The Role of Multidrug Resistance Proteins in Determining Fetal Susceptibility to Drugs of Misuse

A thesis submitted to The University of Manchester for the degree of

PhD in the Faculty of Medical and Human Development

2013

DEIRDRE THAJAM

School of Medicine

Maternal and Fetal Health Research Centre

Institute of Human Development

Word count: 53,732

List of Tables

List of Figures

Candidate: Deirdre Thajam, University of Manchester. **Degree**: PhD, May 2013
Title: The Role of Multidrug Resistance Proteins in Determining Fetal Susceptibility to Drugs of Misuse.

Background- Negative outcomes from fetal exposure to maternal dug use include Neonatal Abstinence Syndrome (NAS) and altered development, the unpredictability of which suggests a biological element as yet not accounted for. The manner in which the human placenta protects the fetus from xenobiotics such as drugs of misuse is not completely characterised. However, Adenosine Triphosphate Binding Cassette (ABC) transporters in placentae have demonstrated their ability to efflux xenobiotics away from the fetal vascular compartment leading to lower concentrations than in the maternal compartment and some commonly used drugs have been shown to be substrates for these proteins, e.g. methadone. It is suggested that polymorphisms in the genes that encode these transporter proteins may alter their expression and/or function.

Hypothesis- Polymorphisms (SNPs) in the ABC transporters ABCB1, ABCG2, ABCC1 and ABCC2 change protein expression and/or function leading to increased fetal exposure demonstrated by increased signs of NAS and/or altered development.

Objectives- To determine if genotype alters protein expression and whether there is a relationship between the level of placental multidrug resistance protein P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP), Multidrug Resistance Associated Proteins (MRP1 and MRP2) expression and neonatal and/or developmental outcomes.

Methods- Drug using women were recruited. In the immediate postnatal period placental tissue, cord blood and maternal hair samples were taken. Hair was analysed to determine drug use in the preceding 3 months, immunoblotting determined the level of P-gp, BCRP, MRP1 and MRP2 protein expression. Sequenom MassExtend Array produced genotypes from DNA obtained from cord blood. Infants were assessed for NAS at birth, 3 days and 3 weeks. At 8 months and 1 year development was assessed using the Griffiths Mental Development Scales. Plink was used to determine statistically significant associations between genotype and outcome phenotypes.

Results- The level of fetal drug exposure did not predict the need for pharmacological treatment for NAS. 32 polymorphisms with significant associations to outcome measures were identified: 4 SNPs significantly altered protein expression, (3 for P-gp and 1 for MRP1). 41 SNPs were associated with changes across 4 of the 5 GMDS subscales.

Discussion- No clear relationship between MDRP protein expression and neonatal outcome was noted. However, fetal genotype did influence the expression of P-gp and MRP1 and genotype across all four proteins was associated with significant changes in the measures of infant development. This was a small study and as such generation of susceptible haplotypes was not possible. However the data generated do support the concept. Further larger and longer term prospective studies, building on the experience reported in this thesis, are necessary to generate more data in order to identify haplotypes leading to increased fetal susceptibility to drug exposure.

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.

Copyright Statement

i. The author of this thesis (including any appendices and/or schedules to this thesis) owns certain copyright or related rights in it (the "Copyright") and s/he has given The University of Manchester certain rights to use such Copyright, including for administrative purposes.

ii. Copies of this thesis, either in full or in extracts and whether in hard or electronic copy, may be made **only** in accordance with the Copyright, Designs and Patents Act 1988 (as amended) and regulations issued under it or, where appropriate, in accordance with licensing agreements which the University has from time to time. This page must form part of any such copies made.

iii. The ownership of certain Copyright, patents, designs, trademarks and other intellectual property (the "Intellectual Property") and any reproductions of copyright works in the thesis, for example graphs and tables ("Reproductions"), which may be described in this thesis, may not be owned by the author and may be owned by third parties. Such Intellectual Property and Reproductions cannot and must not be made available for use without the prior written permission of the owner(s) of the relevant Intellectual Property and/or Reproductions.

iv. Further information on the conditions under which disclosure, publication and commercialisation of this thesis, the Copyright and any Intellectual Property and/or Reproductions described in it may take place is available in the University IPPolicy(see http://www.campus.manchester.ac.uk/medialibrary/policies/intellectual-property.pdf), in any relevant Thesis restriction declarations deposited in the University Library, The University Library's regulations (see http://www.manchester.ac.uk/library/aboutus/regulations) and in The University's policy on presentation of Theses.

Consent

Consent to reproduce the published articles has been obtained from the publishers. Consent to electronically reproduce the Perceived Stress Scale was kindly granted by Dr Cohen.

Alternative Thesis Format

This study has sections that are entirely laboratory based and other sections that are more focussed on the women and their babies. The alternative format appears to be the best way to present a coherent picture to the reader. The format is as the publications/to be submitted publications plus Chapter five written up as a test of concept.

Chapter 2

The author of this thesis was responsible for the review process, analysis of the studies and writing the paper. Her advisor conceived the idea, advised on the process, examined the papers, reviewed the analysis and the paper and her supervisors reviewed the analysis of studies and the paper.

Chapter 3

The author recruited and interviewed the women, collected the samples and wrote the article. Her advisor and supervisors reviewed and edited the article.

Chapter 4

The author, her main supervisor and a senior technician conducted the experiments. The author wrote the article and her supervisors reviewed and edited the article.

Acknowledgements

I would like to thank the women who participated in the study, answered my questions and allowed me access to their lives and their babies. It would not have been possible to complete this study without their continued support.

Professor Graham Dunn cheerfully provided advice on statistics and I thank him for that. Bernadette Baker always shared her knowledge and skills and provided support. Thank you Bo. There are too many people to mention by name but I am grateful for the support I received from everyone over the course of the study. I hope I have thanked them all and they know who they are.

My supervisors, Dr Diane Atkinson and Professor Colin Sibley have provided unstinting support and I will always be grateful to them for giving a midwife the opportunity to do something different and learn a set of completely new skills.

Last, but definitely not least I need to acknowledge the support of family and friends who have been there when I needed them to be and gave me space when I needed that.

The Author

The author trained as a nurse and midwife then practised as a neonatal and community neonatal midwife for many years whilst also studying part time for a BSc (Hons) and PgDip in Psychology. At this time babies born to drug using women were nursed on a neonatal unit and often stayed for several weeks. This sparked the author's interest in drug use in pregnancy and Neonatal Abstinence Syndrome. This led to further training and after successfully completing the "Drug Misuse in Primary Care" course she began working as a Specialist Midwife for women who have a history of drug and /or alcohol use or are currently using drugs and /or alcohol in pregnancy. In 2008, an NIHR Fellowship supported by the Manchester Biomedical Research Centre was awarded to undertake the study reported here. Since completing the research she has returned to clinical practice as a Specialist Midwife- Drugs, Alcohol and Mental Health.

Glossary

Adenosine Triphosphate (ATP)

A nucleotide present in every cell, it stores chemical energy used in metabolism.

Adenosine Diphosphate (ADP)

A nucleotide present in every cell, converted to ATP using energy from respiration.

Allele

One of a number of alternate forms of a gene.

Amnion

The inner of the two membranes that comprise the fluid filled amniotic sac which envelops the fetus.

Amphipathic

A molecule with different affinities, a polar end that is attracted to water and a non-polar end that is repelled by water.

Anionic

A negatively charged ion.

Antinociceptive

Increases tolerance / decreases sensitivity to pain

Apgar score

Method of assessing the physical condition of newborn infants.

Chorionic plate

The portion of the chorion that attaches to the uterine wall.

Chorion

The outer of the two membranes that comprise the fluid filled amniotic sac which envelops the fetus.

Cytokines

Group of chemicals which act as signalling molecules

Deoxyribonucleic Acid (DNA)

Double stranded nucleic acid carrying genetic information required for cell growth, division and function.

Electrophoresis

The separation of charged particles in a fluid or gel using an electric charge.

Endocytosis

The process by which the plasma membrane of a cell folds inward to transport molecules into the cell.

Ex-vivo

Experimental procedures that take place outside the cell or tissue.

Genotype

The individual's full complement of genetic information.

Haplotype

A combination of alleles that are located closely together on the chromosome and inherited together

Heterozygous

Possessing two different alleles coding for a trait.

Homozygous

Possessing identical alleles coding for a trait.

Hydrophobic

Repelling water or incapable of dissolving in water.

In-vivo

Experimental procedures that take place using the living organism.

Linkage disequilibrium

Is the non-random association of alleles on a chromosome which occurs at a greater frequency than chance.

Lipophilic

Has an affinity for or is capable of dissolving in lipids.

Mass spectrometry

An analytical technique to separate the components of a given sample by mass.

mRNA

Messenger RNA is a single stranded nucleic acid which conveys genetic information from DNA. It acts as a template for polypeptide synthesis.

Non- synonymous mutation

A change in the nucleotide sequence that alters the amino acid sequence of a protein.

Phenotype

Is the observed characteristics of the individual based upon the genotype.

Polymorphism

A change in the nucleotide sequence of DNA which can result in different phenotypes.

Stop codon

Known also as a termination codon, it is a triplet of nucleotides in RNA that signal the termination of translation of a protein.

Synonymous mutation

A change in the nucleotide sequence that does not alters the amino acid sequence of a protein.

Uterine decidua

The mucous membrane of the pregnant uterus.

Xenobiotics

A substance that is foreign to an organism.

Chapter One

Introduction

1.1 Introduction

The 2010 United Nations Office on Drugs and Crime (UNODC) report, (UNODC 2010) suggests there is both legal and scientific ambiguity around the terms 'drug misuse' and 'drug abuse'. For the purpose of clarity, this report uses the neutral term 'drug use' to indicate use of legal and illicit drugs rather than the term 'substance use' as this refers to the use of all substances including alcohol. Women who use alcohol in pregnancy are excluded from this study and data within this report pertains to the use of drugs but excludes alcohol. Drug dependence, in this report, is defined as a physical or psychological state in which the woman would display and report signs and symptoms of withdrawal if drug use was suddenly stopped. This distinction is in line with the stance taken by authors nationally, (The National Audit Office 2009) and globally (UNODC 2010). The terms 'drug misuse' and 'drug abuse' appear when an author specifically uses this term to indicate a specific pattern of drug use and are presented in italics. Prevalence rates and relevant health harms are alliterated followed by a brief examination of drug use in women, in pregnancy and associated potential harms to the fetus and infant exposed to maternal drug use.

The placenta as the interface between the maternal and fetal circulations facilitates the wellbeing, growth and development of the fetus, facilitating transfer of compounds essential for growth whilst acting as a barrier to harmful xenobiotics and endotoxins. Its structure and functions are examined, in particular the roles of the Adenosine Triphosphate Binding Cassette (ABC) efflux transporters. Evidence that these transporters interact with commonly used prescribed and non-prescribed drugs is shown and research exploring the role single nucleotide polymorphisms (SNPs) in the genes that transcribe these proteins is reviewed. It is proposed that differences in genotype may alter the amount of drugs a fetus is exposed to which has the potential to alter neonatal and developmental outcomes.

1.2 Drug Use

1.2.1 Prevalence Rates

Drug use remains a global phenomenon that affects individuals, their families and the societies in which they live. It affects individuals' health, public health, social welfare and levels of crime and disorder (HM Government 2010). It is estimated that in the 16-59 age range there are 1.6 million regular users of illicit drugs in the U.K (Dept. of Health 2011). At national levels the financial costs are vast. Based on 2003/4 figures the Home Office estimated Class A drugs alone cost the UK £15.4 billion (Home Office 2006).

Of this sum £488 million (3.1%) was spent on health care and £923 million (6%) on drug related deaths. This did not include the cost of HIV, Hepatitis C and Hepatitis B infection the lifetime costs of which have been estimated to be £23 million, £608,475 and £580,568 respectively. The figures for *drug misuse* in Scotland estimate of the economic and social costs to be 5.1 billion Euros (NHS National Services Scotland 2010).

Accurate, up to date prevalence rates are hard to obtain due to the illicit nature of the activity and methods used to capture and report i.e. household surveys. (Home office 2006). The 2011 statistics for England, report 8.6% of the adult population in 2009 and 8.8% of the adult population in 2010/11 had used one or more illicit drugs within the previous year. Cannabis was the most commonly used illicit substance followed by cocaine and heroin (The Health and Social Care Information Centre 2011).

Despite their authors' best efforts surveys like the National Treatment Agency Report (NTA 2013) and Crime Survey for England and Wales (Home Office 2012) have limitations as they rely on accurate reporting which assumes respondents are willing to respond, have accurate recall of drug use, understand the questions asked of them and are able to complete the forms. The National Treatment Agency report data submitted by drug treatment services and therefore do not include those not in treatment but are able to report on patterns of drug use for those in treatment. Household surveys such as the Crime Survey may not access people whose drug use has become chaotic leading to street homelessness or other manifestations of a chaotic lifestyle e.g. incarceration. Household surveys may therefore, underestimate drug use. An additional problem is the delay in reporting on data so that the most recent available reports may cite data that is several years out of date. For example, the latest figures on drug use in Europe (EMCDDA 2012) provide data based on figures from 2010. Nevertheless these are the most accurate estimations of drug use on a global or regional scale and useful to determine both the scale and trends of drug use. In addition to these difficulties Identifying prevalence rates in pregnancy is difficult as women who fear adverse consequences of reporting their drug use, such as remove their child from their care, may not report it or not report accurately (Thajam *et al* 2012, Scottish Executive 2003, Ostrea *et al* 2001).

Data from the European Monitoring Centre for Drugs and Drug Addiction (EMCDDA 2010) which also uses survey data in addition to information from drug treatment services and criminal justice sources, suggest that in Europe 155-250 million people between the ages of 15 and 64 used an illicit substance at least once in 2008.

This equates to 3.5 to 5.7% of the identified population in that age range. Cannabis remained the most commonly used illicit drug globally, (129-190 million people or 2.9-4.3%) followed by the amphetamine type group of substances, (13.7-52.9 million, 0.3-1.2%), cocaine (15-19.3 million, 0.3-0.4%) and opiates (12.8-21million, 0.3-0.5%). Between 16 and 38 million people were identified as having a dependency on drugs. This equates to 10-15% of those in the age group that reported drug use in 2008, the period in which this study commenced.

1.2.2 Health Harms

Harms directly attributable to drug use include death (Dept. of Health 2011). Bargagli *et al* 2006, followed a cohort of drug users between 1990-98 in eight European sites and calculated a mortality rate directly attributable to opioid use in the 15-49 year age group of 10-23% (Bargagli *et al* 2006). Contaminated drugs increase the risk of death as in the recent fatalities induced by the use of heroin contaminated with anthrax (Powell *et al* 2011). Saving Mothers Lives, the Confidential Enquiry into Maternal Deaths in the United Kingdom (CMACE 2011), showed that drug use still figures highly in the causes of maternal death. In the 2006-8 triennium there were 53 deaths (20.3%) related to substance misuse. Of these 36 women are described as "*known drug addicts*", 16 as occasional users, 6 were alcohol related deaths and 10 women used both alcohol and drugs.

Other health harms associated with drug use are numerous (Dept. of Health 2011). They may be indirectly attributable to drug use i.e. poor health due to the association with drug use and poverty (Scottish Executive 2003) or homelessness (e.g. tuberculosis), sexually transmitted infections or psychiatric morbidity (EMCDDA 2012). In England in 2010/11, hospital admissions for psychiatric problems directly related to drug use numbered 6640, and poisoning by drugs accounted for 12586 admissions in the same time period (The Health and Social Care Information Centre 2011). Injecting drugs use is associated with blood borne infections such as Hepatitis B, C and Human Immunodeficiency Virus (HIV) (EMCDDA 2012), damage to blood vessels associated with repeated trauma, infection, abscesses and thromboembolic disease (NIDA 2007). Repeated use of stimulants such as cocaine can strain the cardiovascular system causing cardiovascular problems such as subacute bacterial-endocarditis and cardiac valve damage (NIDA 2007, Wilbourne *et al* 2001, Day and George 2005, Lloyd-Smith *et al* 2008, Martinez-Selles *et al* 2008).

1.2.3 Women's Drug Use

Women and men have differing requirements of services as they use drugs and treatment services differently (Pinkham *et al* 2012). Women's physiology is different, their reasons for initiating drug use are different and they express more concerns about their children being removed from their care (NTA 2005, Wright and Walker 2007, Macrory and Boyd 2007, NTA 2006, Rosenblaum and Guionnet 2003, Kouimtsidis 2003). For example, in response to psychiatric distress men are more likely to be admitted to an inpatient facility whilst women are more likely to be prescribed antidepressants and stay within primary health care services (Scottish Executive 2003, The Health and Social Care Information Centre 2011).

It has been suggested that women may be underrepresented in treatment services because their specific needs are neither recognised nor catered for. For example if women are the main care provider to dependent children it may make it difficult to access inpatient detoxification services (Becker and Duffy 2002, NTA 2006, Day and George 2005, Burns *et al* 2006). This highlights the need for information and services that are gender specific to achieve equity in service provision (DrugScope 2003). For example, women only clinics, flexible service delivery, support for women with child care responsibilities, more inpatient facilities that allow children to be resident and adequate, accurate information prior to, during and after pregnancy.

Fewer women than men report drug use globally (EMCDDA 2012, UNODC 2010). It has been recognised for some time that the difference in numbers between women and men using illicit drugs is closing as more women use drugs in a dependant manner (Kouimtsidis 2003). National figures for the United Kingdom (The Health and Social Information Centre 2011) report twice the number of men (11.9%) than women (5.4%) using an illicit drug in 2009/10 whilst in 2007, the ratio of men and women accessing treatment services in the UK was 3:1 (Home Office 2007).

In 2005, Wolff *et al* estimated that one in a thousand women in the UK used opiates dependently. In the same year Day and George reported that 90% of women who accessed drug treatment services in the UK were between the ages of 15-39 years (Day and George 2005). Recent data from The Health and Social Care Information Centre (2011) show that the majority of people who accessed drug treatment services were aged 30-34 years. These age ranges fit within women's reproductive lifespan suggesting

implications for both maternity and paediatric service planning and implementation. This is especially relevant if one considers that these figures only represent women who use drugs dependently. They do not reflect numbers of women for whom drug use is not dependent or who choose not to access drug treatment services. Nor do they capture data for women using drugs that do not require substitute prescribing and therefore do not need, or are unable to access, treatment service e.g. cannabis, powdered cocaine, other stimulants and over the counter medications. Therefore, maternity services may significantly underestimate the number of pregnant drug using women and the number of fetuses that are exposed to legal and illicit drugs.

1.2.4 Drug Use in Pregnancy

Prevalence rates for drug use in pregnancy are difficult to obtain and studies often quote very different prevalence rates, making service planning and delivery difficult. Bell and Harvey-Dodds (2008), reported a lack of data, particularly in the UK. In their systematic review, Rayburn and Bogenschutz (2004), suggest this is a consequence of a lack of rigorous epidemiologic studies. The Northern and Yorkshire Region Public Health Observatory Survey (2002) conducted a postal survey of midwifery units in its area and generated an estimate of 7.5 babies per 1000 live births in 2001 born to women who reported drug use. The United States Substance Abuse and Mental Health Services Administration (SAMSHA), cite a 5.1% prevalence rate for maternal *drug misuse* in pregnancy (SAMSHA 2009). Scottish statistics for the year 2008/9 report 592 maternities which equated to a national rate of 10.3 per 1000 for that year (NHS National Services Scotland 2010). The 2011 report on *drug misuse* statistics in England (Health and Social Care Information Centre 2011) reports on both drug treatment services and hospital admissions by gender but does not give rates for drug use in pregnancy. However, Sanaullah *et al* (2006) conducted an anonymous urine screening survey of 150 women attending an antenatal clinic in North East England and found 10.7% of specimens tested positive for at least one substance.

Maternal drug use continues to be associated with adverse pregnancy and neonatal outcomes, which are common to stimulant, opiate and cannabis use. Consequently, in Manchester, all pregnant women with drug and/or alcohol problems are referred to The Manchester Specialist Midwifery Service which provides additional support to women with drug, alcohol and mental health issues. This is an opt in service in which interventions usually take place in the woman's home and does not replace the universal services available to all pregnant women. The Specialist Midwives provide information

with respect to the effects of drug and/or alcohol on the fetus and neonate, to both women and professionals, support women to develop effective strategies to achieve optimal pregnancy and lifestyle outcomes and when necessary act as advocates for the woman and/or the unborn and newborn.

These include pregnancy loss as either miscarriage or stillbirth, preterm birth, intrauterine growth restriction and breech presentation (Goel *et al* 2011, Burns *et al* 2006, Lacroix *et al* 2004, Wright and Walker 2007, Bell and Harvey-Dodds 2008). Other complications such as placental abruption and smaller fetal head circumference are hypothesised to be particularly related to the vasoconstrictive action of stimulants but are also a feature of opiate and cannabis use. (Chiriboga *et al* 1999, Slotkin 1998, Eyler *et al* 1998). Frank *et al* (1990), compared patterns of growth and fat disposition in neonates prenatally exposed to maternal cocaine or cannabis use. Demonstrating different patterns of growth contingent upon drug of choice, they proposed the effects of cannabis were related to maternal/fetal hypoxia and the effects of maternal cocaine use were related to a mechanism that altered nutrient transfer to the fetus. An interesting review of the literature on abnormal fetal growth as a consequence of maternal drug use is presented by Soto and Bahado-Singh (2013).

Pregnant women who are dependent on opiates may be prescribed methadone or buprenorphine to reduce illicit use and to improve neonatal outcomes. However, as they are either partial or full mu-opioid agonists they also affect the fetus and as with opiates, are associated with Neonatal Abstinence Syndrome (NAS) (Farid *et al* 2008, Jansson *et al* 2010, Dashe *et al* 2002, Finnegan *et al* 1975, Cleary *et al* 2012).

The probability of continued cannabis use in pregnancy decreased in a cohort of pregnant drug using women (Bailey *et al* 2008) but a substantial proportion used cannabis throughout pregnancy and women who are prescribed methadone or buprenorphine are advised to continue with this throughout pregnancy to reduce the risk of Intrauterine Abstinence Syndrome (IAS), which in a 2012 review McCarthy suggests is a risk to the fetus. Therefore individual women and their healthcare providers must balance the needs and wishes of the woman against the potential for harm to the developing fetus.

Previous negative experiences of healthcare, fears of negative judgements, multiple agency input or consequences of disclosing drug use may mean a woman delays seeking antenatal care (Scottish Executive 2003, NICE 2010). Delay in registering for, or non-

attendance at, antenatal care has been linked with adverse pregnancy outcomes for all pregnant women (Burns *et al* 2006, CEMACH 2007, Wright and Walker 2007). However, studies also demonstrate that improved antenatal services for *drug misusing* women are associated with improved neonatal outcomes (Goler *et al* 2008, El-Mohandes *et al* 2003, Kahila *et al* 2007, Buckley *et al* 2013).

It is important therefore, that when women present for care they are provided with accurate information and individualised care plans that account for physiology and social circumstances. Unfortunately, the ability to do this is limited by a lack of understanding of how substances of misuse are handled by the human placenta. Mechanisms by which the placenta minimises the transfer of drugs and xenobiotics from the maternal to fetal circulation, especially in placentas that are exposed to licit or illicit drugs throughout pregnancy, are incompletely characterised but there is some evidence from animal (Lankas *et al* 1998, Kalabis *et al* 2007) and human studies (Zharikova *et al* 2007, Nekhayeva *et al* 2005) that multidrug resistance proteins of the ABC family may be involved.

As evidenced by the neonatal behaviours of NAS, (Jansson *et al* 2009, Farid *et al* 2008) many substances of misuse cross the placenta to some extent and affect the fetus. Although the aforementioned antenatal outcome studies (Goler *et al* 2008, El-Mohandes *et al* 2003, Kahila *et al* 2007) demonstrated improved neonatal outcomes, NAS remains a feature of the neonatal period (Farid *et al* 2008) with symptoms that require pharmacological management in 41-81% of babies born to opiate dependent women (McCarthy *et al* 2005). This variability in NAS, despite improved antenatal care, suggests a physiological component that is not being addressed by current treatment services.

1.2.5 Models for Conceptualising Effects of Maternal Drug Use on the Infant.

Wilbourne *et al* (2001), identified five models to conceptualise harms to the fetus and neonate. 1) The **maternal functioning** and 2) the **sociological** models both explain neonatal outcomes in terms of the effect of the woman's environment and her individual characteristics e.g. health, lifestyle and other socioeconomic demographics. 3) The **teratogenic** and 4) **toxic** models use biological mechanisms to explain effects on the infant, i.e. drugs cause direct physical effects on the fetus, or alternatively, they cause physical effects on the mother that then affect the fetus. The fifth model, the **interactive model** synthesises the main elements of the other four models to assume that fetal effects of maternal drug use are multifactorial and cross a variety of the

domains explored in the other models i.e. biological, social, lifestyle and drugs themselves (Wilbourne *et al* 2001).

1.2.6 Neonatal Abstinence Syndrome

The disuse hypersensitivity theory and the neural hyperactivity theory (Robinson and Berridge 1993) seek to explain the underlying mechanisms that lead to the behaviours associated with Neonatal Abstinence Syndrome. The **disuse hypersensitivity** model hypothesises that opiate receptors in the central nervous system develop increased sensitivity in response to maternal opiate use. Once the receptors are no longer blocked by the drug, they are overwhelmed by previously blocked inputs producing signs of withdrawal in the neonate. The **neural hyperactivity** model hypothesises that minor pathways become more active as the opioid blocks more usual pathways. Again, when the infant is born and the system no longer has a supply of opioid, both the minor and major pathways function effectively and the resulting hyperactivity produces the neonatal behaviours classically associated with NAS. **Whatever the mechanism, neither model fully explains why some babies fare less well than others when exposed to similar amounts of maternal drug use, exhibiting more signs of physical distress demonstrated by increased severity of NAS.** Nor why a proportion will require pharmacological intervention to abate their signs whilst others can be managed by supportive measures such environmental adaptations (Velez and Jansson 2008, Velez *et al* 2009).

NAS is a generalized, multisystem disorder in the neonate characterized by a constellation of signs from three systems, the gastrointestinal, central nervous, and the autonomic. Classical signs exhibited by the neonate will be affected by gestation and the drug(s) to which it has been exposed but include excessive and often high-pitched crying, sleep and feeding disturbances, excessive sneezing, yawning, nasal stuffiness, temperature instability, increased muscle tone, tremors, and on occasion convulsions (Finnegan 2004, Seligman *et al* 2008, Dysart *et al* 2007, Jansson *et al* 2009). Johnson *et al* (2003) showed that infants exposed cocaine in addition to opiates experienced a reduction in the severity of NAS. The reasons for this have yet to be elucidated but one hypothesis is that cocaine blocks pathways in the placenta that normally allow methadone to reach the fetus. There is also a growing literature that demonstrates signs of withdrawal in babies exposed to antidepressants (ter Horst, *et al* 2008, Blumenfeld *et al* 2010, Boucher *et al* 2008, Klinger *et al* 2011, Ferreira *et al* 2007).

If severe NAS is left untreated the infant may convulse, with the potential for serious harm. Therefore signs of withdrawal are monitored closely. A scoring tool such as that devised by Finnegan *et al* (1975) may be used to provide a numerical estimate of NAS to determine a) whether signs are abating or increasing and b) whether pharmacological treatment should be instigated or dose of medication reduced.

1.2.6.1 A Relationship Between Maternal Dose of Opiate and Expression of NAS?

Although neonatal outcomes improve when women access specialist multidisciplinary antenatal care services, (Kahila *et al* 2007, El-Mohandes *et al* 2003, Goler *et al* 2008) the potential for the infant to require pharmacological treatment for NAS is still a consideration when formulating a care plan. Clinicians are faced with difficult dilemmas. They must balance the needs of the woman whilst attempting to provide the optimal intrauterine environment to support fetal growth and development whilst minimising the risk of IAS and NAS. Despite best efforts to do this there remain a proportion of women for whom this is not possible as their child will exhibit signs of withdrawal that cannot be managed by environmental adaptation (Velez and Jansson 2008, McCarthy *et al* 2005).

Regardless of attempts to stabilise drug use the lives of many pregnant opioid dependent women often remain blighted by socio-economic factors other than drug use. Homelessness, poverty, domestic abuse, fears of adverse judgements from health care professionals and psychological distress feature in the lives of many women (SAMHSA 2009, The Health and Social Care Information Centre 2011, EMCDDA 2012, Hall and van Teijlingen 2006, DrugScope 2003).

It is recognised that this group of women have complex needs (Finnegan 2004, Scottish Executive 2003). The combination of complex lifestyles, extensive substance misuse histories and individual differences in pharmacokinetics (Ferrari *et al* 2004) lead to a wide range in the dose of prescribed substitute medication. Consequently, there are considerable differences in the amount of medication and/or illicit substances a fetus may be exposed to. This makes predicting the effects of maternal substance misuse on the fetus difficult. Jansson and Velez (2012) provide a review of maternal and fetal factors that affect the presentation of NAS.

Women report conflicting advice and information from healthcare professionals, making it difficult to understand exactly what is required of them to achieve health gains for themselves and their infant (DrugScope 2003). There are many reasons for this, including different philosophies that underpin treatment for example, choice of substitute

prescribed, recommended doses in pregnancy, stabilisation or detoxification in pregnancy. A consequence of debate regarding the safety of detoxification in pregnancy (Luty 2003, McCarthy 2012) means that some women may be facilitated in a request to detoxify whilst others will be discouraged.

The women's experiences reflect the fact that care providers still work with uncertainty about how best to predict which babies will be more vulnerable to their mothers drug use and exhibit a more severe presentation of NAS. Anecdotally, and also from the authors personal experience, women and healthcare providers report instances when a child has exhibited minor signs of NAS not requiring pharmacological intervention despite its mother's chaotic lifestyle, continued illicit polydrug use and high doses of opiate substitute medication. Conversely, there are also women who have a stable lifestyle, remain illicit drug free and take small doses of a prescribed opiate substitute whose infants exhibit NAS to an extent where they require pharmacological intervention and a prolonged stay in hospital.

The reasons for the variability in expression of NAS have yet to be fully understood (Jansson *et al* 2010). There are numerous papers that sought to characterise NAS and its relationship to the dose of maternal opiate/opioid taken in pregnancy. Some report a correlation between NAS and the amount of drug to which the fetus is exposed (Dryden *et al* 2009, Lim *et al* 2009) whilst others do not (Berghella *et al* 2003, Bakstad *et al* 2009, Fischer *et al* 2006, Kacinko *et al* 2008). Evidence from systematic reviews of the literature (Thajam *et al* 2010, Cleary *et al* 2010) demonstrated there was no relationship between the maternal dose of opioid and the severity of expression of NAS. Studies included in the Thajam *et al* (2010) review (presented in Chapter 2 of this thesis) showed that data from studies are difficult to pool due to their heterogeneity, however eight of the ten studies included reported no relationship between NAS and dose of maternal opioid. This finding was consistent across international boundaries and therefore different health care delivery settings regardless of methadone or buprenorphine prescription (Thajam *et al* 2010). Chapter two of this report provides a fuller description of the review and the original article.

1.2.7 Developmental Outcomes

There is a vast and confusing array of data regarding developmental outcomes following human *in-utero* exposure to drugs. Some studies measure physical outcomes (Covington *et al* 2002, Richardson *et al* 2007), others behavioural, cognitive or neurochemical

changes (Sansavini *et al* 2010, Bromley *et al* 2010, Chiang *et* al 2013, Bunikowski *et al* 1998 Richardson *et al* 2008, Richardson *et al* 2009, Richardson *et al* 2011), whilst others have examined the effect of drugs on the transcription of genes (Lee *et al* 2009). There is also a growing body of literature from animal studies (Sanchez *et al* 2008, Campolongo *et al* 2009, Slamberova *et al* 2006, Thompson *et al* 2009).

Different methodologies, measurement tools, small sample sizes and the number of confounding factors such as different patterns of drug use, make replication of studies difficult. This has resulted in conflicting results with unclear messages as to the clinical significance of their findings. For example, Richardson *et al* (2011) found outcomes differed according to the stage of pregnancy at which the fetus was exposed to cocaine whereas Chasnoff *et al* (1989) concluded cocaine had no effect on behavioural or cognitive outcomes at four to six years. However, when Bunikowski *et al* (1998) reviewed the development of 35 opiate exposed infants and 42 controls at one year of age using the Griffiths Mental Development Scales; they found the mean Developmental Quotient was lower in the exposed group. The Locomotor and Performance subscales were particularly affected, this the authors propose, suggests an increased risk of psychomotor impairment with exposure to maternal opiate use.

In an interesting review summarising experimental data from rodent studies using cannabis, Campolongo *et al* (2011) highlight the fact that developmental deficits may be subtle and therefore be difficult to diagnose. Clinically this could lead to misdiagnosis and therefore underreporting of what could be significant numbers of children whose development has been impaired by *in utero* exposure to drugs. This confusing array of data, which often concentrates on the effects of one particular compound in isolation make it difficult for clinicians to offer accurate, evidenced based information to women who are often polydrug users and whose pattern of drug use may change considerably throughout the pregnancy.

1.3 Structure and Function of the Human Placenta

1.3.1 Introduction

The placenta is an organ unique to mammals. A temporary but very complex, organ of pregnancy, it is the interface between the maternal and fetal vascular circulations. It exists to facilitate the continuation of pregnancy, supply the fetus with essential nutrients, transfer waste products back to the maternal circulation and signal to the

maternal physiology to adapt to support the pregnancy and prepare for parturition. It also has a pivotal role in preventing transfer of toxic compounds from maternal to fetal circulations. There are many insightful in-depth reviews that cover all aspects of structure, developments and function (Enders and Blankenship 1999, Atkinson *et al* 2006, Giaginis *et al* 2012, Huppertz 2008, Burton *et al* 2009). This report provides a general overview of its structure and highlights some of its many functions.

1.3.2 Gross Structure

Placentae demonstrate considerable inter-species differences (Sibley and Boyd 2004, Atkinson *et al* 2006a, Myllynen *et al* 2010), which Enders (2009) suggests, reflect divergent evolutionary pathways. They are classified according to shape, the number of layers between the maternal and fetal vascular systems and the sites of interaction between those systems. The human placenta is **discoid** in shape, is **haemomonochorial** i.e. has a single trophoblast layer between the maternal and fetal circulations, with **multivillous flow** i.e. maternal blood circulates between villi in the intervillous space (Sibley and Boyd 2004). Figure 1.1 shows the gross anatomy of the human placenta at term and the orientation of the villi.

At parturition the placenta usually weighs approximately one sixth of the baby's birth weight (500-700grams), thicker centrally at approximately 2.5cms it thins towards its edge and is between 15-20cms in diameter. The **maternal surface**, attached to the uterine decidua is divided into cotyledons by partial septa formed from the decidua basalis and is uneven in texture. The smoother, shinier **fetal surface** is covered by the amnion which is avascular and covers the chorionic plate (Ceckova-Novotna *et al* 2006). The umbilical cord, consisting of two umbilical arteries and one umbilical vein inserts into the chorionic plate, usually in a central position. These cord vessels branch out over the fetal side of the placenta and subdivide into smaller vessels. These eventually form tree like villous structures that lie within the intervillous space. It is at this interface between the maternal and fetal circulations that the exchange of nutrients and waste takes place. Oxygenated, nutrient rich blood from the maternal circulation enters the intervillous space and bathes the villi. Although separated by only a few layers of cells the fetal and maternal circulations do not mix. Transfer of oxygen and nutrients takes place at the syncytiotrophoblast and are transported to the embryo, and later fetus, by the chorionic and umbilical veins. Waste products, endotoxins and xenobiotics are returned to the maternal circulation by the umbilical arteries. A number of mechanisms facilitate or hinder this transfer and include passive diffusion, facilitated diffusion and active

transport facilitated by mechanisms that include the ABC family of transporter proteins (Sibley and Boyd 2004, Atkinson *et al* 2006b, Hamilton and Boyd 1960, Gude *et al* 2004).

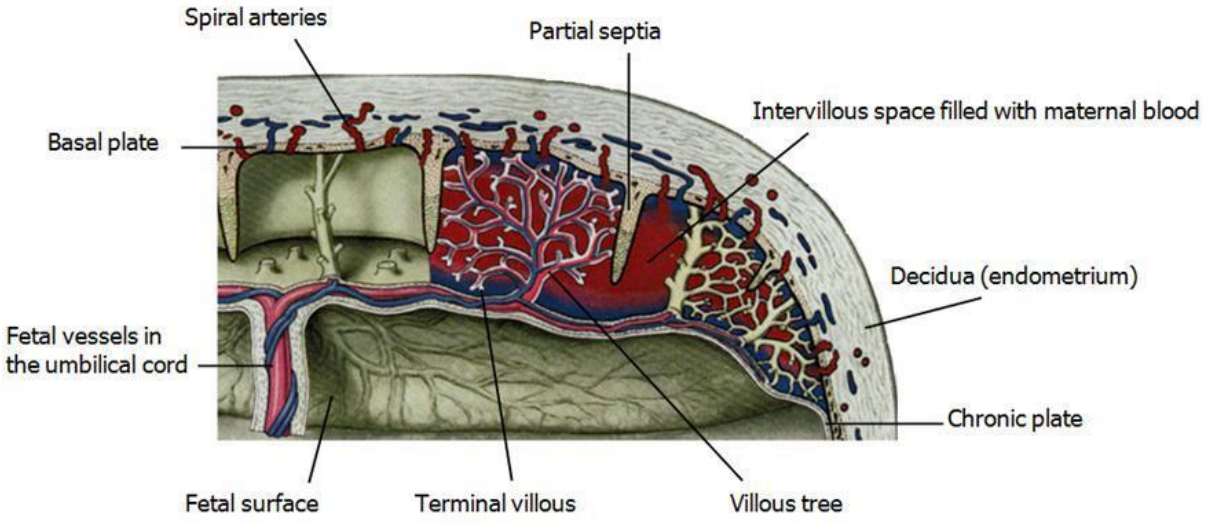


Figure 1.1 The placenta in section showing the progression of subdivision in the fetal vessels and their eventual termination in the villous trees. This subdivision provides closer proximity to the nutrient rich maternal circulation and a much greater surface area to facilitate transfer of nutrient compounds and the waste products from their metabolism. Adapted from Hamilton and Boyd (1960).

1.3.2.1 Villous Trees

The formation of an effective vascular network is essential for effective feto-maternal exchange to take place. It is necessary therefore for this to occur at an early stage and a functioning network develops by eight weeks gestation (Huppertz 2009, Burton *et al* 2009). Jackson *et al* (1992) suggests accelerated growth of the vascular network to facilitate the formation of the villous tree at 25 weeks gestation. The network undergoes continual change, especially in the first and second trimesters of pregnancy to accommodate the changing needs of the fetus with a resulting increase in the number of villi, and surface area. Fox (1997) suggests these develop around 27 weeks gestation and by term account for 60% of villi. As the syncytiotrophoblast, which facilitates transfer to and from the fetus is sited on the terminal villi it is logical that changes in the rate of their proliferation should change to accommodate the changing requirements of the developing fetus. Huppertz and Burton (2006), classify villi as:

- stem villi, the function of which is to provide a scaffold type framework
- intermediate villi which are further divided into immature and mature forms
- terminal villi where exchange functions occur.

In brief villi are composed of a central fetal capillary, villous stroma (fetal connective tissue) and an outer trophoblast layer. It is within the trophoblast layer that the proteins

of interest in this project; P-glycoprotein (P-gp), Breast Cancer Resistance Protein (BCRP) and Multidrug Resistance Associated Proteins 1 and 2 (MRP1 and 2) are situated.

In early pregnancy the syncytiotrophoblast layer of the terminal villous measures between 50-100μm in thickness. In order to meet the challenges brought about by the growing fetus this thins during pregnancy and at term measures approximately 4-5μm (Syme *et al* 2004, Ceckova-Novotna *et al* 2006). This is accompanied by an increase in the number of terminal villi which increases the surface area available for exchange (Evseenko *et al* 2006). The maternal facing, microvillous membrane of the multinucleated syncytiotrophoblast comes into contact with maternal blood in the intervillous space and can be conceptualised as the maternal side of an exchange barrier. The basal membrane the fetal side of the barrier lies closest to the endothelium of the fetal capillary and therefore the fetal vascular system (Figure 1.2). The ABC transporter proteins P-gp, BCRP, MRP1 and MRP2 are situated in this barrier membrane. P-gp, BCRP and MRP2 are located on the maternal facing microvillus membrane and MRP1 on the fetal facing basal membrane.

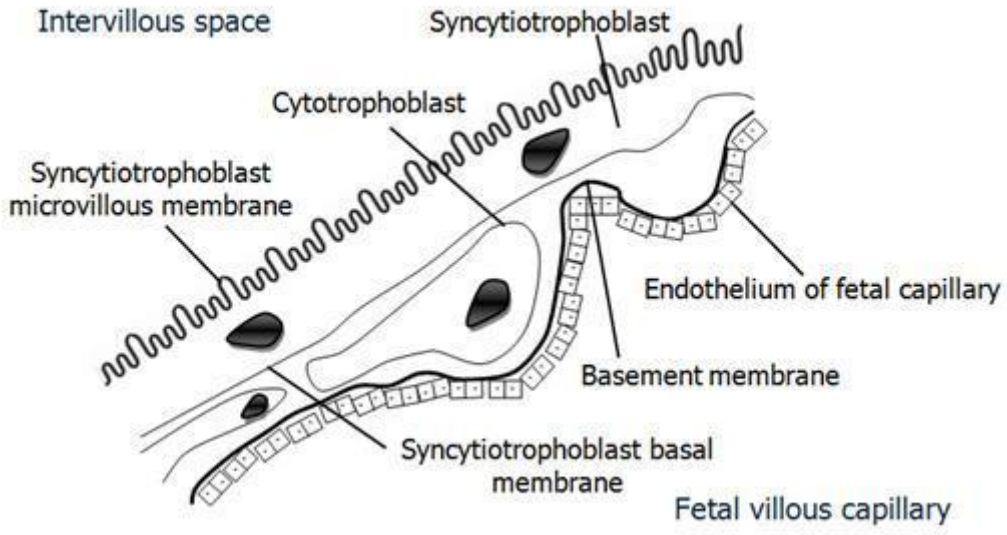


Figure 1.2 A schematic representation of the main layers in the terminal villous which lies in contact with the maternal circulation and is the interface between maternal and fetal circulations. The microvillous membrane of the syncytiotrophoblast is the maternal facing membrane that lies in direct contact with maternal blood in the intervillous space. The cytotrophoblast layer renews, and therefore maintains, the integrity of the multinucleated syncytiotrophoblast layer. The basement membrane lies closes to the endothelium of the fetal capillary. Adapted from Atkinson *et al* (2006b).

1.3.3 Placental Transport

Many factors affect placental transport in an uncompromised placenta. Broadly speaking they can be grouped under the following:

- Blood flow in the placental intervillous space
- Surface area available for exchange e.g. syncytiotrophoblast and fetal capillaries
- Concentrations and chemical composition of a compound within the maternal and/or fetal circulations
- Rate of blood flow in the villous tree
- The ability of the placenta to metabolise the compound
- Different mechanisms of transfer e.g. different diffusion rates and active transfer mechanisms

The physicochemical properties of a compound e.g. its size, degree of lipid solubility, electrochemical gradient between maternal and fetal circulations and ability to bind to plasma proteins, dictate the mechanism by which it can, and how effective it is, at crossing from the maternal to fetal circulations within the placenta. Passive diffusion is an energy neutral process and is one of the main mechanisms by which compounds cross the placental membranes. This is affected by the degree of lipid solubility and the polarity of the compound. Un-ionised, lipophilic substances with a low molecular weight can readily use passive diffusion (Atkinson *et al* 2006b). However, diffusion alone does not have the capacity or ability to support all the requirements of the feto-placental unit. Other mechanisms have evolved therefore to meet the shortfall. These include facilitated diffusion, endocytosis, and carrier mediated transporter proteins such as the ATP Binding Cassette (ABC) Transporter family (Sibley and Boyd 2004, Holcberg *et al* 2003). It is the function of the ABC Transporter proteins that are of interest to this study.

1.3.4 Functions of the Human Placenta.

The relative importance of different placental functions may change over gestation but can be summarised as; transportation of compounds to and from the fetus, acting as an endocrine organ, maintaining the pregnancy and itself, preparation for parturition, metabolism of nutrients and other compounds and protecting the fetus from infections, endotoxins, xenobiotics and exposure to maternal blood.

1.3.4.1 Maintaining the Pregnancy

The placenta and its associated membranes the amnion and chorion ensure the conceptus is not expelled in the early stages of pregnancy by remodelling the spiral arteries in the endometrium. This acts two fold, it helps to lower the maternal immune response locally and ensures adequate blood supply to the fetus before feto-maternal blood flow is fully established (Huppertz 2008, John and Hemberger 2012). The placenta continues to support the continuation of the pregnancy, in part by secreting progesterone which maintains the uterus in a state of quiescence until term.

1.3.4.2 Endocrine

The syncytiotrophoblast excretes a number of hormones e.g. placental lactogen, human chorionic gonadotrophin, and has the capacity to secrete into both the maternal and fetal circulations. Hormones also include both progesterone and oestrogens. As previously stated, progesterone acts to keep the uterus in a state of relative quiescence to allow the pregnancy to continue to a stage whereby the fetus would be viable whilst oestrogens change maternal physiology e.g. breasts and cervix to accommodate the demands of childbirth and breastfeeding. It also produces a number of cytokines that are thought to have a role in the preparation for and timing of parturition (John and Hemberger 2012, Giaginis *et al* 2012, Gude *et al* 2004, De Bonis *et al* 2012).

1.3.4.3 Transfer of Nutrients and Waste Products

The placenta services the fetal need for nutrients to ensure adequate growth and development and facilitates removal of removes waste products e.g. urea and creatinine from the fetal to the maternal circulation. As a functioning organ it metabolises compounds to maintain its integrity and the waste products from this process are also transferred to the maternal circulation. Barker *et al* (1990) assert that fetal nutrition is involved in metabolic programming of the fetus and impaired nutrition in utero can lead to disease in adulthood. If the fetus is to achieve optimal growth and development compounds must pass through the syncytiotrophoblast to reach fetal organs. The physicochemical properties of the compound will dictate the manner in which this occurs e.g. its size, ability to bind to plasma proteins, the electrochemical gradients between the maternal and fetal circulations and it lipid solubility and the physiology of the placenta. Lipophilic compounds such as fat soluble vitamins usually pass to the fetus by passive diffusion whereas amino acids and other compounds rely on an active transport mechanism such as that provided by the transporters of the Human ATP Binding Cassette (ABC) family.

1.3.5 Placenta and Commonly Used Illicit Drugs

The occurrence of Intrauterine Abstinence Syndrome and Neonatal Abstinence Syndrome evidences the fact that the fetus is exposed to maternal drug use when compounds pass through the placenta. In addition it has been shown that fetal motor activity and heart rate change after maternal administration of methadone and buprenorphine (Jansson *et al* 2005, Salisbury *et al* 2012). As previously stated lipophilic compounds readily cross the placenta. Many illicit drugs are lipophilic with a low molecular weight and therefore a high potential for transfer. Table1.1 lists some of the more common substances that are used illicitly, prescribed as an opiate substitute in opiate dependence or prescribed to alleviate chronic pain. They are also the drugs that were used by participants in the study and with the exception of tobacco are the ones that hair samples were tested for.

Table 1.1 Adapted from Little and VanBeveren (1996) this table shows the chemical properties possessed by the drugs used by the participants in the study. These properties ensure they are able to cross the human placenta.

Substance	**Molecular weight**	**Philicity**	**Transfer potential**
Amphetamine	135.20	Lipophilic	High
Benzodiazepines	284.76	Mildly lipophilic	High
Cannabis	314.45	Highly Lipophilic	Very High
Cocaine[1]	303.45	Mildly lipophilic	Moderate to High
Codeine[2]	335.84	Lipo & hydrophilic	Very High
Heroin	369.40	Hydrophilic	Very High
Methadone	345.90	Mild hydrophilic	Very High
Methamphetamine	149.24	Hydrophilic	Very High
Morphine	285.33	Hydrophilic	Very High
Tobacco	123.11	Lipophilic	Very High

[1] Cocaine hydrochloride is water soluble but free base is lipophilic and has a lower molecular weight. Most participants in the study used freebase.[2] Codeine analogues have essentially the same properties as codeine. The placenta has a fluid filled paracellular pathway which allows small hydrophilic molecules to cross by diffusion explaining the high transfer potential of the hydrophilic compounds listed above.

Whilst it is clear from clinical practice that these drugs pass through to the fetus as yet it is unclear as to the mechanisms of by which they reach the fetus, if and how they interact with other drugs and if there are any factors that impede transfer. A number of

studies have sought to answer these questions and used a variety of methods to do so. Examples of methodologies and findings are shown in Table 1.2.

Many studies have used dual perfusion techniques and much of the published literature is output from one research group. Studies have sought to characterise binding sites (Ahmed *et al* 1990, Fares and Gavish 1986), systems that metabolise drugs (Fokina *et al* 2011, Hieronymous *et al* 2006, Nanovskaya *et al* 2004, Zharikova *et al* 2007), transfer kinetics (Bailey *et al* 1987, Nanovskaya *et al* 2009), toxicity and distribution of drugs (Harbison and Mantilla- Plata 1972, Malek *et al* 2009, Myllynen and Vähäkangas 2002). Although many drugs are used recreationally the majority of this body of work has concentrated on methadone and/or buprenorphine (bpn) in cohorts prescribed these for pain relief or opiate dependence. However the studies in Table 1.2 have shown that THC, methadone, diazepam and bpn transfer to the fetus. Fokina *et al* (2011) demonstrated that with buprenorphine this occurs at an early stage of pregnancy and Boskovic *et al* (2001) that dizygotic twins have different levels of cocaine and cannabis metabolites in meconium. This would suggest that there was something specific to the twin's different placentae that accounted for differences as they were exposed to essentially the same *in utero* environment.

Table 1.2 Placental studies involving commonly misused drugs and the role of the placenta in mediating their transfer (presented in alphabetical order according to author). Boskovic et al (2001) examined the level of cocaine and cannabinoids in meconium of mono and dizygotic twins as a model for exploring the role of the placenta in their disposition, others have sought to characterise binding sites, systems that metabolise drugs, transfer kinetics, toxicity and distribution of drugs. Animal and human studies are represented. All showed that drugs of misuse were able to pass to the fetal circulation.

Authors	**Substance**	**Experimental specimen**	**Study**	**Reported Effects**
Ahmed *et al* (1990)	cocaine	human placentae	characterisation of a cocaine binding protein in human placenta	identified binding sites in the villous plasma membrane
Bailey *et al* (1987)	THC*	rhesus monkey placentae	fetal disposition of THC in late pregnancy	THC rapidly crossed the placenta
Boskovic *et al* (2001)	cocaine and cannabinoids	meconium in human twins	role of the placenta in variability of exposure to cocaine and cannabinoids	amount of drug in meconium differed in dizygotic twin pairs
Fares and Gavish (1986)	benzodiazepine (BZ)	term human placentae	characterisation of BZ binding sites	BZ binding sites identified
Fokina *et al* (2011)	bpn**	preterm human placentae (30-34 weeks gestation) dual perfusion	determine if gestational changes affected transfer and metabolism of bpn	no gestational changes identified,
Harbison and Mantilla-Plata (1972)	THC	mouse placentae	to assess toxicity, maternal distribution & placental transfer of THC	THC transferred, measured in fetal tissue and amniotic fluid

Malek *et al* (2009)	cocaine, heroin & methadone	human placentae dual perfusion technique	impact of cocaine and heroin on the placental transfer of methadone	methadone accumulated in placental tissue
Myllynen and Vähäkangas (2002)	diazepam	human placenta dual perfusion	to determine if the human placental perfusion technique can predict placental drug transport	diazepam detected in fetal circulation 15 minutes after administration. Maternal concentrations higher than fetal in all perfusions
Nanovskaya *et al* (2002)	bpn	human placentae dual perfusion	transplacental transfer & metabolism of bpn	transplacental transfer of buprenorphine to fetus was low

*THC is delta–9- tetrahydrocannibinol the psychoactive component of cannabis ** buprenorphine

1.4 ATP Binding Cassette (ABC) Transporters

1.4.1 Introduction

The ATP binding cassette transporters constitute a large family of membrane bound proteins encoded by notably polymorphic genes. Subdivided into seven families, labelled A-G, based on the structure of the proteins which they encode, they are energy dependent transporters. They bind adenosine triphosphate (ATP) in the nucleotide binding domain (NBD) and, it is proposed, use the energy produced by its conversion to adenosine diphosphate (ADP) to transport substrates across the cell membrane against a concentration gradient. They are noted for their capacity to transport a wide variety of chemically and structurally diverse but mainly hydrophobic substrates (Thiebaut *et al* 1987, Tsuji 1992, Sugawara 1997, Cordon- Cardo *et al* 1990). They are found in the liver, blood brain barrier, intestine and placenta. This study is interested in their ability to efflux compounds out of cells. In the context of the human placenta this is their ability to keep concentrations of potentially noxious compounds low in the fetal circulation by reducing their ability to pass through the placenta. Figure 1.3 demonstrates the position of the four proteins of interest to this study within the terminal villi of the term human placenta. In the interests of clarity this report will use the ABCB1, ABCG2, ABCC1 and ABCC2 when discussing the gene and P-glycoprotein/P-gp, BCRP, MRP1 or MRP2 when referring to gene products.

1.4.2 ABC Transporters

To date there have been 49 identified genes in the human genome (Vasiliou *et al* 2009) that encode transporter proteins. Members of this family have been conserved in all organisms along different evolutionary pathways, suggesting that they have important physiological functions (Myllynen *et al* 2010, Vasiliou *et al* 2009). First identified in tumours, studies to determine the cause of poor clinical outcomes of chemotherapy identified the ability of ABC transporters to efflux cytotoxic drugs from target cells and thus maintain intracellular concentrations at sub therapeutic levels (Wu *et al* 2008). They have the ability to efflux a wide range of drugs and xenobiotics from cells irrespective of the original drug of exposure and were thus named multidrug resistance proteins (MDRP) (Schinkel *et al* 1997, Lankas *et al* 1998, Atkinson *et al* 2003, Poller *et al* 2011, Vlaming *et al* 2009a and b).

Subsequently discovered in healthy tissues that function as a protective barriers e.g. blood brain barrier, liver, kidneys, intestine and testes (Cordon-Cardo, *et al* 1990, Maliepaard 2001) it became evident that they are involved in absorption, distribution and excretion (Bodo *et al* 2003). It is now generally accepted that ABC transporters protect tissues from many structurally and chemically diverse xenobiotics by their efflux action (Schinkel *et al* 1997, Behravan and Piquette-Miller 2007) and from endogenous substances such as endogenous steroids (Petropoulos *et al* 2010, Young *et al* 2003, Schinkel and Jonker 2003). Table 1.3 provides examples of some compounds identified as inhibitors or substrates for the four proteins many of which are used in pregnancy.

It has been suggested that some compounds e.g. progesterone can both stimulate and inhibit the protein and therefore its ability to efflux the compound (Young *et al* 2003). The presence of two binding sites, a high affinity site that stimulates and a low affinity site that inhibits (Sharom *et al* 2005) has been proposed. Schinkel and Jonker (2003), assert that two or more substrates can be effluxed concurrently and that this mechanism of co transportation may result in more efficient transport of both substrates.

Following discovery of their presence in the human placenta it was hypothesised that they effluxed xenobiotics and other toxins from the fetal to maternal circulation, thus protecting the development of the fetus (Leslie *et al* 2005). The four multidrug resistance proteins of interest in this study are P-gp, BCRP, MRP1 and MRP2. Although their expression is not confined to the placenta and their location in other tissues may contribute indirectly to protecting the fetus, via its role in other tissues e.g. reducing the absorption and subsequent bioavailability of substances in the maternal circulation. It is their role in the placenta that is of primary interest and is therefore, the main focus of the following characterisation of their expression, structure and function.

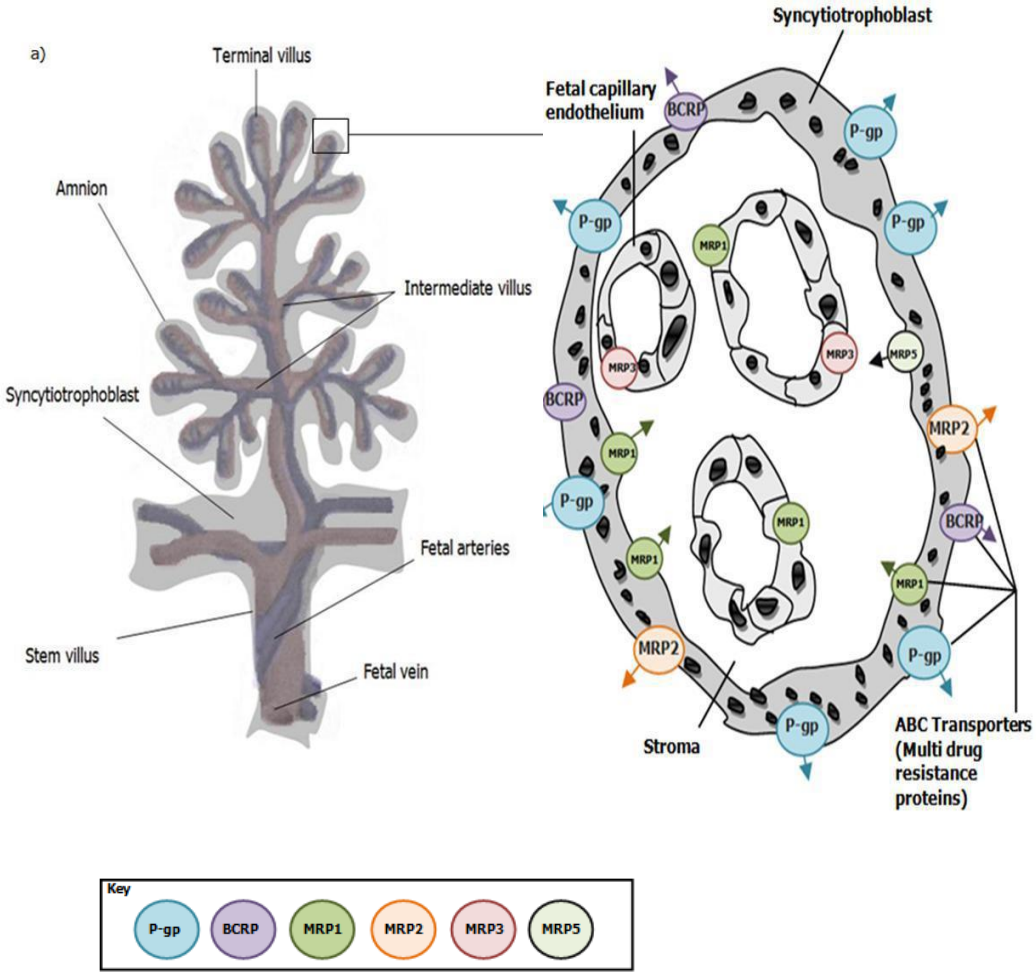


Figure 1.3 A schematic representation of a villous tree. The area represented in the square is enlarged to show a terminal villi in section on which the location of the multidrug resistance proteins P-gp, BRCP, MRP1 & MRP2 are shown. Adapted from Ceckova-Novotna *el al* (2006).

Table 1.3 Example of substrates and inhibitors identified for P-gp, MRP1, MRP2 and BCRP. The lack of substrate specificity for some classes of drug is worthy of note as it impinges on the design of experimental protocols. (Cascorbi et al 2006, Vähäkangas and Myllynen 2009, Hahnova-Cygalova et al 2010, Weiss et al 2007 and Wang et al 2006).

Drug	**P-gp**	**MRP1**	**MRP2**	**BCRP**
Cytotoxics	doxorubicin, vinblastine, vincristine, paclitaxel	doxorubicin, , vincristine vinblastine, methotrexate	doxorubicin, vinblastine, methotrexate, vincristine, ,cisplatin, hormone conjugates	doxorubicin, mitoxantrone methotrexate
HIV protease inhibitors	ritonavir, saquinavir			lopinavir , nelfinavir, saquinavir, atazanavir
Antibiotics	Erythromycin		ampicillin, cefaxidime	nitrofurantoin, erythromycin
Ca2+ channel blockers	verapamil, diltazem			azidopine, dipyridamole
HMG-CoA inhibitors	Lovastatin		pravastatin	rosuvastatin, pitavastin,
Miscellaneous	amitryptiline, , morphine, lansoprazole rifampicin methadone, phenytoin, olanzapine, risperidone	glucuronide, sulphate, glutathione conjugates	bilirubin and some drug conjugates e.g. leukotrine C4	glyburide, cimetidine folic acid, riboflavin biotin (vitamin B7),
fluorescent dyes	rhodamine 123		carboxyfluorescein	Hoechst3342 BBR3390

Although P-gp, BCRP, MRP1 and MRP2 share characteristics they also have important differences. For example in the number of transmembrane helixes in the transmembrane domains, the number of transmembrane domains, the number of nucleotide binding domains and the position of the nucleotide binding domains. A comparison of the proposed structure of P-gp, BCRP, MRP1 and MRP2 is shown in Figure 1.4.

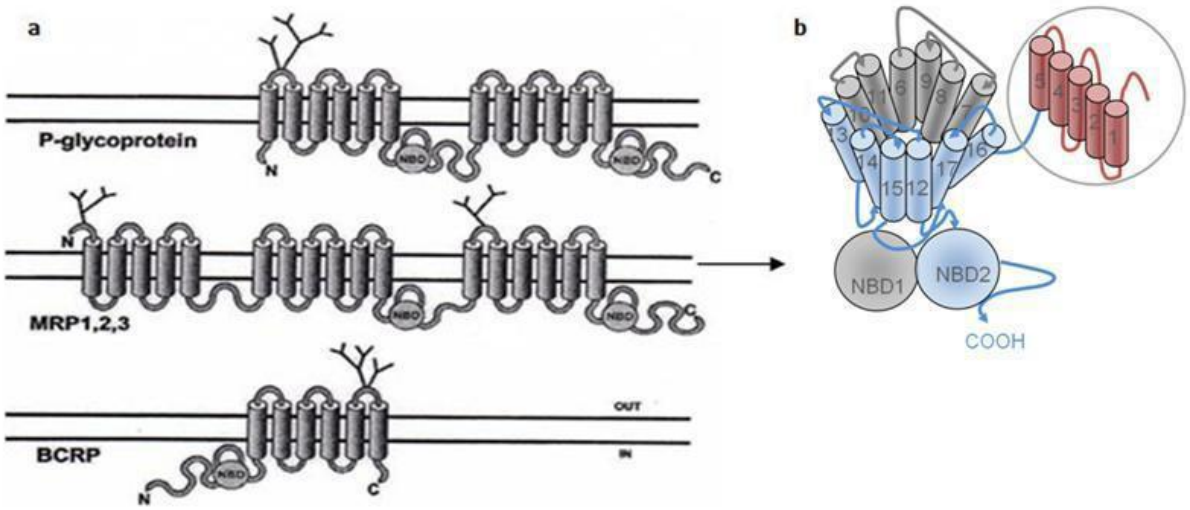


Figure 1.4 Comparative proposed structures of the transmembrane and nucleotide binding domains for P-gp, BCRP, MRP1 and MRP2, a) two dimensional schematic representation of the structure of the four proteins adapted Schinkel and Jonker (2003), b) the predictive packaging of MRP1 adapted from Deeley and Cole (2006).

1.4.2.1 Role of ABC Transporters in the Placenta

Because it is obviously ethically unacceptable to administer compounds with the potential to harm the fetus to pregnant women much of what is known about the role of these transporters has come from *in vivo* experiments performed in animals and in experiments in cell lines. Experiments on human placentae usually use *ex vivo* techniques such as dual perfusion and placental fragment uptakes. The murine orthologues have similar substrate specificity and functions to human P-gp and make this species suitable for functional studies on this protein.

Despite a body of evidence that identified the localisation, expression and function of ABC transporters (Atkinson *et al* 2003, Sun *et al* 2006, Petropoulos *et al* 2007, Evseenko *et al* 2007) work continues to characterise their exact roles within the human placenta. Functional P-gp, MRP2 and BCRP located in the microvillous membrane of the syncytiotrophoblast (Atkinson *et al* 2006b, St. Pierre *et al* 2000), efflux substrates back

to the maternal circulation ensuring that fetal levels are lower than maternal thus ensuring that the fetus is not exposed to compounds potentially detrimental to fetal growth and/or development. This is particularly important at times of critical fetal development. However, it also means that therapeutic agents such as HIV protease inhibitors may not reach the fetus, or not reach therapeutic levels (Wang *et al* 2006, Gulati and Gerk 2009). In contrast, MRP1 located on the basal membrane of the syncytiotrophoblast transports compounds towards the fetal vascular compartment. MRP1 is however, also expressed in fetal capillaries (St. Pierre *et al* 2000) and at this location would efflux substrates in a fetal to maternal direction as can be seen from Figure 1.5.

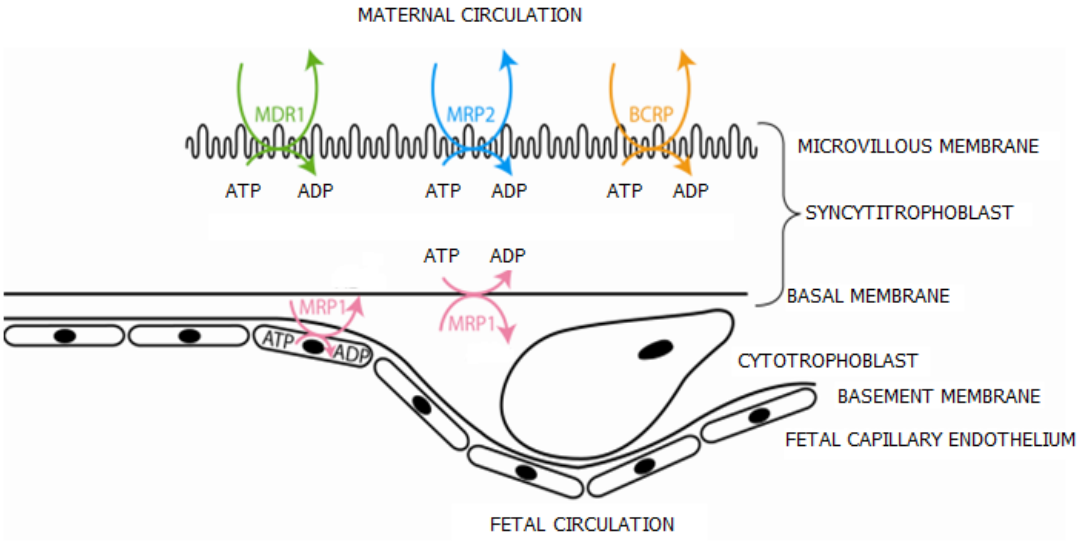


Figure 1.5 Diagram showing the location of P-gp, MRP2 and BCRP on the microvillous membrane of the placental syncytiotrophoblast and MRP1 on the basal membrane. Adapted from Atkinson *et al* (2006b).

1.4.2.2 P-glycoprotein (P-gp. MDR1)

Clinically important because its substrates include many commonly prescribed medications as well as drugs of misuse e.g. cytotoxic drugs, HIV protease inhibitors, analgesics, antibiotics and statins (Cascorbi 2006, Vähäkangas and Myllynen 2009, Hahnova-Cygalova *et al* 2011) P-glycoprotein is encoded by the highly polymorphic ABCB1 gene located on chromosome 7q 21.1.

The first MDRP to be identified in 1976 (Juliano and Ling 1976) it is one of the most abundant in the human placenta and the best characterised efflux transporter of the ABC family. Extensive studies in murine and human tissues have sought to characterise its

expression, structure and function (Smit *et al* 1999, Schinkel *et al* 1997, Lankas *et al* 1998, Leslie *et al* 2005, Chung *et al* 2010).

A 1280 amino acid protein with a molecular mass of 170 kDa, it transports molecules of between 200-1900Da (Gottesman *et al* 1996). Its substrates are diverse but are generally amphipathic, lipid soluble and positively charged (Schinkel and Jonker 2003).

Located on the apical surface of numerous tissues e.g. intestinal epithelial cells, bile canaliculi, renal tubular cells, luminal surface of capillary endothelial cells in the brain, testes and the microvillous membrane of the placenta (Vähäkangas and Myllynen 2009, Atkinson *et al* 2003, St-Pierre *et al* 2000, Callaghan *et al* 2008) it reduces bioavailability of substances to that tissue by its efflux action. In the placenta, its apical location in the microvillous membrane of the syncytiotrophoblast brings it into direct contact with the maternal circulation and thus protects the fetus by effluxing xenobiotics to the maternal vascular circulation and maintaining low concentrations in the fetal circulation (Atkinson *et al* 2003). Research has focussed on the characterisation and manipulation of efflux capacity to improve bioavailability and clinical outcomes with therapeutic agents such as digoxin, cytotoxics and HIV protease inhibitors or reduce bioavailability of harmful substances such as drugs of misuse and xenobiotics.

1.4.2.2.1 Proposed Basic Structure

The two hydrophobic transmembrane domains are linked by loops and nucleotide binding domains that are both hydrophilic and form a dimer in the transmembrane pore through which substrates are effluxed (Figure 1.6).

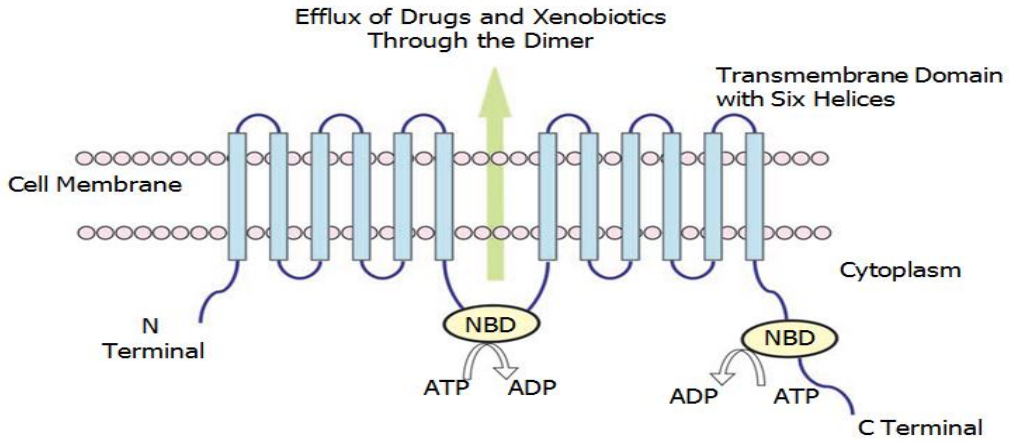


Figure 1.6 A two dimensional representation of P-gp showing the dimer through which compounds are effluxed and the relative positions of the two NBD. Adapted from Lin *et al* 2006.

In P-gp both terminals and the NBDs are intracellular. It is hypothesised that the Walker A & B and the Signature C motifs located in the NBDs are required for the binding and hydrolysis of substrates. Stages of transportation have been described as; partitioning of the substrate to the lipid layer of the membrane, transfer to the binding pocket of the protein and passage through the TMD, this is facilitated by conformational changes in the NBD following ATP hydrolysis, (Sharom 2008, Zhou 2008, Lugo and Sharom 2005). Higgins and Linton (2004) present an informative review summarising the putative roles of ATP binding and ATP hydrolysis in executing the conformational changes necessary for effective functioning.

1.4.2.3 Multidrug Resistance Associated Proteins (MRP)

There are nine proteins in the MRP family. This report focusses on two, MRP1 and MRP2. Both MRP1 and MRP2 transport amphipathic organic anionic and neutral substances conjugated with gluthione, glucuronate or sulphate (Young *et al* 2003 Schinkel and Jonker 2003, Borst *et al* 2000). As with P-gp there are two hydrophobic TMDs containing six helixes that are linked by hydrophilic loops and 2 NBDs. (Rosenberg *et al* 2005) but MRP1 and MRP2 have an additional N terminal extension TMD with 5 helixes This Deeley and Cole (2006), hypothesise is required to maintain the proteins position within the cell membrane and is unnecessary for transportation. An additional role in tissue defence and cell detoxification has also been proposed (Kruh and Belinsky 2003). In the human placenta their substrate profile is very similar but as one is located on the apical and the other on basal sides of membranes it is thought that their functions may vary.

1.4.2.3.1 MRP1

This 190kDa, 1531 amino acid protein was first described by Cole *et al* in 1992. Encoded by the ABCC1 gene located on chromosome 16 p13.1 it is localised on the basolateral membrane of the kidney, skeletal muscles and testes in addition to the basal membrane of the syncytiotrophoblast in human placenta (Borst *et al* 2000, St.Pierre *et al* 2000, Schinkel and Jonker 2003, Atkinson *et al* 2003). It has been hypothesised that this basolateral placement enables it to ensure endogenous toxic compounds such as bilirubin are transported efficiently to the maternal circulation keeping low levels in the fetal circulation (Nagashige *et al* 2003).

1.4.2.3.2 MRP2

A 1545 amino acid protein, encoded by the ABCC2 gene located on chromosome 10, MRP2 localises to the apical surface of polarized membranes. Location is widespread and includes the liver and intestine (Cascorbi 2006, Toyoda *et al* 2008, Jemnitz *et al* 2010) but in humans its location in the hepatocyte canicular membrane (Jedlitschky *et al* 2006) indicates a role in the secretion of bilirubin conjugates into bile as evidenced by Dubin Johnson syndrome in individuals lacking MRP2 (Paulusma *et al* 1997, Borst *et al* 2000). In the human placenta it is expressed on the microvillous (apical) membrane of the syncytiotrophoblast and is thought to facilitate efficient transport of waste products from the fetal to maternal circulations whilst preventing transport of conjugated products from drug metabolism from maternal to fetal.

1.4.2.4 Breast Cancer Resistance Protein (BCRP, ABCG2)

A 75 kDa, 655 amino acid protein encoded by the ABCG2 gene located on chromosome 4q 22 (Bailey-Del *et al* 2001, Kolwankar *et al* 2005) BCRP is expressed in placenta, liver, small intestine, breast, kidney heart and blood brain barrier (Hemauer *et al* 2010, Maliepaard *et al* 2001). It is a half transporter having only 1 TMB with 6 helixes and 1NBD (Figure 1.7). BCRP is known to function as a homodimer and there is some evidence that it also forms a functional protein as a heterodimer but the identity of the other half transporters with which it can dimerize is as yet unknown. There is also some evidence that it can function as a homotetramer (Kolwankar *et al* 2005, Robey *et al* 2009). Located on apical membranes it transports toxic compounds away from tissues. In the human placenta it is expressed on the microvillous membrane of the syncytiotrophoblast. Interestingly, due to its location on the apical membrane of the alveolar in human breast tissue, it secretes substances into breast milk, e.g. riboflavin (van Herwaarden *et al* 2007). Its substrate specificity is wide and Robey *et al* (2009) note it transports both highly negatively and positively charged molecules including sulphate conjugates. Evseenko *et al* 2007 suggest it has a role in trophoblast protection and that reduced expression may result in fetal growth restriction.

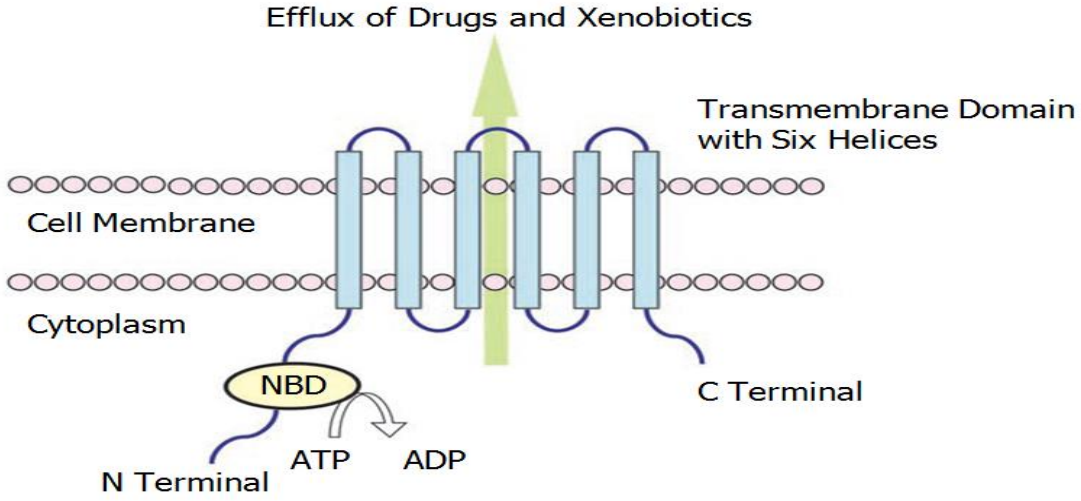


Figure 1.7 A two dimensional diagrammatic representation of BCRP. Unlike P-gp, MRP1 and MRP2 BCRP has only one TMD and one NBD. Commonly called a half transporter because of this it is proposed that BCRP has to form a homo or heterodimer to perform its efflux function. Adapted from Lin *et al* (2006).

1.4.2.5 Evidence for Active Placental Transport

A mechanism to provide evidence for an active transport is to measure what proportion of an administered substrate crosses the placenta in the presence and absence of a transporter inhibitor (Vlaming *et al* 2009a and b, Molsa *et al* 2005, Nanovskaya *et al* 2005). The differential between the two conditions represents the level of activity of the transporter under a particular set of experimental conditions (Chapter 4). If a compound is transferred by diffusion alone at some point equilibrium between the maternal and fetal circulations would be reached and compounds would not cross the concentration gradient. In an active transport model there would be disequilibrium as the transporters efflux compounds away from the fetal circulation to the maternal as reported by Sudhakaran *et al* (2005) and Sudhakaran *et al* (2007). Other models have used transfected cells or animals with a known genetic or chemically induced variation in transporter (Atkinson *et al* 2006b). Known doses of a compound are administered and differences in outcome between the conditions recorded. Seminal works by Lankas *et al* (1998), Smit *et al* (1999) and Schinkel (1998) demonstrated that these are active transporters using these models. Lankas *et al* (1998) administered avermectin to pregnant mice whose genotype for P-gp was known. Fetuses deficient in P-gp (-/-) had 100% susceptibility to chemically induced cleft palate caused by avermectin, heterozygous pups (+/-) 30 % susceptibility and wild type (+/+) litter mates were insensitive to avermectin. As the proportion of affected pups was related to variation in

P-gp present it demonstrates active transfer of avermectin by P-gp, keeping levels in the fetal compartment low and thus protecting from teratogens. Smit *et al* (1999) used P-gp knockout mice and demonstrated active transfer of saquinavir, digoxin and paclitaxel when up to 16 times more compound reached the fetus in the absence of P-gp. In addition, pharmacological inhibition of P-gp by PSC833 and GG918 produced similar levels in wild type and P-gp deficient mice.

Table 1.4 provides examples of other studies that have demonstrated active transport using compounds prescribed in pregnancy, taken in the diet or taken by women. Examples of work with mice, human placentae and cell lines are shown. Data from these studies shows that altering transport capacity by genetic manipulation or chemical inhibition altered the disposition of the compound. To translate these findings to clinical practice, Wang *et al* (2008) showed that commonly prescribed antipsychotics including risperidone, olanzapine and quetiapine inhibited BCRP. If these were prescribed with for example an antibiotic or hypoglycaemic that relied upon BCRP for transportation it would potentially alter the pharmacokinetics and impact upon the efficacy of treatment.

Table 1.4 Examples of studies that demonstrate active transfer of compounds by ABC transporters. Studies using cells lines, mice and human placental tissue have been included.

Author	**Transporter**	**Studied**	**Experimental subject**	**Outcomes**
Kolwankar *et al* (2005)	P-gp, BCRP	maternal smoking,	human placental vesicles	accumulation of ^{3}H mitoxantrone & ^{3}H vinblastine was increased when P-gp and BCRP were inhibited. No statistical difference between smokers and non-smokers.
Zhou *et al* (2008)	Bcrp1*	glyburide**	mice	Bcrp1 significantly limited transfer of glyburide to the fetus
Smit *et al* (1999)	P-gp	^{3}H digoxin, ^{14}C saquinavir, paclitaxel	mice	increased drug accumulation when placental P-gp was inhibited
Jonker *et al* (2000)	Bcrp1	GF120918 (BCRP inhibitor) with topotecan	mice, polarised mammalian cell lines	Bcrp1 mediated apically directed transport
Wang *et al* (2008)	BCRP	5 antipsychotic drugs	Mcf7/Mx100 cell lines	every drug inhibited BCRP showing a potential for drug-drug interactions
Myllynen *et al* (2008)	BCRP	PhIP***	human placenta (dual perfusion), BeWo cells	BCRP decreased placental transfer of ^{14}C-PhIP in perfused human placenta.
Gedeon *et al* (2008)	BCRP, P-gp MRP1	glyburide	human placenta (dual perfusion)	BCRP preferentially transported glyburide

*Bcrp1 indicates animal protein **glibenclamide in UK ***2-amino-1-methyl-6-phenylimiddazol [4, 5-b] pyridine (a dietary carcinogen)

1.4.2.6 Multidrug Resistance Proteins and Drug Use

NAS demonstrates that drugs cross the placenta but the variability of its presentation suggests variability in the amount that is transferred. It is known that some drugs e.g. methadone and morphine are transported by at least some members of the ABC binding cassette family but relationships between drugs and transporters are still unclear. As seen in Table 1.5 research has focussed predominately on P-gp. Holland *et al* (2006, 2007 and 2008) produced a series of papers examining interactions of P-gp, MRP1 and BCRP with cannabinoids and Tournier *et al* (2010) used cells transfected with human P-gp and BCRP. Table 1.5 provides examples of studies and methodologies used. It has been suggested (Tournier *et al* 2010) that it is the differences in methodologies and the use of animal models that is, in part, responsible for a lack of clarity in the literature.

Not all studies in Table 1.5 relate to placental transport. For example, Rodriguez *et al* (2004), Suzuki *et al* (2007) and Wang *et al* (2004) studied transport at the blood brain barrier and Coller *et al* (2006) studied genetically derived variability in MDRPs and how this affected methadone requirements of adults on a methadone treatment programme. Building on previous work (Hassan *et al* 2007) in which they showed oxycodone induced expression of P-gp in the brain, liver and kidneys of rats, Hassan *et al* (2009) investigated the effect of P-gp on the antinociceptive activity and tissue distribution of methadone and buprenorphine in P-gp knockout and wild type mice and Caco-2 cells in the presence and absence of the P-gp inhibitors verapamil and GF120918. They found that the brain distribution and antinociceptive effects of methadone but not buprenorphine were significantly enhanced in P-gp knockout mice. In summary the data shows that commonly prescribed and over the counter medications interact with MDRPs and have the potential to alter the amount of drug the fetus is exposed to and thus contribute to the variability in the severity of NAS.

Table 1.5 Examples of research that explored the interaction of ABC transporters and substances of misuse. Animal and human models are included. Studies are presented in alphabetical order.

Authors	**Substance**	**Protein**	**Experimental Specimen/Vehicle**	**Objectives**	**Reported Effects/Observations**
Beghin *et al* (2010)	methadone	P-gp	rat and human placental trophoblast cells	comparison of rat and human placental P-gp activity	methadone inhibited P-gp activity
Bonhomme-Faivre *et al* (2008)	THC	P-gp	CF1 mice	role of P-gp in distribution of THC	THC exposure was higher in mice deficient in P-gp.
Coles *et al* (2008)	methadone, bpn	P-gp	mouse placentae	distribution of methadone and buprenorphine at day 13 & day18	P-gp expression increased at late gestation. Placental methadone concentrations greater than plasma. No significant difference in placenta –plasma ratio for bpn.
Coller *et al* (2006)	methadone	P-gp	adult opioid dependant individuals	role of genetic variability on methadone dose	ABCB1 genetic variability influenced daily methadone requirements
Hassan *et al* (2007)	oxycodone	P-gp	rats, mice and Caco-2 cells	establish oxycodone's affinity to P-gp	Oxycodone is a P-gp substrate
Hassan *et al* (2009)	methadone, bpn diprenorphine	P-gp	knockout mice	establish the affinity of drugs to P-gp to predict P-gp mediated drug interactions	methadone stimulated P-gp ATPase activity. Brain distribution enhanced in mice lacking P-gp but not bpn or diprenorphine

Hemauer *et al* (2009)	methadone, bpn, morphine	P-gp	human placental microvillous membrane inside out vesicles	establish interactions of P-gp with methadone, bpn and morphine	morphine, methadone and bpn reduced P-gp mediated paclitaxel transport
Holland *et al* (2006)	cannabinoids	P-gp	mouse fibroblast P-gp transfected cells and human leukaemia cell line	to examine the effect of cannabinoids on P-gp	THC and cannabidiol decreased P-gp expression shown by increased intracellular accumulation of Rhodamine 123
Holland *et al* (2007)	cannabinoids	Bcrp1	Cells over-expressing Bcrp1	to explore the interactions of cannabinol, cannabidiol and THC with BCRP	increased mitoxantrone levels indicate cannabinoids inhibit Bcrp1 activity but did not change expression
Holland *et al* (2008)	cannabinoids	MRP1	ovarian carcinoma cells over-expressing MRP1	to examine the effect of cannabinoids on MRP1	THC and cannabidiol decreased MRP1 expression demonstrated by increased intracellular levels of Fluo3 and vincristine
Nanovskaya *et al* (2005)	methadone	P-gp	human placentae, dual perfusion and BeWo cells	to study the effect of P-gp on the disposition of methadone in the presence and absence of the P-gp inhibitor GF182091	P-gp inhibition resulted in 30% more methadone crossing the placenta. Transfer of the substrate paclitaxel increased 50%

Nanovskaya *et al* (2008)	methadone	P-gp	dually perfused preterm human placental lobule	to study gestational age and methadone transfer in relation to P-gp expression	transfer rate and amount of methadone in the fetal compartment lower in the preterm placenta than full term
Nekhayeva *et al* (2005)	methadone	P-gp	dually perfused human placental lobule	to obtain data on methadone pharmacokinetics in pregnancy	methadone had no adverse effects on placental viability and was retained by placental tissue. Methadone transfer fetal-maternal is higher than maternal-fetal
Rodriguez *et al* (2004)	methadone	P-gp	Rat	examine the role of P-gp on the analgesic effect of methadone and its uptake by brain tissues.	analgesic effect of methadone increased in rats pre-treated with a P-gp inhibitor
Suzuki *et al* (2007)	bpn	P-gp	rat	establish role of P-gp in the transfer of bpn across rat blood brain barrier	P-gp inhibitor enhanced the uptake of bpn by the brain
Tournier *et al* (2010)	14 drugs inc. methadone, bpn, cocaine, amphetamine, THC, nicotine,	P-gp, BCRP	hMDR1- and hBCRP-transfected HEK293 cells	to determine if the compounds inhibited or were transported by P-gp and/or BCRP in humans	P-gp was inhibited by bpn, methadone, and THC. BCRP was inhibited by bpn and THC P-gp transported methadone only BCRP did not transport any
Wang *et al* (2004)	methadone	P-gp	knockout mice	role of P-gp in transfer of methadone across the mouse blood brain barrier	brain concentrations of methadone in mice lacking P-gp was significantly higher than wild type mice

1.4.2.7 Gestational Differences in the Human Placenta

Although P-gp protein is abundant at term it is generally agreed that it's expression is greatest in the first trimester of pregnancy and declines with advancing gestation (Sun *et al* 2006, Mathias *et al* 2005, Gil *et al* 2005, Petropoulos *et al* 2007, Kalabis *et al* 2007, Coles *et al* 2008). It is suggested that this profile protects the fetus in early stages of development when exposure to potentially toxic xenobiotics could be teratogenic but allows fetal exposure to endogenous compounds e.g. cortisol in later pregnancy (Sun *et al* 2006). If expression is correlated with function the reduced level of efflux in later pregnancy could leave the fetus vulnerable to compounds that interact with MDRPs. In contrast to the previous findings Chung *et al* (2009) in their study using positron emission tomography (PET) imaging of P-gp activity in pregnant macaques found P-gp activity increased with gestation. Their explanation of this relates to the increase in total villous area as gestation advances. They suggest that even if activity per milligram of placenta decreases the increase in total area ensures an overall increase in P-gp activity.

The evidence for BCRP is less clear. Yeboah *et al* (2006), demonstrated increased expression of BCRP through gestation, Mathias *et al* (2005) showed no change in either BCRP protein or mRNA whereas other authors report reduced protein expression with advancing gestation (Meyer zu Schwabedissen *et al* 2005, Yasuda *et al* 2005). Cygalova *et al* (2009), using a rat model, found reduced Bcrp1 mRNA with advancing gestation as did Kalabis *et al* (2007) using a mouse model. However, Bcrp1 protein levels did not show any significant changes through gestation (Kalabis *et al* 2007). This reflected findings in human placentae whereby no significant change in BCRP protein expression was noted through gestation (Mathias *et al* 2005).

Evidence for MRP1 and MRP2 is less abundant and again the evidence is conflicting. Pascolo *et al* 2003 showed greater expression of MRP1 but not MRP2 with advancing gestation in human placentae and BeWo cells. Later work by May *et al* (2008) produced contrasting results with increased expression of MRP2 with gestation.

1.4.2.8 Variation in Expression and Function

Variation in expression and function has been demonstrated in placenta and other tissues (Keskitalo *et al* 2009, Hoffmeyer *et al* 2000, Atkinson *et al* 2009, Hitzl *et al* 2004). Atkinson *et al* (2009) examined the level of expression of P-gp, BCRP and MRPs1 and 2 in normal, term placentae using the Western Blotting immunoblotting technique. Their

results showed a marked variation in expression of proteins between placentae and variation of individual proteins within each placenta: as shown in Figure 1.8 placentae 1,2,5,6 and 8 express all four proteins whereas placenta 4 which has the most P-gp (as measured in arbitrary optical density units), has among the least MRP1 and no measurable MRP2 or BCRP.

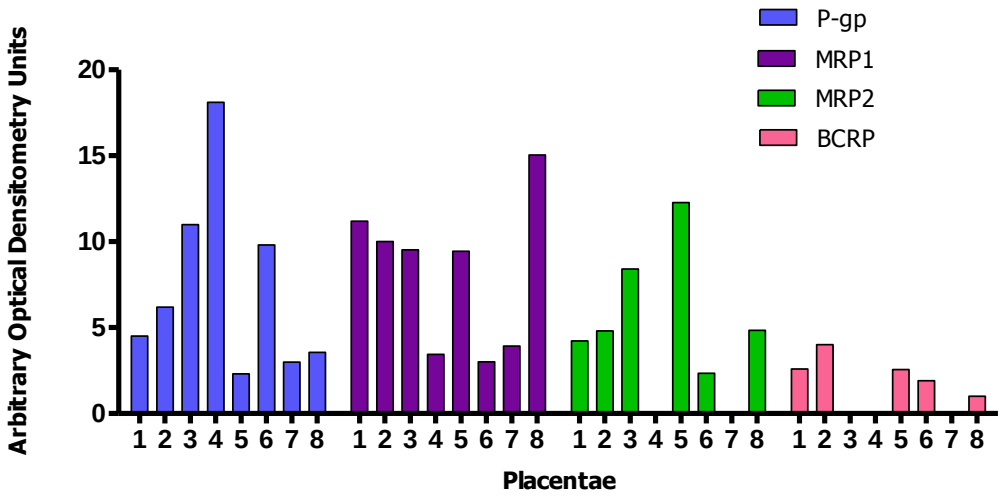


Figure 1.8 The eight term placentae represented in the figure showed considerable variation in expression of the four proteins. Atkinson *et al* 2009.

Hitzl *et al* (2004) demonstrated an eight fold variation in P-gp expression which was not normally distributed and suggested this may be due to genetic factors.

1.4.2.9 Genetic Variation

1.4.2.9.1 Introduction

If the role of these transporter proteins is protection of the fetus from potentially harmful compounds, such wide variation in expression seems counterintuitive as this may render the fetus vulnerable to potential teratogens. Attempts to understand inter-individual differences in expression and function of ABC transporters by identifying naturally occurring variations in the genes that encode the proteins is a rapidly emerging field of study. One hypothesis is that single nucleotide polymorphisms (SNPs) in the encoding genes ABCB1, ABCC1, ABCC2 and ABCG2, alter the expression and or function of the corresponding gene product. This builds on previous work that explored genetic

variation in drug metabolizing enzymes and methadone plasma levels (Crettol *et al* 2006) and THC (Sachse-Seeboth *et al* 2009).

There are numerous polymorphisms identified, the study of which has produced conflicting results and a lack of consensus as to which variations are important and the potential clinical significance of findings. A critical review by Leschziner *et al* (2007), summarizes some of the main limitations of the studies to date e.g. inadequately powered studies, co morbidity, analysis of multiple drugs that may not be substrates, potential confounders and poor definition of disease. Older studies have examined SNPs in isolation but following observations of strong linkage disequilibrium with C1236T and G2677T there is a movement toward studying haplotypes rather than SNPs.

1.4.2.9.2 Single Nucleotide Polymorphisms (SNPs)

SNPs are naturally occurring changes in single nucleotides found in both coding and non-coding regions of genes. Studies have shown that they cause changes in the amount, structure and or function of all four proteins of interest to this study (Hoffmeyer *et al* 2000, Lepper *et al* 2005). Kobayashi *et al* (2005) showed reduced expression of BCRP contingent upon genotype and Meyer zu Schwabedissen *et al* (2005) a similar finding in MRP2. A SNP can be synonymous i.e. the change produces the same amino acid or nonsynonymous i.e. produces a different amino acid (missense) or stop codon (nonsense). It has been suggested that even though synonymous SNPs do not change amino acids, they may change the manner in which the protein functions by affecting the way it folds (Kimchi- Sarfaty *et al* 2007, Sauna *et al* 2007, Fung and Gottesman *et al* 2009). Authors have also described ethnic differences in the frequency of SNPs (Kimchi-Sarfaty *et al* 2007, Kobayashi *et al* 2005).

1.4.2.9.3 Single Nucleotide Polymorphisms and Drugs of Misuse

It has been suggested that polymorphisms in the ABC transporters impact upon different clinical outcomes i.e. in chemotherapy and may also contribute to different neonatal outcomes in human pregnancies by altering fetal exposure to commonly misused drugs (Thadani *et al* 2004).

Coller *et al* (2006), studied the polymorphisms A61G, C1236T, C3435T G1199A, in the ABCB1 gene relating genotype to phenotype i.e. amount of methadone prescribed to abate signs and symptoms of opiate withdrawal in a cohort of 60 opiate dependant adults and 60 controls. Their results showed a statistically significant variation in

required methadone dose dependent upon genotype. The number of copies of wild type haplotype the individual possessed affected outcome. Individual with no copies required the least methadone, 1 copy an intermediate dose and 2 copies required the highest dose indicating that P-gp's ability to transport methadone was affected by genotype. As there was no significant difference in haplotype frequencies between control and methadone groups, they concluded that this was not related to opiate dependence, i.e. a particular haplotype did not indicate a propensity for dependence. The results were broadly in line with work carried in out in mice exposed to avermectin by Lankas *et al* (1998).

This study inferred bioavailability of methadone using genotype the inference being that those with no copies had less transporter protein to efflux methadone allowing more to be absorbed through the intestine and blood brain barrier. This reduced the amount of methadone required to abate signs and symptoms of withdrawal. Conversely participants with the most P-gp would require the most methadone as bioavailability would be reduced. Whilst recognising the limitations of the small sample size the authors suggest haplotyping may ultimately be used to predict dosing requirements of methadone.

Uehlinger *et al* (2007), studied the C3435T SNP on the ABCB1 gene in 14 participants on a methadone maintenance programme. They demonstrated genetically derived differences in the mean increase of plasma concentrations of (R)- methadone after administrating the P-gp inhibitor quetiapine (3% TT, 23% CT and 33% CC). The lowest mean increase was shown by the TT genotype. This indicates this genotype produced the least amount of protein as its inhibition produced the lowest mean level of change in plasma (R)- methadone.

Levran *et al* (2008), also studied methadone requirements but in relation to the C1236T SNP, in an attempt to determine the reasons for inter-individual differences in efficacy of methadone maintenance treatment. They hypothesised that as methadone is a substrate for P-gp, genetic variation within the gene would affect absorption, metabolism and excretion. Their results demonstrated a significant difference in methadone dose dependent upon genotype and suggested these were clinically significance findings.

Investigating the role of the ABCB1 C3435T polymorphism in cannabis dependence Benyamina *et al* (2009) found the CC genotype was independently associated with cannabis dependence. These findings are in concordance with the above studies as Benyamina *et al* (2009) propose that CC carriers have more P-gp at the blood brain

barrier, resulting in a greater efflux of delta 9 tetrahyrdocannabinol (THC). Earlier work by the same group (Bonhomme-Faivre *et al* 2008) showed plasma levels of orally administered THC were higher in P-gp deficient mice than wild type.

1.4.2.9.4 Polymorphisms and the Placenta

A number of SNPs in the ABC transporter genes have been studied to determine their effect on protein functioning and expression levels (Kimchi-Sarfaty *et al* 2007) but as yet there is limited work in the human placenta. Results have demonstrated genetically determined differences but their clinical significance has not been determined. Tanabe *et al* (2001) studied placental polymorphisms in the ABCB1 gene in a cohort of pregnant Japanese women. Nine polymorphisms were detected and a linkage disequilibrium with C3435T & G2677T/A noted. A significant reduction in P-gp expression was noted with -129CT, compared to TT. They also reported a trend to reduced P-gp with 3435TT compared to CT. Reported rates for the C3435T polymorphism vary by ethnicity (Maeda and Sugiyama 2008) making comparison of data difficult. Fung and Gottesman (2009) report ethnically derived differences in the ABCC1 and ABCC2 genes that, they report, may be linked to functional changes in the protein.

Studying C3435T and G2677T/A SNPs in human placentae Hitzl *et al* (2004), found genetically determined differences in protein expression and proposed that both maternal and fetal genotypes determined P-gp expression as when mother and fetus both had the TT genotype there was reduced protein expression. However, Rahi *et al* (2008) produced conflicting results in their study TT increased expression of P-gp but did not produce a significant change in function. Kobayashi *et al* (2005) detected 20 SNPs in ABCG2, 10 amino acid substitutions; 1 synonymous, 2 stop codons and 7 changed amino acids. Eighteen per cent of their sample had the G34A SNP and 35.5% the C421A . Haplotype analysis identified 4 haplotypes.

In summary, whilst it is sometimes inevitable that a woman must take medication when pregnant to maintain her health e.g. anticonvulsants, hypoglycaemics, antibiotics or antipsychotics, the aim of treatment would be to keep fetal exposure low. In contrast some conditions require medication to reach the fetus in addition to treating the woman e.g. HIV medication. As many commonly prescribed medications are substrates or inhibitors of P-gp, BCRP, MRP1 and MRP2 these aims may be difficult to achieve. Knowledge of which genotypes or haplotypes facilitate these strategies in relation to P-gp, BCRP, MRP1 and MRP2 is clinically important. Although there is an increasing body of

research with this as its aim, at present it is not possible to use knowledge of genotype or haplotype to predict which infants are most effectively protected by the efflux action of the placental ABC transporters. This information could be helpful when women are planning a pregnancy or when pregnant to plan medication regimes, especially when women take more than one drug known to interact with these proteins whether they be prescribed or non-prescribed. This would help to ensure fetal drug exposure is low and reduce the fears of women who have to take medication in pregnancy. As yet it remains impossible to offer individualised medication regimes based on sound pharmacogenetic knowledge.

1.5 Hypothesis

The hypothesis for this study is therefore, that polymorphisms in the multidrug resistance genes ABCB1, ABCC1, ABCC2 and ABCG2 cause changes in protein expression, which reduce the capacity of the placenta to efflux drugs of misuse. This in turn leads to increased fetal exposure demonstrated by an increase in signs of NAS and/or altered developmental outcomes.

1.6 Objectives

To test this hypothesis there were two main objectives

1 **To determine whether multidrug resistance protein (MDRP) genotype influences human placental MDRP expression.**

2 **To determine if there is a relationship between the level of placental MDRP expression and neonatal outcomes**.

These objectives were met by a core investigation, ongoing throughout the whole course of the period of PhD study, and for which the outcome is described in Chapter 5 of this thesis. In addition I carried out three studies complementary to the main objectives: (1) a systematic review of whether NAS is related to the amount of maternal opiate use (Chapter2): (2) analysis of how self-reporting of drug use by pregnant women relates to measured levels of drug in hair samples (Chapter 3) and (4) a study of whether drugs misused by pregnant women actually do interact with ABC transporters in the human placenta (Chapter4).

Chapter Two

Is Neonatal Abstinence Syndrome Related to the Amount of Opiate Used?

A systematic review published in the Journal of Obstetric, Gynecologic and Neonatal Nursing (2010), 39 503-509

Authors: Deirdre Thajam, Diane. E. Atkinson, C. P. Sibley, Tina Lavender

Is Neonatal Abstinence Syndrome Related to the Amount of Opiate Used?

Authors Deirdre Thajam, Diane E Atkinson, Colin P Sibley, Tina Lavender

Abstract

Objective: To determine if a relationship exists between the dose of heroin and/or substitute medication used in pregnancy and Neonatal Abstinence Syndrome (NAS).

Data Sources: Ovid online was used to search the following; EMBASE, Ovid MEDLINE, CINHAL, PscyINFO, Cochrane Database of Systematic Reviews.

Study Selection: English language journal articles reporting original research undertaken and published between 1995 and 2009, that examined relationships between Neonatal Abstinence Syndrome (NAS) and opiate use in pregnancy and with patterns of substance abuse that reflect those of the United Kingdom and other high resource settings.

Data extraction: The studies were reviewed independently by two authors using predefined quality criteria.

Data Synthesis: This was a narrative review; key messages from included studies were discussed in the context of the diversity and commonality of findings in relation to NAS.

Conclusions: No correlation between the amount of fetal opioid exposure and expression of NAS was reported in eight of the ten included studies. This observation was consistent across international boundaries and studies included both methadone and buprenorphine.

Key Words: Drug use, Pregnancy, Neonatal Abstinence Syndrome, Dose Relationships, Methadone, Buprenorphine.

Callouts

1. To determine if a relationship exists between the dose of heroin and/or substitute medication used in pregnancy and Neonatal Abstinence Syndrome.

2. Evidence in this review shows no relationship between maternal dose of heroin or substitute medication and Neonatal Abstinence Syndrome.

3. An adequately powered multi-centered, prospective study with Neonatal Abstinence Syndrome as the primary outcome is required to confirm the findings of the review.

Objective

The objective of the review was to determine if a relationship exists between the dose of heroin and/or substitute medication used in pregnancy and Neonatal Abstinence Syndrome (NAS).

Introduction

It has been estimated that there are up to 2 million dependent drug users in the European Union. Of these, opiate use, mainly heroin, is the primary drug responsible for 60% of initial contacts to drug treatment services. In the UK, it is estimated that around one in a thousand women use opioids dependently (Wolff *et al* 2005). In the USA, 5.1% of all live births occur in women who use illicit drugs during pregnancy (Substance Abuse and Mental Health Services Administration (SAMHSA), 2009). Substance abuse presents both clinical and public health problems that include infections and abscesses, subacute bacterioendocarditis, cardiac valve damage, thrombosis, sexually transmitted infections, hepatitis B and C, HIV and mental health problems (Day and George 2005, Martinez-Selles *et al* 2008, Lloyd-Smith *et al* 2008). These become particularly relevant if a woman becomes pregnant as they can affect both maternal and fetal wellbeing. Maternal substance abuse is associated with many adverse pregnancy and neonatal outcomes including preterm delivery, intrauterine growth restriction and Neonatal Abstinence Syndrome (NAS) (Burns *et al* 2006, CEMACH 2007 Wright and Walker 2007).

NAS is a generalized, multisystem disorder of the opioid exposed neonate which may progress to convulsions. It is characterized by gastrointestinal dysfunction, central nervous system irritability and autonomic signs as demonstrated by excessive and often high pitched crying, sleep and feeding disturbances, increased muscle tone and tremors, excessive sneezing, yawning and nasal stuffiness (Seligman *et al* 2008, Finnegan 2004). NAS varies in its presentation, according to the gestation of the infant and the substance from which it is withdrawing (Jansson *et al* 2009, Dysart *et al* 2007). A proportion of these infants can be managed solely by supportive measures such as non-nutritive sucking, and environmental adaptations such as a quiet room and dimmed lights. A fuller explanation of both supportive measures and environmental adaptation is described by Velez & Jansson (2008). Nevertheless 30-80% of infants exposed to maternal opioid use will require pharmacological intervention (Kassim & Greenough 2005) to reduce clinical

signs to a degree where the infant is not in discomfort, can self-settle to sleep and feed adequately (Jansson *et al* 2007). If severe NAS is left untreated there is a risk that the infant may convulse, so physiological signs of withdrawal are closely monitored, usually using a scoring tool such as that devised by Finnegan *et al* 1975. The original Finnegan scale, administered every four hours assesses the severity of NAS by providing a numerical score to guide clinical interventions. Pharmacological interventions are recommended when a score of 8 or more is obtained on more than one successive assessment.

(Callout 1)

Data sources

Search Strategy.

A pre-defined search strategy was used to generate information that would meet the objective of determining whether a relationship exists between the dose of heroin and/or substitute medication used in pregnancy and Neonatal Abstinence Syndrome (NAS). Primary research papers were included if published in peer reviewed journals and reported in English between 1995 and 2009. The time period (1995-2009) was chosen to reflect changed patterns of drug abuse and substitute prescribing. Papers had to examine the relationships between NAS (author defined) and dose of maternal opioid in pregnancy and the included sample had to be obtained from a high resourced setting with patterns of substance misuse that reflect those of the United Kingdom (e.g. America, Western Europe, Australasia). Furthermore, papers were only included if the sample size was greater than 5 and explanation was provided on how maternal opioid dose was estimated.

Ovid online was used to search the following; EMBASE, Ovid MEDLINE, CINHAL, PscyINFO, Cochrane Database of Systematic Reviews. The reference lists of identified articles were reviewed for additional articles that met the inclusion criteria. Searches for the terms methadone, buprenorphine, heroin and opiates were undertaken separately and each combined with the following terms using the advanced search option; Pregnancy, Neonatal outcomes, Neonatal abstinence, Birth weight, Gestational age, Withdrawal and Apgar score. Because the keyword search terms are different, the search in PsycINFO used the terms; Mothers, Neonatal development, Neonatal period, Premature birth and Infant development. Boolean terms 'And' and 'Or' were used as appropriate.

Study Selection

A search of the Cochrane Database of Systematic Reviews did not produce any reviews that explored relationships between the maternal dose of substitute medication (e.g. methadone, buprenorphine) and/or heroin use and expression of NAS. Databases were searched to identify studies related to the objective. Initial limits of English language, human studies and years 1995-2009 were applied. Abstracts were obtained and reviewed according to the inclusion criteria. At this stage, eligibility was often unclear as abstracts lacked detail. Primary papers were then retrieved and each reference list was reviewed for additional papers not obtained by the original search strategy. All the inclusion/exclusion criteria were applied to these papers, leaving ten studies for review.

Data Extraction

The studies were independently reviewed by two of the authors using predefined screening criteria, e.g. definition of NAS, pharmacotherapy, scoring system and amount of opioid, and where appropriate, other substances that the infant was exposed to. Quality was assessed using the Critical Appraisal Skills Program (CASP) tools (CASP, 2006); these were used as the basis of discussions which were continued until consensus was achieved. There are seven tools available, each developed to assist with the critical appraisal of articles presenting different research types. For this review, we used two of the tools; 'Randomized Controlled Trials' and 'Cohort Studies'. Each tool contains ten to twelve questions. The first two questions are screening questions to determine whether it is worth continuing with the article. The first question was 'Did the study ask a clearly focused question?' The second question asks 'Did the authors use an appropriate method to answer their question?' The screening questions are followed by additional questions which assist in decisions regarding the quality of the study. A choice of three responses are included on the tool; 'Yes', 'No' and 'Can't tell.' Detailed discussions focused on the strengths and weaknesses of the study methodology, as guided by the responses to these questions. There were no disagreements regarding paper inclusions, discussions focused solely on our rationale for inclusion and relevance to review objective.

Results

Applying the initial limits of human studies, English Language and years 1995-2009 produced 159 references to potential papers for inclusion. Reading the abstracts led to the exclusion of 118 papers. The main reason for exclusion was no measurement of NAS; this left 41 studies for potential inclusion. Applying all the inclusion/exclusion criteria at this stage identified ten studies. Table 1 provides a summary of included studies.

Description of Studies (Callout 2)

Of the ten studies identified, five were retrospective in design and reviewed clinical records to obtain outcome data (Berghella *et al* 2003; Dryden *et al* 2009, Lim *et al* 2009, McCarthy *et al* 2005). The remaining five studies were prospective with three being observational (Kuschel *et al* 2004, Lejeune *et al* 2006, Bakstad *et al* 2009), and two, double-blind double dummy designs (Fischer *et al* 2006, Kacinko *et al* 2008). Double dummy studies are used when comparing two active treatments, whereby each patient receives one of the active treatments and a placebo treatment (Altman, 2009). Two of the prospective studies were also multi centered (Lejeune *et al* 2006, Bakstad *et al* 2009). Five studies were undertaken in the US and the remainder were conducted in Norway, Austria, UK, France and Australia. The data collection period ranged between one and five and a half years and the number of infants in the studies ranged from 10 to 444. Two studies included women that received methadone for pain relief in addition to women who were prescribed methadone to address addiction to heroin (Kuschel *et al* 2004, Lim *et al* 2009).

Table 1: Summary of studies (Table 2.1 for this thesis)

Author & Year	Substance used and dose	Study aim	Methods	Sample	Conclusions	NAS Scoring System
Berghella et al., 2003	Methadone (in last twelve weeks of pregnancy *and last dose before delivery*) Group1, mean dose <80mgs/day Group 2, mean dose ≥80mgs/day	To determine whether maternal methadone dose correlated with neonatal withdrawal	Retrospective review of all maternal /neonatal records of women attending a multidisciplinary methadone treatment program (Sept 96 - Dec 99)	USA Heroin addicted pregnant women. 100 mother-infant pairs	Maternal methadone dose was not correlated with NAS.	Finnegan
Bakstad et al., 2009	Methadone and buprenorphine. Dose in the last trimester and birth. Reported as mean dose at delivery buprenorphine 13.3 mg/day, methadone 90mg/day.	To describe the national cohort and birth outcomes. To investigate associations between methadone dose and NAS	Prospective Two year period (Jan 05 - Jan 09)	Norway 38 women in drug treatment services on a maintenance program 26 methadone and 12 buprenorphine	Methadone dose did not predict the occurrence of NAS.	Finnegan and Lipsitz

Dryden et al., 2009	Methadone Group 1, 1-29 mg, Group 2, 30-59mg, Group 3 60-68mg Group 4, > 90mg daily	Examine the development of NAS & implications for healthcare resources	Retrospective *cohort* review of clinical records. (*Jan 04 – Dec 06)*	UK 444 infants	Higher methadone is associated with higher *incidence* of NAS.	Lipsitz
Fischer et al., 2006	Methadone 40-100mgs/day. Buprenorphine 8-24mg/day	To evaluate the efficacy & safety of methadone versus buprenorphine in pregnancy for opioid dependent women	Prospective Double blind double dummy comparison study. (00 - 02)	Austria 14 women ~ 6 methadone & 8 buprenorphine ~ enrolled between 24-29 weeks gestation	No positive correlation between mean doses of medication at delivery and the intensity of NAS	Finnegan
Kacinko et al., 2008	Buprenorphine a) mean dose at delivery (18.7mg) b) mean daily dose (15mg/day) c) mean 3rd trimester daily dose (16.1mg) d) cumulative dose (mean 1701.6mg) e) cumulative 3rd trimester dose (mean 12056mg)	To characterize relationships amongst maternal buprenorphine dose, meconium buprenorphine (& metabolites) concentrations & neonatal outcomes	Prospective study (this group was a subgroup of a larger double blind double dummy, flexible, randomized, stratified parallel group controlled study)	USA Opioid dependent women attending a comprehensive treatment program 9 women with 10 infants	No significant correlation between *maternal* buprenorphine *dose* and NAS.	Modified Finnegan

Kuschel et al., 2004	Methadone. Range of 15-105mg daily. Median 55mg at delivery	To determine the usefulness of cord and neonatal serum methadone concentrations in predicting the severity of NAS	Prospective study over 21 months. Infants born between Aug 99 - May 01	Australia 25 mother-infant pairs. (21 receiving methadone for addiction, 4 for chronic pain	No significant correlation between maternal methadone dose and treatment for NAS.	Modified Finnegan
Lejeune et al., 2006	Methadone and high dose buprenorphine at end of pregnancy. Methadone mean 57mg/day, range 10-180mg. Buprenorphine mean 5.4mg/day, range 0.4-24mg	To compare the morbidity and NAS of infants of mothers who are taking methadone or buprenorphine	Prospective, observational study. (Oct 98 - Sept 99)	France 259 women and their infants (260) receiving support for opiate dependency	No relationship for either methadone or buprenorphine in terms of maternal dose at the end of pregnancy and the intensity of NAS.	Lipsitz
Lim et al., 2009	Methadone (last dose before birth) 3 groups 1) ≤70mgs/day 2) 71-139mgs/day 3) ≥140mgs/day	To examine high dose methadone in pregnant women and its effect on the length of NAS	Retrospective cohort study, reviewed medical charts (Mar 02 – Aug 07)	USA. 66 mother/infant pairs. 56 received methadone for addiction, 10 for chronic pain	Higher doses associated with increased incidence of & longer duration of NAS.	Modified Finnegan
McCarthy et al., 2005	Methadone Divided into 2 groups Group 1 less than100mgs/day Group 2.100mgs or more daily	To assess the effect of higher doses of methadone during pregnancy on maternal and fetal outcomes	Retrospectively reviewed clinical data of admissions. (Feb 99 - May 03)	USA 81 women admitted to a specialized methadone maintenance program and their infants	No differences in the rate of pharmacological treatments for NAS. or days of hospitalization	Finnegan

NAS – Neonatal Abstinence Syndrome.

The majority of the included studies (six) reported on maternal methadone use, one on buprenorphine and two on both substances, there were however, differences in how the authors measured the maternal dosage. Some studies reviewed women for a period of time through their pregnancy and therefore had more information available to explore different ways of expressing maternal dosage. For example, dose at delivery, mean daily dose, average third trimester dose, cumulative dose and cumulative third trimester dose are all reported in Kacinko *et al* 2008 whereas Lim *et al* 2009 use only the last dose prior to delivery as their measurement. This demonstrates the heterogeneity between studies.

All studies used NAS as a primary outcome measure although some authors (Dryden *et al* 2009) defined NAS as occurring only if signs of withdrawal were severe enough to require treatment. Some studies used additional outcome measures such as length of hospital stay and length of pharmacological treatment for NAS. Kuschel *et al* 2004 examined cord and neonatal serum methadone concentrations as a predictor of NAS. Similarly, Kacinko *et al* 2008 examined meconium for levels of buprenorphine and its metabolites and their relationship to NAS. Both of these studies (Kuschel *et al* 2004, Kacinko *et al* 2008) focused on the biochemical markers to predict NAS as opposed to measuring NAS per se.

Of the ten studies included, only two found a relationship between the amount of fetal opioid exposure and NAS (Dryden *et al* 2009 Lim *et al* 2009). In these studies, increasing amounts of methadone were associated with more severe expression of NAS. Both studies were retrospective in design and thus limited by the fact that there are many unknown variables that the researcher has no knowledge or control over; such variables may affect the study outcome. Furthermore, although the authors note what measures they took to ensure data were captured accurately, the quality of source documentation remains an issue when relying on records that are completed by others whose priorities lie with delivering care and not the research process.

Conclusions (Callout 3)

The review sought to determine if a relationship exists between the dose of heroin or substitute medication used in pregnancy and Neonatal Abstinence Syndrome. The evidence from the studies identified demonstrates that there is no relationship between the maternal dose of methadone or buprenorphine and the expression of NAS. Although the heterogeneity of the studies makes pooling data difficult, eight of the authors reported no relationship between maternal dose and NAS. These included the five

prospective studies of which two were double dummy double blind studies. Individual studies were limited by small sample sizes and therefore are unlikely to have sufficient power to capture infrequently occurring complications. Furthermore these were not confined to one health care system as the studies occurred in the US, Austria, Australia, France and Norway.

In summary, the heterogeneity of the included studies in terms of their design, inconsistent approaches to recording maternal drug dose and definitions of NAS makes pooling data difficult. This limits the usefulness of the combined findings to inform the delivery of safe and effective care. However, this review demonstrates that the dose of neither maternal methadone nor buprenorphine is predictive of the severity of NAS in the majority of these disparate studies. Practitioners can therefore use this to support their prescribing practices and offer some reassurance to women who worry about the effects of their treatment on their infant. The studies also highlight the difficulties in obtaining a full data set that is not confounded by concomitant use of other substances, for example tobacco. To offset some of our difficulties and confirm these findings, an adequately powered multi- centered, multidisciplinary, prospective study with NAS as the primary outcome is needed to ensure that practice is underpinned by a sound evidence base and inform subsequent research that requires a definitive answer to the question, 'Does maternal dose of opiate predict the severity of NAS?'

Disclosure of Interests

The authors declare no competing interests.

Contribution to Authorship

DT was responsible for the review process, analysis of the studies and writing the paper. TL conceived the idea, advised on the process, examined the papers, reviewed the analysis and the paper. CPS and DEA reviewed the analysis of studies and the paper.

2.1 Potential Further Analysis

Meta-analysis is a statistical technique often applied in systematic reviews to provide a more objective and precise measure of outcome effect than that provided by a narrative review. It compares and combines the results from multiple studies and presents the data, usually in graphical form, relative to a common measure such as relative risk with confidence levels for each study. This visual representation presents data in a manner that can identify how similar or dissimilar the studies and or their findings are. By contrasting the results from individual studies patterns of variance can be identified, quantified and explored and may provide information that suggests exploration of another research question.

Combining results of multiple smaller studies that may not individually have adequate power to detect small or infrequent changes may increase the power of the intervention of interest e.g. a medication to identify those changes. However, as with other techniques there are principles of rigour that must be adhered to if accurate data are to be generated. Attention must be given to criteria that will exclude or include a study in the meta-analysis, to the quality of those studies methodologies, data analysis and reporting of findings. Once selected studies are weighted results can be combined. A sensitivity analysis of the Meta-analysis findings ensures that the predefined criteria e.g. to use only prospective studies, does have an effect.

Although there are benefits of using Meta-analysis if there are very pronounced differences in the studies so that heterogeneity is high, combining them may not be useful as the meta-analysis will not be combining like with like. Ten studies with a high degree of heterogeneity were included in this review. After seeking advice from a statistician a meta-analysis was not included in the publication because the heterogeneity of the studies was determined to be too great. The reasons for this are outlined within the text of the paper and include factors such as; the differences in the manner in which Neonatal Abstinence Syndrome was defined and scored, the inclusion of difference subject populations (e.g. women who were prescribed methadone for pain relief, women who had stopped using methadone prior to giving birth and women with illicit polydrug use), the differences in the manner in which maternal methadone dose was calculated, differences in study design (e.g. blinded and non-blinded, prospective and retrospective studies), and data collection methods to measure fetal exposure. However, these concerns would not exclude a meta-analysis in the future if the quality

of subsequent studies reduced heterogeneity to a degree that would not compromise the validity of findings from a Meta-analysis.

Chapter Three

Using Hair Sample Analysis in Self-Reported and Observed Drug Use in Pregnant Women

Published in: British Journal of Midwifery November (2012); 20: 806-810

Authors: Deirdre Thajam, Colin. P. Sibley, Tina Lavender, Diane. E. Atkinson

Using hair sample analysis in self-reported and observed drug use in pregnant women.

D Thajam, C P Sibley, T Lavender, D E Atkinson

Abstract

Maternal hair samples from 56 drug using women were collected within 24 hours of parturition and assayed using gas chromatography/mass spectrometry for cannabis, heroin, methadone, cocaine, amphetamine, methamphetamine and benzodiazepines. Reported and observed drug use was compared: a Cohens Kappa coefficient of 0.732 was obtained indicating substantial agreement between reported and observed use. Interesting practice issues emerged. These included the wish to please healthcare practitioners and to avoid negative judgements or removal from services. Also concerns that confidentiality would be breached or that children would be removed from their care and dissatisfaction with information provided by healthcare practitioners when prescribing medication.

Keyphrases

1. The majority of women were open and honest about their drug use in pregnancy.

2. Women prescribed mental health medication expressed a wish to please healthcare professionals and avoid negative judgements or removal from services.

3. Fifty per cent of the pregnant opiate dependent women in the sample used illicit drugs in addition to their prescribed medication.

4. Regardless of drug used women in this group had concerns about confidentiality and if their child would be removed from their care.

Introduction

Drug use in pregnancy is associated with negative outcomes for fetal wellbeing, including Neonatal Abstinence Syndrome (NAS) (Kuschel 2007). Members of multidisciplinary teams involved in care need to be aware of which drugs the fetus is exposed to in order to deliver optimal antenatal and postnatal care for both mother and baby (Jansson *et al* 2010). This is of particular importance when caring for an infant exposed to opioids as it may require specialist care such as environmental adaptation or pharmacological intervention (Jansson *et al* 2009). If healthcare providers are unaware

of exposure, signs of NAS may be mistaken for illness and the child submitted to unnecessary tests, or indeed unnecessary admission to a neonatal unit with a resulting separation from its mother. Additionally, women need information and support to provide optimal care when they are at home with their baby. This should start in the antenatal period so that it can be reiterated and reinforced frequently.

It is important therefore, that women feel confident that disclosure of drug use will be used to optimise the care that they receive and not fear the consequences of disclosure. If they fear disclosure will adversely impact upon their care, they may not share this information and wellbeing may be compromised. This is particularly important when women do not access drug treatment services which can provide midwives and obstetricians with information about drug use.

Previous studies, for example, Fendrich and Johnson (2005) have shown that self-report methods may underestimate the amount and number of drugs used by participants but have used data from household surveys rather than pregnant women. Ostrea *et al* (1992) compared self-report of drug use in pregnancy to meconium analysis and found that whilst 11% of participants reported illicit drug use 44% of samples were positive for opiates and cocaine. Data from a later anonymous screening study in pregnant women (Sanaullah *et al* 2006) also demonstrated under reporting of illicit drug use.

As self-report is usually the main source of information available when assessing fetal risk it is important that practitioners are cognisant of maternal concerns when disclosing drug use so that they can work towards engendering a therapeutic relationship that supports optimal outcomes.

As part of a larger study investigating the effects of drug misuse in pregnancy on placental function and neonatal outcomes, we addressed the correlation between self-reported drug use and measurements of drugs in maternal hair samples. It was not originally envisaged that this would yield information of interest other than to the placental research objectives but data provided information that highlighted some maternal concerns that may impact upon that therapeutic relationship and interesting points for practice were observed during conversations.

Our hypothesis and objective lie in the context of the main study. Our hypothesis is that women will under report the number of substances taken and the objective was to use hair testing to obtain a historical record of which substances the fetus was exposed to in the last trimester of pregnancy.

Methods

After obtaining ethical approval, pregnant drug using women who had been referred to The Manchester Specialist Midwifery Service (MSMS) were approached to seek their participation in an investigation into the role of the placenta in determining fetal susceptibility to maternal drug use. MSMS provides support in addition to universal maternity services. Women who were approached had been referred to the service either because they were in drug treatment services and had a confirmed pregnancy or maternity services and had shared the fact that they used a substance of interest to the study. If women were willing to participate, the researcher asked them about past and current drug use, including the frequency, manner and amount of substances used. Women were excluded from the study if they used alcohol, did not use their drug of choice daily or stated that they intended to stop daily use or cease completely.

As previously stated, these results were obtained as part of larger study investigating the effects of drug misuse on the placenta in pregnancy. As interpretation of the results required information as to which substances the fetus had been exposed, to and previous reports suggested that not all women are open about their drug use and may under report the amount or type of substances taken, (Sanaullah *et al*. 2006, Grekin *et al*. 2011) analysis of hair samples was used to provide information about actual drug use in the last three months of gestation.

Drugs and their metabolites are deposited within the hair follicle and remain within the growing hair shaft providing a longer historical record of drug use than provided by urine testing. Musshoff and Madea (2006), suggest that hair samples have advantages over blood and urine samples because it enables detection of a longer period of drug use, therefore maternal hair sample analysis was used in preference to urine testing.

Maternal hair samples were taken within 24 hours of the baby's birth. Samples were collected and stored, according to the protocol supplied by TrichoTech, a commercial testing company accredited by the United Kingdom Accreditation Services, that analysed the samples using gas chromatography/mass spectrometry. Detailed descriptions of procedures used in this technique are available from their company website (www.trichotech.com).

In brief, a section of hair was cut, as close to the root as possible, using sterile scissors and gloved hands. The sample was wrapped in a foil sheet and sealed in an envelope provided by the company. A full length of hair was sent but only the three centimetres

closest to the root were used in analysis to provide data on maternal drug use in the previous three months. Samples were stored at room temperature and posted in batches for analysis. In order to know what additional substances the fetus was exposed to the results of the hair analysis were compared to reported use by application of Cohen's Kappa (Cohen 1968) and discussed with women at the researcher's next planned visit.

Results

More women agreed to participate in the study than the 56 results reported. Of those that were excluded from the study, 2 women terminated their pregnancy (1 major congenital abnormality), 1 child had a major congenital abnormality so was excluded and 10 more woman agreed to participate but the researcher was not informed of the delivery by hospital staff. These women were therefore also excluded from the study. Of those not excluded from the study but for whom results are not available, 1 sample was taken but lost by TrichoTech Ltd. and two women declined a hair test (1 cannabis, 1 methadone and illicit substances). Of the women approached two women declined to participate and three withdrew before giving birth.

Of the samples obtained and reported here only one woman was in employment prior to confirmation of pregnancy, 10 were teenagers (aged 16-19), 26 were in their twenties, 19 were in their thirties and 1 was aged 41 years. There was no prior knowledge of the women's past drug use prior to recruitment into the study. Women who disclose any form of drug use to maternity staff or a pregnancy to drug treatment staff are routinely referred to MSMS for support. Women are not routinely tested for drug use in pregnancy therefore the sample consisted of women who chose to disclose cannabis use and women already in drug treatment services.

Of the 56 samples reported here the assay data of 41 were concordant with reported use leaving 15 samples in which reported and observed use was discordant i.e. reported and observed use did not match. Of these 15 samples, nine were from the 18 women in the study who were receiving prescribed methadone. These samples showed 50% of women prescribed methadone had taken extra illicit substances. Five of the 15 were from women who reported cannabis use exclusively (14.28%), of these three were positive for opiates which were prescribed for pain relief and 2 tested positive for cocaine. The final anomaly was from a woman who reported illicit cannabis and cocaine use with prescribed mental health medication. Her sample tested positive for cocaine as reported but negative for cannabis and mental health medication. It was also positive for

opiates which were prescribed medication as pain relief in pregnancy. Statistical comparison of measured and reported drug use produced a Cohens Kappa coefficient of 0.732, indicating good overall agreement between reported and observed drug use (Figure 1).

OBSERVED	Reclassified Use																				
Initial ReCoDe	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	Total
methadone (1)	1		2										1		1		1				6
methadone, opiates & mental health (2)		1																			1
methadone & opiates (3)			1	1		1															3
methadone, cocaine, opiates & cannabis (4)				1																	1
methadone & cannabis (5)					0			1													1
methadone, opiates & cocaine (6)						4															4
methadone, opiates, cocaine & mental health (7)							1														1
methadone, opiates & cannabis (8)								0												1	1
amphetamines (9)									1												1
cannabis (10)										30				3		2					35
cannabis, cocaine & mental health (11)											0							1			1
subtext (12)												1									1
methadone, benzodiazepines, cocaine & opiates (13)													0								0
cannabis & opiates (14)														0							0
methadone, benzodiazepines & opiates (15)															0						0
cocaine & cannabis (16)																0					0
methadone, cannabis & amphetamines (17)																	0				0
cocaine &opiates (18)																		0			0
opiates (19)																			0		0
benzodiazepines, cannabis, cocaine, methadone & opiates (20)																				0	0
TOTAL	1	1	3	2	0	5	1	1	1	30	0	1	1	3	1	2	1	1	0	1	56
Kappa 0. 732																					

Figure 3.1 (Figure 1 when published) The results of the hair analysis were entered into the grid to provide a measurement of agreement between reported and observed drug use. Reports of individual samples are shown. A Kappa coefficient of 0.732 was obtained indicating good agreement between reported and observed drug use in this sample.

Discussion

Overall the results show that the majority of women in this study were open about their drug use. Whilst some differences between what was reported and what was measured, was due to additional illicit drug use, this was not the only cause of discrepancy. These findings are in contrast to other studies.

There are many possible explanations for this and the small sample size precludes a thorough explanation for all possibilities. For this sample explanations include the fact that the data only included women whose drug use was already known or who had already chosen to share their drug use before being approached by the researcher. Or the fact that they were being asked to participate in a specific research programme in which the main outcome measures objectives were neonatal and developmental rather than a study that sought to prove the veracity of their claims regarding their drug use. Specific findings in this group of women were categorised as follows.

Prescription medication

Three women who reported cannabis use had hair samples that also tested positive for opiates. All three women had been prescribed an opiate based pain relief in pregnancy. At subsequent visits when discussing the results of the analysis, the women expressed concerns that they had not been given this information by their prescriber.

Mental health medications

Three women reported taking mental health medication but only two samples tested positive; the third sample, whilst negative for mental health medication was positive for opiates. In discussion with this woman she shared the information that she had not taken her prescribed mental health medication because she was worried about the effects of the medication on the fetus. She did not want to tell prescribers she was not taking her medication in case she was removed from services and felt it was easier to appear to meet their expectations of her. She used opiates to self-medicate to remove her symptoms of distress as she felt necessary.

These concerns arose with an additional eight women in the study who were also prescribed mental health medication but did not take it. With a greater awareness of a potential neonatal withdrawal syndrome (Levinson-Castiel *et al* 2006) women felt unsure of the safety of their prescribed medication and chose not to take it and to alter their illicit use to relieve symptoms of distress. However, these women had disclosed this in

discussions at recruitment so, although their samples showed what they had reported, this was not what they had been prescribed.

Additional illicit use

Whilst the majority of samples showed only the reported substance(s) there was additional illicit use. Of 18 women in receipt of a methadone prescription nine had used either one or a combination of the following: cocaine, opiates, benzodiazepines or amphetamines. There were a number of reasons for this. Some were temporal in nature in that they reflected changed patterns of use from the time of recruitment to time of sample collection, or intermittent use which was not considered to be important enough to report or had simply been forgotten. One woman stated that she did not report cannabis use because she did not think of it as a drug. Another had used additional drugs to cope with a family crisis. A third woman had been unable to collect her weekend prescription from the chemist and required something to keep her from feeling unwell over a weekend.

Points for practice

Some commonalities arose that are interesting and necessitate reflection on practice when working with women in this client group. Whilst some women were happy to discuss their drug use others expressed fears of judgements by healthcare providers including negative assessments of their ability to successfully parent. This meant that they were unsure of how open they could be, kept worries to themselves and did not always discuss concerns with doctors and midwives. This could increase levels of stress with the potential to increase illicit use to quieten symptoms.

Fears about breeches of confidentiality were raised as a concern. There were worries that staff may tell partners or family members of drug use or that social workers would be informed and their child removed from their care. These fears also extended to the research despite a confidentiality statement on the consent form and the researcher offering assurances. Some women explained that in the past they had disclosed matters in what they thought was a confidential environment, only to have it discussed with others and this had made them wary and distrustful.

Whilst some women were vague about the notion of confidentiality others were more aware of how it worked for or against them. Some women clearly understood that the researcher could not breech confidentiality and were willing to be open for the research.

One woman accepted the confidentiality statement of the research but was aware that any third party in the room would not be covered by this and may pass on information to others involved in their care. In this case the third person was a midwife whom the woman had asked to be present to be a familiar face when the researcher was there. Again there were concerns that this may lead to the removal of their child from their care.

Conclusion

In conclusion, these results show that the majority of women in this sample group were open about their drug use and moreover the pattern of use remained stable for a considerable time. However, a few women had a more variable pattern of use that changed during the pregnancy, often in relation to an emotional crisis. Importantly, there remained some women who had concerns that honesty would have negative consequences for them. These concerns may impact upon the types and level of services that are accessed and patterns of drug use. Worryingly, women with mental health concerns chose to pretend they were taking their prescribed medication, fearing that access to services would be denied if they declined it. As service providers it is incumbent upon us to make services equitable and accessible to obtain optimal health gains for both mother and child. Discussions with the women in this sample show that despite our best efforts, there is still improvement needed. Specialist midwifery services can support families and care providers but may be costly. However, our findings suggest that mindfulness of concerns in all our interactions with drug misusing women could reduce their concerns and thus influence the way these women access and use services.

Conflict of Interests

There are no competing interests to declare.

Contribution to Authorship

DT recruited and interviewed the women, collected the samples and wrote the article. DEA, TL and CPS reviewed and edited the article.

3.1 Self-Report

When women were recruited to the study they were asked what drugs they usually used. As stated in the text of the paper self- report techniques have shown that this may provide an underestimate of drug use. This phenomenon is not specific to this client group. Although self-report measures such as questionnaires and interviews have advantages, such as the ability to economically explore a number of variables with a large number of participants, this places a reliance on the accuracy of personal memory and a willingness to answer honestly which may affect the validity of the findings. Other benefits of self-report techniques, particularly interviews, is the generation of ideas that may not have been apparent at the outset, opportunity for the researcher to present information in different ways to ensure that the participant understands the question and also check understanding of answers. Disadvantages, particularly with anonymous questionnaires are that they may provide too much information and it may generate information from which cause and effect cannot be determined as is impossible to check who gave what information. This in turn may lead to issues with generalizability.

The study's hypothesis is that small changes in genes will alter the amount of placental multidrug resistance proteins in the placenta and this will in turn affect NAS and/or development. It was important therefore to accurately determine which compounds the fetus had been exposed to in the last three months of pregnancy and hair samples were sent for analysis to determine this. This was explained to women when initially seeking their participation in the study along with the limits of confidentiality within the study i.e. that any thoughts/risks of harm to self or others would be shared with appropriate professionals but drug use remained confidential. At this point eight of the eleven women prescribed mental health medication women stated they did not take their prescribed medication and the reasons for this.

When the results of the hair analysis were obtained it became evident that another woman did not take her prescribed antidepressant. Therefore 81.81% of those prescribed mental health medication in this study chose not to take it. This is higher than the 10-60% reported by Lingam and Scott (2002) when they assessed non-compliance in medication prescribed for affective disorders and reflects here the specific concerns about medication in pregnancy.

The findings reported in this chapter resulted from conversations from women rather than planned semi-structured interviews' and as such limits the amount of detailed

analysis that can be performed. Although one woman spoke about her adjustment of illicit drugs to cope with her feelings this was not explored with all women. Due to this it was felt appropriate to present data on reported and observed use as a group using a Cohen's Kappa and provide the reader with general comments and common themes that arose from conversations'. Nevertheless these are important findings and worthy of publication as they suggests there is scope for research to explore this to inform clinical interventions that may increase compliance in pregnancy.

Chapter Four

Interaction of Heroin, Methadone, Buprenorphine, Cocaine, Diazepam and Δ-9 tetrahydrocannibinol with the Multidrug Resistance Proteins P-glycoprotein and Breast Cancer Resistance Protein in the Placenta.

Authors: Deirdre Thajam, Bernadette Baker, Colin. P. Sibley, Diane. E. Atkinson

Ready for submission to the journal *Placenta* in summer 2013

Interaction of Heroin, Methadone, Buprenorphine, Cocaine, Diazepam and Δ-9 tetrahydrocannibinol with the Multidrug Resistance Proteins P-glycoprotein and Breast Cancer Resistance Protein in the Placenta.

Deirdre Thajam, Bernadette Baker, Colin. P. Sibley, Diane. E. Atkinson

Abstract

Objectives: The objective of this study was to explore the interactions of the commonly misused drugs, heroin, methadone, buprenorphine (bpn), cocaine, diazepam and Δ-9 tetrahydrocannabinol (THC) with the Multidrug Resistance Proteins, P-glycoprotein (MDR1, P-gp) and Breast Cancer Resistance Protein (BCRP) in human placenta.

Study Design: A placental fragment uptake technique was used to measure accumulation of ^{3}H-vinblastine and ^{3}H-mitoxantrone, in the presence or absence of the compounds of interest, as a proxy for P-gp and BCRP activity respectively.

Outcome Measures: The amount of radiolabelled isotope uptake by placental fragments in the presence and absence of the compounds of interest, measured as fmol/mg protein and expressed as percentage of control.

Results: Both bpn and THC significantly reduced accumulation of radiolabelled vinblastine, indicating interaction with P-glycoprotein. THC had dose dependant opposing effects on radiolabelled mitoxantrone accumulation whilst diazepam significantly reduced accumulation indicating an interaction of both drugs with BCRP.

Conclusions: Our data demonstrate that drugs misused by some pregnant women do interact with ABC transporters in the placenta. This information regarding the handling by the placenta of drugs of misuse will help provide women with evidence based advice on the use of such substances during pregnancy.

Key words

placenta, ABCB1, ABCG2, drugs, opiates, cannabis

Introduction

It is generally accepted that members of the ATP binding cassette (ABC) family of transporters function to protect tissues from structurally and chemically diverse

xenobiotics and endogenous substances. By virtue of their efflux actions, they have been demonstrated to alter the disposition of a huge variety of compounds in cells and tissues (Schinkel *et al* 1997, Behravan and Piquette-Miller 2007, Petropoulos *et al* 2010, Young *et al* 2003, Schinkel and Jonker 2003). Encoded by the ABCB1 and ABCG2 genes respectively the multidrug resistance proteins, P-glycoprotein (MDR1, P-gp) and Breast Cancer Resistance Protein (BCRP), are members of this group of transporters noted for their ability to transport a variety of chemically and structurally diverse substrates (Thiebaut *et al* 1987, Tsuji 1992, Cordon- Cardo 1990). Located on the microvillus plasma membrane of the human placental syncytiotrophoblast (Ceckova-Novotna *et al* 2006) they are ideally situated to protect the fetus from xenobiotics in the maternal circulation. By their efflux actions they can limit access to the fetus by xenobiotics and other toxins in the maternal circulation, thus protecting the development of the fetus by reducing the bioavailability of noxious substances.

Maternal drug use in pregnancy exposes the growing fetus to a number of potentially harmful compounds and is associated with negative outcomes for the baby. These include preterm delivery, poor intrauterine growth and Neonatal Abstinence Syndrome (Kuschel 2007). The exhibition of signs of Neonatal Abstinence Syndrome (NAS) and detection of drug metabolites in meconium is evidence that drugs pass across the placenta (Boskovic *et al* 2001, Kacinko *et al* 2008). However the ability to ameliorate the consequences of maternal drug use and improve neonatal outcomes is limited by a lack of a complete understanding of how the placenta functions to protect the fetus from such drugs.

With respect to the commonly misused drugs the role of the ABC transporters in reducing fetal exposure is uncertain and for many drugs information is sparse. Different experimental approaches have provided conflicting evidence regarding the affinity of methadone and bpn as inhibitor/ substrates of P-gp (Hassan *et al* 2009). Information on these compounds is particularly relevant as these substances are commonly prescribed in the treatment of drug dependency during pregnancy. Previous authors have demonstrated methadone to be a substrate for P-gp. (Malek *et al* 2009, Nanovskaya *et al* 2002, Nanovskaya *et al* 2004, Nanovskaya *et al* 2008, Zharikova *et al* 2007). However whilst such information is available for methadone, information on other commonly misused compounds, including bpn is sparse (Nekhayeva *et al*, 2006, Nanovskaya *et al* 2009,Tournier *et al* 2010).

Vinblastine, a documented substrate for P-gp (Cascorbi 2006) and mitoxantrone, a substrate for BCRP (Schinkel and Jonker 2003, Syme *et al* 2004) were used here, in a radiolabelled form, to explore the interactions between P-gp and BCRP in placental fragments with several commonly misused drugs. The aim was to determine whether the compounds of interest interact with either of the proteins, as reflected by changes in accumulation of ^{3}H-vinblastine or ^{3}H-mitoxantrone, in order to better understand how the placenta might handle these drugs *in vivo.*

Tolerance, a consequence of repeated and/or chronic drug use requires increased amounts of the compound to produce the same effects. This makes estimation of a therapeutic dose difficult for those who misuse drugs as it will differ by individual and will change at different times in their drug using history. However, reported plasma levels in human adults for the selected drugs used in these series of experiments are reported in Table 4.1.

Table 4.1 Reported plasma concentrations of buprenorphine, cocaine, diazepam, heroin, methadone, and THC in adult humans. THC concentrations will differ according to levels of THC in cannabis and the pattern and depth of inhalation. Cocaine levels will differ by mode of administration and purity of the product. Heroin levels in the table are following administration of a measured dose of pharmaceutical grade heroin.

Compound	**Reported plasma concentrations**
buprenorphine	1-5ng/ml (2-11nM)
cocaine	Intra nasal 0.22mg/ml Smoked cocaine base 0.31mg/l
diazepam	0.1-1mg/l
heroin	Intra nasal 12mg dose gives 0.016mg/l Intravenous 12mg dose gives 0.14 mg/l
methadone	100-1000ng/ml
THC	77ng/ml

The placental fragment technique uses intact villous tissue to measure accumulation of substrates taking into account all placental layers rather than trophoblast cells in isolation (Greenwood and Sibley 2006, Atkinson *et al* 2006b). A series of experiments were performed, using this technique, to test the hypothesis that P-gp and BCRP do interact with compounds commonly prescribed for drug dependence and/or used

recreationally, i.e. methadone, bpn, heroin, diazepam, cocaine and Δ-9 tetrahydrocannabinol (THC), the highly lipophilic psychoactive component of cannabis.

Materials and Methods.

Samples

Following written consent and with ethical approval for the Manchester Maternal and Fetal Health Biobank, placentas from full term, singleton pregnancies were obtained within 30 minutes of delivery (vaginal or caesarean section). Women included did not have a documented pre-existing medical condition, or a complication of pregnancy such as gestational diabetes or pre-eclampsia. Drug use was not disclosed by these women so, as far as can be reasonably determined, the placentas had not been previously exposed to the compounds tested in these experiments.

Materials

All substances of misuse tested in the present study i.e. heroin, methadone, bpn, cocaine, diazepam and THC, were obtained from Sigma Aldrich, Poole, Dorset UK. Radiolabelled tracers ^{3}H-vinblastine and ^{3}H-mitoxantrone were supplied by American Radiolabelled Chemicals Inc. ARC (UK), Cardiff U.K

Accumulation experiments were performed in Tyrodes buffer at Ph7.4 (135mM NaCl, 5mM KCl, 1.8mM $CaCl_2$, 1mM Mg Cl_2, ($6H_2O$), 10 HEPES, 1.8mM Glucose (Sigma Aldrich, Poole, Dorset UK). Placental fragments were dissolved in 0.3M sodium hydroxide (Sigma Aldrich, Poole, Dorset UK) prior to protein determination. Quantification of protein in fragments was achieved using a BioRad protein assay (BioRad, Richmond, C.A.) with Bovine Serum Albumin as standard (Sigma Aldrich, Poole, Dorset UK).

Liquid scintillation fluid (ScintiSafe 2) was obtained from Fischer Scientific, (Loughborough UK) and radioactivity levels were determined using a Tri-Carb 2100 Liquid Scintillation Analyser (Packard Bell)

Methods

Placental fragment uptakes

The method described by Atkinson *et al* (2006b) was used here to measure accumulation of ^{3}H-vinblastine and ^{3}H-mitoxantrone in the presence or absence of the compounds of interest, as a proxy for P-gp and BCRP activity respectively. Activity at equilibrium was determined in all cases. This was achieved by incubating the tissue with substrate for 120 minute as previously established (Atkinson *et al* 2006b).

Small fragments of villous tissue approximately 2mm^{3}, were dissected and washed in Tyrodes solution to remove any traces of contaminants e.g. meconium or maternal blood. They were then tied with thread, in duplicate, to hooks attached to Perspex rods. This facilitated accurate timing of the duplicates and allowed the fragments to be moved between solutions with the minimum of disruption and damage to the tissue.

After equilibration at 37^{o}C for 30 minutes, placental fragments were incubated for 120 minutes in Tyrodes solution containing 0.2µCi/ml (.074MBq/ml) of either ^{3}H-vinblastine or ^{3}H-mitoxantrone in the presence or absence of one of the drugs of interest. Following incubation, fragments were washed for 30 seconds in ice cold Tyrodes solution to remove any extracellular isotope and reduce leakage of the isotope from the tissue. They were then transferred to 4mls of distilled water for a minimum of 18 hours at room temperature to allow complete lysis of the tissue and release of accumulated isotope. The fragments were then cut from the threads and placed into 4mls of 0.3M NaOH at 37^{o}C for 10 hours to dissolve prior to measurement of fragment protein content by BioRad protein assay. Sixteen mls of scintillation fluid was then added to the water lysate and radioactivity determined by liquid scintillation. Using the amount of ^{3}H-substrate accumulated, protein content and the specific activity of the isotope, fragment uptake was calculated in fmol/mg of protein.

A preliminary series of dose response experiments were performed. On the basis of these data, the main series of experiments using selected concentrations of test drugs was then performed. Finally, in order to investigate the concentration dependant effects reported for substrates/ inhibitors of these proteins (Young *et al* 2003, Hassan *et al* 2007, Hassan *et al* 2009) we performed a series of experiments using a subset of the drugs at a higher concentration i.e. 20µM. A control condition, i.e. accumulation in the absence of drugs was included in each placenta.

Statistical Analysis

Data are presented as medians with n= to the number of placentae studied. Data analysis used Graphpad Prism Version 5 (Graph Pad Inc. California USA). Statistical differences between groups was analysed using Wilcoxon signed rank tests.

Results

It has previously been reported that placental expression of P-gp varies greatly between individuals. Hitzl *et al* (2004) demonstrated an 8 fold difference in protein expression that was not normally distributed and Atkinson *et al*. (2009) showed an 8 fold difference in expression resulting in a 70 fold difference in efflux activity. Similarly BCRP expression has been reported to vary 4 fold between individuals (Atkinson *et al*. 2009). The data obtained in the current study supports these previous observations with baseline substrate accumulation (i.e. in the absence of any drug) varying 24 fold and 3.4 fold for P-gp and BCRP respectively (Figure 4.1).

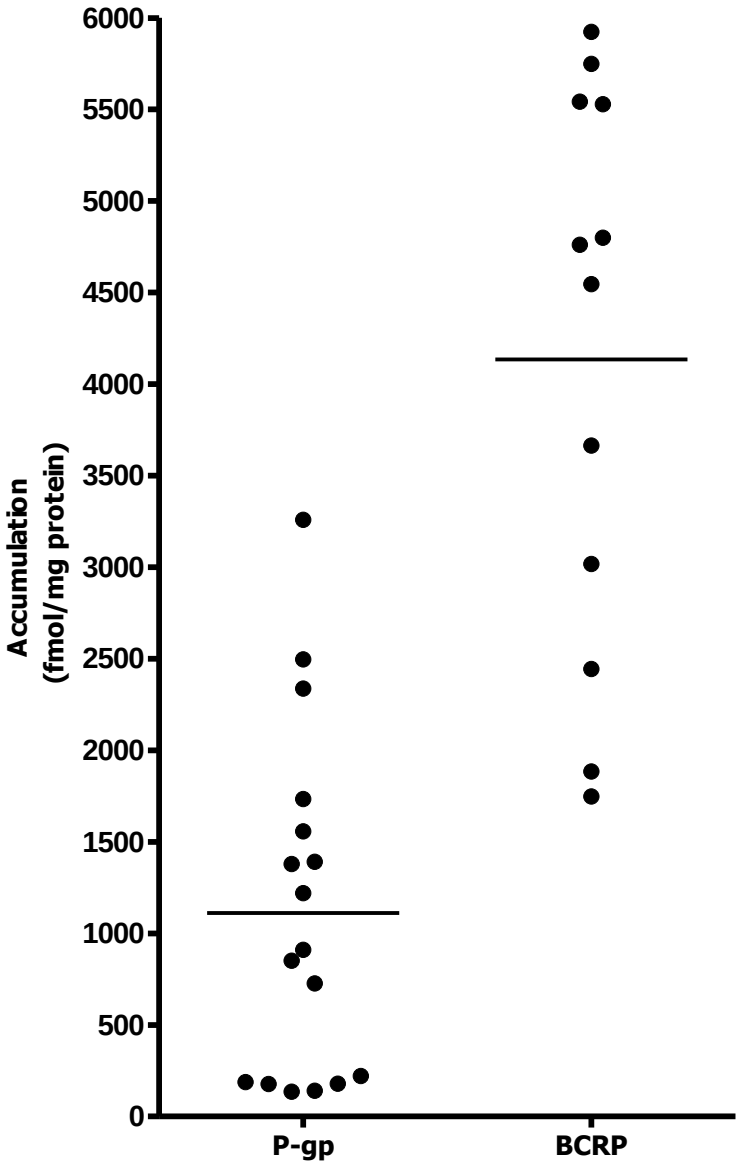


Figure 4.1 Inter placental differences in P-gp and BCRP function. Accumulation of ^{3}H-vinblastine (P-gp) (n=17) and ^{3}H-mitoxantrone (BCRP) (n=12) in the absence of test drugs is shown. The solid black lines indicate the median accumulation value for each condition.

As a result of this variation in baseline protein activity between placentae, all uptakes in the presence of test drugs have been expressed as a percentage of the control uptake for that placenta (i.e. uptake in the absence of drug).

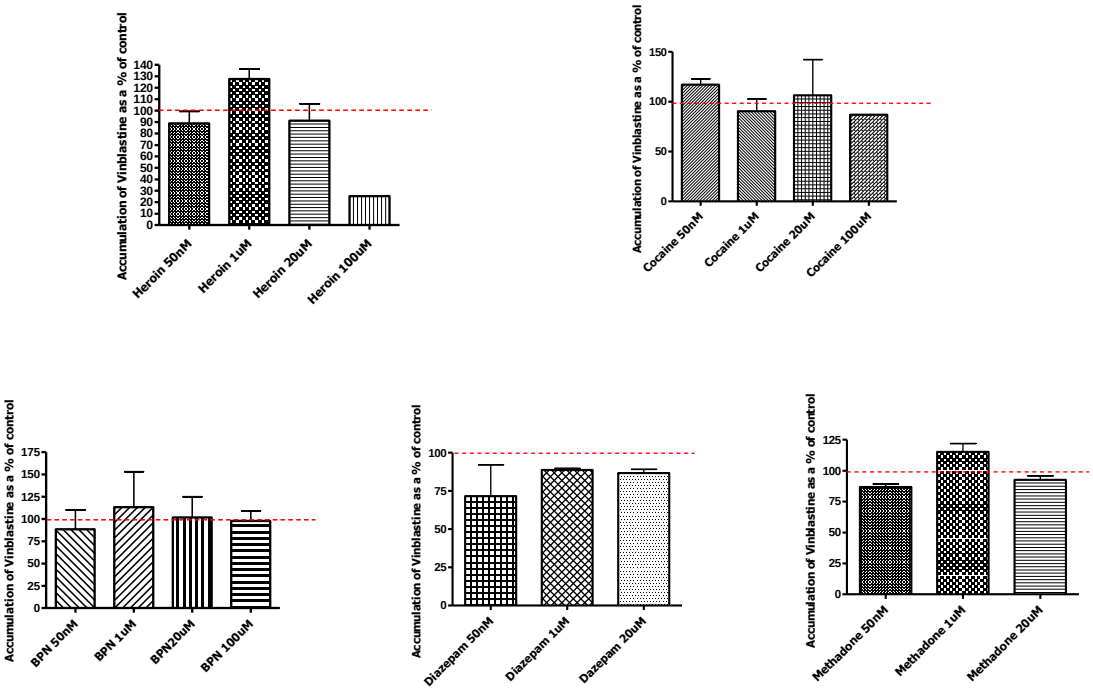


Figure 4.2 Results of the dose response experiments undertaken to determine the selected doses on which to base the uptake experiments. The red dotted line indicates the mean of control i.e. accumulation in the absence of test compound. Results are expressed relative to the control. The dose chosen was the one which gave the maximal change in vinblastine accumulation.

Accumulation of ^{3}H-vinblastine in the presence of selected doses of test substances is shown in Figure 4.2. Significant reductions in ^{3}H-vinblastine accumulation were seen in the presence of 1μM bpn ($p = 0.03$) and 1μM THC ($p= 0.02$).

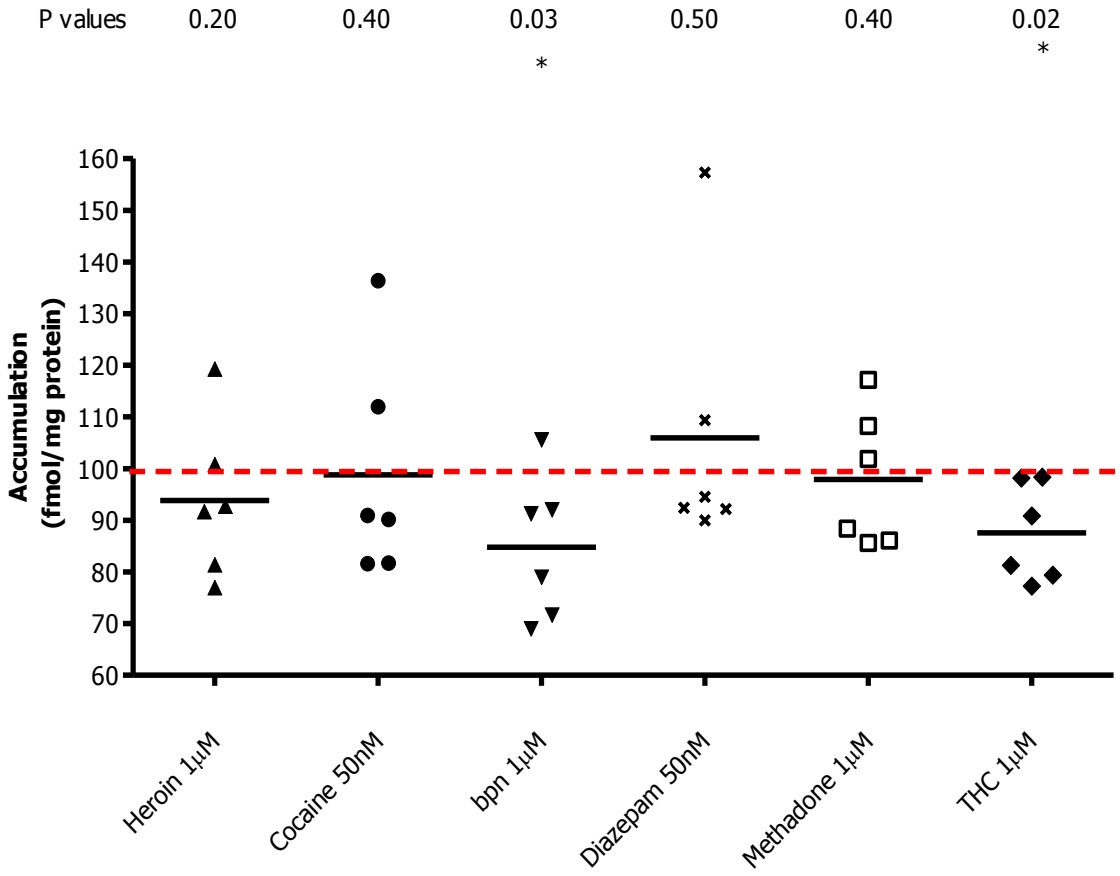


Figure 4.3 3H-vinblastine accumulation in the presence of selected doses of test drugs expressed as a percentage of control (n=6). p values were calculated using a Wilcoxon signed rank test. A significant reduction in 3H-vinblastine accumulation was observed in the presence of 1µM buprenorphine and 1µM THC. The median percentage values are shown as solid black lines.

The same selected doses of drugs were used to investigate their effects on placental accumulation of ^{3}H-mitoxantrone. Figure 4.4 shows a trend towards reduced accumulation with heroin, methadone and THC but only the effect of 50nM diazepam reached statistical significance.

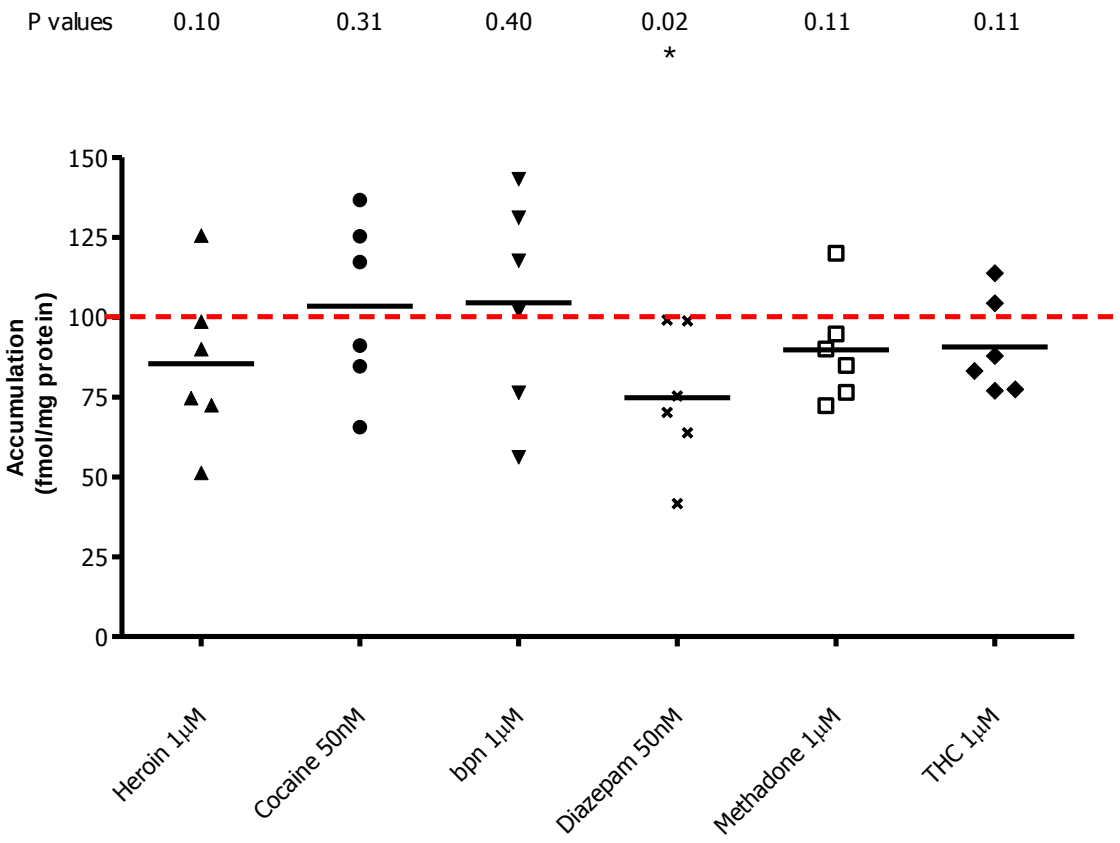
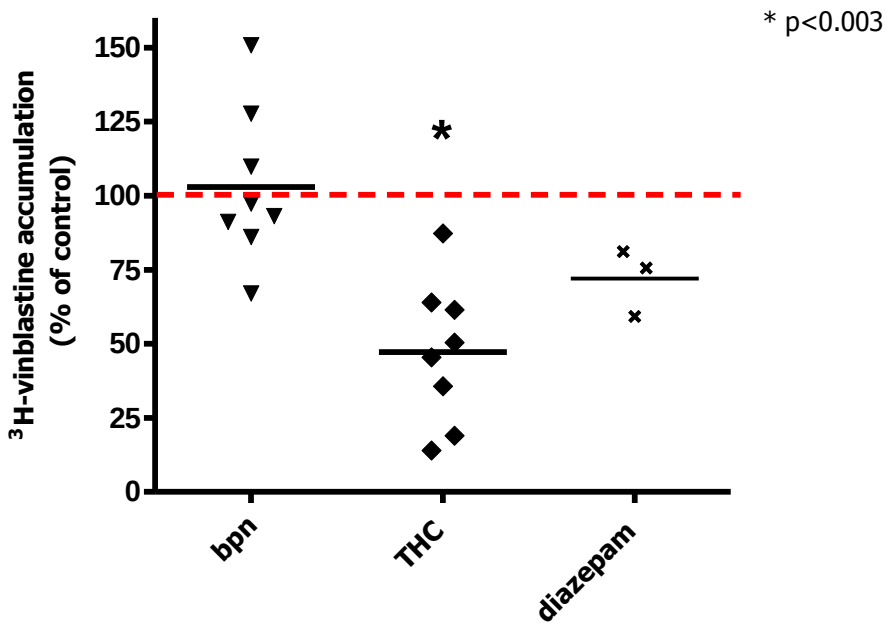


Figure 4.4 3H-mitoxantrone accumulation in the presence of selected doses of test drugs expressed as a percentage of control (n=6). p values were calculated using a Wilcoxon signed rank test. A significant reduction in 3H-mitoxantrone accumulation was observed in the presence of 50nM diazepam. Solid black lines show the median value.

At a higher concentration of 20µM substantially different effects were observed for the drugs tested (Figure 4.5). Considering firstly the effects on ^{3}H-vinblastine accumulation: bpn at 1µM resulted in a significant 15.2% decrease in accumulation whilst 20µM gave no significant change.

a



b

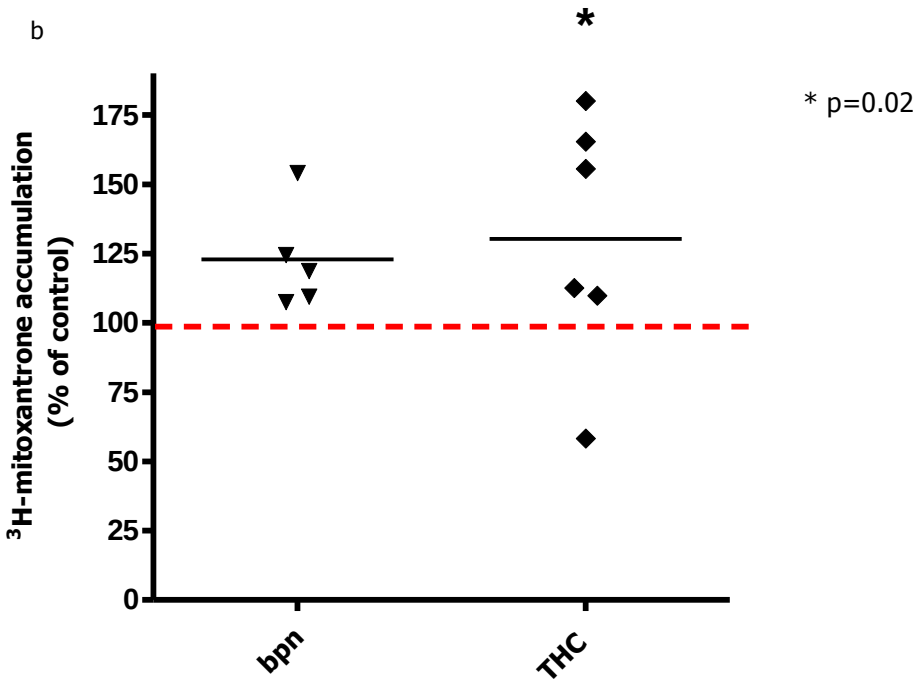


Figure 4.5 ^{3}H-vinblastine a, and ^{3}H-mitoxantrone b, accumulation as a percentage of control in the presence of 20μM bpn, THC and Diazepam (^{3}H-vinblastine only); p values were calculated using a Wilcoxon signed rank test and median values are shown as solid black lines.

In contrast THC resulted in a significant decrease in accumulation at both concentrations with the effect increasing from a 12.4% decrease at 1 μM to a 56.6% decrease at 20μM. Significant effects of the higher dose on mitoxantrone accumulation were restricted to THC but once again markedly different effects were observed at the different concentrations with 1μM a trend towards a decrease in accumulation (9.4%) and 20μM resulting in a 30.3% increase. The significant effect of diazepam found at 50nM was lost at this higher concentration although there remained a trend in the same direction.

Discussion

The variation in control ^{3}H-vinblastine accumulation observed here confirms at a functional level the observation of an eight fold variation in P-gp expression in human placenta (Hitzl *et al* 2004). It further confirms data obtained in this laboratory (Atkinson *et al* 2009) suggesting that an 8 fold difference in P-gp expression results in a 70 fold difference in efflux function. Similarly the 3.4 fold variation in control ^{3}H-mitoxantrone accumulation correlates well with our previously reported 4 fold difference in BCRP expression (Atkinson *et al* 2009).

In the current study the results are expressed as a percentage of the control condition for each individual placenta. A value below 100% therefore indicates reduced accumulation of substrate in the presence of the drug, suggesting an interaction with the protein enabling an increase in its efflux action. Conversely, a value of greater than 100% indicates increased accumulation of substrate and would suggest inhibition of the protein or an interaction that impaired the proteins ability to efflux that compound out of the tissue.

In relation to the feto placental unit, anything that impairs the ability of P-gp or BCRP to efflux potentially harmful compounds away from the fetus has the potential to increase risk of harm to the fetus. Conversely, increased efflux away from the fetus could reduce the risk of harm from xenobiotics.

Accumulation of ^{3}H-vinblastine was used here to measure the activity of P-gp in each placenta and how this is altered in the presence of a range of drugs of misuse. The data presented demonstrate that, in separate experiments, both bpn at 1μM and THC at 1 and 20μM interact with P-gp in such a way as to increase substrate efflux, suggesting stimulation of the protein. Schinkel and Jonker (2003) have suggested that two or more substrates can be effluxed concurrently by this protein and that this mechanism of co-

transport may result in a more efficient transport of both substrates. As ^{3}H-vinblastine is a substrate for P-gp the increase in efflux observed in the presence of bpn could be explained by this mechanism, similarly for THC. This would explain our observed increase in ^{3}H-vinblastine efflux in the presence of bpn and THC and would suggest that both drugs are acting as P-gp substrates.

From our data, which has small n numbers and a wide variation in accumulation in the control condition, it is not possible to speculate which compounds or their metabolites are transported most efficiently but from the literature there is some evidence that these compounds are substrates for these proteins. Our observation that bpn may be acting as a substrate for P-gp contrasts to data reported by Hassan *et al* (2009) who suggested that bpn did not produce a significant change in ATPase activity as measured by an ATPase activity assay, or a Caco 2 monolayer assay. Nekhayeva *et al* (2006), using a dual perfusion technique in human placenta also concluded that bpn was not a P-gp substrate. However, Tournier *et al* (2010) showed inhibition of P-gp by bpn and Suzuki *et al* (2007) concluded that bpn is transported from the rat brain by P-gp, suggesting that this compound does act as a substrate for this multi-drug transporter. The conflicting data reported in the literature is perhaps not surprising when one considers the difference in effect on P-gp of the different doses of bpn reported here. At higher concentrations (20μM) no significant effect on P-gp activity was observed whilst at lower concentrations significant increases in efflux activity were observed.

Our data suggesting that THC increases P-gp efflux activity are consistent with Molnar *et al* (2000), who found that THC reduced drug accumulation in mouse lymphoma cells that were multidrug resistant and Zhu *et al* (2006) who showed THC stimulated P-gp ATPase activity. It also agrees well with the observation of Bonhomme-Faivre *et al* (2008) that THC is a substrate for P-gp.

Hassan *et al* (2009) report that methadone stimulates P-gp ATPase activity in a dose dependent manner. However this effect was only significant at the highest concentration studied (100uM). In data presented here methadone (1μM) did not produce a significant change in P-gp activity. It may be that a larger dose is needed to reach the threshold that stimulates P-gp activity; however the therapeutic range of methadone is 100-1000ng/ml (0.29-2.9μM) so the clinical relevance of an effect at these doses is uncertain. A biphasic result with a threshold for P-gp activation has previously been demonstrated for methadone (Hassan *et al* 2009), oxycodone (Hassan *et al* 2007) and digoxin

(Matsunaga *et al* 2006).

^{3}H-mitoxantrone accumulation was used to measure the activity of BCRP in each placenta and how this altered in the presence of a range of substances of misuse. THC resulted in changes in ^{3}H-mitoxantrone accumulation at both low (1μM) and high (20μM) concentrations. However, these concentrations had opposing effects with 1μM resulting in a 9.5% decrease in ^{3}H-mitoxantrone accumulation, (suggesting stimulation of the protein) and 20μM resulting in a 30.3% increase in accumulation, (suggesting inhibition).

It has been suggested that some compounds can act in a concentration dependant manner to both stimulate and inhibit a transporter. Young *et al* (2003), cite this profile for progesterone, whereby 10uM inhibited P-gp transport and less than 1uM stimulated it. The presence of two binding sites on the BCRP protein has been suggested by Giri *et al* (2009). Whilst using rosuvastatin and canine kidney cells, Huang *et al* (2006) saw evidence of two binding sites in the BCRP protein. They suggest that these are high and low affinity sites, based upon substantially different Km values of 10.8μM for what they suggest is a high affinity site and 307μM for the low affinity site. This model is entirely consistent with the data observed here for THC.

The only other substance that showed any interaction with BCRP in the present study was diazepam, which at 50nM caused a significant decrease in ^{3}H-mitoxantrone accumulation.

Conclusion

In summary, our data show that placental P-gp and BCRP interact with compounds commonly associated with drug dependency in pregnancy i.e. heroin, cocaine, diazepam, bpn and methadone or used recreationally, i.e. cocaine and THC. Whilst statistically significance changes in accumulation of isotope was shown with THC, bpn and diazepam the clinical significance for women who choose to use these drugs in pregnancy remains unclear. The small sample sizes used here together with variation of function between individual placentae and the different responses to varied doses make it difficult to draw conclusions about how to translate this to clinical practice difficult. However, these data do highlight the need for more human placental research, especially with placentas that have been chronically exposed to drug use when women choose to use, or are prescribed, drugs such as cannabis, methadone, bpn, heroin, cocaine or diazepam in pregnancy. There are major benefits of such research in terms of providing women with

evidenced based information on which to base their decisions and the potential to improve outcomes for infants.

Conflict of Interests

There are no competing interests to declare.

Contribution to Authorship

DT, DEA and BB conducted the experiments.

DT wrote the article, DEA and CPS reviewed and edited the article.

Chapter Five

Relationship between Single Nucleotide Polymorphisms in Genes Expressing ABC Proteins in Placenta and the Early Childhood Outcome of Drug Misuse in Pregnancy: a Proof of Concept Study.

5.1 Introduction

Changes to fetal heart rate when exposed to maternal opiate use (Jansson *et al* 2005) and the presence of Neonatal Abstinence Syndrome (NAS) in neonates exposed prenatally to substances of misuse, particularly opiates, demonstrate that drugs pass to the fetus through the placenta. Attempts to identify individual risk factors such as gender, nicotine use, gestation and birth weight (Kaltenbach *et al* 2012, Holbrook and Kaltenbach 2010, Unger *et al* 2011) have failed to produce a definitive model. The variability in the presentation of NAS (Thajam *et al* 2010, Cleary *et al* 2010) suggests that some placentae may work as a more efficient barrier than do others.

As described in Chapter 1, and consistent with data presented in Chapter 4, genes including ABCB1, ABCG2, ABCC1 and ABCC2 have been shown to encode proteins that act as drug transporters altering the absorption and bioavailability of various compounds, e.g. progesterone (Schinkel and Jonker 2003), THC (Molnar *et al* 2000, Zhu *et al* 2006, Bonhomme- Faivre 2008), oxycodone (Hassan *et al* 2007), methadone (Hassan *et al* 2009) and buprenorphine (Suzuki *et al* 2007). The study reported in this Chapter sought to begin testing the hypothesis that Single Nucleotide Polymorphisms (SNPs) in these genes altered protein expression in the human placenta thereby reducing its capacity to efflux drugs of misuse, leading to increased fetal exposure as demonstrated by increased severity of NAS and/or altered developmental outcomes.

Since substance misuse remains common in pregnant women and there is a well-defined NAS with validated tools for assessment this group is an ideal population in which to examine the hypothesis in a prospective test of concept study. However, this group have complex and chaotic lifestyles which meant strategies to minimise confounders had to be introduced. These included taking a comprehensive history at the time of recruitment, analysing hair samples to obtain an accurate record of substances the fetus was exposed to in the three months prior to birth and an assessment of maternal stress.

Maternal stress in pregnancy has been linked with adverse effects on birth weight, reduced occipital frontal circumference (OFC) (Mulder *et al* 2002), changes in neonatal behaviours (Rieger *et al* 2004), developmental delay at 8 months (Huizink *et al* 2003) and placental size (Tegethoff *et al* 2010). These outcomes are also associated with drug use in pregnancy (Hagopian *et al* 1996) therefore an assessment of maternal stress was undertaken. A psychological model of stress, the diathesis model of stress asserts that

an inherited vulnerability, which may be biological or psychological, and one's life experiences work in tandem to determine how one perceives stress in a given situation. For example, Hsu et al (2003) stressed rat pups by vigorous handling and separation from their mother (life experiences) and compared them to a control group of non-stressed rat pups. They hypothesised their early adverse environment would alter the gamma-aminobutyricacid (GABA) system (biological vulnerability). When tested as adults the stressed group showed differences in functioning of the GABA system, which interacted with their stressful early life experiences, to alter the way they the way they coped with a water filled maze learning task. Biochemical measures of stress (corticosterone) differed by group suggesting the animals stressed as pups experienced the test as more stressful when adult. This model allows for protective factors that may moderate the effects of vulnerability suggesting that the perception of stress is subjective. Therefore an event may be perceived and experienced as very stressful by one individual and not another.

Trying to measure perceptions of stress that occurred in the past relies on an accurate recall of events and the feelings that accompanied them. This may have been difficult for this particular group of women as the feelings may be difficult to remember due to their drug use and may relate to illicit activities that they did not want to discuss. Although important factors in lives of women, depression and anxiety were not scored by separate tools in this study. Stress was the only measure of psychological discomfort measured as negative outcomes reviewed in the literature mirror those of reported as negative outcomes of substance use. However a record of medication was noted for all women and remarks about anxiety and depression were noted if mentioned in conversation.

Since women in this client group often have limited contact with education the stress assessment tool was chosen to be quick and easy for women to complete. After a review of potential tools The Perceived Stress Scale (Cohen 1994) was chosen as it enquired about stress in a short specific time frame, was designed to be suitable for people with limited literacy skills and administered in a community setting (appendix 4).

The modified Finnegan NAS scoring tool was chosen as it was already used in clinical practice and was therefore readily available and known to staff should, for any reason, they be required to assess a baby. The Griffiths Mental Development Scale was chosen because is easy to administer in a community setting, provides information over five different subscales and has previously been used with babies exposed *in utero* to

maternal drug use. To reduce differences caused by inter-observer reliability both tools were administered by the same person (Thajam).

5.2 Materials and Methods

5.2.1. Recruitment of Participants

In Manchester any woman who discloses drug use at an antenatal appointment or conversely pregnancy to a drug worker, is referred to The Manchester Specialist Midwifery Service. In the period from April 2009 – February 2011, following ethical approval, these referrals were reviewed to assess eligibility to participate in the study i.e. subjects had to be abstinent from alcohol, use their drug of choice on at least a daily basis and plan to do so throughout their pregnancy. They also needed to be booked to give birth at one of the two participating hospitals and be free from blood borne viruses such as Hepatitis C and Human Immunodeficiency Virus (HIV).

Women were contacted by telephone and a home visit arranged to discuss the project and what would be required of them if they agreed to participate. In essence, a history of their past and present drug use would be taken in the antenatal period from both them and their Specialist Midwife and their levels of stress would be assessed. Immediately following the birth, samples of their hair, the placenta and cord blood would be collected and a history of their recent drug use would be taken. In addition, the baby would be examined. If cord blood was not obtainable a buccal swab would be taken from the baby. Medical records would be reviewed, particularly those sections regarding the progress of labour and fetal wellbeing. At three days and again at three weeks the infant would be assessed for signs of Neonatal Abstinence Syndrome. A further visit would also take place when the child was eight months old to assess development and for some infants this would be repeated when they were twelve months old. Women were given a written information sheet (appendix 1) and encouraged to ask questions about the project. A period of time to reflect was then offered with a view to making telephone contact to arrange gaining consent at a later date. When the consent form was signed (appendix 2) antenatal data, information regarding drug use and stress scores was collected. Agreement to participate in the research project was documented in the hand held maternity notes.

5.2.2 Data Collection

A data collection tool was devised in order to standardize the information collected from each participant (appendix 3). The tool was divided into sub sections dependent on the types of data required. These were; i) demographics and contact details, ii) obstetric history, iii) antenatal and medical history, iv) past and present drug history, v) labour and delivery history, vi) placenta, vii) baby's details, viii) neonatal behaviours and difficulties including any treatment. Table 5.1 illustrates the type of information collected in each section.

Table 5.1 Information recorded on the data collection tool.

Section	**Details include**
Demographics and contact details	Name, address, date of birth, ethnic origin, telephone number, information re baby's father, hospital booked at, housing status i.e. homeless, tenancy or living with family.
Obstetric history	Past obstetric history and outcomes, where those children reside, what substances they were exposed to in pregnancy and maternal perceptions of withdrawal and wellbeing.
Antenatal and medical history	Expected date of delivery for this pregnancy and any concerns or problems including treatment. Any illnesses or blood borne viruses e.g. Hepatitis C, Hepatitis B or HIV. Any history of, or current mental health issues i.e. depression, anxiety and treatments. Perception of stress.
Drug history	Current drug use i.e. drug, mode of usage, how much and pattern of use, if in treatment. Past use, age at commencement and periods of abstinence.
Labour and delivery	Length of labour, complications, pain relief, mode of delivery
Placental details	Weight, general description, measurements (width and length) any abnormalities
Baby	Apgar score, resuscitation, Occipital Frontal Circumference (OFC), length, weight, sex, gestation, ethnicity
Neonatal history	Feeding history, length of stay in hospital, neonatal behaviours including treatment and abstinence scores, who baby went home with

5.2.2.1 Perceived Stress Scale (PSS)

Women were asked about perceived levels of stress in their life and what their stressors were. The narrative answers provided by this approach were too difficult to categorize so a validated, easy to use scale was required. Since women in this client group often have limited contact with education the stress assessment tool was chosen to be quick and easy for women to complete. After a review of potential tools The Perceived Stress Scale

(Cohen 1994) was chosen as it enquired about stress in a short specific time frame, was designed to be suitable for people with limited literacy skills and administered in a community setting (appendix 4). It is also easy to score, had previously been used with women, different ethnicities and in different settings. The PSS was then introduced as a standard way of assessing perceived stress in the four weeks prior to recruitment and acted as a trigger for discussion about general life stressors during the pregnancy (appendix 4). One of the advantages of the scale is that it measures stress over a short, specific time however, this was also a disadvantage as it may have been administered at a time when stresses were low and not representative of actual stress over the course of the pregnancy.

The PSS is a 10 item, 5 point Likert scale developed from a cohort of 2387 adult respondents in the USA. It was designed to be used in a community setting, to be easily understood and has previously been used to measure stress in pregnancy comparing results to birth outcomes (Mulder *et al* 2002). Measuring perception of stress over a 4 week period it explores how overwhelming or uncontrollable stress is perceived to be by that individual. It is a widely used and validated scale with published norms for ethnicity, age and sex. The age norms were used as a reference point for the participants in this study. Each question (figure 5.1) has a potential score of 0-4. Questions 4, 5, 7 and 8 are reverse scored i.e. 0=4 1=3 whilst the others are forward scored i.e. 0=0 1=1etc. Total scores are obtained by adding the responses, giving a potential range of 0-44.

In an effort to enable women to be open about their feelings, women were told why the questionnaire was being used, given the questionnaire to fill in whilst the data collection tool was being completed, and told that it would not be scored until after their participation in the study had ended. Other stressors identified by the women were also recorded.

1. In the last month, how often have you been upset because of something that happened unexpectedly?

2. In the last month, how often have you felt that you were unable to control the important things in your life?

3. In the last month, how often have you felt nervous and stressed?

4. In the last month, how often have you felt confident about your ability to handle your personal problems?

5. In the last month, how often have you felt that things were going your way?

6. In the last month, how often have you found that you could not cope with all the things that you had to do?

7. In the last month, how often have you been able to control irritations in your life?

8. In the last month, how often have you felt that you were on top of things?

9. In the last month, how often have you been angered because of things that were outside your control?

10. In the last month, how often have you felt difficulties were piling up so high that you could not overcome them?

Figure 5.1 Questions on The Perceived Stress Scale. The complete scale as presented to the women is found as appendix 4.

5.2.2.2 Perinatal Observations

Hospital and hand held medical and maternity notes were reviewed to collect data regarding labour and delivery history. Length of labour, complications, pain relief or other medication, mode of delivery and any documented fetal distress were recorded. Interpretations of cardiotocograph recordings were also noted and all information recorded on the data collection tool as was time and amount of last drug use.

At delivery Apgar scores (Apgar 1953) together with a record of any resuscitation required were recorded, as were gestation, ethnicity and sex of the baby. As soon as possible after birth the baby was examined to assess general wellbeing e.g. colour, tone, respirations and to measure OFC and length from the top of the head to the heel. For pregnancies that had a booking BMI recorded, a customised weight centile was calculated using the GROW- centile software available online from the Perinatal Institute. Neonatal measurements on all babies were undertaken by the same person. Measurements were taken twice and the average measurement recorded.

5.2.2.3 Neonatal Observations

The Finnegan Neonatal Abstinence Scoring tool was devised and validated in the 1970s (Finnegan 1975) to assess behaviours of neonates exposed to opiates whilst *in utero* by providing a structure to quantify signs of withdrawal. Originally, a 30 item score, it has been modified to make it easier to administer. Numerous modifications exist. The modification already in use at the hospital, at which the majority of women in the study would give birth, was used in this study (Figure 5.2 and appendix 5). Observed signs of withdrawal are assigned a numerical score. In this modification a score of 5 or more on 3 consecutive occasions is the threshold for pharmacological treatment. For both hospitals the treatment of choice is Oromorph and infants are admitted to a neonatal unit to receive treatment. Infants whose signs of withdrawal can be managed by environmental adaptations stay with their mother, which is reported to facilitate a shorter stay in hospital and fewer neonates requiring treatment (Abrahams *et al* 2007).

Date & Time	
Convulsions	Score 3
High Pitch Cry Tremors Hypertonia	Score 1 if any single symptom or combination of symptoms is noted
Irritable Scratching Excessive Wakefulness	Score 1 if any single symptom or combination of symptoms is noted
Pyrexia >38^{o} C Tachypnoea >60/min	Score1 if either or both are noted
Sweating Dehydration	Score1 if either or both are noted
Vomiting/Diarrhoea/Yawning Hiccups	Score 1 if any single symptom or combination of symptoms is noted*
Salivation Congested Nose Sneezing	Score 1 if any single symptom or combination of symptoms is noted*
Total score	

*when sufficiently repetitive to be out of the ordinary

Figure 5.2 This extract from the modified Finnegan Scoring tool shows the observations that attract a score. The assessments usually take place every four hours and the score should reflect the behaviours exhibited in that time frame rather than a snapshot of behaviours at the time of

assessment. To this end mothers/carers are asked about such behaviours if the child has not been observed by the assessor in the preceding 4 hours.

Although the scoring tool provides a structure for assessment, it is recognised that there remains a degree of subjectivity in the procedure (Kuschel 2007). The behaviours of all infants in this study were therefore assessed by the same person (Thajam). A minimum period of half an hour was spent at each assessment in order to obtain an accurate picture of behaviours. The main caregiver, i.e. parent or foster carer, was asked about behaviours in general and in particular the preceding 4 hours. The scoring tool was then administered and scores documented as were more general comments and observations.

Opiate exposed infants remain on the postnatal ward, between three and five days. This ensured that all infants and any additional information were available for the three day assessment. The three week assessment was undertaken in the home.

Literature examining the effects of maternal cannabis use in pregnancy has not produced any conclusive evidence as to its effects in the immediate postnatal period. However, poorer habituation to visual stimuli and an increase in tremors have been reported (Fried and Maken 1987) as have altered sleep patterns (Scher *et al* 1988) and increased arousal and excitability (de Moraes Barros *et al* 2006). Fried *et al* (1987) suggest the pattern of behaviours exhibited by these infants is similar to a weak Neonatal Abstinence Syndrome from opiates. Perceived wisdom is that these behaviours will not be severe enough to score on a modified Finnegan scoring tool. However, it was used in this study to assess neonates exposed to cannabis as it provided a standard way to measure behaviours across all neonates in the study. In addition, principal care givers were questioned on weight gain and more general behaviours including feeding and sleeping patterns to provide a comprehensive picture of behaviours. Again a minimum of half an hour was spent at each assessment observing the infant and speaking with caregivers. These infants do not have an extended stay in hospital unless there is a medical reason why they or their mother should do so e.g. infection requiring treatment; assessments were therefore generally undertaken in the home.

5.2.2.4 Infant Development

The Griffiths Mental Development Scales (GMDS) is a validated tool used extensively in clinical practice by psychologists and paediatricians to assess infant development (Sansavini *et al* 2010, Bromley *et al* 2010). The original scale, devised in the 1950s, was the first to assess development in children from birth to 2 years of age. It tests a set of

sample skills that reflect underlying skills necessary to perform a task and compares what a specific child does at the time of assessment to what children of a comparable age can do.

Between 1978 and 1982, 447 children were tested to determine if the original scales still reflected the capabilities of children given differences in parenting practices and environment. The results showed a higher mean General Quotient than the original sample (110.2 and 99.7 respectively), with 160 items passed at a younger age (Hanson *et al* 1985). The tool was revised in 1996 (Huntley 1996) to account for these findings and it was this revised tool that was used for the present study.

To ensure that children are assessed in a consistent manner, the assessment kits and record forms can only be purchased by registered assessors who have passed a validated 4 day intensive training course that covers the theoretical knowledge and practical skills required to assess infants using the scales. Children of various ages and abilities (including those attending a school classed as "special needs") are assessed by the trainees under supervision of the trainers who score the accuracy of the assessment. Trainees must pass all elements to become registered. Continued registration is reliant upon attendance at refresher courses. The author undertook this training in Edinburgh during the course of this research.

The directions for administering each task are also detailed in the handbook for reference prior to assessing each child. Table 5.2 itemizes the kit together with an example purpose/usage of each item. In all cases the assessment was administered by the same person (Thajam) when the infant was as close as possible to 8 months of age. Infants who were old enough during the study also had the test repeated as close to 12 months of age as possible.

Table 5.2 The items in the kit are used to assess changing abilities with age. For example a very young infant may only listen to the hand bell but as it matures it would be expected to manipulate it and finally ring it. The items provided are listed in the table along with an example of how the item is used.

Item	**Example task**
Small ball that rattles	Looks for the ball when dropped/throws it
Small rubber ball	Rolls or crawls to the ball when rolled toward it
Wooden ring with cord	Can dangle the ring by the string
Wooden ring with bell	Can grasp and or manipulate the ring
Hand bell	Manipulates and or rings the bell
Torch	Follows a moving light with eyes
Tuning fork	Listens to the bell
Cup and saucer	Helps to hold the cup to drink
Plastic teaspoon	Manipulates the spoon
Plastic beakers	Finds a toy underneath it
Toy with wheels and cord	Walk backwards pulling the toy
Car (silent)	Pushes it along a surface
Mirror	Plays with or smiles at the mirror
Book	Looks at the picture
Red wooden rod	Resists its removal from grasp
Set of form boards	Fits the shape into the one circle board
Set of boxed yellow cubes	Passes a cube from one hand to another
Packet of tissues	Reacts to paper
Set of brick boxes	Removes cubes from the box
Felt	Pulls it to get a toy placed on it but just out of reach
Screw toy	Attempts to undo to obtain an object inside
Hairbrush	Can identify from a row of items
Mini Car	Can identify from a row of items
Car with motor	Can identify from a row of items
Metal teaspoon	Can identify from a row of items
Flow ball	Can identify from a row of items
Baby sock	Can identify from a row of items
Green Brick	Can identify from a row of items
Pair of embroidery rings	Manipulates two objects at once
Sleeping doll- dressed	Identifies 1 part of the doll when asked where it is
Hat	Pulls hat from own head

The assessment was undertaken in the home to enable as many women as possible to continue to participate in the project and also to observe the child in its home environment where it would feel most confident. When the appointment was arranged women were told that the room would need to be quiet, free from distractions and the appointment should be at a time when the child was usually most alert. On arrival the television was turned off and toys removed to a place where the child could not see them. The assessment did not start until the child was happy to interact with the assessor. Parents or foster carers were instructed that there was a set way and number of times to tell the child about the task and they should not try to assist the child by giving cues or paraphrasing instructions. At the same visit notes were also taken about the child's recent health and the child health record reviewed. It should be noted that the GMDS scores reflect a snapshot of the child's ability or willingness to perform a task at a given time. However, all infants were given ample opportunity to become accustomed to the researcher before starting the assessment and tasks were presented at a pace suitable for each child.

5.2.2.4.1 Subscales and presentation of scores.

There are five subscales that measure different aspects of development. These are Locomotor, Personal-Social, Hearing and Language, Eye and Hand Coordination and Performance. A raw score is obtained for each subscale and equates to the number of items a child completes. These raw scores are used to generate other standardized scores; the age equivalent score (which can be viewed as a developmental age), sub and general quotients (an overall age equivalent score) and percentile scores, which are percentile equivalents of the sub quotient score. Subscale and General Quotients are calculated thus:

General/ Subscale Quotient = Age Equivalent (in months) divided by Chronological Age (in months) multiplied by 100

Charts that calculate the standardized scores are published in the handbook therefore eliminating calculation errors. This study used the percentile score as the main measure. A percentile score equal to or less than $2^{1/2}$ is considered statistically significant.

The GMDS is a test of mental rather than physical abilities but a Locomotor subscale is included to reflect the concentration required by the child when developing a skill e.g. the ability to sit, stand, walk. It is a 54 item subscale subdivided into Prone, Dorsal,

Sitting, Walking Climbing, Balance and Running/Jumping items. Example tasks include observing if the child can crawl, roll over, sit in a chair, pull itself up on furniture.

The Personal-Social subscale is divided into, Eating, Drinking, Dressing, Play and Toileting. This subscale assesses the abilities that are required for social development and for the child to gain relative independence from its caregiver. Example items here include observation of how a child reacts to its reflection in a mirror, holds a spoon, drinks from a cup, plays interactive games.

Hearing in the Hearing and Language section of the scale relates to active listening rather than the ability to hear. Within this subscale there are 56 items subdivided into tasks that relate to listening and attention, music, vocalisation and expressive language, the understanding of receptive language, signing and symbolizing. Example scale tasks includes observation of how the child handles a bell, shakes its head for no, knows its name, reacts vocally to music.

The aim of the Eye and Hand Coordination subscale is to assess visual monitoring, fine motor skills and manual dexterity. Within this 54 item scale tasks are subdivided into eye and hand coordination, string and pencil tasks, prehension, throw/ roll ball, toys and cubes (tower) tasks. Example scale tasks include observing how equipment is handled, if complete thumb opposition is obtained, if the child can point with the index finger.

The aim of the 54 item Performance subscale is to assess reasoning ability and uses tasks under the categories of manipulation, permanence of objects, brick boxes, cubes (toys), form boards, screw toys and bricks to do this. Tasks include manipulating cubes, finding a toy hidden under a cup, lifting a lid off a box, taking cubes out of the box.

5.2.2.5 Determination of Drugs Used in Pregnancy

If a potential relationship between placental multidrug resistance protein expression and drug use, neonatal behaviours and developmental outcomes was to be established it was important to obtain an accurate record of what compounds the infant had been exposed to in pregnancy. Drugs and their metabolites are deposited in the growing hair follicle and remain in the hair shaft thus enabling an assessment of drugs used over a longer period than that afforded by urine or blood testing (Musshoff and Madea 2006, Musshoff *et al* 2006, Barbosa *et al* 2013). Depending on the half-life of the compound a substantial period of abstinence has to be maintained before it is not detected in hair. Garcia-Bournissen *et al* (2009) studied the pharmacokinetics of disappearance of cocaine in human hair and demonstrated that a period of 3-4 months abstinence was required

before a negative result was obtained. Maternal self-report measures have consistently shown underreporting of drug use (Sanaullah *et al*. 2006, Ostrea *et al*. 1992, Ostrea et al 2001, Grekin *et al*. 2011). For this reason maternal hair samples were collected at delivery for analysis of drug use in the previous three months.

Whilst acknowledging that the percentage of hair in a growth phase increases in the second and third trimesters of pregnancy and there is variability in the rate of human scalp hair growth (Harkey 1993), it is generally accepted that human scalp hair grows at approximately 1 cm a month (DuPont *et al* 1995, Kintz *et al* 2006), requiring collection of at least three centimetres of hair to detect drug use in the previous three months. As the hair closest to the root reflects the most recent drug use maternal hair was cut as close to the root as possible using gloved hands and sterile scissors. A minimum of 100 hairs were taken, wrapped in foil and placed in a sealed envelope. Samples were stored at room temperature until posted in batches for analysis. Analysis was performed by TrichoTech (TrichoTech Ltd. No1 Pentwyn Business Centre, Cardiff) using gas chromatography. This commercial company has accreditation by the UK Accreditation Service and is used by The Home Office, the General Medical Council, UK Police Forces and Local Authorities.

All hair samples were analysed for cannabis (plus 5 metabolites), amphetamine (and 5 metabolites), methamphetamine, benzodiazepines (10 individual compounds), cocaine (and 5 metabolites), methadone (plus 2 metabolites) and opiates (4 opiates and 2 metabolites). Results were supplied in ng/mg. Metabolites are used to determine passive exposure from the environment or active uptake from the systemic circulation. As the inclusion criteria for the study was regular and daily drug use this information was of limited value but did provide an indication of the amount in the systemic circulation and therefore passing through the placenta.

5.2.3 Genotyping

5.2.3.1 Isolation of Genomic DNA

Cord blood was collected from umbilical cord vessels immediately after delivery of the placenta. Samples were aliquoted into 1ml eppendorfs and stored at -80^{o}C until genomic DNA was extracted, in batches, using a QiAmp Blood Midi Kit, (Quiagen, Crawley, West Sussex, UK). This kit utilises a spin protocol to lyse the sample and bind the DNA to a membrane. This removes contaminants such as proteins before washing in two buffers

to remove residual contamination and improve the purity of the final DNA sample. Finally the DNA is removed from the membrane by use of an elution buffer.

Immediately following extraction, the purity and amount of genomic DNA (µg/ml) in each sample was determined using photometric analysis (Eppendorf Bio Photometer). This technique uses light (in the ultra violet and visible range) and the known absorbance patterns of amino acids and DNA to measure the amount and purity of DNA in a sample. The amount of DNA in the sample was determined by absorbance at 260nm, whilst protein contamination was measured by absorbance at 280nm. An absorbance ratio estimated from these two measurements, i.e. 260nm/280nm, provides a measure of sample purity.

Genomic DNA samples were then stored at -80^{o}C until sent to The Centre for Integrated Genomics In Medical Research (CIGMR) at the University of Manchester for genotyping by Sequenom MassExtend Assay.

Buccal swabs were collected from babies for whom cord blood was unavailable using the SK-2 Isohelix swab (Cell Projects Ltd. Kent, UK). After determining that milk had not been consumed within the previous hour, the mouth was rinsed with water. Using gloved hands, a sterile swab, was rubbed along the cheeks for a minimum of two minutes. The swabs were immediately placed in Proteinase K and a lysis buffer to stabilize them in preparation for later isolation of DNA.

The initial stabilization stage is designed to protect the sample from the enzymatic activity associated with buccal swabs, however once stabilized the swabs can be stored at room temperature, without affecting yield or quality until processed in batches (Hashemian and Dasse 2010). Genomic DNA was isolated from buccal swabs using an Isohelix DNA Isolation Kit (DDK-3/DDK-50, Cell Projects Ltd. Kent, UK). This kit is especially designed for use in buccal cells and uses a spin protocol to lyse and wash samples and to isolate DNA.

Immediately following extraction the purity and amount of genomic DNA (µg/ml) was determined using photometric analysis (Eppendorf BioPhotometer) as above. Again samples were stored at -80^{o}C until sent to CIGMR for genotyping.

5.2.3.2 Selection of Single Nucleotide Polymorphisms

A total of 173 Single Nucleotide Polymorphisms (SNPs) across the four proteins were selected. SNPS were chosen to include:

- SNPs in published literature that demonstrated a significant effect,
- all coding SNPs
- tagging SNPs for linkage blocks

  This approach yielded 51 SNPs for ABCB1 (Pgp), 26 for ABCG2 (BCRP), 79 for ABCC1 (MRP1) and 17 for ABCC2 (MRP2) (see Table 5.3).

Table 5.3 rs numbers of SNPs examined by Sequenom MassExtend Assay. A total of 173 SNPs were chosen.

ABCB1 (P-gp)		**ABCG2 (BCRP)**	**ABCC1 (MRP1**		**ABCC2 (MRP2)**
145a	rs2157930	rs1061018	rs10852377	rs3743527	rs17222723
1517a	rs2214102	rs12505410	rs11075293	rs3765129	rs2002042
41a	rs2229107	rs1564481	rs11864374	rs3784862	rs2073336
755a	rs2235015	rs2046134	rs152022	rs3784867	rs2273697
824a	rs2235067	rs2054576	rs152023	rs3851712	rs2756105
rs1002205	rs2235074	rs2231137	rs152033	rs3887412	rs2756109
rs10264990	rs2373587	rs2231142	rs17205859	rs3887893	rs2756112
rs10274623	rs28401798	rs2231148	rs17287570	rs41395947	rs3740063
rs1045642	rs35023033	rs2622604	rs17501011	rs4148340	rs3740065
rs1055302	rs35657960	rs2622605	rs17501331	rs4148348	rs4148398
rs1128503	rs35730308	rs2622610	rs182482	rs4148349	rs56199535
rs1202170	rs35810889	rs2622621	rs193538	rs4148350	rs56220353
rs1202171	rs3789243	rs2622626	rs1967120	rs4148356	rs56296335
rs1202179	rs4148732	rs2725248	rs2074085	rs4148358	rs72558199
rs1202182	rs4148735	rs2725252	rs212079	rs4148380	rs72558201
rs12720066	rs4148737	rs3114018	rs212081	rs45511401	rs7476245
rs13226726	rs4148739	rs3116448	rs212090	rs4781699	rs8187710
rs13233308	rs4728709	rs3201997	rs212091	rs4781701	
rs17064	rs6949448	rs4148149	rs215052	rs4781709	
rs17149699	rs868755	rs6857600	rs215100	rs4781718	
rs17149792	rs956825	rs72552713	rs215101	rs504348	
rs17327624		rs9999111	rs215105	rs60782127	
rs1858923		rsCT-15622	rs2238476	rs6498594	
rs1882479		rsGA1322	rs2239995	rs6498595	
rs1922240		rsGA1898	rs2283512	rs762775	
rs1922242		rsTC1291	rs246214	rs8044115	
rs1989830			rs246217	rs8054670	
rs2032582			rs246221	rs8187843	
rs2032583			rs246227	rs8187844	
rs2032588			rs246232	rs8187848	
			rs246233	rs875740	
			rs246240	rs903880	
			rs2889517	rs924135	
			rs35593	rs924136	
			rs35597	rs937367	
			rs35600	rs9673292	
			rs35601	rsCT350	
			rs35621	rsCT4535	
			rs35625	rsGT151	
			rs35626		

5.2.3.3 Sequenom MassExtend Assay.

Genomic DNA was supplied to CIGMR at a concentration of 20ng/µl for analysis by Sequenom MassExtend Assay. This technique used locus specific PCR amplification with SNPs of interest and a primer extension process to produce allele specific end products that differ in mass. The differences are determined by mass spectrometry enabling the detection of homozygous wild type, homozygous mutant and heterozygous genotypes. It is a fully automated process that has inbuilt quality control systems and guarantees 99.7% accuracy.

5.2.3.4 Analysis of Genotype

PLINK analysis software (pngu.mgh.harvard.edu/~purcell/plink) a free, open access tool for the analysis of whole genome associations was used to test for associations between genotype and; protein expression, individual Griffiths sub scale scores and NAS. Data for the phenotype e.g. protein expression, to be compared with SNP is entered for each participant. The software runs a quality control, compares the phenotype to genotype and provides a data table showing the SNP, minor allele, number included in the analysis, beta coefficient and p value indicating if there is a statistical difference according to phenotype and the direction of difference i.e. increased or decreased expression relative to the minor allele.

5.2.4 Western Blot Analysis of Placental Tissue

Placental expression of each of the four proteins, P-gp, BCRP, MRP1 and MRP2 was measured using Western blot analysis. The level of expression was then correlated with the outcome measures of the study i.e. genotype, NAS and GMDS.

5.2.4.1 Protein Extraction

After examining, weighing and measuring the placenta four randomly chosen $2cm^3$ full thickness sections were dissected and washed in buffered saline solution (0.15M NaCl, 0.01MTris (base) pH to 8 with HCl). Membranes were removed and the tissue trimmed to remove vessels, obviously fatty or infarcted lesions and gritty areas. Tissue was then snap frozen in liquid nitrogen and stored at -80^{o}C for later protein extraction.

An enriched membrane preparation of all placental samples was prepared using a protocol developed by Atkinson *et al* (2009) to measure P-gp expression. Essentially placental tissue was homogenised using a IKA T18 basic ULTRA-TURRAX homogeniser probe (IKA, Germany) in a buffered solution containing 300mM Mannitol (Sigma Aldrich Dorset, UK) , 10mM HEPES (Sigma Aldrich Dorset, UK) , 1% Protease Inhibitor Cocktail

(Sigma Aldrich, Dorset, UK) and 1mM EDTA (Sigma Aldrich, Dorset, UK), adjusted to pH 7.6 with saturated TRIS. The homogenate was filtered before centrifugation using a Sorval Discovery 100SE centrifuge (Hitachi). Initially the homogenate was spun at 25,000g for 10 minutes at 4^{o}C. The supernatant produced was then transferred to a clean container and respun at 100,000g for a further 30 minutes at 4^{o}C. The supernatant was removed and discarded and the final pellet re-suspended in 500µl of the homogenization buffer. Aliquots of the enriched protein preparation (50µl) were snap frozen in liquid nitrogen before storage at -80^{o}C.

One aliquot from each sample was used to determine protein concentration using a BioRad Protein Assay (Bio-Rad Laboratories Ltd., UK). A standard curve was generated using BSA against which appropriate dilutions of the placental protein samples were measured. Absorbance at 595nm was measured on a VersaMax Microplate reader running SoftMax Pro5 Software. GraphPad Prism was used to generate the standard curve and calculate the amount of protein in each sample.

5.2.4.2 Immunoblotting

5.2.4.2.1 Equipment, Reagents, Buffer Recipes and Antibodies

Bio-Rad Mini-Protean III Electrophoresis system (Bio-Rad Laboratories Ltd., UK)

Bio-Rad PowerPac 200 electrophoresis power supply (Bio-Rad Laboratories Ltd., UK)

Kaleidoscope Precision Plus Protein Standards- pre-stained molecular weight markers (Bio-Rad Laboratories Inc., CA, USA)

Ammonium Persulphate (Sigma Aldrich, Dorset, UK)

Protogel A (Sigma Aldrich, Dorset, UK)

Tetramethylethylenedlamine (TEMED) (Sigma Aldrich, Dorset, UK)

Sodium dodecyl sulphate (SDS) ((Sigma Aldrich, Dorset, UK)

Tween 20 (Sigma Aldrich, Dorset, UK)

SuperSignal West Pico Chemiluminescent Substrate Reagent Kit (ECL) (Thermo Fischer Scientific Ltd, Bishop Meadow Road, Loughborough, UK)

Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes (GE Healthcare, Little Chalfont, UK)

Resolving gel buffer-Solution B (22.7% Tris, 0.5% SDS, pH 8.8 with concentrated HCl)

Stacking gel buffer-Solution C (6.1% Tris0.4% SDS, pH 6.8 with concentrated HCl)

5x Loading Buffer (100% glycerol 2mls (20%), TRIS-HCl pH 6.8 (300mM), 20% SDS 5mls (10%), Dithiothreitol- DTT 3.86% (0.25M), Bromophenol Blue 5mg (0.72mM)

Electrode Buffer (Tris base 50nM, Glycine 400mM, SDS 2% w/v Volume to 2L with dH_2O)

Wet transfer buffer (Trisbase 25mM, Glycine 192mM , Methanol 20% 200mls, made to 1L with distilled water, pH 8.1)

Tris Buffered Saline (TBS) (150mM NaCl 17.52g, 10mM TRIS 2.42g dH_2O to make to 2l pH with HCl 8.0)

Tris Buffered Saline (TBS) (150mM NaCl 17.52g, 10mM TRIS 2.42g dH_2O to make to 2l pH with HCl 8.0)

0.05% Tween/TBS mix (250µl Tween made to 500mls with TBS for Western Blotting)

3% Blotto (15g dried milk powder, 0.05% Tween/TBS mix made to 500mls and filtered)

1^0 Monoclonal Anti BCRP clone BXP 21, (Alexis Biochemicals Nottingham)

1^0 Monoclonal Anti P-glycoprotein clone F4,(Sigma Aldrich, Dorset, UK)

1^0 Monoclonal Anti MRP1 SC773, (C20) (Santa Cruz Biotechnology, USA)

1^0 Monoclonal Anti MRP2 SC5770, (H17) (Santa Cruz Biotechnology, USA)

1^0 Human Anti beta actin antibody (A2228) (Sigma Aldrich, Dorset, UK)

2^0 For Pgp, BCRP and beta actin- horseradish peroxidise conjugated sheep anti mouse (N931) (GE Healthcare, Little Chalfont, UK)

2^0 For MRP1 and MRP2- horseradish peroxidise conjugated donkey anti goat (Santa Cruz Biotechnology, USA)

5.2.4.2.2 Electrophoresis

SDS polyacrylamide gel electrophoresis on discontinuous gels was performed on all placental protein samples. The size of the protein determined the concentration of polyacrylamide used, with smaller proteins requiring a smaller matrix and therefore a higher percentage gel, i.e. 10% for BCRP and 7% for P-gp (MDR1), MRP1 and MRP2. A 3% stacking gel was used in all cases (see Table 5.4).

Table 5.4 Recipes used to make the 10%, 7% and 3% SDS polyacrylamide gels used in the electrophoresis.

	10% gel	7% gel	3% gel
Ammonium Persulphate	15mg	15mg	10mg
Distilled water	4.7mls	5.7mls	6.5mls
Solution B	2mls	2mls	-
Solution C	-	-	2.5mls
Protogel A	3.3ml	2.3ml	1ml
TEMED	10 µl	10 µl	15 µl

Using a dry loading technique, 75µg of protein together with 4µl of loading buffer was loaded into each lane. In addition, 10µl of a prestained protein standard for electrophoresis was loaded onto each gel. Electrophoresis was performed at 120 volts for 70 minutes or until the dye front reached the end of the gel. The resolved proteins were then transferred onto Hybond enhanced chemiluminescence (ECL) nitrocellulose membranes by wet transfer using a voltage of 120V for 70 minutes. Following transfer molecular weight markers were measured before immunoblotting.

5.2.4.2.3 Immunoblotting

Membranes were blocked using 3%Blotto for 1 hour before incubation with the relevant primary antibody for 1 hour. All antibodies were made up in 3% Blotto at the concentrations shown in Table 5.5.

Table 5.5 Concentrations of monoclonal antibodies used in immunoblotting.

Target protein	Antibody	Concentration
P-gp	Monoclonal antibody Anti P-glycoprotein F4	1:500
BCRP	Monoclonal antibody Anti BCRP clone BXP 21	1:100
MRP1	Monoclonal antibody SC773, (C20)	1:500
MRP2	Monoclonal antibody SC5770, (H17)	1:200

After 1 hour incubation the membranes were washed 3 times in Blotto prior to addition of secondary antibodies at a concentration of 1:1000. A further 1 hour incubation was followed by 3 washes in TBS/TWEEN before ECL detection. Record of protein expression was achieved by exposure of photographic film.

5.2.4.2.4 Immunoblotting with Beta Actin

To establish that any differences in expression were not due to differences in protein loading, all membranes were re-probed for beta actin. Membranes were again blocked for 1 hour with 3% Blotto and probed for beta actin using the method described above, with 1:1000 dilution of human beta actin as the primary antibody and a 1:1000 dilution of Sheep AntiMouse secondary antibody.

5.2.4.2.5 Densitometry

ImageJ image processing software version 1.44 (National Institutes of Health) was used to measure the arbitrary optical densitometry score of each band on scanned images of all Western blot films. The expression of each protein was then expressed relative to beta actin expression to give a final corrected expression for each protein in each placenta (arbitrary optical densitometry units).

5.3 Results

5.3.1 Demographics

Of the women eligible for recruitment within the timeframe 87% (n=81) agreed to participate in the study. One woman had a twin pregnancy and gave birth to a set of dizygotic twins.

Of the 81 women recruited:

- 3 women withdrew before giving birth
- 2 women terminated their pregnancy (1 social, 1 congenital abnormality)
- 1 baby had a major congenital abnormality (requiring transfer to another hospital)
- 11 babies' births were not notified to the researcher

After losses therefore there were data available for 64 pregnancies (65 babies).

The general demographics of ethnic origin, tobacco use, employment and age at recruitment are presented in Table 5.6.

Table 5.6 General demographics of those recruited to the study, (n=64). Mode of delivery is also shown.

		At recruitment n=81	%	Active participants n=64	%
Age	teenage	16	19.7	14	21.8
	20-29	43	53.0	30	46.8
	30- 39	21	25.9	19	29.6
	40+	1	1.2	1	1.5
Ethnic origin					
Black British		6	7.4	5	7.8
White British		61	75.3	47	73.4
East European		1	1.2	1	1.5
White British/ Black Caribbean		12	14.8	10	15.6
White British/ Black African		1	1.2	1	1.5
Employed		3		2	
Tobacco	yes	67	82.7	55	85.9
	no	2	2.4	2	3.1
	with cannabis	12	14.8	7	10.9
Mode of delivery n=65					
	forceps			2	3.0
	ventouse			3	4.6
	kiwi			2	3.0
	breech			1	1.5
planned caesarean section				2	3.0
emergency caesarean section				3	4.6
spontaneous vaginal cephalic delivery				52	80.0

5.3.2 Drug Use

At recruitment women were asked to identify their primary drug of choice and other drug usage, Table 5.7 shows the data collected by self-report from the women

Table 5.7 Categories of drugs which women identified as their primary drug of choice at recruitment.

Drug	Active participants
amphetamine	1
opiates *	21
cannabis	42

*this includes methadone, buprenorphine and heroin

The purpose of testing hair for drugs was to produce an accurate picture of fetal exposure and provide a detailed list for individuals. The kappa coefficient table in Chapter 3 of this thesis shows the combinations of drugs babies were exposed to. These are summarised in Table 5.8 below. The Kappa coefficient reported (0.732) indicates good agreement between reported and actual drug used.

Table 5.8 Summary of the combinations of drug use by women in the study (see Chapter 3 for details).

methadone	subutex (buprenorphine)
methadone, opiates, mental health	amphetamines
methadone, opiates	cannabis
methadone, cocaine, opiates, cannabis	cannabis, opiates
methadone, cannabis	cannabis, cocaine, mental health
methadone, opiates, cocaine	
methadone, opiates, cocaine, mental health	
methadone, opiates, cannabis	
methadone, benzodiazepines, opiates	

5.3.3 Perceived Stress Scale (PSS)

A total of 42 women completed the scale. Published norms for the PSS are presented for two age groups; 18-29 years and 30-44 years. As 3 of the participants were under 18 years of age they were excluded from the analysis (appendix 4). Scores ranged from 0-33 from a possible range of 0-44. Scores are viewed as a continuum with low scores representing a low level of perceived stress and higher scores a greater perception of stress. Table 5.9 shows data obtained for all women divided into appropriate age categories for comparison with the published norms supplied with the scale (Cohen 1994).

Table 5.9 Descriptive statistics for the 39 participants aged 18-44 who completed the stress scale separated by age for comparison to published norms.

age	18-29	30-44
participants		
n	26	13
mean	19.35	17.77
range	0-33	4-32
published norms		
mean	14.2	13.0
standard deviation	6.2	6.2

One sample t-tests were performed to determine whether the mean scores of the two age groups were statistically different from the published means (Cohen 1994). For the 18-29 age group significantly higher scores were observed ($p = 0.003$, 2-tailed). Whilst the 30-44 age group also showed trends towards higher stress levels this was not statistically significant ($p= 0.06$, 2-tailed). The data were then analysed by age and drug use to determine whether the differences from the published norms were influenced by the type of drug used (Figure 5.3).

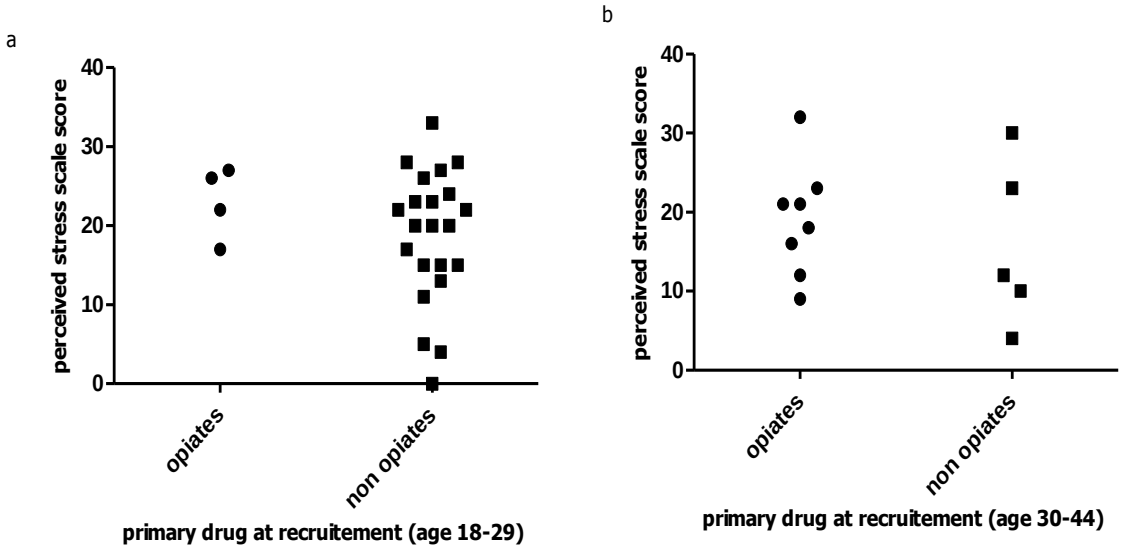


Figure 5.3 a and b Individual Perceived Stress Scale scores presented by age and primary drug at recruitment.

Again one sample t-tests were used to compare the sample means to the published means. In the 18-29 age group a significantly higher level of stress was found in both opiate and non-opiate users (opiate p=0.03 2-tailed, non-opiate p=0.02 2-tailed). In the older age group however, a significant increase in stress levels were found only in those women using opiates (p=0.04 2-tailed) and not in non-opiate i.e. cannabis users (p=0.58 2-tailed).

5.3.4 Perinatal Observations

5.3.4.1 Birth Weight and Customised Centiles

Birth weights of all 65 infants were obtained. These ranged from 1422- 3940 grams in the non-opiate category (cannabis and one amphetamine user in this case) and 1984-3850 grams in infants exposed to opiates. As shown in Figure 5.4 (a) a significant difference in birth weight between opiate and non-opiate exposed infants was observed (p= 0.03 Mann Whitney U) with mean birth weight being reduced by 8.3% in opiate compared to non-opiate exposed infants.

Customised centiles (n=48) ranged from 0-69 in the opiate exposed group and 0-89 in the cannabis exposed group. Overall 40 (83.3%) had a customised centile of less than 50 and 20 (41.66%) below 10. Customised centiles were analysed according to drug use using a Mann Whitney U test (Figure 5.4 b). A significant difference (p= 0.01 2-tailed)

was observed between the groups with the mean customised centile of the opiate exposed group being reduced by 58.07%.

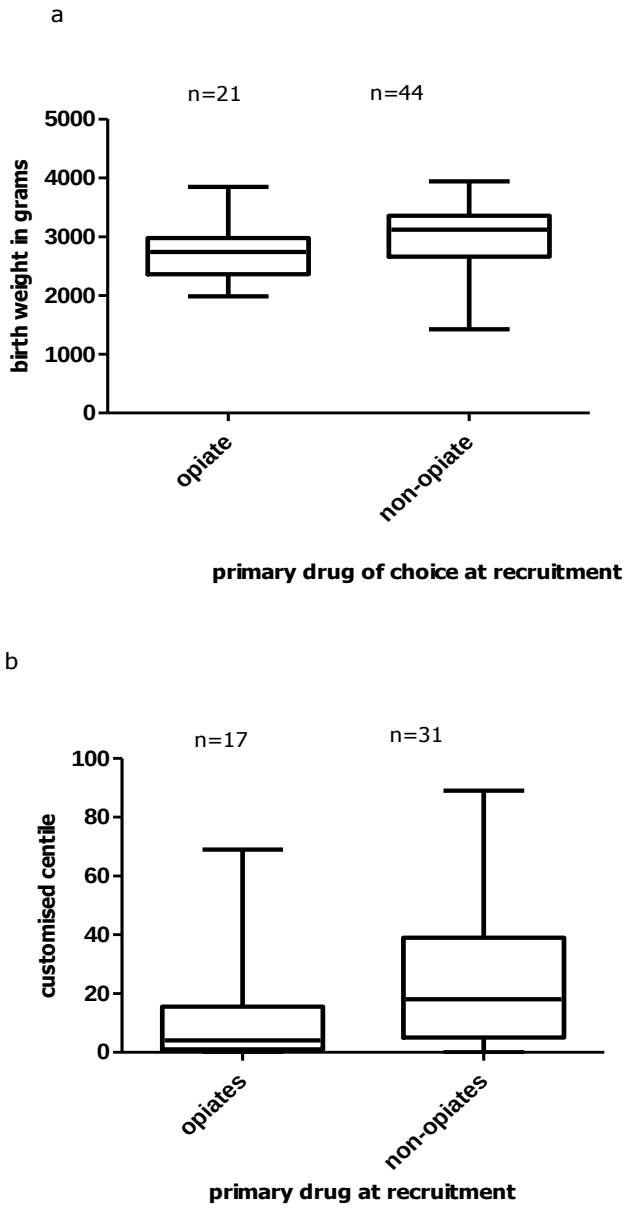


Figure 5.4 (a) Birth weights of infants exposed to opiates and those exposed to other drugs in utero, (the former is significantly lower p=0.03, Mann Whitney U, 2-tailed). (b) Customized centiles of infants exposed to opiates and those exposed to other drugs in utero (the former is significantly lower p=0.01 Mann Whitney U, 2-tailed).

5.3.4.2 OFC and Length

Data for OFC was obtained for 59 babies (21 for opiate use and 38 non–opiate use). Measurements ranged between 30.5-36cms and 29.5-36cms respectively. Length at birth

was obtained for 52 babies (20 opiate exposed and 31 non-opiates exposed) measurements ranged between 43-51.1cms and 41-54cms respectively.

No significant difference in OFC was noted between opiate and non-opiate exposed infants (p=0.41 Mann Whitney U, 2-tailed). However, a comparison of length at birth showed that opiate exposed infants were significantly shorter than non-opiate exposed infants (p=0.014 Mann Whitney U) with a 3.2% decrease in mean length (Figure 5.5). Overall, in comparison to the non-opiate group, the opiate exposed infants were shorter and lighter at birth but with no statistical difference in OFC. This is a small study and whilst confounders were considered it was not feasible to control for all. However, women who used alcohol were excluded from the study, and all the women smoked tobacco daily. Nutrition was enquired about but a specific scale was not used as it was out with the primary focus of the study.

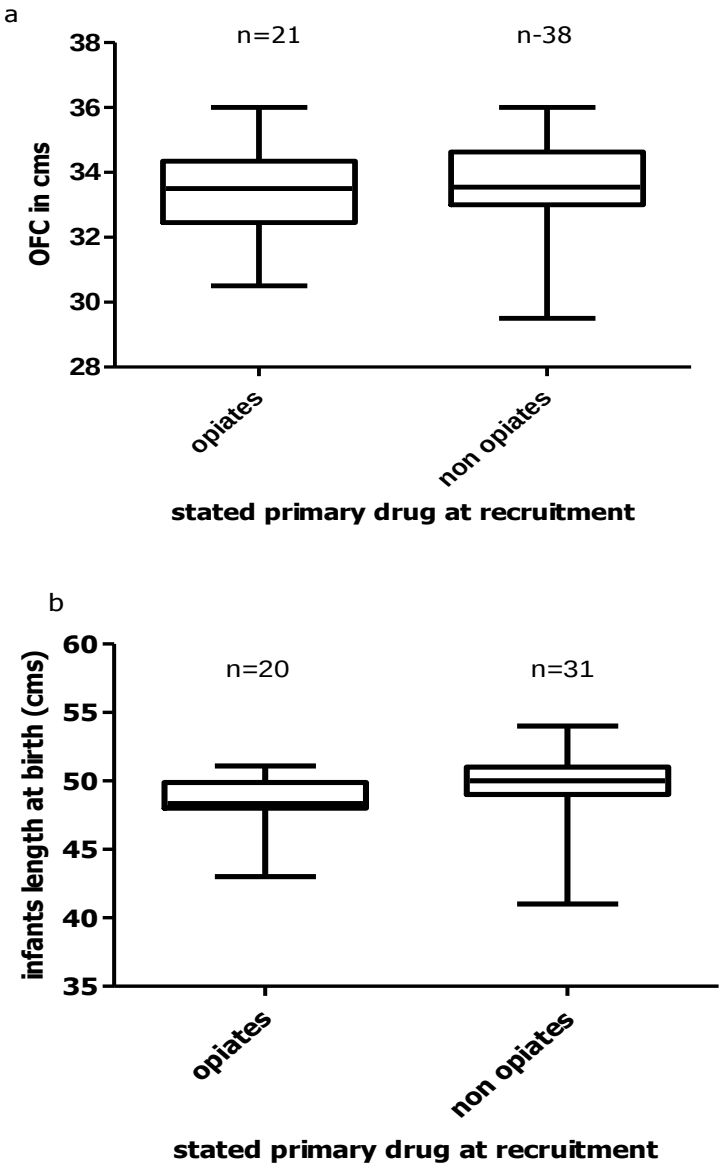


Figure 5.5. OFC (a) and length at birth (b) were compared by drug i.e. opiate exposed or non-opiate exposed. A statistical difference was noted for length (p= 0.014 Mann Whitney U) but not OFC (p=0.414 Mann Whitney U).

Correlating OFC to the same parameters of growth i.e. birth weight, customised centiles and length, showed significant correlations of all three for the non-opiate exposed group (p= 0.0001 birth weight, p= 0.02 customised centile and p=0.0002 length). This pattern did not hold true for opiate exposed infants. There was no statistically significant relationship between OFC and birth weight (p= 0.08) or customised centile (p=0.08) however, correlating OFC and length produced a significant p value of p=0.04.

5.3.4.3 Neonatal Observations

Observations of neonatal behaviours measured by the Finnegan scoring tool (appendix 5) were collected for 65 babies. Table 5.10 shows the number of babies scoring on the Finnegan scale in opiate and non- opiate exposed groups.

Table 5.10 Numbers of babies showing signs of withdrawal i.e. that scored above zero on the modified Finnegan tool.

	Number scoring at 3 days	number scoring at 3 weeks
opiates	19	11
non-opiate	4*	0

*3 cannabis and 1 amphetamine exposed

Of the 21 babies exposed to opiates in utero, 19 had a score of 1 or more on the Finnegan scoring tool at 3 days. Of these 19, 6 required admission to a neonatal unit for treatment of NAS. All babies requiring treatment were treated with Oromorph. Table 5.11 gives Finnegan score at 3 days and 3 weeks and details of drug exposure for each baby requiring treatment.

Table 5.11 NAS scores and maternal drug use for babies requiring treatment for NAS (n=6). Three consecutive scores of 5 initiate the treatment protocol.

NAS score at 3 days	NAS score at 3 weeks	drugs exposed to
5	7	methadone, opiates
5	2	methadone, opiates, cocaine
5	3	benzodiazepines, cocaine, methadone, opiates
5	0	cocaine, methadone, opiates
5	3	cocaine, methadone, opiates
5	3	cocaine, methadone, opiates

As expected no baby whose mother reported cannabis as her primary drug at recruitment required treatment for NAS, however four babies scored on the Finnegan scale details of which are shown in Table 5.12.

Table 5.12 NAS scores and drugs of the non-opiate exposed babies (n=4).

NAS score at 3 days	NAS score at 3 weeks	drugs exposed to
1	0	cannabis and cocaine
1	0	amphetamine
1	0	cannabis
1	0	cannabis

5.3.4.3.1 Admission to the Neonatal Unit

A total of 11 babies were admitted to a neonatal unit. Of these 7 were exposed to opiates and were admitted either for treatment of NAS (n=6) or to observe signs of NAS (n=1). The other 4 infants had been exposed to cannabis and were admitted to observe after a traumatic birth following a placental abruption (n=1) or to receive treatment for infection (n=3). In addition 2 other cannabis exposed infants were readmitted to hospital in the first week of life with signs of an infection.

5.3.5 Infant Development

Infants underwent Griffiths assessment of development at 8 months (n= 41) and 12 months (n=15). The percentile scores are reported across all subscales (table 5.13 a-e) and are presented by category of primary drug at recruitment. As this study used the GMDS as a proxy measure for how well a particular geno/haplotype protected a fetus from drugs of misuse the scores of most interest are the total number with a percentile scores of less than fifty and those in the bottom or top 10% as they potentially show a greater or lesser vulnerability to drugs of misuse.

Table 5.13 a-e GMDS subscale scores at 8 and 12 months. Range of scores is presented together with numbers of infants and percentages of the sample who scored in the top or bottom 10 percentiles or below the 50th percentile.

a)

Locomotor						
8 month	range	≤10 (%)	(n)	≤50 (%)	(n)	≥ 90 (%)	(n)
opiate (n=15)	<1-92	20 .0	(3)	73.3	(11)	6.6	(1)
non-opiate (n=26)	1-92	20.0	(6)	57.6	(15)	3.8	(1)
12 months							
opiate (n=6)	<1-86	50	(3)	66.6	(4)	0	(0)
non-opiate (n=9)	1-83	44.44	(4)	55.5	(5)	0	(0)

b)

Personal-Social						
8 month	range	≤10 (%)	(n)	≤50 (%)	(n)	≥ 90 (%)	(n)
opiate (n=15)	4-77	13	(2)	66.6	(10)	0	(0)
non-opiate (n=26)	2-97	15.3	(4)	76.9	(20)	3.84	(1)
12 months							
opiate (n=6)	<1-62	16.66	(1)	66.6	(4)	0	(0)
non-opiate (n=9)	2-71	11.11	(1)	66.6	(6)	0	(0)

c)

Hearing and Language						
8 month	range	≤10 (%)	(n)	≤50 (%)	(n)	≥ 90 (%)	(n)
opiate (n=15)	38-93	0	0	33.3	(5)	6.66	(1)
non-opiate (n=26)	29-93	0	0	23.07	(6)	3.84	(1)
12 months							
opiate (n=6)	13-75	0	0	66.6	(4)	0	(0)
non-opiate (n=9)	6-75	11.11	(1)	33.3	(3)	0	(0)

d)

Eye-Hand Coordination						
8 month	range	≤10 (%)	(n)	≤50 (%)	(n)	≥ 90 (%)	(n)
opiate (n=15)	1-67	6.66	(1)	66.6	(10)	0	(0)
non-opiate (n=26)	2-87	11.53	(3)	34.61	(9)	0	(0)
12 months							
opiate (n=6)	5-75	33.3	(2)	83.3	(5)	0	(0)
non-opiate (n=9)	5-73	33.3	(3)	66.6	(6)	0	(0)

e)

Performance						
8 month	range	≤10 (%)	(n)	≤50 (%)	(n)	≥ 90 (%)	(n)
opiate (n=15)	2-90	13.33	(2)	66.6	(10)	6.66	(1)
non-opiate (n=26)	<1-90	11.53	(1)	61.5	(17)	3.84	(1)
12 months							
opiate (n=6)	1-42	33.3	(3)	100	(6)	0	(0)
non-opiate (n=9)	4-62	14.2	(1)	77.7	(7)	0	(0)

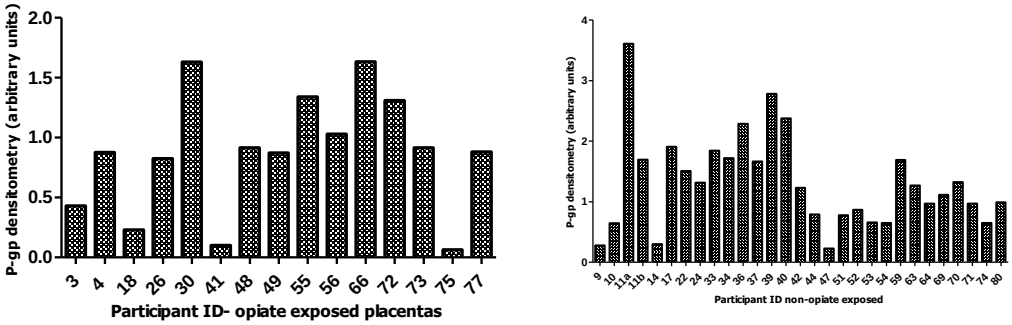
In the opiate exposed group tested at 8 months, 4 of the 5 subscales had more than 66% of the group scoring at, or under, the 50th percentile. The exception to this was Hearing and Language subscale (33%). The percentages of infants scoring at or under the 50th percentile in the non-opiate exposed group were more varied across the 5 subscales. In this group, Personal-Social (76%) had the highest percentage of infants scoring at or below the 50th percentile. The non-opiate exposed group also had the lowest score, Hearing and Language (23%).

Statistical analysis of GMDS Percentile subscale scores at 8 and 12 months, using a Mann Whitney U, did not demonstrate any significant differences between groups. At 8 months there was a trend toward significance (p= 0.08) in Eye-hand Coordination but this was not present at 12 months.

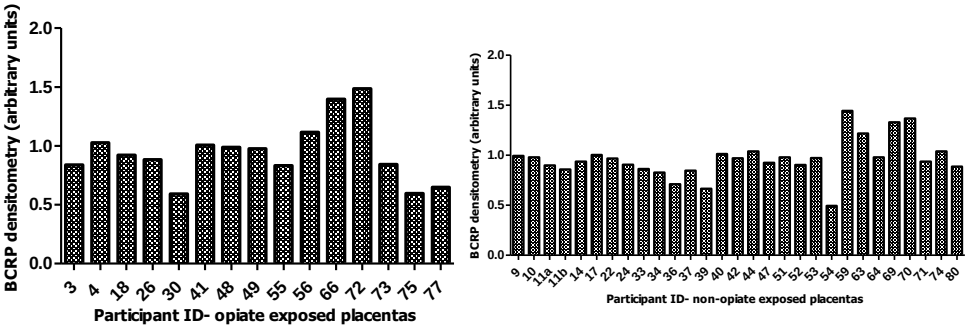
5.3.6 Transporter Protein Expression

There was considerable variation in expression between individual placentae with P-gp showing the widest range in densitometry score (Figure 5.6 a-d, Table 5.14). No significant differences in protein expression were observed between opiate and non-opiate exposed placentae. Beta actin was used as a housekeeping gene and the levels were constant. Sample Western Blot images for each of the proteins are shown in Figure 5.7.

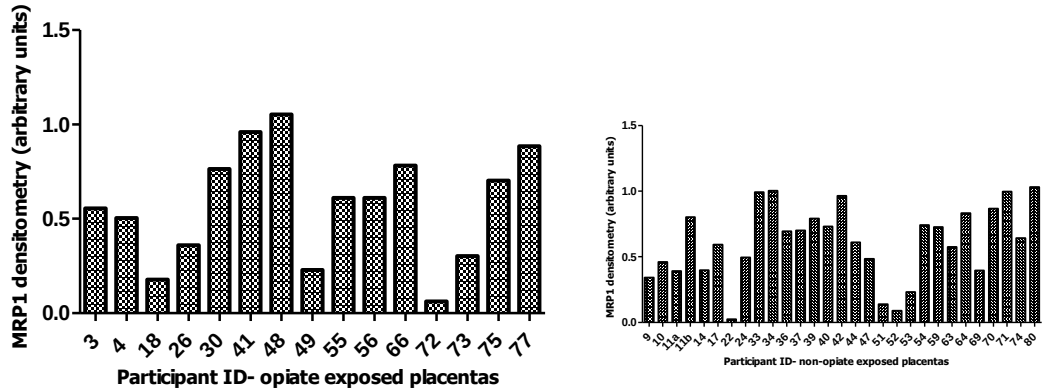
a) P-gp



b) BCRP



c) MRP1



d) MRP2

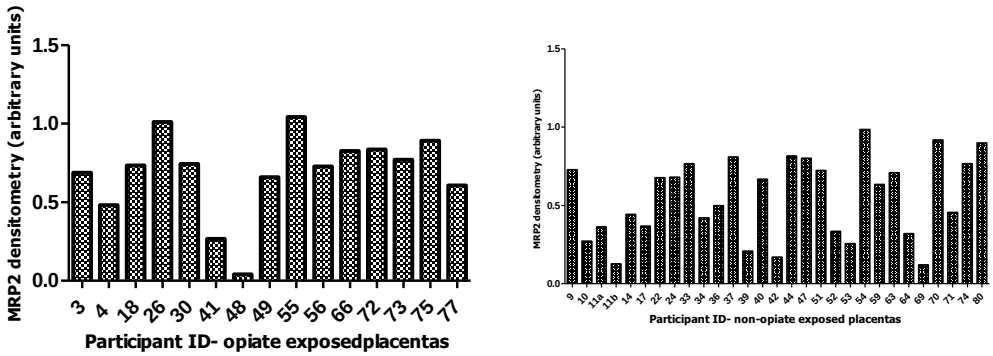


Figure 5.6 a-d Placental protein expression in individual placentae categorised by primary drug at recruitment and individual protein.

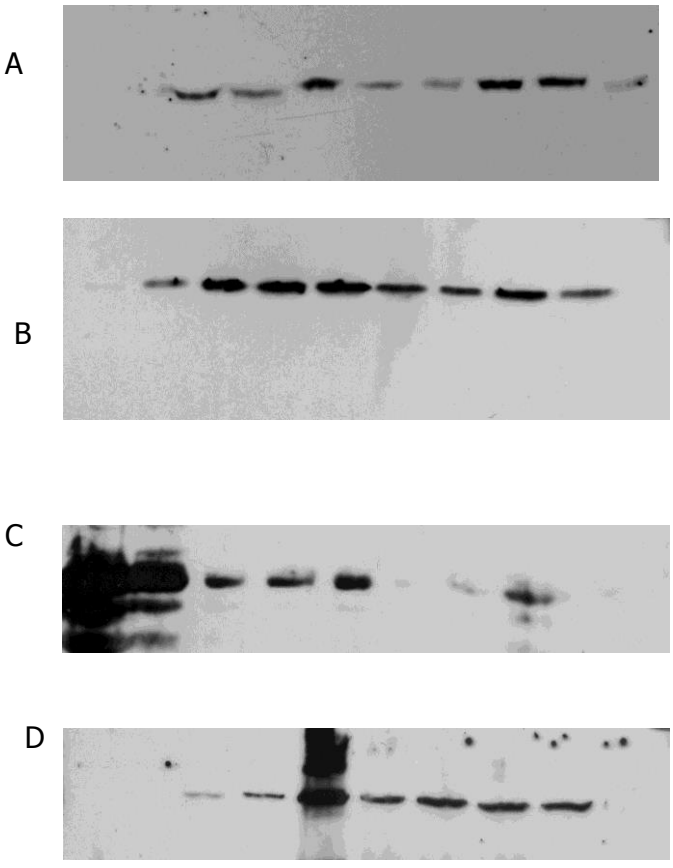


Figure 5.7 A-D Representative Western Blots for P-glycoprotein (A), BCRP (B), MRP1 (C) and MRP2 (D).

Table 5.14 Descriptive statistics of densitometry scores for P-gp, BCRP, MRP1 and MRP2. The range and mean are presented by groups as a whole and by primary drug.

Densitometry scores	P-gp	BCRP	MRP1	MRP2
range				
all	0.063-3.61	0.229-1.485	0.024-1.053	0.041-1.042
opiate	0.063-1.632	0.229-1.485	0.062-1.053	0.041-1.042
non opiate	0.225-3.61	0.494-1.442	0.024-1.032	0.120-0.985
mean				
opiate	0.869	0.966	0.611	0.549
non opiate	1.313	0.896	0.570	0.754

Densitometry scores for each protein were correlated to a measurement of difference between chronological age and overall age equivalent score generated by the GMDS. No significant correlations were obtained for the participants as a whole or by drug use.

5.3.7 Genotyping

Using an 80% quality control, comparing phenotype to genotype PLINK defined SNPs that correlated with a statistically significant change in protein expression (densitometry score) and/or a statistically different GMDS score within genotypes. There were 32 significant SNPs over the four genes shown in Table 5.15 a-d. The arrows within the columns indicate the direction of change in relation to the minor allele i.e. ↑ in the densitometry column indicates increased protein expression, in the GMDS subscales it indicates a higher percentile score relative to the scores with the minor allele.

Table 5.15 a-d SNPS significantly associated with differences in protein expression and/or GMDS scores (p values are shown for significant effects). The arrows within columns indicate the direction of change relative to that of the minor allele.

a)		GMDS subscales				
ABCB1 SNP	Densitometry	Locomotor	Personal-Social	Hearing & Language	Eye Hand Coordination	Performance
rs2032588	0.033 ↓	0.027 ↑			0.03 ↑	
rs17064	0.033 ↑	0.007 ↑			0.025 ↑	
rs2373587	0.04 ↑					
rs17149792		0.007 ↑			0.028 ↑	
rs1026499		0.019 ↓				
rs1055302		0.048 ↑			0.01 ↑	
rs1202171				0.028 ↑		
rs1202179				0.028 ↑		
rs1202182				0.028 ↑		
rs1989830				0.028 ↑		
rs2235015				0.04 ↑		
Total 11	3	5	0	5	4	0

b)		GMDS subscales				
ABCG2 SNP	Densitometry	Locomotor	Personal-Social	Hearing & Language	Eye Hand Coordination	Performance
rs2054576		0.044 ↓				
rs2231142		0.044 ↓				
rs2622610		0.049 ↑				
rs6857600				0.037 ↓		
Total 4	0	3	0	1	0	0

c)		GMDS subscales				
ABCC1 SNP	Densitometry	Locomotor	Personal-Social	Hearing & Language	Eye Hand Coordination	Performance
rs35597	0.03 ↓					
rs152022		0.032 ↑		0.049 ↑		
rs152023		0.049 ↑			0.046 ↑	
rs17205859		0.028 ↑				
rs2151052		0.03 ↑				
rs4781699		0.031 ↑				
rs35621				0.057 ↑	0.011 ↑	
rs35626				0.047 ↑	0.055 ↑	
rs2074085					0.011 ↑	
rs246233					0.029 ↓	
rs35593					0.003 ↑	
rs3851712					0.045 ↑	
rs4148358					0.021 ↑	
rs504348		0.004 ↑			0.018 ↑	0.002 ↑
rs3784862						0.057 ↑
rs875740						0.053 ↓
Total 16	1	6	0	3	9	3

d)		GMDS subscales				
ABCC2 SNP	Densitometry	Locomotor	Personal-Social	Hearing & Language	Eye Hand Coordination	Performance
rs2002042					0.033 ↓	0.033 ↓
Total 1	0	0	0	0	1	1

In summary, there were 32 SNPs identified over the four proteins which generated 45 statistically significant results over the densitometry and GMDS subscales. There were only 4 statistically significant alterations in densitometry scores, 3 for ABCB1 and 1 for ABCC1. Of these 2 reflected a decrease in protein expression and 2 an increase in expression. Where a SNP produced a statistically significant result in more than 1 GMDS subscale the direction of change is consistent with the notable exception of ABCB1 rs2032588 which showed a decrease in densitometry score but an increase in GMDS subscales Locomotor and Eye-Hand Coordination. No SNP showed a significant effect across all subscales. Locomotor and Eye-Hand Coordination both had 14 significant results, Hearing and Language 9, Performance 4 and Personal- Social 0. These effects are summarised in Table 5.16.

Table 5.16 Summary of the numbers generated in Table 5.15 a-d. Numbers of SNPs generating a significant correlation, percentage of SNPs identified, numbers of significant results generated by these SNPs and the direction of change for ABCB1, ABCG2, ABCC1 and ABCC2.

Gene	No of SNPS identified	No with significant correlations	%of SNPs showing significant correlations	No of statistically significant results	Direction of change- increased	Direction of change- decreased
ABCB1	51	11	21.5	17	15	2
ABCG2	26	4	15.38	4	1	3
ABCC1	79	6	20.25	22	19	3
ABCC2	17	1	5.88	2	0	2
Total	173	32	18.49	45	35	10

The pre-adoption medicals of two children reported developmental delay and 4 other children had clinical concerns raised about their development by healthcare professionals. All of these children had a GMDS subscale percentile score of less than 10 in at least one subscale (Table 5.17). The genotype for each SNP found to have a significant effect is presented for these children (Table 5.18 a-d). In addition the genotype is presented for opiate exposed infants treated for NAS, opiate exposed but not treated for NAS and cannabis exposed infants who showed signs severe enough to

score with the Finnegan NAS scoring tool (Table 5.19). There are no obvious differences between those infants that required treatment for NAS and those that did not.

Table 5.17 GMDS subscales (percentile scores) at 8 and 12 months of infants for whom concerns were expressed regarding development. ID 11a and 11b are the twins exposed to cannabis, ID 28 was exposed to amphetamine and the others were exposed to opiates. Infants 28 and 30 were raised in foster care, 28 was placed for adoption before the 12 month assessment.

ID	**Locomotor**		**Personal-Social**		**Hearing & Language**		**Eye Hand Coordination**		**Performance**	
	8	(12)	8	(12)	(8)	(12)	(8)	(12)	(8)	(12)
4	<1	(1)	65	(17)	38	(40)	1	(25)	2	(11)
11a	7	(<1)	16	(48)	48	(6)	29	(14)	19	(40)
11b	2	(<1)	9	(2)	65	(13)	3	(9)	<1	(4)
19	3	(4)	4	(11)	38	(21)	13	(5)	9	(3)
28	3		31		29		2		<1	
30	13	(<1)	31	(<1)	38	(13)	55	(9)	25	(1)

Tables 5.18 a-d Significant SNPs of the 6 children for whom clinical concerns were raised by healthcare professionals.

a) ABCB1

	Participant ID						
SNP ID	**4**	**11a**	**11b**	**19**	**28**	**30**	**Minor Allele**
rs17064	AA	AA	AA	AA	AA	AA	A
rs2032588	CC	CC	CC	CC	CC	CT	T
rs2373587	GG	*	*	GG	GG	*	G
rs17149792	GG	GA	GA	GG	GG	GG	G
rs10264990	AG	*	*	GG	AG	*	C
rs1055302	GG	GG	GG	GG	GG	GA	A
rs1202171	TT	*	*	TA	TT	TA	T
rs1202179	AA	AA	AA	AG	AA	AG	G
rs1202182	TT	*	*	CT	TT	CT	C
rs1989830	CC	*	*	TC	CC	TC	T
rs2235015	GG	GG	GG	GG	GG	GG	T

b) ABCG2

	Participant ID						
SNP ID	**4**	**11a**	**11b**	**19**	**28**	**30**	**Minor Allele**
rs2054576	TC	*	*	TC	TT	*	C
rs2231142	CA	CC	CC	CA	CC	CC	A
rs2622610	CC	CC	CC	CC	CC	CT	T
rs6857600	GG	GG	GG	GG	AG	GG	T

c) ABCC1

	Participant ID						
SNP ID	**4**	**11a**	**11b**	**19**	**28**	**30**	**Minor Allele**
rs35597	TC	TT	TT	CC	TT	TT	A
rs152022	CC	*	*	CG	CC	CC	C
rs152023	AA	AA	AA	GA	AA	GA	G
rs17205859	GG	GG	GG	GG	GG	GG	T
rs215105	AA	AA	AA	AA	AA	AA	G
rs4781699	CC	CC	CC	CA	CC	CC	T
rs35621	GG	GG	GG	GG	GG	GG	T
rs35626	CC	*	*	CA	CC	CC	T
rs2074085	TT	TT	TT	TT	TT	TT	G
rs246233	CC	AA	AA	CA	AA	AA	G
rs35593	AA	*	*	AA	AA	*	C
rs3851712	AA	*	*	GG	AA	*	A
rs4148358	GG	*	*	GG	GG	*	T
rs504348	CC	*	*	CC	CC	CC	C
rs3784862	CC	*	*	CT	TT	*	G
rs875740	GG	TT	TT	GG	TT	TT	G

d) ABCC2

	Participant ID						
SNP ID	**4**	**11a**	**11b**	**19**	**28**	**30**	**Minor Allele**
rs2002042	GA	GG	GG	GA	*	GG	T

Table 5.19 Genotyping results for the SNPs with a statistical significance. Infants are those that were exposed to cannabis and scored on the NAS chart or infants exposed to opiates that needed treatment for NAS and those that scored on the NAS chart but did not require treatment.

	cannabis (scored)				**opiate exposed-treated**						**opiate exposed (scored not treated) max score (in italics)**												
SNP	**ID**										*2*	*1*	*1*	*6*	*4*	*2*	*3*	*3*	*1*	*1*	*1*	*1*	*2*
ABCB1	**21**	**74**	**76**		**4**	**19**	**41**	**55**	**72**	**75**	**3**	**18**	**23**	**26**	**27**	**29**	**30**	**32**	**48**	**56**	**66**	**73**	**77**
rs17064	AA	AA	TA		AA	AA	AA	TA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	TA	AA	AA	AA
rs2032588	CC	CC	CC		CC	CC	CC	CT	CC	CT	CC	CC	CC	CC	CC	CC	CT	CC	CC	CT	CC	CC	CC
rs2373587	GC	*	*		GG	GG	*	*			GG			GG	GG	GC	*	*	*	*	*	*	*
rs17149792	GG	GG	GG		GG	GG	GG	GA	GG	GA	GG	GG	GG	GG	GG	*	GG	GG	GG	GG	GG	GG	GG
rs10264990	AA				AG	GG					AA	*	*	AA	AG	AA	*	*	*	*	*	*	*
rs1055302	GG	GA	GG		GG	GG	GG	GA	GG	GG	GG	GG	GG	GA	GG	GC	GA	GG	GG	GA	GG	GG	GG
rs1202171	TT	*	*		TT	TA	*	*	*	*	AA	*	*	TT	TT	TT	TA	*	*	*	*	*	*
rs1202179	AA	AA	AA		AA	AG	AA	AG	AA	GG	GG	AA	AA	AA	AA	AA	AG	AA	AG	AA	AG	AG	AG
rs1202182	TT	*	*		TT	CT	*	*	*	*	CC	*	*	TT	TT	TT	CT	*	*	*	*	*	*
rs1989830	CC	*	*		CC	TC	*	*	*	*	TT	*	*	CC	CC	CC	TC	*	*	*	*	*	*
rs2235015	GG	GG	GG		GG	GG	*	*	*	*	TT	GG	GG	GG	GG	GG	GG	GG	GG	GT	GT	GT	GG
ABCG2	**21**	**74**	**76**		**4**	**19**	**41**	**55**	**72**	**75**	**3**	**18**	**23**	**26**	**27**	**29**	**30**	**32**	**48**	**56**	**66**	**73**	**77**
rs2054576	TT	*	*		TC	TC	*	*	*	*	TT	*	*	TT	TT	TT	*	*	*	*	*	*	*
rs2231142	CC	CC	CC		CA	CA	CA	CC	CC	CC	CC	CC	CC	CC	CC	CC	CC	CA	CA	CC	CC	CA	CC
rs2622610	CC	CT	CT		CC	CC	CC	CC	CC	CT	CT	CT	CC	CC	CT	*	CT	CC	CC	TT	CC	CC	CC
rs6857600	GG	AA	GG		GG	GG	GG	AG	AG	GG	GG	GG	GG	GG	AG	GG	GG	AG	GG	AA	GG	GG	AG
ABCC1	**21**	**74**	**76**		**4**	**19**	**41**	**55**	**72**	**75**	**3**	**18**	**23**	**26**	**27**	**29**	**30**	**32**	**48**	**56**	**66**	**73**	**77**
rs35597	TC	TC	TT		TC	CC	CC	TC	TC	CC	CC	TT	TC	CC	TC	CC	TT	TC	TT	TC	TC	TC	TC

rs152022	CG	*	*		CC	CG	*	*	*	*	CG	*	*	CG	CC	CG	CC	*	*	*	*	*	*
rs152023	GA	GA	GA		AA	GA	AA	GA	GA	GG	GA	GA	AA	GA	AA	*	GA	AA	AA	GA	GA	FA	GA
rs17205859	GG	GG	GG		GG	GG	GG	GG	GG	GG	AG	GG	GG	GG	GG	AG	GG	GG	GG	GG	GG	GG	GG
rs215105	AA	AA	AA		AA	AA	AA	AA	AA	AA	AA	AG	AA	AA	AA	*	AA	AA	AA	AA	AA	AA	AA
rs4781699	CA	CC	CC		CC	CA	AA	CC	CA	CC	CA	CA	CC	CA	CA	AAA	CC	CC	CC	AA	CC	CA	CC
rs35621	GG	GG	GG		GG	GG	GG	GG	GGG	GA	GA	GG	GG	GG	GA	GA	GG	GG	GG	GG	GG	GG	GG
rs35626	CC	*	*		CC	CA	*	*	*	*	AA	*	*	CA	CA	AA	CC	*	*	*	*	*	*
rs2074085	TT	TT	TT		TT	TT	TT	TT	TT	CT	CT	TT	TT	TT	CT	CT	TT	TT	TT	TT	TT	TT	TT
rs246233	AA	AA	AA		CC	CA	AA	AA	CA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	AA	CA	AA
rs35593	AA	*	*		AA	AA	*	*	*	*	GA	*	*	AA	AA	GG	*	*	*	*	*	*	*
rs3851712	GA	*	*		AA	GG	*	*	*	*	GG	*	*	GG	GA	GG	*	*	*	*	*	*	*
rs4148358	GG	*	*		GG	GG	*	*	*	*	CC	*	*	CC	CC	TC	*	*	*	*	*	*	*
rs504348	CC	*	*		CC	CC	*	*	*	*	CC	*	*	CG	CG	GG	CC	*	*	*	*	*	*
rs3784862	CT	*	*		CC	CT	*	*	*	*	TT	*	*	CT	TT	CT	*	*	*	*	*	*	*
rs875740	GT	GG	TT		GG	GG	TT	TT	GT	GG	TT	GT	TT	GT	TT	*	TT	TT	TT	GT	GT	GT	TT
ABCC2	**21**	**74**	**76**		**4**	**19**	**41**	**55**	**72**	**75**	**3**	**18**	**23**	**26**	**27**	**29**	**30**	**32**	**48**	**56**	**66**	**73**	**77**
rs2002042	GG	GG	GG		GA	GA	AA	GA	GG	GG	GA	GG	GG	GG	GG	GA	GG	GA	GG	GA	GG	GA	GG

5.4 Discussion

5.4.1 Introduction

To recap, the hypothesis for this study is that polymorphisms in the multidrug resistance genes ABCB1, ABCC1, ABCC2 and ABCG2 change placental MDRP expression. This reduces the capacity of the placenta to efflux drugs of misuse leading to increased fetal exposure demonstrated by an increase in signs of NAS and/or altered developmental outcomes. Objectives were:
1 to determine if genotype influenced placental MDRP expression and
2 determine if there was a relationship between placental MDRP expression and neonatal and/or developmental outcomes.
As recruited numbers were too few for a fully powered test of hypothesis, this study is an initial test of concept and methodology. However the results have shown that for these participants, genotype has influenced placental protein expression and changes in measures of infant development.

5.4.2 Demographics

As the study hypothesis is that differences in outcome measures i.e. NAS and infant development relate to differences in genotype that alter protein expression, it was important that a thorough review of possible confounders was undertaken. The demographics, hair analysis, stress scales and basic perinatal observations sections of the report sought to undertake this function. Although there were losses to follow up after recruitment this did not unduly alter the general demographics.

Ethnicity has been shown to alter the frequency of SNPs occurring so the ethnicity of both parents was obtained where possible. When asked how they defined their ethnicity 73.4% responded White British, 17.1% mixed Black/White and 7.8% Black British.

Law *et al* (2003), suggest a withdrawal syndrome for tobacco with a dose response relation to the amount of maternal tobacco used. Kaltenbach *et al* (2012) found a relationship with tobacco use and NAS and Choo *et al* (2004) demonstrated a link with the level of maternal tobacco use and the intensity and timing of peak withdrawal in the neonate. Therefore the amount of daily tobacco use was collected for all participants. The majority of women (85.9%) smoked tobacco, at least 10 cigarettes daily in addition to other drug use. All infants for whom developmental concerns were raised and all infants who required treatment for NAS had mothers who smoked 20 or more cigarettes

daily. Choo *et al* (2004) showed delayed onset of NAS in women who smoked 20 or more cigarettes a day. This did not occur for infants in this study.

5.4.3 Drug Use

Cannabis, the mostly commonly used illicit drug in the UK (EMCDDA 2012) accounted for almost two thirds (65.6%) of those recruited to the study. Opiate use accounted for 32.8%, leaving 1 amphetamine user. There are limited data for this participant as the placenta was not available for collection. Chapter 3 covers the results of hair sample analysis in detail. In brief, results of the Cohens kappa (0.73) which compared reported to observed use, showed substantial agreement between what women reported and the hair sample observed.

5.4.4 Perceived Stress Scale (PSS)

A one sample t-test comparing the mean of the 18-29 age group to the published PSS mean (appendix 4) produced a highly significant result (p= 0.003) indicating that this group were significantly more stressed than the reference group. Using the same statistical test, but comparing drug use to the published mean in this age group, showed that both opiate and non-opiate users (cannabis) were significantly more stressed than the reference population (p=0.03 opiate and p=0.02 non-opiate). The mean stress score of the older age group (30-44years) was also greater than the reference group but this difference was not statistically significant (p=0.06). However, analysis of this age group by category of drug demonstrated a significantly higher mean for the opiate users (p=0.04) but not the non-opiate users (p=0.58). The PSS was developed using a cohort of 2387 adults. Given the rates of illicit drug use it is likely that a proportion of these will have been women using at least one drug. However these data are unknown. Had it been available it would have been interesting to compare PSS scores from pregnant women with and without additional stressors e.g. a long term health condition, to the scores obtained from these women.

There are many reasons that may account for the increased stress levels. As these were not directly related to the study hypothesis the reasons were not explored in depth so possible explanations are essentially speculative but based on clinical experience. However all women used illicit drugs and were at risk of criminal sanctions. All women were daily drug users and most were not in employment. Data on the amount of money spent on drugs was collected. The minimum daily spend was £10 which at £70 a week is a significant proportion of a state benefits allowance. The increase in stress seen in

opiate users, particularly in the older group may arise from specific issues related to opiate use. Their drug using history may result in negative social and financial consequences that impact upon lifestyle stability such as housing, debt and estrangement from family. There were no street homeless women in the study, all women were attempting to manage a "mainstream " lifestyle and accordingly had to develop skills that may have hitherto been undeveloped e.g. budgeting and attending appointments. The combination of different, often negative life experiences and the psychological demands of trying to meet expectations may affect their perception of stress.

Very often opiate using women in their thirties have been using opiates for a considerable time with many negative consequences including the removal of children from their care (NICE 2010, Scottish Executive 2003), guilt about potential damage drug use may cause (Shieh and Kravitz 2002) and input from a number of agencies (NICE 2010). High levels of stress in pregnancy have been associated with poor fetal outcomes including prematurity and low birth weight (Copper *et al* 1996, Rondo *et al* 2003) and developmental delay (Huizink *et al* 2003, Davis and Sandman 2010). This may impose additional stressors in the pregnancy and heighten their perception of stress and it is likely that at both ages the causes of increased stress are multifactorial.

There are also studies however that produced equivocal findings or no association with stress and negative fetal outcomes e.g. Lobel *et al* (2000). In these studies there were no trends with maternal stress and either fetal wellbeing or developmental delay which is consistent with these data. Huizink (2012) provides an interesting review of recent studies and some of the methodological problems that may account for these findings. (Helbig *et al* (2013) concluded that high levels of maternal stress were negatively correlated with feto-placental blood flow in the third trimester of pregnancy resulting in impaired fetal growth. This was not a measure in this study but may be of interest in future studies as placental blood flow may influence either the amount of drug that reaches the fetus and/or the length of time the fetus is exposed to it.

5.4.5 Perinatal Observations

5.4.5.1 Birth Weight

Drug use in pregnancy is reported to have many adverse pregnancy outcomes including a reduction in birth weight (Mulder *et al* 2002), which may affect development (Liu *et al* 2010), and was included in data collection because of this. In addition being small at

birth is associated with an increased risk of hypoglycaemia and hypothermia (Pallotto and Kilbride 2006) the signs of which include tremor and an inability to regulate temperature. Both signs would result in a positive score on the Finnegan scoring tool and therefore be assessed as exhibiting signs of NAS. This was a consideration when assessing neonatal wellbeing. Birth weight is a somewhat crude measure of fetal growth but as data on birth weight was obtained for all babies in the study it was used in addition to customised centiles for the infants for whom these data were possible to calculate. In their 1997 meta-analysis Hulse *et al* calculated mean birth weight to be reduced by 279g if exposed to methadone, 489g if exposed to heroin and 557g if exposed to both. Comparison between opiate and non-opiate exposure in this study showed a statistically significant difference between the groups (p=0.03 Mann Whitney 2-tailed) and an 8.3% reduction in mean birth weight in the opiate exposed group.

Customised centiles are a more sensitive measure of individual fetal growth as the algorithm adjusts for physiological factors that may influence it. Data unique to an individual pregnancy e.g. ethnic origin, parity, height and weight of the mother generate an optimal term weight that the fetus has the potential to obtain if not affected by some pathology such as maternal drug use or illness. When birth weight is added to the equation the software produces a number that represents the centile relative to the optimal birth weight for that pregnancy. Customised centiles were available for 48 infants and demonstrated compromised growth for the group as a whole with 41.66% having a customised centile of 10 or less and 83.3% with a score below 50. The mean customised centile of the opiate exposed infants was 58.07% less than the non-opiate exposed group who were themselves compromised by maternal drug use.

Babies can be symmetrically small i.e. small but in proportion or asymmetrically small where ratios between the OFC and other measurements of growth are disproportionate. Head failing is where the head is smaller than would be expected and head sparing occurs when for example, OFC is within normal range for birth weight but another physiological measurement such as length is shorter. In this study, a comparison of OFC by drug used did not show a statistically significant difference between groups however the infants in the opiate exposed group were significantly shorter, lighter and had lower centiles by comparison to the non-opiate exposed group. The overall pattern therefore, in the opiate exposed group is of lower birth weight, customised centiles and reduced length to maintain the OFC.

5.4.5.2 Neonatal Observations

5.4.5.2.1 Admission to the Neonatal Unit

All 6 babies admitted to a Neonatal Unit for pharmacological treatment of NAS were polydrug exposed but they were not the only babies in the study exposed to these combinations of drugs. Some infants exposed to the same combinations had mild NAS demonstrated by low scores of between 0 and 2. These data are consistent with the findings of the systematic review in Chapter 2 and supports the assertion that some infants are less susceptible to their mothers drug use.

5.4.6 Infant Development

5.4.6.1 Griffiths Mental Development Scales

Hindley (2006) compared the mean scores of males and females in his study and found they did not differ significantly from that of the larger group. Therefore, as neither the opiate nor non-opiate exposed groups were large, this study did not further subdivide by gender.

At 8 months, with the exception of Hearing and Language (33%) in the opiate exposed group all subscales had more than 66% of the group scoring at, or under, the 50th percentile suggesting a global deficit. The number of infants scoring at or under the 50th percentile in the non-opiate exposed group was more varied across the 5 subscales suggesting that cannabis use affects systems differently to opiates, or that the more global deficit demonstrated in the opiate exposed infant results from polydrug use.

McLean *et al* (1991) propose that as the Personal-Social subscale measures behaviours developed by adult-child interactions the child may be more reliant upon its primary carer to develop the skills necessary to pass the tasks in this subscale. If carers are unable to spend time interacting with the infant, have impaired facilities due to the effects of drug use, or have not learned this behaviour it may affect attainment on this subscale and account for the poor attainment in this study.

Hearing and Language produced the best scores for both opiate and non-opiate exposed infants with 33% and 23% of infants scoring less than the 50th percentile respectively. McLean *et al* (1991), suggest this scale measures early reactions to the sounds heard in the environment in addition to speech. This may explain higher attainment on this

subscale by opiate and non-opiate exposed, as infants would be as likely to experience external stimuli as infants not exposed to maternal drug use.

Bunikowski *et al* (1998) compared opiate and tobacco exposed infants to tobacco only exposed infants at 1 year of age and found opiate exposed infants had lower Locomotor and Performance GMDS scores. The data from this study reflects this pattern also with 73% of the opiate exposed infants scoring at the 50th centile or below compared to 57% of cannabis exposed infants. The Performance subscale had a less pronounced difference with scores of 66% and 61% respectively. Bunikowski *et al* (1998) also report that babies who were placed in foster care did not fare better than those placed within the parental home, and also that the infants of women who were polydrug users and not in treatment services fared worse than those on a methadone programme. In this study there were 6 infants where development was a concern. Two of these (ID 28 and 30) were raised in foster care and both were diagnosed with developmental delay at their pre-adoption medicals.

In this study it was not possible to compare the outcomes of infants whose mothers were in treatment with those who were not as the mothers of infants exposed to opiates were in a treatment programme and all infants, with the exception of one whose mother remained illicit drug free, were exposed to polydrug use. The internal GMDS calculations have accounted for social class, gender and other possible considerations that may affect scores. However there remains the possibility that parenting styles, having a partner or other supporter who does not use drugs, attending a nursery or having siblings may affect the rate or pattern of development. Greater exploration of specific factors that may impact upon child development, other than a genetic predisposition, in this group of children would be beneficial but require a longitudinal study of several years with a large cohort of families.

5.4.7 Transporter Protein Expression

Hitzl *et al* (2004) demonstrated an 8 fold variation in expression of P-gp in human placentae which was not normally distributed. Placentae examined in this study also demonstrated a wide variation in densitometry scores by individual protein and within individual placentae as shown in Figure 5.6 a-d. Presuming a change in protein expression is related to a change in activity, this suggests differential capacity to efflux compounds by different placentae. These data are in agreement with the data in Chapter 4 in which placentae unexposed to substances of misuse showed a 24 fold variation in

baseline efflux activity for P-gp and 3.4 fold variation for BCRP. Atkinson *et al* (2009) demonstrated varied expression across proteins and within individual placentae with an 8 fold difference in expression for P-gp, equating to a 70 fold variation in efflux and a 4 fold variation in BCRP expression.

5.4.8 Genotyping

Genetic variations in the ABCB1 gene have been the most extensively studied. Hoffmeyer *et al* (2000), identified 15 SNPs one of which rs1045642 (C3435T), a synonymous mutation in exon 26 was associated with a 2 fold decrease in protein expression in those individuals homozygous for the T allele. This SNP is the most commonly studied but contradictory effects of the T allele have been reported. These are elegantly summarised by Lepper *et al* (2005). This SNP is found in a non-coding region of the ABCB1 gene therefore the mechanism by which it influences P-gp expression is not clear. A discussion of potential mechanisms is presented by Schwab *et al* (2003) and includes linkage of the rs1045642 SNP to other SNPs in the gene. Several studies have shown linkage disequilibrium between this SNP and other SNPs in the ABCB1 gene. These include a synonymous SNP in exon 12 rs1128503 (C1236T) and a non-synonymous SNP in exon 21 rs203582 (G2677T/A). As a result of these associations several groups have performed haplotype analysis: Kroetz *et al* (2003) described 48 SNPs in the ABCB1 gene and have shown significant linkage disequilibrium across the entire gene. This group described 64 statistically inferred haplotypes of which 14 have the rs1045642 (C3435T) allele. Tang *et al* (2002) reported the first haplotype profiling using computational algorithms. Their data indicated strong linkage disequilibrium between the rs1045642 SNP and an unobserved causal SNP. Wang *et al* (2005) showed that in the rs1045642 SNP the C allele resulted in higher levels of mRNA as a result of increasing RNA stability. This suggests one mechanism by which the silent polymorphism could have its effect.

The number of SNPs reported in the ABCB1 gene continues to increase and a useful summary is provided by Lepper *et al* (2005). However, few studies have studied the effect of polymorphisms on placental P-gp expression. Tanabe *et al* (2001) reported reduced protein expression in individuals expressing the G2677(A,T) and /or T-129C alleles whilst Hitzl *et al* (2004) report reduced expression of placental P-gp in individuals homozygous for the rs1045642 T and rs203582 T alleles. However, neither group looked at function in placenta.

In contrast to the large number of investigations into polymorphisms in the ABCB1 gene relatively few studies of ABCG2, ABCC1 and ABCC2 have been reported. Lepper *et al* (2005) provides a comprehensive list of the 43 most common ABCG2 SNPs. Of these the rs2231142 (C421A) variant in exon 5, results in decreased BCRP expression and also reduced ATPase activity of the mutant protein whilst the rs2231137 (G34A) SNP in exon 2 results in poor localization of the protein and a decrease in efflux activity. A third less common SNP, the rs72552713 (C376T) polymorphism in exon 4 substitutes a stop codon for Gln-126 resulting in no BCRP expression. Kobayashi *et al* (2005) demonstrated significantly lower BCRP expression in individuals homozygous for the rs2231142 A allele by comparison to C allele. Few reports of functional effects of MRP1 and MRP2 polymorphisms are available; however Meyer zu Schwabedissen *et al* (2005) report that rs2273697 (G1249A) resulted in a significantly reduced expression of MRP2 mRNA in preterm placentae.

In this study statistical analysis by PLINK produced 32 significant SNPs across the 4 genes. Two of these correspond to the most commonly discussed in the literature; rs2231142 (C421A) on ABCG2 and rs504348 (G260C) on ABCC1.

The rs2231442 SNP has been associated with reduced placental expression of BCRP in human placentae (Kolwankar *et al* 2005) and reduced ATPase activity (Kobayashi *et al* 2005). In this study the ABCG2 SNP rs2231142 was not associated with a statistically significant reduction protein expression but there was a significant reduction in the Locomotor subscale of the GMDS ($p=0.04$). Kobayashi *et al* (2005) examined this SNP in human placenta and found differences in expression dependent upon genotype. Homozygous AA had the lowest expression, homozygous CC the highest and the heterozygous an intermediate level. Investigation of functional activity of this SNP (Morisaki *et al* 2005, Mizuarai *et al* 2004) showed altered transport activity. The influence of this SNP on plasma uric acid levels (Yamagishi *et al* 2010, Wang *et al* 2009, Woodward *et al* 2011) has shown increased levels in heterozygotes and homozygous for the risk allele suggesting reduced functioning of the protein. This fits with this study's hypothesis as reduced functioning of placental BCRP would increase the amount of drug the fetus is exposed to resulting in a lower Locomotor subscale score. Gao *et al* (2008), propose regulation of uric acid from the placenta is vital to fetal development, demonstrating a correlation with reduced birth weight and high levels of uric acid in amniotic fluid. Therefore polymorphisms in the rs2231142 SNP could alter development in this manner also.

In their study examining SNPs and chronic obstructive airways disease (COPD) Budulac *et al* (2010), demonstrated the polymorphic qualities of ABCC1 SNPs rs504348 and rs4781699 and their link with reduced airway wall inflammation. However only rs4781699 was associated with a change in protein expression, with lower MRP1 protein expression in the biopsies of COPD patients. Wang *et al* (2005 and 2009) using an MRP1 promoter assay propose that the major G allele lowers promoter activity when compared to the C allele which would suggest the potential for functional change dependent on genotype. Mafficini *et al* (2011), examined rs504348 in a cohort of cystic fibrosis suffers and showed genotype influenced disease progression with CC (4%) patients faring worse than CG (39%) or GG (57%).

In this study the ABCC1 SNP rs504348 (G260C) produced highly significant increased performance scores on 3 GMDS subscales; Locomotor (p=0.004), Eye-Hand Coordination (p=0.018) and Performance (p=0.002) suggesting that it interacted with expression and/or function whilst rs4781699 had a significant p value (p=0.031) on the Locomotor subscale. Additionally there are 5 ABCB1 SNPs that correspond with data by Atkinson *et al* (2005) who examined polymorphic ABCB1 SNPs in Jar cells. SNPs that demonstrate change across both the Atkinson *et al* study and this study are; rs2373587 which in this study was associated with an increase in protein expression (p=0.04) and 4 SNPs associated with statistically significant increases in GMDS subscales as follows; rs17149792 Locomotor (p=0.007) and Eye-Hand Coordination (p=0.028), rs1989830 Hearing and Language (p=0.028), rs1202182 Hearing and Language (p=0.028) and rs1202179 Hearing and Language (p=0.028).

Participants 11a and 11b are dizygotic twins with different levels of protein expression for P-gp and different developmental outcomes that agree with levels of expression i.e. the twin with most protein fared better. However, for the SNPs studied here the genotype was identical. This hints at the possibility that other SNPs are important for human development.

In summary, whilst these data do not show a clear relationship between MDRP expression and neonatal/developmental outcomes, fetal genotype did influence expression of P-gp and MRP1 and was also associated with significant changes in 4 of the 5 GMDS subscales. This and other themes are discussed further in Chapter 6.

Chapter Six

Overall Discussion

6.1 Introduction

Although the hypothesis of this study relates to the effects of polymorphisms on placental multidrug resistance protein expression and its resulting effects on NAS and infant development there were many points of interest that arose, particularly with the way women embraced and participated in the study. Adverse neonatal outcomes are often associated with maternal drug use in pregnancy e.g. reduced OFC and birth weight, and these were seen within these data, but there are also interesting differences in patterns of fetal growth and infant development dependent upon primary drug of choice and, although the study numbers are small, for reasons discussed later in the chapter, individual SNPs did affect placental protein expression and developmental outcomes and many other research questions have been raised.

6.2 Participants

When designing the study the numbers of referrals to Manchester Specialist Midwifery Service (MSMS) were reviewed to determine if there would be adequate numbers to power the study. In the period April 2007-March 2008, 150 of the 188 women referred to MSMS (79.8%) used drugs daily but did not use alcohol. From this figure it was calculated that it should be possible to recruit the necessary 200 women to adequately power the study. However, the national reduction in drug use in 2008-9 and 2009-10 shown in the 2011 to 2012 Crime Survey for England and Wales (Home Office 2012), was reflected in fewer referrals to the Manchester Specialist Midwifery Service (MSMS). Within those referrals there was also a 19.8% increase in concomitant alcohol use in those who were referred which excluded them from the study. This combination of fewer referrals and an increase in alcohol use prevented recruitment of the required number of participants to adequately power the study. Nevertheless as an initial test of both concept and methodologies I have been able to make useful observations to support future work as well as making some findings that showed trends supporting the hypothesis.

Women in this client group are often described as hard to reach but an 87% recruitment rate and a low dropout rate demonstrates that when approached women are keen to participate and remain engaged for a considerable time, which for some women was almost 18 months. It should be noted that women did not receive any financial remuneration for their participation and had to make themself available to the researcher on at least 5 occasions in the study. Also women with a child that went to foster care who could have been lost to follow up gave permission for their baby to be assessed at

the foster carers. The biggest losses were in the initial stages of the study before midwives were au fait with what they could do to support collection of samples and did not inform the researcher when women were in labour.

6.2.1 Observations and Collection of Samples

In addition to losses due to not being informed when women were admitted to give birth there were times when hospitals were closed to admissions and women were directed to hospitals not covered by the research ethics. This limited the samples that could be taken and contributed to the differences in sample sizes for various components of the data set.

To allow time to process placental and DNA samples, a decision was made to collect tissues and assess infants until February 2011. This limited the number of babies eligible to be assessed by GMDS at 8 months to 45. Ninety-one per cent of these infants (41) were assessed at 8 months. Of the 4 that were not, 2 families had moved away from Manchester and had changed contact details and the researcher did not gain access to two homes. There were 19 babies eligible to be assessed at 12 months, of these 15 were assessed. Again this demonstrates that research with this client group is a viable option.

6.3 Stress

Stress scores in this group of women were statistically higher than the published scale norms for the 18-29 year age group regardless of their drug of choice (appendix 4). However, in the 30-44 year age group this held true only for those who used opiates. This is an interesting finding but the study design precludes any real investigation as to why this may be so. However, there is scope for qualitative investigations to explore whether stress arose from drug use or drug use was used to alleviate stress and if or how drug of choice and ageing affects this. Comparison of perceived stress against biological measures of stress such as cortisol and relating to neonatal outcomes would also be interesting.

As stated in the discussion of Chapter 5, an examination of the effects of maternal stress on feto-placental blood flow by Helbig *et al* (2013) found high levels of maternal stress were negatively correlated with blood flow in the third trimester of pregnancy. This impacted on fetal wellbeing resulting in impaired fetal growth. This was not a measure in this study but may be of interest in future studies given the higher stress levels and

impaired fetal growth in this group. It may also contribute in part to the finding that the severity of NAS is unrelated to maternal opiate dose (Chapter 2) especially if this altered blood flow impacts on the ability of the multidrug resistance proteins to efflux drugs and their metabolites.

6.4 Hair samples

The purpose of testing maternal hair for drugs was to determine what drugs the fetus was exposed to in the last three months of gestation as the literature shows self-report leads to under reporting of drug use (Fendrich and Johnson 2005, Ostrea *et al*. 1992, Sanaullah *et al*. 2006, Grekin *et al*. 2011). Chapter 3 shows that in this group of women this was not generally the case, demonstrated by the Cohens Kappa of 0.73 and some of the discrepancies resulted from prescribed medication taken for pain relief rather than an attempt to hide additional drug use. The higher rate of accuracy in reporting may have been due to the nature of the study which was looking at developmental outcomes in children, or due to the fact that these were women who had already disclosed drug use. Of interest also was the fact that women chose to hide noncompliance with mental health medication due to concerns about lack of evidence on its effects on the fetus and anxiety about removal from services.

In conversations with women following receipt of the results some interesting explanations as to why there were differences in reported and observed use arose and these are summarised in Chapter 3. Although it is easy to find studies that show under reporting of drug use when self-report is used, usually household surveys or anonymous urine screening, there are few, if any, that go back to participants and speak about the results. Despite anxiety about judgements being identified as a concern for drug using pregnant women a decade ago (Scottish Executive 2003), Chapter 3 shows women still worry about judgements. There are few studies that explore professional's judgements, women's perception of judgements and how to improve situation. This study has shown that women are accepting of these types of discussions and this could be used to explore how to improve the service to drug using women. This suggests a series of interesting projects that could stand alone or work in conjunction to inform clinical practice. Study 1 an exploration of attitudes and judgements of health care providers and social workers. Study 2 an exploration of drug using women's experiences e.g. what they value in workers, what judgements they feel are made. Study 3 a prospective study that follows a cohort of drug using women and their health and social care providers to explore their joint experiences. Of particular interest would be to explore how women

feel and experience pregnancy as a drug using woman and if this affects how they experience and feel about parenting as a drug using woman. Data could identify training needs and inform a training programme for drug, health and/or social care providers. Data gathered could inform the development of a best practice model and a brief intervention programme that could be used by those who do not have extensive specialist knowledge of drug use but who work with pregnant drug using women and/or their families.

6.5 Perinatal Observations

The negative neonatal outcomes commonly associated with drug use in pregnancy were demonstrated in this study. Of particular interest is the different pattern of growth by drug of choice in this group of infants. Opiate exposed infants were shorter and lighter at birth than non-opiate exposed but without a statistical difference in OFC. The results of the systematic review in Chapter 2 (Thajam *et al* 2010) predicted that there would not be a relationship with severity of NAS and the maternal drug use and this was indeed the case. This supports the assertion that some babies are more vulnerable to the effects of their mothers drug use and that there may be a biological component to this effect.

6.6 Developmental Outcomes

Statistical analysis of GMDS Percentile subscale scores at 8 and 12 months did not demonstrate any significant differences between groups. At 8 months there was a trend toward significance ($p= 0.08$) in Eye-hand Coordination but this was not present at 12 months, suggesting that the effects of changes in placental protein function may not be observed until later in development when the complexity of skills required to pass GMDS tasks is increased. If this were the case a longer term prospective study to follow up of infants would be required to see changes resulting from different geno/haplotypes. Experiences in this study whereby women expressed the wish for this to happen would lead one to believe that this could be a viable study.

6.7 Genotyping

Comparing phenotype to genotype generated 32 statistically significant SNPs and 45 statistically significant changes in GMDS scores. Significant changes were noted for both protein expression and developmental outcome measure supporting the study's hypothesis.

6.8 Summary

In summary, although the number of participants recruited was too few to adequately power the study this was largely due to the reduction in number of referrals to MSMS and the increased use of alcohol. Future studies would have to factor this into calculations and either recruit over a longer time or use multiple sites as a large sample would be required to mitigate the effects of differences in placental multidrug resistance protein expression and the potential effects of drug use on their activity.

The high recruitment and low dropout rates show this group of women are accepting of a long term commitment to participation in research and may not be as hard to reach as often thought, but recruitment and sample collection were time consuming and would benefit from more than one person undertaking this task. This is particularly true of sample collection as in this study one person was on call throughout the study.

It was easy to obtain hair samples from the participants and the results from hair sample analysis provided interesting data. Exploring the themes found in this study could provide opportunities to support women to be more open about their drug use and provide data to support training for health and social care professionals around judgements, attitudes and beliefs about pregnant drug using women. This would require the introduction of planned qualitative data collection methods in future studies as suggested above.

The study hypothesis is that fetal genotype alters placental expression of P-gp, BCRP, MRP1 and MRP2 and this will be demonstrated by alterations in NAS and/or developmental outcomes. Although no clear haplotypes were identified individual SNPs were shown to significantly affect both placental protein expression and changes in measures of infant development and furthermore significant SNPs were identified that are not previously well defined in the literature.

The Griffiths Mental Development Scales were an appropriate tool that was easy to use in a community setting. In this study assessment took place at 8 and 12 months and delay was noted however there was a wide range of scores in the individual subscale so a larger sample would be useful. In addition, to capture subtle delays a longer term follow up would be beneficial.

The experiments in Chapter 4 provided data on the interaction of commonly misused drugs on placentae not exposed to drugs of abuse. It showed considerable variation in P-gp and BCRP expression and function which is concordant with work by Hiitzl *et al* (2004) and Atkinson *et al* (2009), and significant changes in ^{3}vinblastine and ^{3}mitoxantrone uptake when exposed to THC, buprenorphine and diazepam. This suggests the need for more work on the interaction of SNPs, placental protein expression and drugs of misuse and/or other commonly prescribed medications such as mental health medications which have been shown to result in negative fetal outcomes when taken by a proportion of the population exposed to them. A useful addition to extend this would be comparing the results obtained using placentae not exposed to drugs, as in this study, to results using placentae previously exposed to drugs. This would provide data as to whether chronic exposure affects the expression of placental multi drug resistance proteins. In addition exploration of the genotypes of mother infant dyads could lead to studies that explore potential correlations in haplotype in mother infant dyads and promote the development of interventions to provide women with accurate information on which to calculate relative risk of continuing with medication in pregnancy. Overall the results of this study indicate that it is a satisfactory test of concept and methodologies on which to base further studies.

Although these data are far too preliminary to base clinical decisions on genotype there is a hint of an association between genotype and clinical outcomes. Further multi-site studies looking at mental health medication and or illicit drug use to define a vulnerable geno/haplotype type could provide important data to inform this process. Ideally one would use a prospective study but as it is impractical to genotype a fetus in utero a retrospective study to identify potential susceptible haplotypes would have to be undertaken where babies are genotyped as in this study. A blinded design would be appropriate whereby one researcher would genotype the babies and another assess neonatal behaviours and developmental outcomes. The results would then be used to define a geno or haplotype that is predicative of an outcome. The prospective follow up study would genotype babies and follow up those with the geno/haplotype of interest in order to test the hypothesis that this is predictive of an outcome. This would require a longitudinal study of several years to fully appreciate any effect on the child. It would be important to build in qualitative elements to the study to fully explore some of the confounders.

References

Abrahams, R. R., S. A. Kelly, et al. (2007). "Rooming-in compared with standard care for newborns of mothers using methadone or heroin." Can Fam Physician **53**(10): 1722-30.

Ahmed, M. S., D. H. Zhou, et al. (1990). "Characterization of a cocaine binding protein in human placenta." Life Sci **46**(8): 553-61.

Altman, D. G. (2009). Practical statistics for medical research. London, Chapman and Hall.

Apgar, V. (1953). "A proposal for a new method of evaluation of the newborn infant." Curr Res Anesth Analg **32**(4): 260-7.

Atkinson, D. E., B. C. Baker, et al. (2009). "Pharmacogenetic of placental ABC transporters: Fetal protection from Xenobiotics " Reproductive Sciences **16**(3 (Supplement)): 156A.

Atkinson, D. E., R. D. H. Boyd, et al. (2006a). Placental Transfer. Knobil and Neill's Physiology of Reproduction. J. D. Neill. London, Elsevier**:** 2787-2846.

Atkinson, D. E., S. L. Greenwood, et al. (2003). "Role of MDR1 and MRP1 in trophoblast cells, elucidated using retroviral gene transfer." Am J Physiol Cell Physiol **285**(3): C584-91.

Atkinson, D. E., C. P. Sibley, et al. (2006b). "MDR1 P-gp expression and activity in intact human placental tissue; upregulation by retroviral transduction." Placenta **27**(6-7): 707-14.

Bailey-Dell, K. J., B. Hassel, et al. (2001). "Promoter characterization and genomic organization of the human breast cancer resistance protein (ATP-binding cassette transporter G2) gene." Biochim Biophys Acta **1520**(3): 234-41.

Bailey, J. A., K. G. Hill, et al. (2008). "Men's and women's patterns of substance use around pregnancy." Birth **35**(1): 50-9.

Bailey, J. R., H. C. Cunny, et al. (1987). "Fetal disposition of delta 9-tetrahydrocannabinol (THC) during late pregnancy in the rhesus monkey." Toxicol Appl Pharmacol **90**(2): 315-21.

Barbosa, J., J. Faria, et al. (2013). "Hair as an alternative matrix in bioanalysis." Bioanalysis **5**(8): 895-914.

Bargagli, A. M., M. Hickman, et al. (2006). "Drug-related mortality and its impact on adult mortality in eight European countries." Eur J Public Health **16**(2): 198-202.

Barker, D. J. (1990). "The fetal and infant origins of adult disease." BMJ **301**(6761): 1111.

Bakstad, B., M. Sarfi, et al. (2009). "Opioid maintenance treatment during pregnancy: occurrence and severity of neonatal abstinence syndrome. A national prospective study." Eur Addict Res **15**(3): 128-34.

Becker, J. and C. Duffy (2002). Women Drug Users and Drug Service Provision: service level responses to engagemnent and retention. Report for the Home Office Drugs Strategy Directorate. Report for the Home Office Drugs Strategy Directorate London, Home Office.

Beghin, D., J. L. Delongeas, et al. (2010). "Comparative effects of drugs on P-glycoprotein expression and activity using rat and human trophoblast models." Toxicol In Vitro **24**(2): 630-7.

Behravan, J. and M. Piquette-Miller (2007). "Drug transport across the placenta, role of the ABC drug efflux transporters." Expert Opin Drug Metab Toxicol **3**(6): 819-30.

Bell, J. and L. Harvey-Dodds (2008). "Pregnancy and injecting drug use." BMJ **336**(7656): 1303-5.

Benyamina, A., L. Bonhomme-Faivre, et al. (2009). "Association between ABCB1 C3435T polymorphism and increased risk of cannabis dependence." Prog Neuropsychopharmacol Biol Psychiatry.

Berghella, V., P. J. Lim, et al. (2003). "Maternal methadone dose and neonatal withdrawal." Am J Obstet Gynecol **189**(2): 312-7.

Blumenfeld, Y. J., M. F. Reynolds-May, et al. (2010) "Maternal-fetal and neonatal pharmacogenomics: a review of current literature." J Perinatol **30**(9): 571-9.

Bodo, A., E. Bakos, et al. (2003). "The role of multidrug transporters in drug availability, metabolism and toxicity." Toxicol Lett **140-141**: 133-43.

Bonhomme-Faivre, L., A. Benyamina, et al. (2008). "Disposition of Delta tetrahydrocannabinol in CF1 mice deficient in mdr1a P-glycoprotein." Addict Biol **13**(3-4): 295-300.

Borst, P., R. Evers, et al. (2000). "A family of drug transporters: the multidrug resistance-associated proteins." J Natl Cancer Inst **92**(16): 1295-302.

Boskovic, R., J. Klein, et al. (2001). "The role of the placenta in variability of fetal exposure to cocaine and cannabinoids: a twin study." Can J Physiol Pharmacol **79**(11): 942-5.

Boucher, N., A. Bairam, et al. (2008). "A new look at the neonate's clinical presentation after in utero exposure to antidepressants in late pregnancy." J Clin Psychopharmacol **28**(3): 334-9.

Bromley, R. L., G. Mawer, et al. (2010)."Early cognitive development in children born to women with epilepsy: a prospective report." Epilepsia **51**(10): 2058-65.

Buckley, V., A. Razaghi, et al. (2013)."Predictors of neonatal outcomes amongst a methadone- and/or heroin-dependent population referred to a multidisciplinary Perinatal and Family Drug Health Service." Aust N Z J Obstet Gynaecol.

Budulac, S. E., D. S. Postma, et al. (2010)."Multidrug resistance-associated protein-1 (MRP1) genetic variants, MRP1 protein levels and severity of COPD." Respir Res **11**: 60.

Bunikowski, R., I. Grimmer, et al. (1998). "Neurodevelopmental outcome after prenatal exposure to opiates." Eur J Pediatr **157**(9): 724-30.

Burns, L., R. Mattrick, et al. (2006). "Methadone in Pregnancy: treatment retention and neonatal outcomes." Addiction **102**: 264-270.

Burton, G. J., D. S. Charnock-Jones, et al. (2009). "Regulation of vascular growth and function in the human placenta." Reproduction **138**(6): 895-902.

Callaghan, R., E. Crowley, et al. (2008). "P-glycoprotein: so many ways to turn it on." J Clin Pharmacol **48**(3): 365-78.

Campolongo, P., V. Trezza, et al. (2009). "Developmental exposure to cannabinoids causes subtle and enduring neurofunctional alterations." Int Rev Neurobiol **85**: 117-33.

Campolongo, P., V. Trezza, et al. (2011). "Developmental consequences of perinatal cannabis exposure: behavioral and neuroendocrine effects in adult rodents." Psychopharmacology (Berl) **214**(1): 5-15.

Cascorbi, I. (2006). "Role of pharmacogenetics of ATP-binding cassette transporters in the pharmacokinetics of drugs." Pharmacol Ther **112**(2): 457-73.

CASP (2006) "Critical Appraisal Skill Program " retrieved from http:www.phru.ns.uk February 2009.

Ceckova-Novotna, M., P. Pavek, et al. (2006). "P-glycoprotein in the placenta: expression, localization, regulation and function." Reprod Toxicol **22**(3): 400-10.

CEMACH (2007). Saving Mothers' Lives; Reviewing maternal deaths to make motherhood safer - 2003-2005 The Seventh Report of the Confidential Enquiries into Maternal Deaths in the United Kingdom.

Chasnoff, I. J. (1989). "Cocaine and pregnancy--implications for the child." West J Med **150**(4): 456-8.

Chiang, Y. C., T. W. Hung, et al. (2013). "Development of sensitization to methamphetamine in offspring prenatally exposed to morphine, methadone and buprenorphine." Addict Biol.

Chiriboga, C. A., J. C. Brust, et al. (1999). "Dose-response effect of fetal cocaine exposure on newborn neurologic function." Pediatrics **103**(1): 79-85.

Chiriboga, C. A., L. Kuhn, et al. (2007). "Prenatal cocaine exposures and dose-related cocaine effects on infant tone and behavior." Neurotoxicol Teratol **29**(3): 323-30.

Choo, R. E., M. A. Huestis, et al. (2004). "Neonatal abstinence syndrome in methadone-exposed infants is altered by level of prenatal tobacco exposure." Drug Alcohol Depend **75**(3): 253-60.

Chung, F. S., S. Eyal, et al. (2010). "Positron emission tomography imaging of tissue P-glycoprotein activity during pregnancy in the non-human primate." Br J Pharmacol **159**(2): 394-404.

Cleary, B. J., J. Donnelly, et al. (2010). "Methadone dose and neonatal abstinence syndrome-systematic review and meta-analysis." Addiction **105**(12): 2071-84.

Cleary, B. J., J. M. Donnelly, et al. (2012). "Methadone and perinatal outcomes: a retrospective cohort study." Am J Obstet Gynecol **204**(2): 139 e1-9.

CMACE (2011). "Saving Mothers' Lives Reviewing Maternal Dealths to Make Motherhood Safer: 2006-2008 The Eighth Report of the Confidential Enquiries into Maternal Deaths in the United Kingdom." BJOG **118 Supplement 1**(March).

Cohen, J. (1968). "Weighted kappa: nominal scale agreement with provision for scaled disagreement or partial credit." Psychol Bull **70**(4): 213-20.

Cohen, S. (1994) "Perceived Stress Scale." Mind garden Inc. www.mindgarden.com

Cole, S. P., G. Bhardwaj, et al. (1992). "Overexpression of a transporter gene in a multidrug-resistant human lung cancer cell line." Science **258**(5088): 1650-4.

Coles, L. D., I. J. Lee, et al. (2008). "Distribution of saquinavir, methadone, and buprenorphine in maternal brain, placenta, and fetus during two different gestational stages of pregnancy in mice." J Pharm Sci.

Coller, J. K., D. T. Barratt, et al. (2006). "ABCB1 genetic variability and methadone dosage requirements in opioid-dependent individuals." Clin Pharmacol Ther **80**(6): 682-90.

Copper, R. L., R. L. Goldenberg, et al. (1996). "The preterm prediction study: maternal stress is associated with spontaneous preterm birth at less than thirty-five weeks' gestation. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network." Am J Obstet Gynecol **175**(5): 1286-92.

Cordon-Cardo, C., J. P. O'Brien, et al. (1990). "Expression of the multidrug resistance gene product (P-glycoprotein) in human normal and tumor tissues." J Histochem Cytochem **38**(9): 1277-87.

Covington, C. Y., B. Nordstrom-Klee, et al. (2002). "Birth to age 7 growth of children prenatally exposed to drugs: a prospective cohort study." Neurotoxicol Teratol **24**(4): 489-96.

Crettol, S., J. J. Deglon, et al. (2006). "ABCB1 and cytochrome P450 genotypes and phenotypes: influence on methadone plasma levels and response to treatment." Clin Pharmacol Ther **80**(6): 668-81.

Cygalova, L. H., J. Hofman, et al. (2009). "Transplacental pharmacokinetics of glyburide, rhodamine 123, and BODIPY FL prazosin: effect of drug efflux transporters and lipid solubility." J Pharmacol Exp Ther **331**(3): 1118-25

Dashe, J. S., J. S. Sheffield, et al. (2002). "Relationship between maternal methadone dosage and neonatal withdrawal." Obstet Gynecol **100**(6): 1244-9.

Davis, E. P. and C. A. Sandman (2010)."The timing of prenatal exposure to maternal cortisol and psychosocial stress is associated with human infant cognitive development." Child Dev **81**(1): 131-48.

Day, E. and S. George (2005). "Management of Drug Misuse in Pregnancy " Advances in Psychiatric Treatment **11**: 253-261.

De Bonis, M., M. Torricelli, et al. (2012)."Neuroendocrine aspects of placenta and pregnancy." Gynecol Endocrinol **28 Suppl 1**: 22-6.

de Moraes Barros, M. C., R. Guinsburg, et al. (2006). "Exposure to marijuana during pregnancy alters neurobehavior in the early neonatal period." J Pediatr **149**(6): 781-7.

Deeley, R. G. and S. P. Cole (2006). "Substrate recognition and transport by multidrug resistance protein 1 (ABCC1)." FEBS Lett **580**(4): 1103-11.

Department of Health (2011). A summary of the health harms of drugs, The Centre for Public Health, Faculty of Health & Applied Socai Science, Liverpool John Moore's University, on behalf of the Department of Health & National Treatment Agency for Substance Misuse.

DrugScope (2003). Research into the information and support needs of women drug users. London, DrugScope and the Home Office.

Dryden, C., D. Young, et al. (2009). "Maternal methadone use in pregnancy: factors associated with the development of neonatal abstinence syndrome and implications for healthcare resources." BJOG **116**(5): 665-71.

DuPont, R. L. and W. A. Baumgartner (1995). "Drug testing by urine and hair analysis: complementary features and scientific issues." Forensic Sci Int **70**(1-3): 63-76.

Dysart, K., H. C. Hsieh, et al. (2007). "Sequela of preterm versus term infants born to mothers on a methadone maintenance program: differential course of neonatal abstinence syndrome." J Perinat Med **35**(4): 344-6.

El-Mohandes, A., A. A. Herman, et al. (2003). "Prenatal care reduces the impact of illicit drug use on perinatal outcomes." J Perinatol **23**(5): 354-60

EMCDDA, (2010). European Monitoring Centre for Drugs and Drug Addiction. Annual Report 2010: the state of the drugs problem in Europe. Luxembourg.

EMCDDA, (2012). European Monitoring Centre for Drugs and Drug Addiction. Annual Report 2012: the state of the drugs problem in Europe. Luxembourg.

Enders, A. C. (2009). "Reasons for diversity of placental structure." Placenta **30 Suppl A**: S15-8.

Enders, A. C. and T. N. Blankenship (1999). "Comparative placental structure." Adv Drug Deliv Rev **38**(1): 3-15.

Evseenko, D., J. W. Paxton, et al. (2006). "Active transport across the human placenta: impact on drug efficacy and toxicity." Expert Opin Drug Metab Toxicol **2**(1): 51-69.

Evseenko, D. A., P. Murthi, et al. (2007). "The ABC transporter BCRP/ABCG2 is a placental survival factor, and its expression is reduced in idiopathic human fetal growth restriction." FASEB J **21**(13): 3592-605.

Eyler, F. D., M. Behnke, et al. (1998). "Birth outcome from a prospective, matched study of prenatal crack/cocaine use: I. Interactive and dose effects on health and growth." Pediatrics **101**(2): 229-37.

Fares, F. and M. Gavish (1986). "Characterization of peripheral benzodiazepine binding sites in human term placenta." Biochem Pharmacol **35**(2): 227-30.

Farid, W. O., S. A. Dunlop, et al. (2008). "The effects of maternally administered methadone, buprenorphine and naltrexone on offspring: review of human and animal data." Curr Neuropharmacol **6**(2): 125-50.

Fendrich, M. and T. P. Johnson (2005). "Race/ethnicity differences in the validity of self-reported drug use: results from a household survey." J Urban Health **82**(2 Suppl 3): iii67-81.

Ferrari, A., C. P. Coccia, et al. (2004). "Methadone--metabolism, pharmacokinetics and interactions." Pharmacol Res **50**(6): 551-9.

Ferreira, E., A. M. Carceller, et al. (2007). "Effects of selective serotonin reuptake inhibitors and venlafaxine during pregnancy in term and preterm neonates." Pediatrics **119**(1): 52-9.

Finnegan, L. P. (2004). "The challenges and continued dilemmas in the treatment and diagnosis of children prenatally exposed to opiates." Italian Journal of Pediatrics **30**(2): 73-76.

Finnegan, L. P., R. E. Kron, et al. (1975). "Assessment and treatment of abstinence in the infant of the drug-dependent mother." Int J Clin Pharmacol Biopharm **12**(1-2): 19-32.

Fischer, G., R. Ortner, et al. (2006). "Methadone versus buprenorphine in pregnant addicts: a double-blind, double-dummy comparison study." Addiction **101**(2): 275-81.

Fokina, V. M., S. L. Patrikeeva, et al. (2011)."Transplacental transfer and metabolism of buprenorphine in preterm human placenta." Am J Perinatol **28**(1): 25-32.

Fox, H. (1997). "Aging of the placenta." Arch Dis Child Fetal Neonatal Ed **77**(3): F171-5.

Frank, D. A., H. Bauchner, et al. (1990). "Neonatal body proportionality and body composition after in utero exposure to cocaine and marijuana." J Pediatr **117**(4): 622-6.

Fried, P. A. and J. E. Makin (1987). "Neonatal behavioural correlates of prenatal exposure to marihuana, cigarettes and alcohol in a low risk population." Neurotoxicol Teratol **9**(1): 1-7.

Fried, P. A., B. Watkinson, et al. (1987). "Neonatal neurological status in a low-risk population after prenatal exposure to cigarettes, marijuana, and alcohol." J Dev Behav Pediatr **8**(6): 318-26.

Fung, K. L. and M. M. Gottesman (2009). "A synonymous polymorphism in a common MDR1 (ABCB1) haplotype shapes protein function." Biochim Biophys Acta **1794**(5): 860-71.

Gao, T., N. R. Zablith, et al. (2008). "Second trimester amniotic fluid transferrin and uric acid predict infant birth outcomes." Prenat Diagn **28**(9): 810-4.

Garcia-Bournissen, F., M. Moller, et al. (2009). "Pharmacokinetics of disappearance of cocaine from hair after discontinuation of drug use." Forensic Sci Int **189**(1-3): 24-7.

Gedeon, C., G. Anger, et al. (2008). "Breast cancer resistance protein: mediating the trans-placental transfer of glyburide across the human placenta." Placenta **29**(1): 39-43.

Giaginis, C., S. Theocharis, et al. (2012)."Current toxicological aspects on drug and chemical transport and metabolism across the human placental barrier." Expert Opin Drug Metab Toxicol **8**(10): 1263-75.

Gil, S., R. Saura, et al. (2005). "P-glycoprotein expression of the human placenta during pregnancy." Placenta **26**(2-3): 268-70.

Giri, N., S. Agarwal, et al. (2009). "Substrate-dependent breast cancer resistance protein (Bcrp1/Abcg2)-mediated interactions: consideration of multiple binding sites in in vitro assay design." Drug Metab Dispos **37**(3): 560-70.

Goel, N., D. Beasley, et al. (2011). "Perinatal outcome of illicit substance use in pregnancy--comparative and contemporary socio-clinical profile in the UK." Eur J Pediatr **170**(2): 199-205.

Goler, N. C., M. A. Armstrong, et al. (2008). "Substance abuse treatment linked with prenatal visits improves perinatal outcomes: a new standard." J Perinatol **28**(9): 597-603.

Gottesman, M. M., I. Pastan, et al. (1996). "P-glycoprotein and multidrug resistance." Curr Opin Genet Dev **6**(5): 610-7

Greenwood, S. L. and C. P. Sibley (2006). "In vitro methods for studying human placental amino acid transport placental villous fragments." Methods Mol Med **122**: 253-64.

Grekin, E. R., D. S. Svikis, et al. (2011). "Drug use during pregnancy: validating the Drug Abuse Screening Test against physiological measures." Psychol Addict Behav **24**(4): 719-23.

Gude, N. M., C. T. Roberts, et al. (2004). "Growth and function of the normal human placenta." Thromb Res **114**(5-6): 397-407.

Gulati, A. and P. M. Gerk (2009). "Role of placental ATP-binding cassette (ABC) transporters in antiretroviral therapy during pregnancy." J Pharm Sci **98**(7): 2317-35.

Hagopian, G. S., H. M. Wolfe, et al. (1996). "Neonatal outcome following methadone exposure in utero." J Matern Fetal Med **5**(6): 348-54.

Hahnova-Cygalova, L., M. Ceckova, et al. (2011)."Fetoprotective activity of breast cancer resistance protein (BCRP, ABCG2): expression and function throughout pregnancy." Drug Metab Rev **43**(1): 53-68.

Hall, J. L. and E. R. van Teijlingen (2006). "A qualitative study of an integrated maternity, drugs and social care service for drug-using women." BMC Pregnancy Childbirth **6**: 19.

Hamilton, W. J. and J. D. Boyd (1960). "Development of the human placenta in the first three months of gestation." J Anat **94**: 297-328.

Hanson, R., J. A. Smith, et al. (1985). "Achievement of infants on items of the Griffiths scales: 1980 compared with 1950." Child Care Health Dev **11**(2): 91-104.

Harbison, R. D. and B. Mantilla-Plata (1972). "Prenatal toxicity, maternal distribution and placental transfer of tetrahydrocannabinol." J Pharmacol Exp Ther **180**(2): 446-53.

Harkey, M. R. (1993). "Anatomy and physiology of hair." Forensic Sci Int **63**(1-3): 9-18.

Hashemian, G., Dasse, A. (2010) " Isolation and Evaluation of genonic DNA from Buccal Swabs." Trinity College Dublin Journal of Pharmacy and Pharmaceutical Sciences **1**(1): 8-10.

Hassan, H. E., A. L. Myers, et al. (2009). "Differential involvement of P-glycoprotein (ABCB1) in permeability, tissue distribution, and antinociceptive activity of methadone, buprenorphine, and diprenorphine: In vitro and in vivo evaluation." J Pharm Sci.

Hassan, H. E., A. L. Myers, et al. (2007). "Oxycodone induces overexpression of P-glycoprotein (ABCB1) and affects paclitaxel's tissue distribution in Sprague Dawley rats." J Pharm Sci **96**(9): 2494-506.

Helbig, A., A. Kaasen, et al. (2013). "Does antenatal maternal psychological distress affect placental circulation in the third trimester?" PLoS One **8**(2): e57071.

Hemauer, S. J., S. L. Patrikeeva, et al. (2009). "Opiates inhibit paclitaxel uptake by P-glycoprotein in preparations of human placental inside-out vesicles." Biochem Pharmacol **78**(9): 1272-8.

Hieronymus, T. L., T. N. Nanovskaya, et al. (2006). "Methadone metabolism by early gestational age placentas." Am J Perinatol **23**(5): 287-94.

Higgins, C. F. and K. J. Linton (2004). "The ATP switch model for ABC transporters." Nat Struct Mol Biol **11**(10): 918-26.

Hitzl, M., E. Schaeffeler, et al. (2004). "Variable expression of P-glycoprotein in the human placenta and its association with mutations of the multidrug resistance 1 gene (MDR1, ABCB1)." Pharmacogenetics **14**(5): 309-18.

HM Government (2010). Drug Strategy 2010 Reducing Demand, Restricting Supply, Building Recovery: Supporting People to Live a Drug Free Life. London, HM Government.

Hoffmeyer, S., O. Burk, et al. (2000). "Functional polymorphisms of the human multidrug-resistance gene: multiple sequence variations and correlation of one allele with P-glycoprotein expression and activity in vivo." Proc Natl Acad Sci U S A **97**(7): 3473-8.

Holbrook, A. and K. Kaltenbach (2010). "Gender and NAS: does sex matter?" Drug Alcohol Depend **112**(1-2): 156-9.

Holcberg, G., M. Tsadkin-Tamir, et al. (2003). "New aspects in placental drug transfer." Isr Med Assoc J **5**(12): 873-6.

Holland, M. L., J. D. Allen, et al. (2008). "Interaction of plant cannabinoids with the multidrug transporter ABCC1 (MRP1)." Eur J Pharmacol **591**(1-3): 128-31.

Holland, M. L., D. T. Lau, et al. (2007). "The multidrug transporter ABCG2 (BCRP) is inhibited by plant-derived cannabinoids." Br J Pharmacol **152**(5): 815-24.

Holland, M. L., J. A. Panetta, et al. (2006). "The effects of cannabinoids on P-glycoprotein transport and expression in multidrug resistant cells." Biochem Pharmacol **71**(8): 1146-54.

Home Office (2012). Drug Misuse Declared: findings from the 2011/12 Crime Survey for England and Wales. London HM Government.

Home Office (2006). Measuring different aspects of problem drug use: methodological developments 16/06, Home Office Online Report.

Home Office (2007). Crime in England and Wales - British Crime Survey 2006/07. London, Home Office, Office of National Statistics.

Hsu, F. C., G. J. Zhang, et al. (2003). "Repeated neonatal handling with maternal separation permanently alters hippocampal GABAA receptors and behavioral stress responses." Proc Natl Acad Sci U S A **100**(21): 12213-8.

Huang, L., Y. Wang, et al. (2006). "ATP-dependent transport of rosuvastatin in membrane vesicles expressing breast cancer resistance protein." Drug Metab Dispos **34**(5): 738-42.

Huizink, A. C., P. G. Robles de Medina, et al. (2003). "Stress during pregnancy is associated with developmental outcome in infancy." J Child Psychol Psychiatry **44**(6): 810-8.

Huizink, A. C. (2012). "Prenatal substance use, prenatal stress and offspring behavioural outcomes: considerations for future studies." Nord J Psychiatry **66**(2): 115-22.

Hulse, G. K., E. Milne, et al. (1997). "Assessing the relationship between maternal cocaine use and abruptio placentae." Addiction **92**(11): 1547-51.

Huntley, M. (1996). The Griffiths Mental Development Scales from birth to 2 years. Manual. R. Griffiths. Oxford, The Test Agency. The 1996 Revision.

Huppertz, B. (2008). "The anatomy of the normal placenta." J Clin Pathol **61**(12): 1296-302.

Huppertz, B. and M. Borges (2008). "Placental trophoblast fusion." Methods Mol Biol **475**: 135-47.

Huppertz, B., G. Burton, et al. (2006). "Placental morphology: from molecule to mother -- a dedication to Peter Kaufmann -- a review." Placenta **27 Suppl A**: S3-8.

Huppertz, B., M. Gauster, et al. (2009). "Oxygen as modulator of trophoblast invasion." J Anat **215**(1): 14-20.

Jackson, M. R., T. M. Mayhew, et al. (1992). "Quantitative description of the elaboration and maturation of villi from 10 weeks of gestation to term." Placenta **13**(4): 357-70.

Jansson, L. M., J. Dipietro, et al. (2005). "Fetal response to maternal methadone administration." Am J Obstet Gynecol **193**(3 Pt 1): 611-7.

Jansson, L. M., J. A. Dipietro, et al. (2010). "Infant autonomic functioning and neonatal abstinence syndrome." Drug Alcohol Depend **109**(1-3): 198-204.

Jansson, L. M., J. A. Dipietro, et al. (2007). "Maternal vagal tone change in response to methadone is associated with neonatal abstinence syndrome severity in exposed neonates." J Matern Fetal Neonatal Med **20**(9): 677-85.

Jansson, L. M., J. A. Dipietro, et al. (2009). "Maternal methadone dosing schedule and fetal neurobehaviour." J Matern Fetal Neonatal Med **22**(1): 29-35.

Jansson, L. M., J. A. Dipietro, et al. (2010). "Fetal neurobehavioral effects of exposure to methadone or buprenorphine." Neurotoxicol Teratol **33**(2): 240-3.

Jansson, L. M. and M. Velez (2012). "Neonatal abstinence syndrome." Curr Opin Pediatr **24**(2): 252-8.

Jedlitschky, G., U. Hoffmann, et al. (2006). "Structure and function of the MRP2 (ABCC2) protein and its role in drug disposition." Expert Opin Drug Metab Toxicol **2**(3): 351-66.

Jemnitz, K., K. Heredi-Szabo, et al. (2010)."ABCC2/Abcc2: a multispecific transporter with dominant excretory functions." Drug Metab Rev **42**(3): 402-36.

John, R. and M. Hemberger (2012). "A placenta for life." Reprod Biomed Online **25**(1): 5-11.

Johnson, R. E., H. E. Jones, et al. (2003). "Use of buprenorphine in pregnancy: patient management and effects on the neonate." Drug Alcohol Depend **70**(2 Suppl): S87-S101.

Jonker, J. W., J. W. Smit, et al. (2000). "Role of breast cancer resistance protein in the bioavailability and fetal penetration of topotecan." J Natl Cancer Inst **92**(20): 1651-6.

Juliano, R. L. and V. Ling (1976). "A surface glycoprotein modulating drug permeability in Chinese hamster ovary cell mutants." Biochim Biophys Acta **455**(1): 152-62.

Kacinko, S. L., H. E. Jones, et al. (2008). "Correlations of maternal buprenorphine dose, buprenorphine, and metabolite concentrations in meconium with neonatal outcomes." Clin Pharmacol Ther **84**(5): 604-12.

Kahila, H., T. Saisto, et al. (2007). "A prospective study on buprenorphine use during pregnancy: effects on maternal and neonatal outcome." Acta Obstet Gynecol Scand **86**(2): 185-90.

Kalabis, G. M., S. Petropoulos, et al. (2007). "Breast cancer resistance protein (Bcrp1/Abcg2) in mouse placenta and yolk sac: ontogeny and its regulation by progesterone." Placenta **28**(10): 1073-81.

Kaltenbach, K., A. M. Holbrook, et al. (2012). "Predicting treatment for neonatal abstinence syndrome in infants born to women maintained on opioid agonist medication." Addiction **107 Suppl 1**: 45-52.

Kassim, Z. and A. Greenough (2005). "Effects of substance abuse during pregnancy." J R Soc Promot Health **125**(5): 212-4.

Keskitalo, J. E., O. Zolk, et al. (2009). "ABCG2 polymorphism markedly affects the pharmacokinetics of atorvastatin and rosuvastatin." Clin Pharmacol Ther **86**(2): 197-203.

Kimchi-Sarfaty, C., J. M. Oh, et al. (2007). "A "silent" polymorphism in the MDR1 gene changes substrate specificity." Science **315**(5811): 525-8.

Kintz, P., M. Villain, et al. (2006). "Hair analysis for drug detection." Ther Drug Monit **28**(3): 442-6.

Klinger, G., D. Frankenthal, et al. (2011). "Long-term outcome following selective serotonin reuptake inhibitor induced neonatal abstinence syndrome." J Perinatol **31**(9): 615-20.

Kobayashi, D., I. Ieiri, et al. (2005). "Functional assessment of ABCG2 (BCRP) gene polymorphisms to protein expression in human placenta." Drug Metab Dispos **33**(1): 94-101.

Kolwankar, D., D. D. Glover, et al. (2005). "Expression and function of ABCB1 and ABCG2 in human placental tissue." Drug Metab Dispos **33**(4): 524-9.

Kouimtsidis, C., Baldacchino, A (2003). An overview of service delivery for the pregnant drug user and dependent neonate. European Collaborating Centres in addiction Studies Monograph Series No3. M. R. a. J. C. Alex Baldacchino. London, European Collaborating Centres.

Kroetz, D. L., C. Pauli-Magnus, et al. (2003). "Sequence diversity and haplotype structure in the human ABCB1 (MDR1, multidrug resistance transporter) gene." Pharmacogenetics **13**(8): 481-94.

Kruh, G. D. and M. G. Belinsky (2003). "The MRP family of drug efflux pumps." Oncogene **22**(47): 7537-52.

Kuschel, C. (2007). "Managing drug withdrawal in the newborn infant." Semin Fetal Neonatal Med **12**(2): 127-33.

Kuschel, C. A., L. Austerberry, et al. (2004). "Can methadone concentrations predict the severity of withdrawal in infants at risk of neonatal abstinence syndrome?" Arch Dis Child Fetal Neonatal Ed **89**(5): F390-3.

Lacroix, I., A. Berrebi, et al. (2004). "Buprenorphine in pregnant opioid-dependent women: first results of a prospective study." Addiction **99**(2): 209-14.

Lankas, G. R., L. D. Wise, et al. (1998). "Placental P-glycoprotein deficiency enhances susceptibility to chemically induced birth defects in mice." Reprod Toxicol **12**(4): 457-63.

Law, K. L., L. R. Stroud, et al. (2003). "Smoking during pregnancy and newborn neurobehavior." Pediatrics **111**(6 Pt 1): 1318-23.

Lee, C. T., E. Lehrmann, et al. (2009). "Gene expression profiling reveals distinct cocaine-responsive genes in human fetal CNS cell types." J Addict Med **3**(4): 218-26.

Lejeune, C., L. Simmat-Durand, et al. (2006). "Prospective multicenter observational study of 260 infants born to 259 opiate-dependent mothers on methadone or high-dose buprenophine substitution." Drug Alcohol Depend **82**(3): 250-7.

Lepper, E. R., K. Nooter, et al. (2005). "Mechanisms of resistance to anticancer drugs: the role of the polymorphic ABC transporters ABCB1 and ABCG2." Pharmacogenomics **6**(2): 115-38.

Leschziner, G. D., T. Andrew, et al. (2007). "ABCB1 genotype and PGP expression, function and therapeutic drug response: a critical review and recommendations for future research." Pharmacogenomics J **7**(3): 154-79.

Leslie, E. M., R. G. Deeley, et al. (2005). "Multidrug resistance proteins: role of P-glycoprotein, MRP1, MRP2, and BCRP (ABCG2) in tissue defense." Toxicol Appl Pharmacol **204**(3): 216-37.

Levinson-Castiel, R., P. Merlob, et al. (2006). "Neonatal abstinence syndrome after in utero exposure to selective serotonin reuptake inhibitors in term infants." Arch Pediatr Adolesc Med **160**(2): 173-6.

Levran, O., K. O'Hara, et al. (2008). "ABCB1 (MDR1) genetic variants are associated with methadone doses required for effective treatment of heroin dependence." Hum Mol Genet **17**(14): 2219-27.

Lim, S., M. R. Prasad, et al. (2009). "High-dose methadone in pregnant women and its effect on duration of neonatal abstinence syndrome." Am J Obstet Gynecol.

Lin, T., O. Islam, et al. (2006). "ABC transporters, neural stem cells and neurogenesis--a different perspective." Cell Res **16**(11): 857-71.

Lingam, R. and J. Scott (2002). "Treatment non-adherence in affective disorders." Acta Psychiatr Scand **105**(3): 164-72.

Little, B. B. and T. T. VanBeveren (1996). "Placental transfer of selected substances of abuse." Semin Perinatol **20**(2): 147-53.

Liu, A. J., S. Sithamparanathan, et al. (2010)."Growth restriction in pregnancies of opioid-dependent mothers." Arch Dis Child Fetal Neonatal Ed **95**(4): F258-62.

Lloyd-Smith, E., E. Wood, et al. (2008). "Risk factors for developing a cutaneous injection-related infection among injection drug users: a cohort study." BMC Public Health **8**: 405.

Lobel, M., C. J. DeVincent, et al. (2000). "The impact of prenatal maternal stress and optimistic disposition on birth outcomes in medically high-risk women." Health Psychol **19**(6): 544-53.

Lugo, M. R. and F. J. Sharom (2005). "Interaction of LDS-751 and rhodamine 123 with P-glycoprotein: evidence for simultaneous binding of both drugs." Biochemistry **44**(42): 14020-9.

Luty, J., V. Nikolaou, et al. (2003). "Is opiate detoxification unsafe in pregnancy?" J Subst Abuse Treat **24**(4): 363-7.

Macrory, F. and S. C. Boyd (2007). "Developing primary and secondary services for drug and alcohol dependent mothers." Semin Fetal Neonatal Med **12**(2): 119-26.

Maeda, K. and Y. Sugiyama (2008). "Impact of genetic polymorphisms of transporters on the pharmacokinetic, pharmacodynamic and toxicological properties of anionic drugs." Drug Metab Pharmacokinet **23**(4): 223-35.

Mafficini, A., M. Ortombina, et al. (2011). "Impact of polymorphism of Multidrug Resistance-associated Protein 1 (ABCC1) gene on the severity of cystic fibrosis." J Cyst Fibros **10**(4): 228-33.

Malek, A., C. Obrist, et al. (2009). "The impact of cocaine and heroin on the placental transfer of methadone." Reprod Biol Endocrinol **7**: 61.

Maliepaard, M., G. L. Scheffer, et al. (2001). "Subcellular localization and distribution of the breast cancer resistance protein transporter in normal human tissues." Cancer Res **61**(8): 3458-64.

Maliepaard, M., M. A. van Gastelen, et al. (2001). "Circumvention of breast cancer resistance protein (BCRP)-mediated resistance to camptothecins in vitro using non-substrate drugs or the BCRP inhibitor GF120918." Clin Cancer Res **7**(4): 935-41.

Martinez-Selles, M., P. Munoz, et al. (2008). "Long-term outcome of infective endocarditis in non-intravenous drug users." Mayo Clin Proc **83**(11): 1213-7.

Mathias, A. A., J. Hitti, et al. (2005). "P-glycoprotein and breast cancer resistance protein expression in human placentae of various gestational ages." Am J Physiol Regul Integr Comp Physiol **289**(4): R963-9.

Matsunaga, T., E. Kose, et al. (2006). "Determination of p-glycoprotein ATPase activity using luciferase." Biol Pharm Bull **29**(3): 560-4.

May, K., V. Minarikova, et al (208). "Role of the multidrug transporter proteins ABCB1 and ABCC2 in the diaplacental transport of talinolol in the term human placenta." Drug Metab Dispos **36**(4): 740-4

McCarthy, J. J. (2012). "Intrauterine abstinence syndrome (IAS) during buprenorphine inductions and methadone tapers: Can we assure the safety of the fetus?" J Matern Fetal Neonatal Med **25**(2): 109-12.

McCarthy, J. J., M. H. Leamon, et al. (2005). "High-dose methadone maintenance in pregnancy: maternal and neonatal outcomes." American Journal of Obstetrics & Gynecology **193**(3 Pt 1): 606-10.

McLean, M.E., McCormick K., Baird, S. A. (1991). "Concurrent Validity of the Griffiths' Mental Development Scales with a Population of Children under 24 months." Journal of early Intervention **15**: 338.

McCarthy, J. J., M. H. Leamon, et al. (2008). "Outcomes of neonates conceived on methadone maintenance therapy." J Subst Abuse Treat **35**(2): 202-6.

Meyer Zu Schwabedissen, H. E., G. Jedlitschky,et al. (2005). "variable expression of MRP2 (ABCC2) in human placenta: influence of gestational age and cellular differentiation." Drug Metab Dispos **33** (7): 896-904.

Mizuarai, S., N. Aozasa, et al. (2004). "Single nucleotide polymorphisms result in impaired membrane localization and reduced atpase activity in multidrug transporter ABCG2." Int J Cancer **109**(2): 238-46.

Molnar, J., D. Szabo, et al. (2000). "Membrane associated antitumor effects of crocine-, ginsenoside- and cannabinoid derivates." Anticancer Res **20**(2A): 861-7.

Molsa, M., T. Heikkinen, et al. (2005). "Functional role of P-glycoprotein in the human blood-placental barrier." Clin Pharmacol Ther **78**(2): 123-31.

Morisaki, K., R. W. Robey, et al. (2005). "Single nucleotide polymorphisms modify the transporter activity of ABCG2." Cancer Chemother Pharmacol **56**(2): 161-72.

Mulder, E. J., P. G. Robles de Medina, et al. (2002). "Prenatal maternal stress: effects on pregnancy and the (unborn) child." Early Hum Dev **70**(1-2): 3-14.

Musshoff, F., F. Driever, et al. (2006). "Results of hair analyses for drugs of abuse and comparison with self-reports and urine tests." Forensic Sci Int **156**(2-3): 118-23.

Musshoff, F. and B. Madea (2006). "Review of biologic matrices (urine, blood, hair) as indicators of recent or ongoing cannabis use." Ther Drug Monit **28**(2): 155-63.

Myllynen, P., M. Kummu, et al. (2008). "ABCG2/BCRP decreases the transfer of a food-born chemical carcinogen, 2-amino-1-methyl-6-phenylimidazo[4,5-b]pyridine (PhIP) in perfused term human placenta." Toxicol Appl Pharmacol **232**(2): 210-7.

Myllynen, P., M. Kummu, et al. (2010). "ABCB1 and ABCG2 expression in the placenta and fetus: an interspecies comparison." Expert Opin Drug Metab Toxicol **6**(11): 1385-98.

Myllynen, P. and K. Vahakangas (2002). "An examination of whether human placental perfusion allows accurate prediction of placental drug transport: studies with diazepam." J Pharmacol Toxicol Methods **48**(3): 131-8

Northern and Yorkshire Regional Public Health Observatory. (2002). Drug Misuse in Pregnancy in the Northern and Yorkshire Region. Occasional Paper. Stockton on Tees, Wolfson Research Institute, University of Durham**:** 1-24.

Nagashige, M., F. Ushigome, et al. (2003). "Basal membrane localization of MRP1 in human placental trophoblast." Placenta **24**(10): 951-8.

Nanovskaya, T., S. Deshmukh, et al. (2002). "Transplacental transfer and metabolism of buprenorphine." J Pharmacol Exp Ther **300**(1): 26-33.

Nanovskaya, T., I. Nekhayeva, et al. (2005). "Role of P-glycoprotein in transplacental transfer of methadone." Biochem Pharmacol **69**(12): 1869-78.

Nanovskaya, T. N., R. S. Bowen, et al. (2009). "Effect of plasma proteins on buprenorphine transfer across dually perfused placental lobule." J Matern Fetal Neonatal Med **22**(8): 646-53.

Nanovskaya, T. N., S. V. Deshmukh, et al. (2004). "Methadone metabolism by human placenta." Biochem Pharmacol **68**(3): 583-91.

Nanovskaya, T. N., I. A. Nekhayeva, et al. (2008). "Transfer of methadone across the dually perfused preterm human placental lobule." Am J Obstet Gynecol **198**(1): 126 e1-4.

Nekhayeva, I. A., T. N. Nanovskaya, et al. (2005). "Bidirectional transfer of methadone across human placenta." Biochem Pharmacol **69**(1): 187-97.

Nekhayeva, I. A., T. N. Nanovskaya, et al. (2006). "Role of human placental efflux transporter P-glycoprotein in the transfer of buprenorphine, levo-alpha-acetylmethadol, and paclitaxel." Am J Perinatol **23**(7): 423-30.

Nekhayeva, I. A., T. N. Nanovskaya, et al. (2005). "Effects of nicotine-specific antibodies, Nic311 and Nic-IgG, on the transfer of nicotine across the human placenta." Biochem Pharmacol **70**(11): 1664-72.

NHS National Services Scotland (2010). Drug Misuse Statistics Scotland 2010. Edinburgh, ISD Scotland Publications.

NICE (2010). Pregnancy and complex social factors. A model for service provision for pregnant women with complex social factors, National Institute for Health and Clinical Excellence. **Nice clinical guidance 110**.

NIDA (2007). Drugs, Brains and Behavior. The Science of Addiction, National Institute on Drug Abuse National Institutes of Health.US Dept. of Health and Human Services.

NTA (2005). Women in Drug Treatment Services Research Briefing 6. London, National Treatment Agency for Substance Misuse.

NTA (2006). Models of Care for Treatment of Adult Drug Misusers: Framework for developing local systems of effective drug misuse treatment in England: Update 2006. London, National Treatment Agency.

NTA (2013). National and Regional Estimates of the Prevalence of Opaie and/or Crack Cocaine Use 2010-11: A summary of key Findings. London, National Treatment Agency for Substance Misuse.

Ostrea, E. M., Jr., M. Brady, et al. (1992). "Drug screening of newborns by meconium analysis: a large-scale, prospective, epidemiologic study." Pediatrics **89**(1): 107-13.

Ostrea, E. M., Jr., D. K. Knapp, et al. (2001). "Estimates of illicit drug use during pregnancy by maternal interview, hair analysis, and meconium analysis." J Pediatr **138**(3): 344-8.

Pallotto, E. K. and H. W. Kilbride (2006). "Perinatal outcome and later implications of intrauterine growth restriction." Clin Obstet Gynecol **49**(2): 257-69.

Pascolo, L., C. Fernetti, et al. (2003). "Effects of maturation on RNA transcription and protein expression of four MRP genes in human placenta and in BeWo cells." Biochem Biophys Res Commun **303**(1): 259-65.

Paulusma, C. C., M. Kool, et al. (1997). "A mutation in the human canalicular multispecific organic anion transporter gene causes the Dubin-Johnson syndrome." Hepatology **25**(6): 1539-42.

Petropoulos, S., W. Gibb, et al. (2010). "Effect of glucocorticoids on regulation of placental multidrug resistance phosphoglycoprotein (P-gp) in the mouse." Placenta **31**(9): 803-10.

Petropoulos, S., G. M. Kalabis, et al. (2007). "Functional changes of mouse placental multidrug resistance phosphoglycoprotein (ABCB1) with advancing gestation and regulation by progesterone." Reprod Sci **14**(4): 321-8.

Pinkham, S., C. Stoicescu, et al. (2012). "Developing effective health interventions for women who inject drugs: key areas and recommendations for program development and policy." Adv Prev Med **2012**: 269123.

Poller, B., D. Iusuf, et al. (2011). "Differential impact of P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2) on axitinib brain accumulation and oral plasma pharmacokinetics." Drug Metab Dispos **39**(5): 729-35.

Powell, A. G., J. E. Crozier, et al. (2011). "A case of septicaemic anthrax in an intravenous drug user." BMC Infect Dis **11**: 21.

Rahi, M., T. Heikkinen, et al. (2008). "Influence of adenosine triphosphate and ABCB1 (MDR1) genotype on the P-glycoprotein-dependent transfer of saquinavir in the dually perfused human placenta." Hum Exp Toxicol **27**(1): 65-71.

Rayburn, W. F. and M. P. Bogenschutz (2004). "Pharmacotherapy for pregnant women with addictions." Am J Obstet Gynecol **191**(6): 1885-97.

Richardson, G. A., L. Goldschmidt, et al. (2007). "Effects of prenatal cocaine exposure on growth: a longitudinal analysis." Pediatrics **120**(4): e1017-27.

Richardson, G. A., L. Goldschmidt, et al. (2011). "Prenatal cocaine exposure: Effects on mother- and teacher-rated behavior problems and growth in school-age children." Neurotoxicol Teratol **33**(1): 69-77.

Richardson, G. A., L. Goldschmidt, et al. (2008). "The effects of prenatal cocaine use on infant development." Neurotoxicol Teratol **30**(2): 96-106.

Richardson, G. A., L. Goldschmidt, et al. (2009). "Continued effects of prenatal cocaine use: preschool development." Neurotoxicol Teratol **31**(6): 325-33.

Rieger, M., K. M. Pirke, et al. (2004). "Influence of stree during pregnancy during pregnancy on HPA activity and neonatal behavior." Ann N Y Acad Sci **1032**: 228-30

Robey, R. W., K. K. To, et al. (2009). "ABCG2: a perspective." Adv Drug Deliv Rev **61**(1): 3-13.

Robinson, T. E. and K. C. Berridge (1993). "The neural basis of drug craving: an incentive-sensitization theory of addiction." Brain Res Brain Res Rev **18**(3): 247-91.

Rodriguez, M., I. Ortega, et al. (2004). "Effect of P-glycoprotein inhibition on methadone analgesia and brain distribution in the rat." J Pharm Pharmacol **56**(3): 367-74.

Rondo, P. H., R. F. Ferreira, et al. (2003). "Maternal psychological stress and distress as predictors of low birth weight, prematurity and intrauterine growth retardation." Eur J Clin Nutr **57**(2): 266-72.

Rosenberg, M. F., R. Callaghan, et al. (2005). "Three-dimensional structure of P-glycoprotein: the transmembrane regions adopt an asymmetric configuration in the nucleotide-bound state." J Biol Chem **280**(4): 2857-62.

Rosenblaum, O. and Guionnet. C (2003). Maternal Health and Drug Abuse- A Psychological Perspective. Maternal Health and Drug Abuse Perspectives across

Europe. A. B. M. R. a. J. Corkery. London, European Collaborating Centres in Addiction Studies Monograph

Sachse-Seeboth, C., J. Pfeil, et al. (2009). "Interindividual variation in the pharmacokinetics of Delta9-tetrahydrocannabinol as related to genetic polymorphisms in CYP2C9." Clin Pharmacol Ther **85**(3): 273-6.

Salisbury, A. L., M. G. Coyle, et al. (2012). "Fetal assessment before and after dosing with buprenorphine or methadone." Addiction **107 Suppl 1**: 36-44.

SAMHSA (2009). Substance Abuse and Mental Health Services Administration. (2009). Results from the 2008 National Survey on Drug Use and Health: National Findings (Office of Applied Studies, NSDUH Series H-36, HHS Publication No. SMA 09-4434). Rockville, MD.

Sanaullah, F., M. Gillian, et al. (2006). "Screening of substance misuse during early pregnancy in Blyth: an anonymous unlinked study." J Obstet Gynaecol **26**(3): 187-90.

Sanchez, E. S., J. W. Bigbee, et al. (2008). "Opioid addiction and pregnancy: perinatal exposure to buprenorphine affects myelination in the developing brain." Glia **56**(9): 1017-27.

Sansavini, A., A. Guarini, et al. (2010). "Does preterm birth increase a child's risk for language impairment?" Early Hum Dev **86**(12): 765-72.

Sauna, Z. E., C. Kimchi-Sarfaty, et al. (2007). "The sounds of silence: synonymous mutations affect function." Pharmacogenomics **8**(6): 527-32.

Scher, M. S., G. A. Richardson, et al. (1988). "The effects of prenatal alcohol and marijuana exposure: disturbances in neonatal sleep cycling and arousal." Pediatr Res **24**(1): 101-5.

Schinkel, A. H. (1997). "The physiological function of drug-transporting P-glycoproteins." Semin Cancer Biol **8**(3): 161-70.

Schinkel, A. H. (1998). "Pharmacological insights from P-glycoprotein knockout mice." Int J Clin Pharmacol Ther **36**(1): 9-13.

Schinkel, A. H. and J. W. Jonker (2003). "Mammalian drug efflux transporters of the ATP binding cassette (ABC) family: an overview." Adv Drug Deliv Rev **55**(1): 3-29.

Schwab, M., M. Eichelbaum, et al. (2003). "Genetic polymorphisms of the human MDR1 drug transporter." Annu Rev Pharmacol Toxicol **43**: 285-307.

Scottish-Executive (2003). Good Practice Guidance for working with Children and Families affected by Substance Misuse. Getting our Priorities Right, Edinburgh.

Seligman, N. S., N. Salva, et al. (2008). "Predicting length of treatment for neonatal abstinence syndrome in methadone-exposed neonates." Am J Obstet Gynecol **199**(4): 396 e1-7.

Sharom, F. J. (2008). "ABC multidrug transporters: structure, function and role in chemoresistance." Pharmacogenomics **9**(1): 105-27.

Sharom, F. J., M. R. Lugo, et al. (2005). "New insights into the drug binding, transport and lipid flippase activities of the p-glycoprotein multidrug transporter." J Bioenerg Biomembr **37**(6): 481-7.

Shieh, C. and M. Kravitz (2002). "Maternal-fetal attachment in pregnant women who use illicit drugs." J Obstet Gynecol Neonatal Nurs **31**(2): 156-64.

Sibley, C. P. and J. D. Boyd (2004). Mechanisms of Transfer Across the Human Placenta. Fetal and Neonatal Physiology. P. F. Abman. London, Saunders**:** 111-121.

Slamberova, R., M. Pometlova, et al. (2006). "Postnatal development of rat pups is altered by prenatal methamphetamine exposure." Prog Neuropsychopharmacol Biol Psychiatry **30**(1): 82-8.

Slotkin, T. A. (1998). "Fetal nicotine or cocaine exposure: which one is worse?" J Pharmacol Exp Ther **285**(3): 931-45.

Smit, J. W., M. T. Huisman, et al. (1999). "Absence or pharmacological blocking of placental P-glycoprotein profoundly increases fetal drug exposure." J Clin Invest **104**(10): 1441-7.

Soto, E. and R. Bahado-Singh (2013). "Fetal abnormal growth associated with substance abuse." Clin Obstet Gynecol **56**(1): 142-53.

St-Pierre, M. V., M. A. Serrano, et al. (2000). "Expression of members of the multidrug resistance protein family in human term placenta." Am J Physiol Regul Integr Comp Physiol **279**(4): R1495-503.

Sudhakaran, S., H. Ghabrial, et al. (2005). "Differential bidirectional transfer of indinavir in the isolated perfused human placenta." Antimicrob Agents Chemother **49**(3): 1023-8.

Sudhakaran, S., C. R. Rayner, et al. (2007). "Differential protein binding of indinavir and saquinavir in matched maternal and umbilical cord plasma." Br J Clin Pharmacol **63**(3): 315-21.

Sugawara, I., S. Akiyama, et al. (1997). "Lung resistance protein (LRP) expression in human normal tissues in comparison with that of MDR1 and MRP." Cancer Lett **112**(1): 23-31.

Sun, M., J. Kingdom, et al. (2006). "Expression of the multidrug resistance P-glycoprotein, (ABCB1 glycoprotein) in the human placenta decreases with advancing gestation." Placenta **27**(6-7): 602-9.

Suzuki, T., C. Zaima, et al. (2007). "P-glycoprotein mediates brain-to-blood efflux transport of buprenorphine across the blood-brain barrier." J Drug Target **15**(1): 67-74.

Syme, M. R., J. W. Paxton, et al. (2004). "Drug transfer and metabolism by the human placenta." Clin Pharmacokinet **43**(8): 487-514.

Tanabe, M., I. Ieiri, et al. (2001). "Expression of P-glycoprotein in human placenta: relation to genetic polymorphism of the multidrug resistance (MDR)-1 gene." J Pharmacol Exp Ther **297**(3): 1137-43.

Tang, K., S.M. Ngoi, et al (2002). "Distinct haplotype profiles and strong linkage disequilibrium at the MDR1 multidrug transporter gene locus in three Asian populations." Pharmacogenetics **12**(6): 437-50).

Tegethoff, M., N. Greene, et al. (2010) "Maternal psychosocial stress during pregnancy and placenta weight: evidence from a national cohort study." PLoS One **5**(12): e14478.

ter Horst, P. G., F. G. Jansman, et al. (2008). "Pharmacological aspects of neonatal antidepressant withdrawal." Obstet Gynecol Surv **63**(4): 267-79.

Thadani, P. V., J. F. Strauss, 3rd, et al. (2004). "National Institute on Drug Abuse Conference report on placental proteins, drug transport, and fetal development." Am J Obstet Gynecol **191**(6): 1858-62.

Thajam, D., D. E. Atkinson, et al. (2010). "Is neonatal abstinence syndrome related to the amount of opiate used?" J Obstet Gynecol Neonatal Nurs **39**(5): 503-9.

Thajam, D., Sibley, C.P., Lavender, T., Atkinson, D.E,A. (2012) "Using Hair Sample Analysis in Self Reported and Observed Drug Use in Pregnant Women." British Journal of Midwifery **20** (11): 806-810.

The Health and Social Care Information Centre (2011). Statistics on Drug Misuse: England, 2011 version 1.

The National Audit Office (2009). Tackling Problem Drug use. London, UK National Audit Office.

Thiebaut, F., T. Tsuruo, et al. (1987). "Cellular localization of the multidrug-resistance gene product P-glycoprotein in normal human tissues." Proc Natl Acad Sci U S A **84**(21): 7735-8.

Thompson, B. L., P. Levitt, et al. (2009). "Prenatal exposure to drugs: effects on brain development and implications for policy and education." Nat Rev Neurosci **10**(4): 303-12.

Tournier, N., L. Chevillard, et al.(2010). "Interaction of drugs of abuse and maintenance treatments with human P-glycoprotein (ABCB1) and breast cancer resistance protein (ABCG2)." Int J Neuropsychopharmacol **13**(7): 905-15.

Toyoda, Y., Y. Hagiya, et al. (2008). "MRP class of human ATP binding cassette (ABC) transporters: historical background and new research directions." Xenobiotica **38**(7-8): 833-62.

Tsuji, A., T. Terasaki, et al. (1992). "P-glycoprotein as the drug efflux pump in primary cultured bovine brain capillary endothelial cells." Life Sci **51**(18): 1427-37.

Uehlinger, C., S. Crettol, et al. (2007). "Increased (R)-methadone plasma concentrations by quetiapine in cytochrome P450s and ABCB1 genotyped patients." J Clin Psychopharmacol **27**(3): 273-8.

UNDOC, United Nations Office on Drugs and Crime (2010). World Drug Report 2010. New York, United Nations Office on Drugs & Crime.

Unger, A., R. Jagsch, et al. (2011). "Are males neonates more vulnerable to neonatal abstinence syndrome than female neonates?" Gend Med **8**(6): 355-64.

Vahakangas, K. and P. Myllynen (2009). "Drug transporters in the human blood-placental barrier." Br J Pharmacol **158**(3): 665-78.

van Herwaarden, A. E., E. Wagenaar, et al. (2007). "Multidrug transporter ABCG2/breast cancer resistance protein secretes riboflavin (vitamin B2) into milk." Mol Cell Biol **27**(4): 1247-53.

Vasiliou, V., K. Vasiliou, et al. (2009). "Human ATP-binding cassette (ABC) transporter family." Hum Genomics **3**(3): 281-90.

Velez, M. and L. M. Jansson (2008). "The Opioid dependent mother and newborn dyad: non-pharmacologic care." J Addict Med **2**(3): 113-20.

Velez, M. L., L. M. Jansson, et al. (2009). "Prenatal methadone exposure and neonatal neurobehavioral functioning." Pediatr Res **66**(6): 704-9.

Vlaming, M. L., Z. Pala, et al. (2009a). "Functionally overlapping roles of Abcg2 (Bcrp1) and Abcc2 (Mrp2) in the elimination of methotrexate and its main toxic metabolite 7-hydroxymethotrexate in vivo." Clin Cancer Res **15**(9): 3084-93.

Vlaming, M. L., A. van Esch, et al. (2009b). "Abcc2 (Mrp2), Abcc3 (Mrp3), and Abcg2 (Bcrp1) are the main determinants for rapid elimination of methotrexate and its toxic metabolite 7-hydroxymethotrexate in vivo." Mol Cancer Ther **8**(12): 3350-9.

Wang, B., Z. Miao, et al. (2009). "Genetic analysis of ABCG2 gene C421A polymorphism with gout disease in Chinese Han male population." Hum Genet **127**(2): 245-6.

Wang, D., A. D. Johnson, et al. (2005). "Multidrug resistance polypeptide 1 (MDR1, ABCB1) variant 3435C>T affects mRNA stability." Pharmacogenet Genomics **15**(10): 693-704.

Wang, J. S., Y. Ruan, et al. (2004). "The brain entry of risperidone and 9-hydroxyrisperidone is greatly limited by P-glycoprotein." Int J Neuropsychopharmacol **7**(4): 415-9.

Wang, J. S., H. J. Zhu, et al. (2006). "Evaluation of antipsychotic drugs as inhibitors of multidrug resistance transporter P-glycoprotein." Psychopharmacology (Berl) **187**(4): 415-23.

Wang, J. S., H. J. Zhu, et al. (2008). "Antipsychotic drugs inhibit the function of breast cancer resistance protein." Basic Clin Pharmacol Toxicol **103**(4): 336-41.

Wang, Y., D. Hao, et al. (2006). "A kinetic study of Rhodamine123 pumping by P-glycoprotein." Biochim Biophys Acta **1758**(10): 1671-6.

Weiss, J., D. Theile, et al. (2007). "Inhibition of MRP1/ABCC1, MRP2/ABCC2, and MRP3/ABCC3 by nucleoside, nucleotide, and non-nucleoside reverse transcriptase inhibitors." Drug Metab Dispos **35**(3): 340-4.

Wilbourne, P., C. Wallerstedt, et al. (2001). "Clinical management of methadone dependence during pregnancy." J Perinat Neonatal Nurs **14**(4): 26-45.

Wolff, K., A. Boys, et al. (2005). "Changes to methadone clearance during pregnancy." Eur J Clin Pharmacol **61**(10): 763-8.

Woodward, O. M., A. Kottgen, et al. (2011) "ABCG transporters and disease." FEBS J **278**(18): 3215-25.

Wright, A. and J. Walker (2007). "Management of women who use drugs during pregnancy." Semin Fetal Neonatal Med **12**(2): 114-8.

Wu, C. P., A. M. Calcagno, et al. (2008). "Reversal of ABC drug transporter-mediated multidrug resistance in cancer cells: Evaluation of current strategies." Curr Mol Pharmacol **1**(2): 93-105.

Yamagishi, K., T. Tanigawa, et al. (2010). "The rs2231142 variant of the ABCG2 gene is associated with uric acid levels and gout among Japanese people." Rheumatology (Oxford) **49**(8): 1461-5.

Yasuda, S., S. Itagaki, et al. (2005). "Expression level of ABCG2 in the placenta decreases from the mid stage to the end of gestation." Biosci Biotechnol Biochem **69**(10): 1871-6.

Yeboah, D., M. Sun, et al. (2006). "Expression of breast cancer resistance protein (BCRP/ABCG2) in human placenta throughout gestation and at term before and after labor." Can J Physiol Pharmacol **84**(12): 1251-8.

Young, A. M., C. E. Allen, et al. (2003). "Efflux transporters of the human placenta." Adv Drug Deliv Rev **55**(1): 125-32.

Zharikova, O. L., S. V. Deshmukh, et al. (2007). "The effect of opiates on the activity of human placental aromatase/CYP19." Biochem Pharmacol **73**(2): 279-86.

Zhou, S. F. (2008). "Structure, function and regulation of P-glycoprotein and its clinical relevance in drug disposition." Xenobiotica **38**(7-8): 802-32.

Zhu, H. J., J. S. Wang, et al. (2006). "Characterization of P-glycoprotein inhibition by major cannabinoids from marijuana." J Pharmacol Exp Ther **317**(2): 850-7.

Appendix 1 Study Information Sheet

MOTHERS INFORMATION SHEET

You are being invited to take part in a research study. Before you decide if you would like to take part, it is important for you to understand why the research is being done and what it will involve. Please take time to read the following information carefully and discuss it with others if you wish. Ask us if there is anything that is not clear, or if you would like more information. Take time to decide whether or not you wish to take part.

What is the purpose of the Study?

Some babies exposed to drugs during pregnancy will experience signs of withdrawal after birth. It has been suggested that some may also have problems with development and/or behaviours as they grow. The effects of exposure to drugs is variable and we would like to investigate whether this is because in some cases, drugs can get across the placenta more easily.

Why have I been chosen to participate?

In order to identify mechanisms in the placenta that may be responsible for transferring drugs across the placenta to the growing baby, it will be necessary to obtain information about the baby's genes. We need to compare the genes of babies who experience few difficulties when exposed to various substances, with the genes of babies who experience more difficulties either in the short or longer term. Your participation could therefore, provide valuable information for the study. We hope approximately 200 women will decide to participate.

Who is organising the study?

The study is being organised by the Department of Child Health at St. Mary's Hospital Manchester and the Manchester Specialist Midwifery Service. It is being funded by a grant from the Department of Health.

What will taking part in the study involve for myself and my child?

If you would like to participate we will ask for your consent to

* look at your medical notes and those of your baby, including the results of tests and investigations you may have had.
* ask about aspects of your life, for example, housing, work, your family life and schooling.
* look at the information you have given about your past and present substance use
* weigh you during your pregnancy
* look at your child's developmental record (red book) during the first two years of life and perform a developmental assessment when aged 12-18 months

We will also ask your permission to speak to you once the baby is born and take

* a sample of placenta - to study its ability to transport drugs
* a sample of blood from the placental cord **(or a sample of your baby's saliva if the cord blood is unavailable)** - to examine the baby's DNA
* a hair sample from you - to provide a record of substances that the baby has been exposed to
* a sample of saliva from you, and/or the baby's father, to look at your DNA in relation to your baby's DNA.

This study **will not** involve taking any blood from you or your baby.

Appendix 2 Consent Form

Mothers Consent form: Placental Multi-drug Resistance Protein Genotype

Please initial boxes where applicable.

1. I confirm that I have read and understood the information sheet **version 3 26.09.09** for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw from the study at any time, without giving any reason and without my medical care or legal rights being affected. ☐

3. I give consent for the use of my placenta in this study. ☐

4. I consent to a blood sample being taken from the placental cord to allow DNA analysis of my baby **(or a sample of my baby's saliva if the cord blood is unavailable)**. I understand that this cannot be used as a paternity test. ☐

5. I consent to a sample of my saliva being collected for DNA analysis. ☐

6. I consent to a sample of my hair being taken to assess what drugs my baby has been exposed to. ☐

7. I give consent for the researchers involved in this study to access my medical records. ☐

8. I consent to information about my baby's health at birth and development over a period of 2 years being collected. ☐

9. I agree to the taking, storage and future use of these samples for the present study. ☐

10. I agree to participate in the study. ☐

_____	_____	_____
Name of participant *(Please print)*	Date	Signature
_____	_____	_____
Name of researcher obtaining consent	Date	Signature

☐ **Study ID number Allocated**

Participants signature

Appendix 3 Data Collection Tool

Study: The Role of Placental Multidrug Resistant Proteins. REC Ref Number: 09/H1004/5

Researcher:..... Participant No.: .....

	Consented	Obtained	Comments
Mother's Saliva			
Mother's Hair			
Father's Saliva			
Placenta			

Name: ..... AKA: .....
Address: (1) ..... (2).....
....
....
Tel:..... Tel: .....
Tel:..... Tel: .....

Ethnic Origin: ..... D.O.B:...../...../..... A-Z Ref: ..... Booked at: NMGH | SMH

Housing? _____ Cigarette smoker? Y/N How many daily? _____

GP: ..... Tel.....
Midwife / Health Visitor: ..... Tel.....

Obstetric History

E.D.D		Gravida		Para	

D.O.B	Delivery	M/F	Birth Weight	Resident	Substance misuse	Experience of withdrawal

Medical History

BBV	Mental Health	General

Gestation at booking	/40	Gestation at recruitment	/40	BMI at booking	
Weight gained in pregnancy		Antenatal visits attended		Antenatal visits missed	
Children's services?	Past	Present	Past and present	Referral in pregnancy	

Drug History

Past

Substance						
Mode of use						
Age						
Abstinence						

At Recruitment: ...../...../.....

In treatment?	
Length of treatment	
Substance	
Mode of use	
Dose/frequency	
Duration	

Changes During Pregnancy

	Date	Date	Date	Date	Date	Date
Substance						
Mode of use						
Dose/frequency						
Comments						

Antenatal

Maternal perceptions of stress:	
Illness in pregnancy/ complications:	
Nutrition/diet:	
Admitted to hospital? Y/N ~ Reason(s):	

Labour and Delivery History

Length of labour and second stage				
Time spent in labour	at home		at hospital	
Pain relief				
Complications				
CTG				
Fetal distress				
Mode of delivery		Indications		

Placenta

Weight		Size	
Description			

Child

D.O.B		Gestation	/40	Gender	M / F	Weight		Length	cm	OFC	cm

Ethnicity	Apgar score	Resuscitation	Length of stay in hospital	Home to:	Feeding: Breast / Bottle Difficulties?:

Neonatal Problems

Neonatal Behaviours

Mother using supportive measures				Hospital	Y/N	Home	Y/N
Scores				Hospital		Home	
NNMU	Y/N	Medication	Y/N	Mothers perception of withdrawal			

Developmental milestones

Appendix 4 Perceived Stress Scale

Perceived Stress Scale

The questions in this scale ask you about your feelings and thoughts during the last month. In each case, you will be asked to indicate by circling how often you felt or thought in a certain way.

Name date

Age gender (circle): M F other

0 = never 1= almost never 2= sometimes 3= fairly often 4= very often

1. In the last month, how often have you been upset because of something that happened unexpectedly? 0 1 2 3 4
2. In the last month, how often have you felt you were unable to control the important things in your life? 0 1 2 3 4
3. In the last month, how often have you felt nervous and stressed? 0 1 2 3 4
4. In the last month, how often have you felt confident about your ability to handle your personal problems? 0 1 2 3 4
5. In the last month, how often have you felt that things were going your way? 0 1 2 3 4
6. In the last month, how often have you found that you could not cope with all the things that you have to do? 0 1 2 3 4
7. In the last month, how often have you been able to control irritations in your life? 0 1 2 3 4
8. In the last month, how often have you felt that you were on top of things? 0 1 2 3 4
9 in the last month, how often have you been angered because of things outside of your control? 0 1 2 3 4

Please feel free to use the perceived stress Scale for your research.

Mind garden, Inc.

Info@mindgarden.com

www.mindgarden.com

Appendix 5 Neonatal Abstinence Syndrome Scoring Tool

Chart symptoms 4 hourly. Start treatment if a score of 5 or more is obtained on 3 subsequent occasions.
Commence treatment if two seizures occur.

NAME DOB HOSPITAL NUMBER

Date											
Time											
Convulsion	Score 3										
High pitched cry Tremors Hypertonia	Score 1 if any single symptom or combination of symptoms is noted										
Irritable Scratching Excessive wakefulness	Score 1 if any single symptom or combination of symptoms is noted										
Pyrexia > 38º Tachypneoa > 60/min	Score 1 if either or both are noted										
Sweating Dehydration	Score 1 if either or both are noted										
Vomiting/ diarrhoea/yawning/ hiccups	Score 1 if any single symptom or combination of symptoms is noted*										
Salivation Congested nose Sneezing	Score 1 if any single symptom or combination of symptoms is noted*										
Total Score											

* When sufficiently repetitive to be out of the ordinary