An Investigation of Placental Glutamine and Glutamate Transport in Normal Pregnancy and Fetal Growth Restriction

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Abbreviations

-RT	No reverse transcriptase
^{14}C	Carbon-14
^{1}H NMR	Proton nuclear magnetic resonance
11β-HSD2	11β-hydroxysteroid dehydrogenase 2
4EBP	4E-binding protein
AAR	Amino acid response
AARE	Amino acid response element
AGA	Appropriate for gestational age
akg	α-ketoglutarate
Akt	Protein kinase B
ASC	Alanine serine cysteine-preferring
Asp	Aspartate
ATP	Adenosine triphosphate
BCAA	Branched chain amino acids
BCH	2-Amino-2-norbornanecarboxylic acid
BCKA	Branched chain α-keto acids
BM	Basal membrane
BMI	Body mass index
bp	Base pairs
BSA	Bovine serum albumin
BW:PW ratio	Birth weight:placental weight ratio
$CaCl_2$	Calcium chloride
cDNA	Complementary deoxyribonucleic acid
COMT	Catechol-O-methyl transferase
CT	Cycle threshold
dH_20	Distilled water
DHEAS	Dehydroepiandrosterone-3-sulphate
DMEM	Dulbecco's modified eagles medium
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide triphosphate
dpm	Disintegrations per minute
DTT	Dithiothreitol
E	Embryonic day
EAAT	Excitatory amino acid transporter
eIF4E	Eukaryotic translation initiation factor 4E
ELCS	Elective caesarean section
EMCS	Emergency caesarean section
eNOS	Endothelial nitric oxide synthase
F:P ratio	Fetal:placental weight ratio
FGR	Fetal growth restriction
GABA	γ-amino butyric acid
GC-MS	Gas chromatography-mass spectrometry
GLAST	GLutamate ASpartate Transporter
Gln	Glutamine
Glu	Glutamate
GSK3	Glycogen synthase kinase 3
H^+	Hydrogen

HEPES	4-(2-Hydroxyethyl)piperazine-1-ethanesulfonicacid,N-(2-Hydroxyethyl)piperazine-N′-(2-ethanesulfonic acid)
His	Histidine
HPLC	High performance liquid chromatography
I.U.	International units
IBR	Individualised birth weight ratio
ICM	Inner cell mass
IGF/Igf	Insulin-like growth factor
IL	Interleukin
IPYS	Intraplacental yolk sac
IQR	Interquartile range
IUGR	Intrauterine growth restriction
K^+	Potassium
KCl	Potassium chloride
K_{mf}	Unidirectional maternofetal clearance
KOH	Potassium hydroxide
LAT	Light subunits of Amino acid Transporters
LBW	Low birth weight
LC-MS	Liquid chromatography-mass spectrometry
MCT	Monocarboxylate transporters
MeAIB	Methylaminoisobutyric acid
$MgCl_2$	Magnesium chloride
miR	microRNA
MR	Magnetic resonance
MSTFA	N-methyl-N-(trimethylsilyl)trifluoroacetamide
mTOR	Mechanistic target of rapamycin
mTORC	Mechanistic target of rapamycin complex
MVM	Microvillous membrane
Na^+	Sodium
NaCl	Sodium chloride
NaOH	Sodium hydroxide
NBF	Neutral buffered formalin
NH_3	Ammonia
NHE	Na^+/H^+ exchanger
NHP	Non-human primate
NHS	National Health Service
NTC	No template control
NVD	Normal vaginal delivery
O_2	Oxygen
OAT	Organic anion transporter
OATP	Organic anion transporting polypeptide
P×S	Permeability × surface area product
P13K	Phosphatidylinositol 3-kinase
PBS	Phosphate buffered saline
PCA	Principal components analysis
PCR	Polymerase chain reaction
PDVF	Polyvinylidene fluoride
PIC	Protease inhibitor cocktail
PKC	Protein kinase C
PMCA	Plasma membrane calcium ATPase

pO_2	Partial pressure of oxygen
PTHrP	Parathyroid hormone related peptide
PW:BW ratio	Placental weight:birth weight ratio
QC	Quality control
qRT-PCR	Quantitative real-time polymerase chain reaction
RNA	Ribonucleic acid
rpm	Revolutions per minute
RT	Room temperature
S6K	S6 kinase
SD	Standard deviation
SDS	Sodium dodecyl sulphate
SE	Standard error
SEM	Standard error of the mean
Ser	Serine
SGA	Small for gestational age
SGK1	Serum glucocorticoid–regulated protein kinase 1
SNAT	Sodium-coupled Neutral Amino acid Transporter
SUMO	Small ubiquitin-like modifier
TauT	Taurine transporter
TBP	TATA-box binding protein
TCA cycle	Tricarboxylic acid cycle
TEMED	Tetramethylethylenediamine
TNF	Tumour necrosis factor
TRIZMA	Tris(hydroxymethyl)aminomethane
UmA	Umbilical artery
UmV	Umbilical vein
UtA	Uterine artery
UV	Ultraviolet
VLBW	Very low birth weight
WT	Wild-type
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta

Abstract

A healthy pregnancy depends upon the delivery of amino acids and other essential nutrients to the fetus via the placenta. Placental dysfunction is a major cause of fetal growth restriction (FGR), which is characterised by poor growth *in utero* and most often defined as an individualised birth weight ratio (IBR) of $<5^{th}$ centile. Growth restricted babies are at increased risk of stillbirth, postnatal complications and disease in adulthood. There is an unmet need for efficacious treatment options, the development of which would be aided by improved understanding of the relationship between placental nutrient delivery and fetal growth.

The amino acids glutamine and glutamate are vital for metabolic processes and fetal growth, and are intrinsically linked by interorgan metabolism in the placenta and fetal liver. Studies in normal pregnancy (in women and wild-type (WT) mice) have shown that in the small placenta of a normally grown fetus there is up-regulation (adaptation) of transport of the non-metabolisable amino acid analogue methylaminoisobutyric acid (MeAIB) (per g placenta). This thesis tested the hypothesis that the small, normal placenta up-regulates glutamine and glutamate transport and that in FGR this relationship is disrupted and/or absent.

Unidirectional maternofetal clearance (K_{mf}) of glutamine and glutamate was assessed in normal (WT) mouse pregnancy at embryonic day (E)15.5 and E18.5 (term=E19-20). A method to assess transporter-mediated uptake of glutamine and glutamate by human placental villous fragments *in vitro* was also developed. In normal WT pregnancy, K_{mf} of glutamine and glutamate was significantly higher in the lightest placentas towards term (E18.5), which reinforces the importance of glutamine and glutamate in ensuring appropriate fetal growth is maintained. Contrary to the literature, there was no relationship between placental weight or birth weight and transporter-mediated uptake of glutamine or glutamate in placentas from normal birth weight infants in human pregnancy. However, placentas from male infants had significantly higher glutamine/glutamate uptake compared with females but this was not associated with changes in transporter expression.

Placental transport capacity was next investigated in the *Igf2P0* (P0) knockout mouse, a well-characterised model of FGR. K_{mf} of glutamine and glutamate was higher across P0 versus WT placentas at E15.5. At E18.5 K_{mf} of glutamine remained significantly higher whereas K_{mf} of glutamate was similar between groups. This finding is surprising and suggests that the P0 placenta attempts to adapt to meet fetal nutrient demands. In human FGR where a small dysfunctional placenta is observed, transporter-mediated glutamine uptake was reduced in comparison with normal pregnancy. Glutamate uptake was no different between groups. In contrast, expression of key glutamine and glutamate transporters was significantly higher in FGR, indicating a potential role of post-translational modifications in amino acid transporter activity. Amino acid concentrations in the maternal vein, umbilical vein (UmV) and artery (UmA) were quantified by high performance liquid chromatography (HPLC), and the abundance of small molecule metabolites in UmV and UmA of normal birth weight and FGR infants measured using gas chromatography-mass spectrometry (GC-MS). Glutamine and glutamate concentrations in the maternal circulation were unaltered between normal pregnancy and FGR but glutamate concentration in the UmA was higher in FGR. Levels of lactic acid, pyruvic acid, urea, and others were differentially altered in the UmV and UmA of FGR infants.

In summary, glutamine and glutamate uptake into human placental villous fragments was unrelated to fetal or placental measures, but was influenced by the sex of the fetus. Conversely there is evidence to indicate that K_{mf} of glutamine/glutamate adapts according to placental size in WT mice and that the P0 mouse model of FGR may attempt to modulate its transport capacity in a bid to maintain appropriate fetal growth. HPLC and GC-MS uncovered distinct metabolic changes in FGR compared with normal human pregnancy. Furthermore, glutamine uptake was reduced in FGR despite increased transporter abundance, which suggests that post-translational modifications and/or signaling pathways modify amino acid transporter activity in this pathology. This thesis provides the foundation for future research to investigate the underlying mechanisms that may drive these changes.

Lay abstract

The placenta is the organ responsible for the delivery of nutrients and oxygen from mother to baby during pregnancy. Problems with the placenta can cause reductions in the baby's growth (fetal growth restriction; FGR). This affects between 5-10% of all pregnancies in the UK. These small babies are at an increased risk of stillbirth (the death of a baby before birth) and often have very small placentas too. There are currently no treatment options available for FGR infants, except for premature delivery. To enable the development of strategies to diagnose and treat FGR, we first must improve our understanding of how the placenta delivers nutrients to the baby in normal pregnancy and cases of FGR.

Amino acids are a group of nutrients that join together to make up proteins. Glutamine and glutamate are two amino acids that are essential for the growth and development of the baby. Previous research in human placentas and placentas of normal (wild-type; WT) mice has shown that the size of the placenta is inversely related to the transport of a synthetic amino acid called methylaminoisobutyric acid (MeAIB). This means that a smaller placenta will transport more amino acid relative to a larger placenta, presumably to ensure appropriate growth of the baby. This thesis examined whether this is also true of the amino acids glutamine and glutamate in normal pregnancies, and whether this relationship is lost or altered in FGR.

In WT mice, the transport of glutamine and glutamate from the maternal to the fetal circulation was compared between the lightest and heaviest placenta in a litter at two points in pregnancy (embryonic (E) day 15.5 and E18.5, term is E19-20). In support of previous findings, transport was higher for the lightest placentas in a litter at E18.5. Conversely, the transport of glutamine and glutamate into small sections of human placentas from normal pregnancies was not related to the size (weight) of the placenta. However, in normal human pregnancy, the transport of glutamine and glutamate into placentas from male babies was higher compared to those from female infants.

These experiments were repeated using human placentas from FGR infants and FGR was modelled in mice using the *Igf2P0* (P0) mouse that has mixed litters of normal weight (WT) pups and growth restricted (P0) pups. In the P0 mouse glutamine and glutamate transport was higher earlier in pregnancy (E15.5). The day before term (E18.5) transport of glutamine was still higher for P0 compared with WT placentas, yet increased delivery of glutamine to the P0 fetus was insufficient to support normal growth. Human placentas from FGR infants were significantly smaller than those from normal pregnancies, and they were also unable to transport as much glutamine. The abundance of transporters responsible for the transport of glutamine was higher for FGR placentas, which suggests that there is another, as yet unknown, mechanism responsible for this difference.

Finally, blood samples were collected from the maternal vein, and the vein and artery of the umbilical cord upon delivery of a normal birth weight or FGR infant. The levels of amino acids and small molecules called metabolites were measured in these samples (metabolites were measured in the umbilical vein and artery only) and compared between normal pregnancy and FGR. In FGR levels of glutamate were higher in the umbilical artery compared with normal pregnancy. There were also differences in the levels of metabolites such as lactic acid, pyruvic acid and urea in the umbilical vein and artery of FGR infants, which may indicate altered placental and/or fetal metabolism.

In summary, this thesis has shown that there is no relationship between the weight of the placenta and glutamine or glutamate transport in normal human pregnancy, but there are differences according to the sex of the fetus. In WT mice there is evidence that the function of the placenta is related to placental size, and that the P0 mouse model of FGR attempts to support fetal growth by increasing glutamine transport. In human FGR the transport of glutamine is reduced, and there are specific differences in the levels of amino acids and metabolites between normal pregnancy and WT. These findings should be used as a basis for future experiments which should explore the mechanisms that cause these differences.

Declaration

I declare that 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 of other institute of learning.

Disclaimer

All of the work described in this thesis was carried out by myself except for the following:

Chapter 4: The Western blot experiments were conducted by Ms Kirsty Vincent under my supervision and guidance, for her undergraduate (BSc) project, University of Manchester

Chapter 6: The quantification of amino acid concentrations by high performance liquid chromatography was conducted by the Willink Biochemical Genetics Laboratory, Royal Manchester Children's Hospital, Manchester

Chapter 6: The data extraction and analysis of plasma samples by gas chromatography-mass spectrometry was performed with the technical assistance of Ms Stephanie Church, University of Manchester

Copyright statement

Publications arising from this work

Abstracts

McIntyre K, Hayward C, Sibley, C, Greenwood, S, Dilworth M (2018) Disparity between placental glutamine and glutamate transporter activity and expression in fetal growth restriction *Reproductive Sciences* 25, 227A

McIntyre K, Vincent K, Hayward C, Sibley, C, Greenwood, S, Dilworth M (2017) Activity, but not expression, of placental amino acid transporters specific for glutamine and glutamate is related to fetal sex *Placenta* 57, 249

McIntyre K, Vincent K, Hayward C, Sibley, C, Greenwood, S, Dilworth M (2017) Glutamine and glutamate transport into the placenta is related to fetal sex *BJOG* 124, e264-e278

McIntyre K, Hayward C, Sibley, C, Greenwood, S, Dilworth M (2016) Glutamate transfer (clearance) by the placenta adapts according to placental size in mice in late gestation *Pediatric Research* 80, 619–625

McIntyre K, Hayward C, Sibley, C, Greenwood, S, Dilworth M (2016) Placental transfer (clearance) of glutamine is dependent upon placental size in mice *Placenta* 45, 130

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Dedicated to Jack McIntyre and Malcolm Clements.

Chapter 1 Introduction

1.1 Overview

Normal fetal growth relies upon adequate supply of nutrients via the placenta. Nutrients must be transferred from the maternal circulation into the transporting epithelium of the placenta, the syncytiotrophoblast, where they may either be metabolised to support the metabolic requirements of the placenta or transferred across to the fetal circulation. Failure of the fetus to accrue adequate nutrients during gestation is likely to lead to insufficient fetal growth (fetal growth restriction, FGR). In the UK, FGR affects between 5-10% of pregnancies and is commonly defined as an individualised birth weight ratio (IBR) below the 5th centile. The aetiology of FGR is broad and encompasses a range of maternal and environmental factors; however the majority of cases are believed to be caused by placental dysfunction. Despite the significant medical, societal and economic burden of FGR, there are currently no therapeutic options available. To address this need, a better understanding of the relationship between placental nutrient provision to the fetus and fetal growth is required.

Amino acids are essential for placental and fetal metabolism, biosynthetic pathways and protein anabolism. Two amino acids whose provision is vital for adequate fetal growth and development are glutamine and glutamate. Glutamine is transferred across the placenta at the highest rate of all amino acids. Glutamine and glutamate are of particular interest as their transfer and metabolism are intrinsically linked; interconversion of the two amino acids occurs both in the placenta and fetal liver.

In normal pregnancy (in both women and wild-type (WT) mice), data from several studies suggest that the nutrient transport capacity of the placenta adapts, relative to its size, to support normal fetal growth. If this is a key mechanism to ensure fetal growth in normal pregnancy, failure of this adaptation may underpin cases of FGR. The evidence base for placental adaptation according to placental size is currently stronger for mice than for humans and thus is worthy of further exploration in women. The relationship between placental size (weight) and glutamine and glutamate uptake into the human placenta, or maternofetal clearance of these important amino acids across the mouse placenta, has never been investigated previously.

The main objectives of this project are a) to understand the relationship between placental size (weight) in normal pregnancy and glutamine and glutamate uptake/maternofetal transfer into/across the human and mouse placenta, respectively, b) to assess whether placental glutamine and glutamate transporter activity and expression are altered in human FGR or in an established mouse model of FGR and c) to compare the concentration of amino acids and

metabolites in maternal and fetal umbilical (artery and vein) plasma in normal pregnancies and FGR.

1.2 Fetal growth restriction

Fetal growth restriction (FGR), the failure of the fetus to reach its genetic growth potential, affects between 5-10% of pregnancies in the UK (ONS, 2015). FGR is the strongest risk factor for stillbirth (Gardosi *et al.*, 2013), and for UK births in 2016 placental dysfunction was attributed as a cause of 28.8% of stillbirths (Draper *et al.*, 2018). Stillbirths are a significant economic and societal burden. The stillbirth-related economic costs to the National Health Service (NHS) are estimated to be £13.6 million annually (Campbell *et al.*, 2018). A study by Gardosi *et al.* (2013) reported that the overall stillbirth rate for UK pregnancies was 4.2 per 1000 births but that this rate increased significantly to 16.7 when FGR was present. To examine this further, the stillbirth rate in the presence of FGR can be split according to whether FGR was detected *in utero*. The stillbirth rate was 19.8 per 1000 births for undetected cases versus 9.7 per 1000 births of cases detected antenatally. Furthermore, being born small confers an increased risk of disease in both neonatal life (e.g. developmental delay, cerebral palsy) and in adulthood (metabolic syndrome embracing obesity, type 2 diabetes and cardiovascular disease) (Barker, 2004; Thornton *et al.*, 2004; Veen *et al.*, 1991).

Placental dysfunction accounts for the majority of FGR cases (Mifsud and Sebire, 2014), but this placental dysfunction is likely to present with a myriad of underlying causal mechanisms of which reduced fetal growth is the end result. Established risk factors for FGR include maternal smoking and alcohol consumption (Audette and Kingdom, 2018; Royal College of Obstetricians and Gynaecologists, 2013). The aetiology of FGR is broad and encompasses a range of genetic (e.g. congenital abnormalities), disease (such as pre-eclampsia) and environmental factors. However, many FGR cases are classified as idiopathic because the reasons for placental dysfunction are not fully understood (Audette and Kingdom, 2018; Royal College of Obstetricians and Gynaecologists, 2013).

Broadly, FGR phenotypes are classed either as early- (<32 weeks gestation, ~20-30% of cases) or late-onset (>32 weeks, 70%) (Audette and Kingdom, 2018). Early-onset growth restriction is often symmetrical i.e. overall growth (length, head and abdominal circumference) is reduced proportionally. Infants with late-onset FGR however tend to have a proportionally large head circumference but reduced abdominal circumference compared with appropriately grown infants. This 'brain sparing' phenotype is thought to prioritise brain development (Hindmarsh *et al.*, 2002) at the expense of the growth of abdominal organs such as the liver. Indeed, in fetal sheep, experimental reduction of uterine blood flow by 50% causes preferential redistribution of oxygen to the brain and heart (Jensen *et al.*, 1991).

FGR is diagnosed in the clinic by estimating fetal weight, growth trajectory and fetal parameters such as abdominal and head circumference using serial ultrasonography. Accurate estimation of fetal weight using ultrasound is challenging; in a systematic review by Dudley (2005) the 95% confidence interval exceeded 14% of birth weight across all studies analysed. However, some progress in the field has been made to improve accurate detection of FGR via specialised clinics (Kingdom *et al.*, 2018) and alternative scanning methods such as magnetic resonance imaging (Ingram *et al.*, 2017). The diagnosis and placental phenotypes of FGR are discussed in more detail in section 1.3.3.

Current definitions of FGR are inconsistent. Individualised growth charts have replaced arbitrary birth weight cut offs (previously 2.5 kg was the cutoff used to define low birth weight) (Chiswick, 1985) by including a mixture of maternal and fetal characteristics to calculate individualised birth weight ratio (IBR) (Table 1). This approach has been employed in an attempt to stratify infants that are pathologically small (FGR), from those constitutionally small for gestational age (SGA) infants that do not require intervention, usually defined as an IBR <10th centile. Infants with an IBR of between 10th-90th centile are considered to be of normal size or appropriate for gestational age (AGA). FGR is routinely defined as an IBR below the 5th centile; however a universally accepted clinical threshold (i.e. below the 3rd, 5th or 10th centile +/- the presence of additional indications) does not exist (Unterscheider *et al.*, 2014).

Internationally, different customised standards to calculate IBR are used. In our centre, IBR is calculated using the GROW centile calculator that is based on a UK population (https://www.gestation.net). Elsewhere, INTERGROWTH-21st has been implemented; however the effectiveness of this standard in detecting SGA infants has recently been questioned (Francis *et al.*, 2018; Pritchard, 2018). Whether there is an added benefit of customised (IBR) compared with population growth charts has also been contested (Carberry *et al.*, 2014; Iliodromiti *et al.*, 2017; Stock and Myers, 2017).

Maternal characteristics	**Fetal characteristics**
Height	Gestational age
Weight*	Birth weight
Parity	Sex
Ethnicity	

Table 1: Characteristics required to calculate individualised birth weight centile
*At initial booking appointment (approximately 12 weeks of gestation). Information collated from https://www.gestation.net (Gardosi, 2015).

A recent study applied the Delphi method to reach a consensus on the definition of FGR. Through multiple rounds of questionnaires, a panel of 56 international experts was surveyed

until a consensus was reached. The consensus-based definitions for early- and late-onset FGR are shown in Table 2. The clinical diagnosis and placental phenotypes of FGR are discussed in greater depth in section 1.3.3. For the purpose of this thesis, FGR is defined as an IBR of <5th centile as data collection began in 2014; before the publication of the Delphi procedure study (Gordijn *et al.*, 2016).

Early FGR: GA < 32 weeks, in absence of congenital anomalies	Late FGR: GA ≥ 32 weeks, in absence of congenital anomalies
AC/EFW < 3rd centile or UA-AEDF	AC/EFW < 3rd centile
Or 1. AC/EFW < 10th centile combined with 2. UtA-PI > 95th centile and/or 3. UA-PI > 95th centile	Or at least two out of three of the following 1. AC/EFW < 10th centile 2. AC/EFW crossing centiles > 2 quartiles on growth centiles* 3. CPR < 5th centile or UA-PI > 95th centile

Table 2: Consensus-based definition for early and late-onset FGR in the absence of congenital anomalies
*Growth centiles are non-customised centiles. AC: fetal abdominal circumference, AEDF: absent end-diastolic flow, CPR: cerebroplacental ratio, EFW: estimated fetal weight, GA: gestational age, PI: pulsatility index, UA: umbilical artery, UtA: uterine artery. Reproduced from (Gordijn *et al.*, 2016).

FGR is a significant societal and economic burden (Campbell *et al.*, 2018; Cantwell *et al.*, 2011; Heazell *et al.*, 2016) yet there are currently no approved pharmacological treatment options available (Fisk and Atun, 2008). Current pathways for management of FGR advise early delivery of babies at immediate risk of *in utero* demise (Fisk and Atun, 2008; Royal College of Obstetricians and Gynaecologists, 2013). Being born preterm also carries significant associated risks such as cerebral palsy (Thornton *et al.*, 2004), which underlies the drive to develop effective treatment options for these babies.

The focus of this thesis is to determine the relationship between placental amino acid (glutamine and glutamate) uptake, a determinant of amino acid provision to the fetus, and placental size in babies appropriately grown for gestational age (AGA) and to examine how this relationship is altered in cases of FGR. Understanding of the physiology and mechanisms that underpin normal and perturbed fetal growth, as seen in FGR, is essential to address the clinical need for novel treatments to treat placental dysfunction and thus reduce the incidence of FGR and stillbirth.

1.3 Overview of the placenta in normal human pregnancy

1.3.1 Placental development

Development of the human haemochorial placenta is multi-staged and must be tightly controlled in order to support fetal growth and development. The established placenta functions as a conduit of nutrient exchange, as a producer of hormones to support pregnancy and as an immunological barrier (Nelson, 2015). Placental development begins following the attachment, and subsequent invasion, of the blastocyst into the decidua of the uterine wall at

around 7 days post-fertilisation. The blastocyst consists of an inner cell mass (ICM) that will ultimately form the developing embryo/fetus and a layer of trophectoderm cells that go on to form the placenta. Following invasion, the trophectoderm cells differentiate into an outer layer of multinucleate syncytiotrophoblast cells, supported by an inner layer of proliferating mononuclear cytotophoblast cells (Figure 1) (Georgiades *et al.*, 2002). This syncytiotrophoblast layer next invades the uterine wall. Within the sycytiotrophoblast layer cavities called lacunae form which are the precursors of the intervillous space (Aplin, 1991; Enders and Blankenship, 1999). Uterine gland secretions (histotroph) are a vital nutrient source for the conceptus during the first trimester, before the establishment of maternal blood flow to the intervillous space of the placenta around 12 weeks of gestation (Filant and Spencer, 2014; Rampersad *et al.*, 2011).

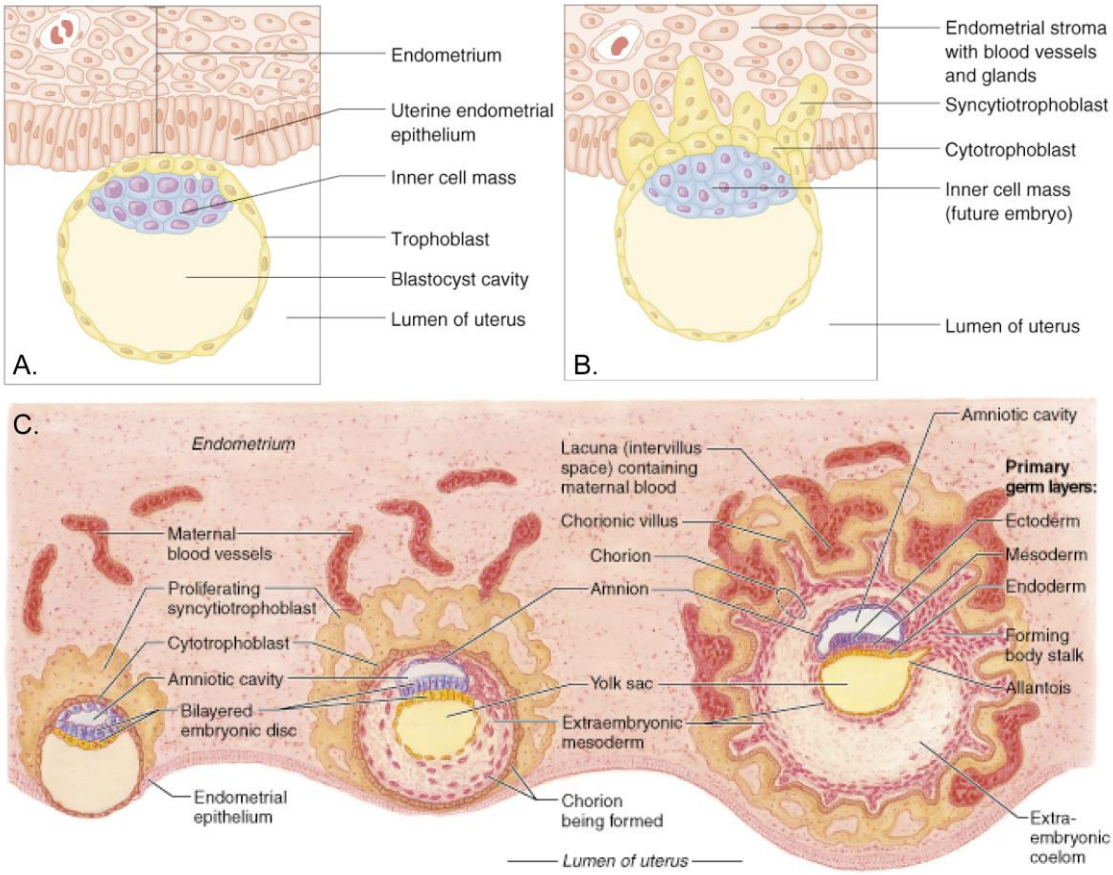


Figure 1: Blastocyst implantation and early placental development
A. The blastocyst consists of an outer trophectoderm layer and inner cell mass (ICM). B. Invasion of the endometrial epithelium occurs approximately 7 days post-fertilisation. C. Multinucleate proliferating syncytiotrophoblast precede the development of chorionic villi and are supported by an inner monolayer of cytotrophoblast cells. Lacunae (cavities filled with maternal blood) are the precursors of the intervillous space. Figure adapted from https://legacy.owensboro.kctcs.edu/gcaplan/anat2/notes/APIINotes2%20human%20development2.htm Accessed 25th June 2018 (Marieb, 2000).

Next, cytotrophoblast cells migrate, forming villous projections that extend towards the maternal basal plate. By the third week of gestation tertiary villi have formed consisting of an outer monolayer of syncytiotrophoblast, invaded by an inner layer of cytotrophoblast cells and vascularised with fetal capillaries (Huppertz, 2008). These tertiary villi will eventually form the site of nutrient exchange (Georgiades *et al.*, 2002). Villi that become attached to the basal plate are called anchoring villi.

A subset of cytotrophoblast cells differentiate to become extravillous trophoblast cells that invade the maternal spiral arteries, remodelling the coiled vessels to become wider, high flow, low resistance vessels. Extravillous trophoblast cells also form intra-arterial plugs to prevent maternal blood flow to the intervillous space until around the 12th week of gestation (Filant and Spencer, 2014; Rampersad *et al.*, 2011).

1.3.2 Placental structure at term

The mature placenta is discoidal and weighs approximately 520 g at term (trimmed of membranes and umbilical cord) (Hayward *et al.*, 2016). At the macroscopic level, the maternal side of the placenta (basal plate) consists of highly vascularised cotyledons (clustered branches of the villous network) surrounded by deep invaginations of tissue called septae (Figure 2). On the maternal side is the chorionic membrane and on the fetal side is the amnion which fills with amniotic fluid to ultimately surround the developing fetus within the amniotic sac (Rampersad *et al.*, 2011). The umbilical cord inserts into the chorionic plate (fetal side) of the placenta and consists of an umbilical vein (delivers oxygenated blood to the fetus) and two umbilical arteries (carrying deoxygenated blood) surrounded by gelatinous Wharton's jelly.

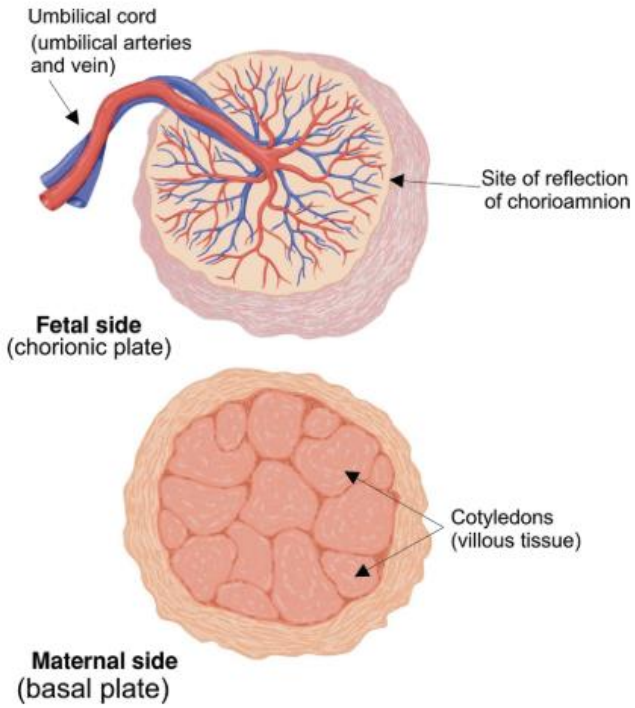


Figure 2: Gross morphology of the term human placenta
The fetal side (chorionic plate) of the placenta is the site of insertion of the umbilical cord. The basal plate (maternal side) of the placenta consists of a cotyledonary network, the site of nutrient exchange. Figure from (Rampersad *et al.*, 2011).

Placental development ensues as the outer chorionic sac develops chorionic villi, microvillous projections of fetal trophoblast cells that extend toward the maternal basal plate (Figure 3) (Aplin, 1991; Rampersad *et al.*, 2011). The exterior villous surface constitutes a bilayer of trophoblastic cells derived from trophoblastic proliferation, fusion and subsequent loss of plasma membranes to form a highly specialised multinucleated syncytium (Enders, 1965b). A monolayer of syncytiotrophoblast is in direct contact with maternal blood, and is the site of nutrient and gas exchange between mother and fetus, supported by an inner layer of mononuclear cytotrophoblasts (Georgiades *et al.*, 2002). The syncytiotrophoblast has an apical maternal-facing microvillous plasma membrane (MVM) and a fetal-facing basal plasma membrane (BM) and is proximal to the fetal capillary endothelium (Desforges and Sibley, 2010). Together, the syncytiotrophoblast and fetal endothelium constitute the placental exchange barrier. Placental stability is supported throughout pregnancy by anchoring villi that branch out to initiate contact with the uterine decidua and are subsequently encapsulated by fibrinoid material (Enders, 1989; Enders and Blankenship, 1999) The syncytiotrophoblast is maintained and renewed by the systematic replacement of the syncytiotrophoblast layer by the incorporation of mononucleate cytotrophoblasts (Aplin, 1991; Mayhew, 2014). Maintenance of the syncytiotrophoblast, by cellular turnover, is key to normal pregnancy and is abnormal in FGR.

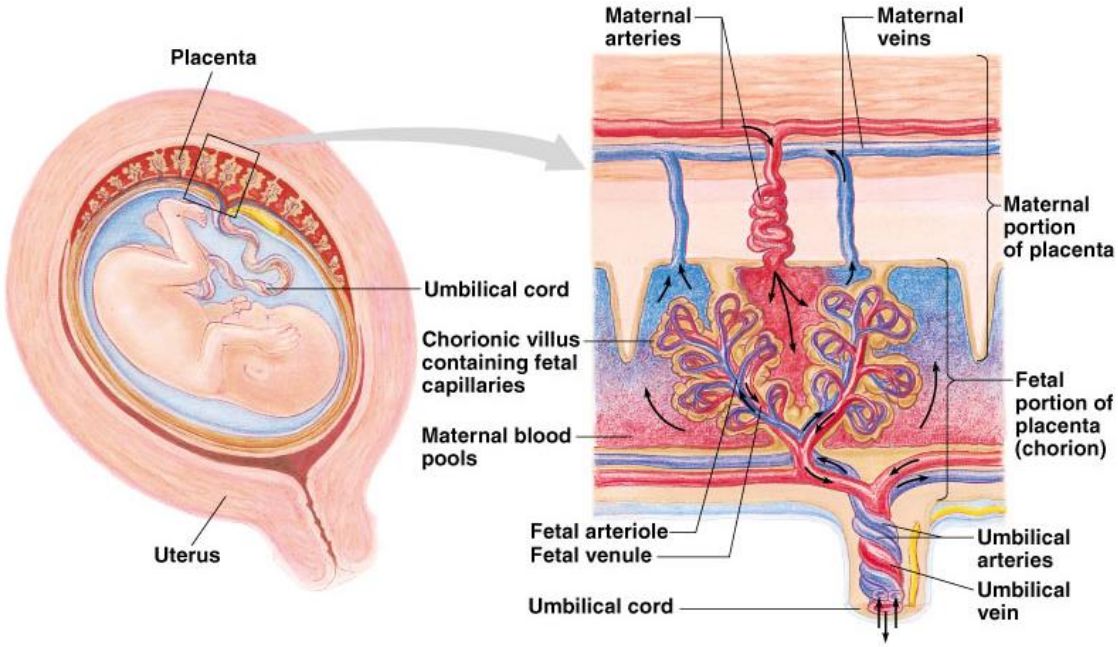


Figure 3: Human placental structure at term depicting the chorionic villi
The fetus is connected to the placenta via the umbilical cord, which has two umbilical arteries and one umbilical vein. Chorionic villi (the villous tree) project from the fetal portion of the placenta. At term the villi are surrounded by a multinucleate syncytiotrophoblast layer and are bathed in maternal blood (intervillous space). Figure is from http://www.zo.utexas.edu/faculty/sjasper/images/46.17.jpg Accessed 25th June 2018.

1.3.3 The placenta in FGR

Both early- and late-onset FGR is associated with a small placenta for gestational age (Mifsud and Sebire, 2014; Silver, 2018). The small placenta in FGR is associated with abnormal angiogenesis/vasculogenesis and development of the placental villous tree (Burton *et al.*, 2009; Mayhew *et al.*, 2004), and abnormal regulation of placental vessel tone which collectively contribute to raised feto-placental vascular resistance and inadequate blood flow between placenta and fetus; this is evident clinically by abnormal umbilical artery (UmA) Doppler waveforms (Baschat, 2011; Giles *et al.*, 1985). Altered villous morphology is accompanied by dysregulated renewal of the syncytiotrophoblast by cellular turnover (Huppertz *et al.*, 2002; Huppertz *et al.*, 2006) and altered nutrient transporter activity and expression in syncytiotrophoblast (Aiko *et al.*, 2014; Glazier *et al.*, 1997; Jansson *et al.*, 1998; Mahendran *et al.*, 1993; Shibata *et al.*, 2008) (discussed further in section 1.10).

The origin of FGR is not clear but it is proposed to arise from failure of the extravillous trophoblasts to adequately invade and remodel the spiral arteries in the first trimester (Baschat, 2011; Bower *et al.*, 1993). Instead of conversion to low resistance high flow vessels, the arteries retain tone, which reduces blood flow and therefore the delivery of oxygen to the intervillous space; the raised vascular resistance can be detected clinically by abnormal uterine artery (UtA) Doppler waveforms. As the spiral arteries retain some contractile ability, the pattern of blood flow to the placenta is altered leading to ischaemia-reperfusion injury (Burton and Jauniaux, 2004; Hung *et al.*, 2001). The consequent hypoxia, inflammation, oxidative and

nitrative stress arising from inadequate and dysregulated blood flow to the intervillous space is thought to underlie abnormal placental vascular development and dysregulated syncytiotrophoblast turnover (Burton and Jauniaux, 2018; Scifres and Nelson, 2009).

Doppler velocimetry scanning is used in the clinic to detect abnormal umbilical and/or uterine blood flow-velocity waveforms which indicate functional and/or structural vascular defects that collectively increase resistance to flow in FGR (Bower *et al.*, 1993). UtA Doppler measurements allow the assessment of blood flow abnormalities within the uteroplacental circulation, a proxy of the quality of the physiological remodelling of the uterine spiral arteries (Figure 4). Though not routinely measured in the clinic, women with three or more minor risk factors for FGR, such as maternal smoking, age >35 years and body mass index (BMI) >30 kg/m^2, are referred for UtA Doppler velocimetry as a screening tool at 20-24 weeks of gestation (Royal College of Obstetricians and Gynaecologists, 2013).

UmA Doppler, which measures blood flow velocity from the fetus back towards the placenta (Savasan *et al.*, 2014) is measured if fetal growth trajectory is identified as abnormal. Early-onset FGR typically presents with an abnormal UmA Doppler waveform (Figure 4). Due to the early gestational age in these cases, serial monitoring can guide expectant management of the pregnancy (Royal College of Obstetricians and Gynaecologists, 2013). In late-onset FGR, fetal growth rate falls towards term and typically presents with normal UmA Dopplers (Whitehead *et al.*, 2016). This fall in fetal growth rate can be detected in the clinic by growth scans or by measuring middle cerebral artery Doppler or fetal heart rate monitoring (Baschat, 2011), but often FGR is only confirmed by determining IBR post-delivery.

FGR is therefore a consequence of a number of differing placental phenotypes (Sibley *et al.*, 2005) and categorising them according to early or late-onset FGR is over simplistic. Rather, it is likely to be a sliding scale with placental pathologies, such as abnormal Doppler waveform indices and nutrient transfer defects, overlapping and contributing both early- and late-onset FGR (Mifsud and Sebire, 2014).

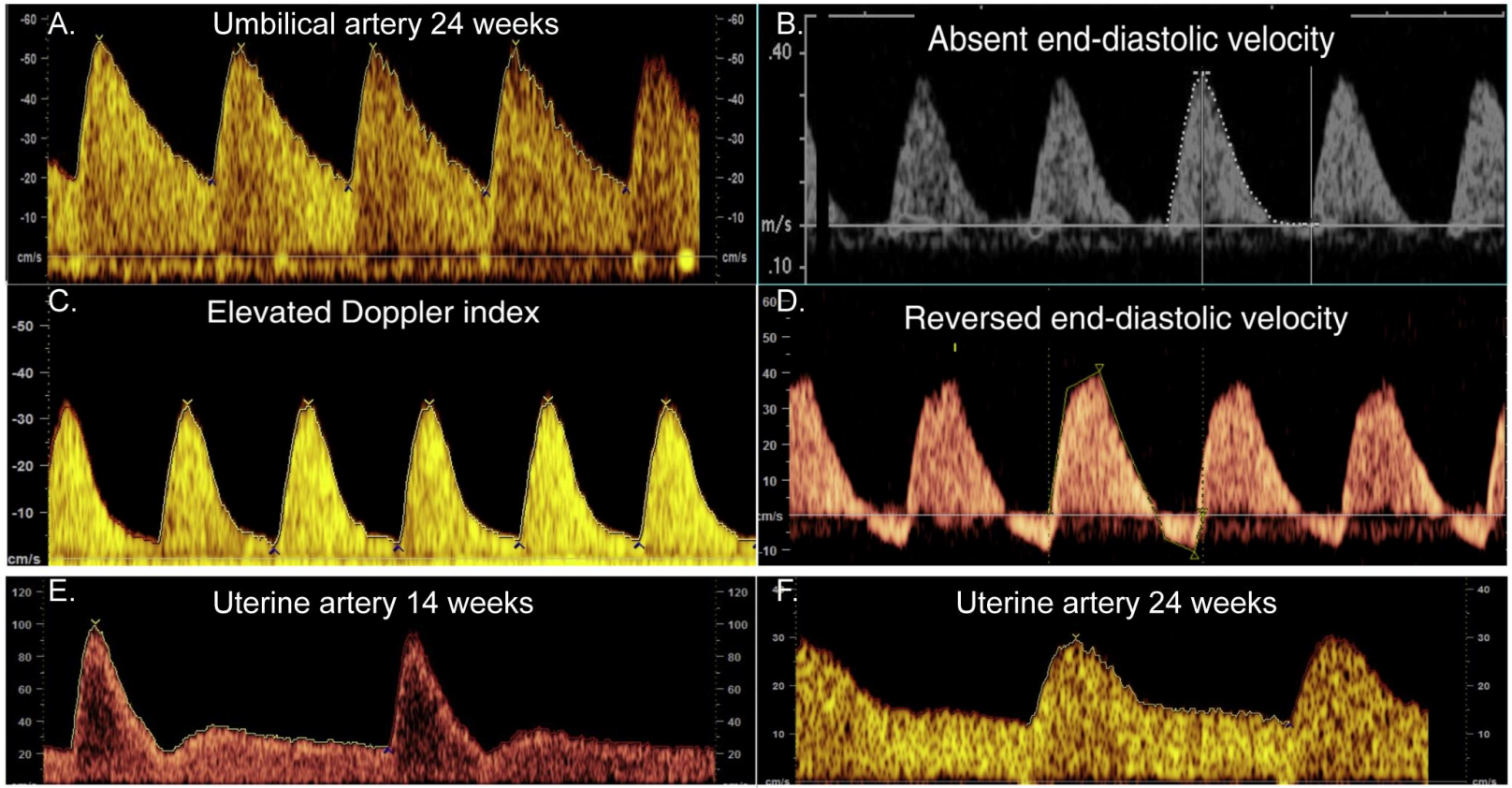


Figure 4 Umbilical (UmA) and uterine artery (UtA) Doppler waveforms in normal pregnancy and pregnancy complications
Normal UmA waveform at 24 weeks gestation (A). In FGR pregnancies, where the fetal villous vascular tree is abnormal, the UmA Doppler index is increased (C) (i.e. the minimum diastolic velocity, the trough in the waveform, is reduced). Abnormalities can also lead to absent (B) or reversed (D) end diastolic velocity. The UtA waveform shows blood flow-velocity in the maternal vessels supplying the placenta. In normal pregnancy there is an early diastolic notch and a high ratio between the systolic (peak) and diastolic (trough) of the velocity of the UtA waveform at 14 weeks (E). By 24 weeks in normal pregnancy (F) the notch and high pulsatility are lost. This transformation will not happen if trophoblast invasion into the spiral arteries is inadequate, as seen in some cases of FGR. Adapted from (Baschat, 2011)

1.4 Animal models of human pregnancy

Many animal species have been used as models to enhance our understanding of human pregnancy such as the rat, mouse, guinea pig, sheep and non-human primate. Animal models can be useful to examine the effects of specific interventions on pregnancy outcome and placental function and, with regards to the focus of this thesis, can be used to measure placental nutrient transport *in vivo*. Placentation is species-specific thus there is no perfect animal model for human placental development and function, and selection of an appropriate model is dependent upon the research question. Several animal models of human pregnancy are considered in the section herein.

1.4.1 Sheep

Sheep are used as a model for human pregnancy since they deliver precocious lambs of a similar size to human babies at term. However, sheep have disadvantages as a model of human placentation since the sheep placenta is structurally distinct. Unlike the human placenta, the sheep placenta is not invasive, rather the fetal membranes (cotyledons) attach to caruncles present in the sheep uterus to form individual placentomes (Filant and Spencer, 2014). The diffusional passive permeability of hydrophilic molecules across the placenta is dependent upon placental surface area and barrier thickness and can be measured as permeability × surface area product (P×S) (Sibley and Boyd, 1988). The P×S of the epitheliochorial sheep placenta is much lower than that of humans and rodents (Knobil and Neill, 2006; Sibley and Boyd, 1988), which does not make it an ideal model for nutrient transport studies. Sheep are used widely for cardiovascular and hypoxia research in pregnancy (Allison *et al.*, 2016). FGR can be experimentally induced in the sheep by over-nourishment in adolescence or following hyperthermic exposure during gestation (Wallace *et al.*, 2005).

1.4.2 Non-human primate

Non-human primates (NHP), such as the baboon and rhesus macaque are used in medical research, predominantly in the United States. The NHP placenta superficially invades in comparison to the human placenta but it is similar in that it is haemomonochorial in structure (Grigsby, 2016). NHPs have been used to investigate the spread of Zika virus (Kublin and Whitney, 2018), and there is a well-established maternal nutrient restriction model of FGR in the baboon (Kavitha *et al.*, 2014; Pantham *et al.*, 2015; Pantham *et al.*, 2016). The use of NHPs in medical research is controversial, expensive and as such represents a very small proportion of scientific procedures involving animals in the UK (0.16% in 2005 – 4,652 procedures carried out on 3,115 animals) (The Royal Academy of Medical Sciences, 2006).

1.4.3 Guinea pig

The guinea pig has been used as a model of human placental nutrient transport and FGR (Jansson and Persson, 1990; Kind *et al.*, 2005). Like humans, guinea pigs have a haemomonochorial placenta (Enders, 1965a) that invades deep into the uterine arteries (Clausen *et al.*, 2003), but they also have a subplacenta (Rodrigues *et al.*, 2006), for which there is no functional equivalent in humans (Carter, 2007). In common with rats and mice, but unlike humans, guinea pigs also retain a functional yolk sac until term (Carter, 2007).

1.4.4 Rodent models of human pregnancy: mice and rats

Rodents are commonly used in pregnancy research and have the practical advantage of a short gestational period (mice 19-21 days; rats 21-24 days) and large litter sizes (Dilworth and Sibley, 2013). This allows relatively quick data collection and matched analyses between littermates within the same maternal environment, increasing statistical power. However, this is at odds with the longer gestational length and propensity towards singleton pregnancy in humans.

In common with humans, the mouse and rat placenta are haemochorial, such that the syncytiotrophoblast is in direct contact with maternal blood (Dilworth and Sibley, 2013). However, in contrast to the haemomonochorial (single layered) human placenta the murine placenta has three layers and is therefore haemotrichorial. Despite this structural difference, the P×S of the mouse, rat and human placenta is within the same order of magnitude (Knobil and Neill, 2006; Sibley and Boyd, 1988). A method is available to isolate pure vesicles of layer II of the rat and mouse placenta (discussed in more detail in section 1.10.2) (Kusinski *et al.*, 2010), which is akin to the maternal-facing MVM of the human placenta (Glazier and Sibley, 2006).

Pregnant rats are a well-established animal model of human pregnancy (Winterhager and Gellhaus, 2017). Like the mouse, the rat placenta is haemotrichorial, but unlike the mouse there is deep trophoblast invasion and remodelling of the spiral arteries (Soares *et al.*, 2012). There is also potential to draw multiple blood samples from the dam due to the larger blood volume in the rat in comparison to the mouse (NC3RS, 2014b). However, genetic modifications remain difficult in this species and are outweighed by those available in the mouse (Jacob *et al.*, 2010). Technological advances and the sequencing of the mouse genome have led to the production of genetic knockout and transgenic mice, which will likely increase with the recent advent of CRISPR-Cas9 techniques (Kherraf *et al.*, 2018). Several well-characterised genetic knockout mouse strains now exist to model pregnancy complications in humans (section 1.11).

The mouse was chosen as an appropriate animal model to address the aims of this thesis following appraisal of the relative costs and benefits of available animal models outlined in Table 3. Primarily mice were chosen due to their haemochorial placental structure, and the

knowledge that the localisation and activity of amino acid transporters studied to date resembles that in the human placenta (Enders, 1965; Georgiades *et al.*, 2002; Jansson *et al.*, 2002; Kusinski *et al.*, 2010; Takata and Hirano, 1997). For example, GLUT1 glucose transporter is expressed on the placental plasma membranes of mice and women (Constância *et al.*, 2005). Activity of the system A amino acid transporter, which is responsible for the transport of small neutral amino acids such as glutamine, is also similar between vesicles of the mouse and human placenta (per g placenta) (Kusinski *et al.*, 2010). However activity of system β, which transports taurine, is lower in the mouse placenta (Kusinski *et al.*, 2010), which highlights a need to directly compare other nutrient transporter systems to fully evaluate the similarities and differences between the two species.

Animal models of poor pregnancy outcome can be broadly characterised as surgical, genetic or environmental (e.g. dietary intervention). A genetic knockout mouse model of FGR was utilised in the current study for comparison with human late-onset FGR and is fully appraised with other mouse models of FGR in section 1.11. The similarities and differences between the mouse and human placenta are appraised in greater depth in the sections that follow.

	Mouse	Rat	Guinea pig	Sheep	Non-human primate
Gestation (days)	19-21	21-24	59-72	~152	~180
Litter size	Multiple, 6-12	Multiple, 10-14	Multiple, 2-5	Singleton/twins	Singleton/twins
Genetic manipulation	Relatively easy	Moderately difficult	Difficult	Difficult	Difficult
***In utero* development**	Altricial	Altricial	Precocial	Precocial	Precocial
Placentation	Haemotrichorial	Haemotrichorial	Haemomonochorial (a subplacenta also exists)	Epitheliochorial (placentomes)	Haemomonochorial
Placental transfer (versus human)	Similar for some solutes	Similar for some solutes	Not well described	Less permeable to solutes	Similar

Table 3: Animal models of human pregnancy

Common animal models of human pregnancy and their advantages/disadvantages. Data collated from (Carter, 2007; Jansson *et al.*, 2006; Knobil and Neill, 2006; Sferruzzi-Perri and Camm, 2016)

1.5 Structure and function of the mouse placenta

Both the human and the mouse placenta are haemochorial as the outer trophoblast layer of the mature placenta is directly bathed in maternal blood (Enders, 1965a; Watson and Cross, 2005). Mouse gestation lasts between 19-21 days, with dams giving birth to altricial young at term. Thus a number of 'fetal' developmental processes (e.g. nephrogenesis) continue postnatally. In contrast, term for human pregnancy is 259 days and women give birth to more developed (precocial) offspring (Carter, 2007).

Placental development in the mouse begins following the establishment of a blastocyst at approximately embryonic day (E) 3.5 (Cross *et al.*, 1994). At the time of implantation (around E4.5) trophectoderm cells that surround the ICM proliferate and differentiate into the extra-embryonic ectoderm and the ectoplacental cone. Trophoblast invasion is shallower in mice compared with women (Carter, 2007). Post-implantation, giant cells derived from the ectoplacental cone surround the conceptus. Next the extra-embryonic ectoderm forms the chorionic epithelium and meets the allantois at E8.5 to form the chorioallantois (Cross *et al.*, 1994; Rossant and Cross, 2001). The fetal portion of the mouse placenta consists of two major and structurally distinct zones: the outer junctional zone, primarily associated with endocrine function, and the inner labyrinthine zone, which is the major site of nutrient exchange in the mouse placenta (Enders and Blankenship, 1999; Malassine *et al.*, 2003). The syncytiotrophoblast of the mouse placenta, evident in the labyrinth zone, consists of three layers: discontinuous (trophoblast giant cell) layer I, syncytial layer II and fetal facing layer III (Figure 5). Thus, the mouse placenta is defined as haemotrichorial in comparison with the human haemomonochorial placenta (Enders, 1965a). Layer II of the labyrinthine zone is thought to be akin to the human syncytiotrophoblast MVM: both stain positively with alkaline phosphatase and transporter proteins such as the GLUT1 glucose transporter have been localised to this layer (Enders, 1965a; Georgiades *et al.*, 2002; Jansson *et al.*, 2002; Takata and Hirano, 1997). Similarly, layer III has been compared to the human syncytiotrophoblast BM, although this has yet to be definitively confirmed due to difficulties facing layer III isolation and characterisation in the mouse (Dilworth and Sibley, 2013). These additional layers in the mouse placenta are not thought to have a major impact on passive permeability due to the similarities in P×S versus human (Knobil and Neill, 2006; Sibley and Boyd, 1988).

Another difference between mouse and human is that the intraplacental yolk sac (IPYS), exclusive to rodents, is thought to play a role in nutrient transport until term. Proteins important for calcium transport such as calbindin-D_{9k} have been localized to the IPYS (Kovacs *et al.*, 2002). In a key study reported by Croy *et al.* (2015) the vitelline artery, which supplies the IPYS, was ligated at various gestational ages to investigate the role of the IPYS in supporting fetal growth. Ligation at E12.5 or E13.5 was embryonically lethal whereas 43% of

fetuses survived to E17.5 when the artery was ligated at E14.5. Whilst all fetuses survived ligation at E15.5 and E16.5, they were growth restricted indicating that the IPYS contributes to normal fetal growth until term.

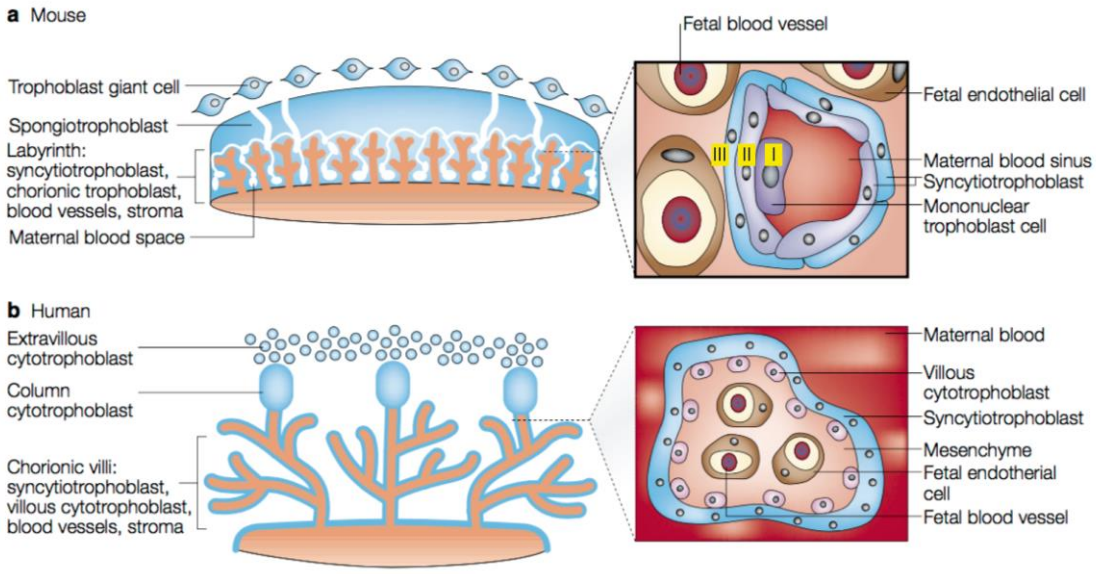


Figure 5: Diagram illustrating the anatomy of the mouse and human placenta
a The labyrinthine layer of the mouse placenta (inset) has three layers (labelled I, II and III, yellow boxes) **b** The chorionic villi of the human placenta are bathed directly in maternal blood. Inset shows a cross-section through a villous. Figure adapted from (Rossant and Cross, 2001).

Genetic knockout and transgenic mice afford the capacity to study the effects of specific genes on placental function and pregnancy outcome. It is also now possible to target the knockout (deletion) of genes within specific tissues. In the field of pregnancy research this provides the exciting opportunity to determine the relative contribution of the fetus or placenta to pregnancy outcomes. The use of mice alongside human studies may facilitate further understanding of the physiology both in normal human pregnancy and in pregnancy pathologies. In addition, mice provide an *in vivo* model in which to test the effectiveness and safety of therapeutic interventions for complications of pregnancy (Dilworth and Sibley, 2013).

1.6 Placental nutrient transport

Exchange of nutrients and respiratory gases between mother and fetus occurs via the placenta during pregnancy. As introduced above, in the human placenta the MVM and BM represent the maternal and fetal-facing barriers of the syncytiotrophoblast. As such, any solutes destined for maternofetal transfer must enter the syncytiotrophoblast cytoplasm from the maternal circulation via the MVM and exit via the BM before finally breaching the fetal capillary endothelium to enter the fetal circulation (Lager and Powell, 2012). There are a number of

different mechanisms by which nutrient exchange across the placental MVM and BM is mediated. These include diffusion, facilitated diffusion, active transport, exchange and co-transport (Figure 6). Endocytosis/exocytosis and paracellular routes also exist (reviewed by Desforges and Sibley, 2010). Diffusion across the syncytiotrophoblast is dependent upon molecular size, charge, solubility and concentration gradient. Oxygen and other respiratory gases diffuse across the syncytiotrophoblast, a process which is blood flow limited: net maternofetal transfer relies upon a concentration gradient down which diffusion occurs (Desforges and Sibley, 2010).

Specific carriers and channels exist on the MVM and BM of the syncytiotrophoblast to mediate the transport of ions, amino acids and other nutrients e.g. glucose and calcium. Ion transport is important for maintenance of electrochemical gradients to support other transport processes such as sodium (Na^+)-co transport. Na^+/K^+/ATPase, present on the MVM and BM (Johansson *et al.*, 2000), actively exchanges Na^+ for potassium (K^+), using ATP as an energy source (Clarson *et al.*, 1996). This action creates a Na^+ gradient across the trophoblast cell membrane (low intracellular Na^+ and high intracellular K^+) (Greenwood *et al.*, 1996) thus facilitating the accumulation of nutrients, via Na^+-dependent mechanisms, required for fetal growth. The Na^+/H^+ exchanger (NHE) is localised predominantly to the MVM (10-fold greater expression than the BM). NHE couples Na^+ influx (into the syncytiotrophoblast) with H^+ efflux creating a transmembrane Na^+ gradient that is exploited by a number of amino acid transporters (Desforges and Sibley, 2010; Johansson *et al.*, 2002). In FGR, NHE activity is reduced (Johansson *et al.*, 2002) which could alter the acid-base status of the syncytiotrophoblast causing the cytosolic pH to become acidic. Activity of Na^+/K^+/ATPase is also reduced in FGR (Johansson *et al.*, 2003) which likely leads to dysfunctional provision of nutrients to the fetus that utilise Na^+-dependent transporters. Indeed, experimental inhibition of Na^+/K^+/ATPase with ouabain inhibits uptake of the non-metabolisable analogue methylaminoisobutyric acid (MeAIB) via Na^+-dependent system A in placental villous tissue fragments (Shibata *et al.*, 2006).

Glucose is transferred across the placenta by facilitated diffusion, via glucose-specific GLUT transporters (GLUT1, GLUT3 and GLUT4), and is an important energy source for the fetus. GLUT3 and GLUT4 expression is low towards term indicating that GLUT1 (localised to the MVM and BM) is the primary isoform responsible for glucose transport in late pregnancy (Ericsson *et al.*, 2005). Nutrients such as calcium pass from maternal to fetal blood via both diffusional (ion channels) and active transport processes, using ATP as an energy source, and require buffering within the syncytiotrophoblast by calcium binding proteins including those of the calbindin family (Bond *et al.*, 2008; Glazier *et al.*, 1992). Many amino acids utilise ion gradients to mediate their transfer (e.g. system A and N-mediated glutamine and system X_{AG}-

mediated glutamate transport utilise a Na^+ gradient) (Desforges and Sibley, 2010; Lager and Powell, 2012). This thesis focuses on transport of the amino acids glutamine and glutamate; therefore the following sections shall consider placental amino acid transport, including regulatory processes, in further detail.

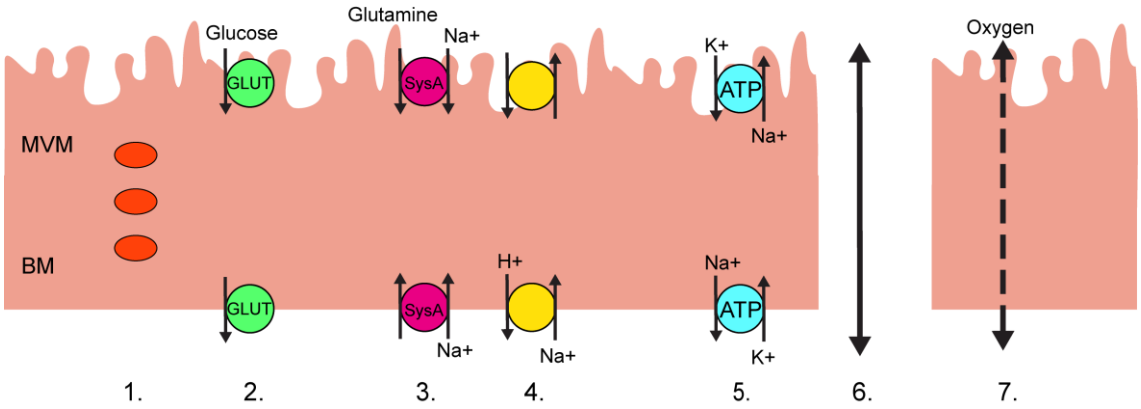


Figure 6: Mechanisms of transfer of nutrients across the microvillous membrane (MVM) and basal membrane (BM) of the syncytiotrophoblast.
Nutrients and gases are transferred across the transporting epithelium of the placenta, the syncytiotrophoblast, via mechanisms such as simple diffusion (7) and facilitated diffusion (2) (e.g. glucose via GLUT transporter proteins). Transporter-mediated mechanisms include co-transport (3) (e.g. system A amino acid transporter, responsible in part for glutamine transport), exchange (4) and active transport (5). Paracellular (6) and endocytosis/exocytosis (1) routes also exist.

1.6.1 Amino acid transport

Several amino acid transporter systems analogous to those present in other epithelia have been characterised in the placenta (syncytiotrophoblast) and localised to either the MVM, BM or both. In normal pregnancy, the concentration of most amino acids in fetal plasma is higher than in maternal plasma (Cetin *et al.*, 1996; Neerhof and Thaete, 2008). Maternofetal amino acid transfer therefore operates against a concentration gradient. Broadly, amino acid transporters are classified according to whether they are Na^+-dependent or Na^+-independent (Desforges and Sibley, 2010). Na^+-dependent transporter systems harness the energy of the electrochemical gradient generated by Na^+/K^+/ATPase. Amino acid transporters are further characterised as co-transporters, exchangers or accumulative transporters (Table 4) (Cleal and Lewis, 2008; Lager and Powell, 2012).

Gene name (protein)	System and mechanism	Substrates	Localisation
SLC38A1 (SNAT1) SLC38A2 (SNAT2) SLC38A4 (SNAT4)	A Na^+ dependent cotransport	**Gln**, Ala, Asn, Cys, His, Ser, MeAIB Ala, Asn, Cys, **Gln**, Gly, His, Pro Ser, MeAIB Ala, Asn, Cys, Gly, Ser, Thr, MeAIB	mRNA, activity and protein on MVM, BM
SLC7A10 (asc1)	asc Exchanger	Gly, Ala, Pro, Ser, Thr, Cys, Met, Leu, Iso, Val, Phe, His	mRNA, no activity on BM
SLC1A4 (ASCT1) SLC1A5 (ASCT2)	ASC Na^+ dependent exchanger	Ala, Ser, Cys, Thr Ala, Cys, **Gln**, Ser, Thr, Asn	mRNA, activity on BM
SLC7A9 ($b^{o,+}$AT)	$b^{o,+}$ Exchanger, Requires rBAT (SLC3A1)	Arg, His, Lys, Phe, Tyr, Trp, Thr, Met, Val, Iso, Leu, His	mRNA, activity inconclusive
SLC7A1 (CAT1) SLC7A2 (CAT2B) SLC7A3P (CAT3) SLC7A4 (CAT4)	y^+ Uniporter	Arg, His, Lys	mRNA, activity on MVM and BM, CAT1 protein on BM
SLC7A5 (LAT1) SLC7A8 (LAT2)	L Na^+ independent exchanger Requires CD98/4F2hc (SLC2A2)	(**Gln**) His, Met, Leu, Iso, Val, Phe, Tyr, Trp, Thr, BCH Ala, Ser, Cys, Thr, Asn, **Gln,** His, Met, Leu, Iso, Val, Phe, Tyr, Trp	mRNA, LAT2 activity on MVM, LAT1 activity on BM, both proteins on MVM and BM
SLC43A1 (LAT3) SLC43A2 (LAT4)	Facilitated diffusion	Leu, Iso, Val, Phe, Met, BCH	mRNA, activity and protein on BM
SLC38A3 (SNAT3) SLC38A5 (SNAT5)	N Na^+/AA cotransport H^+ antiport	**Gln**, His, Ala, Asn **Gln**, His, Asn, Ser	mRNA; activity and protein on MVM
SLC16A10 (TAT1)	TAT1 Facilitated diffusion	Phe, Trp, Tyr, Ala, Leu	mRNA, activity and protein on BM
SLC6A6 (TauT)	TauT; Na^+/Cl- dependent cotransport	β-Ala; Tau	mRNA, activity and protein on MVM, also on BM
SLC1A1 (EAAT3) SLC1A2 (EAAT2) SLC1A3 (EAAT1)	X_{AG^-}; $3Na^+/1H^+/A$ A cotransport/1 K^+ exchange	Asp, **Glu** Asp, **Glu** Asp, **Glu**	mRNA; activity on MVM and BM, protein also expressed
SLC7A6 (y+LAT2) SLC7A7 (y+LAT1)	y^+L; Na dependent exchange	Lys, Arg, **Gln**, His, Met, Leu Lys, Arg, **Gln**, His, Met, Leu, Ala, Cys	mRNA; activity on MVM and BM

Table 4 Amino acid transporters in the placenta

Amino acid transporter systems on the MVM and BM of the placenta. Amino acids are listed using their three letter abbreviation. Adapted from (Cleal *et al.*, 2011; Cleal *et al.*, 2018; Cleal and Lewis, 2008; Desforges *et al.*, 2009; Jansson, 2001)

The fetus grows at a faster rate than the placenta in the third trimester (Figure 7) such that birth weight:placental weight (BW:PW) ratio steadily increases as gestation progresses, or if expressed as PW:BW ratio, decreases from an average of 0.41 at 23-24 weeks to 0.19 at 39-40 weeks (Haavaldsen *et al.*, 2013). Thus, towards term there is an increase in the abundance and activity of transporters (per g placenta) relative to first trimester, in order to meet fetal nutrient demand and maintain appropriate fetal growth (e.g. system A, important for the transport of

small neutral amino acids including glutamine, and plasma membrane calcium ATPase (PMCA), responsible for efflux of calcium from syncytiotrophoblast to fetus across the BM) (Desforges *et al.*, 2009; Mahendran *et al.*, 1994; Strid and Powell, 2000).

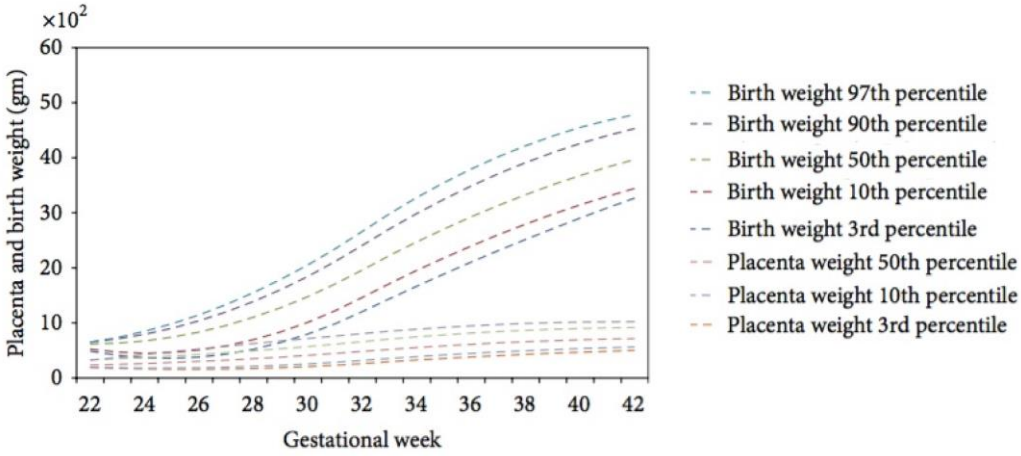


Figure 7: Birth weight and placental weight percentiles of male infants by gestational age (22-42 weeks) The rate of increase in placental weight between 22 and 42 weeks gestation is far exceeded by the rate of fetal growth over the same time period. The placenta must therefore increase its capacity (per g) to supply nutrients to the fetus to support the gain in fetal weight towards term. Figure adapted from (Macdonald *et al.*, 2014)

1.7 Glutamine and glutamate are essential for fetal development and growth

Glutamine is a non-essential amino acid that is termed conditionally essential in pregnancy due to its requirements for fetal growth that exceed dietary intake (Parimi and Kalhan, 2007). Glutamine is transported to the fetal plasma by the placenta at one of the highest rates of all amino acids (Battaglia, 2000). Many essential cell processes such as nucleotide synthesis, pH homeostasis and gluconeogenesis require glutamine (Pochini *et al.*, 2014). In most tissues glutamine is the most abundant extracellular amino acid *in vivo* (concentrations range between 0.2-0.8 mM extracellular and 2-20 mM intracellular), and glutamate the most abundant intracellular amino acid (2-20 mM; see Figure 8 for concentrations in the placenta) (Parimi and Kalhan, 2007; Pochini *et al.*, 2014). Glutamate is a precursor of glutamine and other molecules such as γ-amino butyric acid (GABA), glutathione and 2-oxoglutarate. Glutamate is also a neurotransmitter (Olsen and Sonnewald, 2015) and fetal plasma levels need to be carefully controlled during the development of the fetal brain since high levels of glutamate are neurotoxic (Tian *et al.*, 2012). It has been demonstrated in several species (sheep, pig, non-human primate, human) (Battaglia, 2002; Day *et al.*, 2013; Pitkin *et al.*, 1979; Self *et al.*, 2004; Vaughn *et al.*, 1995) that there is very little net glutamate transfer from maternal to fetal blood across the placenta.

Glutamine is transported across the MVM of the syncytiotrophoblast by systems A, L and N (Table 4); systems A and L also mediate uptake from the fetal blood across the BM

(Desforges and Sibley, 2010; Johnson and Smith, 1988). Although important in other tissues, system y^+L does not contribute to glutamine uptake into the placenta (Hill *et al.*, 2014). Glutamate transport from maternal and fetal blood into the placental syncytiotrophoblast is mediated by system X_{AG-} transporters expressed on the MVM and BM (Hill *et al.*, 2014; Novak *et al.*, 2001) (Table 4). Recent studies have shown that glutamate that has been taken up from the maternal or the fetal circulation can be rapidly exchanged for organic anions such as dehydroepiandrosterone-3-sulphate (DHEAS) via OAT4 and OATP2B1 transporters (see section 1.7.2) (Lofthouse *et al.*, 2015).

Glutamate that accumulates in the placenta is primarily utilised for metabolism (Day *et al.*, 2013; Holzman *et al.*, 1979; Schneider *et al.*, 1979). Previous research in the sheep, pig and guinea pig placenta have indicated that the placenta synthesises glutamine from glutamate (Bloxam *et al.*, 1981; Chung *et al.*, 1998; Self *et al.*, 2004). Early studies in the pregnant rhesus monkey infused radiolabelled glutamate into the maternal circulation and sequentially sampled fetal and maternal plasma to assess placental glutamate transport and metabolism. Stegink *et al.* (1975) demonstrated that the majority of glutamate infused into maternal circulation (69-88%) remained associated with the radiolabel, whereas in fetal plasma the radiolabel was associated with metabolites such as glucose and lactate, and there was little to no radiolabelled glutamate evident. Studies in the perfused human placenta have indicated that as well as glutamine, glutamate is also metabolised to other metabolites such as such as α-ketoglutarate, for entry to the tricarboxylic acid (TCA) cycle, and proline (Day *et al.*, 2013).

During pregnancy, recycling of glutamine and glutamate occurs between the placenta and fetal liver (Figure 8). Whilst there is net release of glutamine from the placenta to the maternal circulation (Self *et al.*, 2004), which is perhaps a strategy to deliver nitrogen generated by placental metabolic processes to the mother, amide nitrogen is also delivered to the fetal liver in the form of glutamine where it is converted to glutamate instead of entering gluconeogenesis as occurs postnatally (Vaughn *et al.*, 1995). Studies in sheep have shown that approximately 45% of glutamine available in fetal plasma is deaminated to glutamate by the fetal liver (Moores *et al.*, 1994; Vaughn *et al.*, 1995). Metabolism of glutamine and glutamate is predominantly mediated by the action of the enzymes glutaminase and glutamine synthetase that catalyses the conversion of glutamine to glutamate, and the synthesis of glutamine from glutamate and ammonia, respectively. Fetal hepatic efflux of glutamate is directly correlated with glutamine influx (Vaughn *et al.*, 1995); 80-90% of this glutamate is taken up by the placenta by high affinity system X_{AG-} transporters on the placental BM (Battaglia, 2000; Noorlander *et al.*, 2004; Schneider *et al.*, 1979). A proportion of glutamate (approximately 6%) taken up from fetal plasma is re-converted to glutamine by the placenta (Figure 8) (Moores *et*

al., 1994). The fetal requirement for glutamate is therefore met by conversion of glutamine in the fetal liver, and not by transfer across the placenta from maternal blood (Day *et al.*, 2013). Impaired provision of glutamine, because of reduced uptake across the MVM of the syncytiotrophoblast, could not only impact fetal levels of glutamine, but also fetal glutamate concentration due to decreased conversion in the fetal liver. Likewise, any reduction in glutamate uptake into the syncytiotrophoblast (from maternal blood) could ultimately impact on levels of glutamate in the fetus, since this would limit conversion to glutamine within the syncytiotrophoblast thereby lowering glutamine delivery to the fetus for subsequent conversion to glutamate in the liver.

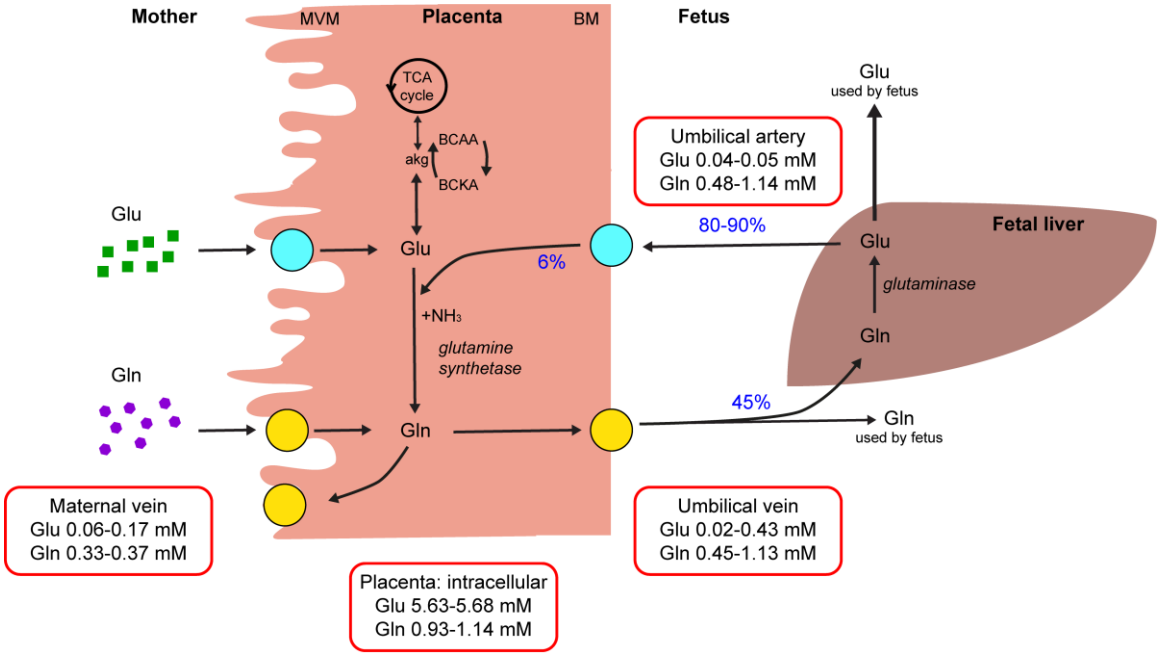


Figure 8 Schematic diagram illustrating glutamine (Gln) and glutamate (Glu) levels in the maternal and fetal compartments, and exchange between the placenta and fetal liver
Glutamine (Gln) is transported into the placenta from maternal blood and effluxes to the fetus via the umbilical vein. Gln is either utilised by the fetus or metabolised in the fetal liver (45%) to glutamate (Glu). 80-90% of Glu made by the fetal liver is transported back into the placenta; 6% of this Glu is metabolised back to Gln which is then either transported back to the fetus or into maternal circulation (see text for details). Glu and Gln levels in the placenta, maternal vein and umbilical vein and artery are from previously published studies (Camelo *et al.*, 2004; Cetin *et al.*, 2005; Holm *et al.*, 2017; Philipps *et al.*, 1978; Steingrimsdóttir *et al.*, 1993). Ranges of the concentrations reported are shown. TCA: tricarboxylic acid; BCAA: branched chain amino acids; BCKA: branched chain α-keto acids; akg: α-ketoglutarate; NH_3: ammonia. Enzymes are written in italics.

1.7.1 Placental transport of glutamine

Glutamine is transported across the plasma membranes of the syncytiotrophoblast by several transport systems that have overlapping specificity for glutamine but distinct modes of transport (Pochini *et al.*, 2014). Glutamine has pleiotropic effects, which may explain why it is a substrate of several transporter families. Glutamine transport across the syncytiotrophoblast

is mediated by the Na^+-dependent systems A and N, and Na^+-independent system L (Table 4). Glutamine is also a substrate of systems y^+L and ASC (Cariappa *et al.*, 2003; Cleal and Lewis, 2008). However, the contribution of system y^+L in mediating glutamine transfer across the MVM is negligible (Hill *et al.*, 2014). The Na^+-dependent system ASC (alanine, serine, cysteine-preferring) has two isoforms ASCT1 (slc1a4) and ASCT2 (slc1a5). ASCT2 plays a critical role in cell growth by supporting exchanger transporters (e.g. LAT1) and is known to be highly expressed by cancerous cells (Reynolds *et al.*, 2014; Tennant *et al.*, 2009). Glutamine is a substrate for ASCT2 which is predominantly localised to the BM of the syncytiotrophoblast (Hoeltzli and Smith, 1989; Johnson and Smith, 1988) but has been identified by immunohistochemistry on the MVM (Aiko *et al.*, 2014). However, functional studies of isolated MVM and BM vesicles have reported system ASC activity on the BM only (Hoeltzli and Smith, 1989). The current study will consider transporter-mediated uptake across the MVM of the human placenta, rather than maternofetal transfer, thus system ASC will not be discussed in depth here. System A, N and L, which are known to contribute to glutamine transport across the MVM are considered in detail in the following subsections.

1.7.1.1 System A

The *SLC38* gene family consists of 11 membrane transporters, of which the most renowned are classified within systems A and N (section 1.7.1.2). System A was first identified in 1965 (Christensen *et al.*, 1965) as a transporter for alanine that could be competitively inhibited by the non-metabolisable specific analogue methylaminoisobutyric acid (MeAIB) (Pochini *et al.*, 2014). System A is Na^+-dependent and mediates amino acid uptake against a concentration gradient by utilising the energy of Na^+/K^+/ATPase. The system A family consists of three isoforms, gene (protein): slc38a1 (Sodium-coupled Neutral Amino acid Transporter (SNAT)1), slc38a2 (SNAT2) and slc38a4 (SNAT4) (Mackenzie and Erickson, 2004). All three isoforms have been localised to both the MVM and BM of the placental syncytiotrophoblast (Figure 9) although they are highly polarised to the MVM (Cleal and Lewis, 2008). System A transports small, zwitterionic, neutral amino acids with short, unbranched side chains (Desforges *et al.*, 2009).

The transporter proteins SNAT1 and SNAT2 are ubiquitous and are functionally similar (Desforges *et al.*, 2009; Pochini *et al.*, 2014). SNAT4 has been identified in the placentas of humans (Desforges *et al.*, 2006), rats (Novak *et al.*, 2006) and mice (Mizuno *et al.*, 2002; Smith *et al.*, 2003), having previously been thought to be liver-specific. SNAT4 activity and protein expression on the MVM is greater in first trimester placenta compared to term (approximately 70% and 33%, respectively) (Desforges *et al.*, 2009). However, SNAT4 is not considered to be a transporter of glutamine (Bröer, 2014). System A has been studied extensively in the

placenta, predominantly due to the utility of the specific non-metabolisable analogue MeAIB. There is substantial evidence indicating that placental system A activity is reduced in FGR and this is discussed in more detail later (section 1.10).

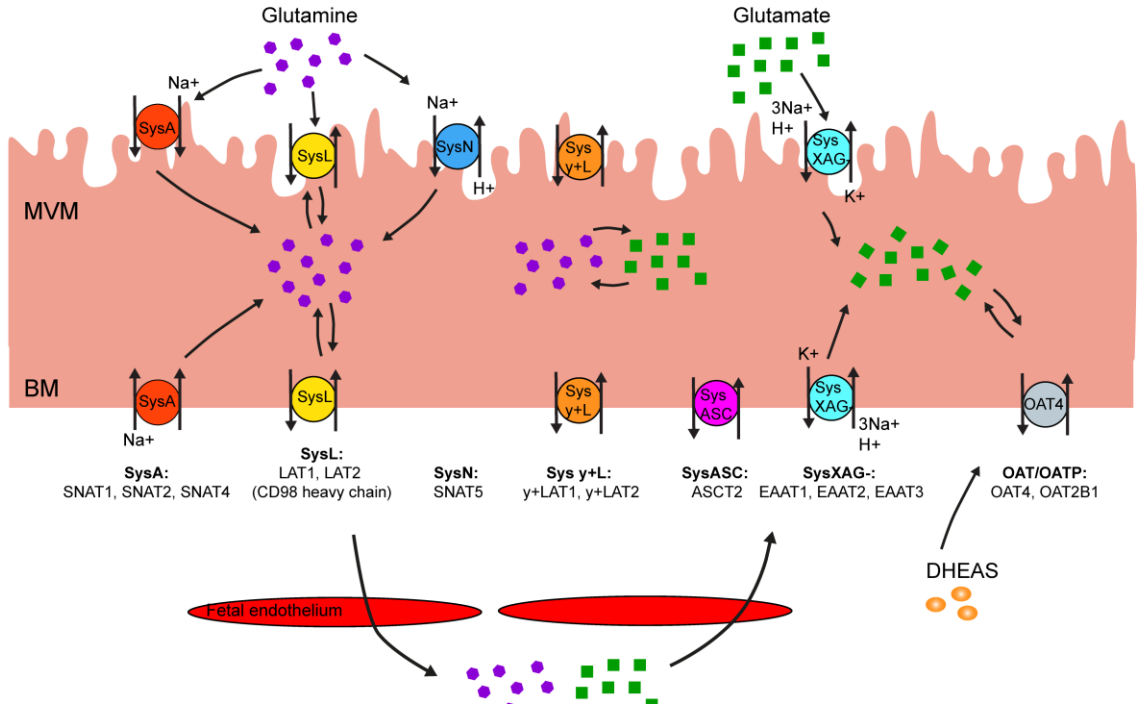


Figure 9: Localisation of glutamine and glutamate transporter systems in the human placental syncytiotrophoblast

Systems A, L and N mediate glutamine uptake from the maternal (MVM) and fetal (BM) circulations. Glutamine is also released into the maternal and fetal circulations via systems L (MVM and BM) and N (MVM only). Glutamate uptake into the placenta is mediated via system X_{AG}-. Glutamate is not thought to be released into the fetal circulation via system X_{AG}- transporters rather it is converted to glutamine or other metabolites. OAT4 and OATP2B1 are thought to play a role in glutamate efflux, via exchange organic anions e.g. DHEAS, see text for details.

1.7.1.2 System N

System N is Na^+-dependent and can be discriminated from system A by the participation of protons in exchange (Na^+-cotransport/H^+-antiport), a narrower substrate specificity (histidine, glutamine and asparagine) and that MeAIB is not a substrate (Bröer, 2002; Jansson, 2001; Novak and Beveridge, 1997). The isoform slc38a5 (SNAT5) exists on the MVM of the syncytiotrophoblast only (Figure 9) (Day *et al.*, 2013; Jansson, 2001). System N remains relatively understudied within the placenta.

1.7.1.3 System L

System L belongs to the *SLC7* family and consists of two isoforms: Light subunits of Amino acid Transporters (LAT)1 (slc7a5) and LAT2 (slc7a8). LAT1 and LAT2 require the presence of the ubiquitously expressed heavy chain CD98 (slc3a2, also known as 4F2hc) to be shuttled

to the plasma membrane and for their functionality (Wagner *et al.*, 2001). The light chains (LAT1 or LAT2) are primarily bound to CD98 by disulphide bonds (Pochini *et al.*, 2014). System L is Na^+-independent, operates via exchange and exists on both the MVM and BM (Figure 9) (Regnault *et al.*, 2002), thus this system may mediate transplacental amino acid transport (Jansson, 2001). LAT1 and LAT2 have broad substrate specificity, transporting amino acids with bulky side chains such as leucine (Jansson, 2001) and are expressed on both the MVM and the BM (Cleal *et al.*, 2018). LAT2 has greater specificity for glutamine than LAT1 (Pochini *et al.*, 2014). The function of LAT1 and LAT2 is important to maintain the amino acid pool within the cell (del Amo *et al.*, 2008). Originally, activity of LAT2 was thought to predominate on the fetal-facing BM (del Amo *et al.*, 2008; Kudo and Boyd, 2001; Verrey, 2003) to contribute mainly to cell efflux. However more recent studies have shown that activity on the BM is consistent with the LAT1 isoform (Cleal *et al.*, 2011). The two system L isoforms are Na^+-independent exchangers and thus activity of unidirectional co-transporters (such as system A and N) is required for system L to be functional (Verrey, 2003).

1.7.2 Placental transport of glutamate

Glutamate uptake into the syncytiotrophoblast is mediated by system X_{AG-} which is localised to both the MVM and BM (Figure 9). System X_{AG-} is the only transporter system known to mediate glutamate uptake from the maternal circulation.

The organic anion transporter OAT4 (slc22a11) and organic anion transporting polypeptide OATP2B1 (slco2b1) are expressed on the BM of the syncytiotrophoblast where they mediate placental uptake of xenobiotics and dehydroepiandrosterone-3-sulphate (DHEAS) essential for placental oestrogen synthesis (Lofthouse *et al.*, 2015; Walker *et al.*, 2017). There is recent evidence to indicate that glutamate efflux via OAT4 and OATP2B1 is coupled with placental uptake of endogenous substrates (e.g. DHEAS) and xenobiotics, prior to re-uptake by system X_{AG-} on the BM (Lofthouse *et al.*, 2015). Since the current project will investigate transporter-mediated uptake across the MVM of the human placenta only, system X_{AG-} will be considered in further detail in the next section.

1.7.2.1 System X_{AG-}

System X_{AG-} transporters are Na^+-dependent co-transporters ($3Na^+$ and $1H^+$-co-transport/$1K^+$-antiport) and belong to the *SLC1* gene family. There are five system X_{AG-} isoforms (Excitatory Amino Acid Transporters, EAATs) of which EAAT1 (slc1a3), EAAT2 (slc1a2) and EAAT3 (slc1a1) have been localised to the placenta (Noorlander *et al.*, 2004). The nomenclature in rats and mice is as follows: GLAST (EAAT1), GLT (EAAT2) and EAAC1 (EAAT3) (Beart and O'Shea, 2007); for simplicity the transporters will be referred to as

EAAT1, EAAT2 and EAAT3 throughout this thesis. EAAT1 and EAAT2 are localised to the MVM of the syncytiotrophoblast throughout gestation. EAAT3 is localised to the syncytiotrophoblast until approximately 8 weeks gestation and towards term EAAT3 is localised to the fetal endothelium (Noorlander *et al.*, 2004).

1.8 Regulation of amino acid transport

Placental amino acid transport is regulated in response to endocrine and metabolic signalling, as well as nutrient and oxygen availability (Vaughan *et al.*, 2017). This section will summarise these regulatory factors, as well as the intracellular signalling pathway mTOR (mechanistic target of rapamycin) which has been proposed to be a key regulator of amino acid transport activity in the placenta.

1.8.1 Substrate availability

The *in vitro* experimental methods introduced in this section are described in full in Table 5. There is evidence in placenta (Jones *et al.*, 2006; Parrott *et al.*, 2007) and other tissues (Bode, 2001; Pochini *et al.*, 2014) for substrate regulation of system A and L, respectively. In villous explants derived from human placentas at term, system A activity is increased in the absence of the system A substrates glutamine, glycine and serine, i.e. system A transporter activity is inversely related to substrate availability (Parrott *et al.*, 2007). Furthermore, Parrott *et al.* (2007) found that the increase in system A was dose-dependent, relative to decreasing concentrations of system A amino acid substrates. Cytotrophoblast cells cultured in leucine-deplete medium have increased system A activity (Roos *et al.*, 2006). In a choriocarcinoma cell line (BeWo cells) cultured in media without non-essential amino acids, system A activity was significantly higher compared with those cultured in amino acid-replete media. This functional adaptation is associated with mechanistic changes: the SNAT2 isoform of system A transporter (of which glutamine is a substrate) is translocated to the plasma membrane after 30 min exposure and slc38a2 gene expression is increased after three hours (Jones *et al.*, 2006). Activity and mRNA expression of taurine transporter (TauT) is decreased in response to exposure of JAR (human placental choriocarcinoma) cells to taurine, a conditionally essential amino acid during pregnancy (Aerts and Van Assche, 2002; Jayanthi *et al.*, 1995). Collectively these studies suggest that the placenta can adapt in terms of its functional capacity (i.e. system A amino acid transporter activity, important for glutamine transport), which may be a strategy to maintain appropriate fetal growth during short-term perturbations in amino acid availability. Whilst these data suggest substrate regulation of glutamine transporter systems in the placenta (namely system A) there is little known regarding the regulation of system X_{AG-}, of which glutamate is a substrate, in the placenta. In other tissue types, system X_{AG-} activity is regulated

by amino acid availability; amino acid deprivation of a renal epithelial cell line NBL-I leads to an increase in Na^+-dependent aspartate uptake. This effect is lost with the addition of cycloheximide indicating that increased aspartate uptake is dependent upon protein synthesis; however EAAT3 mRNA transcript levels initially fall after amino acid deprivation indicating that initial changes are at the post-transcriptional level (Plakidou-Dymock and McGivan, 1993).

Chronic reductions in amino acid availability induced experimentally in animals via maternal calorie or protein restriction, leads to reduced fetal growth. In rats fed an isocaloric protein-deplete diet, activity of amino acid transporters important for glutamine (system A, L and y^+L, on the maternal-facing membrane) and glutamate transport (system X_{AG}^-, on the fetal-facing membrane) is reduced (Malandro *et al.*, 1996; Vaughan *et al.*, 2017). Furthermore, reductions in placental system A transport in rats and baboons fed a low protein diet precede the development of fetal growth restriction (Jansson *et al.*, 2006). This is supported by a further study showing that fetal growth is reduced when system A is blocked pharmacologically (by MeAIB infusion) in rats (Cramer et al 2002). Taken together, these data provide compelling evidence that reduced system A amino acid transport is a cause of FGR, rather than a consequence, in these experimental circumstances.

	MVM/BM vesicles	Primary cytotrophoblast cells	Placental villous tissue fragments	Placental perfusion	Stable isotope
Principle	Pure isolates of the placental syncytiotrophoblast MVM or BM. Can be used to study uptake of substrates across a defined plasma membrane under defined conditions.	Cytotrophoblast cells isolated from placentas after delivery and maintained in culture. Can be treated with potential regulators to assess nutrient transporter activity and expression.	Small (1-2mm^3) fragments of placental villi excised post-delivery. Can be used to study uptake or efflux of substrates, such as amino acids.	A whole cotyledon of the placenta is perfused from the fetal (arterial and venous) and maternal circulations to investigate the transfer of nutrients between these circulations.	Stable isotope tracers (e.g. 1-13C) are infused into the maternal circulation during pregnancy to assess fetal uptake of substrates.
Benefits	Essential to identify localisation of transporter proteins (i.e. to the MVM or BM). Enables determination of transporter kinetics.	Isolated from primary tissue (i.e. from a specific individual, not a cell line). Pure population of trophoblast, which differentiates to form a multinucleated cell resembling syncytiotrophoblast.	Tissue architecture and associated cell signalling mechanisms/driving forces are maintained. Can maintain in culture to examine effect of regulators on transporter activity/expression in the longer term.	Villous architecture and intervillous space maintained. Ability to investigate the transport mechanisms on the BM: villous tissue fragments and cytotrophoblast cells do not give access to this membrane.	Provides insight into the transport of substrates, and placental and fetal metabolism *in vivo*.
Disadvantages	Vesicles lack tissue integrity, intracellular signalling mechanisms and associated driving forces.	Whole tissue architecture is not maintained, cell-cell communication is lost and the cells do not polarise or proliferate in culture.	It is only possible to measure placental uptake, i.e. transfer across the MVM, using this method. Non-specific diffusion must be controlled for. Fragments contain many cell types.	Difficult, time-consuming technique with high failure rate due to damage/tears of placenta post-partum.	Tracer has to be infused into the mother. Maximum of two fetal samples can be collected.

Table 5: Methods used to measure transport of substrates e.g. amino acids by the human placenta

The benefits and disadvantages of the different methods used to assess placental transport of substrates are summarised above. Abbreviations: MVM, microvillous membrane; BM, Basal membrane. Data from references within text and (Cetin, 2001; Greenwood and Sibley, 2006; Huang *et al.*, 2016; Nye *et al.*, 2018).

Based on the substrate regulation experiments *in vitro*, and the studies described in animals above, it is possible that maternal plasma amino acid concentrations could regulate placental transporter activity. Also, fetal umbilical arterial (UmA) plasma amino acid levels could regulate transporters on the BM to influence uptake into the syncytiotrophoblast from the fetal side, in turn influencing intracellular amino acid levels which could regulate transporters on the MVM. Fetal amino acid concentrations are higher than in maternal circulation in normal pregnancy (Cetin *et al.*, 1996; Neerhof and Thaete, 2008). In rodent models and in cases of FGR in humans, maternal plasma amino acid concentrations are higher than in normal pregnancy (Cetin *et al.*, 1996; Jansson *et al.*, 2006; Malandro *et al.*, 1996). However, previous studies have indicated that glutamine concentrations are reduced in the umbilical vein (UmV) compared with normal pregnancy (Cetin *et al.*, 1996; Ivorra *et al.*, 2012). Glutamine concentration is lower in the UmV in FGR, and glutamate concentration is lower in the UmA (Alexandre-Gouabau *et al.*, 2013). Amino acid concentrations in maternal and fetal compartments and studies of small molecule metabolites (metabolomics) are discussed in greater depth in section 1.12. There is a paucity of data from investigations of amino acid concentrations in maternal and fetal (UmV and UmA) plasma in a well-defined population of normal pregnancy and FGR. It would also be valuable to relate amino acid concentrations in the maternal and fetal compartments to placental function i.e. amino acid uptake (transporter activity) in the same women to identify/understand the relationships between these variables in normal pregnancy and how they are affected in FGR.

1.8.2 Endocrine function and oxygen availability

Endocrine signals such as insulin, insulin-like growth factor (IGF)1 and IGF2, and adipose-derived cytokines (adipokines) are known modulators of fetal growth (Qiao *et al.*, 2016; Sferruzzi-Perri *et al.*, 2013). Levels of insulin and IGF1 in the maternal circulation are positively correlated with birth weight (Luo *et al.*, 2012). Although IGF1 does not cross the placenta in physiologically relevant amounts (Sferruzzi-Perri *et al.*, 2007), IGF1 concentrations in the UmV are also correlated with birth weight (Hawkes *et al.*, 2018; Ong *et al.*, 2000; Wiznitzer *et al.*, 1998), whereas IGF2 is weakly associated (Hawkes *et al.*, 2018). However an inverse relationship between IGF1 and birth weight has also been reported (Wang *et al.*, 1991). Insulin and IGF1 stimulate system A activity (Jansson *et al.*, 2003; Karl *et al.*, 1992; Vaughan *et al.*, 2017) via the transmembrane tyrosine kinases insulin receptor and IGF type 1 receptor. Abundance of IGF type 1 receptor, and components of the mTOR signalling pathway P13K and Akt (see section 1.8.3), which act to increase amino acid transporter translation and trafficking to the plasma membrane, are decreased in FGR, suggestive of a role for placental insulin signalling in fetal growth (Vaughan *et al.*, 2017).

IGFs may also act via paracrine mechanisms since *Igf1* and *Igf2* genes are expressed within the placenta (Fowden, 2003; Sferruzzi-Perri et al., 2011). A study of IGF2-null mice determined that IGF2 is required to maintain expression of the glutamate transporters EAAT1, EAAT2, and EAAT3 (Matthews et al., 1999). Targeted knockout of specific *Igf* genes and their transcripts negatively impacts fetal growth and have been well-described in mice (discussed in detail in section 1.11.1 and Chapter 5) (Constância *et al.*, 2002).

In adulthood, pro-inflammatory (e.g. leptin, and tumour necrosis factor (TNF)-α) and anti-inflammatory (e.g. adiponectin) adipokines are secreted from white adipose tissue (Nakamura *et al.*, 2014). Circulating leptin levels are known to be elevated in obesity (Havel *et al.*, 1996), a population more likely to have both large for gestational age (Brett *et al.*, 2016; Ferraro *et al.*, 2012) and FGR babies (Tessier *et al.*, 2013). Experimentally, it has been demonstrated that system A activity increases in a dose-dependent manner when human placental villous fragments are exposed to leptin (Jansson *et al.*, 2003). Conversely, adiponectin is negatively correlated with birth weight (Jansson *et al.*, 2008), and in trophoblast cells cultured in the presence of adiponectin, both system A activity and expression of two of the key transporters for this system (SNAT1 and SNAT2 isoforms) are reduced (Jones *et al.*, 2010).

Oxygen availability also influences fetal growth *in utero*. Low partial pressure of oxygen (pO_2) i.e. at high altitude (generally defined as above 2500 m) (Moore *et al.*, 2011) is associated with low birth weight infants (Julian, 2011). Birth weight decreases with increasing altitude yet communities that have lived at high altitude for multiple generations (such as Tibetans and Andeans) appear better adapted than those who have moved to high altitude comparatively recently where fetal growth is compromised (e.g. Europeans and Han Chinese) (Moore *et al.*, 2011). Hypoxia and oxidative stress induced *in vitro* by 1% and 3% oxygen culture conditions inhibits system A activity in cytotrophoblast cells by 82% and 37%, respectively (Nelson *et al.*, 2003) but does not affect activity of system ASC or system L.

Inadequate remodelling of the maternal spiral arteries post-implantation can lead to reduced placental perfusion, low oxygen levels within the intervillous space, and ischaemia-reperfusion injury. This phenotype of placental dysfunction is known to contribute to severe early-onset FGR (Lyall *et al.*, 2013). In mice differential changes are observed following exposure to 13% and 10% inspired oxygen (equivalent to approximately 3700m and 5800m altitude, respectively) (Higgins *et al.*, 2016). At 13% oxygen morphological and functional changes are evident within the placenta including increased surface area for exchange and increased maternofetal clearance of glucose towards term. Fetal growth of mice exposed to 13% oxygen is slightly reduced (~5%) compared with normoxic conditions (21% oxygen), whereas mice exposed to 10% oxygen have severely growth restricted pups with placentas that failed to adapt, demonstrating increased barrier thickness and reduced maternofetal MeAIB clearance

towards term, as observed in human FGR (Higgins *et al.*, 2016). These findings represent an environmental challenge (10% oxygen) whereby the placenta is unable to functionally and/or morphologically adapt to support adequate fetal growth, and thus severe growth restriction is the end result.

1.8.3 mTOR signaling pathway

The mechanistic target of rapamycin (mTOR) signalling pathway acts as a nutrient sensor, exerting downstream effects according to relative availability of nutrients. mTOR is a serine/threonine kinase which exerts its effect via the phosphorylation of downstream targets. There are two mTOR complexes: mTORC1 and mTORC2 which are associated with the regulatory accessory proteins raptor and rictor, respectively (Alessi *et al.*, 2009). When activated, mTORC1 phosphorylates ribosomal S6 kinase (S6K1 and 2) and eukaryotic initiation factor 4E-binding protein (4EBP1 and 2). mTORC2 phosphorylates Akt (also known as protein kinase B), protein kinase C (PKC) and serum glucocorticoid-regulated protein kinase 1 (SGK1) (Figure 10) (Alessi *et al.*, 2009; Vaughan *et al.*, 2017).

Amino acids and growth factors (such as IGFs) are some of the signals known to stimulate mTORC1 activity (Zheng *et al.*, 2014). mTORC1 is suppressed during periods of low ATP availability (Alessi *et al.*, 2009). mTOR positively regulates the amino acid transporter systems A and L, of which glutamine is a substrate, via the phosphorylation of target proteins as described herein (Jansson *et al.*, 2012).

mTORC1 inhibits the NEDD4-2 complex via SGK1 (Figure 10), which acts to ubiquitinate targets for degradation by the proteasome. This action prevents NEDD4-2 from binding to target transporter proteins thereby enhancing transporter abundance, stability, and activity in epithelial membranes (Alessi *et al.*, 2009). Of specific relevance to the work in this thesis, the inhibitory action of mTORC1 on NEDD4-2 inhibits degradation of MVM-bound amino acid transporters SNAT2 and LAT1, which contribute to glutamine transport (Chen *et al.*, 2015; Rosario *et al.*, 2013). SNAT2 and LAT1 abundance in the MVM of human primary trophoblast cells, but not whole cell lysates, is reduced following silencing of raptor and rictor, resulting in inhibition of mTORC1 and 2 respectively (Rosario *et al.*, 2013). This indicates that reduced mTOR signalling inhibits the trafficking of amino acid transporters (systems A and L, known to contribute to glutamine transport) to the plasma membrane. Another target protein of mTORC1 is 4EBP1, an inhibitor of eukaryotic translation initiation factor 4E (eIF4E). When phosphorylated, 4EBP1 dissociates from eIF4E to stimulate protein translation (Alessi *et al.*, 2009). Conversely, mTORC2 activity is not sensitive to regulation by nutrients or energy availability, instead it is controlled by P13K (phosphatidylinositol 3-kinase) (Alessi *et al.*, 2009).

Recent evidence suggests that mTOR operates as a nutrient sensor and, in the context of the placenta, placental nutrient sensing may act to modulate cell metabolism, growth factor signalling, and subsequent nutrient availability (Jansson *et al.*, 2012). Inhibition of the mTOR pathway via rapamycin causes reduced leucine transport (via system L, of which glutamine is a substrate) in human placental villous fragments (Roos *et al.*, 2007). Amino acid transporter activity (system A, L, glutamine transporters and TauT, taurine transporter) has also been shown to be reduced, but protein expression unaltered, in the same conditions (Roos *et al.*, 2009). That protein expression was unchanged when mTOR was experimentally inhibited suggests that regulation of transporters via this pathway occurs at the post-translational stage.

mTOR is activated and positively correlated with birth weight in the placentas of obese women giving birth to large babies (Jansson *et al.*, 2013). In the same study cohort, system A amino acid transporter activity and SNAT2 isoform expression were positively correlated with birth weight. The authors of this study suggested that the mTOR signalling pathway may be a positive regulator of amino acid transporter activity and expression in this population (Jansson *et al.*, 2013). Consistent with this, in FGR mTORC1 activity is decreased in tandem with increased NEDD4-2 abundance (Chen *et al.*, 2015; Roos *et al.*, 2007), implicating this pathway in reduced placental amino acid transport in FGR. In the same way, rodents and primates on a protein restricted diet have reduced mTORC1 activity (Kavitha *et al.*, 2014; Rosario *et al.*, 2011).

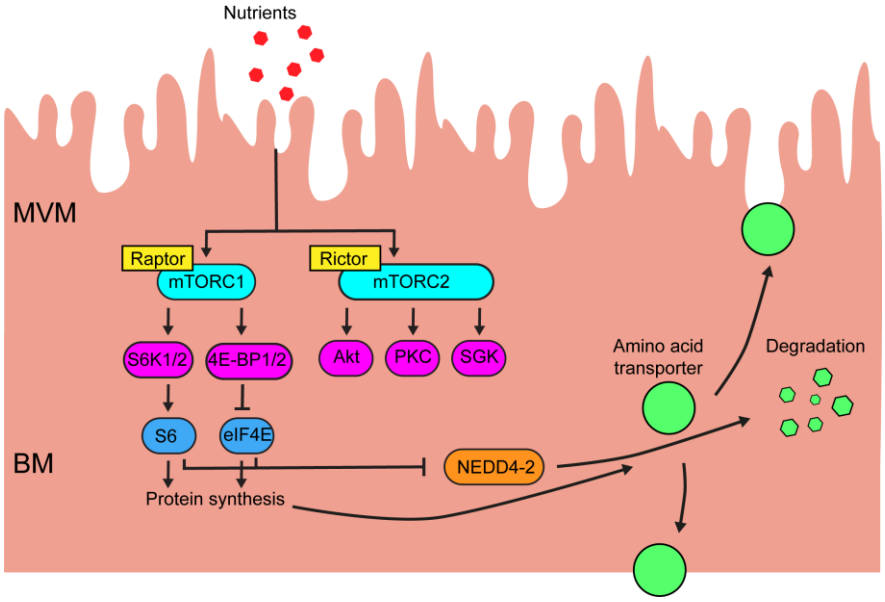


Figure 10: mTOR signaling pathway
The mTOR pathway is a nutrient-sensing pathway that can be stimulated by signals such as amino acids and growth factors. When activated, the downstream effects of mTORC1 depicted above, include inhibition of NEDD4-2 thereby reducing the ubiquitination and degradation by the proteasome of amino acid transporters such as LAT1 and SNAT2 known to transport glutamine.

There is ample evidence that mTOR, via mTORC1 in particular, may impact amino acid transporter function, including placental amino acid transport systems for which glutamine is a substrate (systems A and L). However, there are no studies that have investigated whether the mTOR pathway regulates the activity/expression of the glutamine transporter system N or the glutamate transporter system X_{AG-} in the placenta.

In summary, many factors (substrate and oxygen availability, endocrine function, and mTOR pathway) regulate the function of placental amino acid transporters such as system A and L, for which glutamine is a substrate. It is probable that glutamine transport will also be regulated by these same factors but this remains to be investigated. There are very little data on the regulation of system X_{AG-} in the placenta (of which glutamate is a substrate) and again, this remains to be investigated in the placenta in normal pregnancy and FGR.

1.9 Evidence of placental adaptation relative to placental size

Placental adaptation comprises alterations in placental development (e.g. morphological changes) or function (e.g. changes in nutrient transport) that occur to meet fetal nutritional requirements. These changes are likely to alter placental efficiency, as evidenced by a change in the fetal:placental weight (F:P) ratio. Fetal demand for nutrients alters as gestation progresses. Towards term when fetal nutrient demand is at its peak, fetal weight (grams) per gram placental weight is relatively higher than earlier in gestation (Figure 7).

To optimise fetal growth, the delivery of nutrients to the fetus via the placenta must be sufficient to meet this demand. There is a strong relationship between placental and fetal weight in normal pregnancy (i.e. pregnancy resulting in delivery of an infant appropriately grown for gestational age; AGA) as shown in Figure 11; this implies that in FGR a small placenta could underlie the suboptimal fetal growth, if functional adaptation was insufficient to compensate for small placental size.

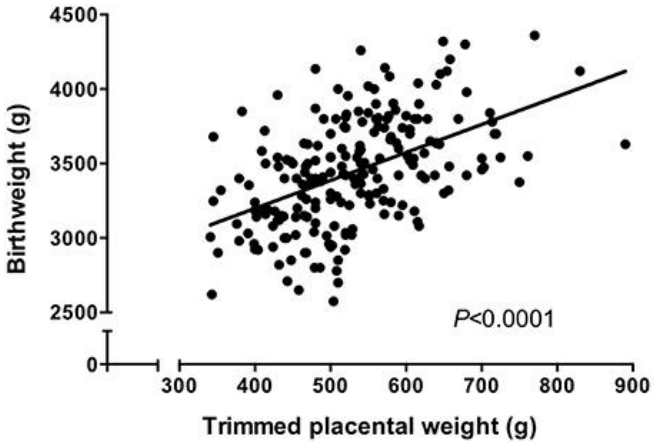


Figure 11: Birth weight versus trimmed placental weight in a normal birth weight (AGA) cohort at term (37-42 weeks)
Figure from (Hayward *et al.*, 2016)

Nutrient delivery to the fetus is influenced by placental morphology, particularly the surface area of the transporting epithelium (syncytiotrophoblast), and the diffusional distance between maternal and fetal circulations. The abundance, localisation and activity of transporters are key determinants of amino acid transfer, and other nutrients that rely upon plasma membrane transport mechanisms (Fowden *et al.*, 2009; Sferruzzi-Perri and Camm, 2016). Changes in any of these variables can significantly alter nutrient delivery to the fetus. Studies in WT mice show that relative to its size, the placenta undergoes both morphological, e.g. placental barrier thickness, and functional adaptations, such as maternofetal clearance of solutes, in order to achieve optimal fetal growth (Coan *et al.*, 2008). This section will outline the evidence to support placental adaptation, with a particular focus on adaptation of amino acid transport capacity in relation to placental size.

There is robust evidence in WT mice that system A amino acid transport adapts according to placental size. The system A amino acid transporter has affinity for small neutral amino acids, including glutamine (Table 4). System A activity can be quantified experimentally using the non-metabolisable synthetic analogue methylaminoisobutyric acid (MeAIB). In this way, Coan *et al.* (2008) compared unidirectional maternofetal clearance (K_{mf}) of MeAIB between the lightest and heaviest placentas in a single WT (C57BL/6J) mouse litter. At both embryonic day (E) 16 and E19 (term E19-20), K_{mf} of MeAIB was significantly higher (per mg placenta) across the lightest compared with the heaviest placentas in a litter but the magnitude of this difference was greater at E19. At E19 raised K_{mf} of MeAIB in the lightest placentas was accompanied by increased expression of the gene slc38a2 (which codes for the SNAT2 isoform of system A). K_{mf} of glucose and inulin were no different between lightest and heaviest placentas at either gestation, in spite of increased GLUT1 glucose transporter (slc2a1) in the lightest placentas at E19, indicating that adaptation is not universal (Coan *et al.*, 2008).

In the study by Coan *et al.* (2008) there was evidence for morphological adaptation at E16 with a relative increase in the labyrinthine zone, important for nutrient exchange, of the lightest placentas at the expense of the junctional zone. Placental morphology was similar between the lightest and heaviest placentas towards term (E19), which suggests that these adaptations, in mice at least, are primarily morphological earlier in gestation and functional towards term. In a similar study, Hayward *et al.*, (2017) reported that K_{mf} of calcium was higher across the lightest versus heaviest placentas in a WT litter at E18.5, which coincided with increased placental calbindin-D_{9K} expression, a calcium binding protein required for calcium transfer, and normalised fetal calcium content following reduced content earlier in gestation (E16.5). The sex of the fetus is important to investigate in these studies since the lightest placentas more often belong to female fetuses, and the heaviest to males. The findings of Hayward *et al.*

(2017) were independent of the sex of the fetus and the effect for calcium was still apparent. The effect of sex was not reported by Coan *et al.* (2008).

Taken together, these studies provide evidence of placental adaptation in terms of functional and/or morphological changes to support appropriate fetal growth in a litter of normally grown (WT) fetuses. Potential environmental challenges, and the placental and fetal morphological and/or functional adaptations that such challenges can induce are summarised in section 1.10.2. Glutamine is a substrate of the system A transporter and whilst there is clear evidence to support up-regulation of this amino acid transport system in small, normal placentas in WT mice, other transport systems important for glutamine (systems L and N) and glutamate transport (system X_{AG}-) have not yet been assessed.

In contrast to the mouse, there are limited data to support functional adaptations of the human placenta. A study by Godfrey *et al.* (1998) quantified system A activity in MVM vesicles isolated from human placentas of normal pregnancy outcome (inclusion criteria: infants without congenital abnormalities born after 36 weeks gestation, mean birth weight 3395 g; individualised charts were not used). MVM vesicles are formed from pure isolated preparations of syncytiotrophoblast MVM and have been used extensively to evaluate transporter-mediated substrate uptake (Glazier and Sibley, 2006). An overview of the *in vitro* methods used to assess transporter activity in the normal and FGR placenta is provided in Table 5. Godfrey *et al.* (1998) found that system A activity was highest in MVM vesicles from placentas of the smallest babies, and that it was inversely correlated with fetal abdominal circumference at birth. Furthermore, Na^+-independent MeAIB (purportedly representative of non-specific diffusional uptake) was also inversely related to placental weight. Others have reported both no relationship (Harrington *et al.*, 1999), and a positive relationship (Jansson *et al.,* 2013) between placental MVM system A activity and birth weight. There are some important differences between these three studies. Jansson *et al.* (2013) included women with high BMI (range 18.5-44.9 kg/m^2) who gave birth to larger infants; birth weight was positively related to BMI. It is possible that the inclusion of infants with a birth weight >4000 g skewed these data. It must also be acknowledged that the definition of 'normal birth weight' was different between the studies of Godfrey *et al.* (1998) and Harrington *et al.* (1999). Harrington *et al.* (1999) defined 'normal' according to IBR using individualised growth charts rather than birth weight per se, as used by Godfrey *et al.* (1998). Taken together, these conflicting findings highlight the unmet need for placental amino acid uptake (transporter activity) to be assessed in relation to placental size in a well-defined normal pregnancy population. This is important to establish because should placental adaptation in response to small size be a key mechanism to ensure appropriate fetal growth in normal pregnancy, it could be that failure of this adaptation may underpin cases of FGR.

Placental efficiency can be estimated as the grams of fetus produced per gram placenta (trimmed placental weight; i.e. with membranes and the umbilical cord removed). A proxy for placental efficiency is the fetal:placental weight ratio (F:P ratio). In mice the lightest placenta in a litter has a higher F:P ratio than the heaviest, i.e. lightest placentas are said to be more efficient (Coan *et al.*, 2008; Hayward *et al.*, 2017). Coan *et al.* (2008) reported a positive correlation between placental weight and birth weight at E16 but not towards term (E19) in normally grown (WT) mice. This signifies that the functional and morphological adaptations evident by E19, and discussed above, compensate for variations in placental weight and means that placental size is not a determinant of fetal weight/growth near term.

Whilst there is substantial evidence to support a positive relationship between placental system A activity and F:P ratio in mice, there is a paucity of evidence to support adaptation (in terms of nutrient transport) in a normal (AGA) human population. In a recent review (Hayward *et al.*, 2016), data relating system A activity from previously published studies (Ditchfield *et al.*, 2010; Hayward *et al.*, 2012; Lean *et al.*, 2014) was positively correlated with placental weight but not with birth weight or birth weight:placental weight ratio (BW:PW ratio, akin to F:P ratio). However this correlation was largely skewed by pregnancies at the extremes of normal placental weight (those with placental weight $<10^{th}$ and $>90^{th}$ centile).

The hypothesis for the current study is that, as in mice, the small human placenta of a normal birth weight (AGA) baby is more efficient and therefore there is increased nutrient transport per g placenta weight and, by current means used to estimate placental efficiency, a high BW:PW ratio. The findings of Hayward *et al.* (2016) differ from Godfrey *et al.* (1998), reporting a positive, and not an inverse, relationship between system A uptake and placental weight in humans. This might have been caused by the different analyses (correlations versus binned weight groups) or techniques used (MVM vesicles versus placental villous fragments). The benefits and disadvantages of methods used to measure transport by the human placenta are presented in Table 5.

In summary, there is evidence that increased activity of system A compensates for small placental size in normally grown WT mice as determined by clearance measurements of the non-metabolisable analogue MeAIB. This has important implications for the clearance of the naturally occurring substrates of system A, which include glutamine. However, glutamine/glutamate clearance (K_{mf}) has not been measured in relation to placental size in mice and there are relatively few studies of placental nutrient adaptation according to placental size in women. In mice the mechanism underlying this adaptation is not well understood. The next section will examine nutrient transport in FGR, covering both *in vitro* studies of human placental tissue and *in vivo* nutritional and experimental manipulations, and their effect on placental function, in animal models.

1.10 Placental nutrient transport in FGR

FGR is a phenotypic endpoint of a heterogeneous disease. The underlying causes of FGR are diverse and include congenital abnormalities and placental dysfunction, which may lead to inadequate placental perfusion and/or insufficient nutrient transport to the fetus (Royal College of Obstetricians and Gynaecologists, 2013). Infants who are small at birth are at an increased risk of morbidities such as developmental delay and cerebral palsy, and are also susceptible to metabolic programming of disease such as type 2 diabetes in later life (Barker, 2004; Thornton *et al.*, 2004; Veen *et al.*, 1991). Yet no treatment for FGR, aside from the decision to deliver the fetus, currently exists (Fisk and Atun, 2008). This thesis will focus on understanding placental nutrient transport in normal pregnancies, and in pregnancies complicated by FGR in which no known cause is apparent other than suspected placental dysfunction (idiopathic FGR). Here, the existing literature on placental nutrient transfer in FGR versus normal pregnancy in both humans and animal models is summarised.

1.10.1 Placental transport studies: FGR and the human placenta

Numerous studies have examined the morphology and function of placentas collected from pregnancies where the infant was growth restricted. The definition of FGR remains opaque which means that inclusion criteria vary from study to study, making comparisons between studies challenging. An IBR <5th centile is a commonly used definition in research studies. A recent Delphi consensus study defined late-onset FGR (the focus of the current study) as >32 weeks gestation and an abdominal circumference or estimated fetal weight <3rd centile, or two out of three of the following: abdominal circumference/estimated fetal weight <10th centile, abdominal circumference/estimated fetal weight crossing more than two quartiles on growth centiles, or cerebroplacental ratio <5th centile/UmA pulsatility index (assessed using Doppler velocimetry) >95th centile (Table 2) (Gordijn *et al.*, 2016). In this section, the existing literature on placental transport in relation to poor fetal growth (FGR) will be discussed, with reference to the definition of FGR used in each study.

System A amino acid transporter activity (of which glutamine is a substrate), assessed by MeAIB uptake into MVM vesicles, is lower in FGR (defined as <10th centile of birth weights) compared with normal (AGA) placentas, and is related to the severity of FGR, as determined by UmA Doppler velocimetry and fetal heart rate monitoring immediately before caesarean section (Glazier *et al.*, 1997). In this study, differences between FGR and normal were only present when umbilical blood flow was abnormal (UmA pulsatility index) in the FGR group (+/- abnormal fetal heart rate). However, the gestational age range in this study (28-40 weeks) is a potential confounding factor: 13/16 FGR babies, but only 4/10 AGA babies were delivered before 36 weeks.

System A activity is also reduced in placentas from small for gestational age (SGA) infants (birth weight <10th centile) compared with AGA, but not when maternal pre-eclampsia co-exists (Mahendran *et al.*, 1993; Shibata *et al.*, 2008). Activity of the amino acid transporter system L (also important for glutamine transport) is reduced in FGR, as evidenced by reduced leucine uptake across both the MVM and the BM of placentas from FGR versus normal birth weight (AGA) infants (Jansson *et al.*, 1998). Contrary to expectations given reduced activity, Aiko *et al.* (2014) has shown that expression of proteins important for system L transport (LAT1 isoform and the associated heavy chain, CD98) as detected by immunohistochemistry, is increased in FGR. This suggests that plasma membrane abundance of system L transporter proteins (LAT1) in FGR may not be directly related to transporter activity as measured by uptake across the syncytiotrophoblast plasma membrane. As a substrate of system A and L, it appears reasonable to hypothesise that glutamine uptake by the placental syncytiotrophoblast is likely to be reduced in FGR, which would have implications not only for fetal glutamine, but also glutamate, provision.

Techniques such as high-performance liquid chromatography (HPLC) enable quantification of amino acid concentrations in maternal and fetal (UmV and UmA) blood. The concentration of several amino acids in maternal plasma, including system A substrates alanine and histidine, is raised in FGR versus normal pregnancies. Yet FGR pregnancies (<10th centile of birth weights) are associated with reduced amino acid concentrations in the UmV (thus the fetal-maternal difference is lower) which is suggestive of a dysfunctional placenta (Cetin *et al.*, 1996). Amino acid concentrations in normal pregnancy are significantly higher in the intervillous space than in maternal plasma in normal (~186%) and preterm deliveries, and levels in the UmV more akin to the levels recorded in the intervillous space than maternal plasma (Camelo *et al.*, 2004; Camelo *et al.*, 2007). The concentration of glutamine in the UmV has been shown to be lower in low birth weight infants (<10th centile) (Ivorra *et al.*, 2012) indicating that the provision of glutamine to the fetus may be compromised in these pregnancies.

In a study by Holm *et al.* (2017), maternal (radial artery and uterine vein) and fetal (UmV and UmA) vessels were sampled *in vivo*, at the time of caesarean section, to calculate arteriovenous differences of amino acid concentrations across the placenta in normal (AGA) pregnancies (using liquid chromatography-mass spectrometry, LC-MS). The study reported fetal uptake (positive venoarterial difference) of 14 amino acids (including glutamine), and fetal release (negative venoarterial difference) of glutamic acid (glutamate). Maternal artery and UmV concentrations were correlated for all amino acids except tryptophan, indicating that there is a relationship between maternal amino acid concentrations and the delivery of amino acids to the fetus via the placenta. A direct comparison between amino acid concentrations in maternal

and fetal (UmV and UmA) plasma in normal pregnancy and FGR would be beneficial to link to (dys)regulation of amino acid transport in FGR.

1.10.2 Placental transport studies: experimental animal models of FGR

Human placental villous tissue fragments are useful to assess transporter-mediated uptake across the maternal-facing membrane (MVM) of the syncytiotrophoblast. A limitation of this technique, however, is that release into the fetal circulation (i.e. across the BM of the syncytiotrophoblast) cannot be measured (see Table 5). In animals such as the mouse however, the unidirectional maternofetal clearance (here referred to as K_{mf}) of substrates can be quantified following injection of a radiolabelled substrate into a maternal vein and subsequent measurement of the amount of radioisotope in individual fetuses, accounting for the amount of radiolabel in maternal plasma. Unidirectional maternofetal clearance (K_{mf}) of substrates such as MeAIB and calcium has been assessed in genetic knockout models of FGR (discussed further in section 1.11.1).

There are three major approaches in which to induce FGR in animals. These are via dietary interventions, surgical interventions and/or the production of genetically modified/knockout models. These types of models are not exclusive and there may be overlap. Such models have been used to investigate factors thought to contribute to FGR such as hypoxia, nutrient insufficiency and small placental size. By exposing animals to these insults under controlled conditions, it is possible to gain a comprehensive insight into the mechanisms underpinning the observed FGR. The most compelling evidence to support suboptimal nutrient transfer as a cause, rather than result, of FGR has come from studies in animals. In rats fed a protein-restricted diet, a reduction in system A amino acid transporter activity precedes the onset of FGR (Jansson *et al.*, 2006). Pharmacologically blocking system A transporter activity (via MeAIB infusion) also reduces fetal growth in rats and is associated with both reduced system A (glutamine transporter) and, unexpectedly, system X_{AG-} (glutamate transporter) activity and EAAT1, EAAT2, EAAT3 and EAAT4 protein expression (Cramer *et al.*, 2002).

In the protein-restricted rat the activity of transporter systems important for amino acid transfer, assessed by preparation of apical and basal membrane vesicles, are reduced on both the maternal-facing (system A) and fetal-facing membrane (system A and X_{AG-}, systems important for glutamine and glutamate transport, respectively) (Malandro *et al.*, 1996). Pregnant baboons fed a calorie-restricted diet (70% of normal nutrient intake) have growth restricted offspring with small placentas which exhibit reduced system A and L activity (in isolated MVM vesicles), as well as reduced protein expression of isoforms which mediate system A and L transport (SNAT2, LAT1, LAT2) (Kavitha *et al.*, 2014; Pantham *et al.*, 2015).

The sheep placenta is comprised of individual placental units, or cotyledons, which attach to caruncles present in the sheep uterus to form placentomes. Removal of caruncles prior to pregnancy results in reduced placental growth and subsequently growth restricted offspring (Owens *et al.*, 1987). However, relative to control animals, carunclectomised placentas support more fetus per gram, and have higher unidirectional maternofetal clearance (K_{mf}) of the non-metabolisable glucose analogue 3-0-methy-D-glucose per gram placenta (Owens *et al.*, 1989).

Animal models can provide valuable insight into the underlying mechanisms of placental insufficiency, via the manipulation of environmental factors such as oxygen levels and maternal diet. There are also several genetic knockout models of FGR that have been well characterised. These will be discussed in the next section.

1.11 Genetic knockout mouse models of human pregnancy complications

Placental dysfunction accounts for the majority of cases of FGR. Phenotypic abnormalities associated with FGR include abnormal morphology and/or reduced placental size, suboptimal invasion or vascular structure/regulation, as evidenced by blood flow abnormalities, and abnormal syncytiotrophoblast morphology, turnover and transporter function (Sibley *et al.*, 2005). These phenotypes often co-exist but the primary focus of this thesis is syncytiotrophoblast function, and specifically placental transport of the amino acids glutamine and glutamate. This section will briefly introduce three commonly used genetic knockout mouse models of FGR and other pregnancy complications such as pre-eclampsia, before focusing on the chosen model for the current study, the *Igf2* P0 knockout mouse.

The endothelial nitric oxide synthase knockout ($eNOS^{-/-}$) mouse is a well-characterised model of FGR associated with vascular dysfunction, hypertension (Hefler *et al.*, 2001) and reduced uteroplacental blood flow (Stanley *et al.*, 2015). In common with human late-onset FGR, $eNOS^{-/-}$ offspring are growth restricted towards term and their placentas demonstrate reduced system A activity (Kusinski *et al.*, 2012).

The enzyme catechol-O-methyl transferase contributes to 17β-oestradiol to 2- and 4-methoxyestradiol16 metabolism (Stanley *et al.*, 2015). The catechol-O-methyl transferase knockout ($COMT^{-/-}$) mouse exhibits a phenotype which mimics pre-eclampsia in humans, such as maternal hypertension and proteinuria towards term (Kanasaki *et al.*, 2008). This model also presents with FGR and abnormal UmA Doppler waveforms (Stanley *et al.*, 2012). The $COMT^{-/-}$ mouse is an inappropriate model for the current study due to the pre-eclamptic phenotype.

The placental-specific *Igf2* knockout mouse (P0), is a well-characterised model of late-onset FGR without vascular defects (Kusinski *et al.*, 2011). The P0 mouse is a model more akin to late-onset FGR (offspring are growth restricted by term) than the $eNOS^{-/-}$ mouse that exhibits

trophoblast dysfunction (reduced system A transport) (Kusinski *et al.*, 2012) but also has a vascular phenotype (Hefler *et al.*, 2001). The P0 mouse has therefore been chosen as an appropriate model of FGR for consideration in the current study and will be discussed further in the section that follows (section 1.11.1).

1.11.1 The placental-specific *Igf2* knockout mouse (P0)

IGF2 plays an important role as a fetal growth hormone during pregnancy. *Igf2* is an imprinted gene, in mice only the paternal allele is expressed (Angiolini *et al.*, 2006; Sferruzzi-Perri, 2018). Under- or over-expression of *Igf2* in humans is associated with Silver-Russell and Beckwith Weidemann syndrome respectively, which include fetal under/overgrowth as one of their symptoms (Burton and Fowden, 2012). During pregnancy, *Igf2* is expressed both in the placenta and the fetus. Complete knockout of *Igf2* from both placental and fetal tissues (*Igf2*-null) leads to severe growth restriction and conceptus loss (Constância *et al.*, 2005). Conversely, over-expression of *Igf2*, achieved via the deletion of the H19 gene which ordinarily imposes a regulatory effect upon *Igf2* expression, results in increased fetal and placental weight and reductions in amino acid and glucose transfer (Angiolini *et al.*, 2011).

Mating wild-type (C57BL/6J) female mice with males heterozygote for the targeted deletion of a placental-specific promoter (P0) of *Igf2* (*Igf2P0* knockout mice, hereafter referred to as P0) produces mixed litters of WT and P0 offspring (Constância *et al.*, 2002). P0 mice are growth restricted at term; more than 90% of P0 fetuses fall below the 5th centile of WT weights at E18.5, as per clinical-type classification of FGR (Dilworth *et al.*, 2011). Additionally, towards term, P0 fetuses demonstrate asymmetric growth, the head-sparing phenotype typical of late-onset FGR. A reduction in placental weight precedes reduced fetal growth in the P0 mouse, which suggests that placental dysfunction is a root cause of suboptimal growth in this model. Mid-gestation (E15.5), placentas from P0 mice have higher system A activity (as determined by maternofetal clearance of MeAIB) compared with WT littermates, an apparent adaptation to maintain appropriate fetal growth. This adaptation is not maintained towards term, thus fetal growth restriction ensues (Constância *et al.*, 2002; Kusinski *et al.*, 2011). In comparison, K_{mf} of MeAIB is similar for *Igf2*-null versus WT mice at E15.5 but by E18.5 K_{mf} of MeAIB is significantly lower across *Igf2*-null placentas; *Igf2*-null fetuses are severely growth restricted as a result.

In support of adaptation seen in the P0 mouse, compensatory adaptation has also been reported in the 11β-hydroxysteroid dehydrogenase 2 knockout (11β-HSD2$^{-/-}$) mouse in response to reduced placental size (Wyrwoll *et al.*, 2009). 11β-HSD2 is responsible for the inactivation and regulation of glucocorticoids (e.g. corticosterone) and is highly expressed in the fetal brain and placenta (Wyrwoll *et al.*, 2011). At E15, 11β-HSD2$^{-/-}$ mice have similar fetal

weights versus 11β-HSD2^{+}/$^{+}$, in conjunction with increased K_{mf} of MeAIB and slc38a2 (SNAT2) gene expression. By E18 this adaptation in K_{mf} of MeAIB and slc38a2 expression is lost, placental and fetal weights of 11β-HSD2^{-}/$^{-}$ mice are reduced and K_{mf} of glucose and slc2a3 (GLUT3) expression are also reduced (Wyrwoll *et al.*, 2009).

The placental phenotype of the P0 mouse has been well characterised. As well as functional differences, the placentas of these animals are also structurally abnormal: the syncytiotrophoblast barrier thickness between maternal and fetal circulations is increased but surface area available for exchange is reduced, akin to human FGR (Mayhew *et al.*, 2003). This is indicative of reduced permeability in P0 mouse placentas, as assessment of the transfer of the radiolabelled solutes mannitol, EDTA and inulin has confirmed (Sibley *et al.*, 2004).

Despite indications that the P0 placenta is functionally and structurally unable to support a fetus of appropriate weight, there is evidence to suggest that, in terms of some nutrient transport systems, the P0 placenta adapts to suboptimal maternofetal nutrient supply in an attempt to optimise fetal growth. Calcium is required to meet the demands of the rapidly mineralizing fetal skeleton during the last third of pregnancy (Kovacs and Kronenberg, 1997). Transport of calcium across the placental BM, against a concentration gradient, is mediated by plasma membrane calcium ATPase (PMCA). In humans, FGR is associated with decreased calcium deposition and bone mineralisation (Namgung *et al.*, 1993). However, PMCA activity is increased in placentas from FGR pregnancies, despite a decrease in protein expression (Strid *et al.*, 2003). P0 fetuses have lower calcium content (determined in dried ash content) at E17. By E19 fetal calcium content is normalised in tandem with an increase in K_{mf} of calcium (similar between groups at E17) (Dilworth *et al.*, 2010). This suggests that the placenta has the capacity to adapt according to specific fetal needs/gestational requirements. Parathyroid hormone related peptide (PTHrP), an activator of PMCA (Strid *et al.*, 2002) has been proposed as a candidate signal that may elicit these changes in calcium acquisition. In humans, PTHrP is increased in umbilical cord (UmV) blood from FGR pregnancies (Strid *et al.*, 2003).

The current literature indicates that the small placenta in the P0 mouse model of FGR fails to sufficiently adapt amino acid (system A) transport towards the end of pregnancy, resulting in FGR (Constância *et al.*, 2002; Kusinski *et al.*, 2011). This contrasts with the adaptation observed in the small but normal WT placenta (Coan *et al.*, 2008) and suggests that the underlying pathology of the small P0 placenta results in a failure of this adaptation, at least with respect to the system A amino acid transporter. This implies that the provision of other substrates of system A (such as glutamine) is also likely to be compromised near term and contribute to the observed FGR in the P0 mouse. Unidirectional maternofetal clearance (K_{mf}) of neither glutamine nor glutamate, amino acids essential for fetal growth and development, has been studied in the P0 mouse.

Whilst it is important to understand how placental amino acid transport compares in normal and FGR pregnancies, more complex untargeted methods offer a whole system perspective as is introduced in the section that follows.

1.12 Metabolomics: a holistic approach to understanding FGR

Metabolomics is the study of small molecule metabolites and as such this technique provides a functional readout of metabolic aspects of cell function and can link mechanism to phenotype (Figure 12). Untargeted, holistic metabolomic techniques can typically measure hundreds to thousands of metabolites, offering the potential to detect unexpected changes in biological samples. There are various techniques used to identify metabolites; typically a biological sample is extracted in a solvent and then separated based on metabolite mass and chromatographic retention – important for metabolites of similar (identical) mass.

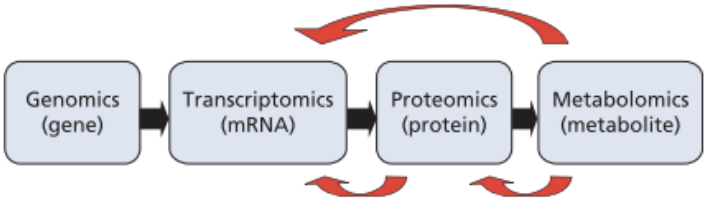


Figure 12: From genome to metabolome
The genetic material of an organism (genome) contains the material for the functional RNA, protein or metabolite product. The relationship is not strictly linear – metabolites for example can interact and influence the proteome. Figure from (Horgan *et al.*, 2009).

Metabolomics can be applied in pregnancy to investigate the metabolomic profile in various tissues and fluids including amniotic fluid, the placenta, vaginal secretions, urine of pregnant women and neonates, plasma of pregnant women and cord blood (Fanos *et al.*, 2013). A summary of the current literature of metabolomic profiles of maternal plasma and/or cord blood in FGR/SGA pregnancies can be found in Table 6.

Metabolomics holds promise to improve the diagnosis of FGR alongside existing techniques (e.g. ultrasonography to assess fetal biometry) (Gaccioli *et al.*, 2017; Horgan *et al.*, 2009). Indeed, potential novel biomarkers for pre-eclampsia and SGA have been identified by metabolomics (Horgan *et al.*, 2011; Kenny *et al.*, 2010). A study by Horgan *et al.* (2011) identified 19 metabolites in maternal plasma that may predict SGA. Furthermore, metabolomic profiling of normal pregnancies may provide a useful comparator for growth restricted pregnancies. In a study profiling normal pregnancy across gestation, Orczyk-Pawilowicz *et al.* (2016) identified a metabolic switch between the 2nd and 3rd trimester in both maternal plasma and amniotic fluid.

Low birth weight (LBW) and growth restricted (FGR) infants appear to have altered metabolomic cord blood profiles compared with normal birth weight infants (Favretto *et al.*, 2012; Ivorra *et al.*, 2012; Tea *et al.*, 2012), although specific profiles appear to be dependent upon gestational age at delivery and clinical subset (i.e. presence of abnormal UmA Doppler waveforms, for example) (Sanz-Cortés *et al.*, 2013). Of significance to the current study, glutamine and glutamate levels are perturbed in very low birth weight (VLBW, <1500 g) neonates born preterm (<32 weeks gestation) compared to full term infants (>37 weeks gestation). Glutamate levels are significantly lower in the UmA of VLBW infants, and the umbilical venous-arterial gradient for glutamine is positive, indicating increased fetal uptake of this amino acid (Alexandre-Gouabau *et al.*, 2013). However, a major limitation when comparing these data is the lack of consensus regarding definition of FGR, and the inclusion/exclusion criteria for case and controls (Table 6) highlighting the need for a systematic evaluation of the metabolomic profile of a well-defined FGR population.

Metabolomics of plasma of pregnant women			
Author	**Population**	**Technique**	**Main Findings**
Horgan *et al.*, 2011	Pregnant women who subsequently delivered an SGA baby (<10th centile) versus AGA (10th-90th centile) were sampled during first trimester between 14-16 weeks gestation.	LC-MS	Differences in 19 metabolites identified which included sphingolipids, phospholipids, carnitines, and fatty acids. Metabolomic profiles may help to predict SGA.
Metabolomics of cord blood			
Author	**Population**	**Technique**	**Main Findings**
Alexandre-Gouabau *et al.*, 2013	VLBW (<32 weeks gestation and/or a birth weight <1500 g) versus control (AGA, >37 weeks gestation).	LC-MS	Differences in carnitines, which indicates enhanced short- and medium chain fatty acid β-oxidation in VLBW class. VLBW neonates had dysregulated glutamate-glutamine shuttling.
Favretto *et al.*, 2012	IUGR (<10th centile) versus AGA (10th-90th centile).	LC-MS	Differences in 22 metabolites identified. Significant differences in phenylalanine, tryptophan, and methionine according to class.
Horgan *et al.*, 2011	Umbilical venous plasma from SGA (<10th centile) and AGA babies (10th-90th centile)	LC-MS	Differences in 19 metabolites identified which included sphingolipids, phospholipids, carnitines, and fatty acids.
Ivorra *et al.*, 2012	LBW (<10th centile) versus control (75th-90th centile)	^{1}H NMR	Differences in 7 metabolites identified. LBW versus control had lower levels of choline, proline, glutamine, alanine and glucose, and higher levels of phenylalanine and citrulline.
Sanz-Cortés *et al.*, 2013	Early IUGR (<10th centile, abnormal UmA Doppler, <35 weeks gestation) versus matched AGA; and late IUGR (<10th centile, normal UmA Doppler, >35 weeks gestation) versus matched AGA	^{1}H NMR	There is no unique metabolic profile for IUGR but changes exist within clinical subsets.
Tea *et al.*, 2012	VLBW (<32 weeks gestation and/or a birth weight <1500 g) versus control (full-term >37 weeks gestation)	^{1}H NMR	Metabolites vary on gestational age at delivery.

Table 6: A summary of metabolomic studies that have analysed maternal and umbilical cord plasma in cases of suboptimal fetal growth.
Criteria set by the researchers are included under the 'Population' heading. Alexandre-Gouabau *et al.* (2013) and Tea *et al.* (2012) sampled from UmV and UmA. All other studies in cord blood assessed UmV only. Abbreviations: ^{1}H NMR, proton nuclear magnetic resonance; AGA, appropriate for gestational age; IUGR, intrauterine growth restriction; LBW, low birth weight; LC-MS, liquid chromatography mass spectrometry; SGA, small for gestational age; UmA, umbilical artery; UmV, umbilical vein; VLBW, very low birth weight.

1.13 Summary

Allocation of nutrients to the growing fetus via placental transport mechanisms is essential for appropriate fetal growth. Approximately 5-10% of fetuses fail to achieve their genetic growth potential (ONS, 2015) based on a definition of IBR <5th centile, predominantly due to placental dysfunction (Mifsud and Sebire, 2014). These growth restricted (FGR) infants are at an increased risk of stillbirth and neonatal morbidity (Cantwell *et al.*, 2011; Gardosi *et al.*, 2014), and an increased risk of disease extends into adulthood (Barker *et al.*, 1990; McIntire *et al.*, 1999). Abnormal syncytiotrophoblast function contributes significantly to the pathogenesis of idiopathic late-onset FGR. Despite this, there are no therapies for FGR (Fisk and Atun, 2008). A major reason for this is that there is an incomplete understanding of the fundamental physiological processes underpinning FGR and indeed normal fetal growth.

Amino acids are essential to support numerous functions, including but not limited to, nucleotide synthesis, protein anabolism and pH homeostasis (Pochini *et al.*, 2014). Glutamine is a conditionally essential amino acid during pregnancy due to fetal demand exceeding dietary intake (Parimi and Kalhan, 2007), and is transported into fetal plasma at one of the highest rates of all amino acids during pregnancy (Battaglia, 2000). Glutamate is a precursor of glutamine; recycling of glutamine and glutamate between the placenta and fetal liver is an essential process during pregnancy to meet fetal nutrient and placental/fetal metabolic requirements (Moores *et al.*, 1994; Vaughn *et al.*, 1995).

Previous work has established that MeAIB transfer, a substrate of the system A transporter which also transports glutamine, is up-regulated in small placentas (per g) of normally grown mouse fetuses consistent with adaptation of nutrient transporters in relation to small placental size in this species (Coan *et al.*, 2008). Furthermore, in a mouse model of late-onset FGR (P0 knockout mouse) which demonstrates reduced placental size, nutrient transfer (MeAIB, reflective of system A activity) is initially higher in P0 versus WT littermates, per g placenta, but this adaptation fails towards term and growth restriction ensues (Constância *et al.*, 2002). There is some evidence of adaptation according to placental size (weight) in humans (Godfrey *et al.*, 1998) but data are relatively lacking. Thus there is an unmet need to assess the relationship between placental glutamine and glutamate uptake/transfer and placental size (weight) in a well-defined population of normal (AGA) and late-onset FGR in humans and mice. Furthermore, it is important that measures of placental function (nutrient uptake/transfer) are related to amino acid concentrations in the maternal and fetal (UmV and UmA) to understand the relationship between these compartments. Untargeted, holistic analyses such as metabolomics offer the potential to investigate the dynamic metabolite profile in an unbiased manner. Only by understanding these fundamental physiological processes can

we maximise our potential to identify novel therapeutic targets and devise strategies with which to treat FGR.

1.14 Hypothesis and aims

The hypotheses for this thesis are:

1. In normal pregnancy, glutamine and glutamate uptake (humans) and clearance (mice) adapts according to placental size, thus ensuring appropriate fetal supply of nutrients in a relatively small but normal placenta.
2. In FGR, these placental adaptations fail to occur, thus contributing to reduced fetal growth.

The major aims of this study were:

1. To determine whether unidirectional maternofetal clearance of, and expression of key transporters for, glutamine and glutamate is different between the lightest and heaviest placentas of a WT (C57BL/6J) mouse litter. To assess whether any of these measures were affected by sex of the fetus.
2. To assess the relationship between transporter-mediated glutamine and glutamate uptake and placental and fetal measures (placental weight, birth weight, F:P ratio, sex of the fetus) in normal pregnancy in women.
3. To determine whether unidirectional maternofetal clearance of, and expression of key transporters for, glutamine and glutamate is altered in the placental-specific *Igf2* (P0) knockout mouse model of FGR.
4. To determine whether transporter-mediated glutamine and glutamate uptake is altered in human FGR.
5. To relate measures of glutamine and glutamate uptake with concentrations of amino acids in maternal and (fetal) umbilical cord plasma in normal and FGR pregnancies in women.

Chapter 2 Methods

2.1 Studies in human placental tissue

2.1.1 Source of chemicals

Unless otherwise stated, all chemicals were from Sigma Aldrich Ltd, Dorset, UK.

2.1.2 Inclusion and exclusion criteria

Inclusion criteria for this study were as follows: maternal age >18<40 years and a body mass index (BMI) >19<30 kg/m^2 at first antenatal appointment. Women were not approached if there was evidence of congenital abnormalities, pre-eclampsia, hypertension, or pre-gestational or gestational diabetes. The demographics of those included in each arm of the study are stated in Chapters 4 and 6. Informed consent (Ethics number 15/NW/0829) for placenta, maternal blood and/or umbilical cord blood was obtained before delivery of the baby and placenta.

2.1.3 Collection of blood samples and placental tissue

All blood samples were collected into vacutainers which were either coated with lithium heparin (BD vacutainer®; BD, Plymouth, UK) or contained lithium heparin beads (S-monovette®, Sarstedt, Leicester, UK; disparity due to change in protocol at Manchester NHS Trust during the study). Maternal venous blood was collected prior to delivery; on admittance to the ward for vaginal births, before anaesthetic for deliveries by caesarean section and after prostaglandin administration for induction of labour. The placenta, and where possible umbilical arterial (UmA) and venous (UmV) blood, were collected immediately following delivery. Each woman was assigned an anonymous number under which all samples were collected and banked.

2.1.4 Dissection of placentas and banking

Untrimmed and trimmed (umbilical cord and placental membranes removed) placental weight was recorded for each placenta. Placental villous tissue was sampled according to a systematic sampling protocol (Figure 13). Tissue was frozen at -80ºC, stored in RNA*later*®, or neutral buffered formalin (NBF, composition in Table 7) for wax embedding. For studies of amino acid uptake by the placenta, six cubes (approximately 1 cm^3) of villous tissue were dissected and chorionic plate and decidua removed. Tissue was maintained in glutamine-free Dulbecco's Modified Eagles Medium (DMEM: 1 g/litre glucose, Life Technologies Ltd, Leicestershire, UK) supplemented with 864 μM glutamine and 120 μM glutamic acid mixed 1:1 with Tyrode's buffer (composition in section 2.1.5), for a final concentration of 432 μM and 60 μM

respectively to mimic the concentration in maternal plasma (according to measurements made in maternal plasma of women having normal pregnancy delivering at term at St Mary's hospital: Greenwood and Desforges, personal communication) before beginning the experimental protocol (section 2.1.5.4).

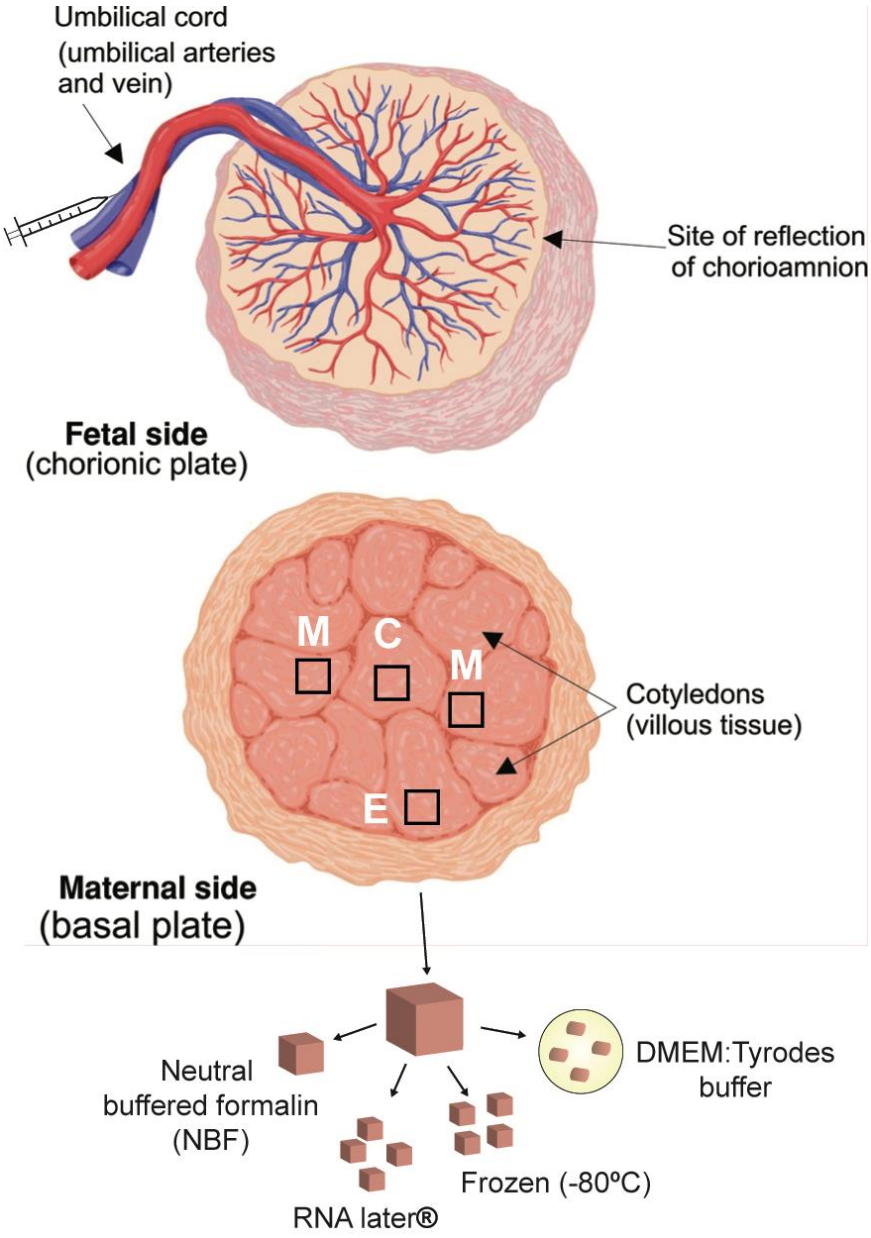


Figure 13: Standardised placental tissue sampling protocol
For each placenta collected, a systematic sampling protocol was followed. Four villous samples were dissected: from the centre (C), 2 x middle (M) and edge (E), and chorionic plate and decidua were removed. From each sample, 1 cube was stored in neutral buffered formalin (NBF) for wax embedding and several smaller sections were frozen at -80ºC or stored in RNA*later*®. For studies of amino acid uptake by the placenta an additional six cubes of villous tissue were dissected (approximately 1 cm^3, chorionic plate and decidua removed) and maintained in a petri dish of 1:1 Dulbecco's Modified Eagles Medium (DMEM):Tyrodes buffer as detailed in section 2.1.4. Where possible, UmV and UmA blood samples were drawn from a double clamped section of the umbilical cord and centrifuged (3,000 rpm (2,056 x g (max)) for 10 min at 4ºC) to obtain plasma (stored at -80ºC). Figure adapted from (Rampersad *et al.*, 2011).

Compound	Quantity
KH_2P0_4	1.36 g
Na_2HP0_4	5.68 g
dH_20	250 ml
Tap water	200 ml
~37% Formaldehyde	50 ml
Total Volume:	500 ml pH to 7.6

Table 7: 10% neutral buffered formalin (NBF)

2.1.5 Blood processing and banking

Maternal venous, UmV and UmA blood was collected in either lithium heparin-coated vacutainers or vacutainers containing lithium heparin beads (as in section 2.1.3). Samples were centrifuged at 3,000 rpm (2,056 x g (max)) for 10 min at 4ºC. The resulting plasma was aliquoted into fresh Eppendorf tubes (Eppendorf; Hamburg, Germany) and stored at -80ºC.

2.1.5.1 Measurement of glutamine and glutamate transporter activity in placental villous tissue

2.1.5.2 Method validation

A pilot experiment was performed to optimise an experimental protocol for measuring the activity of amino acid transporter systems A, L and N (glutamine transporters) and system X_{AG-} (glutamate transporter) in placenta villous tissue.

A method is well established to assess amino acid transporter activity in the microvillous membrane (MVM) of the syncytiotrophoblast by measuring uptake of radiolabelled substrates into placental villous fragments (Greenwood and Sibley, 2006). Measuring amino acid transporter activity in villous tissue fragments has the advantage that intracellular signalling mechanisms that might regulate the transporter activity and/or expression remain intact. On the other hand, when measuring uptake of radiolabelled substrates into tissue fragments dissected from the placenta, it is necessary to distinguish between specific, transporter-mediated uptake of substrates from non-specific diffusion that will occur through areas damaged by dissection. System A is a Na^+-dependent amino acid transporter and its activity has been assessed previously by measuring the Na^+-dependent uptake of ^{14}C-methylaminoisobutyric acid (MeAIB), a non-metabolisable substrate of system A, into villous fragments over 10-30 min (Hayward *et al.*, 2012).

In the pilot experiment, the same concentration of ^{14}C-MeAIB (8.5 nmol/ml) was used as Hayward *et al.* (2012) and ^{14}C-MeAIB uptake was measured in control Tyrode's buffer containing Na^+ (135 mM NaCl, 5.0 mM KCl, 1.8 mM $CaCl_2$, 1.0 mM $MgCl_2$, 10 mM HEPES, 5.6 mM D-glucose, adjusted to pH 7.4 with NaOH; uptake attributed to system A plus non-specific diffusion) and in Na^+-free Tyrode's buffer (as Tyrode's buffer but 135 mM NaCl

replaced with equimolar choline chloride, adjusted to pH 7.4 with KOH; uptake by non-specific diffusion) over 30-90 min. The difference between these measures (the Na^+-dependent uptake) was linear over 30-90 min when expressed per mg fragment protein (Figure 33). Extrapolation of the regression line fitted over this time period intercepted the x,y axis close to the origin (x=0, y=0). Therefore, the Na^+-dependent component of ^{14}C-MeAIB uptake over 30-90 min was taken to represent system A activity in the MVM of the syncytiotrophoblast measured at initial rate.

Transporter-mediated ^{14}C-glutamine and ^{14}C-glutamate uptake has not yet been measured in villous fragments. Therefore a pilot experiment was performed to determine (a) the appropriate concentration of isotope to use to achieve detectable levels of radioactivity in the tissue, (b) a strategy to detect the transporter-mediated component of ^{14}C-glutamine and ^{14}C-glutamate uptake, and (c) the time over which the activity of the glutamine and glutamate transporters could be measured at initial rate. Glutamine transport across the MVM, as determined in human placental MVM vesicle studies, is mediated by the Na^+-dependent systems A and N, and the Na^+-independent system L (Hill *et al.*, 2014). This conclusion was reached because ^{14}C-glutamine uptake into vesicles was inhibited by 5 mM histidine (His, substrate of system N) serine (Ser, substrate of system A) and BCH (2-Amino-2-norbornanecarboxylic acid, non-metabolisable analogue, substrate of system L) (Mastroberardino *et al.*, 1998). In non-placental cell types, glutamine is transported by y^+L, but in MVM vesicles arginine did not affect ^{14}C-glutamine uptake suggesting that this transport system is not important in MVM (Hill *et al.*, 2014). Therefore, in the initial experiment to assess the contribution of systems A, N and L to glutamine transport in fragments, uptake of ^{14}C-glutamine was measured in Tyrode's buffer in the absence of competitive substrates (a measure of total ^{14}C-glutamine uptake comprising non-specific diffusion and transporter-mediated uptake) and in control or Na^+-free Tyrode's buffer with 5 mM His (contribution of system N), 5 mM Ser (contribution of system A) and 5 mM BCH (contribution of system L).

MVM transport of ^{14}C-glutamate is mediated by the Na^+-dependent system X_{AG-}, of which aspartate is a substrate (Hill *et al.*, 2014). Accordingly, in the initial experiment to assess the contribution of system X_{AG-} to glutamate transport, uptake of ^{14}C-glutamate was measured in Tyrode's buffer in the absence of competitive substrates (a measure of total ^{14}C-glutamate uptake comprising non-specific diffusion and transporter-mediated components) and in control or Na^+-free Tyrode's buffer with 5mM aspartic acid (aspartate).

^{14}C-glutamine and ^{14}C-glutamate uptake was measured by exposing placental villous tissue to these radioisotopes for 10-120 min (following the protocols described in detail in section 2.1.5.4) and the transporter-mediated component was determined by subtracting uptake in Na^+-free Tyrode's buffer containing competitive substrates from uptake under control

conditions. ^{14}C-glutamine and ^{14}C-glutamate uptake was expressed in moles per mg fragment protein (see section 2.1.5.5 and 2.1.5.6) and plotted against time (10-120 min). These data were analysed by linear regression to determine the time period over which activity of the transporters was at initial rate (see results section 4.2.2).

2.1.5.3 Experimental preparation

From six villous tissue cubes (Figure 13), an equal number of small fragments of villous tissue (2-3 mm^3) were randomly sampled. Fragments were tied to a comb in triplicate (Figure 14) and suspended in glass vials DMEM:Tyrode's buffer.

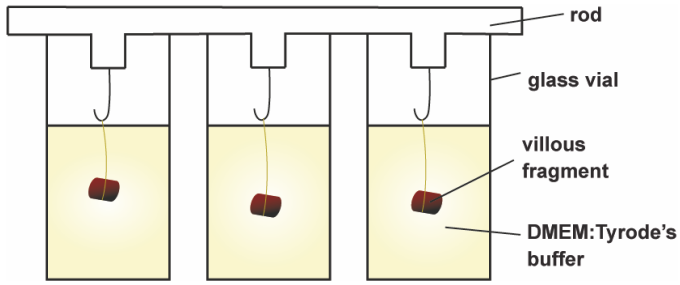


Figure 14: Diagram of placental villous fragments prepared for measurement of amino acid uptake
Placental villous fragments (2-3 mm^3) were randomly sampled from six larger villous tissue cubes. Fragments were tied to a comb in triplicate as shown above and maintained in DMEM:Tyrode's buffer until the experiment commenced.

2.1.5.4 Measurement of amino acid uptake

Following on from the pilot experiment described in section 2.1.5.2 and presented in results section 4.2.2, the following protocol was carried out for all subsequent experiments. A diagrammatic representation of the experimental protocol is shown in Figure 15. After the dissection and preparation of villous tissue (section 2.1.5.3), fragments were maintained at 37ºC (waterbath) in DMEM:Tyrode's buffer for 30 min. Fragments were then incubated in 'prewash' Na^+-containing ('control') or Na^+-free Tyrode's buffer supplemented with substrates to competitively inhibit transporter-mediated uptake (^{14}C-glutamine: 5 mM histidine, serine, BCH, added together to assess the collective contribution of the activities of system A, L and N; ^{14}C-glutamate: 5 mM aspartate; ^{14}C-MeAIB: no substrates added, Na^+-free conditions alone) for 2 min. The prewash step was included to deplete tissue Na^+ before measuring uptake in Na^+-free buffer. Next, fragments were incubated in Tyrode's buffer (+/- Na^+) containing 4 ml ^{14}C-MeAIB (8.5 nmol/ml; 0.0185MBq/ml), ^{14}C-glutamine (0.24 nmol/ml; 0.0024MBq/ml) or ^{14}C-glutamate (0.5 nmol/ml; 0.0048MBq) for 30, 60 or 90 min ('incubation buffer'). After the elapsed time period, fragments were vigorously washed for 2 x 15 sec in 12 ml ice-cold Tyrode's buffer (+/- Na^+) to remove extracellular isotope and to limit efflux of intracellular isotope during the wash process. Samples were subsequently suspended

in 4 ml water for 18 hours to allow release of radioisotope accumulated in the tissue into solution. Lysed tissue samples were then placed in 3 ml 0.3 M NaOH, incubated at 37°C overnight to digest, and stored at 4°C prior to determination of protein content.

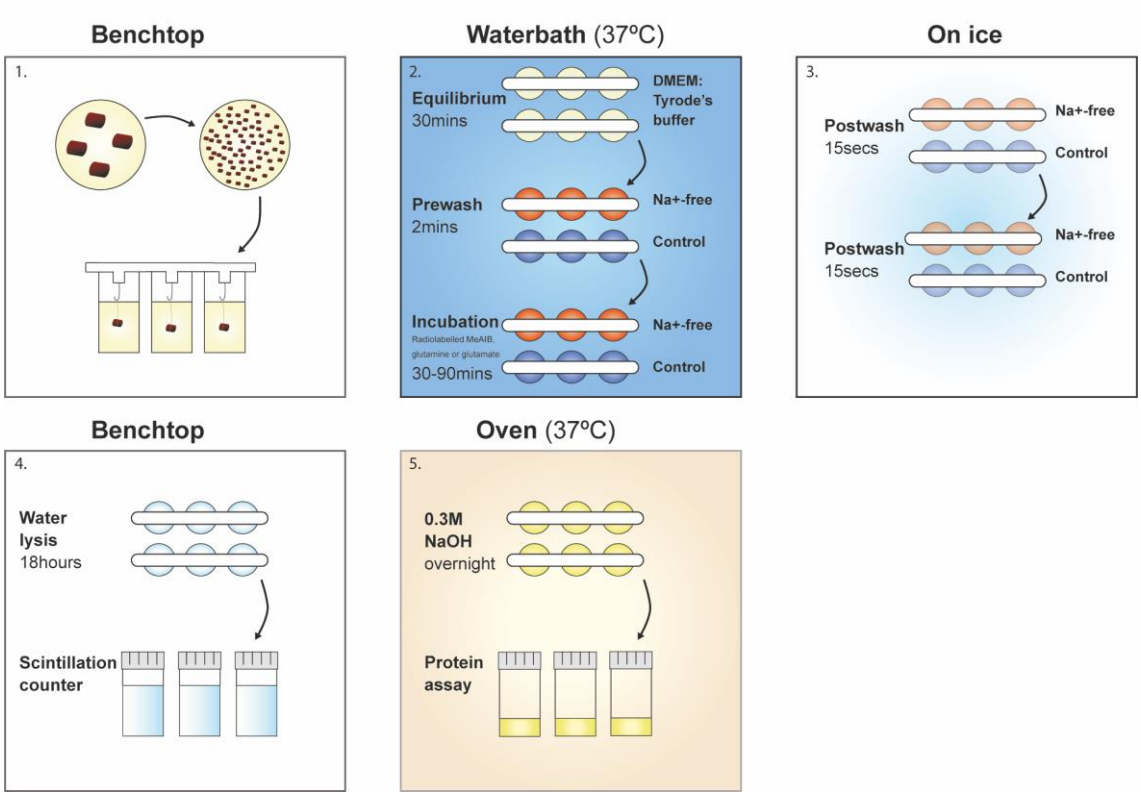


Figure 15: Diagrammatic representation of method to measure amino acid uptake into placental villous fragments

1. Placental villous fragments were randomly sampled, tied to a comb in triplicate and maintained in DMEM:Tyrodes buffer as described in Figure 14. **2.** Once all samples were prepared, all vials were placed in a water bath (set at 37ºC) to equilibrate. Next, each comb (holding triplicate fragments) was moved to prewash Tyrode's buffer (red vials denote Na^+-free Tyrode's buffer, blue represent Na^+-containing 'control' Tyrode's buffer) for 2 min, then into 'incubation buffer' containing radiolabelled amino acids for 30-90 min. See text for full description. **3.** After the allotted time, fragments were washed vigorously ('postwash') for 2 x 15 sec in ice-cold Tyrode's buffer (+/-Na^+) **4.** Samples were suspended in water for 18 hours to lyse the tissue, thus releasing radioisotope into the water to be counted on the scintillation counter. **5.** The fragments were then dissolved in 0.3 M NaOH overnight at 37ºC. The resultant solution was used to calculate protein content (i.e. a proxy measure of fragment size) by protein assay (section 2.1.5.5).

2.1.5.5 Protein assay

Protein content of placental fragments was determined by BioRad protein assay. Protein stock (10 mg bovine serum albumin, BSA, in 10 ml NaOH) was diluted 1:4 with 0.3 M NaOH to produce standards of known protein content (Table 8). Standard and unknown samples (20 µl) were pipetted in duplicate into a 96-well plate. To each sample, 180 µl neutralising solution (1:1.25 of 0.3 M NaOH:0.3 M HCl) and 50 µl BioRad dye reagent (Bio-Rad laboratories Ltd., UK) was added and mixed thoroughly by pipetting. After 10 min incubation, absorbance was

quantified using a microplate reader and Omega software (FLUOstar Omega, BMG Labtech) at 595 nm. A standard curve was generated using GraphPad Prism 7 software, from which protein content (per 20 µl) was determined by interpolation. To determine the total protein content of each fragment (denatured in 3 ml 0.3 M NaOH), the content in µg/20 µl was multiplied by 150 and divided by 1000 to calculate total protein content (mg) in 3 ml NaOH.

0.3M NaOH (µl)	**BSA** 0.25 mg/ml (µl)	**Total volume** (µl)	**BSA content** (µg)	**BSA content** (µg) in 20 µl
160	0	160	0	0
152	8	160	1	0.25
120	40	160	5	1.25
80	80	160	10	2.5
40	120	160	15	2.75
0	160	160	20	5.00

Table 8: Serial dilution of bovine serum albumin (BSA) to prepare standards for Bio-Rad protein assay

2.1.5.6 Calculation of amino acid uptake

Scintillation fluid (16 ml, ScintiSafe, Fisher Scientific, UK or Meridian Biotechnologies, Ltd.) was added to the water lysates for measurement of ^{14}C radioactivity using a β-scintillation counter (Tri-carb 2100TR liquid scintillation analyzer, Packard, disintegrations per minute; dpm). Counts/pmol in the incubation buffer was determined by counting 100 µl samples of incubation buffer, removing the background count and multiplying by 10 to convert to counts/ml. Next, counts/ml were divided by nmol/ml of radioisotope as follows: MeAIB (8.5 nmol/ml), glutamine (0.24-0.28 nmol/ml) and glutamate (0.52 nmol/ml), and finally converted to counts per pmol by dividing by 1000. Uptake of radiolabelled amino acids was expressed as pmol/mg fragment protein content, corrected for background radioactivity. The transporter-mediated component was determined by subtracting uptake in the presence of competing substrates/Na^+-free buffer from total uptake.

2.1.5.7 Statistical analysis

Normal distribution of data was determined using D'Agostino & Pearson omnibus normality test (GraphPad Prism 7 software). Data are expressed as mean ± standard error of the mean (SEM). Regression analysis was performed to determine whether transporter-mediated amino acid uptake was linearly related to time over 30-90 min and to examine the relationship between transporter-mediated uptake at 90 min and placental weight, birth weight and birth weight:placental weight (BW:PW) ratio (Spearman correlation or Linear regression). The correlation was considered significant if $P<0.05$.

2.2 Studies in mouse models of human pregnancy

2.2.1 Animal husbandry

All experimental procedures were performed in accordance with the UK Animal (Scientific Procedures) Act of 1986 under Home Office project licences PPL 40/3385 or PPL 70/8504 and were approved by the local ethical review panel of the University of Manchester. All animals were housed in individually ventilated cages, were provided with nesting material and had free access to food (BK001 diet, Special Diet Services, UK) and water (Hydropac watering system, Lab products Inc, Delaware, US). Animals were housed in rooms with a 12 hour light-dark cycle at 21-23ºC. Rooms were not pathogen-free. C57BL/6J (wild-type, WT) female mice (10-16 weeks old, Envigo, UK) were mated with C57BL/6J (WT) males (10-26 weeks old). Placental-specific *Igf2P0* knockout (P0) mice were kindly gifted by Dr Miguel Constancia and Prof Wolf Reik, University of Cambridge. Males (10-101 weeks old) heterozygote for the *Igf2* P0 gene were mated with WT females (8-16 weeks old). Flow charts and a detailed account of the number of animals used in each experiment can be found in the relevant chapters (Chapter 3 and Chapter 5). The first day of gestation (embryonic day (E) 0.5) for all mice was determined by the discovery of a copulation plug. Experimental procedures were carried out at E15.5 or E18.5 (chosen as peak growth of the fetus occurs between these two time points; term E19-20) (Figure 16).

2.2.2 Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate

Dams were anaesthetised with a 300 µl intraperitoneal injection of 1:1:2 combination of fentanyl/fluanisone (Hypnorm, VetPharma Ltd., Leeds, UK), Midazolam (Roche, UK) and sterile H_20 (Braun medical Inc., Pennsylvania, USA). Additional doses of anaesthetic were given intraperitoneally (50-100 µl per dose) during the experimental procedure as required. Dams were placed in a ventilated hot box (37ºC) for approximately 5 min to dilate the tail vein. Dams were then placed on a heat pad for cannulation of the tail vein with a 25G needle attached to polyethylene tubing (Portex, Hythe, UK). Successful cannulation of the tail vein was confirmed by flushing a small volume of heparinised-saline (500 I.U. heparin/ml saline, Alliance Healthcare, Chessington, UK/Fresenius Kabi Ltd, Runcorn, UK) solution. Dams were tracheotomised to ensure a clear airway and prevent acidosis during the procedure. 100 µl of radioisotope was administered via the tail vein cannula (Figure 16) confirmed by pre- and post-weighing of the syringe. The amount of radioactivity in sterile phosphate buffered saline (PBS) was: 0.023 MBq ^{14}C-glutamine and 0.046 MBq ^{14}C-glutamate. At approximately 2 min post-injection (between 90 sec-4 min ^{14}C-glutamine and 90 sec-3 min for ^{14}C-glutamate), dams were exsanguinated by cardiac puncture, a maternal blood sample obtained and centrifuged at 5000 rpm (1,845 x g) for 5 min to obtain plasma. Death was confirmed by cervical dislocation

and a laparotomy immediately performed. Both uterine horns were exposed and the distribution of conceptuses per horn recorded as well as any fetal resorptions. Fetuses and placentas were trimmed of membranes, blotted and weighed. Biometric measurements were also recorded for C56BL/6J (WT) litters (Chapter 3) as follows: crown:rump length (from the top of the head to beginning of the tail), abdominal circumference (at the level of umbilical cord insertion) and head circumference (above the eyes and ears) using a length of cotton thread. Fetal tail tips were collected and stored at -20ºC for sex and/or genotype determination (section 2.2.6). Fetuses and placentas were placed in an individual scintillation vial, minced and solubilised in 3 ml 3% KOH at 55ºC overnight.

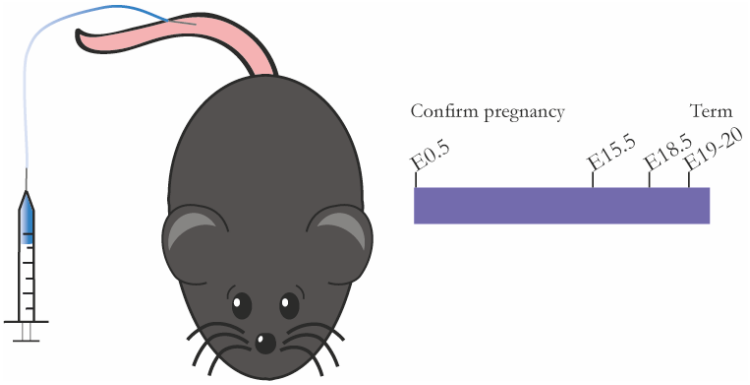


Figure 16: Schematic of unidirectional maternofetal clearance experiments
Pregnancies were confirmed at embryonic day (E)0.5, with experimental procedures performed at E15.5 or E18.5 (term E19-20). Radioisotope was administered via a tail vein cannula as depicted above.

To measure ^{14}C radioactivity in the samples, 15 ml scintillation fluid (ScintiSafe, Fisher Scientific, UK or Meridian Biotechnologies, Ltd., UK) was added to each vial. To measure ^{14}C counts in maternal blood 10 µl of maternal plasma was added to 15 ml scintillation fluid, 140 µl dH_20 and 3 ml 3% KOH. All samples were analysed using a β-scintillation counter (Tri-carb 2100TR liquid scintillation analyzer, Packard, disintegrations per minute; dpm). Counts were monitored over a series of days until a plateau was reached indicating that tissue had been fully digested. Final ^{14}C counts were calculated by subtracting background radioactivity in 'blank' samples (15 ml scintillation fluid, 150 µl dH_20 and 3 ml 3% KOH).

2.2.3 Maternal plasma ^{14}C disappearance curve

The time at which clearance of ^{14}C-glutamine and ^{14}C-glutamate from the maternal circulation was linear was determined by the construction of a ^{14}C maternal plasma disappearance curve. A single maternal blood sample was collected at the time of euthanisation (by cardiac puncture) and processed as described above. Dams were euthanased at timepoints between 22 sec-31 min (glutamine) 26 sec-35 min (glutamate), and data from each radioisotope were collated to construct the disappearance curve (see Figure 18 in Chapter 3 for more details).

The curve was fitted to a one-phase exponential decay model as described previously (Bond *et al.*, 2006a; Bond *et al.*, 2008). Experimental dams were culled within the linear portion of this maternal plasma disappearance curve (90 sec-4 min, glutamine; 90 sec-3 min glutamate).

2.2.4 Calculation of unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate

Unidirectional maternofetal transfer ($^{14C}K_{mf}$, µl/min/g placenta) was calculated as:

$$K_{mf} = \frac{Nx}{W \int_0^x Cm(t)dt}$$

where N_x is total radiolabel accumulation (dpm) by the fetus at *x* minute post-injection of radioisotope via the maternal tail vein. W is placental wet weight (g) and $\int_0^x Cm(t)dt$ is the time integral of radioisotope concentration in maternal plasma (dpm x min/µl from 0-x min area under the curve, from maternal plasma ^{14}C disappearance curve).

2.2.5 Statistical analysis

Data were checked for normality (D'Agostino and Pearson omnibus normality test). Data from C57BL/6J (WT) litters were analysed by comparing the lightest versus heaviest placenta in a litter, or by comparing the litter average of male versus female placentas (a minimum of two of each sex per litter was required). For P0 litters, an average of WT or P0 placentas or fetuses within a single litter was used (a minimum of two of each genotype per litter was required). Data were not normally distributed and therefore presented as median [range]. Data were analysed (GraphPad Prism 7 software) by Wilcoxon signed rank test and were considered statistically significant where $P<0.05$.

2.2.6 Determination of fetal sex and genotype

Fetal tail tips were obtained as described in section 2.2.2. C57BL/6J (WT) tail tips were used to determine the sex of each fetus (from Kunieda *et al.*, 1992). For P0 litters, genomic DNA was extracted from fetal tail tips to determine fetal genotype (DNeasy, Qiagen, Crawley, UK). The sex or genotype of each fetus was determined by PCR using the reagents and primers specified in Table 9. PCR conditions are displayed in Table 10. PCR products were run on a 1% (for fetal sex) or 1.5% (for genotype, bromophenol blue loading dye also required) agarose gel at 120 V for 40 min and visualised under UV light.

Determination of fetal sex	
Reagent/Primer (sequence, 5'-3')	**Volume** (μl)
My Taq Red Mix	25
PCR H_20	5
SRY2 primer (TCTTAAACTCTGAAGAAGAGAC)	4
SRY4 primer (GTCTTGCCTGTATGTGATGG)	4
NDS3 primer (GAGTGCCTCATCTATACTTACAG)	4
NDS4 primer (TCTAGTTCATTGTTGATTAGTTGC)	4
Determination of genotype	
Reagent/Primer (sequence, 5'-3')	**Volume** (μl)
10x PCR buffer without Mg^{2+}	2
dNTPs (final concentration of 200 μM)	2
PCR H_20	8.5
$MgCl_2$ (final concentration of 2 mM)	1.6
Forward primer (common primer; dF) (TCCTGTACCTCCTAACTACCAC)	1.2
Reverse primer (P0; dR) (GAGCCAGAAGCAAACT)	1.2
Reverse primer (WT) (CAATCTGCTCCTGCCTG)	1.2
Taq polymerase enzyme	0.3

Table 9: Reagents and primers used (per PCR reaction) for the identification of male/female fetuses within WT (C57BL/6J) litters and *Igf2* P0$^{+/-}$ and WT fetuses within P0 litters.
My Taq Red Mix was from Bioline Reagents Ltd, London, UK. Primers used for determination of fetal sex had a final concentration of 500 nM. Primers used for determination of genotype had a final concentration of 300 nM.

Determination of fetal sex		
Step 1	94ºC	4 min
Step 2	94ºC	1 min
Step 3	55ºC	1 min
Step 4	72ºC	1 min
Step 5	Repeat steps 2-4 for 35 cycles	
Step 6	72ºC	10 min
Determination of genotype		
Step 1	94ºC	4 min
Step 2	94ºC	1 min
Step 3	56ºC	1 min
Step 4	72ºC	1 min
Step 5	Repeat steps 2-4 for 40 cycles	
Step 6	72ºC	10 min

Table 10: PCR cycle conditions for determination of fetal sex and genotype (male/female fetuses within WT (C57BL/6J) litters and *Igf2* P0$^{+/-}$ and WT fetuses within P0 litters).

2.2.7 Fetal weight distribution curves

Fetal and placental weights were recorded at the time of laparotomy (section 2.2.2). Fetal weight distribution curves were generated from population fetal weights collected in the current study. The 5th percentile of fetal weight was calculated using the equation below:

$$(-z\ score\ x\ SD) + mean$$

Where z score = 1.645 and SD = standard deviation (Dilworth *et al.*, 2011).

2.3 Western blotting

2.3.1 Processing of human placental tissue for Western blotting

Placental villous tissue samples were dissected and stored at -80ºC as described in section 2.1.4.

2.3.1.1 Membrane-enriched placental villous homogenates from human placentas

Frozen placental tissue (approximately 100mg) was homogenised (2-3 x 30 sec homogenisations) in 1:100 protease inhibitor cocktail (PIC, Sigma-Aldrich, UK P8340):Buffer A (300 mM mannitol 54.66 g/litre and 10 mM HEPES 2.38 g/litre, pH 7.6 with saturated Tris (12.114 g/100 ml)). Placental homogenates underwent ultracentrifugation (Hitachi Sorvall™ Discovery™ 100SE, T1250 rotor) for 5 min at 2500 g (4ºC) and the resultant supernatant was centrifuged (in fresh tubes) for 30 min at 100,000 g. The pellet was then re-suspended in 200 µl PIC:Buffer A (1:100), aliquoted and stored at -80ºC.

2.3.2 Collection of mouse placental tissue for Western blotting

WT and P0 dams and fetuses (E15.5 and E18.5, setup as described in section 2.2.1) were euthanised by cervical dislocation in accordance with Home Office approved Schedule 1 methods. Dams were immediately laparotomised, both uterine horns exposed and the distribution of conceptuses per horn recorded as well as any fetal resorptions. Fetuses and placentas were trimmed of membranes, blotted and weighed. Fetal tail tips were collected and stored at -20ºC for determination of fetal sex/genotype (section 2.2.6). Fetuses and placentas were snap frozen on dry ice and stored at -80ºC.

2.3.2.1 Membrane-enriched whole placental homogenates from mouse placentas

Membrane-enriched whole placental homogenates were obtained from the lightest and heaviest placentas within each C57BL/6J (WT) litter, and for one WT and one P0 placenta (as determined by genotyping, chosen randomly) from each P0 litter. Whole placentas were homogenised in 2 ml 1:100 protease inhibitor cocktail (PIC, Sigma-Aldrich, UK P8340):Buffer A (300 mM mannitol 54.66 g/Litre and 10 mM HEPES 2.38 g/Litre, pH 7.6 with saturated Tris (12.114 g/100 ml)). Placental homogenates underwent ultracentrifugation (Hitachi Sorvall™ Discovery™ 100SE, T1250 rotor) for 5 min at 2500 g. 200 µl of supernatant was retained (post-nuclear supernatant) and aliquoted. The remaining supernatant was transferred to fresh tubes and centrifuged at 100,000 g for 30 min. Cytosolic supernatant was aliquoted. The remaining membrane-enriched pellet was then resuspended in 200 µl PIC:Buffer A (1:100) and aliquoted. All samples were labelled and stored at -80ºC.

2.3.3 Reagents and gels

Reagents used for Western blotting are detailed in Table 11. Gels were either homemade or precast (4–15% Mini-PROTEAN® TGX™ Precast Protein Gels, Bio-Rad laboratories Ltd., UK). Homemade gels were prepared as follows: a 10% resolving gel (Table 11) was poured, covered with ethanol and allowed to set. Once set, the ethanol was poured off and replaced with 3% stacking gel (Table 11). A 10 or 15-well comb was inserted immediately. Once set, the comb was removed and the gel was placed in an electrophoresis tank, bathed in running buffer (Table 11) in preparation for sample loading.

Reagent	Composition
Resolving buffer	22.7 g TRIZMA® base 0.5 g SDS 100 ml dH_20 pH to 8.8 with conc. HCl
Stacking buffer	6.1 g TRIZMA® base 0.4 g SDS 100 ml dH_20 pH to 6.8 with conc. HCl
Ammonium persulphate (APS, 10%)	0.1 g/ml in dH_20
Sodium dodecyl sulphate (SDS, 0.1%)	0.1 g SDS 100 ml dH_20
Resolving gel (2 x 10%)	8 ml dH_20 6.6 ml 30% acrylamide 5 ml Resolving buffer 0.2 ml 10% SDS 0.2 ml 10% APS 0.02 ml TEMED
Stacking gel (2 x 3%)	3.05 ml dH_20 0.69 ml 30% acrylamide 1.25 ml Stacking buffer 0.025 ml 10% SDS 0.025 ml 10% APS 0.015 ml TEMED
10x Running buffer (2 litres)	60.6 g TRIS base 288 g Glycine 20 g SDS Make up to 2 L with dH_20 Use at 2 x (dilute with dH_20)
10x Transfer buffer (2 litres)	60.6 g TRIS base 288 g Glycine Make up to 2 L with dH_20 Use at 1 x (100 ml 10 x transfer buffer + 700 ml dH_20 + 200 ml methanol)
Reducing non-boil loading buffer (non-reducing non-boil loading buffer; same composition without DTT)	4.805 g Urea 0.5 g SDS 0.004 g Bromophenol blue 0.7018 g DTT 0.6057 g Tris base 10 ml dH_20 pH to 6.9
Reducing boil loading buffer (non-reducing boil loading buffer; same composition without β-mercaptoethanol)	25 ml Stacking buffer 23.78 g Urea 3.99 g SDS 0.0018 g Bromophenol blue 19.98 ml Glycerol 43 ml dH_20 10% β-mercaptoethanol (add fresh on day) pH to 6.9

Table 11: Western blotting reagents

DTT, Dithiothreitol; TEMED, Tetramethylethylenediamine; TRIZMA® base, Tris(hydroxymethyl)aminomethane.

2.3.4 Gel electrophoresis and transfer

Membrane-enriched homogenates were loaded with the appropriate loading buffer (see Table 12, boil conditions 1:1 sample:loading buffer, non-boil conditions 2:1; alongside a Precision Plus Protein™ All Blue Prestained Protein Standard (Bio-Rad laboratories Ltd., UK)). Gels underwent electrophoresis (10 min at 80 V then 60 min at 120 V homemade gels, 30 min at 200 V for precast gels) in an electrophoresis tank containing running buffer (Table 11).

Gels were then removed, rinsed in transfer buffer and assembled into a transfer cassette for transfer onto a polyvinylidene fluoride (PDVF) membrane (Immobilon®-FL transfer membranes, Merk Millipore Ltd., Ireland; cut to size of the gel, activated in 100% methanol for 15-30 sec) in the following order: sponge, 2 x filter paper (GE Healthcare UK Ltd., Buckinghamshire, UK; pre-soaked in transfer buffer), gel, PDVF membrane, 2 x filter paper, sponge. A small roller was passed over each layer to remove any air bubbles. Cassettes were loaded into a tank containing an ice pack and transfer buffer (Table 11) for 70 min at 120 V (30 min at 100 V for precast gels). The resultant membranes were blocked for 1 hour at room temperature (RT) in either 3% milk (Marvel; London UK) in 100 ml 1 x phosphate buffered saline (PBS) or 5% bovine serum albumin (BSA) in 1 x PBS (see Table 12 for specific conditions). Membranes were then probed with primary antibody (Table 12 for supplier and concentration) in block (3% milk or 3% BSA) and 0.1% Tween overnight at 4ºC on a roller. The next day membranes were washed (4 x 5 min) in 1 x PBS and 0.2% Tween then incubated with fluorescently-conjugated secondary antibody (IRDye 800CW donkey anti-rabbit 1:20,000 in blocking buffer, 0.1% Tween and 0.02% SDS) for 1 hour at RT. Finally, membranes were washed 4 x 5 min in 1 x PBS and 0.2% Tween and kept in 1 x PBS until visualised using a Li-Cor Odyssey machine (Biosciences UK Ltd.).

Antibody (dilution)	**EAAT1** (1:1000)	**EAAT2** (1:1000)	**EAAT3** (1:5000)	**LAT1** (1:200)	**LAT2** (1:500)	**SNAT5** (1:500)	**β-actin** (1:500)	**β-tubulin** (1:500)
Amount of protein loaded	30 μg	30 μg	20 μg	30 μg	40 μg *30 μg	40 μg	-	-
Gel %	10%	Precast	10%	10%	10%	Precast	-	-
Loading buffer	Non-boil reducing	Boil (100ºC, 5 min) reducing	Non-boil reducing	Non-boil reducing	Non-boil reducing	Boil (100ºC, 5 min) reducing	-	-
Blocking buffer	3% milk	3% milk	3% milk	3% milk	5% BSA *3% milk	3% milk	-	-
Antibody manufacturer (catalogue no.)	Abcam (ab416)	Abcam (ab178401)	Abcam (ab124802)	TransGenic Inc (KAL-KE026)	Abbexa (abx121147) *Abcam (ab75610)	Abcam (ab72717)	Abcam (ab8227)	Abcam (ab6046)

Table 12: Experimental conditions for Western blotting

*denotes conditions used in Chapters 5 and 6 using an antibody from Abcam, due to discontinuation of Abbexa antibody.

2.3.5 Densitometry and statistical analysis

Densitometry (semi-quantitative analysis of transporter protein expression as determined by protein band density) was performed using Image Studio™ Lite software. Protein expression intensity was normalised to β-actin or β-tubulin expression. Housekeeper proteins were optimised so that expression fell within the linear range of detection (i.e. was not saturated over the concentrations used). Where the protein of interest was similar in size to β-actin or β-tubulin, IRDye (680LT donkey anti-rabbit 1:40,000) fluorescently-conjugated secondary antibody was used. Data were analysed (GraphPad Prism 7 software) using a Mann-Whitney test. A *P* value <0.05 was considered statistically significant. The full Western blots presented in this thesis can be found in Appendix 9.11.

2.4 Molecular biology techniques

2.4.1 Extraction of total RNA from human placental villous tissue

RNA*later*®-preserved villous tissue samples (stored at -80ºC) from normal (AGA) and FGR pregnancies were selected (from Maternal and Fetal Health Research Centre tissue bank; FGR individualised birth weight ratio (IBR) <5th centile; AGA IBR 20th-80th centile) for analysis by qPCR. Total RNA was isolated using the *mir*Vana™ isolation kit (AM1560, Ambion, LifeTechnologies, UK). To approximately 50 mg of villous tissue, 500 µl Lysis/Binding Buffer was added and homogenised on ice. The manufacturer's protocol was followed; RNA was extracted using organic solvents (Acid-Phenol:Chloroform extraction) then samples were passed through a glass-fibre filter cartridge for purification of total RNA, with a final elution in a volume of 100 µl. Samples were treated with DNAse using TURBO DNA-*free*™ kit (AM1906, Ambion, LifeTechnologies, UK) following the manufacturer's instructions.

2.4.2 Quantification of RNA by Nanodrop

1 µl of DNAse-treated RNA was loaded onto the spectrophotometer (Nanodrop 2000c, ThermoFisher Scientific, UK) to assess RNA purity and quantity. A purity 260/280 ratio of 2.0±0.2 was considered acceptable for further use. Samples were stored at -80ºC.

2.4.3 Generation of cDNA (reverse transcription)

Complementary DNA (cDNA) was synthesised from 500 ng RNA using an AffinityScript Multiple Temperature cDNA Synthesis Kit (Agilent Technologies, Stratagene, UK). A reference total human placental RNA (1 µg, AM7950, Ambion, Life Technologies, UK) and negative controls (no reverse transcriptase, -RT and no RNA template control, NTC) were also included. All samples were reverse transcribed in duplicate. 3 µl of random primers (0.1 µg/µl) was added to each tube, incubated at 65ºC for 5 min (Stratagene MX3005P) and

subsequently cooled at RT for 10 min to allow primers to anneal to the RNA. The following components were then added to each tube to give a final volume of 20 µl: 2 µl 10 x AffinityScript RT Buffer, 0.8 µl dNTP mix (25 mM of each dNTP), 0.5 µl RNase Block Ribonuclease Inhibitor (40 U/µl) and 1 µl AffinityScript Multiple Temperature RT. The reaction was incubated at 25ºC for 10 min to extend the primers, then the reaction temperature was increased to 42ºC for 60 min to generate cDNA. The reaction was terminated by incubation at 70ºC for 15 min. A pooled cDNA sample was generated by collecting 5 µl of each undiluted cDNA sample. The cDNA pool was diluted 1:4 (i.e. 50 µl cDNA in 150 µl PCR H_20). Samples were serially diluted (ie. 100 µl of 1:4 standard added to 100 µl PCR H_20 to generate 1:8 solution, and so on) to generate samples for a standard curve (1:4 – 1:256). All samples were stored at -20ºC. The efficiency of reverse transciption was checked against a housekeeping gene (TBP, TATA-box binding protein) as described below.

2.4.4 Quantitative real-time PCR (qRT-PCR) of mRNA

A mastermix for qRT-PCR was prepared per reaction as follows: 0.2 µl PCR H_20, 5 µl 2X SYBR Green QPCR master mix, 0.3 µl ROX (reference dye, diluted 1:500 in PCR H_20) (Brilliant III Ultra-fast SYBR Green Master Mix, Agilent Technologies, Wokingham, UK) and 0.25 µl of each forward and reverse primer for the gene of interest (diluted 1:10 in PCR H_20, primer sequences in Table 13). 4 µl cDNA samples (diluted 1:10 in PCR H_20, 10 ng cDNA) were mixed with 6 µl master mix and measured in duplicate. A standard curve and negative controls (-RT, NTC) were included in duplicate on each plate. Conditions for qPCR (Stratagene MX3005P) were as follows: 95ºC 5 min, then 95ºC 30 sec, 60ºC 30 sec, 72ºC 30 sec (40 cycles, amplification), 95ºC 1 min, 55ºC 30 sec then a final increase to 95ºC (in 0.2ºC increments) to generate a dissociation curve. For each primer set, a single peak in the dissociation curve and an efficiency between 90-110% was considered acceptable. Cycle threshold (CT) values generated by qPCR were interpolated from the standard curve on each plate, generated from the pooled cDNA sample (section 2.4.3) to calculate mRNA levels. Primer sequences can be found in Table 13. A representative standard curve, dissociation curve and amplification plot are shown in Figure 17.

2.4.5 Determination of suitable housekeeping genes for villous tissue and statistical analysis

Sample expression of housekeeping genes was analysed to confirm that expression was not significantly different between groups. The housekeeping genes TBP (TATA-box binding protein) and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) were selected as these have been previously shown to be stable in placental tissue

(Meller *et al.*, 2005). Expression of TBP and YWHAZ was stable across samples analysed (data not shown). Expression of the gene of interest was normalised to the geometric mean of TBP and YWHAZ since the geometric mean controls for differences in relative gene abundance or expression, unlike the arithmetic mean (Vandesompele *et al.*, 2002). Data were analysed (GraphPad Prism 7 software) using a Mann-Whitney test. A P value <0.05 was considered statistically significant.

Gene	Gene name	Primer sequence (5'-3') Forward (F), Reverse (R)	Annealing temperature (ºC)	Product size (bp)
TBP	TATA box binding protein	F: CACGAACCACGGCACTGATT R: TTTTCTTGCTGCCAGTCTGGAC	60	89
YWHAZ	Tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta	F: CCTGCATGAAGTCTGTAACTGAG R: TTGAGACGACCCTCCAAGATG	60	130
SLC38A1	Solute carrier family 38, member 1	F: GTGTATGCTTTACCCACCATTGC R: GCACGTTGTCATAGAATGTCAAGT	60	187
SLC38A2	Solute carrier family 38, member 2	F: ACGAAACAATAAACACCACCTTAA R: AGATCAGAATTGGCACAGCATA	60	141
SLC38A4	Solute carrier family 38, member 4	F: TTGCCGCCCTCTTTGGTTAC R: GAGGACAATGGGCACAGTTAGT	60	152
SLC3A2	Solute carrier family 3, member 2	F: ACCCCTGTTTTCAGCTACGG R: GGTCTTCACTCTGGCCCTTC	60	160
SLC7A5	Solute carrier family 7, member 5	F: GGAAGGGTGATGTGTCCAATC R: TAATGCCAGCACAATGTTCCC	60	83
SLC7A8	Solute carrier family 7, member 8	F: AGGCTGGAACTTTCTGAATTACG R: ACATAAGCGACATTGGCAAAGA	60	126

Table 13: Primer sequences for qPCR genes of interest and housekeeping genes

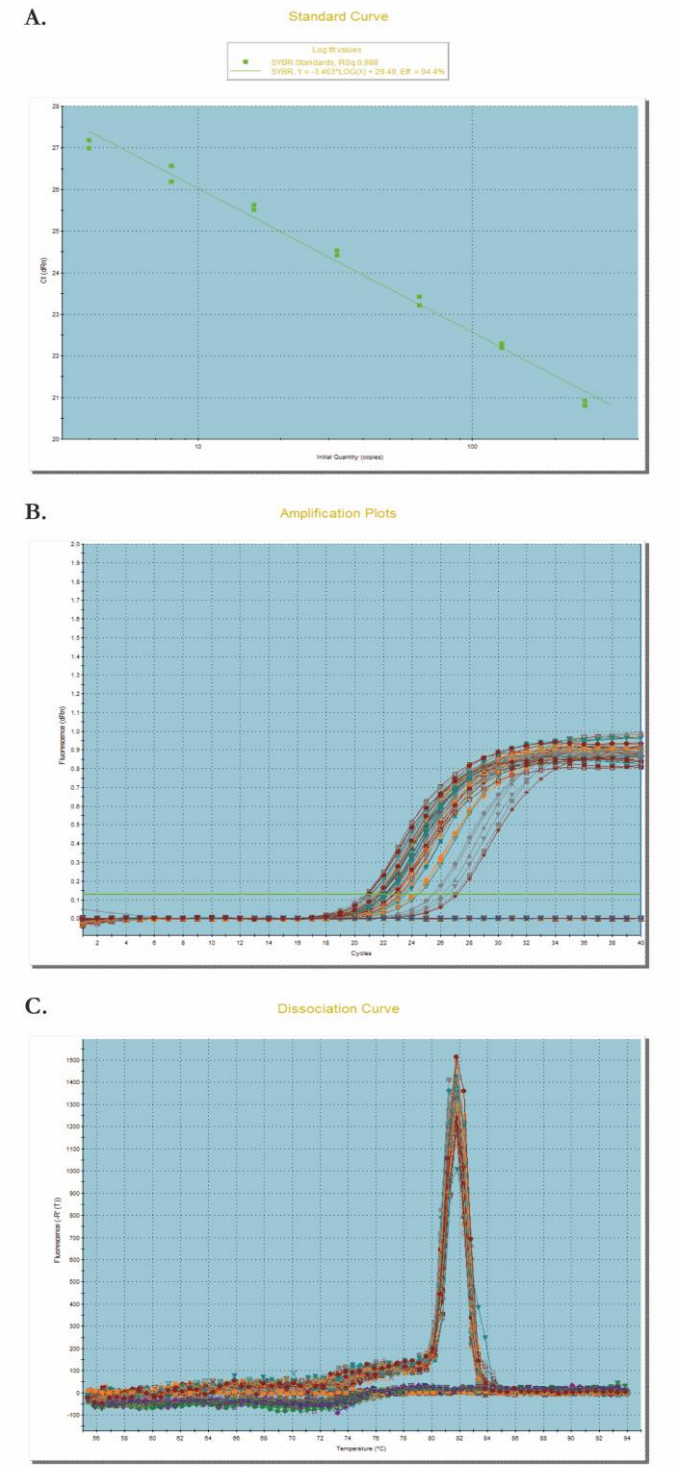


Figure 17: Representative standard curve (1), amplification plot (2) and dissociation curve (3) for SLC38A1

The standard curve (A) illustrates that replicates are consistent and consistency of product amplification. A correlation coefficient value (R^2, above as RSq) of >0.95 an efficiency of between 90-110% was considered acceptable. The amplification curve (B) and dissociation curve (C) confirm successful amplification of product, a single melt curve and absence of primer dimers.

2.5 Gas chromatography-mass spectrometry (GC-MS)

2.5.1 Sample collection

Umbilical arterial (UmA) and venous (UmV) plasma samples which were collected into either tubes coated with lithium heparin (BD vacutainer®; BD, Plymouth, UK) or tubes containing lithium heparin beads (S-monovette®, Sarstedt, Leicester, UK; disparity due to site-wide change in procedure during the study) were taken immediately after delivery of the placenta and stored at -80ºC (described in full in section 2.1.3) prior to sample preparation for GC-MS.

2.5.2 Extraction of small molecules from plasma samples for analysis by GC-MS

Each sample (n=48 total: 24 AGA and 24 FGR matched UmA/UmV plasma pairs) was extracted using chloroform:methanol:water extraction detailed below. Internal standard metabolites (isotopically labelled) were prepared as in Table 14 from which a working standard stock was prepared (1 ml of each internal standard mixture added to 6ml methanol).

Internal standard mixture	Composition
SG	10 mg Succinic acid d_4, 10 mg Glycine d_5 in 10 ml 50:50 (v/v) methanol:water
CFT	10 mg Citric acid d_4, 10 mg Fructose $^{13}C_6$, 10 mg Tryptophan d_5 (all Cambridge Isotope Laboratories Inc.; UK) in 10 ml 50:50 (v/v) methanol:water.
LA	10 mg Leucine d_{10}, 10 mg Alanine d_7 (Cambridge Isotope Laboratories Inc.; UK) in 10 ml 50:50 (v/v) methanol:water.
SB	10 mg Stearic acid d_{35} (Cambridge Isotope Laboratories Inc.; UK), 10 mg Benzoic acid d_5 in 10 ml methanol.

Table 14: Internal standard components of isotopically labelled metabolites

Samples (n=48) were thawed at room temperature. 50 µl of each sample was transferred into a labelled 2 ml microcentrifuge tube (Eppendorf; Hamburg, Germany) held in a cold TissueLyser block (Qiagen, UK, stored at -20ºC). 800 µl cold (refrigerated) chloroform:methanol solution (prepped as 25 ml chloroform, 21.875 ml methanol, 3.125 ml working standard stock) was added to each tube and two empty 2 ml microcentrifuge tubes (extraction blanks). One 3 mm Tungsten carbide bead (Qiagen, UK) was then added to each tube. Samples and blanks were extracted by mechanical disruption for 10 min at a frequency of 25 Hertz on the TissueLyserII (Qiagen, UK), then 400 µl of LC-MS grade water was added to each sample. Samples were vortexed for 5-10 sec and then centrifuged at 2400 x g for 15 min to separate the polar and non-polar phases. From each sample, 100 µl of the chloroform (non-polar) phase was transferred into a fresh labelled 2 ml microcentrifuge tube. A further 100 µl from each sample was pooled into an 8 ml glass vial to create a pooled quality control (QC) sample, which was gently mixed then aliquoted into 14 labelled 2 ml microcentrifuge

tubes. All samples were then dried (ramp 1) using the Savant Speedvac centrifugal concentrator (ThermoFisher Scientific, UK). Dried extracts were stored at 4ºC for potential LC-MS in future.

For GC-MS, the remaining chloroform phase was removed and sample tubes were centrifuged again at 2400 x g for 15 min. From the methanol (polar) phase of each sample, 200 µl was transferred to a 2 ml microcentrifuge tube and the remaining 200 µl into a glass vial to make a pooled QC. Once all samples were added, the pooled QC was gently mixed and 200 µl aliquoted into 14 labelled 2 ml microcentrifuge tubes. Samples were dried overnight using the Savant Speedvac centrifugal concentrator on ramp 5. Dried extracts were stored at 4ºC until ready for derivitisation and analysis by GC-MS.

2.5.3 Gas chromatography-mass spectrometry (GC-MS)

Samples (including blanks and QCs) were chemically derivatised immediately before GC-MS analysis by the following method: 60 µl of 20 mg/ml methoxylamine hydrochloride (Acros Organics, UK) in dry pyridine (Acros Organics, UK) was added to each sample and incubated at 80ºC for 20 min; samples were allowed to cool then 60 µl of N-methyl-N-(trimethylsilyl)trifluoroacetamide (MSTFA) was added to each and heated for another 20 min at 80ºC. Once cooled, 20 µl of retention index markers (n-alkanes from C12-C32) in pyridine was added to each sample tube and centrifuged at 16,000 x g for 5 min. Finally, 95 µl of each sample was transferred to autosampler vials.

Samples were randomised to prevent any bias that may arise from the analysis order. A pooled QC sample was inserted at 11 fixed intervals in the run with the first QC vial sampled from six times to allow the machine to equilibrate.

GS-MS was performed using an Agilent/J&W DB17-MS column (30 m × 0.25 mm × 0.25 µm) with a 3 m × 0.25 mm retention gap, and helium carrier at a constant flow of 1.4 ml/min. The protocol was as follows: 1 µl of sample was injected in Pulse Splitless mode using an 'empty, hot needle' technique (inlet temperature 270ºC; MPS2 autosampler (Gerstel; Germany) and a 7890A Gas Chromatograph with Split/Splitless inlet (Agilent; USA)). The initial column temperature was 50ºC which was held for 6 min before ramping to 300ºC at 10ºC/min, then held for a final 4 min. Total cycle time was therefore 42 min/sample.

Mass spectral data was acquired at 10 spectra/sec over the range of 45-800Da using a Pegasus HT time-of-flight mass spectrometer (LECO; UK) following a 450 sec solvent delay to allow solvent and reagents to elute without damaging the detector.

2.5.4 Data processing

ChromaTOF 4.5 software (LECO; UK) was used to prepare data for analysis (using the 'Reference Compare' method). Putative metabolites were identified from the NIST Mass Spectral Reference Library (NIST08/2008; National Institute of Standards and Technology/Environmental Protection Agency/National Institutes of Health Spectral 262 Library; NIST, Gaithersburg, MD, USA) and an in-house library developed at The University of Manchester.

A reference method was created specific to the mass spectra and expected retention times using the pooled QC samples. The method was edited to remove replicates, low quality spectra and erroneous identifications then applied as a target list to be searched across all samples with the following parameters set: mass spectral match threshold ≥500, retention time tolerance ±12 sec until 700 sec (±6 sec thereafter). The most suitable internal standard (with the lowest variance across all QC injections) was then assigned to each metabolite identified across the sample set. The resulting data (metabolite:internal standard ratio) were exported for statistical analysis.

2.5.5 Data analysis

Data were imported to SIMCA-P software (Umetrics, Sartorius Stedim Biotech; Sweden) for principal component analysis (PCA) of case, control and pooled QC samples to assess the quality of the whole metabolite list. The following variables were assessed: run order, class (case versus control), smoking status, sex of fetus, maternal BMI (</>30 kg/m^2) and gestation (preterm versus term). Next the relative abundance of each identified metabolite in UmA and UmV plasma was analysed according to class (i.e. case (FGR) compared with control (AGA)). Comparisons were made between FGR UmA and AGA UmA and FGR UmV and AGA UmV. All data were analysed by unpaired t test with Welch's correction using GraphPad Prism 7 software. Where differences between case and control groups reached statistical significance (defined as $P<0.05$), $\log_2$ fold change differences (case over control, Log_2 transformed) were calculated and presented visually as a heat map. Differences are described in text using fold change (case over control) (Chapter 6).

Chapter 3 Investigation of maternofetal transfer of glutamine and glutamate relative to placental size and sex of the fetus in wild-type mice

3.1 Introduction

Adequate delivery of nutrients via the placenta to the fetus is essential for appropriate fetal growth. Nutrient transfer, via transporter-mediated mechanisms in the placenta such as facilitated diffusion, active transport or exchange is therefore a key determinant of fetal growth (Desforges and Sibley, 2010). Secondarily active transport mechanisms (e.g. those that utilise the Na^+ electrochemical gradient generated by Na^+/K^+/ATPase) are reliant upon the presence of transporter proteins and their corresponding abundance and activity. It has been demonstrated in WT mice that the activity of the Na^+-dependent amino acid transporter system A, which transports small, neutral amino acids to the fetus against a concentration gradient, is inversely correlated with placental weight (per g placenta, evaluated by comparing the lightest and heaviest placentas in a litter) (Coan *et al.*, 2008).

Amino acid provision is essential to support fetal growth and development. Glutamine and glutamate are important amino acids during pregnancy. Glutamine is transported across the placenta at the highest rates of all the amino acids (Battaglia, 2000) and delivery of glutamine to the developing fetus is essential for normal fetal growth (Parimi and Kalhan, 2007). Glutamate is a precursor of glutamine (Pochini *et al.*, 2014) and key nitrogen resource; however levels in fetal circulation must be tightly controlled since high levels are neurotoxic (Tian *et al.*, 2012). The relationship between the delivery of glutamine and glutamate to the fetus (i.e. unidirectional maternofetal clearance, K_{mf}) and placenta size is therefore of interest but has never been investigated.

Glutamine is a conditionally-essential neutral amino acid that is transported from the maternal to fetal circulation by the transporter systems A, L and N (Battaglia, 2000). Glutamine is the most abundant amino acid involved in many cell processes including nucleotide synthesis, pH homeostasis and gluconeogenesis (Pochini *et al.*, 2014). Glutamine transport is mediated by system A and N transporter proteins (members SNAT1, SNAT2 and SNAT4, and SNAT5, respectively), which are predominantly localised to the maternal-facing MVM, and system L (LAT1 and LAT2, associated with ancillary protein CD98), which resides in both the MVM and fetal-facing BM (Regnault *et al.*, 2002). Uptake of glutamate into the placenta from maternal and fetal circulations is mediated by the high affinity anionic amino acid transport system X_{AG^-}. However, it is generally accepted that glutamate does not cross the placenta (Battaglia, 2002; Day *et al.*, 2013; Pitkin *et al.*, 1979; Self *et al.*, 2004). System X_{AG^-} members

EAAT1, EAAT2 and EAAT3 have been localised to both the apical and basal membranes of the mouse and rat placenta (Matthews *et al.*, 1998; Matthews *et al.*, 1999). Studies in animals and humans have indicated that glutamate in the placenta is primarily used for the synthesis of glutamine (Bloxam *et al.*, 1981; Chung *et al.*, 1998; Day *et al.*, 2013; Self *et al.*, 2004). Conversely, glutamate is generated from glutamine by the fetal liver and subsequently transported to the placenta where it is metabolised primarily to glutamine, to α-ketoglutarate for entry to the Krebs cycle, or to proline (see Figure 8 in Introduction) (Battaglia, 2000; Day *et al.*, 2013; Moores, Vaughn, *et al.*, 1994; Vaughn *et al.*, 1995).

Amino acid transport is dependent upon the expression and activity of specific transporter proteins. Substrate availability, cell signalling pathways and post-translational modifications can all influence transporter abundance and function. For example, mechanistic target of rapamycin (mTOR) signalling pathway is a positive regulator of the amino acid transporter systems A and L, known to transport glutamine, and system β (Vaughan *et al.*, 2017). mTOR operates as a nutrient sensor and plays a role in the trafficking and expression of amino acid transporters, and in itself is influenced by key pathways such as the hexosamine signaling pathway that is reliant upon an adequate supply of nutrients such as glucose and glutamine (Jansson *et al.*, 2012). Therefore not only is glutamine essential for fetal growth per se, but it is also likely to impact on mTOR-mediated nutrient signalling in the placenta, which will influence the provision of other amino acids to the fetus, and is also required for placental synthesis of glutamate (Battaglia, 2000). Understanding factors that regulate glutamine and glutamate provision to the fetus in normal pregnancy, including placental size, is important.

The mouse is a widely-used model of human pregnancy and as such, many dietary, surgical and genetic knockout mouse models of pregnancy-related complications exist. These models enable the investigation of complex mechanisms and the efficacy of novel therapies. The placentas of both mice and women are described as haemochorial since the fetal trophoblast (chorionic villi) are bathed in maternal blood (Enders and Blankenship, 1999). The mouse placenta is formed of two major but distinct zones: the labyrinthine zone, associated with exchange between maternal and fetal circulations, and the junctional zone which is associated with endocrine function (section 1.5) (Dilworth and Sibley, 2013). Nutrient transfer across the mouse placenta takes place in the labyrinthine zone, which is distinct from the villous structure found within the human placenta. Alkaline phosphatase staining has been localised to both the apical (maternal facing) plasma membrane of trophoblast layer II in the mouse placenta and to the microvillous (maternal facing) plasma membrane (MVM) of the human placenta confirming similarities between the two species (Enders and Blankenship, 1999; Jones and Fox, 1976; Kusinski *et al.*, 2010). Furthermore, activity of the amino acid transporter system A has been shown to be comparable in vesicles from the maternal-facing apical

membrane of the mouse placenta and the human MVM (Kusinski *et al.*, 2010). Conversely, system β activity, responsible for the transport of taurine, is significantly lower in the mouse versus the human placenta (Kusinski *et al.*, 2010). This highlights the necessity to evaluate the activity of other transporter systems to establish the overall utility of comparisons between the mouse and human placenta.

Maternofetal nutrient transfer (K_{mf}) can be evaluated in relation to placental size in mice by comparing the extremes of placental weights i.e. the lightest and heaviest placentas within the same WT litter. In WT mouse litters (C57BL/6J strain) the natural variation in placental weight between the lightest and heaviest placenta is on average between 20-30% (Coan *et al.*, 2008; Hayward *et al.*, 2017). Despite having placentas that are at the extremes of normal, fetuses with small and large placentas maintain weights within the normal range: variation is narrower than placental weight, around 5% (Hayward *et al.*, 2017). Investigating K_{mf} in this manner enables paired analysis within the same litter, removing external factors such as gestational age and dam to dam variation that may influence placental function. Previous studies describe placental weight-specific adaptation of maternofetal transfer of glucose, system A amino acid transport and calcium in WT mice insomuch as the lightest placentas transfer more per g placenta compared to the heaviest within a litter towards term (E18.5) (Coan *et al.*, 2008; Hayward *et al.*, 2017). These findings suggest that the mouse placenta can adapt in terms of placental function to ensure that the fetus reaches an appropriate weight. In the case of calcium, increased K_{mf} relative to placental size appeared to normalise reduced fetal calcium content apparent earlier in gestation (E16.5), and points to a role in ensuring appropriate mineralisation of the fetal skeleton.

Since female mice are often lighter than males (Blakley, 1978; Ishikawa *et al.*, 2006) it follows that there are a higher proportion of females with the lightest placenta within a litter, and conversely males with heaviest placentas (Hayward *et al.*, 2017). In human pregnancy fetal sex is not only a determinant of fetal growth (Almog *et al.*, 2011; Misra *et al.*, 2009; Wallace *et al.*, 2012) but males and females may employ different strategies *in utero* in response to adverse environments. Male fetuses are at greater risk of FGR, preterm delivery or *in utero* death compared with females (Clifton, 2010; Vatten and Skjaerven, 2004). Studies indicate that this may be due to sex-specific adaptations of placental function in adverse conditions such as altered glucocorticoid exposure (Stark *et al.*, 2009). In such conditions, the female placenta functionally adapts to reduce fetal growth but maintain a weight within a normal range (10^{th}-90^{th} centile) whereas the male placenta does not adapt gene or protein expression, which leaves the male fetus vulnerable should another adverse event occur (Clifton, 2010). A recent study reported that fetal sex did not influence K_{mf} of calcium in WT mice (Hayward *et al.*,

2017). Nevertheless, regulation of placental function and transfer of other nutrients may differ between males and females and so it is important that the effect of sex is examined.

In summary, the mouse can provide valuable insight into maternofetal nutrient transfer *in vivo* complementary to *in vitro* work on primary human tissue. The objective of this study was to determine the relationship between placental weight and unidirectional maternofetal clearance (K_{mf}) of the amino acids glutamine and glutamate in WT mice. Activity of system A, of which glutamine is a substrate, has been well-described in mice and humans. Studies suggest that system A activity is related to placental size (weight) to support normal fetal growth in a cohort of normal birth weight infants and WT mice (Coan *et al.*, 2008; Godfrey *et al.*, 1998). Given that glutamine is a substrate of system A, it is predicted that K_{mf} of glutamine would be similarly higher in the lightest compared with the heaviest placentas. Here, radiolabelled glutamine or glutamate (^{14}C) were injected into the maternal circulation, as in previous studies (Coan *et al.*, 2008; Kusinski *et al.*, 2010), to monitor K_{mf} in relation to placental weight and sex of the fetus. This study provides the first evaluation of K_{mf} of glutamine and glutamate *in vivo* in mice, with a specific focus upon the contribution of placental size and fetal sex to these transfer processes.

3.1.1 Hypotheses

- Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate will be greater in the lightest compared to the heaviest placenta within a single WT (C57BL/6J) litter and will be accompanied by higher expression of glutamine and glutamate transporters
- Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate will not be influenced by sex of the fetus

3.1.2 Aims

The purpose of this study was to:

- Quantify K_{mf} of ^{14}C-glutamine and ^{14}C-glutamate in the lightest and heaviest placentas in a WT litter
- Examine evidence of sexual dimorphism on fetal and placental growth in a WT population and assess the effect of sex on K_{mf} of ^{14}C-glutamine and ^{14}C-glutamate
- Compare protein expression of glutamine and glutamate transporters in the lightest and heaviest placentas in a WT litter

3.2 Methods

3.2.1 Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate

Unidirectional maternofetal clearance (K_{mf}) of radiolabelled (^{14}C) glutamine and glutamate in the lightest and heaviest placentas in a litter was assessed at two timepoints in pregnancy (E15.5 and E18.5) following the method described in detail in section 2.2. In brief, dams were anaesthetised with a 300 µl intraperitoneal injection of 1:1:2 combination of fentanyl/fluanisone (Hypnorm, VetPharma Ltd., Leeds, UK), Midazolam (Roche, UK) and sterile H_20 (Braun medical Inc., Pennsylvania, USA) and a bolus of radioisotope (100 µl) was administered via a tail vein cannula. At approximately 2 min post-injection (between 90 sec-4 min ^{14}C-glutamine and 90 sec-3 min for ^{14}C-glutamate), dams were exsanguinated by cardiac puncture and a maternal blood sample obtained. Death was confirmed by cervical dislocation, a laparotomy was performed immediately and maternal blood samples were centrifuged centrifuged at 5000 rpm (1,845 x g) for 5 min to obtain plasma. Fetuses and placentas were trimmed of membranes, blotted and weighed. Biometric measurements were also recorded as follows: crown:rump length (from the top of the head to beginning of the tail), abdominal circumference (at the level of umbilical cord insertion) and head circumference (above the eyes and ears) using a length of cotton thread. Fetal tail tips were collected and stored at -20ºC for sex determination (section 2.2.6). Fetuses and placentas were placed in an individual scintillation vial, minced and solubilised in 3 ml 3% KOH at 55ºC overnight. ^{14}C radioactivity in maternal plasma, fetal and placental tissue was measured using a β-scintillation counter (Tri-carb 2100TR liquid scintillation analyzer, Packard, disintegrations per minute; dpm), from which unidirectional maternofetal clearance (K_{mf}) was calculated.

The lightest placentas in a WT litter are predominantly from females and the heaviest from males and so the effect of the sex of the fetus on transfer of ^{14}C-glutamine and ^{14}C-glutamate was also evaluated by comparing litter means of each sex, in litters that contained at least two male and two female fetuses.

A disappearance curve was generated to determine the time at which radioisotope clearance from the maternal circulation was linear as a proxy of transfer at initial rate. ^{14}C disappearance was comparable at E15.5 and E18.5 for both ^{14}C-glutamine and ^{14}C-glutamate (Figure 18) and thus disappearance curves for these gestational time points were pooled. Dams used for experiments were culled during the linear portion of the disappearance curve (90 sec-4 min, ^{14}C-glutamine; 90 sec-3 min ^{14}C-glutamate).

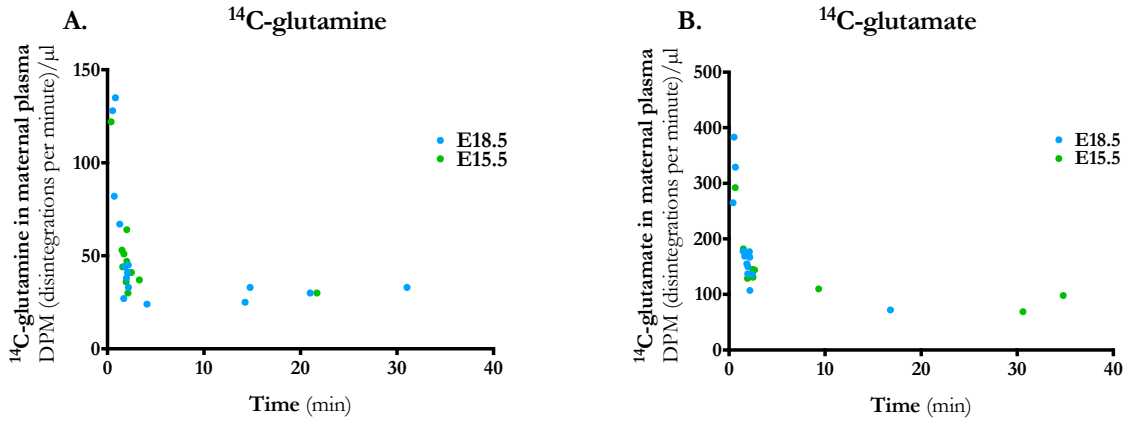


Figure 18: Maternal plasma disappearance curve

^{14}C-glutamine (A) and ^{14}C-glutamate (B) radiolabel counts (disintegrations per min) were measured in maternal plasma over 22 sec-31 min (glutamine), 26 sec-35 min (glutamate). Disappearance was linear at approximately 2 min for both gestational ages. Disappearance curves at E15.5 and E18.5 for each amino acid were therefore collated to calculate area under the curve and subsequently unidirectional maternofetal clearance (K_{mf}). Glutamine E15.5 n=12 E18.5 n=15; Glutamate E15.5 n=10 E18.5 n=13.

3.2.2 Western blotting

Tissue was collected and processed (section 2.3.2) in preparation for Western blot experiments. The expression of glutamine (LAT1, LAT2, SNAT5) and glutamate (EAAT1, EAAT2) transporter proteins in mouse membrane-enriched whole placental homogenates was assessed as described in section 2.3. Specific conditions for all antibodies used can be found in Table 12. A commercially available EAAT1 blocking peptide (ab416, Abcam, Cambridge; UK) was used to evaluate EAAT1 antibody specificity. In brief, the blocking peptide was incubated with the EAAT1 primary antibody (in blocking buffer, Table 12 for specific antibody conditions) for 1 hour at RT at 5 x blocking peptide:antibody concentration. Samples were prepared in two conditions (non-boil reducing and non-boil non-reducing) and loaded onto a gel in duplicate along with a positive (mouse brain whole homogenate) and negative (dH_20) control. Following transfer, the membrane was divided in two, with half of the membrane incubated in primary antibody as per the usual protocol, and the other half incubated with the blocking peptide:antibody solution at 4ºC overnight.

3.2.3 Statistical analysis

Unidirectional maternofetal clearance (K_{mf}) data were analysed by comparing the lightest versus heaviest placenta in a litter, or by comparing the litter average of male versus female placentas (a minimum of two of each sex per litter was required). Data were not normally distributed and therefore presented as median [range]. Data were analysed (GraphPad Prism 7 software) by Wilcoxon signed rank test and were considered statistically significant where $P<0.05$.

Semi-quantitative analysis of transporter protein expression as determined by protein band density (densitometry) was performed for Western blot data using Image Studio™ Lite software. Data were analysed (GraphPad Prism 7 software) using a Mann-Whitney test. A *P* value <0.05 was considered statistically significant.

3.2.4 Experimental flowchart

The number of animals used, and in which experiments, is laid out in the experimental flowchart (Figure 19).

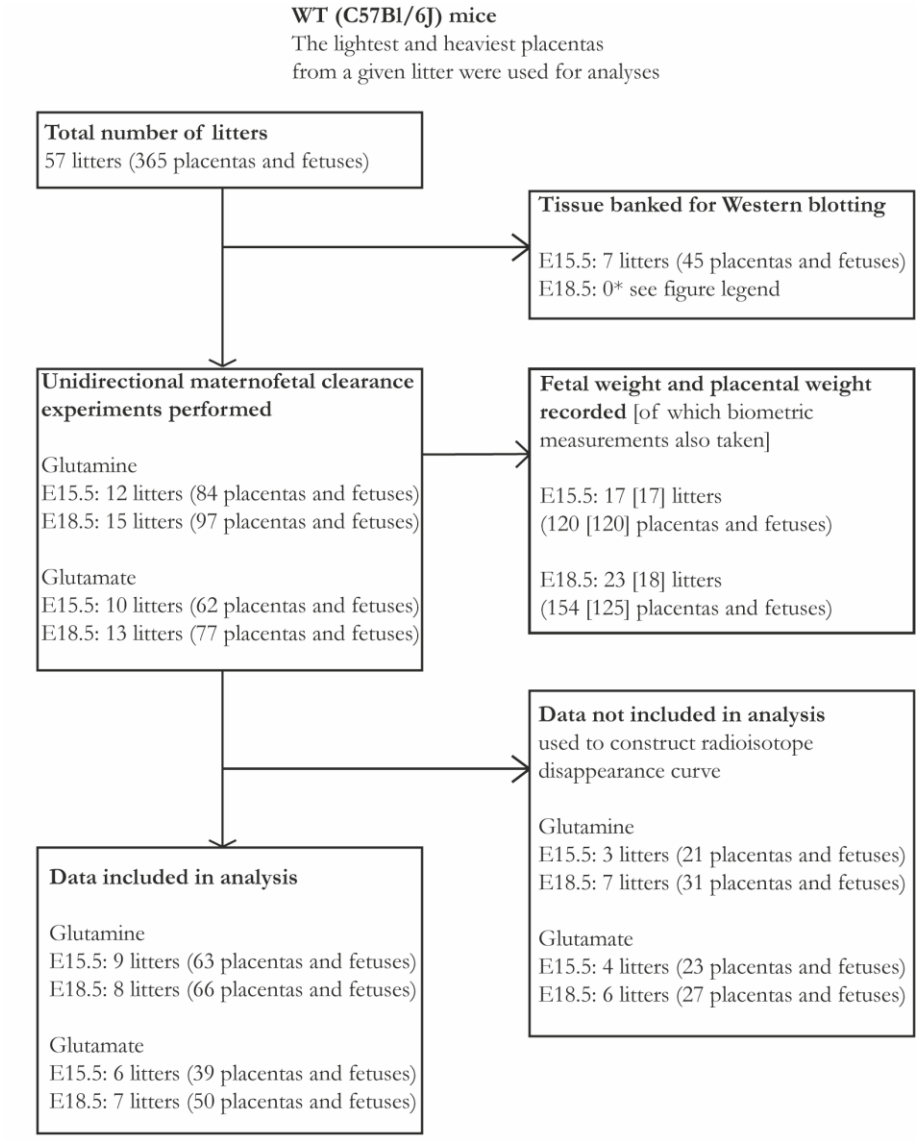


Figure 19: Experimental flowchart illustrating use of animals in experiments
From 57 pregnant dams, 7 litters were harvested for Western blotting experiments and a total of 50 litters used for unidirectional maternofetal clearance (K_{mf}) experiments at E15.5 and E18.5. Fetal and placental weights were recorded where possible (E15.5 n=17; E18.5 n=23 litters). Litters were excluded from further analyses where it was not possible to determine the lightest or heaviest placenta within a given litter (i.e. if two were the same weight). Biometric measurements (crown:rump length, head circumference and abdominal circumference) were also recorded for 17 litters at E15.5 and 18 litters at E18.5. Unidirectional maternofetal clearance (K_{mf}) experiments were split between ^{14}C-glutamine (E15.5 n=12; E18.5 n=15) and ^{14}C-glutamate (E15.5 n=10; E18.5 n=13). Experiments used to construct a radioisotope disappearance curve (^{14}C-glutamine n=10; ^{14}C-glutamate n=10) were excluded from data analysis. *E18.5 tissue used for Western blots (8 litters) was previously banked tissue by our laboratory. Tissue was processed (to generate membrane-enriched whole placental homogenates) at the same time as E15.5 tissue.

3.3 Results

3.3.1 Placental and fetal measures from the lightest and heaviest placentas in a litter

Where comparisons have been made between the lightest and heaviest placenta in a litter, data are presented as the lightest placenta expressed as a percentage of the heaviest placenta

(standardised to a hypothetical value: 100%). Analyses were not made between gestational ages (i.e. E15.5 and E18.5). The lightest placenta in a litter weighed on average 23% less than the heaviest at both E15.5 and E18.5 ($P<0.001$) (Figure 20A). Fetuses with the lightest placentas were also significantly lighter at both gestations; however the magnitude of this difference appears less at E18.5 ($P<0.05$; $P<0.001$ at E15.5) (Figure 20B). Fetuses with the lightest placentas had a significantly higher F:P ratio than those with the heaviest placentas ($P<0.001$) (Figure 20C). Fetuses with the lightest placentas had a significantly shorter crown:rump length (mm) at E15.5 ($P<0.001$) but there was no difference the day before term (Figure 20D). Head circumference (mm) was significantly different between fetuses from lightest and heaviest placentas at E18.5 only, whilst fetuses with the lightest placentas had a significantly reduced abdominal circumference (mm) at both gestations ($P<0.001$) (Figure 20F).

All placental and fetal weights recorded at the two gestational ages investigated (E15.5 and E18.5) are shown in Figure 21, and the raw values comparing the lightest and heaviest placenta in a litter (median [range]) are quoted in Table 15. As shown in the fetal weight distribution curves (Figure 22A, B) fetuses with the lightest placentas were more frequently beneath the 5th centile of birth weights (E15.5 12% lightest; 0% heaviest; E18.5 4% lightest; 4% heaviest; 5th centile is represented by the dotted line; E15.5 = 0.29 g; E18.5 = 1.01 g). However, the mean fetal weight centiles for fetuses with the lightest and heaviest placentas at both gestational ages were within the normal range (i.e. the between the 10th and 90th centiles of fetal weights in this population) (Figure 22C). Fetal weight and placental weight were positively correlated at E18.5 ($P<0.001$) but this failed to reach statistical significance at E15.5 ($P=0.068$) (Figure 23).

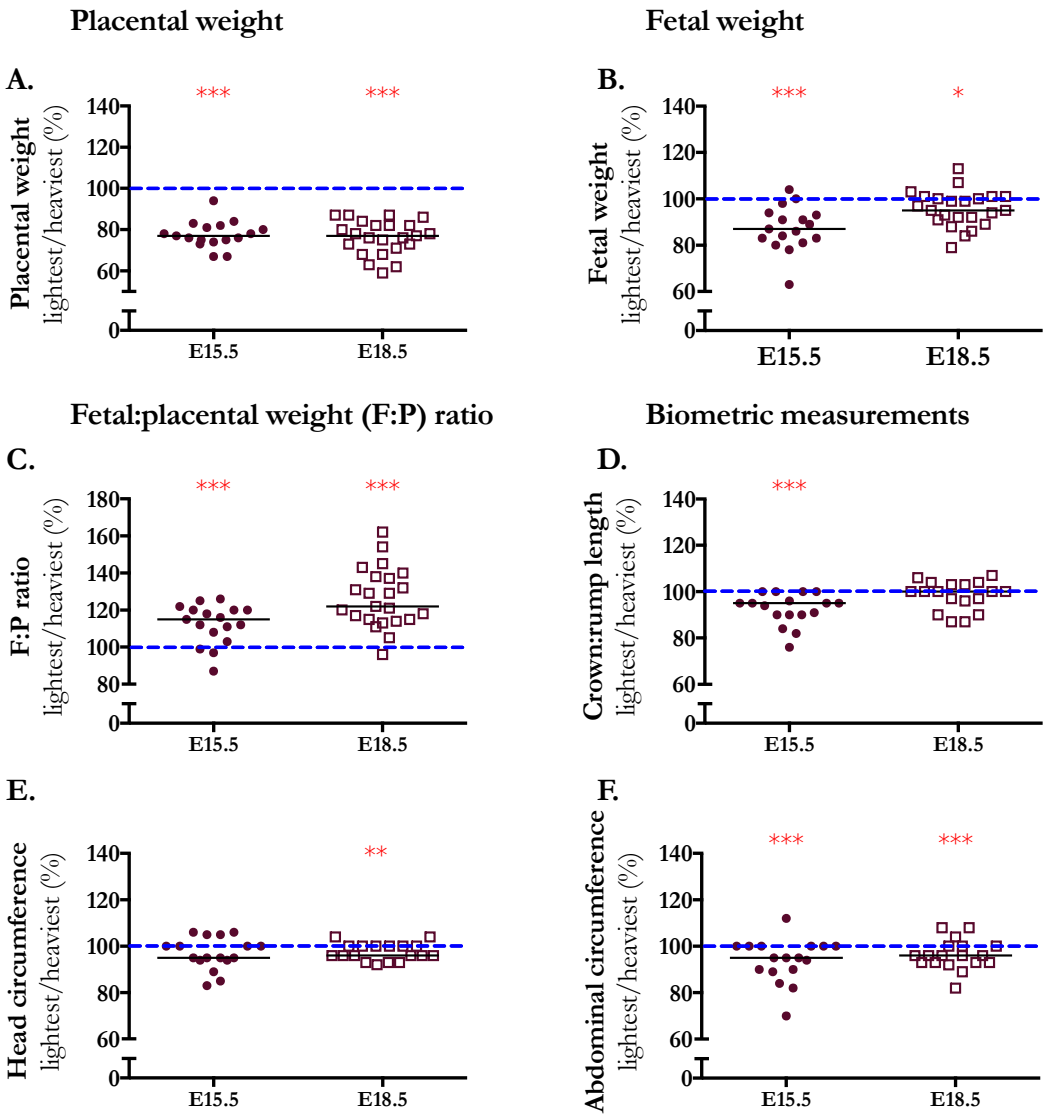


Figure 20: Placental and fetal measures from the lightest and heaviest placentas in WT mice at E15.5 and E18.5

The lightest placenta within a given litter is expressed as a percentage of the heaviest placenta within the same litter (dotted line = 100%; median = solid line). (A) Lightest placentas weighed less than the heaviest within the same litter at E15.5 and E18.5. Fetuses with the lightest placentas were also lighter than those with the heaviest placentas at both gestational ages (B) and therefore had a higher F:P ratio at E15.5 and E18.5 (C). Crown-rump lengths (D) were shorter in fetuses from lightest versus heaviest placentas at E15.5 but not E18.5 whereas head circumference (E) was no different between groups at E15.5 but lower (lightest versus heaviest) at E18.5. Fetuses from the lightest placentas had smaller abdominal circumferences (F) than littermates with the heaviest placentas at both gestational ages studied. It was not possible to record fetal biometric measurements for all litters (see Figure 19) E15.5 n=17 E18.5 n=18/23 *** $P<0.001$; ** $P<0.01$, * $P<0.05$ Wilcoxon signed rank test against a hypothetical value (100%).

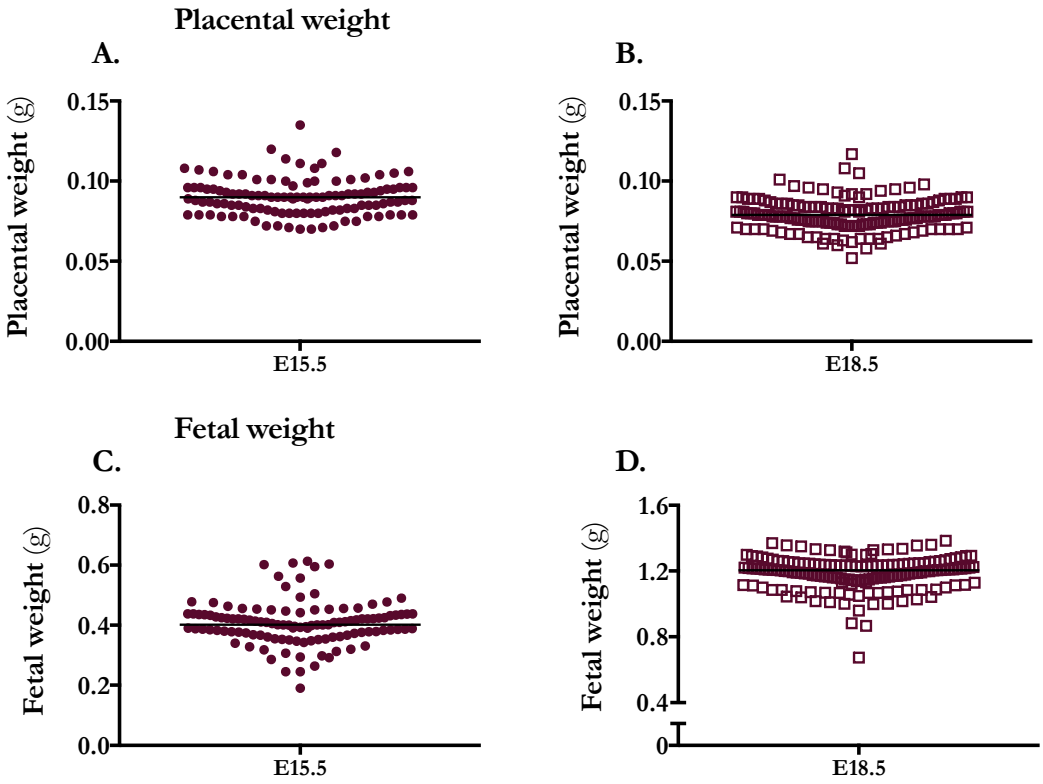
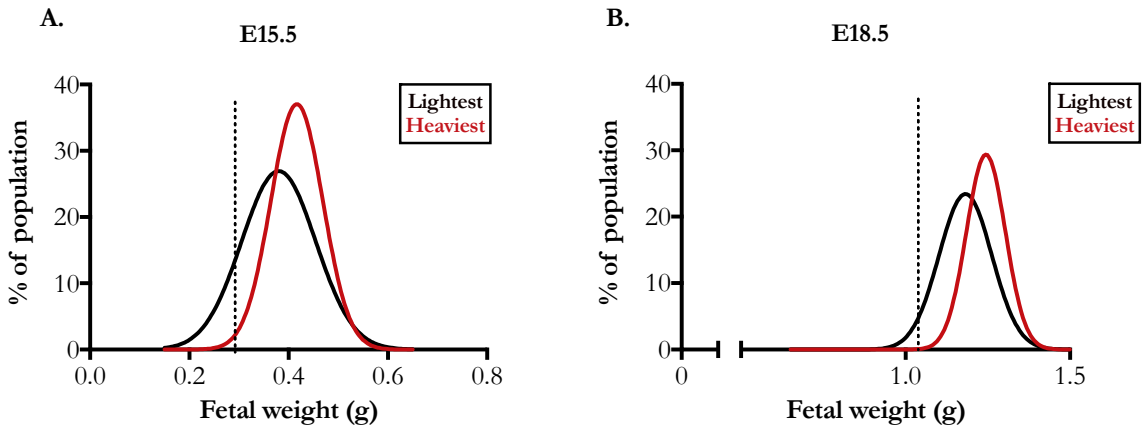


Figure 21 Whole litter placental and fetal weights at E15.5 and E18.5
All placental and fetal weights from a litter are shown in the above graphs. Placental weights at E15.5 (A) and E18.5 (B), and fetal weights at E15.5 (C) and E18.5 (D). E15.5 n=115 E18.5 n=157.

	E15.5			E18.5		
	Lightest	**Heaviest**	***P* value**	**Lightest**	**Heaviest**	***P* value**
Placental weight (g)	0.078 [0.070-0.091]	0.102 [0.080-0.135]	*** <0.001	0.070 [0.052-0.080]	0.090 [0.079-0.117]	*** <0.001
Fetal weight (g)	0.387 [0.245-0.563]	0.422 [0.343-0.602]	*** <0.001	1.184 [0.959-1.355]	1.238 [1.001-1.384]	* 0.014
Fetal weight:placental weight (F:P) ratio	4.8 [3.5-6.5]	4.0 [3.0-5.8]	*** <0.001	17.0 [12.8-21.6]	13.7 [10.5-15.4]	*** <0.001
Crown:rump length (mm)	20 [16-22]	21 [18-23]	*** <0.001	29 [26-33]	30 [28-31]	0.418
Head circumference (mm)	18 [15-21]	19 [16-21]	0.069	25 [24-27]	26 [24-27]	* 0.018
Abdominal circumference (mm)	18 [14-19]	19 [17-20]	*** <0.001	26 [23-28]	26 [24-28]	* 0.033

Table 15: Placental weight, fetal weight, F:P ratio and fetal biometric measurements from the lightest and heaviest placentas in a WT mouse litter at E15.5 and E18.5
Placental weight, fetal weight, F:P ratio and biometric data are presented in the above table as median [range]. Data analysed by Wilcoxon signed rank test *** $P<0.001$ * $P<0.05$ Placental weight, fetal weight and F:P ratio E15.5 n= 17 E18.5 n= 23; Biometric measurements E15.5 n=17 E18.5 n=18.



C.

	Mean fetal weight centile (±SEM)	
Gestation	**Lightest**	**Heaviest**
E15.5	36.2±6.8	57.6±5.6
E18.5	50.2±5.8	67.3±5.2

Figure 22: Fetal weight distribution curves and mean fetal weight centiles in WT mice
Fetal weight distribution curves for fetuses with the lightest (black) and heaviest (red) placentas are shown for E15.5 (A) and E18.5 (B). The dotted line represents the 5th centile of fetal weights, calculated from the entire population of fetal weights (E15.5 = 0.29 g; E18.5 = 1.01 g). The table (C) states the mean fetal weight centile ±standard error of the mean (SEM) for the lightest and heaviest placentas at both gestational ages.

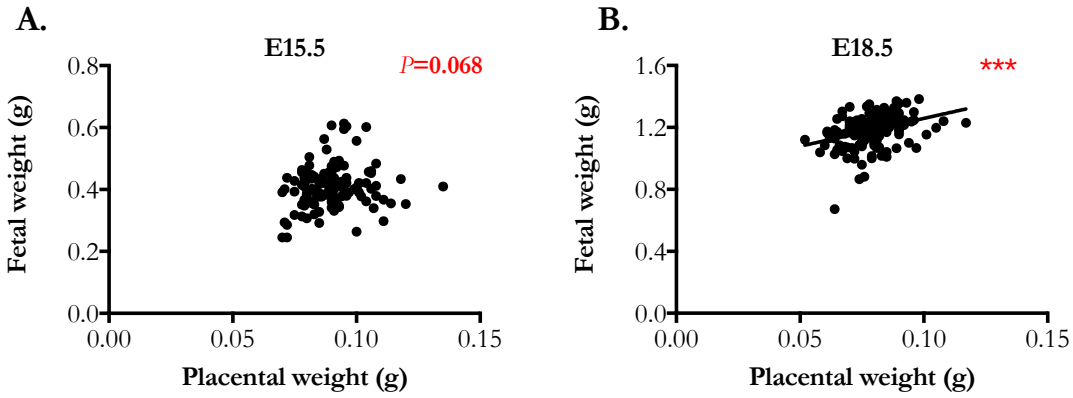


Figure 23: Relationship between placental weight and fetal weight in WT mice at E15.5 and E18.5
Fetal weight and placental weight were significantly correlated at E18.5 (*** $P<0.001$; Linear regression). There was no relationship between fetal weight and placental weight at E15.5. E15.5 n=17 litters, 120 placentas and fetuses; E18.5 n=23 litters, 153 placentas and fetuses.

3.3.2 Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate

Unidirectional maternofetal clearance data are presented as the lightest placenta expressed as a percentage of the heaviest placenta in a given litter with the heaviest placenta represented by the dotted line at 100% in Figure 24, and the raw values (median [range]) are shown in Table 16. Experiments were performed under non-recovery surgery; therefore the same animals could not be followed up from E15.5 to E18.5 and compared directly. Unidirectional

maternofetal clearance (K_{mf}, µl/min/g placenta) of ^{14}C-glutamine was significantly higher in the lightest versus heaviest placentas at E18.5 (P<0.01) (Figure 24A) but not at E15.5. Per g fetus, glutamine transfer (µl/min/g fetus) (Figure 24C) was also higher in the lightest compared with the heaviest placentas at E18.5 (P<0.01) but similar at E15.5. Furthermore, total maternofetal transfer of radioisotope (i.e. irrespective of fetal or placental weight, µl/min) was also higher in lightest versus heaviest placentas at E18.5 (P<0.05) (Figure 24E). There were no differences between groups at E15.5. Unidirectional maternofetal clearance (K_{mf}, µl/min/g placenta) of ^{14}C-glutamate was significantly higher for lightest versus heaviest placentas at E18.5 (P<0.05) (Figure 24B) but not different at E15.5. Maternofetal transfer (µl/min/g fetus) was significantly lower at E15.5 (P<0.05) but normalised towards term (Figure 24D). Total maternofetal transfer (µl/min) was also lower across lightest compared with heaviest placentas at E15.5 (P<0.05) but similar between groups at E18.5 (Figure 24F).

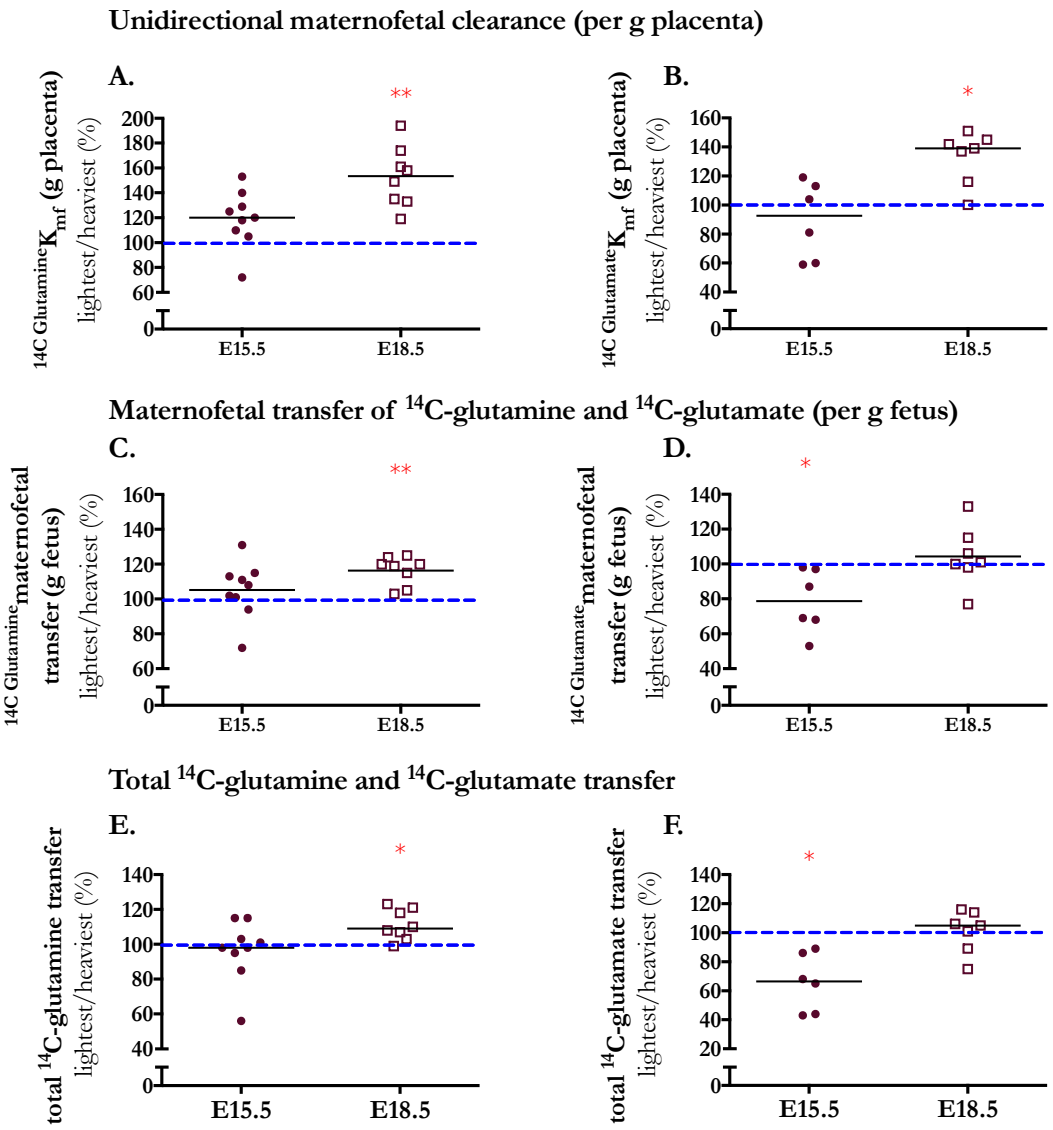


Figure 24: Unidirectional maternofetal clearance/transfer of ^{14}C-glutamine and ^{14}C-glutamate in lightest versus heaviest placentas of WT mice

Maternofetal clearance/transfer from the lightest placenta is expressed as a percentage of the heaviest placenta within the same litter (dotted line = 100%; median = solid line). Unidirectional maternofetal clearance (K_{mf}, per g placenta) of ^{14}C-glutamine (A) and ^{14}C-glutamate (B) was significantly higher in the lightest placentas compared with the heaviest at E18.5. Maternofetal transfer of ^{14}C-glutamine (per g fetus) and total transfer was similar at E15.5 and higher at E18.5 (C, E). ^{14}C-glutamate transfer per g fetus and total transfer was lower at E15.5 but normalised at E18.5 (D, F). Glutamine E15.5 n=9 E18.5 n=8; Glutamate E15.5 n=6 E18.5 n=7; ** P<0.01, * P<0.05 Wilcoxon signed rank test against a hypothetical value (100%).

Unidirectional maternofetal clearance		E15.5			E18.5		
		Lightest	Heaviest	*P* value	Lightest	Heaviest	*P* value
Glutamine	**K_{mf} (µl/min/g placenta)**	506.6 [374.7-754.8]	490.7 [270.0-627.5]	0.097	789.0 [356.6-1301.2]	498.9 [300.3-671.1]	** 0.008
	µl/min/g fetus	113.1 [94.1-130.7]	107.5 [81.7-138.8]	0.250	39.9 [22.3-76.2]	35.1 [21.2-63.8]	** 0.008
	µl/min	38.2 [27.0-65.7]	44.8 [30.0-65.3]	0.680	49.8 [27.1-95.0]	46.2 [26.4-78.5]	* 0.023
Glutamate	**K_{mf} (µl/min/g placenta)**	64.3 [43.2-131.6]	77.1 [64.0-110.4]	0.563	135.4 [56.4-211.2]	109.9 [41.1-145.2]	* 0.016
	µl/min/g fetus	13.3 [8.6-24.7]	16.8 [14.5-25.4]	* 0.031	7.5 [3.3-11.6]	8.1 [3.1-11.5]	0.750
	µl/min	4.8 [3.0-10.3]	7.4 [6.8-11.6]	* 0.031	8.6 [3.9-13.1]	10.4 [3.7-11.9]	0.813

Table 16: Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate

The raw data from unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate experiments are expressed as (median [range]): per g placenta (K_{mf}, µl/min/g placenta), per g fetus (µl/min/g fetus) and as raw transfer (i.e. irrespective of fetal or placental measures; µl/min). The lightest and heaviest placentas within a litter were compared. Analyses were not made between gestational ages i.e. between E15.5 and E18.5. Data analysed by Wilcoxon signed rank test ** $P<0.01$ * $P<0.05$ Glutamine E15.5 n=9; E18.5. n=8; glutamate E15.5 n=6; E18.5 n=7.

3.3.3 Effect of fetal sex on placental and fetal weight

69% (9/13) and 72% (13/18) of the lightest placentas were from female fetuses at E15.5 and E18.5, respectively. Heaviest placentas were from male fetuses 69% of the time at E15.5 (9/13). At E18.5 83% (15/18) of the heaviest placentas were from male fetuses.

Data are presented in Figure 25 as the litter mean of female fetuses as a percentage of the males (minimum of two males, two females in each litter, standardised to a hypothetical value: 100%). There was no difference in placental or fetal weight between males and females at E15.5 (Figure 25A, B). At E18.5 female fetuses had lighter placentas (8% lighter; $P<0.001$) (Figure 25A) and weighed less than male littermates (3% lighter; $P<0.05$) (Figure 25B). Females had a higher F:P ratio relative to males within the same litter at E18.5 ($P<0.05$) but not at E15.5 (Figure 25C). Biometric measurements (crown:rump length, head circumference, abdominal circumference, Figure 25D-F) were no different according to sex at either gestation. All placental and fetal weights recorded from male and female fetuses in a litter (from litters that had a minimum two of each sex) at E15.5 and E18.5 are shown in Figure 26, and the data are collated in Table 17 and expressed as median [range].

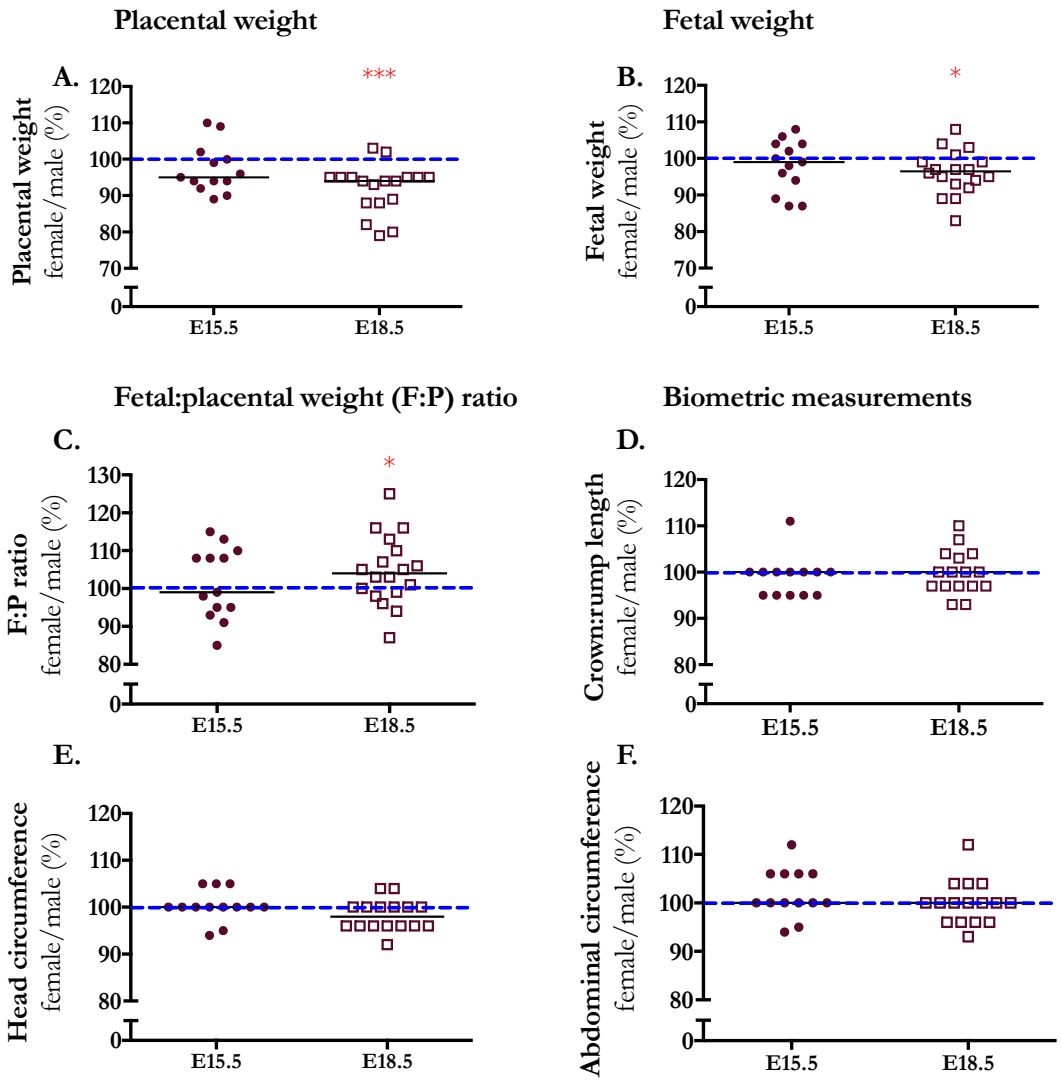


Figure 25: Placental and fetal measures in WT mice in relation to sex of the fetus

Data are expressed as the litter mean of female fetuses as a percentage of the males (minimum of two males, two females in each litter, dotted line = 100%; median = solid line). At E18.5, females had lower placental weight (A), fetal weight (B) and a higher F:P ratio (C). Biometric measurements were not different according to sex of the fetus (D-F). E15.5 n=13 E18.5 n=16/18 ***P<0.001, * P<0.05 Wilcoxon signed rank test against a hypothetical value (100%).

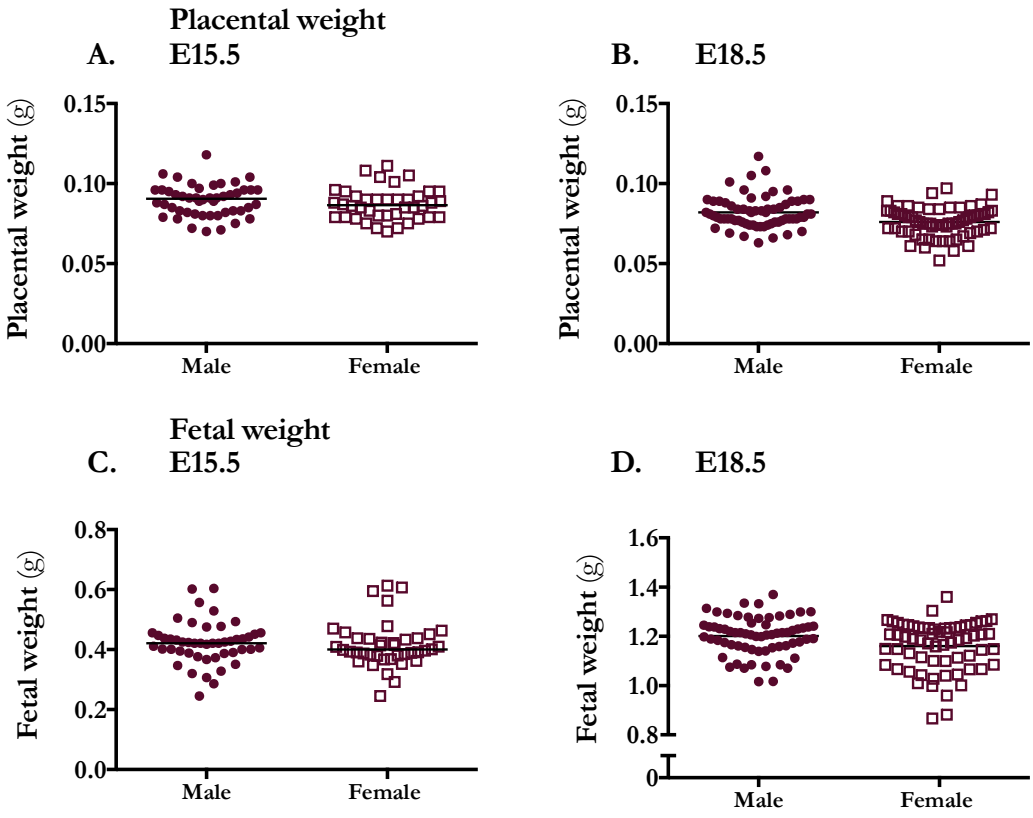


Figure 26 Placental and fetal weights at E15.5 and E18.5 of male and female fetuses
Placental and fetal weights from male and female fetuses in a litter (from litters that had a minimum two of each sex) are shown in the above graphs. Placental weights at E15.5 (A) and E18.5 (B), and fetal weights at E15.5 (C) and E18.5 (D). E15.5 n=48 male, n=40 female, E18.5 n=66 male, n=65 female.

	E15.5			E18.5		
	Female	**Male**	***P*value**	**Female**	**Male**	***P*value**
Placental weight (g)	0.085 [0.077-0.097]	0.089 [0.079-0.095]	0.160	0.078 [0.064-0.084]	0.083 [0.072-0.105]	*** <0.001
Fetal weight (g)	0.406 [0.285-0.595]	0.412 [0.329-0.573]	0.455	1.177 [1.013-1.285]	1.209 [1.14-1.283]	* 0.015
Fetal weight:placental weight (F:P) ratio	4.7 [3.7-6.5]	4.8 [3.9-5.9]	0.435	15.5 [12.4-18.5]	15.1 [11.4-16.5]	* 0.047
Crown:rump length (mm)	20 [18-23]	20 [19-23]	0.531	29 [27-32]	30 [28-31]	0.831
Head circumference (mm)	19 [16-22]	19 [17-21]	0.999	25 [24-27]	26 [25-26]	0.092
Abdominal circumference (mm)	19 [17-20]	18 [17-20]	0.359	26 [25-28]	26 [25-27]	0.999

Table 17: Placental weight, fetal weight and fetal biometric measurements from male and female fetuses in a WT mouse litter at E15.5 and E18.5
The effect of sex on fetal weight, placental weight and biometric measurements was assessed by comparing litter means from male and female fetuses within a litter (minimum two of each). Data are presented as median [range]. Data analysed by Wilcoxon signed rank test *** $P<0.001$ * $P<0.05$ Fetal weight and placental weight E15.5 n=13 E18.5 n=18; Biometric measurements E15.5 n=13 E18.5 n=16.

3.3.4 Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate in female versus male littermates in WT mice

Unidirectional maternofetal clearance data are presented in Figure 27 as the litter mean of female fetuses as a percentage of the males (minimum of two males, two females in each litter, standardised to a hypothetical value: 100%). There were no significant differences in maternofetal clearance of glutamine or glutamate (K_{mf}, μl/min/g placenta) (Figure 27). However, there was a non-significant trend towards higher K_{mf} of ^{14}C-glutamine in female compared with male fetuses at E18.5 (P=0.055). There were no differences between groups in maternofetal transfer per g fetus or total transfer. The data are collated in Table 18 (median [range]).

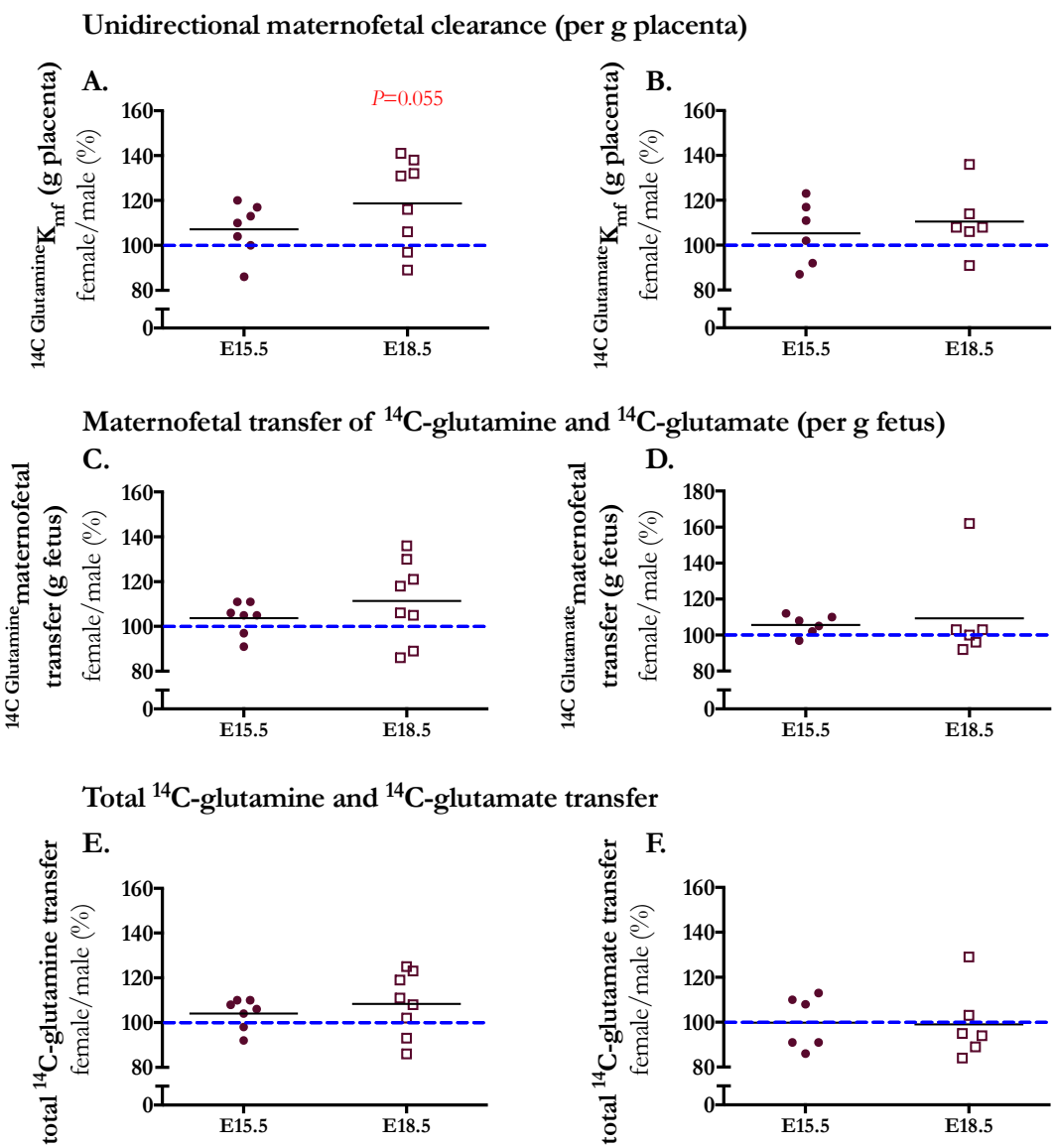


Figure 27: Unidirectional maternofetal clearance/transfer of ^{14}C-glutamine and ^{14}C-glutamate (male versus female)

Data are expressed as the litter mean of female fetuses as a percentage of the males (minimum of two males, two females in each litter, dotted line = 100%; median = solid line). Maternofetal transfer per g placenta (unidirectional maternofetal clearance, K_{mf}), per g fetus, or total transfer of ^{14}C-glutamine and ^{14}C-glutamate was no different between males and females at E15.5 or E18.5. Glutamine E15.5 n=7 E18.5 n=8; Glutamate E15.5 n=6 E18.5 n=6, Wilcoxon signed rank test against a hypothetical value (100%).

Unidirectional maternofetal clearance		E15.5			E18.5		
		Female	Male	*P* value	Female	Male	*P* value
Glutamine	**K_{mf} (µl/min/g placenta)**	53.8 [36.7-72.7]	48.1 [35.5-62.2]	0.156	61.4 [38.0-85.7]	58.1 [32.8-89.7]	0.078
	µl/min/g fetus	112.3 [91.8-133.1]	101.6 [88.1-137.8]	0.375	42.3 [26.4-69.8]	38.8 [21.8-66.5]	0.313
	µl/min	42.5 [34.9-66.7]	40.2 [33.4-60.4]	0.219	48.2 [32.0-87.3]	47.3 [26.8-80.6]	0.195
Glutamate	**K_{mf} (µl/min/g placenta)**	7.7 [6.2-10.6]	7.2 [6.1-12.2]	0.563	13.3 [5.3-15.7]	11.2 [4.9-15.1]	0.219
	µl/min/g fetus	15.8 [13.7-23.8]	15.0 [12.2-23.4]	0.094	8.7 [3.5-10.8]	8.0 [3.4-10.8]	0.999
	µl/min	6.5 [4.8-9.7]	6.3 [5.3-10.7]	0.625	9.6 [3.9-11.5]	9.4 [4.1-12.9]	0.594

Table 18: Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate in relation to sex of the fetus

The effect of sex on unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate was assessed by comparing litter means from male and female fetuses within a litter (minimum two of each). Data are presented as median [range] and expressed per g placenta (K_{mf}, µl/min/g placenta), per g fetus (µl/min/g fetus) and as raw transfer (i.e. irrespective of fetal or placental measures; µl/min). Data analysed by Wilcoxon signed rank test ** $P<0.01$ * $P<0.05$ Glutamine E15.5 n=7; E18.5. n=8; glutamate E15.5 n=6; E18.5 n=6.

3.3.5 Expression of transporter proteins important for the transport of glutamine and glutamate in lightest versus heaviest placentas of WT mice

Expression of known glutamine and glutamate transporter proteins were assessed by Western blot with the intensity of bands detected at the expected molecular weight quantified using densitometry. Representative blots are shown in Figure 28. All data were normalised to the housekeeping proteins β-tubulin or β-actin, the expression of which was consistent between groups (Figure 28). Due to the use of radioisotopes, placental tissue was harvested for Western blot experiments separately to unidirectional maternofetal clearance studies and so it was not possible to perform both techniques using the same animals/tissues. At E15.5 57% (4/7) of the heaviest placentas were from male fetuses, and 71% (5/7) of the lightest placentas from female fetuses. At E18.5 100% (8/8) of the heaviest placentas were from male fetuses and 88% (7/8, unable to determine sex of one fetus) of the lightest placentas from female fetuses.

A MVM isolate (human placental maternal-facing membrane) was included as a positive control during antibody optimisation to validate band location. The specificity of the secondary antibody was confirmed by adding secondary antibody alone (no primary antibody).

Expression of the glutamine transporter proteins LAT1, LAT2 (system L) and SNAT5 (system N) was also assessed. LAT1 expression (band detected at the appropriate molecular weight of 40 kDa) was significantly higher ($P<0.05$) in the lightest versus heaviest placentas at E18.5, with a trend towards higher expression at E15.5 ($P=0.063$) (Figure 29A). A 75 kDa band was also present when probing for LAT1 under reducing conditions (as visible in

representative blot for β-tubulin in Figure 28A) as has been previously described under reducing conditions by Ellinger *et al.* (2016). LAT2 expression (at the expected size of 49 kDa Figure 28B; as reported by Ellinger *et al.*, 2016; Segawa *et al.*, 1999) was unchanged at E18.5 and poor LAT2 expression at E15.5 led to variable and inconsistent values when analysed by densitometry (Figure 29B).

SNAT5 (system N) expression was no different between groups at either gestation (Figure 29C). The band present at 52 kDa (Figure 28C, predicted molecular weight) was used for analysis; a clear band was also present at this molecular weight for the positive controls (human MVM and mouse brain whole homogenate samples). Additional bands were present at 40 kDa. The presence of additional bands has been acknowledged within the antibody manufacturer's datasheet; however the identity of these bands is unknown.

In our laboratory we have been unable to find antibodies for the system A transporters that produce a reliable signal of the correct predicted size. It was therefore not possible to assess the expression of system A transporter proteins (of which MeAIB and glutamine are substrates).

Expression of system X_{AG-} transporter proteins EAAT1 and EAAT2 (responsible for placental glutamate uptake) was no different between groups (Figure 29D, E). EAAT1 has a predicted molecular weight of 60 kDa. Previously published reports using the same anti-EAAT1 antibody (EAAT1 is also known as GLAST: GLutamate ASpartate Transporter) have described the presence of multiple bands at 50 kDa and 150 kDa (Martinez-Lozada *et al.*, 2014). A blocking peptide was used in the current study to ensure the validity of the band present at 35 kDa, where MVM expression was also localised. The 35 kDa band was abolished in the presence of the blocking peptide (Figure 28E), and this band was used for analysis by densitometry. EAAT2 expression was quantified using the band present at the predicted molecular weight of 62 kDa (Figure 28F).

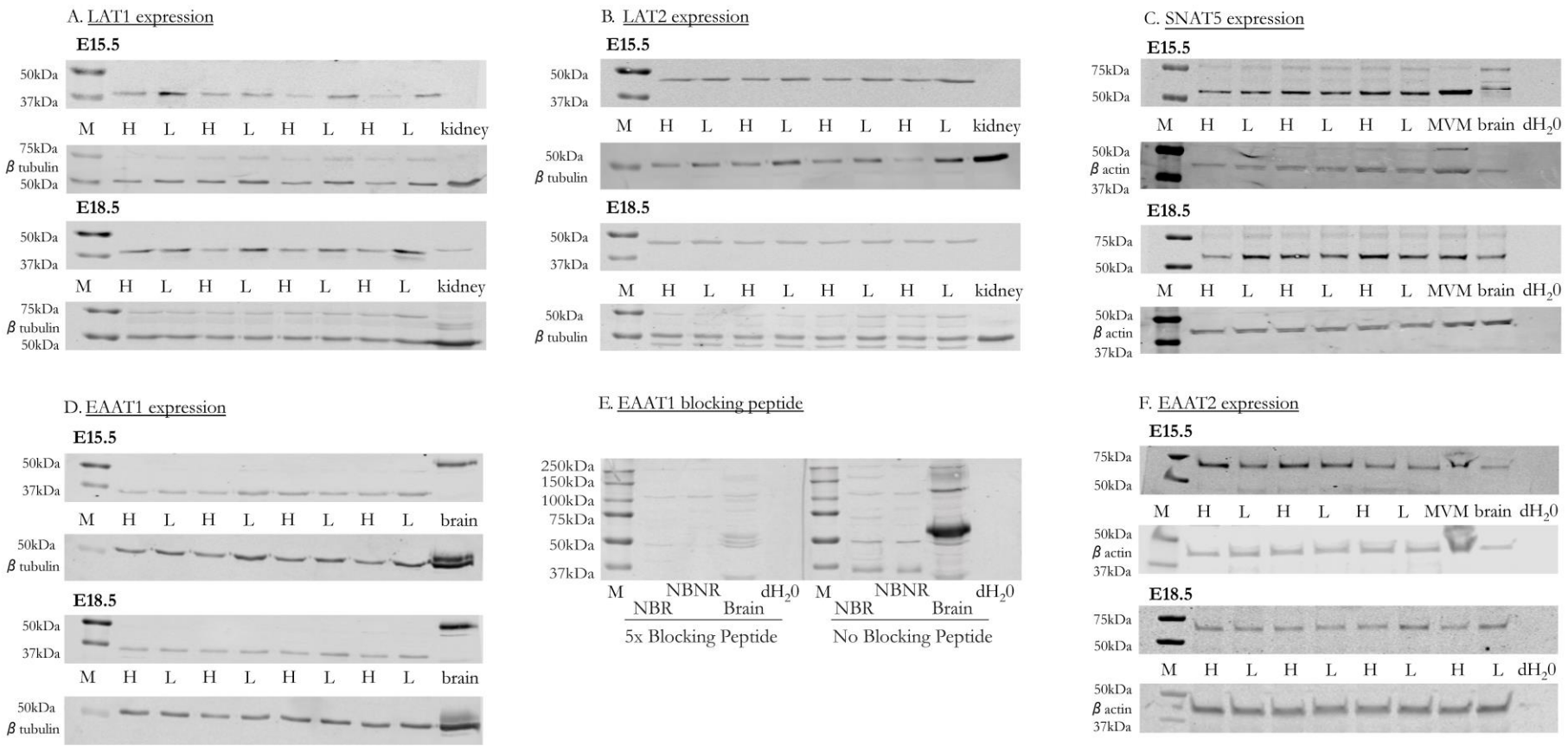


Figure 28: Representative blots illustrating expression of glutamate and glutamine transporter proteins

Shown are representative Western blots of glutamine (system L: LAT1, system N: SNAT5) and glutamate (system X_{AG}-: EAAT1, EAAT2) transporter proteins in the lightest (L) and heaviest (H) placentas at E15.5 and E18.5. Corresponding Western blots for a housekeeping protein β-actin or β-tubulin are shown below each blot. An EAAT1-specific blocking peptide was used to ensure confidence in the EAAT1 antibody (E). The addition of the blocking peptide (5 x) in both non-boil reducing (NBR) and non-boil non-reducing (NBNR) conditions abolished the band at 37 kDa. M= marker, brain= mouse brain whole homogenate, kidney= mouse kidney whole homogenate, MVM= human microvillous membrane, dH_20= deionised water.

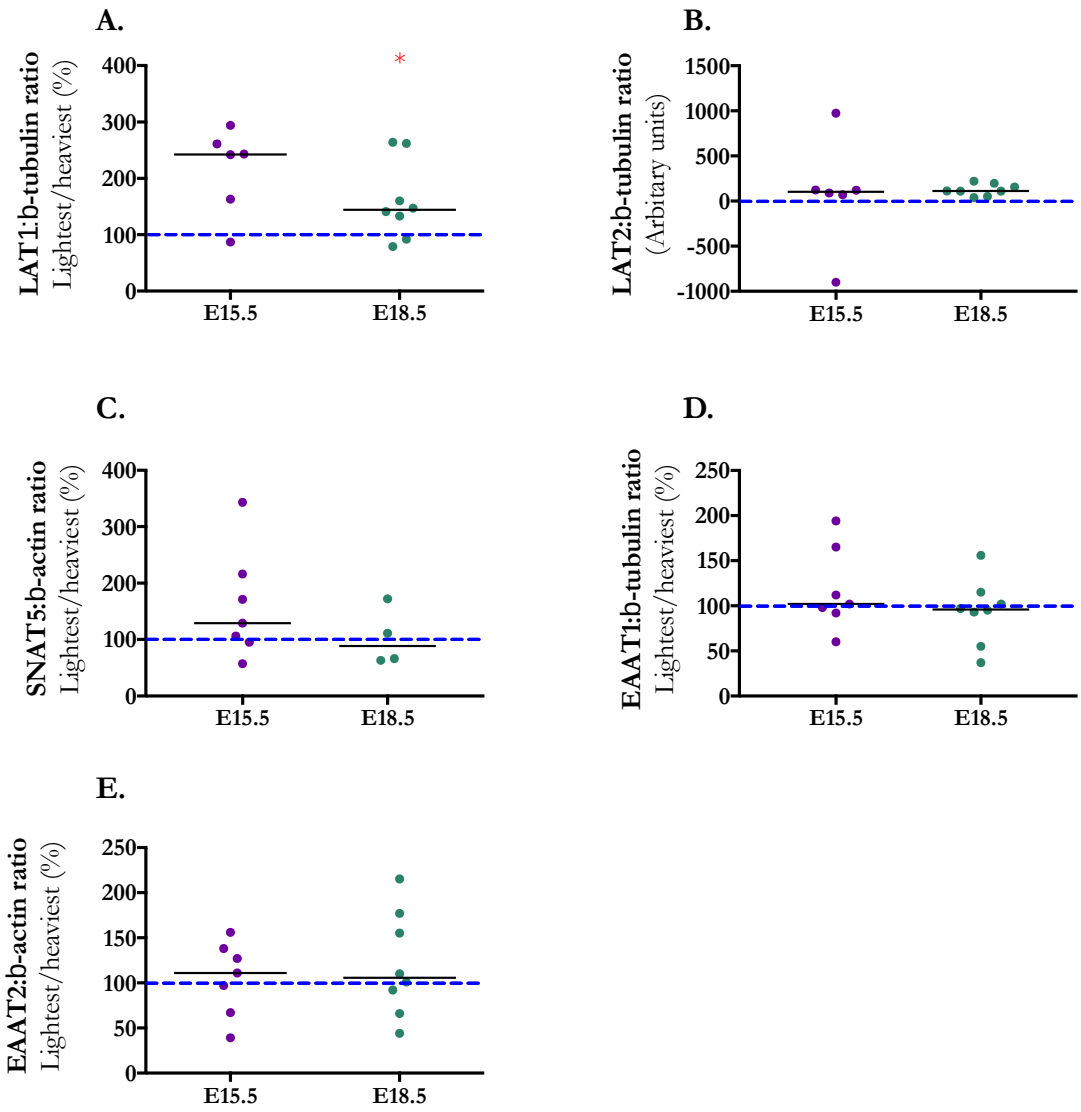


Figure 29: Expression of glutamine and glutamate transporter proteins at E15.5 and E18.5
All data were normalised to β-tubulin or β-actin (see graph axes), the expression of which was stable across groups. Expression of the glutamine transporter protein LAT1 (system L) was significantly higher in the lightest versus heaviest placentas at E18.5. There were no differences in the expression of glutamine transporter proteins LAT 2 (system L), SNAT5 (system N), or glutamate transporter proteins EAAT1 (D) or EAAT2 (E, system X_{AG}), at either gestational age. Poor LAT2 expression at E15.5 led to the variable results when analysed by densitometry (B). E15.5 n=6/7 E18.5 n=4/8 *P<0.05, Wilcoxon signed rank test.

3.4 Discussion

The influence of placental size (weight) on placental transport capacity in a WT mouse litter was examined by assessing K_{mf} across the lightest and the heaviest placentas in a litter, using strategies previously described (Coan *et al.*, 2008; Hayward *et al.*, 2017).

3.4.1 Placental and fetal measures from the lightest and heaviest placentas in a WT mouse litter

The lightest placentas were significantly lighter than the heaviest in a litter at both E15.5 and E18.5 (23% on average). At E15.5 the lightest placenta weighed 4% less and the heaviest placenta 5% more than the median placental weight. At E18.5 the differences were within a narrower range -2%/+3% (-lightest/+heaviest compared to median). Consistent with other studies, peak placental weight was reached at E15.5: median weights were 0.078 g for lightest placentas compared with 0.102 g for heaviest, weights at E18.5 were 90% and 88% of that at E15.5, respectively.

Fetuses from the lightest placentas were significantly lighter at E15.5 and E18.5, yet the magnitude of this difference was smaller towards term (13% at E15.5 and 4% at E18.5). These data are consistent with previous findings (E15.5 12%, E18.5 4% [not statistically significant] and E16.5 11%, E18.5 6%; Coan *et al.*, 2008; Hayward *et al.*, 2017, respectively). The range of fetal weights in the current study are comparable to those reported previously. However, raw values reported by our laboratory (the current study and Hayward *et al.*, 2017; comparing data at E18.5 as E15.5 was not examined in the study by Hayward *et al.*, 2017) are slightly greater than the range reported by Coan *et al.* (2008).

Placental weights at E18.5 reported in this chapter and by Hayward *et al.* (2017) were lighter (range 0.052 g-0.117 g and 0.061 g-0.097 g, respectively) than those reported by others (approximate range 0.060 g-0.190 g, values not quoted in text) (Coan *et al.*, 2008). A potential reason for the discrepancy could be different suppliers: mice used for the purposes of this thesis and by Hayward *et al.* (2017) were obtained from Envigo, UK whilst Coan *et al.* (2008) used an established in-house colony. However, it is reasonable to suggest that in the 10 years between the current study and that of Coan *et al.* (2008) genetic drift may have occurred. Fetal weight:placental weight ratio (F:P ratio) data from our laboratory (data presented in this chapter and by Hayward *et al.*, 2017) were higher than F:P ratio reported by Coan *et al.* (2008). Here, and in all previously published studies (Coan *et al.*, 2008; Hayward *et al.*, 2017) the F:P ratio (g of fetus supported per g placenta) was significantly higher for the lightest placentas at E15.5 (where reported) and E18.5. This indicates that the lightest placentas support more g fetal weight compared with the heaviest placentas in all studies discussed above, despite some differences in raw values. Importantly, in the current study fetuses from both the lightest and heaviest placentas were within the normal fetal weight range (10^{th}-90^{th} centile) (Figure 22). Fetuses with the lightest and heaviest placentas in a litter were therefore not pathologically small or large which suggests that in normal mouse pregnancy a placenta adapts to ensure appropriate fetal growth, thus preventing fetal under- or overgrowth.

Fetal biometry was assessed by measuring crown:rump length, head circumference and abdominal circumference. In humans the growth of pathologically small, growth restricted infants can be described as symmetric or asymmetric; the latter denotes growth that is described as 'head sparing', i.e. brain development is prioritised at the expense of the rest of the body, particularly abdominal organs such as the fetal liver (Hindmarsh *et al.*, 2002). Asymmetric growth is therefore associated with a normal fetal head circumference and reduced abdominal circumference. In the current study, fetuses with the lightest placentas had a reduced abdominal circumference at E15.5 (6%) and E18.5 (4%) and were significantly shorter, as evidenced by a reduced crown:rump length, at E15.5 (7%). Conversely, head circumference was similar between groups at E15.5 but approximately 3% smaller for fetuses with the lightest placentas at E18.5. This suggests that growth was not asymmetrical (since both head and abdominal circumference were reduced towards term). All measurements were made by operators trained using the same standard operating procedures. Although there will be a degree of margin for error, we have nevertheless identified small but consistent differences between groups.

In the present study fetal and placental weight were correlated at E18.5 but not E15.5, which indicates that placental weight is an important determinant of fetal size towards term. These data conflict with previously published findings in mice (Coan *et al.*, 2008), but compare favourably with data that show a positive correlation between placental weight and birth weight in human pregnancy (Hayward *et al.*, 2016; Thame *et al.*, 2001; Thame *et al.*, 2004). Coan *et al.* (2008) reported a correlation between fetal weight and placental weight at E16 (here equivalent to E15.5) but no relationship between measures by E19 (E18.5). This discrepancy may be explained by a wider range of placental weights, specifically much heavier placentas (~0.05-0.19 g at both gestational ages) but similar fetal weight range (~0.20-0.60 g at E15.5 and 0.60-1.40 g at E18.5), compared to the current study (placental weight range of 0.07-0.14 g at E15.5 and 0.05-0.12 g at E18.5) that may have influenced this result. Collating data presented in this chapter with data previously gathered by our laboratory (total n=218 fetuses and placentas) does not alter the strong relationship between fetal weight and placental weight at E18.5. Unfortunately, additional data at E15.5 are unavailable. The extremes of placental weights reported by Coan *et al.* (2008), and a particular skew towards considerably heavier placentas, likely contribute to the lack of correlation between fetal and placental weight at E18.5.

3.4.2 Investigating the effect of placental weight on unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate

The major finding of this chapter was that unidirectional maternofetal clearance (K_{mf} per g placenta) of ^{14}C-glutamine and ^{14}C-glutamate was significantly higher at E18.5 when comparing the lightest and the heaviest placentas in a litter. At E18.5, K_{mf} of glutamine and glutamate in the lightest and heaviest placentas was significantly different from the litter mean (P<0.05; data not shown). To the best of my knowledge, this is the first time that placental transfer of these two amino acids has been assessed in the mouse *in vivo*. K_{mf} was calculated using the interpolated values from the relevant disappearance curve (Figure 18). Curves were generated for both radiolabelled amino acids by combining the single data points from multiple mice euthanased at time points between approximately 20 sec and 35 min post-radioisotope injection. A one-phase exponential decay model was fitted to the curve, and experimental procedures were performed at approximately 2 min; a time at which disappearance from the maternal circulation was linear, as previously described (Bond *et al.*, 2006a).

K_{mf} of MeAIB, a non-metabolisable analogue specific for system A, has previously been assessed in relation to placental weight in WT mice (Coan *et al.*, 2008). K_{mf} of MeAIB is significantly higher across the lightest compared with the heaviest placentas of a WT litter at E15.5 and, to a greater extent, at E18.5 (Coan *et al.*, 2008). Glutamine is a substrate of the system A amino acid transporter family, therefore it is expected that a proportion of total K_{mf} across the intact placenta would be attributed to this transporter system. The relative differences between system A (MeAIB) transport across the lightest and heaviest placentas reported by Coan *et al.* (2008) were greater than the differences in glutamine K_{mf} reported in the current study. However, it is important to stress that direct comparisons cannot be made between results: Coan *et al.* (2008) reported raw radioisotope counts whereas in the current study K_{mf} was calculated relative to radioisotope levels in maternal plasma. It is expected that other transporter systems (L and N on the maternal-facing membrane) also contribute to placental K_{mf} of glutamine. SNAT5 (isoform of system N transporter family) has been localised to the MVM but not the BM in humans (Day *et al.*, 2013; Regnault *et al.*, 2002). Therefore this transporter system likely contributes to placental uptake of glutamine but not release into the fetal circulation. System L transporter members LAT1 and LAT2 facilitate the exchange of substrates to modulate the syncytiotrophoblast amino acid pool (Jansson, 2001; Regnault *et al.*, 2002). The results of the current study indicate that K_{mf} of glutamine across the lightest placenta (versus heaviest in a WT mouse litter) is significantly higher at E18.5. This is likely due to higher abundance or activity of glutamine-specific transporter proteins; however the relative contribution of each transporter system cannot be delineated from these data.

It is generally accepted, in humans and non-human primates at least, that glutamate does not cross the placenta (see section 1.7). In the human placenta, glutamate is taken up from both the maternal and fetal circulations and metabolised within the placenta, primarily to glutamine, for release into the maternal circulation, or to α-ketoglutarate, for entry to the Krebs cycle, or to proline (Day *et al.*, 2013). Glutamate is a substrate of the Na^+-dependent transporter system X_{AG}^-; isoforms EAAT1, EAAT2 and EAAT3 have been localised to the apical and basal membranes of the mouse and rat placenta by immunohistochemistry (Matthews *et al.*, 1998; Matthews *et al.*, 1999). However the activity of glutamate transporters in mice has not been determined. It is not possible to ascertain whether the ^{14}C radiolabel measured in maternal plasma and fetal/placental tissues in the current study remained associated with glutamate or was metabolised within the syncytiotrophoblast. Although some metabolism would be anticipated, data from previous studies in the pregnant rhesus monkey, performed over a series of hours reported that 69-88% of radiolabel infused into the maternal circulation remained associated with glutamate (Stegink *et al.*, 1975). Perfusion of the isolated human placenta for extended periods of time (5 hours) has shown that glutamate is primarily metabolised to glutamine within the placenta (Day *et al.*, 2013). These authors demonstrated that ^{15}N-labelled glutamate inflow reached a steady state at 3 hours of perfusion, although metabolic products were not visible/of very low abundance within the first hour (Day *et al.*, 2013). Therefore, it is unlikely that significant metabolism of ^{14}C-glutamate would occur during the rapid time course of the current experiment (approximately 2 min between radioisotope injection and tissue harvest) and it is expected that the majority of glutamate would have remained intact in the maternal circulation although this remains to be confirmed. That the majority of radioisotope reaches the placenta intact means that the data presented in this chapter are still relevant with regards to placental handling of glutamate (discussed further in section 3.4.5). However, the rate of glutamate metabolism, and metabolic pools into which this feeds has not yet been reported in the mouse placenta.

Glutamate is essential to support mitochondrial function and placental metabolism, providing energy and metabolic intermediates via the tricarboxylic acid (TCA) cycle, and for maintaining the amino acid pool (Day *et al.*, 2013; Tapiero *et al.*, 2002). Glutamate is neurotoxic in high levels and so must be tightly controlled (Tian *et al.*, 2012). Inadequate, or altered glutamate availability will therefore have implications for fetal and placental metabolism. Furthermore, given that glutamate and glutamine are interconverted in both the placenta and fetal liver, glutamate levels in the syncytiotrophoblast and/or in fetal (umbilical) arterial blood could also have knock-on effects for glutamine production and subsequent provision to the fetus.

Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate is the volume of maternal plasma theoretically cleared of isotope per unit time, expressed relative to

individual placental weight (per g placenta). It can also be helpful to express maternofetal transfer relative to fetal weight (μl/min/g fetus) or irrespective of fetal or placental weight (total radioisotope transfer or 'raw transfer') to interrogate the relationship between nutrient transfer and fetal/placental weight. The total amount of nutrients that reach the fetus is a key determinant of fetal growth.

Data were expressed per g fetus to explore whether transfer of glutamine or glutamate was appropriate, relative to the size of the fetus (Figure 24). Maternofetal glutamine transfer (per g fetus) was similar between the lightest and heaviest placentas at E15.5, yet was higher for the lightest placentas at E18.5. This indicates that the lightest placentas over-compensate and deliver more glutamine than their heavier counterparts relative to fetal size, presumably to support a fetus of an appropriate weight. Irrespective of fetal or placental weight, total radioisotope transfer ('raw transfer') of glutamine was also significantly higher across the lightest versus heaviest placentas at E18.5 (no different at E15.5). Towards term (E18.5) the median difference in fetal weight supported by the lightest and heaviest placentas is 5%, compared to 13% at E15.5. This narrowing of fetal weights (by 8%) occurs in parallel with the mechanisms described above; total glutamine transfer across the lightest placentas is 9% higher at E18.5 compared with the heaviest placentas. Taken together, this evidence suggests that the lightest placentas adapt by significantly exceeding total maternofetal glutamine transfer compared with the heaviest placentas in order to meet fetal requirements for growth. Continued compensation into the final 12-24 hours of pregnancy would be expected in order to achieve similar fetal weights between groups.

Conversely, maternofetal transfer of glutamate per g fetus, and total maternofetal transfer irrespective of fetal or placental weight was lower for the lightest placentas at E15.5 but similar between groups at E18.5. Normalisation of glutamate transfer between groups mirrors the catch up in fetal weight towards term, highlighting that glutamate may be an important amino acid for fetal growth. Increased maternofetal glutamate clearance per g placenta (K_{mf}) compensates for the reduced placental size and thus maintains appropriate total transfer of glutamate to the fetus near term.

Taken together, these data are consistent with previous reports that the placenta demonstrates functional adaptations in terms of maternofetal transfer of glucose, calcium and system A amino acid transport to sustain appropriate fetal growth (Coan *et al.*, 2008; Hayward *et al.*, 2017). It has been previously shown in WT mice that the lightest placentas adapt with a relative increase in the labyrinthine (nutrient exchange) zone at E15.5, whilst adaptations are primarily functional towards term; nutrient transport capacity (MeAIB, inulin, glucose) is higher relative to placental size (Coan *et al.*, 2008). The modification of placental nutrient

supply capacity here in terms of K_{mf} of glutamine and glutamate is likely to maintain the fetus on a normal growth trajectory.

3.4.3 Expression of amino acid transporters in the lightest and heaviest placentas

A potential mechanism driving higher K_{mf} in the lightest placentas is increased abundance of transporters known to be important for placental transport of glutamine/glutamate. Thus, expression of putative glutamine and glutamate transporters was next investigated in the lightest and heaviest placentas in a litter.

The sex of the fetus is known to impact on fetal growth and placental function in some instances, and was a significant limitation of the data discussed hereafter. Female fetuses are born lighter than males and respond differently to environmental stress (Blakley, 1978; Clifton, 2010; Ishikawa *et al.*, 2006). The lightest placenta in a WT mouse litter is more likely to belong to a female fetus, and conversely, the heaviest placenta is more often from a male fetus (Hayward *et al.*, 2017). 57% (4/7) of the heaviest placentas assessed by Western blotting were from males and 71% (5/7) of the lightest placentas were from females at E15.5. Close to term (E18.5) 100% (8/8) of the heaviest placentas belonged to male fetuses and 88% (7/8, unable to determine sex from one of the fetuses) of the lightest placentas were from females. The effect of sex cannot be controlled for in the context of the current study; however there was no effect of sex on K_{mf} of glutamine or glutamate (Figure 27, discussed in more detail in section 3.4.4) which supports the hypothesis that transporter expression would be similar between males and females.

Glutamine is a substrate of three distinct transporter systems (system A, L and N) each with unique isoforms of differing affinity for glutamine. The relative abundance of several transporter proteins in membrane-enriched fragments of the lightest and heaviest placentas was assessed by Western blot (Figure 28). System L members LAT1 and LAT2 are Na^+-dependent antiporters that are localised to the apical and basal membranes of the syncytiotrophoblast in mice and humans (Cleal *et al.*, 2018; Regnault *et al.*, 2002). Initially, activity of LAT2 was thought to predominate on the basal membrane (Kudo and Boyd, 2001); however, more recent studies have identified activity consistent with LAT1 activity (Cleal *et al.*, 2011). For LAT1 and LAT2 to be functional, each must be associated with the heavy chain CD98. CD98 is ubiquitous and essential for the shuttling of LAT1 and LAT2 (amongst other light chain transporters) to the membrane and subsequent stability (Wagner *et al.*, 2001). Glutamine is a major substrate of LAT2, whereas LAT1 has less glutamine specificity (Pochini *et al.*, 2014). LAT1 and LAT2 are antiporters (1:1 stoichiometry) working in collaboration with other nutrient transporters to ensure net amino acid uptake is achieved (del Amo *et al.*, 2008; Pochini *et al.*, 2014). A dominant role of LAT1 is to maintain the amino acid pool within the

cell. LAT1 is involved in the uptake of large neutral amino acids and works in concert with Na^+-dependent unidirectional transporters such as systems A and N (Pochini *et al.*, 2014; Verrey, 2003). In the current study LAT1 expression (band detected at the predicted molecular weight of 40 kDa) was significantly higher in the lightest versus heaviest placentas at E18.5 but LAT2 expression (49 kDa, as previously reported) (Ellinger *et al.*, 2016; Segawa *et al.*, 1999) was similar between groups at both gestational ages (Figure 29).

Ingestion of essential amino acids has been shown to induce a transient increase in LAT1, CD98 and SNAT2 mRNA and protein expression in human skeletal muscle, which could be due to mTORC1 activation (Drummond *et al.*, 2010). However, the mechanism which underpins increased LAT1 expression in the lightest versus heaviest placentas reported in the present study is not yet clear. In the context of these results, LAT1 may therefore have a role in maintaining the amino acid pool within the syncytiotrophoblast of the lightest placentas, thus contributing to the amount of amino acids available for exchange between the placenta and fetus, whilst net uptake is achieved via other transporter mechanisms.

SNAT5 is a Na^+-dependent system N co-transporter and also contributes to glutamine transfer. SNAT5 is polarised to the MVM in humans, thus whilst SNAT5 may be important for the uptake of glutamine into the syncytiotrophoblast from the maternal circulation, it is unlikely that it contributes to glutamine efflux to the fetal circulation (Day *et al.*, 2013; Regnault *et al.*, 2002). In this study, expression of SNAT5 (52 kDa) was no different between the lightest and heaviest placentas in a WT mouse litter, suggesting that it is not an underpinning mechanism for the increased K_{mf} of glutamine observed in lightest versus heaviest placentas.

It was not possible to assess expression of system A transporter proteins, of which glutamine and MeAIB are substrates, due to a lack of reliable commercially available antibodies. Therefore, we are unable to comment on the relative abundance of these transporter proteins in membrane-enriched placental fragments. However, increased expression of the gene encoding SNAT2 (slc38a2) has previously been reported in the lightest versus heaviest placentas in a WT litter at E18.5 (other isoforms expressed in the placenta, slc38a1/SNAT1 and slc38a4/SNAT4, were unchanged) (Coan *et al.*, 2008). SNAT1 and SNAT2 have a preference for a broad range of substrates including glutamine whilst SNAT4 is generally not considered to transport glutamine (Schiöth *et al.*, 2013). A study conducted by Palii *et al.* (2004) identified intron 1 as an amino acid response element (AARE) reponsible for regulation of slc38a2 transcription in the human and mouse genes. Collectively, these studies outline a potential role for SNAT2-mediated glutamine transport in the observed changes in the current study, and that slc38a2 expression is up-regulated in response to amino acid deprivation.

System X_{AG-} transporter proteins EAAT1, EAAT2 and EAAT3 mediate placental glutamate uptake across the basal and apical membranes of the placenta (Noorlander *et al.*, 2004). In the current study the relative abundance of EAAT1 and EAAT2 in the lightest versus heaviest placentas was assessed and was not significantly different at E15.5 or E18.5.

Transporter abundance and placental transport capacity do not always correlate since modification of transport proteins, for example phosphorylation, can alter their activity without changing expression (Roos *et al.*, 2009; Vaughan *et al.*, 2017). It was not possible to interrogate this directly by comparing the same placental tissue in K_{mf} and Western analyses since tissues that have been exposed to radioisotopes in clearance studies cannot be used for Western blot. Speculatively, adaptations in placental glutamate transfer may be a result of post-translational modifications or signalling mechanisms that drive up-regulation of transporter activity where placental size is insufficient. Physiological levels of IGF2 maintain normal EAAT1, EAAT2 and EAAT3 expression (Matthews *et al.*, 1999) and system X_{AG-} activity is increased during periods of amino acid deprivation in a kidney cell line (Plakidou-Dymock and McGivan, 1993). In the central nervous system EAAT2 is post-translationally modified by the addition of a small ubiquitin-like modifier (SUMO) to an accessible lysine. SUMOylation causes the transporter to be internalised and available for response from an intracellular pool (Foran *et al.*, 2014). The enzyme protein kinase C (PKC, a downstream target of mTORC2) has also been shown to regulate EAAT3 expression via intracellular trafficking in a cell-type specific manner (Kanai and Hediger, 2003). In human placentas EAAT3 is only expressed in the syncytiotrophoblast in early pregnancy (~8 weeks of gestation) and towards term EAAT3 is localised to the fetal endothelium (Noorlander *et al.*, 2004). If EAAT3 has a similar distribution in the mouse placenta, it is likely that EAAT3 did not make a significant contribution to K_{mf} of glutamate at the time periods investigated in the current study (E15.5 and E18.5). Finally, in rats treated with an infusion of MeAIB to induce FGR, K_{mf} of MeAIB was reduced as was activity of system A (of which glutamine is a substrate) and system X_{AG-} (mediates glutamate transport) in tandem with decreased EAAT1, EAAT2, EAAT3 and EAAT4 expression (Cramer *et al.*, 2002).

The nutrient sensing pathway mTOR (mechanistic target of rapamycin) is a ubiquitously expressed serine/threonine kinase which functions as a 'nutrient sensor', regulating protein translation in response to nutrient availability (particularly amino acids) and other signals such as energy (glucose, ATP) and stress (Roos *et al.*, 2009). mTOR is a potential mechanism by which system A and L transporter activity could be modified in line with the findings of this chapter: i.e. altered K_{mf} of glutamine and glutamate in the absence of differences in overall transporter protein abundance, aside from LAT1. Inhibition of mTOR leads to decreased system A, L and β transporter activity but no difference in protein expression (Roos *et al.*,

2009) which suggests that mTOR regulates amino acid transporters at the post-translational stage. mTOR complex 1 (mTORC1) is a positive regulator of amino acid transporter system A and L transporter activity (Roos *et al.*, 2007; Roos *et al.*, 2009; Rosario *et al.*, 2013). Activity of mTORC1 is regulated by several upstream signals such as AMP kinase, the AAR (amino acid response) signal transduction pathway and GSK3 (glycogen synthase kinase 3), and is altered in response to maternal nutrient availability (Jansson *et al.*, 2012). Cellular glutamine uptake and efflux by exchange, in the presence of other essential amino acids, has been shown to activate the mTOR pathway in other tissues, however this has not yet been shown in placenta (Nicklin *et al.*, 2009). Inhibition of mTORC1 activates NEDD4-2 which ubiquitinates transporter proteins such as LAT1, and this inhibits their insertion (trafficking) to the plasma membrane (Rosario *et al.*, 2013; Rosario *et al.*, 2016). It is not known whether other amino acid transporter systems important for glutamine and glutamate transport (systems N, X_{AG-}) are regulated by mTOR.

The data presented in this chapter reveal altered K_{mf} of glutamine and glutamate in the absence of any differences in overall transporter protein abundance, with the exception of LAT1. It is therefore possible that post-translational modifications and mechanisms such as mTOR are altered between the lightest and heaviest placentas. mTOR activity in relation to placental size has not yet been explored, but is worthy of investigation in light of these results.

3.4.4 Investigating the effect of sex on unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate

The sex of the fetus is a determinant of fetal growth and survival in humans. It has been known for more than 50 years that boys are larger at birth (Lubchenco *et al.*, 1963). Being male is also an independent risk factor for poor pregnancy outcome (Di Renzo *et al.*, 2007). Many studies have disregarded the sex of the fetus, pooling placental samples despite knowledge of the differing strategies employed by the male and female conceptus; there is mounting evidence that male and female fetuses and their placentas employ differing strategies to deal with complications *in utero* (for review see Clifton, 2010). Yet, current understanding of the effect of sex on placental function, in particular placental nutrient transfer, is lacking (Walker *et al.*, 2017). Here we explore the influence of sex on fetal and placental weight and K_{mf} of glutamine and glutamate.

In mice, females are more likely to have the lightest placenta within a litter, and conversely males the heaviest (Blakley, 1978; Ishikawa *et al.*, 2006). In the current study female fetuses and their placentas weighed less than males at E18.5, with no difference observed at E15.5. Females also had a higher F:P ratio compared with males at E18.5. However, biometric measurements did not differ according to sex. These data conflict with some reports in a

human population that males have larger head and abdominal circumferences in correlation with a higher birth weight compared with females (Davis *et al.*, 1993). However, some studies have found no difference in head or abdominal circumference according to sex (Gale *et al.*, 2001). It is feasible that techniques available in the current study to take biometric measurements were not sensitive enough to detect a change this small in mice where the reduction in fetal weight (females versus males) was comparatively small (average 3%).

The data presented in this chapter are in alignment with previous works by this group; Hayward *et al.* (2017) reported that females were lighter than males but that the overall effect on K_{mf} of calcium was slight, just failing to reach statistical significance, and thus not a major contributor to the main findings of the study. In the current study, K_{mf} of glutamine (per g placenta) was 19% higher across placentas of females compared with males, whereas K_{mf} was 53% higher across lightest versus heaviest placentas. Other previous works that have compared transport capacity of the lightest and heaviest placentas in a litter did not report effects of fetal sex (Coan *et al.*, 2008); however, given the data reported here and elsewhere it is likely that there would be a skew within the dataset of Coan *et al* (2008), i.e. a larger proportion of male fetuses with the heaviest placentas, and female fetuses with the lightest placentas.

There have been few studies that directly investigate the effect of fetal sex in mouse pregnancy, though recent data show that females are more susceptible to hypoxia *in utero;* females are more severely growth restricted, have smaller placentas and exhibit evidence of hypoxia-induced oxidative stress compared with males exposed to the same conditions (Cuffe *et al.*, 2014; Matheson *et al.*, 2016).

In this study, the effect of fetal sex on K_{mf} was assessed by comparing the litter mean of females compared with that of males (minimum of two in each group per litter). In an ideal scenario the lightest placenta from a male fetus would be compared with the heaviest placenta from a male fetus within the same litter, with the same analysis carried out for female fetuses. However, this is not possible in practice due to the relatively small litter sizes which results in a narrow range of placental weights between the lightest and heaviest male or female placenta. The sex of the fetus did not significantly influence K_{mf} of glutamine or glutamate although there was a trend towards higher K_{mf} of glutamine for females versus males. Although sex does influence fetal and placental weight and, there is a trend towards an increase in K_{mf} of transfer in females (P=0.055), sexual dimorphism cannot fully account for the differences (section 3.3.2) in K_{mf} of glutamine and glutamate described according to placental weight.

3.4.5 Methodological considerations

The adaptation of a method by Flexner and Pohl (Flexner and Pohl, 1941) has enabled the quantification of unidirectional maternofetal clearance (K_{mf}) of substrates across the intact placenta *in vivo* including mannitol, calcium, glucose and MeAIB (Bond *et al.*, 2006b; Bond *et al.*, 2008; Coan *et al.*, 2008; Dilworth *et al.*, 2010; Hayward *et al.*, 2017). This method is valuable in that K_{mf} can be evaluated *in vivo* in relation to placental weight, sex of the fetus and/or in models of pregnancy pathologies such as fetal growth restriction (FGR). The use of MeAIB in previous studies was advantageous as it is a non-metabolisable analogue specific to system A transporter. It could be that using this method to measure clearance underestimates true transport since the tracer will have to equilibrate with the various pools of amino acids in the placenta (Cleal *et al.*, 2018; Velázquez *et al.*, 1976). Equilibration may be achieved faster in a smaller placenta, but this has not yet been investigated in the mouse.

In the current study, it is possible that there is metabolism of injected ^{14}C-glutamate in the mother and/or in the placenta, and that a radiolabelled metabolite of glutamate was transferred to the fetus. Based on previous studies (Stegink *et al.*, 1975), it is very likely that radiolabelled glutamate is delivered intact to the placenta and that radioactive counts in the fetus will reflect the amount of radiolabelled glutamate taken up by the placenta since any radiolabelled metabolite, e.g. glutamate, glutamine, glucose or lactate, in the fetus must have got there following uptake of glutamate into the placenta. Metabolism of glutamate in the mouse over the rapid timecourse of the experiment (~2 min) is unknown and is a question for future research.

Ideally, radioisotope levels in the maternal circulation would be deduced from an average of serial maternal blood samples drawn during each experiment. This is not possible in the mouse due to the very small blood volume, typically 2-2.5 ml for a pregnant mouse (NC3RS, 2014a). Therefore, a single maternal blood sample was collected at the time of euthanisation by cardiac puncture, and collated with data from various time points between ~20 sec and 35 min to construct a disappearance curve. The area under the curve was then used to estimate radioisotope in maternal blood samples, from which K_{mf} was calculated.

The abundance of putative glutamine and glutamate transporter proteins in membrane-enriched fragments derived from the lightest and the heaviest placentas in a litter was assessed by Western blot to assess the relationship between protein expression and amino acid transfer. A mixed membrane preparation (membrane-enriched fragments drawn from high-speed centrifugation) was used so that the single lightest and heaviest placentas from each litter could be compared. Therefore an inherent limitation of this study is that the abundance of transporter proteins was assessed in a mixed membrane isolate, which will include apical and basal membranes of the syncytiotrophoblast as well as organelle plasma membranes. This was

deemed the most appropriate method given that isolating apical membranes from a single placenta would result in a relatively low protein sample size for these subsequent experiments. There is currently no method published to isolate the basal membrane of the syncytiotrophoblast in mice. By using membrane-enriched fragments from a single mouse placenta, an assessment of the relative protein abundance may be deduced. However, it must be considered that the exact localisation of these proteins cannot be guaranteed. Ideally protein expression would be assessed by comparing the lightest female with the heaviest female, and so on for male fetuses. Unfortunately, in order to maintain the paired nature of these analyses within a single litter, these comparisons were not feasible due to the relatively small litter sizes in this strain of mice; a study of this nature would undoubtedly require far greater numbers of animals in order to encompass a range of placental weights, and would likely not contribute significant further insight into the underlying mechanisms.

3.4.6 Summary

This chapter substantiates previous findings that the lightest placentas within a WT mouse litter are significantly lighter than the heaviest at E15.5 and E18.5, and that these placentas support more fetus per g placenta (as evidenced by a higher F:P ratio) at both gestational ages. Fetuses with the lightest and heaviest placentas are within a normal birth weight range, as determined by fetal weight centile, which may be achieved by the placenta adapting its function to meet fetal growth requirements.

Here we demonstrate for the first time unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate across the WT mouse placenta. K_{mf} of both radioisotopes was significantly higher across the lightest placentas (per g placenta) compared to the heaviest in a litter at E18.5. Provision of glutamine and glutamate is essential during fetal development: availability, as well as fetal and placental metabolism, of these two amino acids is fundamental for maintenance of appropriate fetal growth. That the lightest placentas adapt their transport capacity towards term reinforces the importance of glutamine and glutamate. As has been shown previously for calcium and system A transport in mice (Coan *et al.*, 2008; Hayward *et al.*, 2017), placental adaptation of nutrient transport capacity ensures that the fetus reaches a normal weight at birth.

Due to the skew of female fetuses with the lightest placentas, and conversely, male fetuses with the heaviest, the effect of fetal sex on placental nutrient transfer was also examined. We found that the sex of the fetus, and thus placenta, was not a major determinant of up-regulated K_{mf} of glutamine or glutamate in the lightest placentas.

To investigate the mechanisms underlying the changes in K_{mf} of glutamine and glutamate, protein expression was analysed by Western blot. Semi-quantification of protein expression in

membrane-enriched fragments indicated that expression of the glutamine transporter protein LAT1 was significantly elevated in lightest versus heaviest placentas. Increased LAT1 abundance likely underpins altered K_{mf} of glutamine, and may contribute to changes through altering the composition of the amino acid pool available for exchange. The contribution of system A to these changes is unknown and the expression of SNAT1, SNAT2 and SNAT4 (system A) should also be investigated, should reliable antibodies become commercially available. The mechanisms that regulate and drive changes in K_{mf} of glutamate are as yet undetermined. Potential mechanisms that underpin these changes, such as post-translational modifications and the signalling pathway mTOR, should be explored in future studies.

In summary these data provide evidence that K_{mf} of glutamine and glutamate across the WT mouse placenta (per g placenta) adapts in an effort to maintain appropriate fetal growth. A mechanism for higher K_{mf} of glutamine has been identified (raised LAT1 protein expression), but the mechanisms behind altered glutamate transfer have yet to be identified. Whilst the experiments described in this chapter demonstrate adaptive up-regulation of maternofetal clearance of glutamine and glutamate in relation to small placental size in mice, it is important to assess whether glutamine and glutamate uptake also adapts in relation to placental size in women. Understanding factors that determine amino acid transporter activity and thus the provision of amino acids to the fetus in normal human pregnancy is imperative to improve our understanding of disorders where nutrient transfer is impaired, such as fetal growth restriction (FGR).

Chapter 4 Activity and expression of glutamine and glutamate transporters in placental villous fragments in normal human pregnancy

4.1 Introduction

Appropriate fetal growth is achieved through the net transfer of nutrients from the maternal to fetal circulation by the placental syncytiotrophoblast, via a number of placental transport mechanisms (Figure 6). Regulation of nutrient transport mechanisms, such as transporter protein abundance and activity, is vital to support appropriate fetal growth but the mechanisms responsible are poorly understood.

The amino acids glutamine and glutamate are essential for fetal growth. Aside from fetal requirements per se, glutamine and glutamate are also needed for pH homeostasis and biosynthetic processes (Pochini *et al.*, 2014). Glutamine transport to the fetus is required for the provision of fetal glutamate following glutamine to glutamate conversion in the fetal liver. Glutamate produced in the fetal liver may be subsequently shuttled and re-converted to glutamine within the placenta (Battaglia, 2000; Moores *et al.*, 1994; Vaughn *et al.*, 1995). Although glutamate is taken up by the placenta from maternal blood, it is not thought to cross to the fetus but instead is utilised for placental metabolism and can be converted to glutamine or α-ketoglutarate for entry to the tricarboxylic acid (TCA) cycle for the production of intermediate metabolites and energy (Battaglia, 2002; Day *et al.*, 2013; Pitkin *et al.*, 1979; Self *et al.*, 2004). Glutamine and glutamate should therefore be considered together since their placental transport and metabolism are interlinked.

Placental uptake of glutamine from maternal blood is mediated by one of three amino acid transporter systems; system A, L or N (the isoforms of which are: SNAT1, SNAT2, SNAT4; LAT1, LAT2; and SNAT5, respectively) (Desforges and Sibley, 2010; Johnson and Smith, 1988). Glutamate uptake into the syncytiotrophoblast from maternal blood occurs via the anionic amino acid system X_{AG-} (isoforms EAAT1, EAAT2 and EAAT3) (Hill *et al.*, 2014; Novak *et al.*, 2001).

System A is a Na^+-dependent transporter, responsible for the transfer of small, neutral amino acids such as alanine, serine and glycine, and has been studied extensively in the placenta. Members of the system A family (SNAT1, SNAT2 and SNAT4) are present on both the microvillous (MVM) and basal (BM) membranes of the syncytiotrophoblast (Desforges and Sibley, 2010; Regnault *et al.*, 2002). System A activity (measured using the non-metabolisable analogue MeAIB) has been shown to be inversely related to placental weight in mice and humans (Coan *et al.*, 2008; Godfrey *et al.*, 1998). Since glutamine is a system A substrate, it is

likely that the transport of glutamine is also inversely related to placental size. Activity of other glutamine (system L and N) and glutamate transporters (system X_{AG-}) has not been explored in relation to placental size in humans.

A relationship between placental size and placental transport would ensure that nutrient provision is sufficient to maintain appropriate fetal growth. Functional or morphological changes that lead to a change in placental efficiency (adaptation), as evidenced by fetal:placental weight (F:P) ratio, may include altered abundance or activity of amino acid transporters and/or the molecular pathways (such as mTOR) that regulate this. Adaptations may therefore influence nutrient allocation to the growing fetus in order to achieve appropriate fetal growth. The data in Chapter 3 of this thesis provided evidence that the activity of glutamine and glutamate transporters adapt according to placental size in WT mice. Unidirectional maternofetal clearance (K_{mf}) of glutamine and glutamate was compared in the lightest and heaviest placentas in a WT mouse litter of normally grown fetuses. K_{mf} of glutamine and glutamate was higher for the lightest versus heaviest placenta in a litter (at E18.5). This was associated with higher expression of the glutamine transporter protein LAT1 in the lightest compared with heaviest placentas, but there was no difference in the expression of EAAT1 or EAAT2, transporter proteins important for the transport of glutamate into the syncytiotrophoblast.

In the current study, the activity of glutamine and glutamate transporters in the syncytiotrophoblast was assessed *in vitro* to determine whether placental uptake of glutamine and glutamate is also related to placental size (weight) in human pregnancy. Transporter activity (per mg placental protein) was determined in normal pregnancies resulting in babies that were appropriately grown for gestational age (AGA), and related to placental weight. In several previous studies, syncytiotrophoblast MVM vesicles have been used to assess the activity of amino acid transporters in the maternal-facing membrane of syncytiotrophoblast (Desforges *et al.*, 2009; Dicke *et al.*, 1993; Godfrey *et al.*, 1998; Mahendran *et al.*, 1994). Isolation of the MVM to make vesicles, however, is at the expense of tissue integrity, intracellular signaling mechanisms and associated driving forces (see Table 5). Incubation of placental villous tissue fragments in a solution containing radiolabelled amino acid may instead be used to determine amino acid uptake over time as a measure of transporter activity, whilst maintaining tissue architecture (Greenwood and Sibley, 2006). Placental amino acid transporter activity assessed using these two preparations (MVM vesicles versus villous fragments) has been shown to be comparable, although relative contributions of some transport systems varied (L-serine uptake; Brand *et al.*, 2010). For the current study, villous tissue fragments were used and a method was optimised to measure the activity of glutamine and glutamate transporters (described in full in section 2.1.5.1).

There is evidence that the sex of the fetus, and therefore the placenta, plays a crucial role in response to adverse stimuli and in fetal growth trajectory/birth weight (reviewed in Clifton, 2010). Males are born heavier than females (Clarke, 1788; Misra *et al.*, 2009; Wallace *et al.*, 2012), although this provision could be met by the larger placental size: males have a higher median trimmed placental weight (679 g) than females (668 g) (Almog *et al.*, 2011). Here we have investigated whether the activity and expression of glutamine and glutamate transporters is different in placentas of male and female fetuses. Consideration of the sex of the fetus and its influence on placental function may inform future treatment and diagnostic pathways.

4.1.1 Hypotheses

- Uptake of ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB into placental villous fragments is inversely related to placental weight in normal pregnancy
- Activity and expression of glutamine and glutamate transporters is higher in male compared with female placentas

4.1.2 Aims

The objectives of this study were to:

- Validate a method to determine glutamine and glutamate transporter activity by measuring the transporter-mediated component of ^{14}C-glutamine and ^{14}C-glutamate uptake by placental villous fragments at initial rate
- Assess whether transporter-mediated uptake of ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB (latter used as a positive control and to represent system A transporter activity, see section 2.1.5.2) adapts according to placental or fetal weight in a normal pregnancy cohort
- Determine the effect of fetal sex on transporter-mediated uptake of ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB, and on the expression of transporter proteins, in a normal pregnancy cohort

4.2 Methods

4.2.1 Method validation: measurement of glutamine and glutamate transporter activity in placental villous fragments

A validation experiment was performed to determine the appropriate time course and buffer conditions to assess transporter-mediated ^{14}C-glutamine and ^{14}C-glutamate uptake (see section 2.1.5.2 for full details). Uptake of ^{14}C-glutamine and ^{14}C-glutamate by villous fragments was measured over 10-120 min. Glutamine transport is mediated by systems A, L and N. To distinguish between transporter-mediated and non-specific diffusion, uptake of ^{14}C-glutamine

was measured in Na^+-free buffer (to inhibit system A and N) with 5 mM histidine, (His, to inhibit system N), serine (Ser, to inhibit system A) and 2-Amino-2-norbornanecarboxylic acid (BCH, to inhibit system L). Glutamate is transported by system X_{AG-}. To distinguish between transporter-mediated and non-specific diffusion, uptake of ^{14}C-glutamate was measured in Na^+-free buffer with 5 mM aspartate (Asp, to inhibit system X_{AG-}). Transporter-mediated uptake of ^{14}C-glutamine and ^{14}C-glutamate was calculated as the difference in uptake in control (Na^+-containing) Tyrode's buffer and in Na^+-free buffer with the competitive inhibitor substrates: 5 mM His, Ser and BCH for ^{14}C-glutamine or Asp for ^{14}C-glutamate.

4.2.2 Glutamine and glutamate transporter activity in placental villous fragments from pregnancies with a normal outcome

Women were approached and consented to the study according to the inclusion and exclusion criteria stated in section 2.1.2. Placentas were included in the study where the individualised birth weight ratio (IBR) of the infant was between 10^{th}-90^{th} centile, i.e. considered appropriate for gestational age (AGA) (Figure 30). Placentas were collected and sections of villous tissue were dissected in preparation for the villous fragment uptake experiment.

The full protocol is described in the Methods chapter of this thesis (2.1.5.3-2.1.5.7). In brief, placental villous fragments were exposed to ^{14}C-glutamine or ^{14}C-glutamate, and the initial rate of transporter-mediated uptake of radiolabelled amino acids was measured over 30-90 min and expressed in pmol/mg protein. MeAIB uptake was also measured as a positive control (to assess system A activity), and to confirm and extend previous findings (Ditchfield, 2011; Godfrey *et al.*, 1998; Hayward *et al.*, 2012).

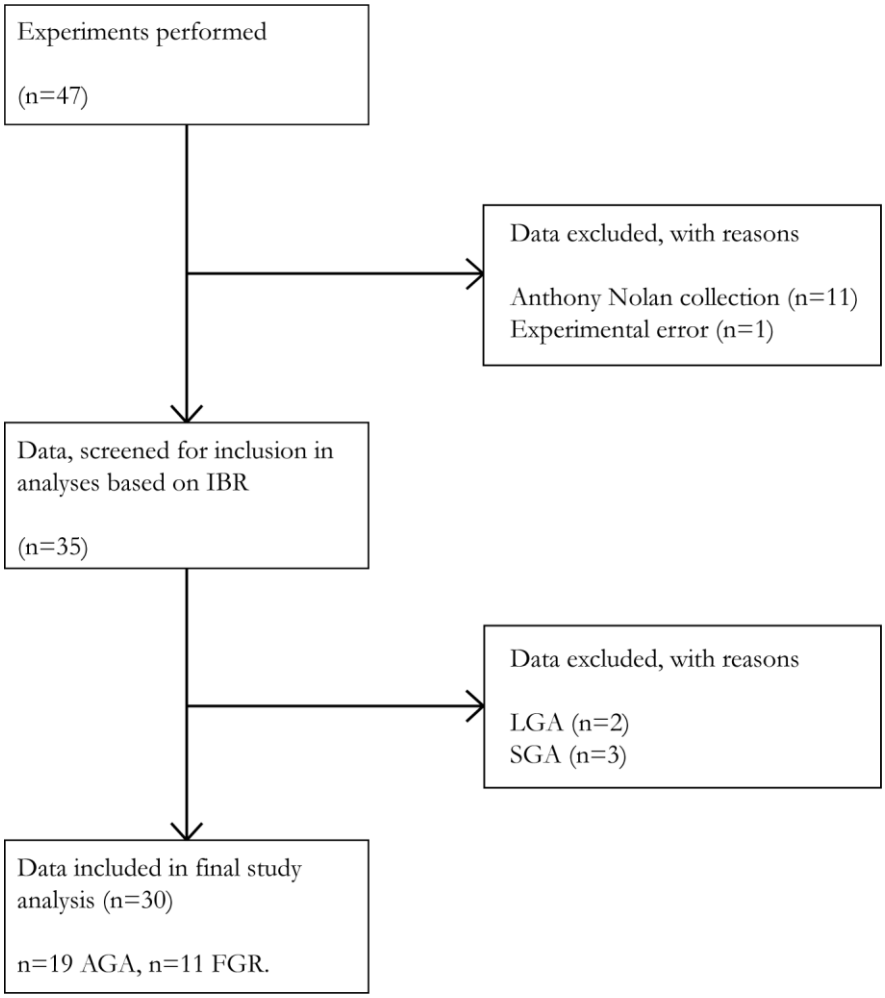


Figure 30 Experimental flowchart
47 amino acid uptake experiments were performed. Placentas collected by the Anthony Nolan charity ('Anthony Nolan collection') were bled for the collection of stem cells. ^{14}C-glutamine uptake by villous fragments was significantly lower in placentas bled by Anthony Nolan (data not shown). Accordingly, data from placentas bled by Anthony Nolan were excluded from final study analysis (n=11). Data were also excluded from final analyses when there was an experimental error (n=1) or if the infant was large for gestational age (LGA, IBR >90th centile, n=2) or small for gestational age (SGA, IBR >5th<10th centile, n=3). Placentas from normal birth weight infants, i.e. those with an IBR of >10th<90th centile (appropriate for gestational age, AGA) were included in the study (n=19). Placentas from FGR (fetal growth restricted) infants, IBR<5th centile (n=11), were included in the study presented in Chapter 6.

4.2.2.1 Glutamine and glutamate transporter activity in relation to fetal and placental measures

Amino acid uptake was measured in placental villous fragments from normal pregnancy outcomes (n=19) (Table 19). A single time point (90 min, when amino acid uptake is still occurring at initial rate) was chosen to correlate placental amino acid uptake with fetal and placental parameters (placental weight, birth weight, F:P ratio, IBR).

	Normal birth weight (appropriate for gestational age, AGA) (n= 19)
Maternal Age (years)	30 (23-40)
Body Mass Index (kg/m^2)	25.9 (18.8-31.2)
Birth Weight (g)	3410 (2690-4400)
Trimmed Placental Weight (g)	495.5 (331.1-719.4)
Individualised Birth Weight Ratio (IBR)	54.0 (13.6-87.7)
Birth weight:placental weight (BW:PW) **ratio**	6.5 (5.2-8.9)
Gestation (days)	273 (260-285)
Mode of Delivery	ELCS (89%) NVD (11%)
Parity	1 (0-8)
Gravidity	2 (1-12)
Ethnicity	Caucasian (58%)
Smoking Status	No (100%)

Table 19 Demographics relating to placentas from normal birth weight (AGA) infants
Placentas included in the study were collected from infants with an IBR >10th<90th centile (appropriate for gestational age, AGA). Data are presented as median (range) or percentage. There were no differences between groups aside from placental weight (those from AN collections were lighter, *P*=0.002, Mann-Whitney test).

4.2.2.2 Effect of sex on glutamine and glutamate transporter activity

The sex of the infant is thought to influence placental function (see section 4.1). To determine whether sex had an effect on amino acid transporter activity, data from normal pregnancy outcomes were stratified according to sex (n=7 male, n=12 female) (Table 20) and analysed as described previously (see section 2.1).

4.2.3 Western blotting of membrane-enriched whole placental homogenates

Membrane-enriched whole placental homogenates were prepared (as described in section 2.3.1) from villous tissue of male (n=6/7) and female (n=5/7/8) placentas. Expression of the transporter proteins LAT1, LAT2, SNAT5, EAAT1, EAAT2 and EAAT3 was assessed by Western blotting according to the method in section 2.3. β-tubulin was used as a housekeeping protein and probed for on each membrane.

	Male (n= 7)	**Female** (n= 12)	***P* value**
Maternal Age (years)	30 (23-38)	31 (27-40)	0.52
Body Mass Index (kg/m^2)	26.9 (21.0-28.2)	25.8 (18.8-31.2)	0.79
Birth Weight (g)	3300 (2820-3540)	3457 (2690-4400)	0.65
Trimmed Placental Weight (g)	517.2 (402.3-719.4)	488.6 (331.1-704.7)	0.90
Individualised Birth Weight Ratio (IBR)	54.0 (26.4-78.9)	55.1 (13.6-87.7)	0.71
Birth weight:placental weight (BW:PW) **ratio**	6.4 (5.5-8.5)	7.2 (5.2-8.9)	0.385
Gestation (days)	271 (260-278)	273 (266-285)	0.52
Mode of Delivery	ELCS (86%) NVD (14%)	ELCS (92%) NVD (8%)	-
Parity	1 (0-2)	1 (0-8)	0.89
Gravidity	2 (1-5)	2 (1-12)	0.88
Ethnicity	Caucasian (57%)	Caucasian (58%)	-
Smoking Status	No (100%)	No (100%)	-

Table 20: Demographics for placentas from male and female infants

Demographics summarised above relate to placentas from normal pregnancy outcomes stratified according to the sex of the infant. Data are presented as median (range) or percentage. There were no significant differences between groups.

4.2.4 Statistical analysis

Normal distribution of data was determined using D'Agostino & Pearson omnibus normality test (GraphPad Prism 7 software). Data from placental villous fragment uptake experiments are expressed as mean ± standard error of the mean (SEM), and regression analyses were performed to determine whether transporter-mediated amino acid uptake was linearly related to time over 30-90 min and to examine the relationship between transporter-mediated uptake at 90 min and fetal and placental measures (Spearman correlation or Linear regression).

Semi-quantitative analysis of transporter protein expression as determined by protein band density (densitometry) was performed for Western blot data using Image Studio™ Lite software. Data were analysed (GraphPad Prism 7 software) using a Mann-Whitney test. For all data a *P* value <0.05 was considered statistically significant.

4.3 Results

4.3.1 Method validation: measurement of glutamine and glutamate transporter activity in placental villous fragments

The results of the validation experiment are shown in Figure 31. The total uptake of radiolabelled glutamine and glutamate into villous tissue in control Tyrode's buffer (Figure 31A, C) is the net result of non-specific diffusion through damaged areas/paracellular routes and uptake mediated by transporter proteins situated in the syncytiotrophopblast MVM.

^{14}C-glutamine uptake was reduced by ~70% in Na^+-free Tyrode's buffer, or in control (Na^+ containing) buffer with 5 mM His, Ser and BCH, and reduced by ~85% in Na^+-free buffer with 5 mM His, Ser and BCH (Figure 31A). This indicates that ~15% of ^{14}C-glutamine uptake occurs by non-specific diffusion and the remaining uptake is by the combined activity of systems A, L and N. ^{14}C-glutamate uptake was reduced by ~95% in Na^+-free Tyrode's buffer +/- 5 mM Asp, or in control buffer with 5 mM Asp (Figure 31C). This indicates that ~5% of ^{14}C-glutamate uptake occurs by non-specific diffusion and the remaining uptake is via the Na^+-dependent activity of system X_{AG-}.

Transporter-mediated uptake of ^{14}C-glutamine and ^{14}C-glutamate by villous fragments increased linearly over 10-120 min (P<0.001, Linear regression) (Figure 31B, D) and extrapolation of the regression line intersected the x,y axes close to the origin, indicating that uptake was at initial rate.

For all subsequent experiments transporter-mediated uptake was assessed as the difference in uptake in control Tyrode's buffer and uptake in Na^+-free buffer + 5 mM His, Ser and BCH (^{14}C-glutamine) or aspartate (^{14}C-glutamate), over 30-90 min. Having completed the pilot experiment it was evident that less isotope could be used to achieve an acceptable tissue count above background and subsequent experiments were performed with lower concentrations of radioisotope (tissue counts ranged from ~10-250 x background); this accounts for the difference in the y axis values in the study results from the plot in Figure 31.

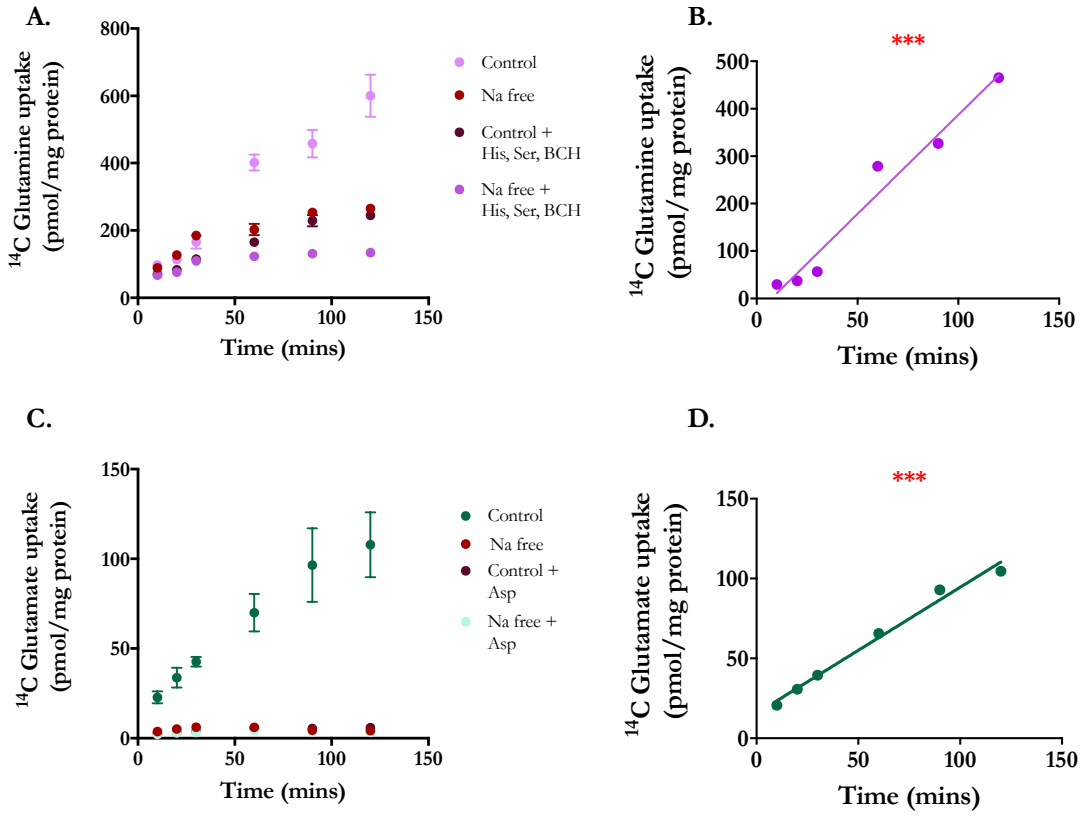


Figure 31 Optimisation of placental amino acid uptake experiment

Uptake of ^{14}C-glutamine (A,B) and ^{14}C-glutamate (C,D) into placental villous fragments over a time period of 10-120 min. Each data point (A,C) represents triplicate measures from one placenta, mean±SEM. Radioisotope uptake was assessed in four conditions: control (Na^+-containing Tyrode's buffer) or Na^+-free buffer +/- 5 mM His, Ser and BCH (histidine, serine, 2-Amino-2-norbornanecarboxylic acid: ^{14}C-glutamine, A) or 5 mM Asp (aspartate: ^{14}C-glutamate, C) to determine the conditions to maximally inhibit transporter-mediated uptake allowing the non-specific component of uptake to be estimated. Transporter-mediated uptake, calculated as the difference in uptake between control conditions and Na^+-free buffer plus 5 mM His, Ser and BCH or 5 mM Asp, increased linearly with time (*** $P<0.001$; Linear regression) for both radiolabelled amino acids as shown in graphs B and D.

4.3.2 Transporter-mediated amino acid uptake is not related to placental weight or birth weight

Birth weight and placental weight were positively correlated with one another (Figure 32) ($P<0.05$, Linear regression). Transporter-mediated uptake (amino acid transporter activity) was calculated as uptake in control (Na^+-containing conditions) minus uptake under Na^+-free conditions plus competitive inhibitors (Figure 33). Transporter-mediated uptake was linear and at initial rate for all experiments that were related to fetal and placental measures. The 90 min time point of transporter-mediated uptake was used for subsequent comparisons.

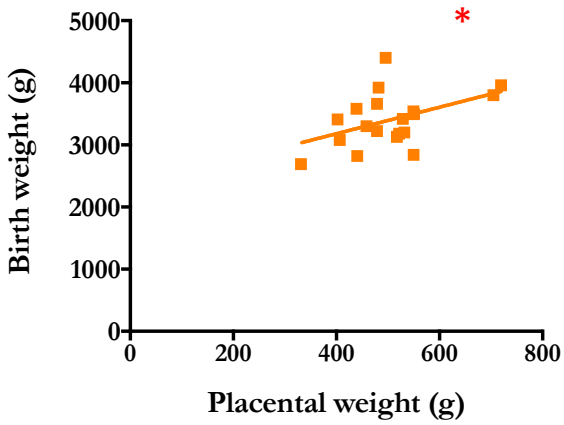


Figure 32 Relationship between placental weight and birth weight from normal birth weight (AGA) infants

Trimmed placental weight and respective birth weight of normal birth weight (AGA) infants with an IBR between 10th-90th centile. Placental weight and birth weight were significantly correlated (n=19; * $P<0.05$; Linear regression).

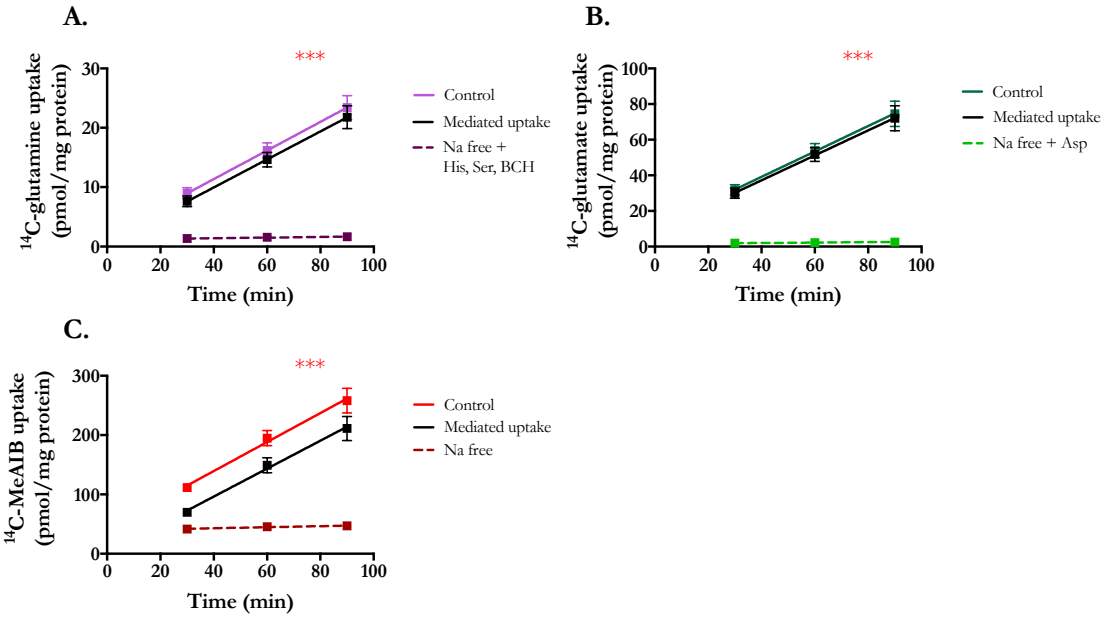


Figure 33: Na^+- free, control and transporter-mediated amino acid uptake in placentas of normal birth weight (AGA) infants

Uptake of amino acids by placentas from normal birth weight (AGA) infants in Na^+-free and control (Na^+-containing) buffer (mean±SEM). Na^+-free buffer was supplemented with 5 mM His, Ser and BCH (histidine, serine, 2-Amino-2-norbornanecarboxylic acid: ^{14}C-glutamine, A) or 5 mM Asp (aspartate: ^{14}C-glutamate, B). Mediated uptake was calculated as the difference between uptake in Na^+-free (representative of non-specific diffusional uptake) and control (uptake via specific and non-specific processes) conditions. Transporter-mediated uptake (amino acid transporter activity) was linear and at initial rate for all the experiments that were subsequently related to placental and fetal measures. n=18/19 *** $P<0.01$ Linear regression.

Transporter-mediated uptake of ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB (90 min time point) in placentas collected from normal pregnancies did not relate to placental weight, birth

weight, F:P ratio or IBR (Figure 34, Linear regression). The non-specific, diffusional component of uptake was not related to any of these variables (see Appendix 9.1).

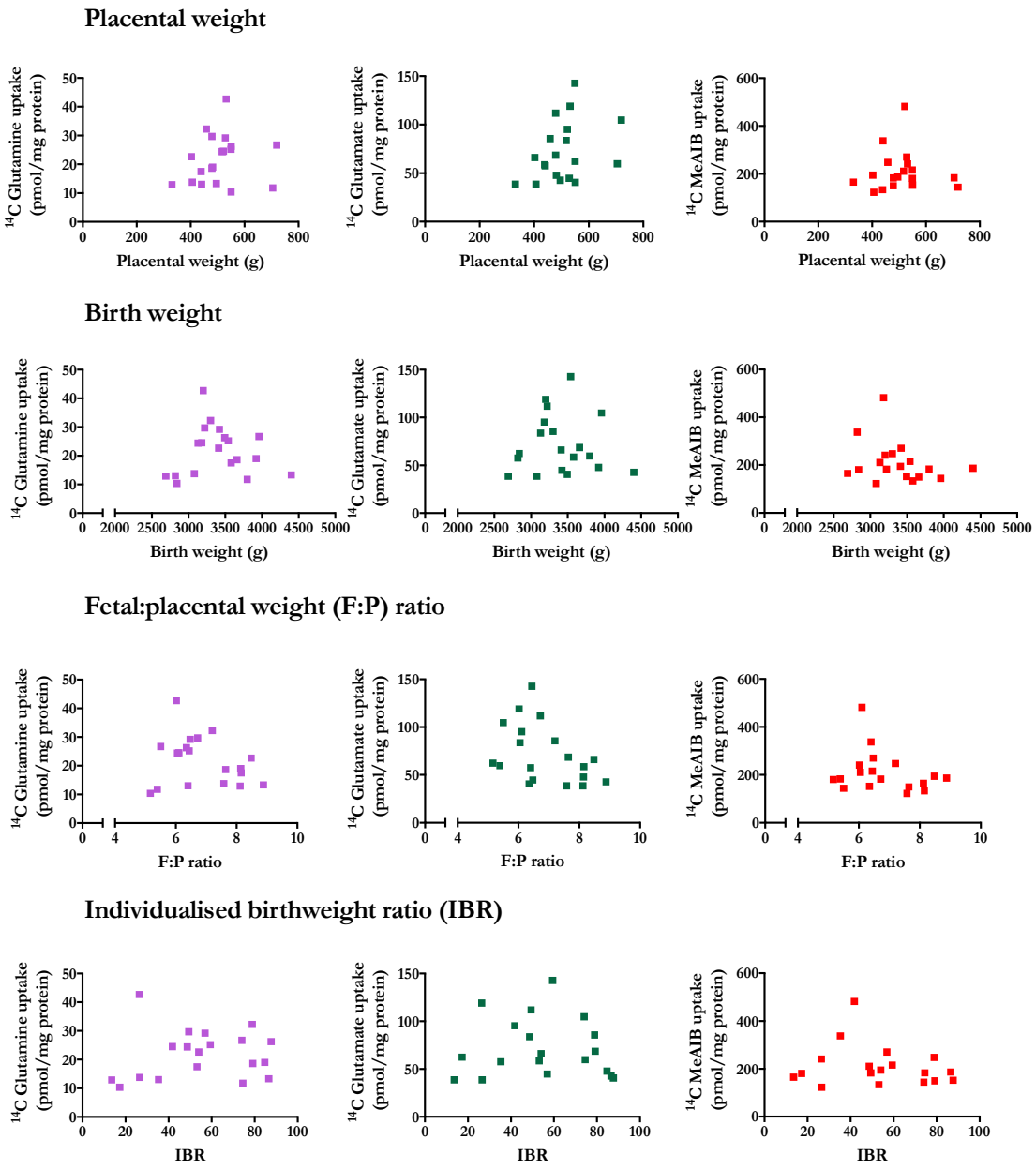


Figure 34: Relationship between transporter-mediated amino acid uptake and fetal and placental measures

There was no relationship between uptake of ^{14}C-glutamate (green symbols), ^{14}C-glutamine (purple) or ^{14}C-MeAIB (red) at 90 min and trimmed placental weight, birth weight, fetal: placental weight (F:P) ratio or individualised birth weight ratio (IBR) (n=18/19, Linear regression).

4.3.3 Effect of sex on transporter mediated amino acid uptake

Placental uptake of ^{14}C-glutamine and ^{14}C-glutamate was significantly lower in placentas of female versus male infants (P<0.05, Linear regression) (Figure 35). The sex of the infant did not have any effect on system A activity (^{14}C-MeAIB uptake) (Figure 35C). The non-specific, diffusional component of uptake was not affected by fetal sex (data not shown).

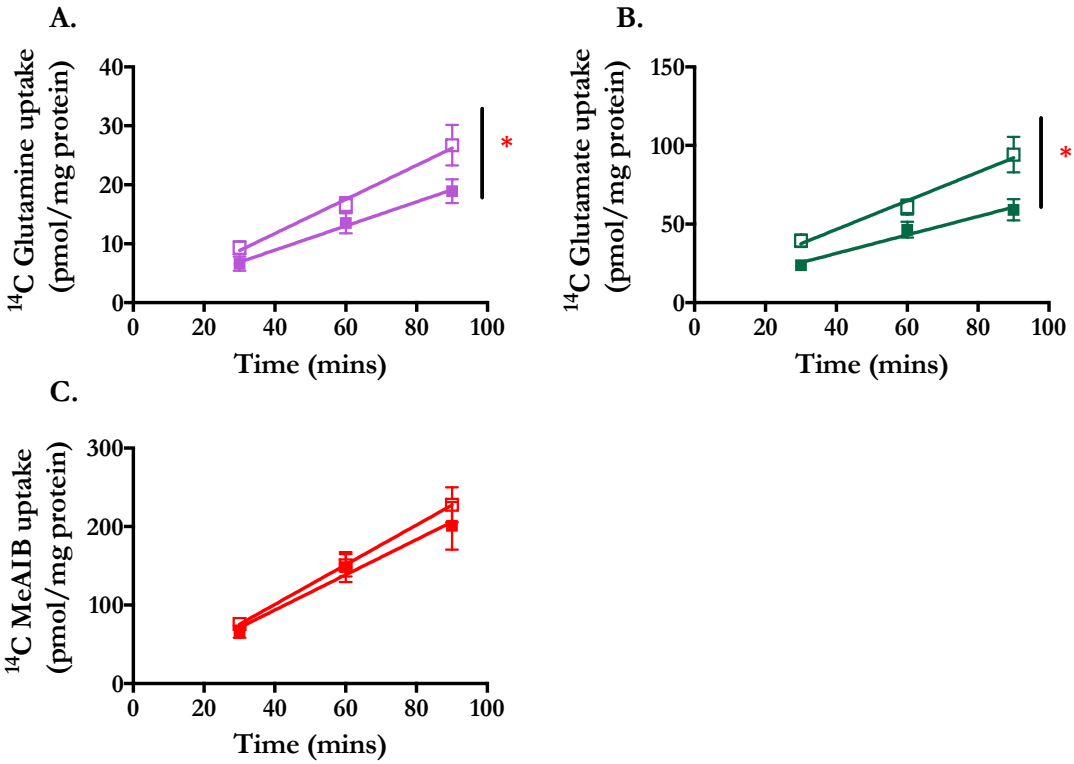


Figure 35: Transporter-mediated amino acid uptake in placentas of male and female infants
Uptake of amino acids by placentas of male (hollow symbols) and female infants (solid symbols) (mean±SEM). Uptake of ^{14}C-glutamine (A) and ^{14}C-glutamate (B) was significantly lower in placentas of female infants compared with those of males (male n=7, female n=11/12; ^{14}C-glutamate and ^{14}C-glutamine $P<0.05$, Linear regression). ^{14}C-MeAIB (C) uptake was unaffected by sex.

4.3.4 Placental expression of amino acid transport proteins is not affected by sex

To determine whether the higher activity of glutamine and glutamate transporters in male compared to female placentas was related to greater transporter protein abundance, the expression of glutamine and glutamate transporter proteins in membrane-enriched placental isolates was assessed by Western blot. Representative Western blots of glutamine (system L: LAT1, system N: SNAT5) and glutamate (system X_{AG}^-: EAAT1, EAAT2, EAAT3) transporter proteins are shown in Figure 36. The intensity of bands detected at the expected molecular weight (kDa) was quantified using densitometry. Positive controls (MVM, human placental maternal-facing membrane) were included during antibody optimisation to validate the location of bands. All data were normalised to β-tubulin expression, and corresponding β-tubulin blots are shown beneath each protein of interest (Figure 36).

LAT1 expression was detected at the predicted molecular weight of 40 kDa. A 75 kDa band was also present under reducing conditions as has been described by others previously (Ellinger *et al.*, 2016). Expression of LAT2 (at the predicted molecular weight of 49 kDa as previously shown by Ellinger *et al.*, 2016; Segawa *et al.*, 1999) was very faint (see Appendix 9.2). This meant that it was not possible to quantify expression/detect any differences

between groups (male versus female). When probing for SNAT5, two bands were detected. During antibody optimisation a clear band at the predicted molecular weight of 52 kDa was found in both positive controls (human MVM isolate and mouse maternal brain whole homogenate), subsequently this band was used for analysis. The identity of the band at 40 kDa, whilst acknowledged by the manufacturer, is unknown.

The predicted molecular weight of EAAT1 is 60 kDa, although previously published data have described multiple bands at 50 kDa and 150 kDa (Martinez-Lozada *et al.*, 2014). During optimisation, MVM EAAT1 expression was localised at 35 kDa and this band was validated by using a EAAT1 blocking peptide (see Figure 28) and subsequently used for analysis by densitometry. EAAT2 expression was present at the predicted molecular weight of 62 kDa. The predicted molecular weight of EAAT3 is 57 kDa. The EAAT3 antibody detects a single band of approximately 70 kDa, according to the manufacturer's datasheet and as shown in Figure 36. There were no differences according to sex in the expression of any of the proteins of interest (Figure 37). System A transporter proteins (of which MeAIB and glutamine are substrates) were not evaluated due to a lack of commercially available antibodies which reliably produce bands at the predicted sizes.

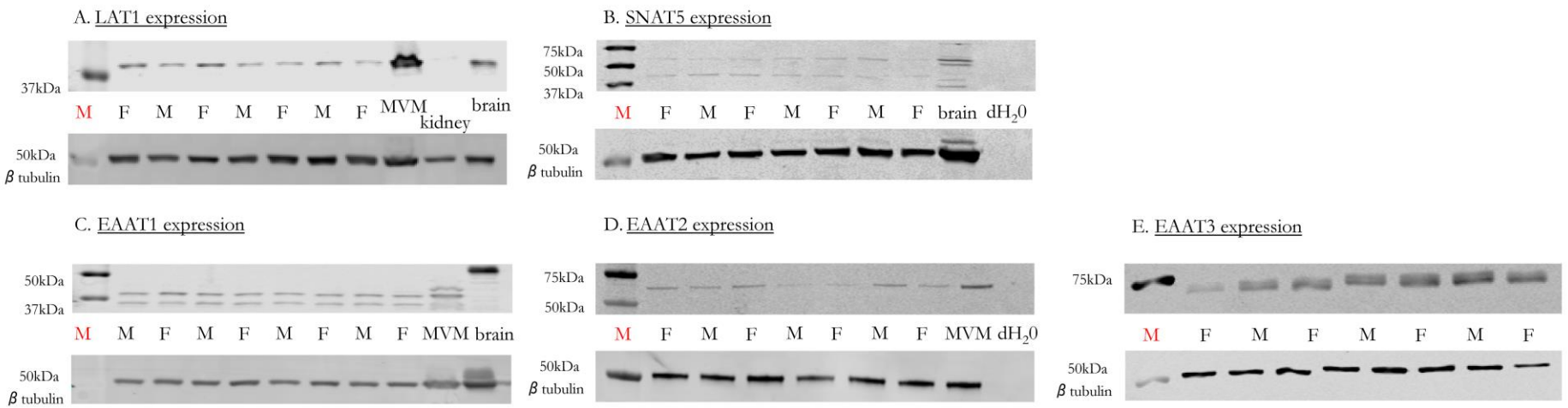


Figure 36: Representative Western blots showing glutamine and glutamate transporter protein expression in membrane-enriched placental samples of male and female infants
Shown are representative Western blots of glutamine (system L: LAT1, system N: SNAT5) and glutamate (system X_{AG}-: EAAT1, EAAT2, EAAT3) transporter proteins in placentas of male and female infants (top blots). Corresponding Western blots for the housekeeping protein β-tubulin are shown below each blot. M= marker, M= male, F= female, brain= mouse maternal brain, kidney= mouse maternal kidney, MVM= human placental microvillous membrane, dH_20= deionised water.

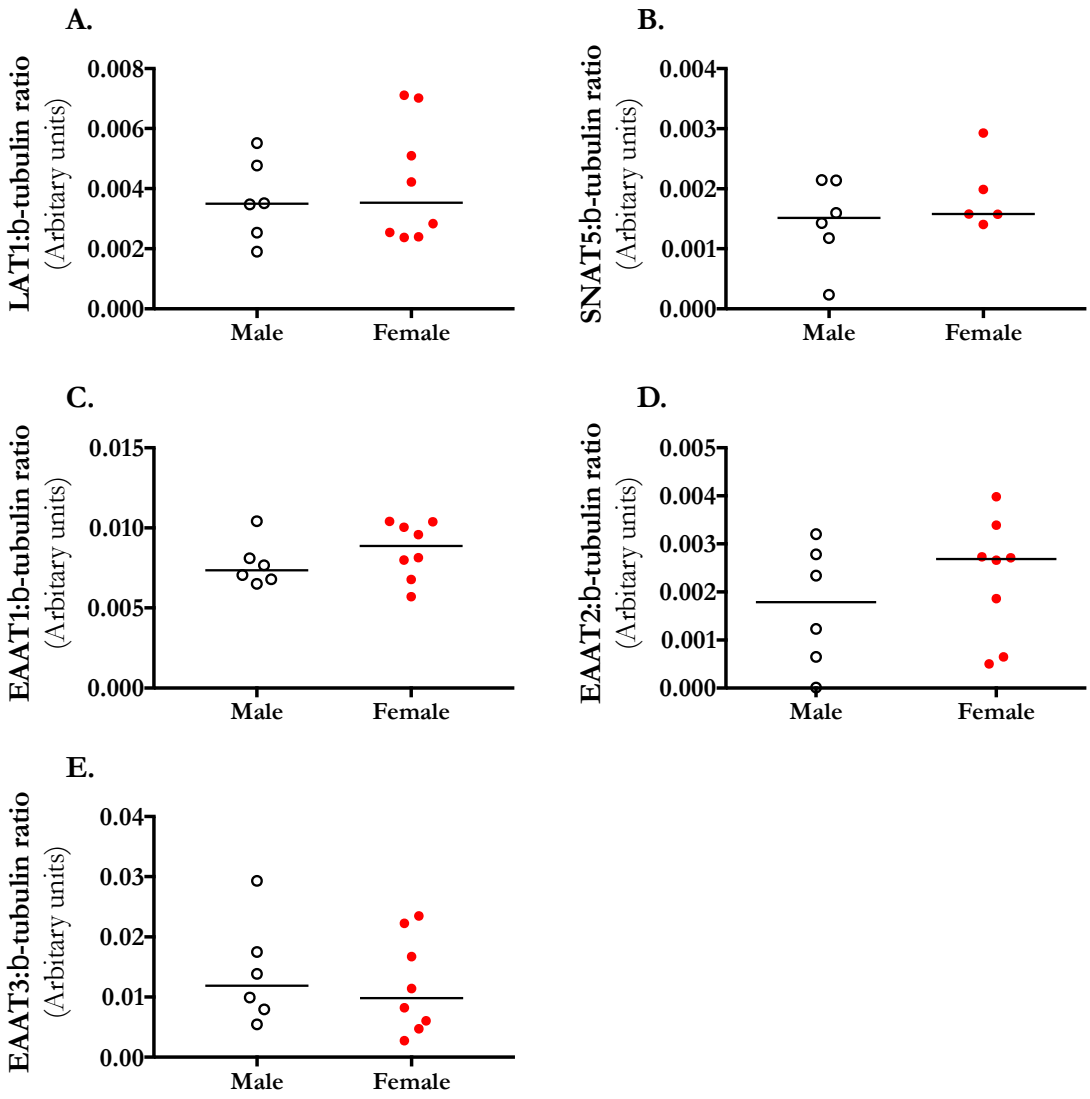


Figure 37: Effect of infant sex on expression of glutamine and glutamate transporter proteins in membrane-enriched placental samples

Expression of all transporter proteins studied (normalised to β-tubulin) was not different in placentas from male and female infants (Mann-Whitney test). LAT1 (D) and SNAT5 (E) are glutamine transporter proteins (system L and N, respectively); EAAT1, EAAT2, EAAT3 (A, B, C) are glutamate transporter proteins (system X_{AG}). LAT2 expression was insufficient to analyse (see Appendix 9.2). Male n=6; Female n=5 (SNAT5)/8 (line represents median).

4.4 Discussion

The villous fragment method has previously been used to assess system A and taurine transporter (TauT) activity in placental syncytiotrophoblast (e.g. Brand *et al.*, 2010; Greenwood and Sibley, 2006; Shibata *et al.*, 2008). Here the method has been successfully adapted to measure the activity of amino acid transporter systems known to contribute to glutamine (systems A, L, N) and glutamate (system X_{AG}-) uptake at initial rate. Known characteristics of glutamine and glutamate transporters were exploited (e.g. Na^+-dependency and substrate specificity) to determine the transporter-mediated component of uptake.

Contrary to the hypothesis, neither glutamine/glutamate transporter activity, nor system A activity (uptake of ^{14}C-MeAIB) (per mg placental protein) was related to placental size (weight) in normal pregnancies resulting in the birth of an infant between the 10th-90th centile IBR. In support of the hypothesis, glutamine and glutamate transporter activity was significantly higher in placentas of male than female fetuses but there was no difference in system A activity. Protein expression of the glutamine transporters LAT1 and SNAT5, and glutamate transporters EAAT1, EAAT2 and EAAT3 in membrane-enriched fractions was not different in placentas of male versus female fetuses.

4.4.1 Measurement of glutamine and glutamate uptake into placental villous fragments at initial rate

A method to measure glutamine and glutamate uptake into human placental villous fragments at initial rate was developed for the purpose of the current study. Incubation of placental villous tissue in solutions containing radiolabelled amino acids/analogues to assess amino acid transporter activity has been used previously by this group and others (Brand *et al.*, 2010; Ditchfield *et al.*, 2015; Hayward *et al.*, 2012; Jansson *et al.*, 2003; Shibata *et al.*, 2008; Warrander *et al.*, 2012). The villous tissue fragment method necessitates distinguishing between non-specific diffusional uptake of the radiolabelled amino acid and the specific carrier-mediated component of total uptake. MeAIB is a specific, non-metabolisable substrate of system A, and system A is Na^+-dependent. Therefore, the specific component of system A-mediated uptake may be determined as the difference between uptake in the presence (total) and absence (diffusion) of Na^+. Glutamine is a substrate of system A, L, N, y+L and ASC (see Introduction; Table 4), and to distinguish between carrier-mediated and non-specific diffusion the contribution of all these components needs to be assessed. System ASC is expressed predominantly on the syncytiotrophoblast BM (Regnault *et al.*, 2002), and 10 mM arginine (a substrate of system y+L) does not alter glutamine uptake into MVM vesicles (Hill *et al.*, 2014). Thus, these two transport systems were not considered in the current experiments. Serine, BCH (2-Amino-2-norbornanecarboxylic acid) and histidine are substrates of Systems A, L and N, respectively. Glutamine uptake was therefore assessed in Na^+-containing and Na^+-deplete buffer containing 5 mM histidine, serine and BCH, with transporter-specific uptake calculated as the difference between the two conditions. An estimate of system L contribution to glutamine transporter-mediated uptake can be made from the pilot data (Figure 31). System L is Na^+-independent, therefore uptake in Na^+-free conditions likely represents system L-mediated glutamine uptake plus non-specific uptake (which constitutes ~15% of total uptake). From these data, system L contributes to approximately 15% of glutamine uptake (~20% of transporter-mediated uptake). Together, the Na^+-dependent systems A and N contribute

~65% of total glutamine uptake, which equates to ~80% of transporter-mediated uptake. However, it is not possible to distinguish the relative contribution of systems A and N from these pilot data.

Glutamate transport across the maternal-facing MVM is mediated by the Na^+-dependent system X_{AG-}. Uptake of radiolabelled glutamate in Na^+-free buffer with high concentrations (5 mM) of system X_{AG-} substrate aspartate (Hill *et al.*, 2014) was used as a measure of non-specific diffusion. During method validation it was noted that removal of Na^+ alone inhibited glutamate uptake by ~95%, and aspartate did not have an additive inhibitory effect on glutamate uptake in Na^+-free conditions confirming the presence of one transporter system for glutamate (Figure 31C).

Membrane-mediated transport of both glutamine and glutamate was linear up to 120 min, and extrapolation of the regression line of uptake (per mg protein versus time) intercepted the x,y axis close to the origin indicating that the carrier mediated uptake was at initial rate. For subsequent experiments ^{14}C-glutamine and ^{14}C-glutamate uptake was measured at 30, 60 and 90 min. MeAIB uptake, representative of system A activity was also included to confirm and extend previous findings. System A activity was in line with previous observations using the same radioisotope concentration (n=25; 30-120 min uptake; Ditchfield, 2011).

4.4.2 The activity of system A, glutamine and glutamate transporters is not related to placental weight or birth weight

4.4.2.1 Placental weight

The current study tested the hypothesis that transporter-mediated uptake of glutamine, a system A substrate, and glutamate is related to placental size. However, contrary to this hypothesis, there was no association between placental weight and glutamine or glutamate uptake (per mg protein). Furthermore, there was no relationship between MeAIB uptake and placental weight. Researchers have previously reported that system A activity (^{14}C-MeAIB uptake into syncytiotrophoblast MVM vesicles, per mg protein) is higher in the placentas of the smallest infants, within a normal birth weight range (mean birth weight of 3395 g) (Godfrey *et al.*, 1998). The current study did not replicate these findings and others, which have demonstrated an inverse relationship between system A activity and placental weight in women and mice (Coan *et al.*, 2008; Godfrey *et al.*, 1998) or placental ratio (placental weight:birth weight (PW:BW) ratio in a study by Harrington *et al.*, 1999).

There are several differences worthy of discussion between the current study and the previous studies described above. Godfrey *et al.* (1998) and Harrington *et al.* (1999) both measured system A activity by uptake of ^{14}C-MeAIB into MVM vesicles, whereas the present study assessed placental system A (MeAIB), glutamine and glutamate uptake into placental villous

tissue samples. Isolated MVM vesicles lack the capacity for intracellular signalling, have lost tissue architecture and interactions between different cell types present in whole villous tissue fragments. These elements could influence amino acid transporter activity and could explain, at least in part, the discrepancies between the current and previous studies (see Introduction; Table 5).

The relationship between system A activity, measured using the villous fragment preparation, placental weight and birth weight was recently reviewed by Hayward *et al.* (2016) using data collated from this laboratory (not including the current study). In contrast to the data of Godfrey *et al.* (1998) on MVM vesicles, a significant positive correlation between system A activity (30 min uptake/mg protein) and trimmed placental weight (range 350-900 g n=60) was observed ($P<0.05$; Spearman correlation). However, Hayward *et al.* (2016) reported that the relationship between system A activity and placental weight was lost when placentas outside the 10^{th}-90^{th} centile of placental weight were excluded (<408 g or >642 g; determined from frequency distributions of trimmed placental weights). In the current study, with fewer numbers (n=18/19), no relationship was observed between system A activity (90 min uptake/mg protein) and trimmed placental weight over a weight range of 331-719 g; 74% of placentas in the current study were within the 10^{th}-90^{th} centiles of placental weights which may explain, at least in part, why no correlation was observed.

The definition of a 'normal' birth weight has evolved since the studies by Godfrey *et al.* (1998) and Harrington *et al.* (1999). Godfrey *et al.* (1998) defined normal pregnancies using absolute birth weight and organised data into weight bins for analysis whilst Harrington *et al.* (1999) assigned birth weight using Gairdner-Pearson population growth charts (Gairdner and Pearson, 1971). Current definitions take into account both maternal and fetal parameters (maternal height, weight, parity, ethnicity, fetal weight, sex and gestational age) to determine an individualised birth weight ratio (IBR, defined as a centile on an individualised chart). We chose to investigate the relationship between transporter activity and placental weight in normal pregnancy, using the definition of 'normal' or AGA as a pregnancy resulting in delivery of an infant with an IBR between 10^{th} and 90^{th} centiles. Instead of organising data into weight bins for comparison (as has been done previously, Godfrey *et al.*, 1998), the data in this study were evaluated using correlation analyses. The recent recognition that infants born at the extremes of birth weight centiles (i.e. ~10^{th} or ~90^{th} centile) are more at risk of adverse outcomes (Iliodromiti *et al.*, 2017), has led to the proposal of a narrower definition of AGA (e.g. 25^{th}-85^{th} centile IBR) in the hope that this will increase the probability of exclusively including 'AGA' infants into study cohorts. There is no relationship between system A activity (MeAIB uptake), glutamine or glutamate uptake and placental weight if the 25^{th}-85^{th} centiles are used to analyse the current data set (excludes n=4).

MeAIB was used to assess system A activity as a positive control and to compare directly to previous studies. The main focus of the study was to assess the relationship between the activity of glutamine (system A, L and N) and glutamate (system X_{AG}^-) transporters and placental weight. In a cohort of placentas collected from normal birth weight (AGA) infants, there was no relationship between placental weight and glutamine or glutamate transporter activity. This indicates that in a population of normal birth weight infants, placental size (weight) is unrelated to the activity of the amino acid transporters measured. The data presented here suggest that transporter activity does not adapt according to placental size per se, rather that other mechanisms may influence amino acid uptake. The contribution of other components, such as placental metabolism, to the ultimate allocation of amino acids to the growing fetus remains to be investigated.

4.4.2.2 Birth weight and fetal:placental weight ratio

Data were also assessed to determine whether birth weight or fetal:placental weight ratio (F:P ratio; frequently referred to as a proxy of placental efficiency) correlated with placental uptake of any of the amino acids measured (glutamine, glutamate or MeAIB). No association existed between birth weight or F:P ratio and amino acid uptake in the current cohort. These findings replicate those drawn by Hayward *et al.* (2016), who also found no correlation between birth weight or BW:PW ratio (F:P ratio) and system A activity at 30 min. Taken together, these data suggest that fetal weight does not correlate with placental uptake of amino acid uptake *in vitro*, indicating that other factors must contribute to fetal weight gain. It is important to note that whilst placental uptake can give an indication of specific transporter activity on the syncytiotrophoblast MVM and thereby infer the availability of amino acids for transfer to the fetus, placental uptake does not represent maternofetal transfer.

F:P ratio is defined as the g of fetus produced per g placenta, and is therefore often referred to as a proxy of placental efficiency insomuch as a higher F:P ratio would indicate that relatively more fetal weight was supported per g placental weight. It is therefore assumed that an elevated F:P ratio is due to higher rates of nutrient transfer (per g placenta), and that the two would therefore be correlated. However, a recently published paper (Christians *et al.*, 2018) has questioned the utility of the F:P ratio. Mathematically the F:P ratio presents a problem since the regression line does not intercept at zero. The implication of this is that as placental weight increases, the F:P ratio will decrease for infants with the expected weight for their placental size. The data presented in this chapter indicate that there is no relationship between glutamine and glutamate uptake and F:P ratio.

Birth weight and placental weight were correlated in this cohort of normal pregnancies resulting in a normal birth weight (AGA) infant ($P<0.05$) (Figure 32). This finding is in

alignment with a recent review that reported a positive correlation between trimmed placental weight and system A activity (Hayward *et al.*, 2016) and suggests that placental weight may be a key determinant of fetal size at birth. The strength of this correlation would likely increase with more numbers available for analysis in the current study (n=19 versus n=210; Hayward *et al.*, 2016).

4.4.3 Adaptation of glutamine and glutamate transport in the mouse compared to human placenta

There is evidence in the mouse (Coan *et al.*, 2008; Dilworth *et al.*, 2010; Hayward *et al.*, 2017) that fetal nutrient requirements are met by functional and morphological adaptations of the placenta in relation to placental size. In the previous chapter, unidirectional maternofetal clearance (K_{mf}) of glutamine and glutamate was measured and compared between the lightest and heaviest placentas from a single WT mouse litter. K_{mf} of both glutamine and glutamate was significantly higher in the lightest versus heaviest placenta within a litter at E18.5 (the day before term in mouse pregnancy). These data suggest that the lightest placentas in a litter transport more glutamine or glutamate (per g placenta) compared with the heaviest placentas (i.e. they are more efficient), and also corroborates previous findings of elevated system A activity (a transporter system important for glutamine transport) at E18.5 in the smallest placentas (Coan *et al.*, 2008). Similarly, system A activity in human placentas is highest in the lightest placentas and inversely correlated with fetal abdominal circumference at birth (Godfrey et al., 1998).

The data in the WT mouse (Chapter 3) were not replicated in human placenta; there was no relationship between the transporter-mediated uptake of glutamine, glutamate or MeAIB and placental weight. However there are important methodological distinctions between the two studies: transplacental flux (K_{mf}) was assessed in WT mice which includes transfer across the fetal-facing basal membrane, layer III of the labyrinth zone, thought to be analogous to the human BM (Dilworth and Sibley, 2013) whereas in this chapter transporter-mediated uptake across the MVM only was quantified. An additional consideration in the mouse studies is the potential metabolism of glutamine or glutamate within the placenta prior to their release into the fetal circulation. However, given the short experimental time period (~2 min) this is unlikely to contribute significantly. These differences between K_{mf} measured in WT mice, and transporter-mediated uptake measured in human placentas may explain the disparity between species in terms of relationship between K_{mf} and uptake, and fetal and placental measures.

Mice also have a more efficient placenta, as illustrated by the higher F:P ratio compared with humans (in the current study median BW:PW ratio for normal birth weight (AGA) infants was 6.5; median F:P ratio at E18.5 in WT mice was 17.0 for lightest placentas and 13.7 for the

heaviest), and therefore may be more able to adapt to meet fetal nutrient requirements. Furthermore, it is not possible to measure uptake in human placentas from normal pregnancies at multiple gestational ages i.e. in the second trimester, therefore data obtained from human placentas at term represent a snapshot of placental function and no direct comparison can be made between data at E15.5 in mouse pregnancy. In summary it appears that, at least in normal pregnancy, the small mouse placenta adapts to meet fetal nutrient requirements (i.e. transports more glutamine/glutamate per g placental weight). This observation was not replicated in human placentas; no relationship exists between glutamine, glutamate or MeAIB uptake and placental weight in normal pregnancy.

4.4.4 Sex specific differences in activity, but not expression, of placental glutamine and glutamate transporters

Emerging data indicate that males are more susceptible than females to adverse conditions *in utero* (Clifton, 2010; Di Renzo *et al.*, 2007) and therefore more likely to die during the neonatal period. In a low birth weight cohort examined by Stevenson *et al.* (2000), mortality for males was 22% compared with 15% for females. Despite being derived from trophectoderm cells that ultimately form the fetal part of the mature placenta, the placenta is often treated as an asexual organ. However, males are larger at birth (both when conceived naturally and by assisted reproductive technology) (Clarke, 1788; Li *et al.*, 2014), and have larger placentas and BW:PW ratio (Wallace *et al.*, 2013) which suggests that male placentas are more efficient.

In line with the proposal that male placentas are more efficient, we hypothesised that transporter activity (per mg placental protein) would be higher in male than female placentas. Activity of transporters specific for both glutamine and glutamate was significantly higher in placentas from male versus female fetuses ($P<0.05$). However, there was no effect of sex on MeAIB uptake. This suggests that the transporters responsible for the sex-specific differences in glutamine uptake (higher in male placentas versus female, per mg protein) are systems L and/or N rather than system A (for which MeAIB is a specific analogue). Data from method development (Figure 31) indicate that system L contributes to approximately 20% of transporter-mediated glutamine uptake. However, it is not possible to determine whether these sex-specific differences are attributable to system L, N or both from the current data. The transfer and metabolism of glutamine and glutamate are intrinsically linked which could underlie, at least in part, why uptake of glutamine and glutamate, but not MeAIB, are influenced by sex. The relationship between uptake of glutamine and glutamate, and uptake of glutamine and MeAIB (system A substrates), is presented and discussed in Chapter 6. Contrary to previous studies, here we observed no difference in placental weight or birth weight between males and females (Clarke, 1788; Li *et al.*, 2014; Wallace *et al.*, 2013), likely due

to the small numbers available for this analysis. Much larger studies are required to relate transport to placental and fetal weight in males and females to assess the significance of these observations. However, the relationships between transporter activity in males and females with placental and fetal weight have been explored in the current study.

Transporter activity (i.e. transporter-mediated glutamine, glutamate or MeAIB uptake) and fetal or placental measures (birth weight, placental weight, F:P ratio or IBR) are not correlated in pregnancies resulting in a normal birth weight (AGA) infant (Figure 34). When stratified according to sex however, MeAIB uptake by male placentas at 90 min was negatively correlated with birth weight in normal pregnancies ($P<0.01$; Linear regression) (Figure 38). There were no other significant relationships within sex between transporter activity at 90 min and birth weight, placental weight, F:P ratio or IBR. Thus these data indicate a negative relationship between MeAIB uptake and birth weight, but not placental weight ($P=0.14$). These preliminary findings (n=7 males) require confirmation with a larger data set in the future.

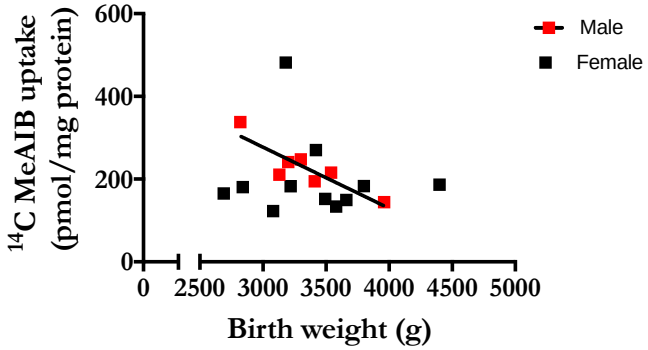


Figure 38: Birth weight versus MeAIB uptake at 90min according to sex

System A activity (transporter-mediated uptake of MeAIB) by male placentas is inversely correlated with birth weight (n=7; $P<0.01$; Linear regression). There was no relationship between birth weight and uptake of MeAIB by female placentas (n=11). There were no other significant relationships between amino acid uptake (glutamine, glutamate or MeAIB) and other fetal or placental parameters (birth weight, placental weight, birth weight:placental weight ratio or IBR) according to sex (data not shown).

In the previous chapter of this thesis, the effect of sex on transporter expression and activity was assessed in WT mice. Female fetuses and placentas weighed significantly less at E18.5 yet the sex of the fetus did not significantly influence unidirectional maternofetal clearance (K_{mf}) of glutamine or glutamate across the WT placenta and therefore sex cannot fully account for evidence of adaptation in terms of placental nutrient transport in the mouse. These data are at odds with that seen in the human placenta in this chapter, which suggests that the mouse adopts different strategies to the human to achieve appropriate fetal growth.

4.4.5 Placental glutamine and glutamate transporter protein expression according to sex of the fetus

Higher activity of glutamine and glutamate transporters in male placentas was not associated with higher expression of transporter proteins that contribute to glutamine and glutamate uptake (LAT1: system L; SNAT5: system N; EAAT1, EAAT2, EAAT3: system X_{AG-}). Expression of the system L transporter protein LAT2 was also evaluated (see Appendix 9.2); however, expression was insufficient to enable semi-quantitative analysis by densitometry. The absence of commercially available antibodies for system A transporter proteins (SNAT1, SNAT2 and SNAT4) which reliably produce bands at the predicted sizes made it impossible to quantify expression of these isoforms. However, it would be predicted that since MeAIB uptake was similar between groups (male versus female), system A transporters do not contribute to the higher glutamine uptake in male placentas.

A limitation of the study is that membrane-enriched placental preparations were used for Western blot analyses rather than pure MVM isolates due to a lack of available banked tissue owing to use in uptake experiments and other usage within the laboratory. Uptake across the maternal-facing MVM of the placenta was assessed in the uptake experiments and therefore transporter expression in this membrane alone would contribute to amino acid uptake. It is therefore possible that the lack of difference in transporter protein expression in males and females are influenced by the presence of transporters on other placental membranes e.g. BM.

There was no relationship between expression and activity of glutamine and glutamate transporters in this study (Appendix 9.3). A possible explanation for this result is that the transporters have been modified post-translationally, altering activity, or that transporters quantified during Western blot analyses were localised to a different membrane (i.e. not the MVM) as described above.

Post-translational modifications of transporters such as transporter ubiquitination (Rosario *et al.*, 2016) or trafficking of transporters to the plasma membrane (Roos *et al.*, 2009) can alter transporter activity. The mechanistic target of rapamycin (mTOR) pathway is a key regulator of system A and L in the placenta (Jansson *et al.*, 2012), therefore it is reasonable to suggest that mTOR could play a role in the regulation of glutamine uptake (a substrate of system A and L). In FGR mTOR activity is lower, and decreased mTOR activity is associated with a reduction in system L activity (Roos *et al.*, 2007). Reduced mTOR signalling leads to NEDD4-2 activation and subsequent ubiquitination of system L transporter LAT1 for degradation by the proteasome (Chen *et al.*, 2015), which reduces its expression in the MVM. It is not known if mTOR regulates glutamate transporters or if mTOR regulation of systems A and L is different in males and females.

4.4.6 Summary

A method for quantifying glutamine and glutamate transporter activity in placental villous fragments at initial rate has been optimised. Using this method, postnatal bleeding of the placenta by AN was found to be associated with lower glutamine transporter activity. Further work is required to establish the mechanisms underlying these observed differences. Glutamine and glutamate transporter activity, and the activity of system A as assessed by the uptake of MeAIB, was not related to placental weight in normal pregnancy. These data indicate that other mechanisms, aside from placental size, may govern amino acid provision to the fetus. Placental glutamine and glutamate transporter activity was greater in males than females which was not associated with changes in transporter expression in membrane-enriched placental homogenates, indicating a potential role of post-translational modifications. A key strength of this work is that both activity and expression were measured in the same placentas. Placental glutamine and glutamate transporter activity and expression in the placentas of normally grown (AGA) and growth restricted (FGR) fetuses is explored in Chapter 6.

Chapter 5 Glutamine and glutamate transfer (clearance) across the *Igf2P0* placenta: a mouse model of fetal growth restriction

5.1 Introduction

Normal fetal growth depends upon the capacity of the placenta to provide adequate nutrition to the developing fetus. Diminished nutrient transfer can be a contributor and not merely a consequence of growth restriction as demonstrated by studies in pregnant rats fed a low protein diet (Jansson *et al.*, 2006) or infused with MeAIB (Cramer *et al.*, 2002). Compared with isocaloric controls, pregnant rats fed a low protein diet have diminished placental system A transport activity at embryonic day (E)19 prior to evident growth restriction at E21 (term E23) (Jansson *et al.*, 2006). Infusion of MeAIB (E7-20) to block system A results in decreased placental amino acid transporter activity including system A and X_{AG-} which precedes a reduction in fetal growth by term (Cramer *et al.*, 2002).

Fetal growth restriction (FGR), characterised by suboptimal fetal growth, can be caused by congenital abnormalities, be associated with complications of pregnancy which include pre-eclampsia, diabetes or maternal obesity, or be idiopathic (Royal College of Obstetricians and Gynaecologists, 2013). Although placental dysfunction is a major cause of FGR (Mifsud and Sebire, 2014), the aetiology is not fully understood. The FGR placenta is smaller than normal for gestational age (Mifsud and Sebire, 2014) and may present with abnormalities in morphology, syncytiotrophoblast function, and/or utero- and fetoplacental blood flow. There are many factors thought to contribute to the placental dysfunction underpinning FGR including hypoxia, oxidative/nitrative stress and elevated inflammation. Each of these conditions could result in alterations in nutrient transporter abundance, localisation and/or activity, and thereby contribute to abnormal syncytiotrophoblast function (Baker *et al.*, 2018; Heyborne *et al.*, 1994; Myatt, 2006; Nadeau-Vallée *et al.*, 2016).

Understanding of how nutrient transport is altered in FGR is incomplete and is hindered by poor knowledge of the physiological processes in normal pregnancy. The hypothesis that nutrient transport adapts according to placental size in normal mouse pregnancy was addressed in Chapter 3. In pregnant WT C57BL/6J mice, a small placenta of a normally grown fetus was shown to up-regulate the maternofetal transport of glutamine and glutamate, amino acids that are essential for fetal growth and metabolism (Pochini *et al.*, 2014). The unidirectional maternofetal clearance (K_{mf}) of glutamine and glutamate was higher in the lightest versus heaviest placentas towards term (E18.5). This adaptation of nutrient transporters ensures that the fetuses with the lightest placentas are born within a normal birth

weight range. However, in mouse models of FGR, the small dysfunctional placenta fails to express the adaptive response evident in normal WT mice towards term, at least in terms of the K_{mf} of MeAIB, a measure of placental transport by system A (Constância *et al.*, 2002; Constância *et al.*, 2005). As glutamine is a substrate of system A, it is likely that the K_{mf} of glutamine is perturbed in FGR; however the K_{mf} of neither glutamine, nor glutamate, has yet to be investigated in a mouse model of FGR.

In mice, *Igf2* expression can be manipulated by targeting one of the many gene promoters for specific transcripts (Figure 39) to generate genetic models of fetal growth disorders. IGF2 is an important hormone for fetal growth. *Igf2* is an imprinted gene and in mice only the paternal allele is expressed (reviewed in detail by Angiolini *et al.*, 2006; Sferruzzi-Perri, 2018). According to Kinship Theory, parental imprinting of genes ensures a balance of maternal resource allocation; simplistically, paternally imprinted genes tend to promote growth whilst maternally imprinted genes tend to repress growth in order to retain resources for the future health of the mother. As a paternally imprinted gene, *Igf2* promotes fetal growth. Humans with Silver-Russell and Beckwith Weidemann syndrome have fetal under/overgrowth due to the under- or over-expression of *Igf2*, respectively (Burton and Fowden, 2012).

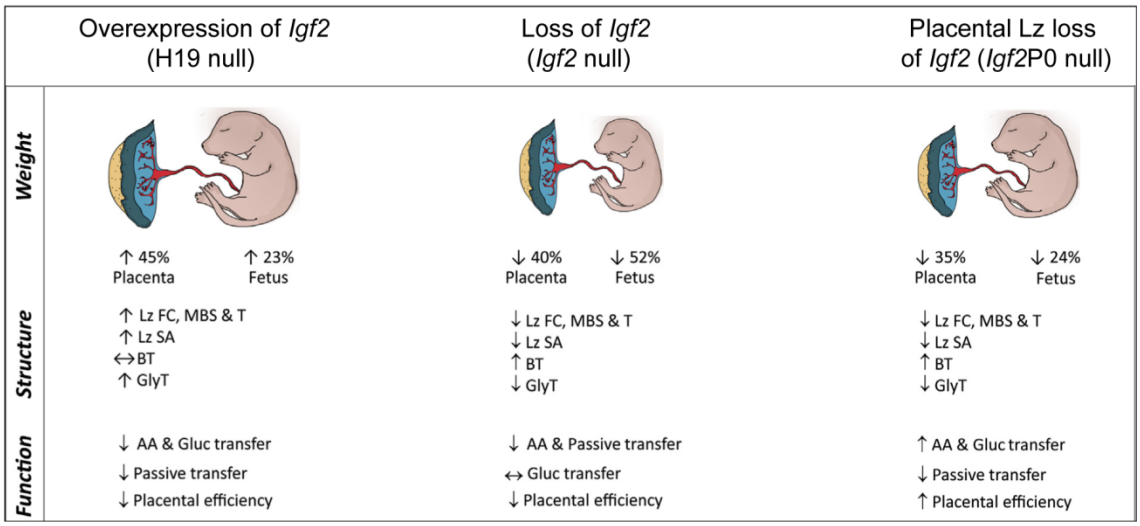


Figure 39 *Igf2*-specific knockout mouse models
Manipulation of the *Igf2* gene, and the subsequent effect on fetal/placental development and function. AA, amino acid; BT, interhaemal membrane barrier thickness (inversely related to the diffusion of oxygen), FC, fetal capillaries; Glu, glucose; GlyT, glycogen cells; Lz, labyrinthine zone; MBS, maternal blood spaces; SA, surface area; T, trophoblast. Table is adapted from Sferruzzi-Perri (2018). Data are from: Angiolini *et al.* (2011); Constância *et al.* (2002); Constância *et al.* (2005); Esquiliano *et al.* (2009); Lopez *et al.* (1996); Sibley *et al.* (2004).

The H19 knockout mouse (Figure 39) is characterised by increased fetal and placental size but a reduced fetal:placental weight (F:P) ratio. H19 is located upstream of *Igf2* and is exclusively expressed from the maternal allele. H19 is responsible for silencing the *Igf2* gene, thus deletion

of 13kb that includes the H19 gene results in increased levels of *Igf2* and fetal overgrowth caused by the doubling of all *Igf2* transcripts. K_{mf} of glucose and MeAIB is reduced in the H19 knockout mouse (versus WT placentas), per g placenta, and is accompanied with reduced gene expression of slc2a3 (GLUT3 glucose transporter) and slc38a4 (SNAT4 system A transporter) (Angiolini *et al.*, 2011). This is thought to be an adaptive response by the placenta to prevent fetal overgrowth.

Igf2-null mice lack both placental and fetal *Igf2*. There is no evidence of adaptations to meet fetal nutrient requirements in the *Igf2*-null mouse. In this model severe growth restriction is observed from E12 onwards and conceptuses are lost before term (Figure 39). By E18.5 *Igf2*-null fetuses weigh 47% of WT and K_{mf} of MeAIB is significantly lower at late-gestation (E18.5) (Constância *et al.*, 2005).

Igf2P0 is a placental-specific *Igf2* transcript. Mating males that are heterozygote for a targeted *Igf2P0* deletion (hereafter referred to as P0) with WT females results in mixed litters of P0 and WT pups (Constância *et al.*, 2005). Importantly, deletion of the P0 transcript does not affect fetal levels of *Igf2* (Constância *et al.*, 2002). In mid-gestation (E15.5-E16.5) K_{mf} of MeAIB (system A activity) and glucose is higher in P0 versus WT littermates (Constância *et al.*, 2002; Constância *et al.*, 2005) and appropriate fetal growth is sustained despite a small placenta. These functional changes (i.e. increased K_{mf}) are matched with increased expression of the system A and glucose isoforms slc38a4 and slc2a3 (Constância *et al.*, 2005). These findings are different to what is reported in *Igf2*-null mice, and indicate that functional adaptation of the *Igf2P0* placenta in mid-gestation involves cross-talk between the placental transcript of *Igf2* and transporter genes (e.g. slc38a4) in an attempt to meet fetal nutrient requirements. However, towards term this adaptation in terms of increased system A and glucose transport ultimately fails, resulting in FGR; P0 fetuses weigh approximately 20% less than WT by E18.5 (Dilworth *et al.*, 2011). Passive permeability is also altered in this model; at E18.5 permeability × surface area product (P×S) is 32% lower for P0 versus WT placentas (Sibley *et al.*, 2004). Thus the P0 mouse is a well-characterised model that expresses a phenotype resembling late onset FGR, having normal uterine and umbilical artery blood flow but abnormal trophoblast structure and function, culminating in reduced nutrient transport to the fetus.

In the current study, P0 mice were used to investigate whether the adaptive up-regulation of glutamine and glutamate transport observed in the small placenta of a normally grown WT fetus (Chapter 3) fails in FGR.

Glutamine is conditionally essential during pregnancy, which evidences its importance for sustaining normal fetal growth (Parimi and Kalhan, 2007). Glutamine is a substrate of system A, the activity of which fails to up-regulate in placentas of growth restricted fetuses towards term in the P0 model of FGR (Constância *et al.*, 2002). However, the K_{mf} of glutamine by the

placenta has not yet been measured in either normally grown, or growth restricted mouse fetuses. In addition, the activity and protein expression of systems L and N, for which glutamine is also a substrate, have not been studied in the mouse placenta. Glutamate provision is also critical for fetal growth and metabolism but glutamate levels must also be tightly controlled since high concentrations are neurotoxic (Pochini *et al.*, 2014; Tian *et al.*, 2012). Glutamine and glutamate transport (influx/efflux across the placental membrane barrier) and metabolism within the placenta and fetal liver are intrinsically linked (Figure 8) and as such these amino acids should be considered together when assessing their provision to the fetus in FGR. The activity (K_{mf}) and expression of the transporter system X_{AG-}, for which glutamate is a substrate, have never been assessed in the placenta of normally grown, or growth restricted mice. The P0 mouse represents a well-characterised model of FGR and allows maternofetal transfer of amino acids to be related to fetal growth at difference time points in gestation.

5.1.1 Hypotheses

- The unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate is higher in placentas of P0 fetuses compared to their WT littermates in mid-gestation, but this adaptation fails nearer term
- Glutamine and glutamate transporter protein expression is higher in P0 versus WT placentas in mid-gestation but similar towards term

5.1.2 Aims

The aims of this chapter were to:

- Compare unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate at mid-gestation (E15.5) and near term (E18.5) in wild type (WT) and growth restricted fetuses in the placental-specific P0 knockout mouse
- Compare the protein expression of key transporters responsible for placental transport of glutamine and glutamate E15.5 and E18.5 in WT and growth restricted fetuses in the P0 mouse.

5.2 Methods

5.2.1 Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate

Male mice that were heterozygote for the P0 knockout were mated with WT females to generate mixed litters of P0 and WT fetuses. Unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine and ^{14}C-glutamate was determined at two timepoints in pregnancy (E15.5 and E18.5) as described in section 2.2. In brief, dams were anaesthetised with a 300 µl

intraperitoneal injection of 1:1:2 combination of fentanyl/fluanisone (Hypnorm, VetPharma Ltd., Leeds, UK), Midazolam (Roche, UK) and sterile H_20 (Braun medical Inc., Pennsylvania, USA) and a bolus of radioisotope (100 µl) was administered via a tail vein cannula. At approximately 2 min post-injection (between 90 sec-4 min ^{14}C-glutamine and 90 sec-3 min for ^{14}C-glutamate), dams were exsanguinated by cardiac puncture and a maternal blood sample obtained. Death was confirmed by cervical dislocation, a laparotomy was performed immediately and maternal blood samples were centrifuged centrifuged at 5000 rpm (1,845 x g) for 5 min to obtain plasma. Fetuses and placentas were trimmed of membranes, blotted and weighed. Fetal tail tips were collected and stored at -20ºC for genotyping (section 2.2.6). Fetuses and placentas were placed in an individual scintillation vial, minced and solubilised in 3 ml 3% KOH at 55ºC overnight. Litters were only included in analyses with a minimum of two P0 and two WT fetuses, as determined by genotyping (section 2.2.6), per litter (seven litters excluded). Data are therefore analysed as the mean of P0 versus WT fetuses within the same litter.

The time at which radioisotope clearance from the maternal circulation was linear was determined by the construction of a ^{14}C maternal plasma disappearance curve. The clearance of radioisotope from maternal blood was comparable between E15.5 and E18.5 for ^{14}C-glutamine and ^{14}C-glutamate and similar to the curve generated for WT mice (Figure 18). Thus, to minimise the number of animals required, ^{14}C disappearance curves from P0 knockout mice and from WT mice were overlaid (Figure 40). Dams used for experiments were culled during the linear portion of the curve (between ~90 sec-3 min for ^{14}C-glutamine and ^{14}C-glutamate).

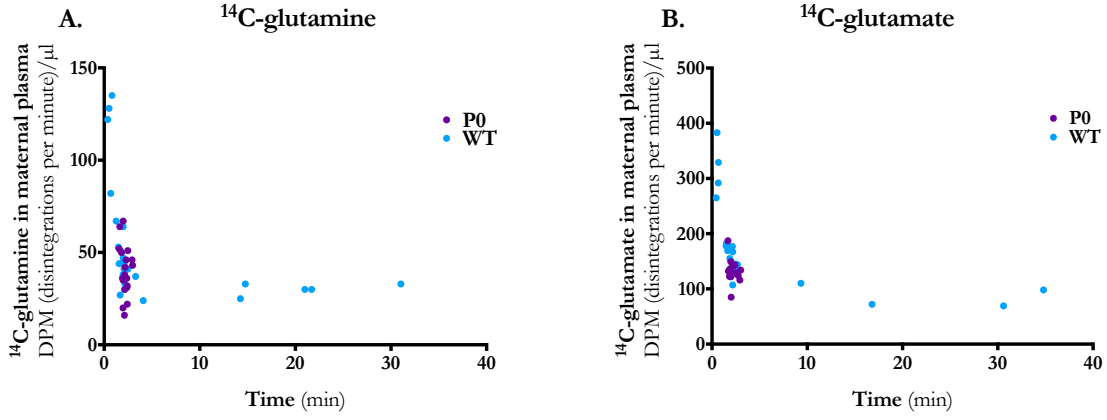


Figure 40 Maternal plasma disappearance curve
Clearance of ^{14}C-glutamine (A) and ^{14}C-glutamate (B) from maternal plasma (disintegrations per min) over time in wild-type (blue; WT data from Chapter 3; Figure 18) and P0 knockout dams (purple; P0). Disappearance was in the linear portion of the curve at approximately 2 min for both WT and P0 mice.

5.2.2 Western blotting

Tissue was harvested from litters that had a minimum of two P0 and two WT fetuses. One P0 and one WT placenta per litter (as determined by genotyping of fetal tail tips, section 2.2.6) were selected at random for use in Western blot analyses. Membrane-enriched whole placental homogenates were extracted and expression of glutamine (LAT1, LAT2, SNAT5) and glutamate (EAAT1, EAAT2) transporter proteins assessed (see Table 12 for Western blot conditions).

5.2.3 Statistical analysis

Unidirectional maternofetal clearance (K_{mf}) data were analysed by comparing the average of WT or P0 placentas or fetuses within a single litter (a minimum of two of each genotype per litter was required). Data were not normally distributed and therefore presented as median [range]. Data were analysed (GraphPad Prism 7 software) by Wilcoxon signed rank test and were considered statistically significant where $P<0.05$.

Semi-quantitative analysis of transporter protein expression as determined by protein band density (densitometry) was performed for Western blot data using Image Studio™ Lite software. Data were analysed (GraphPad Prism 7 software) using a Mann-Whitney test. A *P* value <0.05 was considered statistically significant.

5.2.4 Experimental flowchart

The experimental flowchart overleaf (Figure 41) illustrates the number of animals used for unidirectional maternofetal clearance (K_{mf}) experiments and Western blotting.

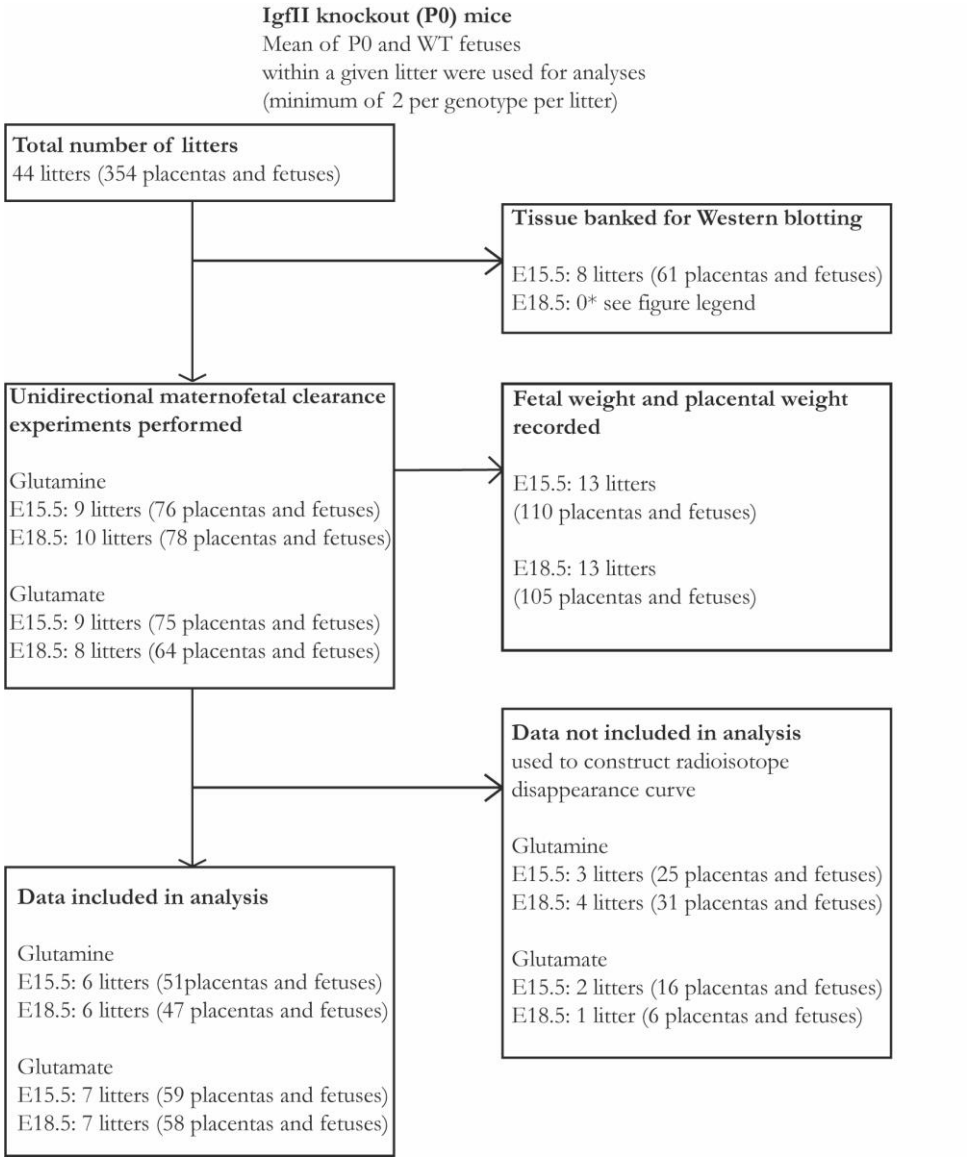


Figure 41 Experimental flowchart of P0 mice used in experiments

From 44 pregnant dams, 8 litters were harvested for Western blotting experiments and a total of 36 litters used for unidirectional maternofetal clearance experiments at E15.5 and E18.5. Fetal weights and placental weights were recorded where possible (E15.5 n=13; E18.5 n=13 litters). Data were excluded from final analyses when there <2 genotype per litter. Unidirectional maternofetal clearance experiments were split between ^{14}C-glutamine (E15.5 n=9; E18.5 n=10) and ^{14}C-glutamate (E15.5 n=9; E18.5 n=8). Experiments used to construct a radioisotope disappearance curve (^{14}C-glutamine n=7; ^{14}C-glutamate n=3) were excluded from data analysis. When found to be no different, the disappearance curve was overlaid with data from WT mice (Figure 18) to reduce the number of animals required. Methods are described in full in section 2.2-2.3. *E18.5 tissue used for Western blots (8 litters) was previously banked tissue by our laboratory. Tissue had already been processed (to generate membrane-enriched whole placental homogenates) and stored at -80ºC. A Biorad protein assay (see section 2.1.5.5) was performed at the same time as E15.5 tissue to determine protein content.

5.3 Results

5.3.1 Placental and fetal measures: P0 versus WT

Average fetal and placental weights of P0 and WT mice were compared directly within a litter (minimum two of each per litter). Data are presented as P0 placentas (litter mean) expressed

as a percentage of the WT placentas (litter mean; standardised to a hypothetical value: 100%). Placentas from P0 fetuses weighed 26% less than WT placentas at both gestational ages (***P<0.001) (Figure 42A). P0 fetuses were significantly lighter at E15.5 (4%; **P<0.01) (Figure 42B) and towards term (a more pronounced difference: 17%; ***P<0.001). P0 fetuses had a higher fetal weight:placental weight (F:P) ratio compared to WT littermates at E15.5 (31%) and E18.5 (12%; ***P<0.001) (Figure 42C). Raw values of placental and fetal measures are quoted (median [range]) in Table 21. At both gestational ages placental and fetal weights were positively correlated for WT and P0 fetuses (Figure 43). Fetal weight distribution curves for E15.5 and E18.5 are shown in Figure 44. The 5th centile of fetal weights is indicated by the dotted line in both instances.

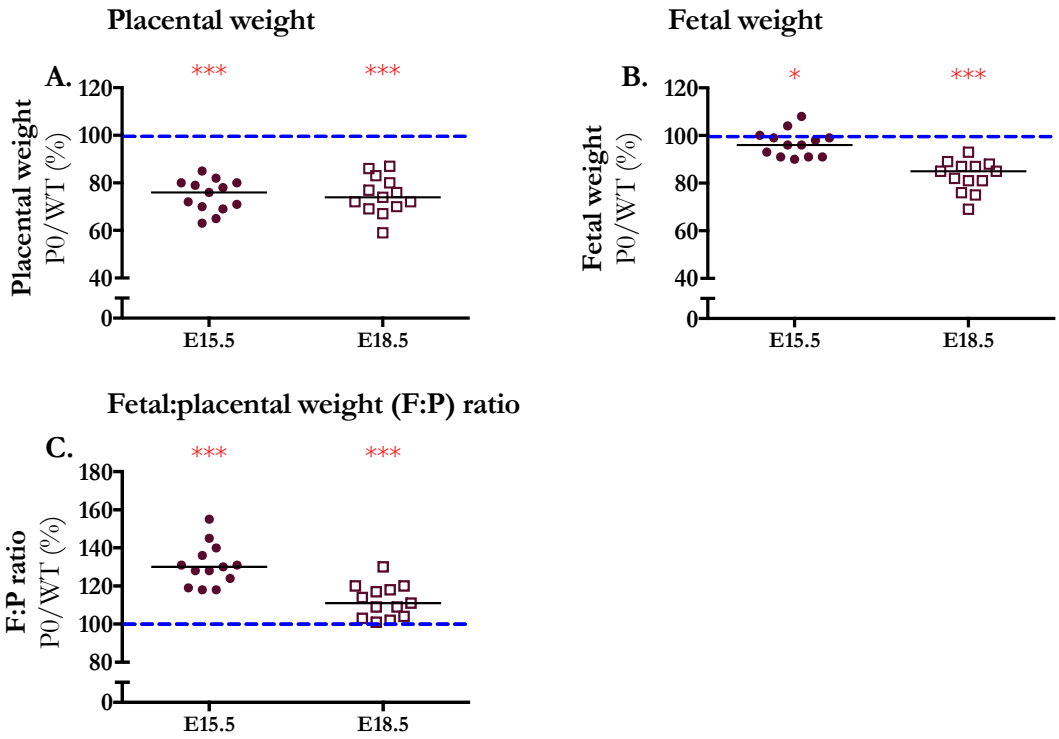


Figure 42 P0 placentas and fetuses weigh less than WT littermates
(A) Placental weights (P0 versus WT) were on average 26% lighter at both gestational ages. (B) P0 fetuses weighed less than their WT littermates at E15.5 (4%) and to a greater extent at E18.5 (17%). (C) F:P ratio was higher for P0 versus WT at both E15.5 and E18.5. E15.5 n=13 E18.5 n=13. Data are litter means with P0 expressed as a percentage of WT (100%) * P<0.05 *** P<0.001 Wilcoxon signed rank test.

	E15.5			E18.5		
	WT E15.5	**P0 E15.5**	***P* value**	**WT E18.5**	**P0 E18.5**	***P* value**
Placental weight (g)	0.104 [0.089-0.112]	0.077 [0.065-0.086]	*** <0.001	0.094 [0.076-0.104]	0.066 [0.057-0.078]	*** <0.001
Fetal weight (g)	0.387 [0.346-0.432]	0.363 [0.351-0.426]	* <0.02	1.220 [1.108-1.381]	1.031 [0.837-1.091]	*** <0.001
F:P ratio	3.9 [3.3-4.6]	4.9 [4.5-6.2]	*** <0.001	13.6 [10.8-15.3]	15.2 [13.4-16.9]	*** <0.001

Table 21 Average placental weight, fetal weight and fetal weight:placental weight (F:P) ratio from P0 and WT placentas in a litter at E15.5 and E18.5
Placental weight, fetal weight and F:P ratio are presented as median [range]. Data analysed by Wilcoxon signed rank test *** $P<0.001$ * $P<0.05$ E15.5 n= 13 E18.5 n= 13.

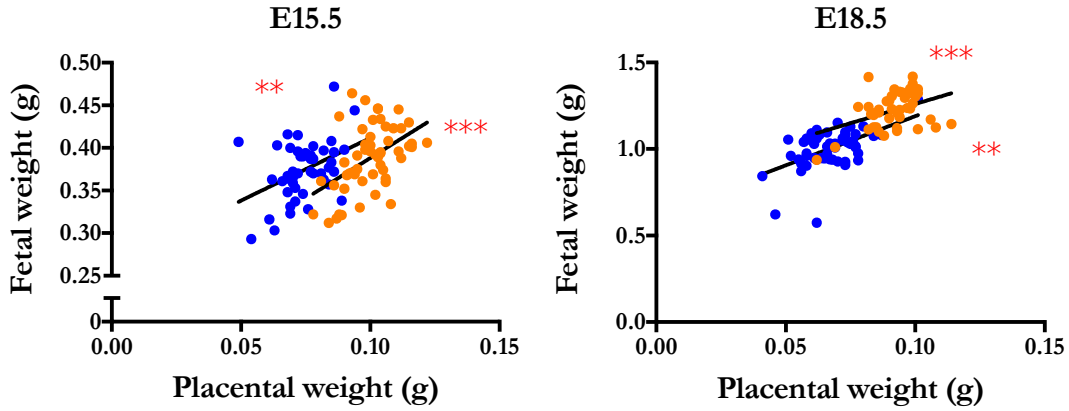


Figure 43 Placental weight versus fetal weight of P0 and WT fetuses at E15.5 and E18.5
For both WT (orange) and P0 (blue) groups, fetal and placental weight are significantly correlated at E15.5 and E18.5 ** $P<0.01$ *** $P<0.001$ Linear regression.

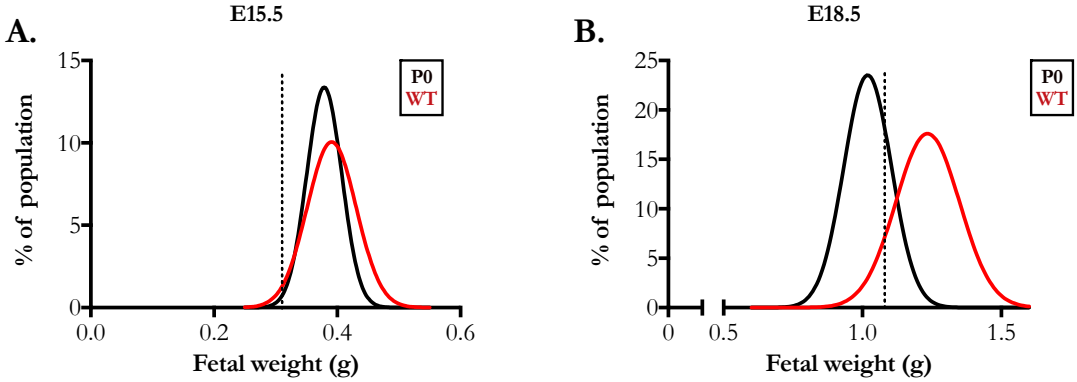


Figure 44 Fetal weight distribution curves for P0 and WT mice
Fetal weight distribution curves for P0 (black) and WT (red) fetuses are shown for E15.5 (A) and E18.5 (B). The dotted line represents the 5th centile of WT fetal weights. 5th centile at E15.5 = 0.325 g and E18.5 = 1.036 g.

5.3.2 Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate

Unidirectional maternofetal clearance (K_{mf}, µl/min/per g placenta) of ^{14}C-glutamine was higher for P0 compared with WT fetuses at E15.5 and E18.5 (*$P<0.05$) (Figure 45A). Per g fetus, maternofetal transfer of ^{14}C-glutamine was also higher across P0 placentas versus WT at E18.5 (*$P<0.05$). There was no difference at E15.5 (Figure 45C). In contrast, total transfer of

^{14}C-glutamine was lower (P0 versus WT) at E18.5 (*P<0.05) but not different at E15.5 (Figure 45E). Unidirectional maternofetal clearance of ^{14}C-glutamate (K_{mf}, µl/min/per g placenta) was higher at E15.5 (*P<0.05) but no different (P0 versus WT) at E18.5 (Figure 45B). There was no difference between groups in either maternofetal transfer of ^{14}C-glutamate (per g fetus) or total ^{14}C-glutamate transfer at either gestational age (Figure 45D, F). Raw values of ^{14}C-glutamine and ^{14}C-glutamate K_{mf} are quoted (median [range]) in Table 22.

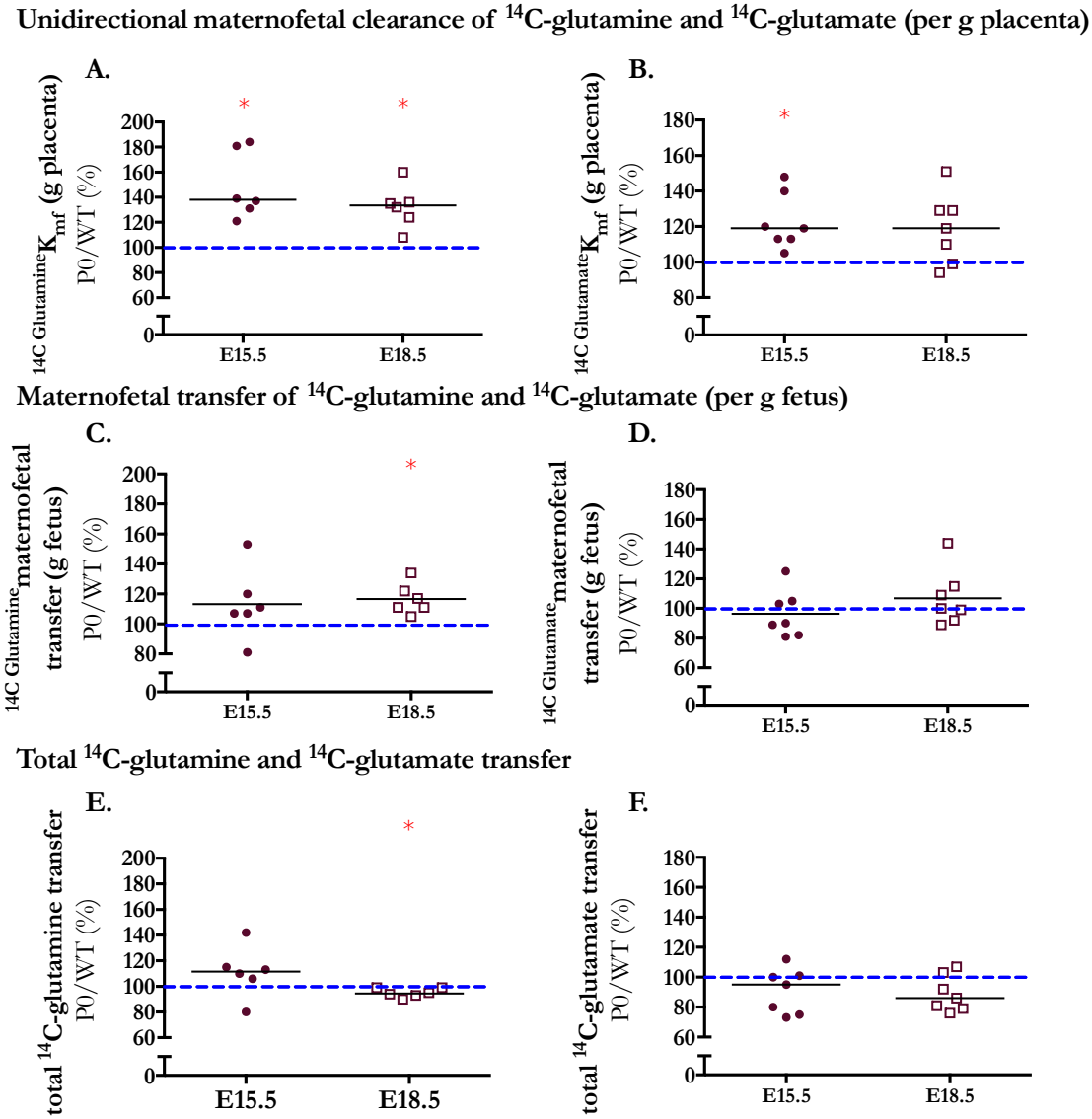


Figure 45 Maternofetal transfer of ^{14}C-glutamine and ^{14}C-glutamate

^{14}C-glutamine unidirectional maternofetal clearance (K_{mf}, per g placenta) was significantly higher across P0 versus WT placentas at E15.5 and E18.5. Maternofetal transfer per g fetus was significantly higher (P0 versus WT) at E18.5. However, total transfer of ^{14}C-glutamine (irrespective of fetal and placenta weight) was significantly lower in P0 compared with WT fetuses at E18.5. ^{14}C-glutamate unidirectional maternofetal clearance (K_{mf}, per g placenta) was higher, P0 versus WT, at E15.5 but similar between groups at E18.5. Maternofetal transfer (per g fetus) and total transfer of ^{14}C-glutamate was similar in P0 and WT at both gestational ages. Glutamine E15.5 n=6 E18.5 n=6, Glutamate E15.5 n=7 E18.5 n=7 * P<0.05 Wilcoxon signed rank test.

Unidirectional maternofetal clearance		E15.5			E18.5		
		WT	**P0**	***P* value**	**WT**	**P0**	***P* value**
Glutamine	**K_{mf} (µl/min/ g placenta)**	306.9 [273.9-376.2]	499.2 [336.9-578.0]	* 0.031	505.9 [240.1-773.6]	748.4 [259.0-1044.0]	* 0.031
	µl/min/g fetus	82.75 [68.2-101.2]	86 [69.2-129.5]	0.312	37.45 [18.1-60.4]	47.1 [19.1-67.2]	* 0.031
	µl/min	31.75 [26.0-39.2]	35.3 [25.0-45.8]	0.312	46.65 [22.4-72.3]	44 [20.1-71.6]	* 0.031
Glutamate	**K_{mf} (µl/min/ g placenta)**	55.55 [29.5-106.3]	77.72 [30.9-127.0]	* 0.016	113.9 [66.5-263.3]	142.8 [66.2-312.0]	0.078
	µl/min/g fetus	14.28 [8.1-27.4]	14.69 [6.7-28.0]	0.578	7.8 [4.8-17.9]	10.3 [4.2-19.5]	0.406
	µl/min	5.2 [3.2-11.3]	5.2 [2.4-11.3]	0.219	10.8 [5.4-19.6]	10.1 [4.2-20.1]	0.093

Table 22 Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate

The raw data from unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate experiments are shown in the above table (median [range]). Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate is expressed (median [range]): per g placenta (K_{mf}, µl/min/g placenta), per g fetus (µl/min/g fetus) and as total transfer (i.e. irrespective of fetal or placental measures; µl/min). P0 and WT placentas from a single litter were compared for statistical analysis. Data analysed by Wilcoxon signed rank test * P<0.05 Glutamine E15.5 n=6; E18.5. n=6; glutamate E15.5 n=7; E18.5 n=7.

5.3.3 Expression of amino acid transporter proteins in P0 versus WT placentas

Placentas were selected for the experiment at random (one P0 and one WT placenta per litter) following genotyping of fetal tail tips from a litter containing a minimum of two P0 and two WT fetuses (section 2.2.6/2.3). Placental tissue harvested for Western blot experiments were from separate litters to those used in unidirectional maternofetal clearance studies due to the use of radioisotopes. It was therefore not possible to perform both techniques using the same animals/tissues. This study was not powered, nor did it aim to, evaluate the effect of the sex of the fetus.

Expression of known glutamine and glutamate transporter proteins was assessed in membrane-enriched whole placental homogenates by Western blot, and protein band density at the predicted molecular weight was determined by semi-quantitative analysis (section 2.3.5). All data were normalised to the housekeeping protein β-tubulin which was consistent between groups. Representative blots of the proteins of interest and housekeeping protein at both gestational ages are shown in Figure 46.

Glutamine is a substrate of systems A, L and N, and in the absence of reliable specific antibodies for the system A isoforms, the expression of the transporter proteins LAT1, LAT2 (system L) and SNAT5 (system N) was assessed. There were no significant differences between P0 and WT fetuses in expression of any glutamine transporter proteins (LAT1, LAT2, SNAT5) (Figure 47A-C) at either gestational age (E15.5 or E18.5). LAT1 expression

was detected at the appropriate molecular weight of 40 kDa (Figure 46). A 75 kDa band was also present under reducing conditions (see Appendix 9.11) as has been previously described by others (Ellinger *et al.*, 2016). The LAT2 antibody used in Chapter 3 (Abbexa abx121147) was discontinued, thus a different antibody, obtained from Abcam (ab75610) was used here (Table 12). The manufacturer's datasheet for the LAT2 antibody predicts expression at 52 kDa (as shown in Figure 46 and analysed here), with additional bands present at 58 kDa and 85 kDa. SNAT5 expression was present at the predicted molecular weight of 52 kDa (Figure 46). Additional bands were also present at 40 kDa, as is mentioned in the antibody manufacturer's datasheet; however the identity of these bands is unknown.

Expression of two transporter proteins important for placental glutamate uptake, EAAT1 and EAAT2 (system X_{AG-}) was assessed. EAAT1 expression was not different between WT and P0 placentas at either gestational age (Figure 47D). The validity of the band present at 35 kDa has previously been confirmed (Figure 28) and was used for analysis by densitometry here. EAAT2 expression (at the predicted molecular weight of ~62 kDa) was significantly lower ($P<0.05$) at E18.5 for P0 versus WT placentas (Figure 47E) but no different at E15.5.

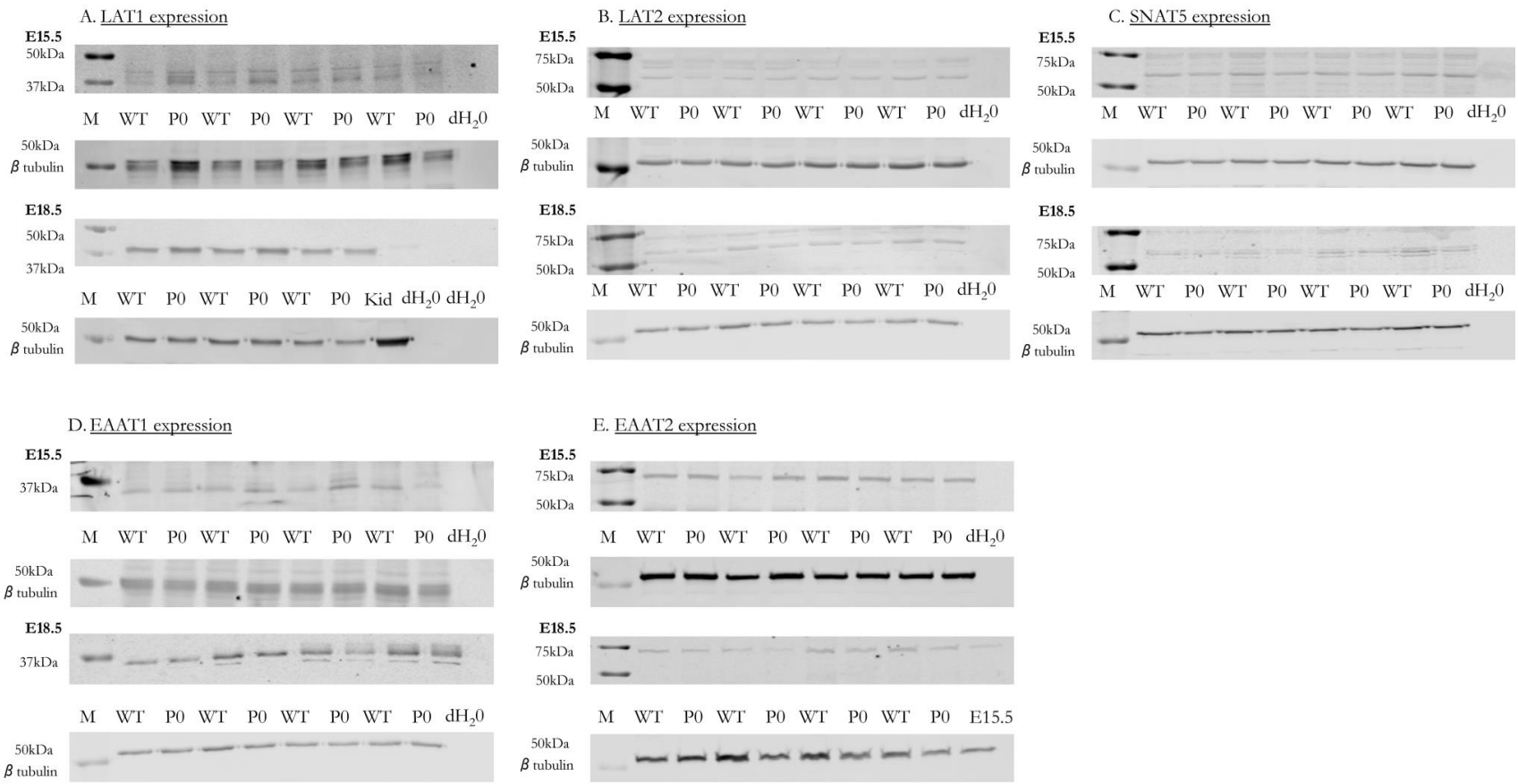


Figure 46 Representative blots illustrating expression of glutamate and glutamine transporter proteins

Representative Western blots of glutamine (system L: LAT1, LAT2 system N: SNAT5) and glutamate (system X_{AG}^-: EAAT1, EAAT2) transporter proteins in the WT and P0 placentas at E15.5 and E18.5. Corresponding Western blots for a housekeeping protein β-tubulin are shown below each blot. M= marker, kid= mouse kidney whole homogenate, E15.5= WT mouse placental membrane isolate from E15.5, dH_20= deionised water.

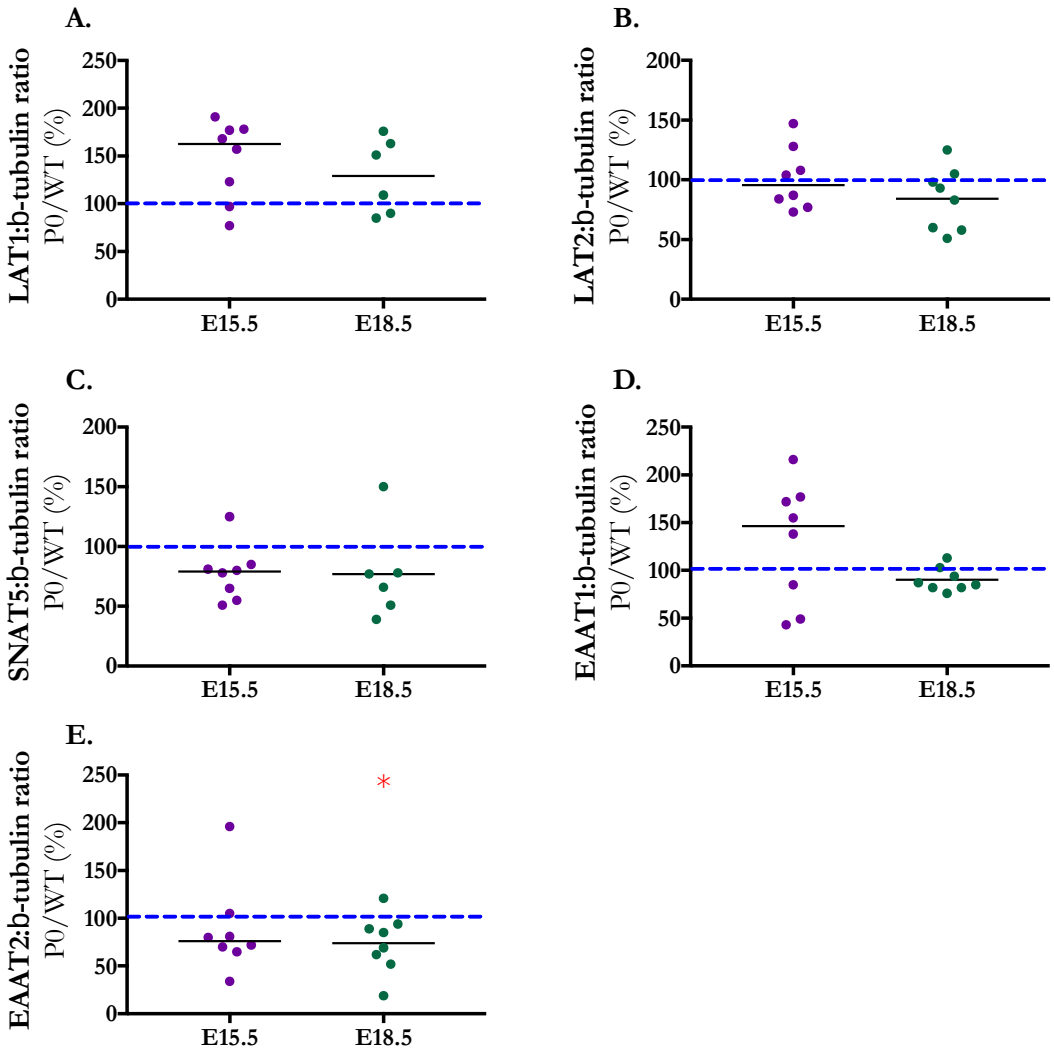


Figure 47 Expression of glutamate and glutamine transporter proteins at E15.5 and E18.5
All data were normalised to β-tubulin, the expression of which was stable across groups. There were no differences in the expression of glutamine transporter proteins LAT1 (A) or LAT2 (B) (system L) or SNAT5 (C) (system N) or glutamate transporter proteins EAAT1 (D) in P0 versus WT placentas at either gestational age. Expression of the glutamate transporter protein EAAT2 (E) (system X_{AG}) was significantly lower in the P0 versus WT placentas at E18.5 (P<0.05, Wilcoxon signed rank test). E15.5 n=8 E18.5 n=8. LAT1 and SNAT5 E18.5 n=6.

5.4 Discussion

This study has reproduced previously published findings and demonstrated that the P0 knockout mouse exhibits FGR in late gestation (Constância *et al.*, 2002; Constância *et al.*, 2005; Dilworth *et al.*, 2010; Dilworth *et al.*, 2011). A major finding of this chapter was that unidirectional maternofetal clearance (K_{mf}) of ^{14}C-glutamine was significantly higher in placentas of P0 fetuses compared with those of WT fetuses both in mid-gestation and towards term (E15.5 and E18.5). ^{14}C-glutamate K_{mf} was significantly higher in placentas of P0 versus WT fetuses at E15.5 only. Expression of transporter proteins important for glutamine and glutamate transport was determined by Western blot of membrane-enriched placental isolates.

Expression of the glutamate transporter EAAT2 was lower in placentas of P0 fetuses at E18.5. The expression of all other glutamate (EAAT1) and glutamine (LAT1, LAT2, SNAT5) transporter proteins was similar between groups at both gestational ages (E15.5 and E18.5).

5.4.1 Placental and fetal weights: P0 versus WT

Timed mating of a WT dam with a male mouse heterozygote for the placental-specific *Igf2P0* knockout results in mixed litters of P0 (*Igf2P0*$^{+}$/$^{-}$) and WT fetuses. This study found that P0 placentas weighed approximately 26% less than WT placentas at both E15.5 and E18.5. The placental weights presented in Table 21 are similar to those previously reported by Dilworth *et al.* (2010) (mean placental weights at E15.5 not reported; E18.5 P0 0.062 g, WT 0.094 g) and Constância *et al.* (2005) (mean placental weights at E15.5 P0 0.075 g, WT 0.105 g; E18.5 P0 0.063 g, WT 0.095 g).

P0 fetuses were 4% lighter than WT at mid-gestation (E15.5) (Figure 42, Table 21). Towards term (E18.5) P0 fetuses weighed approximately 17% less than WT fetuses. These data are in alignment with previous published data by Constância *et al.* (2005) (E15.5 4% E18.5 15%; percentage difference was not reported by Dilworth *et al.* (2010)).

At both gestational ages the fetal weight:placental weight (F:P) ratio was significantly higher for P0 compared with WT fetuses (Figure 42). These data (Table 21) replicate findings by Constância *et al.* (2005) and Dilworth *et al.* (2010) and indicate that at E15.5 and E18.5 the small dysfunctional P0 placenta is supporting significantly more fetal weight per g placental weight but that FGR is still observed towards term.

The 5th centile of fetal weights was calculated using the method described by Dilworth *et al.* (2011) (section 2.2.7). Using the entire population of WT fetal weights recorded for this study, the 5th centile was established as 0.325 g at E15.5 and 1.036 g at E18.5, which is similar to that previously reported (Dilworth *et al.*, 2010; Renshall, 2015) (5th centile in the same mouse model: 1.018 g and 1.070 g at E18.5 respectively; no data for E15.5). The fetal weight distribution curves (Figure 44) show the proportion of P0 and WT fetuses that are below the 5th centile of WT fetal weights (dotted line) at E15.5 and E18.5. By E18.5 there is a shift to the left of P0 fetal weights; towards term 57% of P0 fetuses fall beneath the clinically relevant threshold for FGR (5th centile) compared with 8% at E15.5. Taken together, these similarities provide confidence in the reproducibility of the FGR phenotype in the P0 model.

5.4.2 Methodological considerations

For the first time, unidirectional maternofetal clearance (K_{mf}, per g placenta) of glutamine and glutamate has been assessed in the P0 knockout mouse model of FGR. To calculate K_{mf}, a disappearance curve for each gestational age was generated from single data points from

multiple mice euthanased at time points between approximately 94 sec-3 min after radioisotope injection (section 2.2.3). To reduce the number of animals required in line with the 3Rs (NC3Rs, 2004), the disappearance curves for P0 mice were overlaid with those from WT mice (C57BL/6J) once they were found to be similar (as clearly highlighted in Figure 40). Experimental procedures were carried out at approximately 2 min, during the linear portion of the disappearance curve as previously described (Bond *et al.*, 2006a).

A key difference between the studies reported in this thesis, and the preceding studies of system A in FGR that have measured the maternofetal clearance of MeAIB, is that the latter is not metabolised. The rate of radiolabel appearance in the fetus will depend upon the rate of placental uptake and metabolism, either of which might change in FGR, and radiolabel counts in the fetus reflect the net effect of these processes. Studies in the pregnant rhesus monkey show that the majority of radiolabelled glutamate infused into the maternal circulation (over 1 hour) remains intact as ^{14}C-glutamate (Stegink *et al.*, 1975). It is therefore likely that the majority of isotope that reaches the placenta within the ~2 min time period of the experiment and is subsequently measured in the fetus is representative of glutamate handled by the placenta. Experiments in the current study therefore likely reflect the amount of radiolabelled glutamate taken up into the placenta i.e. activity of the system X_{AG-} isoforms EAAT1, EAAT2 and EAAT3. A mechanism of glutamate efflux in the human placenta is via exchange by the organic anion transporter/transporting polypeptides OAT4/OATP2B1 (see Figure 9). However, OAT4 is not present in the mouse (Koepsell, 2013; Rizwan and Burckhardt, 2007). Determining which metabolites of glutamate were radiolabelled in the fetus following injection into the maternal circulation in the mouse was beyond the scope of the current study and remains to be confirmed in the future.

In all cases where K_{mf} is used to assess amino acid transfer across the placenta in mice, it is possible that time taken for the radiolabelled isotopes to equilibrate with intracellular metabolic compartments/amino acid pools that exist within the placenta (Cleal *et al.*, 2018; Day *et al.*, 2013; Velázquez *et al.*, 1976) could underestimate the true clearance over the time course of the experiment. For the purposes of interpreting data in the current study, it has been assumed that intracellular compartmentalisation does not differ in P0 compared with WT placentas. However, this information is unavailable and it cannot be ruled out that differences in compartmentalisation contribute to the higher glutamine/glutamate clearance by P0 versus WT placentas in the current study.

Expression of transport proteins for glutamine and glutamate was determined by Western blot analyses of membrane-enriched placental homogenates to compare expression in growth restricted (P0) and normally grown (WT) fetuses (discussed fully in section 5.4.5). It is possible that changes in protein expression in the apical maternal-facing plasma membrane are not

detected optimally if expression by other membranes in the homogenate makes a substantial contribution to the transport proteins detected. Future work should examine transporter expression more closely by using pure apical membrane preparations (Kusinski *et al.*, 2010) and/or investigate localisation of putative transporters using immunohistochemistry.

5.4.3 Unidirectional maternofetal clearance in a mouse model of FGR

K_{mf} is a measure of unidirectional maternofetal clearance of radiolabeled amino acid per g placental weight. Expression of maternofetal transfer relative to fetal weight or uncorrected for fetal or placental weight ('raw transfer') can provide additional insight into the relationship between fetal and placental measures and the K_{mf} of glutamine and glutamate, and can help delineate whether the overall amount of glutamine/glutamate reaching the fetus is equivalent between P0 and WT placentas.

5.4.3.1 Unidirectional maternofetal clearance of ^{14}C-glutamine

K_{mf} of glutamine (per g placenta) was significantly higher for P0 versus WT placentas at both E15.5 and E18.5 (Figure 45A). Relative to the size of the fetus there was no difference in maternofetal transfer of glutamine in P0 versus WT at E15.5. At E18.5 maternofetal transfer of glutamine (per g fetus) was increased in P0 (Figure 45C), indicating that, despite more glutamine was being transferred relative to the size of the fetus, fetal weight was not restored to normal. However, expressed as total transfer, i.e. irrespective of fetal or placental size, glutamine delivery to the fetus was significantly lower for P0 versus WT at E18.5, but comparable earlier in gestation (E15.5) (Figure 45E). This indicates that an increase in K_{mf} (per g placenta) did not fully compensate for reduced placental size and thus total delivery to the fetus was lower. This finding is akin to what has been previously described for maternofetal transfer of MeAIB in the P0 mouse (Constância *et al.*, 2002; Constância *et al.*, 2005). Given the importance of glutamine in contributing to fetal growth (Pochini *et al.*, 2014), it is reasonable to suggest that an overall lack of glutamine provision to the P0 fetus may contribute to suboptimal growth.

Previous studies have indicated that K_{mf} of MeAIB, a non-metabolisable analogue for system A, of which glutamine is a substrate, is higher at E15.5 in P0 placentas but similar between groups at E18.5 (Constância *et al.*, 2002). This suggests that other transport systems responsible for placental glutamine transport (e.g. system L, N) are driving the sustained increase in glutamine clearance per g placenta at E18.5.

5.4.3.2 Unidirectional maternofetal clearance of ^{14}C-glutamate

At E15.5 there was higher clearance of glutamate per g placenta (K_{mf}) compared with WT (Figure 45B), and this resulted in the same total delivery of glutamate to the P0 and WT fetus. At E18.5 there was a trend for glutamate clearance to be higher in P0 versus WT, and for total transfer to be reduced, but these differences were not significant.

Glutamate is required for glutamine synthesis within the placenta (Moores *et al.*, 1994; Vaughn *et al.*, 1995). Insufficient uptake of glutamate into the placenta would impair the ability of the placenta to synthesise sufficient glutamine, which could subsequently impact glutamate production from glutamine within the fetal liver (Figure 8), further perpetuating the problem. Maternofetal transfer of glutamate relative to fetal weight (per g fetus), and total maternofetal glutamate transfer was similar between P0 and WT placentas at both E15.5 and E18.5 (Figure 45D, F), indicating that glutamate delivery to the fetus may in fact be sufficient, relative to the size of the fetus. Whilst this study was not designed to test for differences across gestation, there is evidence that transfer of glutamine/glutamate (μl/min) increases towards term as would be expected (Table 22).

5.4.4 Unidirectional maternofetal clearance of ^{14}C-glutamine and ^{14}C-glutamate in normal (WT) small placentas and FGR (P0) placentas

Taken together, the data presented here and in Chapter 3 indicate that there are different strategies to compensate for small placental size in normally grown (WT, C57BL/6J) and growth restricted (P0) fetuses (summarised in Table 23). The P0 placenta weighs less than the lightest placenta in a normal WT litter (0.077 g at E15.5; 0.066 g at E18.5 for P0 versus 0.078 g and 0.070 g for WT lightest). However, the magnitude of this difference is slight, and indicates that perturbed placental function and morphology, in additional to small size, contributes to suboptimal fetal growth in this model of FGR.

At both gestational ages investigated, P0 fetuses and WT (C57BL/6J) fetuses that have the lightest placenta in a litter weigh less than WT fetuses in the P0 model, and WT (C57BL/6J) fetuses with the heaviest placenta in a litter. In the WT mouse, where fetuses with the lightest and heaviest placentas in a litter are compared, the magnitude of difference in fetal weights is reduced towards term. Conversely, in the P0 mouse the difference in fetal weight between P0 and WT fetuses increases towards term. This indicates that in WT litters, the lightest placenta in a litter employs strategies to maintain appropriate fetal growth, which is supported by the findings that K_{mf} of glutamine and glutamate is significantly higher across the lightest placentas towards term (Chapter 3). On the other hand, P0 placentas fail to support a fetus of an appropriate weight by term (57% of fetuses are beneath the 5th centile by E18.5) (Figure 44) and the K_{mf} of glutamate is similar between P0 and WT fetuses at E18.5. K_{mf} of glutamine

is higher across P0 versus WT at E18.5 but this is not sufficient to maintain appropriate fetal weight and growth restriction ensues.

The data presented in this thesis are consistent with previously reported MeAIB clearance in the lightest versus heaviest placentas of a WT litter, which is elevated at E18.5 (no difference at E15.5) (Coan *et al.*, 2008). This shows that in normal pregnancy, several nutrient transporters are up-regulated in a small placenta (per g placenta) to ensure that there is sufficient compensation for small size to sustain normal fetal growth. In the P0 mouse K_{mf} of MeAIB is significantly higher compared to WT at E15.5 but no different between groups at E18.5 (Constância *et al.*, 2002), which is in alignment with K_{mf} of glutamate reported in the current study. However, in contrast to MeAIB, glutamine clearance per g placenta remains elevated in the P0 mouse above WT at E18.5. Taken together, these data suggest that the WT placenta is able to adapt to facilitate appropriate fetal growth towards term. In the P0 mouse close to term, there is higher glutamine but not glutamate or MeAIB (Constância *et al.*, 2005) clearance, indicating that the up-regulation is selective for certain amino acid transporters. However, placental nutrient provision remains insufficient to restore normal fetal growth in the P0 mouse.

	C57BL/6J (WT) lightest versus heaviest				P0 versus WT			
	E15.5		E18.5		E15.5		E18.5	
Placental weight	↓↓↓		↓↓↓		↓↓↓		↓↓↓	
Fetal weight	↓↓↓		↓		↓		↓↓↓	
F:P ratio	↑↑↑		↑↑↑		↑↑↑		↑↑↑	
Unidirectional maternofetal clearance (K_{mf}, µl/min per g placenta)	Gln =	Glu =	Gln ↑↑	Glu ↑	Gln ↑	Glu ↑	Gln ↑	Glu =
Maternofetal transfer (µl/min per g fetus)	Gln =	Glu ↓	Gln ↑↑	Glu =	Gln =	Glu =	Gln ↑	Glu =
Total transfer (µl/min)	Gln =	Glu ↓	Gln ↑	Glu =	Gln =	Glu =	Gln ↓	Glu =

Table 23 Comparison of placental weight, fetal weight, fetal:placental weight ratio, and measures of maternofetal transfer of glutamine and glutamate in WT (lightest versus heaviest placentas: Chapter 3) and P0 (paired P0 versus WT fetuses) mice

Arrows (↑↓) indicate that measurement is significantly higher or lower relative to littermates, i.e. lightest versus heaviest or P0 versus WT. ↑ corresponds to a *P* value of <0.05, ↑↑ *P*<0.01, ↑↑↑ *P*<0.001; same for ↓ arrows. = indicates that there was no difference between groups. Gln: glutamine, Glu: glutamate.

5.4.5 Expression of amino acid transporters in P0 and WT placentas

The expression of transporter proteins known to transfer glutamine (LAT1, LAT2 for system L and SNAT5 for system N) and glutamate (EAAT1, EAAT2 for system X_{AG}^-) was assessed (Western blot) using membrane-enriched placental isolates from paired P0 and WT placentas. To the best of my knowledge, expression of these amino acid transporters has never been evaluated in the P0 mouse.

There were no differences between P0 and WT placentas in the expression of any of the glutamine transporter proteins analysed (LAT1, LAT2, SNAT5) at either E15.5 or E18.5 (Figure 47A-C). Whilst it was not possible to investigate the expression of system A isoforms due to a lack of validated specific antibodies, it is unlikely that system A is wholly responsible for the changes in K_{mf} of glutamine reported here. Previous studies (Constância *et al.*, 2002) have reported in the P0 mouse that K_{mf} of MeAIB (representative of system A activity) is higher for growth restricted (P0) compared with WT placentas at E15.5 (akin to glutamine K_{mf}) but unlike MeAIB, glutamine K_{mf} remains significantly higher in P0 placentas versus WT littermates at E18.5. There was no difference in glutamine transporter abundance in membrane-enriched homogenates between P0 and WT placentas and so the mechanisms that underlie the elevated K_{mf} of glutamine are as yet unknown.

In the current study, expression of the glutamate transporter protein EAAT1 was similar between P0 and WT placentas at both E15.5 and E18.5 (Figure 47D). EAAT2 expression was also similar at E15.5 but was significantly lower across P0 placentas at E18.5 (Figure 47E). Glutamate K_{mf} is higher (P0 versus WT) at E15.5 but similar between groups at E18.5 which may be explained by relatively lower EAAT2 glutamate transporter protein expression.

Previously published work from our group has demonstrated that a sample size of 6-8 litters (one WT and one P0 per litter) is sufficient to detect differences between groups (Dilworth *et al.*, 2010). However, some of the data presented here (Figure 47A, C) appear close to significance, and so more numbers may be required to gain sufficient power to detect changes.

5.4.6 Summary

To summarise, the P0 mouse has a significantly lighter placenta than WT littermates at both E15.5 and E18.5. Compared with WT fetuses, P0 fetuses weigh less at E15.5 (4%); the magnitude of this reduced fetal weight is amplified (17%) by E18.5. Whilst the P0 placenta supports more fetus per g placental weight at both gestational ages than its WT littermates, as evidenced by a higher F:P ratio, it is unable to sustain appropriate fetal growth. By E18.5, 57% of P0 fetuses are below the 5th centile of WT fetal weight, a clinically relevant cut off for FGR (Dilworth *et al.*, 2011).

In alignment with this, unidirectional maternofetal clearance (K_{mf}) of glutamine and glutamate

is significantly higher in P0 compared with WT fetuses at E15.5. At E18.5 K_{mf} of glutamate is similar between groups. K_{mf} of glutamine remains higher at E18.5 in the P0 fetuses but this is insufficient to maintain appropriate total delivery of glutamine to the fetus or to restore fetal weight. Glutamine and glutamate are both fundamental to the metabolic and growth requirements of the fetus (Parimi and Kalhan, 2007; Pochini *et al.*, 2014). That glutamate is required for glutamine synthesis within the placenta suggests that insufficient glutamate could also lead to reduced glutamine availability for transfer to the fetus.

Expression of transporter proteins that affect glutamine and glutamate transfer was assessed as a potential mechanism underpinning changes in glutamine and glutamate clearance. None of the glutamine transporter proteins analysed (LAT1, LAT2, SNAT5) were different between P0 and WT placentas. EAAT1 expression (contributes to placental glutamate uptake) was also unchanged whereas EAAT2 expression was significantly reduced in placentas of P0 compared with WT fetuses at E18.5 only. The up-regulation of glutamine clearance in the P0 placenta is similar to the up-regulation of MeAIB clearance at E15.5 previously reported (Constância *et al.*, 2002). Thus, at the earlier time point the increase in glutamine clearance could be due to system A. However, the higher K_{mf} of glutamine at E18.5, when MeAIB clearance is no longer elevated in FGR, suggests that systems L or N are activated in later gestation. If so, the lack of increase in protein expression of LAT1, LAT1 and SNAT5 at E18.5 in FGR placentas implies an effect on transporter activity and not expression.

The data presented in this chapter illustrate that in the mouse, the placenta of a growth restricted (P0) fetus up-regulates transporter activity for glutamine, providing evidence for placental adaptation of nutrient transporter activity in FGR. This appears to be a functional adaptation in an attempt to support/achieve appropriate fetal weight towards term but this subsequently fails: P0 fetuses weigh 17% less than WT littermates by term. An investigation of glutamine and glutamate uptake (transporter activity), and expression of putative transporter proteins in human pregnancy, comparing placentas from normal birth weight and FGR infants, is the focus of the next chapter.

Chapter 6 Assessment of placental glutamine and glutamate transporter activity, expression and amino acid levels in FGR and normal pregnancies

6.1 Introduction

Fetal growth restriction (FGR) is defined as the failure of the fetus to reach its genetically predetermined growth potential. FGR occurs in around 5-10% of pregnancies in the UK (ONS, 2015) and is associated with an increased risk of stillbirth (Gardosi *et al.*, 2013). Placental dysfunction leads to the majority of FGR cases (Mifsud and Sebire, 2014), yet there are no accurate tests to predict FGR or any treatment options save for delivery, often prematurely, of the fetus.

Fetal growth is dependent upon the provision of nutrients such as amino acids to the fetus via the placenta. The amino acids glutamine and glutamate are essential for pH homeostasis, nucleotide synthesis and protein anabolism (Parimi and Kalhan, 2007; Pochini *et al.*, 2014). Glutamine is a non-essential amino acid that becomes conditionally essential during pregnancy as fetal demand exceeds maternal synthesis (Neu, 2001; Tapiero *et al.*, 2002). Deamination of glutamine in the fetal liver produces glutamate, an important nitrogen resource and precursor of γ-amino butyric acid (GABA), a key inhibitory neurotransmitter (Moores *et al.*, 1994; Tapiero *et al.*, 2002; Vaughn *et al.*, 1995), and results in increased concentration of glutamate in the umbilical artery (UmA) relative to the umbilical vein (UmV) (Holm *et al.*, 2017). Glutamate is transported across the syncytiotrophoblast BM by high affinity EAATs (system X_{AG-}) (Lofthouse *et al.*, 2015) and is subsequently converted to glutamine in the placenta (Day *et al.*, 2013) (Figure 9).

My data in WT mice (Chapter 3) showed that small, normal placentas (lightest versus heaviest) up-regulate K_{mf} of glutamine and glutamate (clearance per g placenta) to support normal fetal growth, but this adaptation was not evident in human placentas from babies that were normally grown (Chapter 4); there was no relationship between glutamine and glutamate uptake (per g placenta) and placental weight. Additionally, in growth restricted (P0) mice (Chapter 5), my data demonstrated that K_{mf} of glutamine (per g placenta) at E15.5 and E18.5, and K_{mf} of glutamate at E15.5 (but not E18.5), was greater than in normally grown WT littermates, indicating an adaptive up-regulation of transporter activity in the growth restricted P0 mouse placenta. However, for glutamine this adaptation was insufficient to compensate for small placental size and the total placental delivery of glutamine to the fetus was significantly lower in P0 versus WT towards the end of pregnancy.

In human FGR, the placenta is smaller than normal for gestational age (Mifsud and Sebire, 2014) and this alone might account for the reduced provision of key amino acids to the fetus. There are as yet no studies of glutamine and glutamate transporter activity in the placenta in human FGR, but substantial evidence to show that system A and system L transporter activity is reduced in FGR (Glazier *et al.*, 1997; Jansson *et al.*, 1998; Mahendran *et al.*, 1993; Shibata *et al.*, 2008). This implies, again in contrast to the P0 mouse, that there is failure of the small abnormal placenta in FGR to up-regulate nutrient transporter activity (per g placenta). As glutamine is a substrate for systems A and L, a reasonable hypothesis is that glutamine uptake will be lower in FGR.

The fundamental reasons behind the reduction in placental system A and system L activity in FGR are poorly understood, not least because the determinants of appropriate provision of amino acids to the fetus in normal pregnancy have not been defined. It is important to evaluate the relationships between amino acid transporter activity and potential determinants of this activity, which include amino acid concentrations, in particular glutamine and glutamate. Measuring amino acid concentrations in maternal and fetal (UmV and UmA) plasma offers insight into the interplay between maternal 'availability' of amino acids (maternal vein), delivery to the fetus (UmV), reflective of what is transported into/across the placenta and of placental utilisation, and the concentrations of amino acids available for transfer back to the placenta (UmA). Analysing amino acid concentrations in UmV and UmA offers the opportunity to assess venoarterial amino acid gradients thus giving insight into fetal utilisation and conversion of amino acids, particularly with regards to glutamine and glutamate. Amino acid concentrations in the UmA are a determinant of amino acids taken up by the placenta from the fetus which in turn, through changing the intracellular concentrations of amino acids, could modulate transporter activity/expression at the MVM of the syncytiotrophoblast since several amino acid transporters are regulated by levels of substrate (section 1.8.1).

Amino acid concentrations in the UmA may act as an indicator of physiological condition of the fetus (Horgan *et al.*, 2011), and in this sense it is possible that the fetus signals to the placenta, through amino acids or other metabolites in the UmA blood, to adapt placental amino acid uptake (MVM transporter activity) to ensure amino acid provision meets fetal demand, as has been suggested for other nutrients such as calcium (Dilworth *et al.*, 2010). Assessment of amino acid and/or metabolite levels in UmA could identify whether potential fetal signals were different in FGR versus normal birth weight (AGA) infants. Should there be no difference, this may imply that the fetus is not able to signal to the placenta by this means, i.e. through altered amino acids or metabolites in UmA, and so this will not be a mechanism to adapt or match placental nutrient provision with fetal demand. If there are differences in potential signals, but no placental response (change in amino acid transporter

activity/expression in the MVM), then the placenta is failing to adapt i.e. react to fetal demand. Glutamate is a likely candidate as a signal/marker of physiological condition as it is produced following the conversion of glutamine by the fetal liver which could be compromised in the growth restricted fetus. Thus, any alteration in glutamate concentrations in the UmA may indicate that fetal physiology is compromised. Cetin *et al.* (1988) and Young and Prenton (1969) found no difference in UmA glutamate concentration in normal pregnancy compared with SGA infants (<10th centile of birth weight); however, whether glutamate concentration is different in well-defined cohorts of FGR versus normal pregnancy remains to be resolved.

Metabolomics is the study of small molecule metabolites which can provide a holistic read-out of the biological system of interest. It has been used previously to evaluate metabolite levels in the placenta, maternal and fetal (umbilical cord) plasma (Fanos *et al.*, 2013) (for a summary of the relevant literature, see Table 6 in Introduction). To date, there are very few studies that have measured the metabolic profile in UmV/UmA plasma in FGR but evidence from these studies suggest that low birth weight and growth restricted infants have altered umbilical cord plasma metabolomic profiles (Table 6). Additionally, very few studies have collected UmA samples, the majority have looked at UmV plasma only.

It is difficult to compare between studies since various gestational cutoffs and definitions of low birth weight/FGR have been used, e.g. <10th centile, <1500 g, and <32 or 35 weeks gestation (Alexandre-Gouabau *et al.*, 2013; Favretto *et al.*, 2012; Ivorra *et al.*, 2012; Sanz-Cortés *et al.*, 2013; Tea *et al.*, 2012). Definitions of normal are also problematic; many studies do not report the inclusion criteria or demographics of the control (normal birth weight) group, and in 1 study only infants with an IBR between 75th-90th centile were accepted in the control arm of the study (Ivorra *et al.*, 2012). Altered amino acid levels in low birth weight (<10th centile) pregnancies, e.g. reduced levels of glutamine in the UmV (Ivorra *et al.*, 2012), could arise from reduced uptake by the syncytiotrophoblast MVM but this has yet to be investigated. Ideally, it would be beneficial to measure amino acid concentrations in maternal and umbilical plasma within the same pregnancy, and to assess differences in well-defined cohorts of normal (AGA) pregnancy and FGR, and relate these to amino acid transporter activity.

In the current study the placental uptake of glutamine and glutamate was compared in normal pregnancy and FGR for the first time. Amino acid concentrations and the levels of metabolites (sugars, intermediate metabolites, and amino acids) in the maternal vein and fetal (UmV and UmA) plasma are potential determinants of placental nutrient transporter activity/expression and might be altered in FGR. Here amino acid concentrations were measured in the maternal vein, UmV and UmA using high performance liquid chromatography (HPLC). Non-targeted metabolomics (GC-MS) assessed levels of metabolites

in the UmV and UmA of FGR and normal birth weight (AGA) infants, to not only reveal potentially novel markers/signals but to also confirm differences in amino acid concentrations observed with HPLC.

6.1.1 Hypotheses

- Uptake of ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB into placental villous fragments is reduced in placentas from FGR versus normal (AGA) pregnancies
- Placental expression of key glutamine and glutamate transporters is reduced in FGR compared with normal pregnancy
- Amino acid concentrations in the maternal and fetal compartments (maternal venous and UmA and UmV plasma) are related to glutamine and glutamate uptake
- The relationship/s between glutamine and glutamate uptake into the placenta and plasma concentration of these amino acids in mother and fetus (UmV and UmA) in normal pregnancy is altered in FGR

6.1.2 Aims

The aims of this chapter were therefore to:

- Determine whether glutamine and glutamate transporter activity and expression of key glutamine and glutamate transporters is altered in FGR compared with normal pregnancy by measuring transporter-mediated ^{14}C-glutamine and ^{14}C-glutamate uptake into placental villous fragments, and protein expression by Western blotting.
- Determine the concentration of amino acids (HPLC) in maternal venous and fetal (UmV and UmA) plasma from normal pregnancy and FGR, and relate concentration to glutamine and glutamate uptake
- Perform metabolomic analysis (GC-MS) to compare paired UmV/UmA (fetal) plasma samples in normal pregnancy and FGR

6.2 Methods

6.2.1 Glutamine and glutamate transporter activity in placental villous fragments from FGR and normal (AGA) pregnancies

Women who met the inclusion criteria (section 2.1.2) were included in the study following informed consent. Infants with an IBR between 10th-90th centile at delivery were classified as normal birth weight (AGA, n=11) and those with an IBR <5th were classed as FGR (n=19) (see Table 24 for demographics). The full experimental details are described in the Methods chapter of this thesis (section 2.1). In brief, maternal venous blood was collected prior to delivery; on admittance to the ward for vaginal births, before anaesthetic for deliveries by

caesarean section and after prostaglandin administration for induction of labour. The placenta, and where possible umbilical arterial (UmA) and venous (UmV) blood, were collected immediately following delivery. Blood samples were centrifuged to obtain plasma (as detailed in section 2.1.5) and stored at -80ºC until required for HPLC or GC-MS analyses (see sections 6.2.4 and 6.2.5). Immediately following collection of placental tissue, sections of villous tissue were dissected in preparation for villous fragment uptake experiments. Placental villous fragments were exposed to ^{14}C-glutamine, ^{14}C-glutamate or ^{14}C-MeAIB, and the initial rate of transporter-mediated uptake of radiolabelled amino acids was measured over 30-90 min and expressed in pmol/mg protein. To test the hypothesis that diminished amino acid uptake may lead to decreased amino acid availability for transfer to the fetus, a proxy measure of amino acid availability was calculated as amino acid uptake at 90 min (per mg placental protein) x trimmed placental weight (g).

	AGA (n= 19)	**FGR** (n= 11)	***P* value**
Maternal Age (years)	30 (23-40)	32 (25-39)	0.694
Body Mass Index (kg/m^2)	25.9 (18.8-31.2)	23.9 (19.8-33.9)	0.196
Birth Weight (g)	3410 (2690-4400)	1730 (717-2550)	*** <0.001
Trimmed Placental Weight (g)	495.5 (331.1-719.4)	301.6 (159.5-429.3)	*** <0.001
Individualised Birth Weight Ratio (IBR)	54 (13.6-87.7)	1 (0-3.5)	*** <0.001
Birth weight:placental weight (BW:PW) **ratio**	6.5 (5.2-8.9)	6.2 (3.2-8.4)	0.232
Gestation (days)	273 (260-285)	247 (204-284)	* <0.05
Mode of Delivery	ELCS (89%) NVD (11%)	ELCS (55%) EMCS (18%) NVD (27%)	-
Parity	1 (0-8)	1 (0-5)	0.268
Gravidity	2 (1-12)	2 (1-6)	0.893
Ethnicity	Caucasian (58%)	Caucasian (64%)	-
Smoking Status	No (100%)	No (73%)	-
Uptake experiment also performed	Yes (100%)	Yes (100%)	-
Sex of fetus	Female (63%) Male (37%)	Female (64%) Male (36%)	-

Table 24: Demographics relating to placentas from normal (AGA) and FGR pregnancies
Placentas were collected from normal (AGA, IBR 10^{th}-90^{th} centile) and FGR infants (<5^{th} centile). Birth weight, placental weight, IBR and gestational age were significantly lower in FGR compared with normal pregnancy (AGA). Data are median (range) or percentage of total. ELCS: elective caesarean section; EMCS, emergency caesarean section; NVD: normal vaginal delivery * $P<0.05$ *** $P<0.001$ Mann-Whitney test.

6.2.2 Western blotting of membrane-enriched whole placental homogenates

Membrane-enriched whole placental homogenates were prepared from 11 AGA and 10 FGR placentas (section 2.3.1.1). Membranes were probed for the amino acid transporter proteins known to contribute to glutamine (system L: LAT1, LAT2, and system N: SNAT5) and glutamate uptake (system X_{AG-}: EAAT1, EAAT2) (see Table 12 for specific antibody conditions). To confirm equal protein loading, β-tubulin (housekeeping protein) was probed for on each membrane.

6.2.3 Quantification of gene expression (qPCR) of glutamine transporters

Currently no reliably validated antibodies exist for determining system A transporter protein expression using Western blotting. Thus, gene expression of system A isoforms slc38a1 (which encodes SNAT1), slc38a2 (SNAT2), slc38a4 (SNAT4) was quantified (section 2.4). The system L isoforms slc7a5 (LAT1), slc7a8 (LAT2), and the associated heavy chain, slc3a2 (CD98/4F2hc) were also quantified by qPCR. Primer sequences are detailed in Methods (Table 13).

6.2.4 High performance liquid chromatography (HPLC)

Maternal venous and UmV and UmA plasma was collected and stored (section 2.1.5), and subsequently sent to the Willink Biochemical Genetics Laboratory (Royal Manchester Children's Hospital, Manchester, UK) for the quantification of amino acid concentration by high-performance liquid chromatography (HPLC). The demographics of the individuals from which these samples were obtained are summarised in Table 25. Table 26 shows the total number of samples collected from the maternal (maternal venous) or fetal (UmA or UmV) compartments as well as the number of paired samples, i.e. samples obtained from the same individual. Maternovenous and venoarterial differences were calculated using the formula below.

$$Maternovenous\ difference = [maternal\ venous] - [umbilical\ venous]$$
$$Venoarterial\ difference = [umbilical\ venous] - [umbilical\ arterial]$$

	AGA (n= 20)	**FGR** (n= 19)	***P* value**
Maternal Age (years)	31 (25-38)	32 (25-39)	0.906
Body Mass Index (kg/m^2)	26.1 (18.8-31.2)	22.8 (19.8-33.9)	0.292
Birth Weight (g)	3230 (2834-3990)	1801 (717-2710)	*** <0.001
Trimmed Placental Weight (g)	516.5 (321.1-719.4)	307.6 (159.5-409.7)^	*** <0.001
Individualised Birth Weight Ratio (IBR)	46.7 (14.8-89.0)	0.9 (0-4.9)	*** <0.001
Birth weight:placental weight (BW:PW) **ratio**	6.4 (5.2-9.1)	6.1 (3.2-8.4)	0.124
Gestation (days)	273 (261-282)	248 (205-279)	*** <0.001
Mode of Delivery	ELCS (100%)	ELCS (58%) EMCS (10%) NVD (32%)	-
Parity	1	1 (0-5)	0.146
Gravidity	2	2 (1-6)	0.270
Ethnicity	Caucasian (60%)	Caucasian (68%)	-
Smoking Status	No (79%)	No (95%)	-
Uptake experiment also performed	Yes (35%)	Yes (53%)	-
Sex of fetus	Female (55%) Male (45%)	Female (68%) Male (32%)	-

Table 25: Demographics relating to maternal venous, and UmV and UmA plasma samples drawn from normal (AGA) and FGR infants subsequently analysed by HPLC

Maternal venous and UmV and UmA blood was collected and centrifuged to obtain plasma (section 2.1.5). Samples were collected from normal (AGA, IBR 10^{th}-90^{th} centile) and FGR infants (<5^{th} centile). ^placental weight was not recorded for one placenta. Data are median (range) or percentage of total. ELCS: elective caesarean section; EMCS, emergency caesarean section; NVD: normal vaginal delivery *** P<0.001 Mann-Whitney test.

Samples		**AGA** (n= 20)	**FGR** (n= 19)
Total	Maternal venous	17	13
	Umbilical venous (UmV)	20	14
	Umbilical arterial (UmA)	11	11
Paired	Maternal venous-UmV	17	8
	UmV-UmA	12	11

Table 26 Samples analysed by HPLC

The total number of samples collected from the maternal venous, UmV or UmA of normal (AGA) and FGR infants. The number of paired samples, i.e. those drawn from the same individual, are also stated.

6.2.5 Gas chromatography-mass spectrometry (GC-MS)

UmV and UmA plasma samples were collected and processed for analysis by GC-MS (section 2.5). 48 samples were collected in total consisting of 12 matched UmV/UmA plasma pairs

from each experimental group, which is a total of 24 normal (AGA) and 24 FGR samples (Table 27).

	AGA (n= 12)	**FGR** (n= 12)	***P* value**
Maternal Age (years)	35 (27-40)	32 (27-38)	0.324
Body Mass Index (kg/m^2)	25.6 (18.8-29.0)	23.3 (19.8-33.9)	0.854
Birth Weight (g)	3210 (2840-3800)	2094 (1058-2580)	*** <0.001
Trimmed Placental Weight (g)	530.3 (406.5-704.7)	311.4 (159.5-409.7)	*** <0.001
Individualised Birth Weight Ratio (IBR)	45.5 (14.8-85.7)	1.3 (0.0-3.6)	*** <0.001
Birth weight:placental weight (BW:PW) **ratio**	6.2 (4.9-7.6)	6.1 (3.7-8.4)	0.745
Gestation (days)	271 (262-277)	253 (205-279)	** <0.01
Mode of Delivery	ELCS (92%) NVD (8%)	ELCS (67%) EMCS (8%) NVD (25%)	-
Parity	1	1	0.371
Gravidity	2	2	0.200
Ethnicity	Caucasian (67%)	Caucasian (83%)	-
Smoking Status	No (100%)	No (75%)	-
Uptake experiment also performed	Yes (75%)	Yes (50%)	-
Sex of fetus	Female (67%) Male (33%)	Female (50%) Male (50%)	-

Table 27: Demographics relating to UmV and UmA blood samples drawn from normal (AGA) and FGR infants subsequently analysed by GC-MS

UmV/UmA blood was collected and centrifuged to obtain plasma (section 2.1.5). Samples were collected from normal (AGA, IBR 10^{th}-90^{th} centile) and FGR infants (<5^{th} centile). Data are median (range) or percentage of total. ELCS: elective caesarean section; EMCS, emergency caesarean section; NVD: normal vaginal delivery ** P<0.01 *** P<0.001 Mann-Whitney test.

6.2.6 Statistical analysis

All data were analysed statistically using GraphPad Prism 7 software and statistical significance was set to P<0.05. Normal distribution of data was determined using D'Agostino & Pearson omnibus normality test. Data from placental villous fragment uptake experiments are expressed as mean ± standard error of the mean (SEM), and regression analyses were performed to determine whether transporter-mediated amino acid uptake was linearly related to time over 30-90 min and to examine the relationship between transporter-mediated uptake at 90 min and fetal and placental measures (Spearman correlation or Linear regression).

Semi-quantitative analysis of transporter protein expression as determined by protein band density (densitometry) was performed for Western blot data using Image Studio™ Lite software. Data were analysed using a Mann-Whitney test.

Gene expression, as quantified using qPCR, was normalised to the geometric mean of the housekeeper genes (TBP and YWHAZ) and analysed statistically using a Mann-Whitney test.

HPLC data were not normally distributed and so were analysed by Mann-Whitney test.

Data generated by GC-MS were analysed by unpaired t test with Welch's correction. Where differences between case (FGR) and control (normal birth weight, AGA, infants) groups reached statistical significance, $\log_2$ fold change differences (case over control, Log_2 transformed) were calculated to allow visual presentation as a heat map. Differences are described in text using fold change (case over control).

6.3 Results

6.3.1 Glutamine and glutamate transporter activity in placental villous fragments from FGR and normal (AGA) pregnancies

Trimmed placental weight and birth weight were correlated for both FGR ($P<0.01$) and normal (AGA) ($P<0.05$) infants (Figure 48). Transporter-mediated uptake of ^{14}C-glutamine was significantly lower in placentas of FGR babies compared with babies appropriately grown for gestational age ($P<0.05$) (Figure 49A). However, there was no significant difference between groups in either ^{14}C-glutamate ($P=0.08$) or ^{14}C-MeAIB ($P=0.07$) uptake (Figure 49B, C).

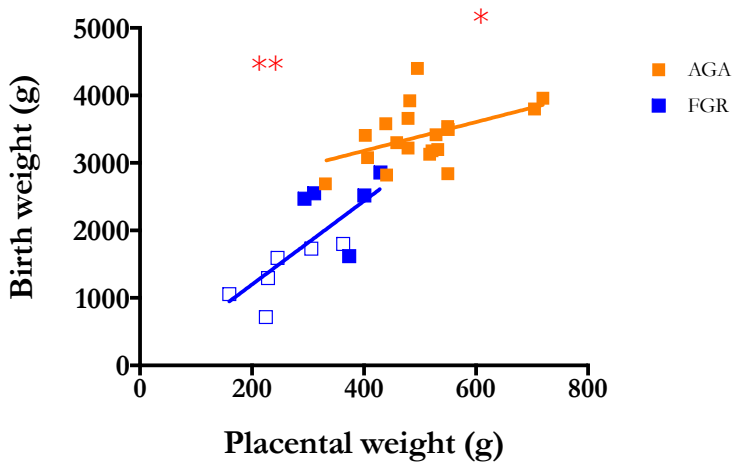


Figure 48: Relationship between placental weight and birth weight from AGA and FGR infants
Trimmed placental weight and respective birth weight of AGA (orange symbols) and FGR (blue symbols) infants. Placental weight and birth weight were positively correlated for both groups. Preterm deliveries (FGR group) are shown as hollow blue squares and term FGR deliveries as solid blue squares. AGA n=19 FGR n=11, of which five were term deliveries (>37 weeks gestation) and six were pre-term (<37 weeks gestation). $^{**}P<0.01$, $^{*}P<0.05$; Linear regression.

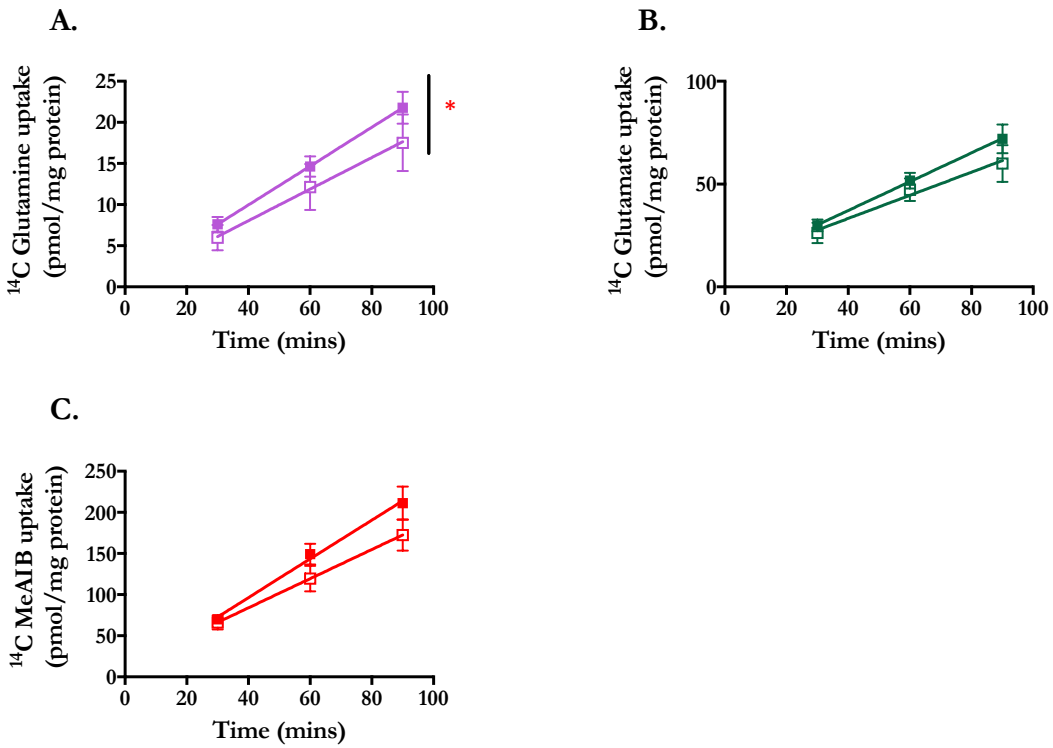


Figure 49: ^{14}C-glutamine uptake was significantly lower in placentas from FGR versus normal (AGA) pregnancies

Placental uptake of ^{14}C-glutamine (A) was significantly lower in placentas from FGR babies (hollow symbols, n=11) compared with placentas from babies appropriately grown for gestational age (solid symbols, n=19) infants. There were no significant differences in ^{14}C-glutamate (B) or ^{14}C-MeAIB (C) uptake between groups. Data are mean±SEM * P<0.05 Linear regression.

^{14}C-glutamine uptake at 90 min was positively correlated with both ^{14}C-MeAIB (P<0.05) (Figure 50A) and ^{14}C-glutamate (P<0.001) uptake (Figure 50B). Linear regression analyses were fitted to all the data, i.e. from both normal and FGR infants.^{14}C-glutamine and ^{14}C-glutamate uptake (Figure 50B) were still positively correlated if data from placentas of normal or FGR infants alone were analysed (normal pregnancy P<0.01, FGR P<0.001). However, there was a positive relationship between ^{14}C-glutamine and ^{14}C-MeAIB uptake for placentas from FGR infants only (P<0.05), but not from babies appropriately grown for gestational age (Figure 50A).

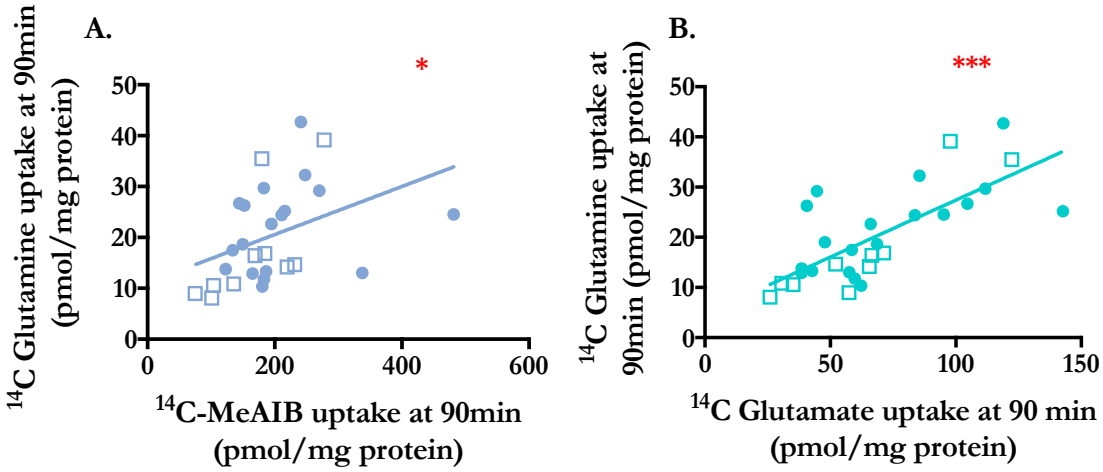


Figure 50: ^{14}C-glutamine uptake correlates with ^{14}C-glutamate and ^{14}C-MeAIB uptake
^{14}C-glutamine uptake at 90 min was correlated with ^{14}C-MeAIB at 90 min (A). ^{14}C-glutamine uptake at 90 min also strongly correlates with ^{14}C-glutamate uptake (90 min). Hollow symbols represent data from FGR infants. * $P<0.05$, *** $P<0.001$ Linear regression (fitted to all the data).

6.3.2 Confounding factors that may influence glutamine and glutamate transporter activity

Potential confounders within this dataset include gestational age, maternal smoking and sex of the fetus, in light of the differences between male and female in normal pregnancy presented in Chapter 4. To determine whether the sex of the fetus biased the difference in uptake between normal and FGR placentas, data were stratified according to sex. Uptake of glutamine was significantly lower for female compared with male placentas within the FGR group (Figure 51). There were no other differences between placentas from males and females. This study did not aim to directly compare between amino acid uptake into placentas from male normal (AGA) and male FGR placentas, or between female normal (AGA) and female FGR placentas. However, a graph of these data can be found in the Appendix (9.4).

Some of the women with FGR infants were smokers or delivered preterm (Table 24). The small numbers in each group precludes statistical analysis of the data. However, there was a trend towards a negative effect of smoking on ^{14}C-MeAIB uptake (AGA n=19, FGR non-smokers n=8, FGR smokers n=3) (Appendix 9.5). Within the FGR group, six infants were delivered preterm (<259 days/37 weeks). Gestational age did not appear to influence ^{14}C-glutamine, ^{14}C-glutamate or ^{14}C-MeAIB uptake (Appendix 9.5).

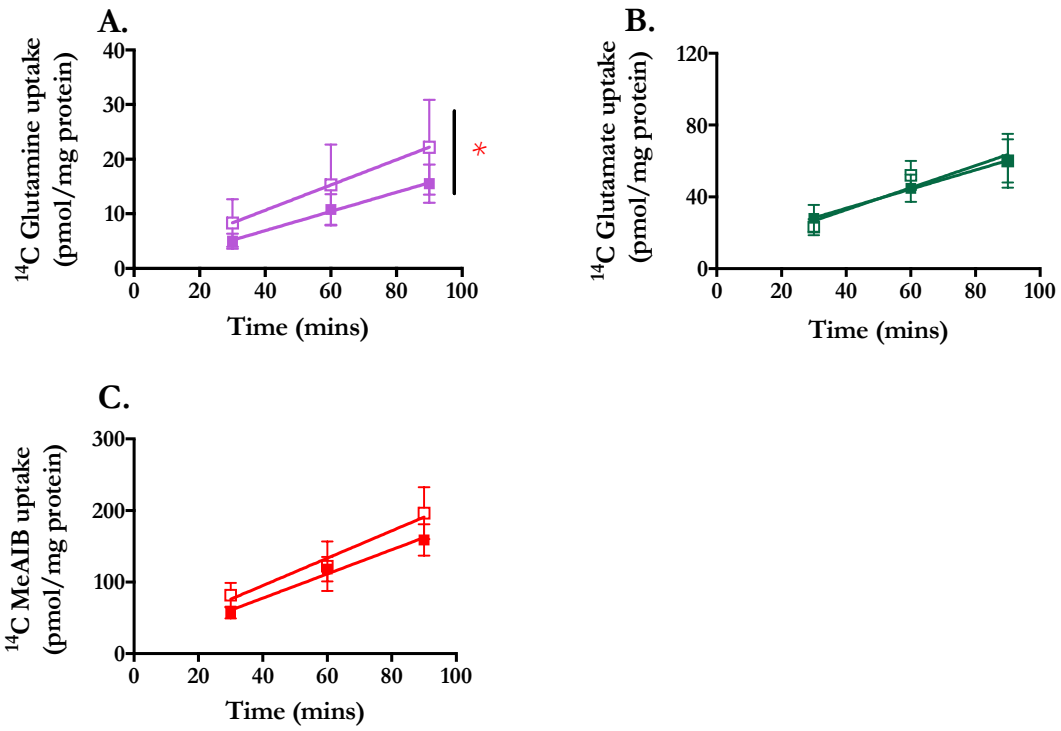


Figure 51 ^{14}C-glutamine uptake was significantly lower in placentas from female versus male placentas in FGR

Placental uptake of ^{14}C-glutamine (A) was significantly lower in placentas from female FGR babies (solid symbols, n=7) compared with placentas from male FGR babies (hollow symbols, n=3/4). There were no significant differences in ^{14}C-glutamate (B) or ^{14}C-MeAIB (C) uptake between groups. Data are mean±SEM * P<0.05 Linear regression.

6.3.3 Total amino acid availability for transfer to the fetus in placentas from normal (AGA) pregnancy and FGR

A proxy measure of placental amino acid availability for transfer to the fetus in normal pregnancy (AGA) and FGR was calculated as amino acid uptake at 90 min (per mg placental protein) x trimmed placental weight (g) (section 6.2.1). FGR placentas weighed significantly less than those from normal pregnancy (Figure 52A). Furthermore, placental amino acid availability (placental weight x amino acid uptake at 90 min (per mg protein) was significantly less in FGR compared to normal pregnancy (Figure 52B-D).

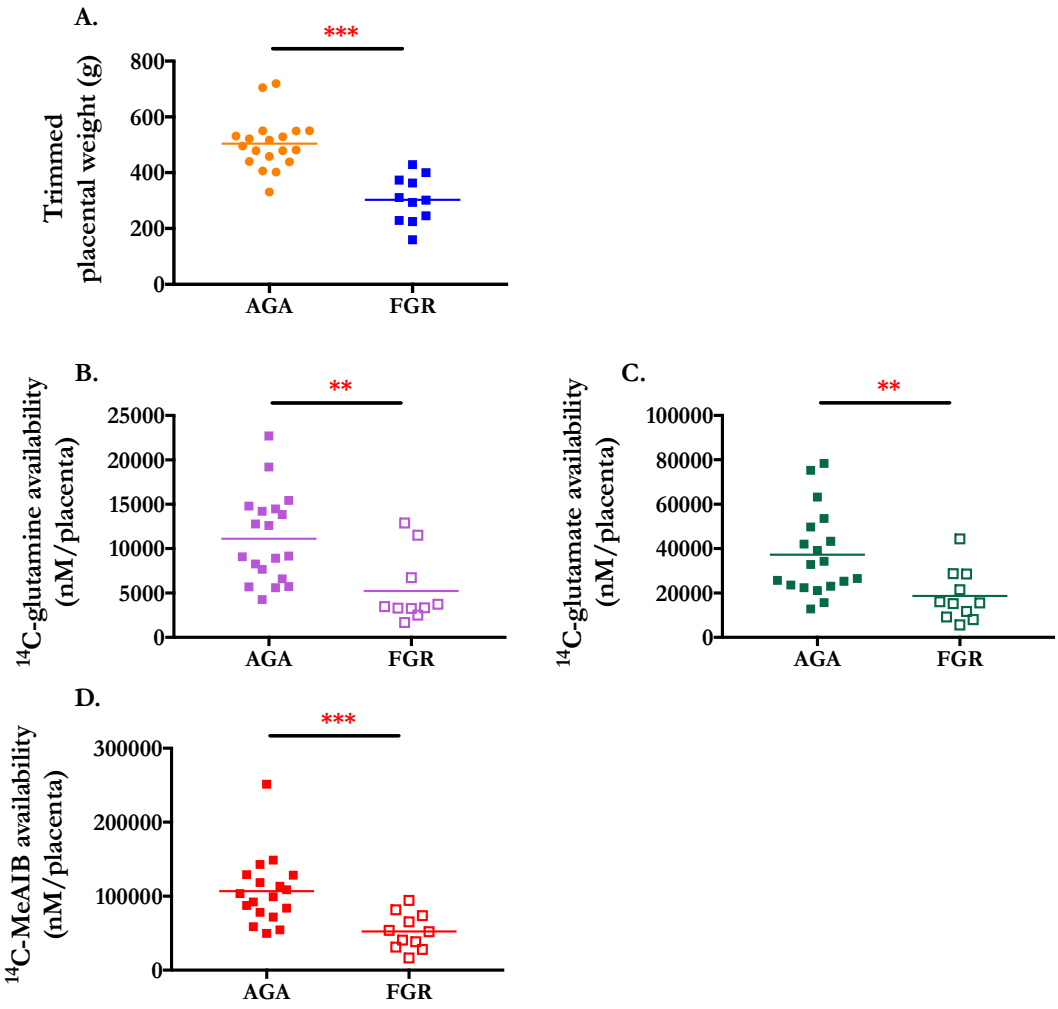


Figure 52: Amino acid availability at 90 min
(A) Placental weight was significantly lower in FGR compared to normal pregnancy (AGA). (B-D) Availability of placental amino acids for delivery to the fetus (placental weight x amino acid uptake/mg placental protein at 90 min) of ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB was also significantly lower in FGR compared to normal pregnancy (AGA). ** $P<0.01$ *** $P<0.001$ Mann-Whitney test.

6.3.4 Expression of amino acid transport proteins is higher in FGR compared with normal (AGA) pregnancy

Representative Western blots of glutamine (system L: LAT1, LAT2, system N: SNAT5) and glutamate (system X_{AG}^-: EAAT1, EAAT2) transporter protein expression in membrane-enriched placental isolates (IBR range: normal pregnancy 27.9-79.1, FGR 0-4) are shown in Figure 53. Bands detected at the predicted molecular weight (kDa) were validated by the inclusion of positive controls (MVM) during antibody optimisation and were quantified using densitometry. Data were normalised to β-tubulin expression (Figure 53), which was no different between groups (data not shown), the corresponding β-tubulin blots are shown beneath each protein of interest.

Expression of the system L transporters LAT1 and LAT2 was detected at the predicted molecular weights of 40 kDa and ~49 kDa, respectively. Under reducing conditions a 75 kDa

band was also present when probing for LAT1, as has been previously reported (Ellinger *et al.*, 2016). SNAT5 expression was present and analysed at the predicted molecular weight of 52 kDa. A second band at 40 kDa, as reported by the manufacturer, was also present.

The glutamate transporter (system X_{AG}^-) EAAT1 is predicted to have a molecular weight of 60 kDa, and previously published data have described the presence of multiple bands, at 50 kDa and 150 kDa (Martinez-Lozada *et al.*, 2014). The top band shown in the EAAT1 blot (Figure 53) was assessed as this is where the positive control (MVM vesicle isolate) was also localised (see also Figure 36 in Chapter 4). Expression of EAAT2 was present at the predicted molecular weight of 62 kDa.

All glutamine transporter proteins analysed (system L: LAT1, LAT2, and system N: SNAT5) were significantly higher in placentas from FGR versus normal (AGA) pregnancies (Figure 54C-E). It was not possible to analyse any system A transporter proteins, known to contribute to MeAIB and glutamine transfer, as there were no suitably validated commercially available antibodies. EAAT1 expression, of which glutamate is a substrate, was significantly higher in placentas from FGR versus normal birth weight infants (Figure 54A). EAAT2 expression was no different between groups (Figure 54B). The sex of the infant did not appear to influence transporter expression (placentas from male infants are denoted by hollow symbols in Figure 54).

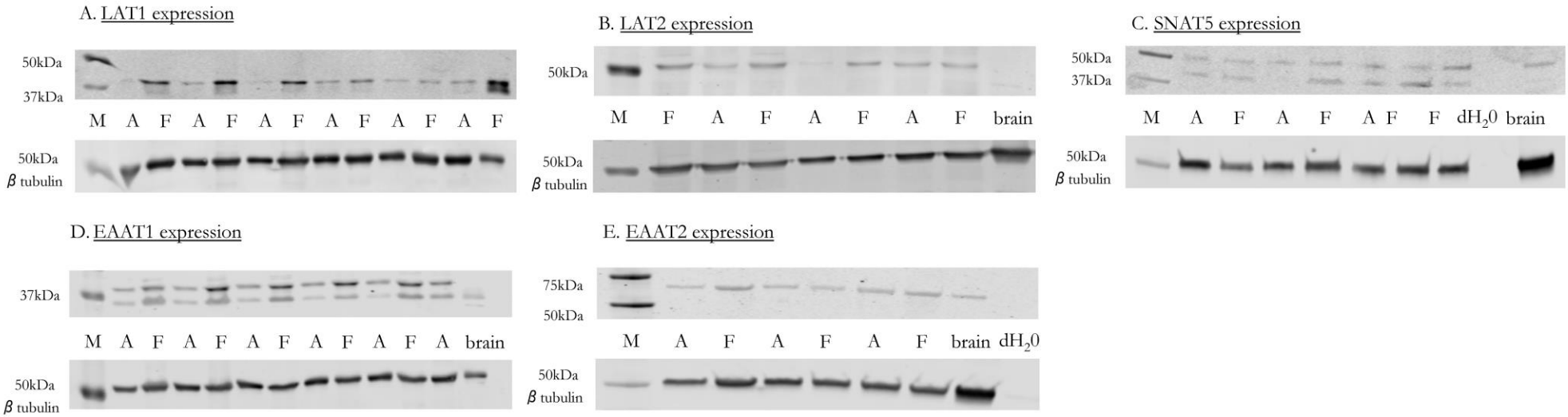


Figure 53: Representative blots illustrating expression of glutamate and glutamine transporter proteins
Shown are representative Western blots of glutamine (system L: LAT1, LAT2 system N: SNAT5) and glutamate (system X_{AG}-: EAAT1, EAAT2) transporter proteins in placentas of normal (AGA) and FGR infants. Corresponding Western blots for the housekeeping protein β-tubulin are shown below each blot. A= AGA, brain= mouse maternal brain, dH_20= deionised water, F= FGR, M= marker.

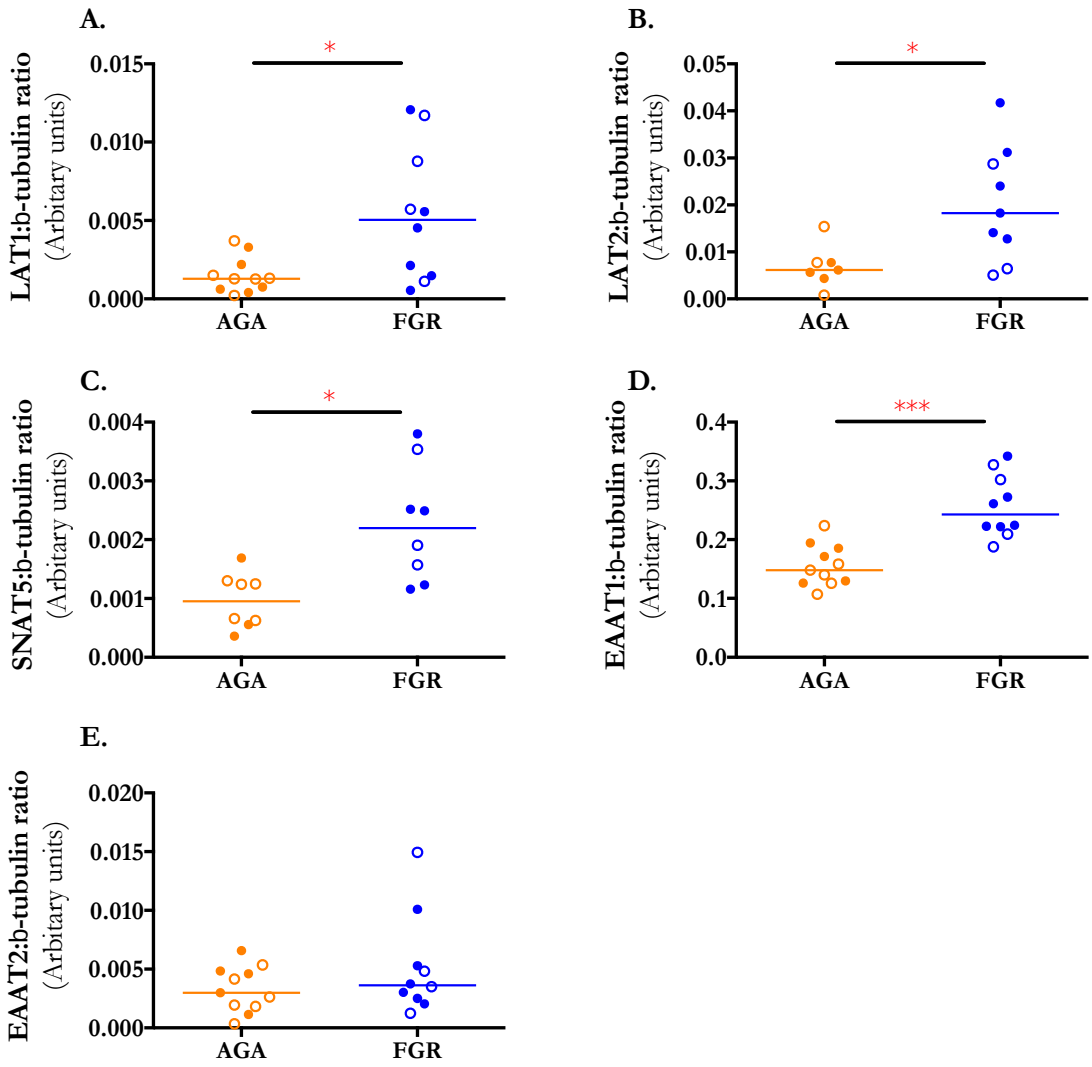


Figure 54: Expression of glutamine and glutamate transporter proteins: AGA versus FGR
Placental expression of the glutamine transporter proteins LAT1, LAT2 (system L) and SNAT5 (system N) was significantly higher in FGR. Placental EAAT1 expression (system X_{AG-}) was also significantly higher in FGR compared with normal pregnancy (AGA). EAAT2 expression was similar between groups. Hollow symbols represent placentas from male infants. AGA n=7/8/11; FGR n=8/9/10. * $P<0.05$ *** $P<0.001$, Mann-Whitney test.

6.3.5 Placental expression of mRNA for amino acid transporters is different in normal pregnancy (AGA) and FGR

The housekeeping genes TBP (TATA-box binding protein) and YWHAZ (tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein zeta) were stable across samples analysed (data not shown). Thus, gene of interest expression was normalised to the geometric mean of TBP and YWHAZ. The geometric mean controls for differences in relative gene abundance or expression, unlike arithmetic mean.

Expression of the gene that encodes the system A isoform SNAT2 (slc38a2) was significantly higher in placentas from FGR than AGA placentas ($P<0.01$) (Figure 55). Conversely, slc38a4 gene expression (encodes SNAT4 protein) was significantly lower when comparing placentas

from FGR and AGA pregnancies (P<0.01). There was a trend towards a significant increase in slc7a5 (LAT1) expression in placentas from FGR pregnancies (P=0.05). The sex of the infant did not appear to contribute significantly to these differences (placentas from male infants have hollow symbols).

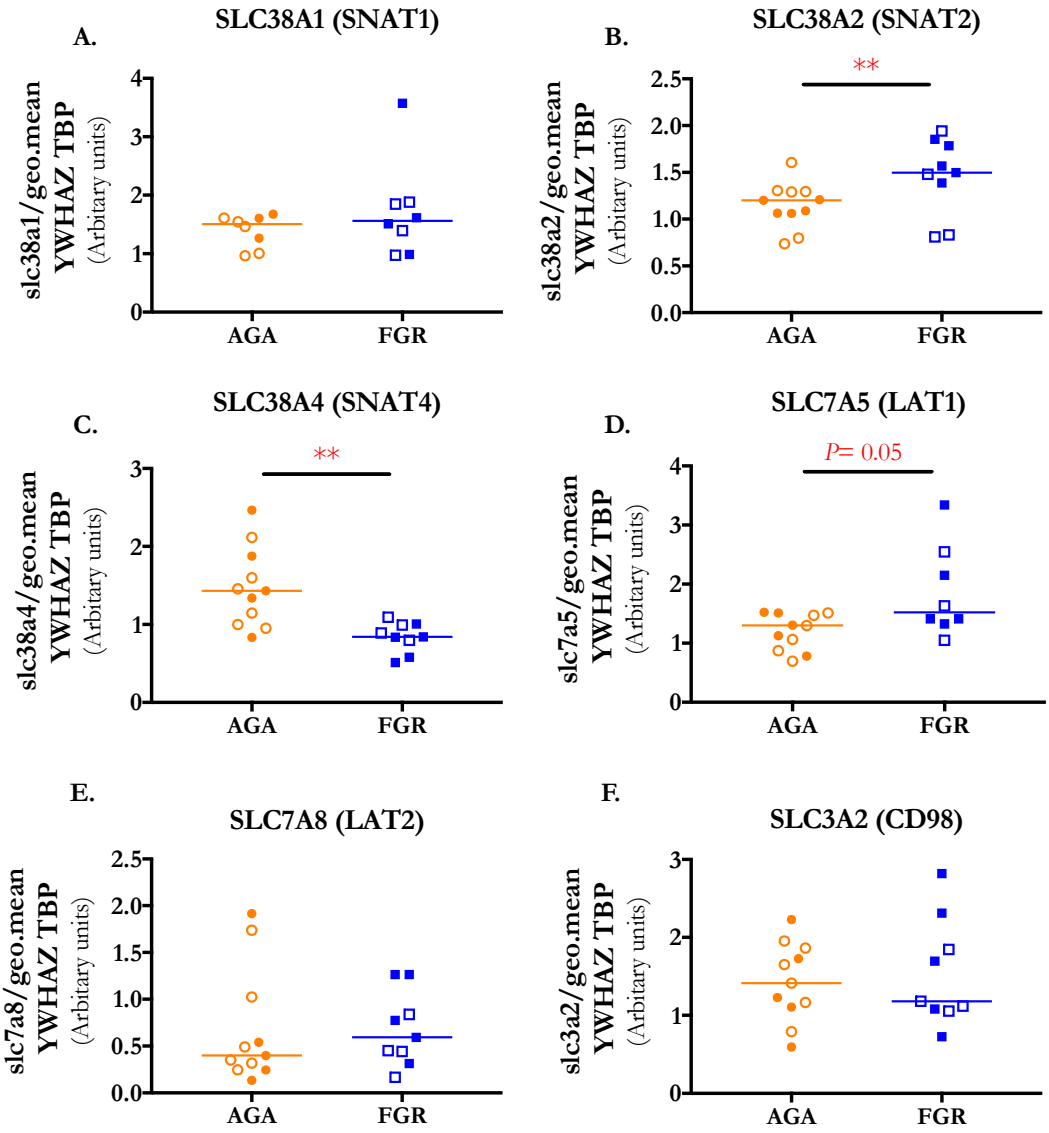


Figure 55: Gene expression of glutamine transporters: AGA versus FGR
Placental gene expression of slc38a1 (system A), slc7a5, slc7a8 (system L) and slc3a2 (CD98 heavy chain) (normalized to the geometric mean of YWHAZ TBP) was not different between normal pregnancy (AGA) and FGR. Expression of slc38a2 was significantly higher in FGR whilst slc38a4 expression was significantly lower, compared with normal pregnancy. Hollow symbols denote placentas from male infants. ** P<0.01 Mann-Whitney test.

6.3.6 Amino acid concentrations in maternal venous and fetal (umbilical) venous and arterial plasma in normal (AGA) and FGR pregnancy

Maternal venous, UmV and UmA plasma was collected, stored and analysed by HPLC to determine the concentration of amino acids in these samples. Patient demographics and the

total number of samples analysed are summarised in Methods (Table 25 and Table 26). To validate the method, duplicate UmV plasma samples from the same centrifuged blood sample were analysed. All replicate values (Figure 56) were within 6% of each other, with the exception of cystine (11%) and tyrosine (19%). All replicate samples were within the widely-accepted clinical threshold of 20%.

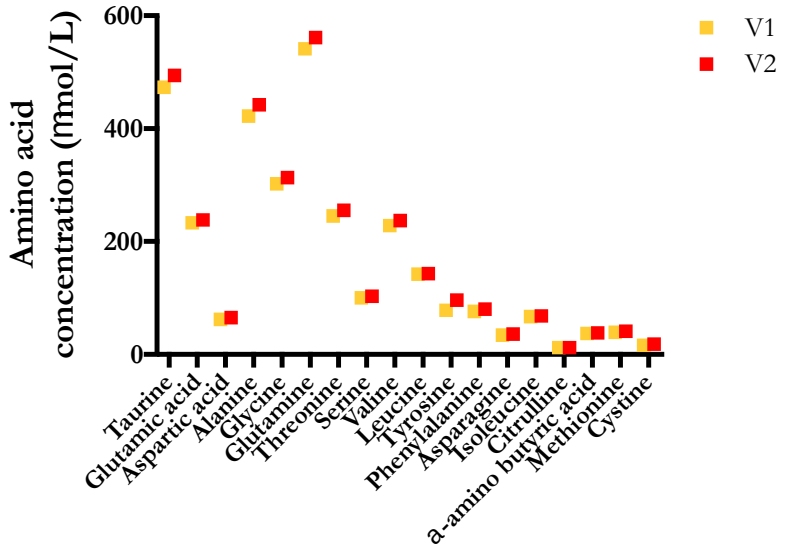


Figure 56 HPLC method validation
Amino acid concentration (μmol/L) in duplicate UmV samples collected from the same individual (V1 and V2). Amino acids are in the order of most to least abundant in the placenta according to Philipps *et al.* (1978).

The concentrations of glutamine, glutamic acid and alanine (a substrate of system A) in the maternal venous, UmV and UmA plasma in normal pregnancy and FGR are shown in Figure 57. Hollow symbols represent preterm deliveries (<259 days/37 weeks). Statistics were performed on all data combined; that is FGR term and preterm deliveries were pooled. With the exception of glutamic acid concentration in the UmA plasma, all statistically significant differences remained if preterm deliveries were excluded.

There were no significant differences in the concentration of glutamine in maternal venous, UmV or UmA plasma between normal pregnancy and FGR (Figure 57). Glutamic acid concentration was significantly higher in the UmA plasma in FGR compared with normal pregnancy ($P<0.05$) (Figure 57). The concentration of alanine was significantly higher in FGR in maternal venous ($P<0.01$), UmV ($P<0.001$) and UmA ($P<0.01$) plasma samples (Figure 57). The concentrations of all the amino acids quantified by HPLC (μmol/L) are stated in full in Appendix 9.6.

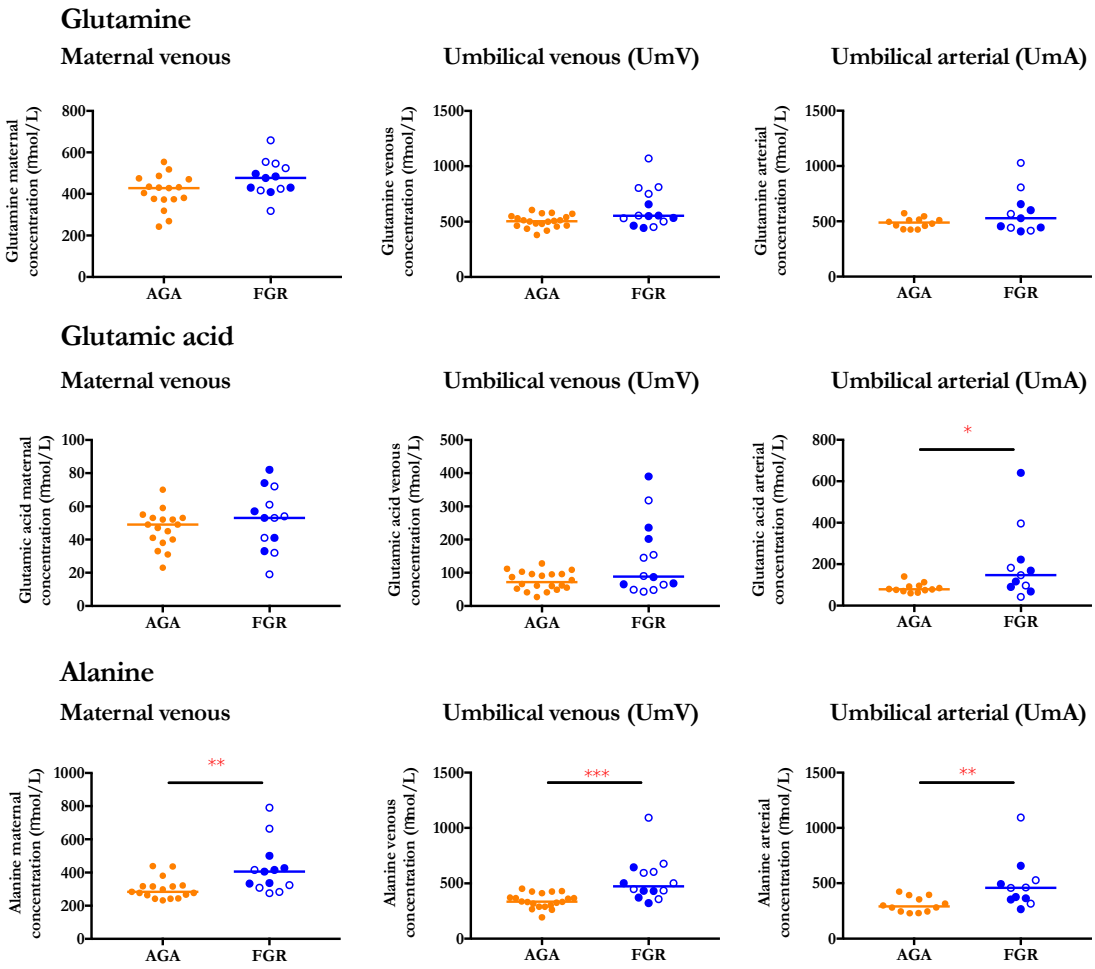


Figure 57 Glutamine, glutamic acid and alanine concentration in maternal venous, UmV and UmA plasma in normal pregnancy (AGA) and FGR

Amino acid concentrations (μmol/L) in maternal venous (n=17 AGA and n=13 FGR), UmV, (n=20 AGA and n=14 FGR) and UmA (n=11 AGA and n=11 FGR) plasma in normal pregnancy (AGA) and FGR. Hollow symbols represent preterm deliveries (FGR: 204-247days) * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Mann-Whitney test.

The concentration of glutamine, glutamic acid and alanine in matched samples obtained from normal pregnancy and FGR are shown in Figure 58. In normal pregnancy, the plasma concentration of glutamine, glutamic acid and alanine was significantly higher in the UmV compared to maternal venous plasma. In FGR, plasma concentrations of glutamine and glutamic acid were also higher in the UmV, but there was no difference in alanine concentration in maternal and UmV plasma. There were no differences in the concentrations of glutamine, glutamic acid or alanine in UmA and UmV plasma in normal pregnancy or in FGR, with the exception of alanine, which was lower in UmA than UmV plasma in normal pregnancy ($P<0.05$).

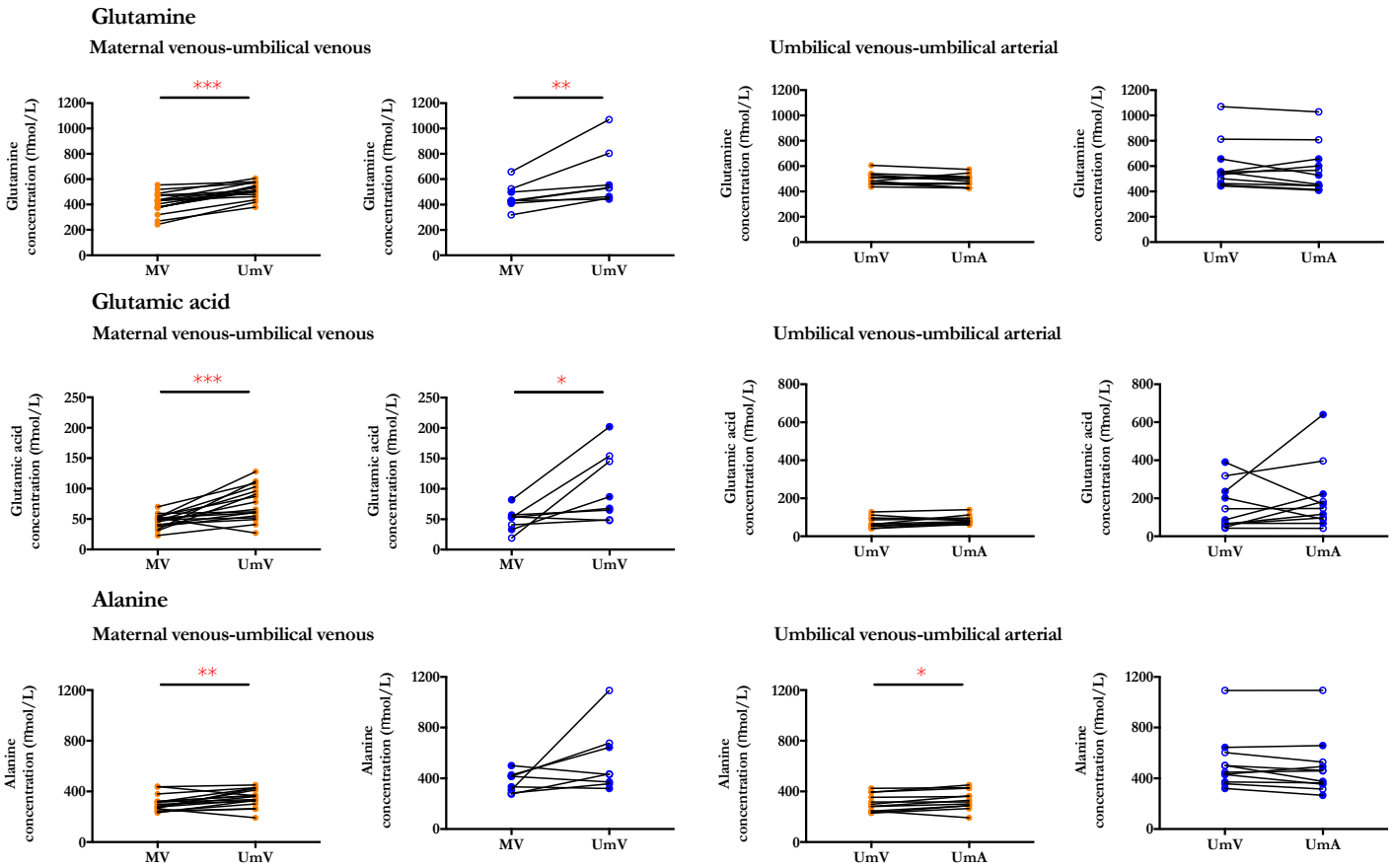


Figure 58 The concentration of glutamine, glutamic acid and alanine in matched samples drawn from the maternal-UmV, and UmV-UmA in normal pregnancy (AGA) and FGR. The concentrations of glutamine, glutamic acid and alanine are shown in matched samples (i.e. from the same individual) in normal pregnancy (AGA: orange data points) and FGR (blue data points). The y axis is set to the same scale to enable direct comparison between normal pregnancy and FGR. Maternal venous (MV) -umbilical venous (UmV) matched n=17 AGA and n=8 FGR. Umbilical venous (UmV) -umbilical arterial (UmA) matched n=12 AGA and n=11 FGR. Hollow symbols represent preterm deliveries. * $P<0.05$, ** $P<0.01$, *** $P<0.001$, Wilcoxon matched-pairs signed rank test.

Maternovenous and venoarterial differences of glutamine, glutamate and alanine (section 6.2.4) were compared between groups (AGA versus FGR) (Figure 59). A negative difference indicates that levels are higher in UmV versus maternal vein, or UmA versus UmV. The venoarterial difference of alanine was significantly different between normal (AGA) and FGR pregnancies ($P<0.01$). In normal pregnancy the venoarterial difference was negative, but it was positive in FGR (Figure 59B).

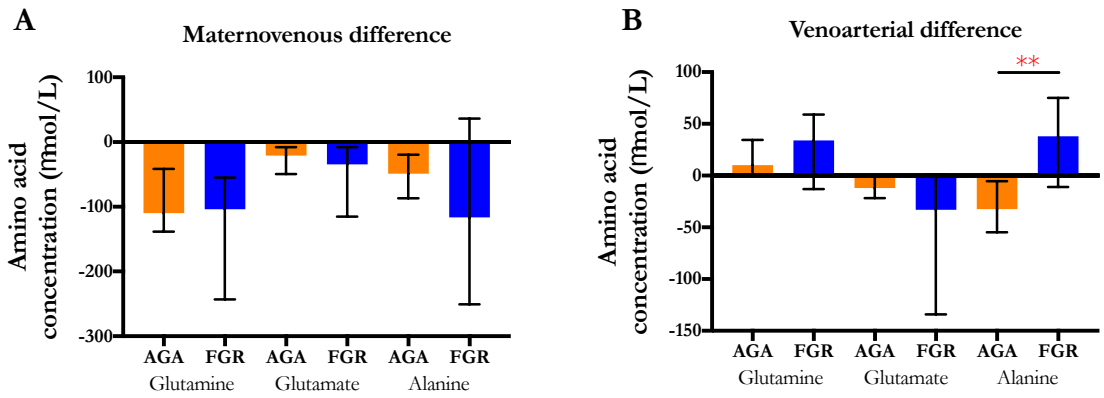


Figure 59 Maternovenous and venoarterial amino acid differences
The maternovenous (A) and venoarterial (B) differences for glutamine, glutamate and alanine in AGA (orange) and FGR (blue) pregnancies. Data are mean±IQR ** $P<0.01$ Mann-Whitney test.

6.3.7 Investigating the relationship between glutamine and glutamate uptake into the placenta and plasma concentration of these amino acids in mother and fetus (UmV and UmA) in normal pregnancy and in FGR

This study aimed to investigate the relationship between amino acid transporter activity (uptake) and amino acid concentrations in the maternal and fetal (umbilical) plasma. Glutamine and glutamate uptake at 90 min are plotted against the corresponding concentration (glutamine and glutamic acid) in maternal venous, UmV, and UmA plasma (Figure 60). The relationship between MeAIB uptake and concentration of alanine was also assessed as both are substrates of system A (Figure 60).

Due to the difficulties encountered obtaining sufficient matched samples, the numbers are too small to make definitive conclusions about these relationships. Currently, there are no correlations between glutamine, glutamate or MeAIB uptake at 90 min, and concentrations of glutamine, glutamic acid, or alanine, respectively.

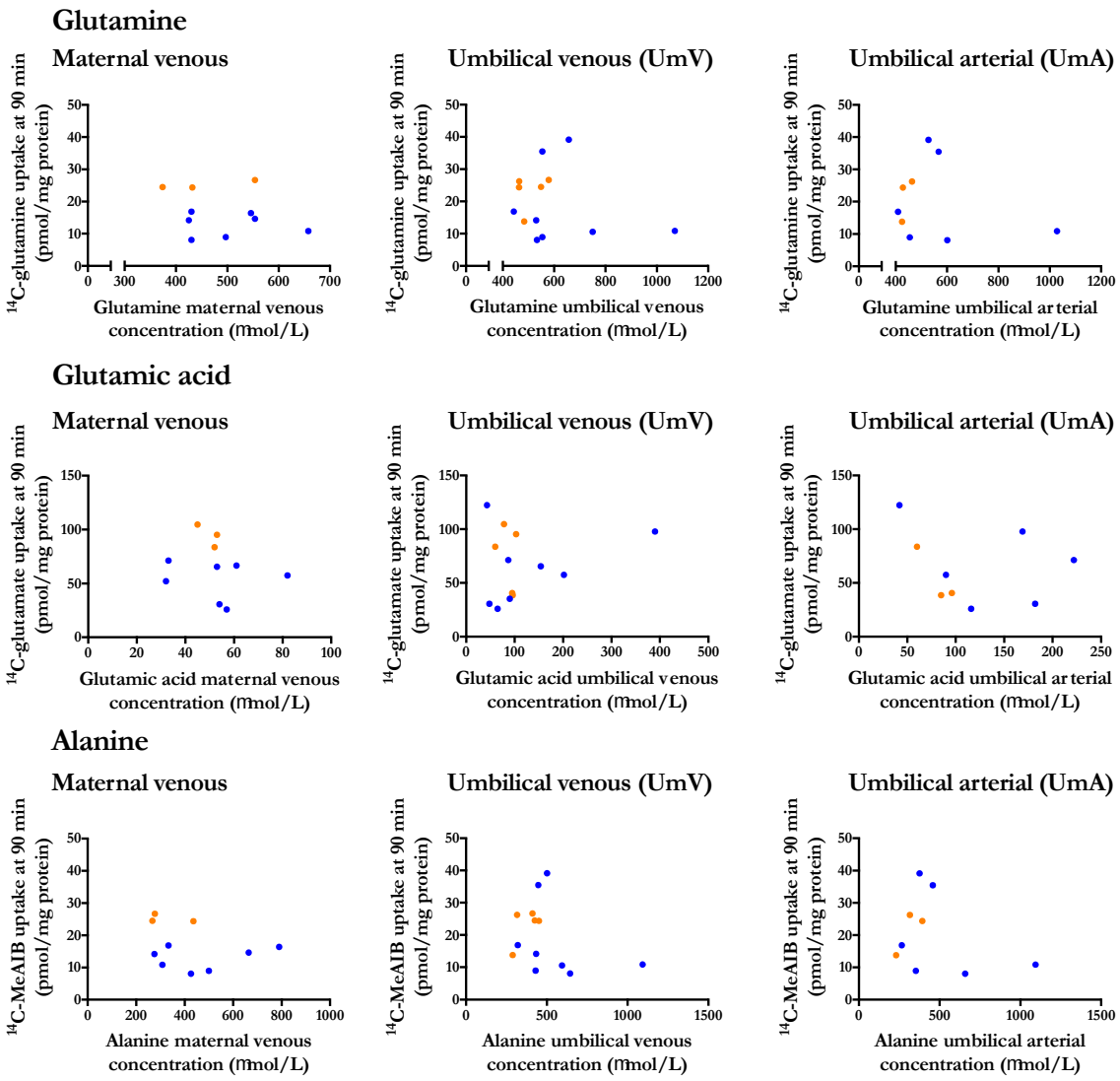


Figure 60 Glutamine, glutamic acid and alanine concentration in maternal venous, UmV and UmA plasma versus glutamine, glutamate and MeAIB uptake at 90 min

The concentrations of glutamine, glutamic acid and alanine are plotted against placental uptake of glutamine, glutamate and MeAIB, respectively. Samples from normal pregnancies (AGA, orange data points) and FGR (blue data points) are shown on the same graphs. Numbers are limited due to difficulties obtaining matched samples. Maternal venous n=3 AGA, n=7 FGR. UmV n=5 AGA, n=8 FGR. UmA n=3 AGA, n=6 FGR. There were no significant relationships between amino acid uptake and concentration. Linear regression.

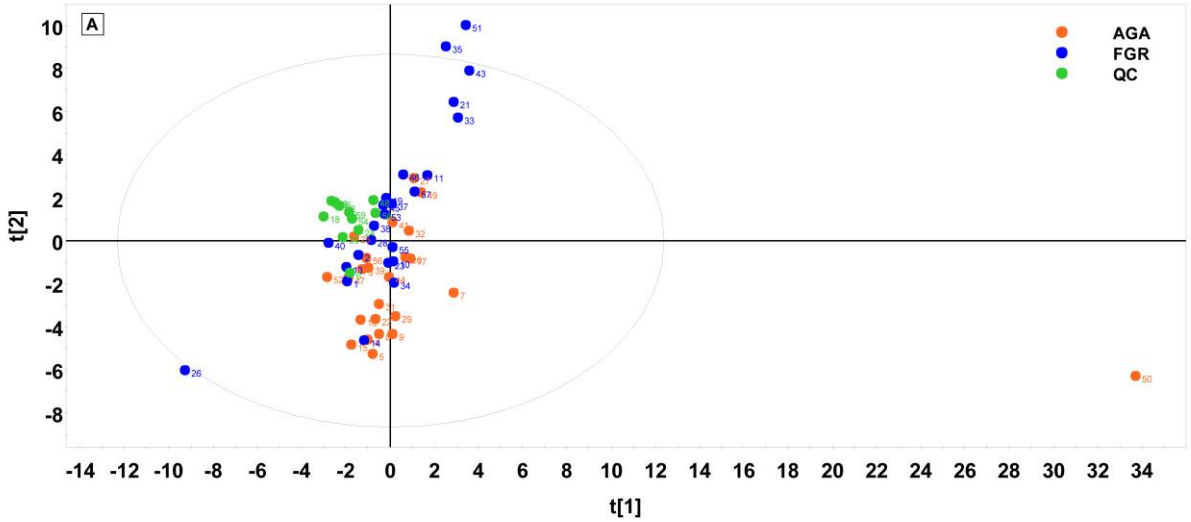
6.3.8 Gas chromatography-mass spectrometry (GC-MS) of UmV and UmA plasma samples from AGA and FGR pregnancies

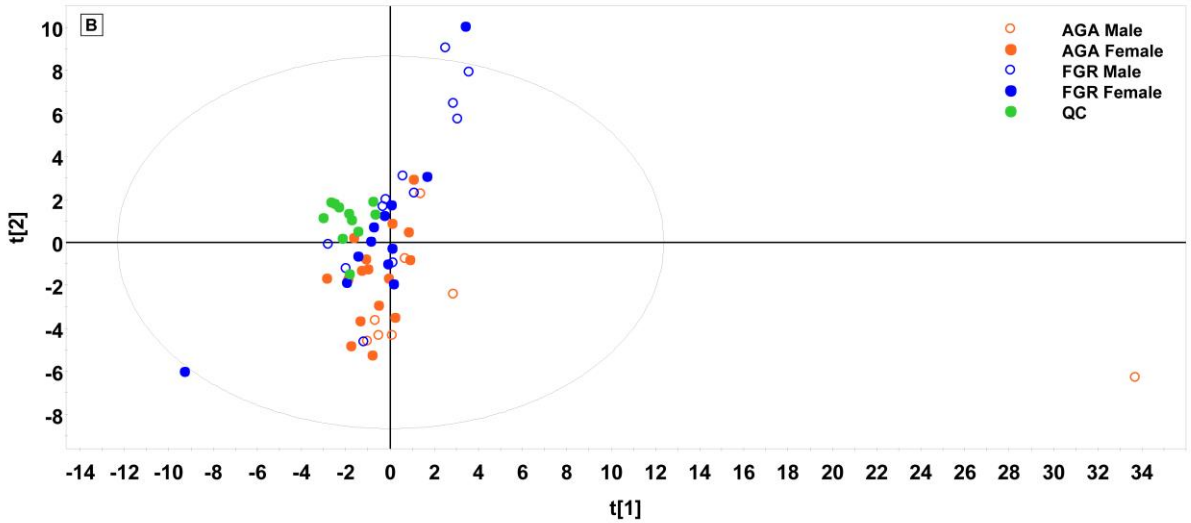
A principal components analysis (PCA) was performed to ensure the integrity of the experiment. During data extraction, the chromatogram of one UmV sample from the AGA group was identified as abnormal. The PCA plot confirmed this sample as an outlier (see Figure 61A, B; number 50 in the run order), and the sample was therefore excluded from data analysis. Run order, sex of the fetus, smoking status and gestation did not have a significant influence on the data set (Figure 61).

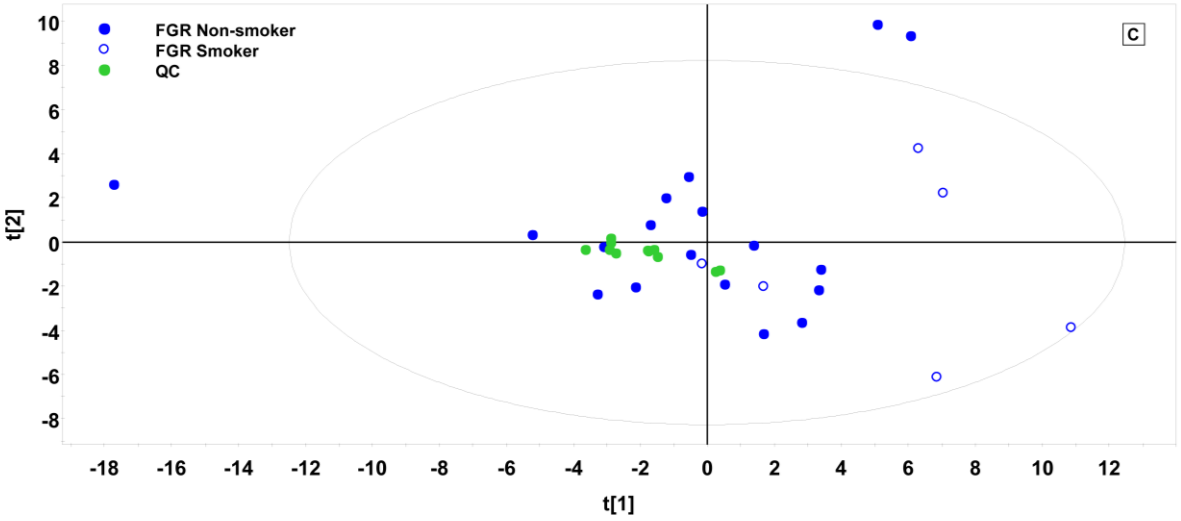
In the GC-MS analysis of paired UmV and UmA plasma samples from 12 normal (AGA) and 12 FGR infants (n=47, one sample excluded as described above), 63 metabolites were identified and quantified. Of these 4 (6%) in the UmA, and 12 (19%) in the UmV were significantly altered in FGR compared to normal pregnancy ($P<0.05$). Log_2 fold change differences (case over control, Log_2 transformed) between groups are visualised in the heat map (Figure 62), and differences are described as fold change differences (case over control) in text (see Appendix 9.7 for full details). Consistent with the HPLC data, alanine was increased (1.7 fold) in UmV and UmA plasma of FGR pregnancies. In the FGR UmV, lactic acid was increased by 2.1 fold. Glutamine was detected but unchanged between groups. Glutamate/glutamic acid was not detected. A full data table of GC-MS data, and a list of identified metabolites can be found in Appendix 9.7.

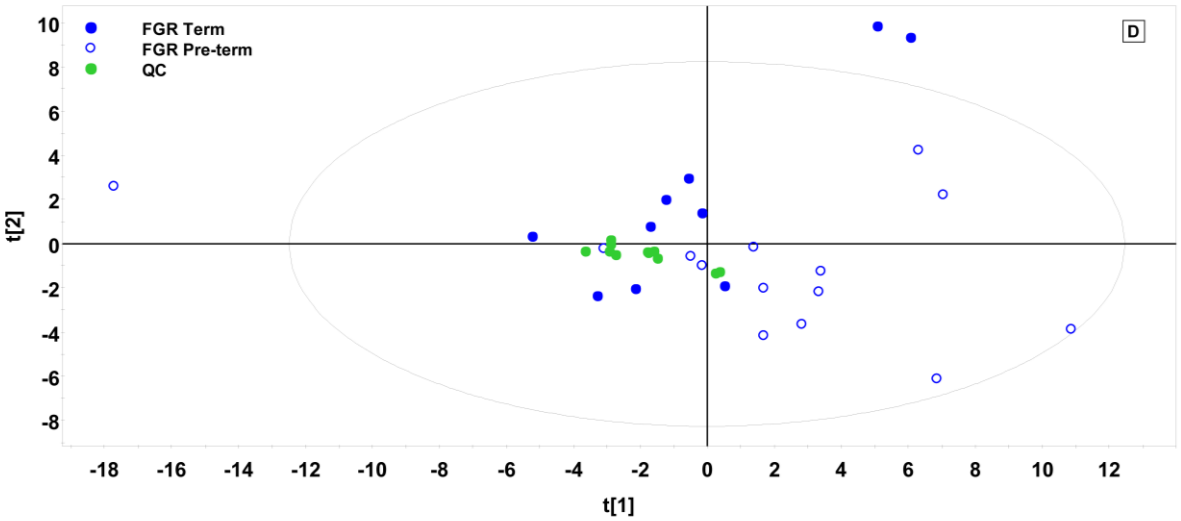
Figure 61 Principal components analysis (PCA) visualisation of samples from normal (AGA) and FGR infants (overleaf)

A principal components analysis was used to visualise the normal (AGA) and FGR groups, and to ensure the integrity of the experiment. (A) There was no bias according to run order of the samples, and the quality controls (QC) clustered together within the analysis. Potential confounding factors were also assessed by PCA: (B) sex of the fetus, (C) smoking status and (D) gestation (term or preterm) (C and D; FGR group only).


A
AGA
FGR
QC
t[1]
t[2]


B
AGA Male
AGA Female
FGR Male
FGR Female
QC
t[1]
t[2]


FGR Non-smoker
FGR Smoker
QC
C
t[1]
t[2]


FGR Term
FGR Pre-term
QC
D
t[1]
t[2]

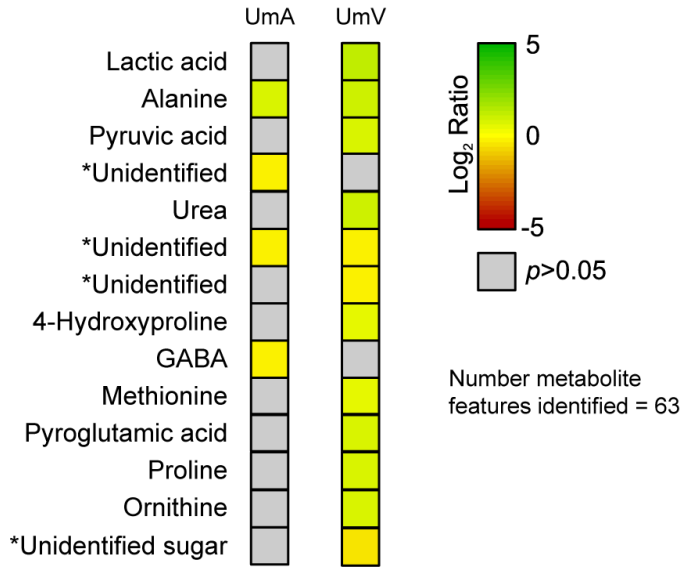


Figure 62: Heat map of log_2 ratio fold change data
Visualisation of log_2 fold change differences between normal (AGA) and FGR pregnancies in UmA and UmV samples. Metabolites were included in the heat map where significance (defined as a *P* value <0.05) was reached by Welch's t-test. Data were log_2 transformed after statistical analysis for expression as log_2 fold change and are shown on a red (-5 log_2 fold change) to green (5 log_2 fold change, yellow = no change, 0) scale. A positive log_2 fold change (green) denotes relative higher levels in plasma from case (FGR) versus control (AGA) pregnancies. *indicates a metabolite that has not been confirmed. A full list of the identified metabolites can be found in the Appendix 9.7.

6.4 Discussion

6.4.1 Placental weight is an important determinant of birth weight in normal pregnancy (AGA) and FGR

Trimmed placental weight was positively correlated with birth weight for normal (AGA) (P<0.05) and FGR (P<0.01) pregnancies (Figure 48), indicating that the size of the placenta is important for fetal growth/birth weight. The relationship between birth weight and placental weight in AGA and FGR is similar to that previously reported in both sheep and humans (Marconi *et al.*, 2006; Regnault *et al.*, 2013). The correlation was stronger for FGR, and statistically different between normal pregnancy and FGR groups (P<0.05), which suggests that placental weight is a stronger determinant of birth weight in FGR than normal pregnancies. However, gestational age range may contribute to this difference since all placentas from normal (AGA) pregnancies were collected at term (>37 weeks gestation) whereas 6/11 FGR infants were delivered pre-term (<37 weeks gestation). It would be pertinent to compare preterm FGR with a preterm control group, such as those with premature rupture of membranes, to establish whether the relationship is due to pathology (i.e. FGR) as opposed to gestational age. However, by definition, preterm controls are not strictly 'normal'.

Birth weight:placental weight ratio (BW:PW or F:P ratio) is a widely used proxy of placental efficiency, thus a higher BW:PW ratio is said to be representative of a more efficient placenta that supports relatively more birth weight per g placental weight. Mean BW:PW ratio in normal pregnancies was similar between AGA and FGR term groups but lower for FGR preterm groups (Table 24); however statistical power to assess these associations when splitting FGR according to gestation is currently too low. Whether a simple ratio such as BW:PW can be an appropriate proxy for placental efficiency is contested given that the regression between the 2 variables, birth weight and placental weight, does not pass through the origin; this means that BW:PW ratio changes along the regression line thus complicating interpretation (Christians *et al.*, 2018).

6.4.2 Transporter-mediated glutamine uptake is lower in FGR compared with normal pregnancies

For the first time, transporter-mediated glutamine and glutamate uptake was compared in placental villous fragments obtained from normal (AGA) pregnancies and pregnancies complicated by FGR. Glutamine uptake was significantly lower for placentas from FGR versus normal birth weight infants (Figure 49). System A activity was measured using the non-metabolisable synthetic analogue MeAIB, a specific substrate for the system A transporter; this was used as a positive control because system A is the most studied transporter in the human placenta and there is substantial evidence to show that system A activity is reduced in FGR (Glazier *et al.*, 1997; Jansson *et al.*, 2002; Mahendran *et al.*, 1993; Shibata *et al.*, 2008). Contrary to expectations, there was no significant difference in MeAIB uptake between placentas of normal pregnancies compared with FGR (Figure 49C).

Several studies that have reported reduced system A uptake have been performed using MVM vesicles (Glazier *et al.*, 1997; Jansson *et al.*, 2002; Mahendran *et al.*, 1993). There are advantages and disadvantages for both villous fragment and vesicle studies (see Table 5 in Introduction) (Glazier and Sibley, 2006; Greenwood and Sibley, 2006). Briefly, villous fragments maintain tissue architecture and associated cell signalling/driving forces whilst vesicles lack tissue integrity but can be used to study uptake across a specific plasma membrane (e.g. the syncytiotrophoblast MVM). However, in normal pregnancy, system A and L activity has been shown to be comparable when measured in MVM vesicles and villous fragments of the same placenta (Brand *et al.*, 2010). Roos *et al.* (2007) showed a significant reduction in system L activity in fragments in IUGR versus normal pregnancy, in line with MVM vesicle data (Jansson *et al.*, 1998). A reduction in system A activity has also been demonstrated in SGA ($<10^{th}$ centile) using fragments (Shibata *et al.*, 2008), in line with several studies that have shown lower system A activity in MVM vesicles in FGR versus normal pregnancy (Glazier *et*

al., 1997; Jansson *et al.*, 2002; Mahendran *et al.*, 1993). It was anticipated that the current study would reproduce these findings, but this was not the case. One inconsistency between studies in general is the criteria used to define both FGR (IUGR) and normal pregnancy (AGA).

In the current study FGR was defined as an individualised birth weight ratio (IBR) of less than the 5th centile using the GROW calculator that is based upon a UK population (https://www.gestation.net). At the time that this study began (2014) this was the definition commonly used by colleagues in the department and in the wider international field, and was therefore selected. Instead of IBR, previous studies have employed a cutoff of <10th centile of birth weight charts as a definition of FGR (Glazier *et al.*, 1997; Jansson *et al.*, 2002) and of SGA (Shibata *et al.*, 2008). Using a cutoff of the 10th centile will likely include a mix of constitutionally small (SGA) infants, that do not require any medical intervention, and pathologically small (FGR) infants. Mahendran *et al.* (1993) defined SGA as <3rd centile using previously published population growth charts (Gairdner and Pearson, 1971). Other studies chose to stratify FGR cases, sub-categorising into bins where group 1 = normal fetal heart rate and UmA pulsatility index, group 2 = normal fetal heart rate and abnormal UmA pulsatility index and group 3 = abnormal fetal heart rate and UmA pulsatility index (Glazier *et al.*, 1997; Jansson *et al.*, 2002). When umbilical blood flow was abnormal (i.e. group 2 or 3; as defined by UmA pulsatility index, +/- abnormal fetal heart rate) system A activity was significantly lower but interestingly, system A activity was no different compared with placentas from normal pregnancy when data from FGR infants was collated (Glazier *et al.*, 1997). However, in this study the effect of gestational age was not investigated by Glazier and colleagues; 13/16 FGR and 4/10 normal birth weight babies were delivered before 36 weeks (range 28-40 weeks) (Glazier *et al.*, 1997). In another study, Jansson *et al.* (2002) showed that system A was only reduced in the preterm FGR group (28-36 weeks) compared with preterm controls (delivered due to placenta previa or spontaneous preterm labour) and not FGR at term (37 weeks). Of note is that 4/19 of the FGR cases included in the study by Jansson *et al.* had co-morbidities: pre-eclampsia, diabetes or hypertension; the effect of these potential confounders were unknown (Jansson *et al.*, 2002).

Maternal smoking might influence system A activity (Appendix 9.5) but has not been investigated in the context of FGR. In a teenage cohort, maternal smoking does not affect villous fragment system A uptake (Hayward *et al.*, 2012). In the studies discussed above, smoking status was often left unreported (Glazier *et al.*, 1997; Jansson *et al.*, 2002). Shibata *et al.* (2008) excluded smokers from their study whereas 50% and 24% of mothers recruited in the SGA and AGA (normal pregnancy) experimental arms of the study by Mahendran *et al.* (1993) were smokers, although the proportion of smokers included in uptake experiments, and its effect, are unclear from the presented data.

Another discussion point of relevance to the current study is the definition of normal/AGA pregnancy to which the FGR data were compared. Several papers do not define the inclusion/exclusion criteria for normal pregnancy, and report only a mean birth weight value (Glazier *et al.*, 1997; Jansson *et al.*, 2002; Shibata *et al.*, 2008), or used a birth weight between 10^{th}-90^{th} centile, as opposed to IBR (Mahendran *et al.*, 1993). For the current study <5^{th} centile IBR was chosen to define FGR in recognition that IBR between 5^{th}-10^{th} likely includes many infants who are constitutionally small, as opposed to pathologically growth restricted (Unterscheider *et al.*, 2014). Similarly, infants with a IBR >90^{th} centile are classified as large for gestational age (Kramer *et al.*, 2001) and were not included here. Normal (AGA) pregnancy was therefore defined as between 10^{th}-90^{th} centiles, to give the widest range of 'normal' placental and birth weights, thereby allowing the hypothesis that adaptation occurs in relation to placental size (weight) to be tested (Chapter 4). However, it could be argued that a narrower range of normal (AGA) pregnancy might be more appropriate to ensure that exclusively 'normal' pregnancies are included in this group. It is likely that a borderline IBR, i.e. ~10^{th}/~90^{th} centile increases the likelihood of including infants that are small or large for gestational age. A recent cohort study in a Scottish population suggests that birth weight <25^{th} and >85^{th} centile is associated with a higher risk of adverse outcomes, such as stillbirth and infant morbidity (Iliodromiti *et al.*, 2017). Thus narrower, more stringent inclusion criteria, e.g. 20^{th}-80^{th} centile, may be more appropriate to compare between normal and FGR pregnancies. Conclusions from the current study are different if AGA is defined using the 20^{th}-80^{th} centile of individualised charts (Appendix 9.8); if this tighter definition is used uptake of MeAIB, glutamine and glutamate is significantly lower in FGR. However, omitting data between 10^{th}-20^{th} and 80^{th}-90^{th} centiles alters the balance of males and females in each group (from AGA 10^{th}-90^{th} n=18/19 of which 7 are male and 11/12 female, to AGA 20^{th}-80^{th} n=13/14 of which 7 are male and 6/7 female). In view of the data reported in Chapter 4, showing that placental uptake of glutamine and glutamate is significantly greater in AGA males than females, more studies are required to confirm that these findings are not an artefact of removing data from placentas of females only.

In the present study uptake of glutamine, a substrate of system A, L and N, was significantly lower for placentas from FGR infants compared with AGA (normal birth weight, 10^{th}-90^{th} centile IBR) (Figure 49A). The lack of a difference in system A activity (MeAIB uptake) between FGR and AGA groups (Figure 49C), suggests that systems L and N likely contribute to the difference in glutamine uptake. In MVM vesicles from FGR placentas, defined as a birth weight of mean - 2 standard deviations, system L-mediated leucine uptake was reduced (Jansson *et al.*, 1998; Roos *et al.*, 2007). In the current study, uptake of glutamate, mediated by

system X_{AG-}, was no different between groups (Figure 49B). System X_{AG-} activity has never before been reported in FGR.

When fitted to all the data (including both normal pregnancy and FGR) glutamine and MeAIB uptake at 90 min are positively correlated. However, when stratified according to pathology this relationship only exists for FGR, and not AGA, infants ($P<0.05$, Figure 50). This suggests that systems L and N contribute significantly to glutamine uptake in placentas from AGA infants, and supports the argument that system A contributes to glutamine uptake in FGR pregnancies, and that it may be systems L or N that are down-regulated in those cases. Similarly, there is a positive relationship between glutamine and glutamate uptake at 90 min for normal pregnancy ($P<0.01$) and FGR ($P<0.001$) (Figure 50), which suggests that there is a relationship between the uptake of these two amino acids.

6.4.3 Confounding variables that may influence transporter-mediated uptake

This study is limited by the practical difficulties in collecting placental tissue from FGR pregnancies without any potential confounding variables, that is from non-smoking women that delivered at term (>37 weeks). Smoking could have been a confounding variable for uptake experiments; 3/11 of mothers who had FGR infants smoked. Smoking status was self-reported by patients and not confirmed by cotinine testing. Due to the small numbers, it was not possible to statistically compare amino acid uptake between smokers and non-smokers; the data are shown in Appendix 9.5.

Jansson *et al.* (2002) previously reported reduced system A activity in preterm FGR (28-36 weeks) compared with preterm controls. As discussed above (section 6.4.1) 55% of the FGR infants in the current study were born preterm (<37 weeks) but in the current study there were no preterm 'normal' control placentas collected for comparison. The effect of gestational age could not be formally assessed statistically; the data are presented in the Appendix 9.5.

Evidence suggests that male and female fetuses have distinct responses to environmental stressors and adverse conditions *in utero* (Clifton, 2010; Di Renzo *et al.*, 2007) and that males are more likely to die during the neonatal period (Stevenson *et al.*, 2000). In Chapter 4 I reported that glutamine and glutamate uptake is significantly lower in placentas from female versus male infants. Likewise in FGR glutamine uptake was significantly lower in placentas of female versus male infants, however uptake of glutamate and MeAIB was similar between sexes (Figure 51, and Appendix 9.4). The numbers in these groups are still very small (n=3/4 males, n=7 females) and would benefit from larger numbers to determine whether there are differential effects of sex on placental amino acid uptake in FGR as those reported in normal pregnancy (Chapter 4).

6.4.4 Total amino acid availability for transfer to the fetus is lower for placentas from FGR compared with normal (AGA) pregnancy

FGR fetuses have smaller placentas than fetuses who are appropriately grown (Mifsud and Sebire, 2014). This finding was reproduced here: placentas collected from FGR pregnancies weighed significantly less than those from babies that were appropriately grown for gestational age (AGA) (Figure 52). A proxy measure of total amino acid within the syncytiotrophoblast theoretically available for exchange, to modify the amino acid pool, or for transfer to the fetus, was calculated as amino acid uptake at 90 min (per mg placental protein) x trimmed placental weight. Total amino acid availability for FGR placentas was significantly lower for glutamine, glutamate and MeAIB in comparison to AGA placentas (Figure 52). Reduced glutamine transport capacity (per mg placental protein) in combination with a smaller placenta therefore likely contributes to reduced glutamine availability for delivery to the fetus. Furthermore, these data indicate that despite the lack of difference in MeAIB and glutamate transport (per mg placental protein) in FGR versus normal pregnancy, there was still a reduced potential for transfer of these amino acids to the fetus because of small placental size.

6.4.5 Expression of key glutamine and glutamate transport proteins is altered in placentas from FGR compared with normal (AGA) pregnancy

Western blot analysis of membrane-enriched whole placental homogenates (Figure 53) revealed higher expression of LAT1, LAT2, SNAT5 and EAAT1 transporter proteins in FGR compared with normally grown (AGA) babies (in this group the AGA babies fell between the 20^{th}-80^{th} centile; Figure 54). EAAT2 expression was unchanged between groups (Figure 54). This is in agreement with previous reports that LAT1 expression, as analysed by immunohistochemistry, is increased in the MVM of the syncytiotrophoblast in FGR (Aiko *et al.*, 2014). This is the first study to show that system N and X_{AG-} (SNAT5 and EAAT1) protein expression is elevated in the placenta in FGR.

Differences in protein expression were not mirrored by changes in gene expression; mRNA expression of slc7a5 (LAT1, P=0.05), slc7a8 (LAT2) and slc3a2 (CD98), required for the functionality and shuttling to the plasma membrane of LAT1 and LAT2 transporter proteins (Wagner *et al.*, 2001), was not different between normal birth weight and FGR infants (Figure 55). Assessment of system A transporter proteins was not possible due to an absence of specific, commercially available and validated antibodies, but evaluation of gene expression showed that placental slc38a2 (SNAT2) expression was higher and slc38a4 (SNAT4) expression lower in FGR compared with normal pregnancy (Figure 55). However, SNAT4 is not considered to be a transporter of glutamine (Bröer, 2014). The expression of genes that

encode the transporter proteins SNAT5 (slc38a5), EAAT1 (slc1a3) and EAAT2 (slc1a2) should be investigated in future.

The disparity between mRNA and protein expression (summarised in Table 28) could be due to differential expression of small non-coding RNAs called microRNAs (miRs) in FGR. miRs are single-stranded RNAs that bind to target mRNA, subsequently repressing translation and protein synthesis (Bartel, 2004). It is not surprising that miRs have been implicated in disease processes such as cancer and cardiovascular disease (Cakmak *et al.*, 2015; Taylor *et al.*, 2013) given that miRs are thought to target more than 60% of coding genes in humans (Slack, 2011). A study by Mouillet *et al.* (2010) found that miR expression was reduced in placentas from FGR infants, but in the same pregnancies circulating miRs in the maternal plasma were raised. Speculatively, higher levels of miRs in the placenta of normal birth weight infants could lower protein translation in these pregnancies, and result in the comparatively higher expression levels of transporter proteins in placentas from FGR infants.

Transporter system		Gene	Protein	Activity
Glutamine	A	slc38a1 = slc38a2 ↑↑ slc38a4 ↓↓	?	= (MeAIB)
	L	slc7a5 = slc7a8 = slc3a2 =	LAT1 ↑ LAT2 ↑	↓?
	N	?	SNAT5 ↑	↓?
Glutamate	X_{AG-}	?	EAAT1 ↑↑↑ EAAT2 =	=

Table 28 Summary of key findings from experiments evaluating gene expression, protein expression, and transporter-mediated uptake

Arrows (↑↓) indicate a relative change in placentas from FGR compared with normal birth weight infants. ↑ corresponds to a *P* value of <0.05, ↑↑ *P*<0.01, ↑↑↑ *P*<0.001; same for ↓ arrows. = indicates that there was no difference between groups. ?were not evaluated in the current study and are therefore unknown.

The expression of transporter proteins important for uptake of glutamine and glutamate was higher in membrane-enriched isolates from the placentas of FGR infants. These data infer that transporter-mediated uptake would be increased in FGR, but this was not the case (section 6.3.1) (Table 28). Uptake of MeAIB and glutamate was unchanged and uptake of glutamine was significantly lower for placentas from FGR infants compared to normal birth weight babies. From the data presented in this chapter it is not possible to determine which glutamine transporter system/s contribute to this observed difference.

A potential reason for the disparity between transporter protein expression and glutamine and glutamate uptake (Table 28) is that transporter-mediated uptake was measured across the MVM using villous fragments, whereas protein expression was assessed in mixed membrane

isolates, which likely contained populations of both MVM, BM and other plasma membranes. It is important in future studies to confirm the exact location of these changes by using MVM and BM isolates and Western blotting. In addition, transporter-mediated uptake and transporter expression were evaluated per mg placental protein, which makes an assumption that each mg membrane protein is a proxy for amount of exchange surface area where transporters may be present. Furthermore, placental size is likely to influence the overall availability of amino acids for transfer to the fetus as it would be assumed that smaller placentas have less surface area available for exchange, and therefore overall fewer transporters due to less membrane, even though expression may not be different per mg protein (Figure 52). Another possible explanation is that increased protein expression is an adaptive response by the placenta in an attempt to facilitate appropriate delivery of nutrients to the fetus, thus also compensating for the small placental size, or that these proteins are not functional, which again raises the importance of understanding what regulates their activity in FGR.

Other factors such as post-translational modifications and substrate availability might also play a role in the regulation of transporter activity and thus amino acid uptake (see section 1.8). Experimental inhibition of the regulatory mTOR pathway using rapamycin leads to reductions in system A and L activity but no change in overall protein expression in cell lysates (Roos *et al.*, 2007; Roos *et al.*, 2009). However, studies have previously shown that mTORC1 activity is reduced in FGR in line with increasing abundance of NEDD4-2, which targets MVM-bound amino acid transporters for the proteasome for degradation (Chen *et al.*, 2015; Roos *et al.*, 2007) (see Figure 10). It would therefore be expected that transporter abundance be reduced, rather than increased in these cases.

Transporter abundance was assessed as a potential mechanistic explanation for changes in transporter activity. However, linear regression analysis revealed that there was no correlation between transporter protein abundance and protein activity in normal pregnancy and FGR (n=11 AGA, n=9 FGR) (Appendix 9.9). This suggests that protein abundance does not correlate with transporter activity, but at this stage it cannot be ruled out at this is due to the presence of other plasma membranes in the membrane-enriched isolates. However, protein abundance does not necessarily correlate with transporter activity; post-translational modifications such as phosphorylation can alter activity without altering expression. For example, previous studies have reported that activity of taurine transporter (TauT) is reduced in FGR, but expression in the MVM is unaltered (Roos *et al.*, 2004). To be certain of whether there are associations between transporter protein abundance and uptake, experiments using MVM and BM isolates would need to be completed and post-translational modification also explored.

6.4.6 Glutamine, glutamic acid and alanine concentrations in maternal venous, UmV and UmA plasma of FGR and normal birth weight (AGA) infants

The concentration of amino acids in the maternal vein, UmV and UmA of FGR and normal birth weight infants were quantified using HPLC. The method was highly replicable (Figure 56). The median concentration of alanine measured in maternal venous and UmV plasma from normal pregnancies were within a similar range to those reported by others previously (Figure 57) (Camelo *et al.*, 2004; Cetin *et al.*, 1996; Holm *et al.*, 2017). There is substantial variation in the literature in the reported concentrations of glutamine and glutamic acid in maternal and umbilical plasma (Camelo *et al.*, 2004; Economides *et al.*, 1989; Holm *et al.*, 2017). Indeed Holm *et al.* (2017) reported significant inter-individual variation within their comparatively large data set (n=179). Amino acid concentrations are dynamic and variations in the maternal, placental or fetal metabolic state at a given time could contribute to the range of values noted here (Figure 58). In addition, maternal samples were not taken under controlled conditions, that is, mothers were not uniformly fasted before maternal plasma was collected. It would be advantageous to standardise the method in this way in order to limit potential differences according to maternal metabolic state.

Within the FGR group several infants were delivered preterm (63%; Table 27) but from the small numbers available this did not appear to skew the data (Figure 57). Others have previously reported changes in amino acid concentrations with gestation (Economides *et al.*, 1989). Future studies would benefit from the inclusion of more numbers to enable power to subclassify and test this effect. The strength of the measurements reported here is that matched samples from the same individual were collected, making it possible to compare between concentrations in the maternal and fetal compartments from the same pregnancy. From a practical perspective it was difficult to coordinate the collection of maternal samples, and the small volume of blood available in the UmA, especially from FGR infants, made collection from this compartment challenging.

In every case, aside from alanine concentrations in FGR pregnancies, amino acid concentrations in UmV plasma were higher than in paired maternal venous plasma which confirms that glutamine, glutamate and alanine transport from maternal to fetal circulation occurs against a concentration gradient (Figure 58) (Camelo *et al.*, 2004; Holm *et al.*, 2017). Concentrations of glutamine and glutamic acid in both maternal and fetal umbilical (UmV/UmA) plasma in FGR demonstrated greater variation compared with normally grown babies (Figure 58). This variability was evident in both term and preterm infants. This may be explained, in part at least, by the fact that within the FGR group there are likely different phenotypes of FGR. For instance, some cases of FGR may present with abnormal uteroplacental or fetoplacental blood flow. Additionally, within our criteria for FGR, it is

possible that some infants may in fact be constitutionally small rather than growth restricted. However, the fact that the majority of our FGR cases were below the 3rd centile of IBRs suggests this is unlikely to be the case.

Exposure to elevated concentrations of substrates down-regulates amino acid transporter activity (Aerts and Van Assche, 2002; Jayanthi *et al.*, 1995; Parrott *et al.*, 2007; Roos *et al.*, 2006). Alanine concentrations in maternal venous plasma were higher in FGR versus normal pregnancies (Figure 57), as has been reported previously (Cetin *et al.*, 1990; Cetin *et al.*, 1996). It might be postulated that increased levels of alanine could lead to the down-regulation of system A in the MVM in FGR that has been shown by several laboratories (Glazier *et al.*, 1997; Jansson *et al.*, 2002; Mahendran *et al.*, 1993; Shibata *et al.*, 2008). However, this finding was not reproduced in the current study; system A activity (MeAIB uptake) was no different between FGR and normal pregnancies (Figure 49). Glutamine concentrations in maternal venous plasma were no different between FGR and normal (AGA) pregnancies (Figure 57), thus the availability of substrate is unlikely to underlie the reduction in glutamine transporter activity in the MVM (Figure 49).

Higher concentrations of the system A substrate serine, and of phenylalanine, tyrosine, and α-amino butyric acid in the maternal plasma of those with FGR infants were also observed (Appendix 9.6). Raised levels of phenylalanine and tyrosine have previously been reported by Cetin *et al.* (1996), however raised levels of leucine, isoleucine and valine were not replicated in the current study.

Previous studies have shown that the concentration of glutamine is reduced in the UmV of FGR infants (Cetin *et al.*, 1996; Ivorra *et al.*, 2012). The data presented here do not support these findings; glutamine and glutamic acid concentrations in the UmV were similar in FGR and normal pregnancy (Figure 57). However, alanine concentrations were significantly raised in the UmV of FGR infants (Figure 57).

Fetal supply of glutamine is from transplacental delivery of glutamine, and from glutamate metabolism within the placenta. The levels of amino acids such as glutamine and glutamate may play a role in facilitating the delivery of other amino acids through accumulative and/or exchanger proteins (Cleal *et al.*, 2007). In the UmA, glutamic acid and alanine concentrations were higher in FGR compared with normal pregnancy (Figure 57). This suggests either increased production of glutamate by the fetal liver, or reduced utilisation of glutamate by the fetus. There was no difference in UmA glutamine concentrations between FGR and normal pregnancies.

It has been suggested that reduced fetal metabolic rate leads to normal or higher than normal amino acid concentrations in the hypoxic FGR fetus (Regnault *et al.*, 2013). In the fetal liver glutamine is deaminated to glutamate through the enzyme glutaminase (see Figure 8 in

Introduction). Glutamate is converted to glutamine via glutamine synthetase or to α-ketoglutarate for entry to the TCA cycle by glutamate dehydrogenase. All three enzymes are predominantly expressed in the Langhans layer of cytotrophoblast cells under the syncytiotrophoblast, but only glutamate dehydrogenase has been found to be decreased in FGR (Jozwik *et al.*, 2009). This could alter the balance of glutamine/glutamate in the syncytiotrophoblast in FGR such that the glutamate/glutamine ratio will be greater in FGR compared with normal pregnancy. The combined spectral contribution of glutamine and glutamate (Glx) has previously been assessed in SGA (defined as abdominal circumference $<10^{th}$ centile at time of scan, confirmed by a birth weight $<10^{th}$ centile at delivery) and normal pregnancies using by ^{1}H MRS (magnetic resonance spectra). Macnaught *et al.* (2015) found that paired Glx/H_20 ratios were altered in SGA and speculated that this might be a marker of placental dysfunction that can be measured *in utero*.

The advantage of collecting paired samples from the same individual is the ability to evaluate matched maternovenous and venoarterial differences. A negative maternovenous difference (Figure 59A) shows that there is either active transfer of amino acids by the placenta from maternal to fetal blood or that the syncytiotrophoblast synthesizes these amino acids (or both). A negative venoarterial difference indicates that the fetus is producing the amino acid (Figure 59B). Amino acid concentrations in the UmV were higher than in the maternal vein in normal pregnancy and in FGR, as evidenced by a negative maternovenous difference, but the data were more variable in FGR (Figure 59A). The venoarterial difference for glutamine was positive, i.e. there was a higher concentration of glutamine in the UmV plasma than in UmA plasma. Consistent with the literature, the concentration of glutamate was higher in the UmA than the UmV (Holm *et al.*, 2017), but this was not affected by FGR. Alanine concentration was also higher in the UmA than the UmV in normal pregnancy, consistent with fetal production of alanine (Holm *et al.*, 2017), but this was not observed in FGR: there was a significant difference in the venoarterial difference in normal pregnancy versus FGR for alanine (Figure 59B).

UmV amino acid concentrations are a composite of transport from maternal circulation and placental metabolism, which may include the synthesis, degradation or transamination of amino acids. To partially address these determinants, it would be pertinent to relate glutamine and glutamate uptake with glutamine and glutamate (glutamic acid) concentrations as assessed by HPLC in the UmV plasma. The relationship between amino acid uptake and concentrations in the maternal and fetal (umbilical) compartments was assessed (Figure 60) but unfortunately this arm of the study was underpowered; only 35% of the plasma samples from normal pregnancy, and 53% from FGR, had matched measures of placental transporter activity. There was no correlation between glutamine, glutamate or MeAIB uptake at 90 min

and concentrations of glutamine, glutamic acid or alanine, in maternal or cord blood (UmV/UmA), respectively (Figure 60). An interesting observation was that for a given amino acid concentration in maternal venous plasma, uptake across the placental MVM was higher in normal pregnancy compared to FGR (Figure 60). This suggests that the response of amino acid transporters to substrate availability may be different in FGR compared with normal pregnancy but more numbers are needed to determine whether this is a robust finding (n=3). Worthy of note was that UmV plasma concentrations do not parallel concentrations in the placenta of normal or FGR pregnancies (Philipps *et al.*, 1978) (Figure 56, see also Appendix 9.10), which suggests a role of the BM as a limiting step for efflux, and/or that amino acids that are taken into the placenta are compartmentalised such that they are not accessible to BM transporters in the syncytiotrophoblast. One way to interrogate the relationship between the concentration of amino acids in the placenta and UmV further would be to combine these data and experiments with mathematical modelling. Using this approach, placental metabolism and efflux across the BM could be explored in greater depth. Whether levels of amino acids in the placenta are altered in FGR is currently unknown. It would be interesting to assess tissue concentrations of amino acids in the FGR placenta to subsequently relate to the net transfer of these amino acids in this pathology.

6.4.7 Gas chromatography-mass spectrometry (GC-MS) of umbilical venous and arterial plasma samples from AGA and FGR pregnancies

In this chapter, data from amino acid uptake experiments and HPLC were supplemented with metabolomics in order to provide further insight into physiological state in FGR. Untargeted GC-MS can detect amino acids, thereby helping to confirm HPLC findings, and also sugars and other small molecule metabolites that may give indicators of fetal physiological condition. 63 metabolites were detected by GC-MS and of these 4 (6%) in UmA and 12 (19%) in UmV were significantly altered ($P<0.05$) in FGR versus AGA (Figure 62). Run order, sex of the fetus, smoking status and gestation did not have a significant influence on the data set as shown by the PCA plots (Figure 61), thus giving confidence that differences seen were due to the presence of a FGR versus an AGA fetus, rather than the effects of potential confounders. In alignment with HPLC findings, alanine was significantly increased (1.7 fold) in UmV and UmA plasma of FGR pregnancies (Figure 62). Components of the urea cycle ornithine (1.4 fold), and urea (1.8 fold), which is a product of amino acid breakdown, were also increased in UmV plasma in FGR compared to AGA (Figure 62). Similar changes in the profile of the IUGR ($<10^{th}$ centile) UmV such as increased levels of uric acid, proline, methionine and glutamate, have been reported by others (Favretto *et al.*, 2012). In the present study, levels of pyroglutamic acid, a precursor of glutamate, were increased (1.5 fold) in UmV of FGR

pregnancies. Conversely, in UmA plasma there was a modest decrease (0.9 fold) in the glutamate precursor GABA (γ-aminobutyric acid). Increased levels of methionine, phenylalanine and tryptophan have been reported in placentas from SGA infants versus AGA cultured *in vitro* in reduced O_2 tension (1%, hypoxic, compared to 6%, normoxic) (Horgan *et al.*, 2010). In the current study methionine levels were raised in the UmV of FGR pregnancies 1.2 fold, but phenylalanine and tryptophan were unaltered between normal pregnancy and FGR when analysed by GC-MS. However HPLC detected no change in methionine concentrations but increased phenylalanine concentrations in the UmV/UmA (Appendix 9.6). Whilst perturbations in metabolite levels can influence metabolic pathways as illustrated in Figure 63, accumulation of metabolites themselves can also have direct toxic effects. Methionine is a precursor of homocysteine, and hyperhomocysteinemia caused by genetic abnormalities of methionine-homocysteine metabolism or a diet deplete in vitamin B6, B12 and folic acid is associated with FGR and cardiovascular disease (Aubard *et al.*, 2000).

Increased levels of proline in the UmV, and alanine in the UmV and UmA, were not expected since previous studies have reported decreases in the UmV of low birth weight infants compared with controls (Ivorra *et al.*, 2012). The current project differs from the study of Ivorra and colleagues (Ivorra *et al.*, 2012) in a number of important ways. A key difference is the inclusion criteria; in this thesis FGR was defined as an IBR $<5^{th}$ centile and normal birth weight (AGA) as an IBR between 10^{th}-90^{th} centiles, conversely Ivorra *et al.* (2012) compared the cord blood profiles of infants with an IBR $<10^{th}$ and controls with an IBR between the 75^{th}-90^{th} centile (Table 6). Furthermore, Ivorra *et al.* (2012) used ^{1}H NMR spectra to detect metabolites; this method is less sensitive than MS and it can be difficult to identify metabolites (Dona *et al.*, 2016).

Figure 63 overlays the metabolite changes detected by GC-MS in the FGR UmV and UmA onto cellular metabolic pathways. Most of the changes observed were in UmV plasma in FGR. Intermediate metabolites of the glycolysis pathway, pyruvic acid (1.6 fold) and lactic acid (2.1 fold) were increased in FGR UmV samples.

Lactic acid (lactate) in UmV plasma comprises lactate transferred across the placenta, as well as lactate produced by the placenta (Burd *et al.*, 1975). Raised lactate levels in the UmV could therefore reflect altered placental metabolism. Lactate is an important energy source for the fetus, contributing to fetal oxidative metabolism in cells that have a high oxidative capacity such as the fetal brain and skeletal muscle (Burd *et al.*, 1975; Settle *et al.*, 2006). Increased lactate levels in the UmV could be a mechanism by which a carbon source/substrate (lactate) is made available to the fetus for gluconeogenesis and other metabolic processes (Brooks, 1986; Settle *et al.*, 2006). In the current study, lactate levels in the UmA were not significantly different in FGR; taken together with the observation of raised levels in the UmV, these

findings imply fetal utilisation of lactate as an energy source by the FGR fetus. Lactate and pyruvate are both substrates of monocarboxylate transporters (MCT)1 and MCT4, present on the placental MVM and BM (Iwanaga and Kishimoto, 2015; Settle *et al.*, 2006). Uptake of lactate over the BM into the placenta is reduced in FGR, despite no difference in transporter expression (Settle *et al.*, 2006). Previous studies have determined that lactate concentrations in cord blood do not change over gestation (Bozzetti *et al.*, 1987; Nicolaides *et al.*, 1989) but there is evidence of increased lactate concentrations in the maternal vein, UmV and UmA in SGA/FGR (Marconi *et al.*, 1990; Nicolaides *et al.*, 1989; Pardi *et al.*, 1993).

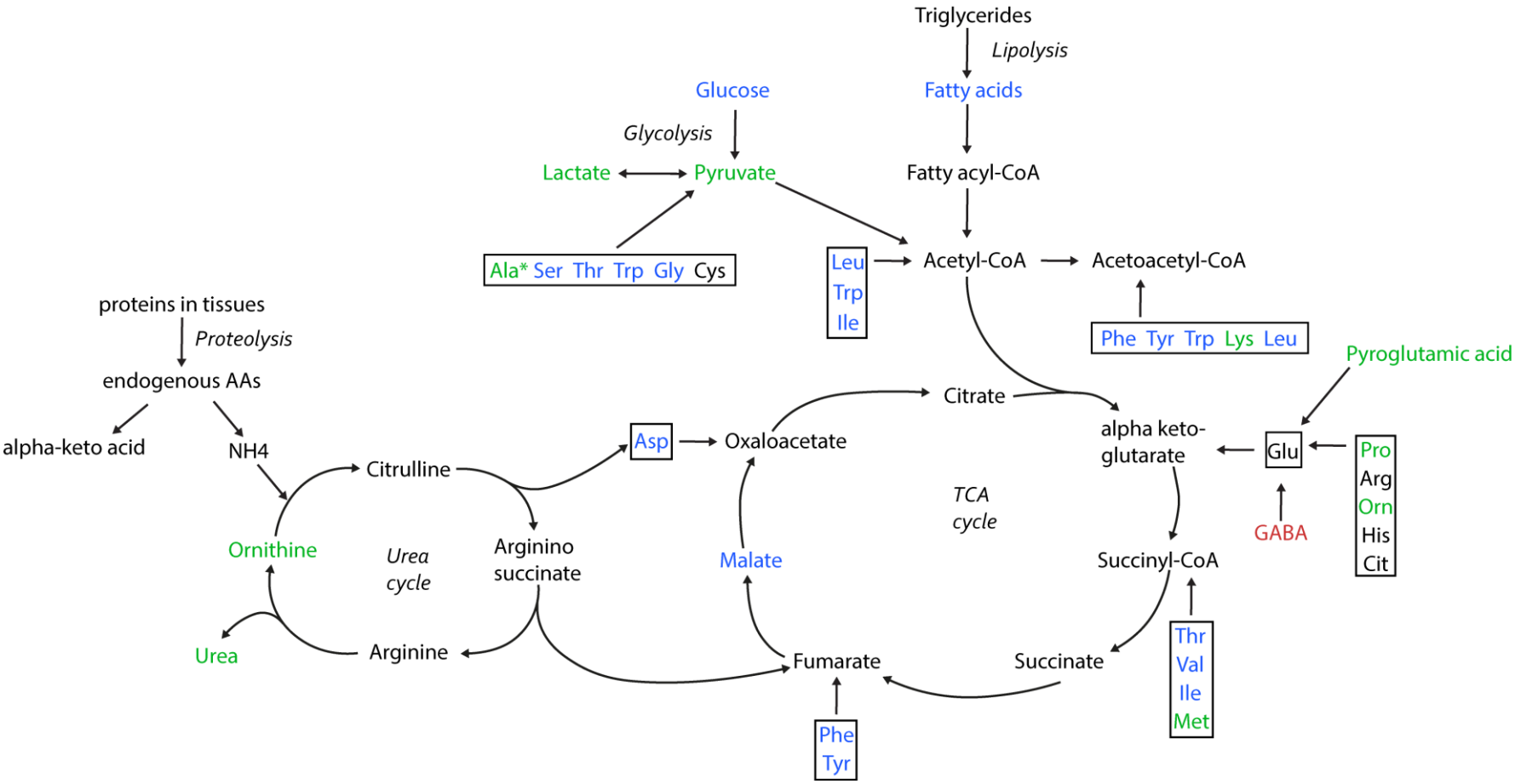


Figure 63 Altered metabolites associated with FGR

Metabolite changes are highlighted in the above schematic. Metabolites that were increased in the UmV in FGR are in green, * indicates those metabolites that were higher in both the UmA and UmV in FGR. Metabolites that were decreased in the UmA are red. Metabolites for which there was no significant difference detected are in blue. Unidentified metabolites are in black. Amino acids are denoted by their three letter abbreviation. Schematic has been adapted from (Zhao *et al.*, 2012).

The GC-MS data presented here complement HPLC analysis which showed that alanine concentrations were higher in FGR but other changes such as elevated phenylalanine concentrations in UmV/UmA, tyrosine in UmV and aspartic acid in UmA (Appendix 9.6) were not detected. Metabolomic analysis has provided additional insight into the metabolic status of the FGR infant and shows that there are distinct metabolic changes in cases of FGR (Figure 63). At this stage it is not possible to distinguish whether these differences are caused by, or are a consequence of the pathology of FGR. The aetiology of FGR remains poorly understood but these data suggest that aberrant control of these metabolic pathways might be a critical factor. However, it is not possible to predict how these cross-sectional measurements taken at delivery relate to the dynamic flux of metabolism during gestation.

6.4.8 Summary

FGR infants have significantly smaller placentas compared with normal birth weight (AGA) infants. A major finding from this chapter was that transporter-mediated uptake of glutamine by the small FGR placenta was lower compared with normal (AGA) placentas but uptake of MeAIB, in contrast to previous reports, was similar between groups (per mg protein). There was also no difference in placental uptake of glutamate between FGR and AGA.

Amino acid transporter protein expression was measured to investigate whether a reduction in expression could be a mechanism of reduced uptake. Contrary to this hypothesis, expression of the transporter proteins, LAT1, LAT2 (system L), and SNAT5 (system N), which mediate glutamine uptake was higher in FGR as was expression of the glutamate transporter protein EAAT1 (system X_{AG}-) in membrane enriched placental samples. The higher expression of amino acid transporter proteins in FGR placentas did not relate to the unchanged or reduced transporter activity. It may be that these transporters are not present on the MVM, since Western blots were conducted using mixed membrane isolates, or that they have been post-translationally modified so that their activity is reduced. Investigation of transporter localisation using MVM isolates, and immunohistochemistry of banked tissue from the same placentas is required to confirm the site of increased protein expression in FGR.

Examination of system A mRNA expression, in the absence of reliable commercially available antibodies, indicated that slc38a2 expression was higher but slc38a4 expression lower in placentas of FGR infants. There was no difference in the mRNA expression of the system A isoform slc38a1, nor any of the system L isoforms (slc7a5, slc7a8), or associated heavy chain (slc3a2) quantified. Thus, there is a disparity between the expression of transporters at the gene and protein level. This could be caused by post-transcriptional regulation by mechanisms such as microRNAs.

The concentration of alanine, a high-affinity substrate of system A, was significantly higher in maternal plasma in FGR. Whilst substrate regulation by maternal plasma levels of relevant substrates might down-regulate system L transporter activity on the MVM, by raised levels of substrates tyrosine, serine and alanine (Appendix 9.6), this does not appear to be the case for system A since MeAIB uptake was unchanged. Concentrations of alanine were also raised in UmV. Despite the reduction in glutamine uptake in FGR, the concentration of glutamine in the UmV was not different to normal pregnancy indicating that initial rate uptake is not directly related to provision of glutamine to the fetus in FGR. Glutamic acid concentration was raised in the UmA of FGR infants which suggests either increased metabolism from glutamine, or lower consumption of glutamic acid by the fetus. Altered glutamine/glutamate balance could be indicative of fetal condition (Macnaught *et al.*, 2015). Fetal physiological condition was further probed using GC-MS to measure relative levels of small molecule metabolites in the UmV and UmA of FGR and normal birth weight infants. Metabolomic analyses showed that lactic acid, pyruvic acid, urea and other metabolites were differentially altered in FGR, indicating potential alterations in placental metabolism and fetal utilisation of these metabolites. Changes uncovered in samples collected from FGR infants are worthy of further investigation in future. There were insufficient data to correlate uptake (transporter activity) and other determinants of amino acid transport (amino acid and metabolite levels) within the same pregnancy. Future research should focus on understanding (a) the disparities between transporter expression and activity, in particular why elevated protein expression in FGR is not associated with increased activity and (b) why uptake of amino acids over the MVM does not always relate to the concentrations of amino acids in the UmV in FGR.

Chapter 7 General discussion

7.1 Main findings

The work presented in this thesis tested two major hypotheses (1) that uptake (humans) and clearance (mice) of glutamine and glutamate adapts according to placental size (weight) in normal pregnancy to ensure an appropriate fetal supply of nutrients and (2) that these placental adaptations fail to occur in FGR.

Studies in WT mice provided evidence of functional changes (up-regulation) in unidirectional maternofetal clearance (K_{mf}, per g placenta) of glutamine and glutamate in the lightest placentas in a litter such that appropriate fetal growth was maintained. Elevated glutamine clearance (K_{mf}) was associated with significantly greater expression of the glutamine transporter LAT1. In contrast, there was no evidence of adaptation according to placental size in humans; placental transporter-mediated uptake of glutamine and glutamate was unrelated to placental weight in normal pregnancy. Analysis of the same cohort according to sex showed that glutamine and glutamate uptake was significantly higher in males. This sex difference in activity was not associated with a change in expression of glutamine and glutamate transporter proteins in membrane-enriched homogenates of male placentas.

In the P0 knockout mouse, a well-characterised model of FGR, K_{mf} of glutamine and glutamate (per g placenta), was higher compared to WT littermates in mid-gestation, and K_{mf} of glutamine remained higher the day before term (E18.5). However, this attempt to normalise delivery of nutrients to the P0 fetus, particularly for glutamine where total maternofetal transfer was reduced near term, was insufficient to rescue fetal growth. A major finding of this thesis was that transporter-mediated uptake of glutamine was lower in human FGR. However, Western blot analysis of membrane-enriched placental homogenates revealed that expression of key glutamine (LAT1, LAT2 and SNAT5), and glutamate transporters (EAAT1) were in fact significantly higher in placentas from FGR infants.

Amino acid concentrations were measured by high performance liquid chromatography (HPLC) in maternal venous, umbilical cord venous (UmV) and arterial (UmA) plasma to test the hypothesis that relationships between the concentrations in these compartments, and their association with uptake of glutamine and glutamate at the MVM, are altered in human FGR. The concentration of alanine (substrate of system A) was significantly higher in all three compartments in FGR compared to normal pregnancy and the concentration of glutamate was higher in UmA plasma in FGR. The strength of this arm of the study was that maternal and fetal (umbilical) plasma samples were measured in the same pregnancies; however, investigation of a direct relationship between amino acid concentration and glutamine/glutamate transporter activity was limited by insufficient numbers. Gas

chromatography-mass spectrometry (GC-MS) analysis of metabolites in UmA and UmV plasma detected 63 metabolites, 4 (6%) and 12 (19%) of which were significantly different in the UmA and UmV respectively in FGR compared with normal pregnancy. In common with the HPLC measurement of amino acids, alanine was significantly higher in the UmA and UmV in FGR. Levels of several amino metabolites for entry to the TCA cycle and products of the urea cycle, were also altered in FGR.

The remainder of this chapter will consider the implications of the major findings of this thesis in greater depth, and how the data contribute new knowledge to our wider understanding of placental nutrient transport in normal pregnancy and FGR. The limitations of this body of work and specific methodological considerations will also be discussed. Finally, this chapter will outline future directions for this research, designed to answer the new hypotheses that this study has generated.

7.2 Addressing differences between mouse and human

This thesis tested the hypothesis that the placenta adapts its transport capacity in relation to its size using *in vivo* mouse models, and *in vitro* techniques using human placental tissue. Placental nutrient uptake experiments can only provide a snapshot of placental function since delivery of the placenta is necessary for these studies. Animal models afford the opportunity to measure placental function *in vivo* at multiple gestational timepoints, thereby giving information on how potential functional adaptations may change over time.

There was no relationship between placental weight and transporter-mediated glutamine/glutamate uptake in human placentas from normal birth weight infants. However, there was evidence of adaptation when comparing the lightest versus heaviest placentas in a WT mouse litter. Studies in human FGR revealed reduced transporter-mediated uptake of glutamine compared with placentas of normal birth weight (AGA) infants. Conversely, the P0 mouse model of late-onset FGR demonstrated significantly higher K_{mf} of glutamine compared with WT littermates at both gestational ages (E15.5 and E18.5), which is indicative of adaptation to meet fetal nutrient demands. This effect was not universal: K_{mf} of glutamate was similar between P0/WT placentas towards term (E18.5). Previous studies in the same mouse model have shown that K_{mf} of MeAIB, reflective of system A-mediated uptake, is higher in mid-gestation (E16) (Constância *et al.*, 2002). The 11β-HSD2^{-}/$^{-}$ mouse shows a similar trend for MeAIB to the P0 mouse, at E15 fetal weights are similar, and K_{mf} of MeAIB higher compared with 11β-HSD2^{+}/$^{+}$ mice, but by E18 this adaptation is lost in parallel with reduced placental and fetal weights for 11β-HSD2^{-}/$^{-}$ mice (Wyrwoll *et al.*, 2009). Conversely, another mouse model of FGR, the eNOS^{-}/$^{-}$mouse, shows reduced system A activity (K_{mf} of MeAIB)

at E17.5 compared to WT (Kusinski *et al.*, 2012) suggesting that the transporter-specific adaptations in the P0 mouse are not universal to all mouse models of FGR.

The differences between placental transport of glutamine/glutamate in normal pregnancy and in FGR, in mouse versus human in the current study, need to be placed in the context of key differences between the methods used in each species. The transporter-mediated component of MeAIB, glutamine and glutamate uptake across the MVM was assessed in human placental villous fragments, representative of uptake into the placenta. Conversely in mice, unidirectional maternofetal clearance (K_{mf}) of a radiolabeled substrate relative to placental weight (per g placenta) was assessed. This measurement is based upon radiolabel counts within the fetus i.e. the transport of the injected substrate into, and subsequent release from, the placenta, and therefore includes as yet undetermined mechanisms of transfer across the fetal-facing BM and any equilibration with cellular amino acid compartments. Thus the data presented in this thesis in human placental tissue, representing uptake (Chapter 4 and Chapter 6), are not directly comparable with those in mice (Chapter 3 and Chapter 5). Potential metabolism of radiolabelled glutamine and glutamate, once within the syncytiotrophoblast, is considered in a later section (section 7.4).

In terms of comparing studies between women and mice, apart from key methodological differences between uptake and clearance, there are also key species differences to consider. Whilst mice offer a controlled environment and means to investigate at multiple gestational timepoints, they also give birth to multiple, altricial young after a relatively short gestation (~20 days) in comparison to humans. Another key difference between species to be considered is the intraplacental yolk sac (IPYS). The human yolk sac plays an important role in early embryonic development (up to ~10 weeks of gestation) (Freyer and Renfree, 2009) whereas in mice, the IPYS contributes to normal fetal growth towards term (Croy *et al.*, 2015), although the exact function of the IPYS is not yet known. Furthermore, the human placenta is haemomonochorial whereas mice have a haemotrichorial placenta. Layer II of the mouse placenta is judged to be akin to the human MVM since both stain positively with alkaline phosphatase, and transporter proteins have been localised to this layer (Jones and Fox, 1976; Kusinski *et al.*, 2010). Layer III is thought to be analogous to the human BM but this has not be confirmed since there is currently no published method to isolate layer III of the mouse placenta (Dilworth and Sibley, 2013). However, these structural differences do not impact upon passive permeability since permeability × surface area product (P×S) is similar between species (Knobil and Neill, 2006; Sibley and Boyd, 1988). Additionally, studies in vesicles from the mouse and human placenta have shown that activity of the system A amino acid transporter, determined using MeAIB, is similar (per g placenta) (Kusinski *et al.*, 2010).

7.3 Potential mechanisms driving changes in amino acid transport in mouse and human

Much of the research focussed upon human placental nutrient transport processes has investigated substrate specificity and localisation of relevant transporter systems/proteins to the MVM or BM of the syncytiotrophoblast. There is much less of an understanding of the underlying regulatory mechanisms that govern expression and subsequent function of these proteins, which may have clinical benefit as therapeutic targets. Amino acid transporter abundance and activity are determined by the regulation of RNA transcription, subsequent translation into protein and cellular localisation, including appropriate trafficking to the plasma membrane (Vogel and Marcotte, 2012). Potential regulatory mechanisms that may mediate changes in activity of transporters important for glutamine and glutamate transport in mouse and human pregnancy are discussed in the section herein.

This thesis revealed a mismatch between transporter protein expression in membrane-enriched homogenates and transporter activity (uptake) in placentas from FGR infants, and in placentas from male versus female babies (Chapter 4 and Chapter 6). Chapter 4 demonstrated that transporter-mediated uptake of glutamine and glutamate was higher in placentas from male babies compared with those from female infants, however transporter abundance did not differ between sexes. That the male and female placenta employs different strategies to support fetal growth is now well described (Clifton, 2010; Eriksson *et al.*, 2010). Males exhibit faster growth *in utero* and could be more sensitive to fluctuating availability of substrates to support growth (Brett *et al.*, 2016; Clifton, 2010; Eriksson *et al.*, 2010). Several lines of evidence support a role of adaptive regulation of transporters (e.g. systems A and L) in response to amino acid availability leading to an up- or down-regulation of transporter function (activity) (Aiko *et al.*, 2014; Jones *et al.*, 2006). Amino acid deprivation can provoke both rapid redistribution of proteins to the plasma membrane from an intracellular compartment and long-term up-regulation of gene transcription (Mackenzie and Erickson, 2004). Amino acid deprivation also increases system X_{AG-} activity, which transports glutamate, in kidney cells (Plakidou-Dymock and McGivan, 1993). Future studies to establish an understanding of the mechanisms that regulate transporter activity in relation to sex are therefore required both in normal pregnancy and in FGR.

Placentas collected from FGR infants exhibited reduced uptake of glutamine compared with normal birth weight infants (Chapter 6). In the current study, pilot data indicated that ~80% of total transporter-mediated glutamine uptake in normal pregnancy was via systems A and N, the remaining ~20% was attributed to system L (Chapter 4). Future studies could establish the contribution of system N to glutamine uptake using the specific inhibitor glutamate-γ-monohydroxamate (Yamakami *et al.*, 1998). System A is the best-described amino acid

transporter system in the placenta but, contrary to expectations, activity of system A (MeAIB uptake) was no different between placentas from FGR and normal birth weight infants in the current study (Chapter 6). The abundance of transporters important for glutamine transport (LAT1, LAT2 and SNAT5) was higher in placentas from FGR infants despite no change in expression of transcripts assessed at the mRNA level (slc7a5, slc7a8 and slc3a2). MicroRNAs (miRs) regulate mRNA transcript stability by binding to target mRNAs and repressing translation and protein synthesis (Bartel, 2004). miR-363 and miR-149, for example, target transcripts of amino acid transporters such as SNAT1, SNAT2, and LAT2 (Thamotharan *et al.*, 2017) and previous studies have reported reductions in overall miR expression in placentas from FGR infants (Mouillet *et al.*, 2010). Differential expression of miRs may underlie the disparity between mRNA and protein expression in FGR. In the context of the results presented in this thesis, reduced miR expression in placentas complicated by FGR could lead to relative increases in protein expression compared to placentas from normal birth weight infants. Higher transporter abundance may be an attempt by the smaller placenta in FGR to rescue suboptimal glutamine uptake, and/or transporter function may be altered by post-translational modifications. Post-translational modifications such as ubiquitination and glycosylation influence membrane targeting, substrate binding and stability of key transporter proteins (Chen *et al.*, 2015; Czuba *et al.*, 2018; Vogel and Marcotte, 2012). For example, disruption of glycosylation sites on the OAT4 transporter, that mediates glutamate efflux on the BM (Lofthouse *et al.*, 2015), results in the protein being trapped in the intracellular compartment rather than being transported to the plasma membrane (Zhou *et al.*, 2005).
The nutrient sensing pathway mTOR is another candidate mechanism, known to down-regulate system A and L activity in cytotrophoblasts by reducing transporter insertion in the plasma membrane without altering overall protein expression (Rosario *et al.*, 2013). There is also some evidence to indicate that protein kinase C (PKC, a downstream target of mTORC2) regulates EAAT3 expression (Kanai and Hediger, 2003). In FGR, mTORC1 activity is decreased in line with increased NEDD4-2 abundance, which ubiquitinates targets such as LAT1 and SNAT2 for degradation by the proteasome (Chen *et al.*, 2015; Roos *et al.*, 2007) and further work should establish whether the reduction in mTOR activity contributes to altered glutamine uptake by placentas from FGR infants.

The results in WT mice showed that the lightest placenta in a litter demonstrates increased placental efficiency; K_{mf}, per g placenta, of glutamine and glutamate was higher compared to the heaviest placenta at E18.5 (Chapter 3). This adds to previous findings focussed upon the system A transporter system (Coan *et al.*, 2008) and indicates that the small but normal mouse placenta can adapt its nutrient transport capacity to achieve optimal fetal growth. Efficient

nutrient exchange is reliant upon placental size, morphology, blood flow and transporter abundance. In the current study, LAT1 expression was increased at E18.5 which could contribute to the changes in K_{mf} of glutamine reported, however expression of glutamate transporters was unchanged. The efflux of glutamate (and/or metabolites) into fetal circulation in the mouse is mediated by as yet unknown mechanisms. This is an important question for future research.

Compared with humans, mice have a higher F:P ratio (in the current study median BW:PW ratio for normal birth weight infants at term was 6.5; the median F:P ratio at E18.5 for lightest placentas in a WT mouse litter was 17.0 and for heaviest placentas was 13.7), which suggests that the mouse placenta is more efficient, and perhaps has more capacity to adapt to the demands of the growing fetus, relative to placental size. A possible explanation for this might be the highly efficient countercurrent blood flow arrangement that enhances fetomaternal exchange in the mouse placenta (Adamson *et al.*, 2002). Previous studies have also shown that morphological adaptations occur earlier in gestation (E16) (Coan *et al.*, 2008) and prior to functional adaptations. The results reported here regarding functional adaptations are consistent with earlier studies focussed upon system A and calcium (Coan *et al.*, 2008; Dilworth *et al.*, 2010), and indicate a role for fetal nutrient demand signals driving these adaptations.

In the P0 knockout mouse, there was increased K_{mf}, per g placenta, of glutamate at E15.5, and of glutamine at both gestational ages assessed (E15.5 and E18.5). However, this was insufficient to support appropriate fetal growth and P0 fetuses were growth restricted at E18.5. As demonstrated in the IGF2-null mouse, IGF2 is required to maintain expression of glutamate transporter proteins EAAT1, EAAT2 and EAAT3 (system X_{AG}^-) (Matthews *et al.*, 1999). In the current study, reduced EAAT2 expression was seen in P0 mice at E18.5, however fetal levels of IGF2 are unaltered in the P0 mouse model (Constância *et al.*, 2002), and so this is not expected to be a mechanism underlying this change.

The findings in the P0 mouse differ to those reported in human FGR. In the P0 mouse, K_{mf} of glutamine was raised in P0 compared with WT placentas whereas glutamine uptake in human FGR was reduced. These results raise intriguing questions with regards to the utility of this model as a tool to better understand human FGR, with the caveat that we currently only have information in human on glutamine uptake into the placenta and not delivery to the fetus as in the mouse studies.

Collectively the studies presented in this thesis set the stage for future investigations into the likely complex interactions and regulatory mechanisms that mediate glutamine and glutamate delivery to the fetus in human pregnancy and raise important questions as to whether adaptive processes seen in mice can translate to human.

7.4 Measuring plasma levels of amino acids and metabolites

Amino acid concentrations were quantified in matched samples of maternal and UmV and UmA plasma from FGR and normal pregnancies. This gave the potential to a) directly compare between these different compartments in the same pregnancy, and b) to relate amino acid concentration to glutamine and glutamate transporter activity at the MVM.

Samples collected from the umbilical cord were drawn as soon as possible after delivery of the infant, and were handled and stored appropriately (-80ºC) prior to analysis. A further advantage of the current study was the collection of both UmV and UmA samples (most previous studies have assessed the UmV only, see Table 6), which enabled measurement of amino acids and metabolite delivery from placenta to fetus and vice versa in the same pregnancy, and allowed venoarterial differences to be compared in normal pregnancy and FGR.

There have been numerous attempts to determine differences in fetal (umbilical) plasma amino acid concentrations in FGR compared with normal pregnancy and yet these data remain contentious signifying the need for the current study in a well-defined population of normal (AGA) pregnancy and FGR. Amino acid concentrations show significant variation between individuals (Economides *et al.*, 1989; Holm *et al.*, 2017), and particularly in FGR, highlighting the necessity for studies to adhere to internationally agreed definitions of FGR and normal pregnancy to enable comparison between studies (Gordijn *et al.*, 2016; Unterscheider *et al.*, 2014). Another key consideration is the multiple phenotypes that underpin FGR; for example those with abnormalities in uteroplacental or fetoplacental blood flow versus those that may present with normal blood flow but aberrant syncytiotrophoblast function. Additionally, according to criteria for FGR, based on individualised birth weight ratios, there may be infants defined as FGR in the current study that were in fact simply constitutionally small. The wide variation in amino acid concentrations observed in the FGR cohort compared with normal pregnancy in the current study may be evidence of these difficulties in defining and profiling cohorts of FGR individuals (Figure 58).

The current study consolidated previous observations that the concentration of alanine is raised in maternal venous plasma of pregnancies with an FGR infant (Cetin *et al.*, 1990; Cetin *et al.*, 1996). Elevated maternal alanine concentrations could conceivably decrease transporter expression on the MVM by substrate regulation since reductions in amino acid availability up-regulates transporter expression (Parrott *et al.*, 2007). This could detrimentally affect i) placental amino acid uptake ii) availability of nutrients for subsequent transfer to the fetus, iii) the composition of the amino acid pool and thus substrates available for exchange. However, a reduction in system A activity (MeAIB uptake) was not observed in the current study which suggests that alanine does not regulate transporter activity in this manner.

Levels of glutamate in the UmA were elevated in FGR whereas glutamine concentrations were similar in all three compartments measured. Higher glutamate levels in the UmA could be due to increased conversion of glutamine via the fetal liver and/or a sign that less is being utilised by the fetus/taken back up by the placenta across the BM. In FGR there is evidence that the abundance of glutamate dehydrogenase, which catalyses the conversion of glutamate to α-ketoglutarate, is decreased in the placenta (Jozwik *et al.*, 2009) but levels in the fetus have not been described. Sophisticated regulatory mechanisms such as the mTOR pathway also act to maintain homeostatic control of amino acid concentrations and thereby support appropriate fetal growth (Carroll *et al.*, 2015). Evidence of interplay between fetal metabolism and placental function in sheep (Teng *et al.*, 2002) indicates that raised UmA glutamate concentrations may influence expression and activity of transporters on the BM. This could be tested experimentally using *ex vivo* placental perfusion techniques.

One aim of the present study was to relate amino acid concentrations, and particularly glutamine and glutamate, to placental uptake of these two amino acids. Disappointingly the collection of plasma and matched placental tissue from the same pregnancy was limited due to the small volume of blood available in the UmA, particularly from FGR infants, and so there was insufficient power to adequately assess these relationships.

Metabolomics can detect significant changes in metabolite levels within a biological system and has been successfully used clinically to predict or diagnose disease status (Horgan *et al.*, 2009; Nicholson *et al.*, 2012). Here GC-MS was conducted as a discovery method to identify potential indicators of fetal physiological condition that may ultimately provide a signal that is able to elicit changes in placental function. Whilst it was not an aim of the current study, recently published data have demonstrated the potential utility of metabolites to act as biomarkers for SGA (Horgan *et al.*, 2011; Macnaught *et al.*, 2015) however the sensitivity and specificity of candidate metabolites is often too low (Powell *et al.*, 2018). The results from GC-MS consolidate the main findings from the HPLC studies discussed above; alanine levels were raised in the UmV/UmA in FGR and glutamine levels were unchanged. Glutamate was not detected by GC-MS but levels of pyroglutamic acid, a precursor of glutamate (Figure 62) were elevated in the UmV. Lactic acid levels were significantly raised in the UmV of FGR infants in alignment with previous studies in humans (Marconi *et al.*, 1990; Pardi *et al.*, 1993) and animals (Owens *et al.*, 1989). Raised lactic acid levels may reflect increased placental amino acid consumption to provide the fetus with an alternative substrate for oxidation and/or a shift to anaerobic conditions favouring conversion of glucose to lactate. Alanine and lactate feed into the pyruvate pool, lactate enters via the enzyme lactate dehydrogenase, the levels of which are raised in SGA (Cox *et al.*, 1988), and pyruvate subsequently feeds into the TCA cycle for production of metabolic intermediates and energy (Brooks, 1986). High levels of urea in the

UmV of FGR infants was also observed which indicate amino acid catabolism (Owens *et al.*, 1989). Raised urea levels in mothers of FGR infants have also previously been reported (Marconi *et al.*, 1990) however the maternal metabolic profile was not investigated in the present study.

The current study has identified that the levels of a number of metabolites and amino acids are altered in pregnancies complicated by FGR. There are now open questions as to the utility of these metabolites as a therapeutic target for FGR. To answer these questions large scale studies with well-defined criteria of FGR and normal pregnancy are required.

7.5 Methodological considerations and limitations

In the studies conducted in mice the identity of the substrate coupled to the ^{14}C radiolabel, as measured in the fetus, is not known. MeAIB, used in a number of placental transport studies previously, is not metabolised, therefore the only limiting factor prior to release into the fetal circulation would be the placental amino acid pool which would be required to equilibrate (Cleal *et al.*, 2018; Velázquez *et al.*, 1976). In a study by Stegink *et al.* (1975), the majority of glutamate infused over 1 hour into the maternal circulation of the pregnant rhesus monkey remained associated with the radiolabel. Given the timecourse of the experiments presented in this thesis (~2 min from injection until harvest of fetal and placental tissue), it is unlikely that injected glutamate is metabolised within maternal circulation before it reaches the placenta, even accounting for the faster metabolic rate of the mouse.

Investigations using placental perfusion techniques suggest that glutamate is not directly transferred from the maternal to fetal circulation, rather it is metabolised to products such as α-ketoglutarate, for entry to the TCA cycle, and proline (Day *et al.*, 2013). Early studies in the pregnant rhesus monkey infused radiolabelled glutamate into the maternal circulation and sequentially sampled fetal and maternal plasma to assess placental glutamate transport and metabolism. Stegink *et al.* (1975) demonstrated that the majority of glutamate infused over 1hr into maternal circulation of the pregnant rhesus monkey (69-88%) remained associated with the radiolabel, whereas in fetal plasma the radiolabel was associated with metabolites such as glucose and lactate, and there was little to no radiolabelled glutamate evident. The proportion of intact ^{14}C-glutamate and the identity of metabolites in the fetal circulation is unknown from the current studies in mice.

Transporter abundance was assessed in placentas from mice and women. This part of the study was limited by the use of membrane-enriched placental homogenates: it was therefore not possible to determine the contribution of the MVM/BM, or other plasma membranes that may have made up the mixed membrane preparations. An analysis of mitochondrial membrane contamination, assayed using the mitochondrial markers succinate dehydrogenase

and cytochrome-c, could help understand the makeup of the membrane-enriched preparation (Jimenez *et al.*, 2004). Future work could also focus upon MVM and BM isolates. This was not possible in the current study primarily due to a lack of available banked human tissue which reflects the villous tissue required for the uptakes in this study and other usage within the laboratory. In addition, there is currently no established method to isolate the BM of the mouse placenta.

Presumably a smaller placenta (by weight) will have less surface area available for exchange. Indeed in FGR, in the presence of a small placenta, the surface area of terminal villi and capillaries is decreased (Mayhew *et al.*, 2003). Correlations between the size of the placenta and surface area for exchange have not been carried out in normal pregnancy. Ideally, surface area available for nutrient transport would be used as the denominator by which to calculate amino acid uptake. However, in the absence of an accurate method of measuring syncytiotrophoblast MVM surface area for this assay, placental uptake was measured per mg protein as a proxy measure of placental size.

Moving forward, there is a necessity within the obstetric field to examine more closely the definition of both normal pregnancy and FGR. In the current study, strict inclusion criteria were used in an effort to have well-defined groups of normal pregnancy and FGR. Women of advanced maternal age (>40 years old) were excluded. The study also set out to exclude women with high and low BMI (<19 or >30 kg/m^2), women who smoked and preterm deliveries; however, difficulties obtaining sufficient tissue from pregnancies complicated by FGR meant that some were included in the study (the range of BMI of women included in the study was 18.8-33.9 kg/m^2)

FGR is a heterogeneous condition that presents with many phenotypes. A recently published consensus-based definition of FGR as an abdominal circumference or estimated fetal weight of $<3^{rd}$ centile, or presence of additional indicators (see Table 2 in Introduction) should be used for all future studies (Gordijn *et al.*, 2016). Individualised growth charts have their own limitations, inasmuch as an infant within the normal birth weight (AGA) range may have dropped centiles over the course of pregnancy and could be legitimately be characterised as FGR. In order to accurately reflect a 'normal' population, it would make sense to include a narrower definition of AGA, thus avoiding the extremes of birth weight centiles, i.e. ~10^{th} or ~90^{th} centile, that are at more likely to be small or large for gestational age, and at risk of adverse outcomes (Iliodromiti *et al.*, 2017). In the current study, if a narrower range of 20^{th}-80^{th} centile is used to define normal birth weight (AGA), the uptake of glutamine, glutamate and MeAIB is significantly lower in FGR compared with normal (AGA) pregnancies (Appendix 9.8).

7.6 Clinical context

FGR affects between 5-10% of pregnancies in the UK, yet there remain no clinical tests to predict the risk of developing nor advise expectant management of FGR (Powell *et al.*, 2018). In addition, there are currently no treatments for FGR (Fisk and Atun, 2008). Whilst dietary models of FGR, such as the protein-restricted rat, and maternal nutrient restricted baboon (Pantham *et al.*, 2015; Rosario *et al.*, 2011) induce the characteristic phenotype, i.e. reduced fetal and placental weight and altered placental function, FGR occurs in well-nourished communities and is therefore not just a feature of malnutrition. This is further supported by the observation in the current study that some amino acid concentrations in fetal circulation are in fact increased in FGR (Figure 57 and Appendix 9.6). Several studies have shown no benefit of administering amino acids to women with SGA infants on fetal growth (Say *et al.*, 2003) and intraumbilical delivery of amino acids via a subcutaneous port system results in amino acid imbalance (Tchirikov *et al.*, 2017). Observations from the current study, that placental glutamine uptake is reduced in FGR, further indicate that amino acid supplementation is unlikely to improve fetal outcomes.

7.7 Main findings of the study

The main aims of the work presented in this thesis were to determine whether glutamine and glutamate transport relates to placental size in normal pregnancy, and to investigate glutamine and glutamate transport in normal pregnancy and FGR. These aims were achieved, and the data presented in this thesis have shown that:

- Towards term in WT mice (E18.5), unidirectional maternofetal clearance (K_{mf}) of glutamine and glutamate was higher across the lightest placentas in a litter compared with the heaviest but was unrelated to the sex of the fetus. The abundance of glutamine (LAT1, LAT2 and SNAT5) and glutamate (EAAT1, EAAT2) transporters in membrane-enriched homogenates from the lightest and heaviest placentas at E15.5 and E18.5 revealed higher expression of LAT1 at E18.5 only.
- Transporter-mediated glutamine and glutamate uptake was not related to placental or fetal weight in normal human pregnancy but glutamine uptake was lower in placentas from female compared with male infants, and this was not due to altered transporter protein abundance.
- In the P0 mouse model of FGR, K_{mf} of glutamine was significantly higher for P0 versus WT placentas at E15.5 and E18.5. Glutamate K_{mf} was higher at E15.5 only. These changes cannot be fully accounted for by changes in transporter protein abundance since only EAAT2 expression was significantly lower in P0 mice at E18.5.

- Transporter-mediated uptake of glutamine was reduced in FGR (uptake of MeAIB and glutamate was unchanged) yet the abundance of glutamine transporters LAT1, LAT2 and SNAT5, and the glutamate transporter EAAT1 in membrane-enriched homogenates was higher.
- A distinct profile of changes in metabolite levels such as glutamate, lactic acid, pyruvic acid, and urea in umbilical vein (UmV) and artery (UmA); and amino acid concentrations such as alanine and glutamate in maternal, UmV and UmA plasma, was reported in samples from FGR compared with normal birth weight infants.

7.8 Future directions

The following are suggested as immediate future works to consolidate current findings:

- Quantify expression of glutamine and glutamate transporter proteins in MVM preparations by Western blot and immunohistochemistry on human placental villous samples to establish whether abundance of these transporters is different between placentas from FGR and normal birth weight infants, and/or according to sex. It would be possible to do immunohistochemistry on tissue banked from placentas already used to explore protein expression by Western blotting in the current study.
- Complete gene expression studies (Chapter 6) by quantifying slc38a5 (SNAT5), and slc1a3 (EAAT1), slc1a2 (EAAT2) and slc1a1 (EAAT3) expression.

The work presented in this thesis has raised many questions in need of further investigation. Longer-term directions for future work could include 1) assessing transporter-mediated uptake in well-defined cohorts of FGR and AGA, and how this relates to amino acid concentrations in the maternal, fetal (umbilical) and placental compartments, and 2) investigating potential mechanisms of regulation of transporter function/abundance in placentas from normal birth weight (AGA) and FGR infants. Transporter-mediated uptake according to sex, and the mechanisms that underlie elevated transporter activity in males could also be explored. Studies of this nature should look to define FGR according to the criteria set by Gordijn *et al.* (2016), and should aim to have a similarly well-defined cohort of AGA infants, e.g. within the 20^{th}-80^{th} centiles to increase the likelihood of including infants that are truly 'normal'.

1) Initial studies should confirm that transporter activity is lower in FGR compared to AGA using the above definitions, ensuring an equal number of males and females in each group. Collection of maternal, UmV and UmA plasma would add to current numbers, and allow transporter activity to be related to amino acid concentration.

- Given the sexually dimorphic differences in transporter activity reported in the current study (Chapter 4) numbers included in each group should be sufficient to detect differences according to sex. Previous studies have found n=10 in each group to be sufficient to detect differences (Aiko *et al.*, 2014). The presence of sex differences in transporter activity in FGR could lead to different strategies for treatment depending on whether the fetus was male or female.
- Based on previous findings (Philipps *et al.*, 1978), the composition of amino acids in the UmV is not akin to the concentrations in placentas from normal birth weight infants (Appendix 9.10). Whether amino acid concentrations in the placenta in FGR are altered, and how this relates to amino acid uptake is unknown and should be investigated.
- HPLC analysis showed that there was a broad range of values in the FGR cohort. This could represent different phenotypes of FGR. This work could be extended to include more matched samples collected from pregnancies with different clinical presentation (e.g. stratified according to evidence of abnormal uterine or umbilical blood flow velocity) to further investigate this. Inclusion of more samples would also allow stratification according to the sex of the infant, the effect of which is currently unknown.

2) The mTOR pathway is a serine/threonine kinase known to modify the function of system A and L transporters in the placenta (Roos *et al.*, 2009), the activity of this pathway in placentas from normal birth weight and FGR infants, and whether sex-specific differences exist, is unknown and should be assessed using Western blot analysis to quantify phosphorylated downstream targets as previously described (Rosario *et al.*, 2013). The effect of experimentally down-regulated/altered mTOR activity on glutamine and glutamate uptake and protein expression could also be tested using known inhibitors of mTOR such as rapamycin, on human placental explants.
- Post-translational modifications are another potential mechanism by which transporter activity could be altered in FGR pregnancies, and/or according to sex. Immunoprecipitated samples could be analysed using Western blot to assess ubiquitination of transporter proteins using previously described methods (Chen *et al.*, 2015).

The findings presented in this thesis have indicated that whilst appropriate for other studies, such as pre-clinical studies to assess potential therapies for FGR, the mouse model used here may not provide an appropriate model to answer the questions raised by this body of work. Evidence of adaptation in both normal and P0 mouse models is at odds with what was reported in normal human pregnancy and FGR, with the caveat that uptake and unidirectional

maternofetal clearance are measuring different physiological parameters, and that other mouse models of FGR have shown reduced amino acid transport akin to human FGR. Understanding the mechanisms which underpin the findings in WT and P0 mice may therefore not have direct relevance to the pathophysiology of human FGR with regards to placental nutrient transport. The $eNOS^{-/-}$ mouse may be worth pursuing in future as a model relating to human FGR with blood flow and trophoblast transporter defects that exhibits reduced system A activity (MeAIB uptake) (Kusinski *et al.*, 2012). Future work should ideally focus upon improving our understanding of the regulatory mechanisms underpinning placental nutrient transport in both normal pregnancy and FGR using techniques in human tissue, and seek to use alternative models as appropriate.

In conclusion, this thesis has provided novel insights into adaptive processes (or lack of) in the placentas of mice and humans, and the knowledge that placental uptake of glutamine is compromised in human FGR. Future studies leading on from this work should focus upon the mechanisms that regulate placental glutamine and glutamate delivery in relation to the sex of the fetus, and in normal and FGR pregnancies. Exploitation of these in the future may provide potential therapeutic avenues for FGR, for which there is no current treatment.

Chapter 8 References

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9.1 Amino acid uptake in Na^+-free conditions

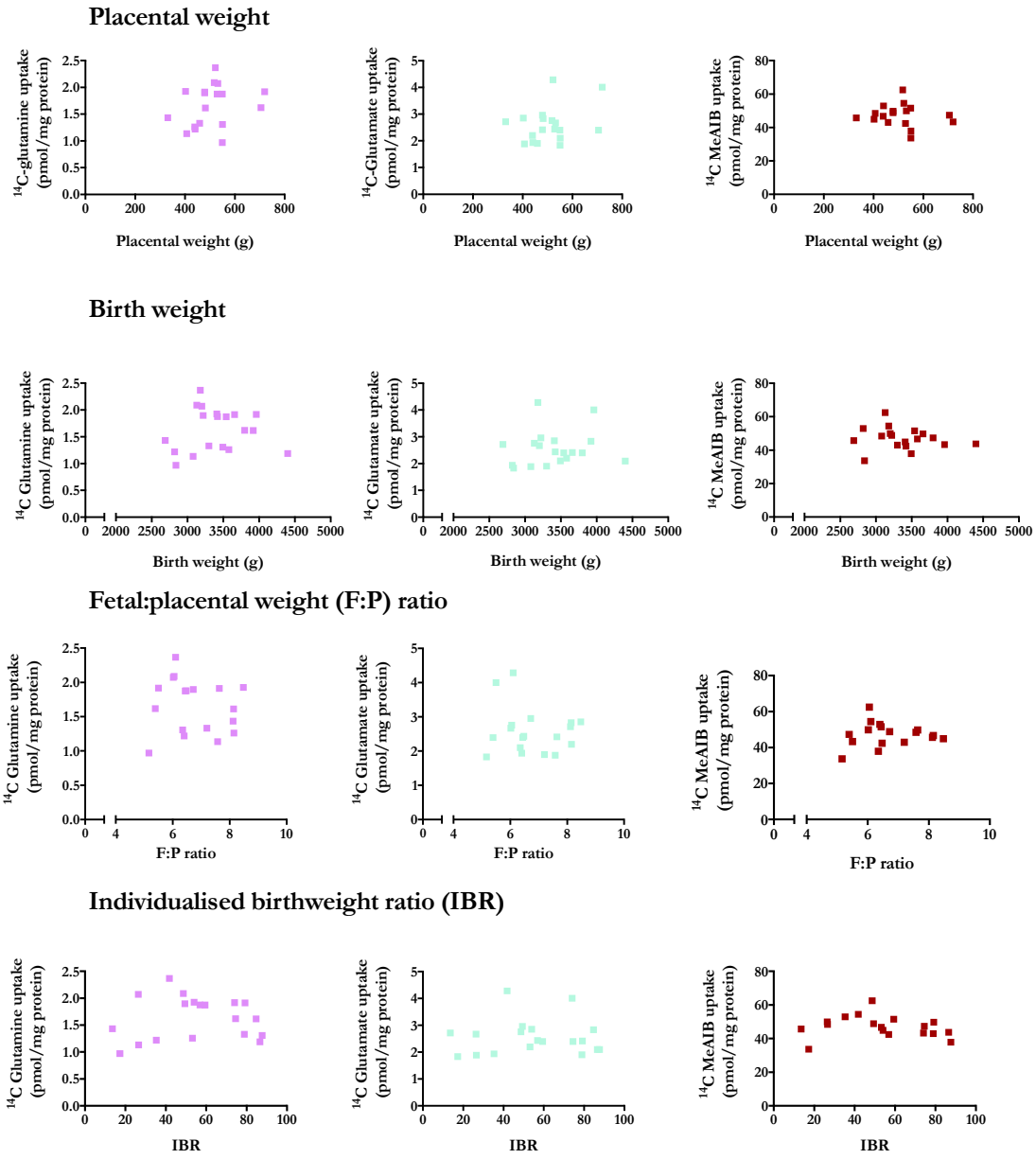

Figure 64 Relationship between amino acid uptake in Na^+-free buffer and fetal and placental measures
There was no relationship between uptake in Na^+-free buffer (plus specific inhibitors; as required) uptake of ^{14}C-glutamine (purple symbols), ^{14}C-glutamate (green) or ^{14}C-MeAIB (red) at 90 min and trimmed placental weight, birth weight, fetal: placental weight (F:P) ratio or individualised birth weight ratio (IBR). Specific substrate inhibitors were histidine, serine and BCH for ^{14}C-glutamine transporters and aspartate for ^{14}C-glutamate transporters (n=18/19, Linear regression).

9.2 LAT2 antibody optimisation

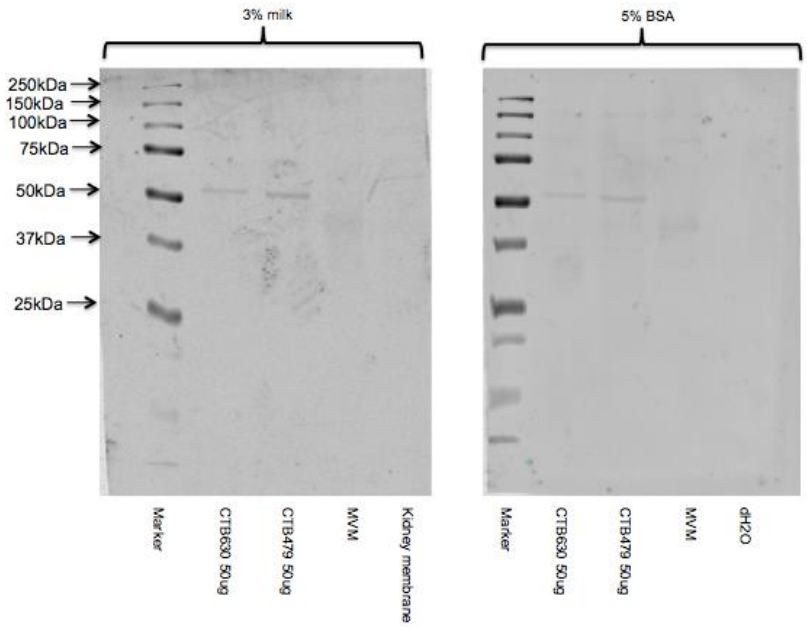

Figure 65 Optimisation of LAT2 antibody

In an attempt to quantify LAT2 protein expression, several conditions were trialled. In the representative blots above, LAT2 was probed for in membrane-enriched human placental homogenates, MVM isolates and kidney. Blots were blocked in either 3% milk (left) or 5% bovine serum albumin (BSA, right). However, even when loading a high concentration of protein (50 μg), expression was faint and did not exist in MVM isolates. It was therefore not feasible to quantify LAT2 expression in these groups (male versus female, normal AGA pregnancy).

9.3 Protein expression in placentas from male and female normal birth weight (AGA) infants versus ^{14}C-glutamine or ^{14}C-glutamate uptake at 90 min in the same placentas

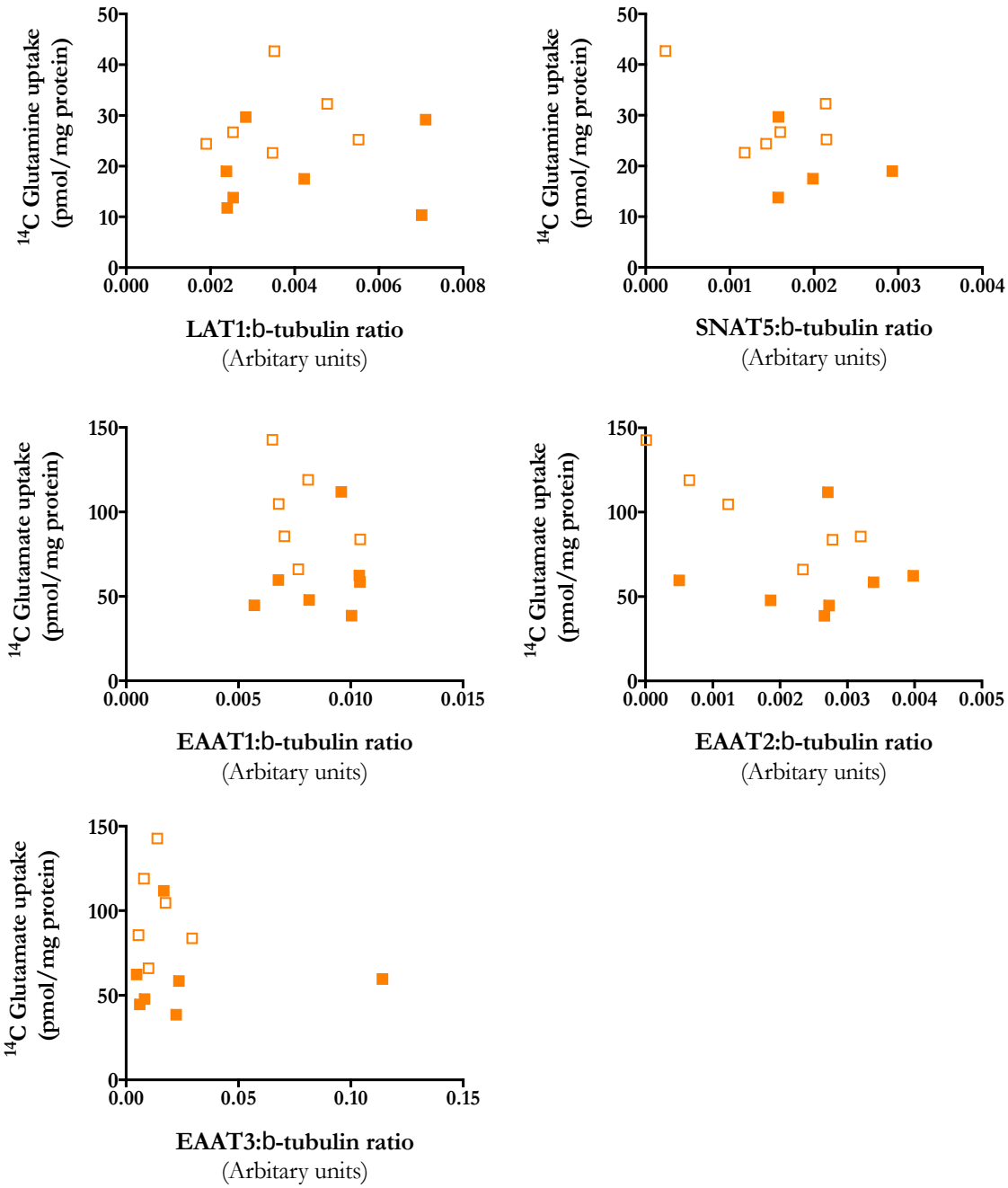

Figure 66 Transporter protein expression in placentas from male and female normal birth weight (AGA) infants plotted against ^{14}C-glutamine or ^{14}C-glutamate uptake at 90 min in the same placentas
There was no significant relationship between expression of transporter proteins and amino acid (^{14}C-glutamine or ^{14}C-glutamate) uptake at 90 min in the same placentas when considering all the data, i.e. pooling data from male and female infants. There was also no correlation if data of male or female infants alone were analysed. Hollow symbols represent placentas from males, solid symbols relate to placentas from females. n=13; n=6 male, n=4/7 female. Linear regression.

9.4 Amino acid uptake by placentas from normal (AGA) pregnancies and FGR split according to sex

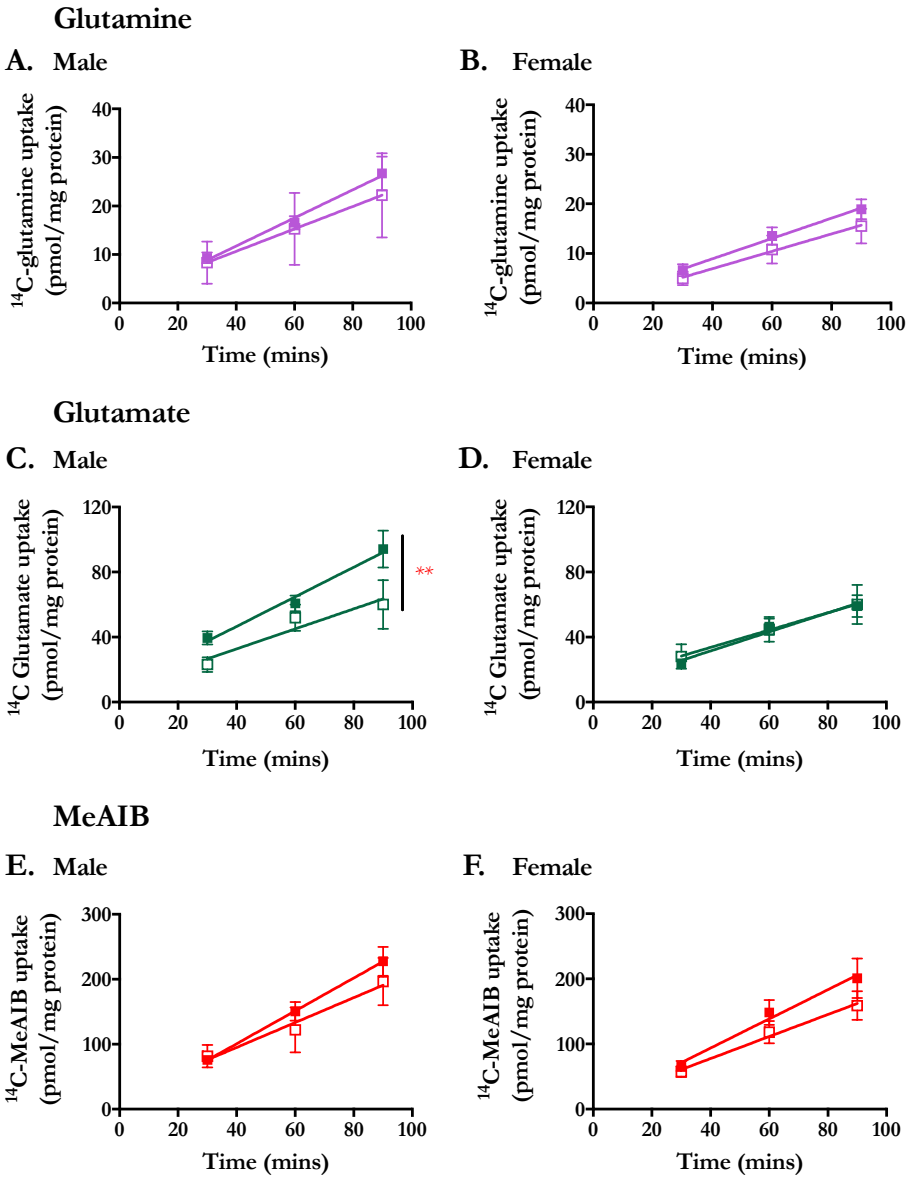

Figure 67 Comparison of transporter-mediated amino acid uptake in placentas from normal (AGA) pregnancies and FGR

Uptake of amino acids by placentas of FGR (hollow symbols) and normal birth weight (solid) infants (mean±SEM) that were male (A, C, E) and female (B, D, F). Uptake of ^{14}C-glutamate (C) by male FGR placentas was significantly lower compared with those of normal birth weight male infants. There were no other significant differences within sex between placentas of normal birth weight (AGA) and FGR infants. Male: AGA n=7, FGR n=3/4; Female: AGA n=11/12, FGR n=7 ** P<0.01, Linear regression.

9.5 Potential confounders of amino acid uptake by placentas from FGR infants

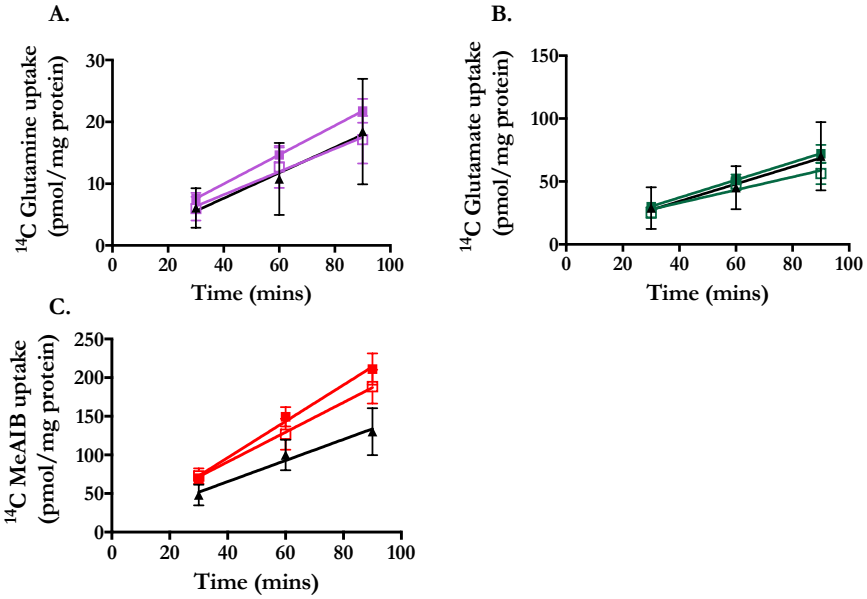

Figure 68: Amino acid uptake according to smoking status
Placental uptake of ^{14}C-glutamine (A), ^{14}C-glutamate (B), and ^{14}C-MeAIB (C) according to smoking status. Solid symbols denote AGA placentas (none of which were smokers, n=19). Hollow, square symbols represent data from FGR infants whose mothers were non-smokers (n=8) whilst black, triangular symbols show placental amino acid uptake in smoking mothers who had an FGR infant (n=3). Numbers were too small to allow statistical analyses; however from these small numbers there is a trend to suggest that smoking negatively influences ^{14}C-MeAIB uptake (C). Data are mean±SEM; Linear regression

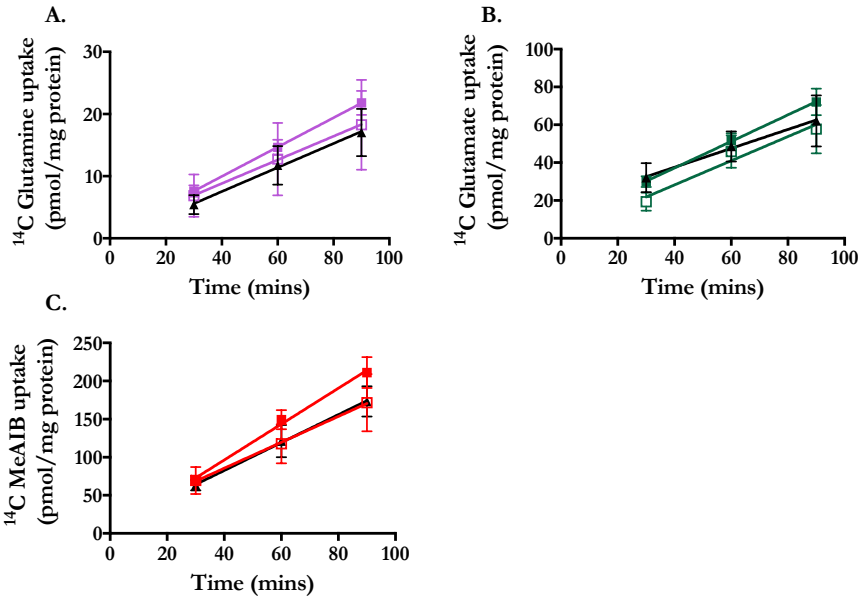

Figure 69: Gestational age does not affect amino acid uptake
All placentas from AGA pregnancies were delivered after 259 days/37 weeks gestation. Within the FGR cohort, six deliveries were preterm (range: 204-247 days) and five were term (range: 260-284 days). Graphs A-C show amino acid uptake by placentas from normal (AGA) pregnancy (solid square symbols, n=19), preterm FGR (black triangle symbols, n=6) and term FGR (hollow square symbols, n=5). Gestational age does not appear to have a significant effect on amino acid uptake. Data are mean±SEM; Linear regression.

9.6 Amino acid concentrations in maternal venous and fetal (umbilical) venous and arterial plasma in AGA and FGR pregnancies

	Maternal venous			Umbilical venous			Umbilical arterial		
	AGA	FGR	*P* value	AGA	FGR	*P* value	AGA	FGR	*P* value
Essential									
Isoleucine	41 [19-58]	42 [31-96]		60 [40-83]	55 [36-74]		61 [40-77]	54 [32-80]	
Leucine	92 [39-116]	104 [69-179]		132 [85-164]	135 [92-162]		130 [82-163]	136 [79-161]	
Methionine	21 [12-41]	34 [13-56]		35 [25-53]	29 [25-71]		30 [23-48]	42 [21-59]	
Phenylalanine	40 [37-52]	55 [29-89]	0.008	65 [54-81]	78 [54-115]	0.020	62 [52-84]	77 [57-98]	0.030
Threonine	192 [95-250]	201 [150-373]		281 [20-417]	255 [170-524]		276 [146-400]	244 [138-516]	
Valine	149 [83-175]	158 [109-263]		221 [172-279]	206 [156-233]		218 [164-265]	185 [147-239]	
Non-essential									
Alanine	284 [232-439]	405 [275-790]	0.004	334 [192-451]	474 [320-1093]	<0.001	291 [229-424]	458 [266-1094]	0.007
Asparagine	49 [32-73]	50 [34-91]		50 [34-86]	54 [35-120]		47 [28-67]	49 [40-110]	
Aspartic acid	8 [6-11]	9 [7-23]		15 [8-48]	22 [9-164]		13 [10-48]	33 [12-197]	0.036
Citrulline	15 [7-26]	18 [11-39]		16 [0-19]	14 [10-21]		14 [9-18]	14 [11-20]	
Cystine	29 [12-40]	24 [8-53]		30 [8-42]	33 [9-60]		32 [10-41]	20 [11-50]	
Glutamic acid	49 [23-70]	53 [19-82]		72 [27-128]	89 [43-390]		79 [60-140]	147 [42-640]	0.023
Glutamine	428 [242-554]	477 [318-658]		505 [379-606]	553 [443-1070]		488 [425-573]	528 [409-1028]	
Glycine	129 [95-247]	148 [91-205]		250 [192-334]	285 [167-462]		259 [187-399]	253 [184-502]	
Serine	75 [48-128]	80 [64-117]	0.042	132 [110-175]	130 [95-192]		132 [108-157]	128 [106-186]	
Taurine	40 [28-78]	36 [17-131]		182 [89-368]	207 [98-892]		194 [134-310]	232 [91-1122]	
Tyrosine	44 [33-57]	60 [34-126]	0.047	69 [54-96]	89 [65-137]	0.044	72 [51-88]	86 [68-141]	
α-amino butyric acid	8 [0-19]	16 [0-25]	0.013	15 [0-28]	26 [0-59]		14 [0-23]	24 [8-58]	0.021

Table 29 Amino acid concentrations in maternal venous and umbilical venous and arterial plasma from AGA and FGR pregnancies

Amino acid concentrations (μmol/L) from AGA and FGR pregnancies. n=17 AGA, n=13 FGR, except for tyrosine and phenylalanine where n=7 AGA and n=12 FGR. Data are median [range].

P value is shown if differences between groups were significant ($P<0.05$ Mann Whitney test).

9.7 GC-MS data tables

Group	Sample	Metabolite	*n-Butylamine	*Hydroxylamine	Lactic acid	Alanine	¹Unidentified	Valine, TMS1	Pyruvic acid	β-Hydroxybutyric acid	Unidentified	Ethanolamine TMS3	Unidentified	Isoleucine, TMS1	Leucine, TMS2	*Butanoic acid	Glycine, TMS3	*2-Ketoisocaproic acid mo-tms	Phosphate (3:1)	*¹Dihydroxybutanoic acid	Threonine, TMS2	Serine TMS3	Urea, N,N'-bis(trimethylsilyl)-
		Retention Index				1142		1184	1178	1204		1256		1278	1298		1331				1372	1388	
AGA	UmA	Mean	0.112	6.407	30.479	0.133	0.020	2.403	0.081	0.152	2.028	9.157	0.038	0.401	0.276	0.019	0.082	3.950	8.854	0.019	0.604	0.042	0.666
FGR	UmA		0.105	5.612	43.464	0.219	0.020	2.138	0.129	0.215	1.725	8.159	0.038	0.397	0.262	0.017	0.085	5.325	8.987	0.023	0.608	0.040	1.031
		P value	0.352	0.142	0.061	0.012	0.948	0.220	0.059	0.446	0.035	0.113	0.949	0.949	0.666	0.239	0.575	0.077	0.903	0.315	0.961	0.716	0.067
		log2 fold change				0.727					-0.234												
		Fold change				1.7					0.9												
AGA	UmV	Mean	0.120	7.419	19.530	0.125	0.019	2.635	0.085	0.189	2.093	9.421	0.037	0.482	0.292	0.017	0.081	4.114	8.275	0.022	0.692	0.037	0.533
FGR	UmV		0.115	6.764	41.323	0.213	0.021	2.517	0.138	0.265	1.918	9.138	0.043	0.517	0.312	0.018	0.105	5.032	10.099	0.031	0.770	0.045	0.985
		P value	0.465	0.133	0.006	0.010	0.741	0.518	0.048	0.343	0.076	0.526	0.583	0.561	0.347	0.487	0.169	0.255	0.087	0.067	0.347	0.053	0.006
		log2 fold change			1.081	0.765			0.707														0.886
		Fold change			2.1	1.7			1.6														1.8

Table 30 GC-MS data table (continued overleaf)

Table of mean levels of identified metabolites from AGA and FGR pregnancies in umbilical arterial (UmA) and venous (UmV) samples. Data were analysed by Welch's t-test and considered significant with a *P* value of <0.05 (highlighted cells). Data from UmA and UmV samples were $\log_2$ transformed to express as $\log_2$ fold change and are highlighted on a red (-5 $\log_2$ fold change) to green (5 $\log_2$ fold change, yellow = no change, 0) scale, as in Figure 62. Fold change differences (case over control) are also presented, and are used to described changes in text. * indicates a metabolite whose identification has not been confirmed. [1] refers to a metabolite that showed more than 20% variation across QC samples indicating that changes seen within this range may be due to inherent technical variation within the data set.

Group	Sample	Metabolite	Benzoic acid	*Nonanoic acid	β-alanine	Unidentified	Unidentified	*2(3H)-Furanone, dihydro-3,4-bis[(trimethylsilyl)oxy]-, trans-	Unidentified	Malic acid	4-Hydroxyproline 3TMS	*GABA	¹Aspartic acid, TMS3	*L-Threonic acid	Methionine	*Ribitol	*Xylitol	¹Glutamine	Pyroglutamic acid 2TMS	2-Ketoglutaric, Mox/TMS	Phenylalanine, TMS1	¹Proline, TMS2	Ornithine, from Citrulline
		Retention Index			1454						1550		1575			1618	1649	1652		1708	1750	1763	1766	1810
AGA	UmA	Mean	0.039	4.063	0.107	2.298	1486.186	0.032	7.092	0.023	0.010	2.785	0.042	0.126	0.010	0.009	0.077	0.061	0.911	0.016	0.282	0.025	0.095
FGR	UmA		0.039	4.612	0.096	1.955	1730.500	0.032	6.415	0.040	0.011	2.442	0.077	0.162	0.010	0.012	0.078	0.119	1.168	0.019	0.254	0.031	0.091
		P value	0.709	0.189	0.094	0.019	0.214	0.997	0.089	0.116	0.547	0.046	0.260	0.086	0.980	0.172	0.939	0.199	0.094	0.169	0.292	0.328	0.826
		log2 fold change				-0.234						-0.189											
		Fold change				0.9						0.9											
AGA	UmV	Mean	0.039	3.823	0.101	2.336	1576.579	0.031	7.745	0.019	0.008	2.788	0.027	0.113	0.008	0.005	0.069	0.021	0.814	0.010	0.292	0.017	0.080
FGR	UmV		0.039	3.998	0.099	2.175	1629.133	0.041	7.144	0.037	0.011	2.594	0.061	0.146	0.010	0.007	0.076	0.049	1.257	0.013	0.283	0.027	0.114
		P value	0.928	0.779	0.487	0.047	0.771	0.216	0.039	0.069	0.010	0.064	0.164	0.081	0.047	0.065	0.572	0.105	0.001	0.081	0.695	0.030	0.018
		log2 fold change				-0.103			-0.117		0.491				0.285				0.628			0.690	0.510
		Fold change				0.9			0.9		1.4				1.2				1.5			1.6	1.4

Group	Sample	Metabolite	¹Mannitol	Fructose (Oxime)	¹Sorbitol	¹Galactitol	Glucose (oxime)	Phosphoethanolamine	L-Lysine 4TMS	*Tetradecanoic acid	Lysine (3TMS)	Glucuronic acid	*Scyllo-inositol	*Erythrose???	Myo-Inositol	¹Unidentified Sugar	Tyrosine, TMS2	Palmitic acid	Stearic acid	Eicosanoic acid	Tryptophan, TMS2	Lactose, minor peak	Maltose
		Retention Index	1817	1825	1830	1834	1846	1848	1887		1900	1908	1934.6		1979		2070	2097	2296		2515	2554	2588
AGA	UmA	Mean	0.024	0.117	0.016	0.002	8.991	0.087	0.211	0.176	0.654	0.009	0.006	12.922	2.530	0.002	0.239	14.321	7.093	0.011	0.048	0.129	0.012
FGR	UmA		0.043	0.122	0.055	0.002	8.298	0.134	0.188	0.167	0.565	0.011	0.008	15.117	3.116	0.002	0.218	14.744	7.630	0.013	0.043	0.359	0.037
		P value	0.643	0.368	0.110	0.550	0.474	0.326	0.589	0.405	0.332	0.238	0.392	0.354	0.566	0.535	0.480	0.604	0.327	0.260	0.411	0.238	0.076
		log2 fold change																					
		Fold change																					
AGA	UmV	Mean	0.015	0.123	0.011	0.001	12.857	0.055	0.170	0.178	0.613	0.009	0.006	15.501	1.576	0.003	0.234	15.036	7.301	0.010	0.034	0.154	0.008
FGR	UmV		0.008	0.133	0.068	0.002	11.545	0.068	0.216	0.185	0.752	0.012	0.007	14.398	2.048	0.002	0.240	15.798	7.938	0.012	0.036	0.485	0.016
		P value	0.447	0.087	0.170	0.327	0.224	0.610	0.150	0.354	0.109	0.082	0.681	0.559	0.383	0.042	0.826	0.124	0.156	0.062	0.772	0.255	0.168
		log2 fold change														-0.505							
		Fold change														0.7							

9.8 ^{14}C-glutamine, ^{14}C-glutamate and ^{14}C-MeAIB uptake by placentas from growth restricted (FGR) babies is significantly lower compared with infants with a normal birth weight, defined as an IBR between 20th-80th centiles

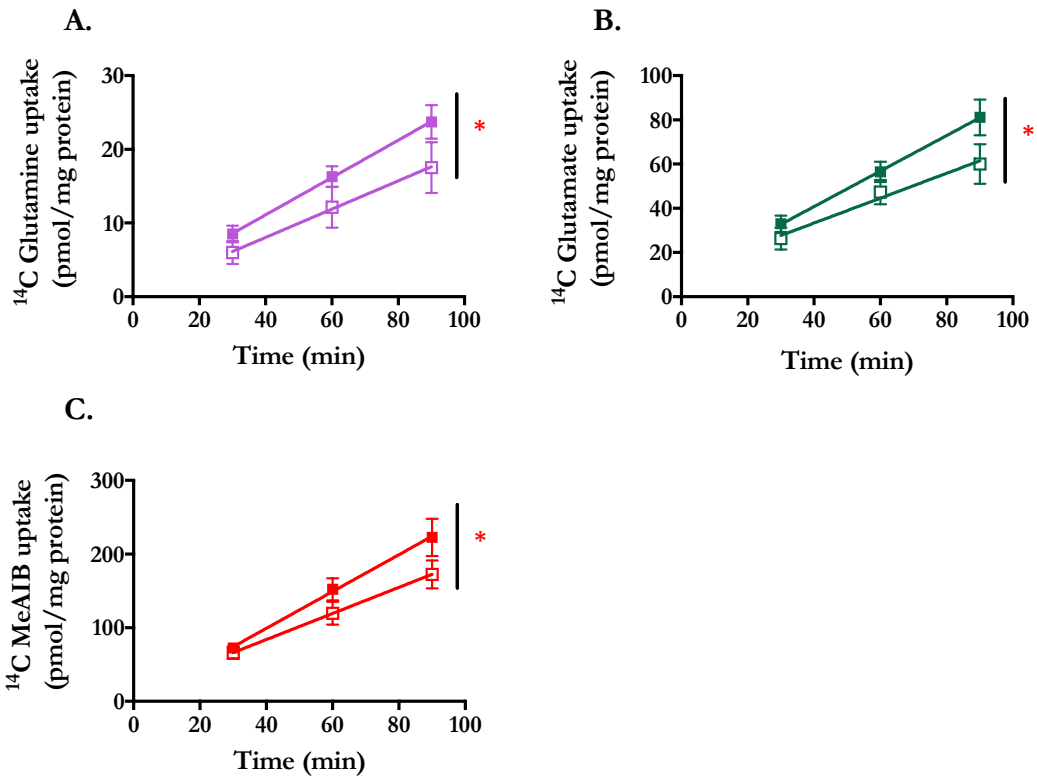

Figure 70 Transporter-mediated uptake by placentas from FGR (IBR <5th centile) and AGA (IBR 20th-80th centile) infants

Placental uptake of ^{14}C-glutamine (A), ^{14}C-glutamate (B) and ^{14}C-MeAIB (A) was significantly lower in placentas from FGR babies (hollow symbols, n=10/11, n=3/4 male, n=7 female) compared with placentas from babies appropriately grown for gestational age, defined as an IBR between 20th-80th centile (solid symbols, n=13/14, n=7 male, n=6/7 female). Data are mean±SEM * P<0.05 Linear regression.

9.9 Protein expression in placentas from normal birth weight (AGA) and FGR infants versus ^{14}C-glutamine or ^{14}C-glutamate uptake at 90 min in the same placentas

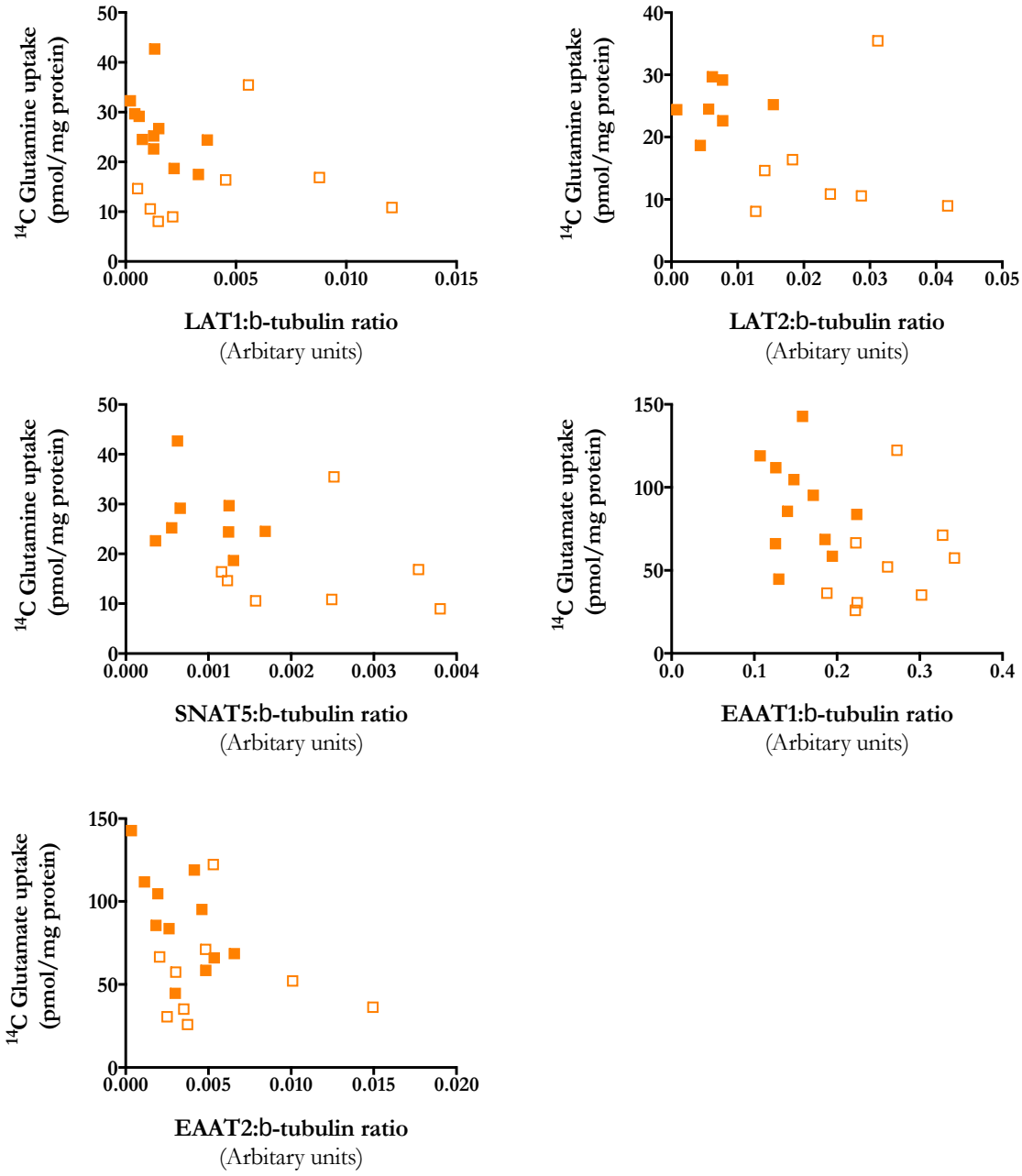

Figure 71 Transporter protein expression in placentas from normal birth weight (AGA) and FGR infants plotted against ^{14}C-glutamine or ^{14}C-glutamate uptake at 90 min in the same placentas

There was no significant relationship between expression of transporter proteins and amino acid (^{14}C-glutamine or ^{14}C-glutamate) uptake at 90 min in the same placentas when were fitted to all the data, i.e. from both normal and FGR infants. There was also no correlation if data of normal or FGR infants alone were analysed. Hollow symbols represent placentas from FGR infants symbols, solid symbols relate to placentas from AGA infants. n=8/9/11 AGA, n=7/8/9 FGR. Linear regression.

9.10 Concentration of amino acids in the UmV of FGR and normal birth weight (AGA) infants as measured by HPLC

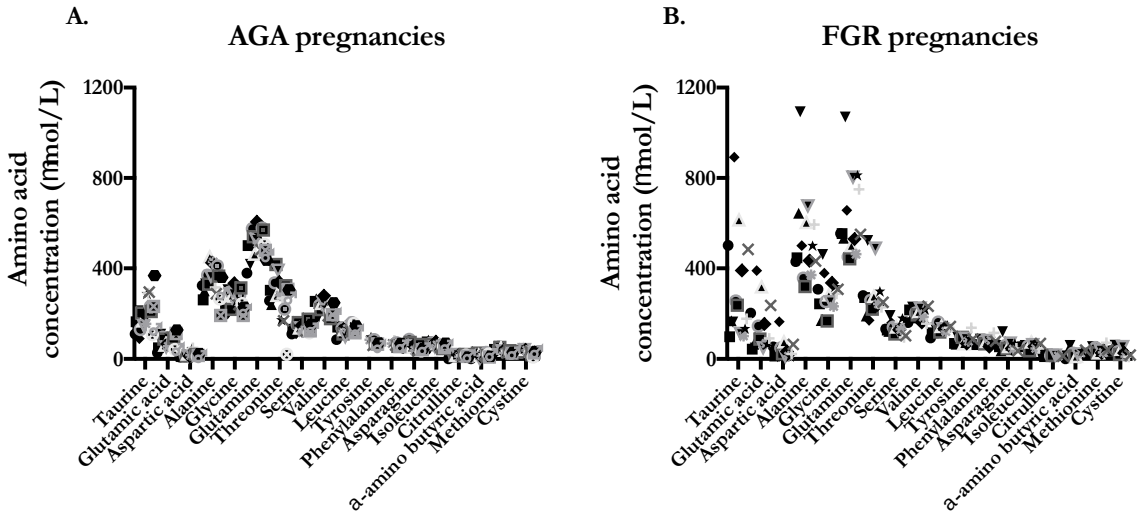

Figure 72 Concentration of amino acids in the UmV of FGR and normal birth weight (AGA) infants as measured by HPLC

Amino acid concentrations in the UmV from (A) AGA and (B) FGR pregnancies. Concentrations are ordered according to the most to least abundant concentrations in normal placentas, as determined by Philipps *et al.* (1978). The order of abundance of amino acids in placental tissues is not the same in the UmV. In addition, the range of values detected in the UmV of FGR infants is wider than those measured in the UmV of normal birth weight infants.

9.11 Western blot appendices

There are 4 powerpoint documents on a CD attached within the back cover of this thesis. The documents contain the full Western blots presented within this thesis. The first slide in each file contains a table of contents. The documents are labelled as follows:

- Chapter 3 Western blot Appendix
- Chapter 4 Western blot Appendix
- Chapter 5 Western blot Appendix
- Chapter 6 Western blot Appendix