear regression coefficients.

Gene name	Stability value (M)
(A)	
PPIA	0.365
RPLP0	0.372
GAPDH	0.433
ACTB	0.510
(B)	
RPLP0	0.437
PPIA	0.460
ACTB	0.522
GAPDH	0.661

Table 17: geNorm^{PLUS} stability values. Average expression stability of each candidate reference gene determined by geNorm^{PLUS} and listed from the most stable to the least stable. Results for time-course (A) and induction (B) experiments.

Gene name	Stability value
(A)	
RPLP0	0.093
PPIA	0.108
GAPDH	0.121
ACTB	0.202
RPLP0 and PPIA	0.074
(B)	
ACTB	0.105
RPLP0	0.127
PPIA	0.253
GAPDH	0.310
ACTB and RPLP0	0.084

Table 18: NormFinder stability values. Stability values, determined by NormFinder, for each candidate reference gene and listed from the most stable to the least stable. Best combination of two genes is also reported. Results for time-course (A) and induction (B) experiments.

7.3.3 Effects of culturing time and prototypical CYP3A inducers on NR mRNA levels

In the time-course study, a decrease of NR1I2 gene expression was observed 48 (p<0.001), 72 (p<0.01), and 96 hrs (p<0.01) after seeding (see Figure 13a); on the contrary an increase (p<0.01), with respect to 48 hrs data, was noticed at 96 hrs. The same behavior (a moderate decrease at 48 hrs followed by an increase at 72 and 96 hrs) was shown by NR1I3. By contrast, NR2B1 and NR3C1 showed a different pattern of gene expression (a trend to up-regulation), reaching the level of statistical significance at 96 hrs (p<0.01 *vs* 24 and 48 hrs). In the induction study, only DEX was shown to up-regulate NR1I2 (p<0.05 *vs* DMSO, PB, and RIF), NR1I3 (p< 0.001 *vs* DMSO, PB, PCN, and RIF) and NR2B1 (p<0.05 *vs* DMSO and RIF; see Figure 13b).

7.3.4 Effects of culturing time and prototypical CYP3A inducers upon CYP2B22, 2C and 3A mRNA levels and apoprotein contents

In the time-course study, all CYP genes were down-regulated to a varying extent (from 81% up to 97%) at 48, 72, and 96 hrs (p<0.001: see Figure 14a). Immunoblotting investigations confirmed qPCR data. In fact, a reduction of CYP2B22 apoprotein was noticed at 72 (p<0.05 *vs* 24 hrs) and 96 hrs (p<0.01 *vs* 24 hrs and p<0.05 *vs* 48 hrs), while for CYP2C such a depletion was significant only at 96 hrs (p<0.05 *vs* 24 hrs: see Figure 14b-c). The cytochrome P450 3A apoprotein amount was similarly reduced, albeit not significantly. Benzyloxyresorufin-*O*-dealkylase and ETDEM catalytic activities, measured on pooled microsomes, confirmed CYP2B and 3A immunoblotting results (see Table 19.A).

In the induction study, no differences in mRNA of target genes were ever noticed between cells incubated with DMSO or medium alone (data not shown). On the other hand, a common trend to transcriptional up-regulation (if compared with DMSO) was observed in CPHs exposed to chosen CYP3A inducers, except for PCN (see Figure 15a). Phenobarbital increased all CYP mRNA levels (p<0.05, p<0.001 and p<0.01 for CYP2B22, 2C and CYP3A, respectively), while DEX and RIF up-regulated only CYP3A (p<0.05). Immunoblotting data largely confirmed transcriptional results: phenobarbital, DEX and RIF increased CYP3A apoprotein (p<0.001: see Figure 15b-c), while no differences were ever found in cells exposed to PCN. On the contrary, PB did not affect CYP2B protein expression, while RIF prompted on a significant increase of CYP2C apoprotein (p<0.05). Likewise to the time-course study, BROD and ETDEM catalytic activitiesconfirmed CYP2B and 3A immunoblotting results (see Table 18.B).

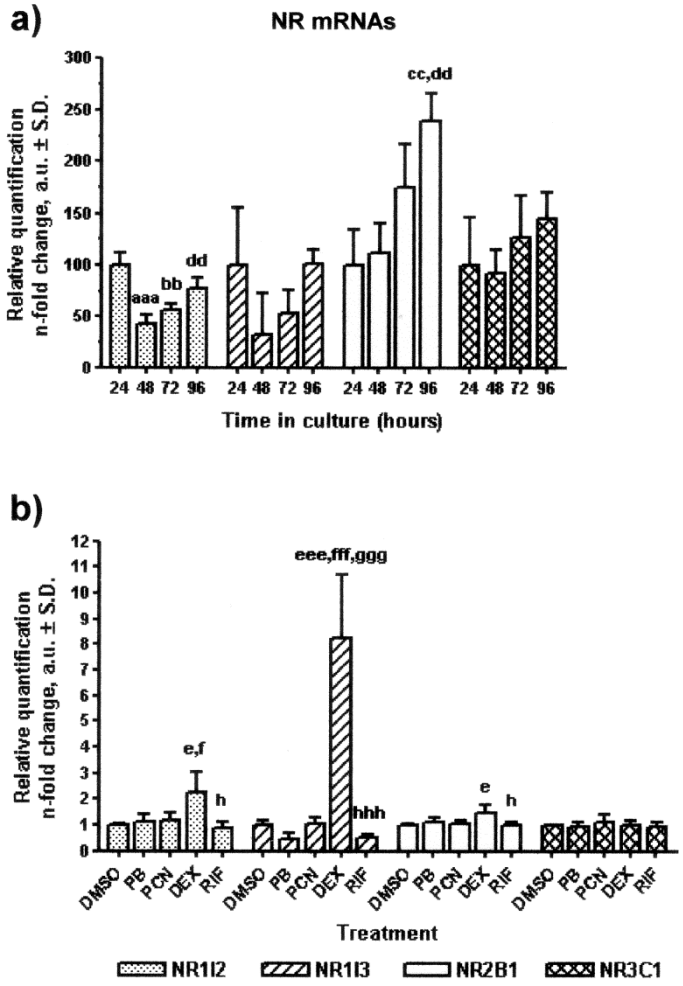

Figura 13: Effects of culturing time and prototypical CYP3A inducers on NR gene expression. Total RNA was isolated from CPH monolayers and target NR mRNA levels were measured by using a qPCR approach. In the time-course study (a), data (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to the $\Delta\Delta C_t$ mean value of cells stopped at 24 hrs, to whom an arbitrary value of 100.00 was assigned. In the induction study (b), data (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to the DMSO $\Delta\Delta C_t$ mean value, to whom an arbitrary value of 1.00 was assigned.

^{aaa}: significant differences ($p < 0.01$) 24 hrs vs 48 hrs

^{bb}: significant differences ($p < 0.01$) 24 hrs vs 72 hrs

$^{c, cc}$: significant differences ($p < 0.05, 0.01$) 24 hrs vs 96 hrs

^{dd}: significant differences ($p < 0.01$) 48 hrs vs 96 hrs

$^{e, eee}$: significant differences ($p < 0.05, 0.001$) DMSO vs DEX

$^{f, fff}$: significant differences ($p < 0.05, 0.001$) PB vs DEX

^{ggg}: significant differences ($p < 0.001$) PCN vs DEX

$^{h, hhh}$: significant differences ($p < 0.05, 0.001$) DEX vs RIF

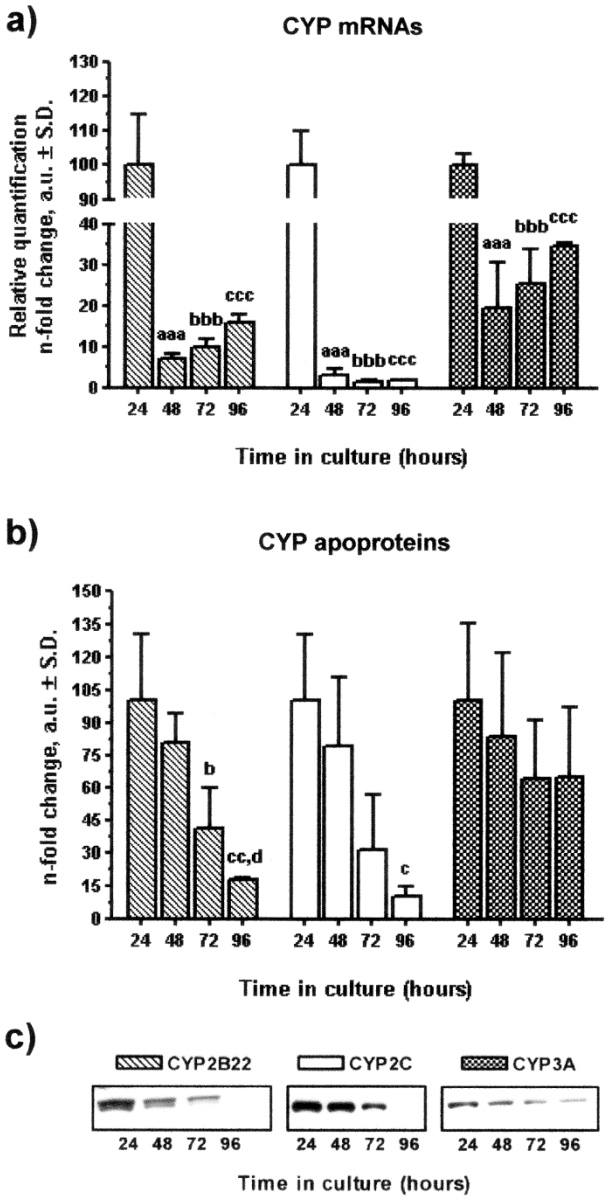

Figure 14: Effects of culturing time on CYP2B22, CYP2C and CYP3A mRNA levels and apoprotein content. Target CYP mRNA levels (a) were measured by using a qPCR approach, and data (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to the $\Delta\Delta C_t$ mean value of cells stopped at 24 hrs, to whom an arbitrary value of 100.00 was assigned. Target CYP apoprotein amounts (b) were measured by immunoblotting, and integrated density data (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to the integrated density mean value of cells stopped at 24 hrs, to whom an arbitrary value of 100.00 was assigned. In (c) a representative CYP2B, 2C and 3A immunoblotting is reported.

^{aaa}: significant differences ($p < 0.01$) 24 hrs vs 48 hrs

$^{b, bbb}$: significant differences ($p < 0.01$) 24 hrs vs 72 hrs

$^{c, cc, ccc}$: significant differences ($p < 0.05, 0.01$) 24 hrs vs 96 hrs

^{d}: significant differences ($p < 0.05$) 72 hrs vs 96 hrs

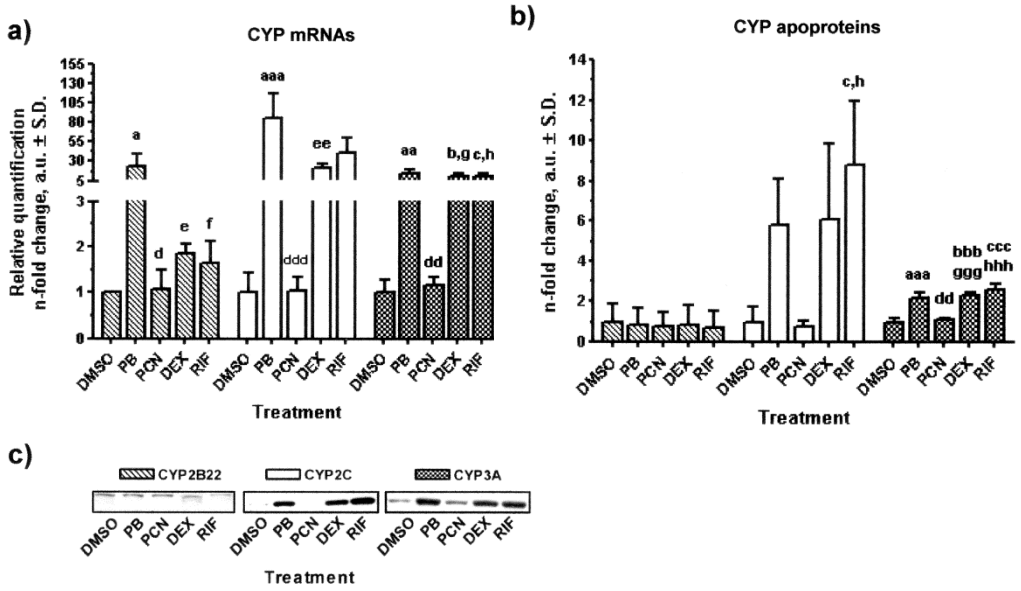

Figure 15: Effects of prototypical CYP3A inducers on CYP2B22, CYP2C and CYP3A mRNA and apoprotein content. Target CYP mRNA levels ***(a)*** *were measured by using a qPCR approach, and data (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to the DMSO* $\Delta\Delta C_t$ *mean value, to whom an arbitrary value of 1.00 was assigned. Target CYP apoprotein amounts* ***(b)*** *were measured by immunoblotting, and integrated density data (arithmetic means ± S.D.) are expressed as n-fold change (a.u.) normalized to the DMSO integrated density mean value, to whom an arbitrary value of 1.00 was assigned. In* ***(c)*** *a representative CYP2B, 2C and 3A immunoblotting is reported.*

[a, aa, aaa]: significant differences (p < 0.05, 0.01, 0.001) DMSO vs PB

[b, bbb]: significant differences (p < 0.05, 0.001) DMSO vs DEX

[c, ccc]: significant differences (p < 0.05, 0.01) DMSO vs RIF

[d, dd, ddd]: significant differences (p < 0.05, 0.01, 0.001) PB vs PCN

[e, ee]: significant differences (p < 0.05, 0.01) PB vs DEX

[f]: significant differences (p < 0.05) PB vs RIF

[g, ggg]: significant differences (p < 0.05, 0.01) PCN vs DEX

[h, hhh]: significant differences (p < 0.05, 0.01) PCN vs RIF

[i, iii]: significant differences (p < 0.05, 0.01) DEX vs RIF

(A)

Catalytic activity	Hrs			
	24	**48**	**72**	**96**
CYP2B	8.08	6.87	8.02	1.78
CYP2C	N.A.			
CYP3A	0.69	0.25	N.D.	0.27

(B)

Catalytic activity	Treatment				
	DMSO	**PB**	**PCN**	**DEX**	**RIF**
CYP2B	10.44	16.31	6.52	13.48	12.63
CYP2C	N.A.				
CYP3A	0.18	1.04	0.47	0.95	1.63

Table 19: Catalytic activity results. Benzyloxyresorufin O-dealkylase (BROD, pmol^{-1} min mg protein^{-1}) and ethylmorphine N-demethylase (ETDEM, nmol^{-1} min mg protein^{-1}) respectively marker activities of CYP2B and 3A, were measured on pooled microsomes of time-course (A) and induction (B) experiments. N.A., not analyzable activity due to the low amount of available proteins;N.D., not detectable activity.

7.4 DISCUSSION

Cryopreserved pig hepatocytes are considered as an alternative to human and rodent hepatocytes for enzyme induction, enzyme inhibition and drug-drug interaction studies. Moreover, and owing to their metabolic similarities with the human species, CPHs are thought as suitable cells for bioartificial liver devices, useful to temporarily support the xenobiotic detoxification pathways in the living organism (Donato et al., 1999; Langsch & Bader, 2001). Human cryopreserved hepatocytes, when appropriately frozen and stored in liquid nitrogen, may stay viable for a long time (up to 4 years or even more) without losing hepato-specific functions (Chesné et al., 1993; Lu et al., 2011). Among these ones there is the potential to respond to CYP inducers, likewise to fresh hepatocytes (Madan et al., 1999). Therefore, the transcriptional effects of culturing time and prototypical CYP3A inducers upon CYP2B22, 2C and 3A as well as on NRs involved in the regulation of DMEs were here investigated in CPHs stored up to ten years in liquid nitrogen.

Long-term CPHs were successfully thawed and cultured, with a mean percentage of viability after thawing of about 77%. Nevertheless, the amount of LDH released in cell medium, particularly 24 and 48 hrs after seeding in the time-course study, was higher if compared to some previously published values (Koebe et al., 1999; Chen et al., 2001).

Although the trypan blue dye exclusion test is a subjective and arbitrary assay, often leading to an overestimation of cell viability (Altman et al., 1993), present results were not astonishing at all. Human and rat cryopreserved hepatocytes show a very poor plating efficiency compared to freshly isolated cells (about 15-20%; Silva et al., 1999), and such an effect is not immediately observable after thawing, being probably due to cells that initially adhere to culture dish but later detach (Terry et al., 2005); in another study, only 28% of viable long-term human cryopreserved hepatocytes attached to collagen-coated dishes within the first 24 hrs of culture (Lu et al., 2011); finally, an early high LDH release followed, after three days of culture, by recovery was observed in a comparative study made in CPHs and freshly isolated hepatocytes (Chen et al., 2001).

During the culturing process, hepatocytes adapt themselves to a new environment through the differential expression of genes involved in the maintenance of cellular functions (i.e., the glutathione status), the sugar metabolism and the production of the extracellular matrix (Baker et al., 2001). Moreover, in the induction study cells were exposed to molecules known to up-regulate the transcription of more than few genes, besides DMEs and NRs. Therefore, a careful evaluation of the best ICG to be used in both studies was undertaken, to avoid mistakes in the normalization of qPCR data. First of all, five candidate ICGs (ACTB, GAPDH, RPLP0, PPIA and 18S), frequently used for the normalization in liver and *in vitro* gene expression studies (Bower & Johnston 2009; Wang & Xu, 2010) were chosen; then, geNorm^{PLUS} (Vandesompele et al., 2002) and NormFinder (Andersen et al., 2004) free algorithms were used for the identification of the best ICGs.

n the time-course study, both algorithms identified ACTB as the less stable gene, followed by GAPDH, PPIA or RPLP0. These results agreed with previously published findings. In fact, primary rat hepatocytes showed a time-dependent up-regulation of genes involved in cytoskeleton and extracellular matrix modifications (ACTB) as well as in sugar metabolism (GAPDH: Baker et al., 2001); furthermore, mRNA levels of the two aforementioned genes and PPIA were altered under different conditions, such as hypoxia or exposure to insulin, DEX and mitogens (Zhong et al., 1999). Nevertheless, in the present experimental conditions PPIA, RPLP0 and GADPH gene expression profiles did not show significant changes between 24 and 96 hrs of culture, and were then chosen to normalize time-course qPCR data.

In the induction study, NormFinder indicated GAPDH as the less stable gene, followed by PPIA, RPLP0 and ACTB; the stability ranking, assessed by geNorm^{PLUS}, was ACTB>PPIA>RPLP0. On a knowledge basis, ACTB has been considered as a suitable ICG as it was not modulated by RIF, DEX or omeprazole in both primary human and rat

hepatocytes (Nishimura et al., 2006). Therefore, and taking into account the present data, PPIA, RPLP0, and ACTB were considered as the most stable genes for the normalization of qPCR data from the induction study.

To date, no information about NRs gene expression in CPHs are available. Two of them, namely NR1I3 and NR1I2, play a crucial role in the regulation of several endogenous or exogenous compounds. Furthermore, they share significant cross-talks in target gene recognition, through the binding to similar xenobiotic responsive elements. Among these genes there are human CYP2B6, CYP2C9, and CYP3A4 (Honkakoski & Negishi, 2000; Pascussi et al., 2003).

In the time-course study, NR1I2 and NR1I3 mRNA levels were decreased to a varying extent from 24 hrs onward. This finding agree with data obtained in human hepatocytes, in which lower NR1I3 mRNA levels were noticed after 120 hrs of culture (Nishimura et al., 2011). Furthermore, this trend was consistent with that observed for target CYPs. By contrast, NR2B1 and NR3C1 gene expression was not affected by the whole culture time; what's more, NR2B1 mRNA was significantly up-regulated at 96 hrs. With regards to NR2B1, its involvement in the liver regeneration has been recently hypothesized; in fact, its deficiency leads to a delay in hepatocyte proliferation, altered growth factor-mediated signaling pathways and an impaired cell cycle progression (Yang et al., 2010). Thus, the present NR2B1 up-regulation might be viewed as a compensatory phenomenon activated following the comparatively higher relevance of cell death occurring in CPHs after thawing.

The constitutive expression and bioactivity of DMEs, and CYPs above all, is a fundamental prerequisite for the use of an in vitro model in drug metabolism studies (Bader et al., 2000). Cytochrome P450 genes were still present in CPHs after ten years of cryopreservation, but their mRNAs were markedly reduced after seeding. In this respect, an early lessening of total CYP expression (mRNA and protein) has been well documented in rodent and human hepatocytes (Kokarek et al., 1993; George et al., 1997). More controversial is whether such a decrease might be CYP isoform specific or not (Kokarek et al., 1993; Binda et al., 2003). A crash and rise effect was here observed for CYP2B22 and 3A, as previously demonstrated in rat primary hepatocytes (Kokarek et al., 1993). Although the CYP2C gene did not show the same behavior, such an effect might be explained by the need of cells to recover from thawing stress, establish the monolayer and adapt themselves to the culture environment. Protein and catalytic activity analysis substantiated transcriptional data, even though they appeared to be less sensitive to detect early CYPs down-regulation. That's the reason why in recent CYP induction studies it

has been suggested to measure mRNA levels, for its greater sensitivity and dynamic range (Fahmi & Ripp, 2010).

Most of CYP inducers (including those here used) are ligands of NRs, and their binding with these transcription factors (in the cytosol) usually constitute the first step of induction process; the ligand-receptor complex is then transferred to the nucleus, where it may heterodimerize or not with NR2B1 and, next, interact with specific DNA responsive elements (i.e., the glucocorticoid responsive elements), whose effect is an increased synthesis of mRNAs coding for target proteins (i.e., CYPs; Timsit & Negishi, 2007). In CPHs, only DEX significantly increased NR mRNA levels (NR1I3<NR1I2~NR2B1). Quite surprisingly, it did not affect NR3C1 gene expression. This pattern of induction is consistent with that observed in human and rodent hepatocytes, where the presence of a dual dose-response effect has been hypothesized. Low DEX concentration (100nM) would increase PXR and CAR mRNAs through a GR-mediated mechanism, while higher ones (>10 μM), would directly activate PXR (Pascussi et al., 2001). Furthermore, 25μM DEX induces PXR in H4IIE cells (Fery et al., 2010), while a wide range of glucocorticoid concentrations (including para-physiological ones, >10 nM) have been shown to enhance the expression of both NR2B1 gene and RXRα protein (Yamaguchi et al., 1999). On the other hand, quite hard to explain is the absence of a modulator effect of PB upon NR1I3, also in light of data here obtained with CYP genes (common up-regulation) and apoproteins (increasing amounts for CYP2C and 3A). Nonetheless, there are some experimental evidences that might justify present results. Species-differences in the ligand affinity and/or binding to NRs exist; this is of particular concern for CAR and PXR, and it is still subject of debate (di Masi et al., 2009; Tolson & Wang, 2010). Phenobarbital, at concentrations of about 0.5 mM, deactivates CAR, while at higher ones (>1 mM) it does not bind to the receptor; furthermore, it has been hypothesized that PB might activate CAR by an alternative mechanism (i.e., phosphorylation), without increasing its mRNA (Moore et al., 2000). Finally, *Bos taurus* and *Sus scrofa* CAR sequences are considered hortologues (di Masi et al., 2009) and, interestingly, the *in vivo* challenge of cattle with PB inducing dosages elicited an up-regulation of liver CYP2B22 but not of CAR and PXR (Zancanella et al., 2009). Undoubtedly, such an argument need to be studied more in depth in forthcoming studies.

Cytochromes P450 induction has been extensively characterized in cryopreserved rat and human cells as well as in CPHs, although in these latter ones only at the post-translational level (protein and enzyme activity). In cryopreserved hepatocytes, PB is considered as a prototypical inducer for human CYP2B6 and rat CYP2B1/2 (Madan et al., 1999; Silva et al., 1999; Abadie-Viollon et al., 2010); furthermore, porcine CYP2B22 responsiveness

has been demonstrated at the protein level (Behnia et al., 2000; Desille et al., 1999). In the present study, conflicting results were obtained: in fact, a CYP2B22 induction was noticed only at the mRNA level, while apoprotein amount and related catalytic activity (BROD) were never modulated by PB. Moreover, RIF did not affect CYP2B22 transcription, in contrast with *in vivo* data (Nannelli et al., 2008). The cytochrome P450 2C mRNA was up-regulated by PB and, albeit not significantly, by DEX and RIF. The transcriptional pattern of response was paralleled at the protein and catalytic activity level, although the order of magnitude was (and not surprising at all) lower than that observed at the mRNA level. As a whole, these results agree with previously published ones. In fact, the exposure to PB and RIF has been shown to induce CYP2C mRNA and tolbutamide (a CYP2C substrate) hydroxylation in primary pig hepatocytes (Monshower et al., 1998), while 10 μM RIF increased CYP2C9 mRNA in cryopreserved human hepatocytes (Abadie-Viollon et al., 2010). What's more, the *in vivo* administration of PB to pigs resulted in a CYP2C6 induction (Desille et al., 1999).

In the long-term stored CPHs, CYP3A expression (gene and protein as well) was significantly up-regulated by PB, DEX and RIF. The catalytic activity (ETDEM) confirmed the expression data. By contrast, and once again, PCN did not provoke effects upon CYP3A as a whole. This was not surprising, cause comparative studies made on hepatocytes from different species revealed that some xenobiotics induce CYP3A in every species, while some other ones act in a more specific way (i.e., PCN induces rodent CYP3A, while RIF up-regulates this gene only in rabbits and humans (Burk & Wojnowski, 2004). Present finding are in line with those previously published either in cryopreserved hepatocytes or in primary cultures of pig hepatocytes. In fact, DEX and RIF induce CYP3A1/2 apoprotein and activity as well as CYP3A4 mRNA in rat (Madan et al., 1999; Silva et al., 1999) and human cryopreserved hepatocytes (Abadie-Viollon et al., 2010), respectively. Moreover, CYP3A mRNA, apoprotein and catalytic activities were increased, in primary pig hepatocytes, following the exposure to PB, RIF and (albeit to a lower extent) DEX (Monshower et al., 1998; Desille et al., 1999).

7.5 CONCLUSIONS

In conclusion, it have been shown that CPHs express NR (mRNA) and CYPs (mRNA, protein and catalytic activity) even after a long-term storage (about ten years) in liquid nitrogen. Despite the relatively high amounts of LDH release in cell medium, when compared to values usually found in primary hepatocytes, CPHs showed an unaltered capacity of attachment and formed confluent monolayers. Likewise to former studies made in primary or cryopreserved hepatocytes obtained from different species (including

the pig, too), a time-dependent down-regulation of CYPs mRNA and protein was observed, whereas NR gene expression data showed some differences in terms of response (crash and rise effect, up-regulation, no effect). When exposed to prototypical CYP3A inducers (RIF, PCN, DEX, PB), both NRs (essentially with DEX) and CYPs gene expression profiles were up-regulated; furthermore, CYPs modulation was confirmed at the post-translational level, although to a lower extent. As a whole, this study provides new data about NRs gene expression in cryopreserved hepatocytes, representing one step forward in the knowledge about CYPs expression and regulation phenomena as well as on the hepatocyte adaptation to the cell culture environment. Furthermore, it confirms that the gene expression profiling might represent an earlier and more sensitive method, compared to protein and catalytic activity assays, to detect a modulation of DMEs expression and function following a xenobiotic exposure. Finally, CPHs have been shown to be, even if stored for longer times than usual, an useful tool to study the expression and regulation of genes (DMEs, NRs) requiring a functional transcription factor machinery.

8. General conclusions and future perspectives

There are more than fourty domestic livestock species in the world and cattle and pigs are considered within the European Union as major food-producing species together with sheep, chicken, turkey and *Salmonidae*. The physiological traits of these different species reflect adaptations that have evolved over the last millennia, not only to promote their survival in local environments but also as a consequence of their usage by man (Toutain et al., 2010). The recent availability of technologies that enable large-scale "shotgun sequencing" of entire genomes on a single chip-based platform, within a relatively short period of time, allowed the release of genome sequences for a number of domestic animal species. In June 2009, the Bovine Genome Sequencing Project, led by the Baylor College of Medicine's Human Genome Sequencing Center, released an update of the genome assembly; likewise, a physical complete map of pig genome is actually available (http://www.sanger.ac.uk/resources/downloads/othervertebrates/pig.html).

For most of veterinary pharmaco-toxicologists, cattle drug metabolism studies are useful for several reasons: (1) for the evaluation of consumers' risk associated with the consumption of foodstuffs collected either from diary or meat cattle treated with drugs or exposed to pesticides and contaminants; (2) to extrapolate pharmaco-toxicological data from this species to other species; (3) to extend licenses of use for certain veterinary drugs from this major species to minor or exotic ones; (4) finally, to investigate the effects of constitutional factors (i.e. species, gender, age, genetic polymorphisms, physio-pathological conditions) as well as induction or inhibition phenomena upon the overall cattle biotransformation capacity (Sivapathasundaram et al., 2001; Ioannides, 2006; Cantiello et al., 2008; Fink-Gremmels, 2008; Giantin et al., 2008; Cantiello et al., 2009; Giantin et al., 2010). Nonetheless, the characterization of the porcine metabolic and toxigenomic profile is essential due to important applications beyond the veterinary field. Pig, in fact, is considered an useful model for several major human diseases, the only non-rodent species in which the generation of transgenic animals is well established, a possible resource in xenotransplantation and bioartificial livers and finally a good animal model in the safety assessment of pharmaceutical or chemical products (Puccinelli et al., 2011).

The first aim of this work was to solve the uncertain and misleading nomenclature of major cattle CYP families (CYP1-4) involved in drug metabolism. Actually, cattle CYPs nomenclature is based upon the human nomenclature and a correct identification of

bovine CYP isoforms is essential to perform further reliable biochemical and molecular investigations. When the true relationships of orthology among different polypeptides is difficult to assess through classical BLAST approach, a phylogenetic analysis is usually required. This is the case of CYP superfamily, one of the most abundant group of genes found in eukaryotic genomes. Its nomenclature, even within a single species, is "naturally" complicated by evolutionary mechanisms (i.e. birth and death process) and gene conversion as well (Thomas, 2007; Goldstone et al., 2006). The phylogenetic analysis here performed revealed several discrepancies in the current nomenclature of CYP2, 3 and 4 families. Consequently, when *Bos taurus* sequence was not orthologous to *H. sapiens* one, a new nomenclature and acronym was proposed. The obtained results offer an explanatory of the several inconsistencies observed in the function of various CYP isoforms (e.g. differences in the substrate specificity and catalytic activity) expressed in cattle, like CYP2C31 and CYP2C88 (previously identified as CYP2C18 and 2C19) or CYP3A28, 3A38 and 3A48. Further steps might be represented by the improvement of here presented phylogenic analysis with new annotated sequences, either from bovine or other veterinary species, and the extension of evolutionary analysis to other DMEs here studied, such as conjugative enzymes and DTs.

Compared to human and rodent model species, for which an extensive literature about the expression of DMEs and DTs in organs involved in xenobiotic metabolism or blood-tissue barriers have been published, a lack of knowledge on the expression and regulation of DMEs and DTs in veterinary species is still present and more fundamental studies are actually needed. In the present thesis and for the first time an overview of tissue distribution of most important bovine phase I and phase II DMEs, NRs as well as of uptake and efflux transporters, has been performed by using a qPCR approach in cattle. In particular, candidate genes were selected due to their known responsiveness to PB or involvement in the PB-mediate induction mechanism in human and rodent species.

Some bovine CYP and phase II transcriptional features were comparable with other ruminants (i.e. the high expression of CYP2B22 achieved in the lung), or humans (such as SULT2A1-*like* tissue distribution). Altogether, even if present results are not suggestive of a clear bovine-specific pattern of gene expression, the existence of a tissue-specific mechanism of cythochromes P450 induction cannot be excluded. Likewise to DMEs, theNRs tissue distribution was comparable to human and rodent species. With regard to major DTs belonging to ABC- and SLC-superfamily DTs, for the first time the gene expression profiles of were investigated in liver and foremost extrahepatic bovine tissues. Obtained results proved that the liver, intestine and testis were the tissues mostly involved in transport activity.

In the past decade, the traditional approach to assess CYP induction was based upon the analysis of the xenobiotic effects upon the liver by immunoblotting and/or enzyme activity analysis as endpoints. With the recent advances on CYP gene regulation, the transcriptional approach has gained increasing importance. The quantitative Real time RT-PCR is the most commonly used method used for this purpose (Wang et al., 2008). Phenobarbital is a prototypical CYP inducer. As mentioned by Handschin and colleagues (2003), DMEs induction is one of PB pleiotropic effects on the liver, which includes liver hypertrophy, tumor promotion, and induction of a number of other genes in addition to those encoding for DMEs and DTs (Handschin and Meyer, 2003). The transcriptional up-regulation by PB of hepatic CYPs has been reported in several animal species (Waxman & Azaroff, 1992) including pig (Puccinelli et al., 2010), rabbit (Marini et al., 2007), dog (Graham et al., 2006; Makino et al., 2009) and frog (Longo et al., 2004); despite this, the knowledge on molecular mechanism of induction is still restricted to human and rodent species (Timsit and Negishi, 2007). In cattle, much less is known about the effects of PB on DMEs, related transcription factors and DTs. Only preliminary results has been reported about PB-modulation on CYP2B, 2C, 3A, GST1A1-*like* and UGT1A1-*like* at post-transductional level by Cantiello and colleagues (2006). We demonstrated that PB induced hepatic CYP2B22, 2Cs and 3A28 in cattle, as well as UGT1A1- and GSTA1-*like*; Interestingly, a possible tissue-specific mechanism of regulation regulation could not be excluded, as demonstrated byresults obtained in small intestine, where a general inhibitory trend was observed, resulting significant for SULT1A1-*like*). Nuclear receptor mRNA levels were never affected by PB, while a significant modulation of hepatic ABCC2 and renal ABCG2 was noticed among DTs. Present results should be considered as a starting point for further molecular studies in cattle, designed to investigate more in depth the presence of species-differences in activation (NRs) and regulation of DMEs and DTs gene expression and function, which might ultimately result in a different kinetic behavior and/or in dangerous drug-drug interactions.

In vitro, liver cell systems represent a good experimental approach to screen potential hepatotoxic compounds and to investigate mechanisms by which chemicals induce liver response. Primary human hepatocytes, are the most recommended *in vitro* tool to study CYP-mediated metabolism and induction of new drugs in humans (Guillouzo, 1998; Gómez-Lechón et al., 2004; Hewitt et al., 2007). Cryopreservation is currently considered the only practical method for the long-term storage of functioning hepatocytes (Strain 1994). Over the last 20 years, many studies have been performed to define a feasible technique allowing the hepatocytes to function normally after thawing, with a particular interest toward DME pathways (Lloyd et al., 2003). Actually only few studies, referring

to human hepatocytes cryopreserved for a long period of time are reported; recently, Lu and colleagues (2011) shown that long-term cryopreserved hepatocytes still maintained liver-specific function after cryopreservation for 4 years. It should here mentioned that despite the fact that porcine CAR and PXRhave been cloned and characterized, the molecular mechanisms involved in pig CYP modulation are still superficial nor yet fully elucidated (Puccinelli et al., 2011), likewise to cattle. Few data about the effect of cryopreservation, time of culture and prototypical inducers upon porcine hepatocytes DMEs have been published so far. Moreover, most data refer to post-translational changes, and data about NRs constitutive gene expression and modulation have never been reported so far. That's the reason why the transcriptional effects of time and prototypical CYP3A inducers upon target CYPs and NRs mostly involved in DMEs regulation were investigated. in CPHs stored up to ten years in liquid nitrogen, Although the viability of CPHs was decreased, cultured viable cells expressed target genes; moreover, once exposed to prototypical CYP3A inducers, these candidate transcriptswere up-regulated. Thus, CPHs can be considered as a promising tool to study the expression and regulation of genes (DMEs, NRs) requiring a functional and complete transcription factor machinery.

Concluding this thesis has provided preliminary data which are important to characterize CYP-dependent biotransformation processes and CYP transcriptional regulation by NRs in bovine and porcine species. Future efforts will be address to functional characterize bovine and pig nuclear receptors as well the specific regulatory regions involved on CYP induction by using advanced biomolecular tools.

9. References

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Zancanella, V., Giantin, M., Lopparelli, R.M., Nebbia, C. & Dacasto, M. Tissue distribution and phenobarbital induction of SLC- and ABC-transporters in cattle tissue. **Paper submitted**.

Zancanella, V., Giantin, M., Lopparelli, R.M., Patarnello, T., Dacasto, M. & Negrisolo, E. (2010) Proposed new nomenclature for Bos taurus cytochromes P450 involved in xenobiotic drug metabolism. *Journal of Veterinary Pharmacology and Therapeutics*, **33**, 528-536.

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10. List of original publications

1. Zancanella, V., Giantin, M., Lopparelli, R.M., Patarnello, T., Dacasto, M. & Negrisolo, E., (2010) Proposed new nomenclature for Bos taurus cytochromes P450 involved in xenobiotic drug metabolism. J vet Pharmacol Therap, 33, 528-536.

2. Zancanella, V., Giantin, M., Lopparelli, R.M., Nebbia, C. & Dacasto, M. Tissue distribution and phenobarbital induction of SLC- and ABC- transporters in cattle tissues. Submitted.

3. Zancanella, V., Giantin, M., Lopparelli, R.M., Nebbia, C. & Dacasto, M. Constitutive expression and phenobarbital modulation of drug metabolizing enzymes and related nuclear receptors in cattle liver and extra-hepatic tissues. Submitted.

4. Giantin, M., Zancanella, V., Lopparelli, R.M., Granato, A., Carletti, M., Vilei, M.T., Muraca, M., Baratto, C. & Dacasto, M. Effects of time culture and prototypical cytochrome P450 3A (CYP3A) inducers on CYP2B22, CYP2C, CYP3A and nuclear receptors (NRs) mRNAs in long-term cryopreserved pig hepatocytes (CPHs). Submitted.

11. Curriculum vitae

Vanessa Zancanella was born on October 24, 1984 in Monselice, Padua, Italy.

In the period May, 2006 – September, 2006 she served an internship (bachelor's degree) in the Pharmacology and Toxicology Laboratory of the Department of Public Health, Comparative Pathology and Veterinary Hygiene, Veterinary Medicine Faculty of Padua (Italy), with Prof. Mauro Dacasto as supervisor. Then, in October, 2006 she graduated in Sanitary Biotechnologies, Interfaculty of Medicine and Surgery, Pharmacy and Veterinary Medicine of Padua (Italy).

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In December, 2008 she won a three years (January, 2009 – December, 2011) PhD fellowship in Veterinary Sciences at the Faculty of Veterinary Medicine of Padua (Italy). She spent seven months (May, 2010 – December, 2010) of her PhD as a guest at the University of Eastern Finland, Kuopio, Faculty of Health Sciences, School of Pharmacy, under the supervision of Prof. Paavo Honkakoski and Dr. Mika Reinisalo.