COMPARATIVE ANALYSES OF ABC TRANSPORTERS AND METABOLISING ENZYMES IN HUMAN AND RAT PLACENTAL MODELS

A Thesis submitted to The University of Manchester for the degree of Doctor of Philosophy (PhD) in the Faculty of Medical and Human Sciences

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List of Abbreviations

ABC	ATP- Binding Cassette
ADME	Absorption, Distribution, Metabolism and Excretion.
AEBSF	(4-(2-aminoethyl) benzenesulfonyl fluoride
AIDS	Acquired Immunodeficiency Syndrome
APS	Ammonium Persulphate
ATP	Adenosine Triphosphate
BCRP	Breast Cancer Resistance Protein
BSA	Bovine serum albumin
Calcein AM	Calcein acetoxymethyl ester
CAPs	3-[cyclohexylamino]-1-propanesulfonic acid
CAR	Constitutive Androstane Recpetor
cRNA	Complementary RNA
CTL	Central Toxicology Laboratory
CYP450/cyp450	Cytochrome p450
DAB	3,3'-diaminobenzidine
dcp	Days post coitum
dH_2O	distilled water
DMEM	Dulbecco's Modified Eagles Medium
DMSO	Dimethyl Sulphoxide
DNA	Deoyribonucleic Acid
ECL	Enhanced Chemo Luminescence
ECVAM	European Centre for the Validation of Alternative Methods
EDTA	Ethylenediaminetetraacetic acid
EOGRTS	Extended one-generation reproductive toxicity study
ERGATT	European Research Group for Alternative Toxicity Testing
EPA	Environmental Protection Agency
EST	Embryonic Stem Cell Test
EU	European Union
EVOM	Electronic Voltohmeter
EURLECVAM	European Union Reference Laboratory for Alternatives to Animal Testing
FBS	Foetal Bovine Serum
FDA	Food and Drug Administration
FMCG	Fast Moving Consumer Goods
FRAME	Fund for the Replacement of Animals in Medical Experiments
FTC	Fumitremorgin C
FC	Foetal Capillary
GAPDH	Glyceraldehyde 3-phosphate dehydrogenase
GD	Gestation Day

GEO	Gene Expression Omnibus
GF120918	Elacridar
GSH	Glutathione
GST/gst	Glutathione-s-Transferase
HEPES	4-(2-hydroxyethyl)-1-piperazineethanesulfonic acid
HIV	Human Immunodeficiency Virus
HRP	Horse-raddish linked Peroxidase
IVS	Intervillous Space
IgG	Immunoglobulin G
ICH	International Conference on Harmonisation
INVITTOX	*In vitro* Techniques in Toxicology
kDa	Dalton
MDR1	Multidrug Resistance Protein 1 (P-gp/ABCB1)
MTT	(methylthiazolydiphenyl-tetrazolium bromide (3-(4, 5-dimethylthiazol-2-yl)-2
mRNA	Messenger Ribonuclease
MRP	Multidrug-resistance associated proteins
MW	Molecular Weight
NBDs	Nucleotide-binding domains
NCBI	National Centre for Biotechnology Information
NCE	New Chemical Entity
NOAEL	No-observed-adverse-effect-level
OATP	Organic Anion Transporting Polypeptides
OECD	Organisation for Economic Co-operation
OPPTS	Office of Prevention, Pesticides and Toxic Substances
PAPS	3'-phosphoadenosine 5'-phosphosulfate
PBS	Phosphate Buffered Saline
PDB	Protein Data Bank
P-gp	P-glycoprotein
PhA	Pheophorbide A
PhIP	2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine
PSA	Polar Surface Area
PVDF	Polyvinylidene Fluoride
RA	Retinoic Acid
RCSB	Research Collaboratory for Structural Bioinformatics
RIPA	Radioimmunoprecipitation assay buffer
RM3	Rat and Mouse No.3 Food
RNA	Ribonucleic Acid
RT-PCR	Real Time Polymerase Chain Reaction
SD	Standard Deviation
SDS	Sodium Dodecyl Sulphate
SDS-PAGE	Sodium Dodecyl Sulphate Polyacrylamide gel electrophoresis
SEM	Standard Error of the Mean

SK	Syncytial Knot
SLCO	Solute Carriers (organic)
SULT/sult	Sulphotransferase
SYN	Syncytiotrophoblast
T1/T2/T3	Trimester 1/Trimester 2/Trimester 3
TBS-T	Tris - buffered saline with tween 20
TEM	Transmission Electron microscopy
TER	Transcellular Electrical Resistance
TG	Test Guideline
TEMED	Tetramethylethylenediamine
TMs	Transmembrane
TMDs	Transmembrane Domains
UDPGA	Uridine-5-diphospho-α-D-glucuronic acid
UGT/ugt	UDP-glucuronosyltransferases
USA	United States of America
WEC	Whole Embryo Culture
WT	Wild Type
XR9576	Tariquidar
ZR	Zona Reticularis

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Abstract

The placenta provides a protective barrier for the developing foetus during gestation. Physiological barriers including the placenta, liver, kidney, intestine and blood-brain barrier are known to express ATP-Binding cassette transporters (ABC transporters) and metabolising enzymes. These specialised proteins have the ability to transport or metabolise xenobiotics. There is evidence to suggest that ABC transporters and metabolising enzymes are located at the interface between the maternal and foetal blood supplies (a cell layer referred to as the syncytiotrophoblast) and therefore may help protect the foetus from harmful xenobiotics.

During new compound development prenatal developmental toxicity testing forms an important part of safety assessment. In order to predict potential toxicity of a new chemical entity to humans, rodent and non-rodent species are currently used. This thesis investigates the rat and human placental barrier properties in order to help facilitate our knowledge of species differences and contribute to our understanding of the limitations of these surrogate models. The approaches taken include: genomic analyses using microarray data to compare the overall expression of ABC transporters and metabolising enzymes throughout gestation in both species, immunohistochemical techniques to localise transporters and metabolising enzymes in the rat placenta, and *in vitro* functionality assays of selected transporters performed in rat and human placental cell line models.

The main findings have shown a similar mRNA expression level of ABCG2/BCRP (breast cancer resistance protein) throughout gestation in the rat and human, however different mRNA expression levels of other transporters (slco4a1/oatp4a1 in particular) and metabolising enzymes were also highlighted. Immunohistochemistry localised selected transporters to the syncytiotrophoblast region of the rat placenta (the interface of maternal and foetal circulations). Functional *in vitro* assays were successfully utilised in rat and human placental cell lines which showed functional ABCB1/P-gp in both species.

Overall, these findings provide a genomic characterisation of the rat and human protective placental barrier properties and show transporter functionality in *in vitro* cell-based assays which will prove useful in prenatal and developmental toxicity tests. Alternatives to using animals have been explored by using functional *in vitro* assays which could potentially be implored during the new compound discovery phase. This could help to make animal testing more selective for given compounds and ensures the new chemical entity is being tested in the model closest resembling the human.

Note:
Human genes and proteins are given in upper case, rodent genes and proteins are given in lower case; genes are given in italic text, proteins are given in non-italics.

Declaration

No portion of the work referred to in this thesis has been submitted in support of an application for another degree or qualification of this or any other university or other institute of learning

Louise Taylor

The Author

Louise Taylor gained a 2.1 (Hons) in Biochemistry from The University of Liverpool in 2008. Throughout her final year, Louise excelled in her dissertation and was inspired by her supervisors there to continue in academia and pursue a PhD.

In 2008, Louise began a PhD in Reproductive Toxicology at The University of Manchester. Throughout the 4 years of Louise's postgraduate training she has been able to showcase her work through poster presentations at various conferences including The Tommy's 3-centre meeting (2008), The *In Vitro* Toxicology Society (2009 and 2010, when she received runner up prize), The Society of Toxicology (2009 and 2012) and The European Teratology Society (2011). After receiving a Travel Award from the European Teratology Society (2011), Louise was invited to Ghent, Belgium to present her findings as an oral presentation. She was also awarded the 'Teddy Edward's Memorial prize' for Research into Congenital Malformations in 2011, awarded to the poster or free communication which best exemplifies the aims of the Society. As a result of being asked to present her findings in an oral presentation at the European Teratology Society, Louise's work was also published as a short communication in Reproductive Toxicology.

Soon after being selected to represent The University of Manchester in an 'Inaugural Open Minds' competition organised by Intelligent Formulation in conjunction with the global FMCG company, Reckitt Benckiser, Louise accepted a Graduate Research Associate position with the company.

Acknowledgments

Throughout my PhD I have had continued support from my supervisors and in particular would like to thank Dr Jeff Penny, Dr Jayne Wright and Dr Pratibha Mistry. A special thank you also to Mr Peter Walker who has been outstanding in sharing his expertise and so encouraging of my future career. The industrial sponsor, Syngenta, has been most generous with their financial support, helping me tremendously in both my professional and personal development throughout my PhD training.

I would also like to thank my family and friends. The continued support from my parents throughout the 8 years of undergraduate and postgraduate study has been unreserved and invaluable. I would also like to thank my friends for their continued generosity, laughter and support.

As a final acknowledgement, I would like to cite a poem that has inspired me throughout my PhD. It seems fitting to 'end' this journey and begin my next with a few words from Rudyard Kipling's timeless poem, "If".

If you can dream - and not make dreams your master;
If you can think - and not make thoughts your aim;
If you can meet with Triumph and Disaster
And treat those two impostors just the same;
If you can bear to hear the truth you've spoken
Twisted by knaves to make a trap for fools,
Or watch the things you gave your life to, broken,
And stoop and build 'em up with worn-out tools:

If you can talk with crowds and keep your virtue,
' Or walk with Kings - nor lose the common touch,
if neither foes nor loving friends can hurt you,
If all men count with you, but none too much;
If you can fill the unforgiving minute
With sixty seconds' worth of distance run,
Yours is the Earth and everything that's in it,
And - which is more - you'll be a Man, my son!

Rudyard Kipling

Chapter 1

Introduction

1.0 Chapter 1; Introduction

1.1 Background

Throughout evolution mammals have constantly been exposed to both harmful endogenous and exogenous substances, for example dietary mutagens such as 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) and aflatoxins (Ferguson and Philpott, 2008). As a result, mammals have developed a number of physical and biological protective systems including immunological processes, the skin, internal barriers that protect against systemic and organ-specific exposure including the blood-brain barrier, the gut and the placenta.

The placenta has an important role in normal foetal development as it provides a protective barrier for the foetus by modulating the exposure of the foetus to xenobiotics. The placenta also facilitates the exchange of nutrients, such as glucose, gases such as oxygen and the removal of waste products. The protective function of the placenta is of particular importance as pregnant women may be exposed to environmental and dietary xenobiotics and also to xenobiotics in the form of medication, for example the antiretroviral drug saquinavir, indicated to treat HIV/AIDS, is used in 0.01 – 0.3 % of pregnancies (Evseenko et al., 2006a). The scale of potential foetal exposure to xenobiotics can be seen from a multi-centre study that reported 64 % of all pregnant women in the USA used at least one prescription drug, other than a vitamin or mineral supplement, in the 270 days before delivery (Andrade et al., 2004). Studies have also shown that a large proportion of pregnant women (5 – 10 %) receive Food and Drug Administration (FDA) category D or X drugs which are potential teratogens (Andrade et al., 2006, Andrade et al., 2004).

It has already been shown that the placenta cannot provide a complete barrier for all xenobiotics; the first recorded evidence of reproductive toxicity caused by foetal exposure to a teratogen was the Thalidomide disaster (1957 – 1961). At the time of the tragedy the manufacturers, Chemie Grunenthal, tested Thalidomide on pregnant animals but only found teratogenic effects in rabbits administered 75 times the therapeutic dose for humans (Taussig, 1962). Further investigation of thalidomide in the mouse (Dipaolo, 1963), hamster (Homburger et al., 1965) and rat (Scott et al., 1977) found thalidomide to be inconsistent at causing teratogenic effects. The effects thalidomide has in different species highlights the extensive

testing procedure that industrial companies must therefore undertake before presenting a new compound to the market.

The way in which the placenta is able to provide a protective barrier is of particular importance in the agrochemical and pharmaceutical industries. The agrochemical and pharmaceutical industries use an array of strict tests in the developmental stages of new compounds. Both industries assess new compounds through toxicology testing in animals including pharmacokinetic and toxico-kinetic assessments, subchronic and chronic exposure studies and reproductive toxicity and neurotoxicity testing (in addition, the agrochemical industry also carries out extensive environmental testing including residual assessments, environmental fate, ecology. Animal and environmental testing provides the information needed to support human health risk assessment, as agrochemical industry products are never directly tested in man. Due to the extensive costs of development of agrochemicals and therapeutic drugs (up to $802 million for development of a single therapeutic drug (DiMasi et al., 2003)) and the impetus to reduce the number of animals used it is important to both the industries to only progress compounds from lead generation with the greatest likelihood of reaching the market. *In vitro* testing of chemicals in lead generation forms an important part of predicting potential *in vivo* toxicities. Early testing aids in candidate selection, it is therefore important that *in vitro* tools are predictive and offer a reliable alternative to animal testing.

Many physiological components contribute to the barrier–like properties of the placenta. There are a series of lipid bilayers that molecules must cross in order to reach the foetus. The processes by which these molecules penetrate the lipid bilayers include simple diffusion, facilitated diffusion and active transport. Recently, attention has focussed on the contribution to the barrier properties of the placenta provided by efflux transporters belonging to the ATP-binding Cassette (ABC) superfamily.

Expression of *ABCB1*, the gene encoding the human ABCB1 (P-glycoprotein, P-gp) transporter and *ABCG2*, the gene encoding human breast cancer resistance protein (BCRP) have been widely studied at the placenta due to their physiologically and toxicologically important contribution to the placental barrier.

Expression and functional activity of ABCB1 have been reported in fresh human placental tissue (Atkinson et al., 2003) and in the JAr human choriocarcinoma cell line (Evseenko et al., 2006b). Expression and functional

activity of ABCG2 have been documented in the JAr and BeWo cell lines (Evseenko et al., 2006b). Attempting to evaluate ABCB1- or ABCG2-mediated transport in these systems is difficult due to co-expression of multiple transporters with overlapping substrate specificities. However, use can be made of inhibitors in an attempt to study the transport contribution of individual transporters.

As developmental toxicity studies are carried out in rat and rabbit models, it is important to study the placenta in both species and evaluate the differences and similarities in transporter expression/function in the rat, rabbit and human placental models. This thesis looks at the direct comparison in transporter expression between the rat and human placenta. In order to be able to compare the molecular basis of barrier properties more fully across species, a similar approach should be used with the rabbit placenta (outside the remit of this thesis).

The TR-TBT 18d-1cell line is derived from the labyrinth region of the rat placenta (Kitano et al., 2004). However, although expression of abcb1 and abcg2 at the mRNA level has been reported (Kitano et al., 2004), there are no reports of functional activity of abcb1 and abcg2 in this cell line. To address this, expression and functional activity of abcb1 and abcg2 in TR-TBT 18d-1 will be investigated in this thesis.

The work carried out in this thesis focuses on investigating the interaction of xenobiotics at the placenta in human and rat models. The placenta is at the interface between the maternal and foetal circulations. During prenatal development it is crucial to understand the processes occurring at the placenta, as these influence embryonic and foetal development. The work and results generated will be able to provide a platform in order to further develop *in vitro* placental models for use in developmental toxicity assays to help predict the extent of xenobiotic transmission across the placental barrier and foetal exposure.

1.2 Toxicity studies

During the stages of new compound development, toxicity studies are performed to assess human safety. Such studies are carried out under guidelines determined by different governing bodies. Several different bodies advise on the type of toxicity testing that is required to adequately assess the effects of a new chemical on human health. The two main governing bodies in the United States are

the Environmental Protection Agency (EPA) and the Organisation for Economic Co-operation and Development (OECD) who perform rigorous risk assessment studies to evaluate the impact of pesticides on human health. The EPA uses the National Research Council's four-step process for human health risk assessment:

Step one: Hazard Identification

Step two: Dose-Response Assessment

Step three: Exposure Assessment

Step four: Risk Characterisation

The first step, hazard identification, looks directly at toxicology testing. Many toxicity tests are conducted and the data generated is used to identify the hazard potential, and to carry out risk assessment for operator exposure and dietary consumption (EPA 2007).

1.2.1 Reproductive Toxicity

Assessment of reproductive toxicology is extremely complex due to the different stages of the reproductive cycle. Test methods in experimental animals cover the essential steps throughout reproduction, including a) growth and maturation of sperm and oocyte; b) fertilisation, e.g. fusion of oocyte and sperm, resulting in a complete, diploid set of chromosomes; c) normal cleavage divisions, implantation, intrauterine development, birth and postnatal development throughout the period of lactation; and d) normal development of the offspring to fertile animals, which are able to produce a second generation (Spielmann, 2009). Due to the complexity of the reproductive system, several tests are stipulated by the OECD, European Union (EU) and the International Conference on Harmonisation (ICH)

Examples of the array of tests stipulated by the OECD during the discovery of new chemical entities are listed in Table 1.1.

Table 1.1 International test guidelines in reproductive toxicology.

	Test guideline (TG)
Industrial and agrochemicals (OECD)	Prenatal developmental toxicity study (TG 414)
	One-generation study (TG 415)
	Two-generation study (TG 416)
	Reproduction and developmental screening (TG 421)
	Combined repeated dose and developmental screening (TG 422)
	Developmental neurotoxicity study (TG 426)
	Uterotrophic assay, oestrogenic properties (TG 440)
	Hershberger assay, androgenic properties (draft TG)
	Human oestrogen receptor-α transcriptional activation assay (draft TG)
	Extended one-generation reproductive toxicity study (EOGRTS; draft TG)

Information taken from (Spielmann 2009). TG; Test guideline.

After the Thalidomide tragedy (1957 – 1961), new regulations in reproductive developmental toxicity testing were enforced; toxicity screens were implemented which included investigational drugs being tested in two species before use in human subjects (Silverman, 2002). As a consequence, the prenatal developmental toxicity test (TG 414) is conducted in one rodent and one non-rodent animal. The rat and rabbit species are routinely used; developmental toxicity studies of thalidomide showed that rats did not present malformed foetuses but rabbits did. This generated the notion, which after 50 years is still used, that rats and rabbits should be used in unison in developmental toxicity to generate a predictor of potential hazards for human health (Janer et al., 2008).

1.2.1.1. Prenatal Developmental Toxicity Study

The Prenatal Developmental Toxicity Study (OPPTS 870.3700, EPA Health Effects guidelines), also referred to as OECD Test 414, or the teratology study, aims to provide information concerning the effects of prenatal exposure on the pregnant test animal and on the developing organism (OECD, 2001).

During the study, a test substance is administered to pregnant animals starting at implantation day until one day prior to the day of scheduled kill. At least three dose levels of the test compound are used; with the dose levels being spaced to produce a graduation of toxic effects. The highest dose should aim to induce some developmental and/or maternal toxicity but not death or suffering, an intermediate dose level should produce minimal observable toxic effects and the lowest dose should not produce any evidence of maternal or developmental toxicity. A

benchmark dose is determined from a maternal no-observed-adverse-effect level (NOAEL) (OECD, 2001). However, if any foetal effects are seen at this maternal NOAEL level, developmental toxicity is implied.

1.2.1.2 Teratogens

Teratogens are compounds which have detrimental effects on the foetus in the first 3 months of pregnancy leading to disfiguring birth defects or malformations. Examples of teratogens include alcohol, some therapeutic drugs (including thalidomide and sodium valporate) and pesticides and insecticides. In 1959, Wilson put forward 'The Six principles of Teratology' which highlight the nature of teratogens (Wilson, 1973). These six principles are:

1. *Susceptibility to teratogenesis depends on the genotype of the embryo*
2. *Susceptibility to a teratogenic agent depends on the developmental stages when the exposure occurs*
3. *Different teratogenic agents affect developing cells and tissues in different ways*
4. *The final manifestations of abnormal development are death, malformation, growth retardation and functional disorder*
5. *The access of adverse environmental influences to developing tissues depends on the nature of the influences (agent)*
6. *Manifestations of deviant development increase in frequency and degree as dosage increases, from the no-effect to the totally lethal level.*

Due to the undesirable effects of teratogens and a need to reduce the number of live animals used in research, it is essential that credible *in vitro* assays be developed to accurately assess and help predict developmental toxicity.

1.2.1.3 Current surrogate developmental toxicity models

In the last 20 years, there has been an increased focus on seeking *in vitro* alternatives for assays that can provide a less expensive and higher throughput means to assess compound toxicity. Also, there are different centres worldwide whose aim is to create more informative and predictive *in vitro* models to help reduce the number of animals currently used in the development of new chemical entities. Centres actively working in this area include The National Institute of Health, The National Institute for Environmental Health Sciences, The Centre for Alternatives to Animal Testing and The John Hopkins School of Public Health.

In Europe, the European Centre for the Validation of Alternative Methods (ECVAM), established in 1992, plays a leading role in identifying developmental toxicity assays. The mission of ECVAM is to promote scientific and regulatory acceptance of non-animal tests thereby reducing the number of animal procedures. Working together with ECVAM; the European Research Group for Alternative Toxicity Testing (ERGATT) and the Fund for the Replacement of Animals in Medical Experiments (FRAME) helped to establish and fund INVITTOX; a database of methods that have been validated by scientists at ECVAM for *in vitro* toxicity testing (Balls and Clothier 1991; Ungar 1993).

Currently under the INVITTOX protocols three *in vitro* assays have been fully endorsed by ECVAM; the Embryonic Stem Cell Test (EST), Embryotoxicity Testing in Post-Implantation Whole Embryo Culture (WEC) – Method of Piersma and The Micromass Test – Method of Brown. Five other *in vitro* tests are currently being further evaluated; Embryotoxicity testing using a whole embryo culture (WEC) procedure, *In vitro* Micromass Teratogen Assay, Lung Cell Assay, Rabbit articular chondrocyte functional toxicity test and Rat Whole Embryo Culture (WEC) (Table 1.2).

There are limitations with each assay which still leads to the notion that animal testing cannot be totally eradicated. One limitation of the EST test is that it only covers a limited window of development (differentiation at the pre-implantation stage) prior to the most susceptible phase of organogenesis. However, the EST assay is the first *in vitro* embryotoxicity test in which no pregnant animals are sacrificed to obtain embryonic tissue for *in vitro* culture (ECVAM, 2010a). Similar to the EST assay, using the method proposed by Piersma, embryotoxicity testing in post implantation whole embryo culture, poses the limitation that there is a lack of

biotransformation capacity (Hareng et al., 2005). However, the advantage of this test is that it can be used as an intermediate between screening and mechanistic studies (ECVAM, 2010b). The final ECVAM-validated Micromass Test – Method of Brown (no.122) utilises primary rat limb buds to assess the teratogenicity of chemical substances. This test is advantageous as it requires less time and fewer animals than *in vivo* teratogenicity studies. However, the test is based on a primary cell culture system which demands individual cell suspensions for each experiment. Also, it has been shown that the system lacks any of the drug metabolising enzymes which transform xenobiotics (Spielmann et al., 2006).

It is evident that new approaches for refining, reducing and replacing animal studies in toxicity testing are constantly emerging. However in order to utilise the best possible predictive model for human outcome it is vital that we understand test species systems and human comprehensively. As the placenta is at the interface between the maternal and foetal circulations it is essential to study the protective placental barrier properties of the test species (rat and rabbit) and human. Having the basis of this knowledge can aid in designing new *in vitro* prenatal developmental toxicity screens for new chemical entities.

Table 1.2 INVITTOX methods approved by ECVAM. Information taken from European Union Reference Laboratory for Alternatives to Animal Testing (INVITTOX,2012)

INVITTOX Protocol	Brief Description
Embryonic Stem Cell Test *Protocol number:113*	The potential embryotoxicity of xenobitoics is assessed by its interference with the cell differentiation of permanent murine embryonic stem cell lines, compared to cytotoxic effects in the cells and 3T3 mouse fibroblasts.
Post - Implantation Whole Embryo Culture (WEC) - Method of Piersma *Protocol number:123*	The potential embryotoxicity of chemical substances in early stages of organogenesis can be assessed by the interference with differentiation and development of whole embryos in in vitro cultures.
Embryotoxicity testing using a whole embryo culture (WEC) procedure *Protocol number:68*	The potential embryotoxicity of chemical substances in early stages of organogenesis can be assessed by the interference with differentiation and development of rat or mouse whole embryos in in vitro culture, either in the presence or in the absence of a biotransforming system prepared from liver.
In vitro Micromass Teratogen Assay *Protocol number: 114*	The potential teratogenicity of chemical substances is assessed by its interference with growth, migration and reaggregation of dissociated rat primary cells of limb buds or the central nervous system, in the presence or absence of a metabolic activation system
Lung Cell Assay *Portocol number: 48*	Potential embryotoxicity is assessed by monitoring the effect of the test compounds on total protein synthesis in culture human foetal lung fibroblasts/ Rat lung epithelial cells can be used to determine cytotoxicity of selected compounds because of their ability to metabolise xenobitoics.
Rabbit articular chondorcyte functional toxicity test *Protocol number: 41*	Rabbit articular androcytes are culutred in the presence of test compound, the toxicity of which is then determined by its effect on the production of proteoglycan by the cells, as detected by the dye Alcian Blue
Rat Whole Embryo Culture (WEC) *Protocol number: 72*	The potential embryotoxicity of chemical substnaces in early stages of organogenesis can be assessed by the interference with differentiation and development of whole rat embryos in in vitro cultures
The Micromass Test - Method of Brown *Protocol number: 122*	The potential teratogenicity of chemical substances is assessed by its interferences with growth, migration and reaggregation of dissociated primary cells of rat limb buds

1.3 The Placenta

1.3.1 Anatomy of the placenta

Many xenobiotics can pass through the placenta to some degree and may therefore influence development of the unborn child. Although a wide range of species have contributed to studies of placental transfer, the most coherent bodies of recent evidence has come from three models; human, rat and sheep.

The basis of the structure of placentas arises from folded layers of tissue providing protection throughout gestation, from the developing fertilised embryo to the pre-term foetus. The maternal side of the placenta is derived from the epithelium or connective tissue of the ovary, oviduct or uterus and the foetal side is a derivative of the ectodermal epithelium (trophoblast or trophoectoderm) which forms the blastocyst surface (Wooding and Burton 2008).

The yolk sac, chorion, amnion and allantois are the key extraembryonic membranes which cumulatively form the placenta and provide the protective barrier between the maternal and foetal circulations (Figure 1.1).

Classification of the placenta is given by the number of extraembryonic foetal layers and is commonly referred to as Grosser's classification (Table 1.3). The number of tissue layers is thought to alter the physiological exchange between the maternal and foetal circulations.

Table 1.3 The anatomical layers of mammalian placentas (Wooding and Flint, 1994)

Classification	Type and number of layers		
	Foetal layers	Maternal layers	Examples
Haemomonochorial	Foetal endothelial layer Foetal connective tissue Chorionic epithlial layer	None	Humans, Rats and Mice
Endotheliochorial	Foetal endothelial layer Foetal connective tissue Chorionic epithelial layer	Uterine endothelial	Dogs and Cats
Epitheliochorial	Foetal endothelial layer Foetal connective tissue Chorionic epithelial layer	Uterine endothelial Connective Tissue Endometrial - Epithelium	Pigs, Cows and Horses

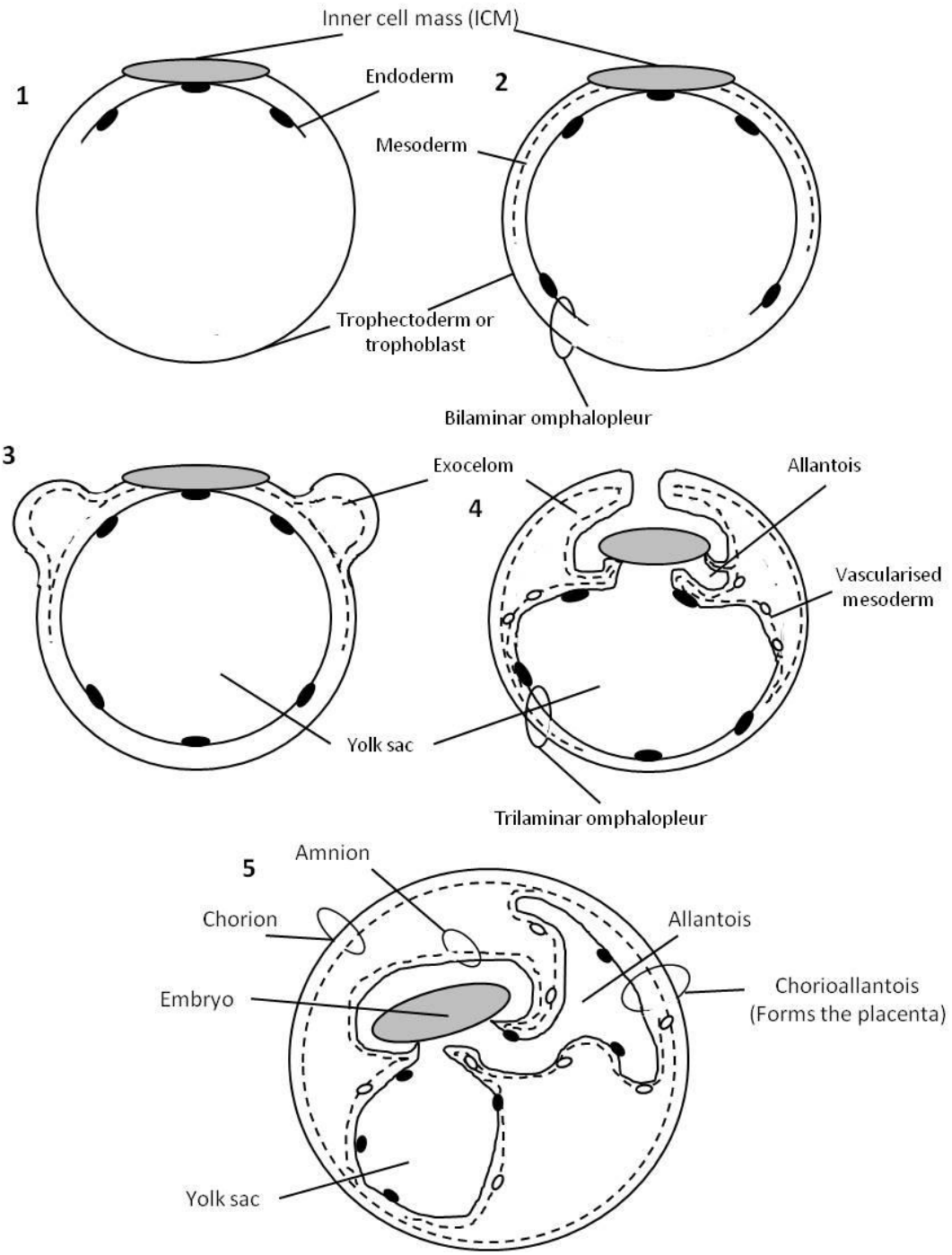


Figure 1.1 Development of the extraembryonic membranes. Stages 1-5 illustrate the development of the yolk sac, the allantois, the amnion and the chorioallantois. Picture adapted from (Wooding and Burton 2008)

Due to the diversity in shape of the placenta across species, further classifications exist; a simple criteria to classify placentas is shape at term (Table 1.4). There are exceptions which do not fall into these categories, such as the human placenta which begins diffuse and ends as discoid (Wooding and Burton 2008).

Table 1.4 Placenta classification by shape. Information taken from Wooding and Burton (2008).

Shape	Description	Species
Diffuse	Folds/villi over entire surface	Pig, horse
Cotyledonary	Chorionic villi grouped into a characteristic number of discrete tufts which can vary from 5 to 150	Ruminants
Zonary	Complex folds restricted to an equatorial band or path	Carnivores
Discoid	Villi restricted in a single or double disc	Rodents, insectivores, anthropoids

The rat and rabbit are the species of choice for developmental toxicity testing due to the short gestation period and their ease of use. They also demonstrate similar properties in terms of placental structure to humans. However, one key difference between the three species is the presence and function of the yolk sac. In eutherian mammals, the yolk sac placenta functions for only a short time, and this varies between species. In humans, a secondary yolk sac develops from the primary yolk sac and by the tenth week of gestation the yolk sac starts to degenerate and ceases to function (Jones and Jauniaux, 1995). In contrast to humans, rats have an inverted yolk sac which is the main source of foetal-placental exchange from its establishment at GD 7 – 7.5 until formation of the chorioallantoic placenta at GD 9. The inverted yolk sac is formed by the invagination of the yolk sac sphere to form a cup, bringing the vascularised endodermal top (or embryonic) region of the sac against the outer non-vascularised bilaminar region, obliterating the yolk sac lumen. It is important to highlight the yolk sac in the rodent as this forms a partial barrier between the mother and foetus, prior to the fully developed chorioallantoic placenta. This is a key anatomical difference between the human and rat placentation and foetal development.

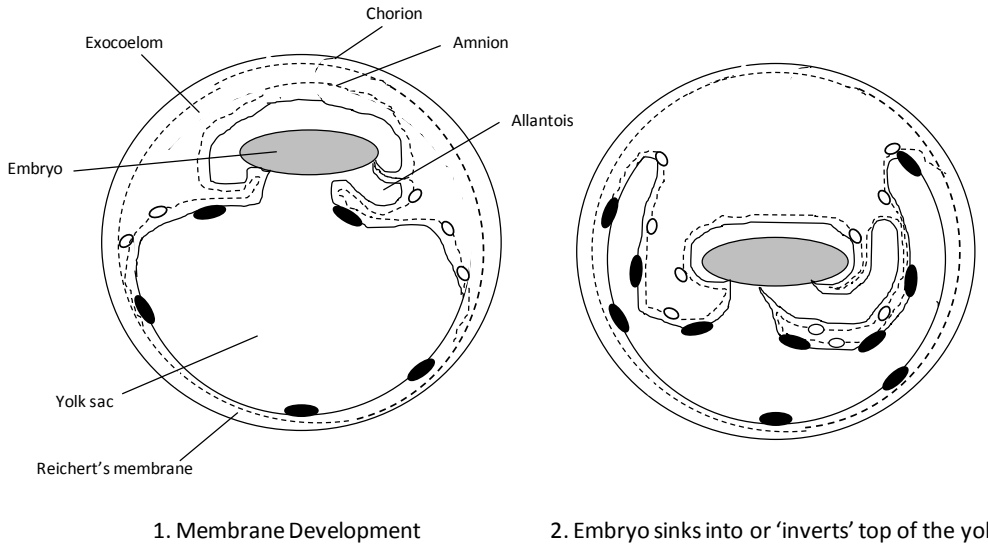


Figure 1.2 Development of the inverted yolk sac found in rat. Picture adapted from Wooding and Burton, (2008)

As prenatal developmental toxicity testing is also performed in the rabbit, it is important to consider the type of placenta this mammal has, as the structural differences in placentas from different species affect their function. A simplistic comparison between human, rat and rabbit gestation and placentation is represented in Table 1.5. As gestation times are shorter in rat and rabbit, this allows experiments to be performed a lot quicker than in a higher organism. Developmental rate of the rodent or rabbit foetus is also much faster than the human.

Table 1.5 Reproductive and Placentation comparisons between human, rat and rabbit. Information taken from Wooding and Burton (2004) and DeSesso et al., (2012)

Species	Sexual cycle	Gestation	Yolk sac	Chorioallantois	Shape	Interhaemal membrane
Human	28 days	270 days	Regresses at ~ 10 weeks of pregnancy	Forms the definitive placenta	Discoid	Haemomonochorial
Rat	4 - 7 days	22 days	(1) Bilaminar yolk sac is primary placenta upto GD 9, then ruptures. (2) Inverted well-vascularised yolk sac persists until term although is redundant once chorioallantois formed	Forms definitive placenta. Allantois sac is vestigal or absent Chorioallantoic placenta forms at about GD 11.5	Discoid, labyrinthine	Haemotrichorial
Rabbit	None	30 - 32 days	(1) Bilaminar yolk sac until 16 dpc, then ruptures (2) Inverted yolk sac placenta, vascularised villous fold adjacent to embryo. Active during pregnancy	Definitive placenta vascularised by the mesoderm of allantoic sac	Discoid, labyrinthine internal structure	Haemodichorial

As more is known about the development of the rat than the rabbit this thesis will now focus on the differences in placentation between the human and rat. The key foetal developmental stages in the rat and human are outlined in Figure 1.3.

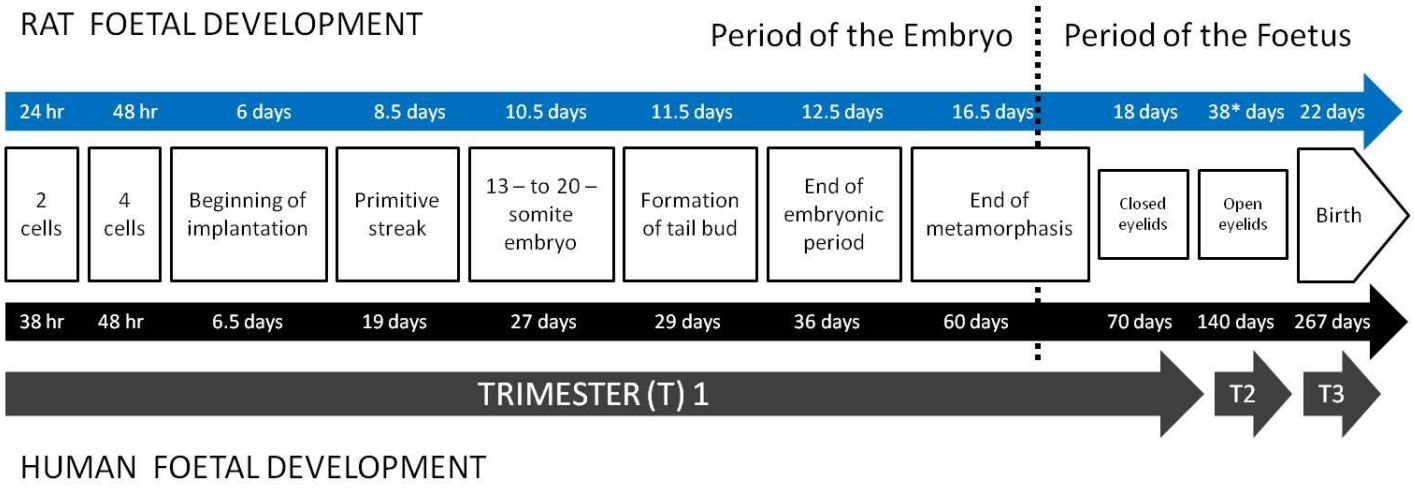


Figure 1.3 A comparative time chart of foetal development in the rat and human. The asterisk at 38 days in rat foetal development denotes this occurs after birth. Human foetal development has been separated into trimesters. Information taken from (Witschi, 1956)

It is of great importance to consider the development of the placenta throughout gestation. As already mentioned, the rat has an inverted yolk sac which acts as an additional barrier within the chorioallantoic placenta until GD 9 (Table 1.5). This anatomical difference between the human and rat placenta is well characterised along with other developmental characteristics such as the presence of Reichert's membrane in the rat embryo. These differences are summarised in Figure 1.4.

Apart from the anatomical differences between the species, it is also important to understand the differences at a molecular level. A basis of understanding at the molecular level will be attempted in this thesis.

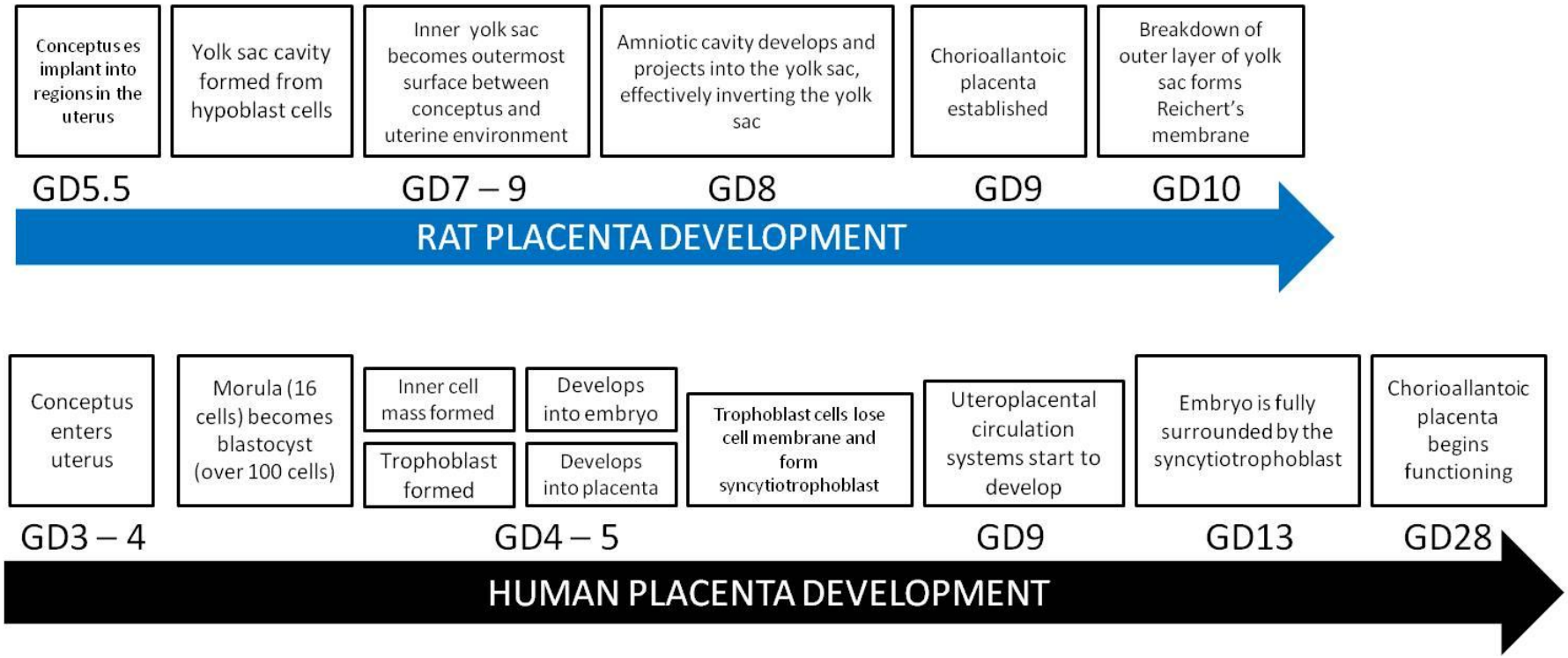


Figure 1.4 Comparative placentation between the rat and human at early development. The key stages of early placental development are highlighted in the rat and human species. In both species the chorioallantoic placenta is eliminated from the uterus at birth (GD 22 in rat and GD 270 in human). Information taken from (Carney et al., 2004)

There have been many articles which have compared the human trimesters to specific gestation days in the rat however the majority of these studies look at the comparison between foetal brain development.

In this thesis the aim is to investigate relative expression levels of transporter proteins and metabolic enzyme in the placenta throughout gestation. The development of the placenta has been used for the basis of the comparison between trimester (in human) and gestation day (in rat). This is numerically outlined in Figure 1.5

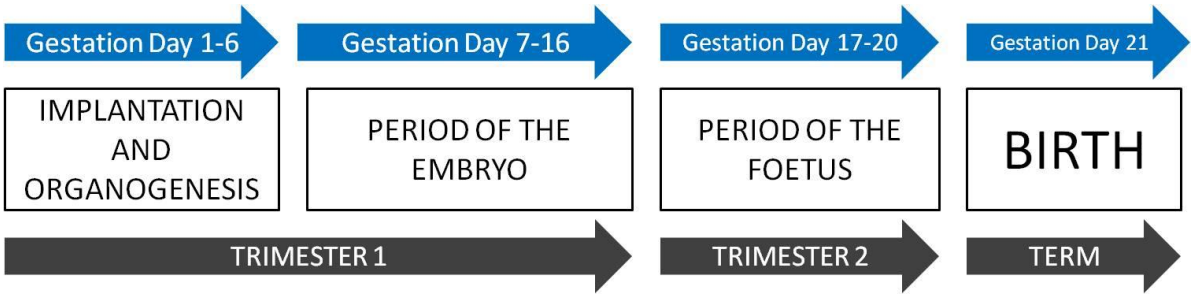


Figure 1.5 Comparison of rat gestation day and human trimester. Timings were evaluated using information from Carney et al., (2004).

At the end of gestation both rat and human have a definitive chorioallantoic placenta, which is discoid in shape (as outlined in Table 1.5) and are represented in Figure 1.6 and 1.7 respectively. The syncytiotrophoblast cells are at the interface between the maternal and foetal circulation, and it is here where localisation of ABCB1 (Atkinson et al., 2003) and ABCG2 (Malipaard et al., 2001) have previously been reported.

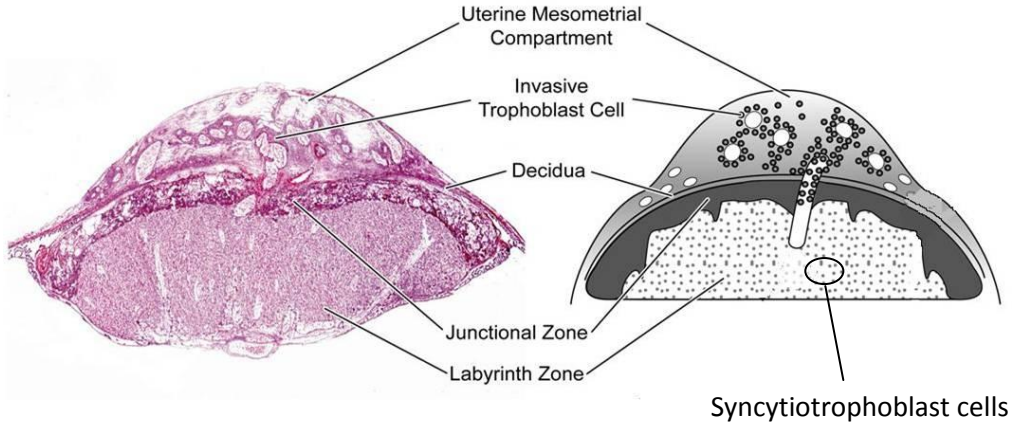


Figure 1.6 Rat placenta at late gestation. Haematoxylin and eosin-stained tissue section of the late gestation rat uteroplacental compartment (left panel, GD 18) and a corresponding schematic diagram (right panel). Picture taken from Ain et al., (2006)

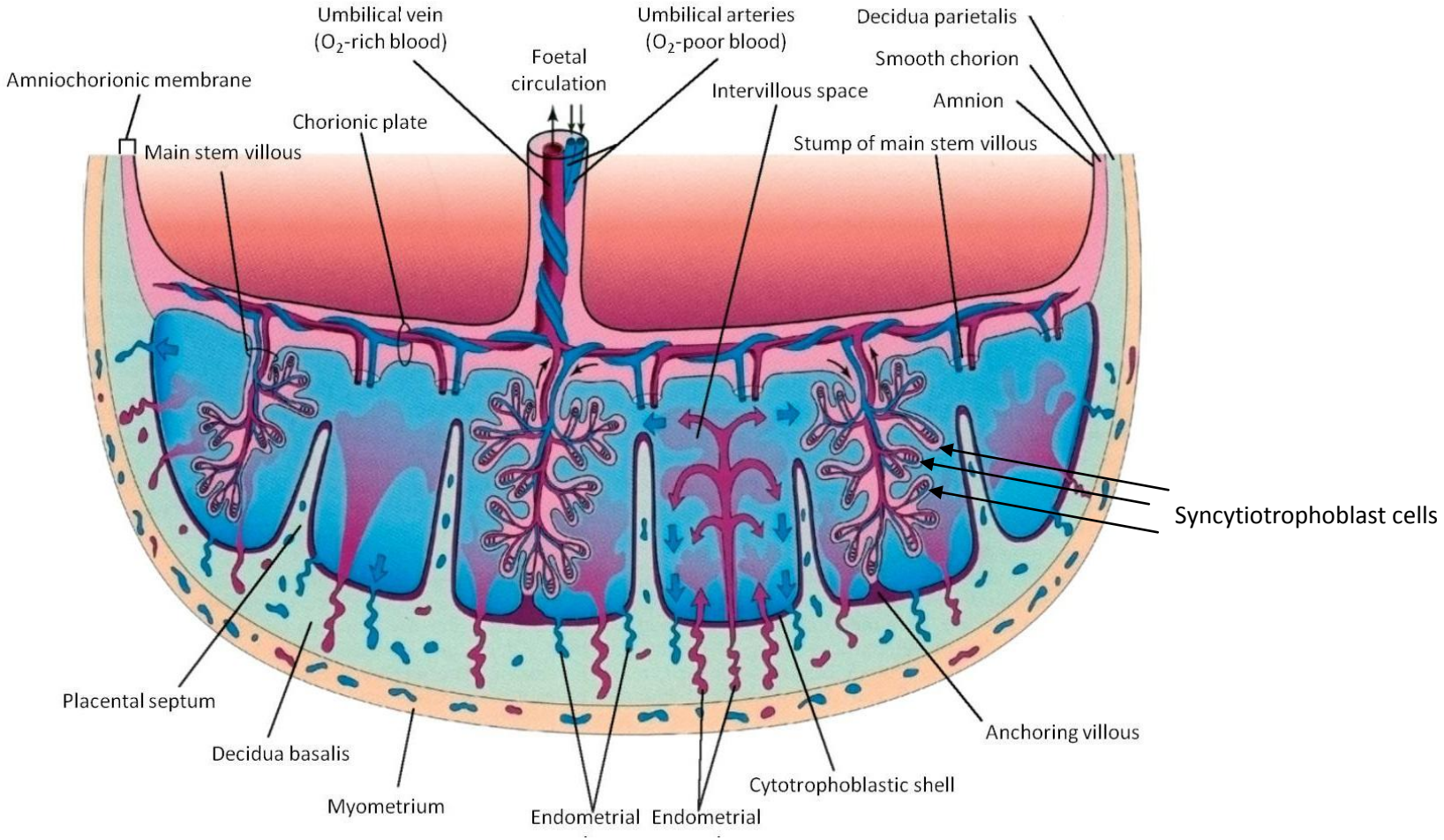


Figure 1.7 Schematic drawing of a transverse section through a full term human placenta. The villous branches are bathed in maternal blood and provide a large surface area for transplacental exchange. Figure adapted from Saunders, (2009)

1.3.2 Maternal and foetal circulation in the placenta

The human placenta is formed by foetal (chorionic plate and chorionic villi) and maternal (deciduas basalis) tissues (Figure 1.7). Foetal blood is supplied from two arteries within the umbilical cord, and the oxygenated blood returns to the foetus via a single cord vein (Page, 1993).

Maternal and foetal circulation are separated by the endothelium of the foetal capillaries and the trophoblast layer, containing the villous stroma, cytotrophoblast cells and syncytiotrophoblast. The trophoblast layer consists of continuous multinucleated syncytiotrophoblasts which arises by fusion of mononucleated cytotrophoblast cells to form a discontinuous layer beneath. The trophoblast layer is bathed by the maternal blood within the intervillous space (Ceckova-Novotna et al., 2006a). It is the uninterrupted syncytiotrophoblast that is the main site of exchange between the maternal and foetal circulations (Bloxam et al., 1997). The apical microvillous membrane of the syncytiotrophoblast is maternal-facing whilst the basal membrane is foetal-facing.

1.3.3 Function of the placenta

The placenta has two main functions: to maximise the transfer of oxygen and nutrients from the mother to the foetus and to minimise immunological rejection mediated by the maternal immune system. As the only source of oxygen and nutrient transfer, the placenta acts as a surrogate foetal lung, gut and kidney (Wooding and Burton 2008). Whilst the maternal and foetal circulations are separate, it is the close proximity of the maternal and foetal blood that allows this efficient exchange. Xenobiotics in the maternal circulation may enter the foetus by a range of transport methods namely passive diffusion, facilitated diffusion, active transport, pinocytosis and phagocytosis (Figure 1.8).

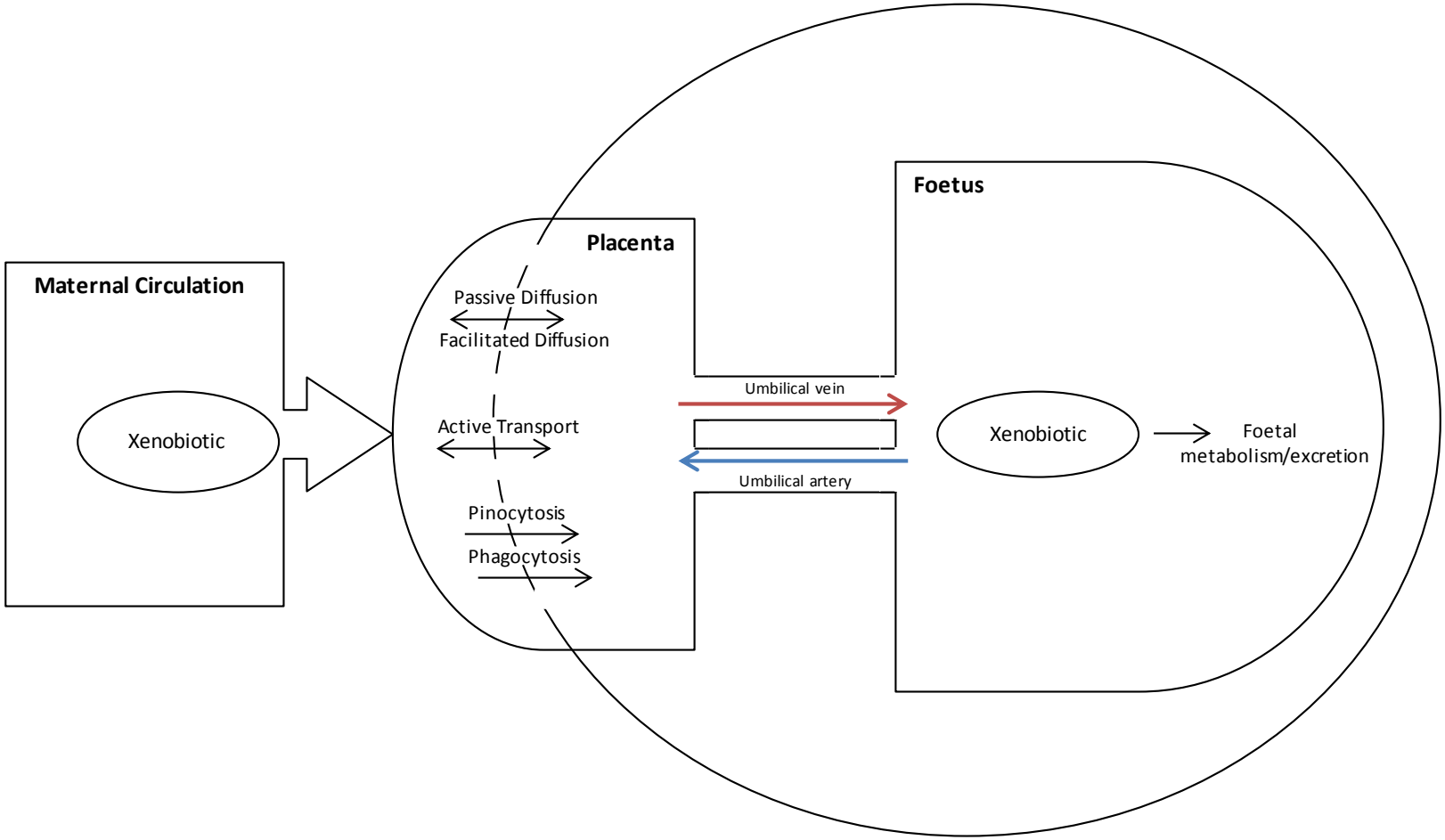


Figure 1.8 Transport of xenobiotics from maternal to foetal circulation. Xenobiotics can transfer from the maternal circulation by diffusion, active transport or via metabolism.

1.4 Efflux transporters and metabolising enzymes at the placental barrier

The placenta forms the materno-foetal interface and therefore has the potential to act as a barrier to transfer of xenobiotics, present in maternal plasma, into the foetus. Both efflux transporters and metabolising enzymes are expressed in the placenta and have the ability to influence exposure of the foetus to xenobiotics. Therefore it is imperative to understand how expression of placental efflux transporters and metabolising enzymes differ between species and throughout gestation.

Efflux transporters can be directly involved in limiting the disposition of drugs and xenobiotics in the foetus. ABCB1 (P-glycoprotein, encoded by the *ABCB1* gene) and ABCG2 (BCRP, encoded by the *ABCG2* gene) are placental efflux transporters able to affect exposure of the foetus to xenobiotics (Smit et al., 1999, Allikmets et al., 1998). In addition to ABCB1 and ABCG2, members of the multidrug-resistance associated proteins (MRPs, encoded by the *ABCC* genes) which extrude hydrophobic uncharged molecules and water-soluble anionic compounds (Bodo et al., 2003), are expressed in the placenta.

Uptake transporters generally participate in the uptake of substrates into cells, and function activity of members of the OATP (SLCO) family (encoded by the *SLCO* genes) have been reported in the human (Loubiere et al., 2010) and rat placenta (St-Pierre et al., 2004). These transporters are thought to transport hormones, e.g. slco4a1 is primarily responsible for transporting thyroid hormones (Fujiwara et al., 2001) however, the role of these transporters in influencing foetal xenobiotic disposition is still unclear.

Xenobiotic metabolising enzymes play a vital part in clearance of xenobiotics. Phase I metabolising enzymes are more commonly referred to as cytochrome p450 (CYP) enzymes. These are well known to catalyze oxidative metabolism of a vast number of compounds including proteratogens, procarcinogens and promutagens (Hakkola et al., 1998). Expression and functional activity of some CYP enzymes have been reported in rat and human placentas; functional activities of human placental CYP1A and CYP2E1 isoforms have been confirmed (Pasanen, 1999) and expression of rat placental cyp3a1 has been found by western blot analysis (Ejiri et al., 2005). Activities of phase II enzymes, for example glutathione-S-transferases, sulfotransferases and UDP-glucuronosyltransferases have also been

reported, however these enzymes have not been linked to detoxification of xenobiotics within the placenta (Pasanen et al., 1999; Syme et al., 2004).

1.4.1 ATP-Binding Cassette (ABC) Transporters

1.4.1.1 The ABC Superfamily

The ABC superfamily of proteins is found in all species from simple prokaryotes to humans. There are 48 human genes belonging to the ABC superfamily, which are categorised into 7 subfamilies, A-G.

1.4.1.1.1 ABCB1

The *ABCB1* gene encodes ABCB1 (P-glycoprotein). Humans possess one gene whereas rodents possess two isoforms of the gene; *abcb1a* and *abcb1b* (Schinkel et al., 1997). ABCB1 is one of the most clinically important transporters in humans (Zhou, 2008) and is expressed throughout the body, typically on pharmacological barriers.

ABCB1 was first discovered in a study conducted on Chinese hamster ovary cells which found that the rate of drug uptake appeared to be controlled, in a reversible manner, by intracellular ATP levels (Juliano and Ling, 1976). The name P-glycoprotein was derived due to the capacity of the protein to alter the permeability of drugs such as vinblastine (an anti-mitotic drug used to treat cancer), colcemid (which is related to cochicine and used to treat gout), and daunomycin (a chemotherapeutic anthracycline) (Juliano and Ling, 1976).

ABCB1 was initially studied as a prominent mechanism conferring multidrug-resistance in cancer cells (Goldstein, 1996, Goldstein et al., 1991); it was later found that ABCB1 also plays an important role in the protection of normal, non-malignant tissue and in influencing human pharmacokinetics (Lankas et al., 1998; Schinkel et al., 1994)

1.4.1.1.1.1 Structure of ABCB1

ABCB1 encodes the 170 kDA ABCB1 (P-glycoprotein) efflux transporter protein containing 1280 amino acids. The protein is comprised of two transmembrane domains (TMDs), each containing six alpha helical regions, and two nucleotide-binding domains (NBDs), joined by an intracellular linker region (Figure 1.9).

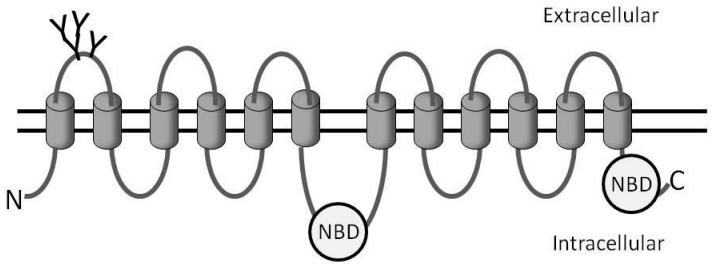


Figure 1.9 Proposed topology of ABCB1. ABCB1 consists of two transmembrane domains; each containing six transmembrane segments. The NBDs are located in the cytoplasmic region. Figure adapted from Schinkel and Jonker (2003).

The NH_2- and COOH-terminals are located intracellularly and the first extracellular loop is N-glycosylated. Based on *in vitro* studies (Schinkel et al., 1993) it appears that N-glycosylation is not necessary for basic transport function. However, N-glycosylation is likely to have an important biological role, possibly helping routing to, and stability in, the plasma membrane. Evidence to support the role of N-glycosylation in aiding protein stability is provided by studies in which inhibition of N-glycosylation increased ubiquitination resulting in the degradation of ABCB1 in MCF-7 cells (Wang et al., 2008)

Substrates are actively 'pumped' using the energy generated by ATP hydrolysis and it has been reported that both NBDs are essential for the proper functioning of ABCB1 (Romsicki and Sharom, 1998). Each NBD consists of two core motifs; Walker A and Walker B, and also a Signature region. These motifs are conserved in most ATPases and are directly involved in the building and hydrolysis of nucleotides (Walker et al., 1982)

It is generally accepted that ABCB1-mediated transport is coupled to hydrolysis of two ATP molecules. The transport process is considered to involve a transient conformational change in the transporter which allows interaction between the drug-binding domain and nucleotide-binding sites, although the precise mechanism of this interaction remains to be clarified.

1.4.1.1.1.2 ABCB1 substrates and inhibitors

ABCB1 is able to transport a wide variety of chemically and structurally diverse compounds. Potential mechanisms by which ABCB1 transports xenobiotics include a hydrophobic 'vacuum cleaner' (Stein, 1997) and a flippase (Higgins and Grottesman, 1992). Briefly, ABCB1 binds hydrophobic compounds located within

the cell membrane and effluxes the substrate to the extracellular compartment preventing or reducing intracellular accumulation of the compound.

ABCB1 has higher affinity for hydrophobic and cationic compounds. Substrates include many therapeutic drugs from different therapeutic classes including antiasthmatics, antibiotics, antidepressants, anti-human immune-deficiency virus (HIV) agents, anti-emetics, and opioids. A list of common substrates and inhibitors are listed in Appendix 3, Table 3A.

Compounds that are likely to interact with ABCB1 can be indicated by their size, shape, solubility and hydrogen bonding characteristics. Lipinski's rule of five (Lipinski et al., 2001) is commonly followed throughout drug development and is considered to be indicative for a compound to transported by ABCB1. The rule of 5 states that poor absorption or permeation is more likely when compounds possess:

a) More than 5 H-bond donors (expressed as the sum of OHs and NHs)
b) A MW of over 500
c) A LogP of over 5
d) More than 10 H-bond acceptors (expressed as the sum of N and O atoms)

1.4.1.1.1.3 Localisation of ABCB1/abcb1

ABCB1/abcb1 is located on the apical membrane of the placental syncytiotrophoblast in humans (Atkinson et al., 2003). Localisation has also been determined in the rat placenta, with immunohistochemistry showing positive abcb1 staining in the labyrinth region of the rat placenta from GD 13 onwards (Novotna et al., 2004). This localisation at the interface of maternal and foetal circulations allows the efflux proteins to transport xenobiotics away from the foetus towards the maternal circulation, thereby protecting the foetus in both human and rat.

1.4.1.1.2 ABCG2

ABCG2 encodes ABCG2 (BCRP, the breast cancer resistance protein). ABCG2 was discovered in 1998 after being cloned from the drug resistance MCF-7/AdrVp breast cancer cell line (Doyle et al., 1998). ABCG2 is also referred to as the mitoxantrone resistance protein as shortly after the discovery of ABCG2, a very similar transporter was identified primarily in S1-M1-80 human colon carcinoma cells and confirmed by Northern analysis using MCF-7 AdVp3000 cells which were

found to express ABCG2 10 -12 fold greater than wild type cells (Miyake et al., 1999).

1.4.1.1.2.1 The structure of ABCG2

ABCG2 encodes the 75 kDa efflux transporter protein possessing a single NBD (Figure 1.10). It is often referred to as a half transporter since, in comparison to ABCB1, it only possesses six TMDs and one NBD. It is generally accepted that for an ABC transporter to be functional at least 2 NBDs and a minimum of 12 TMs are required. It is considered that BCRP functions as either a homodimer or heterodimer (Ozvergy et al., 2001, Janvillisri et al., 2003).

Members of the ABCG subfamily are the only known ABC transporters with a domain arrangement in which the NBD precedes the transmembrane region. As with ABCB1, the Walker A and Walker B regions are located in the NBD, along with the Signature region.

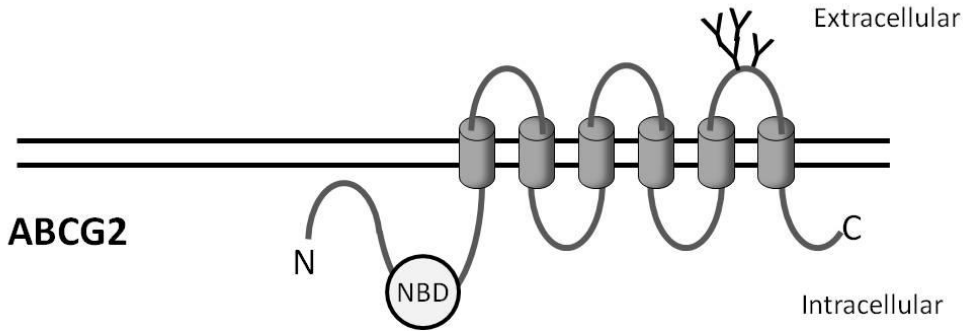


Figure 1.10 Proposed topology of ABCG2. ABCG2 consists of one transmembrane domain containing six transmembrane segments. The one NBD is located in the cytoplasmic region. Picture adapted from Schinkel and Jonker (2003).

1.4.1.1.2.2 ABCG2 Substrates and Inhibitors

The ability of ABCG2/abcg2 to efflux xenobiotics at the placenta has been analysed in studies using *in vitro* cell culture, *ex vivo* perfused placenta and *in vivo* animal models.

ABCG2 possess broad specificity, transporting a range of substrates including the chemotherapeutic agents; mitoxantrone, camptothecin derivatives (e.g. topotecan and irinotecan) and anthracyclines (e.g. daunorubicin and doxorubicin). There is a considerable overlap in substrate specificity between ABCG2 and ABCB1. A list of substrates and inhibitors can be found in Appendix 3, Table 3B.

Key findings demonstrate that fumitremorgin C (FTC) inhibits mitoxantrone uptake into membrane vesicles isolated from human term placenta (Kolwankar et al., 2005). *In vitro* studies using the BeWo and JAr choriocarcinoma cell lines have shown ko143 (Ceckova et al., 2006) and FTC (Evseenko et al., 2006b) to increase the intracellular accumulation of mitoxantrone or Hoechst. Several ABCB1 inhibitors e.g. elacridar (GF120918) (de Bruin et al., 1999) and tariquidar (XR9576) (Robey et al., 2004) have also been reported to inhibit ABCG2, however FTC specifically inhibits ABCG2-mediated transport. The analogue of FTC, ko143, is preferred due to its reduced potency in *in vitro* systems (Allen et al., 2002). Using inhibitors to block the efflux ability of transporters is a useful tool for investigating transporter function; this approach will be utilised in this thesis.

1.4.1.1.2.3 Localisation of ABCG2

ABCG2 has been localised to the membrane of the placental syncytiotrophoblast in human placenta (Malipaard et al., 2001). The localisation of abcg2 has also been verified in the rat term placenta using immunohistochemical analysis, confirming expression at the labyrinth region (Staud at al., 2006). This localisation of ABCG2/abcg2 at the syncytiotrophoblast is at the interface of the maternal and foetal blood exchange, therefore providing a protective barrier to the foetus.

1.4.1.1.3 The ABCC Family

The *ABCC* gene family encodes the multidrug resistance-associated proteins. The ABCC family includes ABCC1-6 and ABCC10-12. Specifically, ABCC1 (Atkinson et al., 2003, St-Pierre et al., 2000), ABCC2 (Jedlitschky et al., 2006, St-Pierre et al., 2000), ABCC3 (St-Pierre et al., 2000), ABCC4 (Serrano et al., 2007), ABCC5 (Meyer Zu Schwabedissen et al., 2005), and ABCC6 (St-Pierre et al., 2000) have been found to be expressed in the human placenta. One study has quantified the mRNA levels of xenobiotic transporters in the rat placenta; abcc1, abcc2, abcc3, abcc4, abcc5 and abcc6 were found (Leazer and Klaassen, 2003).

1.4.1.1.3.1 The Structure and localisation of ABCC family members

As members of the ABC family, the overall architecture between members is similar. ABCC1, ABCC2 and ABCC3 have the same basic structure as ABCB1 however, in addition, they have an N-terminal extension consisting of 5 putative transmembrane segments (Figure 1.11 A). ABCC4 and ABCC5 have the same basic structure as ABCB1, possessing 12 transmembrane segments and 2 nucleotide binding domains (Figure 1.11 B). In ABCC1, ABCC2 and ABCC3 glycosylation occurs at the first and seventh loop (Figure 1.11 A) whereas in ABCC4 and ABCC5 N-glycosylation is thought to occur on the fourth extracellular loop (Figure 1.11 B).

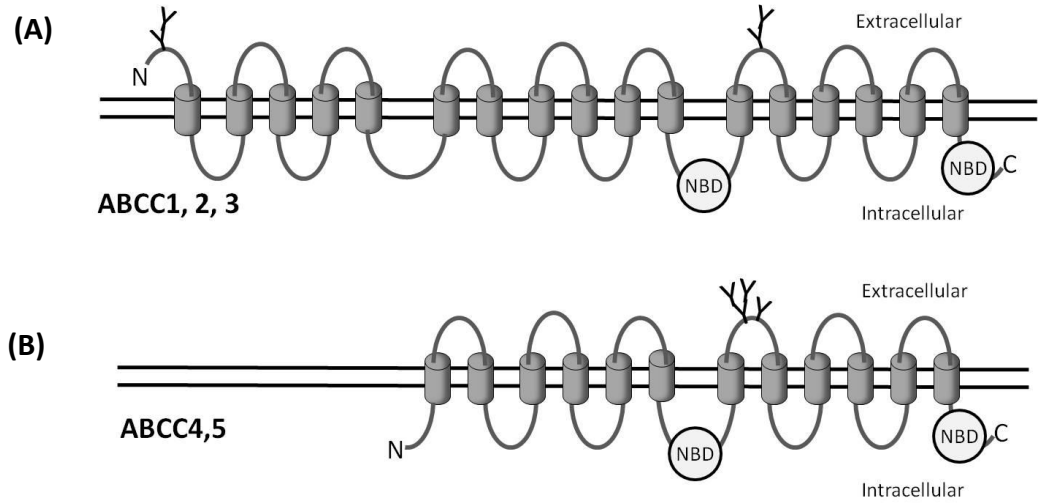


Figure 1.11 Proposed topology of ABCC1 – 5. (A) the additional amino terminal extension containing 5 transmembrane segments which are N-glycosylated at the first and seventh extracellular loops. (B) ABCC4 and ABCC5 lack the amino terminal extension and are N-glycosylated at the fourth extracellular loop. Figure adapted from Schinkel and Jonker (2003).

ABCC transporters transport an extremely large array of structurally diverse substrates including conjugated compounds. Due to the different types of compounds transported by ABCCs it has been suggested that the function of ABCC transporters is to mediate transport of xenobiotics between the mother and foetus (Syme et al., 2004; St-Pierre et al., 2002).

ABCC1 was first identified in 1992 as the cause of multidrug resistance in the anthracycline-selected human small cell lung carcinoma cell line, H69AR (Cole et al., 1992). Substrates of the transporter are structurally diverse and include leukotriene C_4 (Leier et al., 1994), estrone 3-sulfate (Qian et al., 2001) and folic acid (Hooijberg et al., 2004). ABCC1 has been localised to the membrane of the syncytiotrophoblast in humans (St-Pierre et al., 2000) and to the labyrinth region of

the rat placenta (Nagashige et al., 2003). Other members of ABCC family share common substrates with ABCB1, ABCG2 and ABCC1, hence expression of several ABCC transporters in conjunction with ABCB1 and ABCG2 offer a potentially substantial defensive mechanism.

1.4.1.2 The SLCO family

The SLCO superfamily contains the organic anion transporting polypeptide proteins (OATPs). There are 52 members of the SLCO family, of which 36 members have so far been indentified in human, rat and mouse (Hagenbuch and Meier, 2003). SLCO transporters are membrane transporters that mediate sodium–independent transport of amphipathic organic compounds.

Rat *slco1a1* was the first member of the slco gene superfamily to be identified (Jacquemin et al., 1994). A proposed topology of the transporter is illustrated in Figure 1.12, the predicted 12 transmembrane domain model has yet to be proven experimentally (Hagenbuch and Meier, 2003).

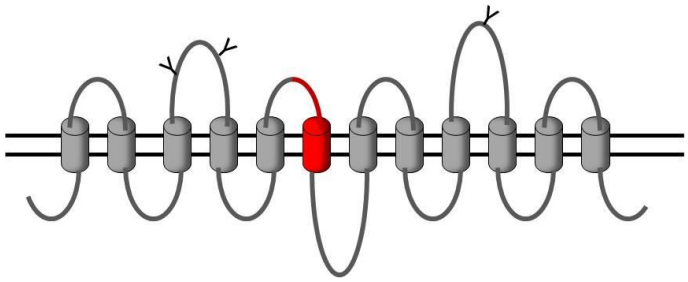


Figure 1.12 Proposed topology of SLCO family members. The SLCO 'superfamily signature' is shown in red. This signature amino acid sequence contains three conserved tryptophan residues and can be used to identify SLCOs in the different databases in other species. Picture adapted from Hagenbuch and Meier (2003).

Common structures of members of the SLCO family are:

1) Large extracellular domains between TMs 9 and 10 (extracellular loop 5)
2) N-glycosylation sites in extracellular loops 2 and 5
3) SLCO 'superfamily signature'.

The degree of glycosylation is thought to have a significant effect on the function of SLCO transporters (Lee et al., 2003) though it is unclear whether the 'superfamily signature' is important for function or proper membrane targeting (Hagenbuch and Meier, 2003).

Of the 11 members of the human SLCO family SLCO1B1 (Ugele et al., 2003), SLCO1B3 (Tertti et al., 2011), SLCO2B1 (Ugele et al., 2003, St-Pierre et al., 2002) and SLCO4A1 (Sato et al., 2003) have been found to be expressed in the placenta. In the rat placenta slco4a1 (at the mRNA level) has been identified as the most abundant slco transporter (St-Pierre et al., 2004). Slco4a1, along with abcc1,

has also been implicated in the placental transfer of conjugated bisphenol A, (Nishikawa et al., 2010).

1.4.2 Phase I and Phase II-metabolising enzymes

Phase I and Phase II metabolising enzymes are categorised in terms of their reactivity with xenobiotics. Phase I enzymes (CYPs) are oxidative enzymes, whereas phase II enzymes (GSTs, SULTs and UGTs) are conjugative enzymes. Both enzyme systems are located in the endoplasmic reticulum and generally have wide substrate specificity (Iyanagi, 2007).

1.4.2.1 Phase I metabolising enzymes: Cytochrome P450 enzymes

Over 100 human cytochrome *P450* (CYP) genes and pseudogenes have been identified to date. In the rat, 88 cytochrome *p450* genes and 69 pseudogenes have been identified (Nelson, 2009). Cyps were initially discovered in rat liver microsomes; their name derives from the haem group within the enzymes and the unusual spectrum at which an absorption band was generated (450 nm) (Garfinkel, 1958, Klingenberg, 1958). Currently there are 32 crystal structures available for nine human CYPs (CYP1A2, 1B1, 2A6, 2B6, 2C8, 2C9, 2D6, 2E1 and 3A4) (Dong et al., 2012). Most CYPs in the 1, 2 and 3 families have a conserved secondary structure and folding pattern (Figure 1.13)

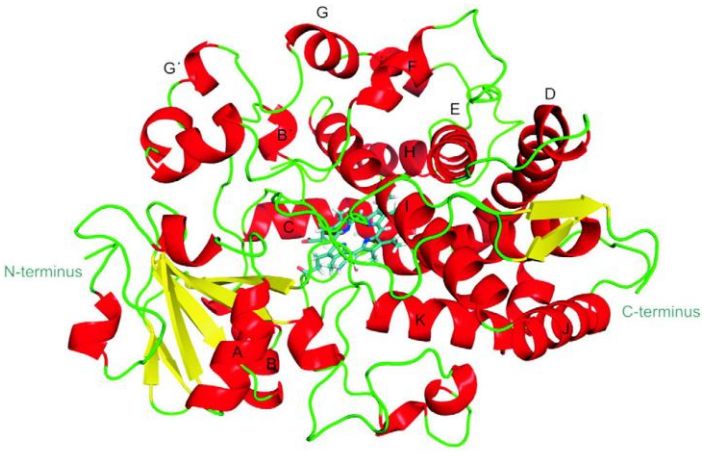


Figure 1.13 Topology of CYP3A4. α-helices are shown in red and labelled by upper case letters, β-strands are in yellow. The heam is located in the interior of the protein in active site near helix I. Figure taken from (Hendrychova et al., 2011)

Xenobiotics have been found to induce expression of CYPs. Ethanol induces CYP2E (Kostrubsky et al., 1995, Upadhya et al., 2000) and valproic acid induces rat cyp2b through the constitutive androstane receptor (CAR) (Takizawa et al., 2010).

CYP polymorphisms have been identified within the population and these can significantly affect xenobiotic metabolism. One example is CYP2C19 which has a polymorphism that alters the enzyme's ability to metabolise mephenytoin. This polymorphism has been identified in 3 % of the Caucasian population but in 20 % of the Asian population (Nakamura et al., 1985). This difference in xenobiotic-metabolism is now playing a major part in drug development (Roche, 2005). A similar approach could be utilised in current developmental toxicity models; exploiting the difference in expression of xenobiotic metabolising enzymes and indeed transporters between species (e.g. human, rat and rabbit) could help filter the discovery of new lead compounds.

Although many CYPs are predominantly expressed in the liver, extrahepatic expression and function has been reported. In the human placenta, RT-PCR analysis has revealed mRNA expression of more CYP isoforms in the first trimester compared to term (Hakkola et al., 1996a, Hakkola et al., 1996b). Cigarette smoke has been found to induce placental CYP1A1 (Whyatt et al., 1995, Zhu et al., 2002, Suter et al., 2010), and elevated levels of CYP1A1 activity have been associated with adverse birth outcomes (Okey et al., 1997).

1.4.2.2 Phase II Metabolising Enzymes

Phase II metabolising enzymes generally serve as a detoxifying step in metabolism. The purpose of phase II enzymes is to perform conjugating reactions. These include glutathione conjugation, sulfation and glucuronidation. The enzymes responsible for these reactions are: glutathione-s-transferases (GSTs/gsts), sulfotransferases (SULTs/sults), UDP-glucuronosyltransferases (UGTs/ugts) respectively.

1.4.2.2.1 Glutathione-S-transferases

Glutathione-S-transferases (GSTs/gsts) play an important role in cellular protection against oxidative stress (Jancova et al., 2010). GSTs catalyze the formation of thioether conjugates between the endogenous tripeptide glutathione and xenobiotics (Figure 1.14)

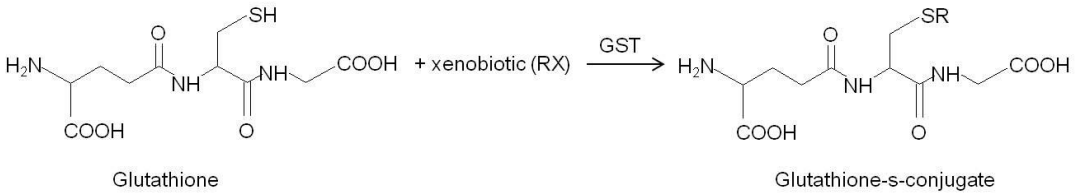


Figure 1.14 Glutathione–S-transferase mediated conjugation.

Substrates of GSTs/gsts include polycyclic aromatic hydrocarbons and endogenous compounds such as prostaglandins and steroids (van Bladeren, 2000).

Localisation of GSTs is predominatly in the cytoplasm, but they are also expressed in the nucleus, mitochondria (Soboll et al., 1995) and in peroxisomes (Morel et al., 2004). GSTs/gsts are widely distributed throughout the human body, including the liver, kidney, brain, pancreas, testis, heart, lung and small intestine (Hayes and Strange, 2000)

Placental expression and function has been investigated. Human villous fragments were utilised to study the formation and efflux of 1-chloro-2,4-dinitrobenzene conjugate 2,4-dinitrophenyl-s-glutathione (Vaidya et al., 2011). Expression of gsts in rat placenta has been reported and evaluated previously using microarray analysis. Sixteen *gst* genes were identified on GD 17 in the placenta (Ejiri et al., 2005). The expression profile of GSTs/gsts throughout gestation in the human and rat is unknown.

1.4.2.2.2 Sulfotransferases

Sulfotranferases (SULTs) are a superfamily of enzymes that catalyse the conjugation of 3′-phosphoadenosine 5′-phosphosulfate with an O-, N-, S- acceptor group of an appropriate molecule (Figure 1.15).

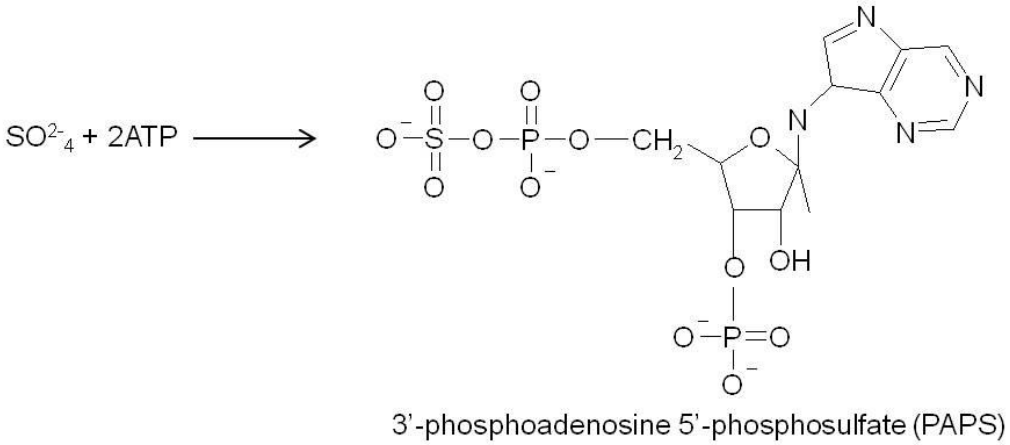


Figure 1.15 Sulfonation mediated conjugation. The two step process shows the formation of PAPS and how PAPS interacts with xenobiotics.

SULTs are localised in the membrane of the Golgi apparatus and in the cytosol. Sulfonation is important in the biotransformation of a number of low-molecular weight compounds, including vitamin D (Glatt and Meinl, 2004). SULTs are also really important in the biotransformation of numerous xenobiotics including drugs and chemicals. Inhibition of SULT activity has been identified in humans exposed to certain xenobiotics including hydroxylated polychlorinated biphenyls (Schuur et al., 1998) and bisphenol A (Kester et al., 2002). Inhibiting SULT enzymes could lead to increased exposure of the foetus to potentially toxic compounds.

SULTs are considered to be one of the major detoxifying enzyme systems in the developing foetus (Strassburg et al., 2002). SULTs are also highly expressed in human and rat placental tissues (Alnouti and Klaassen, 2006, Stanley et al., 2005), yet their expression throughout gestation remains to be elucidated.

1.4.2.2.3 Uridine diphosphate-gluronosyltransferases

Uridine diphosphate-gluronosyltransferases (UGTs) mediate the process known as glucuronidation. UGT enzymes are responsible for the metabolism of many xenobiotics and endogenous compounds (e.g. steroid hormones, thyroid hormones, bile acids and fat soluble vitamins (Kiang et al., 2005, Cashman et al., 1996).

UGTs are a superfamily of membrane-bound enzymes which catalyse the conjugation of a substrate to uridine-5-diphospho-α-D-glucuronic acid (UDPGA). The metabolites, β-D-glucuronides, formed are more readily eliminated via bile or urine than the parent compound (Figure 1.16)

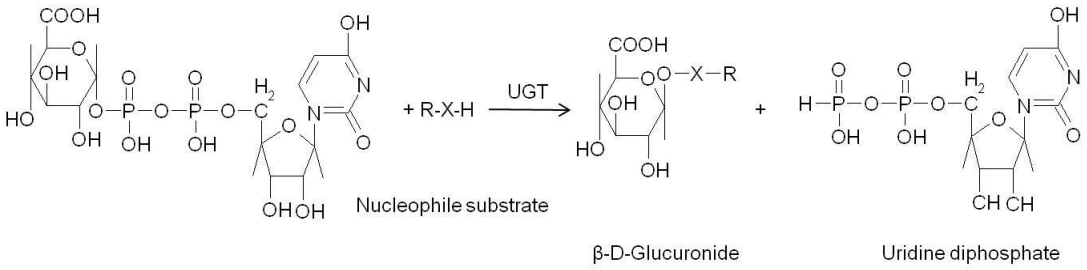


Figure 1.16 Glucuronidation metabolism.

Extrahepatic glucuronidation has been reported, specifically in the intestine (Gregory et al., 2004, Tukey and Strassburg, 2001, Cheng et al., 1999). Placental glucuronidation activity has also been reported (Collier et al., 2002). RT-PCR revealed expression of members of the UGT2 family at term. Expression of UGTs throughout gestation remains to be explored.

1.5 Surrogate *in vitro* models of the placenta

In vitro models of cultured syncytiotrophoblasts are extensively used to study the role and regulation of placental proteins. Current *in vitro* human models that are used to mimic the cyncytiotrophoblast are derived from choriocarcinomas. Choriocarcinoma is a malignant cancer originating from the trophoblast, JAr and BeWo cells derive from the choriocarcinoma and are used as surrogate human placental *in vitro* systems. The structure of the rat placenta is different from the human placenta. The rat placenta has two zones; a distinct junctional zone and a labyrinth zone. The junctional zone is located at the maternal interface, while the labyrinth zone is located at the foetal interface. Fewer cell lines are available to investigate the rat placenta, however the cell line; TR-TBT 18d-1, is suitable to study the syncyntiotrophoblast layer of the rat placenta.

BeWo, JAr and Jeg-3 choriocarcinoma cell lines are highly characteristic of the human placental syncytiotrophoblast. The TR-TBT cell line is a relatively newly established cell line which is derived from the chorioepithelium; the labyrinth region of the rat placenta. Two different sub-lines of TR-TBT cells have been isolated, namely TR-TBT 18d-1 and TR-TBT 18d-2. TR-TBT 18d-1 cells represent the syncytiotrophoblast layer facing the maternal circulation and TR-TBT 18d-2 cells represent the syncytiotrophoblast layer facing foetal circulation. In this respect it is considered that the TR-TBT 18d-1cells provide the equivalent of the maternal blood facing syncytiotrophoblast layer of the human syncytiotrophoblast and the TR-TBT 18d-2 cells provide the foetal-blood facing syncytiotrophoblast layer of the human syncytiotrophoblast (Higuchi et al., 2010). Placental ABC transporters and metabolising enzymes have been studied in the choriocarcinoma cell lines and TR-TBT cell lines. The JAr cell line has predominantly been used to study ABCB1 expression and function (Coles et al., 2009, Evseenko et al., 2007, Atkinson et al., 2003), and the BeWo cell line has been used to study ABCG2 expression and function (Crowe and Keelan, 2012, Wang et al., 2008, Evseenko et al., 2007), although some investigators prefer to use this cell line to explore ABCB1 expression and function (Magnarin et al., 2008, Utoguchi et al., 2000). The TR-TBT cell lines have been characterised using RT-PCR at the mRNA level and have shown expression of abcb1a (Kitano et al., 2004). Further transporter characterisation of the TR-TBT cell line will be performed in this thesis.

1.6 Aim and Objectives

The overall aim of this work is to compare expression profiles of transporters/enzymes in rat and human placentas and to study function of key ABC transporter in *in vitro* models of human and rat placenta. The specific objectives of this work are:-

1. To compare the mRNA expression of placental ABC Transporters and metabolising enzymes throughout gestation in the rat and human placenta.
2. To determine the protein expression of specific placental ABC Transporters and metabolising enzymes in the rat and human placenta.
3. To evaluate surrogate *in vitro* placental models currently used to study abcb1/ABCB1and abcg2/ABCG2 function in rat and human placenta.

Chapter 2

Materials and Methods

2.0 Chapter 2: Materials and Methods

2.1 Materials

2.1.1 Chemicals, solutions and media composition

An alphabetical list of all chemicals and their suppliers is documented in Appendix 4. Solutions and media compositions are documented in Appendix 5.

2.1.2 Embryonic and placental tissue collection

2.1.2.1. Embryonic and placental rat tissue (Syngenta, CTL)

For microarray analysis all rat tissue collection and processing was performed at Central Toxicology Laboratory (CTL), Syngenta. Rat embryonic and placental tissues were collected in-house at Syngenta CTL in 2005 as part of a foetal capability project. Female HsdBr1 Han Wistar rats were obtained from Harlan UK Limited, Bicester, UK. Animals were time-mated at Harlan UK and received at CTL on day 2, 3 or 4 of gestation. Each parent female was housed in solid plastic cages with woodflake bedding and received RM3 (Rat and Mouse No. 3; Special Diet Services Limited, Witham, Essex, UK) and water *ad libitum*.

Pregnant females, three at each time point, were dosed a control solution (0.5 % w/v aqueous carboxymethylcellulose) once daily by gavage. All animals were observed daily throughout the study. Animals were sacrificed on elected gestation days by over exposure to halothane vapour, followed by exsanguination by cardiac puncture. Animal experiments were conducted in accordance with the Home Office Scientific Procedures Act 1986.

2.1.2.2. Human placental data collection

The microarray study of human placental tissue was as described by Mikheev et al., 2008. Placentas were collected by researchers at The University of Washington. Collection was approved by the Institutional Review Board of the University of Washington (Mikheev et al., 2008). Briefly, human term placenta samples were obtained from scheduled uncomplicated caesarean-sections performed at the University of Washington Medical Center, Seattle, Washington. Placenta from first trimester pregnancies (obtained between days 45 and 59) and placenta from second trimester pregnancies (obtained between days 109 and 115) were from

uncomplicated elective terminations (provided by Birth Defects Laboratory of the University of Washington).

2.1.2.3 Placental rat tissue (GD 16) collection (The University of Manchester)

For expression analysis (using Western blots) of selected transporters and metabolising enzymes rat placenta at gestation day (GD) 16 were individually collected (The University of Manchester, 2011). Rats were sacrificed by cervical dislocation, both uterine horns were removed and dissected and the placentas placed in sterile dissociation medium (Medium 199, Dispase 0.5 % (w/v) and Deoxyribonuclease 0.1 % (w/v)) for 60 min at 37 ˚C. The labyrinth region was separated from the junctional zone by careful dissection using watchmaker's forceps. Following this, placentas were snap frozen using liquid nitrogen and stored at -80 °C.

2.1.2.4 Placental human tissue (term) collection (St Mary's Hospital, Manchester)

In order to study the *ex vivo* functional activity of transporters human placenta at term were collected from uncomplicated pregnancies as part of this study (St Mary's Hospital, Manchester). Collection was approved by the Local Ethics Committee of The University of Manchester. The syncytiotrophoblast layer was dissected from the placenta and used immediately for transporter functionality assays (see section 2.2.3.1).

2.2 Methods

2.2.1 Microarray analysis

2.2.1.1 Tissue processing and RNA isolation

Female Han Wistar Rats were time mated and sacrificed on elected days to harvest the embryos and foetuses throughout gestation from days 6 to 21. Embryos and placentas from the right uterine horn were separated and added individually to tubes containing RNAlater reagent (Invitrogen, Paisley, UK). Gene expression levels were measured using Affymetrix Rat Genome 230 2.0 GeneChips (containing 31,042 probe sets). Rat foetal tissues (whole embryos, or placenta only) from three individual animals were analysed at each time point, in order to provide biological triplicate measurements for each gestation date. This work was performed at CTL, Syngenta (2005).

In the study of Mikheev et al., (2008) total RNA from human placental tissue was extracted from frozen samples using the RNeasy kit (Qiagen Ltd., Valencia, CA) according to the manufacturer's protocol. Mikheev et al., (2008) measured gene expression levels using Affymetrix Human Genome U133 Plus 2.0 GeneChips (containing > 54,000 probe sets).

2.2.1.2 Microarray-based transcript profiling

For each GeneChip analysis, biotin labelled complementary RNA (cRNA) target was generated according to the Affymetrix GeneChip Expression Analysis Technical Manual. GeneChip arrays were scanned using a GeneChip Scanner 3000 (Affymetrix) and the probe set intensities were quantified using the GeneChip Operating Software version 1.1 (Affymetrix). This was performed by the respective groups, CTL, Syngenta (UK) or The University of Washington (USA). CEL files were collated and processed in ArrayTrack (FDA, USA).

2.2.1.3 Graphical and Statistical Analysis of Affymetrix Arrays

CEL files were processed in ArrayTrack, a microarray database which incorporates data analysis and data interpretation tools (FDA, USA). The data were normalized using mean/median scaling normalization and gene lists were created. Data-mining was conducted to identify ABC transporters and metabolising enzymes throughout gestation in both species.

Further normalisation of rat and human data was performed; all individual intensities were expressed as a percentage of the housekeeping gene glyceraldehyde 3-phosphate dehydrogenase (GAPDH). Normalisation of all individual intensities to β-actin was also performed to validate the findings obtained using GAPDH. Gene names were derived by matching accession codes using NCBI Gene, UniProtKB and individual scientific references in PubMed (listed in Appendix 6, Tables 6A- 6F). Microsoft Excel (Microsoft Office 2007) was used to sort and filter the data. GraphPad Prism was used to graphically represent the data. Differences in mRNA expression throughout gestation were measured by comparing 2 time points: the earliest time point collected (GD 7 in rat or Trimester 1 in human) and the latest time point collected (GD 21 in rat and term in human). Statistical differences between time-points were measured using a two-tailed Student's t-test (GraphPad Prism version 5.0)

2.2.2 Analysis of expression of placental transporters and metabolising enzymes.

2.2.2.1 Immunohistochemistry

Rat placental tissue from time-mated Han Wistar rats was collected and processed at CTL, Syngenta (2005). Left uterine horns were also taken and submitted intact, containing embryos and placentas into Feiketes fixative. Tissues were fixed in Feiketes fixative and embedded in paraffin prior to immunohistochemistry analysis. Sections (5 µm) were cut and mounted on X-tra® slides (Leica Microsystems, Milton Keynes, UK).

Pre-mounted, ready to use slides of human adrenal gland and placenta (term syncytiotrophoblast tissue at 42 weeks) were custom prepared by Abcam (Cambridge, UK).

Prior to staining, sections were dewaxed and rehydrated by placing in an oven at 60 ˚C for 15 min and then in the following: xylene (5 min) x 2, 100 % ethanol (3 min) x 2, 90 % ethanol (3 min), 70 % ethanol (3 min), 50 % ethanol (3 min) and distilled water (3 min).

Antigen heat-retrieval was performed using citrate-citric acid buffer (0.1 M citric acid and 0.1 M sodium citrate pH 6.0). Sections were placed in pre-warmed citrate buffer and then heated to 95 ºC for 5 min in a microwave oven. After resting

for 5 min, the slides were heated to 95 ºC for a further 5 min. The slides were left to cool to room temperature before being placed in Shandon Sequenza Coverplates (Thermo Shandon, Runcorn, UK). A peroxidise block (10 % (v/v) H_2O_2 in PBS) was applied to each section for 15 min and then sections were washed twice with PBS. Following washing, a protein block (3 % (w/v) BSA in TBS-T) was applied for 1 hour. Sections were then incubated with antibodies specific for abcc1, slco4a1 or cyp26b.

2.2.2.1.2 Immunohistochemical detection of abcc1

Following removal of the protein block, the tissue sections were incubated with the primary monoclonal mouse anti–human ABCC1 antibody (1: 50 in 5 % BSA in PBS) overnight at 4 ˚C (for antibodies used see section 2.2.2.2.6). As an antibody control, sections were also incubated with the monoclonal mouse IgG2a antibody (1: 50 in 5 % BSA in PBS) overnight at 4 ˚C. Following incubation with the primary antibody sections were washed with PBS and a dual mouse and rabbit HRP linked secondary antibody (Dako, Cambridgeshire, UK) was added for 1 h. 3,3'-diaminobenzidine (DAB) was applied to visualise the staining and the sections were counter stained with Papanicolaou's 1b Harris' haematoxylin (Merck, Nottingham, UK).

2.2.2.1.3 Immunohistochemical detection of slco4a1

Following removal of the protein block, the tissue sections were incubated with the primary polyclonal rabbit anti-SLCO4A1 antibody (1: 20 in 5 % (v/v) BSA in PBS) overnight at 4 ˚C. As an antibody control, sections were also incubated with the pre-absorbed SLCO4A1 antibody (using the SLCO4A1 antigen). The pre-absorbed antibody:antigen complex was derived from a 1: 10 dilution (of antibody in antigen) and incubated overnight at 4 ˚C. The antibody:antigen complex was then added to the tissue section at a 1: 20 dilution in 5 % (v/v) BSA in PBS and left overnight at 4 ˚C. Following incubation with the primary antibody sections were washed with PBS and a dual mouse and rabbit HRP linked secondary antibody (Dako, Cambridgeshire, UK) was added for 1 h. 3,3'-diaminobenzidine (DAB) was applied to visualise the staining and the sections were counter stained with Papanicolaou's 1b Harris' haematoxylin (Merck, Nottingham, UK).

2.2.2.1.4 Immunohistochemical detection of cyp26b

Following removal of the protein block, the sections were incubated with the primary rabbit polyclonal anti-cyp26b antibody (1: 100 in 5 %(v/v) BSA in PBS) overnight at 4 ˚ C. As an antibody control, sections were also incubated with the polyclonal rabbit IgG antibody (1: 100 in 5 % BSA in PBS) overnight at 4 ˚C**.** Following incubation with the primary antibody sections were washed with PBS and a dual mouse and rabbit HRP linked secondary antibody (Dako, Cambridgeshire, UK) was added for 1 h. 3,3'-diaminobenzidine (DAB) was applied to visualise the staining and the sections were counter stained with Papanicolaou's 1b Harris' haematoxylin (Merck, Nottingham, UK).

2.2.2.2 Western blot analysis.

2.2.2.2.1 Preparation of rat placental membranes for western blotting

Rat placentae at GD 16 were collected from pregnant Han Wistar dams as described in section 2.1.2. Frozen rat placentae from GD 16 (labyrinth region) were thawed in RIPA (radio immuno-precipitation analysis) buffer (50 mM Tris-HCl pH 7.8, 150 mM NaCl, 0.1 % SDS (w/v), 0.5 % sodium deoxycholate and 1 % Triton-X-100) with 2 µl/ml protease inhibitor cocktail (4-(2-aminoethyl) benzenesulfonyl fluoride (AEBSF), pepstatin A, E-64, bestatin, leupeptin, and aprotinin) (Sigma-Aldrich, UK). The tissue was macerated using a scalpel blade and homogenised in a dounce homogenizer. The homogenate was incubated on ice for 30 min. After 30 min the homogenate was centrifuged at 10,000 x g for 10 min at 4 ˚C. The supernatant was removed and pellet resuspended in RIPA buffer. The suspension was centrifuged again as described above and supernatant was collected. The protein concentration of the cell lysate was determined using the Bradford protein assay (see section 2.2.2.2.3).

2.2.2.2.2 Preparation of JAr, BeWo and TR-TBT cell membranes

Cell membranes were purified from JAr, BeWo and TR-TBT cells. Cells were rapidly thawed at 37 ˚C, resuspended in 1 ml PBS and centrifuged at 1000 x g (Centaur 2, Sanyo, IL, USA) for 5 min. The supernatant was discarded and the pellet was resuspended in 1 ml PBS and the above step repeated. The cell pellet was then resuspended in 1 ml of RIPA lysis buffer with protease inhibitor cocktail and

centrifuged at 1000 x g (Centaur 2, Sanyo, IL, USA) for 5 min. The pellet was resuspended in 500 µl of RIPA lysis buffer with protease inhibitor and centrifuged at 201,458 x g for 15 min. The supernatant was removed and the membrane pellet was resuspended and centrifuged at 204,458 x g to yield a purified membrane pellet. The protein content of the pellet was determined using the Bradford Assay (see section 2.2.2.2.3). Purified membranes were stored at -20 ˚C until use.

2.2.2.2.3 Measure of protein content using the Bradford protein

Bovine serum albumin (BSA) was used as the protein standard at concentrations of 0, 0.1, 0.25, 0.5, 0.75 and 1.0 mg/ml prepared in PBS. Bradford Reagent was freshly prepared at a 1:4 dilution (with dH_2O) at room temperature. To each 10 µl sample of BSA, 1 ml Bradford Reagent (1:4) was added, the sample shaken and left at room temperature for 5 min. Routinely, 10 µl purified membrane preparation was used to determine protein content. Purified membrane samples were treated in the same manner as the BSA standards. The absorbance was measured at 595 nm using a WPA UV1101 Biotech Photometer and a standard curve produced.

2.2.2.2.4 Sodium dodecyl sulphate (SDS) polyacrylamide gel electrophoresis

A 6 % resolving gel was used to separate proteins when detecting abcb1 and abcc1, and an 8 % resolving gel was used to separate proteins when detecting abcg2, cyp26b and slco4a1. The gel apparatus used was the Mini-PROTEAN Tetra Cell, 4 Gel system (Bio-Rad, Hemel Hempstead, UK). The glass plates were cleaned with 70 % (v/v) ethanol in dH_2O and air dried. For gel preparations, a small and a large glass plate were used and held in place using the casting stand and frame.

The resolving (6 % or 8 %) and stacking (4 %) gels were prepared as outlined in Table 2.1. The components were added in the order stated in the table and gently mixed in a beaker by gently swirling in order to prevent the inclusion of air which hinders polymerisation.

Table 2.1. Preparation of gels for SDS-PAGE (10 ml).

Gel	dH_2O (ml)	30 % Acrylamide/Bis (ml)	*Gel Buffer (ml)	10 % w/v SDS (ml)	10 % APS (ml)	TEMED (ml)
4%	6.1	1.3	2.5	0.1	0.05	0.01
6%	5.4	2	2.5	0.1	0.05	0.005
8%	4.7	2.7	2.5	0.1	0.05	0.005

* Resolving Gel Buffer - 1.5 M Tris-HCl, pH8.8
* Stacking Gel Buffer - 0.5 M Tris-HCl, pH 6.8

After the addition of TEMED and APS, the resolving gel was gently poured into the casting cassette of the Bio-Rad Mini PROTEAN Tetra Cell apparatus until the gel reached 1 cm from the bottom of the comb position. A small volume of water was immediately and gently layered on top of the resolving gel to prevent oxygen diffusing into the gel. The gel was left for 45 min at room temperature to set. After 45 min the water was removed and the top of the gel was dried with blotting paper. The stacking gel was then cast on top of the resolving gel in the casting cassette and the Teflon® comb was inserted. The stacking gel was left to set for 45 min at room temperature. The comb was removed and the wells were washed out with running buffer (250 mM glycine, 25 mM Tris pH 8.3 and 0.1 % (v/v) SDS). The glass plates containing the gels were assembled in the electrophoresis tank, the central reservoir was filled with running buffer and running buffer was added to the external chamber to cover the bottom of the plates and complete the electrical circuit.

Protein samples (20 – 30 µg) were mixed with 5 x SDS-sample buffer (60 mM Tris-HCl pH 6.8, 2 % SDS (w/v), 10 % (v/v) glycerol, 5 % (v/v) β-mercaptoethanol, 0.01 % (w/v) bromophenol blue) and loaded into the wells of the gels.

Molecular weight markers (Invitrogen, Paisley, UK) were loaded at positions 1 and 10 (i.e. the end lanes) of each gel. Electrophoresis was carried out at 150 V until the bromophenol blue (present in the sample buffer) had reached 1 cm above the bottom of the resolving gel.

2.2.2.2.5 Electrotransfer of proteins

The BioRad Mini Trans-Blot Cell System (Biorad Laboratories Ltd, Hemel Hempstead, UK) was used to transfer the proteins from the SDS-PAGE gel to Hybond™-P polyvinylidene fluoride (PVDF) membrane.

Following electrophoresis, SDS-PAGE gels were immersed in chilled transfer buffer (10 mM 3-[cyclohexylamino]-1-propanesulfonic acid (CAPS), pH 11) for 20 min to equilibrate. The Hybond™-P PVDF membrane was pre-treated as described by the manufacturers, briefly, the membrane was soaked in methanol for 10 s, distilled water for 5 min and equilibrated in 10 mM CAPS buffer, pH 11 for 10 min. Gels were then placed onto the PVDF membranes and sandwiched between blotting paper and fibre pads, both previously soaked in transfer buffer.

Protein transfer took place in 10 mM CAPs buffer, pH 11 at 200 mA for 1.5 h. Following transfer, the membranes were blocked in 5 % (w/v) Marvel® in TBS-T (10 mM Tris-HCl, 150 mM NaCl, 0.05 % (v/v) Tween-20) for 2 h at 4 ˚C. The membranes were then washed three times in TBS-T for 10 min each time, prior to immunological detection of the transporter or metabolising enzyme.

2.2.2.2.6 Immunological detection of abcb1, abcc1, abcg2, slco4a1 and cyp26b1

The PVDF membranes containing electrotransferred proteins were incubated with either the mouse monoclonal C219 (anti-abcb1) antibody (Cambridge BioScience, Cambridge, UK) (1:100 dilution), goat polyclonal Mdr (C-19) (anti-abcb1) antibody (Santa Cruz, Germany) (1:100), mouse monoclonal anti-ABCC1 antibody [MRPm5] (Abcam, Cambridge, UK) (1:50 dilution), the rabbit polyclonal anti-ABCG2 antibody (Abcam, Cambridge, UK) (1:500 dilution), the rabbit polyclonal anti-SLCO4A1 antibody (Sigma-Aldrich, UK) (1:750 dilution) or the rabbit polyclonal anti-cyp26B antibody (Abcam, Cambridge, UK) (1:3000). Membranes were incubated at 4 ˚C overnight in primary antibody whilst control PVDF membranes were incubated in TBS-T alone. The membranes were then washed three times in excess TBS-T for 10 min and incubated for 45 min at room temperature with horse radish peroxidise-conjugated sheep anti-mouse secondary IgG antibody (GE Healthcare Buckinghamshire, UK) (1:1000 dilution) or goat anti-rabbit secondary IgG antibody (Abcam, Cambridge, UK) (1:2000 dilution) or donkey anti-goat secondary IgG antibody (Santa Cruz, Germany) (1:2000 dilution)

in TBS-T. The PDVF membranes were washed five times in excess TBS-T for 10 min prior to detection of protein by enhanced chemiluminescence.

2.2.2.2.7 Enhanced Chemiluminescent detection of abcc1, slco4a1 and cyp26b1

Enhanced chemiluminescent protein detection was carried out in a dark room. ECL Prime® Western Blotting Detection System (GE Healthcare, Buckinghamshire, UK) was used for protein detection. Solutions A and B were mixed together (1:1) to generate the detection solution.

Excess wash buffer was drained from the PVDF membranes, the detection solution applied to the side of the membranes containing electrotransferred proteins, and the membranes incubated for 5 min at room temperature. The membranes were drained of detection solution and exposed to film (Hyperfilm™ ECL, Amersham Pharmacia Biotech, Buckinghamshire, UK) in an x-ray film cassette for 3-5 min. Following exposure, films were treated with developer (Kodak Developer, Sigma-Aldrich Chemical Company, UK) for 3-5 min, rinsed in dH_2O and treated with fixer (Kodak Fixer, Sigma-Aldrich Chemical Company, Poole, Dorset, UK) for 3-5 min.

2.2.3 Surrogate rat and human placental models

2.2.3.1 *In vitro* models of the rat and human placentas

2.2.3.1.1 Culture of cell lines

The human JAr cell line was obtained from American Type Culture Collection (LCG Standards, Middlesex, UK). JAr cells were rapidly thawed at 37 ˚C in a water bath and seeded in a 25 cm^2 tissue culture flask (T-25) with JAr culture medium (Dulbecco's Modified Eagles Medium (DMEM)/Nutrient F-12 Ham (1:1) supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were incubated at 37 °C in an atmosphere of 5 % CO_2.

The human BeWo cell line was obtained from European Collection of Animal Cell Cultures (ECACC, Salisbury, UK). BeWo cells were rapidly thawed at 37 ˚C in a water bath and seeded in a 25 cm^2 tissue culture flask with BeWo culture medium (Nutrient F-12 Ham supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were incubated at 37 °C in an atmosphere of 5 % CO_2.

The human MCF-7 cell line was obtained from American Type Culture Collection (LCG Standards, Middlesex, UK). MCF-7 cells were rapidly thawed at 37 ˚C in a water bath and seeded in a 25 cm^2 tissue culture flask (T-25) using MCF-7 culture medium (Dulbecco's Modified Eagles Medium (DMEM) supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were incubated at 37 °C in an atmosphere of 5 % CO_2.

The rat TR-TBT 18d-1 cell line was received as a generous gift from Professor Tomi at The University of Keio, Japan. TR-TBT cells were rapidly thawed at 37 ˚C in a water bath and seeded in a 25 cm^2 tissue culture flask (T-25) using TR-TBT culture medium (DMEM supplemented with 10 % (v/v) foetal bovine serum, 2 mM L-glutamine, 100 IU/ml penicillin, and 100 µg/ml streptomycin). Cells were incubated at 33 °C in an atmosphere of 5 % CO_2.

For all cell lines medium was changed on alternate days until the cells reached 80 % confluency (usually 3-4 days post seeding). Growth medium was then removed and the cell monolayers washed with sterile Ca^{2+} and Mg^{2+} free phosphate buffered saline. Cells were detached from the flask by treatment with 1 ml 0.25 % (v/v) trypsin-EDTA solution. Growth medium, 1 ml was added to neutralise the

trypsin. The cell suspension was centrifuged at 1000 x g for 5 min in a Centaur centrifuge (Sanyo) and the pellet was resuspended in growth medium, and either reseeded in T-25 flasks for further culturing or plated into 96- or 24- well flasks for experiments.

Cells were routinely tested for mycoplasma infection using PlasmoTest™ Reagent kit (Source Bioscience Life Sciences, Nottingham, UK).

2.2.3.1.2 Determination of cell viable cell number using the Trypan Blue Assay

Cell number was assessed by trypan blue exclusion analyses, using a haemocytometer. Cell suspension, 20 µl, was added to 20 µl trypan blue (0.4 % w/v) and the sample incubated for 10 min at room temperature. Following the incubation 10 µl of the trypan blue-treated cell suspension was placed in each chamber of a haemocytometer and cells counted. When viewed, live or viable cells appear uncoloured as they exclude the dye whereas dead cells take up the dye and appear blue. The estimated number of viable cells per ml was calculated as follows:

Viable cells (cell/ml) = Average count (unstained cells) per grid square x dilution factor x 10^4

Equation 2.1

2.2.3.1.3 Cryopreservation of Cells

After harvesting cells, as described in section 2.3.3.1.1, the cell pellet was resuspended in freezing medium (FBS 90 % (v/v) and DMSO 10 % (v/v)). This freezing medium was used for all cell lines. Cells were frozen at a cell density of 2 x 10^6 cells/ml. A 1 ml volume of cell suspension was pipetted into individual cryovials and the vials stored overnight at -80 ˚C in a cell cooling box (Nalgene® Labware, Roskilde, Denmark). This allows controlled cell freezing at a rate of -1 ˚C/min, prior to long term storage in liquid nitrogen (-196 ˚C).

2.2.3.1.4 Determination of optimal seeding density using the MTT assay

Cells were required to remain in the exponential growth phase throughout the cell viability MTT (methylthiazolydiphenyl-tetrazolium bromide (3-(4, 5-dimethylthiazol-2-yl)-2, 5-diphenltetrazolium bromide) assay to avoid underestimating drug toxicity. Therefore, it was important to determine optimal

seeding density for each cell type used in these studies. Cells were plated in a 96-well plate at a range of densities between 1 x 10^3 – 4 x 10^4 cells/well in quadruplicate. Cells were incubated at 37 ˚C in a humidified atmosphere of 5 % CO_2 for 48 h. After 48 h, the medium was removed from the wells and MTT (5 mg/ml) in transport medium (culture medium without FBS 10 % v/v) was added for 4 h. After incubation at 37 °C the transport medium containing MTT was removed and the purple formazan crystals formed were dissolved by addition of 100 µl DMSO and agitation on a rocking mixer at room temperature for 90 min. The absorbance was then measured at 540 nm using a Tecan Safire plate reader (Tecan Group Ltd, Switzerland). The seeding density that produced an absorbance of approximately 1 was chosen for subsequent MTT studies.

2.2.3.2 Placental *in vitro* surrogate models employed for studying Abcb1 mediated transport

2.2.3.2.1 Selection of Abcb1 inhibitors

In order to study the functional activities of abcb1, widely reported inhibitors of abcb1 were employed, namely Atovastatin (Wang et al., 2001, Bogman et al., 2001), Quinidine (Pires et al., 2009, Elsby et al., 2008), Saquinavir (Gutmann et al., 1999) and Verapamil (Janneh et al., 2010). The physicochemical properties of the abcb1 inhibitors employed can be found in Appendix 2, Table 2A.

2.2.3.2.2 Determination of non-cytotoxic concentrations of Abcb1 inhibitors

JAr, BeWo and TR-TBT cells were seeded into 96-well plates at a seeding density of 1 x 10^3, 5 x 10^3 and 1 x 10^4 cells/well respectively, and grown until 70 – 80 % confluent (16-18 h). The growth medium was removed and the cells were washed with PBS. Cells were incubated for 2 h with PBS containing Atorvastatin, Quinidine, Saquinavir, or at varying concentrations (0.01 µM, 0.1 µM, 1 µM, 10 µM, 100 µM and 1000 µM). After 2 h, the PBS containing inhibitor was removed and cells were allowed to recover for 24 h in transport medium (growth medium without 10 % (v/v) foetal bovine serum. After 24 h, the medium was removed from the wells and MTT (5 mg/ml) in transport medium was added for 4 h and cell incubated at 37 °C. After incubation at 37 °C the transport medium containing MTT was removed and the purple formazan crystals formed were dissolved by addition of

100 µl DMSO and agitation on a rocking mixer at room temperature for 90 min. The absorbance was then measured at 540 nm using a Tecan Safire plate reader (Tecan Group Ltd, Switzerland).

2.2.3.2.3 Measurement of Abcb1-mediated efflux activity using the Calcein AM accumulation assay

JAr cells were seeded into 24-well plates at a seeding density of 1 x 10^5 cells/well and grown until confluent (16-18 h). BeWo cells were seeded into 96-well plates at a seeding density of 5 x 10^3 cells/well and grown until confluent. TR-TBT cells were seeded into 96-well plates at a seeding density of 1 x 10^4 cells/well and grown until confluent. The growth medium was removed and cells were allowed to equilibrate in PBS for 30 min. The PBS was removed and cells were incubated for 30 min with PBS containing either 10 µM atorvastatin, 10 µM quinidine, 10 µM saquinavir or 10 µM verapamil. After 30 min calcein AM (at a final working concentration of 400 nM) in PBS was added to the cells and the cells incubated for a further 30 min. The PBS containing calcein AM and Abcb1 inhibitor was removed, the cells washed three times with ice cold PBS and ice cold PBS added to each well (200 µl/well for 96-well plates; 800 µl/well for 24-well plates). The intracellular fluorescence was measured at an excitation wavelength of 484 nm and an emission wavelength of 530 nm using a Tecan Safire plate reader (Tecan Group Ltd, Switzerland). Accumulation is expressed as a percentage relative to the control condition (no Abcb1 inhibitor).

2.2.3.3 Placental *in vitro* surrogate models employed for studying Abcg2 mediated transport

2.2.3.3.1 Selection of Abcg2 inhibitors

In order to develop an assay to measure Abcg2 mediated efflux assay a specific inhibitor of Abcg2 was chosen. Due to the overlap in substrate/inhibitor specificity with Abcb1, a smaller range of compounds was available. Ko143, an analogue of FTC was chosen as the specific Abcg2 inhibitor (Allen et al., 2002). The physicochemical properties of ko143 can be found in the Appendix 2, Table 2A

2.2.3.3.2 Measurement of Abcg2-mediated efflux activity using the Pheophorbide A accumulation assay

MCF-7, BeWo and TR-TBT cells were seeded onto 96-well plates at seeding densities of 1 x 10^4, 5 x 10^3 and 1 x 10^4 cells/well respectively and grown until confluent (16-18 h). The growth medium was removed and cells were allowed to equilibrate in Optimem™ for 30 min. The Optimem™ was removed and cells were incubated for 18 h with Optimem™ containing 1 µM pheophorbide A (PhA). Ko143 was used at a concentration of 500 nM. After 18 h the Optimem™ containing PhA ± ko143 was removed and cold PBS, 200 µl/well, was added. The intracellular fluorescence was measured at an excitation wavelength of 417 nm and an emission wavelength of 674 nm using a Tecan Safire plate reader (Tecan Group Ltd, Switzerland). Accumulation was expressed as a percentage relative to the control condition (cells treated with Optimem™ only). Following this treatment, cells were left to recover for 24 h and then treated with MTT to assess their viability (see section 2.2.3.4.4).

2.2.3.4. *In vitro* blood-placental barrier models

2.2.3.4.1 Growth of JAr, BeWo and TR-TBT cells on Transwell® inserts

JAr, BeWo and TR-TBT cells were grown on Transwell® polycarbonate inserts (pore size 0.4 µm, diameter 12 mm, and growth area 1.12 cm^2) in 12 well culture clusters. JAr cells were seeded at a density of 2.5 x 10^4 cells/insert (Vidricaire et al., 2004), BeWo cells were seeded at a density of 1 x 10^3 cells/ insert (Utoguchi et al., 1999) and TR-TBT cells were seeded at a density of 1 x 10^4 cells/ insert. Cells were maintained in respective growth culture. The medium was replaced on alternate days. JAr and BeWo cells were maintained at 37 °C in a humidified atmosphere of 5 % CO_2 whilst TR-TBT cells were maintained at 33 ˚C in a humidified atmosphere of 5 % CO_2 throughout growth on Transwell® inserts. Inserts contained 0.5 ml medium in the upper compartment and 1.5 ml in the lower compartment. During replacement of culture medium, medium was aspirated first from the basolateral compartment of all wells and then carefully and slowly from the apical compartment to ensure monolayer maintenance. Fresh medium was initially replaced in the apical compartment followed by the basolateral compartment

2.2.3.4.2 Measurement of transcellular electrical resistance of cell monolayers

The integrity (reflecting the extent of paracellular and transcellular permeation of ions) of placental cell monolayers was assessed using a voltohmmeter (EVOM) (World Precision Instruments, Aston, Stevenage, UK), to measure TER.

The 'chop-stick' electrodes were placed with the current-passing electrode in the apical compartment and the voltage-measuring electrode in the basolateral compartment of each filter unit and well respectively in order to measure the electrical resistance across the filter and cell monolayer.

Control measurements were also obtained using a filter with no cells seeded (blank filter). Transcellular electrical resistance was calculated (Equation 2.2) by subtraction of the electrical resistance of the blank filter from the electrical resistance of the filter containing the cell monolayer, followed by correction for the filter surface area.

$$TER\ (ohm.cm^2) = A(R_{cell\ monolayer} - R_{filter})$$

Equation 2.2

$R_{cell\ monolayer}$ = Resistance across Transwell® insert with cell monolayer (ohm)
R_{filter} = Resistance across Transwell® insert without cell monolayer (ohm)
A= Surface area of Transwell® insert (cm^2)

2.2.3.4.3 Transmission electron microscopy of confluent cell monolayers

Transmission electron microscopy (TEM) was used for qualitative assessment of JAr monolayers grown on Transwell® inserts. Growth medium was aspirated from inserts containing confluent cell monolayers and the cells were washed twice with PBS.

The filters were carefully cut out of the Transwell® inserts, removed from the Transwell® supports fixed overnight in sodium cacodylate buffer and post-fixed in 1 % (w/v) osmium tetroxide in 0.1 M sodium cacodylate for 30 min. The fixed cells were washed in sodium cacodylate buffer and dehydrated in an increasing concentration series of ethanolic solutions 70 % (v/v) (20 min), 90 % (v/v) (20 min) and 100 % (v/v)(30 min) x 2.

The inserts containing cell monolayers were then treated twice with propylene oxide for 30 min and infiltrated with epoxy resin (Araldite), 50 % (v/v) [40 min at 40 ˚C] and 100 % (v/v) for 1 h at 40 ˚C. The cells were then embedded into fresh Araldite resin, by positioning the filters into the embedding moulds containing a layer of prepolymerised resin to lift the filter away from the bottom of the mould so that transverse sections of the filter could be cut.

Sections, 100 nm thick, of the resin-embedded filters were cut using a diamond knife on a Reichert OMU 4 Ultracut Ultramicrotome (Vienna, Austria) and collected onto 400-mesh copper grids (Agar, Scientific, UK). The sections were stained with 1% (w/v) uranyl acetate (DNA and RNA stain) for 40 min at room temperature, washed with distilled water and stained with Reynolds lead citrate for 10 min at room temperature before a final wash with water. Sections were examined using a transmission electron microscope (Philips CM10, Cambridge, UK) and digital images were recorded on an AMT LR44 digital camera (Deben, UK).

Transmission electron microscopy studies were carried out by Dr Alan Curry at the Department of Clinical Sciences, Manchester Royal Infirmary.

2.2.3.5 *Ex vivo* placental models

2.2.3.5.1 *Ex vivo* uptake model of the human placenta to study Abcb1-mediated transport

Human term placentae were collected as described in 2.1.2. Functional activity of ABCB1 in fresh placental tissue was assessed by measuring accumulation of [^{3}H]Taxol in placental villous fragments as described by Atkinson et al., 2006. Fragments of villous tissue approximately 2 cm x 2 cm square were dissected within 30 min of delivery, washed in Tyrodes solution (133 mM NaCl, 5 mM KCl, 1.8 mM $CaCl_2$, 1 mM $MgCl_2(6H_2O)$, 5.6 mM glucose and 10 mM HEPES pH 7.4) and further dissected into fragments of approximately 2 mm x 2 mm x 2 mm in size. These small fragments were tied with cotton thread to hooks supported by Perspex rods. The uptake of [^{3}H]Taxol was measured in duplicate fragments at 37 °C at 1, 5, 15, 30, 60, 120 min. Incubations were carried out in Tyrodes solution containing 10 nM [^{3}H]Taxol (specific activity 0.2 µCi /ml (stock) 0.8 µCi per 4 ml vial) with or without 50 µM verapamil. After each timed incubation, fragments were washed (2 x 15 s) in ice cold Tyrodes solution and then placed in distilled water to lyse the cells

and release accumulated isotype. Radiation was measured by liquid scintillation counting and the tissue was the dissolved in 0.3 N NaOH and aliquots assayed for protein using a BioRad assay. Accumulation of [^{3}H]Taxol was calculated as pmol/mg protein.

2.2.3.5.2 Determination of protein content of human placental tissue using the Bradford protein assay

The Bradford assay was used to measure the protein content of human placental tissue, with a slight variation from the method described in section 2.2.2.2.3. BSA was used as the protein standard at concentrations of 0, 0.25, 1.25, 3.75, 5.00 µg/ml prepared in 0.3 N NaOH. To each sample 180 µl of a 1:1.25 (0.3 N NaOH:0.3 M HCl) 'neutralising solution' was added, followed by 50 µl of Bradford reagent. The solution was left for 5 min and the absorbance measured at 595 nm using a VersaMax plate reader. Tissue protein to be quantified, 20 µl, was treated in the same manner as the standards and the protein concentration was determined using the standard curve generated.

2.2.3.5.3 Isolation of rat placenta to establish primary cultures of GD16 rat placental cells

Rat placentae were collected from pregnant Han Wistar rats at GD 16 and dissected to yield the labyrinth region (as described in section 2.1.2). In order to isolate syncytiotrophoblast cells from the labyrinth region of the placenta, the method of Kitano et al., 2002 was employed. Placenta tissue was finely minced and transferred to fresh dissociation medium (Medium 199, Dispase 0.5 % (w/v) and Deoxyribonuclease 0.1 % (w/v)) and mixed for 60 min at 37 ˚C. The tissue homogenate, 1 ml, was loaded onto 5 ml of a 40 % Percoll density gradient and the samples centrifuged at 700 x g for 15 min at room temperature. Cells were collected from each fraction and plated in separate wells in a 6-well dish in TR-TBT culture medium (DMEM, 10 % FBS, 100 U/ml penicillin and 100 µg/ml streptomycin) at 37 ˚C and 5 % CO_2.

Chapter 3

A comparative analysis of transporter and metabolising enzyme mRNA expression throughout gestation in human and rat placenta

3.0 Chapter 3; A comparative analysis of transporter and metabolising enzyme mRNA expression throughout gestation in human and rat placenta

3.1 Background

During the discovery of new chemical entities (NCEs), assessment of prenatal and developmental toxicity forms an important part of safety evaluation. Governmental guidelines stipulate the tests which must be performed in order to assess the toxicity of NCEs. The OECD guidelines recommend using two species (one rodent and one non-rodent) when performing test 414 in order to predict potential hazards to human health (OECD, 2001). The rat is most commonly used as the rodent model, and the rabbit as the non-rodent model. In order to understand the true predictive ability of these non-human models, understanding the differences in placental barrier properties between species is of fundamental importance. Transporters and metabolising enzymes are thought to contribute significantly to the protective barrier properties of the placenta (Syme et al., 2004). Although placenta anatomy varies between species, the expression of these transporters and metabolising enzymes, is probably important aspect in providing protection of the developing foetus.

In order to establish how suitable the rat model is for predicting potential hazards of NCEs in human health, it is essential to compare the expression of transporters and metabolising enzymes in the rat and human placentas throughout gestation.

The mRNA expression profiles of several transporters and metabolising enzymes from human placenta were analysed using microarray analysis. Data deposited (CEL files) in the Gene Expression Omnibus (GEO) from the 3 trimesters throughout human pregnancy (Mikheev et al., 2008) were used to profile the expression of human placental transporters and metabolising enzymes. Tissue from rat embryos (both rat embryo and placenta GD 7 - 14, rat placenta only GD 14 - 21) was collected at CTL, Syngenta (2005) and analysis of the microarrays to produce CEL data files was performed as part of this thesis. The CEL files were analysed in order to profile the expression of several rat placental transporters and metabolising enzymes. The change in mRNA expression throughout gestation of individual transporters and metabolising enzymes was scrutinised in both species. The gestation period in the rat was split into trimesters (shown in each graph by different

colours), GD 7 – 16 was considered to be equivalent to trimester 1 in the human (red), GD 16 – 20 was considered to be equivalent to trimester 2 in the human (blue) and GD21 was considered to be equivalent to trimester3/term in the human. The differential expression of members of the transporter and metabolising enzyme families was compared between species.

3.2 Results

3.2.1 The mRNA expression profile of human and rat placental ABCB1/abcb1 throughout gestation

The *ABCB1/abcb1* genes encode the ABCB1/abcb1 (P-glycoprotein, P-gp) efflux transporter. In rats there are two isoforms of abcb1; abcb1a and abcb1b. Previous studies have reported a more prominent role of abcb1b (Pavek et al., 2003). ABCB1 is thought to be one of the most clinically important transporters in humans (Zhou, 2008).

ABCB1/abcb1 is able to bind hydrophobic compounds located within the cell membrane and actively efflux the substrate to the extracellular compartment, preventing or reducing intracellular accumulation of the compound. It has been shown that in addition to providing a prominent mechanism conferring multidrug-resistance in cancer cells (Goldstein et al., 1991, Goldstein, 1996) ABCB1/abcb1 also plays an important role in normal tissue protection, particularly at the placenta (Lankas et al., 1998).

Depending on the expression level of abcb1 and the stage of gestation, the developing foetus may be more susceptible to exposure to compounds which could produce teratogenic effects. In order to compare the potential susceptibility of the rat and human foetuses to exposure to xenobiotics, the mRNA expression levels of placental abcb1/ABCB1 in rat and human were quantified and analysed using ArrayTrack™ (Figure 3.1). The difference between transporter expression intensities in each trimester is shown numerically in Table 3.1. The Genbank accession codes for ABCB1/abcb1 can be found in Appendix 6 Table 6A.

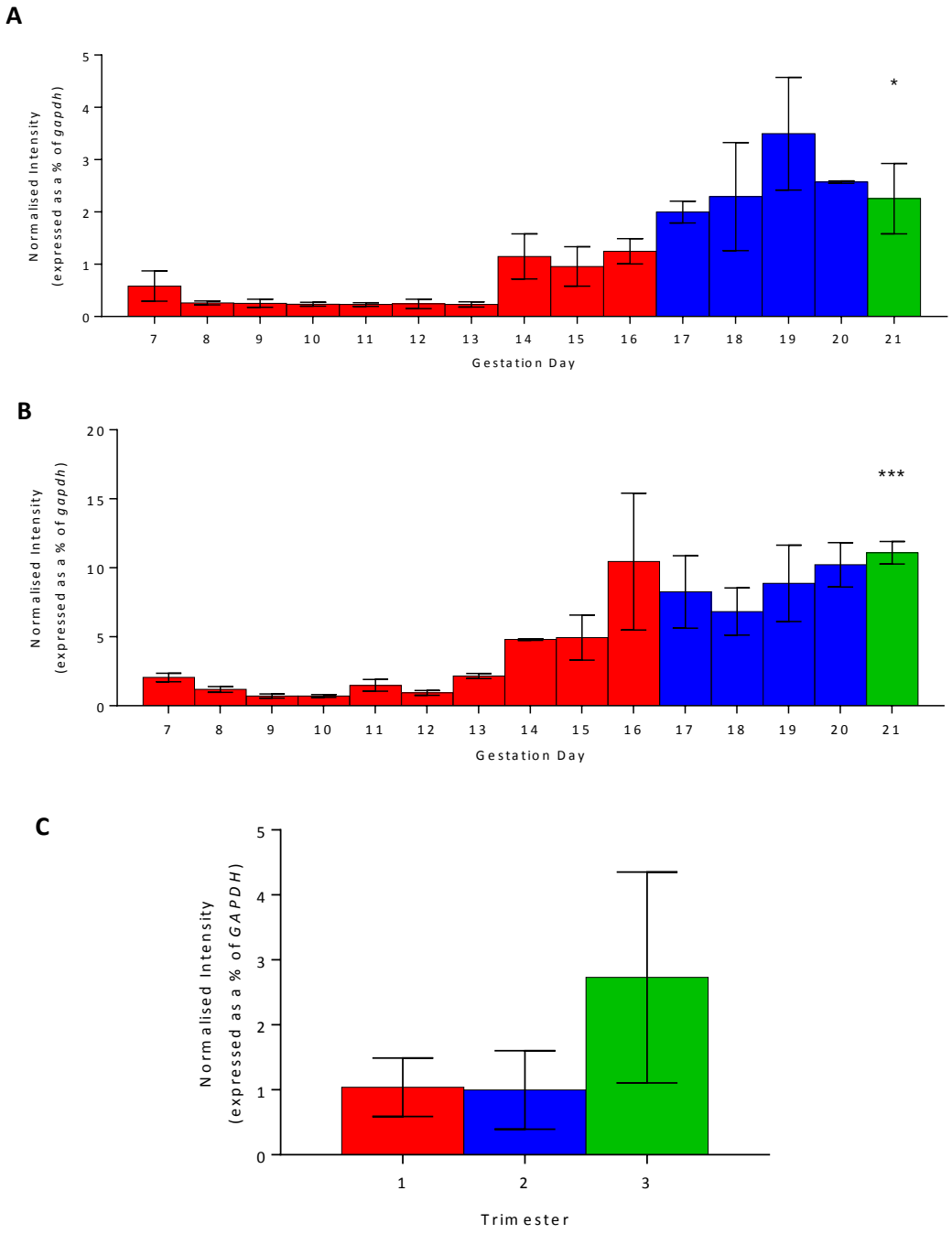


Figure 3.1 The mRNA expression profile of ABCB1 and abcb1 in human and rat placenta throughout gestation. The expression of ABCB1 and abcb1 throughout gestation in the developing embryo (foetus and placenta) in rat (A and B) and developing placenta in human (C). In rats there are two isoforms of abcb1; A represents abcb1a and B represents abcb1b. Expression was normalised using expression of the *gapdh/GAPDH* house keeping gene (to validate the use of normalising expression to a house keeping gene, expression was also normalised using expression of the *β-actin* house keeping gene, an example of this is shown in Appendix 7, Figure 7A). Statistical analysis was carried out using the Student's T-test. Asterisks (* = $p < 0.05$ and *** = $p < 0.001$) denote GD 21 intensity values are statistically different from GD 7. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.1 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) abcb1a/abcb1b and human (developing placenta) ABCB1 throughout gestation.

Abcb1	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat abcb1a	GD 7	0.58 ± 0.289
Rat abcb1b	GD 7	2.05 ± 0.3
Human ABCB1	T1	1.04 ± 0.45
Rat abcb1a	GD 21	2.25 ± 0.67
Rat abcb1b	GD 21	11.09 ± 0.81
Human ABCB1	T3	2.73 ± 1.62

Expression of abcb1/ABCB1 mRNA was detected in both rat and human placenta. The expression level of mRNA was calculated as a percentage of *gapdh/GAPDH* expression. In rats, the abcb1b isoform was more abundantly expressed than abcb1a (Figure 3.1 B). Expression of rat placental abcb1a increased during gestation and at GD21 was significantly higher ($p<0.05$) than at GD7 (Figure 3.1 A). Expression of the abcb1b isoform also increased throughout gestation, at GD 21 the intensity was significantly higher ($p<0.001$) than at GD 7 (Figure 3.1 B). Although there was no significant increase in ABCB1 mRNA expression throughout gestation in the human placenta there was a 2.6 fold increase in expression in T3 compared to T1 (Table 3.1).

There was no substantial change in the level of expression of abcb1a mRNA in the rat in the first trimester until GD 14, when a 2-fold increase (compared to GD 7) in expression was observed. After this point, GD 14, there was only a relatively small change in the level of abcb1a expression during the second and third trimester (Figure 3.1 A) although the level of expression at term (day 21) was significantly higher ($p < 0.05$) than at GD 7 (Figure 3.1 A). A similar expression pattern occurred with rat abcb1b, with relatively small changes in the level of expression observed from GD 7 to GD 13. However at GD 14 a 2-fold increase of abcb1b expression was observed (compared to GD 7) and at GD 16, a 5-fold increase was observed

(compared to GD 7). During the second trimester, on average, a 4-fold increase (compared to GD 7) was observed. At term, (GD 21) a highly significant ($p < 0.001$) increase in abcb1b mRNA expression compared to GD7 was observed (Figure 3.1 B, abcb1b).

Expression of human placental ABCB1 remains relatively constant throughout the first and second trimesters however, in the third trimester there is an increase in ABCB1 mRNA expression compared to the level in trimester 1 (Figure 3.1 C). This increase in expression at term is observed in both rat and human.

Comparing the two species highlights the higher expression of abcb1b than ABCB1 throughout the three trimesters, Figure 3.1 and Table 3.1. The comparison between abcb1a and ABCB1 showed ABCB1 expression to be nearly 2 fold higher than abcb1a expression in the first trimester; however the more predominant isoform in rat, abcb1b, was found to be at least 3-fold higher than abcb1a in all three trimesters.

It is difficult to directly compare the data in this thesis with published microarray data, primarily due to the novel approach used to analyse the microarray data. Analysis of ABCB1/abcb1 expression in this thesis has allowed the generation of time course expression profiles in rat and human. Usual practise in microarray data analysis is to compare two groups, for example control vs. treated, and then generate gene maps to identify genes in the treated group that have been significantly up-regulated or down-regulated compared to the control group.

Generation of an 'mRNA expression time-course' using microarray data was first used in this thesis. Analyses of expression of the genes of interest seemed a logical approach and have allowed the generation of all data for each specific transporter and metabolising enzyme in this thesis.

Defining levels of ABCB1/abcb1 expression will allow investigators to understand the properties of the placenta in greater detail. A direct comparison between the rat and human species highlights the 5-fold difference between rat and human in ABCB1/abcb1b expression in the first trimester, a critical time point during organogenesis when compounds are administered to rodents in order to predict potential hazards for human health. Knowledge of this could aid rationale as to why some compounds fail in the early stages of toxicity testing.

3.2.2 The mRNA expression profile of human and rat placental ABCG2/abcg2 throughout gestation

The *ABCG2/abcg2* genes encode the breast cancer resistance protein (bcrp). The placenta is the site of highest ABCG2/abcg2 expression (Allikmets et al., 1998) and like ABCB1/abcb1, ABCG2/abcg2 provides protection by its ability to efflux a range of xenobiotics away from the intracellular compartment and into the extracellular environment.

Substrates of ABCG2 include drugs that are commonly administered during pregnancy, including nitrofurantoin, ritonaivr and dexamethasone (Evseenko et al., 2006b).

In order to compare placental expression levels between rat and human, the mRNA expression levels of placental ABCG2/abcg2 were quantified using ArrayTrack™ (Figure 3.2). The difference between transporter intensities in each trimester is shown numerically in Table 3.2. The Genbank accession codes for ABCG2/abcg2 can be found in the Appendix 6, Table 6B.

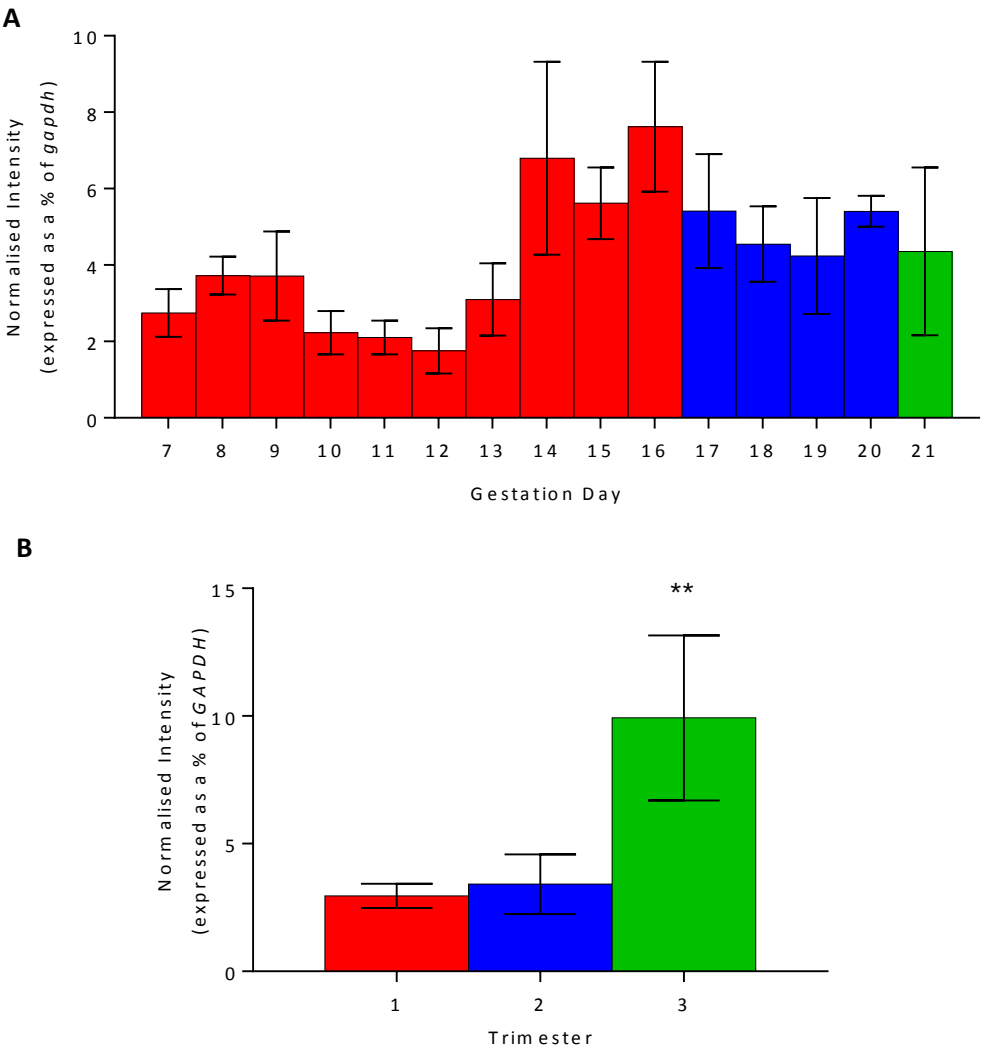


Figure 3.2 The mRNA expression profile of ABCG2 and abcg2 in human and rat placenta throughout gestation. The expression of ABCG2 and abcg2 throughout gestation in the developing embryo (foetus and placenta) in rat (A) and developing placenta in human (B). Statistical analysis was carried out using the Student's T-test. Asterisks (** = $p < 0.01$) denote T 3 intensity values are statistically different from T 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.2 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) abcg2 and human (developing placenta) ABCG2 throughout gestation.

Abcg2	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat abcg2	GD 7	2.75 ± 0.62
Human ABCG2	T1	2.96 ± 0.47
Rat abcg2	GD 21	4.36 ± 2.20
Human ABCG2	T3	9.93 ± 3.23

Expression of abcg2/ABCG2 mRNA was detected in both rat and human placenta. In rat the level of abcg2 expression increased 1.5 fold from GD 7 to GD 21 (Figure 3.2 A). A similar pattern of expression was observed in human placenta with levels of ABCG2 significantly ($p < 0.01$) increasing with expression in trimester 3 3-fold higher than in trimester 1 (Figure 3.2 and Table 3.2).

Comparative levels of ABCG2/abcg2 expression between the human and rat are illustrated in Table 3.2. During the first and second trimester levels of abcg2 expression are higher in rat compared to human. However in the final trimester the mRNA expression level of human placental ABCG2 is more than 2-fold higher than that of rat placental abcg2.

In the rat, during the first trimester from GD7 to GD 13 there was relatively little change in abcg2 expression level, however at GD 14 abcg2 expression increases to more than 2 fold the expression level on GD 7. The level of abcg2 mRNA expression remains fairly constant throughout the second trimester, although the expression level in the second trimester GD 21 is nearly 2-fold higher compared to GD 7 (Figure 3.2 (A)). In human placenta, there is little difference in the levels of ABCG2 expression between the first and second trimesters, unlike in the rat placenta. However, expression of ABCG2 significantly ($P<0.01$) increases between T1 and T3 (Figure 3.2 (B)). In this regard, the increase in expression of abcg2/ABCG2 is similar in both the rat and human. The difference in abcg2/ABCG2 mRNA expression highlights the potential difference in placental barrier properties between the rat and human. As with abcb1b, the rat has a higher level of abcg2 mRNA expression during the first two trimesters than human. Further expression and functional studies are necessary in order to assess the relationship between mRNA expression, protein expression and functional activity.

3.2.3 The mRNA expression profile of members of the ABCC/abcc family in human and rat placenta throughout gestation

The ABCC/abcc family encodes the multidrug resistance-associated proteins (MRPs) and contains 13 members. Nine of the members are MRP-related (abcc1 – 6, and abcc10 – 12) and the remaining proteins are the cystic fibrosis transmembrane conductance regulator (cftr/abcc7) and the sulfonylurea receptors (sur8/abcc8 and sur2/abcc9) (Toyoda et al., 2008).

The mRNA expression levels of members of the ABCC/abcc family in human and rat placenta were quantified using ArrayTrack™ (Figures 3.3 and 3.4 show those members expressed in both species. Appendix 8, Figure 8A shows the members only found in human placenta). The Genbank Accession code for each member of the abcc family can be found in the Appendix 6, Table 6C.

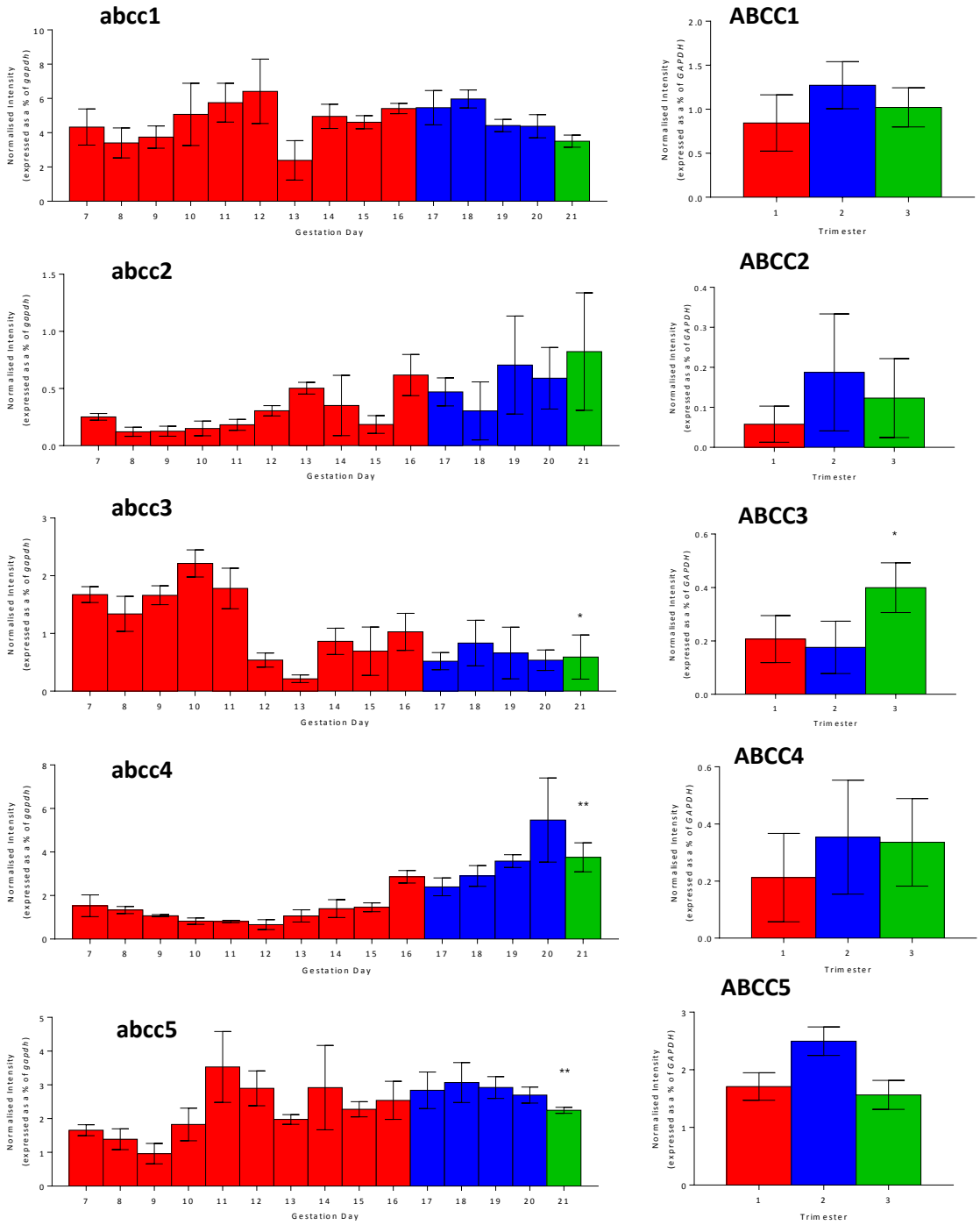


Figure 3.3 The mRNA expression profile of members of the ABCC/abcc family (1-5) in human and rat placenta throughout gestation. The expression of members of the ABCC/abcc family throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = p <0.05, ** = p < 0.01 and *** = p< 0.001) denote GD 21/T3 intensity values are statistically different from GD 7/T1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human..

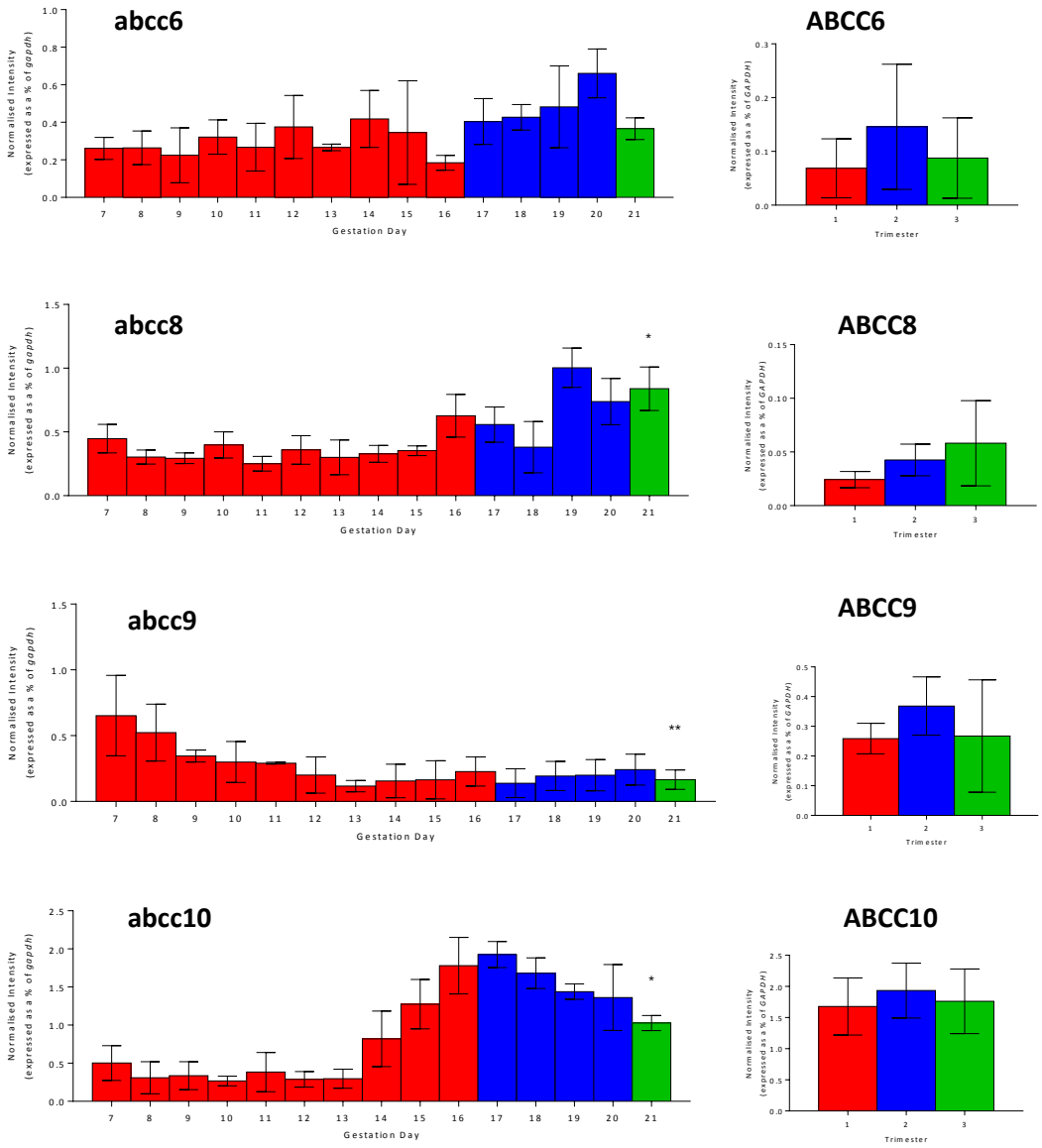


Figure 3.4 The mRNA expression profile of members of the ABCC/abcc family (6, 8, 9 and 10) in human and rat placenta throughout gestation. The expression of members of the ABCC/abcc family throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$) denote GD 21 intensity values are statistically different from GD 7. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.3 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) abcc and human (developing placenta) ABCC family members at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation

Abcc transporter	Gestation Day (GD) or Trimester (T)	Mean intensity (± SD)
Rat abcc1	GD 7	4.3 ± 1.1
Human ABCC1	T 1	0.8 ± 0.3
Rat abcc1	GD 21	3.5 ± 0.4
Human ABCC1	T 3	1.0 ± 0.2
Rat abcc2	GD 7	0.3 ± 0.02
Human ABCC2	T 1	0.06 ± 0.05
Rat abcc2	GD 21	0.8 ± 0.5
Human ABCC2	T 3	0.12 ± 0.09
Rat abcc3	GD 7	1.7 ± 0.13
Human ABCC3	T 1	0.21 ± 0.09
Rat abcc3	GD 21	0.59 ± 0.38
Human ABCC3	T 3	0.4 ± 0.09
Rat abcc4	GD 7	1.5 ± 0.5
Human ABCC4	T 1	0.21 ± 0.15
Rat abcc4	GD 21	3.8 ± 0.66
Human ABCC4	T 3	0.34 ± 0.15
Rat abcc5	GD 7	1.7 ± 0.16
Human ABCC5	T 1	1.7 ± 0.24
Rat abcc5	GD 21	2.2 ± 0.1
Human ABCC5	T 3	1.6 ± 0.25
Rat abcc6	GD 7	0.26 ± 0.06
Human ABCC6	T 1	0.07 ± 0.05
Rat abcc6	GD 21	0.4 ± 0.06
Human ABCC6	T 3	0.09 ± 0.07
Rat abcc8	GD 7	0.45 ± 0.11
Human ABCC8	T 1	0.02 ± 0.01
Rat abcc8	GD 21	0.84 ± 0.17
Human ABCC8	T 3	0.058 ± 0.04
Rat abcc9	GD 7	0.65 ± 0.31
Human ABCC9	T 1	0.26 ± 0.05
Rat abcc9	GD 21	0.17 ± 0.07
Human ABCC9	T 3	0.27 ± 0.19
Rat abcc10	GD 7	0.50 ± 0.23
Human ABCC10	T 1	1.68 ± 0.46
Rat abcc10	GD 21	1.03 ± 0.10
Human ABCC10	T 3	1.76 ± 0.52

Nine members of the abcc family were expressed in both species; abcc1, abcc2, abcc3, abcc4, abcc5, abcc6, abcc8, abcc9 and abcc10 (Figure 3.3 and Figure 3.4). Additional members of the abcc family, namely ABCC11, ABCC12 and ABCC13, were expressed in the human placenta (See Appendix 8, Figure 8A).

A higher level (at least 5-fold) of abcc1 mRNA expression was observed in the rat compared to human at the beginning of gestation (Figure 3.3 and Table 3.3). Low levels of abcc2 mRNA were observed in both species. Abcc3 mRNA expression significantly decreased from the beginning of gestation to term in the rat placenta, and conversely human placental ABCC3 mRNA expression increased significantly from T1 to T3 (Table 3.3 and Figure 3.3). Abcc4 mRNA expression significantly increased from the beginning of gestation to term in the rat placenta, however no significant change in expression was observed in the human placenta (Table 3.3). The expression of abcc5 mRNA increased significantly ($p < 0.01$) in the rat from GD 7 to GD 21 but there was no significant change in expression in the human placenta. There was at least a 3-fold-higher level of abcc6 mRNA expressed in the rat placenta at the beginning and end of gestation, compared to the beginning and end of gestation in the human placenta (Table 3.3), in both species the level of expression remained constant throughout gestation (Figure 3.4). The level of expression of abcc8 was more than 10-fold higher in rat than human at the beginning and end of gestation (Table 3.3) and a significant increase ($p < 0.05$) in expression was also observed in the rat placenta between GD 7 and GD21 (Figure 3.4). No significant change in ABCC8 expression was observed in the human placenta throughout gestation (Figure 3.4). The level of abcc9 mRNA expression decreased throughout gestation in the rat placenta; with levels at GD21 significantly ($p < 0.01$) lower than GD 7. No significant difference in expression in trimester 1 and trimester 3 was seen in the human placenta (Figure 3.4 and Table 3.3). The mRNA expression level of ABCC10 was 3-fold higher in human placenta at the beginning of gestation than in rat placenta (Table 3.3). Whereas a significant increase ($p < 0.05$) in expression of rat placental abcc10 was observed throughout gestation, in human placenta the level remained relatively constant (Figure 3.4).

ABCC1/abcc1 mRNA expression was assessed more in-depth. In the rat placenta throughout the first trimester, lowest expression of abcc1 was observed at GD 13. There was a slight decrease of abcc1 expression between at GD 21 compared to GD 7.

In comparison, in the human placenta there was a 1.5-fold increase in ABCC1 expression in the second trimester compared to the first trimester. Similarly, there was a 1.3 fold increase in the expression of ABCC1 at term compared to the first trimester. These findings demonstrate a difference in the placental mRNA abcc1/ABCC1 expression pattern between the rat and human.

Further work is required to fully determine localisation and function of these ABCC/abcc family members before mRNA expression data can be substantiated and the effects of differential expression between species has upon the outcome of using rodents in developmental toxicity assays to predict potential hazards for human health.

3.2.4 The mRNA expression profile of members of the SLCO/slco family in human and rat placenta throughout gestation

The SLCO/slco family encodes the organic anion transporting polypeptides (oatps) which mediate the uptake of a broad range of substrates. The human OATP family consists of 11 members whereas the rat family consists of 13 members (Hagenbuch and Meier, 2004).

The mRNA expression levels of members of the SLCO/slco family which were expressed in humans and rats were quantified using ArrayTrack™. Those SLCO/slco members that were expressed in both human and rat are shown in Figure 3.5. Those members only expressed in the developing rat placenta or the human placenta are shown in Appendix 8, Figures 8B and 8C respectively. The Genbank accession code for each member of the SLCO/slco family can be found in Appendix 6 Table 6D.

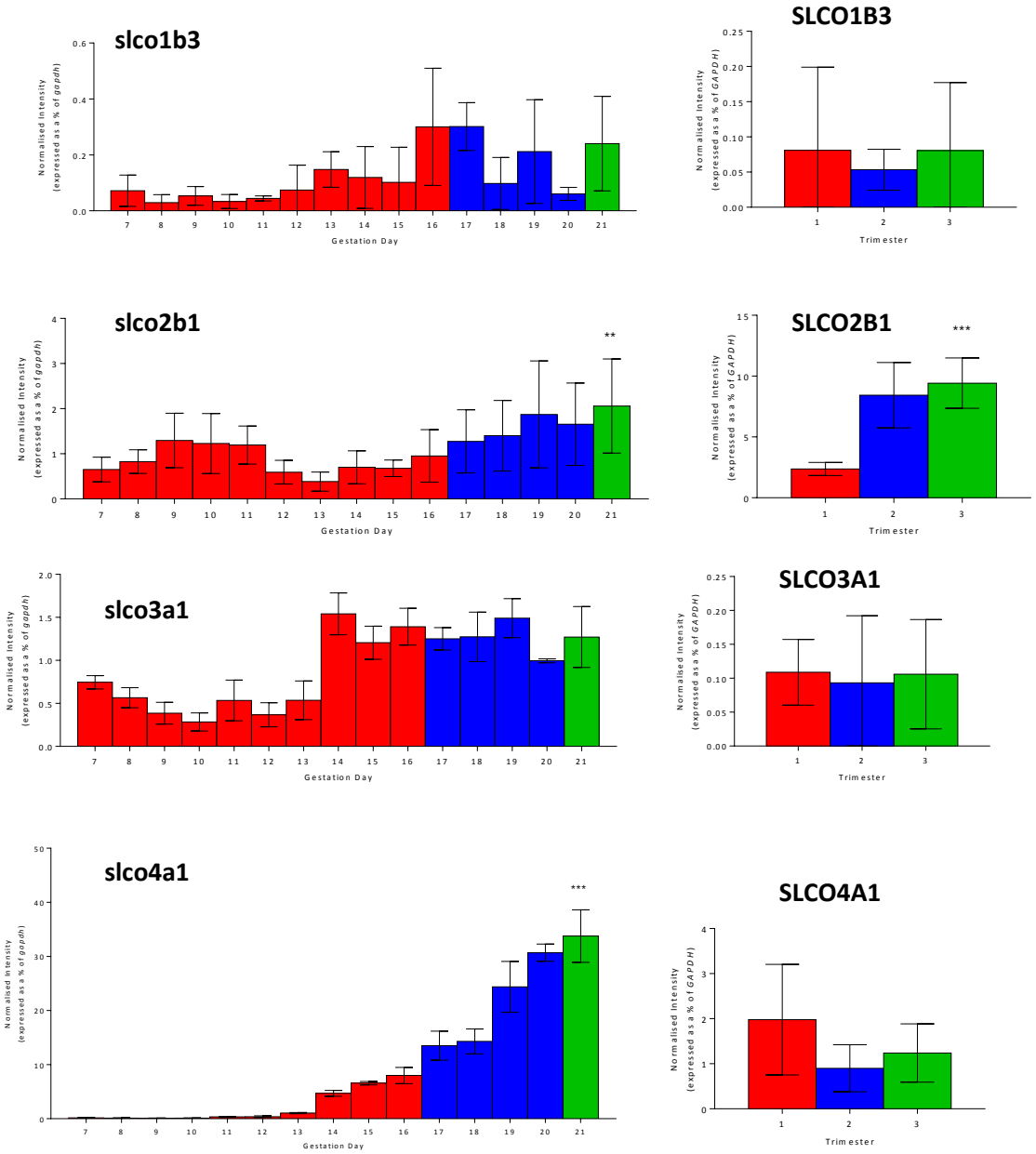


Figure 3.5 The mRNA expression profile of members of the SLCO/slco family in human and rat placenta throughout gestation. The expression of members of the SLCO/slco family throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (** = $p < 0.01$ and *** = $p < 0.001$) denote GD 21/T3 intensity values are statistically different from GD 7/T1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.4 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) slco and human (developing placenta) SLCO family members at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

Abcc transporter	Gestation Day (GD) or Trimester (T)	Mean intensity (± SD)
Rat slco1b3	GD 7	0.07± 0.06
Human SLCO1B3	T 1	0.08 ± 0.12
Rat slco1b3	GD 21	0.24 ± 0.17
Human SLCO1B3	T 3	0.08 ± 0.10
Rat slco2b1	GD 7	0.65 ± 0.27
Human SLCO2B1	T 1	2.40 ± 0.54
Rat slco2b1	GD 21	2.06 ± 1.04
Human SLCO2B1	T 3	9.42 ± 2.07
Rat slco3a1	GD 7	0.75 ± 0.078
Human SLCO3A1	T 1	0.11 ± 0.05
Rat slco3a1	GD 21	1.27 ± 0.35
Human SLCO3A1	T 3	0.11 ± 0.08
Rat slco4a1	GD 7	0.15 ± 0.11
Human SLCO4A1	T 1	1.98 ± 1.23
Rat slco4a1	GD 21	33.76 ± 4.85
Human SLCO4A1	T 3	1.24 ± 0.65

Four members of the slco/SLCO family were expressed in both rat and human placenta: slco1b3, slco2b1, slco3a1 and slco4a1 (Figure 3.5).

Slco1b3 mRNA was expressed in both rat and human. The levels of mRNA expression were found to be similar between species at the beginning of gestation (GD7 and first trimester) (Table 3.4), however at the end of gestation (GD 21 and trimester 3), the level of slco1b3 mRNA expression in rat placenta was 3-fold higher than in human placenta (Figure 3.5 and Table 3.4). At the beginning of gestation (T1), expression of SLCO2B1 mRNA was 3.6-fold higher in human placenta than in rat placenta and at the end of gestation was 4.5-fold higher (Figure 3.5 and Table 3.4) in human placenta than rat placenta. In both species there was significantly higher ($p < 0.01$ rat; $p < 0.001$ human) expression of slco2b1 mRNA in trimester 3 compared to the first trimester.

The mRNA expression level of slco3a1 was 6.8-fold higher in the rat than in human placenta at the beginning of gestation and 10-fold higher in the rat compared to the human at the end of gestation.

SLCO4A1/slco4a1, the gene encoding the thyroid hormone uptake transporter (Loubiere et al., 2010), was found to be expressed at a higher level in the human placenta than the rat at the beginning of gestation (by more than 10-fold higher), but the level of slco4a1 expression at the end of gestation in the rat placenta was nearly 30-fold higher than in human placenta.

Throughout gestation the mRNA level of rat placental slco4a1 significantly increased ($p < 0.001$) from GD 7 to GD 21, more specifically the expression of slco4a1 increased 50-fold between GD 7 and GD 16, 137-fold between trimester 2 and GD7 and 224-fold between term and GD 7. However this trend was not observed in human placenta (Figure 3.5). There was a decrease in SLCO4A1 expression between the first and second trimester, and a 1.6-fold decrease between the third trimester and the first trimester. Highest expression of SLCO4A1 was observed in the first trimester. Significant increases in slco4a1 mRNA have also been observed in rat placenta throughout gestation using real-time PCR (St-Pierre et al., 2004).

Relatively little is known about SLCO4A1/slco4a1 compared to other members of the slco family. Localisation has been reported on the apical membrane of syncytiotrophoblasts in the rat placenta (Nishikawa et al., 2010), however in order to fully understand the role placental slco4a1 could play in influencing exposure to xenobiotics, further functional studies are required. The observation of different mRNA expression levels between rat and human at the end of gestation must be considered by investigators when using the rodent model as a predictor of potential hazards for human health.

3.2.5 The mRNA expression profile of members of the CYPP450/cyp450 family in human and rat placenta throughout gestation.

Cytochrome p450 enzymes are phase I metabolising enzymes which oxidise xenobiotics (Slattery et al., 2004). The oxidised-xenobiotic is then further processed by phase II enzymes including glutathione transferases and UDP-glucuronosyltransferases. The three main xenobiotic metabolising cyp families are the CYP1/cyp1, CYP2/cyp2 and CYP3/cyp3

The mRNA expression levels of members of the CYPP450/cyp450 family expressed in both human and rat placenta were quantified using ArrayTrack™. Those CYP/cyp members that were expressed in both human and rat are shown in Figures 3.6 – 3.11. Those members only expressed in the developing rat placenta or the human placenta, are shown in Appendix 8, Figures 8D – 8J and Figures 8K – 8N respectively. The Genbank Accession code for each CYP450/cyp450 member can be found in Appendix 6 (Human CYP450 Table 6E; rat cyp450 Table 6F).

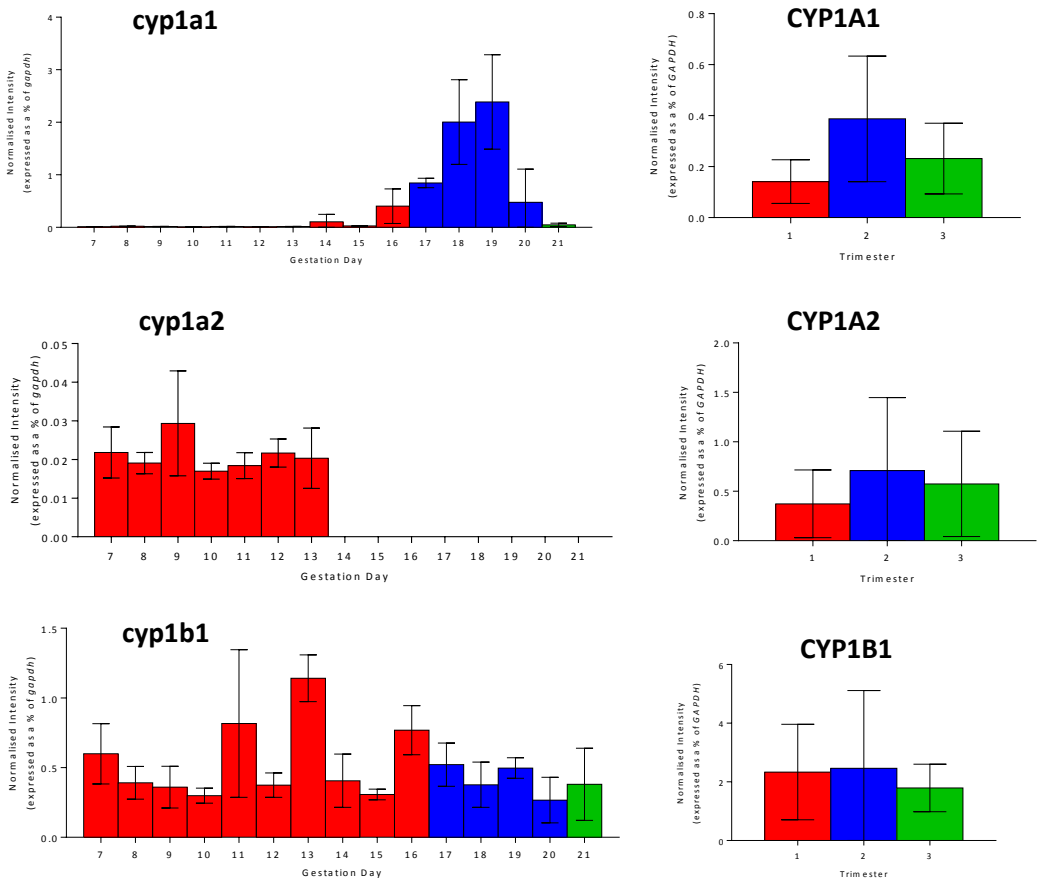


Figure 3.6 The mRNA expression profile of cytochrome p450 enzymes (1a1/1A1, 1a2/1A2 and 1b1/1B1) in the human and rat placenta throughout gestation. mRNA Expression of cytochrome p450 enzymes (1a1/1A1, 1a2/1A2 and 1b1/1B1) throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.5 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) and human (developing placenta) cytochrome p450 members (cyp1a1/CYP1A1, cyp1a2/CYP1A2 and cyp1b1/CYP1B1) at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

cyp450 enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (± SD)
Rat cyp1a1	GD 7	0.014 ± 0.001
Human CYP1A1	T 1	0.14 ± 0.09
Rat cyp1a1	GD 21	0.05 ± 0.03
Human CYP1A1	T 3	0.23 ± 0.14
Rat cyp1a2	GD 7	0.02 ± 0.01
Human CYP1A2	T 1	0.37 ± 0.34
Rat cyp1a2	GD 21	not found
Human CYP1A2	T 3	0.57 ± 0.53
Rat cyp1b1	GD 7	0.60 ± 0.22
Human CYP1B1	T 1	2.34 ± 1.63
Rat cyp1b1	GD 21	0.38 ± 0.26
Human CYP1B1	T 3	1.79 ± 0.81

Expression of mRNA encoding the aryl hydrocarbon hydroxylase, cyp1a1/CYP1A1, was observed in both rat and human throughout gestation (Figure 3.6). This was expressed 10-fold higher in the human placenta than the rat at the beginning of gestation and 4.8-fold higher in the human placenta compared to the rat placenta at the end of gestation (Table 3.5).

From the same cyp1/CYP1 family, cyp1a2/CYP1A2 was expressed in both species during gestation. Substrates include the aromatic amine 2-acetylaminofluorene and heterocyclic amine amino-l-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) (Boobis et al., 1994). Throughout gestation CYP1A2 was expressed in the human placenta however no expression of cyp1a2 was observed in the rat placenta from GD14 to GD21 (Figure 3.6).

One of the more recently characterised members of the cyp1/CYP1 family, cyp1b1/CYP1B1, was expressed in both species. Cyp1b1/CYP1B1 is involved in the metabolism of endogenous oestrogens, and has been specifically linked to the metabolism of 17β-estradiol to 4-hydroxyestradiol (Belous et al., 2007). The human placenta expressed higher levels of CYP1B1 mRNA than the rat at the beginning and end of gestation (3.9-fold and 4.7-fold respectively, Table 3.5 and Figure 3.6).

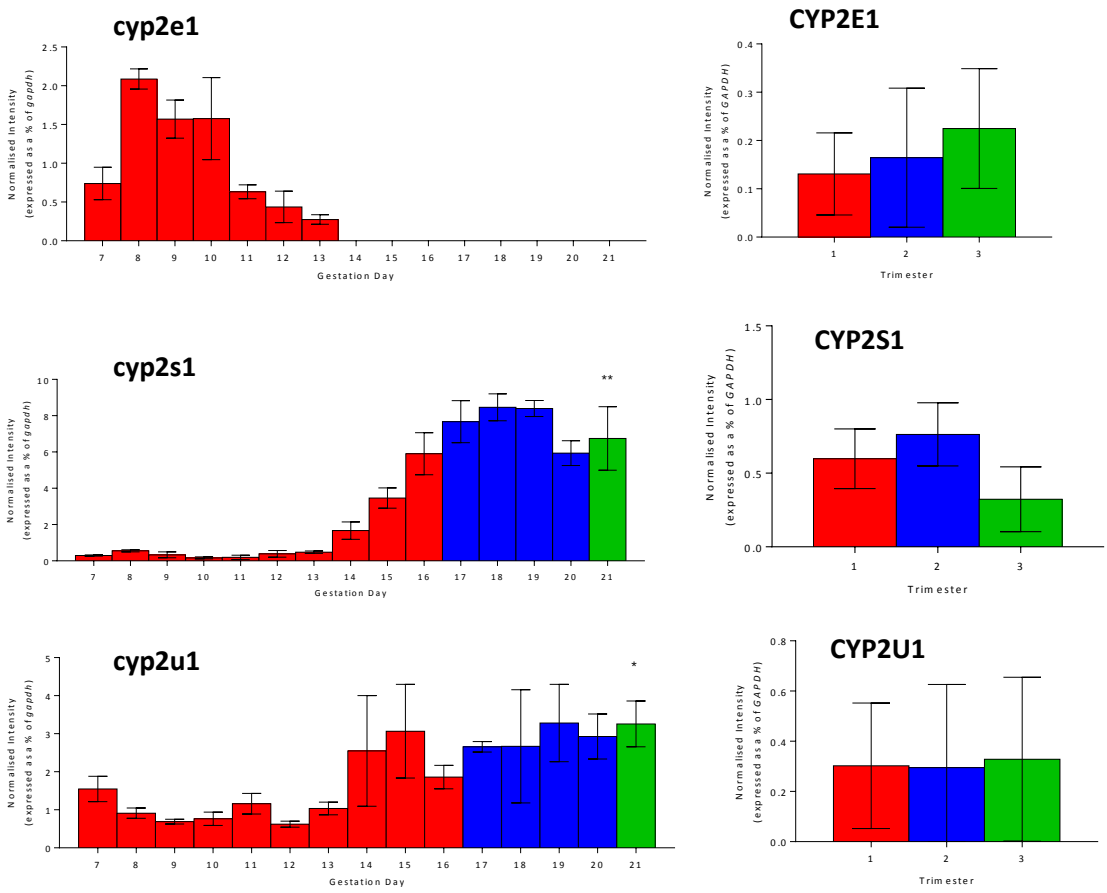


Figure 3.7 The mRNA expression profile of cytochrome p450 enzymes (2e1/2E1, 2s1/2S1 and 2u1/2U1) in the human and rat placenta throughout gestation. mRNA Expression of cytochrome p450 enzymes (2e1/2E1, 2s1/2S1 and 2u1/2U1) throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = $p < 0.05$) denote GD 21 intensity values are statistically different from GD 7. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.6 Comparative mRNA expression intensities of rat (developing embryo: foetus and placenta) and human (developing placenta) cytochrome p450 members (cyp2e1/CYP2E1, cyp2s1/CYP2S1 and cyp2u1/CYP2U1) at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

cyp450 enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (± SD)
Rat cyp2e1	GD 7	0.74 ± 0.21
Human CYP2E1	T 1	0.13 ± 0.08
Rat cyp2e1	GD 21	not found
Human CYP2E1	T 3	0.22 ± 0.12
Rat cyp2s1	GD 7	0.30 ± 0.03
Human CYP2S1	T 1	0.59 ± 0.20
Rat cyp2s1	GD 21	6.7 ± 1.75
Human CYP2S1	T 3	0.32 ± 0.22
Rat cyp2u1	GD 7	1.55 ± 0.33
Human CYP2U1	T 1	0.30 ± 0.25
Rat cyp2u1	GD 21	3.26 ± 0.60
Human CYP2U1	T 3	0.33 ± 0.33

The human CYP2 family is considered to be the largest and most diverse of the CYP families (Nelson et al., 2004). The predominant member of the CYP2E family is CYP2E1 which was expressed at the mRNA level in both human placenta and the developing rat embryo (up to GD 14) (Figure 3.7). At GD 7 a higher level of cyp2e1 mRNA was observed in rat placenta than in trimester 1 human placenta (5.7-fold higher). CYP2E1 is known to metabolise a number of low molecular weight pharmaceutical and xenobiotic compounds including acetaminophen, ethanol, acetone and benzene (Lee et al., 1996 and Lieber, 1997).

The level of cyp2s1 mRNA expression was 20-fold higher in the rat placenta than in the human placenta at term (Table 3.6). The role of cyp2s1 is thought to be in metabolising endogenous substances and in humans CYP2S1 has been found to be highly expressed in respiratory, gastrointestinal tissue, urinary tracts and the skin (Saarikoski et al., 2005).

The expression of cyp2u1/CYP2U1 mRNA significantly increased in the developing rat foetus and placenta from GD7 to term ($p<0.05$, Figure 3.7). This trend was not observed in the human placenta. Previous studies with human CYP2U1 have shown high expression in the brain and thymus (Karlgren et al., 2004). Substrates include arachidonic acid, docosahexaenoic acid and other long chain fatty acids (Chuang et al., 2004).

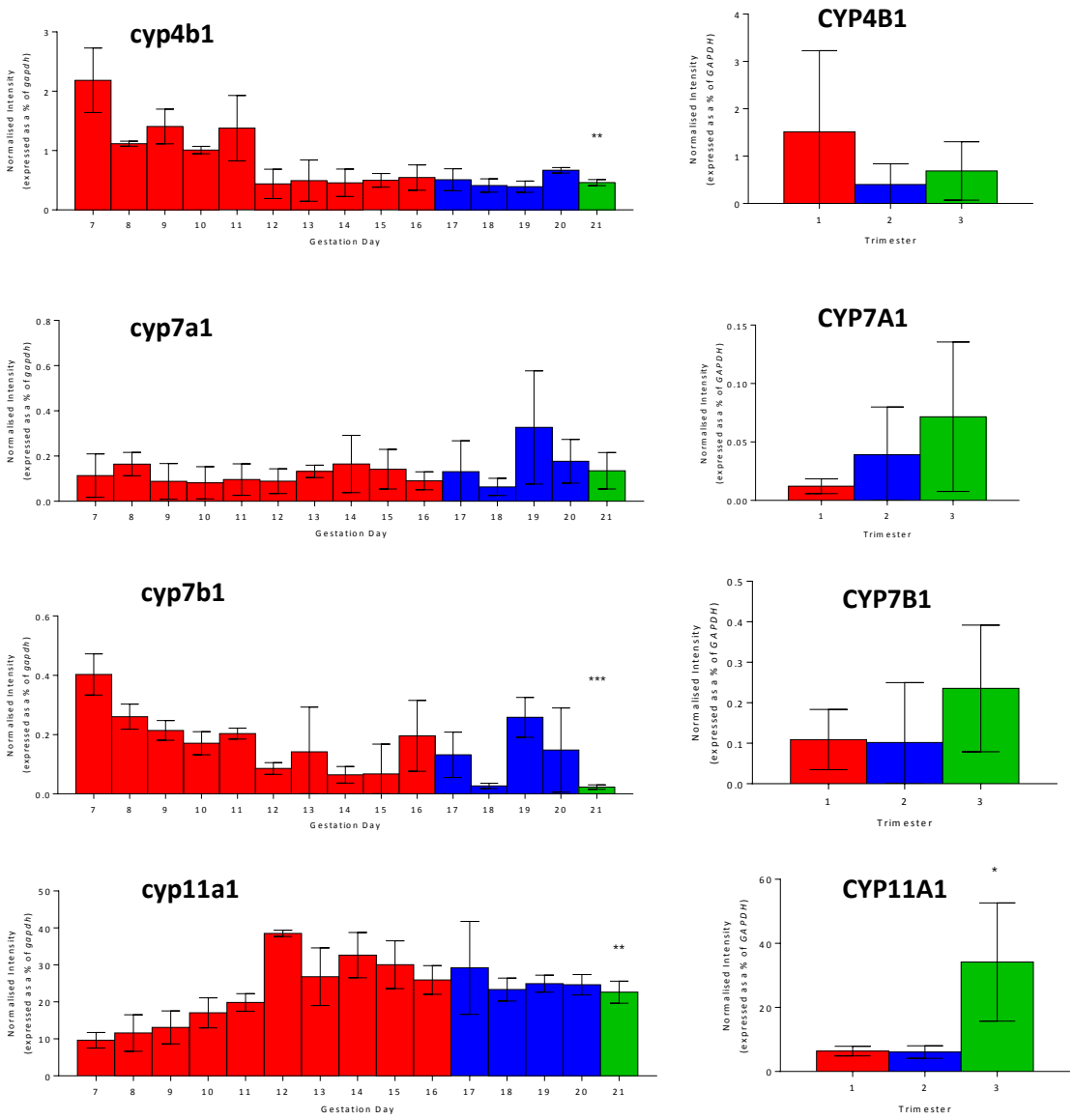


Figure 3.8 The mRNA expression of cytochrome p450 enzymes (cyp4b1/CYP4B1, cyp7a1/CYP7A1, cyp7b1/CYP7B1, cyp11a1/CYP11A1) in the human and rat placenta throughout gestation. mRNA expression of cytochrome p450 enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right hand-side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (** = p< 0.01) denote either GD 21 intensity values are statistically different from GD 7 or term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

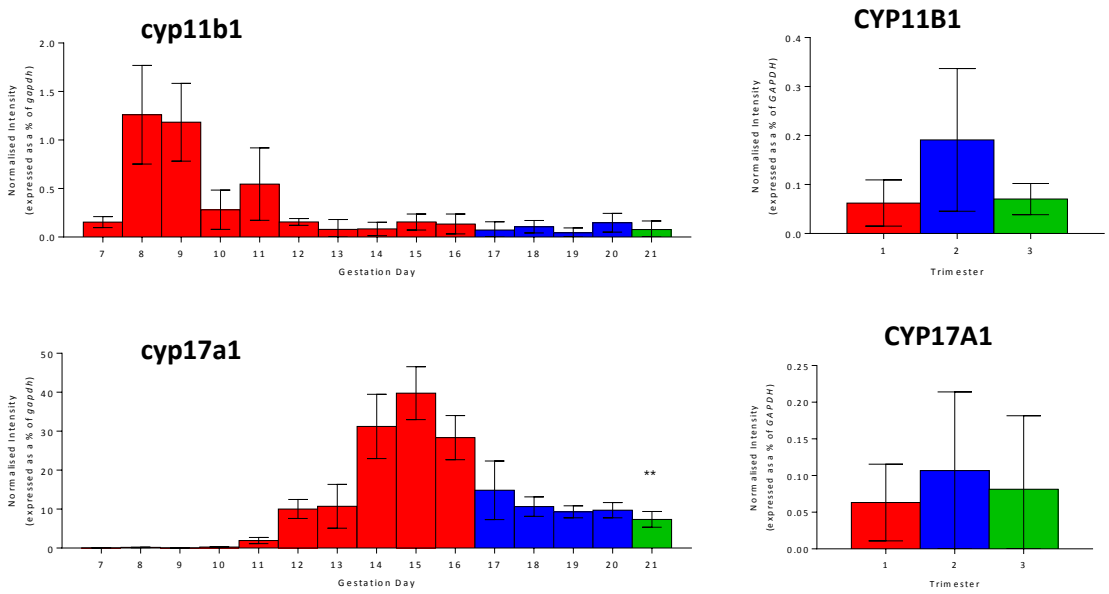


Figure 3.9 The mRNA expression of cytochrome p450 enzymes (cyp11b1/CYP11B1 and cyp17a1/CYP17A1) in the human and rat placenta throughout gestation. mRNA expression of cytochrome p450 enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right hand-side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (** = $p < 0.01$) denote either GD 21 intensity values are statistically different from GD 7 or term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.7 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) and human (developing placenta) cytochrome p450 members (cyp4b1/CYP4B1, cyp7a1/CYP7A1, cyp7b1/CYP7B1, cyp11a1/CYP11A1, cyp11b1/CYP11B1 and cyp17a1/CYP17A1) at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

cyp450 enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat cyp4b1	GD 7	2.184 ± 0.5437
Human CYP4B1	T 1	1.514 ± 1.715
Rat cyp4b1	GD 21	0.4611 ± 0.05128
Human CYP4B1	T 3	0.688 ± 0.6156
Rat cyp7a1	GD 7	0.1139 ± 0.09597
Human CYP7A1	T 1	0.01217 ± 0.006323
Rat cyp7a1	GD 21	0.1352 ± 0.08079
Human CYP7A1	T 3	0.07159 ± 0.06392
Rat cyp7b1	GD 7	0.4028 ± 0.06959
Human CYP7B1	T 1	0.1092 ± 0.07443
Rat cyp7b1	GD 21	0.0226 ± 0.007464
Human CYP7B1	T 3	0.2355 ± 0.1563
Rat cyp11a1	GD 7	9.626 ± 2.102
Human CYP11A1	T 1	6.371 ± 1.51
Rat cyp11a1	GD 21	22.63 ± 2.985
Human CYP11A1	T 3	34.15 ± 18.45
Rat cyp11b1	GD 7	0.1548 ± 0.05682
Human CYP11B1	T 1	0.06232 ± 0.04717
Rat cyp11b1	GD 21	0.07833 ± 0.08728
Human CYP11B1	T 3	0.07037 ± 0.03174
Rat cyp17a1	GD 7	0.07875 ± 0.05675
Human CYP17A1	T 1	0.06329 ± 0.05229
Rat cyp17a1	GD 21	7.365 ± 2.027
Human CYP17A1	T 3	0.08134 ± 0.1003

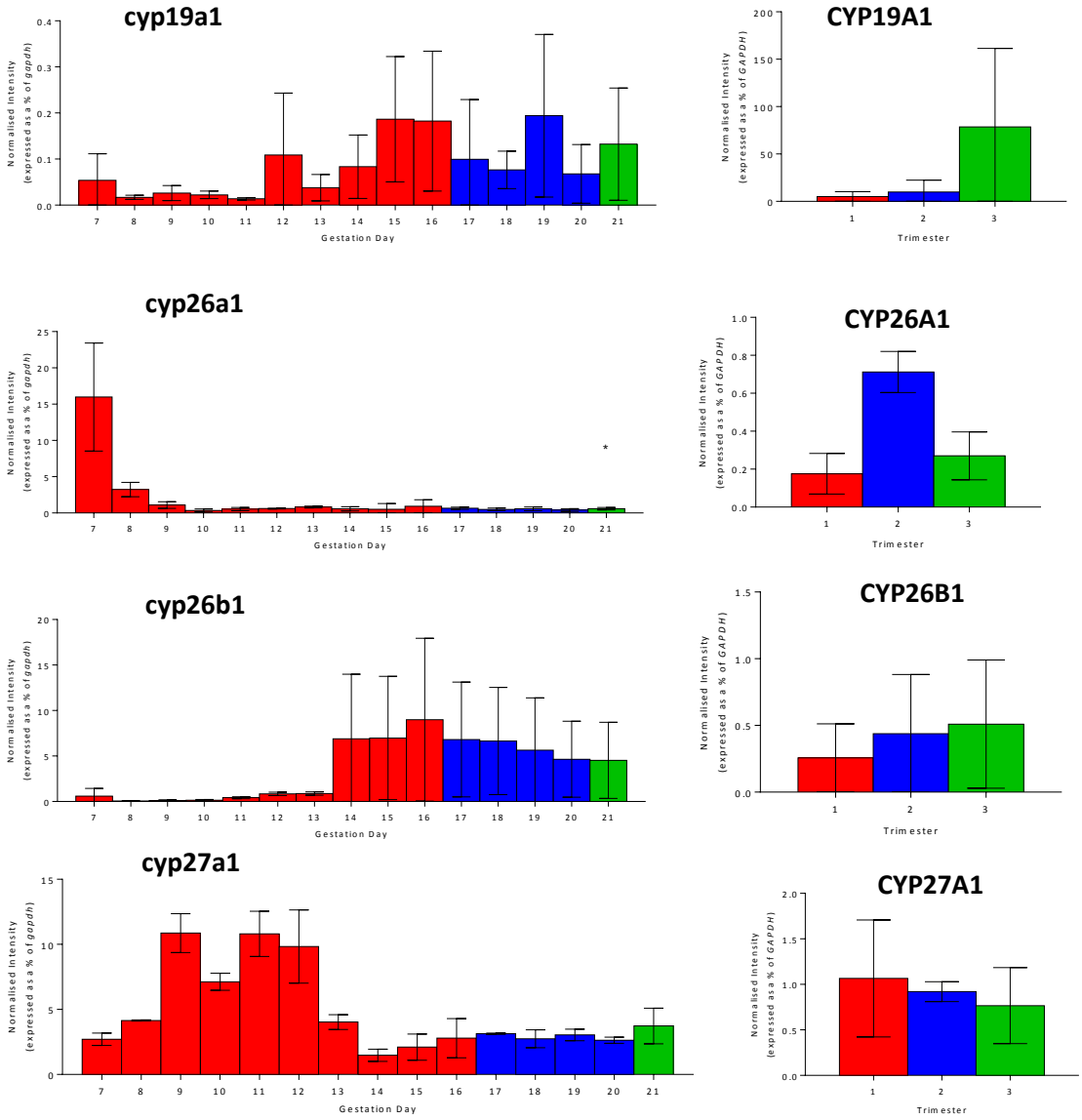


Figure 3.10 The mRNA Expression of cytochrome p450 enzymes (19a1/19A1, 26a1/26A1, 26b1/26B1, 27a1/27A1) in the human and rat placenta throughout gestation mRNA Expression of cytochrome p450 enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = $p < 0.05$) denote either GD 21 intensity values are statistically different from GD 7 or term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

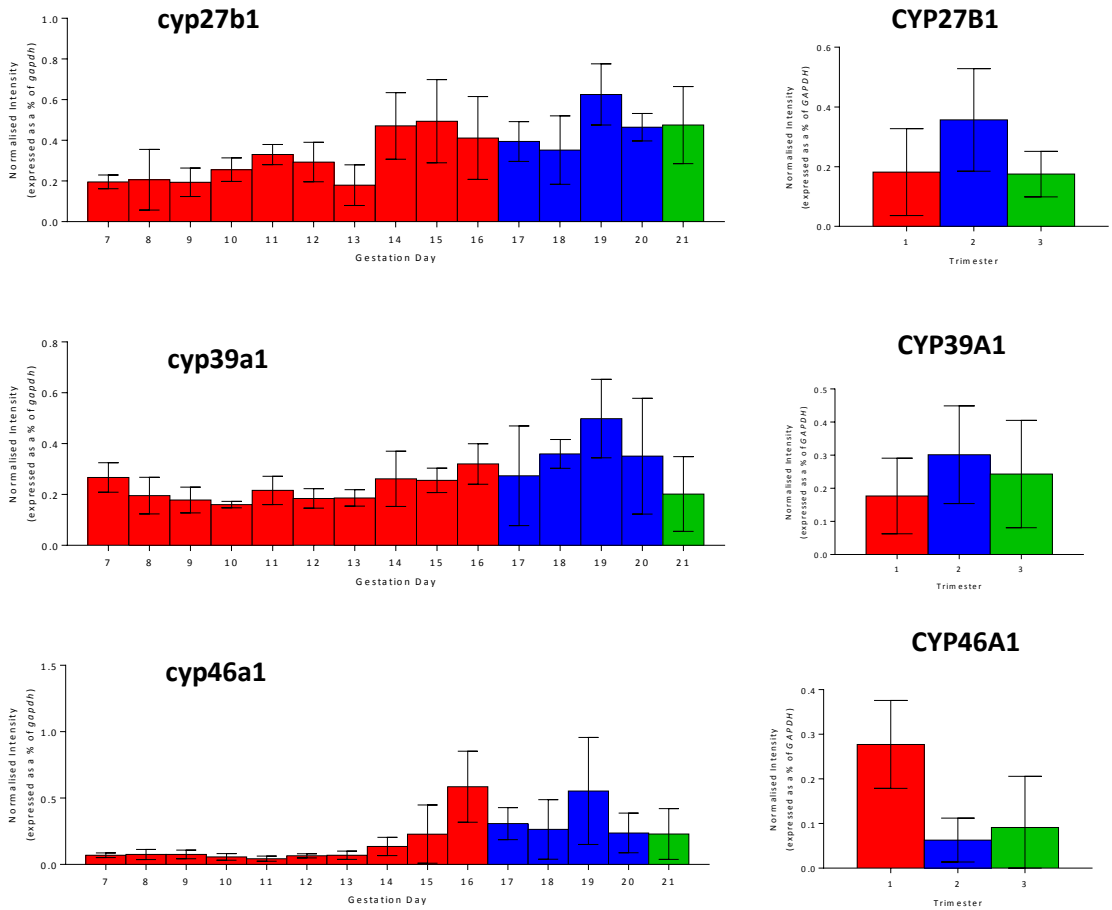


Figure 3.11 The mRNA Expression of cytochrome p450 enzymes (27b1/27B1, 39a1/39A1, 46a1/46A1) in the human and rat placenta throughout gestation. mRNA Expression of cytochrome p450 enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = $p < 0.05$) denote either GD 21 intensity values are statistically different from GD 7 or term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.8 Comparative mRNA expression intensities of rat (developing embryo; foetus and placenta) and human (developing placenta) cytochrome p450 members (cyp19a1/CYP19A1, cyp26a1/CYP26A1, cyp26b1/CYP26B1, cyp27a1/CYP27A1, cyp27b1/CYP27B1, cyp39a1/CYP39A1 and cyp46a1/CYP46A1) at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

cyp450 enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat cyp19a1	GD 7	0.05374 ± 0.0579
Human CYP19A1	T 1	5.135 ± 5.073
Rat cyp19a1	GD 21	0.1324 ± 0.1218
Human CYP19A1	T 3	78.61 ± 82.88
Rat cyp26a1	GD 7	15.99 ± 7.449
Human CYP26A1	T 1	0.1757 ± 0.1074
Rat cyp26a1	GD 21	0.5884 ± 0.167
Human CYP26A1	T 3	0.27 ± 0.1266
Rat cyp26b1	GD 7	0.5857 ± 0.8556
Human CYP26B1	T 1	0.2574 ± 0.2542
Rat cyp26b1	GD 21	4.522 ± 4.189
Human CYP26B1	T 3	0.5096 ± 0.4805
Rat cyp27a1	GD 7	2.705 ± 0.4733
Human CYP27A1	T 1	1.065 ± 0.6419
Rat cyp27a1	GD 21	3.726 ± 1.373
Human CYP27A1	T 3	0.7663 ± 0.4177
Rat cyp27b1	GD 7	0.1955 ± 0.03369
Human CYP27B1	T 1	0.1819 ± 0.1455
Rat cyp27b1	GD 21	0.475 ± 0.1894
Human CYP27B1	T 3	0.175 ± 0.07615
Rat cyp39a1	GD 7	0.2669 ± 0.05782
Human CYP39A1	T 1	0.1769 ± 0.1142
Rat cyp39a1	GD 21	0.2017 ± 0.147
Human CYP39A1	T 3	0.2433 ± 0.1621
Rat cyp46a1	GD 7	0.0696 ± 0.01742
Human CYP46A1	T 1	0.2773 ± 0.09843
Rat cyp46a1	GD 21	0.2297 ± 0.1912
Human CYP46A1	T 3	0.09128 ± 0.1145

The expression of cyp4b1 in the rat significantly decreased throughout gestation ($p < 0.01$) (Figure 3.8). This trend was not observed in human, although there was little difference between the actual mRNA expression levels in both species (Table 3.7). Cyp4b1 has been reported to be involved in the metabolism of several xenobiotics including 2-aminofluorene, 2-naphthylamine and benzidine (Imaoka et al., 1997, Vanderslice et al., 1985).

Cyp7a1/CYP7A1 mRNA and cyp7b1/CYP7B1 mRNA were expressed in both rat and human placenta (Figure 3.8, Table 3.7). Both members of the cyp7/CYP7 family are involved in bile acid synthesis. Cyp7a1/CYP7A1 is involved in the 'classic' bile acid synthesis pathway, whereas cyp7b1/CYP7B1 is involved in the 'alternative' bile acid synthesis pathway (Ren et al., 2003). The developing rat embryo and placenta expressed a higher level of cyp7a1 than human placenta at the beginning and at the end of gestation (9.4-fold and 1.9-fold respectively); however expression of CYP7B1 mRNA in the human placenta was more than 10-fold higher than in rat placenta at the end of gestation (Table 3.7).

High levels of cyp11a1/CYP11A1 mRNA were expressed in the placenta of both species, particularly at the end of gestation where a significant increase was observed in both species compared to the beginning of gestation (Figure 3.8). Cyp11a1/CYP11A1 catalyses the conversion of cholesterol to the steroid hormone pregnenolone (Mast et al., 2010). Cyp11b1/CYP11B1 mRNA was also expressed in both species (Figure 3.9 and Table 3.7). The mRNA levels were higher in the rat than the human placenta at the beginning of gestation (GD 7 in rat, T1 in human) (2.5-higher in the rat), but little difference was seen at the end of gestation (GD 21 in rat, T3 in human) between the species. The cyp11b1/CYP11B1 enzyme is also involved in steroid hormone biosynthesis, converting 11-deoxycortisol to cortisol (Pascoe et al., 1992).

The levels of cyp17a1/CYP17A1 mRNA were relatively comparable at the beginning of gestation in both species (Table 3.7), however at the end of gestation the level of cyp17a1 expression in rat placenta was 90.6-fold higher than in human placenta (Table 3.7). The cyp17a1/CYP17A1 enzyme is often linked with cyp11a1/CYP11A1 as it has a key role in steroid biosynthesis and catalyses the conversion of pregnenolone to 17-α-hydroxypregnenolone and progesterone to 17-α-hydroxyprogesterone (Gilep et al., 2010).

In human placenta expression of CYP19A1 mRNA was 95.6-fold higher at the beginning of gestation and 593.7-fold higher at the end of gestation in comparison to the rat (Figure 3.10 and Table 3.8). The cyp19a1 enzyme is also referred to as aromatase and

is involved in the conversion of androgens to oestrogens (Czajka-Oraniec and Simpson, 2010).

Of the cyp26 family, both cyp26a1/CYP26A1 mRNA and cyp26b1/CYP26B1 mRNA were expressed in the human and rat placenta (Figure 3.10 and Table 3.8). Cyp26a1 mRNA expression in the rat placenta was significantly lower ($p < 0.05$) at the end of gestation than at the start, whereas cyp26b1 mRNA expression in the rat was higher at term than at the start of gestation. The human mRNA expression level CYP26B1 remained relatively unchanged throughout gestation (Figure 3.10). CYP26 enzymes are reported to metabolise retinoic acid (Thatcher and Isoherranen, 2009).

Expression at the mRNA level of two of the three members of the cyp27/CYP27 family, namely cyp27a1/CYP27A1 and cyp27b1/CYP27B1, was observed in both species. There was a higher level of cyp27a1 mRNA in rat placenta compared to human placenta at the beginning of gestation and at the end of gestation (2.5-fold higher and 4.9-fold higher respectively, Figure 3.10). The human CYP27A1 enzyme (also referred to as sterol 27-hydroxylase) is involved in the bile biosynthetic pathway (Cali and Russell, 1991) and human CYP27B1 metabolises vitamin D3 into its bioactive form which has important roles in calcium homeostasis and immune functions (Sundqvist et al., 2010).

Relatively comparable levels of cyp39a1 mRNA and CYP39A1 mRNA were expressed in both species at the beginning and end of gestation (Figure 3.11 and Table 3.8). The enzyme (along with CYP7B1) is important in bile synthesis and is also referred to as sterol 7-α-hydroxylase (Li-Hawkins et al., 2000).

The level of CYP46A1 mRNA expression at the end of gestation was lower than at the start of gestation in the human placenta (Table 3.8). However in the rat there was an increase in expression of cyp46a1 between the start of gestation (GD 7) and term (GD 21) (Figure 3.11 and Table 3.8). Cyp46a1 catalyses the conversion of cholesterol into 24s-hydroxycholesterol (Lund et al., 1999).

To summarise, differences in levels of expression of placental CYP/cyp mRNA in the rat and human at specific points of gestation were observed. The most notable differences were that the human placenta expresses a higher level of CYP1A2, CYP1B1 and CYP19A1 than the rat placenta. The rat placenta expresses a higher level of cyp2s1, cyp2u1, cyp7a1, cyp17a1 and cyp27a1 than the human placenta. In order to relate these differences to placental barrier properties further investigation is required to clarify the function and substrate specificity of the individual enzymes. These differences are

invaluable tools for investigators using the rat as a developmental toxicity screen for potential hazards for human health.

3.2.6 The mRNA expression profile of members of the GST/gst family in human and rat placenta throughout gestation.

The mRNA expression levels of members of the glutathione transferase GST/gst family in humans and rats were quantified using ArrayTrack^{Tm}. Those GST/gst members that were expressed in both human and rat are shown in Figures 3.12 and 3.13. Those members only expressed in the developing rat placenta or the human placenta, are shown in Appendix 8, Figures 8O and 8P respectively. The Genbank Accession code for each gst member can be found in Appendix 6 (Table 6G).

Glutathione S-transferases are phase II metabolising enzymes which catalyse the conjugation of glutathione (GSH) to compounds, making the conjugated metabolite more easily excreted. In the rat and human placenta, mRNA for nine isoforms were observed in both rat and human placenta (Figure 3.12 - Figure 3.13 and Table 3. 9)

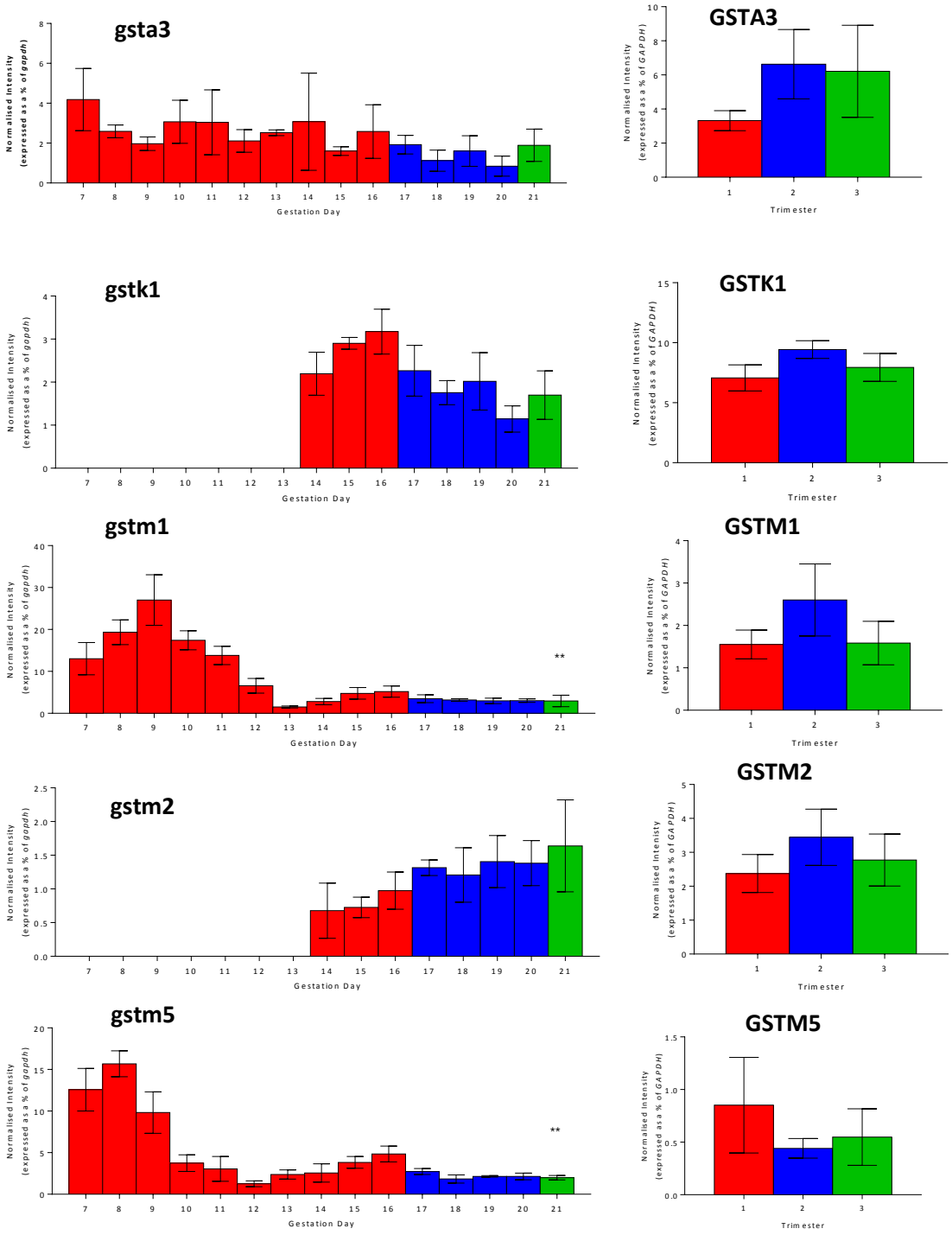


Figure 3.12 The mRNA expression profile of gst/GST enzymes (a3/A3, k1/K1, m1/M1, m2/M2 and m5/M5) in human and rat placenta throughout gestation. The mRNA expression of gst enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side) Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = p <0.05, ** = p< 0.01) denote either GD 21 intensity values are statistically different from GD 7 or term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

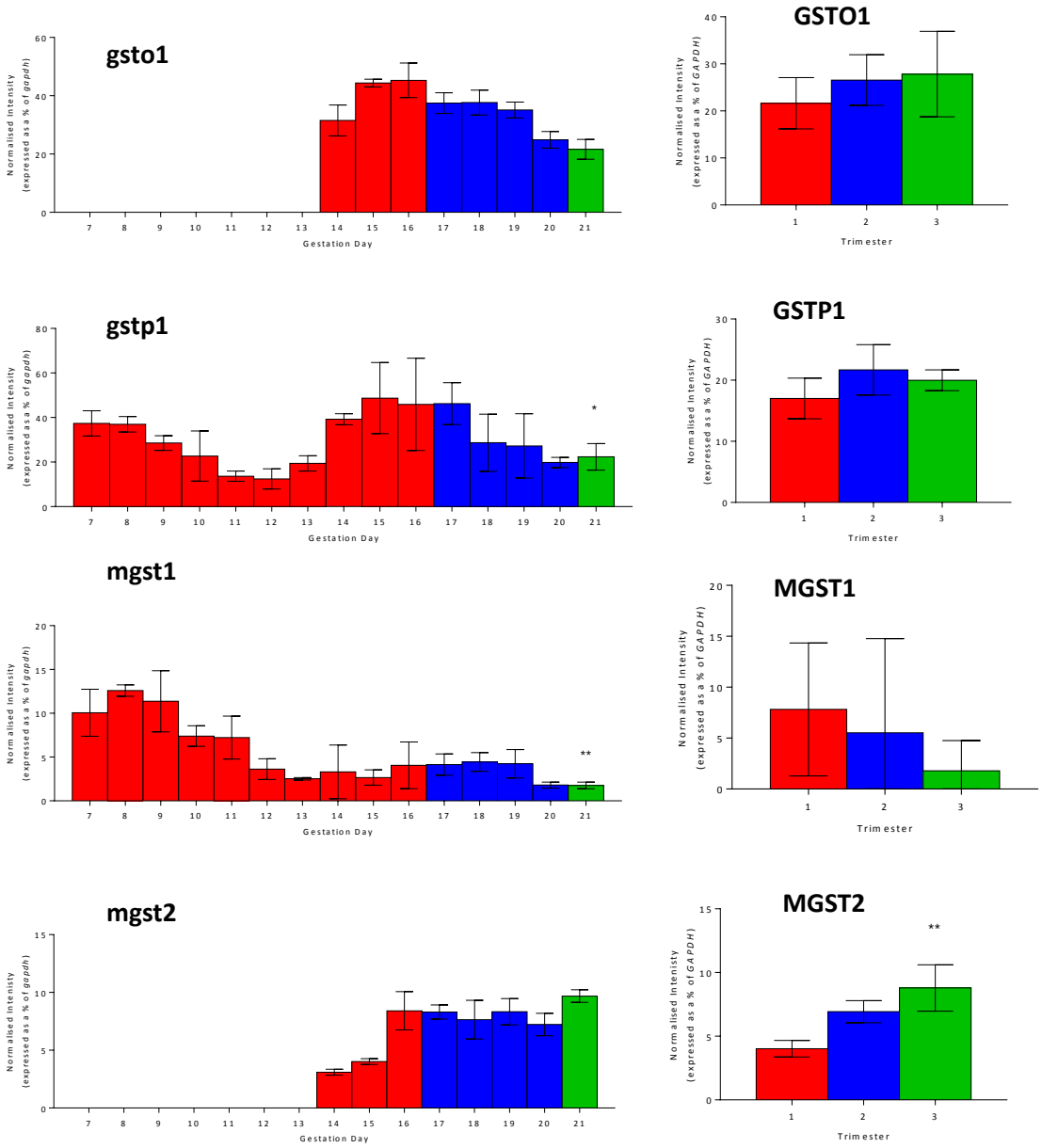


Figure 3.13 The mRNA expression of gst/GST enzymes (o1/O1, p1/P1, mgst1/MGST1 and mgst2/MGST2) in human and rat placenta throughout gestation. The mRNA expression of gst enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = p <0.05, ** = p< 0.01 and *** = p <0.001) denote either GD 21 intensity values are statistically different from GD 7 or term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.9 Comparative mRNA expression intensities between rat (developing embryo; foetus and placenta) and human (developing placenta), of gst enzyme family members at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

gst enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat gsta3	GD 7	4.183 ± 1.56
Human GSTA3	T 1	3.319 ± 0.5883
Rat gsta3	GD 21	1.892 ± 0.81
Human GSTA3	T 3	6.216 ± 2.701
Rat gstk1	GD 7	not found
Human GSTK1	T 1	7.074 ± 1.089
Rat gstk1	GD 21	1.699 ± 0.5628
Human GSTK1	T 3	7.948 ± 1.163
Rat gstm1	GD 7	13.03 ± 3.848
Huamn GSTM1	T 1	1.552 ± 0.3391
Rat gstm1	GD 21	2.948 ± 1.356
Huamn GSTM1	T 3	1.586 ± 0.5139
Rat gstm2	GD 7	not found
Human GSTM2	T 1	2.374 ± 0.5613
Rat gstm2	GD 21	1.639 ± 0.6819
Human GSTM2	T 3	2.773 ± 0.7675
Rat gstm5	GD 7	12.57 ± 2.557
Human GSTM5	T 1	0.8513 ± 0.4546
Rat gstm5	GD 21	1.993 ± 0.2681
Human GSTM5	T 3	0.548 ± 0.2691
Rat gsto1	GD 7	not found
Human GSTO1	T 1	21.63 ± 5.445
Rat gsto1	GD 21	21.61 ± 3.41
Human GSTO1	T 3	27.83 ± 9.097
Rat gstp1	GD 7	37.35 ± 5.661
Human GSTP1	T 1	17.02 ± 3.324
Rat gstp1	GD 21	22.34 ± 5.95
Human GSTP1	T 3	19.99 ± 1.694
Rat mgst1	GD 7	10.06 ± 2.688
Human MGST1	T 1	7.82 ± 6.523
Rat mgst1	GD 21	1.763 ± 0.3737
Human MGST1	T 3	1.795 ± 2.956
Rat mgst2	GD 7	not found
Human MGST2	T 1	4.018 ± 0.6549
Rat mgst2	GD 21	9.686 ± 0.5439
Human MGST2	T 3	8.788 ± 1.824

The mRNA expression levels of members of the gst/GST family differed between the human and rat placenta.

Gsta3/GSTA3 mRNA was expressed in both human and rat placenta (Figure 3.12). At the beginning of gestation similar levels of mRNA expression were observed in human and rat placenta, however at the end of gestation the human placental mRNA expression level was 3.2-fold higher than in rat placenta (Table 3.9).

From the mu subfamily, gstm1/GSTM1, gstm2/GSTM2 and gstm5/GSTM5 were expressed in both species (Figure 3.12). In rat, gstm1 mRNA expression at term (GD21) was significantly lower ($p<0.01$) than at the beginning of gestation (GD7) (Figure 3.12), however similar levels were expressed in each trimester in human (Figure 3.12). At the beginning of gestation gstm1 mRNA expression was 8-fold higher in the rat than in the human placenta (Table 3.9). Expression of GSTM2 in the human was 1.7-fold higher than in the rat at the end of gestation. It is worth noting that gstm2 mRNA was not detected in the rat until GD14.

Expression of gstm5 was 14.8-fold higher in the rat than in human at the beginning of gestation (Table 3.9). A significant decrease ($p < 0.01$) in expression of gstm5 mRNA was observed in rat at GD 21 compared to the beginning of gestation (GD 7) (Figure 3.12). However, the level of expression of GSTM5 mRNA in the human placenta was relatively constant throughout the three trimesters (Figure 3.12).

GSTO1/gsto1 mRNA was expressed in both human and rat placenta, although in the rat gsto1 was only detected from GD14 onwards whereas in human it was expressed in each trimester (Figure 3.13). There was relatively high expression of gsto1/GSTO1 mRNA in both species in the third trimester (human; 21.61 ± 3.41 and human 27.83 ± 9.097 mean intensity values ± SD).

The gst/GST pi family was represented in both species by gstp1/GSTP1. High mRNA expression levels were observed in both species (Figure 3.13). Expression of mRNA encoding microsomal gsts, namely mgst1/MGST1 and mgst2/MGST2, was also noted in both species. A decrease in mgst/MGST1 mRNA expression was observed in both rat and human placenta between the start of gestation (GD 7/T1) and the end of gestation (GD 21/ T3) (Figure 3.13). Although mgst2 mRNA was not expressed at the beginning of gestation in the rat, microarray analysis revealed expression between GD14 and GD21 (Figure 3.13).

To summarise, differences between the expression of placental gsts/GSTs mRNAs between the rat and human at specific points in gestation were observed. The most notable differences were; human placenta expressed a higher intensity of GSTA3 at term compared to the rat, and the rat placenta expressed a higher intensity of gstm1 and gstm5 at specific points in gestation compared to the human placenta. These differences must be considered if the rat is continued to be used as a model for predicting potential hazards for human health.

3.2.7 The mRNA expression profile of members of the SULT/sult family in human and rat placenta throughout gestation.

The mRNA expression levels of members of the sulphotransferase (SULT/sult) family in humans and rats were quantified using ArrayTrack^{Tm}. Overall, four sulphotransferases were found to be expressed in both rat and human placenta (Figure 3.14 and Table 3. 10). Those members only expressed in the developing rat placenta or the human placenta, are shown in Appendix 8, Figures 8Q and 8R respectively. The Genbank Accession code for each SULT/sult member can be found in Appendix 6 (Table 6H). Sulfotransferase enzymes catalyse addition of a sulfate moiety to a substrate and are divided into two subfamilies (i) phenol-sulfating (SULT1) and (ii) hydroxysteroid-sulfating (SULT2) (Nagata and Yamazoe, 2000).

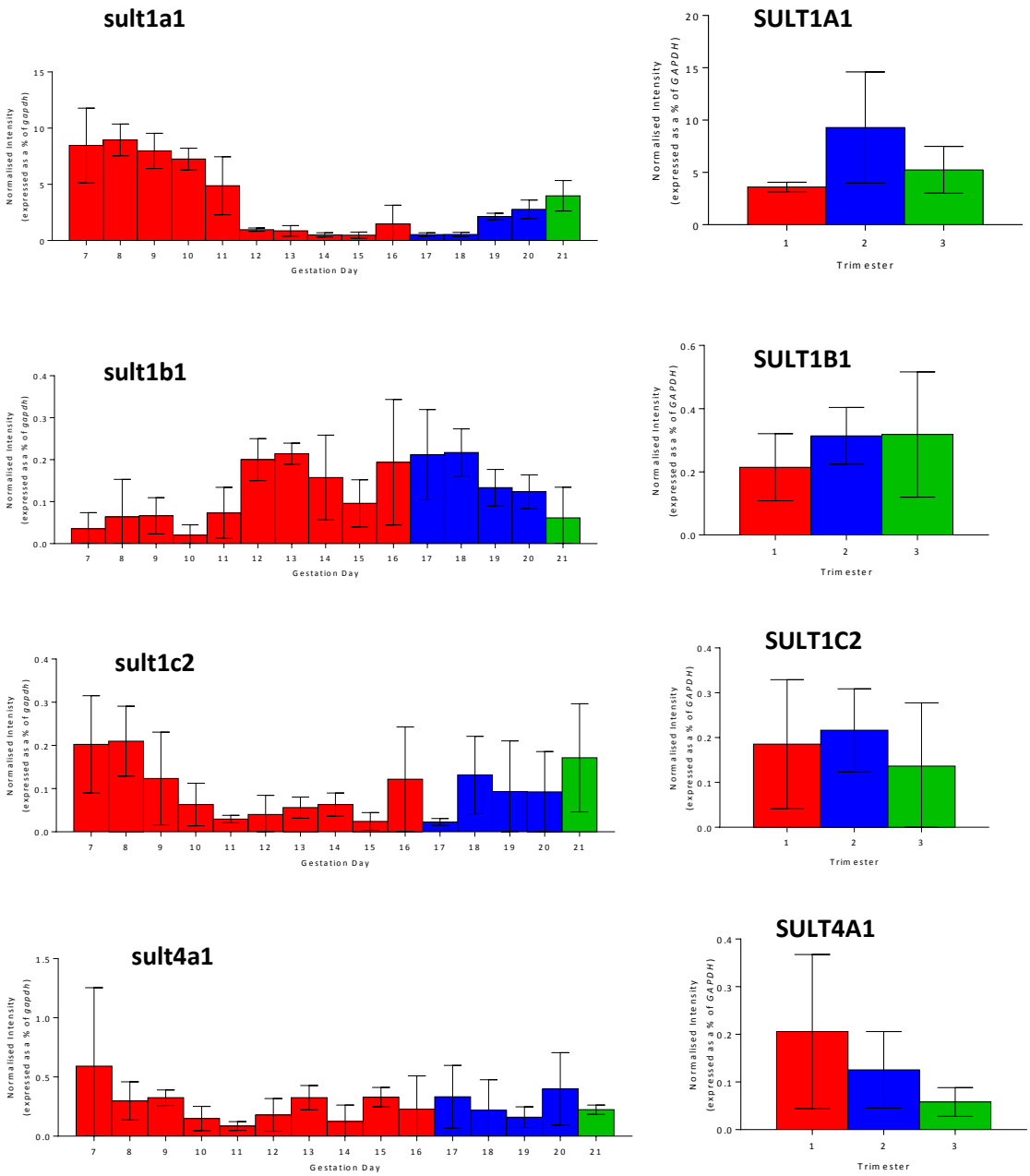


Figure 3.14 The mRNA expression of sult/SULT enzymes (1a1/1A1, 1b1/1B1, 1c2/1C2, 4a1/4A1) in human and rat placenta throughout gestation. The mRNA expression of sult enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.10 Comparative mRNA expression intensities between rat (developing embryo; foetus and placenta) and human (developing placenta), of sult enzyme family members at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

sult enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat sult1a1	GD 7	8.47 ± 3.32
Human SULT1A1	T 1	3.61 ± 0.45
Rat sult1a1	GD 21	3.98 ± 1.35
Human SULT1A1	T 3	5.25 ± 2.25
Rat sult1b1	GD 7	0.04 ± 0.04
Human SULT1B1	T 1	0.21 ± 0.11
Rat sult1b1	GD 21	0.06 ± 0.07
Human SULT1B1	T 3	0.32 ± 0.19
Rat sult1c1	GD 7	0.20 ± 0.11
Human SULT1C1	T 1	0.19 ± 0.14
Rat sult1c1	GD 21	0.17 ± 0.13
Human SULT1C1	T 3	0.14 ± 0.14
Rat sult4a1	GD 7	0.59 ± 0.66
Human SULT4A1	T 1	0.21 ± 0.16
Rat sult4a1	GD 21	0.22 ± 0.039
Human SULT4A1	T 3	0.06 ± 0.03

Of the sult/SULT isoforms expressed in human and rat placenta, different patterns of mRNA expression between species was seen during gestation. At the beginning of gestation the level of sult1a1 mRNA expression was 2.3-fold higher in the rat than in the human placenta (Figure 3. 14 and Table 3.10).

Expression of SULT1B1 mRNA was higher in the human placenta than the rat at the beginning of gestation (6.1-fold higher) and at the end of gestation (5.2-fold higher). In the rat, sult4a1 mRNA expression was 2.8-fold higher than in human at the beginning of gestation and 3.9 fold higher than human in the third trimester.

Due to the detoxifying role sulfotransferases play in xenobiotic metabolism it is necessary that the difference in expression between species is considered. As with the ABCCs/abccs, SLCOs/slocs, CYPs/cyps and GSTs/gsts, function and substrate specificity of sulfotransferases must be investigated in order to gain a true perspective of the implications these differences could have when using the rat model as a predictor of potential hazards for human health.

3.2.8 The mRNA expression profile of members of the ugt/UGT family in human and rat placenta throughout gestation.

The mRNA expression levels of members of the UDP-glucuronosyltransferases (ugt) family in humans and rats were quantified using ArrayTrack^{Tm}. Only one member of the ugt/UGT family was expressed in both species, namely ugt2b17/UGT2B17 (Figure 3.15 and Table 3.11). Those members only expressed in the developing rat placenta or the human placenta, are shown in Appendix 8, Figures 8S and 8T respectively. The Genbank Accession code for each ugt/UGT member can be found in Appendix 6 (Table 6I). UDP-glucuronosyltransferases catalyse conjugation of a glycosyl group from a nucleotide sugar to a small hydrophobic molecule (Mackenzie et al., 1997).

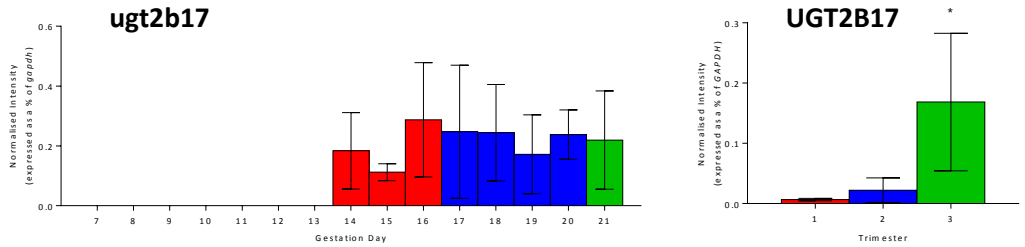


Figure 3.15 The mRNA expression of ugt2b17/UGT2B17 enzymes in human and rat placenta throughout gestation. The mRNA expression of ugt2b17/UGT2B17 enzymes throughout gestation in the developing embryo (foetus and placenta) in rat (left-hand side) and developing placenta in human (right-hand side). Expression was normalised using expression of the *GAPDH* house keeping gene. Statistical analysis was carried out using the Student's T-test. Asterisks (* = $p < 0.05$) denote term intensity values are statistically different from trimester 1. Values are expressed as mean ± SD. N = 3 individual placenta for rat and n = 4 individual placenta for human.

Table 3.11 Comparative mRNA expression intensities between rat (developing embryo; foetus and placenta) and human (developing placenta), of ugt enzyme family members at the beginning (GD 7 or T 1) and end (GD 21 or T 3) of gestation.

ugt enzyme	Gestation Day (GD) or Trimester (T)	Mean intensity (±SD)
Rat ugt2b17	GD 7	not found
Human UGT2B17	T 1	0.006638 ± 0.001991
Rat ugt2b17	GD 21	0.2194 ± 0.1642
Human UGT2B17	T 3	0.1685 ± 0.1141

UGT2B17/ugt2b17 was the only gene to be found in both species during gestation. In human, *UGT2B17* mRNA expression changed significantly during gestation, with mRNA expression increasing significantly ($p<0.05$) from the first to the third trimester (Table 3.11). Comparison of expression levels in rat and human revealed expression of ugt2b17 mRNA in rat was 1.3-fold higher than in human in trimester 3.

It has been reported that activity of UGTs in human placenta is highly variable (Aitio, 1974, Collier et al., 2002a). The mRNA data within this thesis reports high expression of ugt1a7(c) mRNA in the rat placenta (Appendix 8, Figure 8S). Expression of ugt1a7(c) mRNA has previously been reported in the rat liver, duodenum, ovaries, kidneys, testes, spleen and lung (Grove et al., 1997a). No mRNA expression of UGT1A7(c) was detected in the human placenta. This finding at the mRNA level shows a key difference in the two species. This information must

be considered by investigators when using the rat as a predictor of potential hazards for human health.

To summarise the findings from the microarray analyses, differential mRNA expression of transporters and metabolising enzymes in the rat and human placenta have been identified. These differences must be investigated at the protein and functional level before any solid conclusions can be made about how well the rat model could predict potential hazards for human health in developmental toxicity studies when testing a new compound.

Although the methodology employed in this thesis is a novel approach to analysing microarray data, the methodology has been critiqued and peer reviewed at conferences and published as a short communication in Reproductive Toxicology (Taylor et al., 2011). The study of comparative mRNA expression levels of transporters and metabolising enzymes contained in this thesis also received the 'Teddy Edwards Memorial Prize' for research into congenital abnormalities awarded by the European Toxicology Society (2011). In order to support these data it would be interesting to analyse other microarray data of rat and human placenta since only a small population has been used in this study. Further work would also investigate the rabbit model in a similar manner, since rabbit is also used in developmental toxicity studies.

Chapter 4

Expression of transporters and metabolising enzymes in the rat and human placenta

4.0 Chapter 4: Expression of transporters and metabolising enzymes in the rat and human placenta

4.1 Background

Rodent models are routinely used during prenatal developmental toxicity assays in order to predict the human health hazard of test compounds. Characterising the rat placental model at the mRNA level provides a greater insight into the profile of an array of transporters and metabolising enzymes that are expressed. However until the mRNA is translated to protein, it is not possible to determine the definite presence of these transporters and metabolising enzymes within the placenta.

In order to support the mRNA data generated from the microarray analysis (Chapter 3) immunodetection of three well characterised transporters (abcb1, abcg2 and abcc1/ABCC1) and a relatively unknown transporter (slco4a1/SLCO4A1) and metabolising enzyme (cyp26b/CYP26B) using Western blots and immunohistochemical staining was performed on rat embryonic tissue and human syncytiotrophoblast placental tissue.

The term 'Western Blotting' was first coined in 1981, after scientists further developed the Southern and Northern blotting techniques, to allow for whole cell extracts to be analysed by antibodies to detect protein expression (Burnette, 1981). Although tissue can be isolated and homogenised to identify proteins expressed in the tissue, in order to localise expression of a protein within the tissue immunohistochemistry must be employed.

Immunohistochemistry localises antigens in whole tissue sections by the use of labelled antibodies. An antibody is produced in response to the presence of an antigen. The variable F_{ab} domains of the antibody recognise the antigenic epitope and binds through non-covalent forces, such as ionic attraction, hydrogen bonding and hydrophobic interaction (Kiernan, 1999).

Protein expression of abcb1/ABCB1, abcg2/ABCG2, abcc1/ABCC1, slco4a1/SLCO4A1 and cyp26b/CYP26B in placental labyrinthine cells was determined by Western blotting analysis; rat placental tissue at GD 16 was collected from pregnant Han Wistar rats (as outlined in section 2.1.2.3). After isolating the labyrinthine region of the placenta, tissue homogenate was loaded onto gels to undergo separation by SDS-PAGE (see section 2.2.2.2.1) prior to Western blotting.

Localisation of abcc1/ABCC1, slco4a1/SLCO4A1 and cyp26b/CYP26B in placental labyrinthine tissue was determined by immunohistochemical analyses; placental tissue was obtained from the same rats from which tissue was removed and used to obtain mRNA for the microarray analysis (as outlined in section 2.2.2.1) from the Foetal Capability Project (Syngenta, CTL, 2005).

Based on the findings of the mRNA microarray analyses, expression of selected transporters and enzyme at the protein level was carried out for proteins for which modest levels of mRNA were observed. Placentas from rat were isolated at GD 16. This time-point was chosen due to the modest levels of abcb1b (Figure 3.1 A), abcg2 (Figure 3.2 A), abcc1 (Figure 3.3 A), slco4a1 (Figure 3.4 A) and cyp26b (Figure 3.12 A) mRNA generated from the microarray data in Chapter 3. The human placental tissue was obtained commercially from Abcam (Cambridge, UK) and due to the limited availability of tissue, analyses of protein expression by immunohistochemistry was performed on term (42 weeks) human placenta. In order to gain a more comparative study of the difference in expression of the proteins between species, tissue from second trimester placenta would have been preferred.

Previous immunohistochemical studies, using the same rat embryonic tissue sections, have localised expression of the abcb1, abcg2 and abcc2 efflux transporters in the rat placenta (Stratton et al., 2007). Western blotting has previously shown the temporal increase in expression of abcb1 in the rat placenta from GD13 until the end of gestation (Stratton et al., 2007).

In this chapter, further characterisation of expression of placental ABC transporter proteins and metabolising enzyme was performed. Western blots were used to validate protein expression of abcb1 and abcg2 in the labyrinthine rat placenta. For the first time in rat placenta, expression of abcc1, slco4a1 and cyp26b is demonstrated by Western blotting. Immunohistochemistry was used to verify the location of abcc1/ABCC1 and slco4a1/SLCO4A1. The first reported localisation of cyp26b/CYP26B in rat and human placental tissue is documented in this thesis.

4.2 Detection of abcb1 expression in syncytiotrophoblast cells of the rat placenta by Western Blotting.

The importance of abcb1 at the placental barrier was first demonstrated in mice, when pregnant dams deficient in abcb1 gave birth to foetuses with cleft palate following treatment with avermectin Bla (Lankas et al., 1998). Further investigations have demonstrated a 16-fold increase in paclitaxol level in foetuses of pregnant dams deficient in the abcb1 isoforms (abcb1a and abcb1b) compared to wild type control animals (Smit et al., 1999). This evidence supports the notion that abcb1 is extremely important in protecting the developing foetus from exposure to xenobiotics.

Western blot analysis was performed on isolated rat placental tissue at GD 16 to determine expression. This time-point was chosen due to the level of abcb1b mRNA determined from microarray analysis (Chapter 3, Figure 3.1 A).

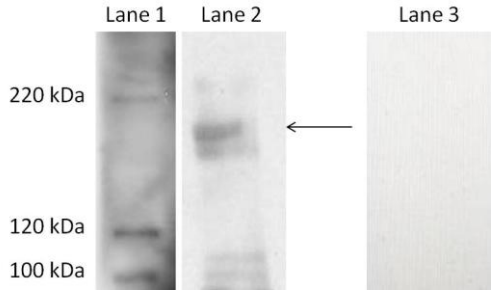


Figure 4.1 Expression of abcb1 in rat placenta at GD 16. Cell membranes were isolated and purified from the labyrinth region of rat placenta by differential centrifugation (see section 2.2.2.2.1). Solubilised protein (30 µg) was loaded into each well of a 6 % (w/v) polyacrylamide gel and subjected to SDS-PAGE at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane at 200 mA for 90 min. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk protein for 120 min and then washed for 3 x 10 min. The PVDF membrane was incubated with mouse monoclonal C219 antibody (1:100) in TBS-T containing 5 % non-fat milk protein overnight at 4 °C. The PVDF membrane was then washed for 3 x 10 min, in TBS-T. The PVDF membrane was then incubated with sheep anti-mouse horse radish peroxidase conjugated IgG (1:2000). Protein detection was performed using an Enhanced Chemiluminesence Prime Western blotting kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Lane 1; molecular weight marker (Invitrogen, Paisley, UK), Lane 2 and 3; rat placenta protein. A negative control was performed in which the PVDF membrane was incubated with only sheep anti-mouse horse radish peroxidise labelled IgG (1:2000) (Lane 3). Western Blot image is a representative of 3 individual experiments.

The expression of abcb1 was confirmed in the rat placenta at GD16 using Western blotting as seen by the band of cross reactivity observed at ~170 kDa depicted by the arrow (Figure 4.1 Lane 2). An additional band is visible under the

band at ~170 kDa which is likely to be the non-glycosylated form of the transporter (Seres et al., 2011). In rat, expression of abcb1 in the placenta by Western blot has been reported previously by Stratton et al., (2007), who demonstrated an increase in expression throughout gestation.

The expression of ABCB1 in human placenta has been confirmed by western blot analysis (Mason et al., 2011) and has been localised to the microvillous border of the syncytiotrophoblast in human placenta (Sun et al., 2006) and further studies have shown decreased expression of ABCB1 as gestation progressed (Gil et al., 2005, Mathias et al., 2005 and Sun et al., 2006)

The main aim of these studies was to determine expression of abcb1 in rat placenta (Figure 4.1). Further analysis is required to interpret the expression levels in comparison to other transporters. A simple approach would be to quantify the expression level of abcb1 using a loading control such as β-actin.

The apparent molecular weight of abcb1 was confirmed from the molecular weight markers (Figure 4.1 Lane 1) and by using cell lines over-expressing abcb1 (MDCK II-MDR1) in Western blots. Unfortunately due to the Intellectual Property rights blots with the MDCK-II MDR1 cells are not permitted to be shown in this thesis.

In conclusion expression of ABCB1/abcb1 in both human and rat placenta has been confirmed. This demonstrates potentially similar protective properties at the placenta between the two species, suggesting that the rat is a good model to use for predicting human health hazards when exposed to substrates (NCEs which directly interfere with the activity) of abcb1.

4.3 Detection of abcg2 expression in syncytiotrophoblast cells of the rat placenta by Western blotting.

Abcg2 is most highly expressed in the placenta (Allikmets et al., 1998). Pregnant abcb1a^{-}/1b^{-} knockout mice have been used to demonstrate the protective function of placental abcg2; after treatment with the abcg2 inhibitor GF120918, relative foetal accumulation of topotecan (a substrate of abcg2) was 2-fold higher than that in the foetuses of pregnant vehicle-treated mice (Jonker et al., 2000).

Further investigations using abcg2 knockout models have shown increased accumulation of genistein (Enokizono et al., 2007), nitrofurantoin (Zhang et al., 2007) and glyburide (Zhou et al., 2008).

Western blot analysis was performed to determine protein expression in rat placenta at GD16. This time-point was chosen due to the level of abcg2 mRNA determined from microarray analysis (Chapter 3, Figure 3.2 A).

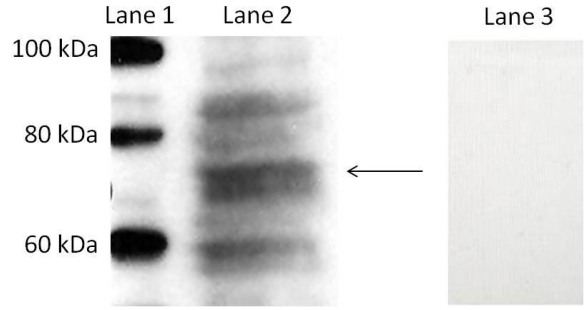


Figure 4.2 Expression of abcg2 in rat placenta at GD 16. Cell membranes were isolated and purified from the labyrinth region of the rat placenta by differential centrifugation (see section 2.2.2.2.1). Solubilised protein (30 µg) was loaded into each well of an 8 % (w/v) polyacrylamide gel and subjected to SDS-PAGE at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane using 200 mA for 90 min. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk protein for 120 min and then washed for 3 x 10 min. The PVDF membrane was incubated with rabbit polyclonal anti-abcg2 antibody (1:500) in TBS-T containing 5 % (w/v) non-fat milk protein overnight at 4 °C. The PVDF membrane was then washed for 3 x 10 min, in TBS-T. The PVDF membrane was then incubated with goat anti-rabbit horse radish peroxidase labelled IgG (1:2000). Protein detection was performed using an Enhanced Chemiluminesence Prime Western blotting kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Lane 1; molecular weight marker (Invitrogen, Paisley, UK), Lane 2 and 3; rat placenta protein. A negative control was performed in which the PVDF membrane was incubated with only goat anti-rabbit horse radish peroxidise labelled IgG (1:2000) (Lane 3). Western Blot is representative from 3 individual experiments.

The expression of abcg2 was confirmed in the labyrinth of the rat placenta at GD 16 using Western blot analysis (Figure 4.2 Lane 2). Previously abcg2 has been shown to be expressed in the rat placenta by Western blot (with an apparent molecular weight of 72 kDa), however the studies used whole homogenised placenta

from GD 15 (Vander Borght et al., 2006). Cross reactivity of the antibody was also observed at ~60 kDa and ~90 kDa (Figure 4.2 Lane 2). Due to the clonality of the antibody (polyclonal) it is possible that similar proteins to abcg2 have been detected.

The expression of ABCG2 in human placenta has been confirmed by western blot analysis (Mason et al., 2011) and localised to the microvillous border of the syncytiotrophoblast (Maliepaard et al., 2001). Immunohistochemistry has also localised ABCG2 to the syncytiotrophoblast region (Mason et al., 2011). In rat abcg2 has been localised to the labyrinth region of the rat placenta by immunohistochemistry (Stratton et al., 2007). Reports from the literature state there is a significant decrease in abcg2 expression from GD 14 to GD 20 (Yasuda et al., 2005). Results of previous studies on the change in human placental ABCG2 protein expression throughout gestation are conflicting. Meyer zu Schwabedissen et al. (2006) reported a higher expression of ABCG2 pre-term (28 ± 1 week, n =10) than at term (39 ± 2 weeks, n = 10) using Western blotting. Other studies concluded that ABCG2 expression did not differ significantly throughout gestation, however the sample population was small (n = 3 for preterm; days 60 – 90 and n = 4 for term) (Mathias et al., 2005). It is clear from these reports that further investigations are required to determine the protein expression levels in the rat and human placenta throughout gestation.

Future work would involve using a blotting for a control protein, such as β-actin, in order for expression levels of transporters to be normalised and compared. The molecular weight marker in this case gave an indication of the apparent weight of abcg2. Previous western blots had been performed in the laboratory and used the over-expressing MDCK II-BRCP cell line which produced a band at 72 kDa. Due to the Intellectual Property rights governing use of the MDCK II-BRCP cell line the blots previously produced are not permitted to be shown in this thesis.

In conclusion, the expression of abcg2 has been identified in the rat placenta. Other studies have also identified placental ABCG2 at the foetal/maternal interface in human (Maliepaard et al., 2001 and Mason et al., 2011).

4.4 Detection of abcc1 expression in syncytiotrophoblast cells of the rat placenta.

In 1992, ABCC1/MRP1 was identified as the cause of multidrug resistance in an anthracycline selected human small cell lung carcinoma cell line, H69AR (Cole et al., 1992). Within the human placenta ABCC1 has been localised to the foetal blood vessel endothelial cells, where the efflux transporter can limit entry of xenobiotics into the foetal circulation, therefore providing a protective barrier to the foetus (St-Pierre et al., 2000). Substrates of ABCC1 include daunorubicin, saquinavir and ritonavir (Hooijberg et al., 2004).

4.4.1 Detection of abcc1 expression in syncytiotrophoblast cells of the rat placenta by Western Blotting.

The expression of abcc1 in rat placenta is yet to be determined. Protein expression by Western blotting analysis has been confirmed in the rat brain (Mercier et al., 2004). The expression of ABCC1 has been confirmed in the human placenta by Western blot analysis (Mason et al., 2011). In this thesis, Western blot analysis was performed on isolated rat placental labyrinthine cells from GD 16 to determine the expression of abcc1. This time-point was chosen due to the level of abcc1 mRNA determined from microarray analysis (Chapter 3, Figure 3.3).

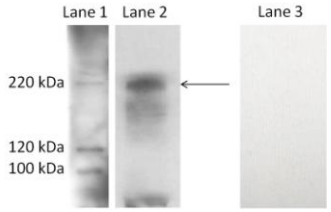


Figure 4.3 Expression of abcc1 in rat placenta at GD 16. Cell membranes were isolated and purified from the labyrinth region of the rat placenta by differential centrifugation (see section 2.2.2.2.1). Solubilised protein (30 µg) was loaded into each well of a 6 % polyacrylamide gel and subjected to SDS-PAGE at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane using 200 mA for 90 min. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk protein for 120 min and then washed for 3 x 10 min. The PVDF membrane was incubated with mouse monoclonal anti-abcc1 antibody (1:100) in TBS-T containing 5 % (w/v) non-fat milk protein overnight at 4 °C. The PVDF membrane was then washed for 3 x 10 min in TBS-T. The PVDF membrane was then incubated with sheep anti-mouse horse radish peroxidase conjugated IgG (1:2000). Protein detection was performed using an Enhanced Chemiluminescence Prime Western blotting kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Lane 1; molecular weight marker (Invitrogen, Paisley, UK), Lane 2 and Lane 3; rat placenta protein. A negative control was performed in which the PVDF membrane was incubated with sheep anti-mouse horse radish peroxidise labelled IgG (1:2000) (Lane 3). Western Blot image is a representative from 4 individual experiments.

Abcc1 appeared to be expressed in cells isolated from the labyrinth of the rat placenta at GD 16 using western blot analysis. Western blotting revealed cross reactivity indicated by the band at an apparent molecular weight of 190 kDa (Figure 4.3 Lane 2). This is thought to be the first report of expression of abcc1 in rat placenta.

Western blotting has shown expression of abcc1 in rat brain, with cross reactivity at the predicted molecular weight of 190 kDa (Mercier et al., 2004).

As already mentioned in sections 4.2 and 4.3 a loading control should be used in further experiments in order to generate a comparison between the level of expression of transporters. At this stage the aim was to validate the expression of the transporter. To support the Western blotting data, immunohistochemistry was performed on rat placenta from the labyrinth region to assess abcc1 expression (Section 4.4.2).

In conclusion, Western blotting has shown expression of abcc1 in the rat placenta, demonstrating that the rat is a good model to use as a predictor of potential hazards for xenobiotics that could potentially interact with ABCC1/abcc1.

4.4.2 Detection of ABCC1/abcc1 expression in syncytiotrophoblast cells of the rat and human placentas by immunohistochemistry.

ABCC1/abcc1 has previously been found to be expressed in the human (Atkinson et al., 2003, Mason et al., 2011) and rat (St-Pierre et al., 2004) placentas using immunohistochemical techniques. To confirm the protein expression of abcc1/ABCC1, immunohistochemical staining was performed on paraffin-embedded embryonic rat tissue and human placental tissue.

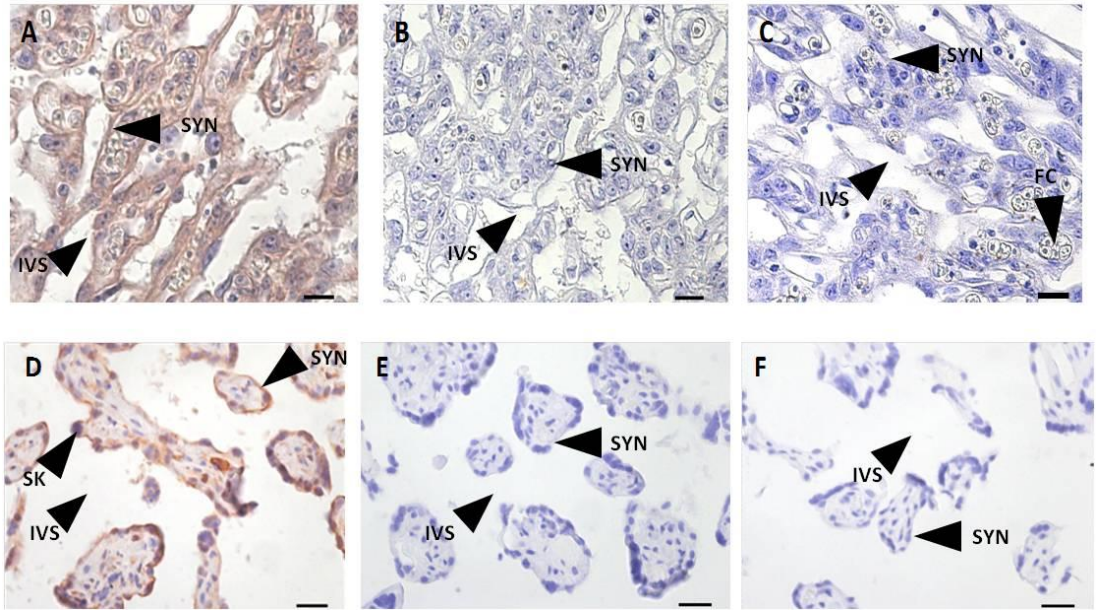


Figure 4.4 Immunohistochemical detection of ABCC1/abcc1. Abcc1/ABCC1 was localised in the rat placenta at GD16 (A, B, C) and in the human placenta at term (42 weeks), (D, E, F). Sections were de-parafinised through a series of xylene and ethanol solutions. Antigen retrieval was performed followed by a peroxidase block (15 mins). A serum block was applied for 1 hour, then either a primary mouse monoclonal anti-MRP antibody (diluted 1: 50 in 3 % (w/v) BSA in PBS) (A, D), a mouse monoclonal IgG isotype antibody (monoclonal IgG2a antibody, diluted 1: 50 in 3 % (w/v) BSA in PBS) (B, E) or PBS (C,F) was applied overnight. After 3 x 5 mins washes in PBS, a secondary HRP-linked antibody (prepared by Dako) was added for 1 hour then DAB was added for visualisation. Specimens were viewed using an *Olympus BX51* upright microscope using a x20 objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij). FC = Foetal Capillary, SYN = syncytiotrophoblast, IVS = intervillous space, SK = Syncytial knot (characteristic of term placenta). Immunohistochemistry images from rat are representative of 5 individual experiments. Images from human placenta are from 1 experiment. Scale bar represents 20 µm.

Immunohistochemical studies confirmed the expression of abcc1/ABCC1 in rat and human placentas respectively (Figure 4.4). Abcc1 was localised to the

plasma membrane of the syncytiotrophoblast in the labyrinth region of the rat placenta (brown staining, Figure 4.4 A), and on the plasma membrane of the syncytiotrophoblast cells of the human placenta (Figure 4.4 D). From this level of analysis it is not possible to localise the membrane staining to the apical or basal region of the cell, however previous studies have shown conflicting reports of the localisation. Apical localisation was reported by St-Pierre et al., (2000), whereas basolateral localisation was reported by Nagashige et al., (2003) and Atkinson et al., (2003). In other tissues (salivary gland, oviduct, uterus and choroid plexus) abcc1/ABCC1 has been reported to be predominantly expressed on the basolateral side of the membrane (Wijnholds et al., 2000). Substrates of ABCC1/abcc1 are more likely to be endogenous compounds, such as leukotrienes; it is thought that the main role of placental ABCC1/abcc1 is to transport these endogenous substrates to the foetus. Also if xenobiotics are not the preferred substrate of ABCC1/abcc1, then xenobiotics would be likely to accumulate in the syncytiotrophoblast, potentially allowing the xenobiotic to be effluxed into the maternal circulation by other transporters such as ABCB1/abcb1 and ABCG2/abcg2 as suggested by Atkinson et al., (2003).

In order to determine the specific localisation of ABCC1/abcc1 in the rat placenta further localisation studies are required. The mouse monoclonal anti-MRP1 antibody used in this thesis did not permit staining to be localised to the apical or basolateral membrane in the rat placenta (Figure 4.4 A), however the staining did seem more intense in the human placenta (Figure 4.4 D) which suggests that the expression level of abcc1 in the rat placenta is less, although the mRNA data did express higher levels of abcc1 in the rat placenta. To further explore this, dual fluorescent staining using abcb1 and abcc1 could be performed. The localisation of abcb1 is well characterised as being apical (Stratton et al., 2007, Sun et al., 2006), therefore staining with abcc1 should show basolateral staining along with apical staining of abcb1 on the syncytiotrophoblast, if the previous reports of abcc1 basolateral expression are correct.

In conclusion, the results presented here prove that both species express ABCC1/abcc1 at the placenta. This indicates that the rat would be a good predictive model for predictors of potential hazards to human health, especially if new compounds interact with abcc1/ABCC1.

4.5 Detection of slco4a1 expression in syncytiotrophoblast cells of the rat placenta.

In the last decade slco4a1/oatp4a1 has been identified as a new member of the slco/oatp uptake transporter family (Fujiwara et al., 2001, Tamai et al., 2000). Slco4a1 has narrow substrate specificity and is primarily responsible for the transport of thyroid hormones (Fujiwara et al., 2001). The expression of slco4a1 mRNA significantly increased throughout gestation in the rat placenta (Chapter 3, Figure 3.5). This finding is consistent with previous findings using RT-PCR which also demonstrated an increase in slco4a1 mRNA in rat placenta (St-Pierre et al., 2004). However from the microarray data analysed in this thesis (original human microarray data are from Mikheev et al., (2008)) this trend in rat slco4a1 mRNA expression was not observed in human placental SLCO4A1. Moreover, little is known of the expression and function of sloc4a1/SLCO4A1 in rat and human placenta, so expression of the proteins was investigated by Western blotting and immunohistochemistry.

4.5.1 Detection of slco4a1 expression in syncytiotrophoblast cells of the rat placenta by Western blotting.

In the present study Western blot analysis was used to confirm expression in rat placenta at GD 16. This time-point was chosen due to the level of slco4a1 mRNA determined from microarray analysis (Chapter 3, Figure 3.5).

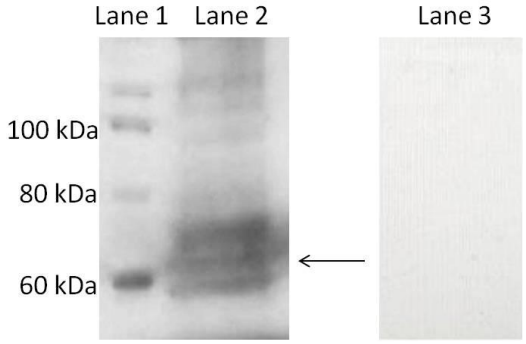


Figure 4.5 Expression of slco4a1 in rat placenta at GD 16. Cell membranes were isolated and purified from the labyrinth region of the rat placenta by differential centrifugation (see section 2.2.2.2.1). Solubilised protein (30 µg) was loaded into each well of an 8 % polyacrylamide gel and subjected to SDS-PAGE at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane using 200 mA for 90 min. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk protein for 120 min and then washed for 3 x 10 min. The PVDF membrane was incubated with rabbit polyclonal anti-slco4a1 antibody (1:250) in TBS-T containing 5 % (w/v) non-fat milk protein overnight at 4 °C. The PVDF membrane was then washed for 3 x 10 min in TBS-T. The PVDF membrane was then incubated with goat anti-rabbit horse radish peroxidase labelled IgG (1:5000). Protein detection was performed using an enhanced chemiluminescence prime Western blotting kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Lane 1; molecular weight marker (Invitrogen, Paisley, UK), Lane 2 and 3; rat placenta protein. A negative control was performed in which the PVDF membrane was incubated with goat anti-rabbit horse radish peroxidise labelled IgG (1:5000) (Lane 3). The image is representative from 2 individual experiments.

The expression of slco4a1 in the rat placenta at GD 16 was confirmed using Western blot analysis (Figure 4.5 Lane 2). Previous studies have identified the expression of SLCO4A1 in the human placenta at ~60 kDa, however slco4a1 has not been detected by Western blotting in the labyrinth region of the rat placenta before. The expression of SLCO4A1 in human placenta has been confirmed by Western blotting, with cross reactivity with a protein of ~60 kDa (Loubiere et al., 2010).

As previously discussed, no loading control was used at this stage for validation of the expression of slco4a1. Further investigations into the expression of slco4a1 in comparison to abcb1/abcg2/abcc1 will require the use of β-actin, however

the use of a molecular weight marker at this stage is sufficient to prove the protein expression of slco4a1.

To investigate the localisation of slco4a1, immunohistochemistry was performed on rat and human placental tissue.

4.5.2 Detection of slco4a1 expression in syncytiotrophoblast cells of the rat placenta by immunohistochemistry.

Localisation of slco4a1 has previously been reported in rat (Nishikawa et al., 2010) and human (Loubiere et al., 2010, Sato et al., 2003) placenta using immunohistochemical techniques. To determine protein expression of slco4a1 in the present study, immunohistochemical analysis was performed on paraffin-embedded embryonic rat tissue and human placental tissue.

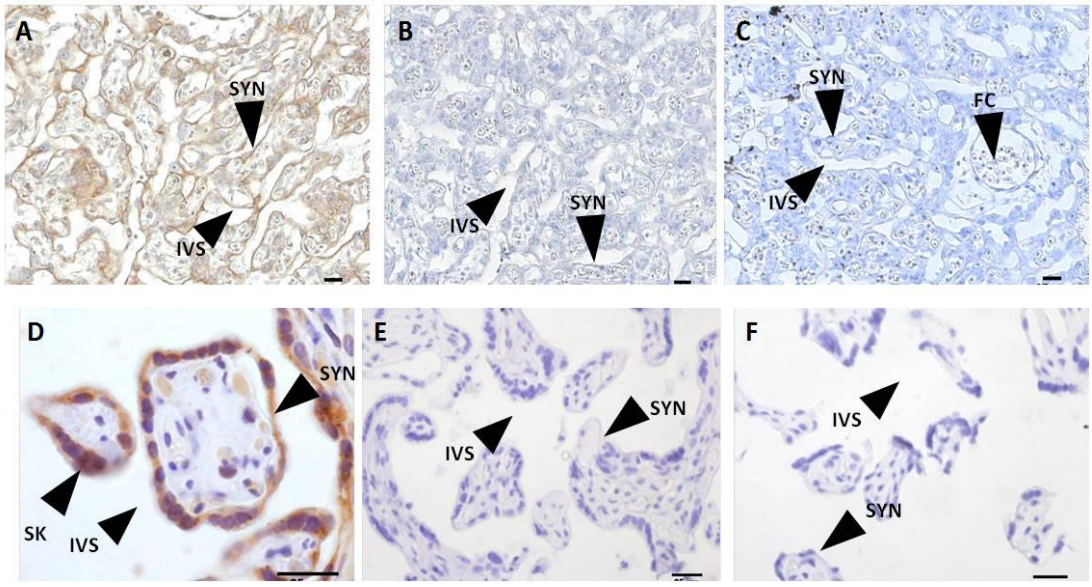


Figure 4.6 Immunohistochemical staining of SLCO4A1/slco4a1. Slco4a1/SLCO4A1 was localised in the rat placenta at GD16 (A, B, C) and in the human placenta at term (42 weeks), (D, E, F)**.** Sections were de-parafinised through a series of xylene and ethanol solutions. Antigen retrieval was performed followed by a peroxidase block (15 mins). A serum block was applied for 1 hour, then a primary polyclonal anti-SLCO4A1 antibody (1: 20 in 5 % (w/v) BSA in PBS) (A, D), a pre-absorbed slco4a1 antibody (using the slco4a1 antigen diluted in 5% (w/v) BSA in PBS) (B, E) or PBS (C, F) was applied overnight. After 3 x 5 mins washes, a secondary HRP-linked antibody (prepared by Dako) was added for 1 hour then DAB was added for visualisation. Specimens were viewed using an Olympus BX51 upright microscope using a 40x objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij). FC = Foetal Capillary, SYN = syncytiotrophoblast, IVS = intervillous space, SK = Syncytial knot (characteristic of term placenta). Immunohistochemistry images from rat are representative of 5 individual experiments. Images from human placenta are from 1 individual experiment. In all images the scale bar represents 25 µm.

Immunohistochemical studies confirmed the expression of slco4a1 in the rat and human placentas (Figure 4.6). Slco4a1 was localised to the plasma membrane of the syncytiotrophoblast cells in the labyrinth region of the rat placenta at GD 16 (Figure 4.6 A) and on the plasma membrane of the syncytiotrophoblast cells in the villi of the human placenta (Figure 4.6 D). These findings are consistent with the membrane localisation of slco4a1/SLCO4A1 reported in previous studies (Loubiere et al., 2010 and Nishikawa et al., 2010). Nishikawa et al., (2010) state that the localisation of slco4a1 is apical, however it is difficult to determine the precise location from the results shown. Further localisation studies would need to be performed, specifically dual labelled-immunofluorescence (previously performed by Nishikawa et al., (2010)) to clarify the precise location.

In conclusion, slco4a1/SLCO4A1 protein expression and localisation has been shown in rat and human placentas. The studies here have confirmed expression in both species, however further work could investigate if the changes in expression of mRNA (as seen in Chapter 3, Figure 3,5) are reflected at the protein level. Substrates of sloc4a1 should also be further investigated as relatively little is known about this uptake transporter. Nishikawa et al., (2010) have suggested the transport of Bisphenol A-glucuronide from maternal to foetal circulation is assisted by slco4a1 (at the maternal interface/apical localisation) and effluxed by abcc1 at the foetal interface/basolateral localisation. This study (Nishikawa et al., 2010) and the findings presented here demonstrate the importance slco4a1 could have at the placental barrier. Further investigations regarding the relationship and function of slco41 and abcc1 should be performed; this combined action of slco4a1uptake and abcc1 efflux could result in the accumulation of xenobiotics in the foetus. An ADME study could also be used to fully determine the function and localisation of the efflux.

4.6 Detection of cyp26b expression in syncytiotrophoblast cells of the rat placenta.

The phase I metabolising enzyme cyp26b was found to be expressed at the mRNA level in both rat and human placenta throughout gestation (Chapter 3, Figure 3.10). Retinoic acid (RA) is essential for normal embryonic development, but in excess can cause birth defects. Genetic evidence indicates that the principle role of the cyp26 enzyme family is the degradation pathway of RA (Niederreither et al., 2002). Studies have shown that cyp26b1 is vital for normal limb development (Yashiro et al., 2004) and phocomelia has been reported in foetuses of cyp26b1$^{-/-}$ mice following RA treatment. Placental cyp26b mRNA expression has previously been identified using RT-PCR (Trofimova-Griffin and Juchau, 1998, Ray et al., 1997), though to date, no localisation of the enzyme in the placenta has been performed.

4.6.1 Detection of cyp26b expression in syncytiotrophoblast cells of the rat placenta by Western blotting.

Currently there are no studies that have investigated expression of cyp26b in the rat placenta. Previous reports of cyp26b expression has determined the molecular weight to be ~58 kDa in human ovaries (Pavone et al., 2011) and 57 kDa in mice testis (Wu et al., 2008). Western blot analysis was used to assess cyp26b expression in the rat placenta at GD16. This time-point was chosen due to the level of cyp26b mRNA determined from microarray analysis (Chapter 3, Figure 3.10).

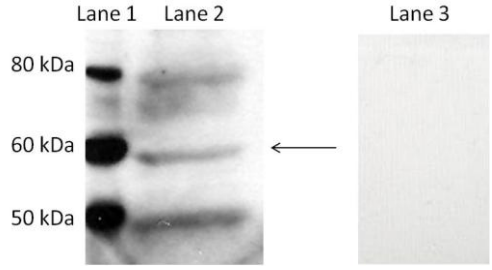


Figure 4.7 Expression of cyp26b in rat placenta at GD 16. Cell membranes were isolated and purified from the labyrinth region of the rat placenta by differential centrifugation (see section 2.2.2.2.1). Solubilised protein (30 µg) was loaded into each well of an 8 % (w/v) polyacrylamide gel and subjected to SDS-PAGE at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane using 200 mA for 90 min. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk protein for 120 min and then washed for 3 x 10 min. The PVDF membrane was incubated with rabbit polyclonal anti-cyp26b antibody (1:750) in TBS-T containing 5 % (w/v) non-fat milk protein overnight at 4 °C. The PVDF membrane was then washed for 3 x 10 min in TBS-T. The PVDF membrane was then incubated with goat anti-rabbit horse radish peroxidase labelled IgG (1:5000). Protein detection was performed using an Enhanced Chemiluminescence Prime Western blotting kit (GE Healthcare Life Sciences, Buckinghamshire, UK). Lane 1; molecular weight marker (Invitrogen), Lanes 2 and 3; rat placenta protein. A negative control was performed in which the PVDF membrane was incubated with goat anti-rabbit horse radish peroxidise labelled IgG (1:5000) (Lane 3). The image is a representative of 2 individual experiments.

The expression of cyp26b in the rat placenta at GD 16 was confirmed using western blot analysis (Figure 4.7 Lane 2). This is the first time expression of cyp26b in the rat placenta has been demonstrated.

To date, only one other study reports detection of expression of cyp26b, in human ovaries, using Western blotting (Pavone et al., 2011).

As previously mentioned, the molecular weight marker was key to identifying and validating the expression of cyp26b. No loading control was used as validation of expression rather than quantification was the main aim of the study.

Further work using β-actin as a loading control could quantify the placental expression level of cyp26b in comparison to abcb1/abcg2/abcc1 and slco4a1.

Unfortunately, due to the lack of human placental tissue available during this project it was not possible to perform Western blots to examine cyp26b expression in human placenta. It was possible to perform immunohistochemistry on rat and human placental tissue.

4.6.2 Detection of cyp26b expression in syncytiotrophoblast cells of the rat placenta by immunohistochemistry

Immunohistochemical studies were performed on paraffin-embedded embryonic rat tissue and human placental tissue. As a positive tissue control immunohistochemistry was performed on human adrenal gland to verify the anti-cyp26b antibody.

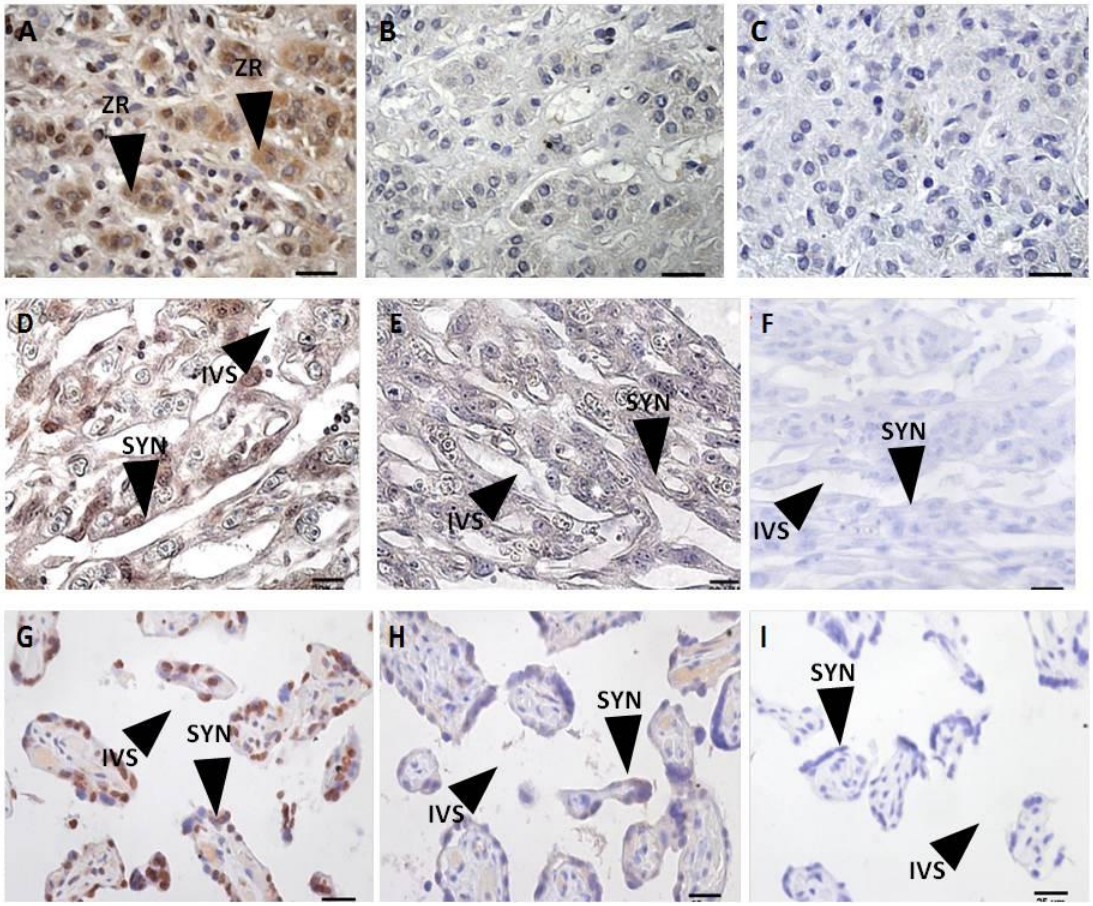


Figure 4.8 Immunohistochemical staining of CYP26B/cyp26b. Cyp26b/CYP26B was localised in the human adrenal gland (A, B, C), in the rat placenta at GD16 (D, E, F) and in the human placenta at term (42 weeks), (G - I)**.** Sections were de-parafinised through a series of xylene and ethanol solutions. Antigen retrieval was performed followed by a peroxidase block (15 mins). A serum block was applied for 1 hour, then the primary polyclonal anti-cyp26b antibody (1:100 in 5 % (w/v) BSA in PBS) (A,D,G) a rabbit polyclonal IgG isotype antibody (diluted 1: 100 in 5 % (w/v) BSA in PBS) (B, E,H) or PBS (C, F,I) was applied overnight. A secondary HRP-linked antibody (prepared by Dako) was added for 1 hour then DAB was added for visualisation of staining. Specimens were viewed using an Olympus BX51 upright microscope using a 40x objective and captured using a Coolsnap ES camera (Photometrics) through MetaVue Software (Molecular Devices). Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij). SYN = syncytiotrophoblst, IVS = intervillous space, ZR = zona reticularis of the adrenal gland. In all images the scale bar represents 20 µm.

Confirmation of the reactivity of the anti-cyp26b antibody towards cyp26b was shown using human adrenal tissue as positive tissue control (brown staining, Figure 4.8 A); slight non-specific staining was seen using the polyclonal IgG isotype (Figure 4.8 B) although no significant non-specific binding of the secondary horse radish peroxidase conjugated antibody was observed (Figure 4.8 C). In the rat placenta (Figure 4.8 D) positive staining was observed but was not to the extent as in the human adrenal tissue (Figure 4.8 A). Immunohistochemistry studies were performed using a range of dilutions of the primary antibody at various incubation temperatures (see Appendix 9, Figure 9A); the optimal staining was generated using a primary antibody dilution of 1: 200 with overnight incubation at 4 °C (Figure 4.8 D).

As an additional control to demonstrate that the primary anti-cyp26b antibody was specific, images from the brain of rat embryo GD 16 were also captured (Appendix 10, Figure 10A). Previously cyp26b expression has been localised in the hindbrain of zebrafish (Hernandez et al., 2007). Definitive staining of cyp26b was observed around the ventricular regions of the developing brain (see Appendix 10, Figure 10A Panel B).

Although positive staining is observed in Figure 4.8 D, there does not appear to be definitive cytoplasmic staining; the expected localisation of cyp26b.

Further studies are required to determine the precise localisation of cyp26b expression in the rat placenta. As already mentioned in sections 4.4 and 4.5 dual fluorescence staining could be performed to visualise the location of cyp26b expression in comparison to one of the membrane-bound transporters.

In conclusion we were able to detect cyp26b in the rat and human placenta. Although the level of cyp26b mRNA expression appears to be greater in the rat than in the human placenta, immunohistochemical studies detected protein in both placentas. This supports the use of the rodent as a predictor of potential hazards for human health due to expression being validated and confirmed in both species. Due to the expression of cyp26b/CYP26B in rat and human, one would expect to see similar metabolites produced.

Chapter 5

Surrogate models of the rat and human placenta.

5.0 Chapter 5; Surrogate models of the rat and human placenta.

5.1 Background

In the last 20 years there has been an added emphasis on reducing, refining and replacing the number of animals used in science. The National Institute of Health, the National Institute for Environmental Health Sciences, the Centre for Alternatives to Animal Testing, the John Hopkins School of Public Health and the European Centre for the Validation of Alternative Methods (ECVAM) play leading roles in identifying replacement developmental toxicity assays. Currently three different *in vitro* developmental toxicity assays, namely the Embryonic Stem Cell Test (EST), Embryotoxicity Testing in Post-Implantation Whole Embryo Culture (WEC) – Method of Piersma and The Micromass Test – Method of Brown have been validated by ECVAM and are ready for consideration for regulatory acceptance and application (Balls, 2002).

The previous work in this thesis (Chapters 3 and 4) has demonstrated the mRNA abundance of placental transporters and metabolising enzymes throughout gestation.

The thalidomide tragedy has highlighted the harmful effect substances can have on the foetus if they are able to cross the placenta. Further studies into thalidomide reported that exposure to the drug just after conception (during weeks 3 – 8 in humans) poses the highest risk for teratogenicity (Franks et al., 2004). What is also interesting about thalidomide is that its use as an anti-angiogenesis drug has been demonstrated in rabbit and human, but not in rodents. This is due to species differences in the ability to metabolise thalidomide into its active metabolite by CYPs. According to Bauer's study (Bauer et al., 1998) the metabolites formed after hydroxylation by microsomal CYPs are not found in rodents but are in higher species (Bauer et al., 1998). This stresses the fact that we should assess the similarities and differences between the human and the test animals before making any sound assumption based on animal data.

Having elucidated the mRNA levels of ABC transporters and metabolising enzymes during gestation in the rat and human placenta (Chapter 3) and confirmed the protein expression of ABCB1/abcb1, ABCG2/abcg2, ABCC1/abcc1, SLCO4A1/slco4a1 and CYP26B1/cyp26b1 in rat and human placenta, it is also important to assess the expression and function of these transporters and metabolising enzymes in placental models. Currently several different surrogate *in*

vitro models are used to study the interaction of xenobiotics with placental transporters. Due to the previous characterisation that has been performed in human placental models on ABCB1 and ABCG2, the *in vitro* functional activity and expression of these two prominent transporters in rat placental models will be the main focus of this thesis when characterising the surrogate *in vitro* placental models. The TR-TBT 18-d1 rat cell line is relatively new, and no functional activity of abcb1 or abcg2 has been investigated in this cell line to date.

ABCB1/abcb1 has been shown to contribute to the placental barrier property by influencing penetration of xenobiotics which are substrates of abcb1 (Smit et al., 1999). Substrates of placental ABCB1/abcb1 are less likely to cross the maternal-foetal barrier (due to the efflux action of ABCB1/abcb1), and cause teratogenicity. Along with ABCB1/abcb1, ABCG2/abcg2 has been widely studied and is known to be expressed at the syncytiotrophoblast in both human (Evseenko et al., 2007) and rat (Cygalova et al., 2008) placenta. Similar to ABCB1/abcb1, the apical membrane localisation of ABCG2/abcg2 and its efflux action provide protection at the placenta against xenobiotics (Cygalova et al., 2008). Utilising *in vitro* models of the placenta to identify ABCB1/abcb1 and ABCG2/abcg2 substrates could help to identify the extent of transfer of NCEs across the placenta.

In vitro models of the human and rat placenta, which show similar characteristics to the syncytiotrophoblast layer of the tissue, were investigated in terms of their transporter expression and function. Human placental cell lines which represent the syncytiotrophoblast were employed for the work in this thesis. Choriocarcinoma is a malignant tumour of the human placental trophoblast. Three cell lines have been isolated and immortalised from the choriocarcinoma; BeWo, JAr and Jeg-3 (Hochberg et al., 1991). Only one rat cell line represents the syncytiotrophoblast of the rat placenta; the TR-TBT 18d-1 cell line is a relatively newly established cell line which is derived from the chorioepithelium; the labyrinth region of the rat placenta (Kitano et al., 2004).

To assess the expression of placental ABCB1/abcb1 in human and rat *in vitro* models Western blots were performed using antibodies raised against ABCB1. To assess the expression of placental ABCG2/abcg2 in human and rat *in vitro* models Western blots were performed using antibodies raised against ABCG2.

In order to assess the functionality of ABCB1/abcb1 in human placental cells (JAr and BeWo) and in rat cells (TR-TBT 18d-1), the intracellular fluorescent

calcein AM assay was implemented, using well documented inhibitors of ABCB1/abcb1 to confirm ABCB1/abcb1-mediated transport.

The functionality of ABCG2 in human cells (BeWo) and rat cells (TR-TBT 18d-1) was assessed by measuring the intracellular accumulation of the fluorescent pheophorbide A employing an inhibitor of ABCG2.

An alternative approach to measuring transporter activity was attempted using an *ex vivo* human placental model: verapamil-sensitive uptake of the ABCB1 substrate ([^{3}H]Taxol) was determined in placental villous fragments from term.

To further investigate the expression and function of transporters in the rat placenta, the isolation of primary placental labyrinth rat cells was also attempted.

5.2 Abcb1/ABCB1 activity in *in vitro* models of rat and human placenta.

5.2.1. Determination of optimum seeding density of cells employed in *in vitro* placental models using the methylthiazolyldiphenyl-tetrazolium bromide (MTT) assay

Prior to performing studies to determine the functional activity of ABCB1/abcb1 and ABCG2/abcg2, preliminary studies were performed to determine the optimum seeding density of each cell type (JAr, BeWo and TR-TBT 18d-1) using the methylthiazolyldiphenyl-tetrazolium bromide (3-(4, 5-dimethyl-2-thiazolyl)-2, 5-diphenyl-tetrazolium bromide, MTT) assay. Optimum sensitivity of the assay is achieved using near confluent cells that are in the exponential growth phase (this will avoid underestimating the toxicity of the ABCB1/abcb1 and ABCG2/abcg2 inhibitors used in this thesis). Following 24 h in culture in a 96-well plate, the seeding density for each cell type that gave an absorbance of approximately 1 was chosen as the optimum seeding density for subsequent assays. Optimum seeding densities were as follows: JAr 1 x 10^3 cells/well, BeWo 5 x 10^3 cells/well and TR-TBT 18d-1 1 x 10^5 cells/well.

5.2.2 Assessment of non-cytotoxic concentrations of the compounds used in functional ABCB1/abcb1 assays in human and rat *in vitro* placental models

Prior to measurement of ABCB1 functional activity in each *in vitro* placental model, an MTT assay was performed to determine non-cytotoxic concentrations of the ABCB1 inhibitors Atorvastatin (Wang et al., 2001, Bogman et al., 2001), Quinidine (Pires et al., 2009, Elsby et al., 2008), Saquinavir (Gutmann et al., 1999) and Verapamil (Janneh et al., 2010). Cell viability was measured following incubation of cells with a range of concentrations (1 µM, 10 µM, 100 µM and 1000 µM), see section 2.2.3.5.1 for details. Plasma levels of each xenobiotic from the literature is listed in Appendix 11, Table 11A.

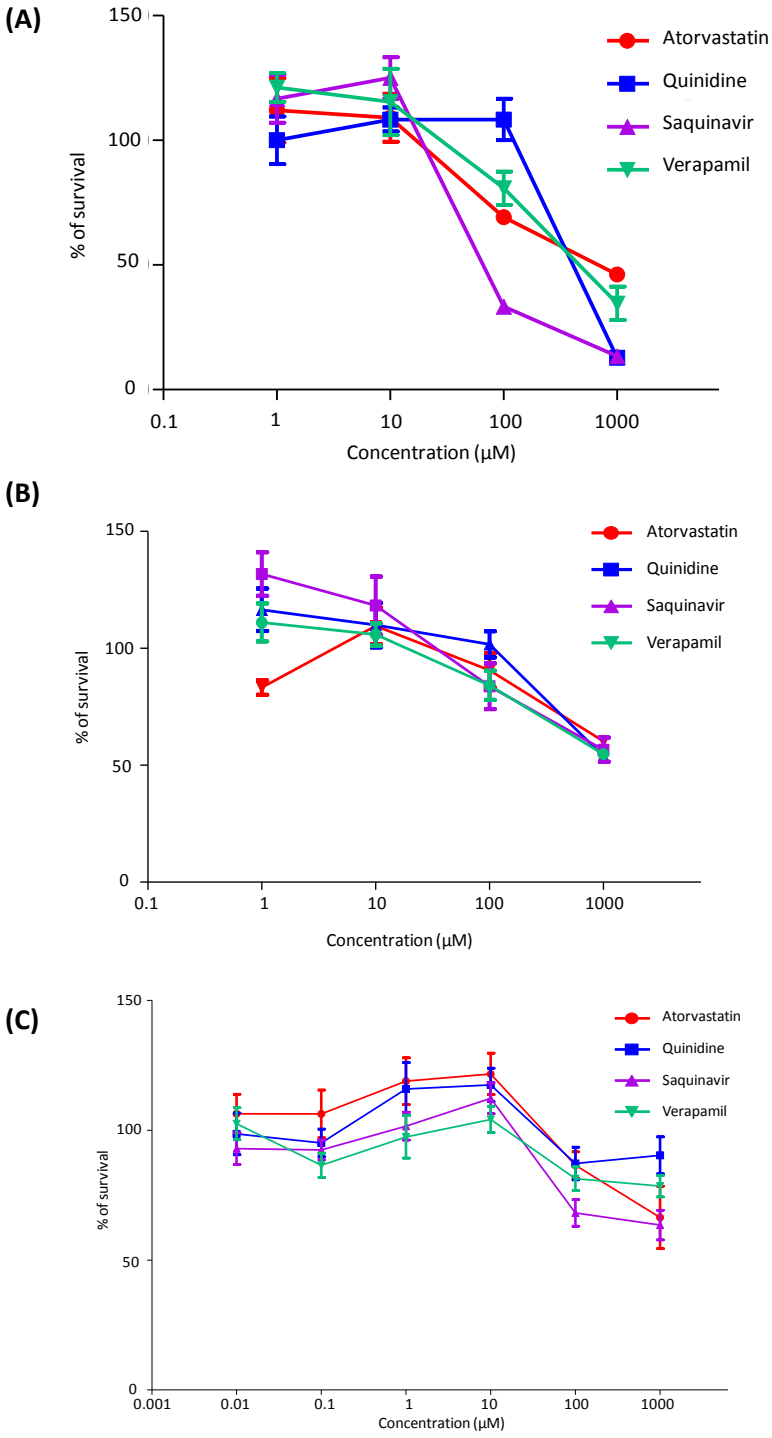


Figure 5.1 Effect of ABCB1/abcb1 inhibitor on the viability of human and rat placental cells. Cells (A) JAr, (B) BeWo and (C) TR-TBT 18d-1 were seeded onto 96-well plates at a pre-determined optimal cell density (see section 5.2.1) and incubated for 24 h. Cells were then treated with compound for 2 h, rinsed with PBS and incubated for a further 24 h in growth medium. MTT dissolved in PBS (10 µl of a 5 mg/ml stock solution) was added to the growth medium in each well and cells incubated for 4 h. The growth medium was carefully aspirated and the insoluble formazan produced solubilised by addition of 100 µl DMSO. The formazan produced was quantified colorimetrically by measuring the absorbance at 590 nm using a Tecan Safire spectrophotometer. The data are mean ± SEM (n=2 independent experiments with 4 replicates in each experiment)

In the JAr (Figure 5.1 A), BeWo (Figure 5.1 B) and TR-TBT 18d-1 (Figure 5.1 C) cell lines, 1000 µM of all inhibitors proved substantially cytotoxic to the cell whereas 10 µM of all inhibitors proved to be non-cytotoxic.

In the JAr human placental cell line all four ABCB1 inhibitors were found to be non-cytotoxic at 10 µM (Figure 5.1 A). As the concentration of Atorvastatin increased, the cell viability decreased; at 100 µM there was 69.2 % ± 0 % cell survival and only 46.2 % ± 0 % cell survival at 1000 µM. Quinidine did not reduce the cell viability at 100 µM (108 % ± 8.33 % cell survival) but at 1000 µM the cell viability was decreased to 12 % ± 1.8 %. Saquinavir decreased the cell viability to 33.3 % ± 0 % at 100 µM and this further decreased to 13.4 % ± 2.1 % at 1000 µM. Verapamil at 100µM decreased the cell viability to 80.8 % ± 6.6 % and at 1000 µM the cell viability further decreased to 34.6 % ± 6.6 %.

In the BeWo human placental cell line all four ABCB1 inhibitors were found to be non-cytotoxic at 10 µM (Figure 5.1 B). As the concentration of Atorvastatin increased, the cell viability decreased; at 100 µM there was 90.6 % ± 7.3 % cell survival and only 60.2 % ± 1.2 % cell survival at 1000 µM. Quinidine did not reduce the cell viability in BeWo cells at 100 µM (101.7 % ± 5.6 % cell survival) but at 1000 µM the cell viability was decreased to 53.7 % ± 0.7 %. Saquinavir decreased the cell viability to 83.7 % ± 9.7 % at 100 µM and this further decreased to 56.7 % ± 5.1 % at 1000 µM. Verapamil, at 100µM decreased the cell viability to 84.2 % ± 6.2 % and at 1000 µM the cell viability further decreased to 54.8 % ± 0.9 %.

In the TR-TBT 18d-1 rat placental cell line all four abcb1 inhibitors were found to be non-cytotoxic at 10 µM (Figure 5.1 C). As the concentration of atorvastatin increased, the TR-TBT 18d-1 cell viability decreased; at 100 µM there was 86.5 % ± 5.1 % cell survival and at 1000 µM only 66.4 % ± 12.0 % cell survival. Quinidine did not affect the cell viability to the same extent as Atorvastatin; at 100 µM cell viability was 87.2 % ± 6.3 % and at 1000 µM was 90.3 % ± 7.1 %. The viability of TR-TBT 18d-1 cells exposed to 100 µM Saquinavir was 68.1 % ± 5.1 % which decreased to 63.5 % ± 5.7 % when exposed to 1000 µM. Verapamil, at 100 µM decreased the cell viability to 81.3 % ± 4.6 % and at 1000 µM further decreased cell viability to 78.5 % ± 4.1 %. This study confirmed that all ABCB1/abcb1 inhibitors could be used at 10 µM in the calcein-AM assay in each cell line to measure functional activity of ABCB1/abcb1.

5.2.3 Measurement of ABCB1/abcb1 functional activity in human and rat placental cells *in vitro*

Calcein acetoxymethyl ester (calcein AM) is a non-fluorescent, membrane permeable compound which, following entry into the cell undergoes hydrolysis by intracellular esterases to produce calcein, a strongly fluorescent membrane impermeable compound which is retained in the cell. Calcein AM is a substrate of ABCB1/abcb1 (Tiberghien and Loor, 1996). ABCB1 activity in the human placental cells (JAr and BeWo) and rat placental cells (TR-TBT 18d-1) was assessed by measuring intracellular calcein accumulation with or without the ABCB1/abcb1 inhibitors (Atorvastatin, Quinidine, Saquinavir and Verapamil).

In order for the JAr cells to remain attached to the wells for the entirety of the experiment, method development was performed to investigate the ability of different extracellular matrices (collagen and fibronectin) to improve cell adhesion. The MTT assay was utilised to compare the viability of JAr cells on different coated 96 well plates. No difference in JAr cell adherence was observed in collagen or fibronectin-coated 96 well plates compared to JAr cells plated on non-coated 96 wells plates. Evseenko et al., (2006a) successfully performed similar experiments using JAr cells in 24 well plates, this approach was subsequently followed. With the larger surface area, the effect of washing appeared to be much reduced and there was a marked improvement in adhesion of JAr cells to the well. This complication in cell adhering was not observed for BeWo and TR-TBT 18d-1 cells and the experiments using BeWo and TR-TBT 18d-1 cells were performed in 96-well plates.

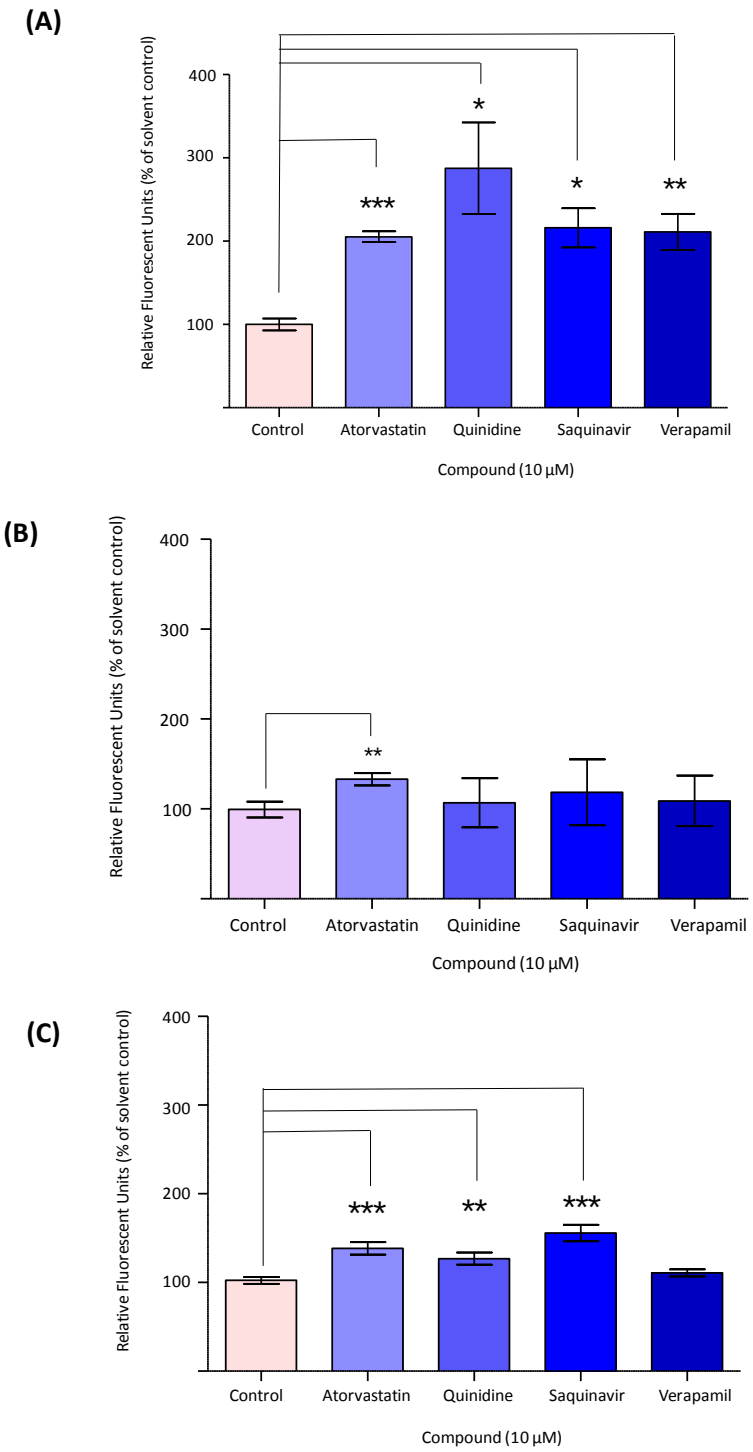


Figure 5.2 Measurement of ABCB1/abcb1 efflux activity in human and rat placental cells. Cells (A) JAr, (B) BeWo and (C) TR-TBT 18d-1 were seeded onto 24-well (JAr) or 96-well (BeWo and TR-TBT 18d-1) plates at a pre-determined optimal cell density (see section 5.2.1) and allowed to adhere overnight. Cells were then pre-incubated in PBS with 1 % solvent control or PBS containing 10 µM of compound for 30 min prior to incubation with calcein AM (final concentration 0.4 µM) for 30 min. Intracellular calcein accumulation was measured by fluorescence spectroscopy (484 excitation and 530 nm emission) using a Tecan Safire spectrophotometer. Data are expressed as the mean ± SEM (n = 3 independent experiments with 4 replicates). Statistical significance was determined using a student's t-test: * = $p < 0.05$, ** = $p < 0.01$ and *** = $p < 0.001$

In JAr cells, atorvastatin significantly increased calcein accumulation from 100 % in control (no atorvastatin) to 205 % ± 13.1 % ($p<0.001$) (Figure 5.2 A).

In BeWo cells, Atorvastatin increased calcein accumulation from 100 % to 133 % ± 26.8 % (Figure 5.2 B), and in the rat cell line, TR-TBT 18d-1, Atorvastatin increased calcein accumulation from 100 % to 138.5 % ± 24.6 % (Figure 5.2 C).

In JAr cells and TR-TBT 18d-1 cells Quinidine and Saquinavir significantly increased the calcein accumulation, however this was not observed in the BeWo cells.

This suggests that JAr and TR-TBT 18d-1 cells have functional ABCB1/abcb1, however the results from the calcein AM assay in the BeWo cell line are inconclusive as not all ABCB1 inhibitors were able to increase the intracellular accumulation.

These studies have shown that it is possible to measure functional activity in the JAr and TR-TBT 18d-1 cell lines and that the activity is sensitive to well-documented ABCB1 inhibitors. The findings also suggest that JAr and TR-TBT 18d-1 cell lines may prove useful in *in vitro* assays to study the role of ABCB1/abcb1 in placenta.

5.2.4 Expression of ABCB1/abcb1 in placental cell lines; JAr, BeWo and TR-TBT 18D-1.

The ABCB1 efflux transporter is known to be expressed in the JAr cell line and at lower levels in the BeWo cell line (Atkinson et al., 2003). The rat placental cell line; TR-TBT 18d-1, has shown mRNA expression of one of the isoforms of abcb1 in rat (mdr1a) by RT-PCR (Kitano et al., 2004). Whilst expression of *abcb1a* at the mRNA level in the TR-TBT 18d-1 rat placental cell line has been reported previously using RT-PCR studies (Kitano et al., 2004), it is not known if abcb1 is expressed at the protein level. To determine if TR-TBT 18d-1 cells express abcb1, Western blots, were performed.

In the human placental cell lines, JAr and BeWo, expression of ABCB1 was determined by using the polyclonal goat anti-MDR1 antibody. This antibody produced a single cross reactive band with an apparent molecular weight of approximately 170 kDa (Figure 5.3 (A), lanes 2 and 3) confirming the expression of ABCB1 at the protein level in both JAr and BeWo cells. In the rat placental cell line (TR-TBT 18d-1) the mouse monoclonal C219 antibody produced a band at approximately 170 kDa (Figure 5.3 (B), lane 2) confirming the expression of abcb1 at the protein level in TR-TBT 18d-1 cells.

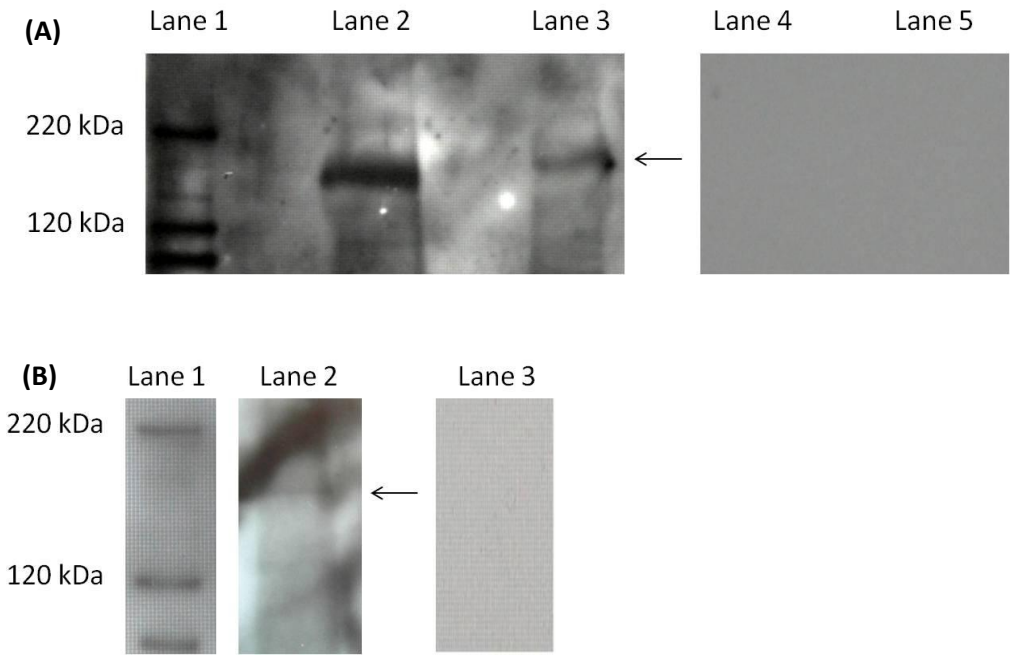


Figure 5.3 Expression of ABCB1/abcb1 in placental cell lines; JAr, BeWo and TR-TBT 18d-1. Cell membranes were isolated and purified from JAr (Panel A, lane 2), BeWo (Panel A, lane 3) and TR-TBT 18d-1 (Panel B, lane 2) cells by differential centrifugation (see section 2.2.2.2.2). Solubilised protein (20 µg protein) was loaded into each well of a 6 % (w/v) polyacrylamide gel and subjected to SDS-polyacrylamide gel electrophoresis at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane using 200 mA for 90 min. The PVDF membrane was incubated in TBS-T containing 5 % (w/v) non-fat milk protein for 120 min and then washed for 3 x 10 min. The PVDF membrane was incubated with polyclonal goat anti-MDR1 antibody (1:100) (Panel A) or mouse monoclonal C219 antibody (1:100) (Panel B) in TBS-T containing 5 % (w/v) non-fat milk protein overnight at 4 ºC. The PVDF membrane was then washed for 3 x 10 min in TBS-T and incubated with donkey anti-goat horse radish peroxidase labelled IgG (1:1000) (Panel A) or sheep anti-mouse horse radish peroxidase labelled IgG (1:2000) (Panel B)). Protein detection was performed using an enhanced chemiluminesence prime Western blotting kit (Amersham). A negative control (Panel A, lanes 4 and 5, Panel B lane 3) was performed in which the PVDF membrane was incubated with donkey anti-goat horse radish peroxidase labelled IgG (1:1000) or sheep anti-mouse horse radish peroxidase labelled IgG (1:2000) alone. Lane 1 in Panel A and Panel B are molecular weight markers.

Western blots confirmed the expression of ABCB1 in human JAr and BeWo cells (Figure 5.3 (A), Lanes 2 and 3) and in the rat TR-TBT 18d-1 cell lines (Figure 5.3 (B), Lane 2).

5.3 Placental rat and human *in vitro* abcg2/ABCG2 activity

Pheophorbide A (PhA) is a fluorescent chlorophyll catabolite. It is reported to be a specific substrate of abcg2 and knockout mice studies reveal plasma levels of PhA were 17 fold higher in abcg2 -/- mice than WT mice (Jonker et al., 2002). Several studies have measured the efflux ability of ABCG2/abcg2 by flow cytometry using PhA as a specific substrate and ko143 as an inhibitor (Pick et al., 2010, Asashima et al., 2006). In order to establish an *in vitro* assay to measure ABCG2-mediated transport, experiments in a 96-well format were performed in the laboratory using MCF-7 cells. Measuring ABCG2 efflux activity by this method (see section 2.2.3.3.2) had been performed previously in the laboratory using MDCKII-BCRP cells.

5.3.1 Evaluating the cytotoxicity of Pheophorbide A and ko143 in MCF-7 cells

Previous experiments conducted in the laboratory had determined non-cytotoxic concentrations of PhA and ko143 using MDCKII-BCRP cells. In order to confirm non-cytotoxicity for the MCF-7 cells, an MTT assay was performed.

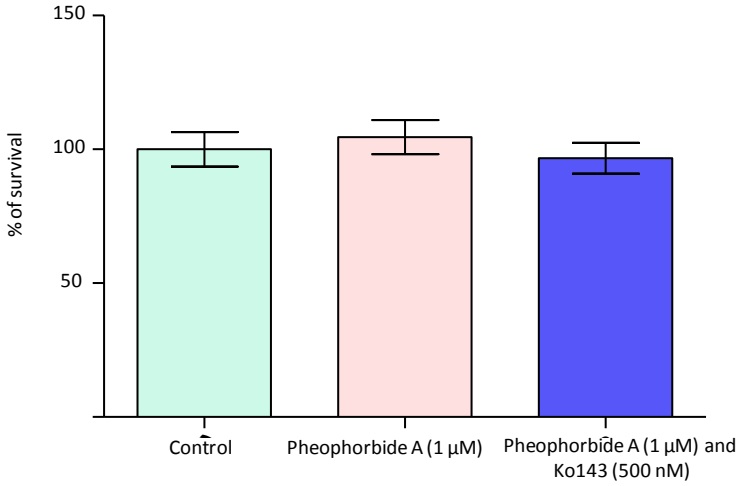


Figure 5.4 The cytotoxic effect of PhA ± ko143 on MCF-7 cells.
MCF-7 cells were seeded onto 96-well plates at a pre-determined optimal cell density (see section 5.2.1) and incubated for a further 24 h in Optimem™. MTT dissolved in PBS (10 µl of a 5 mg/ml stock solution) was added to the Optimem™ in each well and cells were incubated for 4 h. The Optimem™ was carefully aspirated, the insoluble formazan solubilised by addition of 100 µl DMSO and the formazan quantified colorimetrically by measuring the absorbance at 590 nm using a Tecan Safire. Control is Optimem™ only. The data are mean ± SEM (n=2 with 8 replicates).

The combination of 1 µM PhA with 500 nM ko143 was found to be non-cytotoxic towards MCF-7 cells (Figure 5.4). The percentage of cell survival following exposure to PhA at 1 µM was 104 % ± 25.41 and for PhA (1 µM) + ko143 (500 nM) was 96.7 % ± 26.93 %.

5.3.2 Establishing a reliable *in vitro* assay to measure ABCG2/abcg2-mediated efflux activity in the MCF-7 cell line.

ABCG2-mediated transport was investigated in the MCF-7 cell line. PhA was used as the specific substrate and ko143 was used as the specific inhibitor.

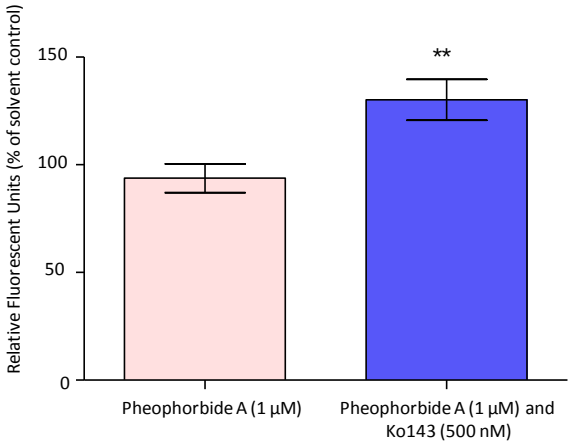
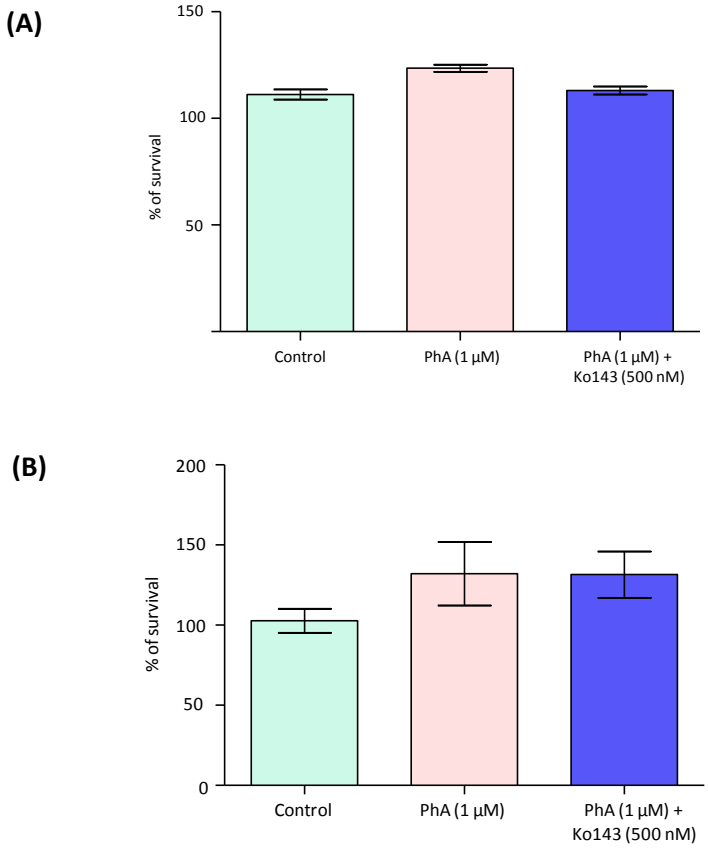


Figure 5.5 Measurement of ABCG2 efflux activity in MCF-7 cells. MCF-7 cells were seeded onto a 96 well plate at 1 x 10^4 cells/well. Cells were incubated for 24 h and then washed in PBS. Cells were treated with either 1 µM PhA in Optimem™ or 1 µM and 500 nM ko143 in Optimem™ for 18 h. After 18 h, cells were washed with ice-cold PBS and the intracellular PhA fluorescence was measured at an excitation wavelength of 417 nm and an emission wavelength of 674 nm using a Tecan Safire spectrophotometer. Data are mean ± SEM (n = 2 independent experiments with 8 replicates). Statistical significance was determined using a Student's t-test: ** = $p < 0.01$

The ABCG2/abcg2 inhibitor, ko143, significantly increased the accumulation of PhA in MCF-7 cells from 93.8 % ± 26.94 % (control –ko143) to 130.2 % ± 37.9 % (+ko143), Figure 5.5. This shows the fluorescence-based assay can be carried out in the 96 well format and can successfully measure functional activity of ABCG2 in the MCF-7 cell line.

5.3.3 Evaluating the cytotoxicity of PhA or PhA + ko143 on BeWo and TR-TBT 18D-1 cells

To determine if the concentrations of PhA (1 µM) and PhA (1 µM) + ko143 (500 nM) used in the standard assay conditions were non-cytotoxic for BeWo and TR-TBT 18d-1 cells an MTT assay was performed.



5.6 The effect of PhA or PhA + ko143 on BeWo and TR-TBT 18d-1 cell viability.
BeWo and TR-TBT 18d-1 cells were seeded onto 96-well plates at a predetermined optimal density (see section 5.2.1) and incubated for a further 24 h in Optimem™. MTT dissolved in PBS (10 µl of a 5 mg/ml stock solution) was added to the Optimem™ in each well and cells were incubated for 4 h. The Optimem™ was carefully aspirated, the insoluble formazan solubilised by addition of 100 µl DMSO and the formazan quantified colorimetrically by measuring the absorbance at 590 nm using a Tecan Safire. Control is Optimem™ only. The data are mean ± SEM (n=2 with 8 replicates).

The concentration of PhA (1 µM) and PhA (1 µM) + ko143 (500 nM) was found to be non-cytotoxic in the BeWo and TR-TBT 18d-1 cells (Figure 5.6 (A) and (B) respectively). The proportion of viable BeWo cells when treated with PhA (1 µM) was 123.5 % ± 6.3 % and 113.1 % ± 6.9 % when treated with PhA (1 µM) + ko143 (500 nM). The proportion of viable TR-TBT 18d-1 cells when treated with PhA (1 µM) was 132 % ± 48.43 % and 131 % ± 38.06 % when treated with PhA (1 µM) + ko143 (500 nM).

5.3.4 Measurement of ABCG2/abcg2 efflux activity in BeWo and TR-TBT 18d-1 cells.

ABCG2/abcg2 functionality was measured in the placental cell lines using the substrate PhA (1 µM) and the inhibitor ko143 (500 nM) as used previously in the MCF-7 cell line (Figure 5.5). The BeWo cell line was used to measure the human placental ABCG2 function and the TR-TBT 18d-1 cell line was used to determine the rat placental abcg2 function.

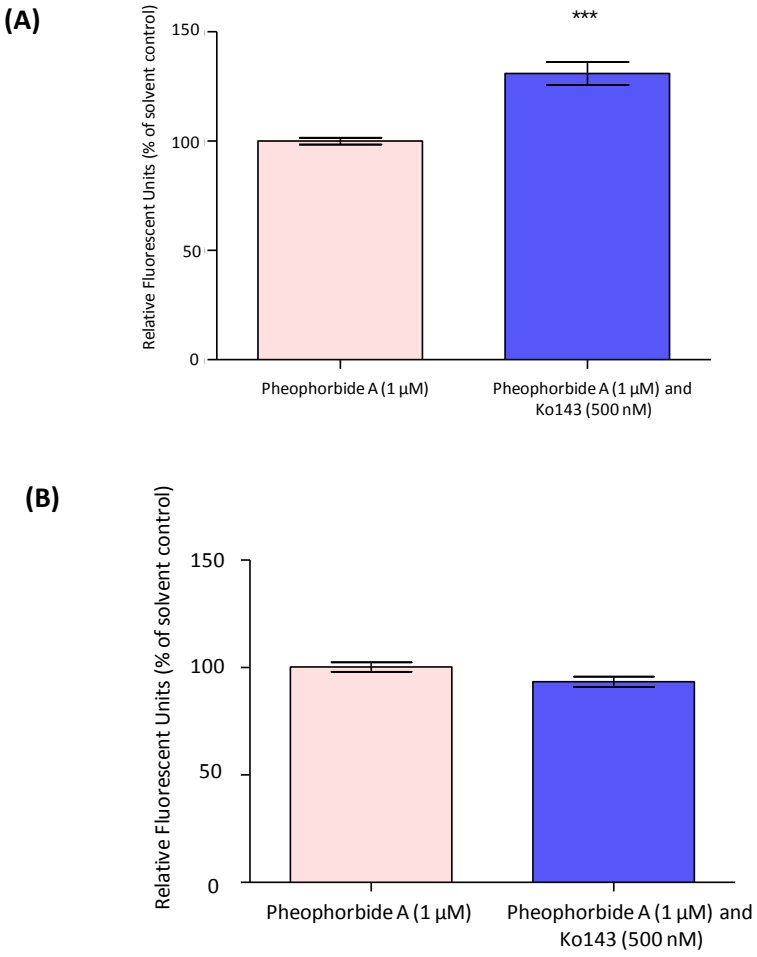


Figure 5.7 Measurement of ABCG2/abcg2 efflux activity in BeWo and TR-TBT 18d-1 cells. BeWo cells (A) or TR-TBT 18d-1 cells (B) were seeded onto a 96 well plate at densities of 5 x 10^3 cells/well or 1 x 10^4 cells/well respectively. Cells were incubated in Optimem™ for 24 h and then washed in PBS. Cells were treated with PhA (1 µM) in Optimem™ or PhA (1 µM) + ko143 (500 nM) in Optimem™ for 18 h. After 18 h, cells were washed with ice-cold PBS and the intracellular PhA fluorescence was read at excitation 417 nm and emission 674 nm using a Tecan Safire spectrophotometer. Data are mean ± SEM (n = 3 with 8 replicates). Statistical significance was determined using a Student's t-test: *** = $p < 0.001$

ko143 significantly increased the intracellular accumulation of PhA from 100 % ± 11.9 % to 130.9 % ± 36.51 % in the BeWo cell line (Figure 5.7 (A)). This confirms functional activity for ABCG2 in the BeWo cell line. No significant difference in accumulation of PhA between the two conditions (±ko143) was observed in the TR-TBT 18d-1 rat placental cell line (Figure 5.6 (B).

5.3.5 Expression of ABCG2/abcg2 in human and rat placental cell lines

The ABCG2 efflux transporter is known to be expressed in the BeWo human placental cell line (Evseenko et al., 2007). However, expression of abcg2 in the TR-TBT 18d-1 rat placental cell line at the protein level has not yet been reported. To investigate the expression of ABCG2/abcg2 in the human and rat placental cell lines used in this thesis, Western blots were performed.

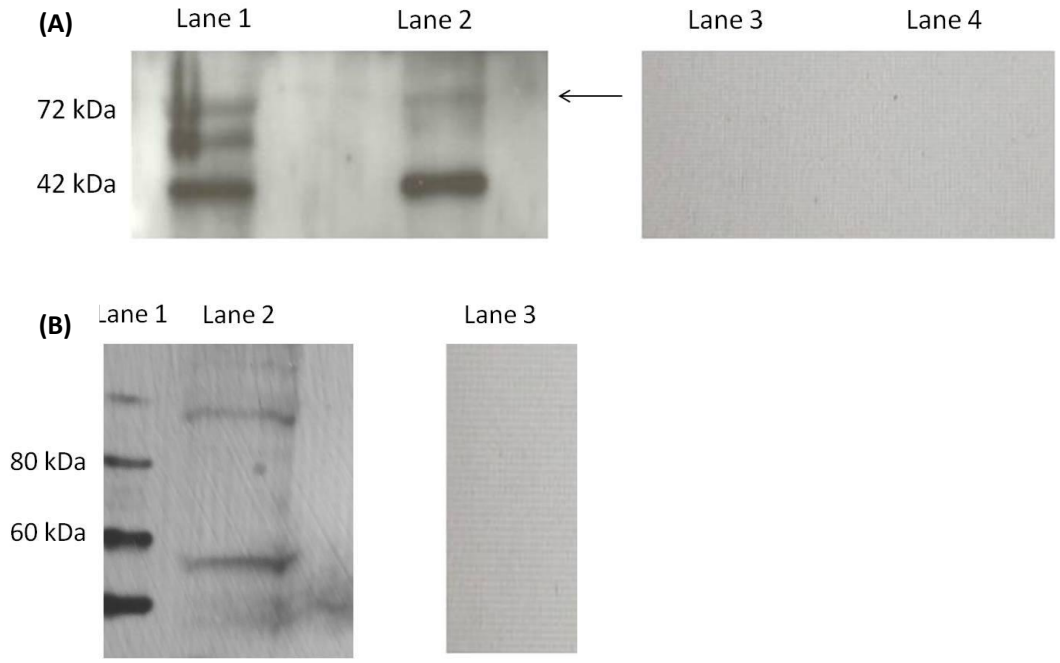


Figure 5.8 Expression of ABCG2/abcg2 in placental cell lines; BeWo and TR-TBT 18d-1. Cell membranes were isolated and purified from MCF-7 (Panel (A), Lane 1), BeWo (Panel (A), lane 2) and TR-TBT 18d-1 (Panel (B), lane 2) cells by differential centrifugation (see section 2.2.2.2.2). Solubilised protein (20 µg protein) was loaded into each well of an 8 % (w/v) polyacrylamide gel and subjected to SDS-polyacrylamide gel electrophoresis at 150 volts for 60 min. Separated proteins were then electrotransferred to PVDF membrane using 200 mA for 90 min. The PVDF membrane was incubated with TBS-T containing 5% (w/v) non-fat milk protein for 120 min at room temperature in a shaking incubator. The PVDF membrane was then washed for 3 x 10 min, in TBS-T. The PVDF membrane was then incubated in polyclonal rabbit anti-BCRP antibody (1:250) in TBS-T containing 5 % (w/v) non-fat milk protein overnight at 4 ºC. The PVDF membrane was washed for 3 x 10 min, in TBS-T and then incubated with mouse anti-β actin antibody for 1 h (in TBS-T) (BeWo and MCF-7 membrane only). The PVDF membrane was washed for 3 x 10 min, in TBS-T before adding goat anti-rabbit horse radish peroxidase labelled IgG (1:1000) for 45 min. Sheep anti-mouse horse radish peroxidise labelled IgG (1:2000) was then added for 45 min (for the BeWo and MCF-7 membrane only). Protein detection was performed using an Enhanced Chemiluminescence Prime Western blotting detection kit (Amersham). A negative control (Panel A, lanes 3 and 4 and Panel B, lane 3) was performed in which the PVDF membrane was incubated with goat anti-rabbit horse radish peroxidise labelled IgG (1:1000) where only goat anti-rabbit horse radish peroxidase was added. In both panels A and B, lane 1 is a molecular weight marker.

Western blotting confirmed the expression of ABCG2 in the BeWo cell line (Figure 5.8 Panel A lane 2). No expression of abcg2 was found in the TR-TBT 18d-1 cell line (Figure 5.8 (B) lane 2). The lack of abcg2 expression was not surprising as no functional abcg2 activity had been observed in the functional abcg2 assay (Section 5.3.4, Figure 5.7)

5.4 *In vitro* blood-placental models

For *in vitro* systems to fully represent a physiological barrier, the apical and basolateral surfaces of the cell should be exposed to the culture medium. By growing cell monolayers on polycarbonate permeable membranes, *in vitro* models of the placenta can be used to study the directional transport of compounds (Utoguchi et al., 1999, Liu et al., 1997), including pharmaceuticals and agrochemicals.

There are contradictory reports in the literature as to whether JAr cells form a polarised monolayer, however the BeWo cell line reportedly forms a confluent, polarized monolayer that provides a good *in vitro* model system to study the transcellular distribution of nutrients and drugs across the placental trophoblast (Liu et al., 1997).

5.4.1 Transcellular electrical resistance of placental cell lines

Preliminary studies were performed to assess the integrity of the *in vitro* placental models. Transcellular electrical resistance (TER) readings were recorded over 7 days for the 3 different cell lines.

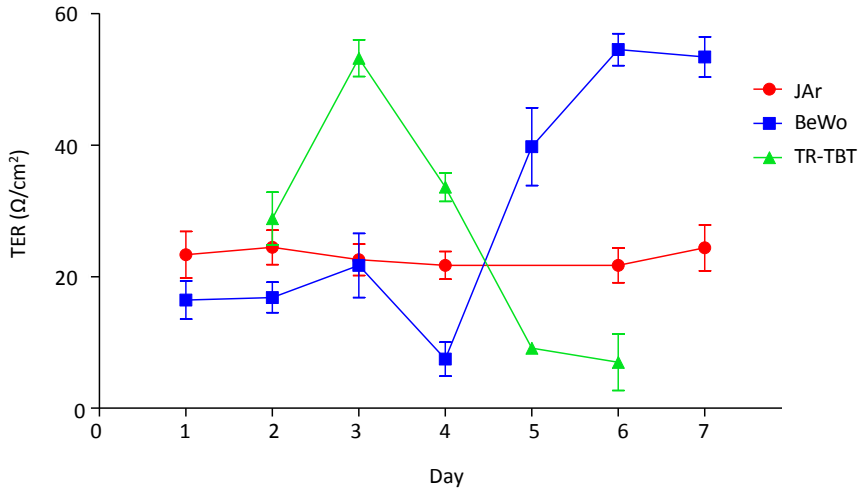


Figure 5.9 Transcellular electrical resistance of placental cell lines. Placental cell lines were seeded (JAr: 100,000 cells/cm^2, BeWo: 10,000 cells/cm^2 and TR-TBT 18d-1:100,000 cells/cm^2) and grown over 7 days on Transwell® inserts. TER readings were taken from Day 1-7. Data are mean ± SEM (n=3 with at least 4 replicates for JAr and BeWo, n=1 with 4 replicates for TR-TBT 18d-1). Readings from blank wells were subtracted.

The TER values did not exceed 60 Ω/cm^2 for all three cell lines. The rat placental cell line, TR-TBT 18d-1, detached from the Transwell® after 6 days. The TER readings in the JAr human placental cells were relatively unchanged over the 7

days (Figure 5.9). The TER values are representative of the placenta, which is regarded to be a leaky barrier. Further investigations to characterise JAr cells were performed.

5.4.2 Ultrastructural morphology of JAr cells grown on Transwell® inserts

Transmission electron microscopy was used to determine the morphology of JAr cells grown on Transwell® inserts.

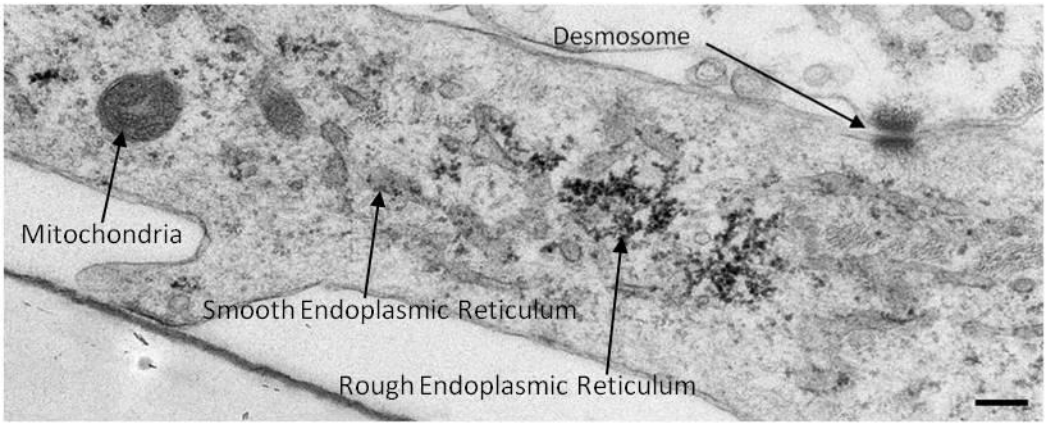


Figure 5.10 TEM image of JAr cell on Transwell®. A cross section is shown of the human JAr cell, with leg like structures attaching to the Transwell® insert and desmosome-like junctions between the cells. Scale bar is 500 nm. Magnification 17,000 x.

Transmission electron micrographs confirmed the formation of desmosome-like junctions between cells (Figure 5.10). No tight junctions, were observed.

In many epithelial cell types, tight junctions, or zona occludens connect adjacent cells. The MDCK cell line has been extensively studied and used as a model for permeability assays as they possess higher TER values (upto 1000 Ω/cm^2) (van Meer and Simons, 1986, Gonzalez-Mariscal et al., 1985, Dukes et al., 2011).

The lack of tight junctions (shown in Figure 5.10) suggest that the placenta is a 'leaky' organ, so when performing any type of permeability studies this must be taken into consideration. However, this does show that the *in vitro* models studied here are representative of the placenta, so these monolayers should be investigated further as they could be a valuable tool in predictive *in vitro* prenantal toxicity assays.

5.5 *Ex vivo* models of the placenta

The aim of an *in vitro* system is to mimic the whole tissue as best as possible. A unique advantage of studying the placenta is its availability and viability (up to 4 h post-partum) (Sooranna et al., 1999). Many studies have been performed utilising freshly collected human placenta; the collection and study of placental villous fragments is particularly advantageous due to retained syncytiotrophoblast metabolism and signalling (Greenwood and Sibley, 2006).

5.5.1 Functional activity of ABCB1 in human placental fragments

The functional activity of ABCB1 was assessed using villous fragments obtained from healthy term placentas. The accumulation of the ABCB1 substrate [^{3}H]taxol was measured over a time period of 2 h (± the ABCB1 inhibitor verapamil), Figure 5.11.

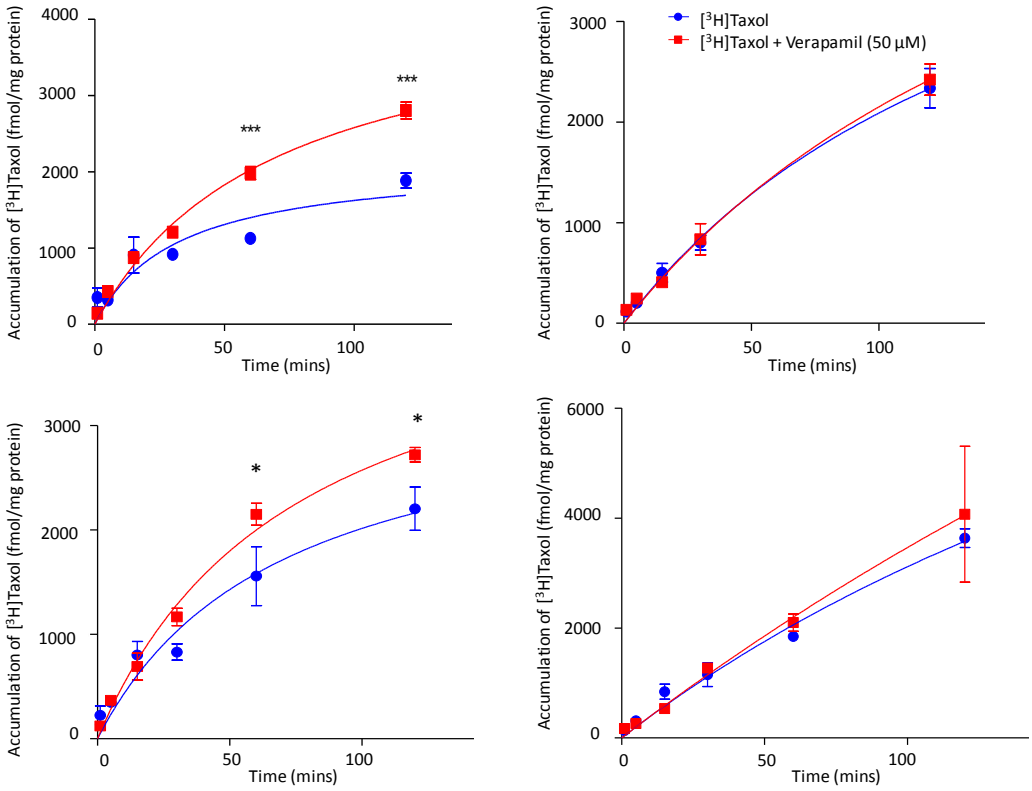


Figure 5.11 The effect of verapamil on [^{3}H]taxol accumulation in fresh placental villous tissue. [^{3}H]taxol accumulation was studied in the absence (blue circle) or presence (red square) of verapamil. [^{3}H]taxol accumulation in the tissue was measured by liquid scintillation spectroscopy. The protein content of tissue was measured by the Bio-Rad protein assay. Data are mean ± SD. Each panel represents one placenta; each time point was carried out in duplicate. Statistical significance was determined using a 2-way ANOVA:* = $p<0.05$, *** = $p< 0.001$

[^{3}H]taxol accumulation within placental fragments increased with time (Figure 5.11). Differing degrees of verapamil sensitive [^{3}H]taxol accumulation were observed in fragments isolated from four placentas.

The 120 min data from the four placentas were combined to see if there was any difference in [^{3}H]taxol accumulation between the two conditions.

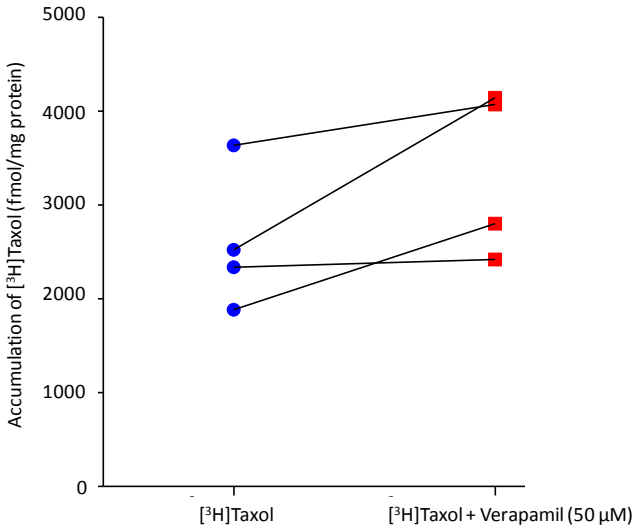


Figure 5.12 The effect of verapamil on [^{3}H]taxol accumulation in fresh placental villous tissue at 120 min. The 120 min data from Figure 5.11 were pooled and a Wilcoxon-signed rank test performed. There was no statistical difference found between the two conditions.

The combined data showed no significance in verapamil sensitive [^{3}H]taxol accumulation using a Wilcoxon-signed rank test (Figure 5.12).

This method was performed on up to 20 placentas, however the difference in ABCB1 functionality (shown by performing uptake experiments) was vast. An uptake model for the study of ABCG2 efflux was also tried, using 3H-mitoxantrone as the substrate and FTC or Ko143 as the specific inhibitor. However, the experimental approach was perhaps too crude for the level of analysis and detail this project was wanting to achieve. The availability of placenta tissue was on average one placenta per week (performed at St Mary's Hospital, Manchester), and under the ethics approved for the project, no background information of the patient was collected. Further work in this area is required as utilising *ex vivo* human placenta is a good source; refinement of the experimental technique is required for the type of information this thesis wanted.

5.5.2 Isolation of primary rat placental cells

The isolation of syncytiotrophoblast cells from the labyrinth region of rat placentas at GD 16 was attempted as described by Kitano *et al.* (2002). On three separate occasions, placentas from pregnant Han Wistar rats were isolated. The metrial gland and decidua were separated from the placenta to leave the junctional and labyrinth regions (Figure 5.13). After separation by centrifugation and Percoll gradient (see section 2.2.3.5.3), the isolated cell pellet was seeded into 96 and 24 well plates. After 24 hours no cell growth was observed.

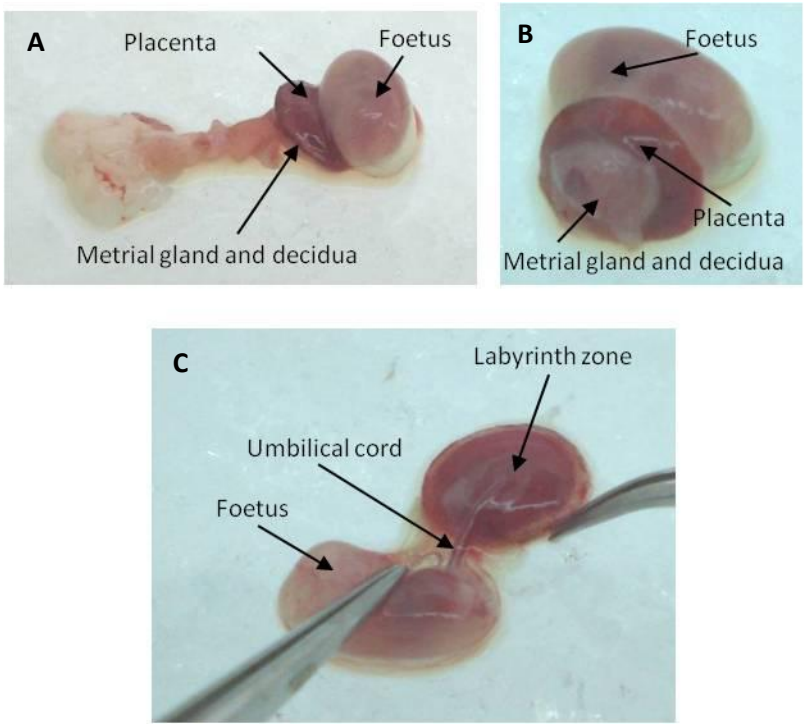


Figure 5.13 Isolation of the labyrinth zone from the rat placenta at GD 16. Placentas were collected from time-mated pregnant Han Wistar rats at GD 16. The labyrinth zone was dissected from the junctional zone of the placenta (Panel C) and finely minced on ice. The minced tissue was then subjected to enzyme digest by Dispase (0.5 % (w/v)) and deoxyribonuclease (0.1 % (w/v)) in Medium 199 for 60 min at 37 °C. 1 ml of tissue homogenate was loaded onto pre-prepared 5 ml 40 % (w/v) Percoll density gradients. The samples (resting on top of the Percoll gradient) were centrifuged at 700 x g for 15 min at room temperature. Sequential 1 ml fractions were removed from the Percoll gradient and placed into separate wells of 24 well plates. Cells were allowed to grow at 37 °C in 5 % CO_2 for upto 7 days, cell growth was monitored daily.

On all three separate occasions the isolation and growth of the labyrinth cells were not successful. This area needs further development to be able to achieve results.

Chapter 6

Discussion

6.0 Chapter 6; Discussion

6.1 Evaluation of methods used to compare the barrier properties of rat and human placenta.

In terms of investigating the expression of transporters and metabolising enzymes in the rat and human placentas mRNA expression was investigated throughout gestation by analysing microarray data (Chapter 3). To validate and explore the expression of particular transporters and metabolising enzymes (abcb1, abcg2, ABCC1/abcc1, SLCO4A1/slco4a1 and CYP26B/cyp26b) the protein expression was assessed via Western blotting and immunohistochemistry. Due to the extent of the work involved (validating the techniques, validating and optimising the antibodies and timed tissue collection) the protein expression data was only performed at one time-point; GD 16 in rat and term in human.

Gestational Day 16 was chosen for rat as mRNA levels of the selected transporter and metabolising enzymes (abcb1, abcg2, ABCC1/abcc1, SLCO4A1/slco4a1 and CYP26B/cyp26b) were considered to be at a detectable level (Chapter 3, Figures 3.1, 3.2, 3.3, 3.5 and 3.10) . Also, previous work conducted by Stratton et al., (2005) as part of the Foetal Capability project had shown localised expression of abcb1, abcg2 and abcc2 by immunohistochemical analysis at GD 16. The aim of this work was to determine expression of the transporter/metabolising enzyme at the protein level. Using a time-point that had already shown positive expression for abcb1, abcg2 and abcc2, it seemed logical to investigate whether other transporters and metabolising enzymes are expressed at this time-point.

One limitation of part of this study was the collection of human placental tissue. This was obtained commercially (Abcam, Cambridge, UK) and the only placental tissue available at the time was term (42 weeks). As the primary aim of this study was to determine protein expression of the specific transporter or metabolising enzyme, the difference in gestation age of the tissue samples between species was not a hindrance. However, this is an area that would be very interesting to further investigate in order to gain a true comparison between the rat and human placental expression levels. If tissues were able to be collected from similar time points, e.g. GD 16 from rat and trimester 2 from humans, levels of protein expression could be directly compared. β-actin can be used as a loading control in the Western blots, which can also be used to semi-quantify expression levels. As the primary aim of the work in Chapter 3 was to determine protein expression, this

approach was not used at this time. Optimising the antibodies required extensive work and utilised a lot of the time for the project.

Elucidating the protein expression of the selected transporters and metabolising enzymes throughout gestation could be the next step for continuation of this work, especially as tissue collection, antibody validation and technique validation have been optimised. Showing the temporal expression of slco4a1/SLCO4A1 at the protein level in both rat and human would be particularly interesting due to the differences highlighted between the species at the mRNA level (Chapter 3, Figure 3.4, at GD 21 rat slco4a1 mRNA expression is more than 27 times higher than human expression of SLCO4A1 at term). Not only will investigating the change in temporal expression at the protein level provide investigators with a greater understanding, it will also verify the mRNA data generated in Chapter 3.

One of the obstacles that was faced in the early stages if the work performed in this thesis was being able to directly compare the rat and human gestation time periods. No studies to date have directly compared the rat gestational time period with the human gestational time period in terms of placental development. Comparisons between rat and human foetal development have been made; these focus on foetal neurodevelopment and limb development (Witschi, 1956), however as the focus of this thesis is placentation in rat and human, a comparison between human placentation and rat placentation was performed. Foetal development was taken into consideration, for example from the beginning of implantation to the formation of the tail bud to the end of metamorphosis and up to birth. However, some processes, such as opening of the eyelids occur after birth in the rat, compared to the human in utero (Chapter 1, Figure 1.3) but the main focus during this thesis was placental development (Chapter 1, section 1.3.1, Figure 1.4). The three main stages of foetal development was taken into consideration (organogenesis, period of embryo and period of foetus) and a direct placentation time-course comparison was generated (Chapter 1, section 1.3.1, Figure 1.5).

This comparison between gestation day in the rat and trimesters in humans has been followed throughout the thesis. As the first comparison of its kind this does stand alone and is open for critique. With the current knowledge regarding placentation in both species this ‘gestation day vs. trimester’ comparison does seem to be the most logical to date, however there is no doubt that further analysis and

critique should be employed to validate or dis-prove the 'gestation day vs. trimester' comparison.

6.2 Differences in temporal expression of transporters and metabolising enzymes between species

One of the novel findings is the elucidation of the temporal expression of ABC transporter and metabolising enzymes throughout gestation in the rat and human placenta.

Key differences between species have been found in mRNA expression (for example high expression of CYP19A1 in human, very low expression of cyp19a1 in rat, and high expression of cyp17a1 in rat and very low expression of CYP17A1 in human) and differences in the temporal expression throughout gestation; for example the significant increase of placental mRNA expression throughout gestation in the rat; at GD 21 the expression of slco4a1 was found to be 225 times higher than at GD 7. This informs the investigator of how the placenta is changing its barrier properties throughout gestation. Under the OECD guidelines (414), the rat is dosed everyday from GD 6 to the day before scheduled killing (GD 19 or GD 20). The data presented in Chapter 3 demonstrates how the developing placenta is changing its barrier properties, therefore allowing the investigator to accommodate and appreciate how the given NCE may interact when perhaps 200 times higher expression of slco4a1 is present compared to when only 1 times the level of expression is present.

It must be taken into consideration however that the data shown in Chapter 3 is at the mRNA level. Although Chapter 4 has shown the protein expression of selected transporters and metabolising enzymes, the temporal change of the transporters and metabolising enzymes at the protein level must be investigated before any solid conclusion can be made. The data presented in Chapter 3 merely provides an insight as to what is potentially occurring during placental development in the rat and human.

Another factor that ought to be considered regarding the temporal changes in mRNA expression is the way in which the data was collected. The rat data, part of a Foetal Capability study performed in house at CTL, by Syngenta was carried out in biological triplicate. All analyses of the data, to provide the raw CEL files (which have been analysed in this thesis) were performed at CTL in 2005. The human data was taken from a study by Mikheev et al., (2008), and was performed in biological

quadruple. The data shown in Chapter 3 only represents a very small population of the Han Wistar rat species, and a very small population of human species. Also regarding collection of the human data, nothing is known regarding the background of the patients, e.g. if the patients smoked or not, the age of the patients, if they had given birth before, if they were on any medication. All of these factors could potentially alter the expression of transporters at the placental barrier. Polymorphisms of ABCB1 and how they can affect levels of expression of ABCB1 have previously been investigated. It has been considered that variations in single nucleotide polymorphisms (SNPs) can alter the level of expression of ABCB1 (Tanabe et al., 2001). The single nucleotide polymorphism C3435T in exon 26 of ABCB1 has been associated with altered drug disposition and drug effects (Fellay et al., 2002); plasma levels of antiretrovirals (efavirenz and nalfinavir) were found to significantly differ in patients with different allele types. There is also a possible link between polymorphisms and ethnicity. Single nucleotide polymorphisms have identified two low frequency ABCB1 promotoer polymorphisms found more frequently in Japanese than Caucasian populations (Takane et al., 2004). Homozygosity of the C3435T ABCB1 polymorphism has also been reported in 24 % of a German population (Hoffmeyer et al., 2000).

Realising the importance of ABCB1 and other transporters and metabolising enzymes is just the beginning of understanding the protective role being played by these proteins and enzymes at physiological barriers. Due to the very small collection of both rat and human tissue, a bigger population from both species is required. This would help to validate the results found from this study and would also increase the represented population in each species.

One of the other major findings of this thesis is the difference in cyp mRNA expression throughout gestation between the rat and human. The differential expression of cyps between species highlights the different endocrine function of the placenta during pregnancy in the human and rat. The mRNA data showed high expression of cyp11a1 mRNA and cyp17a1 mRNA in rat placenta (Chapter 3, Figure 3.11) and CYP11A1 and CYP19A1 in human placenta (Chapter 3, Figure 3.11 and 3.12). The high expression of cyp11a1/CYP11A1 in both rat and human placenta is expected due to the function it has in catalysis of the first and rate-limiting step in the biosynthesis of all primary steroid hormones. A 3-step conversion of cholesterol to pregnenolone via a side-chain cleavage reaction constitutes the rate-limiting step in

the biosynthesis of biologically active progestins, oestrogen, androgens and glucocorticoids. CYP19A1 is also referred to as aromatase, and is not expressed in rodents. In humans, it is well characterised that CYP19A1 is expressed in the placenta. CYP19A1 is located in the corpus luteum, which is redundant in rodents. The corpus luteum acts as the oestrogen production centre during the early stages of pregnancy in humans. In rats, oestrogen is produced by the ovaries. CYP19A1 catalyses the conversion of C-19 steroids such as androstenedione and testosterone to steroidal C-18 estrogens via hydroxylation of the angular 19-methyl group to give 19-hydroxy, 19-*gem*-diol, and 19-oxo intermediates. The lack of cyp19a1 in the rat placenta highlights the difference between the two endocrine organs, and demonstrates the need for another test species, such as the rabbit.

6.3 Assessment and exploration of the predictive *in vitro* and *in vivo* studies in rat and human surrogate placental systems.

The work outlined in this thesis has compared placental transporters and metabolising enzymes expression (and function in the case of ABCB1/abcb1 and ABCG2/abcg2) in the rat and human placenta. The key findings have centred on the similar expression and function of ABCB1/abcb1 in *in vitro* models of the rat (TR-TBT 18d-1 cells) and human (JAr cells) (Chapter 5, Figures 5.2 and 5.3). This similarity is of importance as ABCB1 expression has previously been demonstrated in *ex vivo* human placenta (Atkinson et al., 2003). The first report of abcb1 deficiency in mice, leading to cleft palate formation in foetuses after exposure to avermectin (Lankas et al., 1998) highlights the great importance of ABCB1/abcb1 at the placenta. The expression and function of placental ABCB1/abcb1in both rat and human *in vitro* models can help significantly in early compound development. The calcein AM assay could be used to identify whether or not new compounds are substrates of ABCB1/abcb1 in both human and rat *in vitro* systems before the new compounds are evaluated in the rat and rabbit animal models. The advantages of this are; the calcein AM assay is relatively high throughput, up to 4 compounds can be tested in the JAr cell line (using 24 well plates) and up to 10 compounds can be tested in the TR-TBT 18d-1 cell lines (using 96 well plates) in one experiment. The length of time the assay takes is 24 hours (from plating cells to reading the fluoresences of accumulated calcein) and due to the absence of animal models being used, the expense is remarkably less. Utilising *in vitro* models also fits in part of the

worldwide initiative of the 3Rs; refining, replacing and reducing the amount of animals used in experimental toxicology. However there are disadvantages to the study. Calcein AM is a substrate of ABCB1/abcb1. Once calcein AM enters the cell, esterases cleave calcein AM to produce calcein, the fluorophore, which is also an ABCC1/abcc1 substrate. Therefore the interaction of the NCE with ABCC1/abcc1 should also be considered. If the xenobiotic/NCE is a substrate of ABCC1/abcc1, there would be an increased accumulation of calcein in the cell. This assay does not just identify substrates of ABCB1/abcb1. This issue was considered when utilising the calcein AM assay in Chapter 5 (Section 5.2.3); to avoid this test compounds were chosen that were considered to have a greater affinity for ABCB1/abcb1. To help eliminate this issue an ABCC1/abcc1 inhibitor could also be utilised in the assay. The MK-571 compound has been utilised as an ABCC1/abcc1 inhibitor (van der Kolk et al., 1998), but this is an unpredictable approach as if another compound is introduced to the assay there is likely to be a drug-drug interaction.

To summarise an *in vitro* option has been proposed that could potentially reduce the amount of animals used in early developmental toxicity studies. Animal studies would undoubtedly still be a requisite, but implementing preliminary *in vitro* studies can help to streamline and isolate the compounds which would hopefully go further into the next stages of compound development.

The work presented in Chapter 3 does provide investigators with a great insight as what is potentially occurring in terms of transporter and metabolising expression at the rat and human placenta throughout gestation. However the key question is whether this mRNA data is providing the investigator with a sound background knowledge of what is occurring at the protein level in the animal systems, and in *in vitro* systems that could potentially be used to 'pre-screen' compounds. Chapter 4 has validated expression of some of these transporters and metabolising enzymes, and Chapter 5 has looked at current *in vitro* models. The human cell line, JAr, and the rat cell line, TR-TBT 18d-1 have proven to be viable candidates for studying the interaction of xenobiotics/new compounds with ABCB1/abcb1, however other *in vitro* surrogate models were not proven to be as successful. Use of *ex vivo* human placental tissue using radio-labelled substrates with or without inhibitors proved to be inconclusive of ABCB1 functionality. The experimental approach seemed somewhat crude, in order to evaluate such a precise

interaction with one transporter. Many obstacles were encountered including the viability of the tissue, the bottles in which the experiments were performed (specific glass bottles were used, as it was found that binding of the radio-labelled taxol was occurring on certain glass bottles) and size of the placental tissue isolated; although this was corrected for by evaluating the mg of protein, the surface area of the tissue could not be monitored. Isolating primary rat placental cells was attempted, but unfortunately this didn't yield any conclusive results. The isolation was only performed 3 times, and it involved the very precise dissection of the labyrinth region and differential centrifugation steps. This technique was not fully optimised during this project and should be further investigated. Primary cells are considered to be a truer representative of the *in vivo* system than cell lines that have been immortalised. After a number of passages immortalised cell lines phenotypes can begin to change. The whole point of using *in vitro* systems is to mimic the *in vivo* system, so using the cells that represent this closest is the natural choice.

6.4 The implications of this study and how it can add to the current developmental toxicity guidelines

Currently the OECD guidelines (414) stipulate that two animal species must be used; one rodent and one non-rodent, in order to predict potential hazards for human health. However using animals is costly, time consuming and does not receive positive support from the public. Implementing 'pre-screening' *in vitro* assays can help to reduce the number of animal studies performed. In Europe, ECVAM have endorsed three *in vitro* toxicity tests; the Embryonic Stem Cell Test (EST), Embryotoxicity Testing in Post-Implantation Whole Embryo Culture (WEC) – Method of Piersma and The Micromass Test – Method of Brown. In theory, these can be used either before testing new compounds in the animal models or to gain a better understanding of the potential risks the new compound could pose for human health. However, as previously mentioned, these endorsed *in vitro* toxicity tests do have limitations. The EST only covers a limited window of development (differentiation at the pre-implantation stage), the WEC – Method of Piersma test has a lack of biotransformation and the Micromass Test – Method of Brown is a primary cell culture system which does lack drug metabolising enzymes.

This thesis has highlighted the significant role transporters and metabolising enzymes have at the placental barrier, often playing a part in the disposition of a

xenobiotic from the maternal circulation to the foetal circulation. Employing a pre-screening *in vitro* assay (of human origin) for the interaction of xenobiotics/new compounds with transporters would give a prediction of any interaction between xenobiotics/new compounds and transporters in humans. As the expression and function of ABCB1/abcb1 has been found to be similar in both human and rat *in vitro* models, the two *in vitro* systems could be employed to give a predictor before deciding to further investigate the new compound in the rat and rabbit whole animal systems.

In short employing two predictive *in vitro* assays as a pre-screen for animal models can provide a more in depth evaluation of the NCE and how it interacts with transporters. Knowing this interaction can actually provide the investigator with more knowledge on how the NCE could affect the developing foetus prior to applying the NCE in the whole animal system, thereby saving time, money and resources (animals).

6.5 Future Work

The work performed in this thesis has focussed directly on the rat as a predictor for human health risk assessment. However, the OECD guideline (for 414) clearly state that two species must be used in developmental toxicity studies. Routinely the rat and rabbit are the two species of choice. Expression of ABC transporters and metabolising enzymes has not been investigated in the rabbit placenta. A logical place to start is by addressing the mRNA expression of ABC transporters and metabolising enzymes to build upon the 'expression profiles library' generated in Chapter 3. However, there are issues regarding this; such as the availability of a suitable rabbit microarray chip (which would preferably be Affymetrix, in order to provide a comparison between the 3 different species). The next logical steps to follow are to investigate the protein expression levels of transporters and metabolising enzymes in the rabbit, and then to explore the current *in vitro* rabbit placental models available. Utilising this information will help investigators be much more selective in further tests for human health assessment, will help to save time and money, and most importantly will steer the investigator to a truly predictive outcome of the test compound, after having a greater understanding of the potential interactions the xenobiotics/new compound is being exposed to at the placental level.

Further development of the current *in vitro* systems is also very important. The BeWo cell line has showed ABCG2 expression and function (Chapter 5, Figure 5.7), and has also previously been used to assess transport across a monolayer as BeWo cells are able to polarise (Liu et al., 1997). It would be interesting to study the transport of ABCG2 substrates in these monolayers using the ABCG2 inhibitor, ko143. As the TR-TBT 18d-1 cells are relatively new, it would be interesting to further investigate these, especially through protein expression. As the mRNA data from Chapter 3 highlighted such a difference in the cyp expression between species it would be really interesting to see the difference in protein expression of the cyps in the JAr, BeWo and TR-TBT 18d-1 cell lines. Western blotting could be employed, and the use of β-actin could be used to semi-quantify the expression levels of the cyps in order to compare these levels across species.

This naturally leads to the idea that more of the transporters and metabolising enzymes from the rat and human placenta should be determined at the protein level by Western blotting and immunohistochemistry. As already mentioned, β-actin

should be used as a loading control so that expression levels can be semi-quantified in order to provide comparative protein expression levels between the species.

Outside the scope of this thesis but work that would successfully follow from the *in vitro* assays is the development of *in silico* toxicology modelling. The work performed in this thesis (mRNA expression data, protein expression and functionality of ABC transporters and metabolising enzymes) has in some respects set the background for the computational models. The findings in this thesis must be replicated in the *in silico* models if the *in silico* models are going to be representative of the whole animal or *in vitro* system. *In silico* toxicology models are becoming increasingly popular; High Throughput Screening (HTS) is being implemented by ToxCast™, an initiative launched in 2007 by the EPA to develop ways to predict the potential toxicity of chemicals that require toxicity testing. Prenatal developmental toxicity has been targeted by one group who have assessed the rat and rabbit animal models in terms of their ability to predict prenatal developmental toxicity by evaluating specific assay targets (for example retinoic acid receptors, G-protein coupled receptor, interleukins and chemokines) and also evaluating toxicity endpoints (for example cleft palate, embryonic development and placental development) (Sipes et al., 2011). The information gathered in this thesis would fit appropriately in these ToxCast™ models

Temporal expression of selected transporters or metabolising enzymes can be fully investigated at the protein level; this should primarily be performed on ABCB1/abcb1 and ABCG2/abcb2 as the reports that are currently available are very inconclusive and contradictory. As already mentioned, a bigger population must be tested in order to provide a sound conclusion.

It is hoped that this thesis has provided a basis of understanding for the underlying role transporters and metabolising enzymes play throughout gestation. Further work can build upon this basis, for example comparing placentas from teratogenic foetuses with placentas from ‘normal’ foetuses.

6.6 Conclusion

The overall aim of this thesis was to compare expression and/or functional activity of selected transporters and enzymes in currently available models of rat and human placenta. Realising this aim has provided an insight into the suitability of the models in predicting potential hazards for human health of new compounds. As the main contributors to the protective placental barrier, the protein expression and function of ABC transporters (and a metabolic enzyme of interest), throughout gestation in the human and rat placenta were evaluated.

The first part of the work in this thesis involved exploring the mRNA expression of ABC transporters and metabolising enzymes in the human and rat placenta throughout gestation by microarray analysis. Transporters and metabolising enzymes were identified in both species; key findings highlighted the difference in the trend of the mRNA expression of transporters and metabolising enzymes throughout gestation (e.g. SLCO4A1/slco4a1; this significantly increased in the rat placenta but did not increase in the human placenta) or the difference in mRNA expression of transporters and metabolising enzymes (e.g. significantly high expression of CYP19A1 mRNA was observed in the human placenta, but very low, almost none, expression was found in the rat placenta).

After exploring the expression of transporters and metabolising enzymes at the mRNA level, the protein expression of abcb1, abcg2, ABCC1/abcc1, SLCO4A1/slco4a1 and CYP26B/cyp26b was investigated in both species using Western blotting and immunohistochemical techniques. The expression of ABCB1/abcb1, ABCG2/abcg2, ABCC1/abcc1, SLCO4A1/slco4a1 and CYP26B/cyp26b at the mRNA level was reflected by expression at the protein level at GD 16 in the rat placenta and at term (42 weeks) in the human placenta.

The final part of this thesis characterised current *in vitro* surrogate placental models. The functional activities and expression of ABCB1/abcb1 and ABCG2/abcg2 was explored in human and rat cell lines. The human JAr cell line was found to be a suitable model to assess functional activity of ABCB1, however future work is required to determine the suitability of the cell line for use in a monolayer model system. Abcb1 expression and functional activity was observed in the rat TR-TBT 18d-1 and again, future work is required to assess the potential of establishing a monolayer model using these cells. Overall the JAr and TR-TBT 18d-

1 cell lines have potential to be used as a pre-screen to assess interactions of xenobiotics with ABCB1/abcb1.

Overall the work generated from this thesis provides a strong understanding as to the interactions NCEs could encounter when being transferred between the maternal and foetal circulation in the rat and human species. Further work (as outlined in section 6.5) should be employed in order to build upon this knowledge and help investigators towards more precise predictors of potential hazards for human health in the developmental toxicity area.

Chapter 7

Appendix

7.0 APPENDIX

Appendix 1

Table 1A. Classification of drugs according to the FDA

Category	Risk to foetus/mother
A	After controlled studies in women no risk to the foetus in first trimester or in the later trimesters. The possibility of fetal harm appears remote.
B	Animal reporduction studies not demonstrated a foetal risk, or no controlled study in pregnant women has been carried out. Animal reproduction studies may have shown an adverse effect, but has not been confirmed in controlled studies in women in the first trimester.
C	Studies in animals have revealed adverse effects on the foetus. No controlled study in pregannt women. Drug should only be given if the potential benefit justifies the risk to the foetus
D	There is positive evidence of human foetal risk, but benefit from use may be acceptable dispite the risk e.g. Life-threatening.
X	Studies in animals or human beings have demonstrated foetal abnormalities. The risk of using the drug clearly out-weighs any possible benefit. The drug is contraindictaed in women who are or may become pregnant.

Appendix 2

Table 2A Physiocochemical properties of ABCB1/abcb1 inhibitors (Aotrovastatin, Quinidine, Saquinavir and Verapamil) and ABCG2/abcg2 inihbitor (ko143).

Compound	Molecular weight	Log P	Log D (at pH7.4)	PSA	Hydrogen bond donors	Hydrogen bond acceptors	IC_{50} (µM) (for Abcb1)	IC_{50} (µM) (for Abcg2)
Atorvastatin	558.64	5.39	1.00 - 1.25	111.79	4	5	~ 10[c]	
Quinidine	324.417	2.51	2.4	45.59	1	4	2.1[d]	
ko143	469.57	3.6	3.6					0.5[a]
Saquinavir	670.841	2.58	4.6	174.72	5	7	6.5[d]	
Verapamil	454.59	5.04	3.7	63.95	0	6	5.2 ± 2.0[b]	

[a] Taken from Matsson et al., (2009)
[b] Taken from Pajeva and Wiese, (2009)
[c] Taken from Chen et al., (2005)
[d] Taken from FDA, (2011)

Appendix 3

Table 3A. Substrate and Inhibitors of ABCB1(Marzolini et al., 2004, Zhou et al., 2007, Seelig and Landwojtowicz, 2000)

Substrate	Inhibitor
Amitriptyline	Astemizole
Amprenavir	Atorvastatin
Celiprolol	Bepridil
Chloropromazine	Biricodar
Cimetidine	Bromocriptine
Cortisol	Carvedilol
Cyclosporine	Chlorpromazine
Daunorubicin	Clarithriomycin
Dexamethasone	Cortisol
Digoxin	Cyclosporine
Digoxin	Diltiazem
Diltiazem	Dipyidamole
Docetaxel	Disulfiram
Docetaxel	Elacridar (GF120918)
Domperidon	Erythromycin
Doxorubicin	Felodipine
Doxycycline	Fluoxetine
Erythromycin	Itraconazole
Etoposide	Ketoconazole
Imatinib	LY335979
Indinavir	Midazolam
Irinotecan	Nicardipine
Itraconazole	Nitrendipine
Ivermectin	OC144-093
Ketoconazole	Paroxetine
Levofloxacin	Progesterone
Loperamide	Propafenone
Losartan	Quinidine
Methylprednisolone	Quinine
Mitomycin C	R101933
Mitoxantrone	Reserpine
Morphine	Ritonavir
Nelfinavir	Saquinavir
Ondansetron	Sertraline
Paclitaxel	Tacrolimus
Pentazocine	Tamoxifen
Phenobarbital	Terfenadine

Appendix 3 continued

Table 3A. Continued. Substrates and Inhibitors of ABCB1 (Marzolini et al., 2004, Zhou et al., 2007, Seelig and Landwojtowicz, 2000)

Substrate	Inhibitor
Phenothiazine	Tetrabenzine
Phenytoin	Valinomycin
Quinidine	Valspodar (PSC833)
Ranitidine	Verapamil
Rifampin	Vinblastine
Ritonavir	XR9051
Saquinavir	
Sirolimus	
Sparfloxacin	
Tacrolimus	
Talinolol	
Teniposide	
Terfenadine	
Tetracycline	
Topotecan	
Valspodar	
Vecuronium	
Verapamil	
Vinblastine	
Vincristine	

Table 3B. Substrates and Inhibitors of ABCG2/abcg2 (Robey et al., 2007)

Substrate	Inhibitor
9-aminocamptothecin	17{beta}-estradiol
Bisantrenea	6-prenylchrysin
BNP1350	Acacetin
Chlorin e6	Biricodar (VX-710)
CI1033	Chrysin
Daunorubicina	Curcumin
Diflomotecana	Cyclosporin A
Doxorubicina	Dipyridamole
DX-8951f	Elacridar (GF120918)
Epirubicina	Estrone
Etoposide	Fumitremorgin C
Flavopiridol	Gefitinib
Gefitinib	Genistein
GW1843	Imatinib
Homocamptothecin	Ko143
Imatinib	Naringenin
Irinotecan	Nicardipene
J-107088	Nimodipene
Methotrexate, methotrexate di- and triglutamate	Nitrendipene
Mitoxantrone	Novobiocina
NB-506	Ortataxel
Pheophorbide a	Quercetin
Protoporphyrin IX	Reserpine
Pyropheophorbide a methyl ester	Silymarin
SN-38	Sirolimus
Teniposide	Tacrolimus
Tomudex	Tamoxifen
Topotecan	Tariquidar (XR9576)
UCN-01	Tectochrysin

Appendix 4

Acrylamide/bisacrylamide	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Bovine serum albumin	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Bradford reagent	BioRad Laboratories Ltd, Hertforshire, UK
Bromophenol blue	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Calcein acetoxymethyl ester (Calcein-AM)	In Vitrogen, Paisley, Scotland, UK
Calcium chloride	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
CAPS: (3-(cyclohexylamine)-1-propane sulfonic acid)	VWR International (Leicestershire, UK).
CL-Xposure Film	Thermo Fisher Scientific, Northumberland, UK
Citric Acid	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
DakoCytomation EnVision + Dual Link system HRP (+DAB)	Dako, Cambridgeshire, UK
Dulbecco's Modified Medium Eagle Medium	Invitrogen, Paisley, Scotland, UK
ECL Prime western blotting reagent	GE Healthcare Life Sciences, Buckinghamshire, UK
Foetal Bovine Serum	Invitrogen, Paisley, Scotland, UK
Glycerol	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Glycine	Fisher Scientific, Leicestershire, UK
Glucose	Sigma-Aldrich Chemical Co, Poole, Dorset, UK

HEPES 1M buffer solution	Invitrogen, Paisley, Scotland, UK
Hybond™-P PDVF membrane	GE Healthcare Life Sciences, Buckinghamshire, UK
L-glutamine 200mM 100 X	Invitrogen, Paisley, Scotland, UK
M199	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Magnesium Chloride	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Methanol	Fisher Scientific, Leicestershire, UK
MTT (3-(4, 5-dimethylthiazol-2-yl)-2,5-diphenyl tetrazolium bromide	Invitrogen, Paisley, Scotland, UK
2-mercaptoethanol	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Non-essential amino acids 100 X	Invitrogen, Paisley, Scotland, UK
Spectra™ Multicolour High Range Protein marker, broad range (40 – 300 kDa)	Fermentas Life Sciences,
Papnicolaou's Solution 1a Harris' Haematoxylin	Merck
Penicillin G sodium 10,000 units/ml Streptomycin sulphate 10.000 µg/ml	Sigma-Aldrich Chemical Co, Poole Dorset, UK
PlasmoTest™ reagent kit	Source BioScience Lifesciences, Nottingham, UK
Percoll	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Potassium Chloride	Sigma-Aldrich Chemical Co, Poole, Dorset, UK

Potassium Phosphate	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Primary antibody: mouse monoclonal C219	Cambridge BioScience, Cambridge, UK
Primary antibody: mouse monoclonal anti-MRP1	Abcam, Cambridge, UK
Primary antibody: rabbit polyclonal anti-cyp26	Abcam, Cambridge, UK
Primary antibody; rabbit polyclonal anti-bcrp	Abcam, Cambridge, UK
Primary antibody: rabbit polyclonal anti-slco4a1	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Primary antibody: goat polyclonal anti-MDR1	Santa Cruz Biotechnology, Heidelberg, Germany
Protease inhibitor cocktail	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Secondary antibody: HRP-linked sheep anti mouse IgG	GE Healthcare Life Sciences, Buckinghamshire, UK
Secondary antibody: HRP-linked goat anti rabbit IgG	Abcam, Cambridge, UK
Secondary antibody: HRP-linked donkey anti goat IgG	Santa Cruz Biotechnology, Heidelberg, Germany
Sodium chloride	Analab, Dorset, UK
Sodium phosphate	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Sodium deoxycholate	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Sodium hydroxide	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
[^{3}H]Taxol	ARC UK Limited, Cardiff, UK

TEMED (N,N,N',N'-tetramethylethylenediame)	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
T-Flask -25 cm^2	Greiner Biosciences, Stonehouse, UK
T-Flask-75 cm^2	Greiner Biosciences, Stonehouse, UK
Tissue culture-treated 24-well flat bottomed plate	Corning Costar, High Wycombe, UK
Tissue culture-treated 96-well flat bottomed plate	Corning Costar, High Wycombe, UK
Transwell™ polycarbonate inserts (pore size 0.4 µm, diameter 12 mm, growth area 1.12 cm^2, 12-well cell culture cluster)	Corning Costar, High Wycombe, UK
Tris (Tris[hydroxymethyl]aminomethane)	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Tri-sodium citrate	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Triton x-100	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Trypan Blue solution 0.4 %	Sigma-Aldrich Chemical Co, Poole, Dorset, UK
Trypsin-EDTA solution (1x 500 Na-Benzoyl-Arginine Ethyl Ester Units porcein trypsin and EDTA, 180 µg/ml)	Invitrogen, Paisley, Scotland,UK
X-tra® slides	Leica, Microsystems, Milton Keynes, UK

Appendix 5

Reagents for Immunohistochemistry

Citrate buffer pH 6.0

Citric Acid	0.1 M
Dissolve in 100 ml dH_2O	
Sodium citrate	0.1 M
Dissolve in 500 ml dH_2O	

To prepare the citrate buffer mix 9 ml of citric acid with 41 ml of sodium citrate. Make up to 500 ml with dH_2O. Adjust to pH 6.

Phosphate buffered saline pH 7.4

NaCl	137.0 mM
KCl	2.7 mM
Na_2HPO_4	10.0 mM
KH_2PO_4	2.0 mM

Dissolve in dH_2O and adjust to pH 7.4. Autoclave

Peroxidase block (10 %)

H_2O_2 (30 %)	10 % (v/v)
PBS	90 % (v/v)

Make up fresh prior to use

Protein block (3 %)

BSA	30 mg/ml
PBS	10 ml

Make up fresh prior to use

Reagents for Western blotting

RIPA buffer with protease inhibitors

Tris-HCl (pH 7.8)	50 mM
NaCl	150 mM
SDS	0.1 % (w/v)
Sodium deoxycholate	0.5 % (w/v)
Triton-X-100	1 % (v/v)
Protease inhibitor cocktail	2 µl/ml

Sonicate and heat if required. Once prepared keep on ice or at 4 ºC

1.5 M Tris – pH 8.8

Tris Base	340 mg/ml

Adjust pH with HCl and store at 4 ˚C

0.5 M Tris – pH 6.8

Tris Base	76.25 mg/ml

Adjust pH with HCl and store at 4 ˚C

5 x SDS Sample buffer

Tris HCl pH 6.8	60 mM
SDS (2 %)	20 µg/ml
Glycerol (10 %)	100 µl/ml
β-mercaptoethanol (5 %)	50 µl/ml
Bromophenol blue (0.01 %)	0.1 µg/ml

Stored at room temperature

10 x Running buffer

Tris Base	25 mM
Glycine	192 mM
SDS (10 %)	100g/L

Dissolved in 1 L dH_2O and stored at 4 ºC.

Transfer buffer – pH 11

CAPs	10 mM

Dissolved in 1 L dH_2O and pH adjusted to pH 11 using NaOH. Stored at 4 ºC.

TBS-T

Tris Base	10 mM
NaCl	150 mM
Tween-20	0.05% v/v

Made up to 1 L with dH_2O

Blocking buffer (5 %)

Low fat powdered milk (Marvel)	50 mg/ml
TBS-T	100 ml

Only use freshly prepared blocking buffer

Cell growth media

JAr

D-MEM/F-12 (1:1)	
FBS	10 % (v/v)
Glutamine	2 mM
Penicillin G sodium	100 u/ml
Streptomycin sulphate	100 µg/ml

BeWo

F-12 Nutrient Mixture (Ham's)	
FBS	10 % (v/v)
Glutamine	2 mM
Penicillin G sodium	100 u/ml
Streptomycin sulphate	100 µg/ml

MCF-7

D-MEM (4.5 glucose)	
FBS	10 % (v/v)
Glutamine	2 mM
Penicillin G sodium	100 u/ml
Streptomycin sulphate	100 µg/ml

TR-TBT

D-MEM (1.5 glucose)	
FBS	10 % (v/v)
Glutamine	2 mM
Penicillin G sodium	100 u/ml
Streptomycin sulphate	100 µg/ml

Tyrodes solution pH7.4

NaCl	135 mM
KCl	5 mM
$CaCl_2$	1.8 mM
$MgCl_2(6H_2O)$	1 mM
HEPES	10 mM
Glucose	5.6 mM

Make up to 1 L with dH_2O and store at 4ºC

Dissociation medium

Medium 199	
Dispase	0.5 % (w/v)
Deoxyribonuclease	0.1 % (w/v)

Made up fresh prior to use.

Appendix 6

Table 6A. ABCB1/abcb1 isoforms and corresponding accession codes. The isoforms of Abcb1 in rat were verified using GeneBank accession codes (NCB1 Gene). The human ABCB1 gene was also verified using GeneBank accession codes

Transporter	Human accession code	Rat accession code
abcb1a		AF257746
abcb1b		AY082609
ABCB1	AF016535	

Table 6B ABCG2/abcg2 and corresponding accession codes. Abcg2 in both species was verified using the GeneBank accession codes listed below.

Transporter	Human accession code	Rat accession code
abcg2		AI175616
ABCG2	AF098951	

Table 6C Members of the ABCC/abcc family and their accession codes. Members of the abcc family were verified using GeneBank Accession codes (NCB1 Gene)

Transporter	Human accession code	Rat accession code
abcc1	NM_004996	AI059506
abcc2	NM_000392	NM_012833
abcc3	AK000791	AF072816
abcc4	BC041560 and AY133679	BE100533
abcc5	AF146074	NM_053924
abcc6	AI074459	NM_031013
abcc8	L78207	AB052294
abcc9	NM_020297 and AK056519	NM_013040 and D83598
abcc10	AK000002 and AK024446	BM390369
abcc11	BC039085	
abcc12	AF395909	
abcc13	AP001660	

Table 6D Members of the SLCO/slco family and their accession codes. Members were identified using GeneBank Accession codes (NCBI Gene or UniProtKB)

Transporter	Human accession code	Rat accession code
slco1a1		NM_017111
SLCO1A2	AF085224 and NM_021094	
slco1a4		NM_131906 and U95011
slco1a6		NM_130736
SLCO1B1	AB026257	
slco1b2		NM_031650
SLCO1B3	NM_019844	AF147740
SLCO1C1	NM_017435	
SLCO2A1	NM_005630	
SLCO2B1	NM_007256	NM_080786 and AF169410
SLCO3A1	NM_013272	AF239219
SLCO4A1	NM_016354	NM_133608
SLCO5A1	NM_030958	
SLCO6A1	NM_173488	
slco6b1		NM_133412

Table 6E Members of the cytochrome p450 enzyme family and their accession codes found in the human placenta. Members were identified using the accession codes listed below.

Cytochrome p450 enzyme	Human accession code
CYP1A1	NM_000499
CYP1A2	NM_000761 and AF182274
CYP1B1	NM_000104 and AU144855
CYP2A6	M33318 and AF182275
CYP2A7	NM_000764
CYP2A13	NM_000766
CYP2B6	NM_000767 and X06399
CYP2C8	NM_000770
CYP2C9	NM_000771 and M21940 and M15331 and AV646536
CYP2C18	NM_000772
CYP2C19	X65962
CYP2D6	X07618 and NM_000106 and X16866
CYP2E1	AF182276 and J02843
CYP2F1	NM_000774
CYP2J2	NM_000775
CYP2R1	NM_024514
CYP2S1	AF335278
CYP2U1	BC012027 and AK026498
CYP2W1	NM_017781
CYP3A4	AF182273 and J04449 and NM_017460
CYP3A5	X90579 and NM_000777 and X90579
CYP3A7	AF315325 and NM_000765
CYP3A43	AF280111 and AF280113 and NM_022820 and AF280110
CYP4A11	D13705 and NM_000778 and BC022851
CYP4B1	J02871 and AY151049
CYP4F2	D26480
CYP4F3	NM_000896
CYP4F8	AF133298
CYP4F11	NM_021187
CYP4F12	NM_023944
CYP7A1	NM_000780
CYP7B1	NM_004820
CYP11A1	NM_000781
CYP11B1	NM_000497
CYP11B2	X54741
CYP17A1	NM_000102 and AK094106
CYP19A1	BC035714 and NM_000103
CYP21A2	M17252
CYP24A1	NM_000782
CYP26A1	NM_000783
CYP26B1	AC007002 and NM_019885
CYP27A1	NM_000784
CYP27B1	NM_000785
CYP27C1	BC039307
CYP39A1	BC010358 and NM_016593
CYP46A1	NM_006668
CYP51A1	NM_000786 and U40053

Table 6F Members of the cytochrome p450 enzyme family and their accession codes found in the rat palcenta. Members were identified using the accession codes listed below.

Cytochrome p450 enzyme	Rat accession code
Cyp1a1	X00469
Cyp1a2	K02422
Cyp1b1	NM_012940
Cyp2a2	NM_012693
Cyp2a3	NM_012542
Cyp2b3	M20406
Cyp2b12	NM_017156
Cyp2b21	AF159245
Cyp2c6	M18336
Cyp2c7	AA800502
Cyp2c11	NM_019184
Cyp2c12	NM_031572
Cyp2c13	J02861
Cyp2c22	M58041
Cyp2c23	U04733
Cyp2d2	NM_012730
Cyp2d3	AB008424
Cyp2d4	U48220
Cyp2d4v1	U48219
Cyp2e1	NM_031543
Cyp2f4	NM_019303
Cyp2g1	M33296
Cyp2j3	U39943
Cyp2j4	NM_023025
Cyp2j10	BG374493
Cyp2s1	BI274639
Cyp2t1	NM_134369
Cyp2u1	BF392959
Cyp3a2	U09742
Cyp3a9	U46118
Cyp3a18	D38381
Cyp4a3	M33936
Cyp4a8	AW142784 and NM_031605
Cyp4b1	M29853
Cyp4f1	NM_019623
Cyp4f4	U39206
Cyp4f5	U39207
Cyp4f6	U39208
Cyp4f17	BG376949
Cyp4v3	AI170346 and AF311886
Cyp7a1	NM_012942
Cyp7b1	BF283070
Cyp8b1	NM_031241
Cyp11a1	NM_017286
Cyp11b1	D11354
Cyp11b3	U17082
Cyp17a1	NM_012753
Cyp19a1	NM_017085 and AI407180
Cyp20a1	BE118606
Cyp21a1	NM_057101
Cyp26a1	NM_130408
Cyp26b1	BE105541 and BF397093
Cyp27a1	M73231
Cyp27b1	NM_053763
Cyp39a1	BE115944
Cyp46a1	AI111519

Table 6G Members of the GST/gst family and their accession codes. Members were identified using GeneBank Accession codes (NCBI Gene or UniProtKB)

Transporter	Human Acession code	Rat accession code
GSTA1	NM_000846	
gsta2 /// LOC494499		NM_017013
GSTA3	NM_000847	
gsta3		NM_031509
GSTA4	NM_001512	
GSTCD	BC032942	
GSTK1	NM_015917	
GSTM1	NM_000561 and X08020	
gstm1		M28241
GSTM2	NM_000848	
gstm3		NM_020540
GSTM4	NM_000850	
GSTM5	NM_000851	
gstm5		U86635
gstm7		NM_031154
GSTO1	NM_004832	
GSTO2	AL162742	
GSTP1	NM_000852	
gstp1		X02904
GSTT1	NM_000853	
gstt1		NM_053293
GSTT2	NM_000854	
gstt2		NM_012796
GSTZ1	BC001453	
MGST1	NM_020300	
mgst1		NM_134349
MGST2	NM_002413	
MGST3	NM_004528	

Table 6H Members of the SULT/sult family and their accession codes. Members were identified using GeneBank Accession codes (NCBI Gene or UniProtKB)

Transporter	Human Acession code	Rat Acession code
SULT1A1	NM_001055 and U37025	
sult1a1		AF394783
SULT1A2	NM_001054 and U28169	
SULT1B1	NM_014465	
sult1b1		NM_022513
SULT1C2	NM_001056	
sult1c2		NM_133547
sult1c3		NM_031732
SULT1C4	NM_006588	
sult1e1		NM_012883
SULT2A1	U08024	
sult2a2		D14989
SULT2B1	NM_004605	
SULT4A1	NM_014351	
sult4a1		NM_031641
sult5a1		BF283670

Table 6I Members of the UGT/ugt family and their accession codes. Members were identified using GeneBank Accession codes (NCBI Gene or UniProtKB)

Transporter	Human Acession code	Rat Acesssion code
ugt1a7(c)		AF461738
ugt2a1		NM_022228
UGT2A3	NM_024743	
UGT2B4	NM_021139	
ugt2b5		M31109
UGT2B15	U06641	
UGT2B15	NM_001076	
UGT2B17	NM_001077	
UGT2B28	AF177272	
UGT8	NM_003360	

Appendix 7

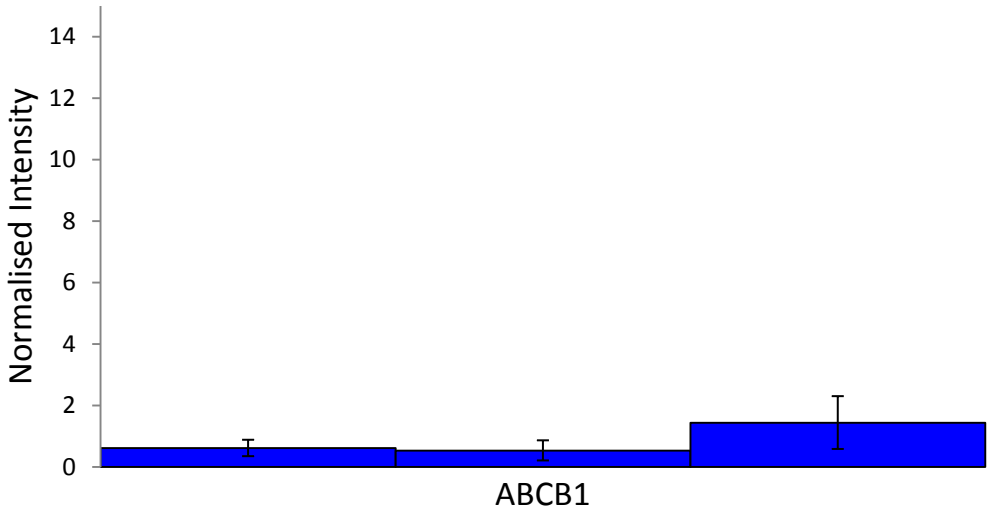


Figure 7A. The mRNA expression profile of ABCB1 in human placenta throughout gestation. The expression of ABCB1 throughout gestation in the developing placenta in human. Expression was normalised to *β-actin*. Values are expressed as mean ± SD of triplicate (rat) and quadruple (human) microarrays. On the graph, the bars, left to right are T1, T2 and T3 (T = trimester)

Appendix 8

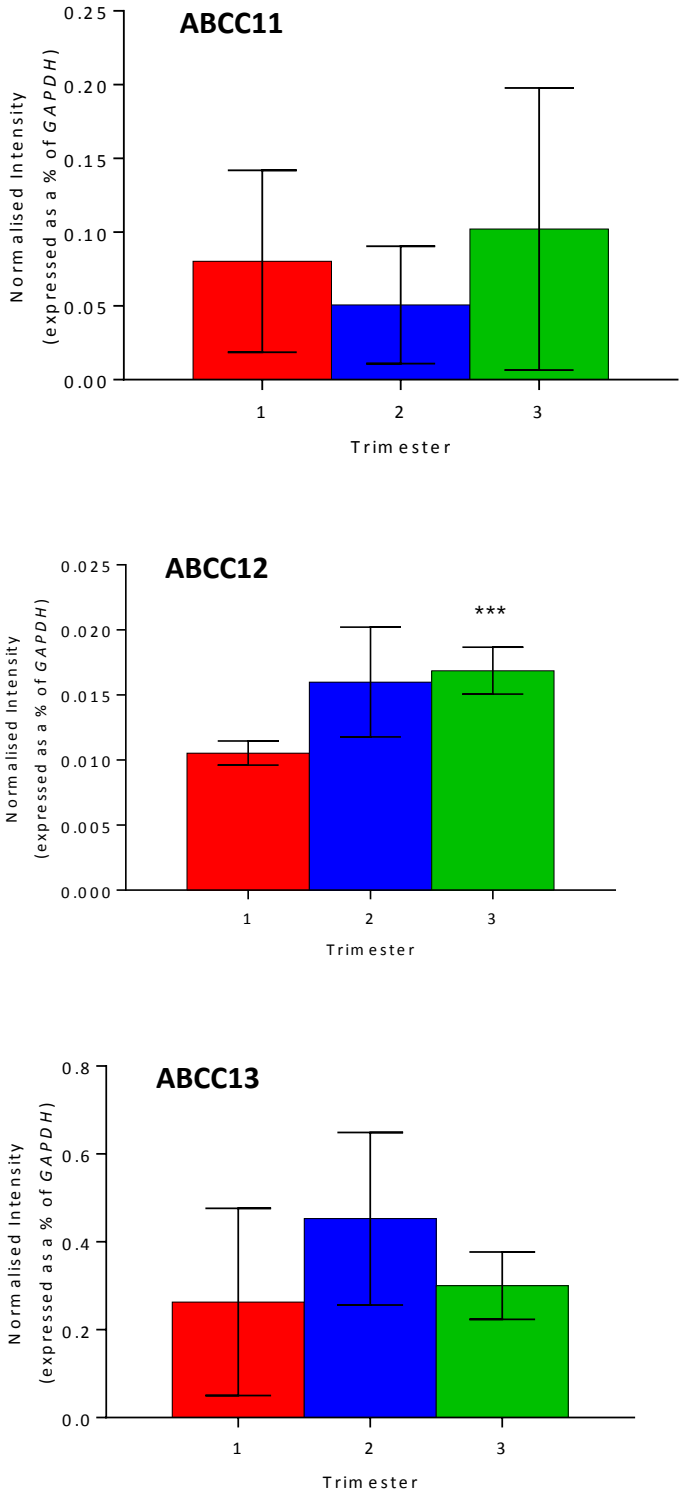


Figure 8A. The mRNA expression profile of ABCC11, ABCC12 and ABCC13 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Statistical analysis was performed using the Student's T-test. Asterisks (*** = p< 0.001) denote T3 intensity values are statistically different from T1. Values are expressed as mean ± SD. N = 4 individual placenta for human

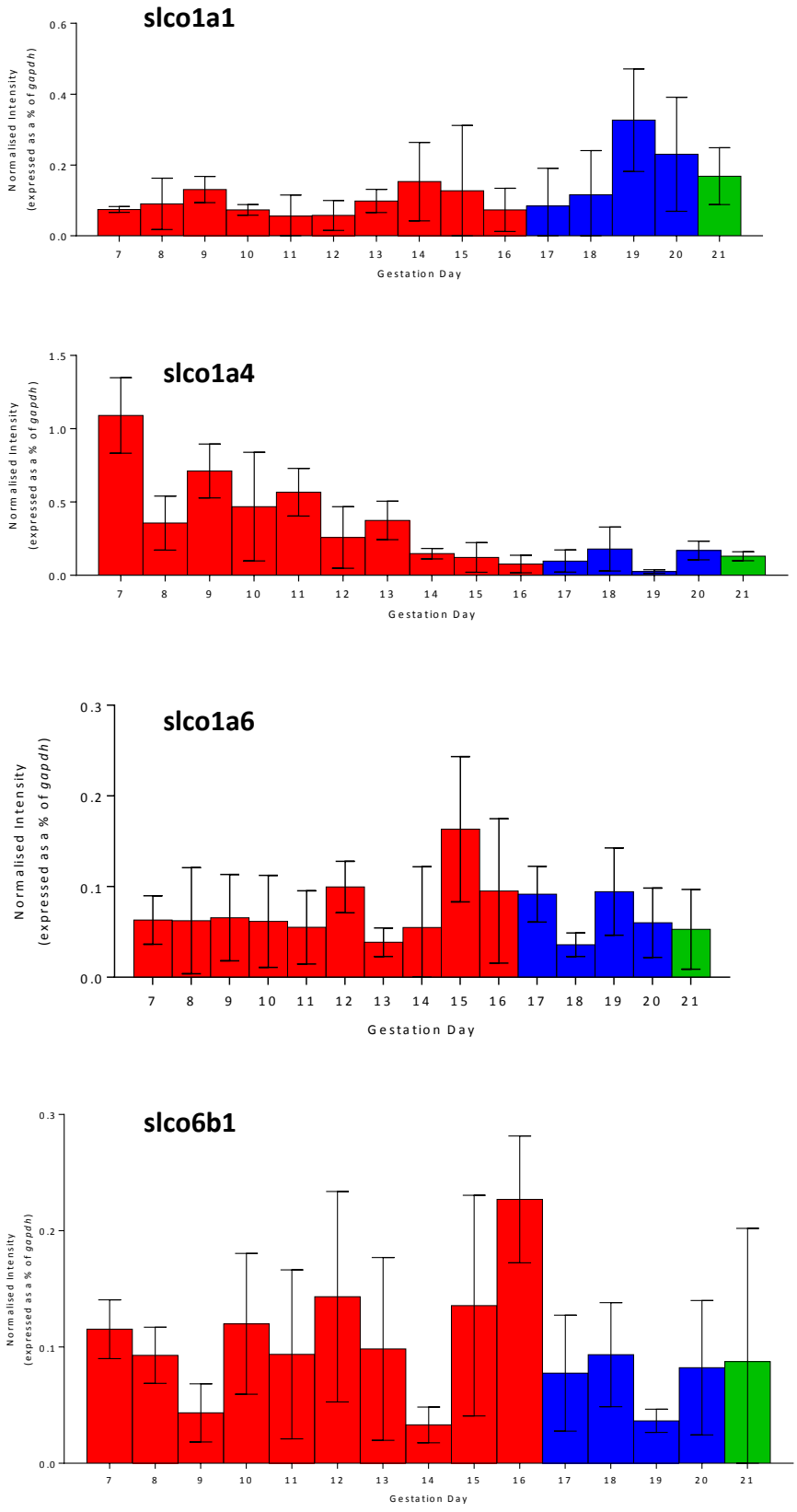


Figure 8B. The mRNA expression profile of slco1a1, slco1a4, slco1a6 and slco6b1 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

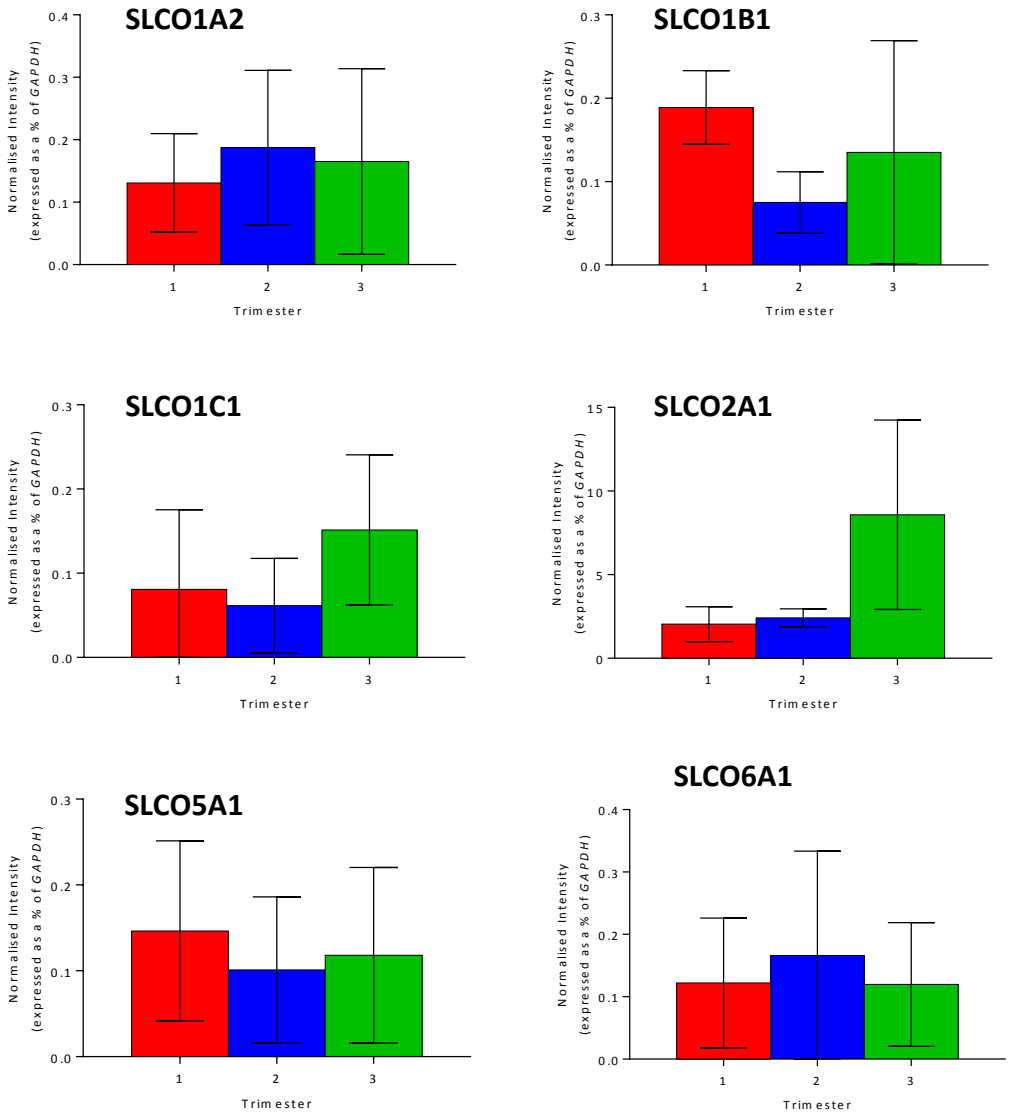


Figure 8C. The mRNA expression profile of SLCO1A2, SLCO1B1, SLCO1C1, SLCO2A1, SLCO5A1 and SLCO6A1 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

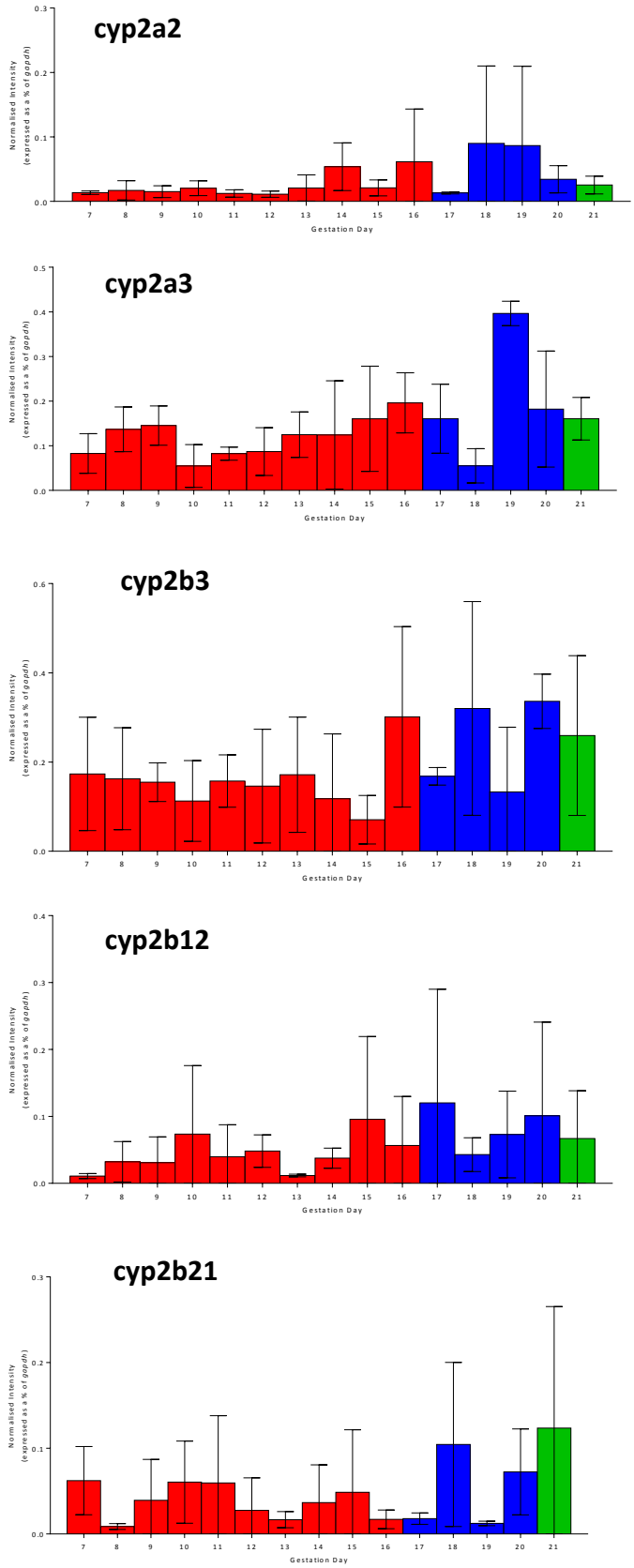


Figure 8D. The mRNA expression profile of cyp2a2, 2a3, 2b3, 2b12 and 2b21 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

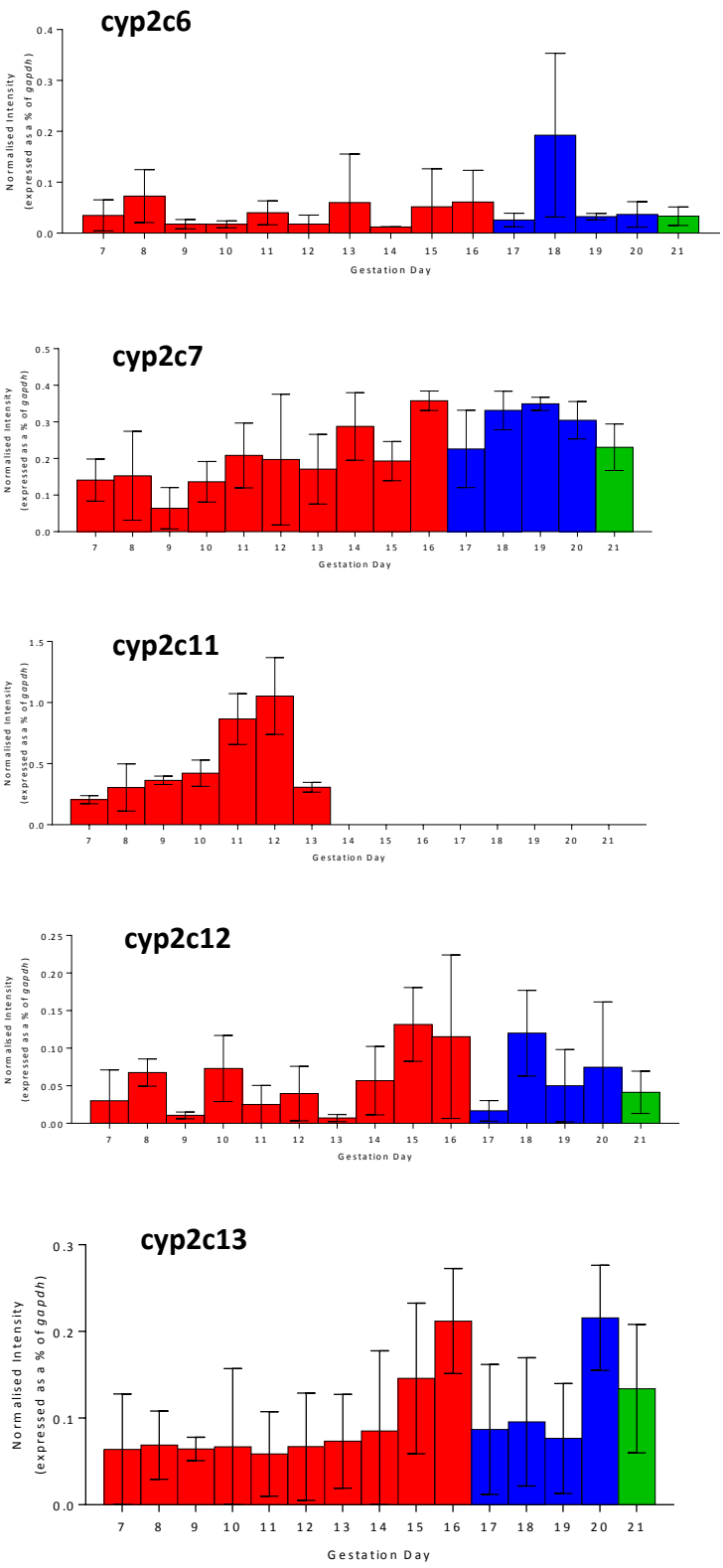


Figure 8E. The mRNA expression profile of cyp2c6, 2c7, 2c11, 2c12 and 2c13 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

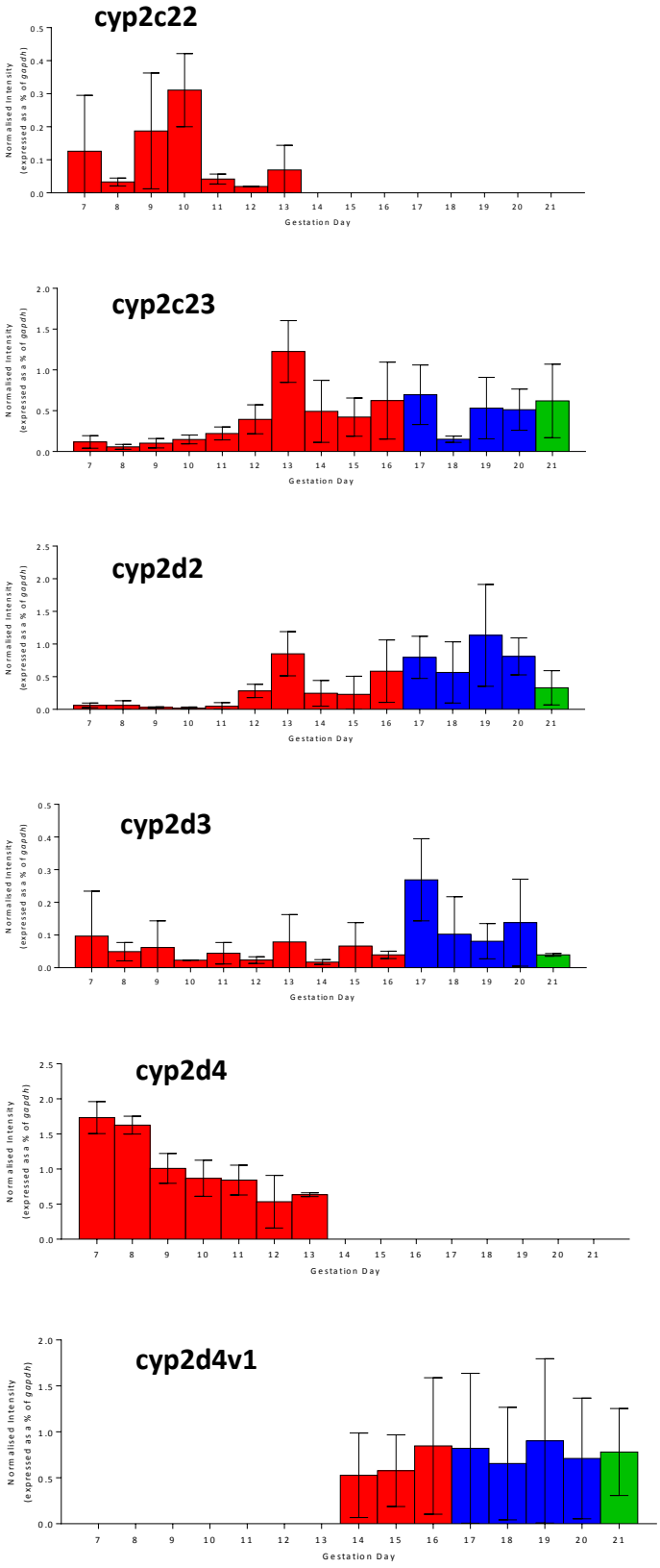


Figure 8F. The mRNA expression profile of cyp2c22, 2c23, 2d2, 2d3, 2d4 and 2d4v1 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

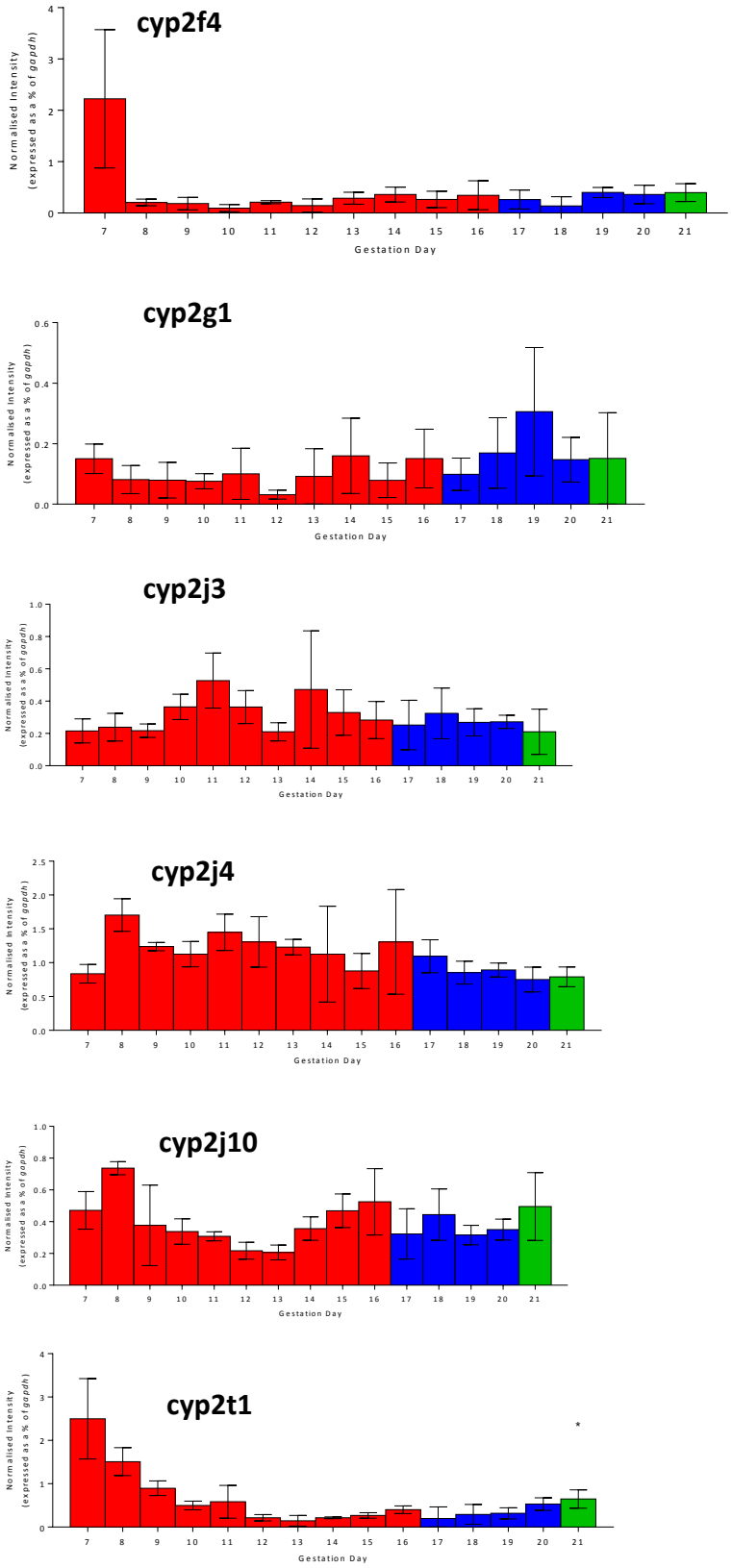


Figure 8G. The mRNA expression profile of cyp2f4, 2g1, 2j3, 2j4, 2j10 and 2t1 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Statistical analysis was performed using the Student's T-test. Asterisks (* = p <0.05) denote GD 21 intensity values are statistically different from GD 7 Values are expressed as mean ± SD of triplicate microarrays.

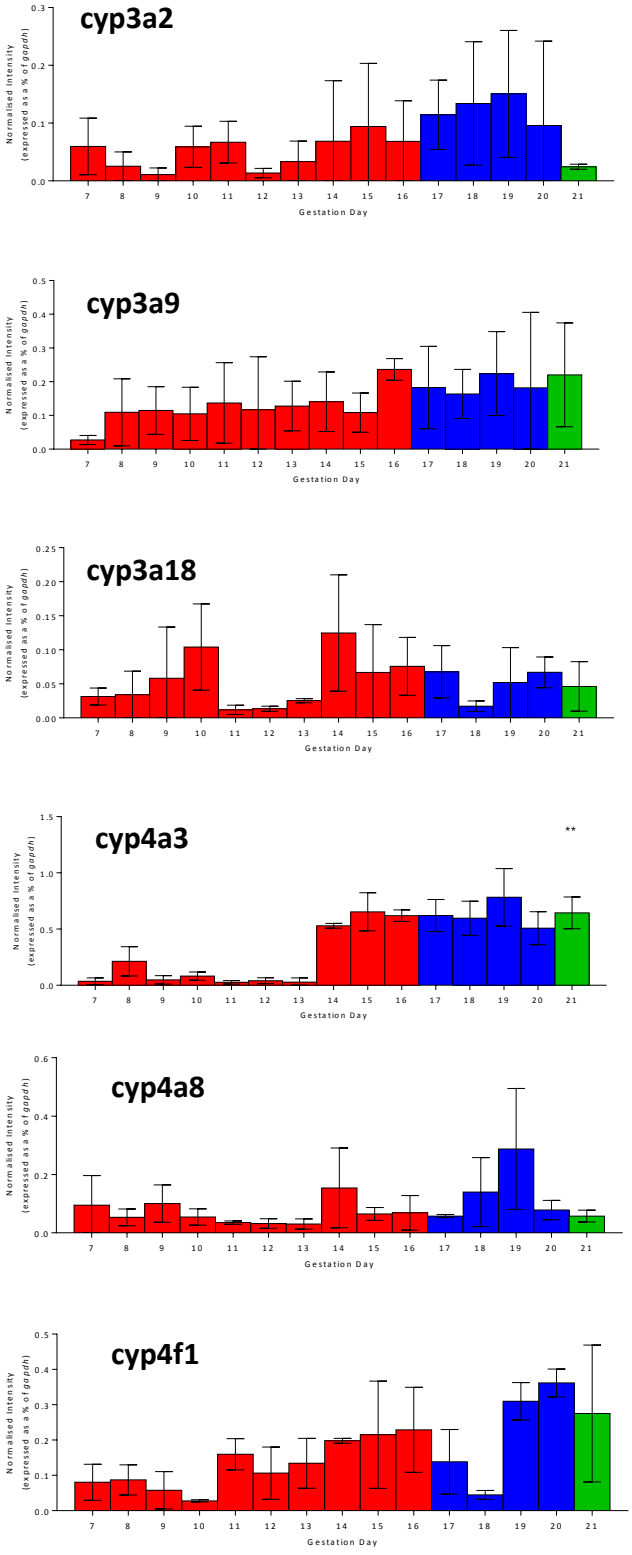


Figure 8H. The mRNA expression profile of cyp3a2, 3a9, 3a18, 4a3, 4a8 and 4f1 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Statistical analysis was performed using the Student's T-test. Asterisks (** = $p < 0.01$) denote GD 21 intensity values are statistically different from GD 7 Values are expressed as mean ± SD of triplicate microarrays.

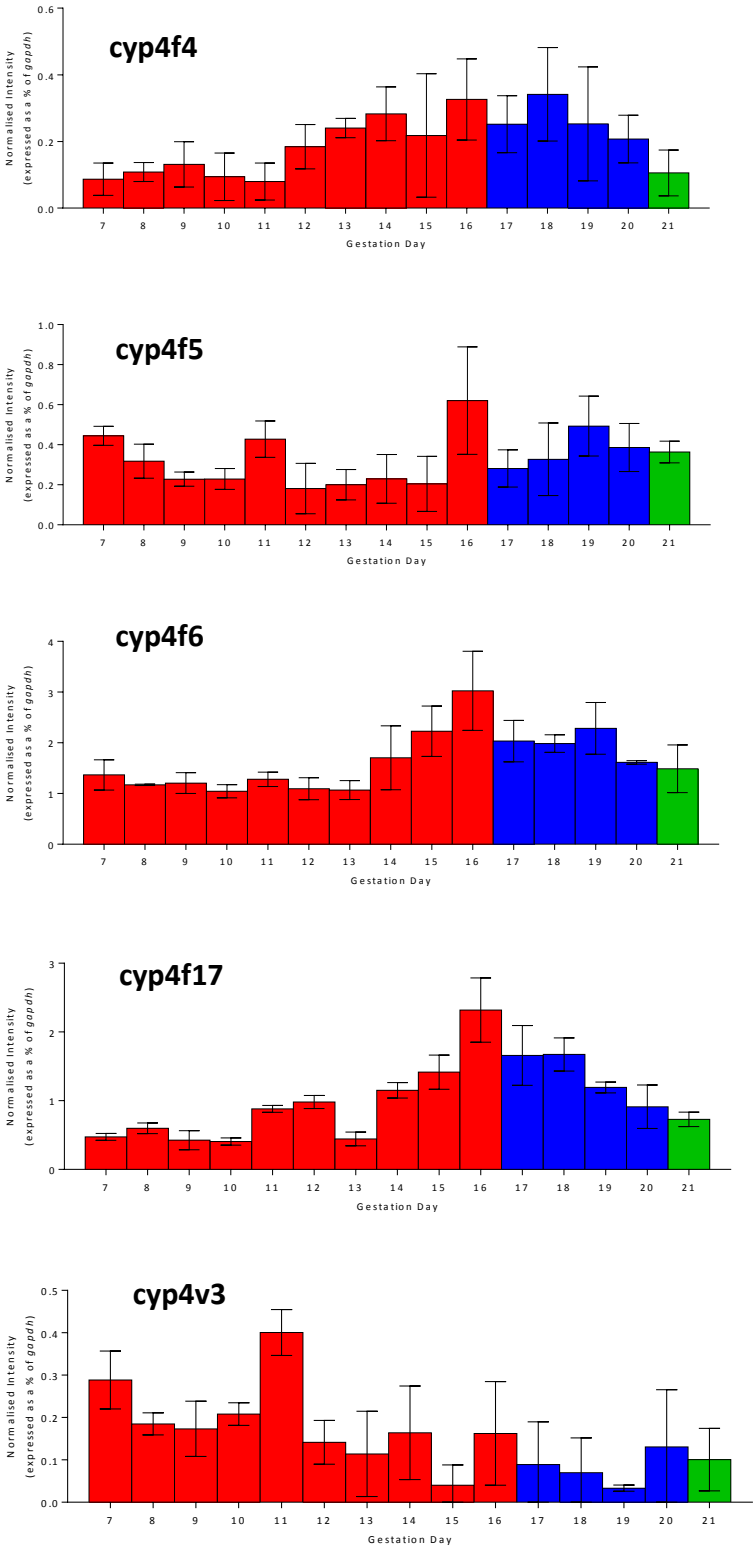


Figure 8I. The mRNA expression profile of cyp4f4, 4f5, 4f6, 4f17 and 4v3 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

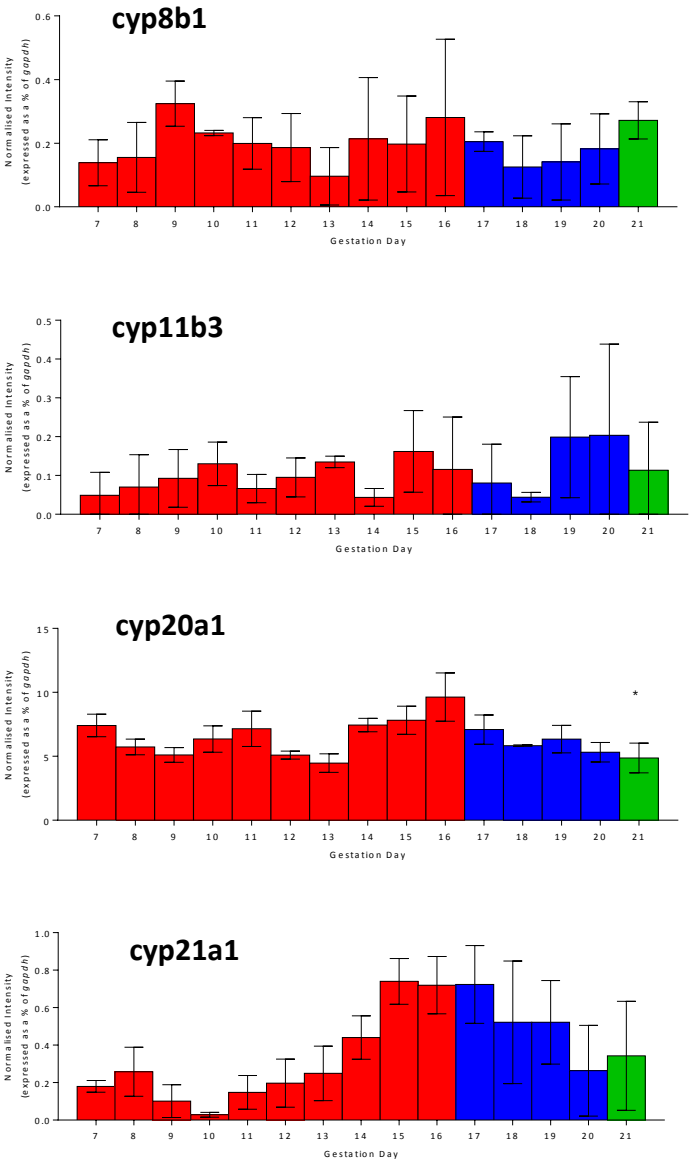


Figure 8J. The mRNA expression profile of cyp8b1, 11b3, 20a1, and 21a1 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Statistical analysis was performed using the Student's T-test. Asterisks (* = $p < 0.05$) denote GD 21 intensity values are statistically different from GD 7. Values are expressed as mean ± SD of triplicate microarrays.

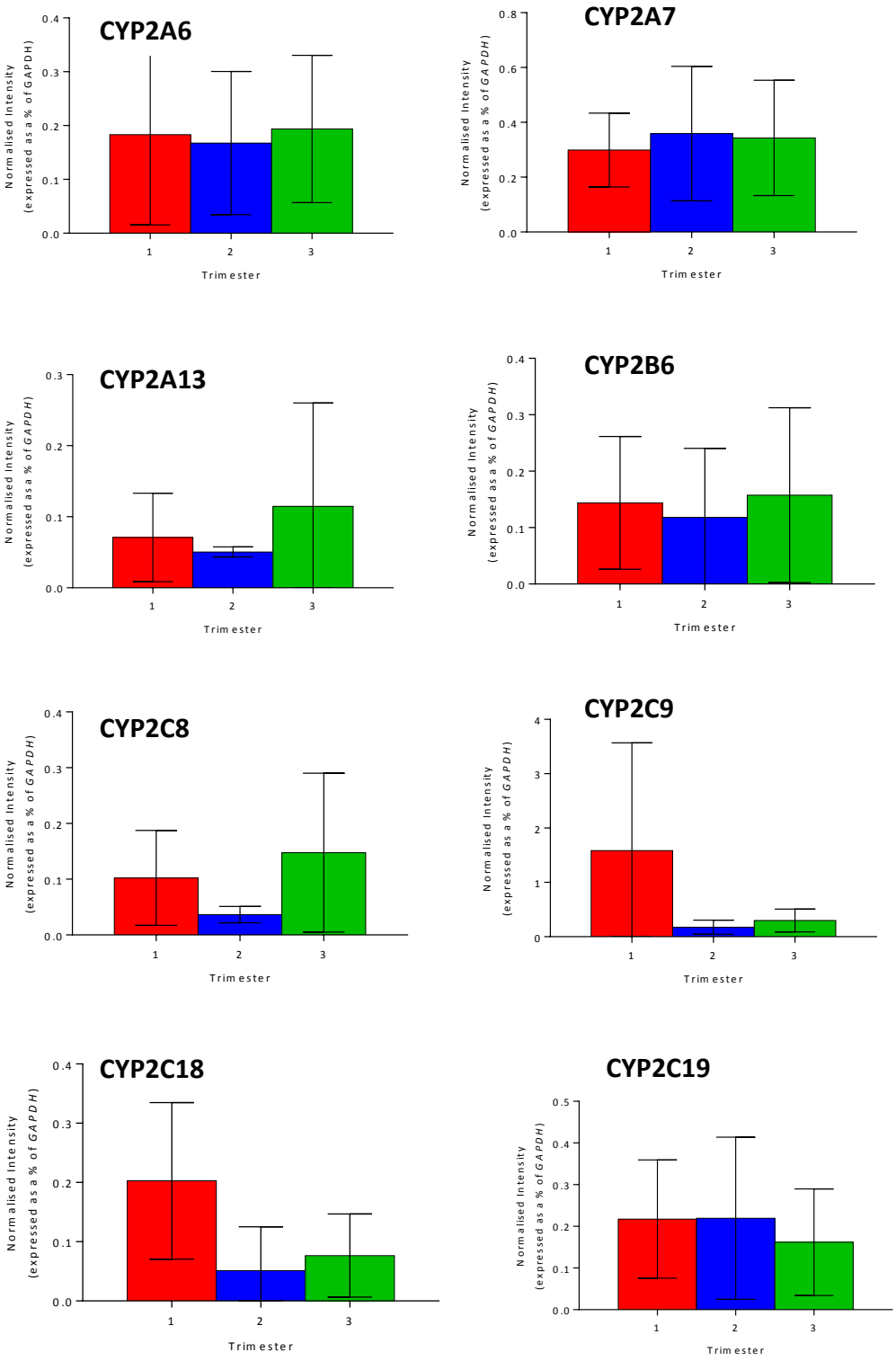


Figure 8K. The mRNA expression profile of CYP2A6, 2A7, 2A13, 2B6, 2C8, 2C9, 2C18 and 2C19 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

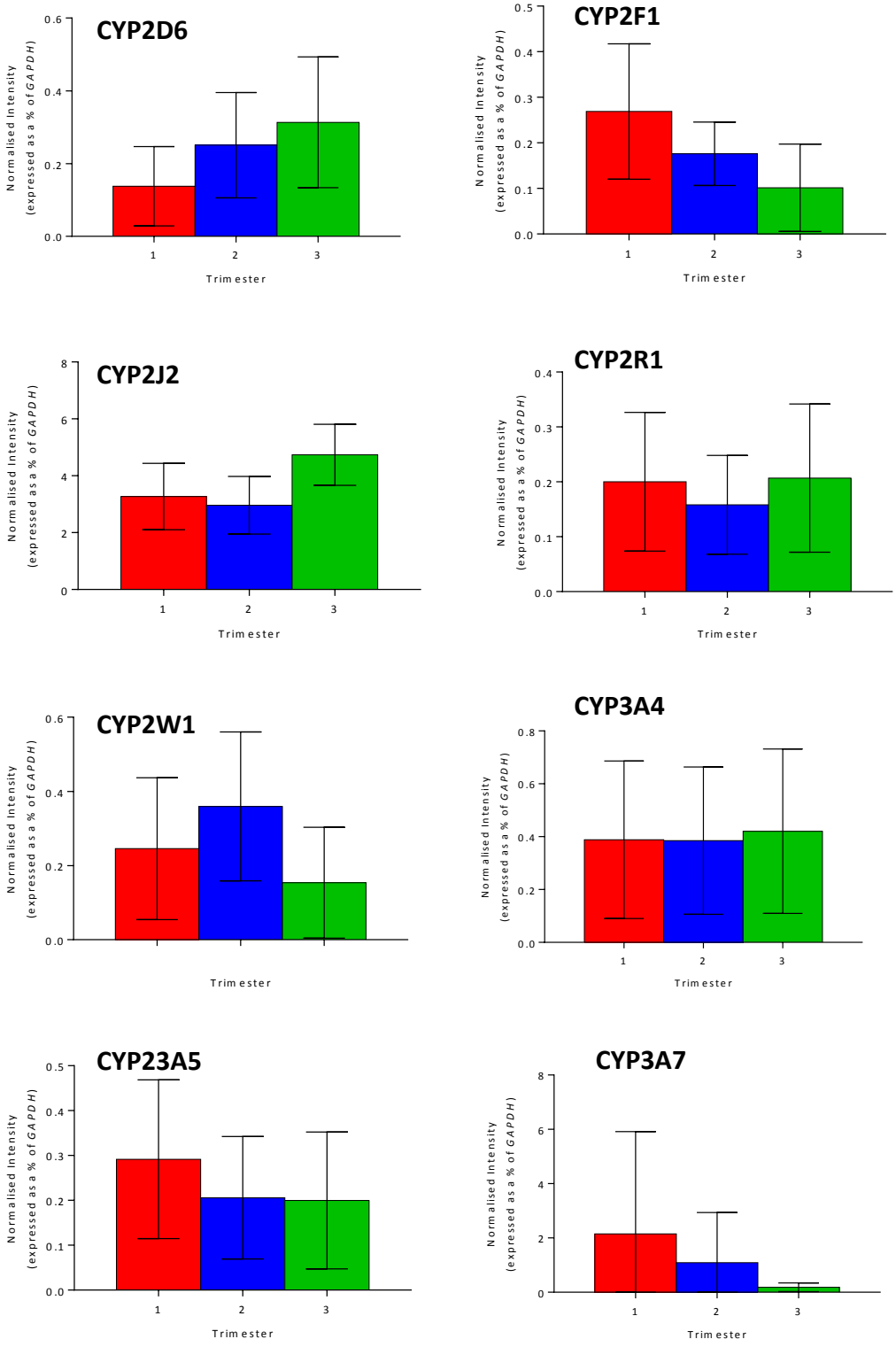


Figure 8L. The mRNA expression profile of CYP2D6, 2F1, 2J2, 2R1, 2W1, 3A4, 3A5 and 3A7 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

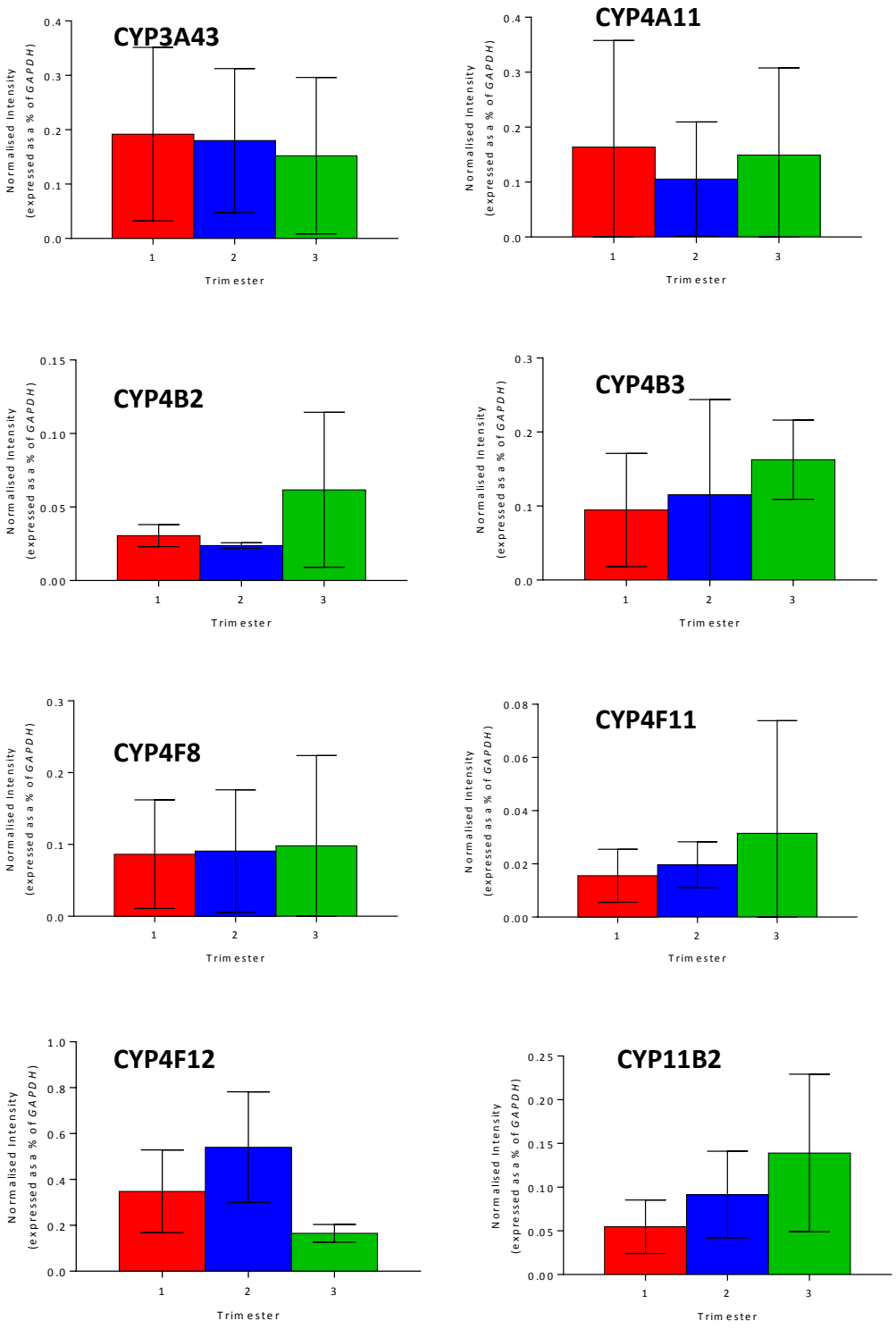


Figure 8M. The mRNA expression profile of CYP3A43, 4A11, 4B2, 4B3, 4F8, 4F11, 4F12 and 11B2 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

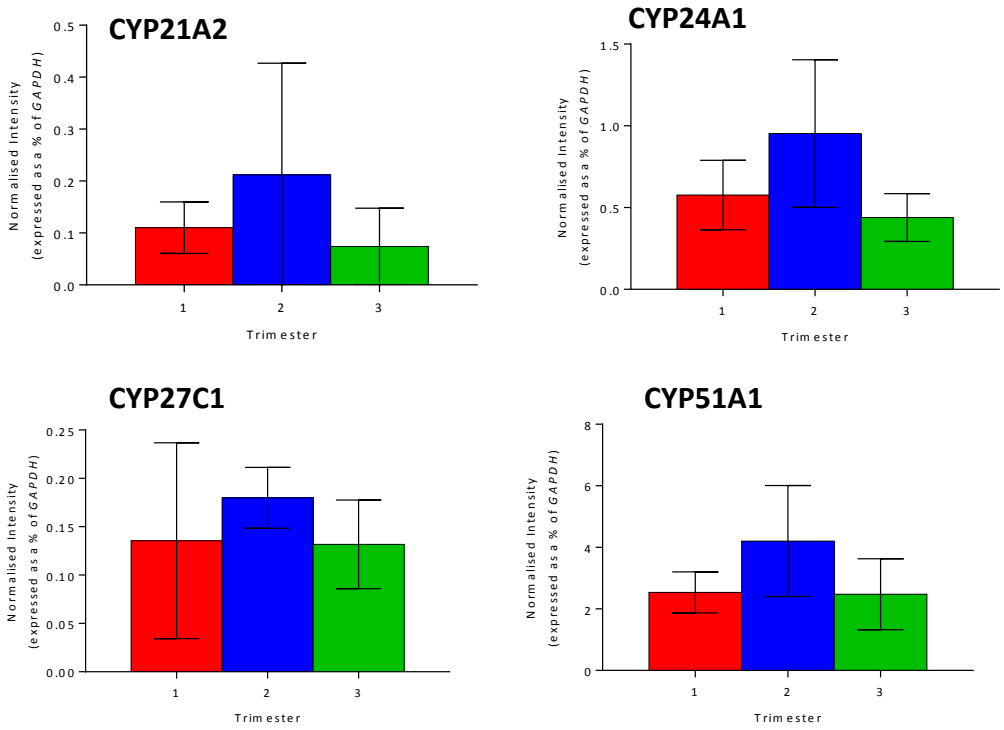


Figure 8N. The mRNA expression profile of CYP21A2, 24A1, 27C1 and 51A1 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

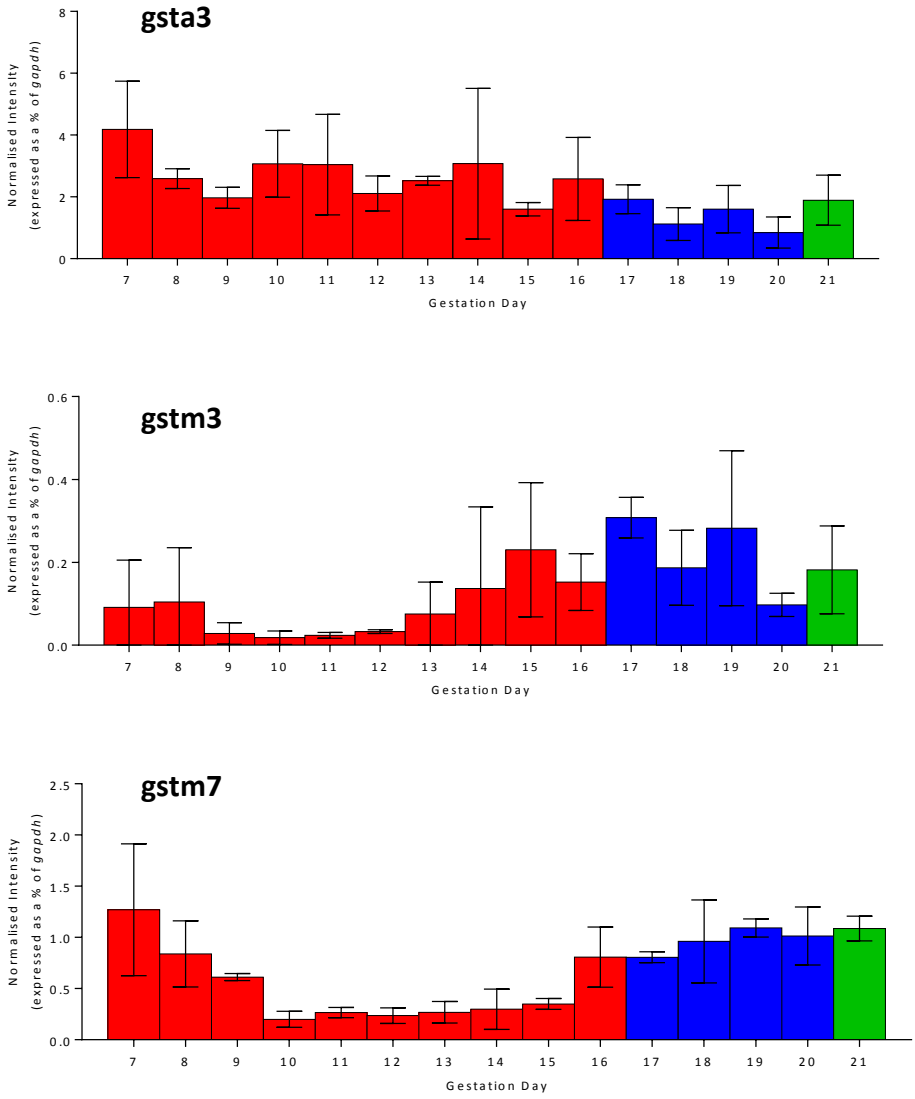


Figure 8O. The mRNA expression profile of gsta3, gstm3 and gstm7 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

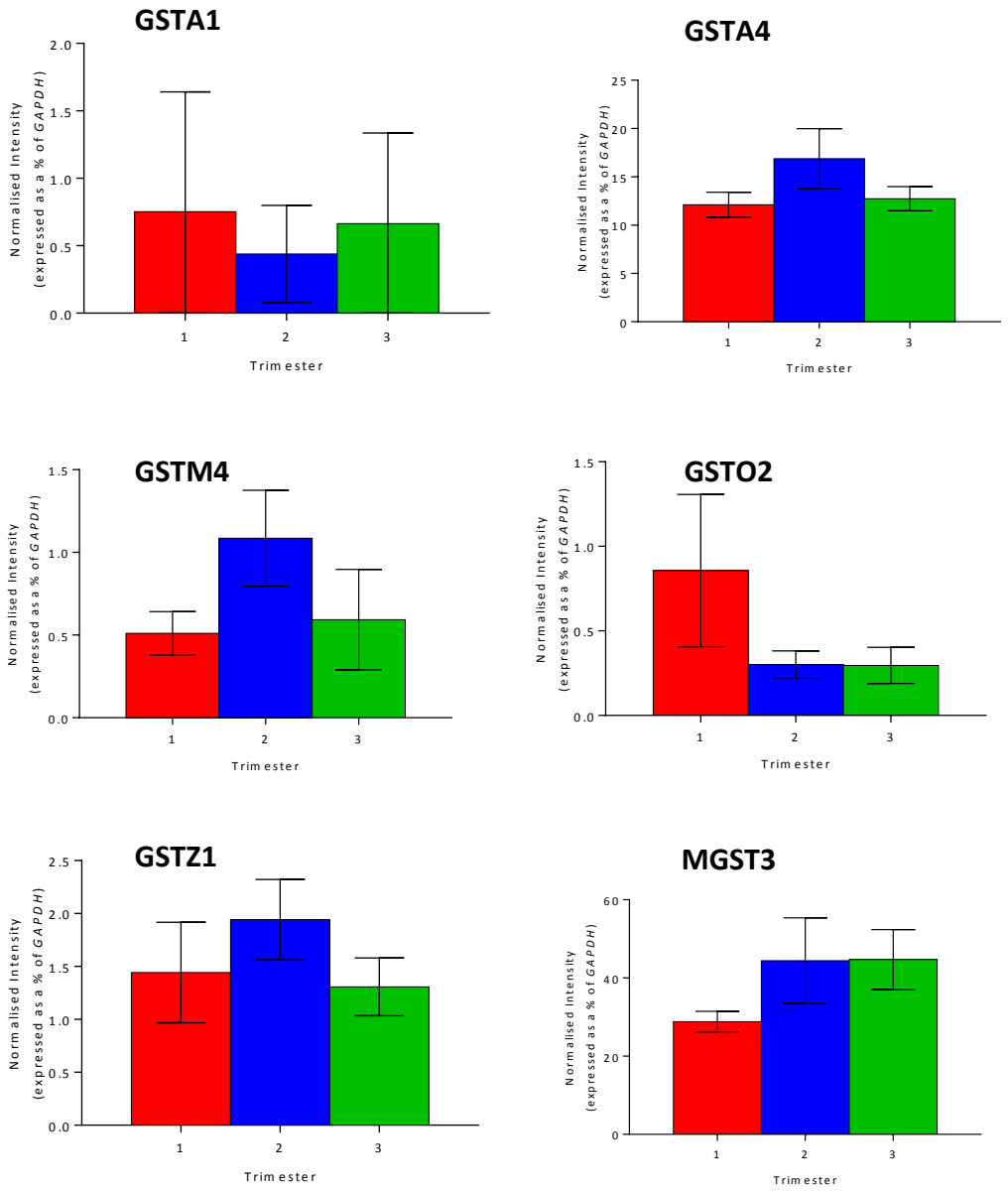


Figure 8P. The mRNA expression profile of GSTA1, A4, M4, O2, Z1 and MGST3 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

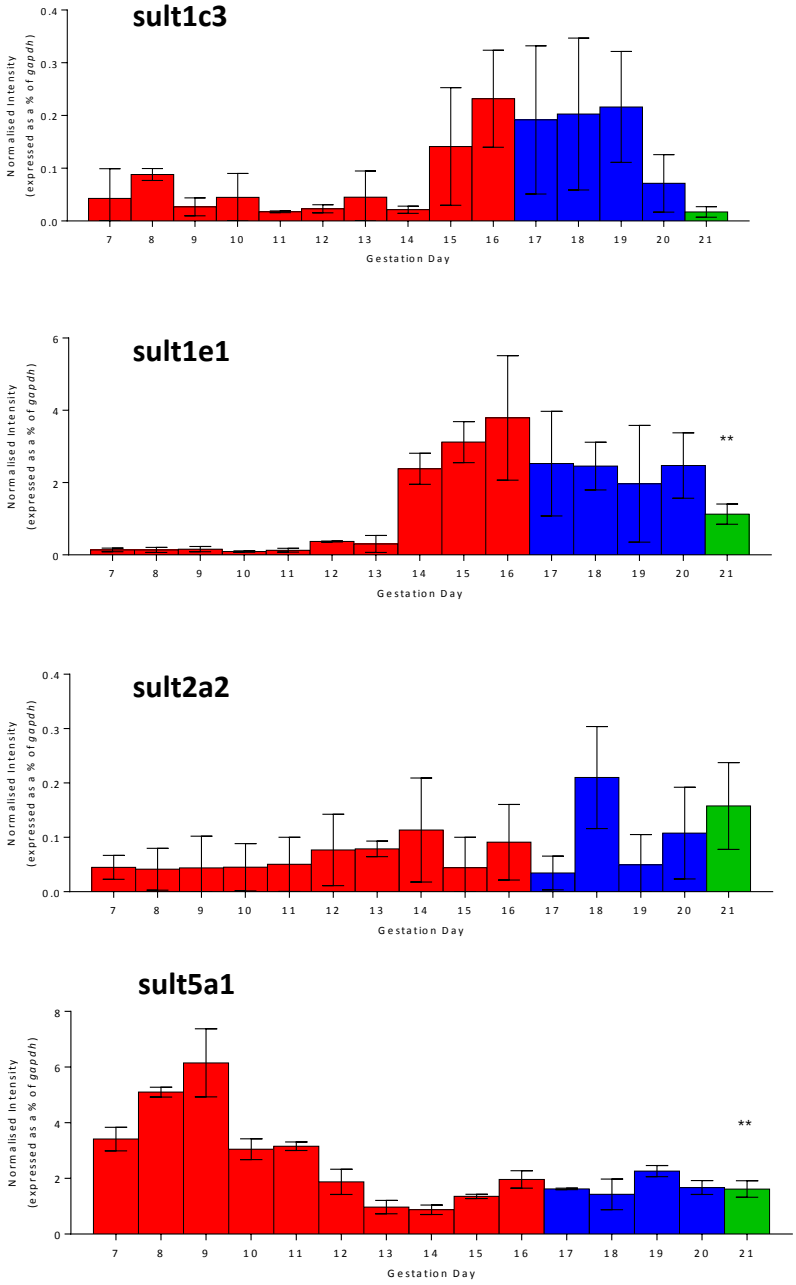


Figure 8Q. The mRNA expression profile of sult1c3, 1e1, 2a2 and 5a1 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Statistical analysis was performed using the Student's T-test. Asterisks (** = $p < 0.01$) denote GD 21 intensity values are statistically different from GD 7 Values are expressed as mean ± SD of triplicate microarrays.

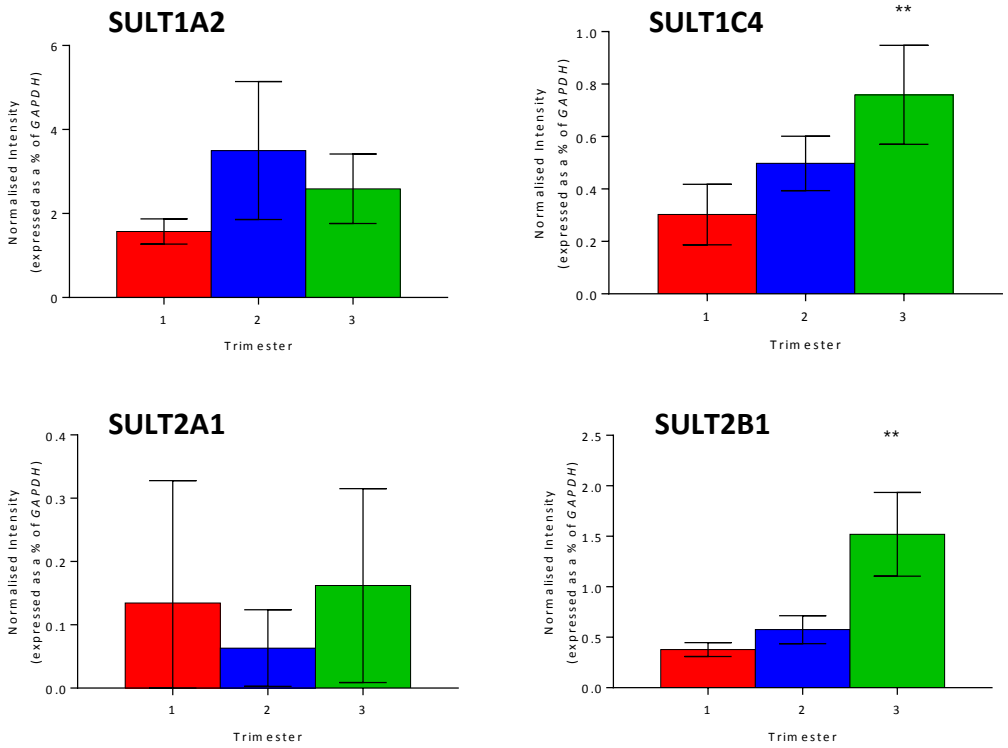


Figure 8R. The mRNA expression profile of SULT1A2, 1C4, 2A1 and 2B1 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Statistical analysis was performed using the Student's T-test. Asterisks (** = p< 0.01) denote T3 intensity values are statistically different from T1. Values are expressed as mean ± SD. N = 4 individual placenta for human

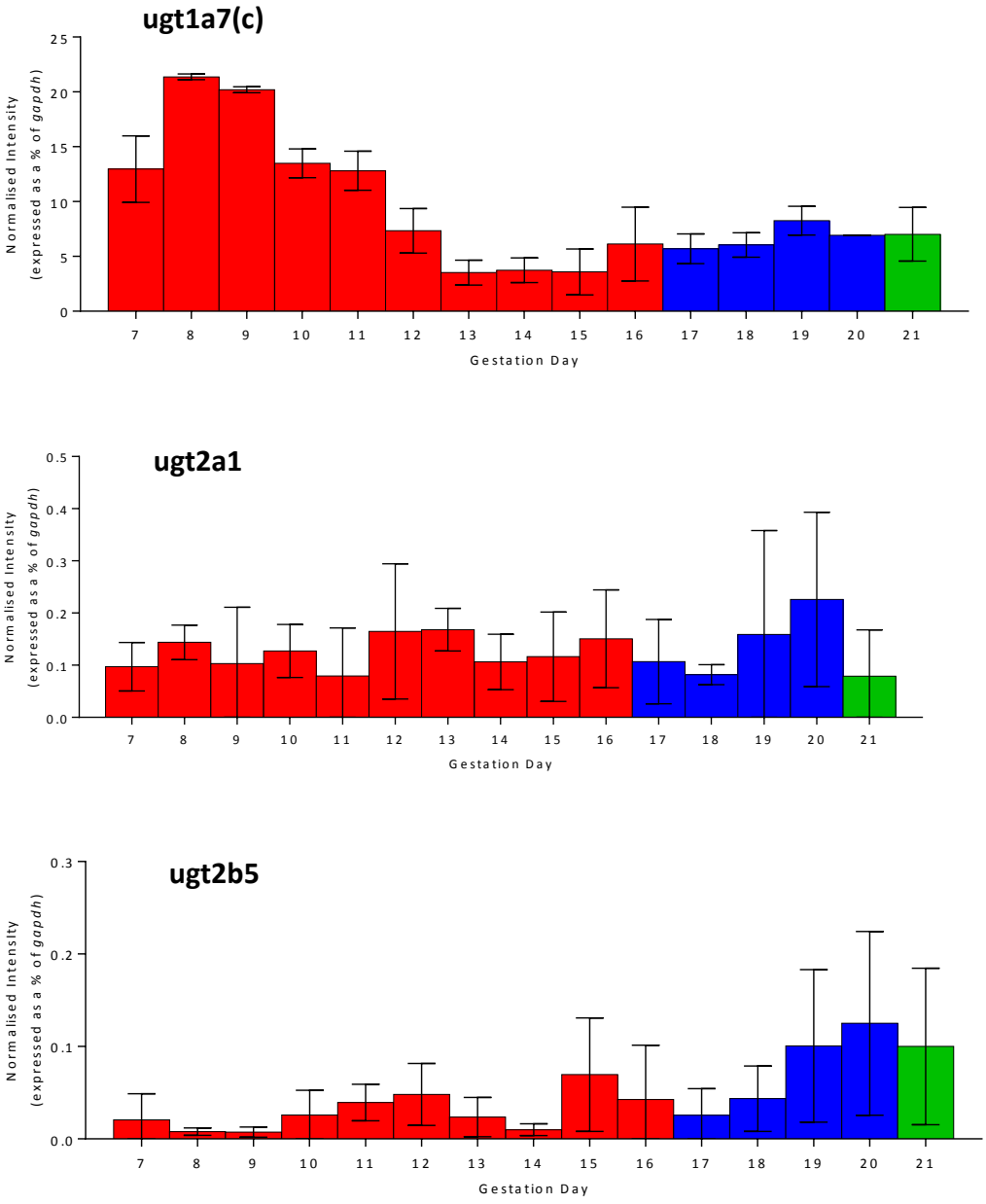


Figure 8S. The mRNA expression profile of ugt1a7(c), 2a1 and 2b5 in the rat placenta throughout gestation. Expression was normalised using expression of the *gapdh* housekeeping gene. Values are expressed as mean ± SD of triplicate microarrays.

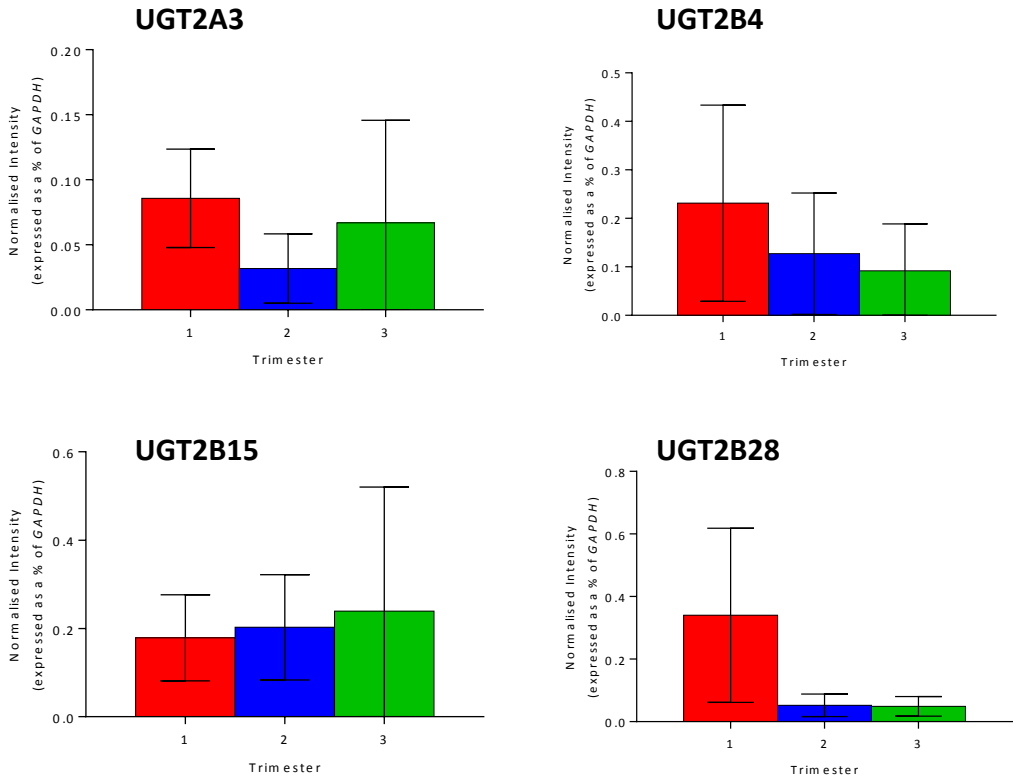


Figure 8T. The mRNA expression profile of UGT2A3, 2B4, 2B15 and 2B28 in the human placenta throughout gestation. Expression was normalised using expression of the *GAPDH* housekeeping gene. Values are expressed as mean ± SD. N = 4 individual placenta for human

Appendix 9

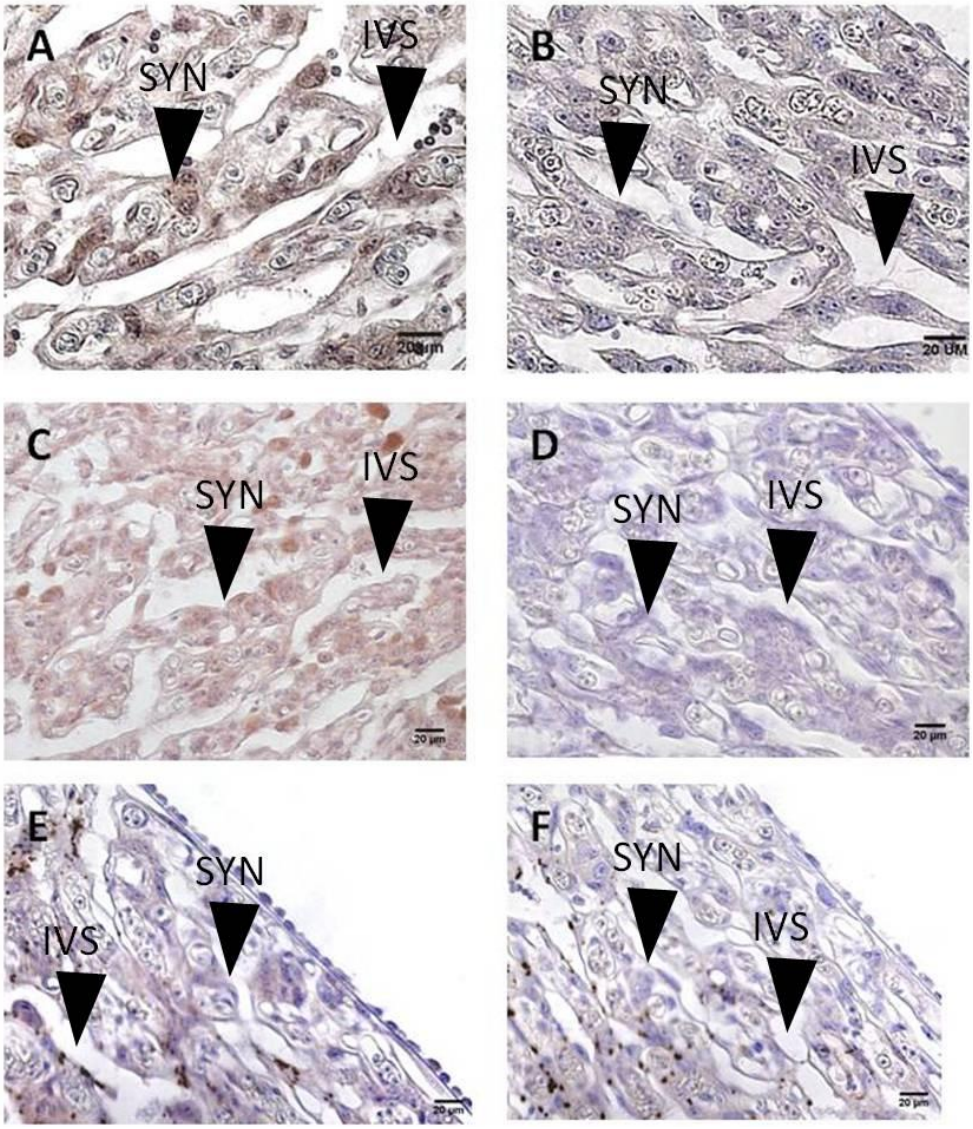


Figure 9A Optimisation of cyp26b staining using immunohistochemistry. Cyp26b was localised in the rat placenta at GD 16 (A - F), in the rat placenta at GD16 Sections were de-parafinised through a series of xylene and ethanol solutions. Antigen retrieval was performed followed by a peroxidase block (15 mins). A serum block was applied for 1 hour, then the primary polyclonal anti-cyp26b antibody (1 in 100 in 5 % (w/v) BSA in PBS at 4°C overnight) (A) or primary polyclonal anti-cyp26b antibody (1:100 in 5 % (w/v) BSA in PBS at RT overnight) (C) or primary polyclonal anti-cyp26b antibody (1 in 200 in 5 % (w/v) BSA in PBS at RT overnight) (E) or a rabbit polyclonal IgG isotype antibody (diluted 1:100 in 5 % (w/v) BSA in PBS at 4°C overnight) (B) or a rabbit polyclonal IgG isotype antibody (diluted 1 in 100 in 5 % (w/v) BSA in PBS at RT overnight (D) or a rabbit polyclonal IgG isotype antibody (diluted 1 in 200 in 5 % (w/v) BSA in PBS at RT overnight (F) was applied. A secondary HRP-linked antibody (prepared by Dako) was added for 1 hour then DAB was added for visualisation of staining. Specimens were viewed using an *Olympus BX51* upright microscope using a 4*0x* objective and captured using a *Coolsnap ES camera (Photometrics)* through *MetaVue Software (Molecular Devices).* Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij).

Appendix 10

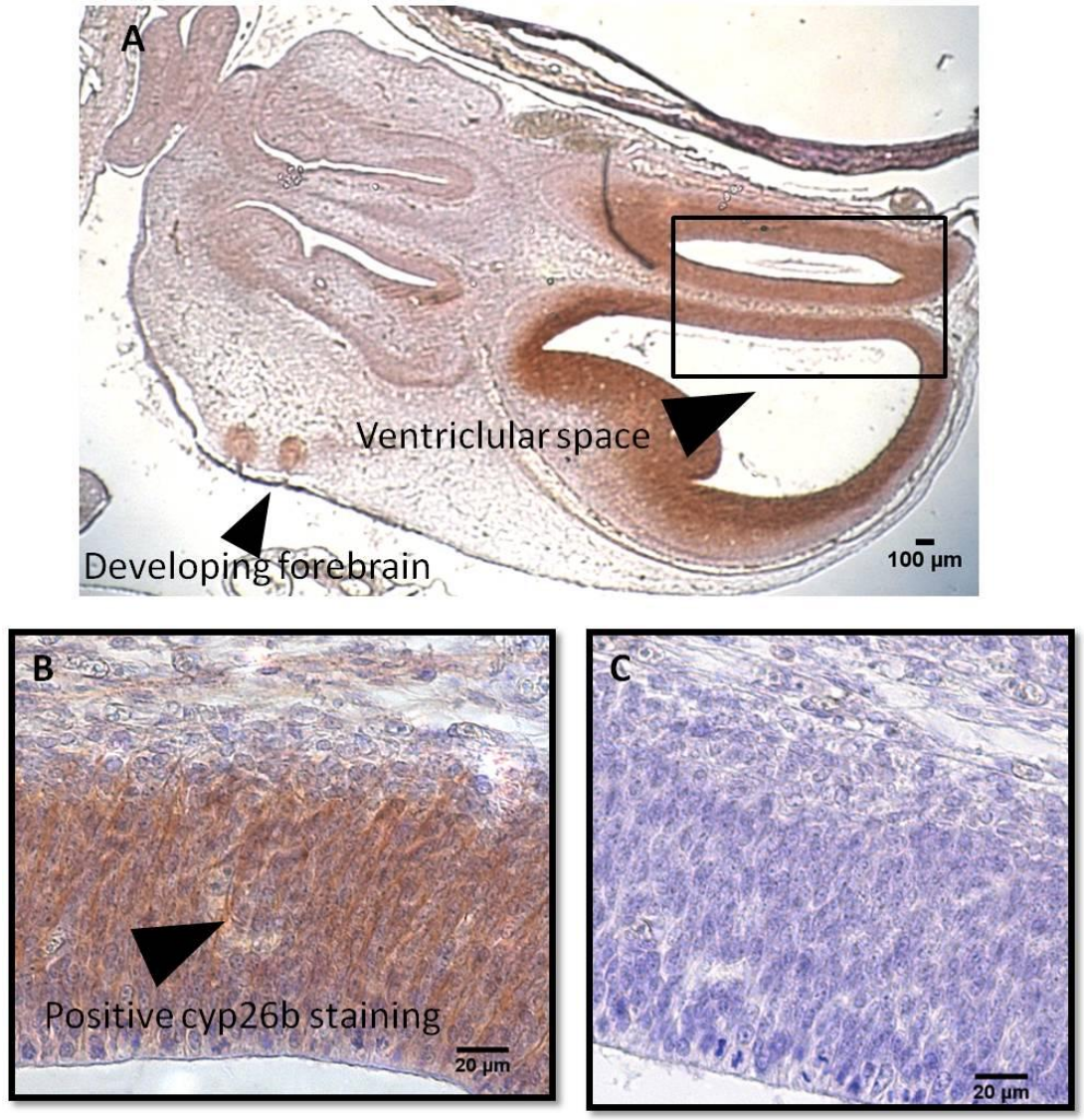


Figure 10A Figure 9A Optimisation of cyp26b staining using immunohistochemistry. Cyp26b was localised in the developing rat foetus brain (A and B) at GD 16. Sections were de-parafinised through a series of xylene and ethanol solutions. Antigen retrieval was performed followed by a peroxidase block (15 mins). A serum block was applied for 1 hour, then the primary polyclonal anti-cyp26b antibody (1:100 in 5 % (w/v) BSA in PBS at 4°C overnight) (A and B) or a rabbit polyclonal IgG isotype antibody (diluted 1: 100 in 5 % (w/v) BSA in PBS at 4°C overnight) (C) was applied. A secondary HRP-linked antibody (prepared by Dako) was added for 1 hour then DAB was added for visualisation of staining. Specimens were viewed using an *Olympus BX51* upright microscope using a 4*0x* objective and captured using a *Coolsnap ES camera (Photometrics)* through *MetaVue Software (Molecular Devices)*. Images were then processed and analysed using ImageJ (http://rsb.info.nih.gov/ij).

Appendix 11

Table 11A Plasma levels of Atorvastation, Quinidine, Saquinavir and Verapamil. Values taken from the literature.

Xenobiotic	MW	Human Plasma Levels[a] (C_{max})	fu_p[b]	Concentration[c] (μM) (corrected for using fu_p)	Ref[d]
Atorvastatin	558.64	66.2 ± 46.2 ng/ml (80 mg dose)		0.002	Lins et al., 2003
		65.55 ± 46.74 ng/ml (20 mg dose)	0.02	0.002	Kruger et al., 2009
Quinidine	324.417	2.78 ± 0.87 ug/ml (4 mg/kg IV)		2.228	Benton et al., 2000
		2.42 ug/ml (200 mg tablet)	0.26	1.939	Frigo et al. 1977
Saquinavir	670.841	6.1 ng/ml (600 mg dose)		0.0003	Burhenne et al., 2003
		14 ± 22 ng/ml (600 mg dose)	0.028	0.001	Kupferschmidt et al., 1998
Verapamil	454.59	282.8 ng/ml (160 mg dose)		0.058	Voegelgesang et al., 1984
		57.35 ng/ml (80 mg dose)	0.093	0.126	Borges et al., 2005

[a] Mean ± SD.
[b] fu_p values taken from (Gertz et al., 2010)
[c] Mean C_{max} value used.
[d] Ref listed for C_{max} value

Appendix 12

The following 'PDF' copy was published in Reproductive Toxicology. The work outlines preliminary findings from Chapter 3.

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Short communications

Differential expression of xenobiotic transporters and metabolising enzymes in the rat placental barrier throughout gestation

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The placenta can protect the developing foetus from xenobiotics through a variety of transporter proteins and metabolising enzymes. The expression and function of these proteins have yet to be fully elucidated, particularly in animal models that are used to evaluate developmental toxicity. The primary objective of this study was to explore the pattern of expression of xenobiotic transporter proteins and metabolising enzymes in rat placenta throughout gestation. Utilising placental/foetal tissue from time mated female rats (gestation days 6–21), genomic analysis was carried out, using Affymetric microarray and bioinformatic tools (ArrayTrack™), to explore the expression profiles of xenobiotic transporters and metabolising enzymes throughout the gestation period. A range of transporters were identified, and then further explored using western blot and immunohistochemical analysis of placental/foetal tissue obtained from the same animals. Changes in mRNA expression levels were observed in 100+ transporter and metabolising enzyme genes from a number of families as gestation progressed. These included the multidrug resistance protein (P-glycoprotein, abcb1), the breast cancer resistance protein (bcrp, abcg2), multi-drug resistance-associated proteins (mrps; abcc transporters) and organic-anion transporting polypeptides (oatps) transporter families, and uridine diphosphate glucuronosyltransferase, sulfotransferases, glutathione S-transferases, and cytochrome P450 metabolising enzymes families. Individual genes from the different families showed differential mRNA expression as gestation progressed predominantly from days 14 to 21 (time points where the placenta was separated from the foetus). For example Cyp17a1 mRNA expression levels showed a 4-fold decrease at day 21 compared to day 14, Oatp4a1 mRNA expression levels were 7-fold higher at day 21 compared to day 14 and no change in expression was observed in mrp1 and abcg2. Western blot analysis confirmed expression of abcb1 and immunohistochemistry confirmed localisation of the transporter proteins abcb1, abcg2, and mrp2 within the placenta. Abcb1 and abcg2 were localised in the endothelial cells of the labyrinth region, whereas mrp2 was localised to the yolk sac mesoderm. Further work is ongoing to explore the expression and localisation of Oatp4a1 between days 14 and 21. This work will aid our understanding of the changes in expression patterns of transporter proteins and metabolising enzymes throughout gestation in the rat. It will also help to improve our understanding of the protective properties of the rat placenta, and provide a foundation for comparison of the differential protective properties of the placenta between other mammalian species. These data will be utilised to aid the validation of a surrogate *in vitro* model system.

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Chemical class-specific gene expression changes in the zebrafish embryo after exposure to three glycol ether alkoxy acids and two 1,2,4-triazole antifungals

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The zebrafish embryotoxicity test (ZET) is an alternative test to predict embryotoxicity of substances. The classical read-out of this assay is based on morphological assessment of the embryos. Implementing transcriptomic techniques such as microarray may increase the sensitivity and objectivity of the test system. In this study we applied the category approach to study the common effects of compounds from a similar class. 8–64 cell stage zebrafish embryos were exposed to two 1,2,4-triazole derivatives, flusilazole (FLU) and cyproconazole (CYP), and three glycol ether metabolites, methoxyacetic acid (MAA), ethoxyacetic acid (EAA) and phenoxyacetic acid (PAA) of which PAA was a negative control. Equipotent concentrations of all compounds were used, as was previously determined by the general morphology score (GMS) at 72 h post fertilization (hpf). After 24 hpf embryos were collected and total RNA was isolated. Samples were prepared and hybridized to microarrays and analysis showed that MAA and EAA revealed several thousands of transcriptionally responsive genes, as for the triazoles only several hundreds of genes were regulated after expo-

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Chapter 8

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