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UNIVERSITY OF SOUTHAMPTON  
FACULTY OF MEDICINE  
Institute of Developmental Sciences

# **The transfer and metabolism of glucose and amino acids by the human placenta**

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by

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UNIVERSITY OF SOUTHAMPTON

**Abstract**

FACULTY OF MEDICINE: INSTITUTE OF DEVELOPMENTAL SCIENCES

Doctor of Philosophy

THE TRANSFER AND METABOLISM OF GLUCOSE AND AMINO ACIDS BY  
THE HUMAN PLACENTA

**By Priscilla Elly Liwawa Day**

Identifying the mechanisms underlying placental nutrient transfer is important to understand the processes determining fetal growth. Impaired fetal growth is associated with increased rates of perinatal morbidity and mortality and chronic disease in adulthood.

Significant questions regarding placental glucose and amino acid transfer across the human placenta remain unresolved. This thesis investigated aspects of placental glucose and amino acid transfer in the human placenta and whether maternal factors affect placental expression of genes involved in amino acid transfer.

Transfer of  $^3\text{H}$ -3MG and  $^{14}\text{C}$ -L-glucose at various maternal and fetal D-glucose concentrations and metabolism of  $^{15}\text{N}$  and U- $^{13}\text{C}$ -glutamate and  $^{15}\text{N}$ -leucine were investigated using an isolated perfused human placenta. Glucose, creatinine and lactate concentrations were measured using calorimetric assays. Radiolabelled tracers were measured by liquid scintillation counting. Enrichments and concentrations of amino acids were measured using GC-MS and HPLC respectively and placental gene expression by rt-PCR.

D-glucose had little or no effect on the placental transfer of  $^3\text{H}$ -3MG, suggesting a high capacity for glucose transport that is not transporter-limited. As glucose concentrations did not reach equilibrium between the maternal and fetal circulations, glucose transfer is limited. The factors limiting glucose transfer may be diffusive resistance, the endothelial barrier or placental blood flow.

$^{15}\text{N}$  from  $^{15}\text{N}$ -glutamate or  $^{15}\text{N}$ -leucine was incorporated into glutamine, aspartate, alanine, proline, valine and branched-chain amino acids.  $^{13}\text{C}$  from U- $^{13}\text{C}$ -glutamate appeared in glutamine, aspartate and proline isotopomers. These observations demonstrate placental amino acid metabolism which may allow synthesis of amino acids to meet fetal requirements.

Placental mRNA levels of genes involved in amino acid transfer were associated with a range of maternal factors, including smoking, exercise and parity, but relatively few fetal or neonatal parameters. This provides further evidence that maternal factors influence the placenta and provide interesting avenues for future research.

These findings have implications for our understanding of the mechanisms underlying altered fetal growth. These findings provide targets for interventions to optimise fetal growth and prevent chronic disease.

# Table of Contents

Abstract .....	iii
Table of Contents .....	iv
Table of figures.....	x
Table of Tables.....	xvii
Academic thesis: declaration of authorship .....	xx
Publications arising.....	xxi
Acknowledgements .....	xxii
Abbreviations.....	xxiii

## Chapter 1: General introduction

1.1: Background .....	1
1.2: Epidemiological studies and Developmental Origins of Health and Disease (DOHaD).....	2
1.2.1: Predictive adaptive responses, Match and Mismatch theories .....	3
1.2.2: Animal experimental studies.....	4
1.3: Fetal growth and development .....	5
1.3.1: The peri-implantation period.....	5
1.3.2: The embryonic period.....	6
1.3.3: The fetal period .....	6
1.3.4: Intrauterine growth restriction (IUGR).....	7
1.3.5: Factors regulating fetal growth .....	8
1.3.6: Nutritional supplements to improve fetal growth.....	8
1.3.7: Large for gestation age fetus.....	9
1.4: The placenta.....	10
1.4.1: Placental development and structure .....	10
1.4.2: Hormonal secretion by the placenta .....	16
1.4.3: Simple diffusion of nutrients across the placenta .....	18
1.4.4: Transport of nutrients across the placenta .....	18
1.5: Placental glucose transfer .....	20
1.5.1: Normal maternal and fetal plasma glucose levels .....	20
1.5.2: Glucose transfer across cell membranes .....	21
1.5.3: Placental glucose transporters .....	21
1.5.4: Placental glucose metabolism.....	23
1.5.5: Unanswered questions about placental glucose transfer .....	23
1.6: Placental amino acid transfer .....	24

1.6.1: Mechanisms of amino acid transport across human placental syncytiotrophoblast membranes.....	24
1.7: Amino acid metabolism.....	29
1.7.1: The metabolism of amino nitrogen .....	29
1.7.2: Catabolism of amino acid carbon .....	32
1.7.3: Essential and Non-essential amino acids .....	36
1.7.4: Inter-organ amino acid metabolism .....	37
1.7.5: Amino acid metabolism during pregnancy.....	42
1.7.6: Placental amino acid metabolism .....	43
1.8: Regulation of amino acid metabolic enzymes and transporters.....	44
1.8.1: Maternal influences on the placenta.....	45
1.8.2: Mechanisms of maternal influences on the placenta.....	45
1.8.3: Interactions between the placenta and the fetus .....	46
1.9: Scope of this thesis.....	47

## **Chapter 2: General methods**

2.1: Background .....	51
2.2: Placental perfusion methodology.....	51
2.2.1: Equipment and reagents .....	51
2.2.2: Working solutions .....	52
2.2.3: Perfusion set up .....	53
2.2.4: Placental tissue homogenisation .....	54
2.3: Calorimetric assays and scintillation counting .....	54
2.3.1: Equipment and reagents .....	55
2.3.2: Glucose assay.....	55
2.3.3: Creatinine assay.....	56
2.3.4: Lactate assay .....	56
2.3.5: Stable isotope analysis.....	56
2.3.6: Liquid scintillation counting.....	57
2.4: Principles of chromatographic separation.....	58
2.4.1: High Pressure Liquid Chromatography (HPLC).....	59
2.4.2: Gas Chromatography – Mass Spectrometry. ....	61
2.5: Analysis of amino acid concentration by reverse phase High Pressure Liquid Chromatography .....	63
2.5.1: Equipment and materials.....	64
2.5.2: Working reagents .....	65
2.5.3: Sample preparation, injection and analysis .....	65

2. 6: Analysis of branched keto acids and pyruvate concentrations by reverse phase HPLC .....	67
2.6.1: Equipment and reagents .....	67
2.6.2: Working reagents .....	68
2.6.3: Sample preparation, injection and analysis .....	69
2.7: Analysis of isotopic abundance in amino acids by GC-MS.....	70
2.7.1: Equipment and reagents .....	70
2.7.2: Resin preparation .....	71
2.7.3: Sample preparation and derivatisation .....	71
2.8: GC-MS analysis of isotopic abundance in glucose.....	73
2.8.1: Equipment and reagents .....	73
2.8.2: Sample preparation .....	73
2.9: Cohort studies: The Southampton Women’s Survey and placental gene expression .....	76
2.9.1: Maternal measurements in the SWS.....	78
2.9.2: Fetal and neonatal measurements in the SWS .....	78
2.9.3: Principles of mRNA expression analysis .....	79
2.9.4: Equipment and reagents .....	80
2.9.5: RNA extraction and purification .....	80
2.9.6: RNA quality control.....	81
2.9.7: The synthesis of cDNA.....	81
2.9.8: Choosing control genes for quantitative PCR.....	82
2.9.9: Quantitative polymerase chain reaction (qPCR) for the housekeeping genes .....	83
2.9.10: Primer design for the genes of interest.....	85
2.9.11: Quantitative polymerase chain reaction (qPCR) for the genes of interest .....	85
2.10: Methodology development.....	87
2.10.1: Sampling time during placental perfusion.....	87
2.10.2: Optimum conditions for determining amino acid concentration by HPLC.....	87
2.10.3: Optimal conditions for GC-MS sample preparation. ....	89
2.11: Statistics .....	95

### **Chapter 3: Glucose transfer and metabolism**

3.1: Background .....	99
3.2: Methods.....	100
3.2.1: Equilibration.....	101
3.2.2: Placental characteristics.....	101

3.2.3: Glucose and creatinine only experiments.....	102
3.2.4: Tracer experiments.....	103
3.2.5: Statistical analysis .....	105
3.2.6: Calculations for uptake, transfer, transport, diffusion, consumption and venous ratios .....	106
3.3: Results.....	108
3.3.1: D-glucose experiments without tracer .....	108
3.3.2: Tracer appearance preliminary results .....	108
3.3.3: Maternal to fetal tracer studies .....	113
3.3.4: Fetal to maternal tracer studies .....	116
3.3.5: Lactate production and transfer .....	118
3.4: Discussion .....	134
3.4.1: Glucose transfer across the human placenta .....	134
3.4.2: What is rate-limiting for placental glucose transfer? .....	135
3.4.3: Paracellular diffusion of glucose .....	137
3.4.4: Placental consumption and glucose transfer .....	139
3.4.5: Implications .....	141
3.4.6: Summary .....	142

## **Chapter 4: Amino acid transfer and metabolism**

4.1: Background .....	145
4.2: Methods.....	147
4.2.1: Placental characteristics.....	147
4.2.2: Fetal and maternal <sup>15</sup> N-glutamate arterial perfusions. ....	147
4.2.3: Maternal <sup>15</sup> N-leucine arterial perfusion .....	148
4.2.4: U- <sup>13</sup> C-glutamate perfusions .....	149
4.2.5: Statistics .....	150
4.2.6: Calculations .....	151
4.3: Results.....	152
4.3.1: Concentrations of free amino acids in the intracellular placental pool. .....	153
4.3.2: Infusate uptake into the placenta from the maternal and the fetal circulation .....	153
4.3.3: The fate of nitrogen from <sup>15</sup> N-glutamate infused in the fetal artery .	155
4.3.4: The fate of nitrogen from <sup>15</sup> N-glutamate infused in the maternal artery .....	157
4.3.5: The fate of nitrogen form <sup>15</sup> N-leucine infused in the maternal artery .....	158

4.3.6: Concentration differences in the <sup>15</sup> N labelled amino acids between the three experiments.....	160
4.3.7: Concentrations of BCKAs (α-keto-isocaproic acid, α-keto-isovaleric acid and α-keto-β-methylvaleric acid) and pyruvate .....	161
4.3.8: The fate of <sup>13</sup> C carbon from glutamate in other amino acids .....	162
4.4: Discussion .....	180
4.4.1: Placental free amino acid concentrations .....	180
4.4.2: Infusate uptake and transfer by the human placenta.....	181
4.4.3: The metabolism of nitrogen from glutamate and leucine .....	181
4.4.4: U- <sup>13</sup> C-glutamate is used for amino acid synthesis and intermediary metabolism.....	188
4.4.5: Evidence for metabolic compartmentalisation .....	193
4.4.6: Evidence for novel amino acid efflux mechanisms in the placenta.	195
4.4.7: Summary.....	197

## **Chapter 5: Associations between maternal factors, placental mRNA levels and fetal growth parameters**

5.1: Background .....	201
5.2: Methods.....	203
5.2.1: Gene expression analysis .....	203
5.2.2: Statistics.....	206
5.3: Results.....	208
5.3.1: Maternal and neonatal characteristics.....	208
5.3.2: Genes that were not detected at mRNA level.....	208
5.3.3: Gene expression data .....	208
5.3.4: Sex differences in mRNA levels of housekeeping genes in the placenta.....	209
5.3.5: Maternal smoking before pregnancy .....	209
5.3.6: Maternal smoking during pregnancy .....	209
5.3.7: Maternal exercise .....	210
5.3.8: Maternal parity.....	211
5.3.9: Maternal body composition .....	211
5.3.10: Maternal dietary prudence.....	213
5.3.11: Maternal high energy diet .....	213
5.3.12: Maternal social class .....	214
5.3.13: Maternal depression.....	214
5.3.14: Paternal birth weight.....	214
5.3.15: Placental weight .....	215
5.3.16: Placental to birth weight ratio .....	215

5.3.17: Birth weight.....	215
5.3.18: Fetal abdominal circumference growth rate.....	215
5.3.19: Fetal head circumference .....	215
5.3.20: Fetal femur length.....	215
5.3.21: Subscapular skinfold thickness .....	216
5.4: Discussion .....	239
5.4.1: Amino acid transporter and metabolic enzyme mRNA expression .....	239
5.4.2: Sex differences in mRNA levels .....	240
5.4.3: Maternal environment and placental gene expression .....	241
5.4.4: Paternal birth weight.....	246
5.4.5: Placental mRNA levels and fetal growth parameters .....	246
5.4.6: Other associations from a parallel study.....	248
5.4.7: Additional analysis.....	248
5.5: Study limitations.....	249
5.6: Implications.....	251
5.7: Future work.....	251
5.8: Summary .....	252

## **Chapter 6: General discussion**

6.1: Background .....	255
6.2: Transfer and metabolism of glucose across the human placenta .....	256
6.2.1: Implications of glucose transfer for fetal growth .....	258
6.3: Placental amino acid metabolism .....	258
6.3.1: Implications of placental amino acid metabolism for fetal growth ...	261
6.4: Maternal influences on the placenta and placental gene expression....	262
6.4.1: Implications of placental gene expression studies.....	262
6.4.2: Implications of glucose and amino acid metabolism on fetal growth .....	263
6.5: Future studies.....	264
6.5.1: Placental glucose transfer follow up studies.....	264
6.5.2: Placental amino acid transfer follow up studies.....	266
6.5.3: Placental gene expression follow up studies .....	267
6.6: Summary .....	267

## **Appendices**

Appendix 1: Reference list.....	273
Appendix 2: Glucose tracer buffers .....	305
Appendix 3: Supplementary material for amino acid metabolism .....	309
Appendix 4: Supplementary materials for gene expression data.....	316

# Table of figures

## Chapter 1

<b>Figure 1.1:</b> Illustration, demonstrating the differences between nutrient transfer and transport .....	2
<b>Figure 1.2:</b> Stages of human growth and development in utero.....	5
<b>Figure 1.3:</b> In utero fetal weight increase chart .....	7
<b>Figure 1.4:</b> Illustration of stages of early placental villous development, showing different types of villi at different stages.....	12
<b>Figure 1.5:</b> An illustration of placental structure .....	14
<b>Figure 1.6:</b> MicroCT of a placental cotyledon.....	15
<b>Figure 1.7:</b> Electron micrograph of sectioned placental villi .....	16
<b>Figure 1.8:</b> Placental endothelial junctions.....	17
<b>Figure 1.9:</b> A summary of amino acid metabolism .....	33
<b>Figure 1.10:</b> Amino acid metabolisms in the Krebs's cycle .....	34
<b>Figure 1.11:</b> An illustration of the aspartate-malate shuttle .....	36
<b>Figure 1.12:</b> An illustration of the glucose alanine cycle .....	40
<b>Figure 1.13:</b> Inter-organ amino acid metabolism and exchange. ....	42

## Chapter 2

<b>Figure 2.1:</b> An illustration of an isolated perfused placenta methodology set up .....	54
<b>Figure 2.2:</b> Beta energies for $^3\text{H}$ and $^{14}\text{C}$ .....	58
<b>Figure 2.3:</b> Amino acid chromatogram showing retention times .....	67
<b>Figure 2.4:</b> HPLC profile of the derivatives of branched BCKAs and pyruvate .....	70
<b>Figure 2.5:</b> Standard curves for U- $^{13}\text{C}$ glutamate and $^{15}\text{N}$ -alanine .....	77
<b>Figure 2.6:</b> Example of variation in GLUD1 amino acid transporter mRNA.....	82
<b>Figure 2.7:</b> Expression stability of housekeeping genes .....	84
<b>Figure 2.8:</b> Glutaminase gene expression standard curve.....	86
<b>Figure 2.9:</b> Mean time points for sample collection .....	88
<b>Figure 2.10:</b> The changes in the ionic abundance of asparagine.....	90
<b>Figure 2.11:</b> Glutamine spectrum showing ionic peak response.....	91
<b>Figure 2.12:</b> Lysine spectrum showing ionic peak response.....	92

<b>Figure 2.13:</b> Aspartate spectrum showing ionic peak response.....	93
<b>Figure 2.14:</b> Asparagine spectrum showing ionic peak response .....	94

### **Chapter 3**

<b>Figure 3.1:</b> Experimental outline for experiments without tracers.....	102
<b>Figure 3.2:</b> Maternal arterial tracer perfusion experimental outline: .....	104
<b>Figure 3.3:</b> Experimental outline for fetal arterial tracer perfusion experiments. .....	105
<b>Figure 3.4:</b> An illustration of the pathways through which D-glucose, L-glucose, and 3MG ( <sup>3</sup> H-3-O-methyl-D-glucose), are transferred across the placenta. .....	107
<b>Figure 3.5:</b> Transfer of D-glucose from the maternal arterial circulation to the fetal circulation.....	108
<b>Figure 3.6:</b> Appearance of D-glucose and creatinine from maternal artery.....	109
<b>Figure 3.7:</b> Time course for the appearance of maternal arterial .....	110
<b>Figure 3.8:</b> Time course for the appearance of fetal arterial tracer .....	111
<b>Figure 3.9:</b> Tracer remaining in the maternal vein and appearing in the fetal vein .....	119
<b>Figure 3.10:</b> Tracer appearance in the maternal and fetal vein.....	120
<b>Figure 3.11:</b> Maternal to fetal transfer of creatinine and <sup>14</sup> C-L-glucose. ....	121
<b>Figure 3.12:</b> D-glucose in the fetal and maternal vein of the tracer experiments. .....	122
<b>Figure 3.13:</b> Vein to vein ratios for D-glucose, <sup>3</sup> H-3-O-methyl-D-glucose and L- glucose. ....	123
<b>Figure 3.14:</b> Maternal tracer experiments <sup>14</sup> C-L-glucose transfer to the fetal venous circulation from maternal artery.....	124
<b>Figure 3.15:</b> Placental uptake of <sup>3</sup> H-3-O-methyl-D-glucose from the maternal artery.....	124
<b>Figure 3.16:</b> Transfer of <sup>3</sup> H-3-O-methyl-D-glucose from maternal artery circulation to the fetal vein. ....	125
<b>Figure 3.17:</b> Maternal to fetal transfer of <sup>3</sup> H-3-O-methyl-D-glucose. ....	125
<b>Figure 3.18:</b> Placental uptake of D-glucose from the maternal artery.....	126
<b>Figure 3.19:</b> Maternal to fetal transfer of D-glucose.....	126

<b>Figure 3.20:</b> Estimated unidirectional maternal to fetal transport of D-glucose. .....	127
<b>Figure 3.21:</b> Estimated maternal to fetal unidirectional paracellular diffusion of D-glucose.....	127
<b>Figure 3.22:</b> Consumption of D-glucose by the placenta.....	128
<b>Figure 3.23:</b> Fetal to maternal paracellular diffusion of <sup>14</sup> C-L-glucose.....	128
<b>Figure 3.24:</b> Placental uptake of <sup>3</sup> H-3-O-methyl-D-glucose from the fetal circulation .....	129
<b>Figure 3.25:</b> Fetal to maternal <sup>3</sup> H-3-O-methyl-D-glucose transfer.....	129
<b>Figure 3.26:</b> Fetal to maternal transport of <sup>3</sup> H-3-O-methyl-D-glucose.....	130
<b>Figure 3.27:</b> Placental uptake of D-glucose from fetal arterial circulation.....	130
<b>Figure 3.28:</b> Fetal to maternal placental transfer of D-glucose. ....	131
<b>Figure 3.29:</b> Estimated fetal to maternal unidirectional transport of D-glucose. .....	131
<b>Figure 3.30:</b> Estimated fetal to maternal unidirectional paracellular diffusion of D-glucose.....	132
<b>Figure 3.31:</b> Consumption of D-glucose by the placenta in the fetal tracer experiments. ....	132
<b>Figure 3.32:</b> Lactate release from glucose perfused placentas into the maternal and the fetal vein. ....	133

## **Chapter 4**

<b>Figure 4.1:</b> Placental intracellular free amino acid concentrations. ....	154
<b>Figure 4.2:</b> Equilibration of enriched amino acids in the fetal vein .....	166
<b>Figure 4.3:</b> Uptake of the infused isotope by the placenta .....	166
<b>Figure 4.4:</b> Amino acid enrichments and concentrations .....	167
<b>Figure 4.5:</b> The uptake and metabolic distribution to other amino acids of <sup>15</sup> N from glutamate infused in the fetal arterial circulation.....	168
<b>Figure 4.6:</b> Amino acid enrichments and concentrations following 5 hrs maternal arterial <sup>15</sup> N-glutamate perfusion.....	169
<b>Figure 4.7:</b> The uptake and metabolic distribution to other amino acids of <sup>15</sup> N from glutamate.....	170
<b>Figure 4.8:</b> Amino acid enrichments and concentrations following 5 hrs maternal arterial <sup>15</sup> N-leucine perfusion. ....	171

<b>Figure 4.9:</b> The uptake and metabolic distribution to other amino acids of <sup>15</sup> N from leucine .....	172
<b>Figure 4.10:</b> Rate of transfer of BCKAs and pyruvate in the maternal and fetal veins and concentrations in the placental tissue.....	173
<b>Figure 4.11:</b> Concentrations of Pyruvate and BCKAs in the placental tissue .	174
<b>Figure 4.12:</b> Enrichments and concentrations of <sup>13</sup> C labelled glutamate.....	175
<b>Figure 4.13:</b> Enrichments and concentrations of glutamine .....	176
<b>Figure 4.14:</b> Enrichments and concentrations of <sup>13</sup> C labelled aspartate .....	177
<b>Figure 4.15:</b> Enrichments of <sup>13</sup> C labelled proline isotopomers .....	178
<b>Figure 4.16:</b> The uptake and metabolic distribution to other amino acids of <sup>13</sup> C from U- <sup>13</sup> C-glutamate.....	179
<b>Figure 4.17:</b> A summary of metabolic pathways involved in the metabolism of <sup>13</sup> C glutamate, <sup>15</sup> N-glutamate and <sup>15</sup> N-leucine in the normal term human placenta. ....	190
<b>Figure 4.18:</b> Simplified schematic of Krebs's cycle for U- <sup>13</sup> C-glutamate metabolism .....	191

## **Chapter 5**

<b>Figure 5.1:</b> Average expression stability value (M) for each amino acid transporter and metabolic gene, ranked according to increasing stability with the most stable genes on the right. ....	216
<b>Figure 5.2:</b> Sex differences in the expression of stably expressed genes.....	217
<b>Figure 5.3:</b> Associations between maternal pre-pregnancy smoking and placental mRNA levels.....	218
<b>Figure 5.4:</b> Association between maternal pre-pregnancy smoking and placental y <sup>+</sup> LAT1 mRNA levels.....	219
<b>Figure 5.5:</b> Associations between maternal smoking during pregnancy and placental y <sup>+</sup> LAT2 mRNA levels.....	219
<b>Figure 5.6:</b> Associations between maternal strenuous exercise and placental ASC1 mRNA levels.....	220
<b>Figure 5.7:</b> Sex differences in the associations between maternal strenuous exercise and placental glutaminase mRNA expression. ....	220

<b>Figure 5.8:</b> Associations between parity and placental mRNA levels for ASC2, EAAT3, cytosolic branched chain aminotransferase, glutamate dehydrogenase 1, and aspartate aminotransferase 2.....	221
<b>Figure 5.9:</b> Sex differences in the associations between maternal parity and placental alanine aminotransferase and LAT2 mRNA levels.....	222
<b>Figure 5.10:</b> The association between maternal birth weight and placental mitochondrial branched chain aminotransferase mRNA levels.....	223
<b>Figure 5.11:</b> The association between maternal birth weight and placental ASC1 mRNA levels.....	223
<b>Figure 5.12:</b> The association between maternal birth weight and placental LAT2 mRNA levels.....	224
<b>Figure 5.13:</b> The association between maternal height and placental aspartate aminotransferase mRNA levels. ....	224
<b>Figure 5.14:</b> The association between maternal height and placental cytosolic branched chain aminotransferase mRNA levels. ....	225
<b>Figure 5.15:</b> The association between maternal height and placental cytosolic branched chain aminotransferase mRNA levels placental mitochondrial branched chain aminotransferase mRNA levels in male placentas. ....	225
<b>Figure 5.16:</b> The association between maternal BMI and placental ASC1 mRNA levels.....	226
<b>Figure 5.17:</b> The association between maternal BMI and placental $\gamma^+$ LAT2 mRNA levels.....	226
<b>Figure 5.18:</b> The association between maternal BMI and placental glutaminase 1 mRNA levels.....	227
<b>Figure 5.19:</b> The association between pre-pregnancy mid-upper arm circumference and placental ASC1.....	227
<b>Figure 5.20:</b> The association between 11 week gestation mid-upper arm circumference and placental ASC1 mRNA levels.....	228
<b>Figure 5.21:</b> The association between mid-upper arm circumference at 34 weeks gestation and placental ASC1 mRNA levels.....	228
<b>Figure 5.22:</b> The association between pre-pregnancy arm muscle area and placental ASC1 mRNA levels. ....	229
<b>Figure 5.23:</b> The association between arm muscle area at 11 weeks gestation and placental ASC1 mRNA levels. ....	229

<b>Figure 5.24:</b> The association between arm muscle area at 34 weeks gestation and placental ASC1 mRNA levels. ....	230
<b>Figure 5.25:</b> The association between maternal pre-pregnancy calf circumference and placental ASC1 mRNA levels. ....	230
<b>Figure 5.26:</b> The association between pre-pregnancy sum of skinfold thickness and placental ASC1 mRNA levels. ....	231
<b>Figure 5.27:</b> The association between maternal derived fat mass and placental ASC1 mRNA levels. ....	231
<b>Figure 5.28:</b> The association between paternal birth weight and placental EAAT3 mRNA levels. ....	232
<b>Figure 5.29:</b> The association between paternal birth weight and placental glutaminase 1 mRNA levels. ....	232
<b>Figure 5.30:</b> The association between paternal birth weight and placental glutamine synthetase mRNA levels. ....	233
<b>Figure 5.31:</b> The association between placental weight and placental alanine aminotransferase mRNA levels. ....	233
<b>Figure 5.32:</b> The association between placental weight and placental LAT2 mRNA levels. ....	234
<b>Figure 5.33:</b> The association between placental Birth weight ratio and placental LAT2 mRNA levels. ....	234
<b>Figure 5.34:</b> The association between child's birth weight and placental alanine aminotransferase mRNA levels. ....	235
<b>Figure 5.35:</b> The association between fetal abdominal circumference growth rate and placental ASC1 mRNA levels. ....	235
<b>Figure 5.36:</b> The association between fetal abdominal circumference growth rate and placental glutamine synthetase mRNA levels. ....	236
<b>Figure 5.37:</b> The association between 19-34 week gestation fetal head circumference and placental LAT2 mRNA levels. ....	236
<b>Figure 5.38:</b> The association between 19 week gestation fetal head circumference and placental $\gamma$ <sup>+</sup> LAT2 mRNA levels. ....	237
<b>Figure 5.39:</b> The association between 34 week gestation fetal femur length and placental glutamine synthetase mRNA levels. ....	237
<b>Figure 5.40:</b> The association between fetal sub-scapular skinfold thickness and placental alanine aminotransferase mRNA levels. ....	238

## **Appendices**

<b>Figure I:</b> Mass-spectrum finger print ions of beta-D-galactofuranose-pentaacetate.....	309
<b>Figure II:</b> Mass-spectrum finger print ions of alpha D-glucose-pentaacetate..	310
<b>Figure III:</b> Mass-spectrum finger print ions of D-glucose, 2, 3, 4, 5, 6-pentaacetate.....	311
<b>Figure IV:</b> Placental uptake vs inter-conversion of $^{15}\text{N}$ -glutamate from the fetal arterial circulation.....	312
<b>Figure V:</b> Placental uptake vs. transfer of $^{15}\text{N}$ -glutamate from the fetal arterial circulation .....	312
<b>Figure VI:</b> Placental uptake vs. inter-conversion of $^{15}\text{N}$ -glutamate from the maternal arterial circulation.....	313
<b>Figure VII:</b> Placental uptake vs. transfer of $^{15}\text{N}$ -glutamate from the maternal arterial circulation.....	313
<b>Figure VIII:</b> Placental uptake vs inter-conversion of $^{15}\text{N}$ -glutamate from the maternal arterial circulation.....	314
<b>Figure IX:</b> Placental uptake vs. transfer of $^{15}\text{N}$ -leucine from the maternal arterial circulation .....	314
<b>Figure X:</b> Placental uptake vs inter-conversion of U- $^{13}\text{C}$ -glutamate from the fetal arterial circulation.....	315
<b>Figure XI:</b> Placental uptake vs. transfer of U- $^{13}\text{C}$ -glutamate from the fetal arterial circulation.....	315

# Table of Tables

## Chapter 1

<b>Table 1.1:</b> Recommended values for the diagnosis of gestational diabetes in the UK.....	21
<b>Table 1.2:</b> Amino acid transporter systems in the human placenta .....	28
<b>Table 1.3:</b> The classification and properties of amino acids .....	30

## Chapter 2

<b>Table 2.1:</b> Running solvent gradient for the of amino acid separation.....	66
<b>Table 2.2:</b> Amino acids molecular fragmentation ion m/z .....	73
<b>Table 2.3:</b> Housekeeping genes tested for stability in the placental tissue.....	84
<b>Table 2.4:</b> Coefficient variation of the amino acids measured by HPLC .....	89

## Chapter 3

<b>Table 3.1:</b> Average placental weights and fetal flow recovery rates .....	101
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## Chapter 4

<b>Table 4.1:</b> Perfused cotyledon weights and fetal venous flow rate .....	148
<b>Table 4.2:</b> Concentrations of <sup>15</sup> N enriched of amino acids in the placental tissue after 5 hrs <sup>15</sup> N-glutamate perfusion into the fetal arterial circulation .....	156
<b>Table 4.3:</b> Concentrations of amino acids and <sup>15</sup> N labelled amino acids in the placental tissue after 5 hrs maternal arterial <sup>15</sup> N-glutamate .....	158
<b>Table 4.4:</b> Concentrations of amino acids and <sup>15</sup> N labelled amino acids in the placental tissue after 5 hrs maternal arterial <sup>15</sup> N-leucine perfusion.....	160
<b>Table 4.5:</b> Concentrations of <sup>13</sup> C labelled amino acid isotopomers in the placenta .....	164
<b>Table 4.6:</b> Amino acids that were detected but were not enriched .....	165

## **Chapter 5**

<b>Table 5.1:</b> Primers and probes used for amino acid transporter genes .....	204
<b>Table 5.2:</b> Primers and probes used for amino acid metabolic enzyme genes .....	205
<b>Table 5.3:</b> Pre-pregnant maternal and neonatal characteristics .....	208

## **Appendices**

<b>Table I:</b> Amino acid concentrations in the placental tissue. ....	316
<b>Table II:</b> Relationship between placental mRNA levels and maternal lifestyle	317
<b>Table III:</b> Relationship between placental mRNA levels and maternal lifestyle .....	318
<b>Table IV:</b> Relationship between placental mRNA levels and maternal lifestyle .....	319
<b>Table V:</b> Relationship between placental mRNA levels at birth and maternal lifestyle.....	320
<b>Table VI:</b> Relationship between placental mRNA levels at birth and maternal lifestyle.....	321
<b>Table VII:</b> Relationship between placental mRNA levels and parental body composition .....	322
<b>Table VIII:</b> Relationship between placental mRNA levels and parental body composition .....	323
<b>Table IX:</b> Relationship between placental mRNA levels and parental body composition .....	324
<b>Table X:</b> Relationship between placental mRNA levels and parental body composition .....	325
<b>Table XI:</b> Relationship between placental mRNA levels and fetal growth parameters .....	326
<b>Table XII:</b> Relationship between placental mRNA levels at birth and fetal growth parameters .....	327
<b>Table XII:</b> Relationship between placental mRNA levels and fetal growth parameters .....	328
<b>Table XIV:</b> Relationship between placental mRNA levels and neonatal parameters .....	329

<b>Table XV:</b> Relationship between placental mRNA levels and neonatal parameters.....	330
<b>Table XVI:</b> Relationship between placental mRNA levels at birth and neonatal parameters.....	331
<b>Table XVII:</b> Relationship between placental mRNA levels and neonatal parameters.....	332
<b>Table XVIII:</b> Relationship between placental mRNA levels and 4 year parameters.....	333
<b>Table XIX:</b> Additional table for relationship between placental mRNA levels and 4 year parameters.....	334
<b>Table XX:</b> The relationships between placental genes and pre-pregnancy smoking .....	335
<b>Table XXI:</b> The relationships between placental genes and smoking in pregnancy .....	335
<b>Table XXII:</b> The relationships between placental genes and parity .....	336
<b>Table XXIII:</b> The relationships between placental genes and strenuous exercise .....	336
<b>Table XXIV:</b> The relationships between placental genes and maternal diet ...	337
<b>Table XXIV:</b> The relationships between placental genes and maternal body composition.....	338
<b>Table XXV:</b> The relationships between placental genes and fetal head circumference .....	338
<b>Table XXV:</b> The relationships between placental genes and neonatal growth parameters.....	339

**Academic thesis: declaration of authorship**

I, **PRISCILLA ELLY LIAWA DAY** declare that this thesis entitled

**The transfer and metabolism of glucose and amino acids by the human placenta**

and the work presented in it is my own and has been generated by me as the result of my own original research.

I confirm that:

1. This work was done wholly or mainly while in candidature for a research degree at this University;
2. Where any part of this thesis has previously been submitted for a degree or any other qualification at this University or any other institution, this has been clearly stated;
3. Where I have consulted the published work of others, this is always clearly attributed;
4. Where I have quoted from the work of others, the source is always given. With the exception of such quotations, this thesis is entirely my own work;
5. I have acknowledged all main sources of help;
6. Where the thesis is based on work done by myself jointly with others, I have made clear exactly what was done by others and what I have contributed myself;
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## **Publications arising**

### **Papers in peer reviewed journals**

- Cleal JK, **Day PE**, Hanson MA, & Lewis RM (2009). Measurement of housekeeping genes in human placenta. *Placenta* 30, 1002-1003.
- Cleal JK, **Day PE**, Hanson MA, & Lewis RM (2010). Sex differences in the mRNA levels of housekeeping genes in human placenta. *Placenta* 31, 556-557.

### **Abstracts presented at conferences**

- **Day, PE**, Jackson J, Cleal JK, Jackson A, Hanson MA and Lewis RM (2010) Amino acid metabolism by the isolated perfused human placenta. *J Physiol*, 19 C142
- **Day PE** Cleal JK, Hanson MA and Lewis RM (2010) Maternal environment and associations with placental gene expression (IFPA).
- **Day PE**, Cleal JK, Field EM, Hanson MA, Lewis RM (2011) Transport and paracellular diffusion of 3-o-methyl glucose across the isolated perfused human placenta (IFPA).

### **Papers in preparation**

- **Day PE**, Cleal JK, Jackson JJ, Jackson AA, Hanson MA and Lewis RM. Amino acid metabolism and transfer by the human placenta
- **Day PE**, Cleal JK, Field EM, Hanson MA and Lewis RM. Mechanisms of glucose transfer across the placenta
- **Day, PE**, Cleal, JK, Crosier, SR, Hanson, MA, Harvey, NCH, Robinson, SM, Cooper, C, Godfrey, KM, and Lewis, RM. Associations between maternal factors and placental genes involved in amino nitrogen transfer and metabolism
- **Day PE**, Cleal JK, Jackson JJ, Jackson AA, Hanson MA and Lewis RM. Methods for amino acid, branched chain keto acids and pyruvate extraction from placental tissue.

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## Abbreviations

Abbreviation	Full terminology	Abbreviation	Full terminology
KIC	$\alpha$ -ketoisocaproate	GLU	Glutamate dehydrogenase
KIV	$\alpha$ -ketoisovalerate	GLS	glutaminase
KMV	$\alpha$ -keto- $\beta$ -methylvalerate	GLUL	glutamine synthetase
KV	$\alpha$ -ketovalerate	HCL	Hydrochloric acid
ATP	Adenosine triphosphate	IGF	Insulin-like growth factor
ALT	Alanine aminotrasferase	IUGR	Intrauterine growth restriction
NH <sub>4</sub> <sup>+</sup>	Ammonium	LGA	Large for gestational age
ANOVA	Analysis of variance	LC	Liquid Chromatography
AST1/2	Aspartate aminotransferase 1/2	Val	L-valine
BG	Background	mTOR	Mammalian target of rapamycin
BCKA	Brached chain keto acids	MA	Maternal artery
BCAA	Branched chain amino acids	MFR	Maternal flow rate
Co <sub>2</sub>	Carbon dioxide	MV	Maternal vein
CV	Coefficient variation	mRNA	Messenger Ribonucleic acid
CoA	Co-enzyme A	MVM	Microvillus membrane
cDNA	complementary deoxyribonucleic acid	BCATm	Mitochondrial branched chain aminotransferase
CHD	Coronary heart disease	MMLV -RT	Moloney Murine Leukemia Virus Reverse Transcriptase
BCATc	Cytosolic branched chain aminotransferase	NA	Natural abundance
dATP	Deoxyadenosine triphosphate	NAD/NADH	Nicotinamide adenine dinucleotide
dCTP	Deoxycytidine triphosphate	MTBSTFA	N-Methyl-N- (tert-butyl-dimethyl-silyl) trifluoroacetimide
dGTP	Deoxyguanosine triphosphate	OPA	o-Phthaldialdehyde
dNTP	Deoxynucleoside triphosphates	O <sub>2</sub>	Oxygen
DNA	Deoxyribonucleic acid	PA	Placental uptake
dTTP	Deoxythymidine triphosphate	PAR	predictive Adaptive Response'
DOHaD	Developmental Origins of Health and Disease	q-rt-PCR	Quantitative real time-Polymerase Chain Reaction
DA	Donor artery	CPM	Counts per minute
DV	Donor vein	RA	Receipient artery
DEXA	Dual-energy x-ray absorptiometry	RV	Receipient vein
EBB	Earle's bicarbonate buffer	RT	Retention time
EJ	Endothelial junction	RT-HPLC	Reverse-Phase High Pressure Liquid Chromatography
Enr	Enrichment	SHMT	Serine hydroxymethyltransferase
FA	Fetal artery	SLC	Solute carrier
FFR	Fetal flow rate	SWS	Southampton Women's Survey
FV	Fetal vein	SEM	Standard error of mean
GC-MS	Gas Chromatograph-Mass spectrometry	STK	Stock
GDM	Gestational diabetes mellitus	TTR	Tracer/Tracee Ratio
GLUT	Glucose transporter	WHO	World Health Organisation



# **Chapter 1**

## **General introduction**



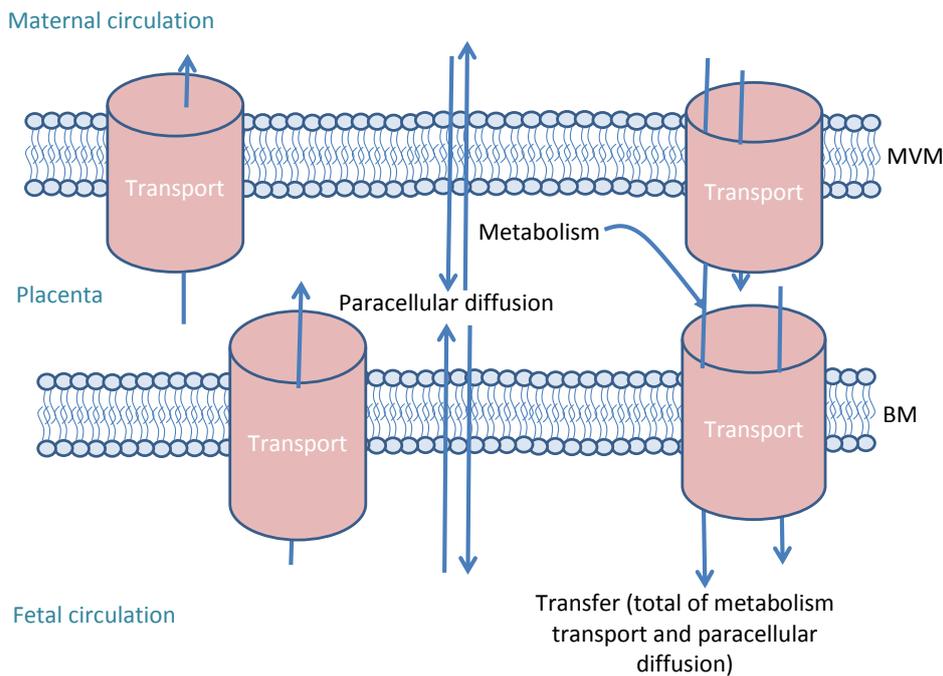
## 1.1: Background

The placenta is an important organ during fetal growth and altered placental nutrient transfer can cause intrauterine growth restriction (IUGR) and fetal macrosomia. Such altered fetal growth is associated with chronic disease such as cardiovascular disease, diabetes and obesity in adulthood (Barker, 1997c;Gluckman *et al.*, 2008). Chronic disease has major costs to societies worldwide, both at the personal and economic level. Developing a deeper understanding of placental function is necessary if we are to understand the determinants of fetal growth and develop interventions to prevent poor fetal growth and its long term sequel.

Placental nutrient transfer is the sum of membrane transport and placental metabolism. Nutrients from the mother must be transported across the maternal facing microvillus membrane (MVM) and the fetal facing basal membrane (BM) of the placenta to reach the fetus (Figure 1.1) (Jansson, 2001). Nutrients must also cross the fetal capillary endothelium but it is thought that water soluble nutrients cross paracellularly via endothelial cell junctions (Leach & Firth, 1992;Michel & Neal, 1999). Placental metabolism may consume nutrients taken up by the placenta, preventing them reaching the fetus, or produce new nutrients therefore altering the composition of nutrients being transferred to the fetus. Thus the placenta is not a simple conduit but actively determines which nutrients cross to the fetus.

Two of the most important nutrients for which transfer to the fetus has been most closely studied in association with fetal growth are glucose and amino acids (Osmond *et al.*, 2000;Jansson *et al.*, 1993;Cetin, 2003). Reduced glucose transfer to the fetus may result in fetal growth restriction while excessive glucose transfer may result in fetal macrosomia (Challis *et al.*, 2000;Osmond *et al.*, 2000). Impaired amino acid transfer is associated with IUGR in humans and it has been suggested that amino acids may play an important role in fetal macrosomia (Kalkhoff *et al.*, 1988;Cetin, 2003). Although the transfer of glucose and amino acids has been studied for some time there are contradicting data concerning these nutrients and important questions remain regarding their transfer across the placenta.

The broad aim of this study was to investigate placental transfer of glucose and amino acids in order to address these uncertainties. This was done by studying the transfer and metabolism of glucose and amino acids by the human placenta. Having established the pathways for metabolism and transfer, an exploratory study was carried out to determine whether placental transporters and metabolic enzymes involved in amino nitrogen transfer are subject to maternal environmental influences at the mRNA level.



**Figure 1.1: Illustration, demonstrating the differences between nutrient transfer and transport**

## 1.2: Epidemiological studies and Developmental Origins of Health and Disease (DOHaD)

The DOHaD hypothesis was developed based on epidemiological observations linking poor growth *in utero* to higher rates of chronic disease in later life. The DOHaD hypothesis suggests that the risk of developing some chronic diseases in adulthood is influenced not only by genetic and lifestyle factors but by environmental factors acting in fetal and infant life (Barker, 1995; Gluckman & Hanson, 2004a). Studies in a Hertfordshire cohort demonstrated that low birth weight is associated with higher risk of coronary heart disease (CHD) and

diabetes in later life (Barker *et al.*, 1989). Subsequent studies in Finland then found an association between postnatal growth and increased risk of CHD, with boys being at a higher risk than girls, if they have rapid growth by age of one (Eriksson *et al.*, 2001). Further epidemiological studies have suggested that poor maternal nutrition is the factor underlying the association between low birth weight and postnatal disease. These studies have led to the hypothesis that in order to survive periods of maternal under-nutrition *in utero*, the fetus makes physiological or metabolic adaptations that result in permanent alterations to the development of organs and tissues.

Studies on the effects of the Dutch Famine of World War II (November, 1944 to May, 1945) have supported this hypothesis by indicating that maternal under-nutrition can have long term effects on the offspring (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000). These studies also suggested that the effects of *intra-uterine* environment depend on the time at which the insult occurred. They showed that although birth weight was not reduced in individuals who were exposed to under-nutrition during the first trimester of pregnancy, they had a higher risk of obesity and CHD than those exposed later in pregnancy (Ravelli *et al.*, 1999). This led to the suggestion that the timing of maternal nutrition has varying effects on the different organs of the offspring depending on what tissue is growing at the time of the insult.

Various theories have been proposed to explain the mechanisms involved in developmental adaptation that may lead to long term health consequences. In addition, various studies have been carried out in animals and humans to determine mechanisms involved in these developmental adaptations and determine whether nutritional supplementation may improve fetal growth.

### **1.2.1: Predictive adaptive responses, Match and Mismatch theories**

As well as the fetal responses that provide an immediate survival advantage, it has been suggested that adaptations may occur with no immediate benefit to the fetus, but aimed at enabling future reproduction (Ravelli *et al.*, 1999; Roseboom *et al.*, 2000). These adaptations are termed 'predictive adaptive responses' (PARs). Health complications may however arise if the

environment *in utero* does not match the postnatal environment (Match and Mismatch concept). It has been suggested that the effects of a mismatched environment may particularly be evident in individuals who have moved from a poorer environment to a richer environment (Gluckman & Hanson, 2004a).

### **1.2.2: Animal experimental studies**

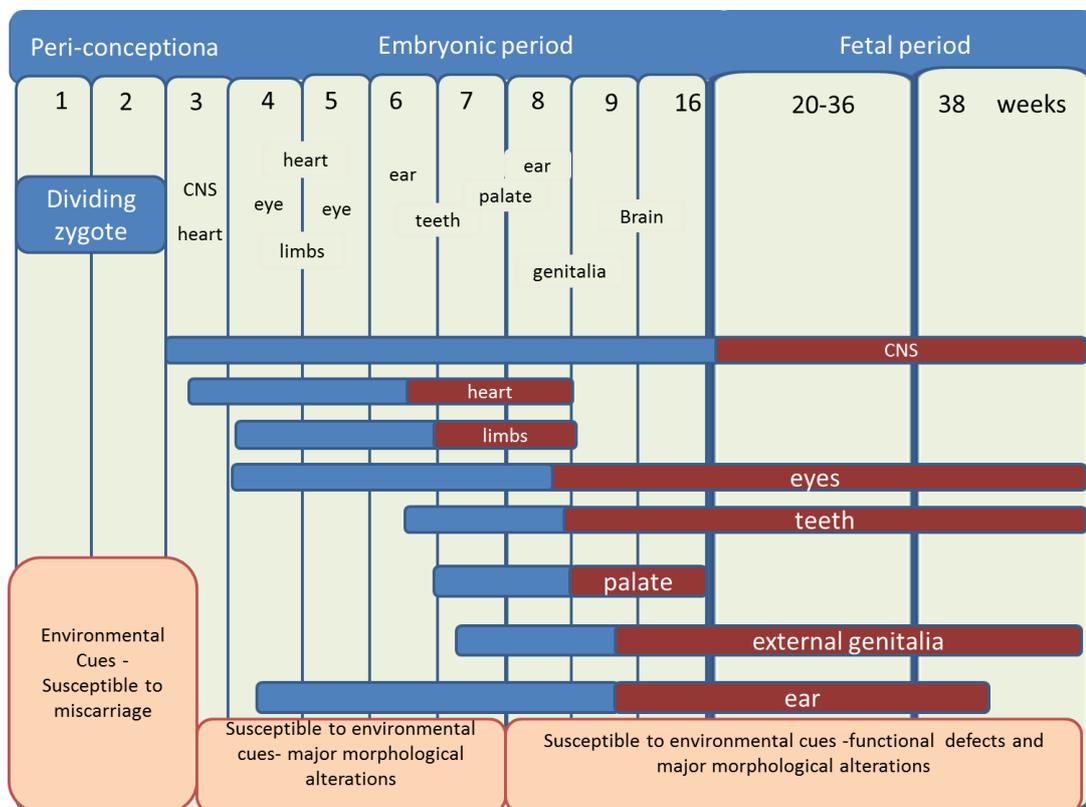
A range of animal studies have been carried out to investigate mechanisms involved in developmental adaptations that lead to chronic diseases in adult life. An association between perturbations of the peri-implantation environment and altered postnatal cardiovascular and renal function have been confirmed in both rats and sheep (Gluckman *et al.*, 2007). It has been demonstrated that these perturbations may be regulated by hormones. Evidence for this comes from the observations that maternal under-nutrition post-conception influences fetal insulin secretion in late gestation and that brief fetal exposure to glucocorticoids alters glucose metabolism and blood pressure in adult sheep (Cleal *et al.*, 2007b; Rumball *et al.*, 2009; Connor *et al.*, 2009). Changes in glucose regulation, insulin secretion and action, blood pressure regulation and renal and endothelial function have been reported to influence fetal physiological and metabolic changes (Oliver *et al.*, 2001; Moss *et al.*, 2001; Gatford *et al.*, 2000).

Although the mechanisms through which the *in-utero* environment affects the fetus are not yet fully understood, there is evidence that alterations in gene expression via epigenetic mechanisms may be one of the ways through which maternal environment affects the placenta and the fetus. For example, poor maternal environment such as poor nutrition, hypoxia and diabetes, is associated with altered gene expression in the rat and sheep offspring and the placenta (Torrens *et al.*, 2008; Dilworth *et al.*, 2010; Hay, Jr., 2006). Alterations in gene expression in response to maternal environment have been shown to occur through inheritable epigenetic mechanisms involving histone modifications, and DNA methylation (Hay, Jr., 2006; Gheorghe *et al.*, 2007; Rouse *et al.*, 2007; Gheorghe *et al.*, 2006). Further evidence for this also comes from the observation that there is a strong association between epigenetic changes, birth weight and the risk of developing chronic disease in adulthood (Lillycrop & Burdge, 2010; Weaver *et al.*, 2004). Further studies are

required to determine how maternal environment affects placental gene expression in humans and whether this is through epigenetic mechanisms.

### 1.3: Fetal growth and development

Fetal growth is measured in terms of weight and size in relation to gestational age as well as changes in body composition and functional development of tissues or organs (Godfrey *et al.*, 2011; Hanson *et al.*, 2011). The gestation period for humans is 266 days (38 weeks) and consists of three periods of growth and development; the pre-implantation period, the embryonic and the fetal period as shown in Figure 1.2.



**Figure 1.2: Stages of human growth and development *in utero*** showing major events involved in fetal growth and development throughout pregnancy and the response at different periods to environmental cues. The numbers represent, gestation age in weeks. Image was adapted from Hill, 2010.

#### 1.3.1: The peri-implantation period

Within 24 hours of fertilisation, the zygote undergoes mitotic cell division called cell cleavage and forms many daughter cells called the blastomere. By the 32

cell stage, the zygote is called the morula, from which the blastocyst develops (Brar & Rutherford, 1988; Moore & Persaud, 2003). At this stage a fluid filled blastocoel forms and the cells become differentiated into the inner cell mass and the trophoctoderm (Larsen, 1997). The inner cell mass, eventually develops into the fetus, while the trophoctoderm, becomes the fetal membranes and the placenta (Moore & Persaud, 2003). On the 6<sup>th</sup> day after fertilisation, the blastocyst implants into the uterine wall and this is followed by the embryonic period which lasts until the 8<sup>th</sup> week of gestation (Moore & Persaud, 2003).

### **1.3.2: The embryonic period**

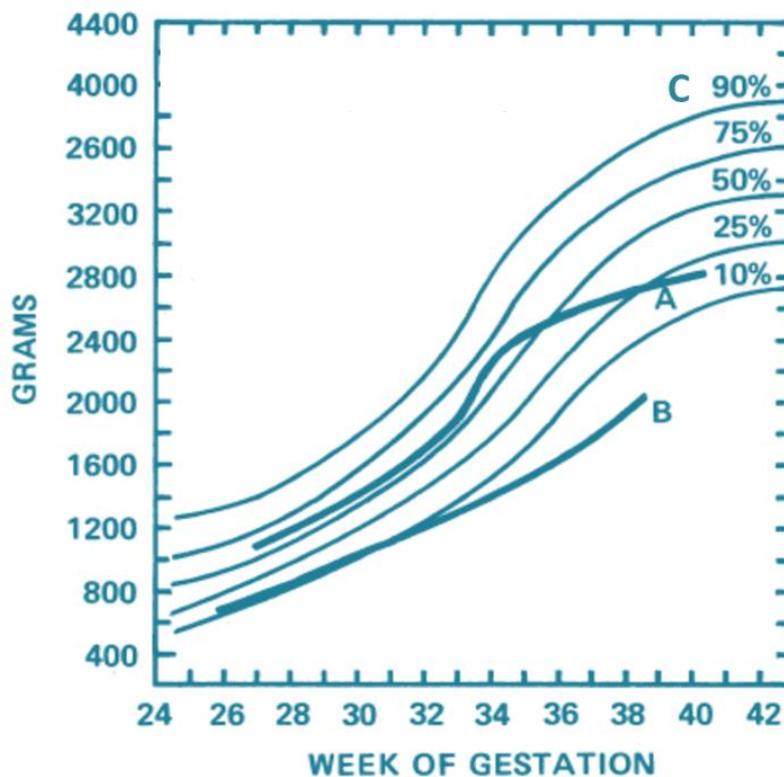
The embryonic period is a period of organ growth and development involving cellular hyperplasia and hypertrophy (Fowden *et al.*, 2006; Brown, 2009). Although by the embryonic period, the embryo is only 1.25 cm, its central nervous system is developed, the embryo has a beating heart, a digestive tract and facial appearances are beginning to form (Larsen, 1997). The embryonic period is followed by the fetal period.

### **1.3.3: The fetal period**

The fetal period begins from the 8<sup>th</sup> week of gestation until term (second to third trimester) and it is in this period that organ development, maturation and growth occurs. Although by the 28<sup>th</sup> week, the fetal lung is not fully developed, babies born around this week can survive if breathing is aided (Brown, 2009). The fetal period is also sub-divided into four stages which include the period of slow growth, accelerated growth, maximal growth and the period of decelerating growth. The period of slow growth occurs at around 15 to 16 weeks gestation where the fetus only gains 10 grams weight per week (Brown, 2009). Towards the end of 16<sup>th</sup> to the 27<sup>th</sup> week there is accelerated fetal growth and the fetus gains about 85 grams of weight per week. This is followed by the period of maximum growth which occurs between the 26<sup>th</sup> - 27<sup>th</sup> to the 38<sup>th</sup> week and during this stage, the fetus gains about 200 grams of weight per week. From the 37<sup>th</sup> week up until the last week of pregnancy, fetal growth decelerates and the fetus only gains about 70 grams per week.

Environmental insult at any time during gestation may have different effects on the fetus depending on the organs growing at that time and this may lead to

IUGR or fetal macrosomia (Neumann & Carroll, 1984). At different stages of gestation, measures of fetal body composition such as crown-rump length and femoral length are used to derive fetal weight and size. The percentiles of fetal weight at different gestations are then used to determine whether the fetus is normal for gestational age, small for gestational age or large for gestational age (Figure 1.3) (Fowden *et al.*, 2006).



**Figure 1.3: *In utero* fetal weight increase chart** with A and B representing a profile for growth restriction in utero and C representing a profile for large for gestational age weight (Figure adapted from Neumann & Carroll, 1984).

#### 1.3.4: Intrauterine growth restriction (IUGR)

IUGR babies are those who have not reached their full genetic growth potential and whose estimated birth weight is below the 10<sup>th</sup> percentile for their gestation age (Larsen, 1997; Neumann & Carroll, 1984; Zamorski & Biggs, 2001). IUGR is a clinical concern as it is associated with increased rates of neonatal morbidity and mortality as well as an increased risk of developing chronic disease in adulthood (Cross & Mickelson, 2006; Wollmann, 1998).

IUGR is categorised into asymmetrical and symmetrical IUGR with symmetrical IUGR accounting for 20 to 30% of all IUGR pregnancy outcomes and asymmetrical for 70 to 80%. (Figure 1.3) (Barker, 1997a;Neumann & Carroll, 1984). Symmetrical IUGR occurs during the first and second trimester of pregnancy when cells are multiplying and differentiating and thus characterised by the impairment of body and head growth (Sindi *et al.*, 2008). Asymmetrical IUGR is characterised by a continued head growth and compromised growth for the rest of the body. Asymmetrical IUGR occurs during late gestation when cells are increasing in size for the maturation of body organs as well as body weight and size (Cross & Mickelson, 2006;Wollmann, 1998).

### **1.3.5: Factors regulating fetal growth**

For optimal fetal growth all nutrients must be available at the required level. Any nutrient can become growth limiting if its level drops below that required by the fetus. This means that any alterations in the capacity for nutrient transfer across the placenta may become limiting for fetal growth. The capacity for nutrient transfer may be determined by maternal factors such as disease and smoking which may alter nutrients' availability and transfer across the placenta to the fetus (Peleg *et al.*, 1998;Wollmann, 1998). In addition, fetal weight may be determined by maternal constraints such as small maternal size, parity and maternal age which may indirectly alter the level of nutrients being transferred to the fetus (Mayhew *et al.*, 2008;Scholl *et al.*, 2001;Sastry *et al.*, 1989).

While the causes of IUGR are not always clear, impaired placental function is likely to be a major contributor. In IUGR there is impaired amino acid transfer to the human fetus and fetal hypoglycaemia (Gluckman & Hanson, 2004b). In addition, in rats the decrease in placental amino acid transfer has been shown to precede the development of IUGR suggesting that it is a cause rather than a consequence of IUGR (Cetin *et al.*, 1992;Jansson *et al.*, 2006).

### **1.3.6: Nutritional supplements to improve fetal growth**

An adequate nutrient supply from the mother, via the placenta, is essential for fetal growth. Intervention studies have been undertaken to determine whether fetal growth can be improved by maternal nutritional supplementation. While

animal studies have shown that fetal growth may be improved by nutritional supplementation, in humans nutritional supplementation has been shown to have varying effects and at times has proved to worsen fetal growth outcomes (Jansson *et al.*, 2006). For example the supplementation of glucose has been shown to increase fetal oxygen demand which leads to impaired oxygen partitioning between organs and thus may worsen fetal growth (Sloana *et al.*, 2001;Rush D, 1989). In addition, high protein supplementation has been shown to lead to increased perinatal mortality and IUGR (Nicolini *et al.*, 1990). This has been attributed to adverse effects of high amino acid concentrations such as phenylalanine in phenylketonuria and competitive transport of amino acids across the placental membranes, which may reduce the supply of amino acids essential for fetal growth.

Problems faced during supplementation studies indicate that there is limited knowledge on the handling of nutrients by the mother, the placenta and the fetus. Placental metabolism may determine the delivery of supplemented nutrients to the fetus. It is important to investigate how glucose and amino acids are changed in the human placenta before they reach the fetal circulation. Knowing the extent of placental metabolism for individual amino acids will help establish which nutrients can be supplemented through the mother, the placenta or the fetus without having detrimental consequences on the mother or the fetus.

### **1.3.7: Large for gestation age fetus**

Large for gestation age (LGA) or fetal macrosomia is defined as babies with a birth weight in the 90<sup>th</sup> percentile or above and a weight of 4000 g or more regardless of their gestation age (Figure 1.3) (Sloana *et al.*, 2001;Rush D, 1989). LGA occurs commonly as a result of maternal diabetes which is thought to lead to increased glucose and amino acids transfer to the fetus (Zamorski & Biggs, 2001).

LGA is associated with a number of serious pregnancy complications including prolonged labour, caesarean delivery, postpartum haemorrhage, infection and thromboembolic events (Bibee *et al.*, 2011;Osmond *et al.*, 2001). In addition,

LGA babies are at a greater risk of becoming obese and developing chronic disease in adulthood (Gurel SA *et al.*, 1996).

It is clear that fetal growth involves complex mechanisms that require optimum supply of nutrients from the mother via the placenta. If the capacity for the placenta to transfer nutrients is altered, fetal growth will also be altered. It is therefore important to understand mechanisms involved in placental nutrient transfer to the fetus.

## **1.4: The placenta**

The placenta is essential for the sustenance of fetal growth and development. The placental barrier protects the fetus from the substances in maternal blood and controls the flow of maternal nutrients to the fetus. As an endocrine organ the placenta regulates both maternal and fetal physiology. In performing these essential functions the placenta enables the mother to adapt to the pregnancy and determines the health of the fetus, both *in utero* and in later life.

The placenta is a fetal tissue embedded in the wall of the uterus where it is perfused with maternal blood. It transports maternal nutrients to the fetus and fetal waste products to the mother. Maternal blood enters the placenta from the terminal ends of the uterine spiral arteries and flows around the placental villi before exiting back into the maternal uterine veins. The placental villi contain fetal blood vessels bringing fetal and maternal blood into close proximity but preventing any mixing of the two circulations. This protects the fetus from many unwanted substances but also this means that mechanisms must exist to transport the nutrients required by the fetus across the placental barrier (Figure 1.1). The following sections will outline the development of the placenta and then discuss placental function.

### **1.4.1: Placental development and structure**

Placental growth is prerequisite to fetal growth and development and as such it is the first organ to develop. The placenta develops from the cells of the trophoblast surrounding the peri-implantation embryo. At the time of implantation, nutrient demand by the embryo is met by simple diffusion (Wang *et al.*, 2009; Wallace & McEwan, 2007). However, as gestation progresses, with

transformation from embryo to fetus, a specialised exchange system which is capable of exchanging nutrients and gases as well as removing fetal waste products is required. This demand is met by the placenta; of which capacity to supply substrates must also increase as gestation progresses. This occurs by the development of the placenta which must first establish in *utero-placental* blood circulation and differentiate into specialised placental villi. Following the peri-implantation and implantation period, human placental development has three stages which correspond to the three trimesters of pregnancy.

#### **1.4.1.1: First trimester placental development**

Immediately after implantation, the trophoblast cells differentiate into the innermost cytotrophoblast and the outer syncytiotrophoblast. This is followed by the formation of cytotrophoblast projections into the trophoblastic lacunae and at this stage they are called the primary villi (Kliman, 2000). These are subsequently invaded by extra-embryonic connective tissue which transforms them into secondary villi containing a mesenchyme core surrounded by the inner cytotrophoblast cell layer and the outer syncytiotrophoblast layer (Boyd & Hamilton, 1970). This is followed by the penetration of the mesenchymal core with blood vessels and the formation of new branches. The formation of fetal capillaries marks the transformation of secondary villi into tertiary villi, which are eventually transformed into mesenchymal villi (King, 1987). These processes are repeated throughout pregnancy to allow the expansion of the placental villi. Mesenchymal villi continue to differentiate into immature intermediate villi, stem villi, mature intermediate villi and terminal villi (Demir *et al.*, 1989; Kaufmann *et al.*, 1979). By week 7 of gestation, mesenchymal villi are no longer formed from the original blastocystic cells but they are formed by sprouting from their own surface and that of immature intermediate villi (Castellucci *et al.*, 2000; Kaufmann *et al.*, 1979).

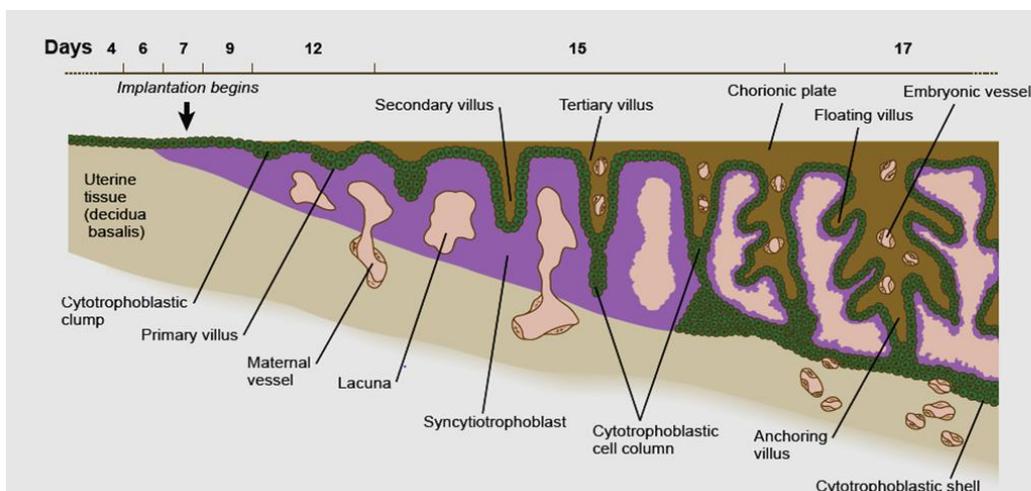
#### **1.4.1.2: Second trimester placental development**

Immature intermediate villi develop from mesenchymal villi and are characterized by their spherical structure, large numbers of cytotrophoblast cells, a thick layer of the syncytiotrophoblast and longitudinal connective tissue channels (stromal channels) (Kaufmann *et al.*, 1985). Immature intermediate

villi only form the major site for exchange in the first and second trimester, when the other specialised villi are not fully differentiated. Further formation of fibrous connective tissue transforms immature intermediate villi into stem villi. Stem villi are better capillarised, have no stromal channels and they branch out to form the villous tree and anchoring villi.

#### 1.4.1.3: Third trimester placental development

At this stage mesenchymal villi are no longer transformed into immature intermediate and stem villi (Castellucci *et al.*, 2000; Kaufmann *et al.*, 1979). Instead, they are transformed into mature intermediate villi, which have fewer cytotrophoblast cells, a thin layer of syncytiotrophoblast and no stromal channels. These do not transform into stem villi but instead they transform into terminal villi. Because of the highly dilated capillaries and thin syncytiotrophoblast layer, terminal villi provide a better surface area for maternal-fetal exchange (Castellucci *et al.*, 2000). Depending on the location of the cytotrophoblast cells, they can differentiate to give rise to floating villi or anchoring villi (Figure 1.4).



**Figure 1.4: Illustration of stages of early placental villous development, showing different types of villi at different stages** (Figure adapted from Bruce, 2008) (Castellucci *et al.*, 2000).

#### 1.4.1.4: Establishment of utero-placental circulation

Following attachment of the blastocyst to the endometrium, cells of the trophoctoderm divide into cytotrophoblast and invade the maternal uterine tissue (Figure 1.4). The *utero-placental* circulation does not become established

until the end of the 1st trimester (Bruce, 2008). The vascular connections form earlier in gestation but the ends of the spiral arteries are blocked by cytotrophoblast plugs. Prior to this nutrients are released into the placenta by uterine glands in a process known as histiotrophic nutrition (Burton *et al.*, 1999).

Prior to the start of placental blood flow, the extravillous trophoblast is responsible for remodelling the spiral arteries. The extravillous trophoblast still contains a shell of cytotrophoblast columnar cells, which under the influence of hypoxia begin to bore into the syncytiotrophoblast layer, penetrate into the endometrial cells, comingle with the endometrial cells and form a cytotrophoblastic shell surrounding the developing embryo. The villi containing cytotrophoblast cells that invade the endometrial wall are called anchoring villi as they physically attach the placenta to the maternal uterine wall.

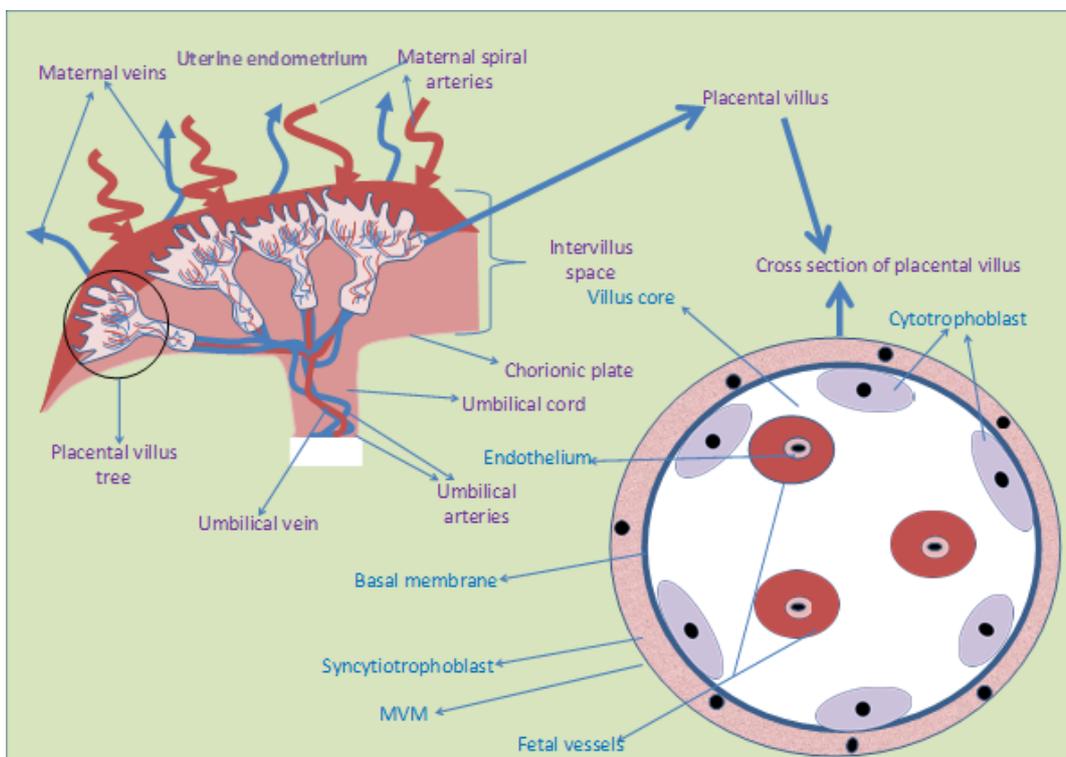
It was thought that the erosion of the smooth muscle around the spiral arteries reduced the vascular resistance of the spiral arteries allowing more blood flow to the placenta. However recent work has shown that the dilation of the ends of the spiral arteries by the extravillous trophoblast slows blood flow into the placenta (Burton *et al.*, 2002). This therefore ensures that high blood flow rate does not damage the villi thus increasing the efficiency of nutrient transfer.

#### **1.4.1.5: Structure of the placenta at term**

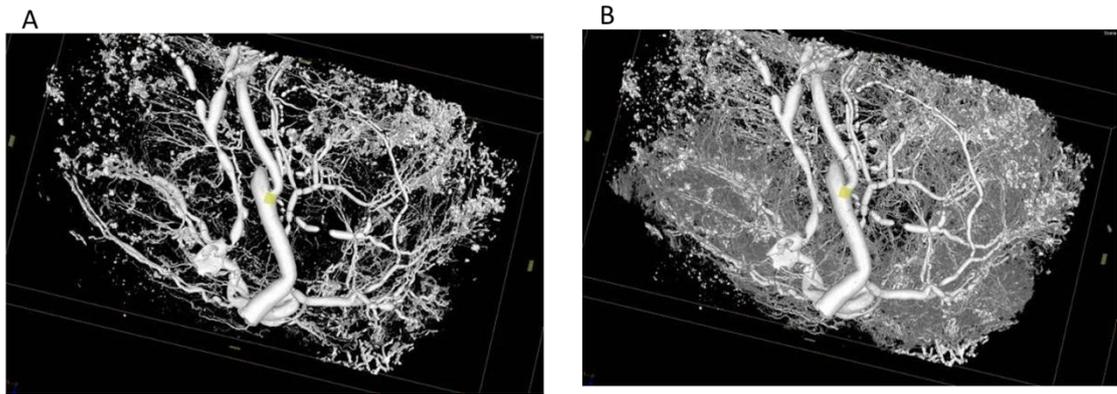
The placenta is a discoid organ of about 20 to 25 cm in diameter and about 2 to 3 cm thick. It is divided into functional units called cotyledons or lobules and there are about 20 to 40 cotyledons per placenta, each having its own maternal and fetal blood supply. The maternal side implants in the wall of the uterus and the fetal side is attached to the fetal membranes and umbilical cord which delivers fetal blood. The umbilical cord is suspended in the chorionic cavity which is enclosed in the chorionic plate from which the chorionic villi extend outwards. As the placental villi and the outer layer of the chorionic plate are bathed in maternal blood, the human placenta is termed a haemochorial placenta.

### 1.4.1.6: Maternal and fetal blood supply

The placenta has both maternal and fetal blood supplies which do not mix within the placenta. Maternal blood is delivered to the placenta via the spiral arteries. As it exits the spiral arteries it leaves the maternal vascular system and comes into direct contact with the placental villi. After flowing around the placental villi maternal blood re-enters the maternal vasculature at venous openings (Figure 1.5). Fetal blood is delivered to the placenta through the umbilical cord via two umbilical arteries and taken back to the fetus via the umbilical vein. Inside the placenta fetal blood is contained within the fetal vascular network (Figure 1.6).



**Figure 1.5: An illustration of placental structure and a cross section of a placental villous.**



**Figure 1.6: MicroCT of a placental cotyledon** showing the fetal vascular network A) without smaller vessels and B) including smaller vessels. The fetal chorionic plate arteries and veins are seen in the foreground. (From RM Lewis)

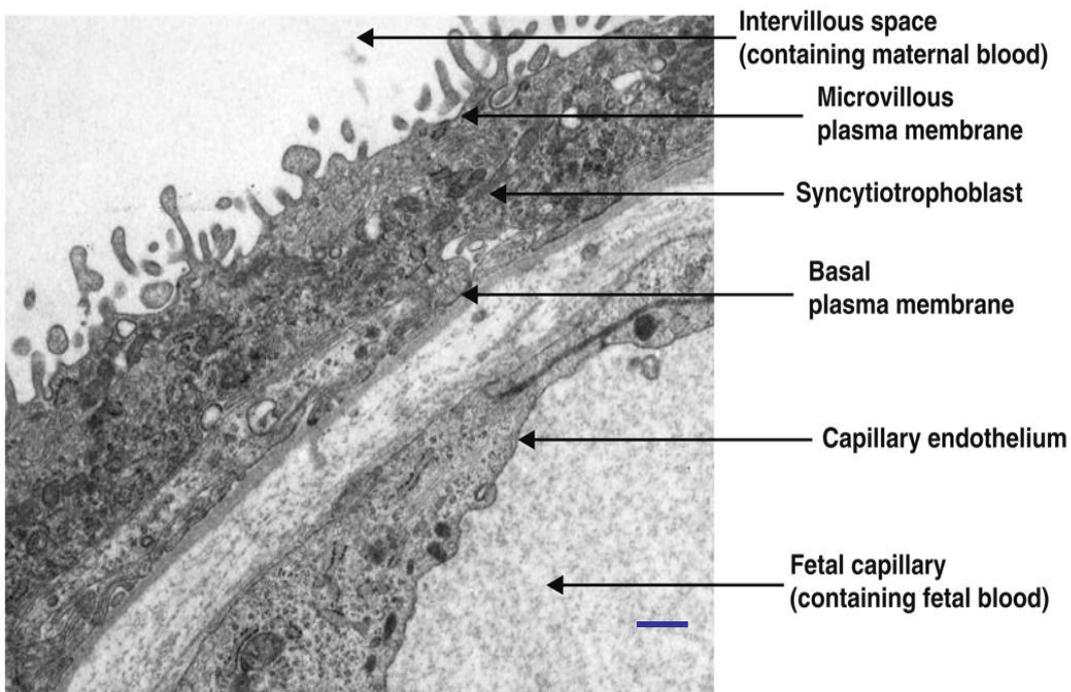
#### 1.4.1.7: The placental barrier

The placental syncytiotrophoblast forms a physical barrier between the maternal and fetal circulations. Maternal and fetal bloods are separated by the syncytiotrophoblast (in direct contact with maternal blood), a discontinuous layer of cytotrophoblast, connective tissue and finally the fetal capillary endothelium (in direct contact with fetal blood) (Figure 1.7). Together these block the transfer of cells and molecules which are not able to diffuse through lipid membranes. However, small lipophilic molecules, such as oxygen and carbon dioxide, can diffuse across the placenta relatively easily.

The syncytiotrophoblast is an outer layer of the placental villi formed from the fusion of cytotrophoblast cells. The syncytiotrophoblast is a continuous syncytium and therefore is the primary barrier of the placenta. On the maternal side of the placenta, the syncytiotrophoblast is covered in abundant microvilli which provide a large surface area for substrate exchange (Burton *et al.*, 2009).

Cytotrophoblast cells form a discontinuous cell layer between the syncytiotrophoblast and placental connective tissue. Continuous fusion of the cytotrophoblast throughout pregnancy is important for the replenishment of the syncytiotrophoblast. In comparison to early gestation, the villi later in gestation contain very small numbers of cytotrophoblast cells and contribute to a very small proportion of total placental volume. The cytotrophoblast is separated from the fetal capillaries by a basement membrane.

Between the trophoblast layers and the fetal endothelium is the BM which is also separated from the fetal endothelium by connective tissue, containing stromal cells. Although nutrients are able to pass through the stromal cells via simple diffusion to reach the fetus, nutrients must first cross the fetal endothelial barrier. There are conflicting ideas as to whether the fetal endothelium is permeable to nutrients, although there is clear evidence that there are gap junctions which may allow passive diffusion of small molecules (Figure 1.8) (Cantle *et al.*, 1987). However this may not apply to molecules such as lipids and immunoglobulin G (IgG) which need to be transported across the fetal endothelial barrier.

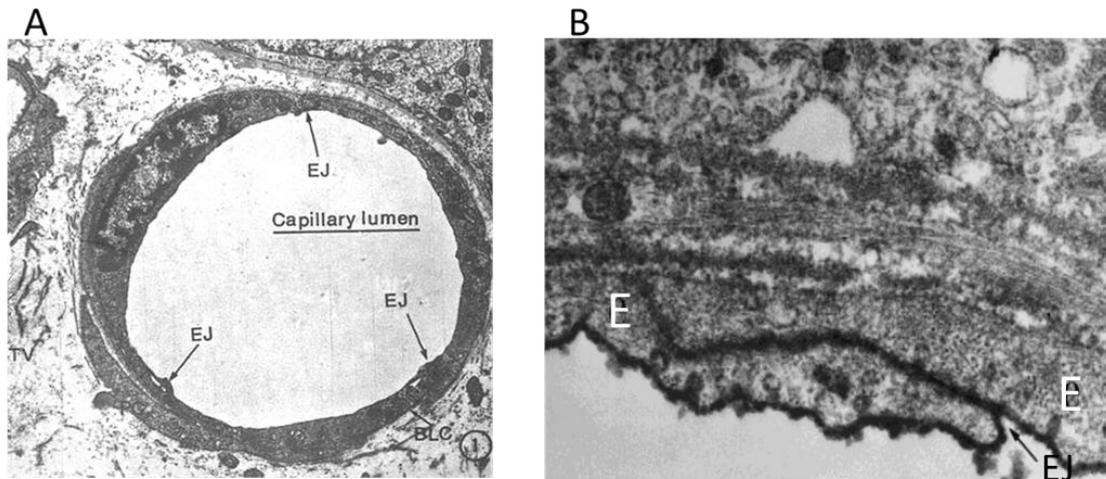


**Figure 1.7: Electron micrograph of sectioned placental villi**, showing various exchange barriers involved in the transfer of nutrients from the maternal circulation to the fetus. Bar = 2 $\mu$ m, figure adapted from Jones, by (Leach & Firth, 1992).

### 1.4.2: Hormonal secretion by the placenta

The secretion of hormones by the placenta into both the maternal and fetal circulation is also important as the hormones so produced enable the mother to adapt to pregnancy. Some of these adaptations include the modification of the

cardiovascular system, substrate metabolic adaptations and the modification of the uterus to ensure that sufficient substrates are transferred to the fetus.



**Figure 1.8: Placental endothelial junctions** A) Endothelial junctions within the placental capillary endothelium, B) Capillary endothelium which may allow paracellular diffusion of nutrients, EJ (endothelial junction), E (endothelial cells) (Tsitsiou *et al.*, 2011).

Earlier on in pregnancy, the embryonic trophoblast cells, which eventually develop into the placental tissue, secrete human chorionic gonadotropin (hCG) hormone (Leach & Firth, 1992). It is this hormone that also stimulates the secretion of placental oestrogen and progesterone and is commonly measured in the urine during a pregnancy test (Lopata *et al.*, 1997). Although the placenta is capable of synthesising progesterone, it requires the enzymatic activity of the fetal adrenal gland and the liver to synthesise oestrogen (Blackburn, 2007).

From the 15<sup>th</sup> week of pregnancy the placenta also takes over the secretion of growth hormone by the mother and becomes a sole secretor of this hormone. Growth hormone is thought to be important for the regulation of maternal blood glucose levels and ensure that there is more maternal gluconeogenesis for fetal glucose supply (Blackburn, 2007). The placenta also produces lactogen, otherwise called chorionic somatomammotropin, which is important for regulating fetal growth, lactation and the metabolism of lipids and carbohydrates. It has been reported that the levels of lactogen and growth hormone positively correlate with fetal growth and this indicates that they are capable of influencing fetal growth (Blackburn, 2007). Although the placenta is capable of evading the

maternal circulation and diverting nutrients to itself and the fetus by modifying maternal blood vessels and secreting hormones, it still remains questionable as to how the mother does not reject the placental and fetal tissue. Evidence is however emerging that the placenta expresses immunomodulatory cells and factors such as regulatory T- cells and indoleamine 2,3, dioxygenase (enzyme for the metabolism of tryptophan) that have been shown suppress maternal immune response in response to fetal tissue (Mannik *et al.*, 2010).

### **1.4.3: Simple diffusion of nutrients across the placenta**

Nutrients can be transferred across the placenta via passive diffusion and through transporters. During passive diffusion, nutrients can either diffuse between the cells (paracellular diffusion) or diffuse across the cells (transcellular diffusion). Paracellular diffusion of substances is dependent on the size of the molecule, with smaller lipophilic molecules diffusing more easily than larger lipophobic ones. Transcellular diffusion has been shown to mediate the transfer of small molecules such as oxygen and carbon dioxide.

Despite the apparently continuous nature of the syncytiotrophoblast there is a paracellular route across the placenta which has not been clearly identified at the anatomical level. The paracellular routes could be the areas of fibrinoid rich placental denudations where the syncytium has become damaged or the trans-syncytial channels (Munn *et al.*, 1999; Lee *et al.*, 2002; Mellor *et al.*, 2002). It has not yet been established whether transtrophoblastic channels extend across the whole placental syncytium (Brownbill *et al.*, 2000; Kertschanska & Kosanke, 1994). The rate of paracellular diffusion appears to be proportional to molecular size (Kertschanska & Kosanke, 1994). It should be remembered that diffusion via this route will be bi-directional. There are two ways in which a substance can cross via this route; via diffusion alone (in the absence of any movement of solute) or via bulk flow if a pressure difference is driving net movement of fluid in the maternal to fetal or fetal to maternal direction (Bain *et al.*, 1990).

### **1.4.4: Transport of nutrients across the placenta**

Due to limited diffusion of most substrates across the placenta, specialised transporters are required to transport nutrients to the fetus. The transfer of

nutrients across the placenta is bi-directional although most but not all of substances transferred to the mother from the fetus are waste products and toxins. The transport of both macro and micro nutrients across the placenta is mediated by specific proteins which are located on both the MVM and the BM of the syncytiotrophoblast layer. Transport of substrates may be either facilitated transport requiring protein transporters, without the need for energy and down a concentration gradient as with glucose, or via active transport such as those involved in amino acid transport which require both a transporter protein and energy (Brownbill *et al.*, 1995;Kertschanska *et al.*, 1997). Maternal immune cells such as IgG are also transported to the fetus to protect the fetus against infection (Manolescu *et al.*, 2007;Economides *et al.*, 1989).

To ensure that the fetus is able to grow to its genetic potential, nutrients must be transferred in the right composition and quantity. Insufficient amounts of any substrate available to the fetus may become limiting and thus alterations in any nutrient transport system may result in poor fetal growth. On the other hand, increased nutrient transfer as seen with glucose in maternal diabetes may result in fetal overgrowth, which is associated with maternal complications at birth and chronic disease such as type 2 diabetes in offspring's adulthood (Virella *et al.*, 1972). Recent studies have suggested that transport of nutrients across the placenta may be regulated by maternal and fetal signals through mechanisms that may involve hormonal and nutrient signalling (Leguizamon & von, 2003;Pettitt *et al.*, 1993). It is thought that fetal signals up-regulate the capacity for the placenta to transport nutrients required for its growth. However, maternal signals may be complex as there is a need for the mother to balance the support of the fetus whilst maintaining her own health to ensure her future reproductivity (Jansson *et al.*, 2003;Liu *et al.*, 1994;Constancia *et al.*, 2005).

It is clear that the transfer of nutrients across the placenta is much more complex than is currently known. It is important to understand how principle nutrients such as glucose and amino acids are transferred across the placenta and which mechanisms are involved.

## 1.5: Placental glucose transfer

Glucose is an important substrate for cellular metabolism and it is as important to the fetus as it is to the adult. Maternal plasma glucose is derived from the mother's diet and is sustained between meals by hepatic glycogenolysis and gluconeogenesis (King, 2003). While the mother can obtain glucose from her diet, there is conflicting evidence on whether gluconeogenesis occurs in the human fetus. One study has shown an increase in gluconeogenic enzymes; fructose 1,6-bisphosphatase and phosphoenolpyruvate carboxykinase from 20 weeks throughout gestation while another study using labelled  $^{13}\text{C}$ -alanine in severe growth-retarded fetuses was unable to demonstrate the availability of fetal gluconeogenesis (Anousis *et al.*, 2004). It may therefore be possible that the fetus depends on placental glucose transfer from the mother.

### 1.5.1: Normal maternal and fetal plasma glucose levels

According to the World Health Organisation (WHO), individuals with glucose levels between 6.1 mmol/L and 7.8 mmol/L are considered normal while those with fasting glucose levels above 7 mmol/L and 2 hour postprandial glucose levels above 11.1 mmol/L are considered diabetic (Sadava *et al.*, 1992; Marconi *et al.*, 1993). In pregnancy, fasting plasma glucose levels of 5.0 to 5.5 mmol/L are considered normal and in the UK, the values in Table 1.1 are used as a guide for the diagnosis of gestational diabetes. There are no clear classifications for hypoglycaemia by the WHO, but it has been suggested that individuals with plasma glucose levels below 3.9 mmol/L should be considered as hypoglycaemic (WHO, 2006). There are limited data, on fetal arterial and venous glucose concentrations, however using data from a high altitude study, with umbilical glucose concentrations of 3.5 mmol/L and fetal venous /fetal arterial difference of 0.7 mmol/L, arterial glucose concentration may be about 2.8 mmol/L (Mazze R *et al.*, 2012; Raju & Cryer, 2005).

**Table 1.1: Recommended values for the diagnosis of gestational diabetes in the UK.** Gestational diabetes is diagnosed if two or more measurements in one column are abnormal, i.e. if they exceed the levels indicated (Zamudio *et al.*, 2010).

Time	O'sullivan and Mahan (whole blood)	National Diabetes Data Group (NDDG) recommendations (plasma glucose in mmol/L)	Carpenter and Coustan adaptation (plasma glucose in mmol/L)
Fasting	≥ 5.0	≥ 5.8	≥ 5.3
1 hour	≥ 9.1	≥ 10.0	≥ 10.0
2 hour	≥ 8.0	≥ 9.1	≥ 8.6
3 hour	≥ 6.9	≥ 8.0	≥ 7.8

### 1.5.2: Glucose transfer across cell membranes

Glucose cannot diffuse across biological membranes and its transport must be mediated by transport proteins. There are two types of glucose transporters, secondary active Na<sup>+</sup> dependent glucose transporters found only in the gut and kidney epithelium and facilitated glucose transporter proteins called GLUTs which are found in all cell types. 13 members of the GLUT transporter family have been identified in mammalian tissue, with a tissue specific distribution. Of the 13 known transporters, the functional characteristics of GLUT1, 2, 3, and 4 are the most understood.

### 1.5.3: Placental glucose transporters

To be transported from the mother to the fetus glucose must be transported across the MVM and the BM of the syncytiotrophoblast. Glucose is thought to be able to diffuse through the endothelial junctions between the fetal capillary endothelial cells. It is therefore the glucose transporters in the MVM and the BM of the syncytiotrophoblast which are crucial for placental glucose transfer to the fetus. There are glucose transporters in other placental cell types but while these will take up glucose to support placental metabolism, thus decreasing supply to the fetus, they do not directly mediate placental glucose transfer themselves.

The primary glucose transporter in the human placenta is GLUT1, which is present at high levels on both the MVM and the BM (O'sullivan & Mahan, 1964;Carpenter & Coustan, 1982;Kahn, 2003). In addition to GLUT1, there is

evidence that several other glucose transporters are expressed in the human placenta. At mRNA level there is evidence that *GLUT3*, *GLUT4*, *GLUT9a*, *GLUT9b* and *GLUT12* are expressed in the human placenta.

*GLUT3* has been shown to be expressed at protein level on the MVM and BM of the syncytiotrophoblast in early gestation where it is thought to contribute to maternal-fetal glucose transfer but at term it is thought to make a minor contribution as it is not localised on the syncytiotrophoblast membranes (Jansson *et al.*, 1993; Magnusson *et al.*, 2004). At term *GLUT3* has been shown to be present in placental, endothelial and stromal cells. As *GLUT3* co-localises with glycogenin it is thought that *GLUT3* may play a role in mediating glucose supply from placental glycogen stores to the fetus during a period of metabolic demand (Illsley, 2000).

*GLUT4* mRNA has also been shown to be expressed in the human placental stromal cells at term. However as *GLUT4* protein is not found on the MVM or BM, it is unlikely to contribute to glucose transfer across the syncytiotrophoblast.

It has also been demonstrated that both *GLUT9a* and *GLUT9b* isoforms are present on the MVM and the BM of the placenta (Hahn *et al.*, 2001; Hauguel-De *et al.*, 1997). The extent to which *GLUT9a* and *b* play a role in mediating glucose transfer across the placenta is not clear. Further studies are required in this area to determine the role of these glucose transporters.

Further studies have shown that both the mRNA and protein for *GLUT12* is also expressed in the syncytiotrophoblast and extravillous trophoblast cells during the first trimester and in the stromal and endothelial cells at term in the human placenta (Bibee *et al.*, 2011). The role and substrate specificity of *GLUT12* is not known.

While several different glucose transporters are now known to be present in the placenta it is thought that *GLUT1* is by far the most abundant transporter and it is likely to mediate the majority of glucose transport across the placenta.

#### **1.5.4: Placental glucose metabolism**

It is not known how much maternally derived glucose is utilised by the human placenta but the sheep placenta consumes between 40% and 60% of the total glucose taken up from the maternal circulation (Gude *et al.*, 2003). In both humans and sheep the placenta is also known to produce lactate from glycolysis and release this back into the blood (Jansson *et al.*, 2003;Liu *et al.*, 1994;Bauer *et al.*, 1998). It is not known whether the human placenta also makes and releases pyruvate into either the maternal or fetal circulation.

Placental glucose metabolism is closely associated with amino acid metabolism. For example metabolites of glucose are introduced into the Krebs's cycle and may exit the Krebs's cycle to form the carbon skeleton of many amino acids; for instance pyruvate is used for alanine synthesis and oxaloacetate for aspartate synthesis. These amino acids may also be used for the synthesis of glucose. It has been speculated that placental amino acid metabolism may affect the availability of glucose in the fetal circulation (Bauer *et al.*, 1998;Battaglia & Meschia, 1986).

#### **1.5.5: Unanswered questions about placental glucose transfer**

It is clear that placental glucose transfer is mediated via facilitated glucose transporters, primarily GLUT1 (Lin *et al.*, 2003;Nehlig, 1997; Jansson *et al.*, 1993). However the reports that glucose transport across the placenta shows no signs of saturation kinetics up to a maternal plasma glucose concentration of 20 mmol/L do not seem to fit with the idea that the BM is rate-limiting for transport of glucose across the placenta (Jansson *et al.*, 1993). The failure to observe saturation kinetics in the human placenta is also in contrast to work in sheep where glucose transfer saturates at a maternal glucose concentration of 8 mmol/L (Osmond *et al.*, 2001;Hauguel *et al.*, 1986;Jansson *et al.*, 1993).

One paper has suggested that glucose transfer across the placenta may be flow-limited (Hay, 2008). Although there are no current studies showing the link between altered blood flow and glucose transfer, reduced placental glucose transfer has been attributed to altered blood flow at high altitude (Illsley, 1987).

In order to understand placental glucose transfer and improve fetal growth outcomes a clear picture of mechanisms involved in placental glucose transfer is required.

## **1.6: Placental amino acid transfer**

Placental amino acid transfer provides the fetus with an essential class of nutrients required for protein accretion, energy metabolism and as precursors for a wide range of biosynthetic pathways. Amino acids cannot diffuse across biological membranes and their passage must be mediated by membrane transport proteins. The availability of amino acids for the fetus is thought to depend on both their transport across the MVM and BM of the placental syncytiotrophoblast and on their metabolism by the placenta (Zamudio *et al.*, 2010). While the mechanisms of amino acid transport across the human placenta have recently been described, the metabolism of amino acids in the human placenta is not well understood (Vaughn *et al.*, 1995; Moores, Jr. *et al.*, 1994; Cleal & Lewis, 2008).

### **1.6.1: Mechanisms of amino acid transport across human placental syncytiotrophoblast membranes**

Amino acid transfer to the fetus across the placenta involves transport from the maternal circulation across the MVM into the placental syncytiotrophoblast and transport from the syncytiotrophoblast across the BM to the fetal circulation (Cleal & Lewis, 2008). Placental amino acid concentrations are higher than the maternal and fetal concentrations, and those of the fetal plasma are higher than the maternal plasma concentrations (Jansson, 2001). This demonstrates that amino acids are actively transported from the maternal circulation to the fetal circulation via the placenta (Phillips *et al.*, 1978).

There are three classes of amino acid transporters in the human placenta, namely; accumulative, exchangers and facilitated transporters (Cetin *et al.*, 2005) (Table 1.2).

### 1.6.1.1: Accumulative amino acid transporters

Accumulative transporters mediate the active transport of extracellular amino acids into the cell (influx). Accumulative amino acid transporters in the placenta include members of the system A amino acid transporter family (SLC38A1, SLC38A2 and SLC38A4),  $\gamma^+$  transporters and the system  $X_{AG}^-$  transporter family (Cleal & Lewis, 2008).

System A mediates  $\text{Na}^+$  dependent uptake of small neutral amino acids via a secondary active mechanism. The three system A transporters have been shown to be present in the placenta at the mRNA and functional levels (Cleal *et al.*, 2007a). Although system SLC38A4 has been localised on both the MVM and the BM at protein level, the precise membrane localisation of systems SLC38A1 and A2 is not known (Cleal & Lewis, 2008; Johnson & Smith, 1988).

System  $\gamma^+$  transporters mediate uptake of cationic amino acids and are dependent on the membrane potential of the cell to drive directional uptake. In the placenta system  $\gamma^+$  is localised on the MVM (Desforages *et al.*, 2006).

The main substrates for  $X_{AG}^-$  transporters are glutamate and aspartate. There are five types of  $X_{AG}^-$  transporters, four of which are expressed in the human placenta, namely EAAT1 (SLC1A3), EAAT2 (SLC1A2) and EAAT3 (SLC1A1) EAAT4 (SLC1A6) (Jansson *et al.*, 1998; Dicke *et al.*, 1993). While EAAT1 has been shown to be expressed in the syncytiotrophoblast, EAAT2 has been localised in both the syncytiotrophoblast and the endothelial cells and EAAT3 only in the endothelial cells (Noorlander *et al.*, 2004).

### 1.6.1.2: Amino acid exchangers

Amino acid exchangers are obligate exchangers and must exchange one amino acid for another across the membrane (Noorlander *et al.*, 2004). They alter the composition of amino acids within the cell but not the concentrations of the amino acids, allowing the cell to take up scarce amino acids without increasing the total amino acid concentrations. Amino acid exchangers in the placenta include the system L amino acid transporters, system ASC, system asc and system  $\gamma^+L$  transporters (Cleal *et al.*, 2007a).

There are two system L amino acid transporters; LAT1 (SLC7A5) and LAT2 (SLC7A8). Both isoforms transport large neutral amino acids, with LAT1 having a higher affinity than LAT2 (Cleal & Lewis, 2008). LAT2 also transports small neutral amino acids (Segawa *et al.*, 1999). They must both form heterodimeric complexes with 4F2hc (SLC3A2) to function (Segawa *et al.*, 1999). The mRNA and activity for both isoforms has been shown to be expressed in the placenta (Kim *et al.*, 2004) (Pineda *et al.*, 1999; Segawa *et al.*, 1999).

ASCT1 (SLC1A4) transports glutamate, alanine, proline and hydroxyproline (Cleal & Lewis, 2008). It has been suggested that ASCT1 may function to equilibrate amino acid concentration gradients between cell membranes (Pinilla-Tenas *et al.*, 2003; Pinilla *et al.*, 2001). *ASCT1* gene expression has been shown to be very low in the human placenta (Zerangue & Kavanaugh, 1996). ASCT2 (SLC1A5) transports anionic and neutral amino acids and it is also a receptor for syncytin (protein involved in syncytiotrophoblast formation) (Shafqat *et al.*, 1993; Kudo & Boyd, 2001).

System  $y^+L$  transports cationic amino acids and there are two transporters;  $y^+LAT1$  and  $y^+LAT2$  (SLC7A7 & SLC7A6) (Chen *et al.*, 2006). Both isoforms have been shown to be active and expressed at mRNA levels in the human placenta (Pfeiffer *et al.*, 1999).

### 1.6.1.3: Facilitated amino acid transporters

Facilitated transporters are  $Na^+$ -independent transporters and they mediate bi-directional transport of amino acids. In the placenta they are thought to allow efflux transport of amino acids across the BM of the placental syncytiotrophoblast in the direction of the concentration gradient. The facilitated transporters TAT1 (SLC16A10), LAT3 (SLC43A41) and LAT4 (SLC43A42) are expressed in the human placenta at both the protein and mRNA levels (Kamath *et al.*, 1999; Cleal *et al.*, 2011). In order to mediate the transfer of amino acids across the placenta the different classes of amino acid transporters must work together. On the MVM accumulative transporters mediate uptake of a subset of amino acids into the placenta. These amino acids taken up by the MVM accumulative transporters can then be exchanged for other amino acids transported by MVM exchangers. In this way all the amino acids the fetus needs

can be taken up by the placenta. On the BM accumulative transporters are present but they do not play an obvious role in mediating amino acid transfer to the fetus. This is because in the placenta accumulative transporters mediate uptake of amino acids into the cell (in the direction of  $\text{Na}^+$  gradient) (Cleal *et al.*, 2011). Net efflux of specific amino acids from the placenta to the fetus is mediated by facilitated transporters (Cleal *et al.*, 2007a). Some of the amino acids released into the fetal circulation by the BM facilitated transporters are then exchanged by BM exchangers for all the other amino acids required by the fetus.

**Table 1.2: Amino acid transporter systems in the human placenta** (Figure adapted from Cleal et al, 2008)

Human Gene Name (protein)	System	Membrane localization	Mechanism	Substrates
SLC38A1 (SNAT1)		mRNA present		Gly, Ala, Ser, Cys, Gln, Asn, His, Met, MeAIB
SLC38A2 (SNAT2)		Activity: MVM, BM (Hoeltzli & Smith, 1989c)		Gly, Pro, Ala, Ser, Cys, Gln, Asn, His, Met, MeAIB
SLC38A4 (SNAT4)	A	mRNA present Protein: MVM, BM (Desforges et al. 2001)	1Na <sup>+</sup> /AA cotransporter	Gly, Pro, Ala, Ser, Cys, Asn, (Met, MeAIB)
SLC1A1 (EAAT3)				Glu, Asp, Cys
SLC1A2 (EAAT2)	X-AG	Activity: MVM, BM	3Na <sup>+</sup> /1H <sup>+</sup> /AA-cotransport/1K <sup>+</sup> -exchange	Glu, Asp
SLC1A3 (EAAT1)				Glu, Asp
SLC1A4 (ASCT1)				Ala, Ser, Cys
SLC1A5 (ASCT2)	ASC	Activity: BM	Na <sup>+</sup> -dependent exchanger	Ala, Ser, Cys, Thr, Gln
SLC7A5 (LAT1)		Activity: MVM, BM (Ramadan et al, 2007; Ramadan et al., 2006; Verrey et al., 2004)		Gln, His, Met, Leu, Iso, Val, Phe, Tyr, Trp, BCH, L-DOPA
SLC7A8 (LAT2)	L		Exchanger	Ala, Ser, Cys, Thr, Asn, Gln, His, Met, Leu, Iso, Val, Phe, Tyr, Trp
SLC43A1 (LAT3)		Low/ mRNA (Cleal et al., 2007a)		Leu, Phe, Iso, Val, Met, BCH
SLC43A2 (LAT4)			Facilitated diffusion	Phe, Leu, Iso, Met, BCH
SLC7A7 (y <sup>+</sup> LAT1)		Activity: MVM, BM (Ayuk, et al, 2000); mRNA	Exchanger Na <sup>+</sup> -dependent for neutral amino acids	Lys, R, Gln, His, Met, LeuA
SLC7A6 (y <sup>+</sup> LAT2)	y <sup>+</sup> L	mRNA (Kim et al., 2002, (Cleal et al., 2011)		Lys, Arg, Gln, His, Met, LeuA, Ala, Cys
SLC16A10 (TAT1)			Facilitated diffusion	Phe, Tyr, Trp, Ala, Leu, L-DOPA

**Key :** BCH, 2-aminobicyclo-(2,2,1)-heptane-2-carboxylic acid; Ala, L-alanine; Arg, L-arginine; Asn, L-asparagine; Asp, L-aspartate; Cys, L-cysteine; Cys-, L-cystine; Glu, L-glutamate; Gln, L-glutamine; Gly, glycine; His, L-histidine; Iso, L-isoleucine; Leu, L-leucine; Lys, L-lysine; Met, L-methionine; Phe, L-phenylalanine; Pro, L-Proline; Ser, L-serine; Thr, L-threonine; Trp, L-tryptophan; Tyr, L-tyrosine; Val, L-valine (Val).

## 1.7: Amino acid metabolism

Amino acid synthesis and catabolism are important processes for whole body metabolism and they provide a link between carbon and nitrogen metabolism. The metabolism of amino acids and their inter-organ exchange is important for protein and nucleotide synthesis as well as for energy metabolism required to maintain cellular homeostasis.

All amino acids contain an amine group, a hydroxyl group and a side chain functional group which varies depending on the amino acid. In addition, all amino acids except glycine exist in two optical isomers; the L-isomer and D-isomer with the L-amino acids representing the metabolically important amino acids found in most mammals (Cleal *et al.*, 2011). Amino acids can be classified into various groups depending on their functional groups or depending on whether their carbon and nitrogen can be synthesised in the body (Table 1.3).

The catabolism of amino acids involves the removal of their nitrogen which may be incorporated into other amino acids or may be used in other pathways such as the urea cycle and the purine nucleotide synthesis cycle. Once the nitrogen has been removed, the carbon chain of the amino acid, called the keto acid, is also catabolised into various pathways, one of which is the Krebs's cycle, from which other amino acids are synthesised (Figure 1.9).

### 1.7.1: The metabolism of amino nitrogen

The metabolism of nitrogen is important for the synthesis of amino acids as well as polyamines (Spermidine and spermine) and nucleotides (purines and pyrimidine), which are essential for rapidly growing cells (Nelson DL & Cox MM, 2005). The nitrogen of amino acids may be metabolised through aminotransferase reactions which involve the exchange of nitrogen between amino acids and their keto acids or via deamination, whereby the nitrogen is lost from amino acids as ammonia. The incorporation of this ammonia into amino acids such as glutamate and aspartate to form glutamine and asparagine is termed amidation.

**Table 1.3: The classification and properties of amino acids**

Amino acids	Conditionally essential	charge	Other properties
Alanine			Aliphatic
Arginine	Conditionally essential	Basic	
Asparagine		Neutral	Non-polar
Aspartate		Acidic	
Glutamate		Acidic	
Glutamine	Conditionally essential	Neutral	Non-polar
Glycine	Conditionally essential		Aliphatic
Histidine	Essential (carbon backbone)	Basic	
Isoleucine	Essential (carbon backbone)		Aliphatic, branched chain
Leucine	Essential (carbon backbone)		Aliphatic, branched chain
Lysine	Essential (carbon backbone + nitrogen)	Basic	
Methionine	Essential (carbon backbone)		Sulphur containing
Phenylalanine	Essential (carbon backbone)		Aromatic
Proline	Conditionally essential		Imino acid
Serine		Neutral	
Threonine	Essential (nitrogen carbon backbone)	Neutral	Non-polar
Tryptophan	Essential (carbon backbone)		Aromatic
Tyrosine	Conditionally essential		Aromatic, Non-polar
Valine	essential (carbon backbone)		Aliphatic, branched chain
Cysteine	Conditionally essential		Sulphur containing

**Key :** Ala, L-alanine; Arg, L-arginine; Asn, L-asparagine; Asp, L-aspartate; Cys, L-cysteine; Cys-, L-cystine; Glu, L-glutamate; Gln, L-glutamine; Gly, glycine; His, L-histidine; Iso, L-isoleucine; Leu, L-leucine; Lys, L-lysine; Met, L-methionine; Phe, L-phenylalanine; Pro, L-Proline; Ser, L-serine; Thr, L-threonine; Trp, L-tryptophan; Tyr, L-tyrosine; Val, L-valine (Val).

Aminotransferase reactions reversibly transfer an amino group from an amino acid to a keto acid forming a second amino acid and the keto acid of the first amino acid (e.g. glutamate + the keto acid pyruvate  $\leftrightarrow$   $\alpha$ -ketoglutarate + alanine) (Figure 1.9). Most amino acids are involved in transamination reactions although to varying degrees (Wu *et al.*, 2008; Grimble & Grimble, 1998). For example glutamate, alanine, aspartate and branched chain amino acids (BCAAs) are readily involved in transamination reactions, with glutamate

playing a central role for nitrogen exchange between amino acids (Aqvist SEG, 1951; Jahoor *et al.*, 1988).

Amino acids, such as glycine, serine, histidine and threonine, are not readily involved in aminotransferase reactions (Jackson & Golden, 1980). The nitrogen from these amino acids is thought to be introduced into the free amino acid pool via their deamination to produce ammonia, which is then incorporated into other amino acids. In addition, glutamate may also be catabolised by glutamate dehydrogenase to form ammonia but with varying capacities depending on the tissue (Jackson, 1981). The extent at which the ammonia is incorporated into different amino acids also varies depending on the amino acid and the origin of the ammonia. Evidence for this comes from a study in rats where various <sup>15</sup>N labelled amino acids were administered and the <sup>15</sup>N enrichments of various amino acids were determined. It was found that amino acids such as histidine, threonine and lysine barely received nitrogen from any other amino acid, while serine and glycine predominantly transferred nitrogen to each other. Very low enrichments were observed in glutamate, aspartate and glutamine amide following infusion of <sup>15</sup>N-labelled glycine and when the transaminating amino acids were administered, very low enrichments were observed in glycine and serine (Jahoor *et al.*, 1988).

Amino acids such as glutamine and asparagine have also been shown to be enriched after the infusion of transaminating amino acids and amino acids that are catabolised by deamination (Aqvist SEG, 1951; Matthews *et al.*, 1981). Both glutamine and asparagine are formed after the transfer of ammonia to glutamate and aspartate via amidation reactions involving glutamine synthetase and asparagine synthetase respectively. Glutamine and asparagine may also be deaminated to form glutamate and aspartate via glutaminase and asparaginase respectively.

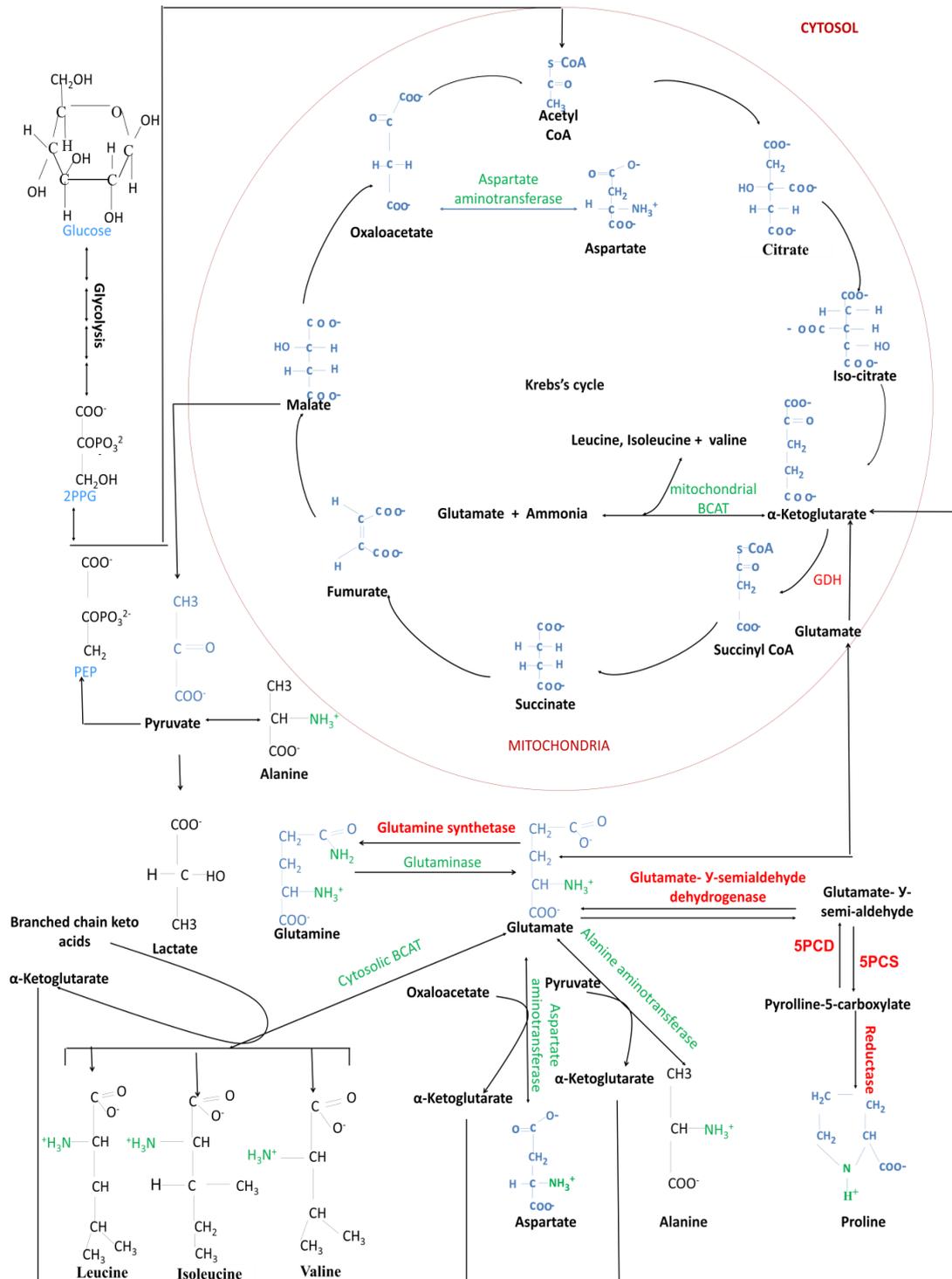
Aspartate nitrogen may also be incorporated into glutamine if aspartate is catabolised via the purine nucleotide cycle. However the provision of free ammonia from glutamate and the purine nucleotide cycle has been shown to be stoichiometrically insufficient to provide for the large amounts of glutamine required by the body (Matthews *et al.*, 1981). This therefore renders the source

of glutamine amide unknown. Other studies have postulated that the breakdown of glutathione, proteins and peptides may be another source of ammonia but this is subject to further investigation (Jackson, 1981). This also suggests that there are yet more studies required to fully understand interrelationships between amino acid nitrogen metabolism.

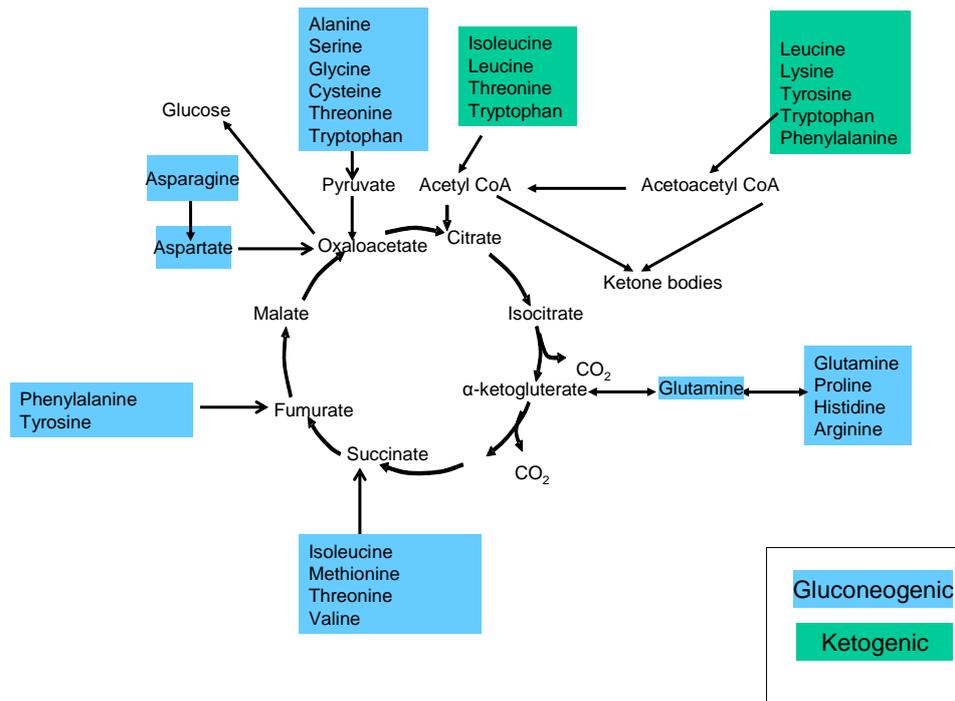
Glutamate can also be catabolised to form arginine or proline. The first committed step for proline and arginine synthesis requires the formation of  $\gamma$ -glutamate semialdehyde from glutamate (Tizianello *et al.*, 1978). Through cyclisation reaction,  $\gamma$ -glutamate semialdehyde, forms  $\Delta^1$ -pyrroline-5-carboxylate and is eventually metabolised to proline via  $\Delta^1$ -pyrroline-5-carboxylate reductase. Alternatively  $\Delta^1$ -pyrroline-5-carboxylate is catabolised by ornithine aminotransferase to form ornithine, which is then incorporated into the urea cycle and converted to citrulline and arginine as it goes through the cycle (figure 1.12).

### 1.7.2: Catabolism of amino acid carbon

When the amino nitrogen is taken off, the remaining keto acid will ultimately be broken down to  $\text{CO}_2$  and energy. This happens by different pathways for different amino acids. Amino acids that form glucose are called gluconeogenic amino acids, while those that form ketone bodies are called ketogenic amino acids (Figure 1.10). It should be noted in Figure 1.10 that some amino acids are both gluconeogenic and ketogenic. Both ketogenic and gluconeogenic amino acids are eventually metabolised in the Krebs's cycle through their conversion to Krebs's cycle intermediates such as  $\alpha$ -ketoglutarate, oxaloacetate, fumarate, succinyl-CoA and acetoacetate (Figure 1.10) (Bicknell *et al.*, 2008).



**Figure 1.9: A summary of amino acid metabolism** via the Krebs's cycle and aminotransferase reaction which involve glutamate as the central amino acid for carbon and nitrogen redistribution.



**Figure 1.10: Amino acid metabolisms in the Krebs's cycle**, in blue are all the gluconeogenic amino acids and in green are all the ketogenic amino acids.

### 1.7.2.1: Amino acid carbon metabolism in the Krebs's cycle

To enter the Krebs's cycle, glutamine and proline are first converted to glutamate, which is metabolised by glutamate dehydrogenase or aminotransferases to form  $\alpha$ -ketoglutarate (Felig *et al.*, 1970; Cahill, Jr. *et al.*, 1970; Noda & Ichihara, 1976) (Figure 1.9 & 1.10). Once in the Krebs's cycle  $\alpha$ -ketoglutarate is converted to other Krebs's cycle intermediates including oxaloacetate and malate. Oxaloacetate can continue on the cycle by condensing with acetyl-CoA or can be reversibly transaminated to form aspartate. Through a process known as pyruvate recycling, malate and oxaloacetate can also be converted to pyruvate by malic enzymes and phosphoenolpyruvate carboxykinase (plus pyruvate kinase) respectively which re-enters the Krebs's cycle via acetyl CoA (Waagepetersen *et al.*, 2008; Bak *et al.*, 2008; Reeds *et al.*, 2000). As aspartate and alanine interconvert with oxaloacetate and pyruvate respectively they may also subsequently be metabolised to form glutamine and glutamate (Olstad *et al.*, 2007b). Glutamate and aspartate are also important for the aspartate-malate shuttle which

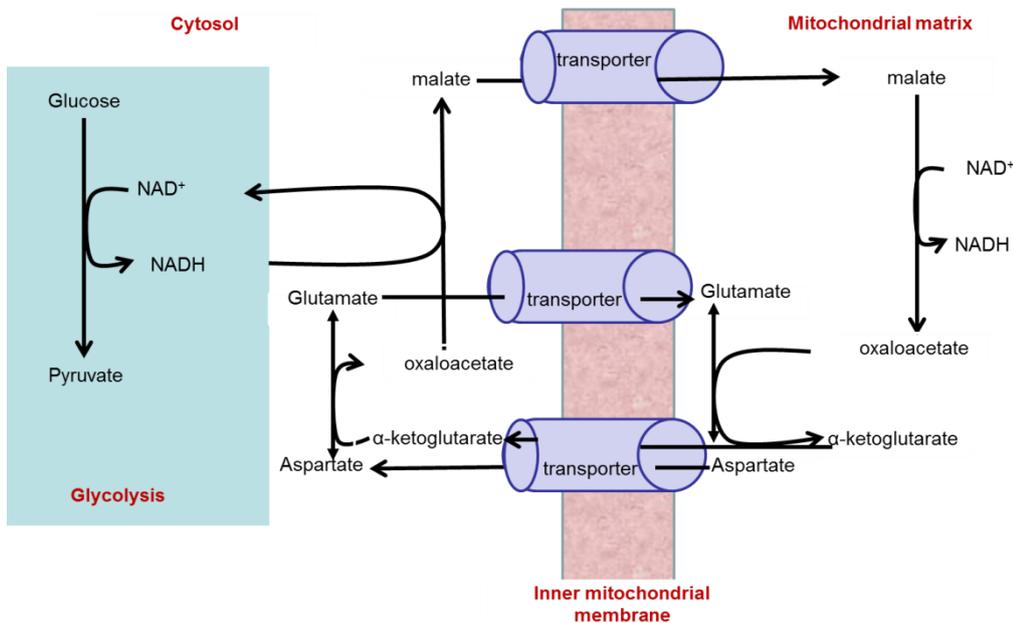
mediates the transfer of NADH from the cytosol to the mitochondria by reducing it to NAD<sup>+</sup> (Figure 1.11) (Olstad *et al.*, 2007b).

### **1.7.2.2: Serine and glycine carbon catabolism**

Serine and glycine may be catabolised to pyruvate and their carbon metabolism is thought to be the major pathway for folate co-enzyme and one carbon metabolism. Through their inter-conversions 5,10-methylene-tetrahydrofolate (a folate-co-enzyme) is formed via serine hydroxymethyltransferase (SHMT) (Salway, 2004). Although both serine and glycine can also be formed from glyoxylate and hydroxypyruvate, it has been suggested that there is yet more to be understood about the formation of serine and glycine carbon. This is because glyoxylate and hydroxypyruvate are only present at lower levels to satisfy the requirement for the major metabolic pathways they are involved in (Pasternack *et al.*, 1992).

### **1.7.2.3: The metabolism of BCAAs**

BCAAs are also catabolised through the Krebs's cycle by branched chain-  $\alpha$ -keto acid dehydrogenase enzyme, which exists in both active and inactive forms. When there is a low protein diet, the enzyme becomes inactive to limit the degradation of the keto acids and allow protein synthesis (Jackson, 1983). When there is sufficient protein the enzyme becomes active and oxidises the keto acids to their co-enzyme derivatives and eventually to glucose and acetoacetate via the Krebs's cycle (Harris *et al.*, 1985).



**Figure 1.11: An illustration of the aspartate-malate shuttle** which mediates the transfer of NADH from the cytosol to the mitochondria for the electron transport chain and enables energy replenishment.

Labelled carbon from BCAAs has also been shown to appear in glutamate, glutamine and aspartate (Harris *et al.*, 1985). This suggests that  $\alpha$ -ketoglutarate and oxaloacetate formed in the Krebs's cycle from the BCAAs are transaminated to glutamate and aspartate respectively.

### 1.7.3: Essential and Non-essential amino acids

As amino acids are synthesised and catabolised to various degrees they are classified as essential or non-essential depending on whether their carbon skeleton can be synthesised in the body (Table 1.3) (Bixel *et al.*, 2004). However, it has been demonstrated that some amino acids may become conditionally essential in certain disease conditions and during growth and development (Rose & Rice, 1939). In addition, it has also been demonstrated that while the body cannot synthesise the carbon skeleton of some amino acids (e.g. valine and leucine), if the carbon skeleton is provided in the diet it can be transaminated to produce valine or leucine (Jackson, 1983). This is in contrast to histidine and lysine, which cannot be synthesised in the body even when both the nitrogen and the carbon are supplied in the diet. Recent studies however suggest that there may be *de novo* synthesis of some essential amino acids such as lysine as a result of recycling via the activity of gut microflora (Walser *et*

*al.*, 1973;Richards *et al.*, 1977). Further studies are required to investigate whether amino acids can indeed be synthesised through the microflora pathway and whether this can meet the body's requirements.

#### **1.7.4: Inter-organ amino acid metabolism**

As the metabolism of amino acids is complex and requires specialised cells and enzymes, some amino acids may be synthesised in one tissue and transferred to another tissue and this is called inter-organ metabolism. The main tissues involved in inter-organ amino acid metabolism include, the gut, liver, muscle and kidney. Most studies on inter-organ metabolism have been carried out in animals rather than humans and if in humans, they have been carried out in various pathological conditions. It should therefore be noted that metabolic processes explained in this section may not be representative of normal human physiological conditions.

##### **1.7.4.1: The role of the gut in inter-organ metabolism**

Extensive studies in rats and pigs as well as humans have demonstrated that the gut is an important site for amino acid metabolism. The gut is the first site at which dietary protein and amino acids are catabolised and used for the synthesis of other amino acids or for energy metabolism (Torrallardona *et al.*, 2003;Millward *et al.*, 2000;Jackson, 1995). As a result amino acid compositions in the circulation do not represent amino acid compositions in the diet.

In humans, the gut preferentially extracts non-essential amino acids rather than essential amino acids. For example about 50% of enterally delivered glutamine and 90% of glutamate are extracted by the gut, while only 17% of leucine and 29% of phenylalanine are extracted by the gut (Baracos, 2004). Both in animal and human studies, most of the glutamine and glutamate absorbed by the gut are used for energy metabolism (Matthews *et al.*, 1993a;Matthews *et al.*, 1993b). Studies in humans have also demonstrated that the nitrogen from glutamate and glutamine is incorporated into transaminating amino acids such as alanine, proline, aspartate and the BCAAs which are then transferred to the liver (Windmueller & Spaeth, 1977;Reeds *et al.*, 1996). Studies using the pig's intestine have also suggested that glutamate is catabolised to glutathione which

is essential for maintaining the integrity of the gut epithelium (Reeds *et al.*, 1997). The gut microflora has also been implicated in the salvaging of urea nitrogen for the synthesis of essential and conditionally essential amino acids such as lysine and glycine during pregnancy (Figure 1.10) (Reeds *et al.*, 1997).

#### **1.7.4.2: The role of the liver in inter-organ metabolism**

The liver is an important site for the regulation of amino acid degradation and biosynthesis (Jackson, 1995; Tanaka *et al.*, 1980; Millward *et al.*, 2000; Torrallardona *et al.*, 1996). As excess amino acids cannot be stored, they are degraded in the liver to form glucose and ammonia (Jungas *et al.*, 1992). The glucose is stored as glycogen which is converted back to glucose when the glucose levels are low. In addition the liver may degrade amino acids from the muscle protein break down to make glucose when glucose levels are low (Salway, 2004). The ammonia from most amino acids is converted to glutamate in the cytosol of liver hepatocytes and the glutamate is then degraded to  $\alpha$ -ketoglutarate and ammonia by glutamate dehydrogenase in the mitochondria. The ammonia is then disposed of in the urea cycle or used for glutamine synthesis (Figure 1.12).

The presence of specialised cells that synthesise and degrade certain amino acids enables the liver to deal with varying metabolic states. For example, excess glutamine is degraded in the portal hepatocytes to glutamate while during fasting glutamine is synthesised from glutamate in the perivenous hepatocytes (Salway, 2004). This also enables the liver to deal with excess toxic ammonia in other tissues. In the muscle, the kidney and the intestine, ammonia is converted to non-toxic glutamine which is transferred to the liver for detoxification through the urea cycle. Ammonia in the liver is disposed of by the urea cycle either via carbamoyl phosphate synthetase or through aspartate via argininosuccinate (Figure 1.12) (Haussinger, 1998).

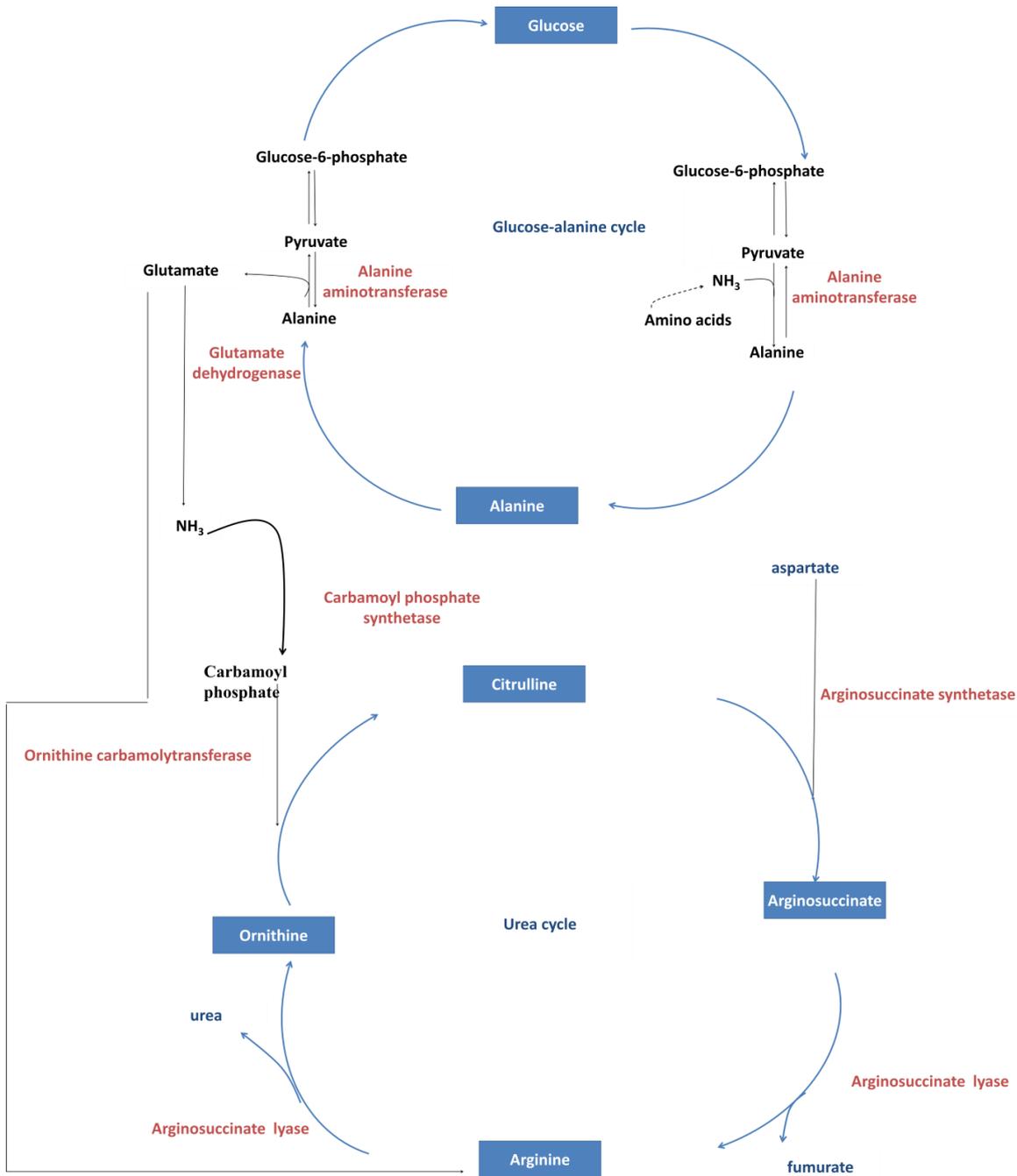
#### **1.7.4.3: The role of the muscle in inter-organ metabolism**

Although skeletal muscle has large proportions of free amino acids and protein turnover rate, only a few of the muscle amino acids namely, BCAAs, glutamine, aspartate, and alanine are significantly involved in inter-organ amino acid

metabolism (Guffon *et al.*, 1995). The degradation and synthesis of these amino acids is also regulated by different metabolic demand. For example, after a meal amino acid concentrations exceed their requirement for protein synthesis and this leads to a rapid transamination of BCAA to alanine, glutamine and aspartate (Rennie *et al.*, 1996). Glutamine, aspartate and alanine, are then transferred to the liver where they are used for glucose synthesis and storage.

During the post-absorptive state, there is protein breakdown to BCAAs, which are metabolised to alanine and glutamine, with glutamine release at post-absorptive state exceeding that after a meal (Bergstrom *et al.*, 1990; Pozefsky *et al.*, 1969; Felig *et al.*, 1969). The synthesis and release of glutamine and alanine also increase during exercise and this is in parallel to an increase in the production of BCAAs and ammonia from muscle protein breakdown (Harris *et al.*, 1985; Consoli *et al.*, 1990).

Glutamate also contributes to the synthesis of both alanine and glutamine which occurs due to increased glutamate dehydrogenase and aminotransferase activity. It has been speculated that during exercise increased glutamate dehydrogenase activity may be important to ensure a steady supply of Krebs's cycle intermediates and thus energy during exercise (Sahlin *et al.*, 1990). Other studies have suggested that the utilisation of glutamate for gluconeogenesis in the liver may be more important than the utilisation of alanine (Sahlin *et al.*, 1990). However it should be noted that the formation of glucose from alanine in the glucose alanine cycle is an important pathway for glucose homeostasis during exercise. In addition, the nitrogen from alanine forms a link between carbon metabolism and nitrogen metabolism in the urea cycle ( Figure 1.12) (Sahlin *et al.*, 1990).



**Figure 1.12: An illustration of the glucose alanine cycle and the urea cycle and the contribution of glutamate's and aspartate's nitrogen to the urea cycle.**

#### 1.7.4.4: The role of the Kidney in inter-organ metabolism

The coordination of inter-organ amino acid metabolism is particularly appreciated during metabolic acidosis when hepatic urea synthesis is reduced to enhance glutamine synthesis (Felig, 1973). Glutamine from the liver and the gut is taken by the kidney where it is degraded to produce ammonia, which is

used to buffer the urine and increase the kidney's capacity to excrete acids (Figure 1.13) (Salway, 2004).

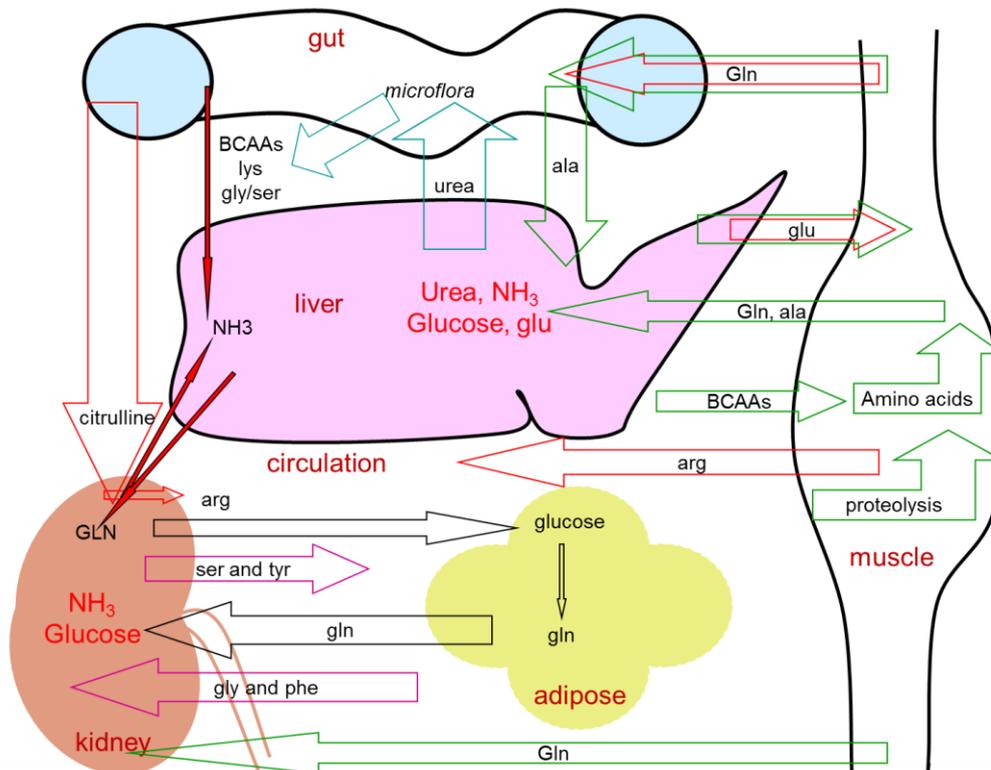
In a separate process glutamine and glutamate may also be degraded to citrulline and eventually arginine as a diet rich in glutamine results in renal citrulline uptake from the gut and arginine release by the kidney (Figure 1.13) (Halperin *et al.*, 1989; Cheema-Dhadli *et al.*, 1987). The kidney has also been shown to be the major site for the metabolism of phenylalanine to tyrosine and impaired renal phenylalanine metabolism may lead to a deficiency in tyrosine which is manifested by extensive protein breakdown (Houdijk *et al.*, 1994; Prins *et al.*, 1999).

Other organs such as adipose tissue and the brain are also important for inter-organ amino acid metabolism (Figure 1.13). Adipose tissue has been shown to extensively metabolise BCAAs, glutamate and glucose to produce glutamine and alanine and in obese and insulin-resistant individuals, metabolic enzymes for BCAAs are down-regulated (Garibotto *et al.*, 2002). This suggests that the adipose tissue is important when considering inter-organ amino acid metabolism as glutamine from the adipose tissue is taken up by the kidney where it may play a role in acid base regulation (Herman *et al.*, 2010).

Although not considered a major pathway for amino acid metabolism, the brain may play a part in inter-organ metabolism in pathological conditions such as during starvation. The metabolism of amino acids in the brain has been shown to occur in specialised cells such as neurons and astrocytes, which catabolise, synthesise and exchange amino acids with each other (Frayn *et al.*, 1991). For example, glutamine produced in astrocytes is transferred to the neurons where it is metabolised to glutamate. The brain is also involved in aminotransferase reactions of aspartate and alanine, and the BCAAs which eventually result in the formation of glutamine, and arginine (Yudkoff *et al.*, 1993). Amino acid metabolism in the liver during starvation also provides the brain with glucose (Yudkoff *et al.*, 1987).

It is clear that inter-organ amino acid metabolism is an essential part of maintaining the body's homeostasis. However, amino acid metabolism

observed in adult humans and animals may not be similar to amino acid metabolism during pregnancy, as there are extra compartments such as the fetal organs and the placenta which may alter whole body amino acid metabolism. There are limited studies in amino acid metabolism during pregnancy as will be discussed in the next section.



**Figure 1.13: Inter-organ amino acid metabolism and exchange.** Green arrow represents amino acid exchange during fasting and exercise, clear red arrows represent the cycle for glutamine and glutamate catabolism to form citrulline and arginine, solid red arrows represent glutamine exchange for acid base regulation, black arrows represent exchange interrelationship between the kidney and adipose tissue, blue arrows represent amino acid salvaging by the gut microflora and pink arrows represent metabolism of glycine

### 1.7.5: Amino acid metabolism during pregnancy

During pregnancy both maternal and fetal protein synthesis increase, without any changes in maternal protein intake (Waagepetersen *et al.*, 2008; Zwingmann *et al.*, 2001). Protein synthesis requirements are met by adaptive changes in urea nitrogen through changes in urea kinetics and amino acid metabolism (Cross & Mickelson, 2006; Duggleby & Jackson, 2002b). This is

manifested by a decrease in urea production, a decrease in plasma amine nitrogen, a lower rate of BCAA oxidation and an increase in urea nitrogen salvaging by the gut microflora (Duggleby & Jackson, 2002b). In addition, there is evidence that decreased maternal amino acid oxidation is associated with increased birth weight of the neonates (Forrester *et al.*, 1994; Kalhan *et al.*, 2000).

### **1.7.6: Placental amino acid metabolism**

Although it is thought that poor fetal growth is attributed to altered placental amino acid metabolism, there are no studies in the human placenta that give a clear indication of whether placental metabolism is important for amino acid transfer (Duggleby & Jackson, 2002a). If placental metabolism is altered, the fetus may not get amino acids in the right quantities and compositions, and its growth may be restricted. It is therefore important to investigate the extent to which amino acids are metabolised in the human placenta.

#### **1.7.6.1: What is known about placental metabolism in other species?**

Placental amino acid metabolism has been best characterised in sheep where there is significant interconversion of glutamate and glutamine as well as of serine and glycine (Cetin *et al.*, 1992; Jansson *et al.*, 2002b). Placental conversion of glutamate to glutamine has also been observed in rats, pigs and to a lesser extent in guinea pigs (Cetin *et al.*, 1991; Vaughn *et al.*, 1995).

Ovine placental metabolism contributes to glutamine and glutamate cycling between the placenta and the fetal liver which may have implications for fetal metabolism (Self *et al.*, 2004; Bloxam *et al.*, 1981). Nitrogen for the synthesis of glutamate, and subsequently glutamine, is derived from BCAA transamination within the ovine placenta. The amine nitrogen of the BCAAs is also thought to be recycled back to the BCAAs by the transamination of their BCKAs in the fetal liver (Vaughn *et al.*, 1995; Self *et al.*, 2004).

#### **1.7.6.2: What is known about placental metabolism in humans?**

The first evidence of amino acid metabolism in the human placenta came from studies which showed that maternal glutamate was converted to another amino

acid as it crossed the perfused placenta (Loy *et al.*, 1990). It was suggested that glutamate could be converted to either glutamine or asparagine, but these amino acids could not be distinguished at the time. Further evidence has come from studies of the singly labelled  $^{13}\text{C}$ -glutamate metabolism in cultured human trophoblast which did show no evidence for the incorporation of  $^{13}\text{C}$  to any other amino acids and proteins (Schneider *et al.*, 1979). However, they showed that glutamate's  $^{13}\text{C}$  was incorporated into  $\text{CO}_2$  and Krebs's cycle intermediates suggesting that the carbon skeleton was being catabolised for energy. The fate of glutamate's amino nitrogen in these experiments is unclear.

It is clear that there are species differences in placental amino acid metabolism as, although in sheep placental conversion of serine to glycine by SHMT is a major pathway, this does not seem to be the case in humans (Broeder *et al.*, 1994). Levels of SHMT are 25 times lower in the human placenta than in the ovine placenta suggesting that this might not be a major pathway for glycine synthesis in the human placenta (Cetin *et al.*, 1991; Lewis *et al.*, 2005).

As there are species differences in the handling of amino acids, it cannot be assumed that amino acid metabolism observed in animal placentas occurs in the human placenta. Understanding the extent to which placental metabolism contributes to amino acid transfer will help in establishing mechanisms involved in poor fetal growth. This may be of clinical benefit as future intervention may aim to enhance the supply and metabolism of amino acids when poor fetal growth is suspected. Further studies are therefore required in the human placenta to provide a clear picture of how amino acid metabolism contributes to amino acid transfer across the placenta.

## **1.8: Regulation of amino acid metabolic enzymes and transporters**

The availability of amino acids in various tissues is dependent on the supply from either tissue protein or plasma amino acids. The availability of specific amino acids in individual tissues will also depend on the metabolic capacity of the tissue or the exchange between different tissues. Alterations in amino acid dietary supply or protein synthesis and breakdown may lead to altered amino acid quantity and composition in specific organs and tissues as well as whole

body nitrogen balance (Lewis *et al.*, 2005). In addition, alterations in amino acid availability may also lead to altered regulation of hormones involved in cell growth (Walser *et al.*, 1976; Fouillet *et al.*, 2008; Pacy *et al.*, 1994). As a result adaptive and defensive mechanisms may occur in order to maintain cellular amino acid and nitrogen homeostasis. These adaptive and defensive mechanisms may involve complex interaction between genes, hormones and nutrients and they may be short term or long term (Bower & Johnston, 2010).

In simple organisms, adaptations may only require allosteric regulation, while in mammals adaptations at the gene transcription, protein synthesis and protein activity levels are important (Block & Buse, 1990; Liu *et al.*, 2004). For example glutamate metabolism is regulated by glucocorticoids which act via changes in gene transcription (Desvergne *et al.*, 2006). It has been suggested that the regulation of amino acids via transporters and metabolic enzyme genes is more effective for long term adaptation (Timmerman *et al.*, 2003).

### **1.8.1: Maternal influences on the placenta**

Maternal environment has been shown to affect both placental function and structure. For example maternal smoking and gestational diabetes reduce both placental transport of amino acids and transporter activity (Desvergne *et al.*, 2006). Furthermore, maternal factors such as body composition have also been shown to affect placental system A amino acid transporter activity (Pastrakuljic *et al.*, 2000; Kuruvilla *et al.*, 1994; Jansson *et al.*, 2002a). Over the longer term altered placenta function may be a secondary effect of chronic exposure to a maternal environment that affects placental structure such as size, surface area and thickness (Lewis *et al.*, 2010). Indeed placental structure has been shown to be altered in placentas from asthmatic and diabetic mothers (Higgins *et al.*, 2011).

### **1.8.2: Mechanisms of maternal influences on the placenta**

Mechanisms through which the maternal environment affects the placenta are not well understood. However, there is evidence that the capacity for the placenta to transfer nutrients is subject to maternal nutrient and hormonal regulation (Babawale *et al.*, 2000; Mayhew *et al.*, 2008). Amino acid transfer is

an example of a placental function that has been shown to be differentially affected by a range of hormones such as leptin, growth hormone and insulin (Shibata *et al.*, 2006;Jansson *et al.*, 2003;Giovannelli *et al.*, 2011).

It has been suggested that maternal nutrient levels may be sensed by the placenta via mammalian target of rapamycin (mTOR), through mechanisms involving hormones such as insulin and IGF1 (Jones *et al.*, 2010;Jansson *et al.*, 2003). BCAAs and glutamine are examples of such nutrients known to be sensed and regulated by mTOR signalling molecules (Roos *et al.*, 2009). In turn, mTOR regulates mRNA expression of genes involved in nitrogen metabolism (Dickinson *et al.*, 2011). For example nutritional deprivation (which may be sensed through mTOR) is associated with reduced mRNA levels of placental amino acid transporters such as system A. The activity of placental system A may also be altered in response to maternal body composition, which may reflect maternal nutritional status (Cardenas *et al.*, 1999). Further studies are therefore required to investigate the influence of maternal factors that may reflect maternal nutritional status on placental mRNA expression of genes involved in amino acid metabolism and transfer.

### **1.8.3: Interactions between the placenta and the fetus**

It is clear that placental function can affect fetal growth. For example there is evidence in rats that decreased amino acid transport precedes fetal growth restriction suggesting that it is a causal relationship (Lewis *et al.*, 2010). But there is also evidence that the fetus may influence placental function. In a model of placental insufficiency, the P0 *Igf2* knockout model in mice, there is up-regulation of placental transporters within the smaller placenta which, for a time, enables normal fetal growth to continue (Jansson *et al.*, 2006). This has been interpreted to suggest that the placenta is responding to fetal signals to increase placental nutrient supply. How placental function may be regulated by fetal signals is far from clear but it is something which needs to be considered when investigating the regulation of placental function.

## 1.9: Scope of this thesis

Studies presented in this thesis focus on the placental transport and metabolism of glucose and amino acids. Three studies are presented in this thesis and their findings discussed in relation to the role of the placenta in sustaining fetal growth and development. Chapter three will investigate placental glucose transfer and chapter four will focus on placental amino acid transfer and metabolism. Chapter five will address maternal environmental influences on placental mRNA expression of genes involved in amino acid transport and metabolism. This will be followed by a general discussion in chapter six which will discuss the main findings of the studies in this thesis and how they may relate to fetal growth.

**Study 1:** Glucose is an important substrate for fetal growth. There are contradictory data on placental factors determining glucose transfer across the placenta. While some studies have suggested that glucose transfer is proportional to maternal glucose concentration, others have suggested that the BM is limiting for glucose transfer. Chapter three will investigate glucose transfer and metabolism in the human placenta and will explore whether these or other factors determine glucose transfer to the fetus.

**Study 2:** Understanding placental transport and metabolism of amino acids is crucial as amino acids are important substrates for fetal growth. The mechanisms of amino acid transport across the placenta have recently been described. However, amino acid transfer is likely to be dependent on both membrane transport and placental metabolism. It is therefore important to determine the extent to which amino acid metabolism occurs in the human placenta. Chapter four will therefore investigate carbon flux from U-<sup>13</sup>C-glutamate, and nitrogen transfer from <sup>15</sup>N-glutamate and <sup>15</sup>N-leucine to other amino acids in the human placenta.

**Study 3:** Maternal environment affects both placental function and structure through unknown mechanisms. Placental amino acid transfer is known to be affected by maternal factors. Chapter five will therefore investigate whether maternal factors are associated with the expression of placental genes involved

in placental amino acid transfer and metabolism and whether the expression of these genes is associated with fetal growth.

## **Chapter 2**

### **General methods**



## 2.1: Background

In order to investigate the transfer and metabolism of glucose and amino acids in the human placenta, an isolated placental perfusion methodology was used. Colorimetric assays were used to measure the concentrations of glucose, creatinine and lactate in the maternal and fetal venous outflow samples. Gas-chromatography mass spectrometry (GC-MS) analysis and High Performance Liquid Chromatography (HPLC) analysis were used for measuring isotopic abundance and concentrations respectively. To determine whether maternal environment relates to placental gene expression and whether placental gene expression relates to fetal growth, quantitative real time polymerase chain reaction (rt-qPCR) was used. This chapter will give a background and an outline of the methods used in this thesis and the validations carried out prior to analyses.

## 2.2: Placental perfusion methodology

Human placentas from uncomplicated term deliveries were obtained immediately after delivery from Princess Ann Maternity Hospital with a given consent and ethical approval of South and West Hants Local Research ethical committee. The placental perfusion methodology used in our laboratory was adapted from Schneider et al, 1972 (Figure 2.1). Placentas are collected within 30 mins after delivery to ensure that its viability is not compromised.

### 2.2.1: Equipment and reagents

- Perfusion Roller pumps (Watson Marlow, Falmouth, UK)
- Perfusion water baths (Thermal electron corporation, Cheshire, UK)
- Portex clear PVC Tubing (30m coil length, (Smiths Medical, Ashford, UK)
- Unlabelled amino acids (Sigma, Dorset, UK)
- $^{15}\text{N}$ -glutamate,  $^{15}\text{N}$ -leucine,  $^{15}\text{N}$ -alanine and U- $^{13}\text{C}$ -glutamate (Cambridge isotopes/ CK gas, Cambridge, UK)

- Sodium dihydrogen phosphate ( $\text{NaH}_2\text{PO}_4$ ; VWR- BDH Chemical Suppliers, Lutterworth, UK)
- Sodium chloride (NaCl; Sigma, Dorset, UK)
- Sodium bicarbonate ( $\text{NaHCO}_3$ ; Sigma, Dorset, UK)
- Potassium chloride (KCl; Fisher, Loughborough, UK)
- Calcium chloride dihydrate ( $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$ ; Sigma, Dorset, UK)
- Creatinine (Acros organics, Geel, Belgium)
- Magnesium sulphate heptahydrate ( $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$ ; Sigma, Dorset, UK)
- D-glucose (Sigma, Dorset, UK)
- Heparin sodium (NHS supplies, Wrexham, UK)
- Microtubes (Fisher, Loughborough, UK)
- Ultra-turax tissue homogeniser (Janke and Kunkel IKA-labortechnik, Staufen, Germany)
- Bovine serum albumin (BSA; Sigma, Dorset, UK)

### 2.2.2: Working solutions

**Perfusion stock solution A:** 2.65 g of  $\text{CaCl}_2 \cdot 2\text{H}_2\text{O}$  and 1 g of  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  were dissolved in deionised water.

**Perfusion stock solution B:** 5 g of D-glucose, 34 g of NaCl, 2 g of KCl, 0.7g  $\text{NaH}_2\text{PO}_4$  and 11 g of  $\text{NaHCO}_3$  were dissolved in deionised water and 1 mL of heparin (25,000 I.U. /mL) was added to the solution.

**Creatinine stock solution:** A 200 mL of 200 mmol/L creatinine stock solution was prepared by adding 5.656 g creatinine in 100 mL water, 50 mL perfusion stock solution B and making it up to 250 mL with water.

**Earle's bicarbonate buffer (EBB) preparation:** For maternal perfusions, 2 L of EBB was prepared using 200 mL of perfusion stock solution A, 400 mL of perfusion stock solution B, 200 mL of 1% BSA (2 g dissolved in 200 mL water) and 20 mL of the 200 mmol/L creatinine solution. Fetal EBB was also prepared in the same way as the maternal EBB but creatinine was not added to it. The final EBB contained 1.8 mM  $\text{CaCl}_2$ , 0.4 mM  $\text{MgSO}_4$ , 116.4 mM  $\text{NaCl}$ , 5.4 mM  $\text{KCl}$ , 26.2 mM  $\text{NaHCO}_3$ , 0.9 mM  $\text{NaH}_2\text{PO}_4$ , 5.5 mM glucose, 0.1% BSA and 5000 I.U./L heparin. Buffers were kept at 37°C for 30 min prior to perfusions until the end of the experiments and were gassed with 95%  $\text{O}_2$  / 5%  $\text{CO}_2$  5 min prior to perfusions, until the end of the experiments. Perfusion experiments were carried out in a perfusion chamber which was maintained at 37°C and a controlled humidity. It is noteworthy that dextran was traditionally added to EBB as an osmotic agent. Our laboratory has stopped using dextran and has not noticed any changes in placental function since doing so.

### 2.2.3: Perfusion set up

Placentas were obtained immediately after delivery and catheters of 15 cm in length were inserted in the fetal-placental artery (Portex PVC tubing; inside diameter (i.d) 1.0 mm, outside diameter (o.d.) 1.6 mm) and fetal-placental vein (Portex PVC tubing; i.d. 2 mm, o.d. 3 mm) of an intact placental cotyledon and sutured in place (Figure 2.1). EBB was first perfused through the fetal artery at 6 mL/min using a roller pump. After 15 min, if the fetal venous out flow recovery was  $\geq 95\%$  of fetal arterial inflow (to exclude physically damaged cotyledons), five 10 cm length of PVC tubing (Portex; i.d. 0.58 mm, o.d. 0.96 mm) were inserted through the decidua and into the intervillous space on the maternal side of the placenta. EBB was first perfused through the maternal catheters at 14 mL/min using a roller pump (Figure 2.1). If maternal outflow rate was 14 mL/min approximately 2 mL baseline samples of maternal and the fetal venous outflow were collected. Following 30 min of initial perfusion the maternal or fetal arterial circulation was perfused with the required nutrient substrates and samples were collected as will be described in the individual chapters.

The maternal and fetal flow rates chosen in this study were chosen on the assumption that the average cotyledon weight was 30g and that they would be

equivalent to the flow rates in the whole placenta with average weight of 750g and blood flow rate of 600/min. Five maternal arterial catheters were chosen as they were shown to cover the whole placental area being perfused.

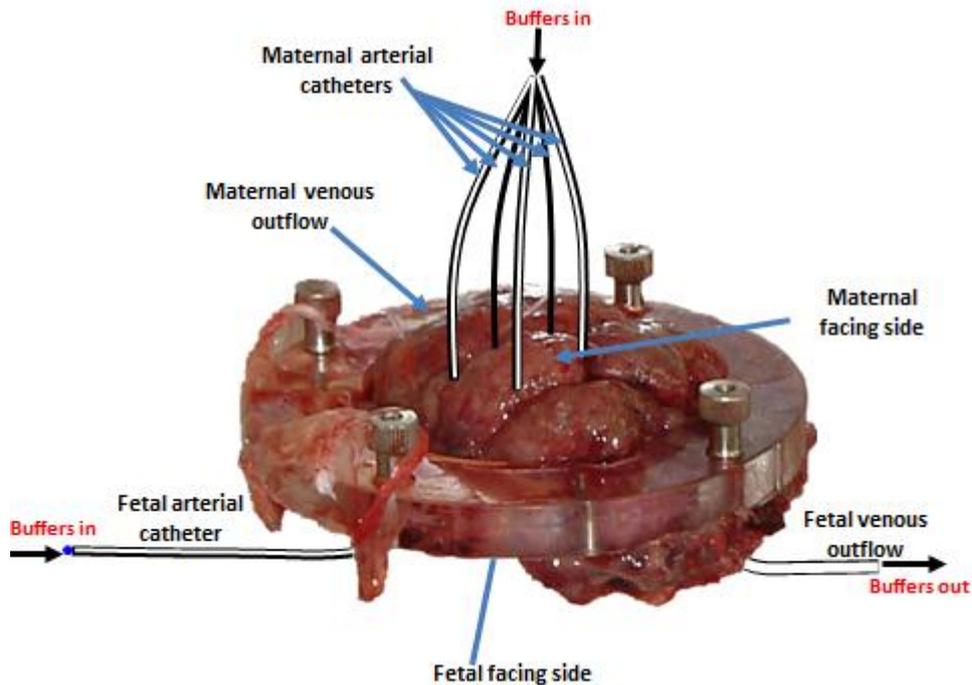


Figure 2.1: An illustration of an isolated perfused placenta methodology set up

#### 2.2.4: Placental tissue homogenisation

At the end of each experiment, dark unperfused placental regions, lining the white perfused cotyledon tissue were trimmed off and the white perfused tissue was blotted with a tissue and its wet weight was recorded. Placental tissue was directly stored at  $-80^{\circ}\text{C}$  or homogenised in 2 volumes of ice cold EBB using an Ultra-turax tissue homogenizer (USA), centrifuged for 20 min and the supernatant was stored at  $-80^{\circ}\text{C}$  until analysis.

### 2.3: Calorimetric assays and scintillation counting

Calorimetric assays were used for the determination of glucose, creatinine and lactate concentrations in the maternal and fetal venous outflow samples. Liquid scintillation counting was used to determine the  $\beta$  emissions from the  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose of glucose tracers in the maternal and fetal venous samples.

### 2.3.1: Equipment and reagents

- Glucose liquid reagents (Alpha Laboratories, Eastleigh, UK)
- Creatinine liquid reagents (Alpha Laboratories, Eastleigh, UK)
- Lactate Dry fast reagents (Alpha Laboratories, Eastleigh, UK)
- UV plate reader (Dynex Technologies/Fisher, Loughborough, UK)
- $^{14}\text{C}$ -L-glucose and  $^3\text{H}$ -O-methyl-D-glucose (Perkin Elmer, Massachusetts, USA)
- Optiphase Hisafe 2 Liquid scintillation fluid cocktail (Perkin Elmer, Massachusetts, USA)
- Centrifuge (Hermle labortechnik, Wohigen, Germany)
- Packard liquid scintillation analyser (Packard-Perkin Elmer, Massachusetts USA)

### 2.3.2: Glucose assay

Glucose calorimetric enzymatic assay is based on the oxidation of glucose in the sample by glucose oxidase in the reagent to form gluconic acid and hydrogen peroxide. The hydrogen peroxide then reacts with 4-aminoantipyrine and hydroxybenzoic acid via peroxidase activity to form a red compound, whose colour intensity if measured at 490 to 505 nm is proportional to glucose concentration in the sample.

A stock solution of 200 mmol/L glucose was prepared by dissolving 9 g of D-glucose in 100 mL of water and 50 mL of perfusion solution B and 2.5 mL of glucose (200 mmol/L) and this was made up to 250 mL with deionised water. This was aliquoted into 2 mL microtubes. Standards of concentrations ranging from 0 mmol/L to 20 mmol/L were then prepared using this stock solution.

For the assay, 5  $\mu\text{L}$  of the standards (2.5, 5, 10, 20 mmol/L), the maternal and fetal venous outflow samples together with the maternal and fetal EBB stocks were added to individual wells of a 96 well microplate and 200  $\mu\text{L}$  glucose

reagent was added to the wells using an 8 x multi-pipettor. The plate was incubated at room temperature and on a shaker for 10 minutes, and the absorbance was read at 490 nm.

### **2.3.3: Creatinine assay**

The creatinine assay is based on the reaction of creatinine in the sample with picrate alkaline to form a red compound with colour which has intensity proportional to creatinine in the sample at 490 - 505 nm. Creatinine stock solution was prepared as described in the previous section and

5  $\mu$ L of 2 mmol/L creatinine standard was added to the first and last lanes of the microplate. For maternal samples and stocks, 5  $\mu$ L were also added to individual microplate wells in duplicates and for the fetal samples 10  $\mu$ L were also added to the individual microplate wells in duplicates. 100  $\mu$ L of creatinine reagent 1 was added to the microplate using a multi-pipettor and incubated at room temperature for 1 minute. 100  $\mu$ L of creatinine reagent 2 was then added to the microplate wells and shaken for 20 sec. The absorbance was measured at 30 s and 1 min at 490 nm.

### **2.3.4: Lactate assay**

The lactate assay is based on the oxidation of lactate in the sample by lactate oxidase in the reagents to form pyruvate and hydrogen peroxide. The hydrogen peroxide then reacts with a compound in the lactate reagents in the presence of peroxidase to form a purple compound, which has intensity proportional to that of lactate in the sample at 540 nm.

Lactate was measured using a Lactate Dry Fast reagent. 10  $\mu$ L of the 8 mmol/L lactate standard and maternal and fetal venous samples were pipetted into the micropipette and lactate reagents were added to the samples. The samples were incubated at room temperature for 5 min and the microplate was read at 540 nm.

### **2.3.5: Stable isotope analysis**

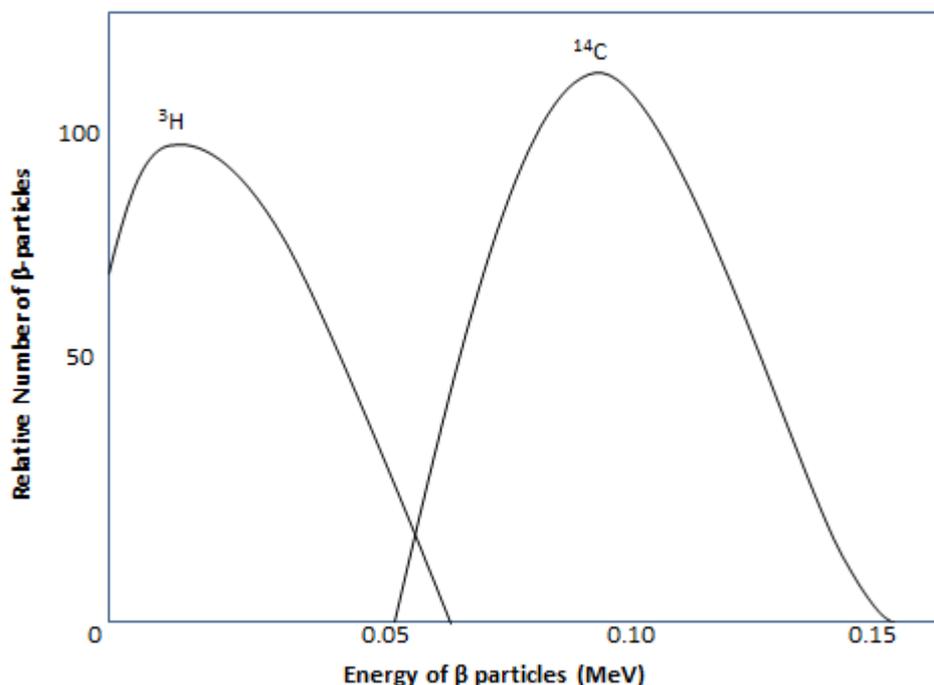
Isotopes are forms of an element that has the same number of proton in the nucleus of their atom but different numbers of neutrons (Constancia *et al.*,

2002). Isotopes that are capable of decaying over a period of time are known as radioisotopes and those that do not are termed stable isotopes. Carbon is a good example of an element existing in a number of isotopes ( $^{12}\text{C}$ ,  $^{13}\text{C}$  (stable) and  $^{14}\text{C}$  (radioactive)) with the superscript representing the sum of neutrons and protons. Stable isotopes and radioisotopes occur in very low percentages (< 1.1%) in nature. This if they are introduced into a biological system within a biological molecule their transfer or incorporation into other metabolites can be determined as they can be distinguished from naturally occurring isotopes.

### 2.3.6: Liquid scintillation counting

Scintillation counting is a method that takes advantage of the use of beta-emitting atoms that disintegrate to form beta particles and energy. As the spectrum of energy produced is continuous and distinctive for each isotope, scintillation counting allows simultaneous measurements of the activities for both  $^3\text{H}$  and  $^{14}\text{C}$ . In order for the decaying particles to be detected, a liquid scintillator is added which absorbs the energy released by the isotope and result in the formation of excited molecules which, when they return to their ground state, produce a photon proportional to the energy radiated by the isotope. Figure 2.2 shows the energy spectra produced from  $^3\text{H}$  and  $^{14}\text{C}$ .

In dual counting, the liquid scintillation counter has two channels; Channel 1 is set to only record the  $^{14}\text{C}$  spectrum of the sample and exclude any radiation contribution from  $^3\text{H}$  of the sample and channel 2 is set to include energies from both  $^3\text{H}$  and  $^{14}\text{C}$  of the spectrum sample. The scintillation counter adjusts for background contribution and the overlapping interference from each isotope by using the counts obtained from a pure standard and the sample.



**Figure 2.2: Beta energies for  $^3\text{H}$  and  $^{14}\text{C}$**  generated by the liquid scintillation counter. There is some overlap between  $^3\text{H}$  and  $^{14}\text{C}$  regions, for instance within the  $^3\text{H}$  region there is a contribution of 7-14% of the total counts which are  $^{14}\text{C}$ , and this overlap is taken into account when the values of the two regions are calculated.

## 2.4: Principles of chromatographic separation

In chromatographic sample separation, compounds are separated on the basis of their partition coefficients in two immiscible phases; the mobile phase and the stationary phase. The stationary phase is usually held in a column but may also be on a plate and can be a gel, liquid, solid or a mixture of liquid and solid. Depending on the mode of chromatographic separation the stationary phase may be attached to an insoluble support such as silica, glass, metal or a metal foil plate.

The mobile phase can be either a liquid or a gas, which flows over or through the stationary phase. The choice of the liquid phase and the mobile phase is dependent on the chemical properties of the compounds to be separated. The time taken (retention time) for an analyte to exit the column will depend on its solubility and interaction with the mobile phase relative to the stationary phase. The eluted components are quantified by a detector and the response is presented as a series of peaks in a chromatogram.

The degree to which eluted peaks are separated is termed resolution. The resolution of components in a mixture may be improved by changing a property of the mobile phase, such as pH, polarity or temperature, during the process. The preparation and introduction of a sample into the chromatographic system and the detection and quantification of the separated components depends on the type of chromatography used. In the next sections the separation and detection of samples using high pressure liquid chromatography and gas chromatography-mass spectrometry (GC-MS), as applied to the studies reported in this thesis, will be described.

## **2.4.1: High Pressure Liquid Chromatography (HPLC)**

### **2.4.1.1: Basic principles of Instrumentation**

The basic components of a typical automated HPLC system are;

1. Reservoir containing the mobile phase or phases.
2. Single or dual pumping system to pump the mobile phase through the column containing the stationary phase and capable of producing a high pressure.
3. Autosampler/ injector to inject the sample into the mobile phase immediately before the analytical column. The sample is introduced into the mobile phase by alternately loading the sample into a sample loop and then switching the mobile phase circuit from loop by-pass to flow through the loop and wash out the sample.
4. Analytical column typically made of stainless steel, containing the stationary phase. In the applications described here a non-polar, long chain octadecylsilyl stationary phase, bonded to closely packed fine (5 micron) silica particles, was used. The small size provides good resolution but provides a resistance to the flow of the mobile phase that requires the high pressure pumps. The analytical column should be preceded by a small guard column containing a similar material to the analytical column. This

preserves the working life of the analytical column by collecting and strongly retaining the debris in the sample components and can be replaced at less cost than a full column. A non-polar stationary phase, (commonly called reverse phase) is usually operated with a relatively polar mobile phase which might typically contain solvents such as water or an aqueous buffer, methanol or acetonitrile either alone or in combination. With a non-polar stationary phase the more polar components are eluted first and strongly retained non-polar components can be eluted more rapidly by providing a mobile phase gradient of increasing non-polarity. In contrast, a normal phase column is relatively polar, it is operated with a non-polar mobile phase such as hexane and the less polar components are eluted first. Many different types of stationary phases are available, the choice of which is dependent on the nature of the compounds to be separated.

5. Detector. In the applications described here the detector was a fluorimeter, other types include absorbance, electrochemical or refractive index meters. Fluorescence is a sensitive form of detection but, as not many compounds are naturally fluorescent, it may be necessary to chemically modify the sample to make the components fluorescence.
6. Computer for control of the system components and analysis of the detector output.

#### **2.4.1.2: Basic principles of sample preparation for HPLC.**

For HPLC separations, samples of biological origin frequently require a prior clean-up to remove protein and other components that are either incompatible with the column performance, sample derivatisation or interfere with the detection by giving overlapping peaks. Such procedures include acid precipitation of protein and solvent extraction to remove unwanted components.

It may be necessary to chemically modify the sample to make the components detectable at the concentrations in which they are present in the sample. This

can be done either before or after separation on the column, but post-column derivatisation can lead to unacceptable loss of resolution. Many HPLC systems have autosamplers which can also automate some pre-column derivatisation procedures, thus simplifying the method and removing some of the manual processing.

## **2.4.2: Gas Chromatography – Mass Spectrometry.**

### **2.4.2.1: Basic principles of instrumentation.**

In this technique, sample components are initially separated by gas chromatography in which a gas mobile phase passes over a liquid stationary phase bonded most usually to the inside wall of a long narrow silica capillary column. The column eluate passes into a mass spectrometer in which molecular ionisation and fragmentation occurs. Ionised molecules and molecular fragments of the separated components are further separated in the mass spectrometer flight tube according to their mass to charge ratio ( $m/z$ ) and measured to give quantitative and structural information. This includes information on the relative distribution of isotopes within the molecule.

The basic components of a typical automated GC-MS system are;

1. Gas cylinder supplying the mobile phase. This is usually helium.
2. Autosampler to select and inject the sample into the gas stream in the heated inlet of the gas chromatograph.
3. Chromatograph inlet is used to vaporise the sample in the carrier gas before it flows onto the column containing the stationary phase. The proportion of the vaporised sample reaching the column may be varied by splitting the flow and diverting some of it to waste. A high split ratio is used to divert sample away from the column to prevent overload and loss of resolution. A splitless injection is used when it is necessary to maximise the sensitivity of the analysis.
4. Column containing the stationary phase. This is typically a thin (e.g. 25  $\mu\text{m}$ ) film of a waxy liquid bonded to the inside of a long (e.g. 30 m)

narrow (e.g. 0.25 mm i.d) silica capillary column. The choice of stationary phase depends on the nature of compounds to be separated.

5. Oven to closely regulate the temperature of the column. Elevated temperatures are frequently required to maintain the sample components in a vapour phase and precision of temperature control is necessary for reproducible retention times. A temperature gradient may be used to ensure elution of a range of compounds with widely differing volatilities.
6. Mass spectrometer The mass spectrometer source produces a stream of electrons that collide either directly with the molecules eluted from the gas chromatograph (electron impact mode) or with a secondary ionising agent such as methane which then collides with the eluate (chemical ionisation mode). Electron impact ionises molecules and causes considerable fragmentation of the molecules, many fragments also being ionised. Chemical ionisation is less energetic and causes less fragmentation, often allowing measurement of the ionised intact molecule. This is generally a cleaner and thus more sensitive mode than electron impact for measuring changes in isotope distribution. However, electron impact mode has the advantage of producing fragments that may be helpful in locating the molecular position of an observed change of isotope. Generation of a precisely controlled varying magnetic field in a flight tube allows separation and quantification of ions according to their mass (or mass to charge ratio or  $m/z$  ratio). The information generated in this way can be used to identify compounds from their fragmentation pattern, quantify compounds by comparison with a suitable standard and identify changes in the isotopic composition of the compound that result in a change in the  $m/z$  of the fragments observed.

7. Computer to control the operation of the gas chromatograph and mass spectrometer to collect and record the mass spectrographic information generated.

#### **2.4.2.2: Basic issues relating to sample preparation for GC-MS**

Although some samples may be injected directly into the GC many samples, particularly those of biological origin require an initial sample preparation that may be quite extensive. This is necessary firstly to remove compounds such as protein and water that are incompatible with the column, secondly to remove compounds that might give interfering peaks and thirdly to increase the volatility of the compounds of interest so that they can enter the vapour phase and pass down the column.

Removal of unwanted components can be carried out by a variety of techniques including acid precipitation, solvent extraction, ion exchange purification and vacuum drying. A variety of reagents are available for sample derivatisation to increase volatility. They generally react with polar groups on the molecules and replace them with non-polar groups thus reducing inter-molecular interactions and increasing volatility. Frequently used agents for the analysis of amino acids include those producing N-acetyl N-propyl esters, N-perfluoroacyl alkyl esters and silyl derivatives (Tykot, 2006). Some of these methods are good for derivatising most of the amino acids except glutamine and asparagine but the tert-butyldimethylsilyl derivative is useful for all amino acids including glutamine and asparagine (Brand, 1996). After derivatisation samples are analysed by GC-MS but sample size, peak identification by retention time and choice of selected ions for MS monitoring must also be considered before analysis.

### **2.5: Analysis of amino acid concentration by reverse phase High Pressure Liquid Chromatography**

The concentration of the amino acids in the fetal and the maternal venous outflow were measured by reverse-phase high performance liquid chromatography (RP-HPLC) with fluorescence detection using a gradient elution method adapted from Turnell & Cooper (Brand, 1996).

### 2.5.1: Equipment and materials

- Pumps: Gilson 305 and Gilson 307 high pressure pumps (Anachem, Luton, UK)
- Autosampler / Prep. Unit Gilson 231 sampler/ Gilson 401 dilutor (Anachem, Luton, UK)
- Column: Supelcosil LC18 (25 x .46 cm, 5 micron) (Sigma Aldrich Chemical Company, UK)
- Detector: Perkin Elmer LC 240 fluorimeter (Perkin Elmer, Beconsfield, UK)
- Software control system Gilson 715 HPLC Controller v1.21. Gilson Medical electronics, Villiers-le-Bel, France)
- Di-sodium hydrogen orthophosphate (Fisher, Loughborough, UK)
- Acetonitrile (Fisher, Loughborough, UK)
- Methanol (Fisher, Loughborough, UK)
- Propionic acid (Fluka, Dorset, UK)
- phthaldialdehyde (OPA) (Sigma-Aldrich, Dorset, UK)
- 0.45  $\mu$  nylon filter (Fisher, Loughborough, UK)
- Vacuum brand ME2 pump (Fisher, Loughborough, UK)
- Boric acid (Fisher, Loughborough, UK)
- 3-mecaptopropionic acid (Fluka, Dorset, UK)
- Chromacol vials (Fisher, Loughborough, UK)
- 10 ml Glass extraction tubes/caps (Fisher, Loughborough, UK)
- Pasteur pipettes (Fisher, Loughborough, UK)
- Tetrahydrofuran (Fisher, Loughborough, UK)
- Sulphosalicylic acid (Fisher, Loughborough, UK)

### 2.5.2: Working reagents

Mobile phase Solvent A: 6.4 g di-sodium hydrogen phosphate was dissolved in 460 mL distilled water and adjusted to pH 6.3 with propionic acid and 40 mL methanol added before degassing by vacuum filtration through a 0.45  $\mu$  nylon filter. After filtration, 5 mL tetrahydrofuran was added.

Mobile phase Solvent B: 0.5 g disodium hydrogen orthophosphate was dissolved in 400 mL distilled water and adjusted to pH 6.8 with propionic acid, followed by addition of 300 mL methanol and 300 mL acetonitrile and degassed as above.

Borate buffer was prepared by adding 2.5 g of boric acid to 100 mL distilled water and was adjusted to pH 10 by addition of sodium hydroxide.

Derivatising reagent *o*-phthalaldehyde (OPA) was prepared by adding 5 mg OPA to 100  $\mu$ L methanol, 15  $\mu$ L 3-mercaptopropionic acid and 1 mL borate buffer at pH 10.

Internal standard /precipitant was prepared by dissolving 6 g sulphosalicylic acid in 100 mL distilled water and adding nor-valine to a final concentration of 100  $\mu$ mol/L.

Amino acid standard solutions. A stock amino acid standard solution containing all amino acids to be measured was prepared by weighing to give a concentration of 100  $\mu$ mol/L in 0.01 mol/L HCl. Further standard solutions in the range 3.125 to 100  $\mu$ mol/L were prepared by dilution and stored in aliquots at -20°C.

### 2.5.3: Sample preparation, injection and analysis

Samples or standards were thawed, mixed and a 200  $\mu$ L portion added to 200  $\mu$ L of internal standard / precipitant. The mixture was vortexed and allowed to stand at room temperature for 10 min. before centrifugation at 9000 x g for 10 min at 4°C. The supernatant was transferred to glass chromatography vials (Fisher, UK) and placed in the autosampler.

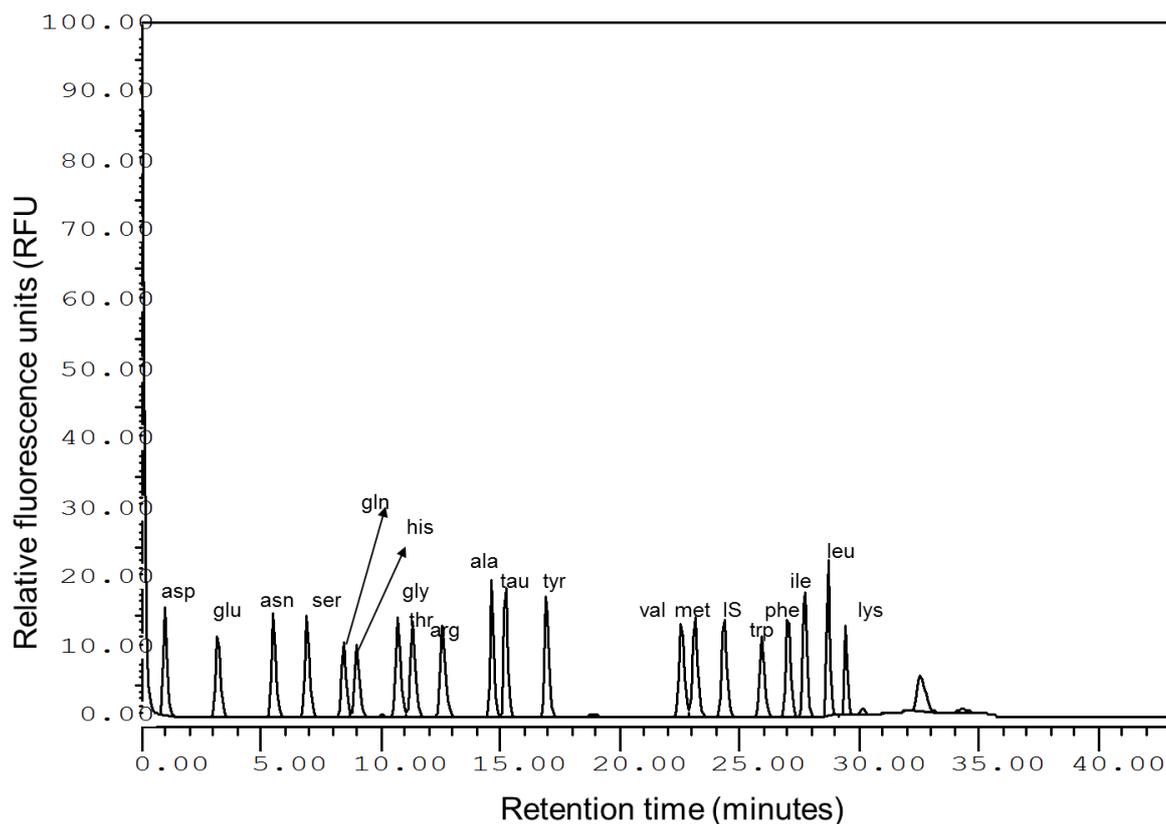
Sample derivatisation was carried out automatically by the autosampler/preparation unit for each sample or standard immediately before injection. 10  $\mu\text{L}$  derivatisation reagent, 40  $\mu\text{L}$  sample or standard and 290  $\mu\text{L}$  borate buffer were added to a secondary vial on the sampler, mixed by flushing and allowed to react for 100 sec. An 80  $\mu\text{L}$  portion of the mixture was then flushed through a 20  $\mu\text{L}$  sample loading loop and the 20  $\mu\text{L}$  sample residing in the loop was injected onto the column by timed operation of the injector valve. The derivatised amino acids in the column eluate were detected by fluorescence with excitation at 335 nm and emission at 455 nm. For quantification, the response ratio of each amino acid peak was calculated as the area ratio to the nor-valine internal standard peak. Individual amino acid concentrations of samples were calculated from the calibration of a series of standards in the range 3.125  $\mu\text{mol/L}$  to 100  $\mu\text{mol/L}$  analysed in the same batch.

The required resolution of the amino acids in the samples and standards was obtained by using the elution profile shown in Table 2.1. These conditions were obtained after a series of adjustments of timing, and were found to be reproducible between several analytical columns of the same type.

The identity of individual amino acid peaks in the chromatogram of the mixture was confirmed by analysis of each amino acid alone and individual retention times determined (Figure 2.3).

**Table 2.1: Running solvent gradient for the of amino acid separation using RT-HPLC**

Time (min)	Flow (mL/min)	% Solvent B
0	1.2	0
2	1.2	0
6	1.2	10
12	1.2	20
13	1.2	30
15	1.2	35
20	1.2	40
26	1.2	50
31	1.2	100
35	1.2	100
36	1.2	0



**Figure 2.3: Amino acid chromatogram showing retention times and the order of their elution for each amino acid IS = internal standard.**

## 2. 6: Analysis of branched keto acids and pyruvate concentrations by reverse phase HPLC

Concentrations of the keto acids in the maternal and fetal venous outflow and placental tissue homogenates were measured by HPLC using the modified method of Pailla (Turnell & Cooper, 1982).

### 2.6.1: Equipment and reagents

- Pump: Beckman Coulter System Gold 125 solvent delivery system (Beckman Coulter High Wycombe, UK)
- Autosampler: Beckman Coulter System Gold 508 autosampler (Beckman Coulter High Wycombe, UK)
- Column: Supelcosil LC18 (25 x .46 cm, 5 $\mu$ ) (Sigma Aldrich Chemical Company, UK)

- Detector: Perkin Elmer LC 240 fluorimeter (Perkin Elmer, Beaconsfield, UK)
- Software control system Beckman 32 Karat Gold v8.0 (Beckman Coulter High Wycombe, UK)
- 0.45 micron nylon filter (Fisher, Loughborough, UK) Sodium 3-methyl-2-oxobutyrate, Sodium 3-methyl-2-oxovalerate, Sodium 4-methyl-2-oxovalerate, Sodium 4-methyl-2-oxovalerate Sodium bicarbonate ( $\text{NaHCO}_3$ ) and sodium pyruvate (Sigma Dorset, UK)
- Sodium phosphate ( $\text{NaH}_2\text{PO}_4$ ; Sigma Aldrich, Dorset, UK)
- Phenylenediamine (Sigma Aldrich, Dorset, UK)

### 2.6.2: Working reagents

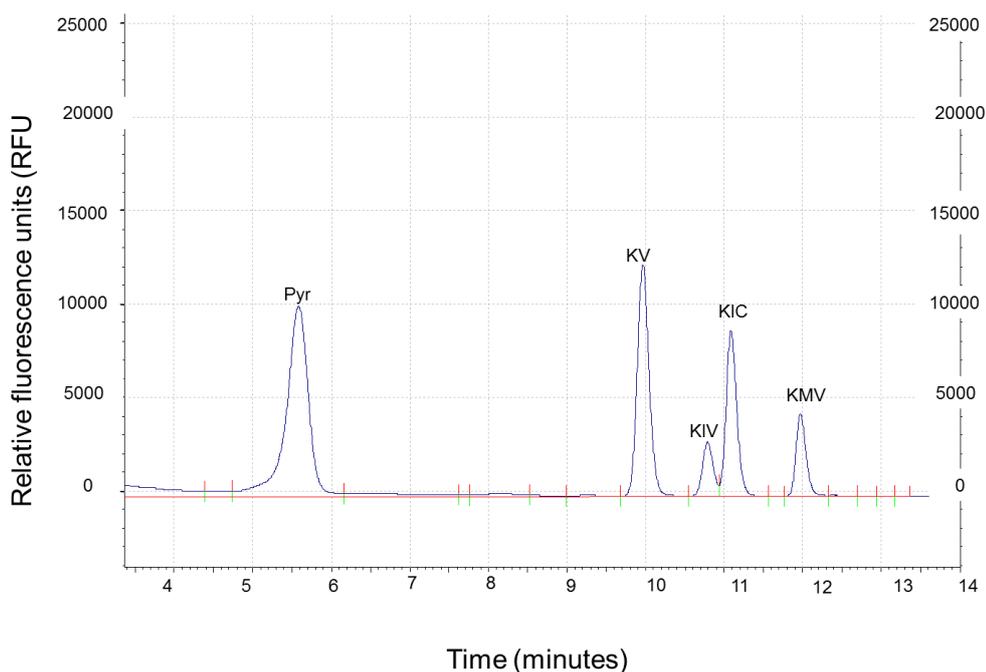
Mobile phase Solvent A: Methanol: Water 20:80 v/v. degassed by vacuum filtration through a 0.45  $\mu$  nylon filter .

Mobile phase Solvent B: Methanol 100% degassed as above.

Standard solutions: a stock standard solution containing 100  $\mu\text{mol/L}$   $\alpha$ -ketoisocaproate (KIC),  $\alpha$ -ketoisovalerate (KIV),  $\alpha$ -keto- $\beta$ -methylvalerate (KMV) and pyruvate was prepared in EBB buffer containing BSA or in water by weighing. Further standard solutions containing 50, 25, 12.5 and 6.25 and 3,125  $\mu\text{mol/L}$  of each keto acid were prepared from the stock solution by dilution. All standard solutions were stored at  $-20^\circ\text{C}$ .

- Internal Standard  $\alpha$ -ketovalerate (KV) 250  $\mu\text{mol/L}$  was prepared in distilled water.
- Phenylenediamine (OPA) derivatising agent of 4 mmol/L was prepared by dissolving a 20 mg tablet in 50 mL 3 mol/L HCL.
- Sulphosalicylic acid 6%: (6% SSA) 6 g sulphosalicylic acid was dissolved in 100 mL distilled water.
- Ethyl acetate, sodium sulphate (anhydrous,  $\text{Na}_2\text{SO}_4$ ) and methanol.





**Figure 2.4: HPLC profile of the derivatives of branched BCKAs and pyruvate in standard samples.** Pyr (pyruvate /alanine keto acids), KV ( $\alpha$ -ketovalerate/internal standard), KIV ( $\alpha$ -ketoisovalerate/ valine keto acid), KIC ( $\alpha$ -ketoisocaproate/leucine keto acid) and KMV ( $\alpha$ -keto- $\beta$ -methylvalerate/isoleucine keto acid).

## 2.7: Analysis of isotopic abundance in amino acids by GC-MS.

Before analysis, samples were extracted using a cation exchange chromatograph and derivatised as described below

### 2.7.1: Equipment and reagents

- A cation exchange resin Dowex 50W X8 – 200 mesh (Sigma Aldrich, Dorset, UK).
- GC-MS Agilent 6890/5962 gas chromatograph/ mass spectrometer (Agilent, Stockport, UK)
- GC-MS Column CPSIL 5 MS (30 m x 0.5 mm id, 0.25  $\mu$ m film thickness)
- Ethyl acetate (Fisher, Loughborough, UK)

- N-Methyl-N-(tert-butyldimethyl-silyl)trifluoroacetimide (MTBSTFA) derivatising agent (Sigma Aldrich, Dorset, UK).
- Ammonia solution 3 mol/L prepared by a 1 plus 5 dilution of 35% ammonium hydroxide (Sigma Aldrich, Dorset, UK) stock with distilled/deionised water.
- Acetic acid (Fisher, Loughborough, UK) 1 mol/L prepared by a 1 plus 16.5 dilution of acetic acid 99.5% with distilled/deionised water.

### 2.7.2: Resin preparation

A cation exchange resin Dowex 50W X8 – 200 mesh was used to extract and purify the amino acids. Wash resin 3 times with distilled water, decanting the supernatant, to remove fines. Store in 1 mol/L NaOH until use. For use, dispense a 1 mL bed volume of resin into a disposable extraction column and attach a column extender. Wash with 50 mL distilled water followed by 10 mL 1 mol/L HCl and a further 50 mL distilled water to convert the resin to the H<sup>+</sup> form. Plug the column to prevent it running dry.

### 2.7.3: Sample preparation and derivatisation

Add 1 mL of acetic acid to 1 mL of sample and mix. Carefully apply the whole mixture to the top of the resin in a prepared ion exchange extraction column and allow to drip through under gravity until the liquid is level with the top of the resin. Amino acids are retained on the column. Wash carefully with 20 mL distilled water and allow the eluate to run to waste without the top of the resin drying out. Place a glass 4 mL collection tube beneath each column tip and carefully apply 3 mL 3 mol/L ammonia to the column and collect the eluate which contains the released amino acids. Place the open glass tubes containing the ammonia eluate in a centrifugal drier and evaporate to dryness at ambient temperature.

When dry, add 100 µL MTBSTFA and 100 µL acetonitrile to each tube, cap and carefully dissolve the residue. Heat the tubes at 80°C for 1 hr in a heating block in the fume cabinet. Remove, cool for 10 min at 4°C and add a further 100 µL acetonitrile. Mix and transfer the solution to a 300 µL fixed insert chromatography vial and load on GC-MS autosampler for analysis as below.

## GC-MS operation

Ionisation    Electron impact

Injection    1  $\mu$ L Splitless for perfusate samples, 1/50 split for tissue homogenates

## Temperature program

Initial	80°C
Hold	1 min
Ramp	20°C/min to 160°C
Ramp	8°C/min to 290°C
Hold	2 min
Reset	80°C

## Monitor mode

For peak identification; full scan 50m/z to 800 m/z, for isotopomer analysis; in a selected ion mode as in Table 2.2.

**Table 2.2: Amino acids molecular fragmentation ion m/z**

Amino acid	m/z ion
Alanine	260-262
Glycine	246-248
Valine	288-289/
Proline	286-301
Leucine	302-303
Isoleucine	302-303
Asparagine	417-418
Serine	390-391
Threonine	404-405
Aspartate	418-422
Glutamate	432-438
Lysine	431-432
Glutamine	431-437

## 2.8: GC-MS analysis of isotopic abundance in glucose

### 2.8.1: Equipment and reagents

- GC-MS      Agilent 6890/5962 gas chromatograph/ mass spectrometer (Agilent, Stockport, UK)
- Column      CPSIL 5 MS (30 m x 0.5 mm id, 0.25 µm film thickness)
- Glucose stock solution      10 mmol/L in distilled water
- Methanol
- Acetic anhydride      (Sigma Aldrich, Dorset, UK)
- Ethyl acetate      (Fisher, UK)

### 2.8.2: Sample preparation

1.5 mL sample (or standard for identification studies) was added to 10 mL of ice-cold methanol, mixed and centrifuged at 4°C for 10 min at 12,000 x g. The supernatant was transferred to a clean glass tube and evaporated to dryness under vacuum.

In a fume cabinet, 50  $\mu\text{L}$  acetic anhydride and 50  $\mu\text{L}$  pyridine was added to the dried residue. The tube was securely capped, mixed and heated in a heating block for 10 min at 60°C. After cooling, the sample was dried under nitrogen, reconstituted with 300  $\mu\text{L}$  ethyl acetate and 200  $\mu\text{L}$  of clear solution transferred to a 300  $\mu\text{L}$  fixed insert chromatography vial.

### GC-MS operation

Ionisation    Electron impact or Positive Chemical Ionisation (Methane)

Injection     1  $\mu\text{L}$  with 1/50 split for standard, splitless for perfusates

### Temperature program

Initial	100°C
Hold	1 min
Ramp	20°C/min to 215°C
Ramp	5°C/min to 240°C
Ramp	20°C/min to 290°C
Hold	5 min
Reset	100°C

### Monitor mode

For peak identification    EI mode full scan 50m/z to 800 m/z

For isotopomer analysis    SIM EI m/z 200, 201, 202, 203

                                     SIM CI m/z 331, 332, 333, 334

### **Calculation of enrichment as moles % excess from GC-MS**

Isotopomer enrichment was expressed as moles % excess (MPE) using the relationship  $\text{MPE} = \text{moles isotopomer above baseline occurrence} \times 100 / \text{total moles of all isotopomers}$ .

In the cases of amino acids containing a single atom enriched by a single mass unit this was calculated as;

$$\text{MPE} = (A(t)_{m+1} - A(t)_{m^*R}) / (A(t)_{m+1} + A(t)_{m^*R})$$

Where;

$A(0)_m$  = observed abundance at baseline of isotopomer mass  $m$

$A(0)_{m+1}$  = observed abundance at baseline of isotopomer mass  $m + 1$

$A(t)_m$  = observed abundance at time  $t$  of isotopomer mass  $m$

$A(t)_{m+1}$  = observed abundance at time  $t$  of isotopomer mass  $m + 1$

$$R = A(0)_{m+1} / A(0)_m$$

In the more complicated case of the quantification of isotopomer over a range of up to 5 additional mass units the following general scheme was used.

$$R1 = A(0)_{m+1} / A(0)_m: R2 = A(0)_{m+2} / A(0)_m: R3 = A(0)_{m+3} / A(0)_m:$$

$$R4 = A(0)_{m+4} / A(0)_m: R5 = A(0)_{m+5} / A(0)_m$$

Enrichment at time  $t$  in isotopomer  $M + 1$

$$E(t)_{(m+1)} = (A(t)_{m+1} - A(t)_m * R1) / A(\text{total})$$

Enrichment at time  $t$  in isotopomer  $M + 2$

$$E(t)_{(m+2)} = (A(t)_{m+2} - A(t)_m * R2 - E(t)_{(m+1)} * R1) / A(\text{total})$$

Enrichment at time  $t$  in isotopomer  $M + 3$

$$E(t)_{(m+3)} = (A(t)_{m+3} - A(t)_m * R3 - E(t)_{(m+2)} * R1 - E(t)_{(m+1)} * R2) / A(\text{total})$$

Enrichment at time  $t$  in isotopomer  $M + 4$

$$E(t)_{(m+4)} = (A(t)_{m+4} - A(t)_m * R4 - E(t)_{(m+3)} * R1 - E(t)_{(m+2)} * R2 - E(t)_{(m+1)} * R3) / A(\text{total})$$

Enrichment at time  $t$  in isotopomer  $M + 5$

$$E(t)_{(m+5)} = (A(t)_{m+5} - A(t)_m * R5 - E(t)_{(m+4)} * R1 - E(t)_{(m+3)} * R2 - E(t)_{(m+2)} * R3 - E(t)_{(m+1)} * R4) / A(\text{total})$$

Where;

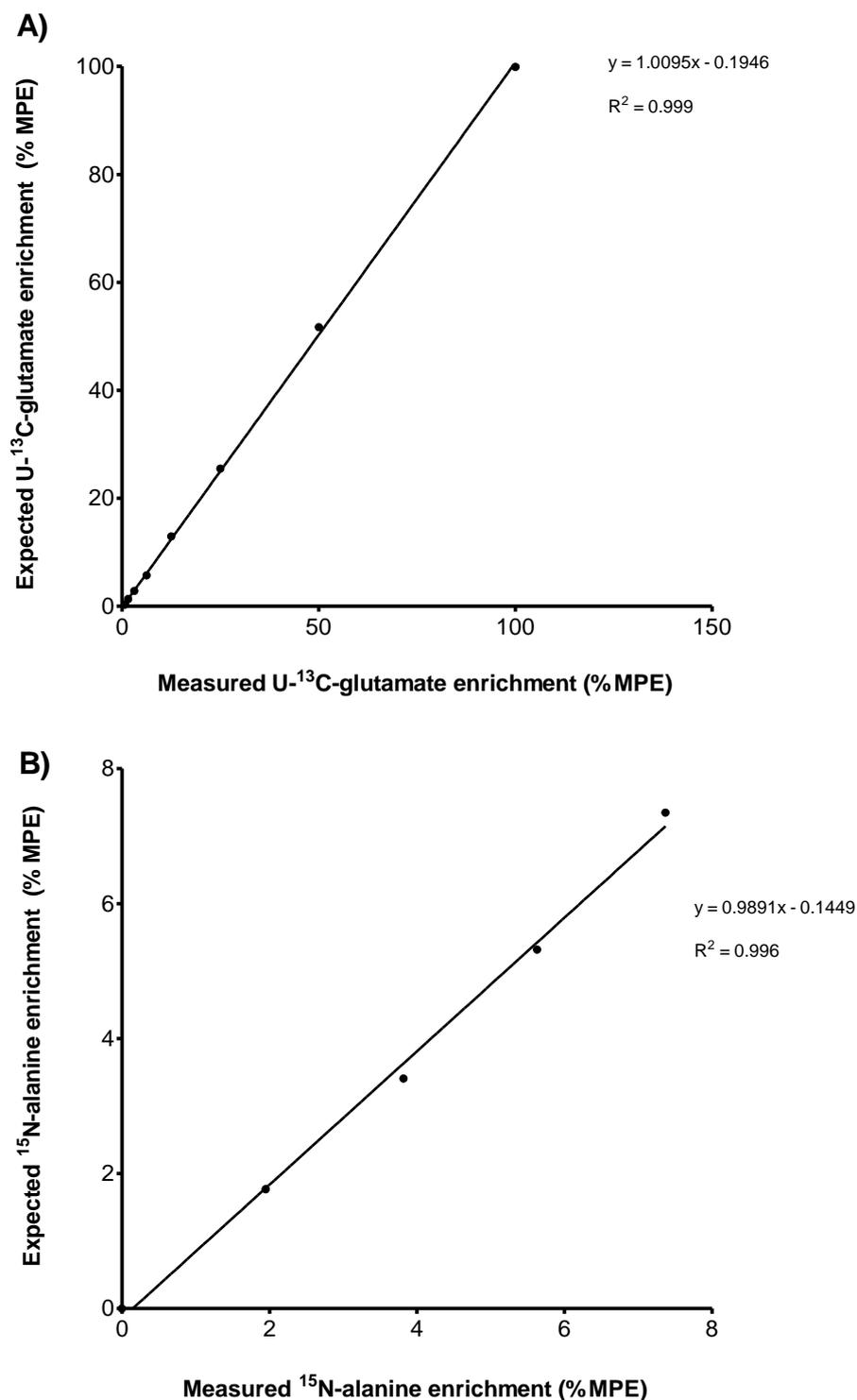
$$A(\text{total}) = A(t)_m + E(t)_{(m+1)} + E(t)_{(m+2)} + E(t)_{(m+3)} + E(t)_{(m+4)} + E(t)_{(m+5)}$$

In some mass spectrometer conditions, the output response as total counts for a given abundance may be different between the isotopomers because of mass discrimination effects in the mass spectrometer. For the calculations above, the observed abundances should be obtained from a calibration of MPE calculated directly from the mass spectrometer output in total counts against a series of standards containing known MPE prepared by weighing and dilution of the natural and enriched materials of known purity. However, standards of known enrichment were not available for all the amino acids to be investigated.

Figures 2.5A and 2.5B show such calibrations for  $^{15}\text{N}$ -alanine and  $\text{U-}^{13}\text{C}$ -glutamate. They show very good linearity and a slope close to 1. This indicates negligible mass discrimination effects and confirms that the mass spectrometer output of total counts for each isotopomer may be used directly for the calculation of abundance and enrichment. Therefore, the results presented here were calculated using mass spectrometer total counts as a direct surrogate for abundance.

## **2.9: Cohort studies: The Southampton Women's Survey and placental gene expression**

In order to determine the associations between maternal and fetal factors and placental mRNA expression levels, placentas from the Southampton Women's Survey (SWS) were used. The details and the aims of the SWS cohort study have previously been described (Inskip et al., 2008). Briefly, 12,583 non-pregnant women aged 20-34 years were recruited via their General Practitioners; assessments of lifestyle and diet (by validated food frequency questionnaire) were carried out (Crozier et al., 2006). Maternal anthropometry measurements were performed by trained research nurses at study entry and then in early (11 weeks) and late (34 weeks) gestation among those who became pregnant (Inskip et al., 2008). Fetal and neonatal growth parameters were recorded as well as the mother's and the father's birth weight.



**Figure 2.5: Standard curves for U-<sup>13</sup>C glutamate and <sup>15</sup>N-alanine** showing assay linearity and accuracy for enrichment determination using total isotopomer counts after MTBSTFA derivatisation and measurement by GC-MS. A) U-<sup>13</sup>C glutamate standard curve, B) <sup>15</sup>N-alanine standard curve.

### **2.9.1: Maternal measurements in the SWS**

Maternal measurements for the SWS placentas were collected by SWS research staff. Four skinfold thicknesses (triceps, biceps, subscapular and supra-iliac) were measured to the nearest 0.1 mm in triplicate on the non-dominant side using Harpenden skinfold callipers, and mid-upper arm circumference was measured using a tape measure (Harrison et al., 1988). Fat mass was estimated from skinfold thickness measurements using the method of (Durnin & Womersley, 1974) and arm muscle area was derived using a formula (mid-upper arm circumference –  $\pi \times$  triceps skinfold thickness)  $^2 / 4\pi$ ] - 6.5) (Heymsfield et al., 1982).

A score summarizing dietary patterns (prudent diet score) was obtained as described previously (Robinson et al., 2004). Women were asked how often they had taken strenuous exercise over the previous three months and a dichotomous variable for ever having taken such exercise was derived. Mothers and fathers were also asked for their birth weight and if unknown they were asked to obtain this information from their parents. The mother's social class, education, whether they smoked before or during pregnancy and whether they have had children were also recorded.

### **2.9.2: Fetal and neonatal measurements in the SWS**

Infant data for SWS pregnancies was collected by SWS midwives. The infants' gestational age at birth was calculated from the combination of the mother's last menstrual period data and ultrasonography. Trained research midwives recorded neonatal anthropometric measures (birth weight, abdominal and mid-upper arm circumferences and crown–heel length).

A subset of mothers gave written informed consent for the baby to undergo a Dual-energy x-ray absorptiometry (DEXA) within 2 weeks of birth. A Lunar DPX instrument with neonatal scan mode and specific paediatric software (GE Corporation, Madison, Wisconsin, USA) was used. Measurement of whole body bone area, bone mineral content, areal bone mineral density and body composition was performed. The baby was weighed at the end of the visit, and this weight and the previously recorded birth-length were entered into the DEXA

record. The short-term and long-term coefficients of variation (CV) for whole body bone mineral density for the DEXA instrument were 0.8% and 1.4% respectively. Placental weights were also recorded after delivery.

### **2.9.3: Principles of mRNA expression analysis**

In order to determine mRNA levels in tissue samples, RNA is first extracted from the tissue. RNA is extracted by first separating it from the DNA and proteins using an acidic solution which retains RNA in an upper aqueous phase. The RNA is then precipitated from the aqueous phase using ethanol or isopropanol (Pailla *et al.*, 2000). The RNA is checked for quality and quantity by measuring the absorbance ratio of RNA at 260 and 280 nm (with ratio of 1.8 to 2.0 indicating purity). Gel electrophoresis can also be used to determine the quality of the RNA. Typically, two bands corresponding to 18S and 28S ribosomal RNA species can be visualised on the gel to demonstrate the integrity of the RNA.

The RNA must then be transcribed to complementary DNA using commercially available kits which utilise a reverse transcriptase enzyme. The RNA structure is first disrupted by heating and this allows the primers supplied in the reaction mix to anneal to the RNA strand. In the presence of deoxynucleoside triphosphates (dNTPs) and reverse transcriptase, the cDNA strand is extended on the mRNA strand to form a hybrid RNA/DNA strand.

Quantitative real-time polymerase chain reaction (Q-RT-PCR) is then used to determine the mRNA expression levels in each sample. In RT-PCR, DNA polymerase, primers and probes are used. The probes have a reporter fluorescent dye attached to the 5' end and a quencher at the 3' end and it anneals to a target sequence on the cDNA strand downstream of the site where the primer anneals. While the probe is intact the quencher, stops the reporter dye from fluorescing. As the polymerase extends primers over the cDNA strand, it cleaves the probe and this releases the reporter dye which fluoresces. The intensity of the fluorescent is proportional to the quantity of the amplicons produced. DNA binding dyes such as SYBR<sup>®</sup> Green that bind to any double stranded DNA can also be used to determine amount of PCR products.

#### 2.9.4: Equipment and reagents

- Nanodrop™ 1000 Spectrophotometer (Thermal Fisher, Loughborough, UK)
- Vortexer (SI Scientific industries, USA)
- ABI PRISM 7700 sequence detector system (Applied Bio-systems, California, USA).
- LC480 light cycler (Roche diagnostics, Sussex, UK)
- Primers (Eurogentec, Seraing, Belgium)
- Probes, Roche human Universal Probe library (Roche diagnostics, Sussex, UK)
- Housekeeping gene primers and perfect probes, geNorm housekeeping genes kit and programme (Primerdesign, Southampton, UK)
- Reverse transcription reagents (Promega, Southampton, UK)
- Reverse transcription thermal reactor (Hybaid, Cambridge, UK)
- RNeasy fibrous tissue RNA isolation mini kit (Qiagen, Sussex, UK)
- Microtubes (Fisher, Loughborough, UK)

#### 2.9.5: RNA extraction and purification

Prior to the start of this thesis 300 placentas from SWS pregnancies were collected within 30 min of delivery. Placental weight was measured after removing obvious blood clots, cutting the umbilical cord flush with its insertion into the placenta, trimming away surrounding membranes and removing the amnion from the basal plate. Analysis of gene expression was carried out in 102 of the 300 placentas which were selected based on availability of neonatal DEXA data and amino acid system A activity data (Lewis et al., 2010).

To ensure that the RNA extracted was representative of the placentas as a whole 5 villous tissue samples were selected using a stratified random sampling

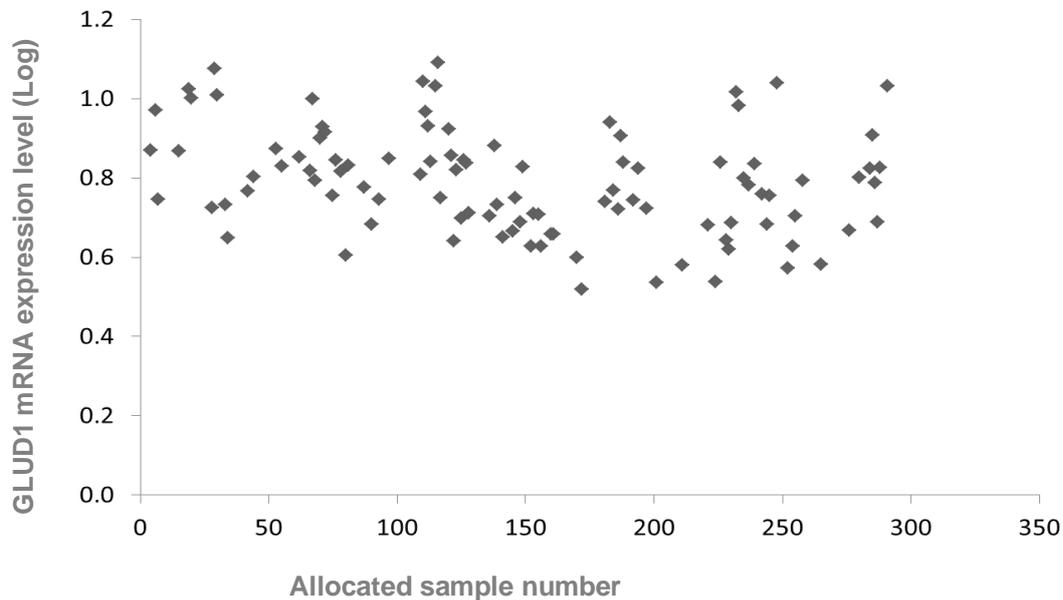
method. The snap frozen samples were pooled and ground while frozen and stored at  $-80^{\circ}\text{C}$ . Total RNA was extracted from 30 mg powdered placental tissue using the RNeasy fibrous tissue RNA isolation mini kit according to the manufacturer's instructions. Briefly, tissue was lysed in a guanidine-isothiocyanate buffer after dilution and the lysate samples were treated with proteinase K. The debris was pelleted by centrifugation. Ethanol was added to the cleared lysate and RNA was bound to the RNeasy silica-gel membrane. Traces of DNA that may co-purify were removed by a DNase treatment on the RNeasy spin column. The column was then washed three times and total RNA was eluted in 50  $\mu\text{L}$  RNase-free water.

### **2.9.6: RNA quality control**

The concentration and quality of RNA was assessed by taking the OD 260/280 ratio and concentration measurements using a Nanodrop™ 1000 Spectrophotometer and visualised on an agarose gel using ethidium bromide under ultra violet light to assess the integrity of the 18S and 28S rRNA bands.

### **2.9.7: The synthesis of cDNA**

cDNA synthesis is a process where total RNA is reverse transcribed into complimentary DNA. The reaction mixture of 5  $\mu\text{L}$  of 0.2  $\mu\text{g}$  RNA, 9  $\mu\text{L}$  nuclease free water and 1  $\mu\text{L}$  of 500 ng/L random hexamer primers was incubated at  $70^{\circ}\text{C}$  for 5 min to disrupt the secondary structures using a thermal reactor. This was followed by cooling to allow the primers to anneal to the complementary RNA on ice. A second master mix (10  $\mu\text{L}$ ) containing 1  $\mu\text{L}$  (200 units) of MMLV reverse transcriptase, 0.625  $\mu\text{L}$  (25 units) recombinant RNasin ribonuclease inhibitor, 1.25  $\mu\text{L}$  (0.5 mM) each of dATP, dCTP, dGTP and dTTP, 5  $\mu\text{L}$  of 5  $\times$  MMLV reaction buffer and 2.125  $\mu\text{L}$  nuclease free water was added to the RNA mix. Heating was carried out at  $37^{\circ}\text{C}$  for 1 hour,  $42^{\circ}\text{C}$  for 10 min and  $75^{\circ}\text{C}$  for 10 min using a thermal reactor. All 102 samples were produced in one batch to reduce variation and Figure 2.6 shows that there were negligible sample variations.



**Figure 2.6: Example of variation in GLUD1 amino acid transporter mRNA levels in the 102 samples.** This demonstrates there are no systematic differences due to extraction in different batches or date of collection.

### 2.9.8: Choosing control genes for quantitative PCR

Housekeeping genes were used for the normalisation of mRNA expression levels of the genes of interest. Normalisation accounts for the amount of RNA loaded into the reverse transcription reaction, efficiency of reverse transcription reaction, presence of inhibitors or enhancers in a reaction tube and experimental conditions (Vandesompele et al., 2002).

Housekeeping genes are constitutively expressed in tissues and they code for proteins that are involved in the basic function of the cell such as glycolysis which occurs in all cells (Vandesompele et al., 2002). Originally, only one housekeeping gene was used for the normalisation of gene expression but several studies compared the expression levels of several housekeeping genes in different tissues and showed that their levels of expression differ between tissues and cell types (Vandesompele et al., 2002; Warrington et al., 2000; Thellin et al., 1999; Suzuki et al., 2000; Bustin, 2000). Further studies have shown that there is not a specific gene that can be used for normalization but it

is more accurate to use the geometric mean expression level of between three and five genes depending on the tissue being investigated (Meller et al., 2005; Vandesompele et al., 2002). In this study, the expression levels and stability of 12 housekeeping genes were determined using the geNorm kit.

### **2.9.9: Quantitative polymerase chain reaction (qPCR) for the housekeeping genes**

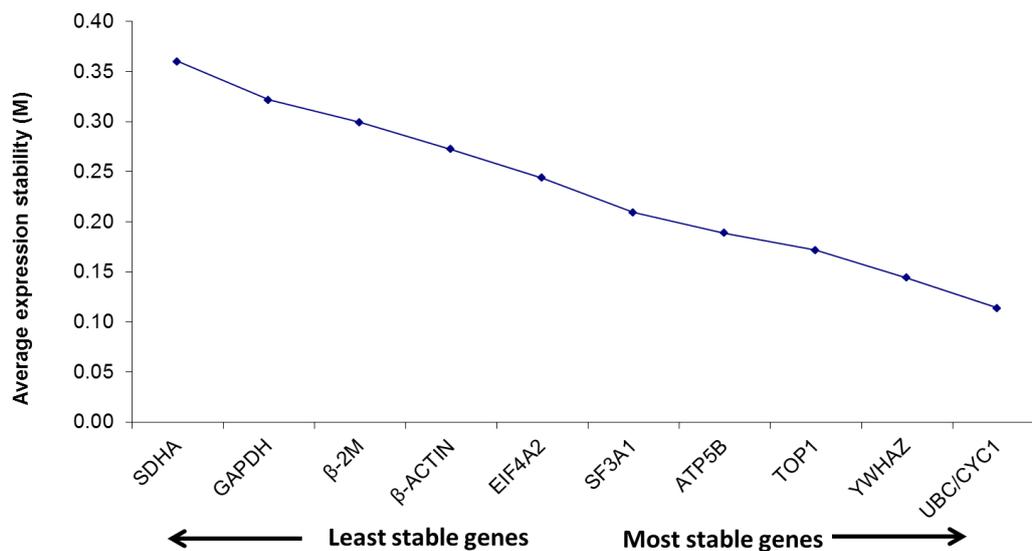
Two different qPCR methods were used; one for determining the expression stability of the housekeeping genes and the other for the analysis of genes of interest together with the chosen housekeeping genes.

**Housekeeping genes:** The GeNorm housekeeping selection kit was used to analyse twelve housekeeping genes (Table 2.3) by quantitative real-time PCR. Briefly cDNA from 10 placentas was diluted 1 in 10 and the housekeeping genes were measured by real-time PCR using primers and perfect probes (Primerdesign UK). 1  $\mu$ L perfect probe/primer was mixed with 10  $\mu$ L qPCR master mix and 4  $\mu$ L of nuclease free water. The the mixture was then mixed with 5  $\mu$ L cDNA. The sample mixtures were measured in duplicate using an ABI PRISM 7700 sequence detector system.

The cycle parameters were; enzyme activation at 95°C for 10 min, followed by 50 cycles at 95°C for 15 s and 50°C for 30 s and extension at 72°C for 15 s. The cycle number at which the increase in fluorescence and thus PCR product crossed a threshold was recorded as a CT value for each sample. CT values were transformed into relative quantification data using the deltaCT method (as explained below) and expression stability and the optimum number of control genes required was calculated using geNorm software. The geNorm software calculated the expression stability of each housekeeping gene and ranked them in order of stability (Figure 2.7). The software also gave the optimum number of housekeeping genes required in order to achieve the best normalization strategy. For this study we compared results from two different extraction protocols; Trizol or RNeasy fibrous tissue RNA isolation mini kit and the geNorm software suggested we use UBC, YWHAZ and TOP1 as normalization genes as they were the most stably expressed genes (Cleal et al., 2009).

**Table 2.3: Housekeeping genes tested for stability in the placental tissue**

Gene	Gene Name	Accession Number (Genbank)
<i>SDHA</i>	Succinate dehydrogenase complex, subunit A,	NM_004168
<i>GAPDH</i>	Glyceraldehyde-3-phosphate dehydrogenase	NM_002046
<i>18S RNA</i>	N'-adenosyl (rRNA) dimethyltransferase 18S rRNA dimethylase	X03205
<i>UBC</i>	Ubiquitin C	NM_021009
<i>B2M</i>	Beta-2-microglobulin form pl 5.3	NC-000015.8
<i>EIF4A2</i>	Homo sapiens eukaryotic initiation factor 4A-II	NC_000003.10
<i>SF3A1</i>	Splicing factor 3a subunit 1	NM_001005409
<i>TOP1</i>	Topoisomerase (DNA) I	NM_003286.2
<i>ATP5B</i>	ATP synthase	NM_001686
<i>CYC1</i>	Homo sapiens Cytochrome c	NM_018947.4
<i>YWHAZ</i>	Phospholipase A2	NM_001135699
<i>SDHA</i>	Succinate dehydrogenase complex	NM_004168



**Figure 2.7: Expression stability of housekeeping genes** in the normal term human placentas. The average expression stability values (M) for each housekeeping gene are ordered according to increasing stability with the least stably expressed genes on the left and the most stable genes on the right.

### **2.9.10: Primer design for the genes of interest**

Oligonucleotide probes and primers for the genes of interest were designed using the Roche ProbeFinder version 2.45 and universal probe library and primers were synthesised by Eurogentec. To ensure that genomic DNA was not amplified in the PCR reaction all the primers were intron-spanning and they all had a small amplicon size such that they are more likely to generate reproducible and robust assays (Mouritzen, 2005). The primer design software also uses *in silico* PCR which eliminate assays that target small introns as well as assays that could amplify irrelevant sequences such as pseudogenes (Roche universal probe library). Primers were also designed to ensure that they cover all transcript variants.

### **2.9.11: Quantitative polymerase chain reaction (qPCR) for the genes of interest**

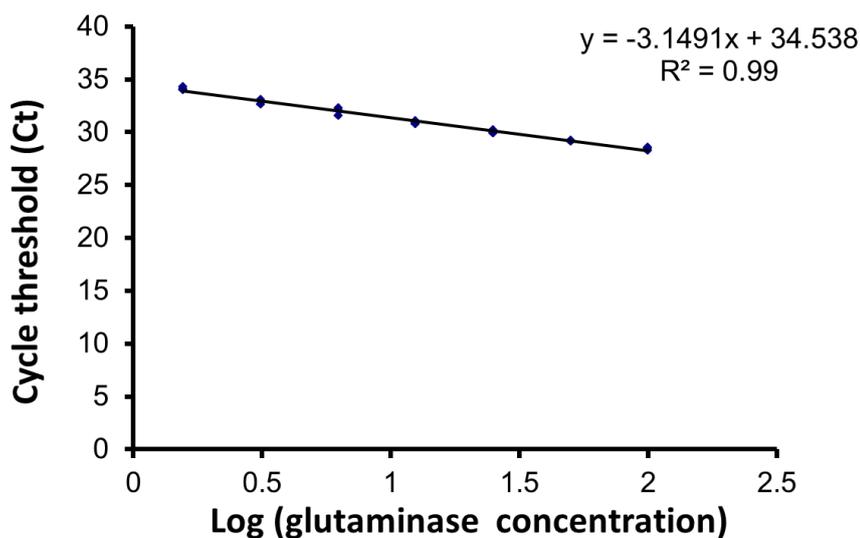
Real time PCR was carried out for the genes of interest and the housekeeping genes in the 102 SWS cDNA samples diluted 1 in 15. For genes of interest the real-time RT-PCR reaction mixture consisted of 10 µL cDNA, 0.8 µL of 200 nM for each of the reverse and forward primers, 4 µL of 100 nm probe, 20 µL PCR master mix and 5.2 µL of deionised water. For the housekeeping genes the reaction mixture consisted of 1 µL Primer/perfect probe, 10 µL qPCR master mix and 4 µL nuclease free water. The mixture was then mixed with 10 µL cDNA. Each of the 102 samples was run on the same plate in triplicate using an LC480 light cycler. The cycle parameters for Roche universal probe library probes and primers were 95°C for 10 min, followed by 40 cycles of 95°C for 15 s and 60°C for 1 min. The cycle parameters for Perfect Probes (UBC, YWHAZ and TOP1) were 95°C for 10 min, followed by 40 cycles of 95°C for 10 s and 60°C and 72°C for 15 s. Intra-assay CV's for each gene were 5-8%. The results for each sample were given as the cycle number (Ct), calculated by the second derivative method (Chomczynski & Rymaszewski, 2006) by the Light Cycler quantification software 3.5; Roche Diagnostics .

The Second Derivative Maximum method identifies the Ct of a sample as the point where the sample's fluorescence curve turns sharply upward. This turning point corresponds to the maximum of the second derivative of the amplification

curve. Thus, this method is called “Second Derivative Maximum method”. The big advantage of this method is that it requires little user input as the software performs the calculation automatically. For both genes of interest and housekeeping genes a standard curve was used to determine the expression levels of the each gene (an example is shown in Figure 2.8). The geometric mean of the three housekeeping genes (UBC, YWHAZ and TOP1) was calculated for each sample. This was then used to correct for experimental and sample errors when calculating the expression levels for target genes.

***Calculations for determining suitable housekeeping genes and normalisation of target genes***

1. To calculate of deltaCT value for the housekeeping genes; determine the highest Ct value for each gene and subtract this number from each of the rest of the Ct values and apply the formula ( $2^{-\text{deltaCT}}$ ) to each Ct value.
2. Use the geNorm software to rank the expression stability of the genes and determine how many housekeeping genes to use.
3. Obtain the Ct values for the chosen housekeeping genes and the target genes in the samples.
4. Calculate expression levels using the standard curve.
5. Carry out relative quantification by; amount of target gene/geometric mean of the housekeeping genes.



**Figure 2.8: Glutaminase gene expression standard curve** as an example of standard curves obtained from all genes by RT-PCR analysis.

## **2.10: Methodology development**

Inherent to any scientific project it is essential to determine the optimal experimental conditions and to ensure that the chosen methodology is sensitive, specific, and reproducible. Among the conditions necessary to determine the optimal experimental conditions in this project were: time points for sample collection during placental perfusion methodology, conditions for sample purification, derivatisation when measuring the concentration of amino acids by HPLC and the enrichment of amino acids by GC-MS. Detection limits for the two chromatographic methods had to be determined.

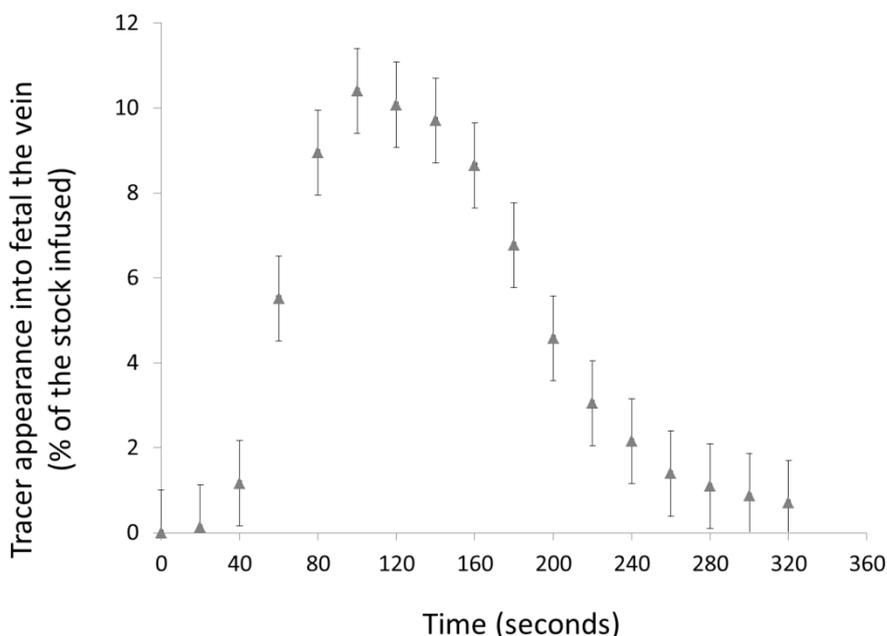
### **2.10.1: Sampling time during placental perfusion**

One aspect of this methodology was to determine the time a bolus would take from time of injection to the time it reaches the fetal or the maternal circulation. This is of interest because whether any exchange or placental metabolism is observed depends on the time at which the bolus takes to reach the placenta and whether it is metabolised or transported to the fetal circulation as well as the time of sampling. If sampling is done too early the injected bolus has not reached the placenta and no effect is observed; if sampling is done too late, little or no effect is observed. To determine the points for sample collection, the fetal circulation was perfused with EBB and a bolus of radio labelled phenylalanine was injected into the fetal circulation. Samples were collected prior to and every 20 s for 6 min following a bolus injection. Samples were mixed with scintillation fluid and counted in a  $\beta$ -counter. From this experiment, it was determined that the optimal times for sample collection are between 100 to 140 s after bolus injection (Figure 2.9).

### **2.10.2: Optimum conditions for determining amino acid concentration by HPLC**

Initially solvent A was prepared using disodium hydrogen orthophosphate and by adding propionic acid drop wise to adjust the pH to 6.0 and buffer B was prepared without disodium hydrogen orthophosphate buffer salt. However the resolution between a valine peak and methionine peak was very unstable. To ensure stable resolution of the peaks, buffer A was prepared with a pH of 6.3 and buffer B was prepared by adding 0.5 g disodium hydrogen orthophosphate

and adding propionic acid to set the pH to 6.8. This modification resolved the two peaks and it gave a good resolution for other amino acids but it was still not possible to detect cysteine and proline because they do not react with the derivatising agent. Peaks for tryptophan and histidine had insufficient sensitivity at lower concentrations.



**Figure 2.9: Mean time points for sample collection** after a bolus injection in an isolated perfused placenta (n = 5) Data is presented as mean  $\pm$  SEM.

To determine the concentration limits and analytical reproducibility, a series of standard solutions containing all amino acids to be measured, excluding proline and cysteine at concentrations of 50  $\mu\text{mol/L}$ , 25  $\mu\text{mol/L}$ , 12.5  $\mu\text{mol/L}$ , 6.25  $\mu\text{mol/L}$ , 3.125  $\mu\text{mol/L}$  and 0  $\mu\text{mol/L}$  were analysed with nor-valine as an internal standard. Reproducibility was determined by multiple analyses and the CV of the measurements was calculated (Table 2.4). In order to identify the amino acids individually, each amino acid was run separately and its chromatogram was matched to the peak in the chromatogram containing all the amino acids. The linearity was studied in the range of 3.125  $\mu\text{mol/L}$  to 50  $\mu\text{mol/L}$  of standard amino acids. Five concentration points were assayed in duplicate and both the amino acid standards and the test products showed good linearity in the tested range. R square coefficient ( $R^2$ ) was always greater than 0.995 for all the amino acids. The CVs for all amino acids were below 10% at concentrations 25  $\mu\text{mol/L}$ ,

12.5  $\mu\text{mol/L}$  and 6.25  $\mu\text{mol/L}$  which is acceptable for this method (Table 2.4). However taurine, histidine, tryptophan and lysine had CVs of greater than 10% at 3.125  $\mu\text{mol/L}$  and at 50  $\mu\text{mol/L}$  the CV of all the amino acids were less than 10% except for serine and glycine which had CVs of 11.1% and 13.3% at concentrations of 25  $\mu\text{mol/L}$  and 50  $\mu\text{mol/L}$  respectively.

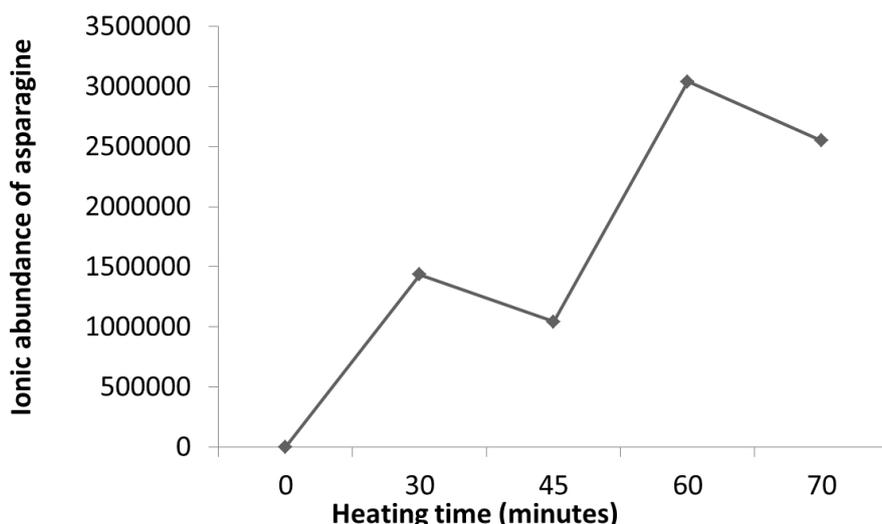
### 2.10.3: Optimal conditions for GC-MS sample preparation.

Prior to GC-MS samples undergo a series of modifications such as purification and derivatisation. Initially, samples for GC-MS analysis were analysed as the n-propyl, heptafluorobutyrate derivatives. It was found that the peaks for the glutamine and lysine derivatives co-eluted and because these two compounds share the same molecular weight it was not possible to determine their individual ionic abundance. Initial attempts to improve separation were not successful. Additionally, no peak for asparagine was observed which was probably due to it being converted to aspartate. For these reasons, that initial derivatisation method was abandoned in favour of the MTBSTFA method described previously. The time of heating for this method was varied to determine the optimum time for asparagine recovery (Figure 2.10). On the basis of these results a heating time of 60 min, was subsequently used for all samples.

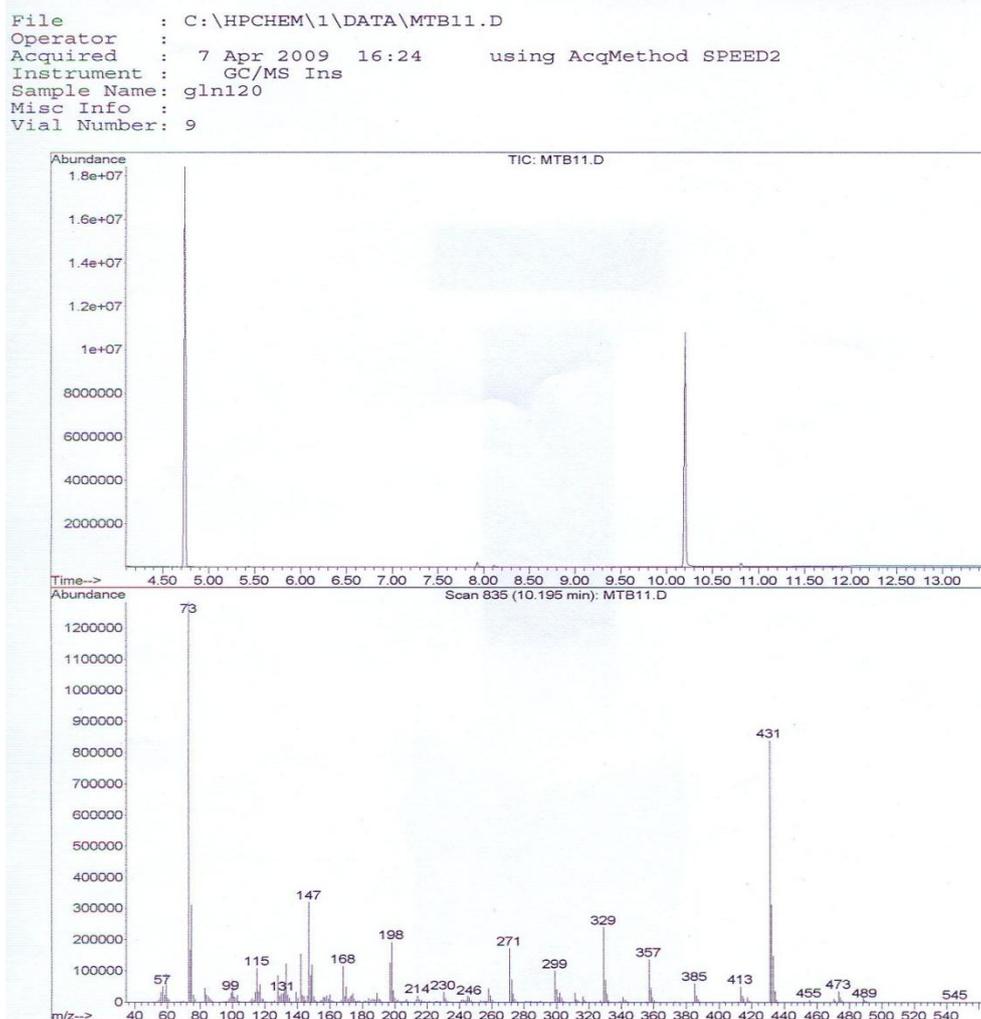
**Table 2.4: Coefficient variation of the amino acids measured by HPLC**

Amino acids	3.125 ( $\mu\text{mol/L}$ )	6.25 ( $\mu\text{mol/L}$ )	12.5 ( $\mu\text{mol/L}$ )	25 ( $\mu\text{mol/L}$ )	50 ( $\mu\text{mol/L}$ )
asp	7.3	2.6	2.9	6.5	6.6
glu	13.5	5	4.9	6.2	2.1
asn	2	2.1	3.1	6.7	2.4
ser	6.3	1.2	3.7	11.1	19
gln	8.2	5.2	10.1	9.6	8.7
his	35.2	5.4	3.6	8.5	7.2
gly	8.7	4	3.4	9.2	13.3
thr	6.9	1.8	3.7	6.9	5.8
arg	6.5	3	3.5	7.3	3.6
ala	5.9	2.5	2.8	6.8	7.6
tau	12.2	9.6	4.5	6.9	3.2
tyr	3.3	1.4	3.7	5.9	2.2
val	4	2.5	3.8	6.8	4.4
met	4.8	3.7	7.2	6	2.9
trp	29.3	3.6	5.9	6.3	2.6
phe	6.9	2.7	1.9	5.4	2.5
iso	6.2	3.9	2.5	6.6	2.4
leu	5.7	3.3	2	5.8	3.5
lys	19.8	5.6	3.2	6.6	2.8

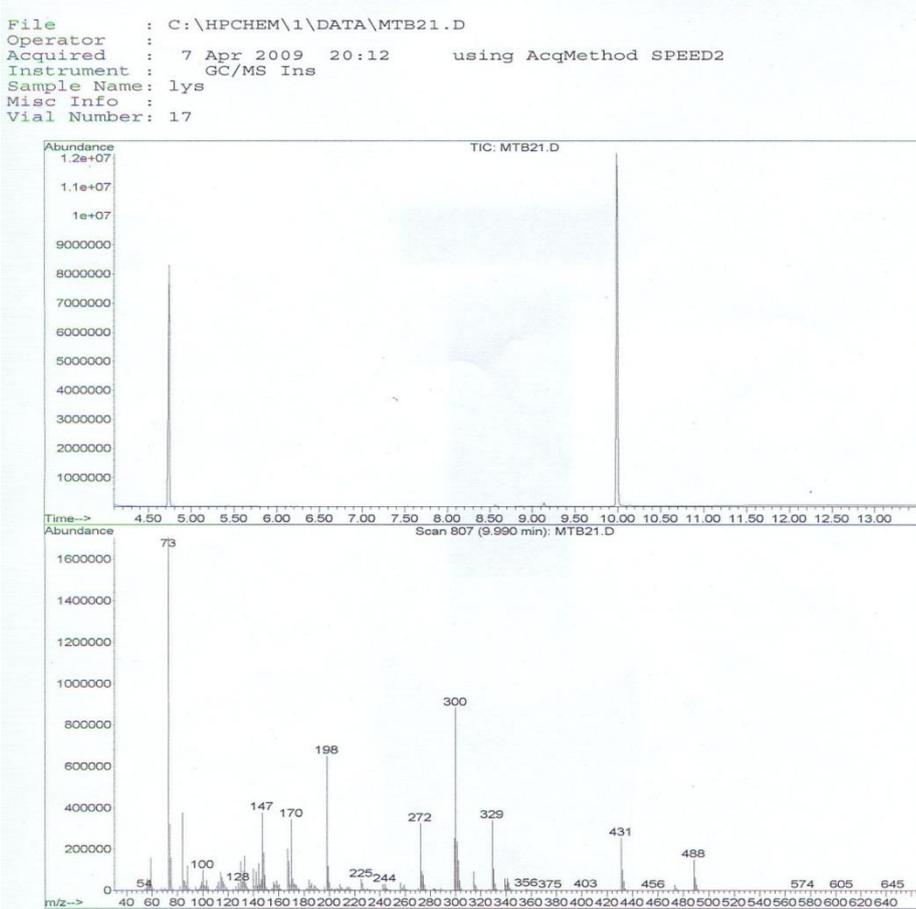
In other studies, it was found that when asparagine and glutamine were derivatised by heating with MTBSTFA at 90°C for 1 hour (more extreme conditions than the routine procedure) they could successfully be measured by GC-MS without their degradation to aspartate and glutamate respectively. It was also shown that leaving the samples overnight had no effect on asparagine degradation or conversion to aspartate. The chromatograms in Figures 2.10 to 2.13 were obtained with electron impact ionisation in scan mode using an  $m/z$  range of 50 -800. Figure 2.11 shows that glutamine (Retention time or RT 10.2 min), when derivatised at 90°C for 1 hr is not decomposed to glutamate as there is no glutamate peak at retention time (RT) 9.5 min (top panel). Figure 2.12 illustrates that lysine in the top panel (RT 10.0 min) can be distinguished from glutamine as there is no glutamine peak at RT 10.2 min in the top panel. Figure 2.13 shows that aspartate in the top panel RT 9.0 min is not converted to asparagine as there is no asparagine peak at RT 9.7 min and Figure 2.14 shows an asparagine peak (RT 9.7), showing that asparagine is not being converted to aspartate (No aspartate peak at RT 9.0). However peak at 9.5 could not be characterised and as none of the amino acids has similar molecular weight and retention time as asparagine, this peak could not have risen from any other amino acid and affect the results for other amino acids in subsequent studies.



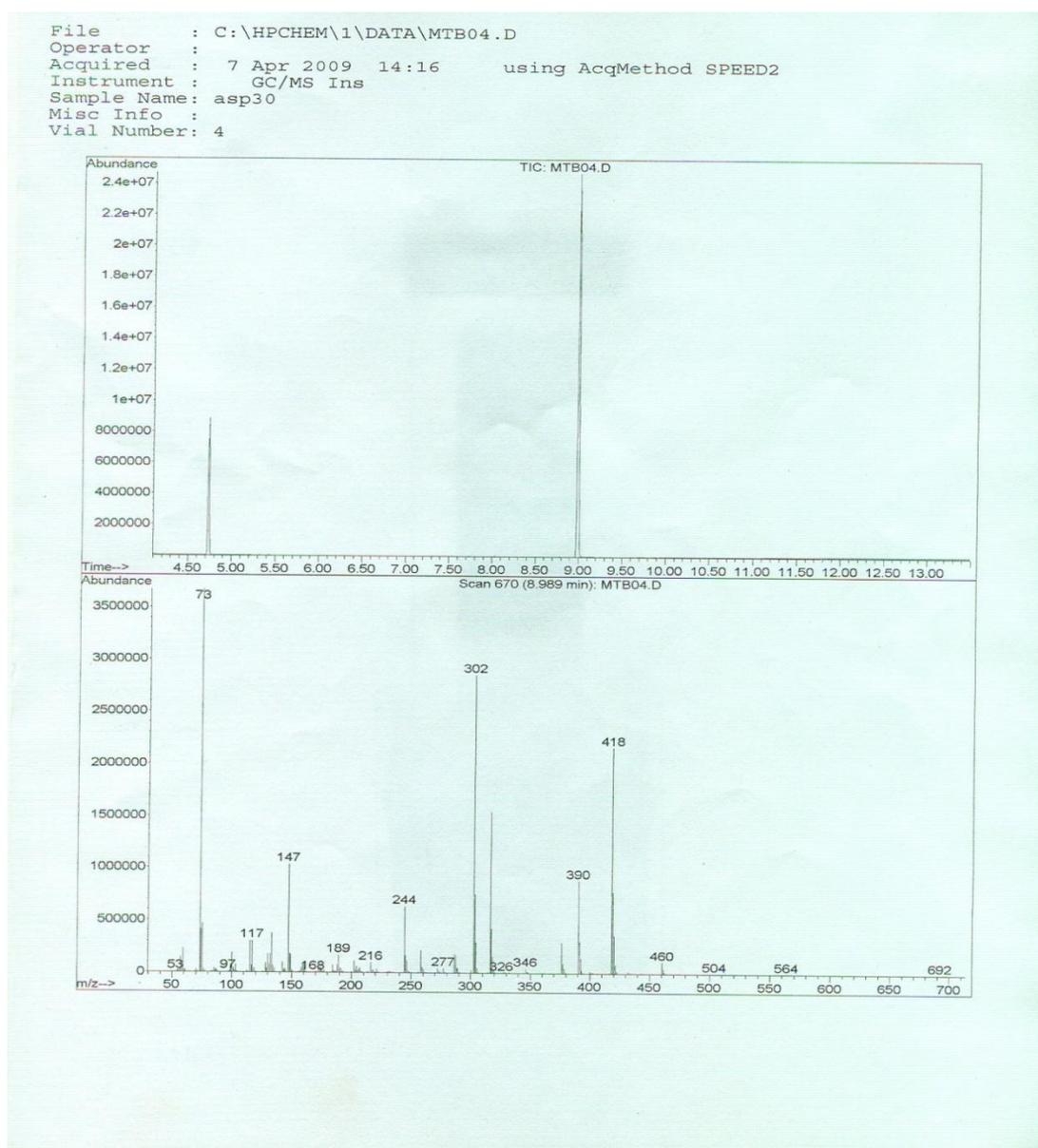
**Figure 2.10: The changes in the ionic abundance of asparagine** measured by GC-MS after derivatisation with MTBTFA and acetonitrile at 90°C for the duration of 30, 45, 60 and 70 min.



**Figure 2.11: Glutamine spectrum showing ionic peak response** for glutamine in a standard sample (top panel) and ionic fingerprint of glutamine ionization (bottom panel) from the NIST library. Only a peak for glutamine at retention time 10.2 min is visible in the top panel indicating that there is no conversion of glutamine to glutamate (no glutamate peak at 9.5).

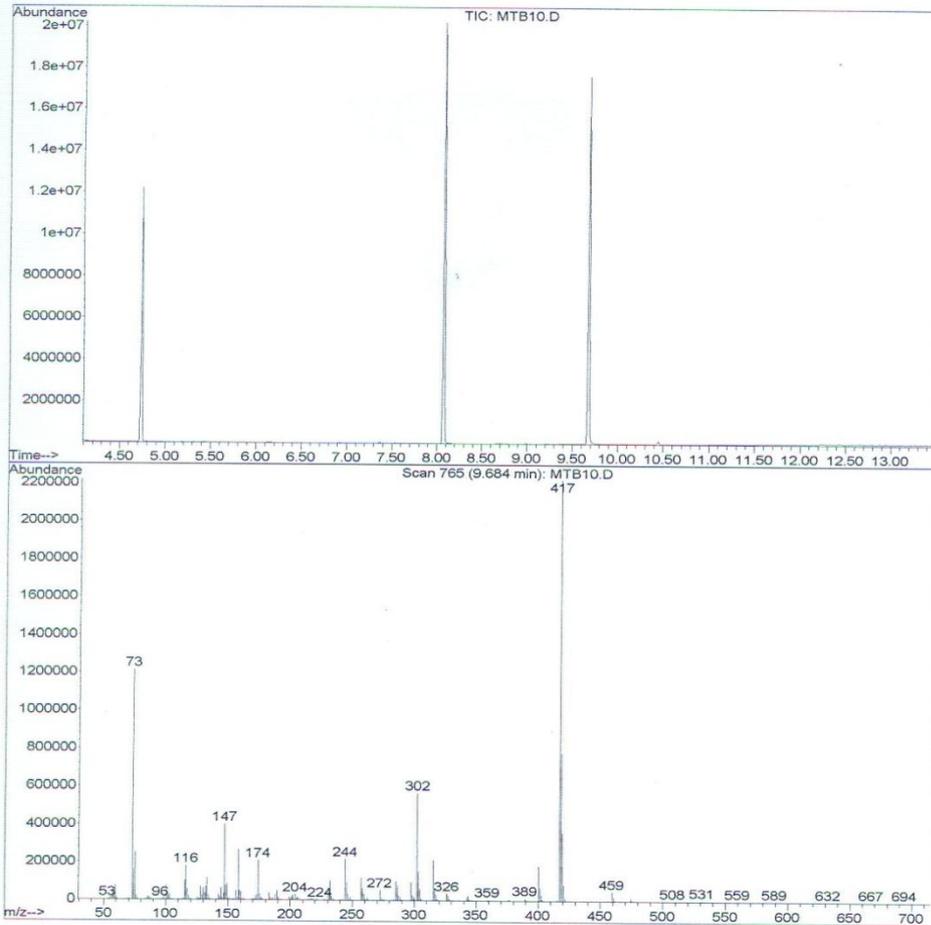


**Figure 2.12: Lysine spectrum showing ionic peak response** for lysine in a standard sample (top panel) and ionic fingerprint of lysine ionisation (bottom panel) from the NIST library. Only a peak for lysine at RT 10 min is visible in the top panel indicating that lysine can be distinguished from glutamine (no glutamine peak at RT 10).



**Figure 2.13: Aspartate spectrum showing ionic peak response** for aspartate in a standard sample (top panel) and ionic fingerprint of aspartate ionization (bottom panel) from the NIST library. Only a peak for aspartate at retention time 9.0 min is visible in the top panel indicating that there is no conversion of aspartate to asparagine (no asparagine peak at 9.7).

File : C:\HPCHEM\1\DATA\MTB10.D  
Operator :  
Acquired : 7 Apr 2009 16:06 using AcqMethod SPEED2  
Instrument : GC/MS Ins  
Sample Name: asn70  
Misc Info :  
Vial Number: 8



**Figure 2.14: Asparagine spectrum showing ionic peak response** for asparagine (RT 9.7) in a standard sample (top panel) and ionic fingerprint of aspartate ionization (bottom panel) from the NIST library. Only a peak for asparagine at retention time 9.7 min is visible in the top panel indicating that there is no conversion of aspartate to asparagine (no aspartate peak at 9.0).

## **2.11: Statistics**

Statistical analyses were carried out using PASW SPSS19 (IBM, Chicago, IL, USA) and Stata version 11.0 (Statacorp, Texas, USA). All graphs for the glucose and amino acid data were drawn using Graphpad Prism 5 (GraphPad Software, California, USA) while the SWS and placental gene expression data were drawn using either GraphPad 5 or Stata version 11.0. All glucose and amino acid data are presented as mean  $\pm$  SEM while some of the placental gene expression data are presented as mean  $\pm$  SEM or as mean (SD) or median (inter-quartile range). Levene and Kurtosis tests were used to check group variance and normality respectively. One-Way and Two way-ANOVA with Bonferonni posthoc and Pearson's Correlation test were used for the analysis of the glucose data as described in the statistics section of Chapter 3. One-way ANOVA with Bonferonni or Dunnett's posthoc tests was used for amino acid data as described in the statistics section of Chapter 4. Non-parametric tests such as Wilcoxon signed rank test were also used for comparing pairs of data that were not normally distributed and parametric tests such as paired T test were used for the data that were normally distributed as described in Chapter 4. SWS data and placental mRNA data were transformed by Fisher-Yates to Z-scores or logarithmically transformed. Relationships between gene expression levels and the SWS data were analysed by linear regression and analysis of gene expression levels between different categories of maternal lifestyle were tested by one-way ANOVA.



## **Chapter 3**

### **Glucose transfer and metabolism**



### 3.1: Background

Alterations in placental glucose transfer are implicated in both IUGR and fetal macrosomia (Tichopad *et al.*, 2003). In order to determine the role of glucose transfer in the pathogenesis of these conditions it is important to understand the factors which determine placental glucose transfer. This chapter will investigate the uncertainties in the current understanding of glucose transfer across the human placenta using the isolated perfused placental cotyledon.

There are conflicting ideas on the factors determining glucose transfer across the placenta. Some studies have demonstrated that glucose transport is proportional to maternal glucose concentration and suggest that there is no saturation of transport below maternal D-glucose concentrations of 20mmol/L. In contrast other studies have suggested that the BM is rate-limiting for placental glucose transfer as it has lower levels of GLUT1 and a smaller surface area than the MVM (Osmond *et al.*, 2000;Hahn *et al.*, 1998;Magnusson *et al.*, 2004). The suggestion that the BM is rate-limiting for glucose transfer appears to contradict the observation that placental glucose transfer is proportional to maternal glucose concentration. Further studies are therefore required to investigate this point. One other study has suggested that maternal and fetal placental blood flow is rate-limiting for glucose transfer (Jansson *et al.*, 1993;Gaither *et al.*, 1999).

Glucose transfer across the placenta is determined by membrane transporters, metabolism within the placenta and diffusion via paracellular routes (Illsley, 1987). Transport of glucose across the MVM and BM of the placental syncytiotrophoblast is primarily mediated by the facilitated transporter GLUT1 (Jansson *et al.*, 1993;Magnusson *et al.*, 2004). However, as described in the introduction, other members of the GLUT family may play a lesser role in mediating glucose transport. Placental consumption of glucose will limit transfer to the fetus although placental release of glucose metabolites, pyruvate and lactate, may provide an additional energy source for the fetus. Paracellular diffusion is thought to play a minor role in transferring glucose to the fetus (Jansson *et al.*, 1993). However, paracellular diffusion of L-glucose is reduced in gestational diabetes, suggesting that it is a regulated process and that

paracellular diffusion may be important in regulating the trans-placental glucose gradient (Osmond *et al.*, 2000). It is therefore important to determine the extent to which paracellular diffusion of glucose occurs across the human placenta.

There are also conflicting data on whether glucose transfer across the placenta is limited by placental glucose consumption. While some studies have shown that placental glucose consumption is decreased in IUGR, others have shown that placental glucose consumption is increased in IUGR (Osmond *et al.*, 2000). Another study has contradicted these observations by showing that placental glucose consumption is neither reduced nor increased in IUGR (Challis *et al.*, 2000; Malek, 1995).

These apparent contradictions suggest that further clarification of factors determining placental glucose transfer across the human placenta is required. The main focus of this study is to investigate glucose transfer and metabolism in the human placenta and explore whether there are other placental factors that may determine glucose transfer to the fetus.

### **3.2: Methods**

In order to determine mechanisms of placental glucose transfer, the placental perfusion methodology was used as explained in Chapter 2. Two groups of glucose perfusion experiments were carried out. In the first group, glucose transfer was investigated using creatinine as a marker for paracellular diffusion and without any radiolabelled tracers (Figure 3.1). In the second group of experiments, D-glucose, radiolabelled  $^{14}\text{C}$ -L-glucose (paracellular marker) and  $^3\text{H}$ -3-O-methyl-D-glucose (non-metabolisable) were infused into the maternal or fetal arterial circulation to measure glucose flux (Figures 3.2 and 3.3). Samples were collected on ice and stored at  $-20^\circ\text{C}$  until analysis. 1 ml of each sample was mixed with 5 ml of Hi safe 2 scintillation fluid and analysed using a 2100TR Packard liquid scintillation analyser. Glucose, lactate and creatinine concentrations were measured using glucose assay, lactate assay and creatinine assays as described in Chapter 2. Lactate assay was carried out to determine whether lactate release to maternal and fetal vein was dependent on glucose concentration.

### 3.2.1: Equilibration

After changing perfusion conditions it is necessary to let the system reach a new equilibrium before sampling. To determine the time taken for transfer of D-glucose and the tracers appearing in the fetal vein or maternal vein to reach equilibrium, glucose and the tracers were infused into the fetal or the maternal arterial circulation and samples were collected from both the maternal and the fetal vein every two minutes for 18 minutes. The appearances of glucose, creatinine and tracers in the fetal or maternal vein had equilibrated by 14 minutes after the start of infusion (Figures 3.6 to 3.8). From this, it was decided that samples to be analysed should be collected at 15, 17 and 18 minutes after the start of infusion. The fetal arterial circulation recoveries for the fetal tracer data were  $5.9 \pm 0.5$  ml/L and  $5.9 \pm 0.05$  ml for maternal tracer data. The average placental weight for all the glucose experiments and fetal flow rate recoveries are shown in table 3.1.

### 3.2.2: Placental characteristics

All placentas used for the studies in this chapter had a fetal arterial flow rate recovery of 95% or greater and maternal arterial circulation flow rates were 14 mL/min in all the experiments. At the end of the experiments, the perfused area of placental cotyledon that assumed a white colour was obtained by trimming off the non-perfused tissue, then blotted and weighed. Cotyledon weights together with the fetal venous flow rates at the start of perfusions are shown in Table 3.1.

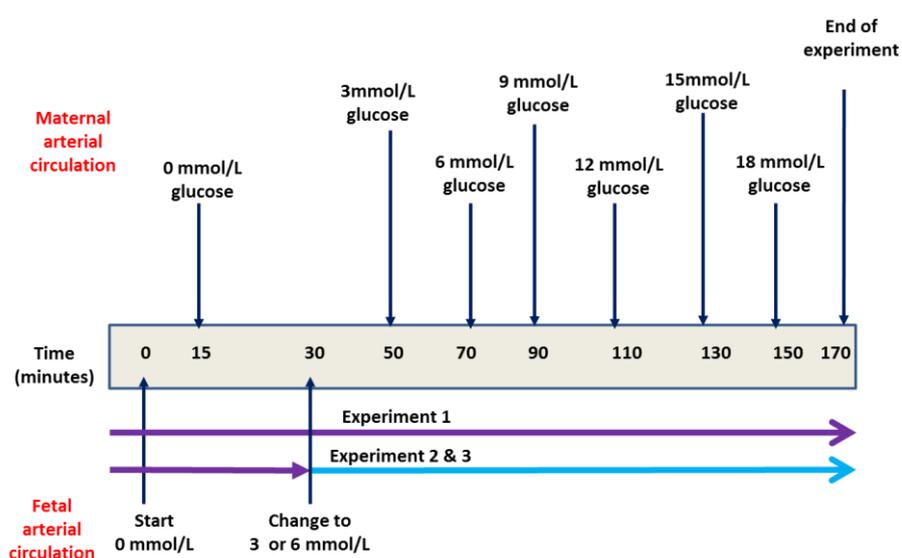
**Table 3.1: Average placental weights and fetal flow recovery rates** in the 5 glucose experiments carried out in this study

Experiment	Flow rates (ml/min)	Average cotyledon weight (g)	Number of experiments
0 mmol/L glucose fetal arterial perfusions	5.93 (0.04)	35.77 (5.95)	6
3 mmol/L glucose fetal arterial perfusions	5.97 (0.03)	31.27 (4.31)	5
6 mmol/L glucose fetal arterial perfusions	6.00 (0.00)	28.45 (4.58)	5
Maternal tracer glucose perfusions	6.00 (0.03)	30.53 (5.88)	5
Fetal tracer glucose perfusions	5.93 (0.04)	36.48 (3.73)	5

*Data are expressed as mean (SEM)*

### 3.2.3: Glucose and creatinine only experiments

Three sets of experiments using D-glucose and creatinine only were conducted (Figure 3.1). In all experiments maternal D-glucose was started at 0 mmol/l and increased every 20 minutes in 3 mmol/L increments up to 18 mmol/L. In the first set of experiments the fetal artery was continuously perfused at 6 ml/min with EBB containing sucrose (to maintain buffer composition and viscosity) instead of D-glucose while the maternal side was perfused with EBB containing 1.8 mmol/L creatinine. In the second set of experiments, the fetal arterial circulation was continuously infused with 3 mmol/L D-glucose. In the third set of experiments the fetal arterial circulation was perfused continuously with 6 mmol/L of D-glucose and the maternal arterial circulation was increased from 0 to 18 mmol/l (representing hypoglycaemia, normal glycaemia and above hyperglycaemia) as described above. In all three experiments approximately 2 ml samples were collected from maternal and fetal venous outflow at 15 min and 18 min after the start of each infusion.



**Figure 3.1: Experimental outline for experiments without tracers** (Group 1 experiment): Experiment 1 had a continuous perfusion of 0 mmol/L D-glucose into the fetal circulation from the start of experiment to the end. Experiments 2 and 3 had initial perfusion of 0 mmol/L up to 30 minutes, from then onwards fetal arterial circulation had a continuous perfusion of 3 or 6 mmol/L D-glucose until the end of the experiment. In all three experiments, maternal arterial glucose concentrations were changed as described above.

### 3.2.4: Tracer experiments

#### 3.2.4.1: Maternal side tracer perfusion

To estimate paracellular diffusion of glucose a paracellular marker  $^{14}\text{C}$ -L-glucose was used and to estimate glucose transport, the non-metabolisable  $^3\text{H}$ -3-O-methyl-D-glucose was used (Figure 3.4 shows transfer routes for glucose and the tracers). Two batches of EBB were prepared; the first EBB (batch A) was prepared in EBB without D-glucose and tracer. The second EBB (batch B) was prepared in EBB containing 4  $\mu\text{Ci}$  of 0.1mCi/mL  $^{14}\text{C}$ -L-glucose (Perkin Elmer, Boston, USA) and 40  $\mu\text{Ci}$  of 1 mCi/ml  $^3\text{H}$ -3-O-methyl-D-glucose (Perkin Elmer, Boston, USA). The concentrations of the tracers remained the same in all the experiments.

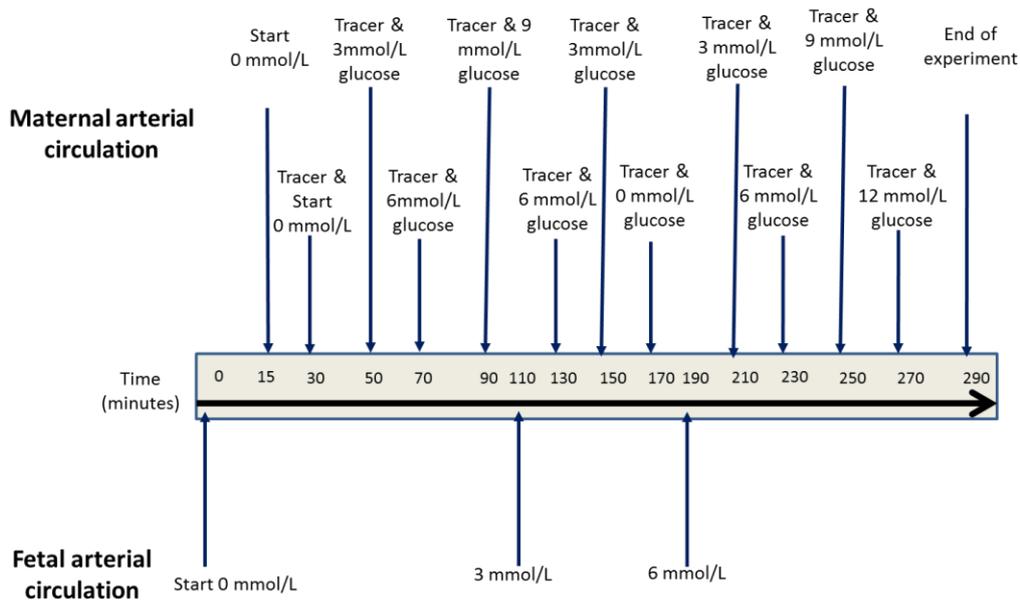
Batch A was used to make up 0, 3 and 6 mmol/L D-glucose buffers which were infused into the fetal arterial circulation as shown in Figure 3.2. Batch B was used to make up 0, 3, 6, 9 and 12 mmol/L buffers containing tracers which were infused into the maternal arterial circulation. 15 minutes after establishing fetal-side arterial perfusion with recovery of  $\geq 95\%$ , the maternal-side arterial perfusion was established. 15 minutes later, tracer concentrations of  $^{14}\text{C}$ -L-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose were perfused into the maternal arterial circulation. In these experiments maternal and fetal arterial D-glucose concentrations were (maternal: fetal (mmol/L)) 0:0, 3:0, 6:0, 9:0, 9:3, 6:3, 3:3, 0:3, 0:6, 3:6, 6:6, 9:6 and 12:6. Maternal and fetal venous samples were analysed by liquid scintillation counting and glucose assay. For a detailed protocol see appendix 2.

#### 3.2.4.2: Fetal side tracer perfusion

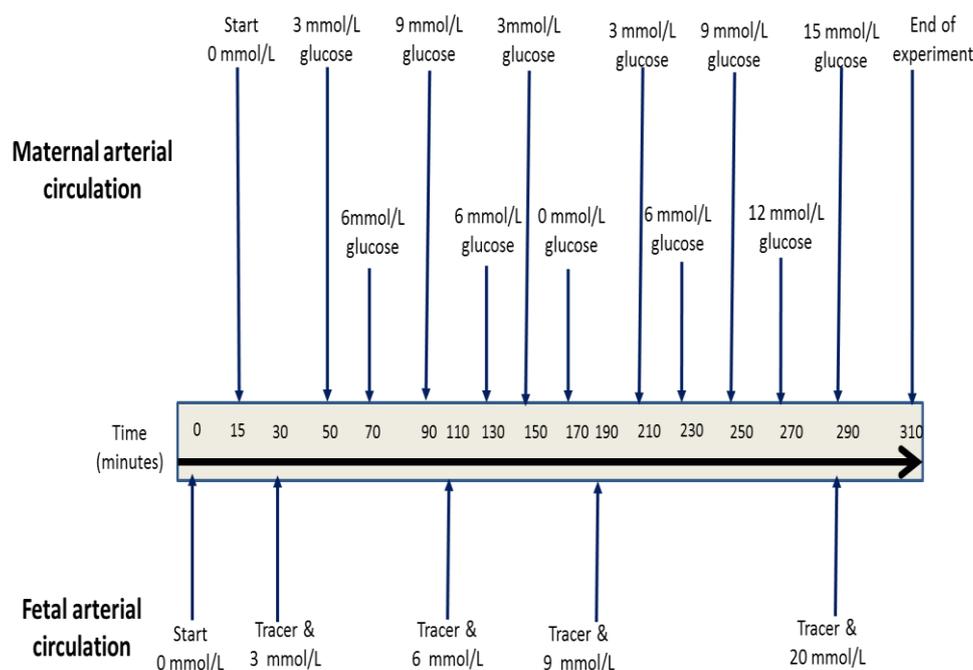
$^{14}\text{C}$ -L-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose were used as described above. Two batches of EBB were also prepared as described above; the first EBB (batch A) was prepared in EBB without D-glucose and tracer. The second EBB (batch B) was prepared in EBB containing 4  $\mu\text{Ci}$   $^{14}\text{C}$ -L-glucose and 40  $\mu\text{Ci}$   $^3\text{H}$ -3-O-methyl-D-glucose.

Batch A was used to make up 0, 3, 6, 9, 12 and 15 mmol/L D-glucose which were infused into the maternal arterial circulation as shown in figure 3.3. Batch

B was used to make up 0, 3, 6, 9 and 20 mmol/L buffers containing tracer which were infused into the fetal arterial circulation as shown in Figure 3.3. 15 minutes after establishing fetal-side arterial perfusion with recovery of  $\geq 95\%$ , the maternal-side arterial perfusion was established. 15 minutes later, tracer concentrations of  $^{14}\text{C}$ -L-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose were perfused into the fetal arterial circulation. In these experiments maternal and fetal arterial D-glucose concentration were (maternal: fetal (mmol/l)) 0:3, 3:3, 6:3, 9:3, 9:6, 6:6, 3:6, 0:6, 0:9, 3:9, 6:9, 9:9, 12:9, 15:9, 15:20. Maternal and fetal venous samples were analysed by liquid scintillation counting and glucose assay.



**Figure 3.2: Maternal arterial tracer perfusion experimental outline:** The fetal circulation was perfused from time 0 with EBB and the maternal circulation was infused from 15 minutes. At 30 minutes, the maternal buffer was changed to buffer containing radiolabelled  $^{14}\text{C}$ -L-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose tracers. Infusions of various D-glucose concentrations were perfused into the maternal circulation and the fetal circulation as indicated by the arrows.



**Figure 3.3: Experimental outline for fetal arterial tracer perfusion experiments.**

The fetal circulation was perfused from time 0 with EBB and the maternal circulation was infused from 15 minutes. At 30 minutes, the fetal buffer was changed to buffer containing radiolabelled  $^{14}\text{C}$ -L-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose tracers. Infusions of various D-glucose concentrations were perfused into the maternal circulation and the fetal circulation as indicated by the arrows.

### 3.2.5: Statistical analysis

Statistical analyses were carried out using PASW SPSS19 (IBM, Chicago, IL, USA). Effects of maternal and fetal arterial D-glucose concentration and D-glucose gradient on the uptake, transfer and paracellular diffusion of tracers and D-glucose as well as the interaction between maternal and fetal D-glucose, were determined using two-way analysis of variance (ANOVA). When maternal or fetal arterial D-glucose had an effect on D-glucose, L-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose placental uptake, transfer, transport, and paracellular diffusion and placental consumption the differences at various maternal or fetal arterial concentrations were tested using a Bonferroni Posthoc test. The maternal venous to fetal venous or fetal venous to maternal venous ratios were compared using one-way ANOVA with a Bonferroni Posthoc test. A P value of less than 0.05 was considered significant. Pearson Correlation was used to test

the correlation between arterial levels of glucose and placental D-glucose uptake, transfer, transport, paracellular diffusion, consumption and lactate concentrations. All data are presented as mean  $\pm$  standard error of means (SEM). As in these studies mechanisms of glucose transfer were being determined rather than comparing two groups of data, sample number and power of the study could not be calculated. However, for future studies, using data from Osmond et al, 2000, sample size of  $n = 8$ , would be sufficient for a statistical power of 80.5%.

### 3.2.6: Calculations for uptake, transfer, transport, diffusion, consumption and venous ratios

In these calculations the following abbreviations are used;

DA (donor artery), DV (donor vein), RA (recipient artery), RV (recipient vein), BG (background reading), 3MG ( $^3\text{H}$ -3-O-methyl-D-glucose), L-gluc ( $^{14}\text{C}$ -L-glucose), D-gluc (D-glucose), FR (flow rate – 0.014 L/min for maternal circulation and 0.006L/min for fetal circulation).

For the sake of these calculations, Figure 3.4 shows the pathways through which each tracer and D-glucose takes during their transfer across the placenta.

- **Placental uptake of 3MG (%)** =  $(\text{DA}^{3\text{MG (CPM)}} - \text{BG}^{3\text{MG (CPM)}}) - (\text{DV}^{3\text{MG (CPM)}} - \text{BG}^{3\text{MG (CPM)}}) / (\text{DA}^{3\text{MG (CPM)}} - \text{BG}^{3\text{MG (CPM)}}) * 100$
- **Transfer of 3MG to recipient vein (%)** =  $((\text{RV}^{3\text{MG (CPM)}} - \text{BG}^{3\text{MG (CPM)}}) * \text{FR}) / ((\text{DA}^{3\text{MG (CPM)}} - \text{BG}^{3\text{MG (CPM)}}) * \text{FR}) * 100$
- **Placental uptake of  $^{14}\text{C}$ -L-glucose (%)** =  $(\text{DA}^{\text{L-gluc (CPM)}} - \text{BG}^{\text{L-gluc (CPM)}}) - (\text{DV}^{\text{L-gluc (CPM)}} - \text{BG}^{\text{L-gluc (CPM)}}) / (\text{DA}^{\text{L-gluc (CPM)}} - \text{BG}^{\text{L-gluc (CPM)}}) * 100$
- **Transfer of  $^{14}\text{C}$ -L-glucose to recipient vein (%)** =  $((\text{RV}^{\text{L-gluc (CPM)}} - \text{BG}^{\text{L-gluc (CPM)}}) * \text{FR}) / ((\text{DA}^{\text{L-gluc (CPM)}} - \text{BG}^{\text{L-gluc (CPM)}}) * \text{FR}) * 100$
- **Total paracellular diffusion (%)** = (Transfer of  $^{14}\text{C}$ -L-glucose to RV - Transfer of 3MG to RV) x 100
- **Transport of 3MG to recipient vein (%)** = Transfer of 3MG to RV - Transfer of  $^{14}\text{C}$ -L-glucose to RV \* 100
- **Placental uptake of D-glucose (mmol/min)** =  $(\text{DA}^{\text{D-gluc (mmol/L)}} - \text{DV}^{\text{D-gluc (mmol/L)}}) * \text{FR}$

- **Transfer of D-glucose to RV (mmol/min)** =  $(RV^{D\text{-gluc (mmol/L)}} - RA^{D\text{-gluc (mmol/L)}})$
- **Paracellular diffusion of D-glucose to RV (mmol/min)** = Transfer of  $^{14}\text{C-L-glucose}$  to RV (%) \*  $(DA^{D\text{-gluc (mmol/L)}}) * DA^{FR}$
- **Transport of -D-glucose to RV (mmol/min)** = Transfer of 3MG to RV (%) \*  $(DA^{D\text{-gluc (mmol/L)}}) * DA^{FR}$
- **Placental consumption of D-glucose (mmol/min)** =  $(DA^{D\text{-gluc (mmol/L)}} * DA^{FR} + RA^{D\text{-gluc (mmol/L)}} * RA^{FR}) - ((DV^{D\text{-gluc (mmol/L)}} * DV^{FR} + RV^{D\text{-gluc (mmol/L)}} * RV^{FR})$
- **Donor vein: recipient vein ratios** =  $DV^{\text{substrate}} / RV^{\text{substrate}}$

Note: The ratios were calculated when the recipient side had 0 mmol/L glucose, hence the ratios for 12mmol/L maternal glucose and 20 mmol/L fetal glucose are not included in Figure 3.12.

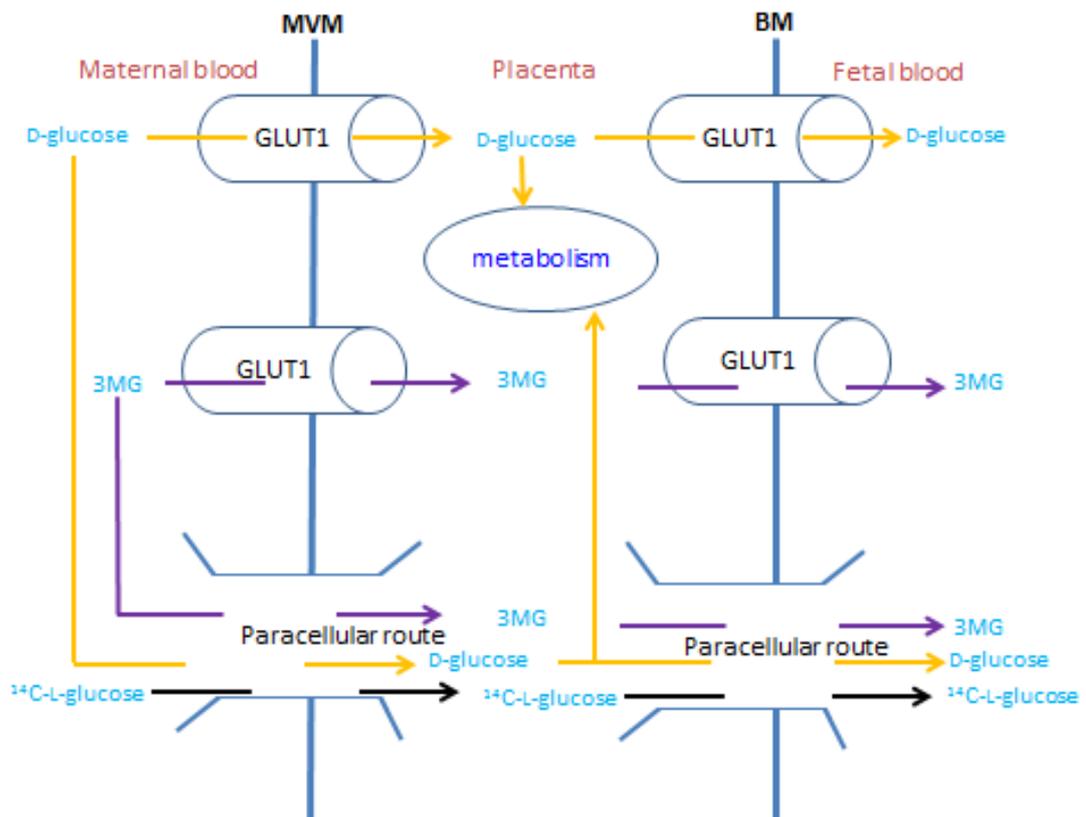
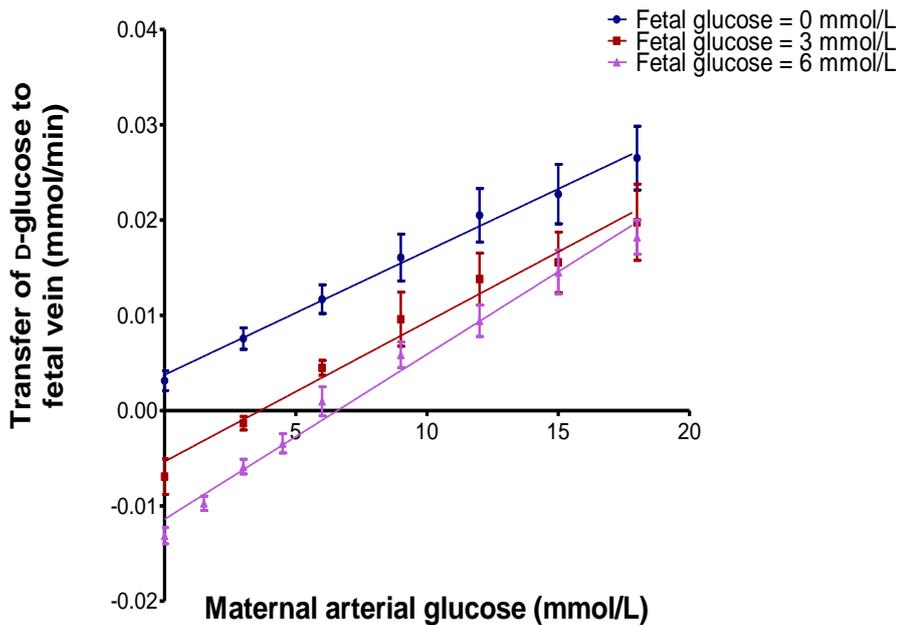


Figure 3.4: An illustration of the pathways through which D-glucose, L-glucose, and 3MG ( $^3\text{H-3-o-methyl-D-glucose}$ ) are transferred across the placenta.

### 3.3: Results

#### 3.3.1: D-glucose experiments without tracer

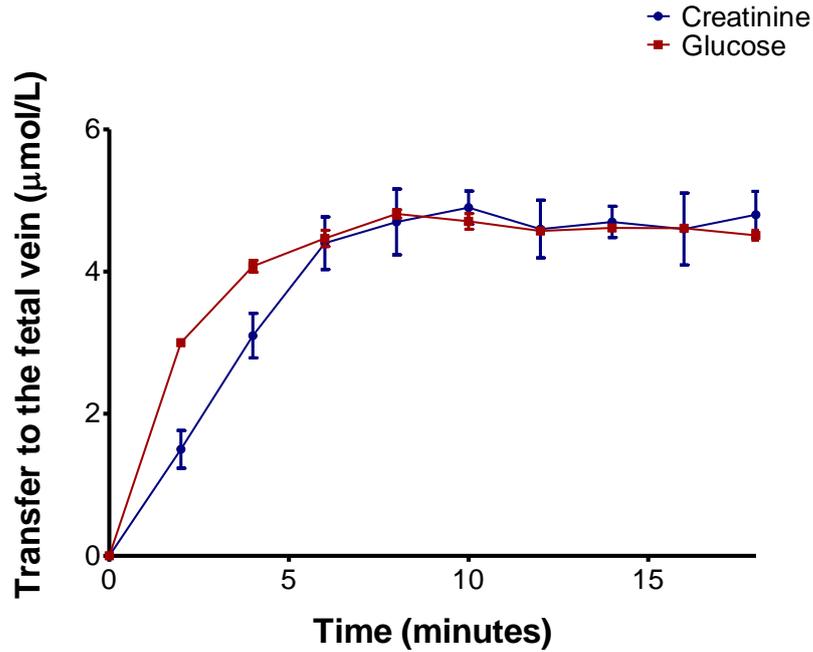
Transfer of D-glucose to the fetal vein was positively correlated to maternal arterial D-glucose concentration in 0 mmol/L ( $P < 0.0001$ ,  $R^2 = 0.98$ ), 3 mmol/L fetal arterial D-glucose ( $P < 0.0001$ ,  $R^2 = 0.98$ ) and in 6 mmol/L fetal arterial D-glucose experiments ( $P < 0.0001$ ,  $R^2 = 0.97$ ) (Figure 3.5).



**Figure 3.5: Transfer of D-glucose from the maternal arterial circulation to the fetal circulation** when fetal D-glucose was 0 mmol/L, 3 mmol/L and 6 mmol/L. Transfer to the fetal vein at 0 mmol/L, 3 mmol/L and 6 mmol/L fetal glucose was positively correlated to maternal arterial D-glucose concentration ( $R^2 = 0.98$ ,  $0.98$ ,  $0.97$  respectively). Data are presented as mean  $\pm$  SEM,  $n = 5$ .

#### 3.3.2: Tracer appearance preliminary results

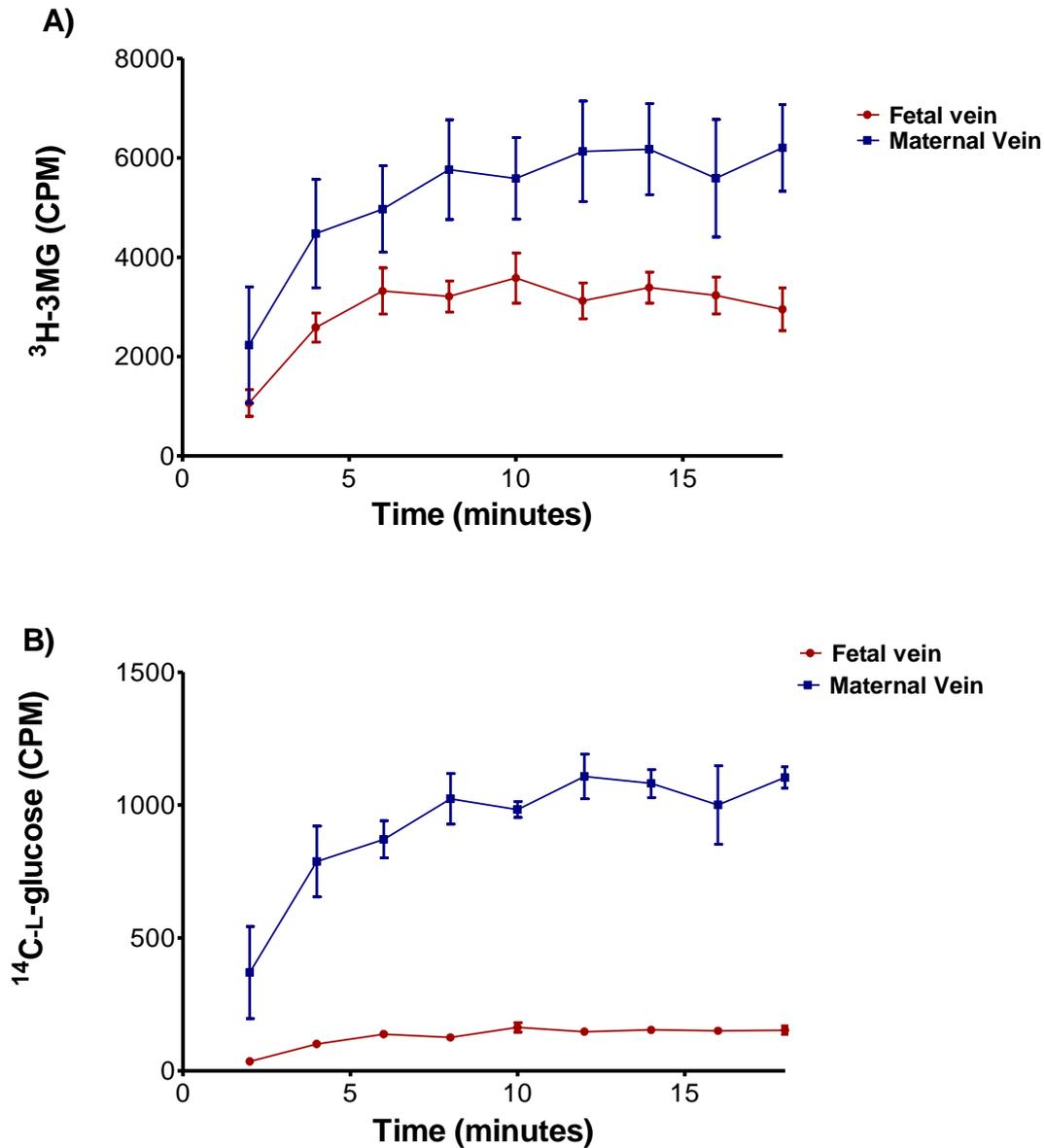
After starting infusion with tracers the appearances of D-glucose, creatinine and the tracers were measured over an 18 minute period with sampling at every 2 min. D-glucose and creatinine levels in the fetal vein plateaued at 12-14 min as shown in Figure 3.6 and the levels of tracers appearing in the maternal and fetal vein are shown in Figures 3.7 and 3.8. As a steady state had been reached by 14 min, samples were collected from 15 min onwards.



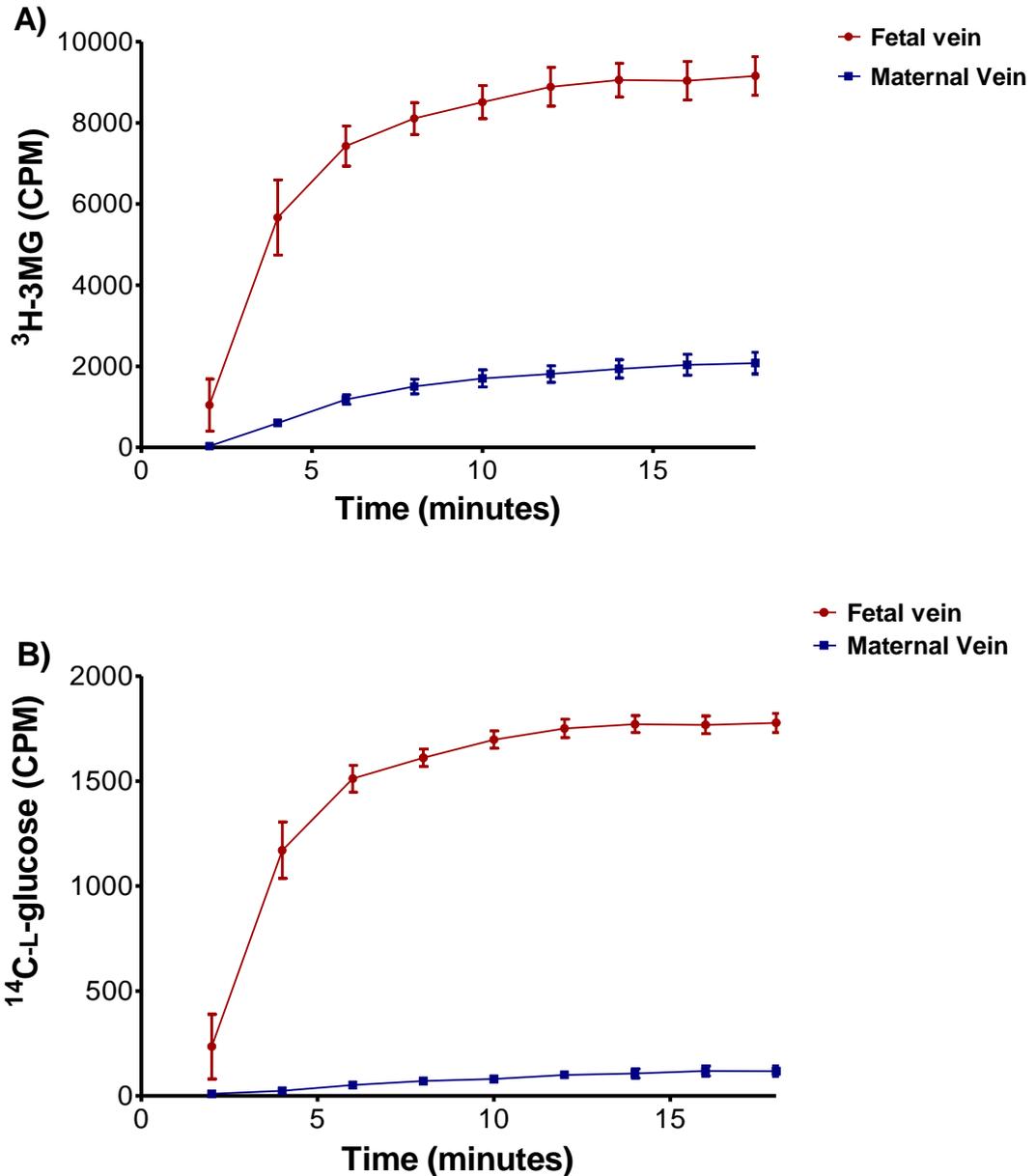
**Figure 3.6: Appearance of D-glucose and creatinine from maternal artery in the fetal venous circulation over an 18 minute period.** This shows that the appearance of creatinine and D-glucose reached equilibrium at 12-14 min; therefore samples were collected from 15 min onwards. Data are presented as mean  $\pm$  SEM,  $n = 5$ .

### 3.3.2.1: Maternal tracer experiments: Tracer appearance and transfer over the course of experiments

The levels of  $^3\text{H}$ -3-O-methyl-D-glucose remaining in the maternal vein were significantly higher than those of  $^{14}\text{C}$ -L-glucose  $^3\text{H}$ -3-O-methyl-D-glucose in the fetal vein ( $P < 0.001$  Figure 3.9A). The levels of  $^{14}\text{C}$ -L-glucose remaining in the maternal vein were higher than those of  $^{14}\text{C}$ -L-glucose appearing in the fetal vein ( $P < 0.001$ , Figure 3.9B). The levels of  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose remaining in the maternal vein did not change throughout the experiment ( $P = 1.00$  for both). Neither did their levels appearing in the fetal vein despite the changes in arterial glucose concentrations ( $P = 1.00$  and  $0.074$  respectively).



**Figure 3.7: Time course for the appearance of maternal arterial tracer in the fetal and maternal venous circulation over 18 min period.** A) Appearance of  $^3\text{H-3-O-methyl-D-glucose}$  in the fetal and maternal vein, B) Appearance of  $^{14}\text{C-L-glucose}$  in the fetal and maternal vein. This shows that the appearance of tracer on both sides plateaued by 10 min; therefore samples were collected from 15 min onwards. Data are presented as mean  $\pm$  SEM,  $n = 5$ .



**Figure 3.8: Time course for the appearance of fetal arterial tracer in the maternal and fetal vein.** A) Appearance of  $^3\text{H}$ -3-O-methyl-D-glucose and of  $^{14}\text{C}$ -L-glucose A) Appearance of  $^3\text{H}$ -3-O-methyl-D-glucose in the fetal and maternal vein, B) Appearance of  $^{14}\text{C}$ -L-glucose in the fetal and maternal vein. This shows that the appearance of tracer on both sides reached equilibrium by 10 min; therefore samples were collected from 15 min onwards. Data are presented as mean  $\pm$  SEM, n = 5.

### 3.3.2.2 Fetal tracer data: Tracer appearance and transfer over the course of experiments

The levels of  $^3\text{H}$ -3-O-methyl-D-glucose remaining in the fetal vein were significantly higher than those of appearing in the maternal vein ( $P < 0.001$  Figure 3.10A). The levels of  $^{14}\text{C}$ -L-glucose remaining in the fetal vein were higher than those of  $^{14}\text{C}$ -L-glucose appearing in the maternal vein ( $P < 0.001$ , Figure 3.10B). The levels of  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose remaining in the fetal vein did not change throughout the experiment ( $P = 0.985$  and  $0.937$  respectively). There were no changes in the levels of  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose appearing in the maternal vein over the course of the experiments despite changes in maternal and fetal arterial glucose concentrations ( $P = 0.999$  and  $1$  respectively).

The diffusion of creatinine from the maternal artery to the fetal vein was significantly higher than that of L-glucose ( $P < 0.001$ , 3.11). The percentage of creatinine appearing in the fetal circulation did not change throughout the experiments ( $P = 0.150$  and  $0.969$  respectively, Figures 3.11).

### 3.3.2.3: Raw glucose values in maternal and fetal veins of the maternal tracer experiments and maternal venous to fetal venous ratios

When maternal and fetal arterial glucose concentrations were changed, the glucose concentration changes were observed in both the maternal and the fetal vein. Figure 3.12A shows the raw values from the maternal tracer experiments. When the maternal artery was used as the donor circulation the maternal venous to fetal venous ratios for D-glucose,  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose were  $2.16 \pm 0.08$ ,  $2.36 \pm 0.06$  and  $7.89 \pm 0.15$  respectively (Figure 3.13A). In the maternal tracer perfusions, there were no differences within groups of D-glucose  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose for 3, 6 and 9 mmol/L glucose ( $P = 0.381$ ,  $0.868$  and  $0.960$  respectively). The maternal venous to fetal venous ratios of  $^{14}\text{C}$ -L-glucose were significantly higher than the ratios for D-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose ( $P = 0.012$  and  $0.015$  respectively for 3 mmol/L,  $P = 0.018$  and  $0.019$  respectively for 6 mmol/L and  $0.03$  and  $0.018$  respectively for 9 mmol/L).

### 3.3.2.4: Raw glucose values in maternal and fetal vein in the fetal tracer perfusions and fetal venous to maternal venous ratios

When maternal and fetal arterial glucose concentrations were changed, the glucose concentration changes were observed in both the maternal and the fetal vein. Figure 3.12B shows the raw values from the fetal tracer experiments. When the fetal artery was used as the donor circulation, the fetal venous to maternal venous ratios were; D-glucose  $3.35 \pm 0.20$ ,  $^3\text{H-3-O-methyl-D-glucose}$   $3.96 \pm 0.42$  and  $^{14}\text{C-L-glucose}$   $14.39 \pm 1.87$  (Figures 3.13B). There were no differences in the groups of D-glucose,  $^3\text{H-3-O-methyl-D-glucose}$  and  $^{14}\text{C-L-glucose}$  in the fetal tracer perfusions ( $P = 0.981, 0.203$  and  $0.112$ ). The fetal venous to maternal venous ratios of  $^{14}\text{C-L-glucose}$  were significantly higher than the ratios for D-glucose and  $^3\text{H-3-O-methyl-D-glucose}$  ( $P < 0.001$  both and at all concentrations).

### 3.3.3: Maternal to fetal tracer studies

#### 3.3.3.1: Maternal tracer data: Paracellular diffusion of $^{14}\text{C-L-glucose}$

Average transfer of  $^{14}\text{C-L-glucose}$  to the fetal vein was  $4.4\% \pm 0.2\%$  of the maternal arterial  $^{14}\text{C-L-glucose}$  (Figure 3.14). Maternal and fetal arterial D-glucose had no effect on  $^{14}\text{C-L-glucose}$  transfer to the fetal vein ( $P = 1.00$  and  $0.191$  respectively) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.885$ ). Paracellular diffusion and transport of D-glucose and  $^3\text{H-3-O-methyl-D-glucose}$  were estimated based on percentage of  $^{14}\text{C-L-glucose}$  appearing in the fetal vein as a percentage of maternal arterial  $^{14}\text{C-L-glucose}$  infusion. When expressed as a percentage of total  $^{14}\text{C-L-glucose}$  transfer, estimated paracellular diffusion of D-glucose was  $27.2\% \pm 0.5\%$  (data not shown). Total unidirectional paracellular diffusion in the fetal circulation to the maternal circulation direction was higher than total unidirectional paracellular diffusion in the maternal to the fetal circulation direction ( $P < 0.001$ , data not shown).

#### 3.3.3.2: Maternal tracer data: Placental uptake of $^3\text{H-3-o-methyl-D-glucose}$ from maternal artery

Neither maternal nor fetal D-glucose had a significant effect on  $^3\text{H-3-O-methyl-D-glucose}$  uptake by the placenta ( $P = 0.414$  and  $0.142$  respectively) and there

was no interaction between fetal and maternal arterial D-glucose ( $P = 0.992$ , Figure 3.15).

### **3.3.3.3: Maternal tracer data: Transfer of $^3\text{H}$ -3-O-methyl-D-glucose from maternal artery to fetal vein**

Maternal arterial D-glucose significantly decreased transfer of  $^3\text{H}$ -3-O-methyl-D-glucose transfer to the fetal vein ( $P = 0.007$ ) while fetal arterial D-glucose increased  $^3\text{H}$ -3-O-methyl-D-glucose transfer to the fetal vein ( $P = 0.032$ , Figure 3.16). There was no interaction between maternal and fetal D-glucose ( $P = 0.918$ ). Transfer of  $^3\text{H}$ -3-O-methyl-D-glucose when fetal D-glucose was 0 mmol/L was lower than transfer when fetal D-glucose was 3 mmol/L and 6 mmol/L ( $P = 0.033$  and  $0.046$  respectively). No differences in  $^3\text{H}$ -3-O-methyl-D-glucose transfer were observed at different maternal arterial D-glucose concentrations (Figure 3.16).

### **3.3.3.4: Maternal tracer data: Transport of $^3\text{H}$ -3-O-methyl-D-glucose from maternal artery to fetal vein**

Maternal arterial D-glucose decreased  $^3\text{H}$ -3-O-methyl-D-glucose transport to the fetal vein ( $P = 0.015$ ) but fetal arterial D-glucose had no effect on  $^3\text{H}$ -3-O-methyl-D-glucose transport ( $P = 0.166$ ) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.982$ , Figure 3.17).

### **3.3.3.5: Maternal tracer data: Placental uptake of D-glucose from maternal artery**

D-glucose uptake from the maternal arterial circulation increased with increasing maternal D-glucose concentration ( $P < 0.001$ ) and decreased with increasing fetal arterial D-glucose concentrations ( $P < 0.001$ ) but there was no interaction between maternal and arterial fetal D-glucose ( $P = 0.825$ ). Uptake of D-glucose when fetal glucose was 0 mmol/L was higher than uptake when fetal glucose was 3 mmol/L ( $P < 0.001$ ) and 6 mmol/L ( $P < 0.001$ , Figure 3.18).

### **3.3.3.6: Maternal tracer data: Transfer of D-glucose from maternal artery to fetal vein**

Transfer of D-glucose from the maternal arterial circulation to the fetal vein increased with increasing maternal arterial D-glucose concentration ( $P < 0.001$ ) and decreased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ).

There was no interaction between the two ( $P = 0.365$ , Figure 3.19). When D-glucose gradient was included as a covariate, maternal arterial D-glucose had an effect on transfer ( $P = 0.030$ ) but the fetal arterial D-glucose effect disappeared ( $P = 0.211$ ). Transfer of D-glucose to the fetal vein was higher when fetal arterial D-glucose was 0 mmol/L than when fetal arterial D-glucose was 3 mmol/L and 6 mmol/L ( $P < 0.001$  for both) and transfer of D-glucose to the fetal vein when fetal arterial D-glucose was 3 mmol/L was higher than when fetal arterial D-glucose was 6 mmol/L. There were strong positive correlations between transfer of D-glucose to the fetus and maternal arterial D-glucose concentration at 0 mmol/L, 3 mmol/L and 6 mmol/L fetal arterial D-glucose concentrations ( $R^2 = 0.98, 0.98, 0.97$  respectively, Figure 3.19).

### **3.3.3.7: Maternal tracer data: Transport of D-glucose from maternal artery to fetal vein**

Transport of D-glucose from the maternal arterial circulation to the fetal vein increased with increasing maternal D-glucose ( $P = 0.003$ ) but was not affected by fetal arterial D-glucose ( $P = 0.993$ ) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.340$ , Figure 3.20).

### **3.3.3.8: Maternal tracer data: Paracellular diffusion of D-glucose from maternal artery to fetal vein**

Paracellular diffusion of D-glucose from the maternal arterial circulation to the fetal vein increased with increasing maternal D-glucose ( $P = 0.048$ ) but was not affected by fetal arterial D-glucose ( $P = 0.991$ ) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 1.00$ , Figure 3.21). The maternal effect disappeared when the D-glucose gradient was used as a covariate ( $P = 0.308$ ).

### **3.3.3.9: Maternal tracer data: Consumption of D-glucose by the placenta**

Neither maternal nor fetal arterial D-glucose had an effect on placental consumption of D-glucose ( $P = 0.052$  and  $0.441$  respectively). There was no interaction between maternal and fetal arterial D-glucose ( $P = 0.420$ , Figure 3.22).

### 3.3.4: Fetal to maternal tracer studies

#### 3.3.4.1: Fetal tracer data: Paracellular diffusion of $^{14}\text{C}$ -L-glucose from fetal artery

Average transfer of  $^{14}\text{C}$ -L-glucose from fetal arterial circulation to the maternal vein was  $15\% \pm 0.5\%$  (Figure 3.23). Maternal and fetal arterial D-glucose had no effect on L-glucose transfer to the fetal vein ( $P = 0.987$  and  $0.782$  respectively) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.995$ ). Paracellular diffusion and transport of D-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose were estimated based on percentage of  $^{14}\text{C}$ -L-glucose appearing in the maternal vein as a percentage of fetal arterial  $^{14}\text{C}$ -L-glucose infusion. When expressed as a percentage of total transfer, paracellular diffusion of  $^{14}\text{C}$ -L-glucose to the maternal vein was  $35.8\% \pm 0.6\%$  (data not shown).

#### 3.3.4.2: Fetal tracer data: Placental uptake of $^3\text{H}$ -3-O-methyl-D-glucose from the fetal artery

There was no significant effect of maternal and fetal D-glucose on  $^3\text{H}$ -3-O-methyl-D-glucose uptake ( $P = 0.999$  and  $0.375$  respectively) and there was no interaction between maternal and fetal arterial glucose ( $P = 0.974$ , Figure 3.24).

#### 3.3.4.3: Fetal tracer data: Transfer and transport of $^3\text{H}$ -3-O-methyl-D-glucose to maternal vein

There was no significant effect of either maternal or fetal glucose on  $^3\text{H}$ -3-O-methyl-D-glucose transfer to the maternal arterial circulation ( $P = 0.999$  and  $0.434$  respectively) and there was no interaction between maternal and fetal arterial glucose ( $P = 0.991$ , Figure 3.25).

There was no effect of maternal and fetal arterial glucose on the transport of  $^3\text{H}$ -3-O-methyl-D-glucose to the maternal venous circulation ( $P = 0.994$  and  $0.540$  respectively). There was no interaction between maternal and fetal glucose ( $P = 0.997$ , Figure 3.26).

#### 3.3.4.4: Fetal tracer data: Placental uptake of D-glucose from fetal artery

Uptake of D-glucose increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) and decreased with increasing maternal D-glucose concentration ( $P = 0.003$ ). There was no interaction between maternal and fetal

arterial D-glucose ( $P = 0.999$ , Figure 3.27). When maternal arterial D-glucose was 0 mmol/L placental uptake of D-glucose was not significantly different to uptake when maternal arterial D-glucose was 3 mmol/L ( $P = 1.00$ ) and 6 mmol/L ( $P = 0.118$ ) but was significantly higher than uptake when maternal arterial D-glucose was 9 mmol/L ( $P = 0.005$ ), 12 mmol/L ( $P = 0.011$ ) and 15 mmol/L ( $P = 0.022$ ). There were no differences in placental uptake when maternal arterial D-glucose was 3 mmol/L or higher.

#### **3.3.4.5: Fetal tracer data: Transfer of D-glucose from the fetal artery to the maternal vein**

Transfer of D-glucose from the fetal arterial circulation to the maternal vein increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) and decreased with increasing maternal D-glucose concentration ( $P < 0.001$ ). There was no interaction between maternal and fetal arterial D-glucose ( $P = 1.00$ , Figure 3.28). D-glucose transfer when maternal arterial D-glucose was 0 mmol/L was significantly higher than transfer when maternal arterial D-glucose was higher ( $P < 0.001$  for all). D-glucose transfer when maternal arterial D-glucose was 3 mmol/L was higher than transfer when maternal arterial D-glucose was 6 mmol/L or higher ( $P = 0.001$  for 9, 12 and 15 mmol/L). D-glucose transfer at 6 mmol/L was higher than transfer at 9 mmol/L ( $P = 0.028$ ) and 12 mmol/L ( $P = 0.009$ ) but not when maternal arterial D-glucose was 15 mmol/L ( $P = 0.105$ ).

#### **3.3.4.6: Fetal tracer data: D-glucose transport from the fetal artery to the maternal vein**

Unidirectional transport of D-glucose increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) but was not affected by maternal arterial D-glucose ( $P = 0.983$ ) and there was no interaction between maternal and fetal D-glucose ( $P = 0.997$ , Figure 3.29).

#### **3.3.4.7: Fetal tracer data: D-glucose paracellular diffusion from the fetal artery to the maternal vein**

Unidirectional paracellular diffusion of D-glucose increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) but was not affected by maternal

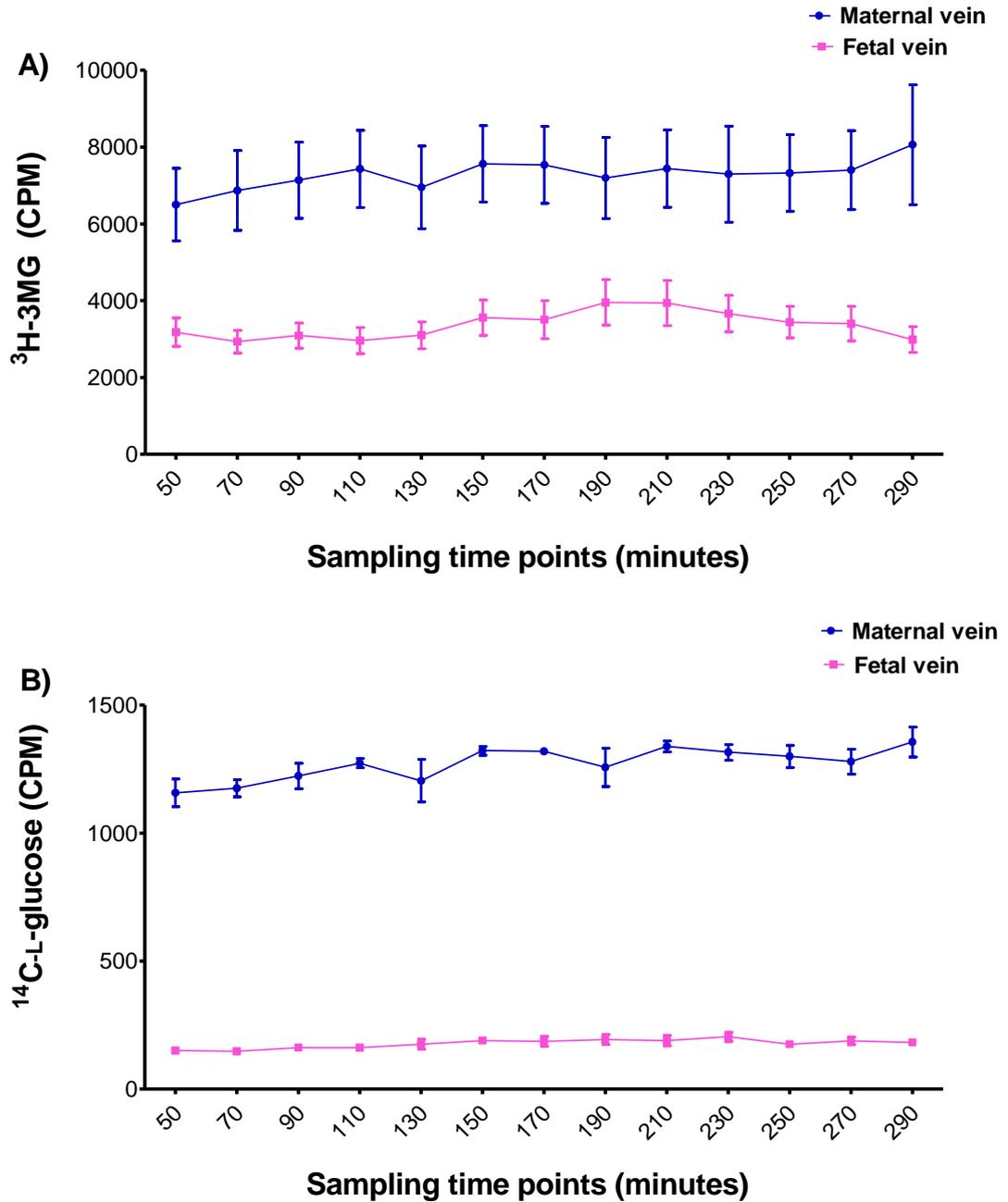
arterial D-glucose ( $P = 0.976$ ) and there was no interaction between maternal and fetal D-glucose ( $P = 0.999$ , Figure 3.30).

#### **3.3.4.8: Fetal tracer data: Effects of D-glucose on placental D-glucose consumption**

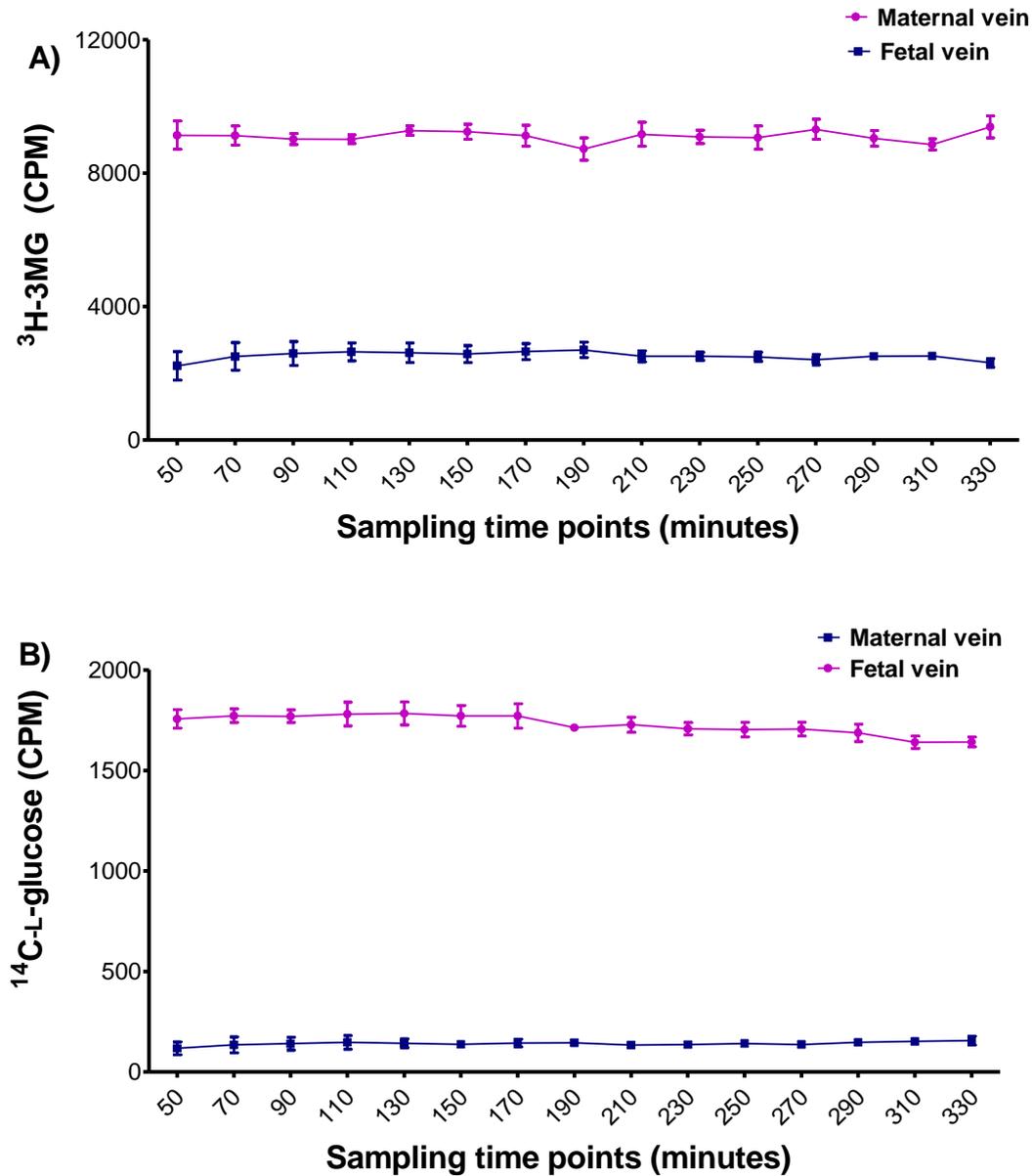
There were no significant effects of maternal and fetal arterial D-glucose concentrations on placental consumption ( $P = 0.986$  and  $0.739$  respectively) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 1.00$ , Figure 3.31).

#### **3.3.5: Lactate production and transfer**

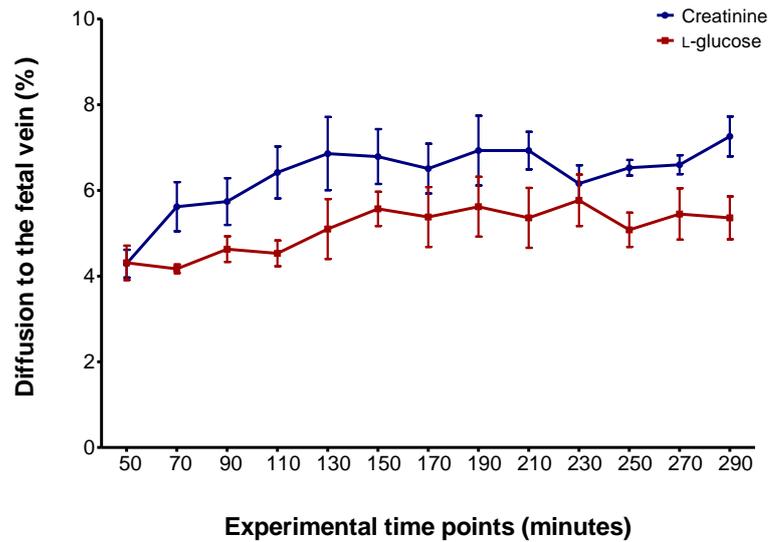
The concentration of lactate appearing in the maternal vein was significantly higher than that in the fetal vein ( $P < 0.001$ ) in both the 3 mmol/L and 6 mmol/L fetal arterial glucose experiments (Figure 3.32). Appearance of lactate in the maternal or the fetal vein did not change over the experimental time course in both the 3 mmol/L ( $P = 0.933$  and  $0.931$  respectively) and the 6 mmol/L perfusions ( $P = 0.972$  and  $0.888$  respectively). Appearance of lactate in the maternal vein of the 3 mmol/L fetal D-glucose experiments was significantly higher than that in the 6 mmol/L fetal arterial D-glucose experiments ( $P = 0.001$ ) but the concentrations in the fetal vein of the 3 mmol/L and 6 mmol/L fetal arterial D-glucose were not different ( $P < 0.382$ ).



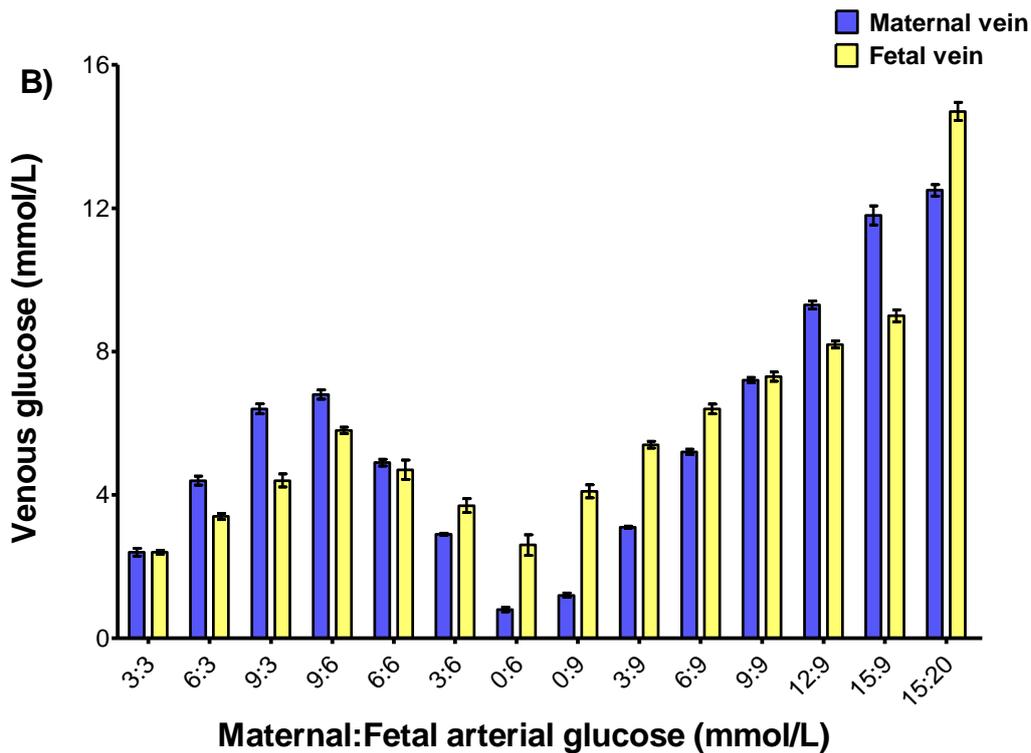
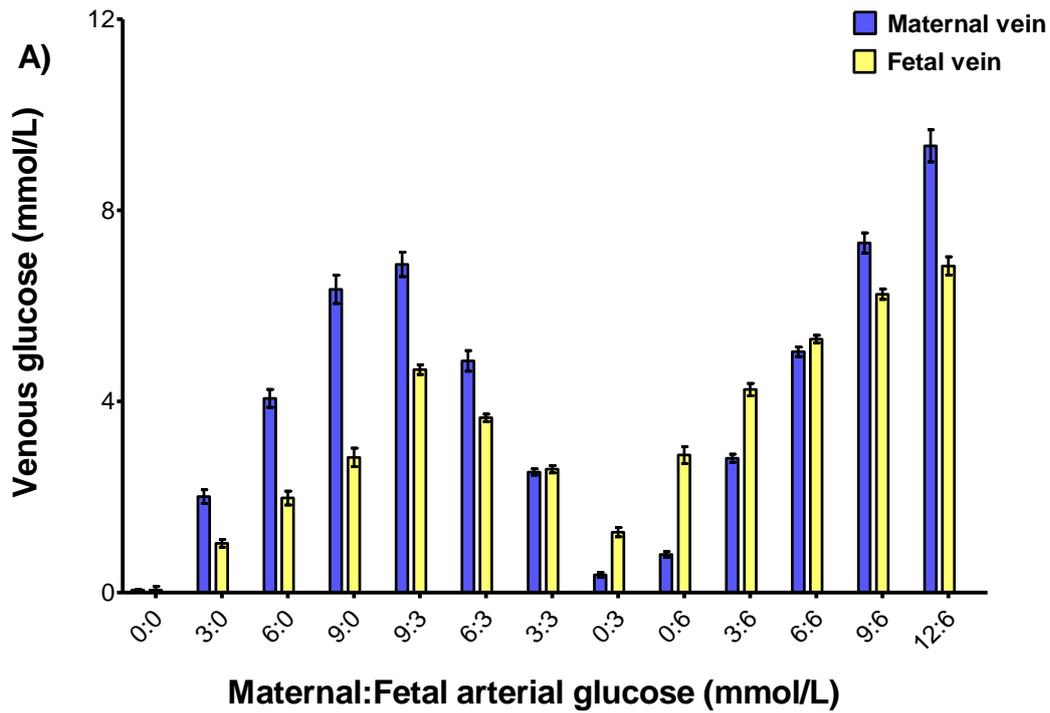
**Figure 3.9: Tracer remaining in the maternal vein and appearing in the fetal vein** after maternal arterial tracer infusion. A) Appearance of  $^3\text{H-3-O-methyl-D-glucose}$  in the fetal venous circulation and remaining in the maternal venous circulation over 5 hr and 10 min B). Appearance of  $^{14}\text{C-L-glucose}$  in the fetal venous circulation and remaining in the maternal venous circulation and appearing in the fetal venous circulation over 5 hr and 10 min. Despite changes in D-glucose concentrations, the percentage of  $^3\text{H-3-O-methyl-D-glucose}$  and  $^{14}\text{C-L-glucose}$  remaining in the maternal vein and appearing in the fetal vein did not change over time Data are expressed as mean  $\pm$  SEM, n = 5.



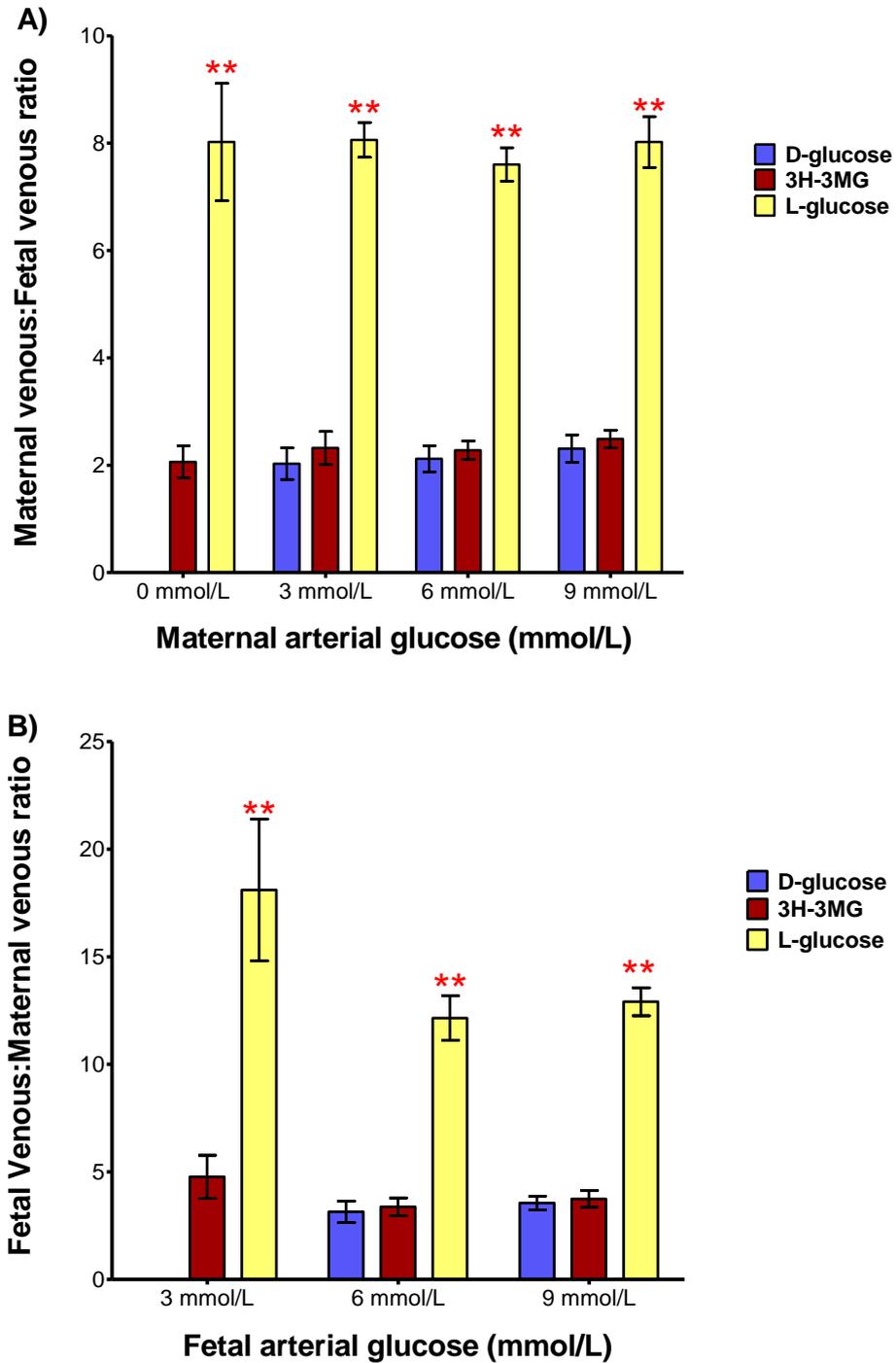
**Figure 3.10: Tracer appearance in the maternal and fetal vein after fetal arterial tracer infusion.** A) Appearance of  $^3\text{H}$ -3-O-methyl-D-glucose in the maternal vein and remaining in the fetal venous circulation over 5 hr 10 min and B). Appearance of  $^{14}\text{C}$ -L-glucose in the maternal vein and remaining in the fetal venous circulation over 5 hr and 10 min). Despite changes in D-glucose concentrations, the percentage of  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose remaining in the fetal vein and appearing in the maternal vein did not change over time. Data are expressed as mean  $\pm$  SEM, n = 5.



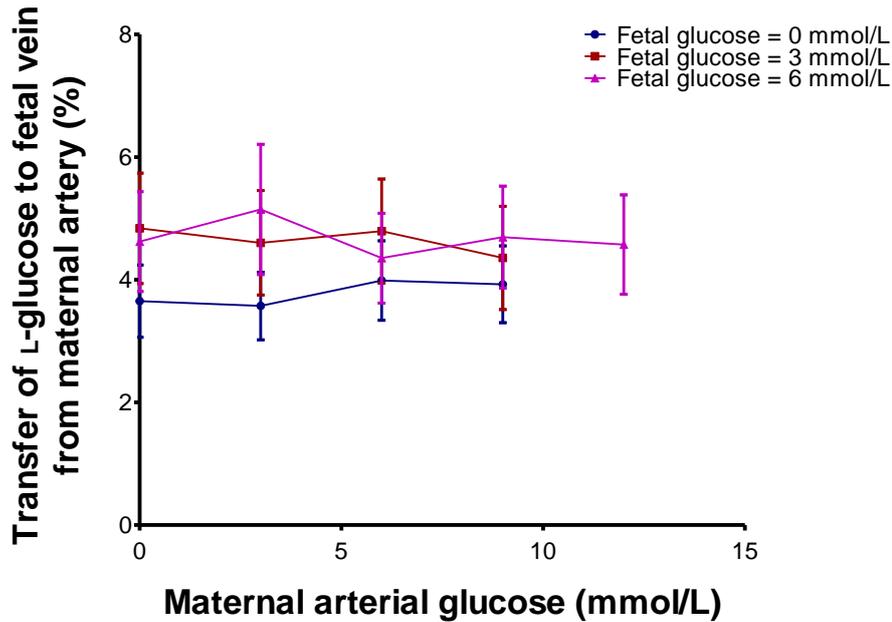
**Figure 3.11: Maternal to fetal transfer of creatinine and  $^{14}\text{C}$ -L-glucose.** The diffusion creatinine to the fetal vein from maternal artery was significantly higher than that  $^{14}\text{C}$ -L-glucose ( $P < 0.001$ ). The transfer of both creatinine and  $^{14}\text{C}$ -L-glucose did not change over time ( $P = 0.150$  and  $0.969$  respectively). Data are expressed as mean  $\pm$  SEM,  $n = 5$ .



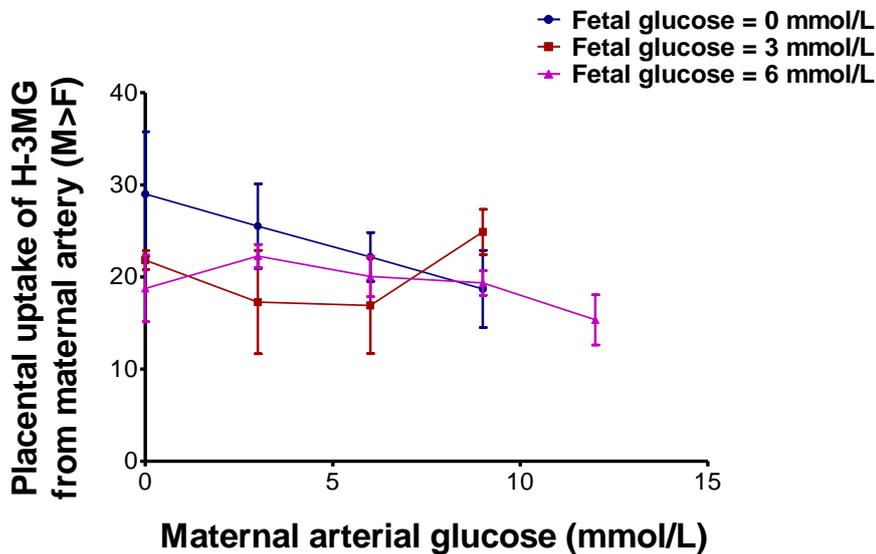
**Figure 3.12: D-glucose concentrations in the fetal and maternal vein of the tracer experiments.** A) Maternal side tracer experiments showing D-glucose appearing in the maternal and fetal vein, B) Fetal side tracer experiments showing D-glucose appearing in the maternal and fetal vein. Data are expressed as mean  $\pm$  SEM, n = 5.



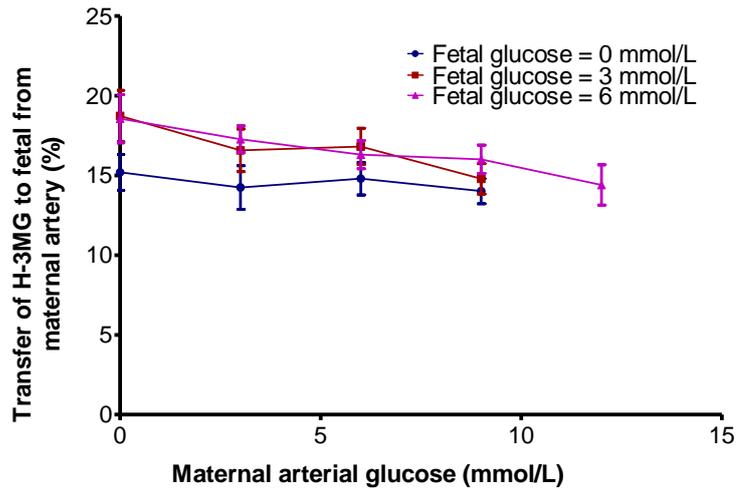
**Figure 3.13: Vein to vein ratios for D-glucose, <sup>3</sup>H-3-O-methyl-D-glucose and L-glucose.** A) Maternal venous to fetal venous ratios for D-glucose, <sup>3</sup>H-3-O-methyl-D-glucose and L-glucose in the maternal side tracer experiments, B) Fetal venous to maternal venous ratios for D-glucose, <sup>3</sup>H-3-O-methyl-D-glucose and L-glucose in the fetal side tracer experiments. The ratios are greater than 1 indicating that the placenta is acting as a barrier for transfer. Data are expressed as mean ± SEM, n = 5, \*\* indicates, P < 0.001 vs. both D-glucose and 3MG, n = 5.



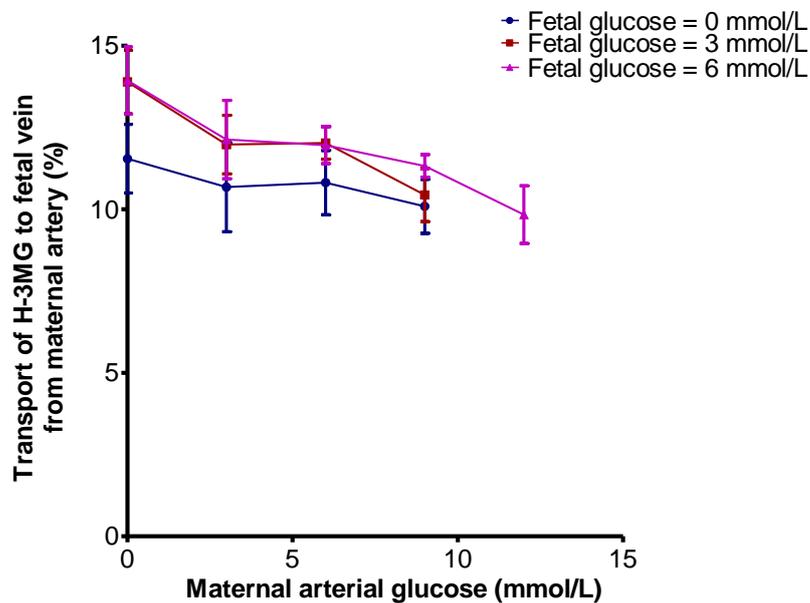
**Figure 3.14: Maternal tracer experiments  $^{14}\text{C}$ -L-glucose transfer to the fetal venous circulation from maternal artery.** Neither maternal nor fetal arterial D-glucose had an effect on  $^{14}\text{C}$ -L-glucose diffusion from the maternal arterial circulation to the fetal vein ( $P = 1.00$  and  $0.191$  respectively). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



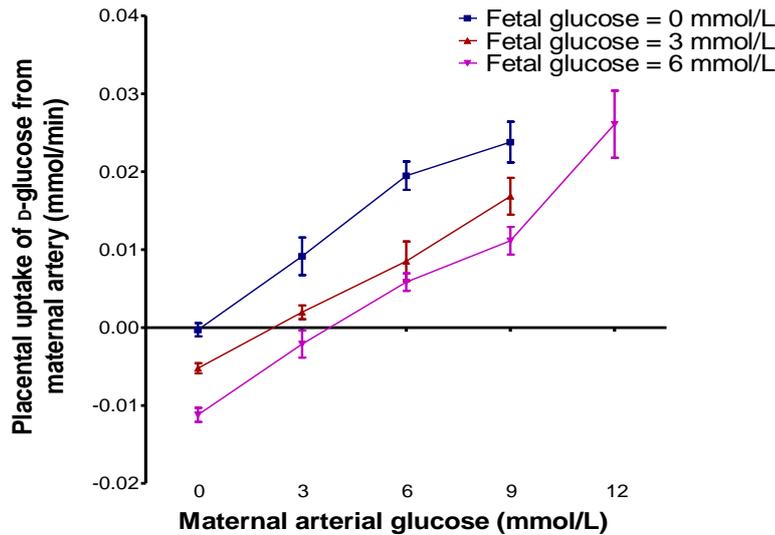
**Figure 3.15: Placental uptake of  $^3\text{H}$ -3-O-methyl-D-glucose from the maternal artery.** Neither maternal nor fetal arterial D-glucose had an effect on  $^3\text{H}$ -3-O-methyl-D-glucose uptake ( $P = 0.414$  and  $0.142$  respectively) and there was no interaction between fetal and maternal arterial D-glucose ( $P = 0.992$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



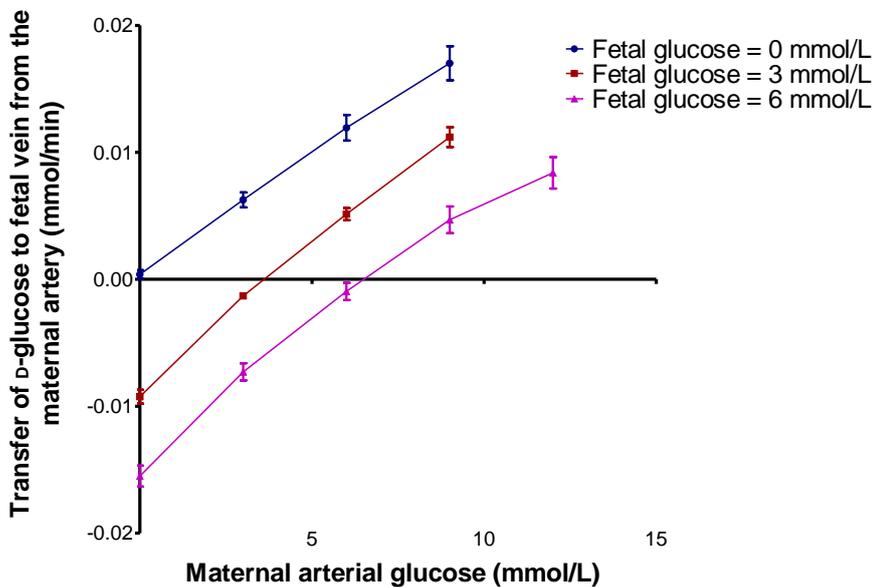
**Figure 3.16: Transfer of  $^3\text{H}$ -3-O-methyl-D-glucose from maternal artery circulation to the fetal vein.** The transfer of  $^3\text{H}$ -3-O-methyl-D-glucose to the fetal vein decreased with increasing maternal D-glucose concentration ( $P = 0.007$ ) and increased with increasing fetal D-glucose concentration ( $P = 0.032$ ). There was no interaction between maternal and fetal arterial D-glucose ( $P = 0.918$ ). Data are expressed as mean  $\pm$  SEM,  $n = 5$ .



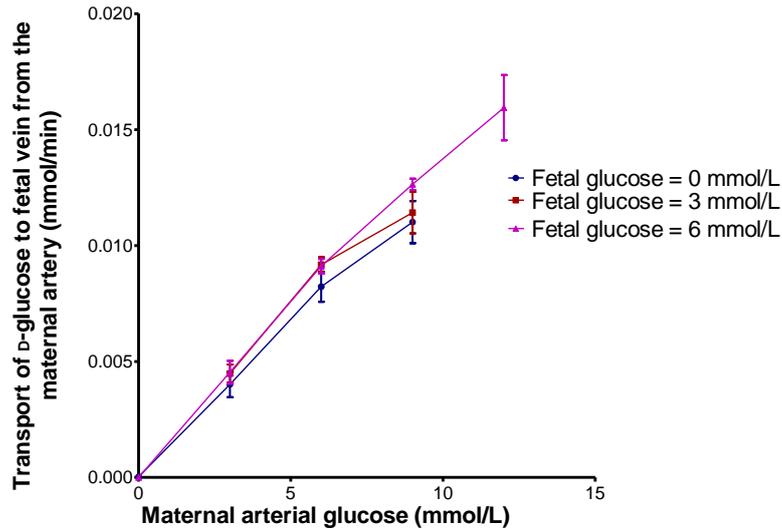
**Figure 3.17: Maternal to fetal transfer of  $^3\text{H}$ -3-O-methyl-D-glucose.** Transport of  $^3\text{H}$ -3-O-methyl-D-glucose to the fetal vein was decreased with increasing maternal D-glucose concentration ( $P = 0.015$ ) but was not affected by fetal arterial D-glucose concentration ( $P = 0.166$ ) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.982$ ). Data are expressed as mean  $\pm$  SEM,  $n = 5$ .



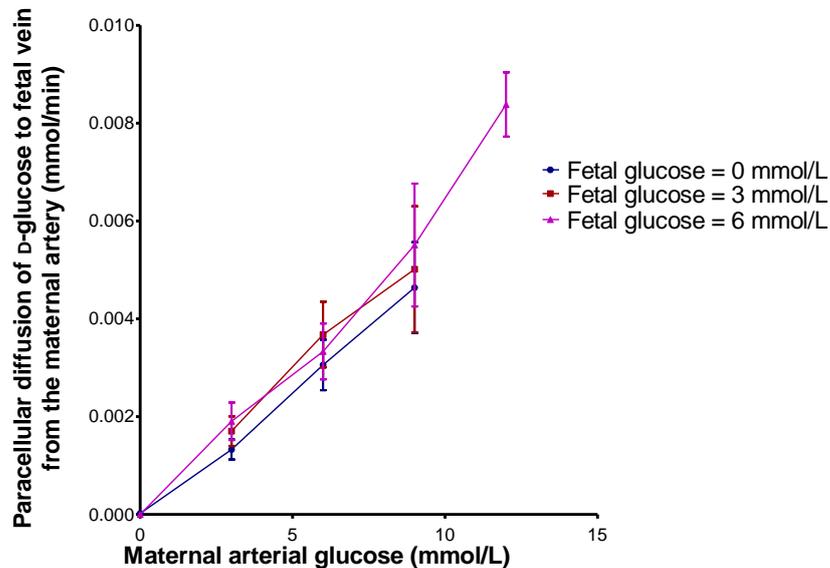
**Figure 3.18: Placental uptake of D-glucose from the maternal artery.** Glucose uptake from the maternal arterial circulation increased with increasing maternal glucose concentration ( $P < 0.001$ ) and decreased with increasing fetal arterial D-glucose concentrations ( $P < 0.001$ ) but there was no interaction between maternal and arterial fetal D-glucose ( $P = 0.825$ ). Data are expressed as mean  $\pm$  SEM,  $n = 5$ .



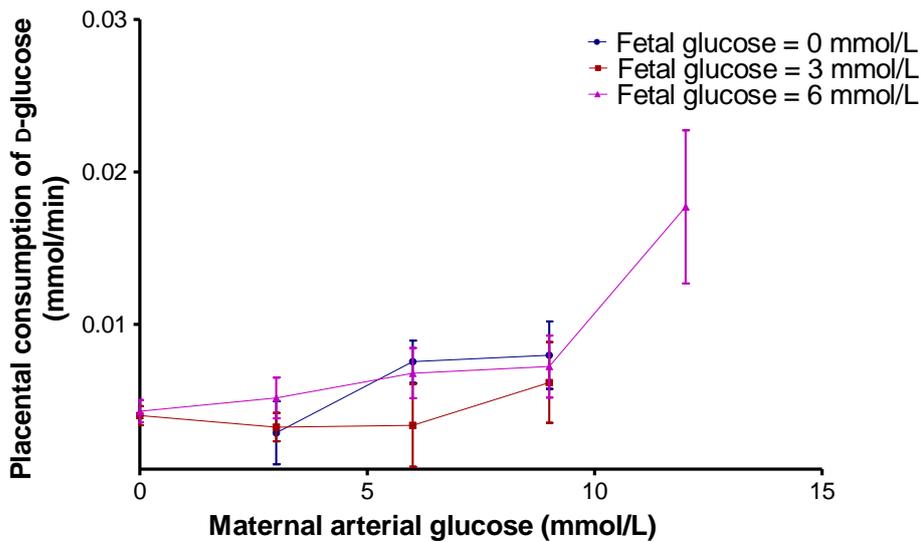
**Figure 3.19: Maternal to fetal transfer of D-glucose.** Transfer of D-glucose from the maternal arterial circulation to the fetal vein increased with increasing maternal arterial D-glucose concentration ( $P < 0.001$ ) and decreased with increasing fetal arterial of D-glucose concentration ( $P < 0.001$ ). There was no interaction between maternal and fetal arterial D-glucose concentration ( $P = 0.365$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



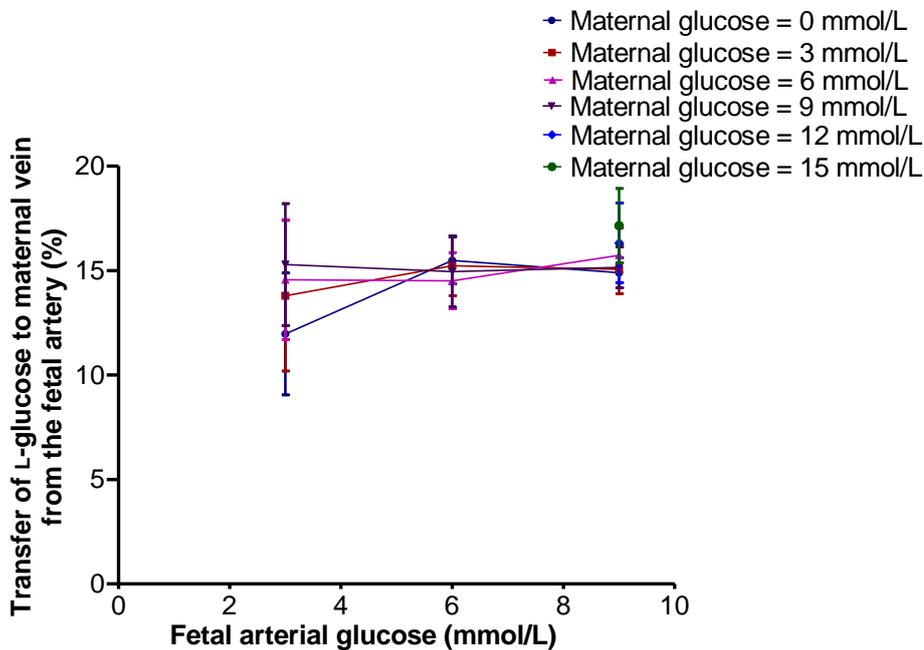
**Figure 3.20: Estimated unidirectional maternal to fetal transport of D-glucose.** Transport of D-glucose from the maternal arterial circulation to the fetal vein increased with increasing maternal D-glucose ( $P = 0.003$ ) but was not affected by fetal arterial D-glucose ( $P = 0.993$ ) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.340$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



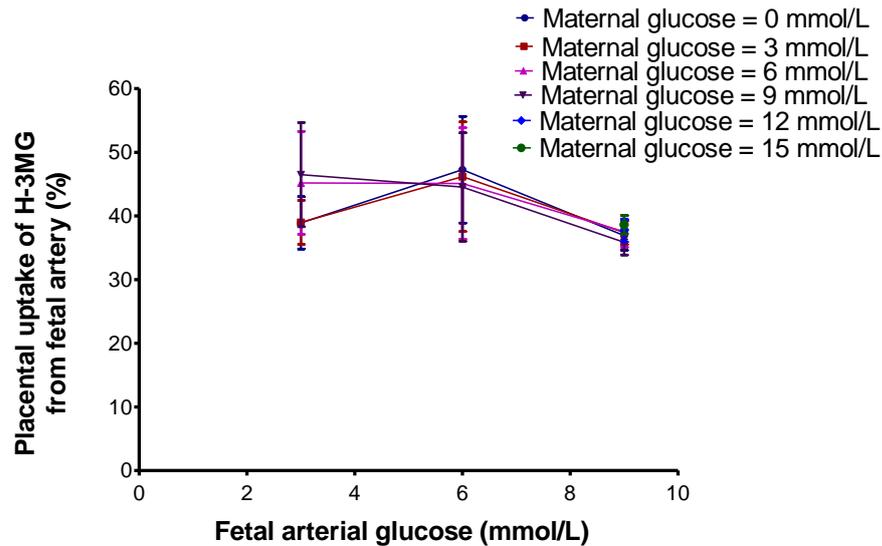
**Figure 3.21: Estimated maternal to fetal unidirectional paracellular diffusion of D-glucose.** Estimated paracellular diffusion of D-glucose from the maternal arterial circulation to the fetal vein increased with increasing maternal D-glucose ( $P = 0.048$ ) but was not affected by fetal arterial D-glucose ( $P = 0.991$ ) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 1.00$ ). Both D-glucose transport and paracellular diffusion are estimated based on  $^{14}\text{C}$ -L-glucose transfer to the fetal vein. Data are presented as mean  $\pm$  SEM,  $n = 5$ .



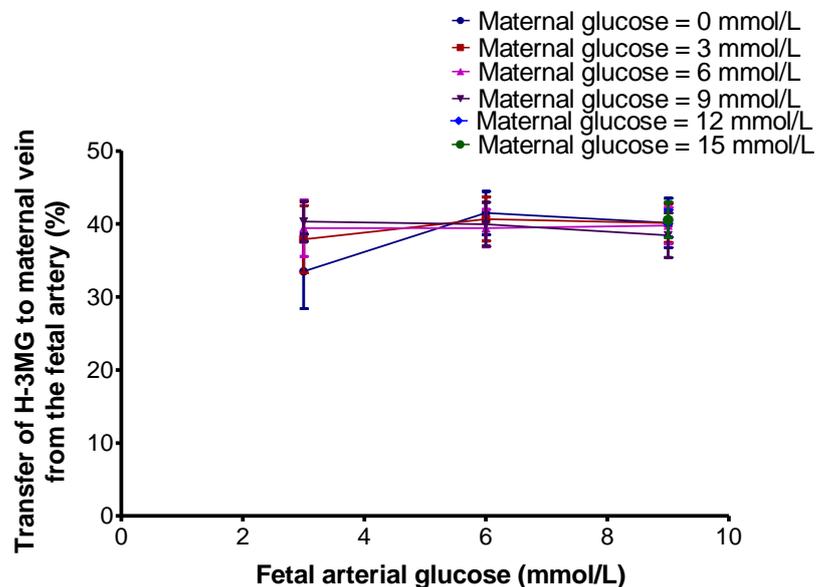
**Figure 3.22: Consumption of D-glucose by the placenta** in the maternal tracer experiments. Both maternal and fetal arterial D-glucose had no effect on placental D-glucose consumption ( $P = 0.052$  and  $0.441$  respectively). There was no interaction between maternal and fetal arterial D-glucose ( $P = 0.420$ ). Data are expressed as mean  $\pm$  SEM,  $n = 5$ .



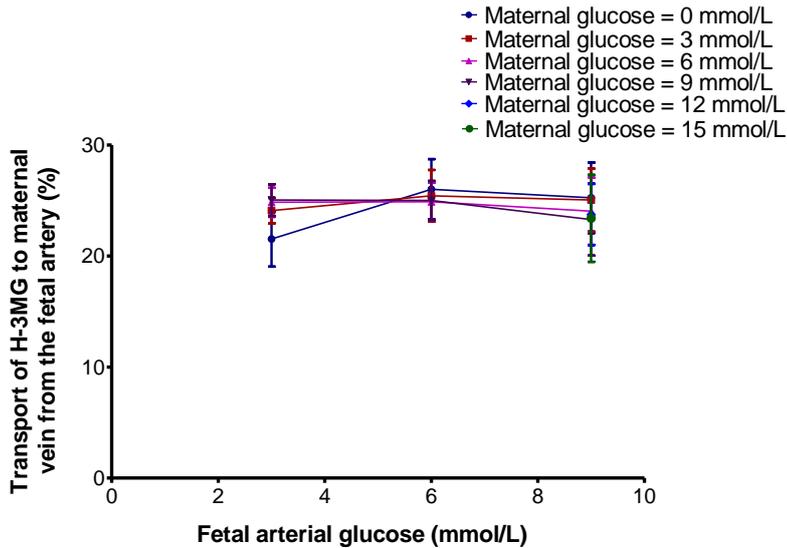
**Figure 3.23: Fetal to maternal paracellular diffusion of  $^{14}\text{C}$ -L-glucose.** Neither maternal nor fetal D-glucose had a significant effect on the transfer of  $^{14}\text{C}$ -L-glucose to the maternal vein ( $P = 0.987$  and  $0.782$  respectively). There was no significant interaction between maternal and fetal arterial D-glucose ( $P = 0.995$ ). Data are expressed as mean  $\pm$  SEM,  $n = 5$ .



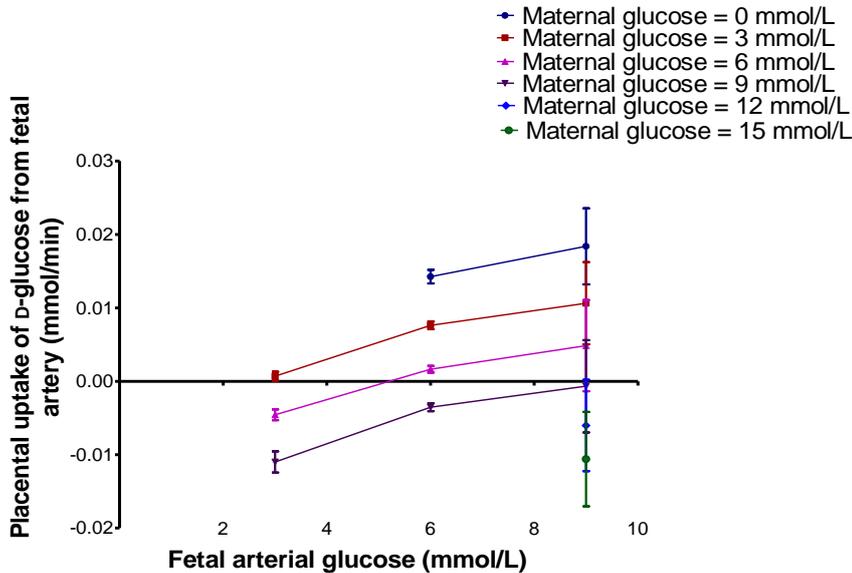
**Figure 3.24: Placental uptake of  $^3\text{H}$ -3-O-methyl-D-glucose from the fetal circulation.** Placental uptake of  $^3\text{H}$ -3-O-methyl-D-glucose from fetal arterial circulation. There was no significant effect of maternal or fetal D-glucose on placental  $^3\text{H}$ -3-O-methyl-D-glucose uptake from the fetal circulation ( $P = 0.999$  and  $0.375$  respectively) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 0.974$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



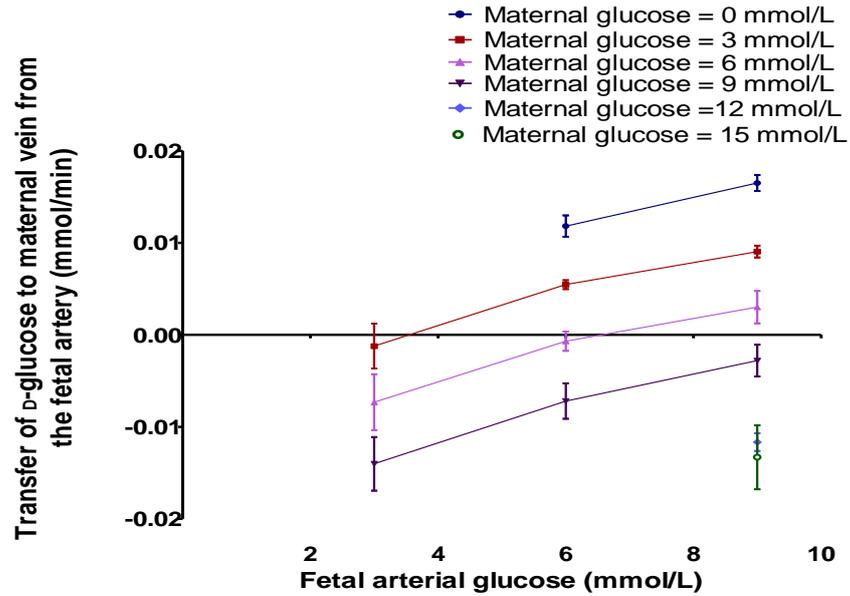
**Figure 3.25: Fetal to maternal  $^3\text{H}$ -3-O-methyl-D-glucose transfer.** There was no significant effect of either maternal or fetal D-glucose on  $^3\text{H}$ -3-O-methyl-D-glucose transfer to the maternal arterial circulation ( $P = 0.999$  and  $0.434$  respectively) nor was there any interaction between maternal and fetal arterial D-glucose ( $P = 0.991$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



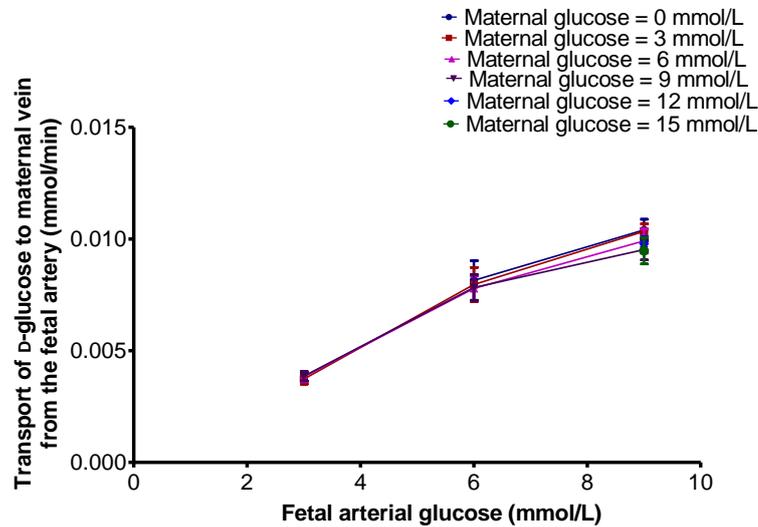
**Figure 3.26: Fetal to maternal transport of <sup>3</sup>H-3-O-methyl-D-glucose.** There was no effect of maternal or fetal arterial D-glucose concentration on the transport of <sup>3</sup>H-3-O-methyl-D-glucose to the maternal venous circulation (P = 0.994 and 0.540 respectively). There was no interaction between maternal and fetal D-glucose (P = 0.997). Data are presented as mean ± SEM, n = 5.



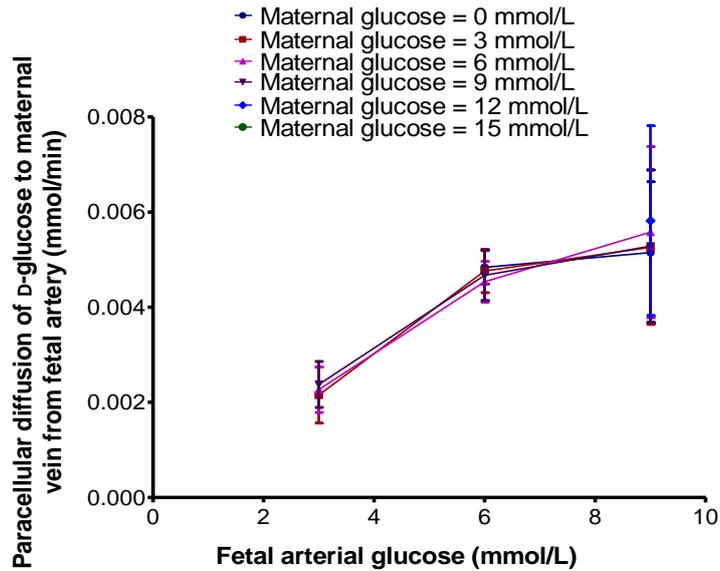
**Figure 3.27: Placental uptake of D-glucose from fetal arterial circulation.** Uptake of D-glucose increased with increasing fetal arterial D-glucose concentration (P < 0.001) and decreased with increasing maternal glucose concentration (P = 0.003). There was no interaction between maternal and fetal arterial D-glucose (P = 0.999). Data are presented as mean ± SEM, n = 5.



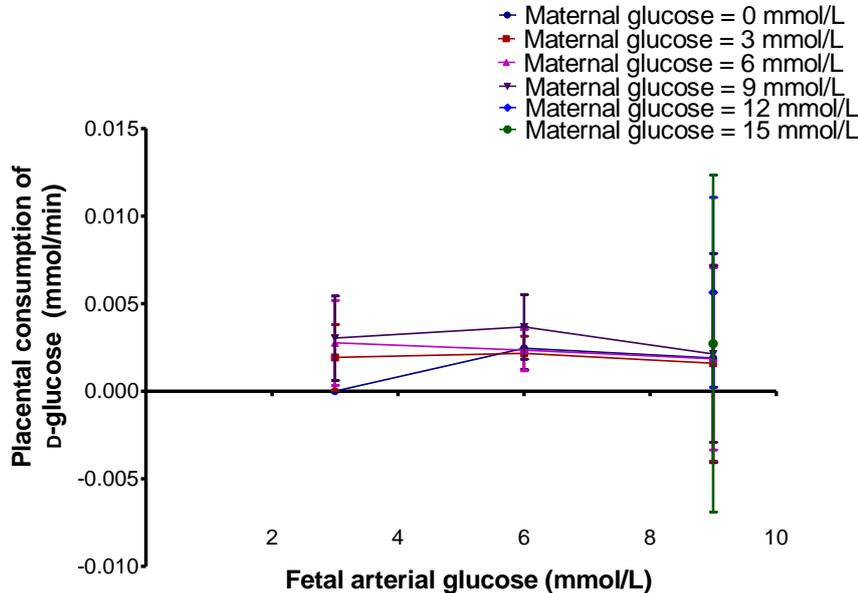
**Figure 3.28: Fetal to maternal placental transfer of D-glucose.** Transfer of D-glucose from fetal arterial circulation to maternal vein increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) and decreased with increasing maternal D-glucose concentration ( $P < 0.001$ ). There was no interaction between maternal and fetal arterial D-glucose ( $P = 1.00$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



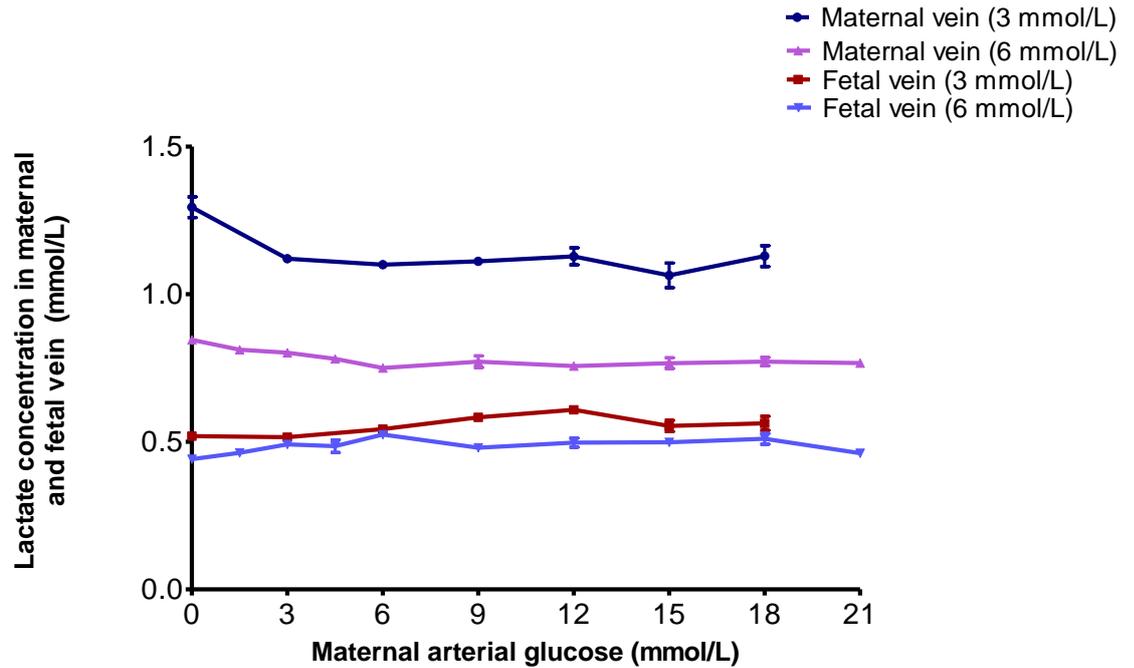
**Figure 3.29: Estimated fetal to maternal unidirectional transport of D-glucose.** Estimated unidirectional transport of D-glucose increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) but was not affected by maternal arterial D-glucose ( $P = 0.983$ ) and there was no interaction between maternal and fetal D-glucose ( $P = 0.997$ ). Estimated transport was determined based on the rate  $^3\text{H}$ -3-O-methyl-D-glucose and  $^{14}\text{C}$ -L-glucose transfer. Data are presented as mean  $\pm$  SEM,  $n = 5$ .



**Figure 3.30: Estimated fetal to maternal unidirectional paracellular diffusion of D-glucose.** Unidirectional paracellular diffusion of D-glucose increased with increasing fetal arterial D-glucose concentration ( $P < 0.001$ ) but was not affected by maternal arterial D-glucose ( $P = 0.976$ ) and there was no interaction between maternal and fetal D-glucose concentrations ( $P = 0.999$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



**Figure 3.31: Consumption of D-glucose by the placenta in the fetal tracer experiments.** There were no significant effects of maternal or fetal arterial D-glucose concentrations on placental D-glucose consumption ( $P = 0.986$  and  $0.739$  respectively) and there was no interaction between maternal and fetal arterial D-glucose ( $P = 1.00$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .



**Figure 3.32: Lactate release from glucose perfused placentas into the maternal and the fetal vein.** Concentrations of lactate appearing in the maternal vein were significantly higher than those in the fetal vein ( $P < 0.001$ ) in both the 3 mmol/L and 6 mmol/L fetal arterial glucose experiments. Concentrations of lactate appearing in the maternal or the fetal vein did not change over the experimental time course. Concentrations of lactate in the maternal vein of the 3 mmol/L fetal D-glucose experiments were significantly higher than those in the 6 mmol/L fetal arterial D-glucose experiments ( $P < 0.001$ ) but the concentrations in the fetal vein of the 3 mmol/L and 6 mmol/L fetal arterial glucose were not different ( $P = 0.382$ ). Data are presented as mean  $\pm$  SEM,  $n = 5$ .

### 3.4: Discussion

This study suggests that there is a high capacity for glucose transport across the human placenta and that glucose transporters are not the limiting factor for glucose transfer. However as the glucose gradient across the placenta is maintained regardless of whether glucose is infused into the maternal or the fetal circulation, it suggests that there are unknown factors that are rate-limiting for glucose transfer. In addition, a high capacity for glucose transfer via paracellular routes was observed, both by diffusion and convection (bulk flow of fluid through paracellular routes), which also contributes to placental glucose transfer. This section will discuss possible factors that may be limiting for glucose transfer and the implications they may have on net placental glucose transfer to the fetus and fetal growth.

#### 3.4.1: Glucose transfer across the human placenta

Glucose transfer across the placenta was observed by both paracellular and transcellular routes. Transfer by paracellular routes was lower than transfer by transcellular routes as indicated by the greater maternal to fetal gradient for D-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose compared to L-glucose and the difference in the calculated transfer rates. The similarities in the maternal to fetal gradients for the transfer of D-glucose and of  $^3\text{H}$ -3-O-methyl-D-glucose indicate that they are transferred with similar affinities in both directions. However the lack of inhibition of  $^3\text{H}$ -3-O-methyl-D-glucose transfer by D-glucose suggests that there is little or no saturation of placental glucose transporters. This is consistent with the observations that glucose transfer across the placenta is directly proportional to maternal glucose concentrations up to at least 20 mmol/L but inconsistent with suggestions that glucose transport across the BM is rate-limiting (Magnusson *et al.*, 2004). The suggestion that the BM was rate-limiting was based on observations that glucose uptake by BM vesicles was much lower than uptake by the MVM vesicles and the fact that the BM has one third the surface area of the MVM (Jansson *et al.*, 2002b; Osmond *et al.*, 2001; Hauguel *et al.*, 1986). It is possible that the MVM versus BM data do not provide a good representation of *in vivo* glucose transport across the placenta as it is not possible to show unidirectional transfer. Alternatively the vesicle data

may be representative but the rate of transfer is still so great that transport is not a rate-limiting step. Whatever the case,  $^3\text{H}$ -3-O-methyl-D-glucose transfer was not inhibited by D-glucose concentrations even at concentrations higher than those in poorly controlled GDM.

This demonstrates that there is a high capacity for glucose transfer across the human placenta which is not limited by transporters on either the MVM or the BM.

While the transporters on the placental membrane must provide a barrier to glucose transport as opposed to free diffusion, this study suggests that the syncytiotrophoblast MVM and BM transport barrier may be smaller than other barriers such as diffusive resistance or blood flow rate. If we think of glucose transporters as holes in a membrane then glucose molecules moving randomly may hit the membrane and bounce off or hit the hole and go through. In the experiments in this chapter adding maternal or fetal glucose is like blocking some of these holes, but however many holes were blocked glucose transport was not inhibited. For this reason it was concluded that it is not the transport of glucose through the glucose transporters which was limiting to glucose transfer but the diffusion across the placental tissue once the glucose has been taken up by the placenta.

As protein synthesis is a costly process it is not clear why the placenta would make so many transporter proteins that they are not saturable even at concentrations twice the physiological concentrations. As glucose is a principal substrate for energy for both the placenta and the fetus, it is possible that having as many GLUT1 transporters allows the placenta to take up as much glucose from the maternal circulation as possible such that some of this glucose is stored and can be readily available for the fetus during starvation thereby limiting the chances of starving the growing fetus.

### **3.4.2: What is rate-limiting for placental glucose transfer?**

If transport is not rate-limiting for glucose transfer, what is? If there were no effective resistance to glucose transfer then the system would reach equilibrium and concentration in the fetal vein would be the same as in the maternal vein.

However when  $^{14}\text{C}$ -L-glucose, D-glucose and  $^3\text{H}$ -3-O-methyl-D-glucose were perfused into the maternal circulation or the fetal circulation the concentrations in venous outflow of the donor circulation were greater than in the recipient venous outflow. This indicates that there is resistance to glucose transfer across the placenta.

Resistance via the paracellular route, as indicated by L-glucose transfer, was greater than via the transcellular route, as indicated by the maternal to fetal ratios for 3MG. Resistance to glucose transfer could occur for several reasons. Simple diffusive resistance; the glucose molecules are not moving in a set direction but effectively randomly. Each glucose molecule is on a 'random walk'; some of the time it will go in the direction needed to cross the placenta and other times not. This increases the time that it takes to cross the distance between the maternal and fetal circulations. Another limiting factor may be the rate at which glucose can diffuse through the fetal capillary endothelial junctions. Small water soluble molecules should be able to diffuse through endothelial junctions relatively rapidly but this is difficult to measure. Studies of amino acid transfer suggest that it occurs rapidly (Jansson *et al.*, 1993). A further point may be in the mixing of the fluids at tissue interfaces, both at the MVM and fetal capillary endothelium. At these interfaces you may get unstirred layers of fluid which act to limit the rate of diffusion.

Glucose metabolism will also act to maintain the gradient although this cannot be the whole story as glucose consumption was seen to remain constant as glucose levels rose. If metabolism were the rate-limiting step then as glucose concentration rose the gradient would become progressively smaller and this was not what was observed.

If the transfer of glucose is not transporter-limited as the data presented in this study suggest, this raises the question as to whether glucose transfer may also be flow-limited. A previous study has suggested that placental glucose transfer is flow limited and future research should be focused in this direction (Sengers *et al.*, 2010).

Placental blood flow through the maternal and fetal circulations depends on arterial pressure and the resistance to blood flow through the respective circulations. Poor placental villous development may increase placental vascular resistance resulting in absent or reversed end diastolic flow in the placenta. This could happen if fetal cardiac output cannot sustain the pressures needed to push blood through the placenta throughout the cardiac cycle. Absent or reversed end diastolic flow is associated with severe IUGR and if glucose transfer is flow-limited then impaired glucose transfer would be a significant contributor (Illsley, 1987).

It has been suggested that poor cytotrophoblast invasion and failure to transform the uterine spiral arteries may decrease maternal-placental blood supply although recent work suggests that this is not the case (Krebs *et al.*, 1996; Macara *et al.*, 1996). Further studies are required to investigate this further and determine whether placental glucose transfer correlates with blood flow in pathological pregnancies.

Factors affecting the structure or morphology of placental compartments will affect glucose transfer and thus fetal growth. For example there is evidence that high altitude which is characterized by hypoxia alters placental structure and size and there is also evidence that glucose transfer is reduced and is related to fetal growth at high altitude (DiFederico *et al.*, 1999; Burton *et al.*, 2009). However there is no evidence linking altered placenta structure and morphology to altered glucose transfer. Further studies should therefore aim to investigate whether altered placental structure and morphology may be associated with altered placental glucose transfer.

### **3.4.3: Paracellular diffusion of glucose**

A high capacity for paracellular diffusion of glucose across the human placenta was observed in this study. Although the anatomical nature of the paracellular route across the human placental syncytiotrophoblast has yet to be clearly defined these data provide further physiological evidence for its existence. Paracellular transfer can occur either by diffusion, where there is simple diffusion of solute through the paracellular route, or convection where there is bulk flow of fluid through the paracellular route driven by a pressure difference.

Paracellular diffusion of L-glucose was observed in both directions but was greater in the fetal to maternal direction. This difference is likely to be due to conductive transfer of fluid and solutes from the fetal to maternal circulations due to higher pressure in the fetal capillaries of the perfused placenta. *In vivo* it is likely that fetal capillary pressure is also higher than in the intervillous space as otherwise the villous capillaries would collapse. However as these pressures have not been determined experimentally the extent to which this will drive conductive transfer *in vivo* is not clear.

As maternal glucose concentrations are higher than fetal glucose concentrations diffusive transfer via the paracellular route would be expected to result in net transfer from the maternal to fetal circulation down the concentration gradient. Apart from concentration, diffusive transfer may also be affected by maternal and fetal blood flow rates as they affect the concentration gradient. Another factor which may be important is placental size, as larger placentas may have a greater number of paracellular routes.

In pathological placentas where surface area and size is altered, paracellular diffusion may influence the total transfer of glucose across the placenta and therefore glucose gradient. Indeed placental L-glucose transfer has been shown to be reduced in placentas from GDM mothers suggesting that paracellular diffusion may be regulated to protect the fetus (Zamudio *et al.*, 2010; Zamudio, 2003)

The extent to which paracellular diffusion contributes to net placental glucose transfer remains unclear. Diffusive transfer is likely to lead to net transfer to the fetus while conductive transfer is likely to mediate net transfer out of the fetus and the balance between the two is not known. Further studies are required to investigate this further. It is also not clear as to what the paracellular routes for transferring glucose are, but they could be the fibrin-containing fibrinoid-filled denudations or the trans-trophoblastic channels which have been shown to be present in the human placenta (Osmond *et al.*, 2001). Future studies should determine whether these paracellular routes are responsible for glucose transfer and whether they are subject to regulation in different pathological pregnancies.

#### 3.4.4: Placental consumption and glucose transfer

Glucose consumption by the placenta in this study was  $0.05 \pm 0.002$   $\mu\text{mol}/\text{min}/\text{g}$  which is similar to the values reported in a previous study using villous tissue (Brownbill *et al.*, 2000;Kertschanska & Kosanke, 1994). However, in this study placental glucose consumption was not related to either maternal or fetal glucose concentrations. This is in contrast to a study which showed a direct relationship between maternal glucose concentration and placental glucose consumption (Magnusson *et al.*, 2004). However consistent with the latter study, in the studies in this chapter lactate release was not related to maternal and fetal glucose arterial concentrations (Osmond *et al.*, 2000). The reasons for the discrepancies in the placental consumption between the latter study and the studies in this chapter are not clear but may be related to experimental conditions such as the use of placentas from gestational diabetic mothers in the latter study.

Placental glucose metabolism occurred at a constant rate in these experiments, it is not clear if this reflects an inherent capacity for glucose metabolism or whether placental glucose metabolism simply reflects placental demand which would have been relatively constant in the perfusion system. It would be interesting to alter placental metabolism, possibly using hormones such as insulin or adding substrates which require active transport, to see if this increases glucose consumption. Alternatively, the constant rate of lactate release may be because placental lactate production is not only dependent on glucose but may be dependent on other factors such as amino acids and tissue aerobic conditions. Consistent with this suggestion is the observation that in IUGR, placental glucose consumption is not altered although lactate production is reduced in parallel with reduced glycolytic enzymes (Osmond *et al.*, 2000).

Neurons incubated under hypoglycaemic conditions exhibit reduced glucose catabolism to lactate; instead, the oxidation of amino acids such as glutamine and glutamate becomes enhanced to produce lactate and pyruvate required for energy production (Magnusson *et al.*, 2004). This occurs through pyruvate recycling. As the data to be discussed in Chapter 4 suggest the presence of pyruvate recycling from amino acids in the human placenta, it is possible that

lactate is also produced from amino acids. This may explain the reduced activity for phosphofructokinase observed in IUGR placentas in a previous study possibly due to a mechanism preventing the committed step of glucose catabolism to ATP (Amaral *et al.*, 2011). This would thus preserve glucose required by the fetus.

Placental glucose consumption and lactate production may be regulated differently in different conditions. For example in contrast to IUGR placentas, placental glucose consumption is reduced in parallel to lactate production in placentas from gestational diabetic mothers (Magnusson *et al.*, 2004). Evidence for hormonal regulation of lactate production comes from studies using ovine placentas where growth hormone and IGF-I have been shown to alter placental lactate production (Osmond *et al.*, 2000). In addition, in human placentas of GDM mothers and macrosomic babies, maternal and fetal plasma levels of a range of hormones are elevated (Liu *et al.*, 1994; Harding *et al.*, 1994). It is not clear how hormonal levels may affect overall glucose transfer to the fetus and further studies are required to determine whether placental glucose consumption and metabolism is subject to hormonal regulation.

In contrast to the ovine placenta, this study has demonstrated that lactate is predominantly transferred to the maternal circulation in the human placenta (Grissa *et al.*, 2010). This is consistent with previous studies some of which have shown that lactate is transferred from the fetal circulation to the maternal circulation (Carter *et al.*, 1993).

The direction of lactate flux will be determined by the localisation and characteristics of monocarboxylate transporters on the MVM and BM of the placental syncytiotrophoblast (Piquard *et al.*, 1990; Inuyama *et al.*, 2002). These transporters also transport pyruvate and branched chain keto acids and these findings may suggest the likely direction of transport of these substances from the human placenta.

As placentally derived lactate is primarily transported to the mother it is unlikely to be a major energy substrate for the human fetus. By undertaking anaerobic glycolysis but not aerobic metabolism via the Krebs's cycle, the placenta may

be minimising its O<sub>2</sub> consumption to maximise transfer to the fetus. This would be consistent with the suggestion that placentas at high altitude shift their glucose metabolism away from aerobic metabolism to preserve O<sub>2</sub> supply to the fetus (Settle *et al.*, 2006; Inuyama *et al.*, 2002).

Lactate production and release may be stimulated by poor tissue perfusion conditions. In this case, these data suggest that the integrity of the placental tissue was not compromised during perfusion. It must be remembered that the placental perfusion set-up exposes placental tissue to supra-physiological oxygen concentrations which may suppress lactate production. In future experiments it would be interesting to determine the effect of O<sub>2</sub> concentration on lactate production.

### **3.4.5: Implications**

The finding that placental glucose uptake and transfer are proportional to maternal and fetal arterial concentrations has implications for our understanding of fetal growth in both IUGR and macrosomia. Although IUGR is associated with fetal hypoglycaemia neither placental glucose transporters nor placental glucose consumption are reduced in IUGR (Zamudio *et al.*, 2010). Studies in this chapter suggest there is a high capacity for glucose transfer across the placenta and that glucose transporter levels would have to fall significantly to reduce glucose transfer. This is consistent with studies which show glucose transporter levels are not altered in placentas from IUGR pregnancies (Jansson *et al.*, 2002b; Magnusson *et al.*, 2004). If glucose transporters are not the causative factor in IUGR then the role of other factors needs to be considered. Factors such as blood flow and placental diffusive barrier may regulate glucose transfer to the fetus and their impairment may result in IUGR.

As there is no saturation of glucose transport high maternal glucose levels in diabetes will drive excess transfer of glucose to the fetus. This study suggests that given the high capacity for glucose transport any attempts to inhibit glucose transporters in diabetic pregnancies are unlikely to be successful. For this reason other factors may be altered to reduce glucose transfer to the fetus. Interestingly paracellular diffusion is reported to be reduced in placentas from

GDM mothers suggesting that some form of adaptive regulation may be occurring (Jansson *et al.*, 2002b).

### **3.4.6: Summary**

This study has demonstrated that glucose transfer across the human placenta is not limited by transport across the placental membranes. This suggests that other factors are responsible for determining glucose transfer across the placenta. These factors may include placental diffusive barrier, placental blood flow, placental glucose consumption and paracellular diffusion. Future studies should therefore investigate how these factors may be affected in pathological pregnancies and how these factors could be altered to improve glucose transfer to the fetus.

As well as highlighting important factors that may determine glucose transfer to the fetus, it is also possible that amino acid metabolism may be implicated in determining glucose availability. Like glucose, amino acids are also important substrates for fetal growth. The next chapter will therefore investigate factors that determine amino acid transfer and how these may be related to placental glucose metabolism.

## **Chapter 4**

### **Amino acid transfer and metabolism**



## 4.1: Background

The transfer of amino acids across the placenta involves both membrane transport and metabolism. While the mechanisms of amino acid transport via transporters on the MVM and the BM of the human placental syncytiotrophoblast have been described, the extent to which amino acid metabolism occurs in the human placenta is not well understood (Osmond *et al.*, 2000). Placental metabolism may affect the quantities and composition of the amino acids supplied to the fetus, and may affect fetal growth.

The majority of studies on placental amino acid metabolism have been carried out on animals, particularly sheep. These studies have demonstrated that there are inter-conversions of amino acids including glutamate to glutamine and serine to glycine in the placenta (Cleal & Lewis, 2008). It has further been demonstrated that nitrogen from essential amino acids such as leucine is used for the synthesis of glutamine and their keto acids are transferred to the fetus (Cetin *et al.*, 1991; Vaughn *et al.*, 1995). However much less is known about amino acid metabolism in the human placenta. For example, in contrast to the sheep placenta, the human placenta has low levels of SHMT responsible for glycine and serine metabolism (Loy *et al.*, 1990). However, as glycine is a conditionally essential amino acid and very low levels of glycine are transferred to the fetus, it is not clear from where the human fetus derives its glycine (Lewis *et al.*, 2005). It has been speculated that the fetus may derive its glycine from the catabolism of serine within its tissue (Paolini *et al.*, 2001; Cetin *et al.*, 1995; Jackson, 1991).

A previous study has also shown that glutamate is metabolised in the human placenta, but it did not conclusively demonstrate what it was metabolised into (Lewis *et al.*, 2005). Other studies using placental mitochondria or cultured trophoblast cells suggest that glutamate catabolism does occur in the human placenta (Schneider *et al.*, 1979). Although glutamate is central to amino acid nitrogen and carbon metabolism the fate of nitrogen and carbon from glutamate in these studies is not clear. It is important to understand glutamate metabolism as in most tissues, glutamate is involved in the synthesis of non-essential amino acids such as glutamine and alanine.

Non-essential nitrogen has been shown to be rate-limiting for growth (Broeder *et al.*, 1994). The nitrogen from leucine transamination with glutamate has also been shown to be important for the provision of this non-essential nitrogen via the synthesis of alanine and glutamine (Snyderman *et al.*, 1962; Jackson *et al.*, 1990). Indeed, in human pregnant mothers, leucine oxidation is reduced in response to maternal and fetal protein demands and its reamination is proportional to glutamine flux (Darmaun & Dechelotte, 1991). This suggests that the nitrogen from leucine is used for glutamine synthesis. Although studies have shown that maternally-derived leucine is transferred to the fetus, it is not clear whether some of this leucine is metabolised to other amino acids by the human placenta.

As it has been demonstrated that there are species differences in the handling of amino acids, it cannot be assumed that the amino acid metabolism observed in animals occurs in the human placenta (Kalhan & Parimi, 2006). Further studies of the human placenta are required to provide a clear picture of how amino acid metabolism contributes to amino acid transfer across it. Placental amino acid consumption could prevent amino acids from reaching the fetus, while amino acid inter-conversion could alter the composition of amino acids transferred to the fetus. Knowledge of the extent to which placental metabolism contributes to amino acid transfer may help to understand processes underlying poor fetal growth. This may be of clinical benefit as future intervention may aim to enhance the supply and metabolism of amino acids when poor fetal growth is suspected.

The aim of the studies reported in this chapter was therefore to investigate the nature of glutamate and leucine metabolism occurring in the normal term human placenta and whether metabolites of glutamate are transferred across the placenta. This study also aimed to investigate whether nitrogen from glutamate and leucine is used for the synthesis of non-essential amino acids such as glutamine by transamination and whether glutamate carbon skeleton is used for the synthesis of glutamine and other non-essential amino acids. In addition, this study investigated the extent to which BCKAs and pyruvate are released from the placenta.

## 4.2: Methods

To study placental amino acid metabolism, placentas were collected from normal term pregnancies immediately after delivery, with ethical approval from the South and West Hampshire Local Research Ethics Committee. They were perfused using the isolated placental cotyledon perfusion methodology as described in Chapter 2. Samples were collected from the fetal and maternal venous outflows and placental tissue homogenates and kept on ice or stored at -80°C until analysis. The concentrations of individual amino acids were determined by HPLC and their isotopic abundance was determined by GC-MS as described in Chapter 2. The enrichments in each amino acid isotopomer were calculated by correcting for the natural abundance determined in the baseline sample. The concentrations of isotopically labelled component of each amino acid were calculated as a product of total concentration and enrichment.

### 4.2.1: Placental characteristics

All placentas used for the isotopic enrichment studies had a fetal arterial flow rate recovery of 95% or greater. Placentas that had a fetal arterial flow rate recovery of less than 95% were perfused through both circulations for 15 minutes with EBB containing 1.8 mmol/L creatinine and were used for the determination of initial placental amino acid concentrations. At the end of the experiments the perfused area of placental cotyledon, identified by the white colour after the red cells are washed out, was obtained by trimming off the non-perfused tissue, blotted and weighed. Cotyledon weights together with the fetal venous flow rates at the end of perfusions are shown in Table 4.1.

### 4.2.2: Fetal and maternal <sup>15</sup>N-glutamate arterial perfusions.

Placental amino acid perfusions were carried out using a dually perfused isolated placental cotyledon methodology as described in Chapter 2. The metabolic fate of the nitrogen from glutamate was studied by continuously perfusing 200 µmol/L of 98% enriched <sup>15</sup>N-glutamate into an isolated human placental cotyledon for 5 hrs. Two sets of experiments were carried out; in the first set <sup>15</sup>N-glutamate was infused into the fetal arterial circulation and in the second <sup>15</sup>N-glutamate was infused into the maternal arterial circulation. During

perfusion, a bolus of unlabelled serine was injected into the fetal circulation, upstream of the pump, once every hour for 5 hrs. Samples were collected from the fetal and maternal venous outflow at 1 min 20 s, 2 min, 55 min and 58 min following each serine bolus.

**Table 4.1: Perfused cotyledon weights and fetal venous flow rate** before the start of the substrate perfusions

Experiment	Flow rates (ml/min)	Cotyledon weight (g)	Number of experiments
<sup>15</sup> N-glutamate fetal arterial perfusions	5.85 (0.10)	38.75 (10.06)	4
<sup>15</sup> N-glutamate maternal arterial perfusions	5.95 (0.05)	33.98 (8.33)	4
<sup>15</sup> N-leucine maternal arterial perfusions	5.92 (0.05)	39.29 (7.12)	5
U- <sup>13</sup> C-glutamate fetal arterial perfusions	5.92 (0.05)	27.55 (6.00)	5

*Data are expressed as mean (SEM).*

Placental tissue from the perfused area was obtained and homogenised as explained in Chapter 2. The incorporation of <sup>15</sup>N from glutamate into other amino acids in the maternal and fetal samples and in the placental tissue homogenates was determined by measuring the amino acid concentrations and isotopomer enrichment in each sample by HPLC and GC-MS respectively as described in Chapter 2. Glutamate was perfused at 200 µmol/L, which is around twice the average physiological umbilical cord arterial concentration taken from the literature (95 µmol/L) to ensure rapid uptake of labelled glutamate into the placenta (Lewis *et al.*, 2005). The serine bolus was infused into the fetal inflow to stimulate transport of amino acids by exchange across the BM as previous experiments suggested that key products of glutamate metabolism, such as glutamine, were only released from the placenta by exchange transport (Economides *et al.*, 1989; Cetin *et al.*, 1988; Ronzoni *et al.*, 1999; Young & Prenton, 1969; Hayashi *et al.*, 1978; Evans *et al.*, 2003).

#### 4.2.3: Maternal <sup>15</sup>N-leucine arterial perfusion

To investigate the fate of <sup>15</sup>N from leucine, 146 µmol/L of 98 % labelled <sup>15</sup>N-leucine was continuously infused into the maternal arterial circulation of an

isolated human placental cotyledon for 5 hrs. During perfusion, a bolus of unlabelled serine was injected into the fetal circulation upstream of the pump, once every hour for 5 hrs. Samples were collected from the fetal and maternal venous outflow at 1 min 20 s, 2 min, 55 min and 58 min following each serine bolus. Incorporation of  $^{15}\text{N}$  from leucine into other amino acids in the maternal vein, fetal vein and the placental samples was determined by measuring the amino acid concentrations and isotopomer enrichments in each sample by HPLC and GC-MS respectively as described in Chapter 2. Leucine was perfused at  $146\ \mu\text{mol/L}$  which is twice the average physiological umbilical cord arterial concentration reported in the literature ( $73\ \mu\text{mol/L}$ ) to ensure that it is rapidly taken up by the placenta (Cleal & Lewis, 2008). The serine bolus was infused into the fetal arterial inflow to stimulate exchange transport of amino acids across the BM as previous experiments suggested that key products of leucine metabolism such as glutamine were only released from the placenta by exchange (Economides *et al.*, 1989; Cetin *et al.*, 1988; Ronzoni *et al.*, 1999; Young & Prenton, 1969; Hayashi *et al.*, 1978; Evans *et al.*, 2003).

The concentrations of BCKAs and pyruvate in the maternal and fetal venous samples and in the homogenised placental tissue samples were also determined in this study using HPLC as described in Chapter 2.

#### **4.2.4: U- $^{13}\text{C}$ -glutamate perfusions**

To study the metabolism of the glutamate carbon skeleton,  $100\ \mu\text{mol/L}$  of 99% labelled U- $^{13}\text{C}$ -glutamate was continuously infused into the fetal arterial circulation of an isolated human placental cotyledon for 5 hrs. Samples were collected from the fetal and maternal vein at 55 min and 58 min every hour. At the end of the experiment placental tissue was homogenised as described in Chapter 2. Incorporation of  $^{13}\text{C}$  from U- $^{13}\text{C}$ -glutamate into other amino acids was determined by measuring amino acid concentrations and isotopomer enrichments in each maternal and fetal sample and the homogenised placental tissue by HPLC and GC-MS respectively as described in Chapter 2. As a serine bolus had little or no effect on the transfer of glutamate) metabolites across the placenta in the  $^{15}\text{N}$  studies (possibly due to competitive inhibition of its transfer or the transfer of substrate it exchanges for, serine bolus infusions were omitted

in this study.  $^{13}\text{C}$  was infused in the fetal arterial circulation as the previous studies in sheep have shown glutamate-glutamine cycling between the placenta and the fetal liver and we have shown in the  $^{15}\text{N}$  studies that glutamate from the fetal arterial circulation is taken up and metabolised by the human placenta (Cleal & Lewis, 2008).

#### 4.2.5: Statistics

Data were analysed using SPSS (SPSS Inc, Illinois, USA). Levene and Kurtosis tests were first used to check if there was equal variance and normal distribution of the groups being tested. One Way ANOVA was used to compare differences between groups that were normally distributed. A related samples test, the Wilcoxon signed rank test (non-parametric), was used for the comparison of samples that were not normally distributed. Finally, a paired T test (parametric) was used to compare data that were normally distributed. Results from both Wilcoxon and paired T test were adjusted for multiple comparisons using a Bonferroni adjusted value ( $0.05/\text{number of tests}$ ), which was used as a new cut-off point for significance (Vaughn *et al.*, 1995). Amino acid comparisons between the maternal and the fetal vein concentrations (placental estimated concentration values not included), were carried out using either a paired sample T test or Wilcoxon signed rank test, both with a Bonferroni adjustment. Initially paired T test and Wilcoxon signed rank test were considered for testing the differences between maternal vein, fetal vein and placental variables but as three groups were being compared, the Paired T test or the related sample test has no posthoc tests so it was decided to use One Way ANOVA, followed by posthoc tests. When the ANOVA test was significant at a P value less than 0.05, a Bonferroni posthoc test was used for all the samples that had equal variance, otherwise a Dunnett's tests was used and a P value of less than 0.05 was considered significant. The total concentrations of the  $^{15}\text{N}$  labelled amino acids in the three sets of the  $^{15}\text{N}$  experiments were compared using One Way ANOVA followed by a posthoc test. All the amino acid enrichment data and the keto acid concentrations data were analysed by One Way ANOVA followed by either a Bonferroni or a Dunnett's test. All data were presented as mean  $\pm$  SEM.

### 4.2.6: Calculations

$^{15}\text{N}$  and  $^{13}\text{C}$  enrichments were calculated as described in the methods chapter. Placental uptake and transfer of the infusates and the substrates were calculated as described below. The side on which the infusate was perfused is treated as a donor circulation and the side to which the infusate was transferred is treated as the recipient circulation in these calculations.

- Rate of infusion (mol/min) = [Donor arterial infusion] (mol/L) \* flow rate (L/min)
- Rate of infusate remaining in the donor circulation (mol/min) = [Donor vein] (mol/L) \* flow rate (L/min)
- Rate of infusate appearance in recipient circulation ( $\mu\text{mol}/\text{min}$ ) = [Recipient vein] (mol/L) \* flow rate (L/min)
- Rate of accumulation in placental tissue (mol/min) = [Placental tissue] (mol/L) \* placental intracellular volume (29%)/300 (assuming steady state at 300 minutes) (L/min)
- Rate of placental uptake (mol/min) = Rate of infusion (mol/min) - Infusate remaining in the donor circulation (mol/min)
- Rate of transfer of infusate to the metabolic pool (mol/min) = Rate of placental uptake (mol/min) – (Rate of infusate transfer to recipient circulation (mol/min) + Rate of infusate accumulation in placental tissue (mol/min))
- Rate of  $^{15}\text{N}$  labelled metabolite production = [metabolite donor vein] (mol/min) + [metabolite recipient vein] (mol/min) + [metabolite placental tissue homogenates] (mol/min) (assuming steady state at 300mins)
- Rate of  $^{15}\text{N}$  labelled metabolite transfer = [metabolite donor vein] (mol/min) + [metabolite recipient vein] (mol/min)
- Percentage placental volume was determined by subtracting percentage placental extracellular space (determined using creatinine concentrations) in the placental tissue from 100%.

### 4.3: Results

Studies in this chapter were carried out to investigate the metabolic fate of nitrogen from  $^{15}\text{N}$ -glutamate and  $^{15}\text{N}$ -leucine and the metabolic fate of carbon from  $\text{U-}^{13}\text{C}$ -glutamate in the perfused normal term human placenta. The intracellular concentrations of amino acids in the placenta that was perfused for 15 minutes with creatinine only were first determined and compared to those in a previous study (Field A, 2005). This was followed by  $^{15}\text{N}$  labelled infusate studies, in which  $^{15}\text{N}$ -glutamate or  $^{15}\text{N}$ -leucine were continually perfused in either the maternal or the fetal arterial circulation, with a fetal arterial serine bolus infusion at every hour. Both  $^{15}\text{N}$ -glutamate and  $^{15}\text{N}$ -leucine were taken up by the placenta and their  $^{15}\text{N}$  was incorporated into glutamine, aspartate, alanine, proline and the BCAAs.

Having established a working placental perfusion and biochemical protocols for amino acid analyses as well as the  $^{15}\text{N}$  metabolic pathways in the human placenta, the next step was to investigate whether there is net synthesis of amino acids from glutamate. This was carried out by infusing  $\text{U-}^{13}\text{C}$ -glutamate into the fetal arterial circulation, which was taken up by the placenta and its labelled carbon was incorporated into glutamine, aspartate and proline.

This section will present results from studies in this chapter. It should be noted that in the  $^{15}\text{N}$  infusate studies it was anticipated that the infusion of a serine bolus would have an effect on the transfer of  $^{15}\text{N}$  labelled metabolites across the placenta and so the results presented in these studies are from the samples taken at 55 min and 58 min after the last serine bolus (5 and 2 minutes before the end of the experiments). As a serine bolus was found to be unnecessary for the transfer of these metabolites across the placenta, it was omitted in the  $\text{U-}^{13}\text{C}$ -glutamate experiments. Although a serine bolus was not infused in the  $\text{U-}^{13}\text{C}$ -glutamate experiments, the results from the  $\text{U-}^{13}\text{C}$ -glutamate experiments are also from samples taken at 5 min and 2 min before the end of the 1 hourly intervals as equilibrium had been reached at these time points (Figure 4.2).

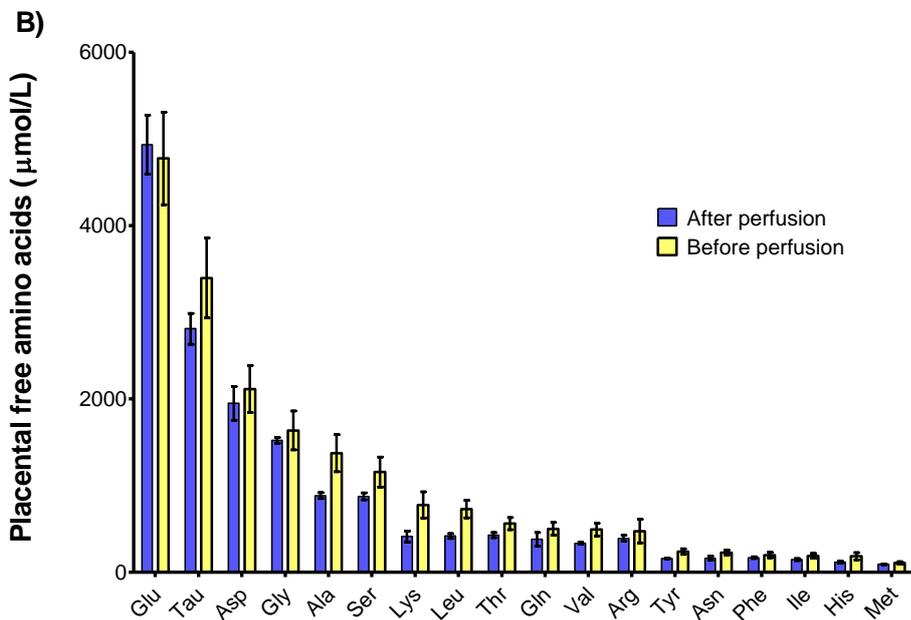
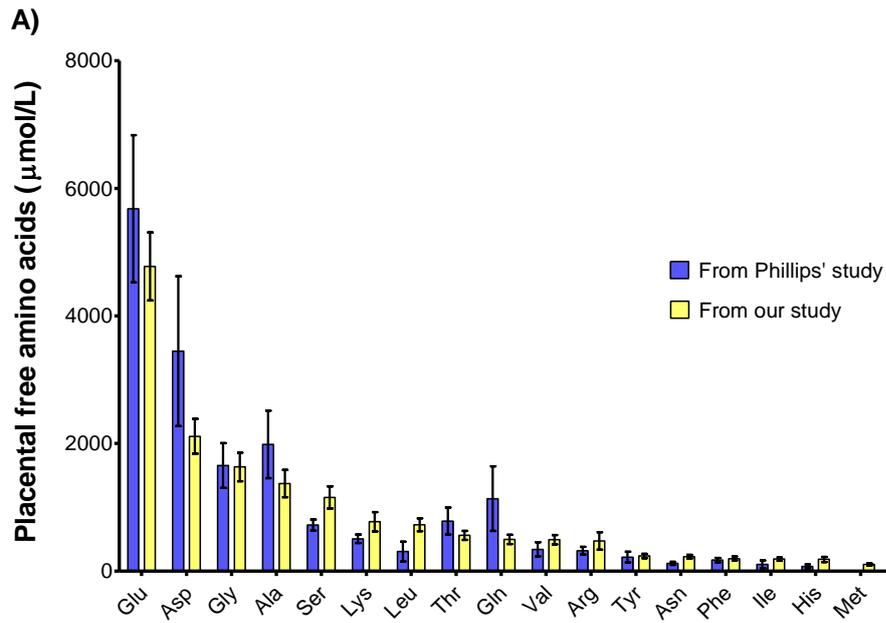
### **4.3.1: Concentrations of free amino acids in the intracellular placental pool.**

Free amino acid concentrations in placental tissue that was perfused for 15 minutes with EBB containing creatinine were similar to those measured in a previous study (Figures 4.1 A)(Phillips *et al.*, 1978). Free placental amino acid concentrations were also measured at the end of the 5 hour perfusions with labelled substrates and these were not significantly different from the tissue concentrations measured after 15 minute perfusions with EBB containing creatinine alone (Figure 4.1B).

### **4.3.2: Infusate uptake into the placenta from the maternal and the fetal circulation**

Placental uptake from the  $^{15}\text{N}$ -glutamate maternal arterial perfusion experiments was not significantly different from placental uptake from the  $^{15}\text{N}$ -glutamate fetal arterial perfusions ( $P = 0.243$ , Figure 4.3). Placental uptake from the fetal arterial U- $^{13}\text{C}$ -glutamate perfusion and that from maternal arterial  $^{15}\text{N}$ -leucine perfusion could not be compared to the uptakes in any of the experiments, as the infused amino acid concentrations were different (Figure 4.3).

When  $^{15}\text{N}$ -glutamate was infused into the fetal arterial circulation,  $31 \pm 5\%$  was taken up by the placenta and  $32 \pm 13\%$  of the total  $^{15}\text{N}$ -glutamate placental uptake was transferred to the maternal venous circulation. When  $^{15}\text{N}$ -glutamate was infused into the maternal arterial circulation,  $28 \pm 9\%$  was taken up by the placenta and only  $0.2 \pm 0.1\%$  of the total  $^{15}\text{N}$ -glutamate placental uptake was transferred to the fetal venous circulation. When  $^{15}\text{N}$ -leucine was infused into the maternal circulation,  $54 \pm 20\%$  of the  $^{15}\text{N}$ -leucine was taken up by the placenta and  $24 \pm 7\%$  of the total  $^{15}\text{N}$ -leucine placental uptake was transferred to the fetal venous circulation. When U- $^{13}\text{C}$ -glutamate was infused into the fetal arterial circulation at a near physiological concentration,  $47 \pm 5\%$  of the labelled glutamate was taken up by the placenta and  $11 \pm 2\%$  of the total glutamate placental uptake was transferred to the maternal venous circulation.



**Figure 4.1: Placental intracellular free amino acid concentrations.** A) Placental intracellular free amino acid concentrations from this study and those from a previous study (Phillips *et al.*, 1978). B) Placental intracellular free amino acid concentration before and after 5 hrs perfusion (n = 6 and n = 20 respectively (*One way ANOVA*)). Data are expressed as mean  $\pm$  SEM.

### 4.3.3: The fate of nitrogen from <sup>15</sup>N-glutamate infused in the fetal artery

When 200 μmol/L of <sup>15</sup>N-glutamate was infused into the fetal arterial circulation, of the isolated perfused human placenta, the <sup>15</sup>N labelled nitrogen was found to be incorporated into glutamine, alanine, aspartate, proline and the BCAAs.

The enrichment of glutamate was higher in the fetal venous samples than in the maternal venous and placental homogenised samples as expected ( $P < 0.001$  and  $P = 0.003$  respectively, Figure 4.4A). The enrichments of glutamate in the maternal venous samples were not significantly different from those in the placental samples ( $P = 0.184$ ).

The enrichment of glutamine was significantly higher in the fetal vein than in the maternal vein ( $P = 0.016$ ) and placental tissue ( $P < 0.001$ ) which were not different ( $P = 0.216$ , Figure 4.4A).

The enrichment of aspartate was higher in the fetal vein than in the maternal vein ( $P = 0.015$ ) but not different from that in the placental tissue ( $P = 0.284$ ) and there were no differences in the enrichment of aspartate between the maternal vein and the placental tissue ( $P = 0.312$ , Figure 4.4A).

The enrichment of proline was significantly higher in the fetal vein than in the placental tissue ( $P < 0.01$ ) but not different from the enrichment in the maternal vein ( $P = 0.112$ , Figure 4.4A). There were no differences in the enrichment of proline between the maternal vein and the placental tissue ( $P = 0.346$ ).

The enrichment of isoleucine was higher in the fetal vein than in the maternal vein ( $P = 0.023$ ) and the placental tissue ( $P = 0.002$ ) but there were no differences in the enrichments of isoleucine between the maternal vein and the placental tissue ( $P = 0.315$ ). The enrichment of leucine in the maternal vein and the fetal vein were higher than the enrichments in the placental tissue ( $P = 0.045$  and  $0.001$  respectively) and that in the fetal vein was higher than that in the maternal vein ( $P = 0.048$  Figure 4.4A). The enrichments of alanine and valine were also higher in the fetal vein than in the maternal vein and the placental tissue ( $P < 0.05$ ).

There were no differences in the concentrations of  $^{15}\text{N}$  enriched amino acids between the maternal and the fetal venous samples ( $P > 0.05$ , Figure 4.4B). As placental tissue amino acid concentrations were based on steady state estimates at 300 minutes, they were not compared to the maternal and fetal vein concentrations of enriched amino acids but the total concentration and enriched concentration values are given in Table 4.2.

**Table 4.2: Concentrations of  $^{15}\text{N}$  enriched amino acids in the placental tissue after 5 hrs  $^{15}\text{N}$ -glutamate perfusion into the fetal arterial circulation**

Amino acids	Total concentrations of amino acids in placental tissue ( $\mu\text{mol/L}$ )	Concentration of $^{15}\text{N}$ labelled amino acids ( $\mu\text{mol/L}$ )
Glutamate	4900 (794.5)	1292 (574.3)
Aspartate	2014 (447.2)	645 (179.9)
Glutamine	399 (197.6)	31 (19.1)
Alanine	1004 (187.1)	15 (0.7)
Valine	379 (89.8)	8 (2.5)
Isoleucine	173 (64.0)	5 (1.4)
Leucine	439 (117.5)	4 (1.0)

*Data are expressed as mean (SEM), n = 4.*

The total rate of production of each  $^{15}\text{N}$  labelled amino acid was calculated as the sum of the rate of each metabolite appearing in the maternal and fetal venous samples and estimated in the placental tissue (Figure 4.5). The total concentration of  $^{15}\text{N}$  labelled glutamine was higher than that of alanine, isoleucine, valine and leucine ( $P < 0.05$ ) but was not significantly different from the aspartate total  $^{15}\text{N}$  concentration ( $P = 0.677$ ). The total concentration of  $^{15}\text{N}$  labelled aspartate was higher than the concentrations of all other  $^{15}\text{N}$  labelled amino acids ( $P < 0.01$ ) except for glutamine.

#### 4.3.4: The fate of nitrogen from <sup>15</sup>N-glutamate infused in the maternal artery

When 200 µmol/L of <sup>15</sup>N-glutamate was infused into the maternal arterial circulation of the isolated perfused human placenta, the <sup>15</sup>N labelled nitrogen was found to be incorporated into glutamine, alanine, aspartate, proline and the BCAAs.

The enrichments of glutamate were higher in the fetal and maternal venous samples than in the placental tissue ( $P < 0.001$ ) as expected but there were no differences in the enrichments of glutamate between the maternal and fetal venous samples ( $P = 1.00$ , Figure 4.6A).

The enrichments of glutamine in the maternal and fetal vein were higher than those in placental tissue ( $P < 0.001$ , Figure 4.6A) but there were no differences in the enrichment of glutamine between the maternal and the fetal vein ( $P = 1.00$ ).

The enrichment of proline in the fetal vein was higher than that in the placental tissue ( $P = 0.007$ , Figure 4.6A) but there were no differences in the enrichments of proline between the maternal and the fetal venous samples ( $P = 0.237$ ), nor were there differences between the maternal venous and the placental tissue samples ( $P = 0.169$ ). The enrichment of leucine was higher in the maternal vein than in the placental tissue and fetal vein ( $P < 0.05$ ) but there were no differences in the enrichment of leucine between the fetal and the placental tissue. The enrichment of isoleucine was higher in the maternal and the fetal vein than in the placental tissue ( $P < 0.05$ ) but there were no differences between the maternal and the fetal vein ( $P > 0.05$ ). There were no other differences in the enrichments of amino acids between the maternal and the fetal venous and the placental tissue samples (Figure 4.6A).

Concentrations of all <sup>15</sup>N enriched amino acids except those of valine were higher in the maternal vein than in the fetal venous samples ( $P > 0.05$ , Figure 4.6B). As placental tissue <sup>15</sup>N labelled amino acid production rates were based on steady state estimates at 300 minutes, they were not compared to the maternal and fetal venous concentrations of enriched samples but the total

placental amino acid concentrations and enriched concentrations are given in Table 4.3.

The total rate of appearance of  $^{15}\text{N}$  labelled amino acids was calculated as the sum of the rate of each metabolite appearing in the maternal and fetal venous and estimated in the placental tissue (Figure 4.7). The total concentrations of  $^{15}\text{N}$  labelled glutamine and aspartate were significantly higher than those of other  $^{15}\text{N}$  labelled amino acids ( $P < 0.01$ ) and there were no differences in the concentrations of  $^{15}\text{N}$  labelled aspartate and  $^{15}\text{N}$  labelled glutamine ( $P = 0.527$ ) and among the rest of  $^{15}\text{N}$  labelled amino acids ( $P > 0.05$ ).

**Table 4.3: Concentrations of amino acids and  $^{15}\text{N}$  labelled amino acids in the placental tissue after 5 hrs maternal arterial  $^{15}\text{N}$ -glutamate (200  $\mu\text{mol/L}$ ) perfusion.**

Amino acids	Total concentrations of amino acids in placental tissue ( $\mu\text{mol/L}$ )	Concentration of $^{15}\text{N}$ labelled amino acids ( $\mu\text{mol/L}$ )
Glutamate	5438 (1181)	1952 (493.4)
Aspartate	2344 (747)	903 (417)
Glutamine	366 (53)	41 (5.8)
Alanine	824 (114)	41 (18)
Leucine	328 (33)	11 (0.7)
Isoleucine	118 (16)	4 (0.1)
Valine	401 (37)	2 (0.1)

*Data are expressed as mean (SEM), n = 4.*

#### 4.3.5: The fate of nitrogen from $^{15}\text{N}$ -leucine infused in the maternal artery

To study the transfer of nitrogen, 146  $\mu\text{mol/L}$  of  $^{15}\text{N}$ -leucine was perfused into the maternal arterial circulation of the isolated perfused human placenta for 5 hrs.  $^{15}\text{N}$ -leucine was taken up from the maternal circulation and its nitrogen was incorporated into glutamine, glutamate, alanine, aspartate and proline as well as valine and isoleucine (Figure 4.8A).

The enrichments of leucine in the maternal venous samples were higher than in the fetal venous samples ( $P = 0.05$ ) and in placental homogenised samples ( $P < 0.001$ , Figure 4.4A). The enrichment of leucine in the maternal venous

samples was also higher than those in the placental homogenised samples ( $P < 0.001$ ).

The enrichments of glutamine in the maternal and the fetal vein were significantly higher than the enrichment in the placental tissue ( $P = 0.008$  and  $0.029$  respectively) and there were no significant differences in the enrichment of glutamine between the maternal and the fetal vein ( $P = 0.913$ , Figure 4.8A).

The enrichment of valine in the maternal vein was significantly higher than the enrichment in the fetal vein ( $P = 0.025$ ) and the placental tissue ( $P = 0.011$ ) but there were no differences in the enrichment of valine between the fetal vein and the placental tissue ( $P = 0.617$ , Figure 4.8A).

The enrichment of aspartate was higher in the placental tissue than in the fetal vein ( $P < 0.05$ ) and that in the maternal vein was negligible (Figure 4.8A).

The enrichment of isoleucine in the fetal vein was significantly higher than the enrichment in the placental tissue ( $P = 0.009$ ), but the enrichment in the maternal was negligible (Figure 4.8A).

The concentrations of  $^{15}\text{N}$  labelled glutamate, glutamine, alanine and valine were higher in the maternal venous samples than in fetal venous samples ( $P < 0.05$ , Figure 4.8B) while concentrations of isoleucine in the maternal vein were negligible. As the rate of  $^{15}\text{N}$  labelled amino acids in the placental tissue were based on calculations using steady state estimates at 300 minutes, they were not compared to the maternal and fetal vein concentrations of enriched samples but the total placental amino acid concentrations and enriched concentrations are given in Table 4.4.

The total rate of appearance of  $^{15}\text{N}$  labelled amino acids was calculated as the sum of the rate of each metabolite appearing in the maternal and fetal venous and estimated in the placental tissue (Figure 4.9). The total concentrations of  $^{15}\text{N}$  labelled glutamine and  $^{15}\text{N}$  labelled glutamate were significantly higher than those of alanine, aspartate, isoleucine and valine ( $P < 0.01$ ). The total concentrations of  $^{15}\text{N}$  labelled alanine were higher than those of  $^{15}\text{N}$  labelled isoleucine and valine. The total concentrations of  $^{15}\text{N}$  labelled aspartate were

lower than those of isoleucine ( $P < 0.01$ ) but not different from those of  $^{15}\text{N}$  labelled valine and alanine ( $P > 0.05$ , Figure 4.9).

**Table 4.4: Concentrations of amino acids and  $^{15}\text{N}$  labelled amino acids in the placental tissue after 5 hrs maternal arterial  $^{15}\text{N}$ -leucine perfusion.**

Amino acids	Total concentrations ( $\mu\text{mol/L}$ )	Concentration of $^{15}\text{N}$ labelled amino acids ( $\mu\text{mol/L}$ )
Glutamate	4005.5 (810.8)	643.5 (104.0)
Aspartate	1423.8 (330.5)	237.4 (52.4)
Leucine	496.1 (101.9)	40.8 (12.8)
Alanine	886.6 (205.0)	25.1 (4.3)
Glutamine	215.6 (40.0)	9.4 (2.6)
Isoleucine	128.7 (26.3)	7.1 (1.2)
Valine	335.3 (72.1)	5.6 (1.3)

*Data are expressed as mean (SEM),  $n = 5$ .*

#### 4.3.6: Concentration differences in the $^{15}\text{N}$ labelled amino acids between the three experiments

When the concentrations of individual  $^{15}\text{N}$  labelled amino acids were compared between different experiments, the concentrations of  $^{15}\text{N}$  labelled aspartate in the fetal and maternal  $^{15}\text{N}$ -glutamate perfusions were higher than those in the maternal  $^{15}\text{N}$ -leucine perfusions ( $P < 0.01$ ). However, those in the fetal and maternal  $^{15}\text{N}$ -glutamate perfusions were not different ( $P = 1.00$ ). The concentrations of  $^{15}\text{N}$  labelled isoleucine were lower in the maternal  $^{15}\text{N}$ -leucine perfusions than in the maternal glutamate perfusions ( $P < 0.01$ ) and there was a trend towards lower concentrations of  $^{15}\text{N}$  labelled isoleucine in the maternal  $^{15}\text{N}$ -leucine perfusions than in the fetal  $^{15}\text{N}$ -glutamate perfusions ( $P > 0.05$ ). In contrast, the concentrations of  $^{15}\text{N}$  labelled valine were higher in the maternal  $^{15}\text{N}$ -leucine perfusions than in the  $^{15}\text{N}$ -glutamate maternal and fetal perfusions ( $P < 0.01$ ). There were no differences in the concentrations of  $^{15}\text{N}$  labelled alanine in the three experiments and there were no differences in the concentrations of  $^{15}\text{N}$  labelled leucine between the two  $^{15}\text{N}$ -glutamate experiments.

In the maternal  $^{15}\text{N}$ -glutamate perfusion experiments the serine bolus increased the enrichment of isoleucine ( $P = 0.012$ ) and decreased the enrichment of aspartate ( $P < 0.001$ ) but had little or no effect on any other amino acids. In addition, a serine bolus had no effect on the enrichments and concentrations of the amino acids in the fetal  $^{15}\text{N}$ -glutamate perfusions and in the maternal  $^{15}\text{N}$ -leucine perfusions. As a serine bolus was found to be unnecessary for the stimulation of metabolite transfer across the placenta, serine boluses were not administered in the  $^{13}\text{C}$ -glutamate experiments.

#### **4.3.7: Concentrations of BCKAs ( $\alpha$ -keto-isocaproic acid, $\alpha$ -keto-isovaleric acid and $\alpha$ -keto- $\beta$ -methylvaleric acid) and pyruvate**

The concentrations of the BCKAs and pyruvate were measured in the placental tissue, maternal and fetal vein of the leucine perfusion experiments (Figures, 4.10 & 4.11). Overall the concentrations of the BCKAs were very low.

The concentrations of  $\alpha$ -keto-isocaproic acid (keto acid of leucine) measured in maternal and fetal veins, were higher than the concentrations of  $\alpha$ -keto-isovaleric acid (keto acid of valine) and  $\alpha$ -keto- $\beta$ -methylvaleric acid (keto acid of isoleucine) (Data not shown). The concentrations of  $\alpha$ -keto-isovaleric acid and  $\alpha$ -keto- $\beta$ -methylvaleric acid were not different in either the placental samples ( $P = 0.844$ ), the maternal vein ( $P = 0.394$ ) or the fetal vein ( $P = 0.771$ ) (Data not shown). The rate of release of  $\alpha$ -keto-isocaproic acid,  $\alpha$ -keto-isovaleric acid and  $\alpha$ -keto- $\beta$ -methylvaleric acid into the maternal vein was higher than the rate of release into the fetal vein ( $P < 0.001$ , Figure 4.10). Placental concentrations could not be compared to those in the maternal and fetal vein as those in the maternal and fetal vein are calculated based on the flow rates.

The concentrations of pyruvate in the maternal and fetal veins ( $22.4 \pm 2.3$   $\mu\text{mol/L}$ ) were over twenty-five times higher than the concentrations of the BCKAs ( $P < 0.05$ , Data not shown). The rate of pyruvate release into the maternal vein was significantly higher than into the fetal vein ( $P < 0.001$ , Figure 4.10). Placental concentrations could not be compared to those in the maternal and fetal vein as those in the maternal and fetal vein are calculated based on the flow rates. However the concentrations of pyruvate in the placental tissue were higher than those of  $\alpha$ -keto-isocaproic acid,  $\alpha$ -keto-isovaleric acid and  $\alpha$ -

keto- $\beta$ -methylvaleric acid ( $P < 0.0001$ , Figure 4.11) and those of  $\alpha$ -keto-isovaleric acid were higher than placental concentrations of  $\alpha$ -keto-isocaproic acid and  $\alpha$ -keto- $\beta$ -methylvaleric acid ( $P < 0.05$ , Figure 4.11).

### **4.3.8: The fate of $^{13}\text{C}$ carbon from glutamate in other amino acids**

#### **4.3.8.1: $^{13}\text{C}$ -Glutamate enrichments and concentrations**

As expected, the enrichment of the M + 5 glutamate isotopomer was higher in the fetal vein than in the maternal vein and the placental tissue ( $P < 0.001$ ) but there were no differences in the M + 5 enrichments between the maternal vein and placenta ( $P = 0.453$ , Figure 4.12A). In addition, the enrichment of the M + 4 glutamate isotopomer was higher in the fetal vein than in the maternal vein and the placenta ( $P < 0.001$ ) which were not different ( $P = 1.00$ ). The enrichment of the M + 3 glutamate isotopomer was higher in the placental tissue than in the maternal and fetal vein ( $P < 0.05$ ) which were not different ( $P = 0.762$ ). The enrichments of the M + 1 and the M + 2 glutamate isotopomers were higher in the maternal vein and the placental tissue than in the fetal vein ( $P < 0.01$ ) and there were no differences in the enrichments of M + 1 and M + 2 between the placental tissue and the maternal vein ( $P > 0.05$ ).

As expected, the concentrations of the M + 5 labelled glutamate were higher in the fetal vein than in the maternal vein ( $P < 0.01$ , Figure 4.12B). In addition, the concentrations of the M + 4 labelled glutamate were higher in the fetal vein than in the maternal vein ( $P < 0.01$ ). There were no other differences in the concentrations of different  $^{13}\text{C}$  labelled glutamate isotopomers between the maternal and the fetal vein.

#### **4.3.8.2: $^{13}\text{C}$ -Glutamine enrichments and concentrations**

The M + 5 enrichments of glutamine were higher in the maternal and fetal venous samples than in the placental tissue ( $P < 0.01$ ) and those in the maternal vein were higher than those in the fetal vein ( $P < 0.01$ , Figure 4.13A). The enrichments of the M + 3 isotopomers in the fetal and maternal vein were also higher than those in the placental tissue which were negligible ( $P < 0.01$ ) but they were not differences between the maternal and the fetal vein ( $P =$

0.072) while the enrichments of the M + 4 glutamine isotopomers in the placental tissue were negligible and as such, they were lower than those in the maternal and the fetal vein. There were no differences in the enrichment of the M + 4 glutamine isotomers between the maternal and the fetal vein ( $P = 0.185$ ). The M + 2 enrichment of glutamine in the maternal vein was negligible as such it was higher in the fetal vein and the placental tissue than in the maternal vein ( $P < 0.01$ ). The enrichment of M + 1  $^{13}\text{C}$  glutamine isotopomer was higher in the fetal vein than in the maternal vein ( $P < 0.01$ ) and the placental tissue ( $P = 0.04$ ). There were no differences in the M + 1 enrichments of glutamine between the maternal vein and the placental tissue ( $P = 0.06$ ).

The concentrations of the M + 5 and M + 4, M + 3  $^{13}\text{C}$  enriched glutamine in the maternal vein were higher than in the fetal vein ( $P < 0.01$ , Figure 4.13B) and that of M + 2 glutamine was negligible in the maternal vein and as such they were higher in the fetal vein than in the maternal vein ( $P < 0.01$ ). There were no significant differences in the concentrations of the M + 1 glutamine isotopomers between the fetal and maternal vein ( $P > 0.05$  Figure 4.13B).

#### **4.3.8.3: $^{13}\text{C}$ -aspartate enrichments and concentrations**

The enrichment of the M + 4 aspartate isotopomer in the fetal vein was higher than that in the maternal vein and the placental tissue ( $P < 0.01$ ) and that in the placental tissue was higher than that in the maternal vein ( $P < 0.01$ , Figure 4.14A). The enrichment of the M + 2 aspartate isotopomer was lower in the maternal vein than in the fetal vein and the placental tissue ( $P < 0.01$ ) but that in the fetal vein was not significantly different from that in the placental tissue ( $P = 0.09$ ). There were no other differences in the enrichments of the other aspartate isotopomers between the maternal vein, fetal vein and the placental tissue ( $P > 0.05$ ).

The concentrations of M + 4 enriched aspartate isotopomers were higher in the fetal vein than in the maternal vein ( $P < 0.01$ ), Figure 4.14B). There were no other differences in the concentrations of the aspartate isotopomers ( $P > 0.05$ ).

#### 4.3.8.4: $^{13}\text{C}$ -Proline enrichments

The enrichments of the M + 5, M + 4 and M + 1 proline isotopomers were higher in the fetal vein than in the maternal vein and the placental tissue ( $P < 0.01$ ) and there were no differences in the enrichments of the M + 5 and M + 4 proline isotopomers between the maternal vein and the placental tissue although the enrichment of the M + 1 proline were lower in the maternal vein than in placental tissue ( $P > 0.05$ , Figure 4.15). The enrichments of the M + 2 and M + 3 proline isotopomers were higher in the placental tissue than in the maternal and fetal vein ( $p < 0.05$ ) but they were not different between the placenta and the fetal vein ( $P = 0.20$ ).

Concentrations of proline could not be measured in this study because the derivatising reagent OPA does not allow the detection of molecules with a secondary amine structure. However, overall, the carbon from glutamate was predominantly used for glutamine synthesis rather than for proline and aspartate synthesis.

#### 4.3.8.5: Rate of appearance of $^{15}\text{N}$ labelled amino acids

The total rate of appearance of  $^{13}\text{C}$  labelled amino acids was calculated as the sum of the rate of each metabolite appearing in the maternal and fetal vein and estimated in the placental tissue (Figure 4.9).

The concentrations of labelled amino acids in the placental tissue from the U- $^{13}\text{C}$ -glutamate perfusions were estimated from 300 minute steady state assumptions and therefore were not compared to the maternal and fetal vein concentrations but they are given in table 4.5.

**Table 4.5: Concentrations of  $^{13}\text{C}$  labelled amino acid isotopomers in the placenta after 4 hrs fetal arterial  $^{13}\text{C}$ -glutamate perfusion ( $\mu\text{mol/L}$ ).**

Amino acids	Isotopomer				
Amino acids	M + 1	M + 2	M + 3	M + 4	M + 5
Glutamate	101.2 (94.4)	157.4 (70.6)	364.6 (66.1)	23.3 (3.4)	535.2 (75.5)
Glutamine	15.3 (0.9)	7.9 (1.2)	1.3 (1.9)	NA	29.8 (4.5)
Aspartate	133.3 (27.4)	158.9 (30.6)	27.9 (5.7)	252.7 (47.9)	NA

*Data are expressed as mean (SEM). n = 5, NA = not applicable.*

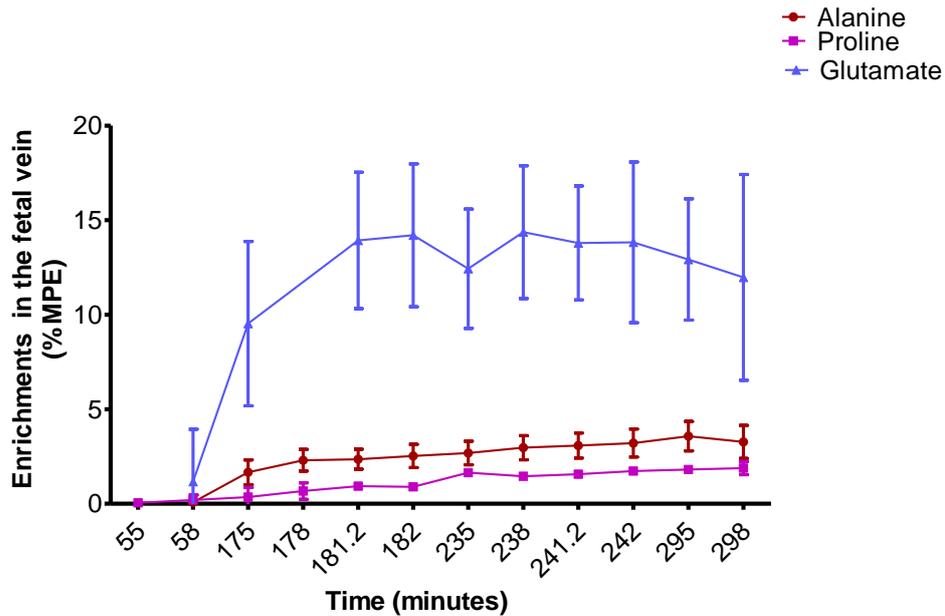
#### 4.3.8.6: Other amino acids and glucose

In this study the enrichments of arginine, and cysteine could not be measured because of methodological reasons. Amino acids that were detected in the  $^{13}\text{C}$ -glutamate experiments but in which the enrichment could not be detected are shown in Table 4.6.

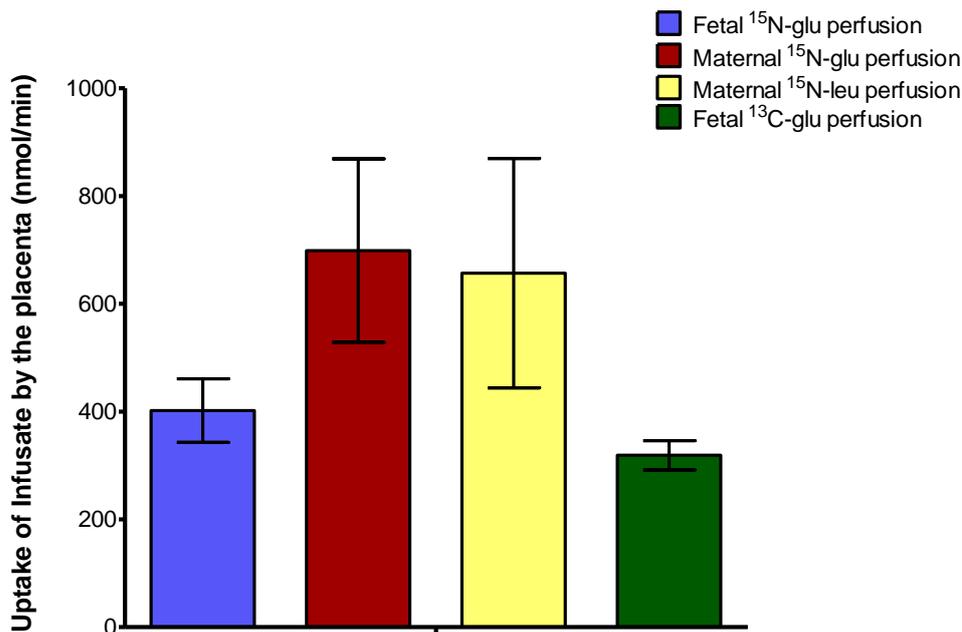
Although glucose, which was not infused in this study, was identified in the maternal and fetal venous samples and the placental tissue homogenates, no incorporation of  $^{13}\text{C}$  labelled carbon from the infused glutamate into glucose could be detected.

**Table 4.6: Amino acids that were detected but were not enriched** in the U- $^{13}\text{C}$ -glutamate perfusion experiments. The m/z ions represent the base or parent ions looked for, for each amino acid

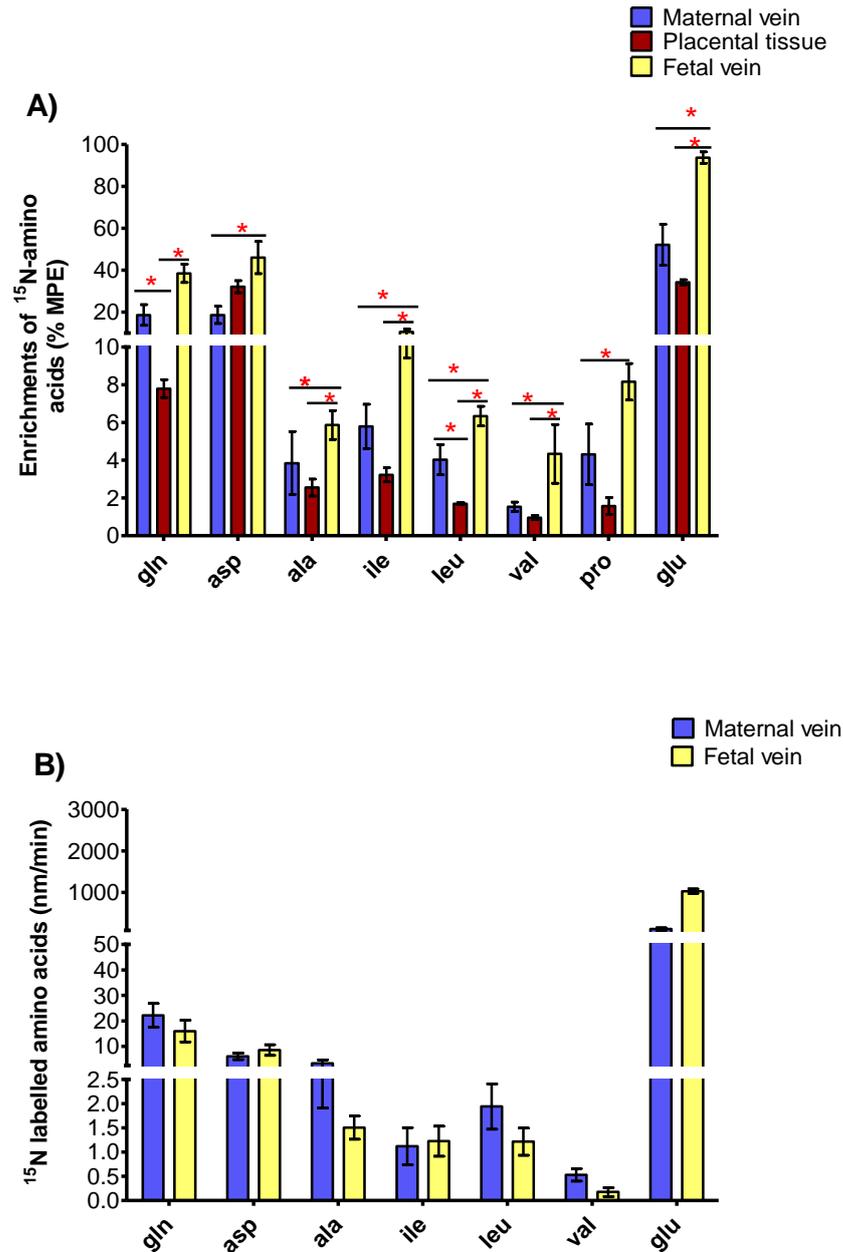
Amino acid	m/z ion	Retention time (minutes)
Alanine	260	7.5
Glycine	246	7.8
Valine	288	8.7
Leucine	302	9.2
Isoleucine	302	9.6
Asparagine	417	9.7
Serine	390	12.4
Threonine	404	12.7
Lysine	431	16.8
Phenylalanine	336	14.6
Tyrosine	466	19.3



**Figure 4.2: Equilibration of enriched amino acids in the fetal vein** during a 5 hr perfusion of  $^{15}\text{N}$ -leucine in the maternal arterial circulation. Data are mean  $\pm$  SEM



**Figure 4.3: Uptake of the infused isotope by the placenta**, calculated as arterial-venous differences. Placental uptake from the  $^{15}\text{N}$ -glutamate maternal arterial perfusion experiments was not significantly different from placental uptake from the  $^{15}\text{N}$ -glutamate fetal arterial perfusions ( $P = 0.243$ ). Uptake from fetal  $\text{U-}^{13}\text{C}$ -glutamate perfusions and maternal  $^{15}\text{N}$ -leucine perfusions could not be compared to the uptakes in any of the other experiments due to concentration differences. Data are expressed as mean  $\pm$  SEM.



**Figure 4.4: Amino acid enrichments and concentrations** following 5 hrs fetal arterial  $^{15}\text{N}$ -glutamate perfusion. A)  $^{15}\text{N}$  enrichments of amino acids in the maternal vein, placental tissue and fetal vein following  $^{15}\text{N}$ -glutamate (200  $\mu\text{mol/L}$ ) infusion into the fetal arterial circulation. B) Concentrations of enriched amino acids in the maternal vein and fetal vein after 5 hrs  $^{15}\text{N}$ -glutamate perfusion into the fetal arterial circulation. There were no significant differences in enriched amino acid concentrations between the maternal and the fetal vein. \* indicates significant difference ( $P < 0.05$ ,  $n = 4$ ). Data are expressed as mean  $\pm$  SEM.

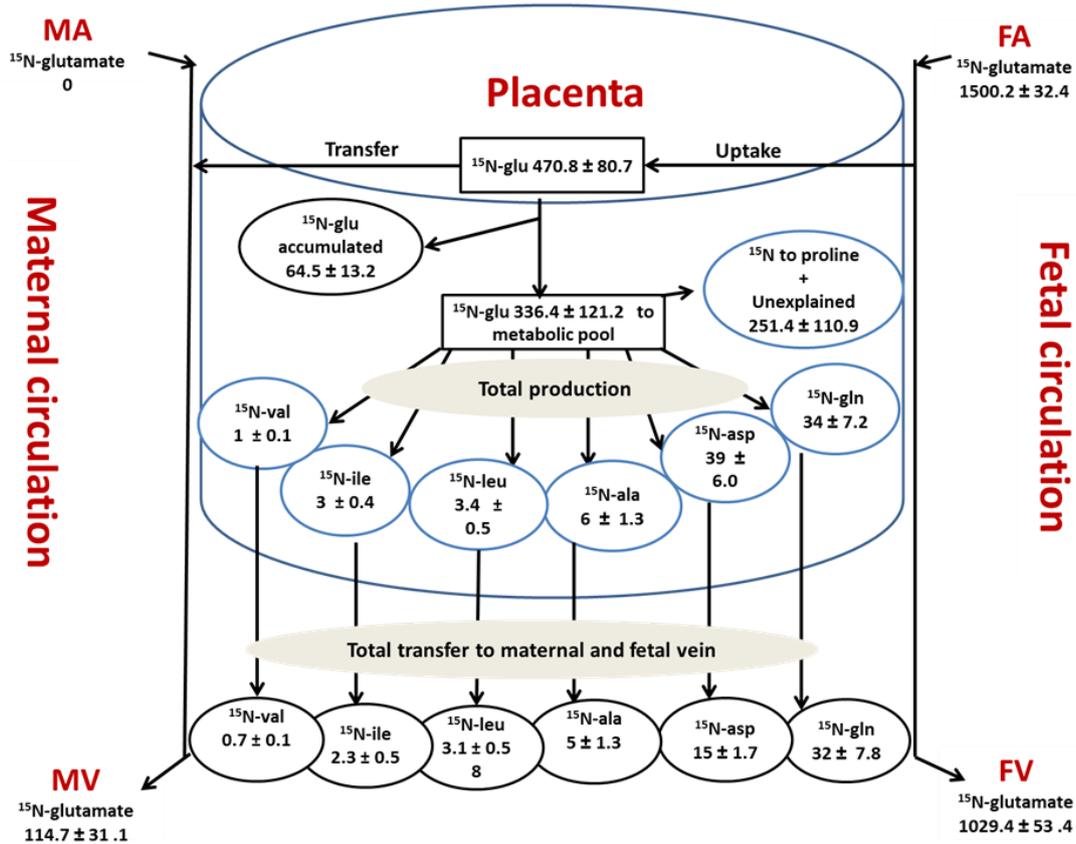
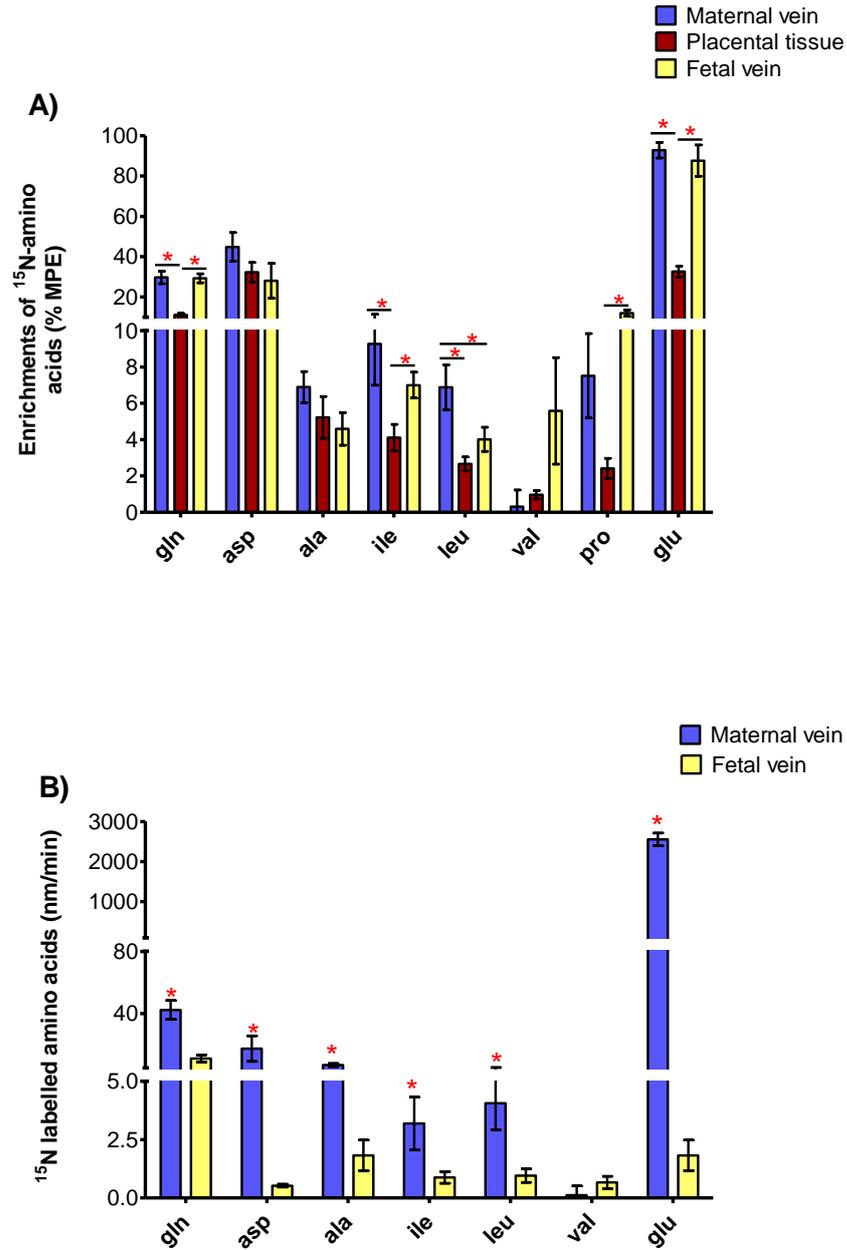


Figure 4.5: The uptake and metabolic distribution to other amino acids of  $^{15}\text{N}$  from glutamate infused in the fetal arterial circulation. MA is maternal artery, MV is maternal vein, FA is fetal artery and FV is fetal vein. Data are expressed as mean  $\pm$  SEM (nmol/min).



**Figure 4.6: Amino acid enrichments and concentrations following 5 hrs maternal arterial <sup>15</sup>N-glutamate perfusion.** A) <sup>15</sup>N enrichments of amino acids in the maternal vein, placental tissue and fetal vein 5 hrs following <sup>15</sup>N-glutamate (200 μmol/L) infusion into the maternal arterial circulation. Enrichments of glutamine were higher in the maternal and fetal vein than in the placental tissue and that of proline was higher in the fetal vein than in the maternal vein and the placental tissue B) Concentrations of enriched of amino acids in the maternal vein and fetal vein after 5 hrs <sup>15</sup>N-glutamate perfusion into the maternal arterial circulation. Data are expressed as mean ± SEM, \* significant difference (P < 0.05, n = 4).

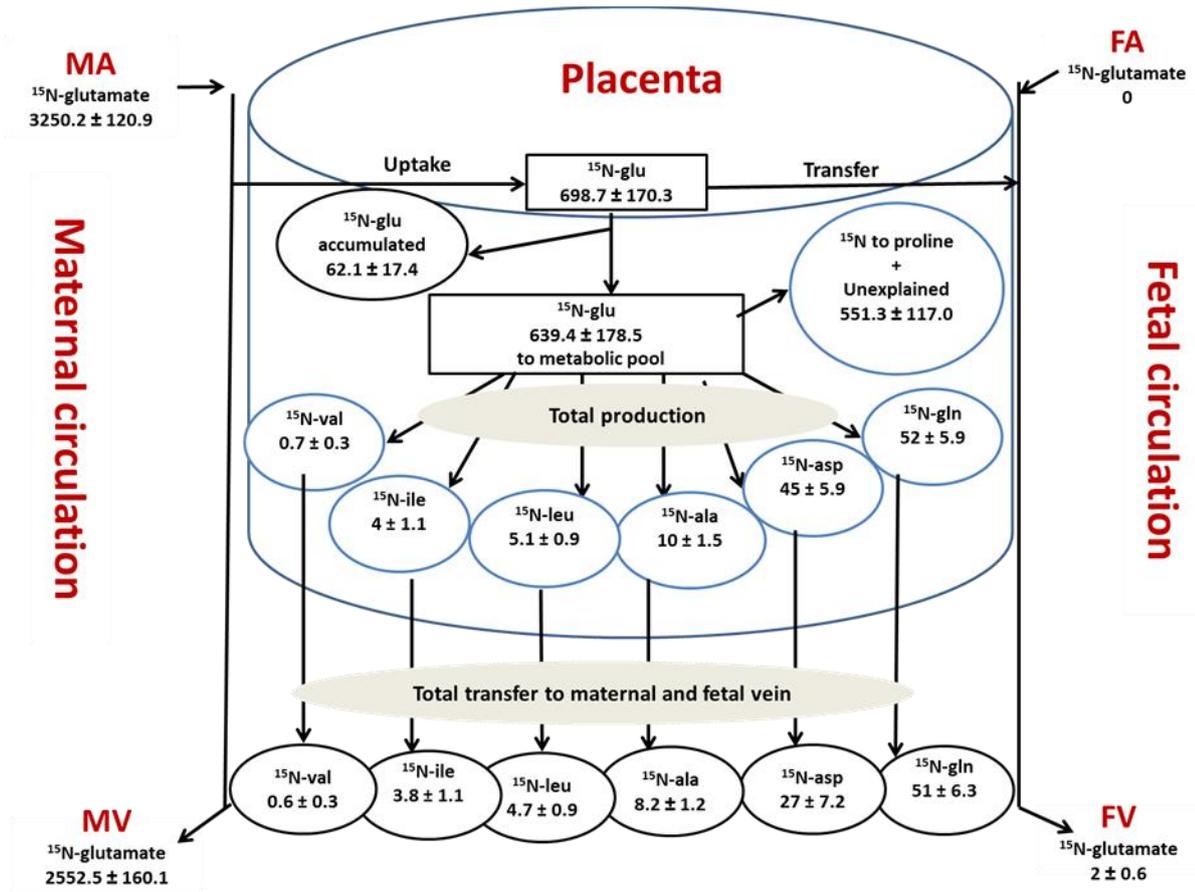
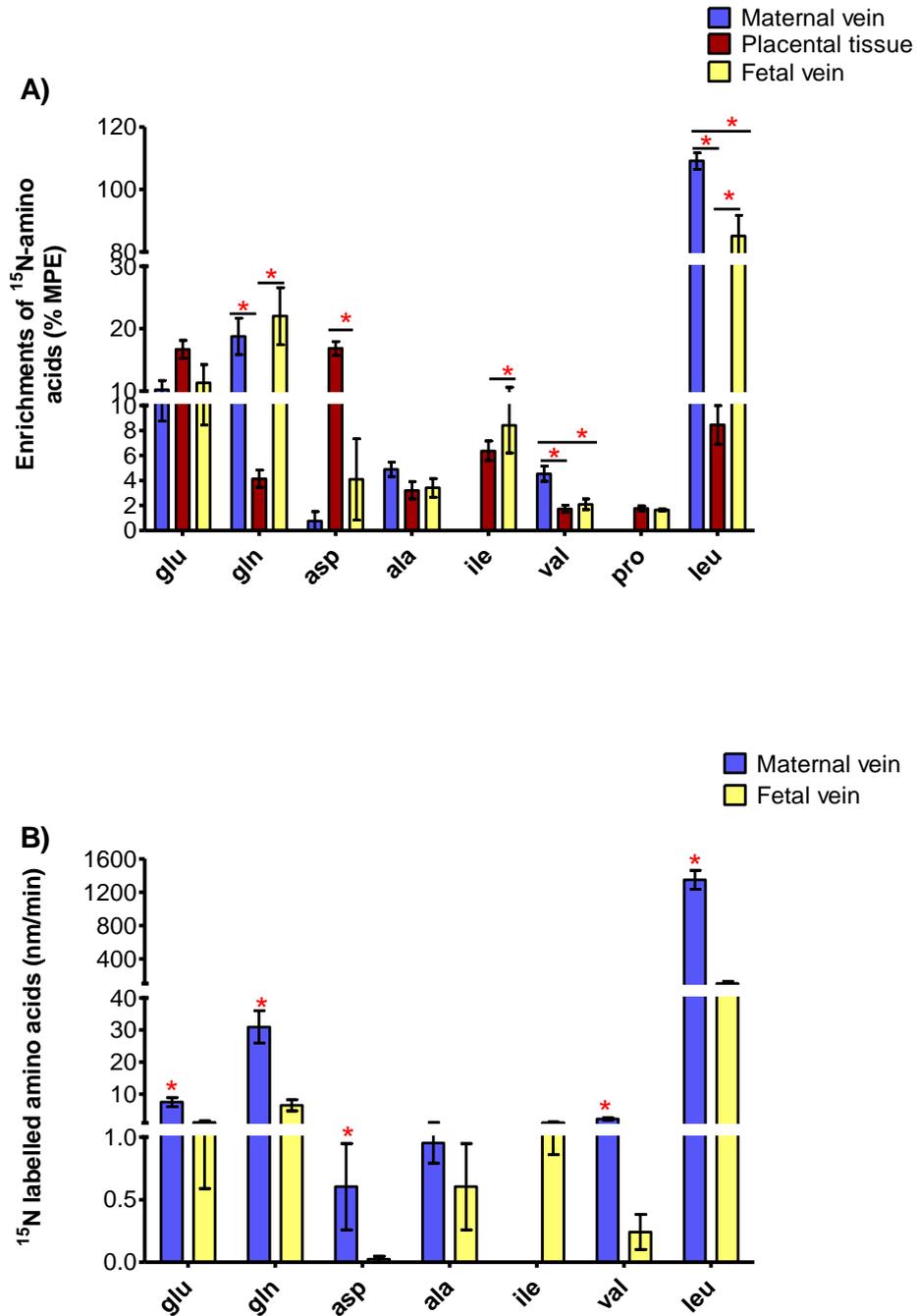
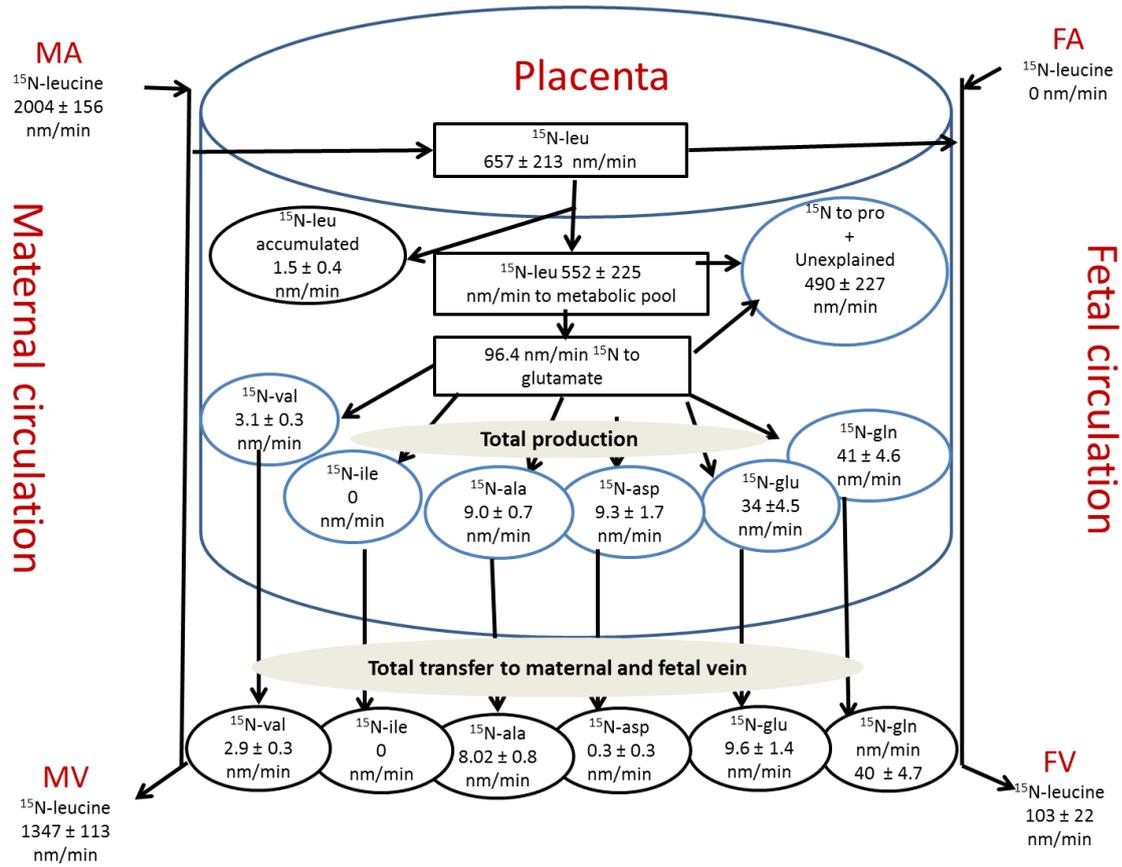


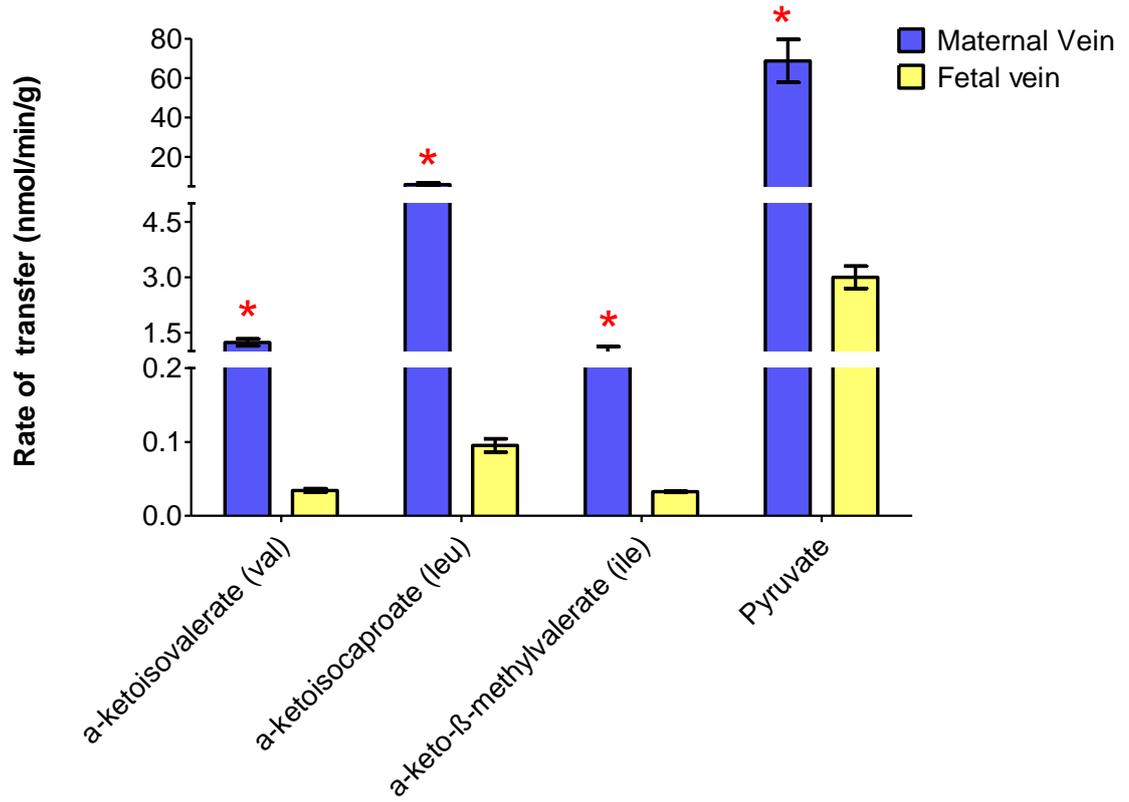
Figure 4.7: The uptake and metabolic distribution to other amino acids of  $^{15}\text{N}$  from glutamate infused in the maternal arterial circulation. MA is maternal artery, MV is maternal vein, FA is fetal artery and FV is fetal vein. Data are expressed as mean  $\pm$  SEM (nmol/min).



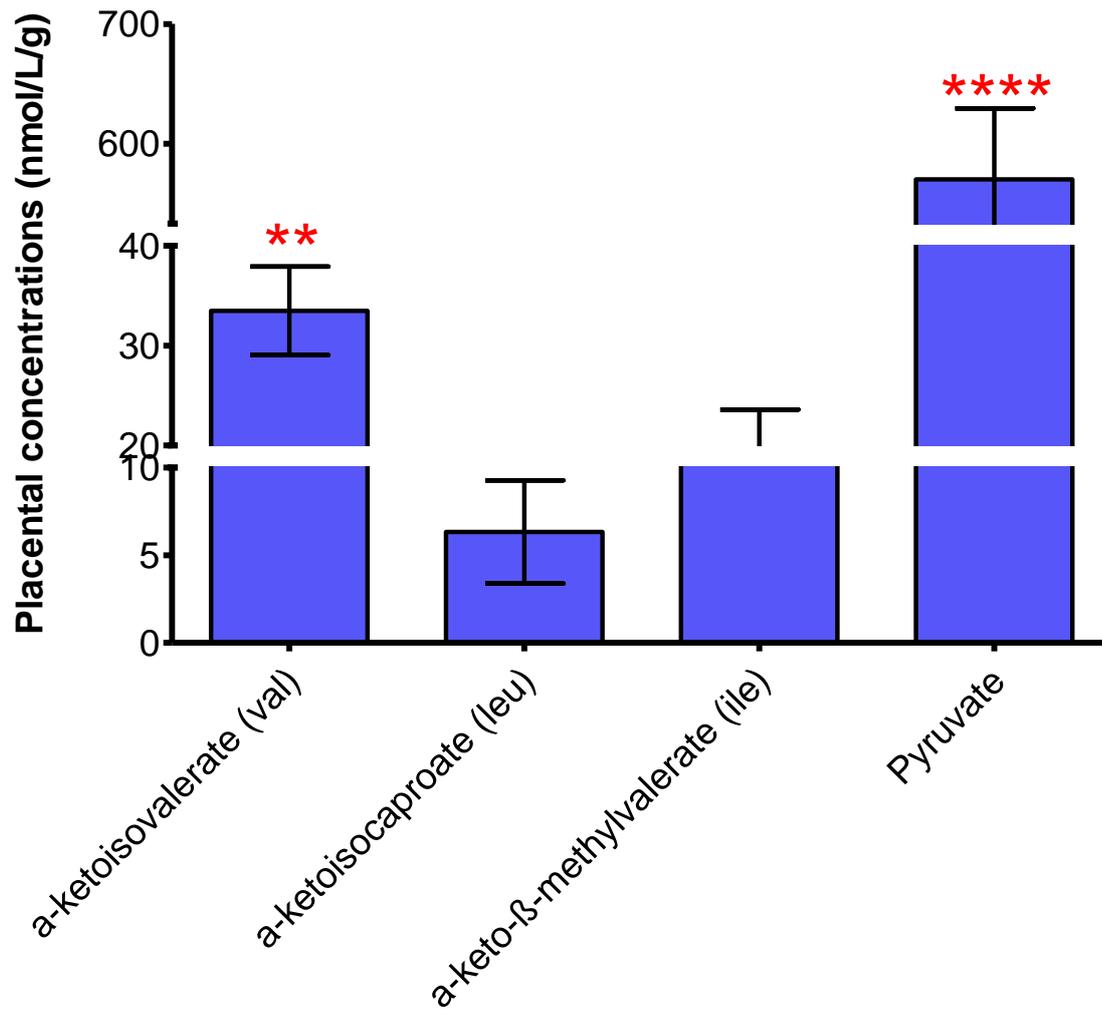
**Figure 4.8: Amino acid enrichments and concentrations following 5 hrs maternal arterial <sup>15</sup>N-leucine perfusion.** A) <sup>15</sup>N enrichments of amino acids in the maternal vein, placental tissue and fetal vein 5 hrs following 146 μmol/L <sup>15</sup>N-leucine infusion into the maternal arterial circulation, indicating nitrogen flux from <sup>15</sup>N-leucine to glutamate, glutamine, alanine, aspartate, proline and BCAAs. B) Concentrations of enriched amino acids in the maternal vein and fetal vein after 5 hrs <sup>15</sup>N-leucine perfusion into the maternal arterial circulation. Data are expressed as mean ± SEM, \* significant difference P < 0.05, n = 5.



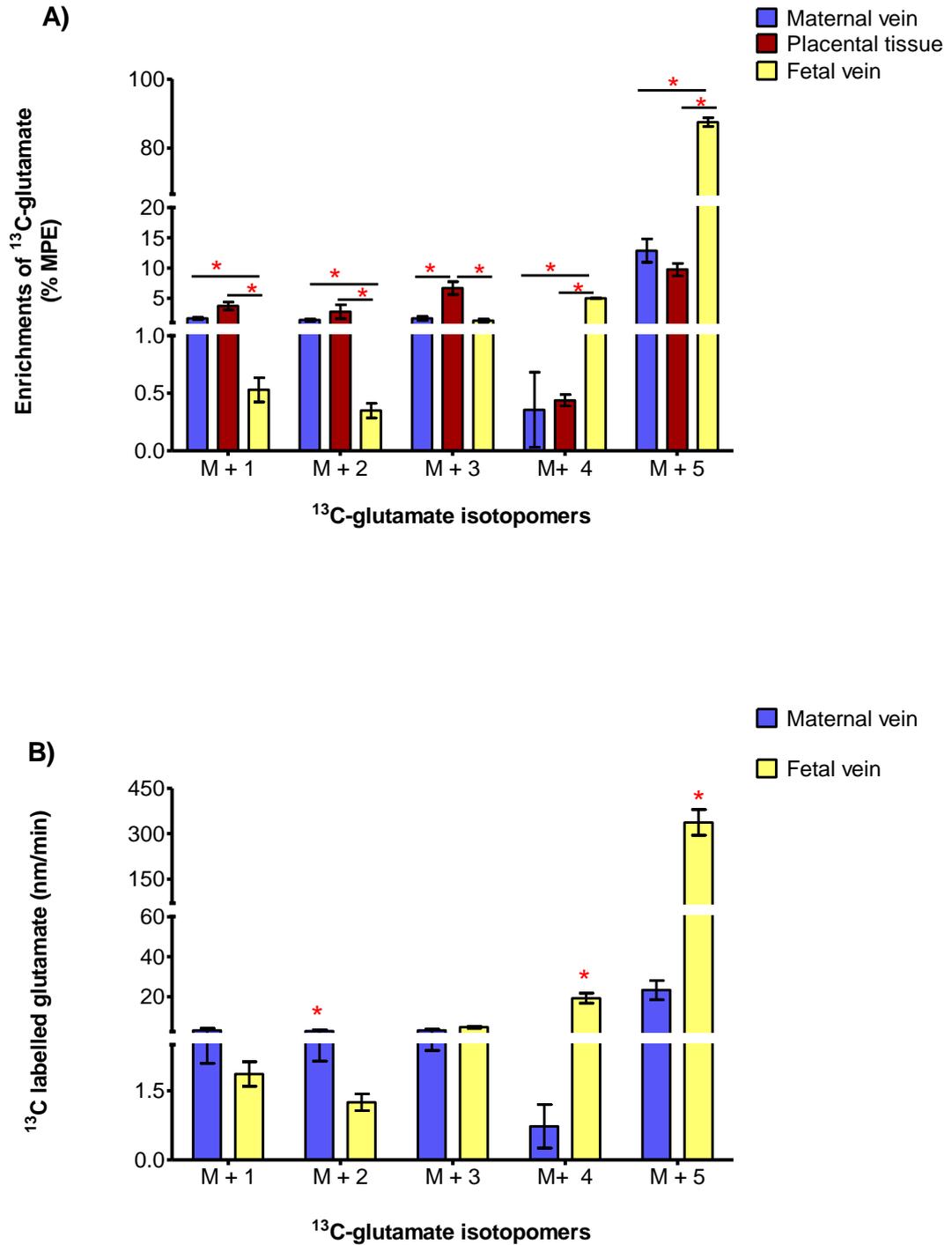
**Figure 4.9: The uptake and metabolic distribution to other amino acids of <sup>15</sup>N from leucine** infused in the maternal arterial circulation. MA is maternal artery, MV is maternal vein, FA is fetal artery and FV is fetal vein. Data are expressed as mean ± SEM (nmol/min).



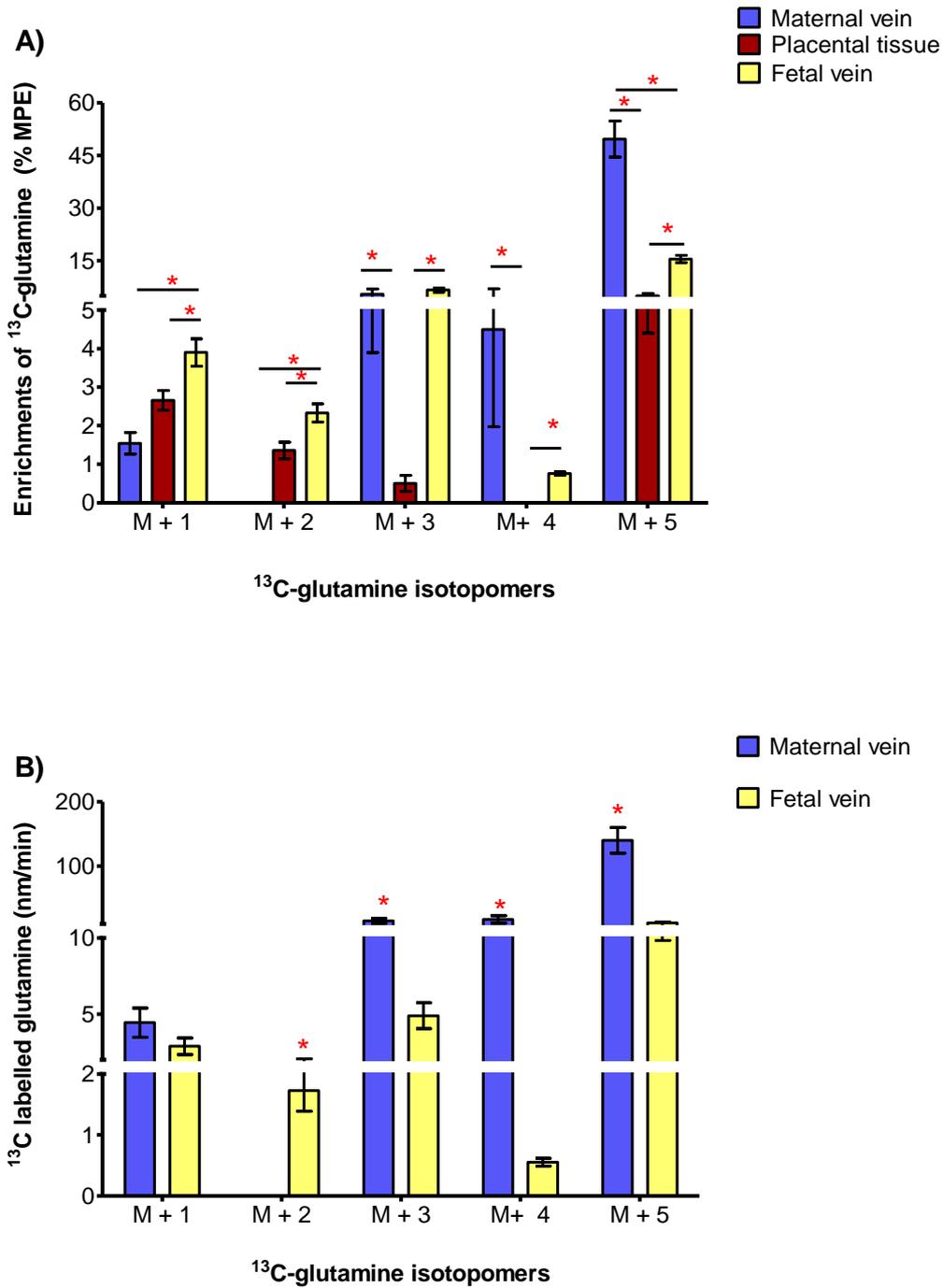
**Figure 4.10: Rate of transfer of BCKAs and pyruvate in the maternal and fetal veins 5 hrs after leucine perfusion into the maternal arterial circulation.** Data are presented as mean  $\pm$  SEM, \*\* indicates P < 0.001 and \* indicates P < 0.05 (n = 5).



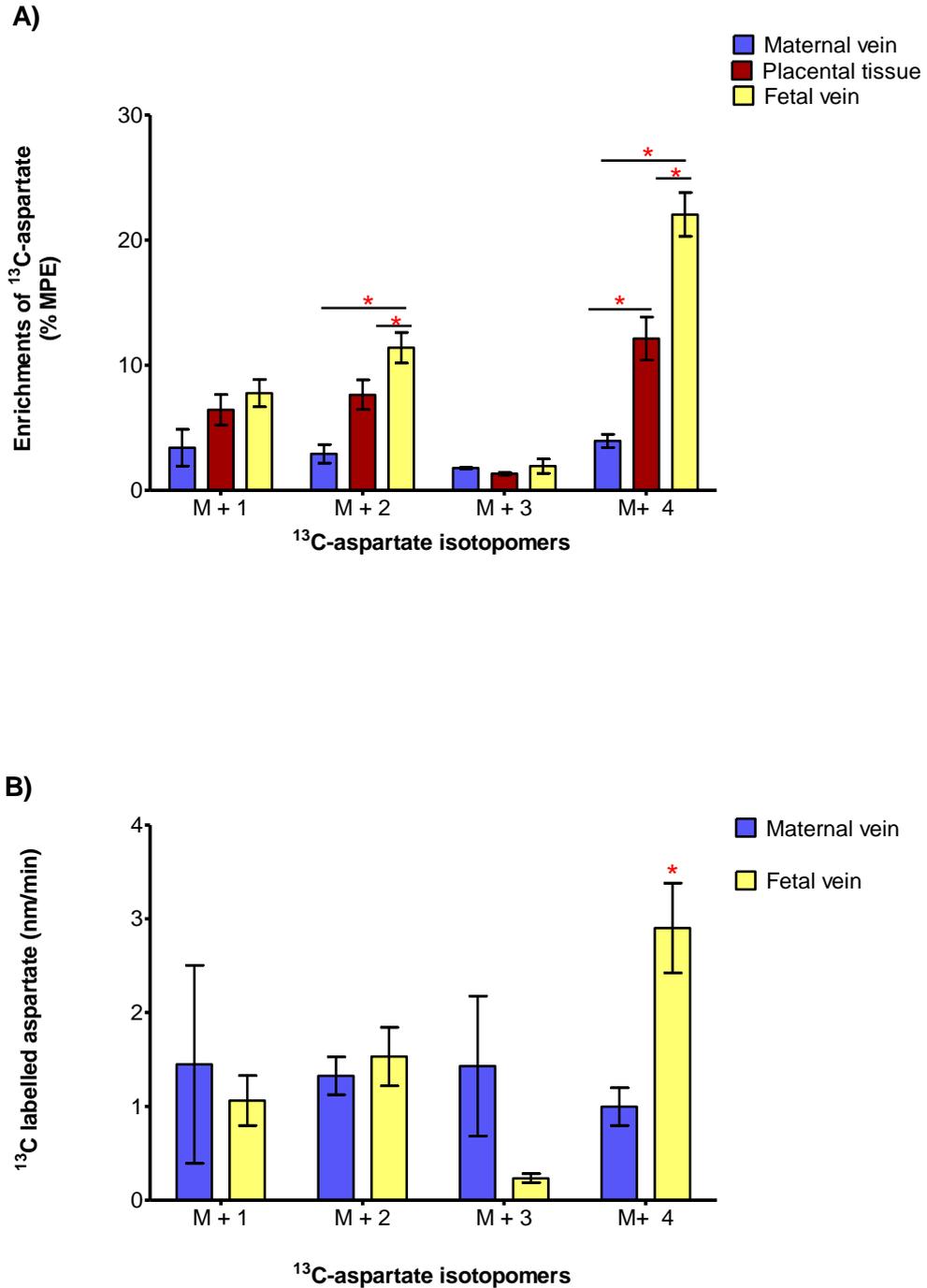
**Figure 4.11: Concentrations of Pyruvate and BCKAs in the placental tissue.** \*\*\*\* indicates difference from all the BCKAs and \*\* indicates significantly different from α-ketoisovalerate and α-keto-β-methylvalerate with ( $P < 0.05$ ). Data are presented as mean  $\pm$  SEM. ( $n = 5$ ).



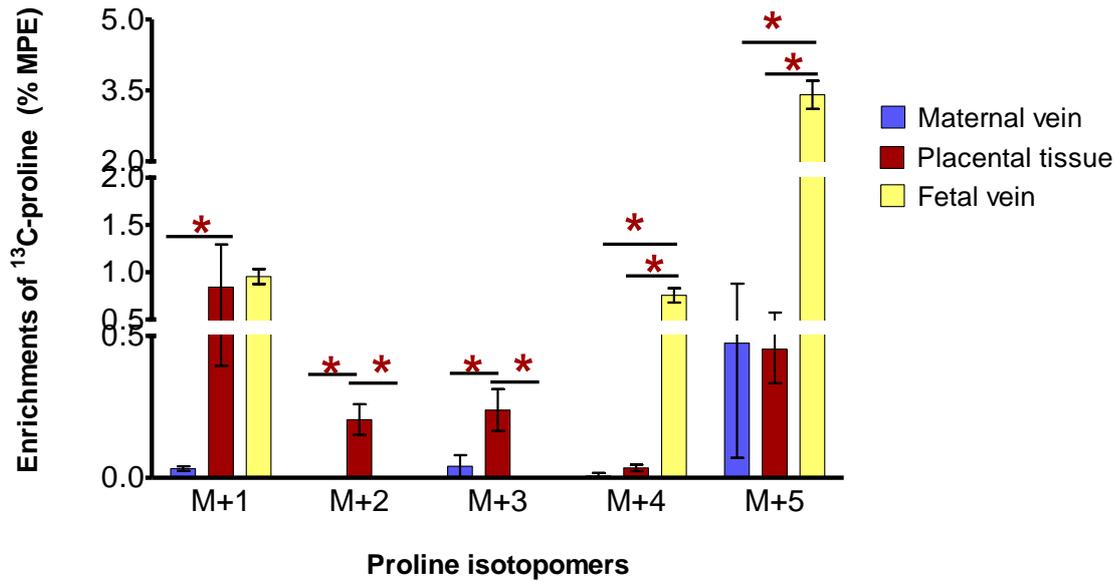
**Figure 4.12: Enrichments and concentrations of  $^{13}\text{C}$  labelled glutamate isotopomers in the fetal arterial  $^{13}\text{C}$ -glutamate perfusion experiments** A) Enrichments of  $^{13}\text{C}$  labelled isotopomers of glutamate in maternal vein, placental tissue and fetal vein after 5 hrs fetal arterial  $^{13}\text{C}$ -glutamate perfusion. B) Concentrations of  $^{13}\text{C}$  labelled glutamate isotopomers in the maternal and fetal vein after 5 hrs fetal arterial  $^{13}\text{C}$ -glutamate perfusion.. Data are expressed as mean  $\pm$  SEM, \* indicates significant difference ( $P < 0.05$ ,  $n = 5$ ).



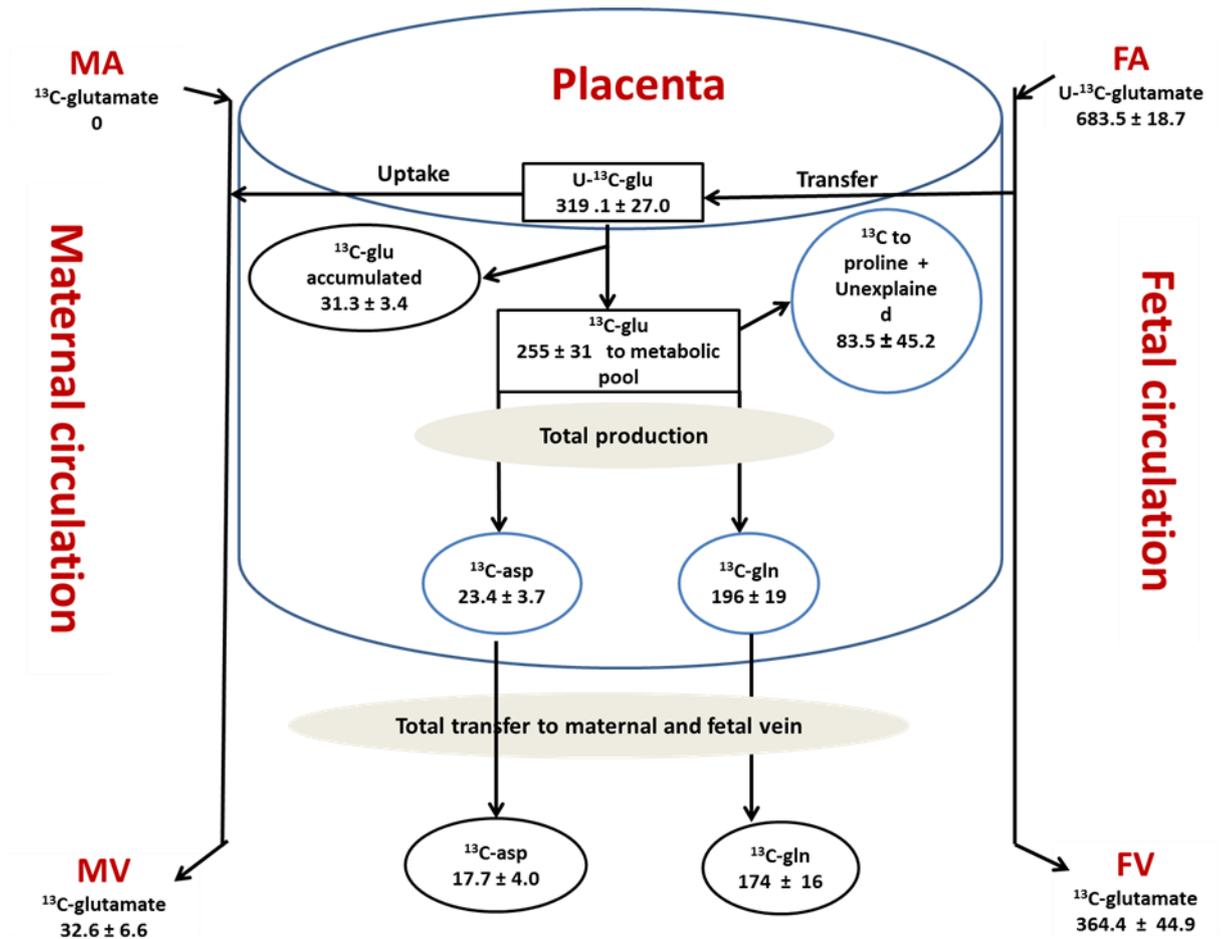
**Figure 4.13: Enrichments and concentrations of glutamine  $^{13}\text{C}$  labelled isotopomers in the fetal arterial  $^{13}\text{C}$ -glutamate perfusion experiments** A) Enrichments of  $^{13}\text{C}$  labelled isotopomers of glutamine in maternal vein, placental tissue and fetal vein after 5 hrs fetal arterial  $^{13}\text{C}$ -glutamate perfusion. B) Concentrations of  $^{13}\text{C}$  labelled glutamine isotopomers in the maternal and fetal vein after 5 hrs fetal arterial  $^{13}\text{C}$ -glutamate perfusion. Data are expressed as mean  $\pm$  SEM, \* indicates significant difference ( $P < 0.05$ ,  $n = 5$ ).



**Figure 4.14: Enrichments and concentrations of <sup>13</sup>C labelled aspartate isotopomers in the fetal arterial <sup>13</sup>C-glutamate perfusion experiments.** A) Enrichments of <sup>13</sup>C labelled isotopomers of aspartate in maternal vein, placental tissue and fetal vein after 5 hrs fetal arterial <sup>13</sup>C-glutamate perfusion. B) Release of <sup>13</sup>C labelled aspartate isotopomers in the maternal and fetal vein after 5 hrs fetal arterial <sup>13</sup>C-glutamate perfusion. Data are expressed as mean  $\pm$  SEM, \* indicates significant difference between maternal and fetal vein ( $P < 0.05$ ,  $n = 5$ ).



**Figure 4.15: Enrichments of  $^{13}\text{C}$  labelled proline isotopomers** in the fetal arterial  $^{13}\text{C}$ -glutamate perfusion experiments. Enrichments of  $^{13}\text{C}$  labelled isotopomers of proline in maternal vein, placental tissue and fetal vein after 5hrs fetal arterial  $^{13}\text{C}$ -glutamate perfusion. Data are expressed as mean  $\pm$  SEM, \* indicates significant difference ( $P < 0.05$ ,  $n = 5$ ).



**Figure 4.16:** The uptake and metabolic distribution to other amino acids of  $^{13}\text{C}$  from U- $^{13}\text{C}$ -glutamate infused in the maternal arterial circulation. MA is maternal artery, MV is maternal vein, FA is fetal artery and FV is fetal vein. Data are expressed as mean  $\pm$  SEM (nmol/min).

## 4.4: Discussion

Studies in this chapter were carried out to investigate the nature of glutamate and leucine metabolism occurring in the normal term human placenta. The nitrogen from both glutamate and leucine was transferred to glutamine and aspartate and to a lesser extent, alanine, valine and isoleucine. It was also demonstrated that the carbon from glutamate is primarily used for glutamine synthesis rather than for the synthesis of other amino acids.

Catabolism of glutamate through the Krebs's cycle was demonstrated, suggesting glutamate is used as an energy metabolite. In addition to producing energy it suggests that there is pyruvate recycling in the human placenta which may be important for the replenishment of intermediary metabolites.

This study provides evidence for the activity of system N amino acid transporter which was previously not thought to be expressed in the human placenta. In addition, contrary to the current literature this study has shown that glutamate and aspartate are transferred out of the placenta, and this suggests the existence of unknown transporters for these amino acids.

This section will first discuss the concentrations of amino acids in the placental pool and this will be followed by a discussion of placental nitrogen metabolism and how this led to further questions on whether the whole carbon skeleton from glutamate is also catabolised in the human placenta. This section will conclude by discussing the implications of the findings of this study for the overall amino acid transfer across the placenta and how this may be related to fetal growth.

### 4.4.1: Placental free amino acid concentrations

The finding that the amino acid concentrations measured in the placentas that were perfused with creatinine to establish extracellular volume are similar to those reported in a previous study, suggest that the placental amino acid extraction and analysis protocols are valid (Phillips *et al.*, 1978). In addition, the similarities between the amino acid concentrations in placentas that were perfused with creatinine and those that were perfused for 5 hrs with glutamate or leucine suggest that the tissue integrity was maintained over the time course

of the experiments and there was negligible leakage of amino acids out of the placenta.

#### **4.4.2: Infusate uptake and transfer by the human placenta**

The similarities in the uptake of glutamate from the maternal and the fetal circulation infused with  $^{15}\text{N}$ -glutamate at a double physiological concentration indicate that the MVM and the BM have a similar capacity for glutamate uptake. While 13% of glutamate taken up from the fetal circulation appeared in the maternal vein, only 0.2% of glutamate taken up from the maternal circulation appeared in the fetal vein. This is consistent with the evidence for bulk flow in the fetal to maternal direction presented in Chapter 3. Taking transfer by paracellular routes into account, the MVM is likely to have the greater total capacity while the BM, with only 1/3 the surface area of the MVM, is likely to have the greatest transporter density. It is clear that both maternal and fetal glutamate are likely to contribute to the placental metabolic pool.

Although the uptake and appearance of leucine could not be compared to the uptake and transfer from the  $^{15}\text{N}$ -glutamate studies, it is clear that there was a high capacity for leucine uptake by the placenta, allowing its entry into the placental metabolic pool.

The percentage placental uptake of  $\text{U-}^{13}\text{C}$ -glutamate infused at a near physiological concentration into the fetal arterial circulation and the percentage appearance observed in this study are similar to the 49% placental uptake and 10% appearance of glutamate observed in a previous study (Phillips *et al.*, 1978). This further validates the perfusion and the analytical methods used in this study.

#### **4.4.3: The metabolism of nitrogen from glutamate and leucine**

The appearance of  $^{15}\text{N}$  from glutamate in glutamine, aspartate, alanine, proline and the BCAAs (leucine, isoleucine and valine) indicates that glutamate taken up from both the maternal and the fetal circulation was involved in aminotransferase reactions in the human placenta. In addition, the appearance of  $^{15}\text{N}$  from leucine suggests that leucine is indeed important for the synthesis of non-essential amino acids with glutamate as a central substrate for nitrogen

exchange. It should be made clear that the appearance of  $^{15}\text{N}$  in other amino acid pools does not necessarily indicate net synthesis as aminotransferase reactions are reversible and there will be equilibration of  $^{15}\text{N}$  through the amino acids involved in these reactions. The net synthesis of amino acids by transamination will be determined by the availability of the relevant keto acids and donor amino acids. The observation that the  $^{15}\text{N}$  nitrogen predominantly appeared in glutamine and aspartate in the glutamate experiments and in glutamate and glutamine in the leucine experiments does however suggest that these may be the most active pathways in the human placenta. Alternatively this may suggest that the pools for the keto acids of these amino acids are higher than those of alanine, and the BCAAs, which had very little  $^{15}\text{N}$  nitrogen transferred to them. This would be in keeping with the low levels of the ketoacids within the placenta and transferred to the maternal and the fetal vein (pyruvate and the BCKAs) observed in this study. The following section will discuss the possible implications of the differential nitrogen transfer to individual amino acids and whether this may have implications for fetal growth.

#### **4.4.3.1: Why is nitrogen from leucine transferred to glutamate?**

The appearance of  $^{15}\text{N}$  in other amino acids from leucine can only occur via the aminotransferase reaction of leucine and  $\alpha$ -ketoglutarate to form  $\alpha$ -ketoisocaproate and glutamate. The glutamate is then metabolised in different pathways to form the amino acids that received nitrogen from leucine (Figure 4.17). Glutamate therefore acts as an intermediate for nitrogen transfer to other amino acids.

#### **4.4.3.2: Why is nitrogen predominantly transferred to glutamine?**

The appearance of  $^{15}\text{N}$  in glutamine from  $^{15}\text{N}$ -leucine as observed in studies in this chapter can occur through glutamate synthesis from leucine as discussed in the previous section. This is then followed by glutamine synthesis by glutamine synthetase which catalyses the incorporation of either labelled or unlabelled ammonia into glutamate. If the unlabelled ammonia is incorporated into labelled glutamate it is the amine nitrogen that is labelled. Alternatively glutamate catabolism by glutamate dehydrogenase may produce  $^{15}\text{N}$  labelled ammonia which may again be incorporated into  $^{15}\text{N}$ -glutamate to form labelled glutamine

with two  $^{15}\text{N}$  labelled nitrogens. In this process both the amine and the amide are labelled. The  $^{15}\text{N}$  labelled ammonia may also be incorporated into unlabelled glutamate and thus only the amide nitrogen would be labelled. Although the amine and amide could not be distinguished in this study, the formation of glutamine also requires the whole glutamate carbon chain.

Previous studies have suggested that non-essential nitrogen is limiting for growth and that, during pregnancy and in growing infants, BCAAs are important for the synthesis of non-essential nitrogen via the production of glutamine (Schneider *et al.*, 1979). The demand for glutamine may be higher than can be provided from the maternal circulation alone and placental synthesis may be important to supply enough glutamine and meet the high demand.

Although the rate of glutamine consumption in the human fetus is not known, in cultured cells the rate of its consumption exceeds that of any other amino acid and it is clear that cultured cells cannot grow without it (Snyderman *et al.*, 1962; Kalhan & Parimi, 2006).

In the fetus, as in other tissues, the amide from glutamine may be used for nucleotide biosynthesis or may be used for the hexamine and glycosylation reactions which are required for protein and lipid modifications prior to their role in signal transduction and trafficking (Eagle, 1955).

Glutamine is also an important signalling molecule during cell growth and it is rate-limiting for the cell growth stimulatory effects induced by leucine via the mTOR pathway (Gaglio *et al.*, 2009; Wu *et al.*, 2001). The stimulation of cell growth by essential amino acids occurs in two stages. First, glutamine must be transferred out of the cell in exchange for an essential amino acid. Once in the cell the essential amino acid then activates the mTOR pathway. The transfer of glutamine to the maternal circulation as observed in this study may therefore be important for the uptake of essential amino acids and their activation of the mTOR pathway. Studies are required to investigate this further as they may provide evidence of mechanisms through which both placental and fetal growth are regulated by amino acid availability.

#### 4.4.3.3: Why is nitrogen from glutamate predominantly transferred to aspartate?

The appearance of  $^{15}\text{N}$  in aspartate from  $^{15}\text{N}$  labelled leucine as observed in studies from this chapter can occur via  $^{15}\text{N}$  labelled glutamate synthesis as described in the previous section. The glutamate so produced can then undergo a reversible aspartate aminotransferase reaction with oxaloacetate to form  $\alpha$ -ketoglutarate and  $^{15}\text{N}$  labelled aspartate (Figure 4.17). It should be noted that as this reaction is reversible, there may not necessarily be net synthesis of aspartate.

Unlike in the glutamate perfusion studies where aspartate had similar enriched concentrations to those of glutamine, in the leucine perfusions, aspartate had very low enriched concentrations. It is unlikely that this could be due to the steps involved in their formation, as in both studies, both glutamine and aspartate are synthesised from glutamate. It is possible that in the leucine study, where there is no glutamate influx, there is preferential utilisation of nitrogen for glutamine synthesis rather than aspartate.

Previous studies have also shown that the transfer of nitrogen to aspartate is reduced in the presence of leucine as some of the leucine is metabolised to acetyl CoA and the entry of acetyl CoA into the Krebs's cycle requires oxaloacetate (Nakajo *et al.*, 2005; Nicklin *et al.*, 2009). This reduces the amount of oxaloacetate that is transaminated to aspartate and as the Krebs's cycle continues, more  $\alpha$ -ketoglutarate is formed and some must be removed by forming glutamate and eventually glutamine, thereby increasing glutamate and glutamine but reducing aspartate (Li *et al.*, 2003). As substrate composition can affect the flux through the different metabolic pathways it will be important for future studies to investigate this and how changes in maternal substrate concentrations affect placental amino acid production and supply to the fetus.

As aspartate is not efficiently transferred to the fetus, the transfer of nitrogen from glutamate to aspartate may be important for purine and nucleotide synthesis within the placenta or it may be used in the aspartate-malate shuttle which is required for the electron transfer from glycolysis (Figure 1.7). As the placenta requires energy for its function, the participation of aspartate in this

shuttle may ensure that there is a continuous replenishment of ATP for placental energy demands.

#### **4.4.3.4: Nitrogen transfer to alanine**

The appearance of  $^{15}\text{N}$  in alanine from  $^{15}\text{N}$  labelled leucine as observed in studies in this chapter occurs via the formation of  $^{15}\text{N}$  labelled glutamate from labelled leucine as described previously and then via a reversible alanine aminotransferase reaction of glutamate and pyruvate to form  $\alpha$ -ketoglutarate and  $^{15}\text{N}$  labelled alanine (Figure 4.17). Whether there is net synthesis of alanine in this reaction, as opposed to equilibration of  $^{15}\text{N}$  through the exchangeable amino N pool via reversible transaminase reactions, will depend on the balance of metabolites within the placenta. For example in a more hypoxic placenta increased pyruvate levels may increase alanine synthesis.

After lysine, more alanine is transferred to the fetus than any other amino acid suggesting it is able to be readily transferred into the fetal circulation (Kelly *et al.*, 2002; Li *et al.*, 2003). The transfer of nitrogen to alanine may ensure that non-essential nitrogen is efficiently transferred to the fetus. In all three studies there was relatively little  $^{15}\text{N}$  transfer to alanine in comparison to glutamine. However, given the relatively large amount of alanine transferred to the fetus *in vivo* it would be interesting to investigate placental alanine production under more physiological conditions. In particular it would be interesting to see if there was more alanine production under more hypoxic conditions, where intracellular pyruvate levels might be higher. In light of the discussion above regarding metabolic control and nutritional status it may also be interesting to determine the effect of insulin and glucagon on the production of alanine in the perfused placenta.

#### **4.4.3.5: Nitrogen transfer to proline**

The appearance of  $^{15}\text{N}$  in proline from  $^{15}\text{N}$  labelled leucine as observed in this study can also occur via the formation of  $^{15}\text{N}$  labelled glutamate from labelled leucine as already described. Glutamate can then undergo transformation to glutamate- $\gamma$ -semialdehyde, by pyrroline 5-carboxylate synthetase which is spontaneously transformed into pyrroline 5-carboxylate and reduced to proline.

Because of its rigid ring structure, proline is an important amino acid for maintaining protein structure and it is particularly important for collagen formation during growth (Cetin *et al.*, 1988). Studies in sheep and pigs have shown that proline is a major substrate for polyamine synthesis and its transfer is altered in human IUGR (Watford, 2008). A previous study has also shown that very small amounts of maternally derived proline are transferred to the fetus and no transporters capable of mediating proline efflux from the BM are known, suggesting that transfer which is observed may occur by paracellular routes (Nicklin *et al.*, 2009; Paolini *et al.*, 2001). Placental proline production from glutamate and leucine observed in studies in this chapter may provide proline required by the fetus for polyamine and collagen synthesis (Cleal & Lewis, 2008; Paolini *et al.*, 2001). While it is not clear how proline would be transported across the BM of the syncytiotrophoblast into the fetus, the appearance of  $^{15}\text{N}$  and  $^{13}\text{C}$  labelled proline in the fetal circulation observed in these experiments demonstrate that this does occur. Further studies quantifying placental proline production and transfer are required to investigate this hypothesis.

#### 4.4.3.6: Transfer of nitrogen to and from BCAAs

The appearance of  $^{15}\text{N}$  in valine and isoleucine, also requires the formation of  $^{15}\text{N}$ -glutamate from  $^{15}\text{N}$  leucine via branched chain aminotransferase reaction. The glutamate is then metabolised to valine and isoleucine by branched chain aminotransferase.

Although BCAAs are essential amino acids, their nitrogen is not essential. It is therefore not surprising that the nitrogen from leucine is transferred to other amino acids and the nitrogen from glutamate is transferred to BCAAs. However, as leucine is an essential amino acid, it is surprising that it is not reserved from catabolism to other amino acids by the placenta.

So why does the placenta degrade BCAAs when it has to produce them again? It should be noted that in all three  $^{15}\text{N}$  studies there was relatively little  $^{15}\text{N}$  transfer from either glutamate or leucine to the BCAAs. However, it seems that the main purpose for BCAA catabolism is to make glutamate which is eventually metabolised to glutamine. As the aminotransferase reactions may not

necessarily mean there is net synthesis, the transfer of nitrogen to BCAAs, may be important for the regulation of nitrogen homeostasis in the maternal, fetal and placental compartments. Previous studies have suggested that the exchange of nitrogen between BCAAs and non-essential amino acids is important for redistributing nitrogen during pregnancy when the need to closely regulate protein accretion and catabolism and nitrogen balance is crucial (Nicklin *et al.*, 2009; Paolini *et al.*, 2001).

It is also possible that once the nitrogen from leucine has been removed, the remaining carbon skeleton may be utilised for energy production via its entry into the Krebs's cycle as acetyl CoA. Evidence for this comes from the observations in this study that the concentrations of BCKAs, especially that of  $\alpha$ -keto-isocaproate in the maternal vein, fetal vein and the placenta were very low, although leucine was infused at a double physiological umbilical concentration. This is in contrast to studies in porcine placentas where BCKAs are released into the fetal tissue where they are thought to be converted back to their respective amino acids thus providing an alternative route for BCAA transfer to the fetus (Kalhan & Parimi, 2006). As the human placenta has a high capacity to transfer BCAAs to the fetus, the pathways for BCKAs reamination in the fetal tissue may not be necessary (Jozwik *et al.*, 1999). The pattern observed for the BCKAs and pyruvate is similar to that of lactate observed in the previous chapter, in that there are higher concentrations of these substrates being released to the maternal than the fetal circulation

Overall, the nitrogen studies have demonstrated that nitrogen from leucine is transferred to glutamate and redistributed to a range of amino acids, but predominantly to glutamine. However, as proline concentrations could not be determined, it is not clear to what extent nitrogen from both glutamate and leucine is used for proline synthesis. In addition, about 82% of the nitrogen from leucine and glutamate could not be accounted for and thereby suggesting that there are other substrates that received nitrogen from glutamate or leucine. It is possible that glutamate perfused or formed from leucine may have been catabolised to ammonia, arginine, citrulline and glutathione which were not

measured in this study. Future studies should therefore aim to investigate the fate of the nitrogen that was unaccounted for in this study.

Having established the metabolic pathways for placental metabolism of nitrogen from glutamate and leucine, the next step was to confirm that there is net synthesis of glutamine and proline and whether there is net synthesis of amino acids from glutamate.

#### **4.4.4: U-<sup>13</sup>C-glutamate is used for amino acid synthesis and intermediary metabolism**

The enrichment of M + 5 labelled glutamine and proline observed in this study suggest that U-<sup>13</sup>C-glutamate was directly metabolised to form glutamine and proline while the multiple labelling of various isotopomers of glutamine, proline and aspartate suggest that catabolism of glutamate was also occurring via the Krebs's cycle (Figures 4.17 & 4.18). In addition, the enrichments of M + 4 glutamine and proline and that of M + 3 aspartate suggest the carbon from glutamate was used for pyruvate synthesis which re-entered the cycle via acetyl CoA in a process called pyruvate recycling (Figure 4.17). The expected labelling patterns for glutamate, glutamine, proline and aspartate in different rounds of the Krebs's cycle are discussed in the following sections and shown in Figure 4.18.

##### **4.4.4.1: Direct synthesis of glutamine and proline**

U-<sup>13</sup>C-glutamate (M + 5) can be catabolised via an irreversible reaction involving glutamine synthetase to form M + 5 labelled glutamine observed in this study (Figure 4.18) (Paolini *et al.*, 2001). Alternatively, U-<sup>13</sup>C-glutamate can be catabolised via another irreversible reaction involving pyrroline-5-carboxylate synthase to form  $\gamma$ -glutamate semialdehyde which is eventually metabolised by pyrroline-5-carboxylase to form M + 5 labelled proline observed in this study (Olstad *et al.*, 2007a). For the other isotopomers of glutamine, proline and aspartate to be formed, glutamate must enter the Krebs's cycle as  $\alpha$ -ketoglutarate.

#### 4.4.4.2: Metabolism of $^{13}\text{C}$ -glutamate in the first round of the Krebs's cycle

To enter the Krebs's cycle, U- $^{13}\text{C}$ -glutamate must first be deaminated by glutamate dehydrogenase or aminotransferases to form  $\alpha$ -ketoglutarate (Bicknell *et al.*, 2008). The  $\alpha$ -ketoglutarate then enters the Krebs's cycle where the labelled carbon skeleton is utilised in the cycle until it is catabolised to  $\text{CO}_2$  or leaves the cycle via a different pathway (Figure 4.17).

Once in the Krebs's cycle  $\alpha$ -ketoglutarate (M + 5) is oxidatively decarboxylated to uniformly labelled succinyl-CoA, which is eventually converted to M + 4 labelled succinate, fumarate, malate and oxaloacetate. Oxaloacetate can be transaminated to M + 4 labelled aspartate and leave the cycle or it can condense with unlabelled acetyl CoA to form M + 4 labelled citrate (Olstad *et al.*, 2007a). This is metabolised to form isocitrate and the cycle completes with the conversion of isocitrate to M + 3 labelled  $\alpha$ -ketoglutarate. M + 3 labelled  $\alpha$ -ketoglutarate can then be transaminated to M + 3 labelled glutamate, which is either metabolised to M + 3 labelled glutamine or proline, observed in this study.

#### 4.4.4.3: Metabolism of $^{13}\text{C}$ -glutamate in the second round of the Krebs's cycle

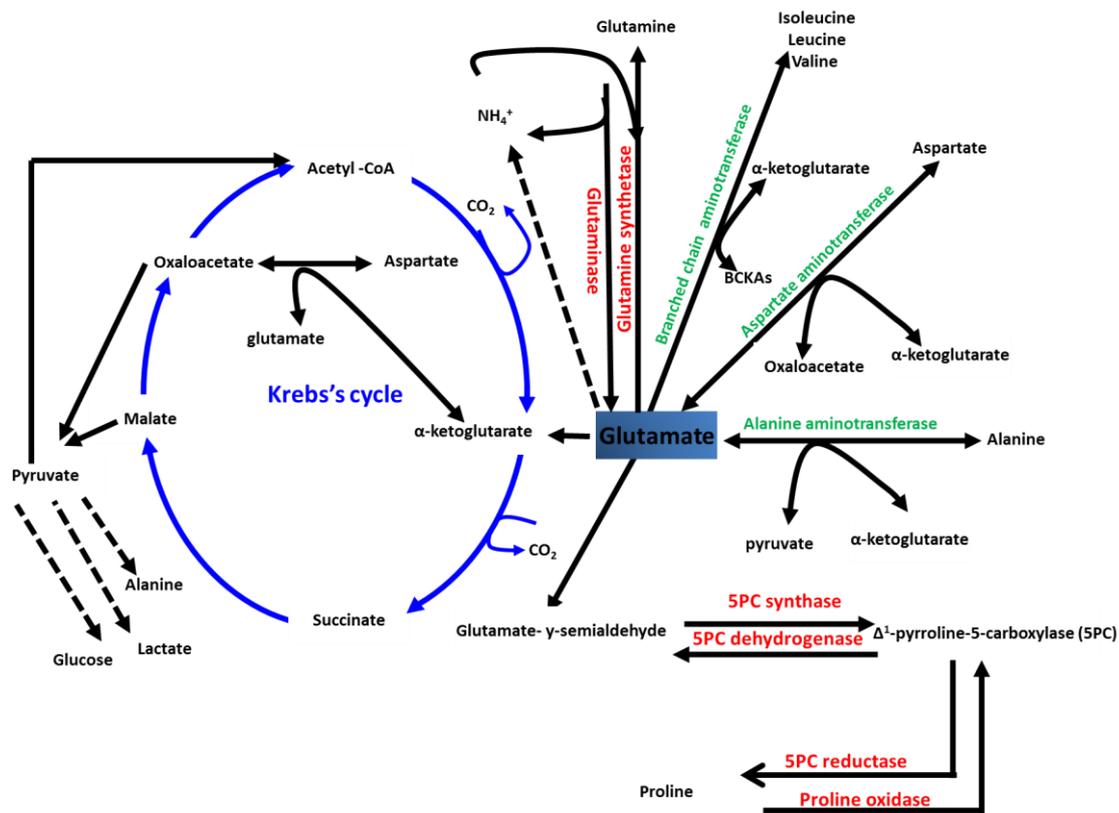
The carbon skeleton of glutamate can remain within the Krebs's cycle for a second cycle, during which some of the M + 3  $\alpha$ -ketoglutarate is decarboxylated to two forms of M + 2 succinyl-CoA; one with labelled carbon at position one and two and the other with the labelled carbon at positions three and four. The two forms of labelled succinyl-CoA are metabolised to oxaloacetate, which is transaminated to form the two forms of M + 2 aspartate as observed in this study. The M + 3 oxaloacetate can also continue in the cycle and form M + 3 labelled citrate and eventually M + 2 and M + 1  $\alpha$ -ketoglutarate which is metabolised to M + 2 and M + 1 glutamate, glutamine and proline as observed in this study.

The two forms of M + 2 labelled aspartate as shown in Figure 4.18 are formed due to the symmetrical structure of succinyl-CoA. It is thought that the symmetrical labelling may occur due to reorientation of these molecules in solution. However studies have suggested that there may not be reorientation in

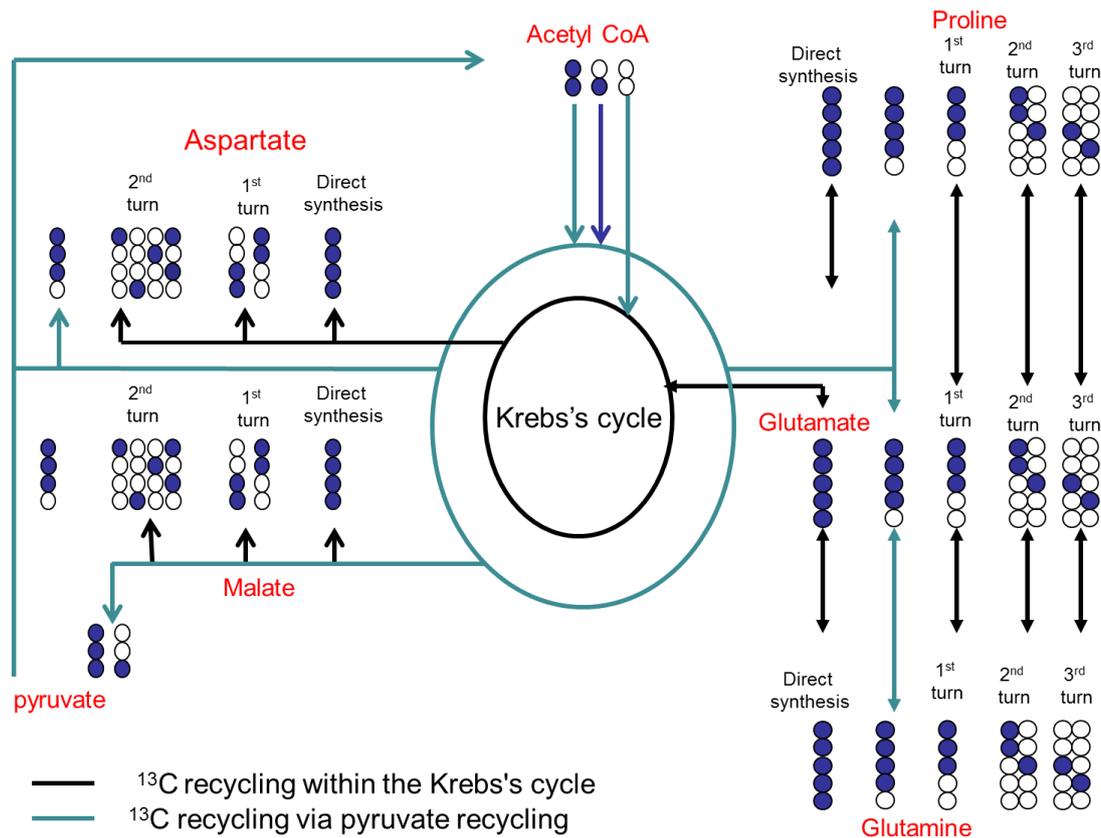
solution meaning that only one form of labelling should be expected (Olstad *et al.*, 2007a). This could not be investigated in our study using GC-MS.

#### 4.4.4.4: Metabolism of $^{13}\text{C}$ -glutamate in the third round of the Krebs's cycle

The  $\text{M} + 1/\text{M} + 2$   $\alpha$ -ketoglutarate can still continue in the third cycle and subsequently form singly labelled oxaloacetate and aspartate, or continue in the cycle to form  $\text{M} + 1$  glutamate (Sherry *et al.*, 1994). Glutamate is then metabolised to  $\text{M} + 1$  glutamine and proline as observed in this study.



**Figure 4.17: A summary of metabolic pathways involved in the metabolism of  $^{13}\text{C}$  glutamate,  $^{15}\text{N}$ -glutamate and  $^{15}\text{N}$ -leucine in the normal term human placenta. The dashed lines represent metabolic pathways that are possible but were not evident or not measured in this study.**



**Figure 4.18: Simplified schematic of Krebs's cycle for U-<sup>13</sup>C-glutamate metabolism** showing the pattern of labelling in glutamate, glutamine, proline and aspartate during three turns of the Krebs's cycle.

#### 4.4.4.5: Pyruvate recycling

An important aspect of this study was the evidence supporting the existence of pyruvate recycling within the human placenta. The M + 3 labelling of aspartate and M + 4 labelling of glutamate, glutamine and proline observed in this study can only occur if there is pyruvate recycling (Olstad *et al.*, 2007a). For pyruvate recycling to occur, labelled carbon must leave the Krebs's cycle and re-enter as acetyl CoA (Figure 4.18). Labelled pyruvate may be formed from any of the labelled forms of malate via pyruvate-malate carboxylase or from oxaloacetate to phosphoenolpyruvate through pyruvate carboxykinase and pyruvate kinase. This pyruvate is then metabolised by the pyruvate dehydrogenase complex to form NADH and acetyl CoA with different labels. The different forms of acetyl CoA may then form different forms of labelled Krebs's cycle intermediates and amino acids. It is noteworthy that other forms of labelled isotopomers rather than just the M + 3 aspartate and M + 4 glutamate, glutamine and proline are

formed from pyruvate recycling, but they cannot be distinguished from those formed in the absence of pyruvate recycling using GC-MS.

Pyruvate recycling was first observed in lactate metabolism studies carried out in cerebral tissues (Olstad *et al.*, 2007a). It is thought that for efficient oxidation of glutamate, pyruvate must be formed from Krebs's cycle intermediates and re-enter via acetyl CoA (Cerdan *et al.*, 1990). Studies using cultured neurons have demonstrated that pyruvate recycling is an important process which is predominant when glucose levels are low and therefore utilising amino acids for energy (Olstad *et al.*, 2007a). Pyruvate recycling in the human placenta may therefore be important during hypoglycaemia to ensure continual energy production. Further studies are required to investigate this.

Overall, carbon from glutamate was predominantly used for glutamine synthesis. Only 14% of the infused  $^{13}\text{C}$  could not be accounted for and it is likely that this 14% may have gone to proline or been utilised in the Krebs's cycle for energy.

#### **4.4.4.6: Lack of evidence for $^{13}\text{C}$ incorporation into alanine and glucose**

This study suggests that pyruvate is formed from labelled glutamate in the human placenta. It is, however, surprising that there was no evidence for incorporation of labelled carbon into alanine and glucose.

In the presence of pyruvate, alanine can be formed through transamination reaction with glutamate. As shown in the nitrogen study, alanine transamination does occur in the human placenta. It may be that there was incorporation into alanine but that the high intracellular alanine concentrations ( $\approx 2$  mmol/L) mean that it was too dilute to detect. This is unlikely, since the intracellular concentrations of alanine in the  $^{15}\text{N}$  perfusion studies, where alanine was enriched, were similar to those in this study.

Another explanation for the lack of evidence for the incorporation of  $^{13}\text{C}$  into alanine and glucose may be that pyruvate recycling is metabolically preferred over the formation of alanine and glucose to aid complete oxidation of glutamate in the Krebs's cycle. Preferential utilisation of carbon in the Krebs's

cycle may be an efficient way of utilising glutamate's carbon for energy, as the formation of glucose would require energy.

In several mammalian tissues the conversion of pyruvate to acetyl CoA or glucose is also subject to regulation by cellular glucose and energy status. When glucose levels are high as in the human placenta, pyruvate dehydrogenase is active and pyruvate conversion to acetyl CoA is preferred to aid the utilisation of glucose for energy (Amaral *et al.*, 2011; Cleal & Lewis, 2008). However, when glucose levels are low, fatty acid oxidation occurs to produce energy, with acetyl CoA being one of their intermediate metabolites. Once produced, acetyl CoA inactivates pyruvate dehydrogenase and activates pyruvate dehydrogenase kinase which conserves pyruvate for glucose synthesis. It is not clear whether these processes operate in the placenta to regulate amino acid metabolism and glucose availability. Further studies are required to investigate whether placental amino acid metabolism is subject to such regulation and whether hormones that are involved in glucose regulation such as insulin and glucagon affect these pathways.

#### **4.4.5: Evidence for metabolic compartmentalisation**

The enrichments of some amino acids released from the placenta were greater than their enrichments in the placenta. For example, depending on the experiment, the enrichment of labelled glutamine released into the circulation was from twice (maternal  $^{15}\text{N}$ -glutamate and fetal  $^{13}\text{C}$ -glutamate experiments) to five times (fetal  $^{15}\text{N}$ -glutamate experiments, maternal  $^{15}\text{N}$ -leucine experiments) the average placental enrichment. This suggests metabolic compartmentalisation within the placenta as the enrichment of amino acids released from a cell cannot be greater than that within the cell (in the absence of a selective transport mechanism). However there could be a cellular compartment within the placenta where amino acid enrichment was greater than in other compartments. If this was the case even though the average cellular enrichment might be low (what is measured in placental homogenate) if amino acids were primarily released from the more highly enriched compartment, then they could have a greater enrichment than in the placenta as a whole.

Compartmentalisation could occur at the cellular level, with some cell types having high enrichment and others not, or within cellular compartments such as mitochondria. Placental cell types include cytotrophoblast, fibroblasts, macrophages, fetal endothelial capillary cells and the placental syncytiotrophoblast which is one big cell. It would seem most likely that the metabolism was occurring within the syncytiotrophoblast as there is a high concentration of glutamine being transferred to the maternal circulation which is closer to the syncytiotrophoblast.

Given that the syncytiotrophoblast is a significant proportion of total placental cell volume (which contributes to 19.5 % of the total placental volume) there would need to be metabolically distinct compartments within the syncytiotrophoblast and it is difficult to see how this would occur within a true syncytium (Wu *et al.*, 1999). If this could be demonstrated it would suggest a much more nuanced view of syncytial function than currently exists. Alternatively, compartmentalisation could occur within a cellular compartment such as the mitochondria. If this were the case the subcellular compartment would need to contain large amounts of the non-enriched amino acid. This is because, for efflux from the cell to occur, the enriched amino acid must enter the cytosol and if it were coming from a subcellular compartment it would be diluted.

While these data do suggest metabolic compartmentalisation, the possibility of an experimental artefact needs to be considered. For instance, if the cotyledons were only partially perfused then non-enriched glutamine in the non-perfused region would dilute the enriched glutamine from the perfused regions when the cotyledon was homogenised. Yet if this were the case the same pattern would be expected for most amino acids but other amino acids, such as aspartate, did not follow this pattern of enrichment.

If this compartmentalised metabolism is occurring within the syncytiotrophoblast the localisation of the metabolically active compartment may suggest its regulation. If the compartment is on the fetal side of the placenta it may suggest the mechanism of regulation by the fetal factors, whereas if it is within the syncytiotrophoblast it would be more available to regulation by maternal signals.

Amino acid metabolic compartmentalisation may be important for the regulation of amino acid transfer between the mother and the fetus and further studies could be carried out to investigate what these compartments and their role may be. In the liver and the brain, metabolic compartmentalisation has been observed for glutamine and glutamate and it is thought to be important for the homeostasis of nitrogen metabolism (Sala *et al.*, 1983).

#### **4.4.6: Evidence for novel amino acid efflux mechanisms in the placenta**

Although experiments in this chapter were focused on amino acid metabolism they have also provided novel information about amino acid transport. In the <sup>13</sup>C-glutamate experiment where serine as a known substrate that exchanges with glutamine was not infused into the fetal arterial circulation; there was glutamine transfer to the maternal and fetal circulation. Previous studies have not identified efflux transporters for glutamate, aspartate or proline so these would not have been expected to be observed in the circulation (Stoll *et al.*, 1991; Haussinger *et al.*, 1989; Schousboe *et al.*, 1993). In addition previous studies in our laboratory have suggested that glutamine efflux to the fetal circulation can only occur in the presence of a substrate for exchange (Cleal *et al.*, 2011). However in this study efflux of labelled glutamine was observed in the absence of substrates for exchange. This therefore suggests that there may be additional amino acid efflux transporters that are responsible for transferring glutamine, glutamate, aspartate and proline out of the placenta.

##### **4.4.6.1: Glutamine efflux may occur via system N**

This study has demonstrated that there is glutamine efflux to the maternal and fetal arterial circulation. Efflux of glutamine was not expected based on known amino acid transporters in the placenta. There is a transport system known to mediate the efflux of glutamine, system N, but this was not thought to be expressed in the human placenta (Cleal *et al.*, 2011). However, further studies in our laboratory following this observation have demonstrated that mRNAs for the two system N amino acid transporters, SLC38A3 and SLC38A5 are expressed in the human placenta and the protein for SLC38A5 is localised to the MVM and BM of placental syncytiotrophoblast by western blotting (Personal

communication, Emma Field, Southampton). The presence of System N in the placenta may also explain previous observations that there is a sodium dependent and system A independent component of serine uptake which mediates the majority of serine uptake in villous fragments but a very small proportion in MVM vesicles (Nakanishi *et al.*, 2001). As system N exchanges amino acids and  $\text{Na}^+$  for  $\text{H}^+$  it seems likely that the explanation for the difference in serine uptake between fragments and MVM vesicles is that in fragments cellular metabolism is producing  $\text{H}^+$  which drives system N activity. System N also transports asparagine of which transport to the fetus could not be explained by the model proposed by Cleal and Lewis (Brand *et al.*, 2010).

#### 4.4.6.2: Unexplained efflux of glutamate aspartate and proline

The fact that aspartate, glutamate and proline are released from the placenta is an unexpected finding since there is no clearly identified mechanism by which this might occur (Cleal *et al.*, 2011). As such it is not clear how this efflux was mediated. There are several possibilities; one is that they are relatively low affinity substrates of a known transporter, another that they are transported by a novel transporter; alternatively the lower efflux of aspartate from the placenta in the leucine perfusions compared to the glutamate perfusions is consistent with the hypothesis that glutamate might be exchanging for aspartate. A recent study following these observations has suggested that glutamate may exchange for itself and cysteine via amino acid exchanger system  $\text{X}_C^-$  (Personal communication, Emma Field, Southampton) but this would not explain the efflux of aspartate or proline.

Aspartate, glutamate and proline are reported to be low affinity substrates of the exchanger ASC (Cleal & Lewis, 2008) and it is possible that their efflux may be mediated by this system. The high intracellular levels of glutamate and aspartate may help overcome their low affinity for the transporter. However, as system ASC transporters are obligate transporters, if the efflux of these amino acids is mediated via this route it is not clear what they are exchanging for.

Another possible route for aspartate and glutamate efflux is via volume gated cation channels which have been reported in trophoblastic culture systems (Dall'Asta *et al.*, 1983). This may especially be true as placental amino acid

uptake strongly correlates with their consumption by the placenta, rather than their transfer across the placenta (Appendix 3, Figures IV to IX). If this were the case it may suggest that these amino acids are involved in cell volume regulation.

Whatever the route by which these amino acids are transferred out of the placenta, these data demonstrate that we do not yet have a complete understanding of the mechanisms underlying placental amino acid transfer.

#### **4.4.7: Summary**

Studies in this chapter were carried out to investigate the nature and extent of glutamate and leucine metabolism occurring in the normal term human placenta and whether metabolites of glutamate and leucine are transferred across the placenta. Overall, the three studies have demonstrated that glutamate is central to nitrogen metabolism and that BCAAs are catabolised by the placenta to produce non-essential amino acids. As non-essential nitrogen is limiting for growth, it is possible that placental amino acid metabolism may play a role in providing this non-essential nitrogen. However, more studies are required to investigate this further and explore which other amino acids may be important for supplying this non-essential nitrogen.

While previous studies were unable to show the fate of nitrogen from glutamate, this study has shown that there are a range of metabolic pathways through which nitrogen from glutamate is transferred to other amino acids, each with varying capacities. This study has further demonstrated that the carbon skeleton from glutamate is mainly used for glutamine synthesis and to a lesser extent for energy metabolism. For the first time, this study also suggests that there is pyruvate recycling in the human placenta which may be important for the replenishment of the Krebs's cycle intermediates. In addition, it has highlighted important aspects of amino acid transport across the placenta that may require further investigation. Understanding amino acid metabolic pathways in the human placenta is important, as the metabolic pathways shown to be active in this chapter may be altered in poor fetal growth. Future studies may therefore aim to investigate whether these pathways are indeed altered in poor fetal growth and whether intervention can be sought by supplying the

amino acids that are metabolised in the human placenta, in order to increase those required by the fetus.

As metabolic genes involved in nitrogen metabolism are altered at transcription level in response to the availability of good or bad nitrogen sources, the next chapter investigated whether genes involved in nitrogen metabolism and transfer, as observed in this chapter, may be subject to maternal influences and whether this may be associated with fetal growth parameters.

## **Chapter 5**

### **Associations between maternal factors, placental mRNA levels and fetal growth parameters**



## 5.1: Background

Placental function is subject to regulation by both maternal and fetal signals. The mother may be able to limit fetal growth in line with her ability to support the pregnancy, a process referred to as maternal constraint (Levitan & Garber, 1998). However the signals and mechanisms underlying maternal constraint are not clear. Recent work has suggested that maternal muscle mass may be a determinant of placental amino acid transport capacity (Gluckman & Hanson, 2004b). It has been hypothesised that maternal factors such as muscle mass may act as measures of maternal nutrition and the mother's ability to support the pregnancy. In this chapter the relationships between maternal factors such as diet, body composition and lifestyle are investigated in relation to mRNA levels of genes in the placenta related to amino acid transport and metabolism.

There is evidence that placental function is influenced by both maternal and fetal signals (Lewis *et al.*, 2010). Maternal signals to the placenta will reflect the mother's nutritional status and her capacity to support fetal growth, while fetal signals will reflect its metabolic demands and growth capacity. In response to these signals, the placenta will determine nutrient partitioning between the mother and the fetus and therefore fetal growth outcomes.

For the fetus to achieve its genetic growth potential the placenta must supply it with sufficient nutrients. While optimal growth in the womb is associated with better health in adult life there may be situations where it is better for the mother and/or the fetus if fetal growth is limited. In these circumstances the level of nutrient transfer across the placenta may be limited to reduce the growth of the fetus. One obvious reason is that a large fetus may have difficulty being born, particularly to a smaller mother, endangering both the mother and the fetus. A second reason is that if the mother is living in an environment where food is scarce being smaller may be an advantage. Growing slowly means that the fetus and the baby will require less food and so will place fewer demands on the mother. It has been shown that very slow growing fetuses are better able to adapt to maternal nutrient restriction (Constancia *et al.*, 2005; Ericsson *et al.*, 2007).

There is evidence that the capacity of the placenta to transport nutrients is regulated by maternal hormones and plasma nutrient levels (Harding & Johnston, 1995). There is also evidence that the placental nutrient transport capacity can be regulated in response to nutrient levels in the maternal blood. While plasma nutrient levels will indicate a short term maternal nutritional status, maternal body composition may indicate long term maternal nutritional status (Shibata *et al.*, 2006; Jansson *et al.*, 2003; Giovannelli *et al.*, 2011). Supporting this is a recent study that has demonstrated that placental system A amino acid transporter activity may be influenced by maternal body composition (Lewis *et al.*, 2006). Maternal nutrient levels may be sensed by the placenta via mammalian target of rapamycin (mTOR), through mechanisms involving hormones (Lewis *et al.*, 2010).

One way in which the placenta may adjust its capacity to transfer nutrients is through changes in gene expression. For example the mTOR pathway, which is sensitive to nutritional changes, has been shown to regulate genes involved in nitrogen metabolism through multiple steps involved in mRNA processing (Roos *et al.*, 2009). As changes in placental mRNA levels may be more effective for long term adaptations, it is important to investigate whether maternal factors such as diet, body composition and lifestyle affect placental mRNA levels of genes involved in nitrogen metabolism and transfer.

Studies in mice have also provided evidence that placental function may be determined by fetal signals (Cardenas *et al.*, 1999; Kilberg *et al.*, 2005). Alterations in placental mRNA expression levels may therefore be reflected in fetal growth parameters. Alternatively, fetal signals may also influence placental mRNA levels. It is therefore important to determine whether there are associations between placental mRNA levels and fetal growth parameters.

The aim of this chapter was therefore to carry out an exploratory investigation of whether maternal factors are associated with placental mRNA levels of genes involved in nitrogen transfer to the fetus. In addition, this chapter also investigated whether placental mRNA levels of genes involved in nitrogen transfer are associated with fetal growth parameters.

## 5.2: Methods

Placentas had been collected previously from the Southampton Women's Survey (SWS) as described in Chapter 2. The SWS team collected data on the women, their partners and babies. In this thesis mRNA levels were measured using quantitative rtPCR and expression was normalised using the geometric mean of three housekeeping genes.

### 5.2.1: Gene expression analysis

For RNA studies 102 SWS placentas were selected from 300 collected in total, based on availability of neonatal Dual-emission X-ray absorptiometry (DXA) data and previous measurement of system A activity on those placentas. Messenger RNA expression analyses were carried out as described in Chapter 2; briefly, RNA extraction and cDNA synthesis was carried out using a group of 102 SWS placentas. At the time of collection, 5 samples of placental villous tissue were collected from each placenta, using a stratified random system, and snap frozen. The 5 tissue samples from each placenta were powdered together in a frozen press and total RNA was extracted from 30 mg powdered tissue using the RNeasy Fibrous Tissue Mini Kit (Qiagen, Crawley, West Sussex, UK) according to the manufacturer's instructions. 0.2 µg RNA was reverse transcribed as explained in Chapter 2 and according to the manufacturer's instructions. As initial experiments showed large inter-batch variation between batches of cDNA, to ensure consistency all samples to be analysed were converted to cDNA on the same day using the same reagent master mix. This was followed by quantification using real-time quantitative reverse transcriptase PCR as described in Chapter 2. Tables 5.1 and 5.2 show the oligonucleotide probes and primers designed for the genes of interest using the Roche ProbeFinder v. 2.45 for humans. Probes were supplied by Roche from the human universal probe library and primers were synthesised by Eurogentec (Seraing, Belgium).

**Table 5.1: Primers and probes used for amino acid transporter genes**

Gene	Genebank accession number	Primers	Roche universal probe library number	Intron spanning region (base pairs)
<i>LAT1</i> ( <i>SLC7A5</i> )	NM_003486.5	F- 5'-gtggaaaaacaagcccaagt-3' R- 5'-gcatgagcttctgacacagg-3'	25	1493 -1583
<i>LAT2</i> ( <i>SLC7A8</i> )	NM_182728.1 NM_012244.2	F - 5'-ttgccaatgtcgcttatgtc-3' R - 5'-ggagctctctccaaaagtac-3'	17	1561 - 1667 470 - 576
<i>ASC1</i> ( <i>SLC1A4</i> )	NM_003038.2	F - 5'-ttgcgacagcattgtctac-3' R - 5'-gcactcatcatagaggaagg-3'	78	345 – 737 1318 - 1410
<i>ASC2</i> ( <i>SLC1A5</i> )	NM_005628.2 NM_001145144.1 NM_001145145.1	F - 5'-gaggaatatcaccggaacca-3' R – 5'-aggatgttcatcccctcca-3'	43	1240 – 1325 104 – 189 294 – 379
<i>y+LAT1</i> ( <i>SLC7A7</i> )	NM_001126105.1 NM_001126106.1 NM_003982.3	F - 5'-acactgccgtgagaacctg-3' R – 5'-aggagaggaaaccttcacc-3'	72	117 – 212 252 - 347 496 - 591
<i>y+LAT2</i> ( <i>SLC7A6</i> )	NM_001076785.1 NM_003983.4	F - 5'-gctgtgatccccatacct-3' R – 5'-ggcacagttcacaatgtcag-3'	66	685 -781 772-868
<i>4F2HC</i> ( <i>SLC3A2</i> )	NM_001012661.1 NM_001012662.1 NM_002394.4 NM_001012663.1 NM_001012664.1 NM_001013251.1	F – 5'-tggttctcactcaggtga-3' R -5'-cagccaaaactccagagcat-3'	49	1241 – 1341 1151 – 1251 1148 – 1248 1055 – 1155 962 – 1062 830 - 930
<i>EAAT1</i> ( <i>SLC1A3</i> )	NM_004172.4	F- 5'-ttgaactgaactcggacaaatta-3' R- 5'-attccagctgcccataact-3'	76	1722 - 1814
<i>EAAT2</i> ( <i>SLC1A2</i> )	NM_004171.3	F - 5'-aaaatgctcattctccctaatc-3' R –5'-gccactagccttagcatcca-3'	78	850 - 944
<i>EAAT3</i> ( <i>SLC1A1</i> )	NM_004170.4	F- 5'-agttgaatgacctggacttg-3' R –5 gcagatgtggccgtgatac-3'	9	1374 - 1457
<i>EAAT4</i> ( <i>SLC1A6</i> )	NM_005071.1	F - 5'-tgcagatgctggtgttacct-3' R –5'- gttgtccagggatgccata-3'	19	297 - 381
<i>EAAT5</i> ( <i>SLC1A7</i> )	NM_006671.4	F – 5'-cgcccaggtcaacaactac-3' R –5'-gctgcagtggtgtgatact-3'	9	1334 - 1422

*Note: The rest of the transporters found in the placenta and not investigated in this study are shown in figure 1.2.*

**Table 5.2: Primers and probes used for amino acid metabolic enzyme genes**

Gene	Genebank accession number	Primers	Roche universal probe library number	Intron spanning region (base pairs)
Branched chain aminotransferase cytosolic EC 2.6.1.42	NM_005504.5	F – 5'-gatgttgctctggtacagc-3' R –5'-ggaccattctccatagttgaa -3'	61	1413 - 1528
Glutaminase (GLS1) kidney isoform EC 3.5.1.2	NM_014905.3	F –5'- tgcagagggatcatgtgaag-3' R – 5'-catccatgggagtgattacc -3'	11	2036 - 2150
Glutamine synthetase ( <i>GLUL</i> ) EC 6.3.1. 2	NM_002065.4 NM_001033044 .1 NM_001033056 .1	F – 5'-ccataccaactcagcacca-3' R –5'-caatggcctcctcgatga-3'	52	1252 – 1359 896 – 983 956 - 1045
Glutamate dehydrogenase ( <i>GLUD</i> ) mitochondrial EC 1.4.1.3	NM_005271.2 NM_012084.3	F –5'- cactctgctggcatacac-3' R – 5'-tcaggtccaatcccaggta -3'	76	1606 - 1710
Alanine aminotransferase 1 ( <i>ALT1</i> ) EC 2.6.1.2	NM_005309.2	F – 5'-catagtcagcagccttg-3' R – 5'-ggatgacctcggtgaaagg -3'	15	333 - 426
Alanine aminotransferase 2 ( <i>ALT2</i> ) EC 2.6.1.2	NM_133443.1	F –5'- ggatcttattcctgccaaa-3' R – 5'-acatgtctggagccattga -3'	75	1415 - 1497
Aspartate aminotransferase 1 ( <i>AST1</i> ) Cytoplasmic EC 2.6.1.1	NM_002079.1	F – 5'-caactgggattgaccaact-3' R – 5'-ggaacagaaaccggtgctt -3'	38	601 - 692
Aspartate aminotransferase 2 ( <i>AST2</i> ) Mitochondrial EC 2.6.1.1	NM_002080.2	F – 5'-ccattctgaacccccagat-3' R - 5'-ggtcagccatgacttcactt -3'	46	1090 - 1172

## 5.2.2: Statistics

### 5.2.2.1: Variable comparisons

Statistical analyses in this chapter were carried out by statisticians from the MRC Life course Epidemiology Unit in Southampton.

SWS maternal variables that were not normally distributed were transformed logarithmically. Placental mRNA data were transformed to normality using Fisher-Yates transformation which converts the data to Z scores (Constancia *et al.*, 2002) and adjusted for fetal sex. Fetal variables were adjusted for sex. Neonatal birth weight, crown-heel length, abdominal circumference and mid-upper arm circumference were adjusted for sex and gestational age. Summary data are presented as mean (SD) or median (inter-quartile range) depending on whether or not the data were normally distributed.

Analysis of gene expression levels between different categories of maternal lifestyle were tested by one-way ANOVA. Relationships between placental genes expression levels and maternal, fetal or neonatal variables were analysed by linear regression. Data were analysed using Stata version 11.0 (Statacorp, Texas, USA).

### 5.2.2.2: Sex differences in gene expression

Following initial analysis of the data it was determined that gene expression in the control genes and many of the target genes was higher in male than in female placentas (Royston, 1995). To avoid introducing a systematic bias due to the control genes all mRNA data was adjusted to remove any sex effects.

To investigate whether there were sex differences in the relationship between mRNA expression and the variables, sex was included in regression analyses as appropriate and where an interaction was found, data were analysed separately by sex.

### 5.2.2.3: Sample size

Sample size calculations are difficult, as, in order to be meaningful, they require a good estimate of the variation in the population to be studied. This information was not available for the genes studied in this thesis. However, data were available from studies on the expression of *PMCA3* in the SWS placentas (Cleal *et al.*, 2010). To provide an estimate of the likely numbers required for studies in this chapter, power calculations were performed for t-tests and correlations using Sigma stat version 3.5 and the data from the *PMCA3* study. This suggested that when comparing categorical data (for instance smokers and non-smokers) to identify a 20% difference between the groups with a P value of 0.05 a sample size of 40 would be required, and for correlation studies (for instance relating gene expression to maternal BMI) to obtain an R value of 0.3 with a power of 0.8 and a P value of 0.05, a sample size of 85 would be required. Given the inherent uncertainties in these calculations it was decided to analyse as many samples as it was possible to fit on a PCR plate which accommodates 102 samples in triplicate together with the required controls and standards.

## 5.3: Results

### 5.3.1: Maternal and neonatal characteristics

Of the 102 placentas from SWS pregnancies studied here, 53 of the infants were male and 49 were female. Maternal pre-pregnancy characteristics for the pregnant women and the neonatal characteristics of the children are described in Table 5.3.

**Table 5.3: Pre-pregnant maternal and neonatal characteristics**

Characheristic	Mean or median (SD or Inter Quartile Range)	Number of subjects
Maternal age (years)	30.9 (3.9)	102
Maternal height (cm)	162.3 (6.5)	101
Maternal body mass index (kg/cm <sup>2</sup> )	25.2 (23.0-29.3)	101
Maternal mid upper arm circumference (cm)	29.8 (27.2-32.7)	101
Maternal arm muscle area (cm <sup>2</sup> )	36.7 (31.1-43.5)	101
Offspring's birth weight (g)	3503 (453)	102
Placental weight (g)	470 (96)	101
Birth weight / Placental weight ratio	7.5 (6.8-8.4)	101
Neonatal abdominal circumference (cm)	31.7 (1.8)	102
Neonatal crown heel length (cm)	49.7 (1.8)	102
Neonatal mid upper arm circumference (cm)	11.6 (1.0)	102

### 5.3.2: Genes that were not detected at mRNA level

All isoforms of transporters and enzymes were analysed as they may be expressed differently or not at all in human placentas. Of the genes shown in Tables 5.1 and 5.2, mRNA for alanine aminotransferase 1 (*ALT1*), liver glutaminase (*GLS2*), *EAAT1*, *EAAT4* and *EAAT5* were not detected in the human placenta. These results only show mRNA expression levels for the genes that were detected in the human placenta.

### 5.3.3: Gene expression data

In this exploratory study the placental mRNA expression of 20 genes was compared to 22 maternal factors and 17 fetal factors. This led to 780 comparisons of which approximately 75 were statistically significant. Given the large number of comparisons undertaken here it was decided to present only those significant differences where there was a pattern of significant differences

for a gene or maternal factor, as it was thought that these would be the most robust. However the results of all comparisons are presented in Appendix 4.

#### **5.3.4: Sex differences in mRNA levels of housekeeping genes in the placenta**

Placental gene expression levels of the housekeeping genes *UBC*, *TOP1* and *YWHAZ* (mean (SEM) arbitrary units) were significantly higher in the placentas of male fetuses (n = 53) compared to those of females (n = 49): *UBC* 19.77 (0.58) vs. 17.76 (0.75), p = 0.035; *TOP1* 22.76 (0.53) vs. 19.66 (0.61), p = 0.0002; *YWHAZ* 19.25 (0.43) vs. 17.64 (0.48), p = 0.014 (Figure 5.1). Further analyses for the transporter and metabolic enzyme genes using the geNorm demonstrated that mRNA levels of the most stably expressed genes are higher in male than in female placentas (Figure 5.2 A & 5 2B) (Martin *et al.*, 2007).

#### **5.3.5: Maternal smoking before pregnancy**

Pre-pregnancy maternal smoking was reported in 26 out of 102 women and was associated with elevated mRNA levels for *LAT2* (for non-smokers mean (SD) = -0.14(0.96), for smokers mean (SD) = 0.41 (0.82), P = 0.011),  $\gamma^+LAT2$  (non-smokers mean (SD) = -0.10 (0.89), smokers mean (SD) = 0.35 (0.89), P = 0.026, Figure 5.3), aspartate aminotransferase 2 (non-smokers -0.13 (0.94), smokers mean (SD) = 0.39 (0.95), P = 0.018) and aspartate aminotransferase 1 (non-smokers mean (SD) = 0.10 (0.89), smokers mean (SD) = -0.33 (0.94), P = 0.038, Figure 5.3). There was an interaction between pre-pregnancy smoking and sex for  $\gamma^+LAT1$  (P < 0.05 for interaction). For  $\gamma^+LAT1$  pre-pregnancy maternal smoking was associated with higher mRNA levels in female placentas P = 0.001 (for non-smokers mean (SD) = 1.119 (0.892), n = 37; for smokers mean (SD) = -0.849 (0.738), n = 12) but not in male placentas (for non-smokers mean (SD) = 0.123 (0.944), n = 39, for smokers mean (SD) = -0.070 (1.107) n = 14, P = 0.86, Figure 5.4).

#### **5.3.6: Maternal smoking during pregnancy**

Smoking during pregnancy was reported in 14 out of 95 women and there was an interaction between smoking and sex for  $\gamma^+LAT2$  and alanine aminotransferase mRNA levels (P < 0.05 for interaction).  $\gamma^+LAT2$  mRNA levels

were higher in female placentas (for non-smokers mean (SD) = 0.19 (0.95), n = 37; for smokers mean (SD) = 1.24 (0.94), n = 6, P = 0.013) than in male placentas (for non-smokers mean (SD) = -0.31 (0.89), n = 44; for smokers mean (SD) = -0.044 (0.98), n = 8, P = 0.70 Figure 5.5). Alanine aminotransferase levels were different (as indicated by interaction) but did not reach significance individually in female placentas (for non-smokers mean (SD) = -0.12 (0.91), n = 37; for smokers mean (SD) = 0.55 (0.79), n = 6, P = 0.11) or male placentas (for non-smokers -0.06 (0.98), n = 44; for smokers mean (SD) = -0.63 (1.0), n = 8 P = 0.08, data not shown). It should be noted that the numbers in each group are small once the data is split by sex.

### 5.3.7: Maternal exercise

At recruitment 60 out of 102 women reported that they undertook strenuous exercise. Placentas from these women had elevated mRNA levels of *ASC1* (for no strenuous exercise -0.24 (1.02), for yes strenuous exercise 0.17 (0.85), P = 0.031), mitochondrial branched chain aminotransferase (for no strenuous exercise -0.28 (0.91), for yes strenuous exercise 0.18 (0.95), (P = 0.017), and glutamine synthetase (for no strenuous exercise -0.27 (0.86), for yes strenuous exercise 0.15 (0.96), P = 0.049, Figure 5.6). There was a differential relationship between strenuous exercise and fetal sex for glutaminase mRNA levels (P < 0.05 for interaction). Glutaminase mRNA levels were higher in male placentas (for no strenuous exercise, mean (SD) = -0.65 (0.73), n = 23; for yes strenuous exercise, mean (SD) = 0.17 (0.96), n = 30; P = 0.001) but not female placentas (for no strenuous exercise, mean (SD) = 0.19 (0.78), n = 16, for yes strenuous exercise, mean (SD) = s 0.21 (1.03), n = 33; P = 0.95, Figure 5.7)

At recruitment 47 out of 102 women had reported that they had faster than normal walking speed. These women had decreased mRNA levels for aspartate aminotransferase 2 (mean (SD) = - 21 (0.88) vs mean (SD) = - -0.24 (1.01), P = 0.019) and EAAT3 (mean (SD) = -0.19 (0.92) vs mean (SD) = -0.20(0.91), P = 0.036). *ASC2* (R = 0.25, P = 0.01) and aspartate aminotransferase 1 (R = -0.22, P = 0.03).

### 5.3.8: Maternal parity

For analysis, placentas from nulliparous women (no previous live births,  $n = 46$ ) were compared to multiparous women (1 or more previous births,  $n = 56$ , Figure 5.8). Being multiparous was associated with increased placental mRNA levels for *ASC2* (for nulliparous, mean (SD) = -0.3 (0.93); for multiparous, mean (SD) = 0.27 (0.88),  $P = 0.001$ ), aspartate aminotransferase 2 (nulliparous, mean (SD) = -0.26 (0.98); for multiparous, mean (SD) = 0.22 (0.90),  $P = 0.011$ ), cytosolic branched chain aminotransferase (nulliparous, mean (SD) = -0.24 (0.98); multiparous, mean (SD) = 0.21 (0.90),  $P = 0.018$ ), and *EAAT3* (for nulliparous, mean (SD) = -0.21 (1.00); for multiparous, 0.19 (0.84),  $P = 0.03$ ) while mRNA levels of glutamate dehydrogenase were decreased (for nulliparous, mean (SD) = 0.23 (1.01); for multiparous, mean (SD) = -0.19 (0.89),  $P = 0.027$ , Figure 5.8 ). There were interactions between parity and fetal sex and the relationship of alanine aminotransferase and *LAT2* with maternal body composition ( $P < 0.05$  for interaction, Figures 5.9 A and 5.9B). Being multiparous was associated with lower levels of placental alanine aminotransferase mRNA levels in male placentas (for nulliparous mean (SD) = 0.399 (0.760),  $n = 23$ , multiparous mean (SD) = -0.340 (1.079),  $n = 30$ ,  $P = 0.007$ ) but not female placentas (for nulliparous mean (SD) = 0.004 (1.013);  $n = 23$ , for multiparous, mean (SD) = 0.037 (0.849),  $n = 26$ ,  $P = 0.902$  Figure 5.9A). For *LAT2* there was a trend towards higher mRNA levels in male placentas with increasing parity (for nulliparous mean (SD) = -0.199 (1.030),  $n = 23$ , for multiparous, mean (SD) = 0.361 (0.793),  $n = 30$ ,  $P = 0.051$ ) but not in female placentas (for nulliparous mean (SD) = 0.017(0.871),  $n = 23$ ; for multiparous mean (SD) = -0.321 (1.081),  $n = 25$ ,  $P = 0.242$ , Figure 5.9B).

### 5.3.9: Maternal body composition

There were many associations between maternal body compositions and placental mRNA levels. However the associations between placental *ASC1* mRNA levels and maternal body composition were the most predominant.

#### 5.3.9.1: Maternal birth weight

Maternal birth weight was negatively associated with placental mitochondrial branched chain aminotransferase mRNA levels ( $R = -0.27$ ,  $P = 0.011$ , Figure

5.10). There was an interaction with sex for *ASC1* and *LAT2* mRNA levels ( $P < 0.05$  for interaction). *ASC1* mRNA levels were positively related to maternal birth weight in female placentas ( $R = 0.35$ ,  $P = 0.018$ ,  $n = 44$ , Figure 5.11) and there was a trend towards a negative relationship in male placentas ( $R = -0.28$ ,  $P = 0.069$ ,  $n = 44$ ). *LAT2* mRNA levels were negatively related to maternal birth weight in male placentas ( $R = -0.453$ ,  $P = 0.002$ ,  $n = 44$ ) but not in female placentas ( $R = 0.17$ ,  $P = 0.28$ ,  $n = 43$  Figure 5.12).

#### **5.3.9.2: Maternal height**

Maternal height was positively associated with placental aspartate aminotransferase levels ( $R = 0.25$ ,  $P = 0.014$ ,  $n = 101$ , Figure 5.13) and negatively associated with placental cytosolic branched chain aminotransferase levels ( $R = -0.20$ ,  $P = 0.05$ ,  $n = 101$ , Figure 5.14). There was an interaction between maternal height and sex for mitochondrial branched chain aminotransferase mRNA levels. This gene was negatively associated with maternal height in male placentas ( $R = -0.37$ ,  $P = 0.01$ ,  $n = 53$ ) but not in female placentas ( $R = 0.07$ ,  $P = 0.64$ ,  $n = 48$ , Figure 5.15).

#### **5.3.9.3: Maternal pre-pregnant BMI**

Maternal pre-pregnant BMI was negatively associated with placental mRNA levels for *ASC1* ( $R = -0.26$ ,  $P = 0.01$ ,  $n = 10$ , Figure 5.16),  $\gamma^+$ *LAT2* ( $R = -0.20$ ,  $P = 0.055$ ,  $n = 101$ , Figure 5.17) and positively associated with glutaminase 1 ( $R = 0.21$ ,  $P = 0.042$ ,  $n = 10$ , Figure 5.18).

#### **5.3.9.4: Maternal pre-pregnancy mid-upper arm circumference**

Maternal pre-pregnancy mid-arm upper circumference was negatively associated with placental *ASC1* mRNA levels ( $R = -0.26$ ,  $P = 0.01$ ,  $n = 101$ , Figure 5.19), also mid-arm upper circumference at 11 weeks gestation was negatively related to placental *ASC1* mRNA levels ( $R = -0.23$ ,  $P = 0.045$ ,  $n = 76$ , Figure 5.20) and mid-arm upper circumference at 34 weeks gestation was also negatively related to placental *ASC1* mRNA levels ( $R = -0.27$ ,  $P = 0.009$ ,  $n = 95$ , Figure 5.21).

#### **5.3.9.5: Maternal arm muscle area**

Maternal pre-pregnancy arm muscle area was negatively associated with placental *ASC1* mRNA levels ( $R = -0.21$ ,  $P = 0.04$ ,  $n = 101$  Figure 5.22). Maternal arm muscle area was also negatively related to placental *ASC1* mRNA levels at 11 weeks gestation ( $R = -0.23$ ,  $P = 0.046$ ,  $n = 76$ , Figure 5.23) and at 34 weeks gestation ( $R = -0.21$ ,  $P = 0.04$ ,  $n = 95$ , Figure 5.24).

#### **5.3.9.6: Maternal pre pregnancy calf circumference**

Maternal pre-pregnancy calf circumference was negatively associated with placental *ASC1* mRNA levels ( $R = -0.34$ ,  $P < 0.001$ ,  $n = 100$ , Figure 5.25).

#### **5.3.9.7: Maternal pre-pregnancy sum of skinfold thickness**

Sum of skinfold thickness was negatively associated with placental *ASC1* mRNA levels ( $R = -0.20$ ,  $P = 0.042$ ,  $n = 100$ , Figure 5.26).

#### **5.3.9.8: Maternal pre-pregnancy derived fat mass**

Maternal pre-pregnancy derived fat mass was negatively associated with placental *ASC1* mRNA levels ( $R = -0.27$ ,  $P = 0.007$ ,  $n = 101$ , Figure 5.27).

#### **5.3.10: Maternal dietary prudence**

Maternal dietary prudence before pregnancy was positively related to placental glutamine synthetase mRNA levels ( $R = 0.21$ ,  $P = 0.03$ ). Maternal dietary prudence at 11 weeks gestation (PC1) was associated with placental mRNA levels for *LAT4* ( $R = -0.27$ ,  $P = 0.02$ ) and aspartate aminotransferase 2 ( $R = -0.28$ ,  $P = 0.01$ ).

#### **5.3.11: Maternal high energy diet**

There were interactions with sex and high energy diet for *LAT2*, *ASC2*, mitochondrial branched chain aminotransferase, aspartate aminotransferase 1 and *EAAT2* ( $P < 0.05$  for interaction). Pre-pregnancy high energy diet and *ASC2* mRNA levels were positively associated in male placentas ( $R = 0.22$ ,  $P = 0.109$ ,  $n = 53$ ) and negatively associated in female placentas ( $R = -0.251$ ,  $P =$

0.081,  $n = 49$ , data not shown). Aspartate aminotransferase 2 mRNA levels were not associated with pre-pregnancy high energy diet in males ( $R = -0.142$ ,  $P = 0.311$ ,  $n = 53$ ) but were positively associated in females ( $R = 0.29$ ,  $P = 0.044$ ,  $n = 49$ ). *EAAT2* mRNA levels were positively associated with pre-pregnancy high energy diet in males ( $R = -0.27$ ,  $P = 0.048$ ,  $n = 53$ ) and not associated in females ( $R = -0.15$ ,  $P = 0.31$ ,  $n = 49$ ).

### 5.3.12: Maternal social class

Maternal social class was recorded for 101 women and was associated with placental *4f2hc* mRNA levels (I/II  $-0.27(0.87)$ ,  $n = 47$ ; IIIN/M  $0.23(1.04)$ ,  $n = 43$ ; IV/V  $0.29(0.66)$ ,  $n = 11$ ,  $P = 0.026$ , data not shown). The definition for these classifications are; I/II class (Professional occupations, managerial and technical occupations), IIIN/M (Skilled occupations, N – non-manual, M – manual), IV/V (Partly-skilled and unskilled occupations (Cleal *et al.*, 2010).

### 5.3.13: Maternal depression

Maternal depression was reported in 16 out of 68 women and was associated with decreased glutamine synthetase mRNA levels (for those not depressed mean (SD) =  $0.13 (0.94)$ ,  $n = 52$ , for those depressed mean (SD) =  $-0.46 (1.10)$ ,  $n = 16$ ,  $P = 0.04$ , data not shown). Maternal educational attainment was not related to the expression of any of the genes reported here.

### 5.3.14: Paternal birth weight

Paternal birth weight was negatively associated with placental mRNA levels for *EAAT3* ( $R = -0.40$ ,  $P = 0.005$ ,  $n = 47$ , Figure 5.28) and glutaminase ( $R = -0.40$ ,  $P = 0.005$ ,  $n = 47$  Figure 5.29). Paternal birth weight was also negatively associated with placental glutamine synthetase mRNA levels in the female placentas ( $P = 0.016$ ,  $R = -0.45$ ,  $n = 28$ , Figure 5.30). However when the paternal birth weight of 1107 g which seemed to be an outlier was taken out, the association was not significant ( $P = 0.147$ ,  $R = -0.29$ ,  $n = 27$ ). A few outliers were also observed for placental glutaminase and *EAAT3* mRNA levels, but they were not taken out as shown in Figures 5.28 & 5.29.

### 5.3.15: Placental weight

Placental weight was negatively related to placental mRNA levels for alanine aminotransferase mRNA levels ( $R = -0.242$ ,  $P = 0.015$ ,  $n = 10$ , Figure 5.31) and *LAT2* ( $R = -0.22$ ,  $P = 0.03$ ,  $n = 101$ , Figure 5.32).

### 5.3.16: Placental to birth weight ratio

Placental to birth weight ratio was negatively related to *LAT2* mRNA levels ( $R = -0.223$ ,  $P = 0.026$ ,  $n = 102$ , Figure 5.33).

### 5.3.17: Birth weight

Birth weight was negatively associated with expression of alanine aminotransferase ( $R = -0.199$ ,  $P = 0.045$ ,  $n = 102$ , Figure 5.34).

### 5.3.18: Fetal abdominal circumference growth rate

There were no significant associations between 19 week and 34 week abdominal circumference and placental gene expression. However 19-34 week abdominal circumference growth rate was positively associated with *ASC1* mRNA levels ( $R = 0.29$ ,  $P = 0.027$ ,  $n = 58$ , Figure 5.35) and glutamine synthetase mRNA levels ( $R = 0.39$ ,  $P = 0.002$ ,  $n = 58$ , Figure 5.36).

### 5.3.19: Fetal head circumference

19 - 34 week head circumference was negatively related to *LAT2* mRNA levels ( $R = -0.272$ ,  $P = 0.040$ ,  $n = 58$ , Figure 5.37). 19 - 34 week head circumference was positively associated with  $y^+LAT2$  mRNA levels ( $R = 0.284$ ,  $P = 0.031$ ,  $n = 58$ , Figure 5.38).

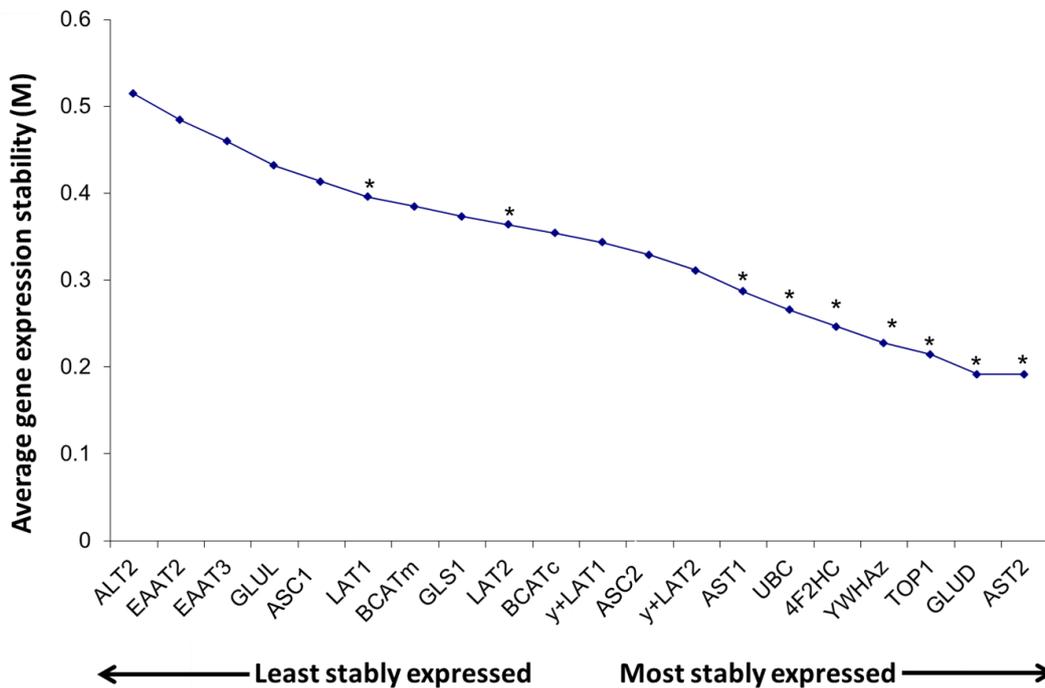
### 5.3.20: Fetal femur length

There were differential relationships with sex between 19 week fetal femur length and placental mitochondrial branched chain aminotransferase mRNA levels in male placentas  $R = -0.35$ ,  $P = 0.07$ ,  $n = 29$  and female placentas ( $R = 0.31$ ,  $P = 0.11$ , data not shown). At 34 weeks fetal femur length was negatively associated with glutamine synthetase mRNA levels ( $R = -0.288$ ,  $P = 0.027$ ,  $n = 59$  Figure 5.39) and 19-34 week fetal femur growth rate ( $R = -0.266$ ,  $P = 0.043$ ,

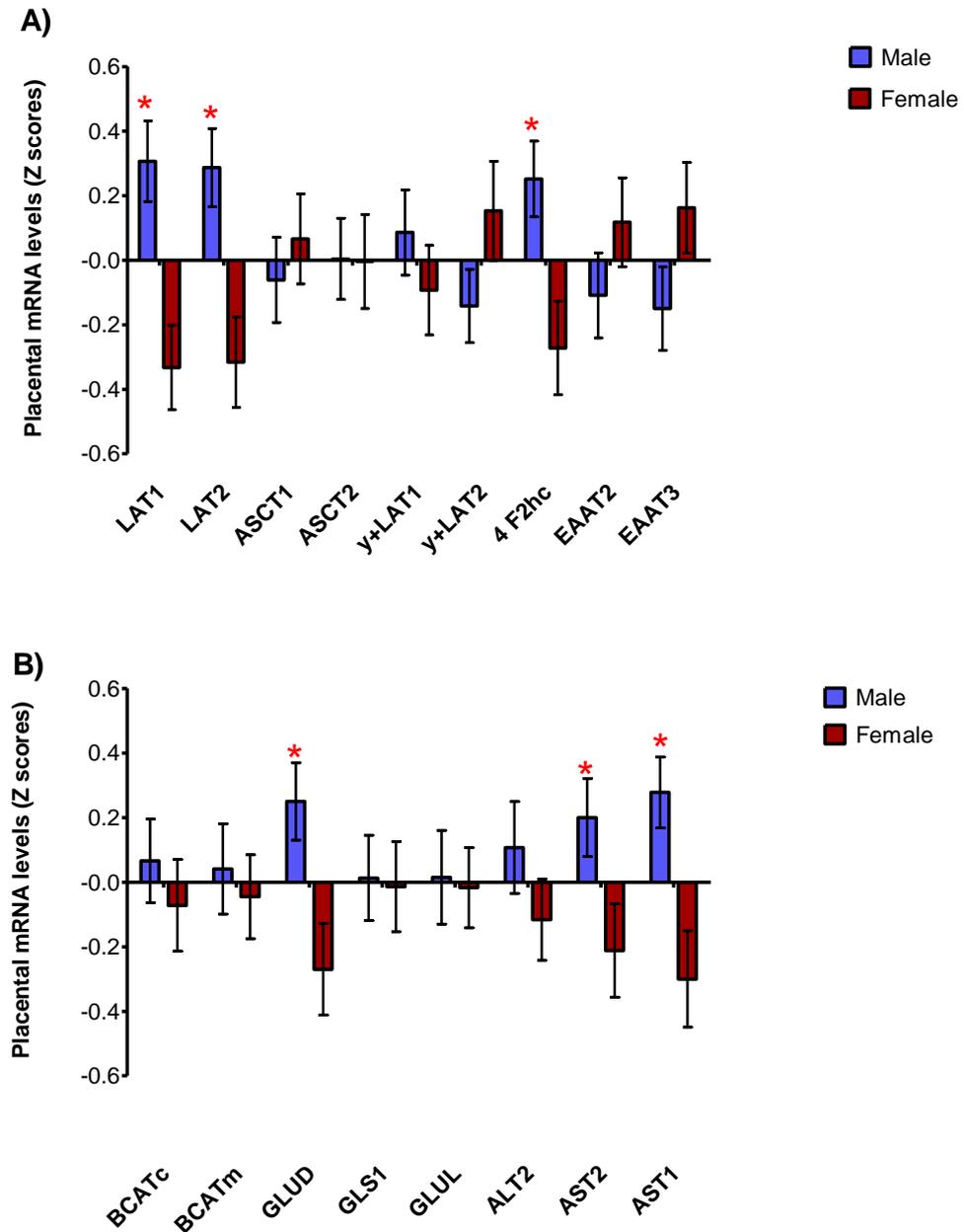
data not shown). At 34 weeks fetal femur length was correlated to  $y^+LAT2$  ( $R = -0.272$ ,  $P = 0.039$ ).

### 5.3.21: Subscapular skinfold thickness

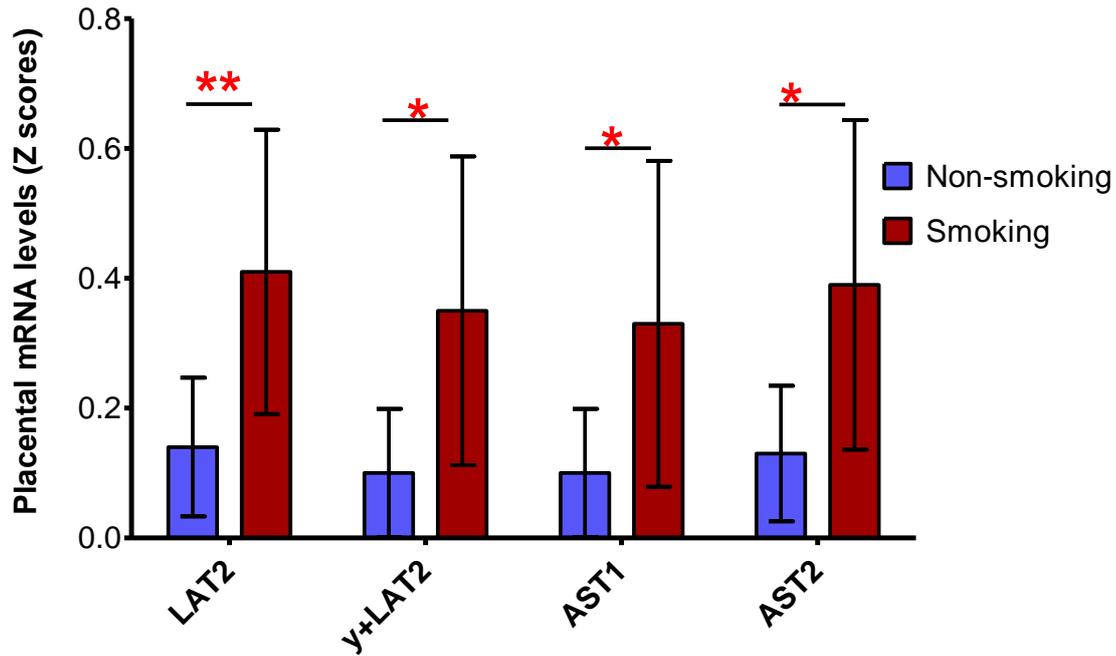
Subscapular skinfold thickness was negatively related to mRNA levels for *LAT1* ( $R = -0.294$ ,  $P = 0.003$ ,  $n = 102$ , data not shown) and alanine aminotransferase ( $R = -0.330$ ,  $P = 0.001$ ,  $n = 102$ , Figure 5.40).



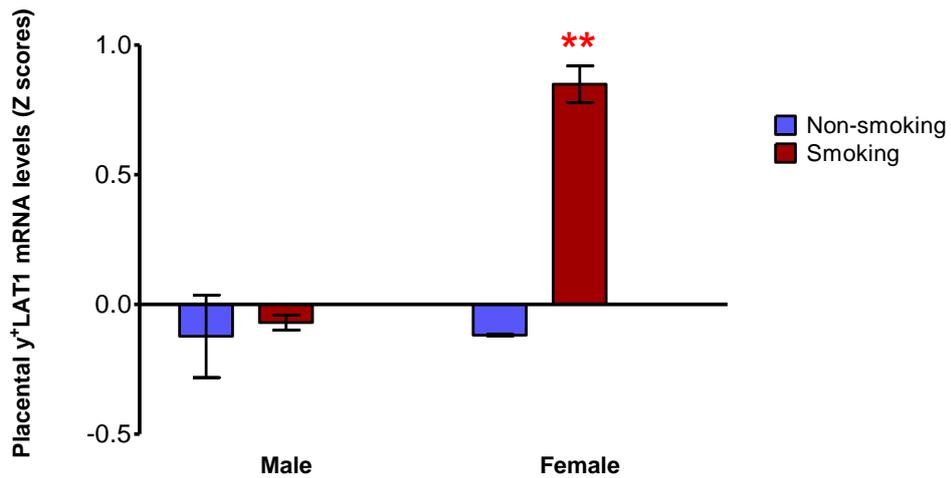
**Figure 5.1: Average expression stability value (M) for each amino acid transporter and metabolic gene, ranked according to increasing stability with the most stable genes on the right.** \* Indicates gene is significantly different in male compared to female placentas. *LAT1*, (large neutral amino acids transporter small subunit 1), *LAT2* (large neutral amino acids transporter small subunit 2), *EAAT2* (excitatory amino acid transporter 2), *EAAT3* (Excitatory amino acid transporter 3), *ASC1* (serine/cysteine transporter 1), *ASC2* (alanine/serine/cysteine transporter 2), *y+LAT1* ( $y^+$  L-type amino acid transporter 1) *y+LAT2* ( $y^+$  L-type amino acid transporter 2), *4F2hc* (4F2 cell-surface antigen heavy chain), *GPT2* (alanine aminotransferase 2), *AST1* ( aspartate aminotransferase 1), *AST2* (aspartate aminotransferase), *GLUL* (glutamine synthetase), *GLUD* (glutamate dehydrogenase), *GLS* (glutaminase), *BCATc* (cytosolic branched chain aminotransferase), *BCATm* (mitochondrial branched chain aminotransferase).



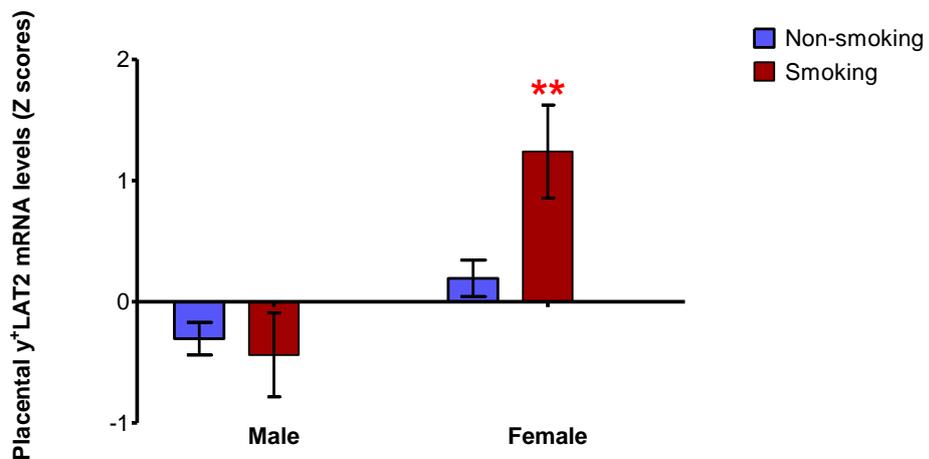
**Figure 5.2: Sex differences in the expression of stably expressed genes.** A) Expression of placental amino acid transporter genes in the male and female placentas. The most stably expressed; *LAT1*, *LAT2* and *4F2hc* transporter genes are more highly expressed in male placentas than in female placentas. B) Expression of placental amino acid metabolic enzyme genes in the male and female placentas. The most stably expressed; *GLUD 1*, *ASC1* and *ASC2* metabolic enzyme genes are more highly expressed in male than in female placentas. Data are mean  $\pm$  SEM, n = 102. \* indicates P < 0.05.



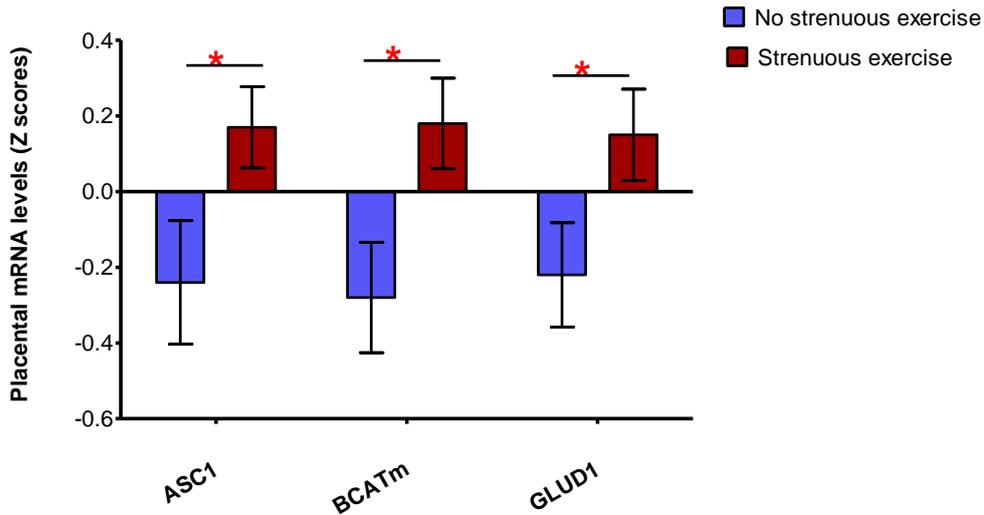
**Figure 5.3: Associations between maternal pre-pregnancy smoking and placental mRNA levels.** Maternal pre-pregnancy smoking was associated with increased placental mRNA levels for *LAT2*, *y+LAT2*, aspartate aminotransferase 1 and aspartate aminotransferase 2. Data is mean  $\pm$  SEM (Z scores),  $n = 76$  for not smoking and 26 for smoking. \*\* indicates  $P < 0.01$  and \* indicates  $P < 0.05$ .



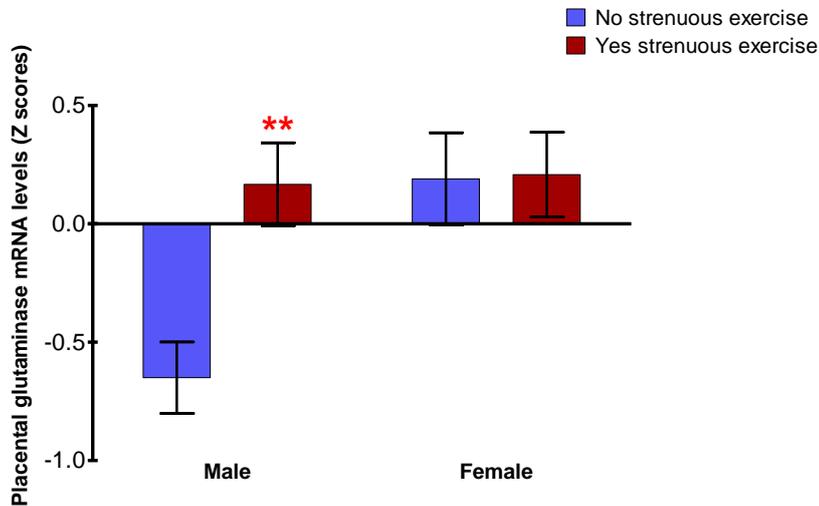
**Figure 5.4: Association between maternal pre-pregnancy smoking and placental  $y^+LAT1$  mRNA levels.** Maternal pre-pregnancy smoking was associated with increased levels of placental  $y^+LAT1$  mRNA expression in female ( $P = 0.001$ ) but not in male placentas. Data are mean  $\pm$  SEM (Z scores),  $n = 39$  for not smoking and  $n = 14$  for smoking in male placentas,  $n = 37$  for not smoking and  $n = 12$  for smoking in female placentas. \*\* indicates  $P < 0.01$ .



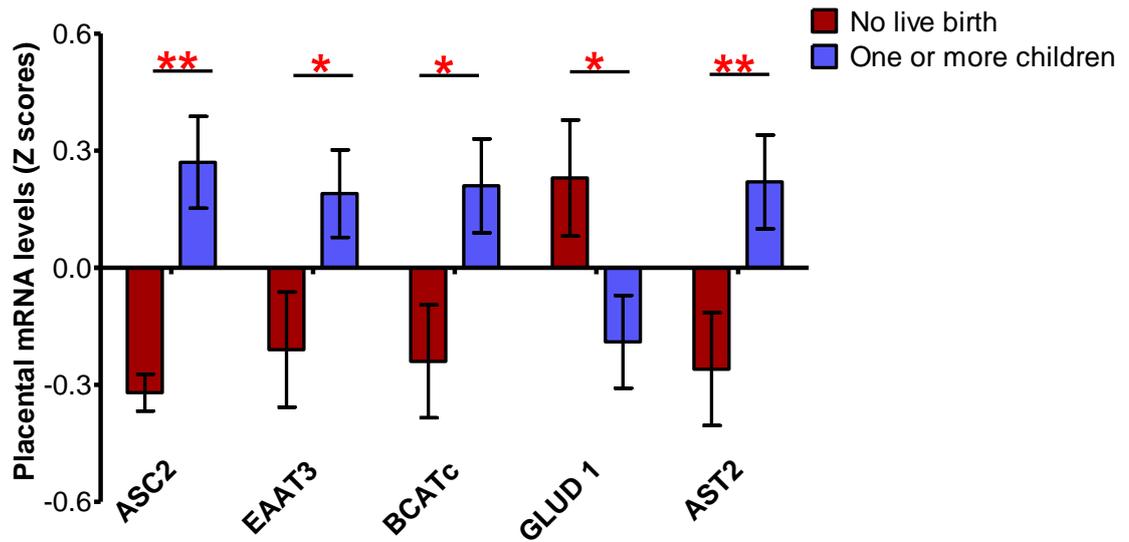
**Figure 5.5: Associations between maternal smoking during pregnancy and placental  $y^+LAT2$  mRNA levels.** Maternal in-pregnancy smoking was associated with increased levels of placental  $y^+LAT2$  mRNA expression in female but not in male placentas ( $P = 0.702$ ). Data are mean  $\pm$  SEM (Z scores)  $n = 44$  for not smoking and  $n = 8$  for smoking in male placentas,  $n = 37$  for not smoking and  $n = 6$  for smoking in female placentas. \*\* indicates  $P < 0.01$  vs. non-smoking.



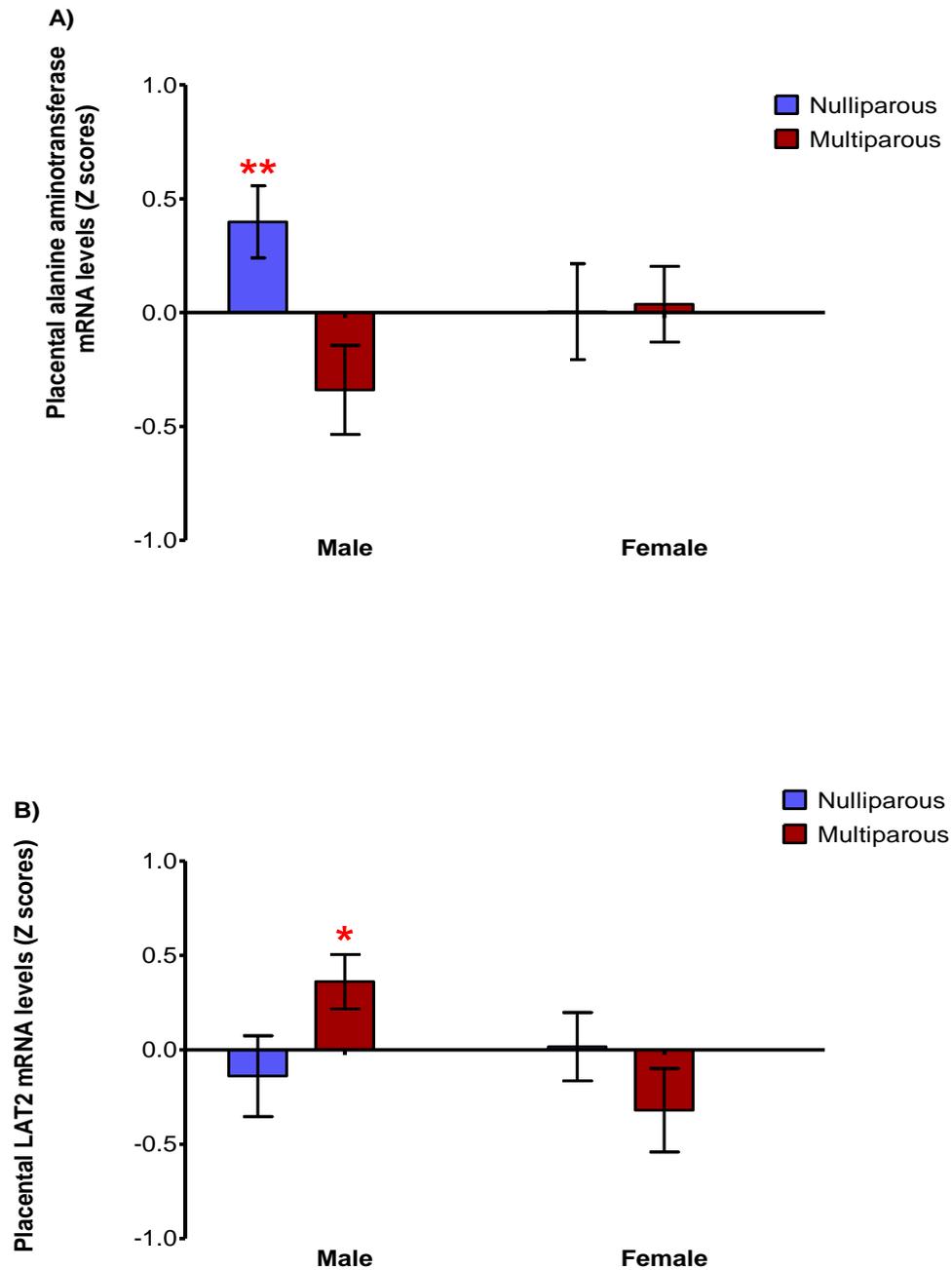
**Figure 5.6: Associations between maternal strenuous exercise and placental *ASC1* mRNA levels.** Maternal strenuous exercise was associated with increased levels of placental mRNA levels for *ASC1*, mitochondrial branched chain aminotransferase and glutamate dehydrogenase. Data are mean  $\pm$  SEM (Z scores),  $n = 39$  for no strenuous exercise and  $n = 63$  for strenuous exercise \*\* indicates  $P < 0.01$  and \* indicates  $P < 0.05$ .



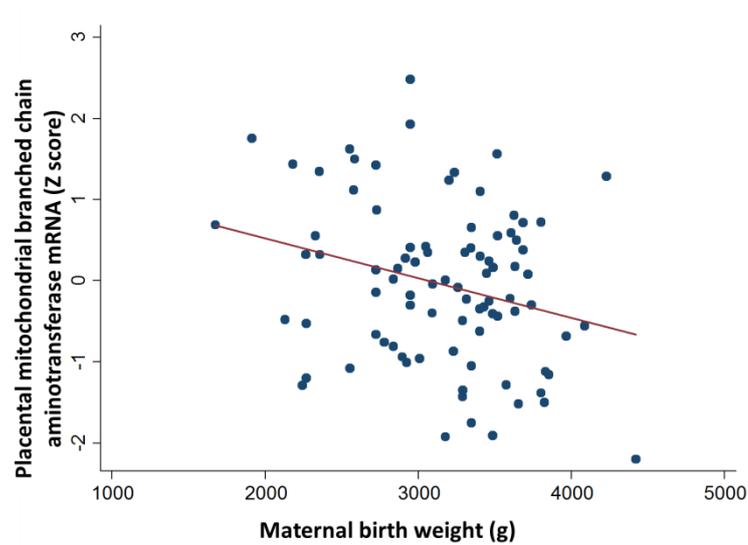
**Figure 5.7: Sex differences in the associations between maternal strenuous exercise and placental glutaminase mRNA expression.** Maternal strenuous exercise was positively associated with levels of placental glutaminase mRNA expression in males but not in females. Data are mean  $\pm$  SEM (Z scores),  $n = 23$  for no strenuous exercise and  $n = 30$  for strenuous exercise for males and  $n = 16$  for no strenuous exercise and  $n = 33$  for strenuous exercise for females \*\* indicates  $P < 0.01$ .



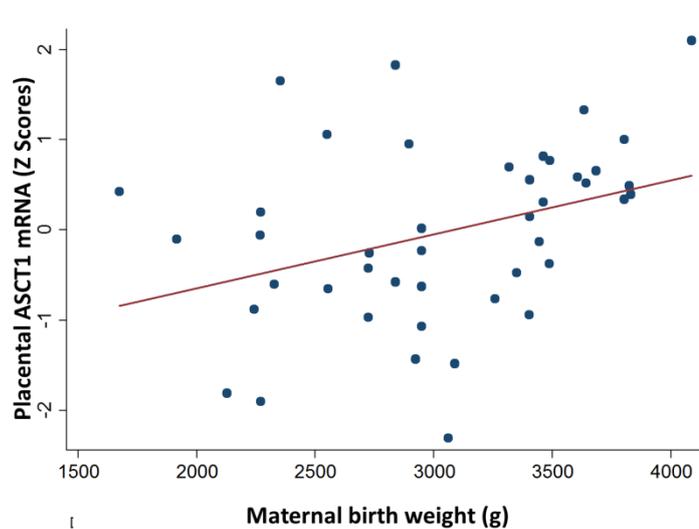
**Figure 5.8: Associations between parity and placental mRNA levels for *ASC2*, *EAAT3*, cytosolic branched chain aminotransferase, glutamate dehydrogenase 1, and aspartate aminotransferase 2.** Being multiparous was associated with increased placental mRNA expression for *ASC2*, *EAAT3*, cytosolic branched chain aminotransferase, glutamate dehydrogenase 1, and aspartate aminotransferase 2. Data are mean  $\pm$  SEM (Z scores),  $n = 46$  for nulliparous and  $56$  for multiparous. \*\* indicates  $P < 0.01$  and \* indicates  $P < 0.05$ .



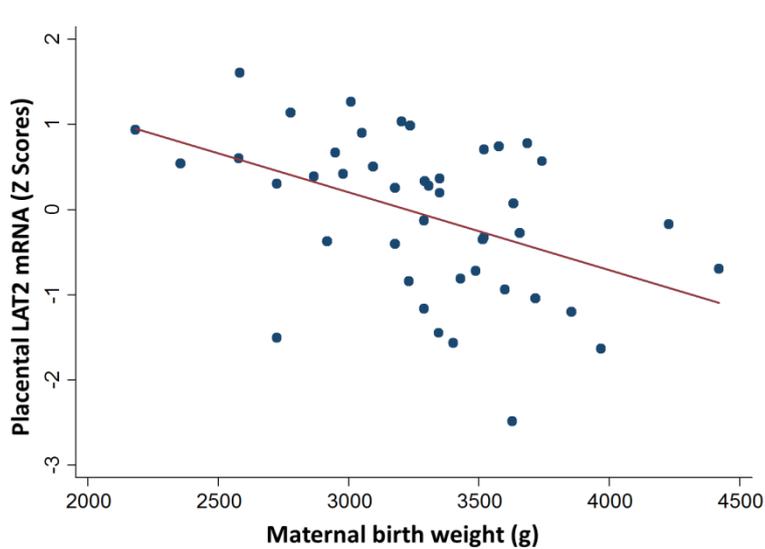
**Figure 5.9: Sex differences in the associations between maternal parity and placental alanine aminotransferase and *LAT2* mRNA levels.** A) Being multiparous was associated with decreased levels of placental alanine aminotransferase mRNA in male but not in female placentas, B) Being multiparous was associated with increased levels of placental *LAT2* mRNA in male but not in females. Data are mean  $\pm$  SEM (Z scores),  $n = 23$  nulliparous and  $n = 30$  multiparous in males and  $n = 23$  for nulliparous and  $n = 25$  for multiparous in female placentas (*LAT2*) and  $n = 26$  for multiparous (alanine aminotransferase). \*\* =  $P < 0.01$ , \* indicates  $P < 0.05$ .



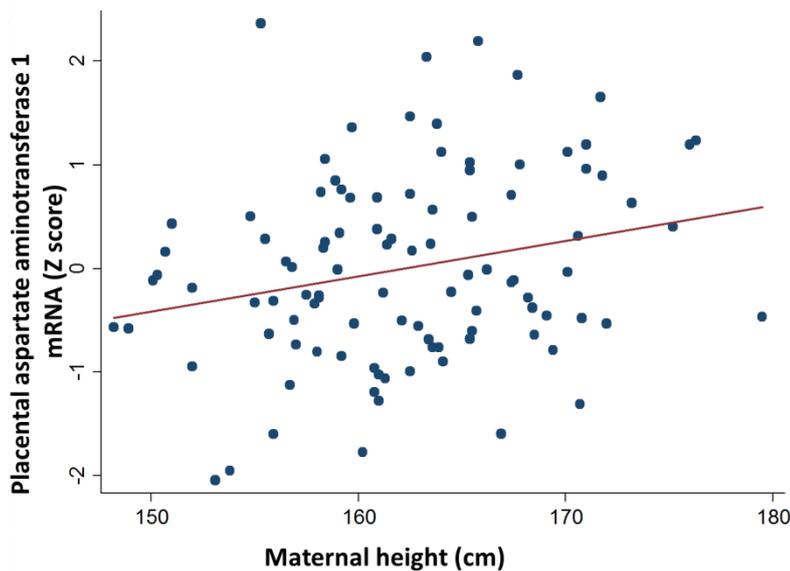
**Figure 5.10: The association between maternal birth weight and placental mitochondrial branched chain aminotransferase mRNA levels.** Maternal birth weight was negatively associated with placental mitochondrial branched chain aminotransferase mRNA levels ( $P = 0.011$ ,  $R = -0.27$ ,  $n = 88$ ). Placental mitochondrial branched chain aminotransferase mRNA data are presented as Z scores.



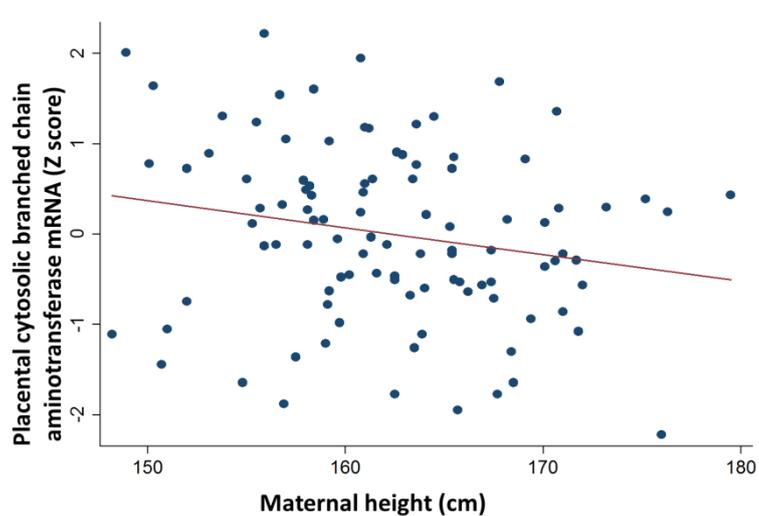
**Figure 5.11: The association between maternal birth weight and placental ASC1 mRNA levels.** Maternal birth weight was positively associated with placental ASC1 mRNA levels in female placentas ( $P = 0.018$ ,  $R = 0.35$ ,  $n = 44$ ) but not in male placentas ( $P = 0.069$ ,  $R = -0.28$ ,  $n = 44$ ). Placental ASC1 mRNA data are presented as Z scores.



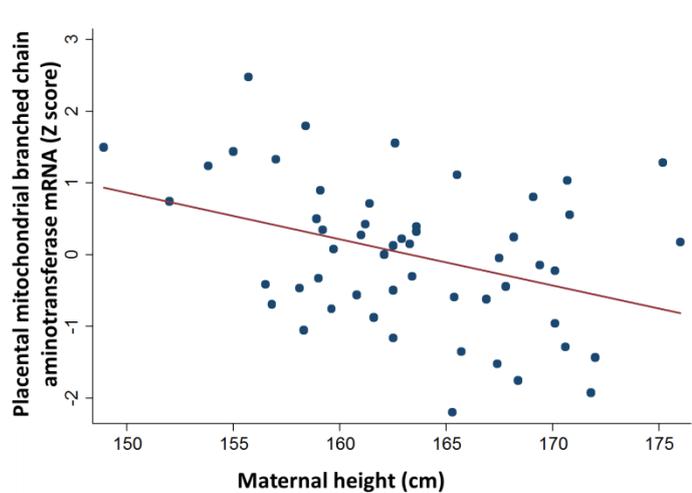
**Figure 5.12: The association between maternal birth weight and placental *LAT2* mRNA levels.** Maternal birth weight was negatively associated with placental *LAT2* mRNA levels in male placentas ( $P = 0.002$ ,  $R = 0.45$ ,  $n = 44$ ). Placental *LAT2* mRNA data are presented as Z scores.



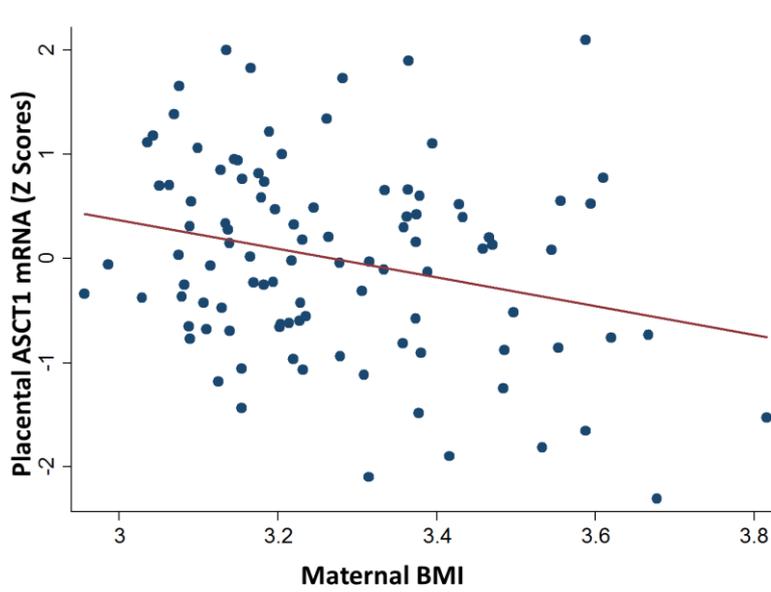
**Figure 5.13: The association between maternal height and placental aspartate aminotransferase mRNA levels.** Maternal height was positively associated with placental aspartate aminotransferase mRNA levels ( $P = 0.014$ ,  $R = 0.25$ ,  $n = 101$ ). Aspartate aminotransferase 1 mRNA data are presented as Z scores.



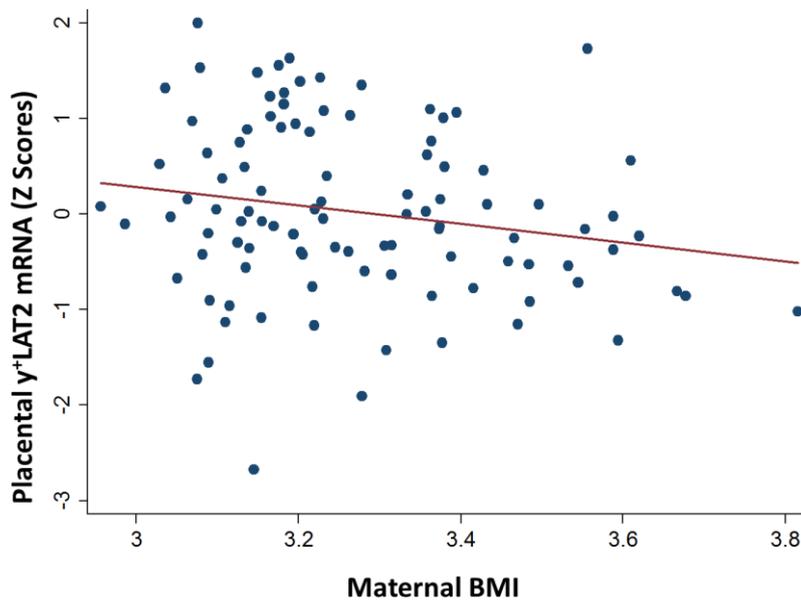
**Figure 5.14: The association between maternal height and placental cytosolic branched chain aminotransferase mRNA levels.** Maternal height was negatively associated with placental cytosolic branched chain aminotransferase mRNA levels ( $P = 0.045$ ,  $R = -0.20$ ,  $n = 101$ ) Cytosolic branched chain aminotransferase mRNA data are presented as Z scores.



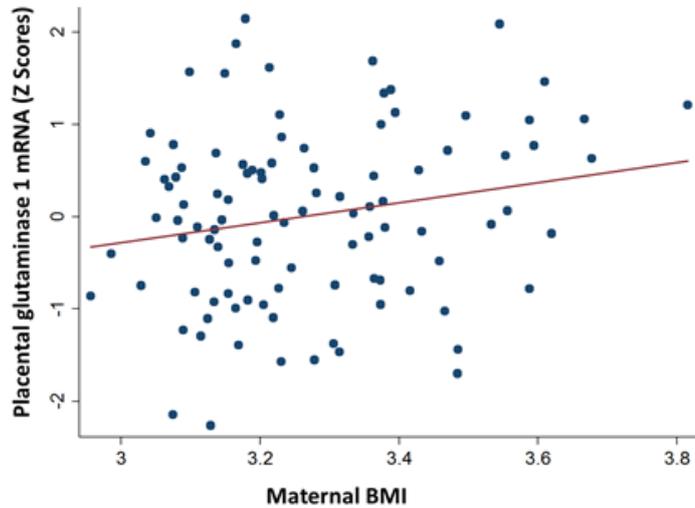
**Figure 5.15: The association between maternal height and placental cytosolic branched chain aminotransferase mRNA levels placental mitochondrial branched chain aminotransferase mRNA levels in male placentas.** Maternal height was negatively associated with placental mitochondrial branched chain aminotransferase mRNA levels in male placentas ( $P = 0.006$ ,  $R = -0.37$ ,  $n = 53$ ). Mitochondrial branched chain aminotransferase mRNA data are presented as Z scores.



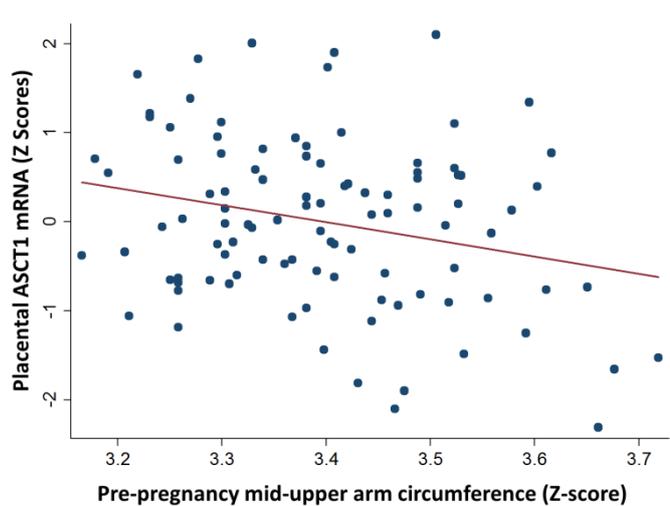
**Figure 5.16: The association between maternal BMI and placental *ASC1* mRNA levels.** Maternal BMI was negatively associated with placental *ASC1* mRNA levels ( $P = 0.008$ ,  $R = -0.26$ ,  $n = 101$ ). Maternal BMI data were log transformed and *ASC1* mRNA data are presented as Z scores.



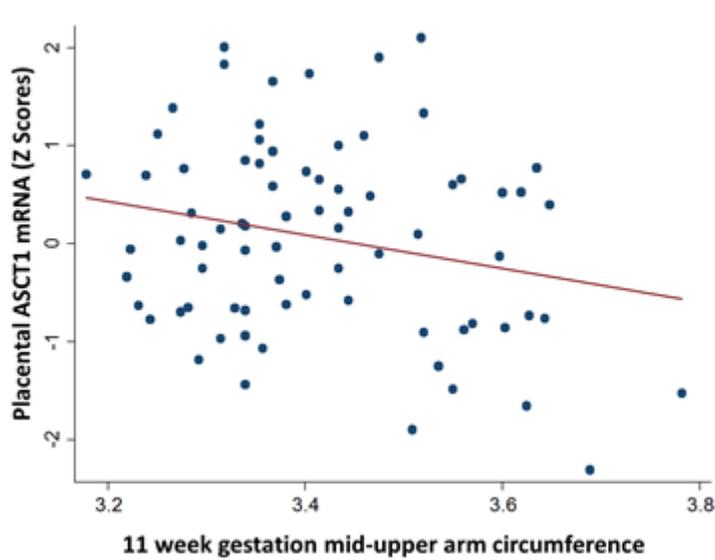
**Figure 5.17: The association between maternal BMI and placental *y<sup>+</sup>LAT2* mRNA levels.** Maternal BMI was negatively associated with *y<sup>+</sup>LAT2* mRNA levels ( $P = 0.042$ ,  $R = -0.20$ ,  $n = 101$ ). Maternal BMI data were log transformed and *y<sup>+</sup>LAT2* mRNA data are presented as Z scores.



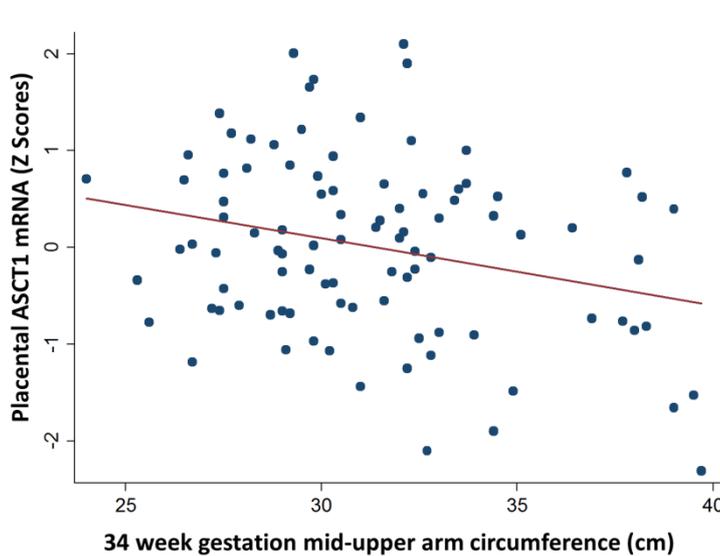
**Figure 5.18: The association between maternal BMI and placental glutaminase 1 mRNA levels.** Maternal BMI was positively associated with placental glutaminase1 mRNA levels ( $P = 0.042$ ,  $R = -0.20$ ,  $n = 101$ ). Maternal BMI data was log transformed and placental glutaminase 1 mRNA data are presented as Z scores.



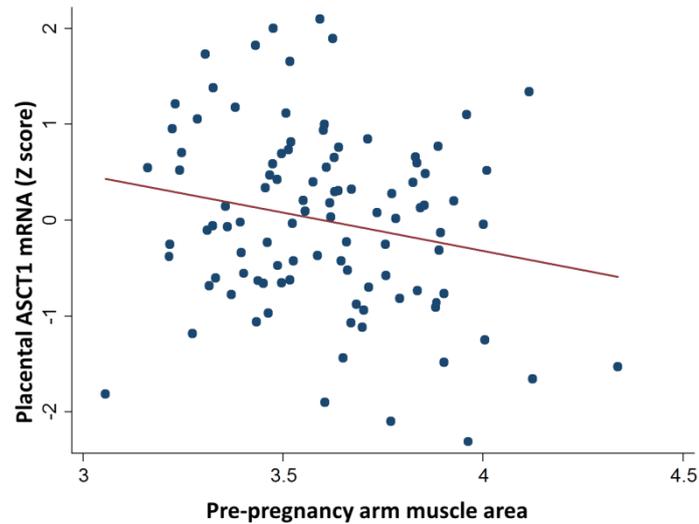
**Figure 5.19: The association between pre-pregnancy mid-upper arm circumference and placental ASC1.** Pre-pregnancy mid-upper arm circumference was negatively associated with placental ASC1 mRNA levels ( $P = 0.01$ ,  $R = -0.26$ ,  $n = 101$ ). Pre-pregnancy mid-upper arm circumference data were log transformed and placental ASC1 mRNA data are presented as Z scores.



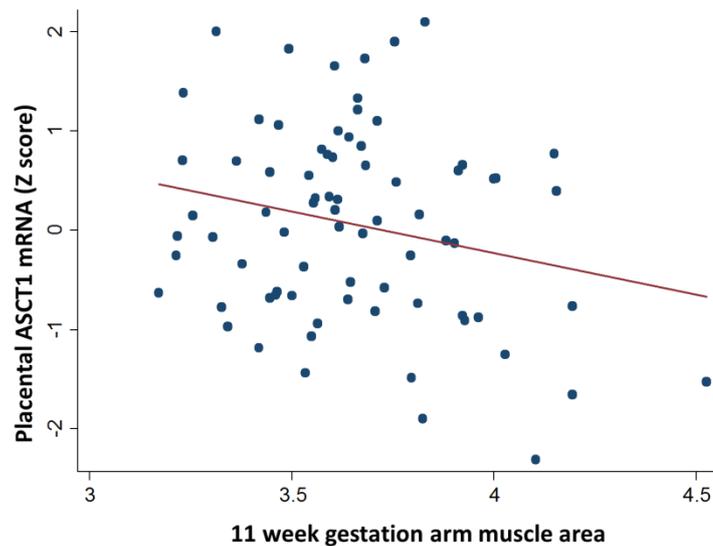
**Figure 5.20: The association between 11 week gestation mid-upper arm circumference and placental *ASC1* mRNA levels.** Mid-upper arm circumference at 11 week gestation was negatively associated with placental *ASC1* mRNA levels ( $P = 0.045$ ,  $R = -0.23$ ,  $n = 76$ ). 11 week gestation mid-upper arm circumference data were log transformed and placental *ASC1* mRNA data are presented as Z scores.



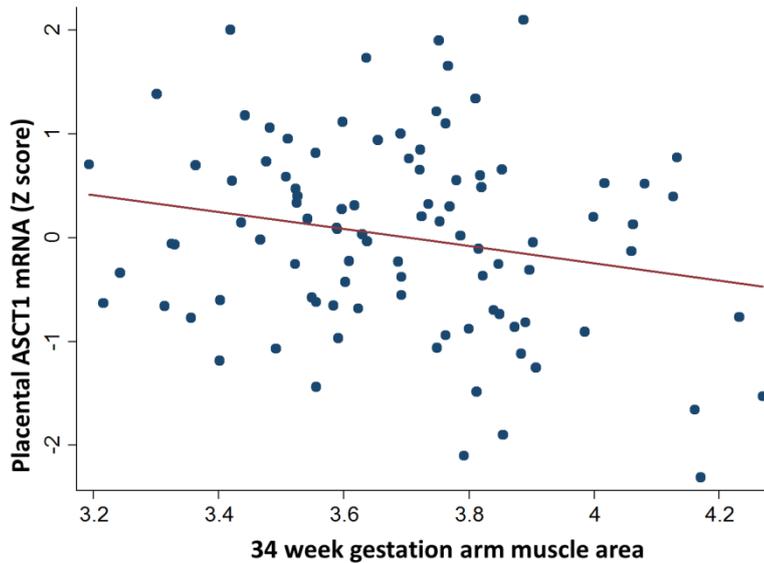
**Figure 5.21: The association between mid-upper arm circumference at 34 weeks gestation and placental *ASC1* mRNA levels.** Mid-upper arm circumference at 34 weeks gestation was negatively associated with placental *ASC1* mRNA levels ( $P = 0.009$ ,  $R = -0.27$ ,  $n = 95$ ). *ASC1* mRNA data are presented as Z scores.



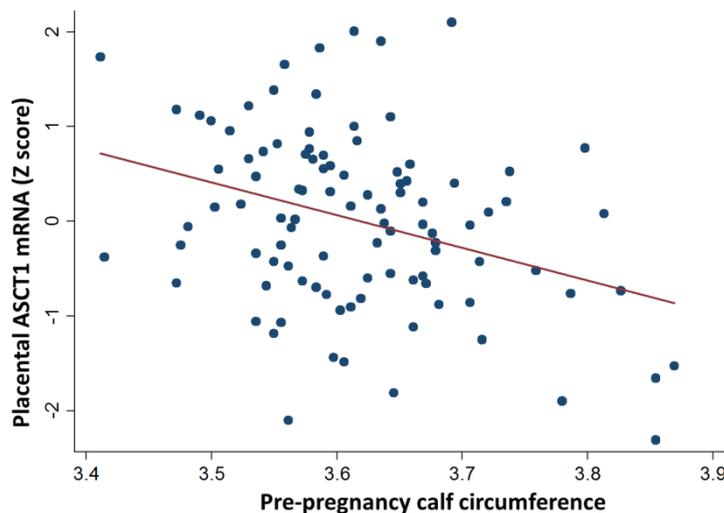
**Figure 5.22: The association between pre-pregnancy arm muscle area and placental *ASC1* mRNA levels.** Pre-pregnancy arm muscle area was negatively associated with placental *ASC1* mRNA levels ( $P = 0.04$ ,  $R = -0.21$ ,  $n = 101$ ). Pre-pregnancy arm muscle area data were log transformed and placental *ASC1* mRNA data are presented as Z scores.



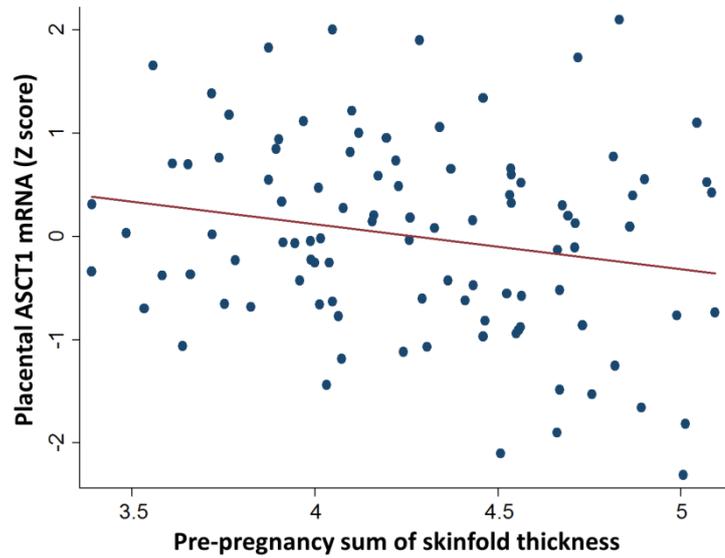
**Figure 5.23: The association between arm muscle area at 11 weeks gestation and placental *ASC1* mRNA levels.** Arm muscle area at 11 weeks gestation was negatively associated with placental *ASC1* mRNA levels ( $P = 0.05$ ,  $R = -0.21$ ,  $n = 76$ ). 11 week gestation arm muscle area data were log transformed and placental *ASC1* mRNA data are presented as Z scores.



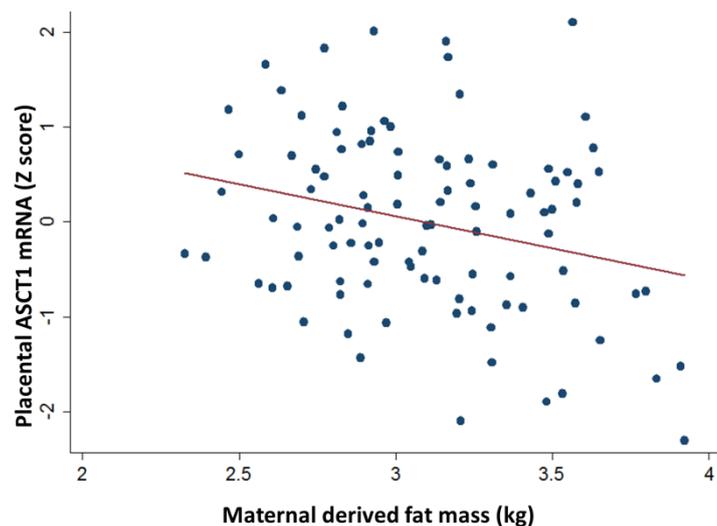
**Figure 5.24: The association between arm muscle area at 34 weeks gestation and placental ASC1 mRNA levels.** Arm muscle area at 34 weeks gestation was negatively associated with placental ASC1 mRNA levels ( $P = 0.04$ ,  $R = -0.21$ ,  $n = 95$ ). 34 weeks gestation arm muscle data were log transformed by Fisher-Yates and placental ASC1 mRNA data are presented as Z scores.



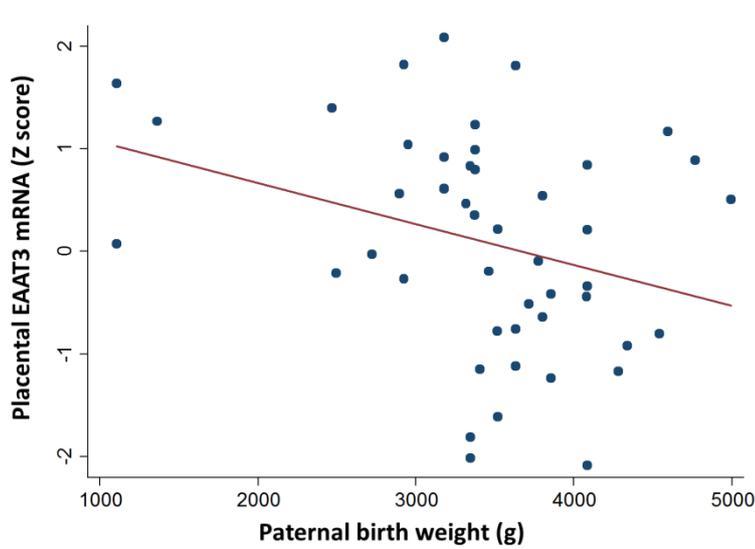
**Figure 5.25: The association between maternal pre-pregnancy calf circumference and placental ASC1 mRNA levels.** Maternal pre-pregnancy calf circumference was negatively associated with placental ASC1 mRNA levels ( $P < 0.001$ ,  $R = -0.34$ ,  $n = 100$ ). Maternal pre-pregnancy calf circumference data were log transformed and ASC1 mRNA data are presented as Z scores.



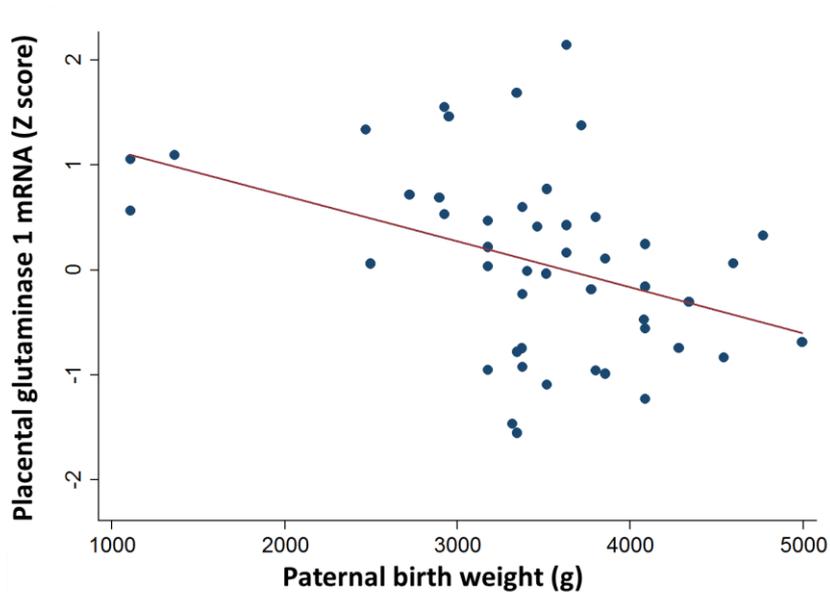
**Figure 5.26: The association between pre-pregnancy sum of skinfold thickness and placental *ASC1* mRNA levels.** Pre-pregnancy sum of skinfold thickness was negatively associated with placental *ASC1* mRNA levels ( $P = 0.042$ ,  $R = -0.20$ ,  $n = 100$ ). Pre-pregnancy sum of skinfold thickness data were log transformed by Fisher-Yates and placental *ASC1* mRNA data are presented as Z scores.



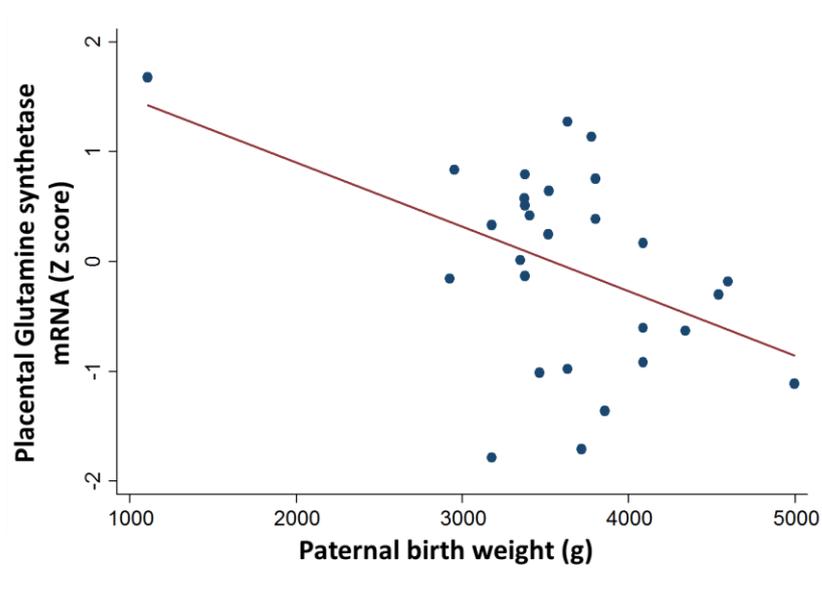
**Figure 5.27: The association between maternal derived fat mass and placental *ASC1* mRNA levels.** Maternal derived fat mass was negatively associated with placental *ASC1* mRNA levels ( $P = 0.007$ ,  $R = -0.27$ ,  $n = 101$ ). Maternal derived fat mass data were log transformed and placental *ASC1* mRNA data are presented as Z scores.



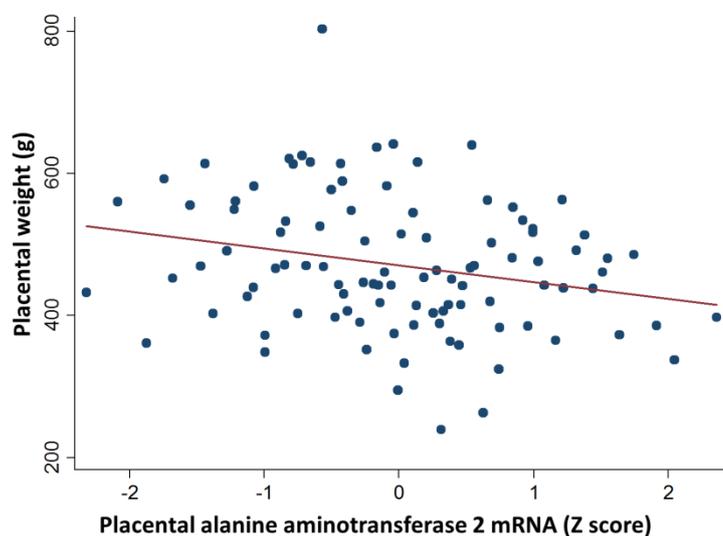
**Figure 5.28: The association between paternal birth weight and placental *EAAT3* mRNA levels.** Paternal birth weight was negatively associated with placental *EAAT3* mRNA levels ( $P = 0.036$ ,  $R = -0.31$ ,  $n = 47$ ). Placental *EAAT3* mRNA data are presented as Z scores.



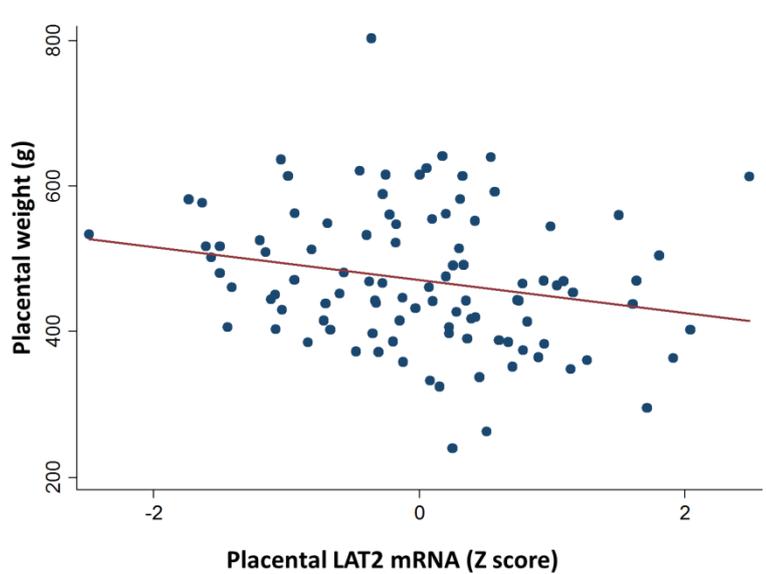
**Figure 5.29: The association between paternal birth weight and placental glutaminase 1 mRNA levels.** Paternal birth weight was negatively associated with placental glutaminase 1 mRNA levels ( $P = 0.005$ ,  $R = -0.40$ ,  $n = 47$ ). Placental glutaminase 1 mRNA data are presented as Z scores.



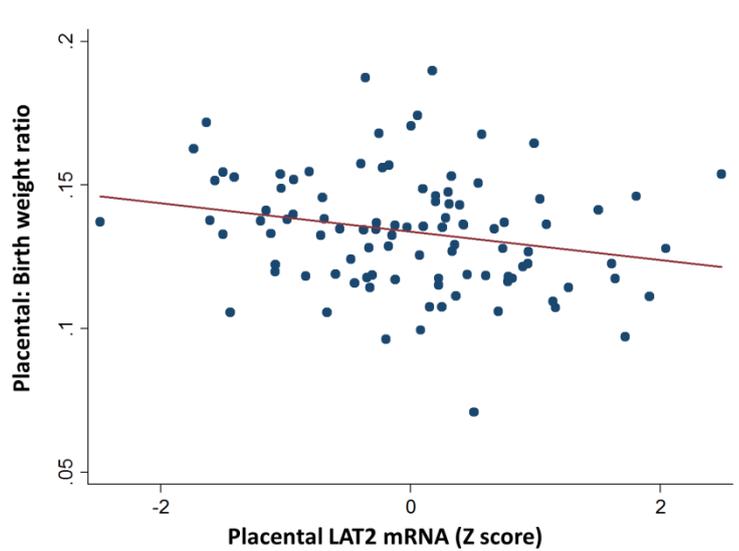
**Figure 5.30: The association between paternal birth weight and placental glutamine synthetase mRNA levels.** Paternal birth weight was negatively associated with placental glutamine synthetase mRNA levels in female placentas ( $P = 0.016$ ,  $R = -0.45$ ,  $n = 28$ ). However when the paternal birth weight of 1107 g which seemed to be an outlier was taken out, the association was not significant ( $P = 0.147$ ,  $R = -0.29$ ,  $n = 27$ ) Placental glutamine synthetase mRNA data are presented as Z scores.



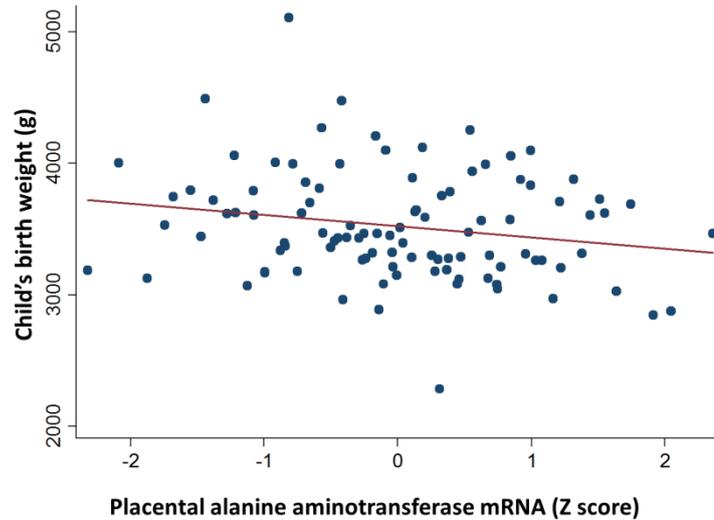
**Figure 5.31: The association between placental weight and placental alanine aminotransferase mRNA levels.** Placental weight was negatively associated with placental alanine aminotransferase mRNA levels ( $P = 0.015$ ,  $R = -0.24$ ,  $n = 102$ ). Placental weight data were adjusted for gestational age and sex and placental alanine aminotransferase mRNA data are presented as Z scores.



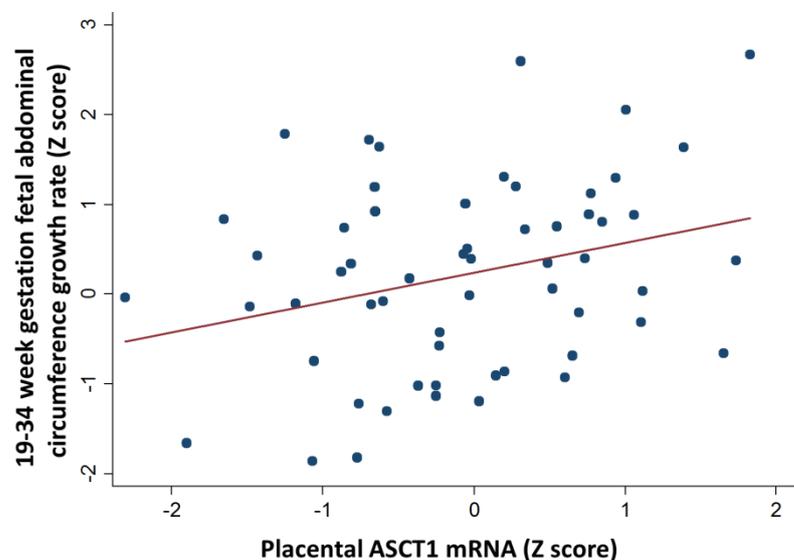
**Figure 5.32: The association between placental weight and placental *LAT2* mRNA levels.** Placental weight was negatively associated with placental *LAT2* mRNA levels ( $P = 0.026$ ,  $R = -0.22$ ,  $n = 102$ ). Placental weight data were adjusted for gestational age and sex and placental *LAT2* mRNA data are presented as Z scores.



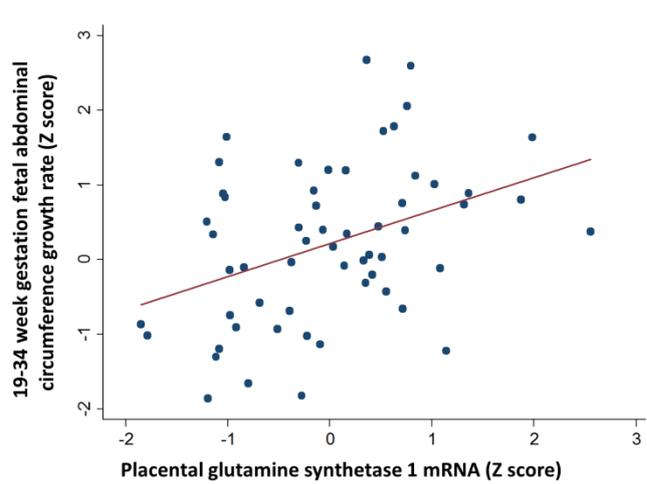
**Figure 5.33: The association between placental Birth weight ratio and placental *LAT2* mRNA levels.** Placental: Birth weight ratio was negatively associated with placental *LAT2* mRNA levels ( $P = 0.022$ ,  $R = -0.23$ ,  $n = 102$ ). Placental: Birth weight data were adjusted for gestational age and sex and log transformed and placental *LAT2* mRNA data are presented as Z scores.



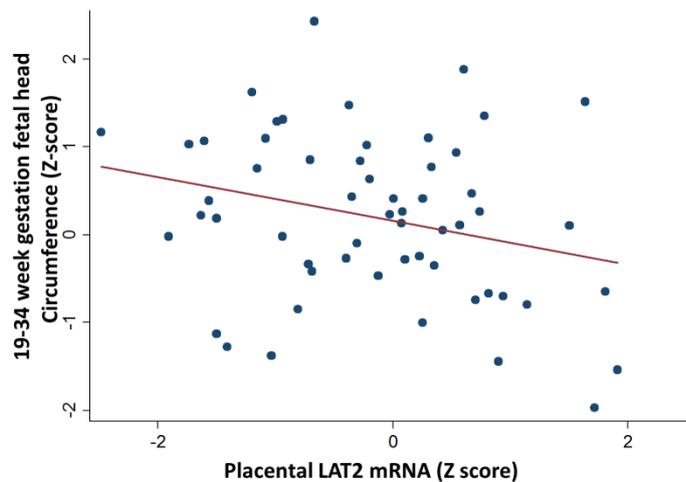
**Figure 5.34: The association between child's birth weight and placental alanine aminotransferase mRNA levels.** Child's birth weight was negatively associated with placental alanine aminotransferase mRNA levels ( $P = 0.045$ ,  $R = -0.20$ ,  $n = 102$ ). Child's birth weight data were adjusted for gestational age and sex and placental mRNA data are presented as Z-scores.



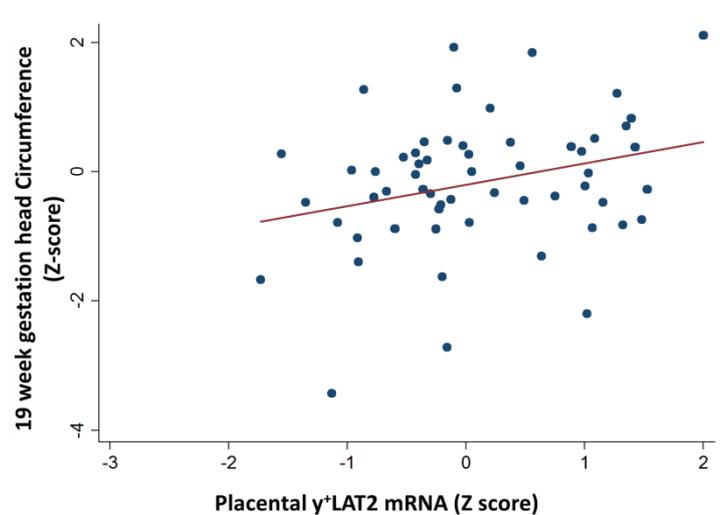
**Figure 5.35: The association between fetal abdominal circumference growth rate and placental ASC1 mRNA levels.** Fetal abdominal circumference growth rate was positively associated with placental ASC1 mRNA levels ( $P = 0.027$ ,  $R = 0.27$ ,  $n = 58$ ). Fetal abdominal circumference growth rate data were log transformed and placental ASC1 mRNA data are presented as Z scores.



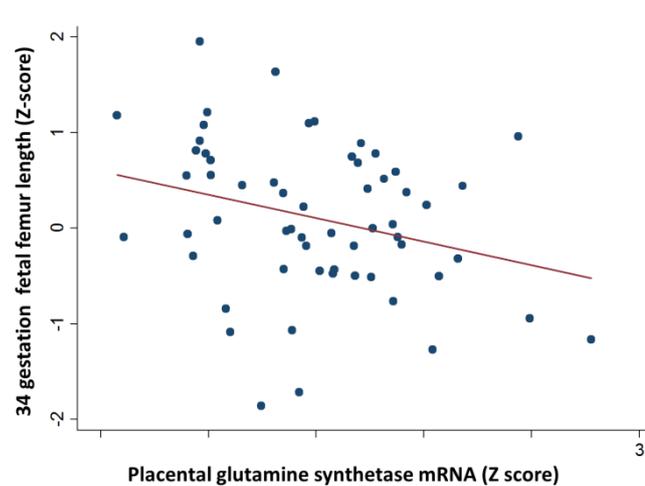
**Figure 5.36: The association between fetal abdominal circumference growth rate and placental glutamine synthetase mRNA levels.** Fetal abdominal circumference growth rate was positively associated with placental glutamine synthetase mRNA levels ( $P = 0.002$ ,  $R = 0.39$ ,  $n = 58$ ). Fetal abdominal circumference growth rate data were log transformed. Both fetal abdominal circumference growth rate and placental glutamine synthetase mRNA data are presented as Z scores.



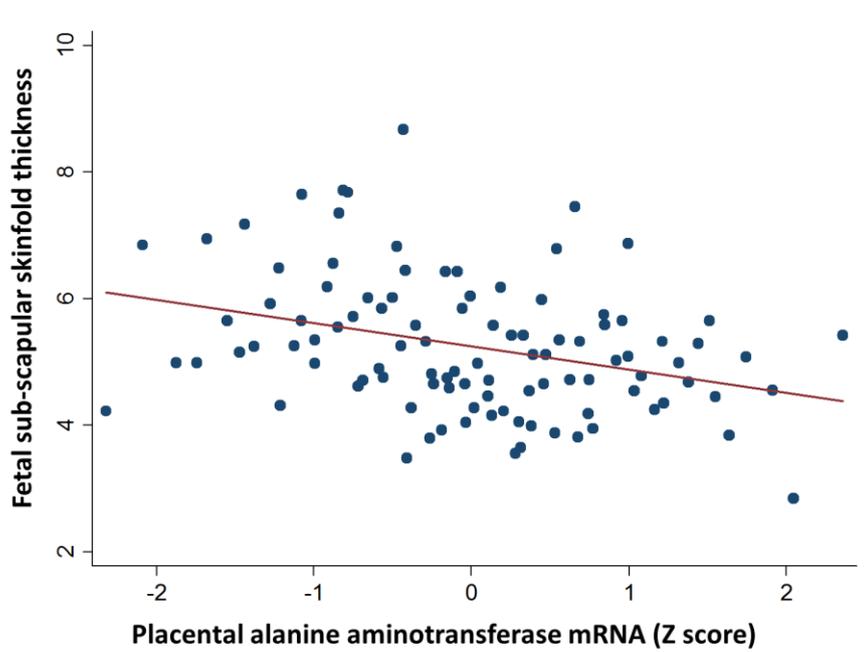
**Figure 5.37: The association between 19-34 week gestation fetal head circumference and placental *LAT2* mRNA levels.** Fetal head circumference at 19-34 week gestation was negatively associated with placental *LAT2* mRNA levels ( $P = 0.041$ ,  $R = -0.27$ ,  $n = 102$ ). Fetal 19-34 week gestation fetal head circumference data were log transformed and placental *LAT2* mRNA data are presented as Z scores.



**Figure 5.38: The association between 19 week gestation fetal head circumference and placental  $y^+LAT2$  mRNA levels.** 19 week gestation fetal head circumference was negatively associated with placental  $y^+LAT2$  mRNA levels ( $P = 0.031$ ,  $R = -0.28$ ,  $n = 102$ ). Fetal 19 week gestation fetal head circumference data were log transformed and placental  $y^+LAT2$  mRNA data are presented as Z scores.



**Figure 5.39: The association between 34 week gestation fetal femur length and placental glutamine synthetase mRNA levels.** Fetal femur length at 34 weeks gestation was negatively associated with placental glutamine synthetase mRNA levels ( $P = 0.027$ ,  $R = -0.29$ ,  $n = 102$ ). 34 week gestation fetal femur length data were log transformed and placental glutamine synthetase mRNA data are presented as Z scores.



**Figure 5.40: The association between fetal sub-scapular skinfold thickness and placental alanine aminotransferase mRNA levels.** Fetal sub-scapular skinfold thickness was negatively associated with placental alanine aminotransferase mRNA levels ( $P = 0.001$ ,  $R = -0.32$ ,  $n = 102$ ). Data are adjusted for sex and presented as Z scores. Fetal sub-scapular skinfold thickness data were log transformed and placental alanine aminotransferase mRNA data are presented as Z scores.

## 5.4: Discussion

This exploratory study has identified the most stably expressed housekeeping genes, amino acid exchangers and isoforms of amino nitrogen metabolic genes that are expressed in the human placenta (Standard Occupational Classification, 1990). It has been demonstrated in this study that the mRNA levels for the most stably expressed genes are higher in the male placentas than in the female placentas (Cleal *et al.*, 2009). This study has also identified relationships between maternal factors and the expression of multiple genes in the human placenta suggesting key areas for further investigation. Maternal parity, pre-pregnancy smoking and exercise were all associated with altered expression of multiple placental genes. The associations with parity may help us to understand the relationship between parity and birth weight and the ways in which a prior pregnancy helps prepare the uterus for future pregnancies. Maternal smoking and exercise are interesting in terms of our understanding of the placental biology and as these are potentially modifiable lifestyle factors. The very few associations between fetal parameters and placental mRNA levels may indicate placental adaptability in response to maternal factors.

### 5.4.1: Amino acid transporter and metabolic enzyme mRNA expression

The expression of the kidney isoform glutaminase 1 and the absence of the liver isoform glutaminase 2 are consistent with a previous study which also showed that both glutamate dehydrogenase and glutamine synthetase are expressed in the human placenta (Cleal *et al.*, 2010). Consistent with a previous study, this study has demonstrated that mitochondrial branched chain aminotransferase is expressed in the human placenta and for the first time, this study has also shown that the cytosolic branched chain aminotransferase is expressed at mRNA level in the human placenta (Jozwik *et al.*, 2009). While the mRNA for both the cytosolic (*AST1*) and the mitochondrial (*AST2*) forms of aspartate aminotransferases were detected in this study, only the mitochondrial (*ALT2*) form of alanine aminotransferase was detected in the in this study. The mRNA for all the amino acid transporters previously detected in the human placenta was also detected in this study, except for amino acid transporter *EAAT1*. This

is in contrast to a previous study which showed that EAAT1 is expressed at protein level in the human placenta (Than *et al.*, 2001).

#### **5.4.2: Sex differences in mRNA levels**

An unexpected finding in this study was that the mRNA expression levels of the three housekeeping genes were between 9% and 16% higher in male placentas than in female placentas.

This raised questions as to whether these three housekeeping genes were really stably expressed as initial experiments had suggested. However, when all genes measured in this study were reanalysed using the geNorm software (n = 102), it was confirmed that the three housekeeping genes were stably expressed relative to other genes even though they differed by sex (Noorlander *et al.*, 2004). At this point it became clear that the seven most stably expressed genes all differed by sex. This may suggest that there is a generalized sex difference in the expression of placental genes, one that is present for most genes but that is only observed in the stably expressed genes where variation due to other factors is lower.

It is unlikely that the sex differences are due to methodological problem as all cDNA was prepared in the same batch and PCR was run in triplicates in the same 384 well plate. In addition, sample preparation and analysis was blinded to sex. One potential methodological source of this difference could be if male placentas have less ribosomal RNA than female placentas. This would mean that in the 0.2 µg total RNA added to the cDNA synthesis reaction there was less mRNA in the female placental samples. Consistent with this hypothesis when 18s rRNA expression levels were analysed they were found to be higher in females than in males. To test this hypothesis further the expression of these genes could be measured in samples where mRNA rather than total RNA was extracted as this would remove any potential bias due to rRNA.

These findings have implications for future studies of placental gene expression which need to be designed and powered to account for sex differences in gene expression. In addition if the observations with regard to ribosomal RNA hold

true in future studies this would suggest that instead of purifying total RNA it may be necessary to extract mRNA to avoid the confounding sex differences.

#### **5.4.2.1: Sex differences in response to maternal environment**

A number of studies have suggested that there are significant differences between the placentas of male and female fetuses and the way in which they respond to maternal environment (Cleal *et al.*, 2009). Male placentas have been shown to respond differently to under-nutrition and to the administration of steroids (Verthelyi, 2001;Sood *et al.*, 2006;Eriksson *et al.*, 2009;Scott *et al.*, 2009). In addition, sex specific differences in gene expression have been observed in the human placenta (Vu *et al.*, 2009;Scott *et al.*, 2009).

This study also found differences in the ways in which mRNA levels in male and female placentas were associated with factors such as maternal smoking and exercise as discussed below. It should be noted that at this point these were subgroup analyses and the numbers were quite small, particularly in the maternal smoking during pregnancy group. However the observations are interesting in the light of the broader literature and provide avenues for further studies. The data presented here therefore provide further evidence that differences between male and female placentas need to be taken into account when designing and analysing studies involving male and female placentas.

#### **5.4.3: Maternal environment and placental gene expression**

The clusters of significance identified by this study were related to factors which were specific to maternal parity, smoking and exercise rather than more general measures such as education and social class where there were few significant associations with placental gene expression. Of great interest are also the relationships between placental *ASC1* mRNA levels and the 7 markers of maternal body composition (maternal body composition reflects maternal nutritional status).

##### **5.4.3.1: Smoking**

Maternal smoking has been shown to be associated with preterm birth and poor fetal growth outcomes (Sood *et al.*, 2006). Previous studies have demonstrated

structural changes in the placentas of smoking mothers (Velez *et al.*, 2008; Voigt *et al.*, 2011). The current study suggests that there was differential regulation of five of the genes studied in mothers who smoked before and/or during pregnancy. A parallel study on the same samples also found elevated mRNA levels for the facilitative amino acid transporter *LAT3* in the placentas of smoking mothers, although only in female placentas (Personal communication, Dr Jane Cleal, University of Southampton).

The mechanism by which smoking, particularly pre-pregnancy smoking, affects placental gene expression is not clear. However previous studies have shown that smoking exposes the body to multiple toxins many of which may persist in the body. In rats it is clear that peri-conceptual nutrition may affect later development and it is possible that peri-conceptual smoking may also have detrimental effects on the development of the oocyte or early embryo (Rath *et al.*, 2011). If this were the case, the possibility that many of these women did not give up smoking until after they had become pregnant may explain how pre-pregnancy smoking has an effect.

The association between maternal smoking and placental gene expression was significant for pre-pregnancy maternal smoking but not in mothers who smoked during pregnancy. This may be because those who reported stopping smoking during pregnancy were included as non-smokers and may also be due to the low sample size in the smoking group where sex interaction was observed.

The fewer associations observed between maternal smoking during pregnancy and placental mRNA levels may also be due to other factors, such as change in behaviour during pregnancy. Alternatively, the associations of pre-pregnancy smoking and placental mRNA levels may be due to other confounding factors such as social class, obesity and dietary prudence. However there was no association with the expression of these genes and social class, obesity or dietary prudence so there was no evidence for this in our study.

As gene expression was up-regulated by maternal pre-pregnancy smoking it is possible that this is an adaptive response increasing nutrient transport to the fetus. The transporters  $y^+LAT1$  and  $y^+LAT2$  transport arginine which is a

substrate for nitric oxide which regulates vascular tone (Kwong *et al.*, 2000). The increase of  $y^+LAT1$  in response to maternal smoking may enable the placenta to adapt to altered placental vascular tone as well as reduced maternal blood flow which is seen in smoking mothers (Galley & Webster, 2004). Alternatively, this could be a response due to fetal signals if the fetus was deprived of amino acids that are transported by  $y^+LAT1$ . There is evidence that the transporter systems in the placenta may be altered in response to fetal signals (Clausen *et al.*, 1999).

There were opposing effects of pre-pregnancy smoking on the cytoplasmic and mitochondrial isoforms of aspartate aminotransferase; it is not clear why this is but it has been suggested that maternal smoking may cause oxidative damage to the placenta which may result in mitochondrial dysfunction (Constancia *et al.*, 2005). Differential alterations in the amino acid metabolic enzyme gene expression observed in this study may therefore depend on whether the enzymes are expressed in the mitochondria or the cytosol.

#### **5.4.3.2: Maternal exercise**

Given the previous observations that activity of the amino acid transporter system A is associated with maternal muscle mass, it was interesting to see that mothers reporting that they had undertaken strenuous exercise and mothers with a faster than normal walking speed both had altered placental expression of multiple genes (Wiktor *et al.*, 2004; Bouhours-Nouet *et al.*, 2005). Strenuous exercise was associated with up regulation of genes and increased walking speed associated with a down regulation of placental genes.

During pregnancy nitrogen requirements for net tissue deposition in both the mother and the fetus is met by changes in nitrogen kinetics that occur to increase nitrogen availability (Lewis *et al.*, 2010). At this time the mother and the fetus may be in competition for resources such as nitrogen. The associations observed could be due to increased maternal nitrogen utilisation or diversion of maternal blood flow away from the placenta during exercise (Duggleby & Jackson, 2002b). Increases in placental gene expression for the genes involved in nitrogen transport may therefore aim to compete with the

mother for the available nitrogen. This may lead to increased nitrogen partitioning into the fetal compartment.

In general, exercise increases the expression of a wide range of metabolic genes which increase the efficiency of energy use and it is possible that the same signals that drive this process in the adult are also affecting the placenta (Clapp *et al.*, 2000).

It is not clear why the two measures of exercise studied here had different effects. The differences in the response of strenuous exercise and walking speed may be surrogate for other less obvious factors. On the other hand walking speed may not have similar physiological effects on blood flow or nitrogen balance as seen with strenuous exercise.

#### **5.4.3.3: Parity**

Nulliparous women have higher rates of low birth weight and reduced placental weight (Clapp *et al.*, 2000;Booth & Shanely, 2004). It is therefore interesting that there are changes in placental gene expression in the placentas of multiparous mothers which may reflect placental responses to these uterine changes and provide clues to their nature. Uterine changes during the first pregnancy may modify blood supply so the changes in placental gene expression may reflect changes in blood supply and thus nutrient availability (Wilsher & Allen, 2003;Shah, 2010;Naeye, 1987).

In males, multiparous women had decreased levels of placental *LAT2* mRNA levels. This may be because, in multiparous women, there is extensive trophoblast invasion which may be accompanied by increased transporter mRNA levels and therefore an increased capacity to transport nutrients. The reduced alanine aminotransferase mRNA levels, also observed in males in multiparous women, may be in response to the increased capacity for amino acid transfer so that the need to synthesise and catabolise amino acids becomes less (Prefumo *et al.*, 2004).

#### **5.4.3.4: Maternal body composition and ASC1**

The negative association between *ASC1* mRNA levels and the seven measures of maternal body composition suggest that this gene may be particularly responsive to maternal body composition. It has been suggested that body composition may reflect maternal nutrient reserves and the mother's capacity to support the pregnancy. Mothers with more muscle or fat had lower placental *ASC1* mRNA levels. Maternal fat and lean mass may influence the mother's ability to support the pregnancy especially if food becomes scarce during the pregnancy.

#### **5.4.3.5: Maternal diet**

Maternal diet is one of the most important factors determining fetal growth as well as the risk of chronic disease in adult life (Prefumo *et al.*, 2006). In this study two types of maternal diet were assessed, prudent diet which involved intakes of food such as fruit, vegetables, yogurt, whole-meal bread, rice and pasta. The high-energy diet on the other hand involved high consumption of fruit and vegetables, puddings, meat and fish, eggs and egg dishes, cakes, biscuits, full-fat spread, potatoes, crisps, and confectionery (Barker, 1997b; Godfrey *et al.*, 1997).

There were eight associations between maternal diet and placental gene expression. The sex differences observed in maternal high-energy diet are similar to observations in mice placentas where different diets have differential effects on male and female placentas (Crozier *et al.*, 2010). However in contrast to the mice placenta where females were more responsive to diet, this study has shown that maternal diet was to a larger extent associated with mRNA levels in male placentas. These results may also implicate the role of the placenta on the sex differences observed in the Dutch Winter Hunger studies (Mao *et al.*, 2010). It is not clear why there are sex differences in response to maternal diet but it may be due to metabolic demand differences between males and females as well as levels of sex hormones.

The effects on glutamate and aspartate transporters (*EAAT2* and *ASC2*) in parallel with their metabolic enzymes may be an attempt by the placenta to take

up and metabolise these amino acids efficiently. In the rat splanchnic bed, activity and mRNA of *EAAT2* has been shown to be up-regulated by protein diet (Ravelli *et al.*, 1999).

It is noteworthy that there was a negative association between dietary prudence and aspartate aminotransferase 2 and a positive association between maternal high-energy and aspartate aminotransferase in females. This is consistent with observations that aspartate aminotransferase transcription is negatively regulated by low-energy diet through mechanisms involving insulin (Jean *et al.*, 2001).

#### **5.4.4: Paternal birth weight**

Paternal birth weight was negatively associated with the expression of the accumulative transporter *EAAT3* and glutaminase 1 mRNA levels. It is interesting to observe that the strength of these relationships is in part dependent on the high expression of these genes in those with very low birth weight fathers (< 1250 g). However, as the number of low birth weight fathers is low it is not possible to draw conclusions from this observation and further studies are required to investigate this. If the observation were sustained by further work it would suggest that there is paternal transmission of an environmentally induced epigenetic signal, consistent with work in animals (Plee-Gautier *et al.*, 1998).

#### **5.4.5: Placental mRNA levels and fetal growth parameters**

There were relatively few significant relationships between placental mRNA levels and fetal or neonatal parameters compared to the number of associations observed for maternal parameters outlined above. The mRNA levels of two genes were negatively associated with placental weight, one was negatively associated with birth weight and two genes were positively associated with fetal abdominal circumference growth rate and abdominal circumference growth rate. The fewer associations observed between fetal parameters and placental genes may be because placental gene expression is less related to fetal growth or that changes in gene expression are adaptive and normalise fetal growth. Evidence for placental normalisation of fetal growth comes from studies in Saudi Arabia,

which have shown that during Ramadan fasting, placental weight is reduced, but its efficiency is increased (Carone *et al.*, 2010). As a result, fetal growth is not affected in response to changes in maternal diet. Whether this may manifest itself in adulthood is not clear. As there were very few associations between placental mRNA levels and fetal growth parameters, the following discussions are grouped in relation to what they might mean for future studies.

#### **5.4.5.1: Fetal head circumference and placental to birth weight ratio**

It is interesting that maternal birth weight and parity were negatively associated with placental *LAT2* mRNA expression, which was also negatively associated with fetal head circumference and placental to birth weight ratio. The negative association of *LAT2* mRNA with nulliparity and then fetal head circumference may be a protective mechanism against increased head circumference observed in first born babies, which may lead to birth complications (Alwasel *et al.*, 2010). A preserved head circumference is also observed in babies from mothers who were small at birth, and in this study maternal birth weight was negatively associated with *LAT2* mRNA levels. These observations may present a true effect of maternal birth weight and parity on placental *LAT2* mRNA levels, which may then affect amino acid transfer and subsequently fetal head circumference and placental to fetal birth weight ratio. However, due to sample numbers and the possibility of confounding factors, these associations could not be investigated further.

#### **5.4.5.2: Femur length and fetal abdominal circumference growth rate**

The associations of placental *ASC1* mRNA with maternal body composition and fetal abdominal circumference growth rate may also present a true maternal effect on the placenta and then the fetus. In contrast to *ASC1*, placental glutamine synthetase was positively associated with abdominal circumference growth rate and negatively associated with femur length and paternal birth weight. It is not clear whether this may also present a true paternal effect on placental genes and fetal growth parameters or not. However both femur length and fetal abdominal circumference growth rate are known to be better markers of fetal growth. Future studies should therefore investigate how the genes

associated with femur length and fetal abdominal circumference growth rate are regulated.

#### **5.4.5.3: Placenta weight, fetal birth weight and sub-scapular skinfold thickness**

Alanine aminotransferase 2 protein levels are associated with markers of metabolic syndrome such as abdominal visceral obesity, impaired insulin sensitivity, elevated fasting glucose and in addition, alanine aminotransferases are good indicator of type 2 diabetes (Ong *et al.*, 2002). Although this study did not measure protein levels of alanine aminotransferase 2, the associations between alanine aminotransferase 2 mRNA levels and birth parameters, such as placental weight, fetal birth weight and sub-scapular skinfold thickness, may reflect placental response to fetal fat and glycogen stores. In addition, in the fatty liver of an obese mouse, the gene expression of alanine aminotransferase 2 is increased two-fold (Iacobellis *et al.*, 2008; Schindhelm *et al.*, 2005; Vozarova *et al.*, 2002). The placenta may be responding to fetal alanine aminotransferase levels which may reflect fetal adiposity by reducing its alanine aminotransferase levels. Further studies are required using mouse models to determine the associations between fetal and placenta alanine aminotransferase levels, birth weight and metabolic syndrome markers.

#### **5.4.6: Other associations from a parallel study**

In a parallel study on the same samples, correlations were also found between the mRNA levels of the facilitative amino acid transporters *LAT3* and *LAT4* in the placenta and calf circumference (Personal communication, Dr Jane Cleal, University of Southampton).

#### **5.4.7: Additional analysis**

The possibility of making predictions from these data and testing them using the SWS data sets was considered as a way of testing and making further use of the placental gene expression data set. In particular, the 27 cases in which placental gene expression was related to both a maternal factor and a fetal factor could be investigated further to demonstrate whether there was also a relationship between the maternal factor and the fetal factor. If factor A

(maternal) is related to factor B (placental) and factor B is also related to factor C (fetal) then, if these were real relationships, A should be related to C. Whether or not the predicted relationships were present could be investigated within the SWS cohort. However, after statistical advice it was decided that this approach should not be adopted. The reason for this is that we could not adequately account for confounding variables without a great deal of statistical analyses within the SWS data set for each individual factor. For example in this study, maternal body composition was associated with placental *ASC1* amino acid transporter mRNA levels, which was also related to fetal abdominal circumference growth rate. This may have been due to a direct relationship between maternal factors, the placenta and fetal growth parameters or it may have been due to other confounding factors. Perhaps women who are thin might have a specific diet or might smoke and it may be the diet or the smoking that have an effect on the fetal abdominal circumference growth rate rather than the actual maternal body composition. If a straight comparison between maternal body composition and fetal abdominal circumference were performed without adjusting for confounding factors there could not be very much confidence that a positive result was indeed positive or that a negative result was in reality negative. However, in a dataset of only 27 cases, exploration of all possible confounding factors would throw up many false positives and the statistical power to test the direct relationships between maternal and fetal factors would be very low. Moreover, these direct relationships are being explored in the much larger SWS datasets as part of other projects, and that work is beyond the scope of this thesis.

## **5.5: Study limitations**

This was an exploratory study and there are two primary limitations inherent in its design. The first is that it involves multiple statistical comparisons and the second is that confounding variables make interpretation difficult. In addition there were the issues that mRNA levels do not necessarily correspond to protein activity and that mRNA expression was seen to be different in males and females.

The problem with multiple statistical comparisons is that for every 20 statistical tests done one will come up positive by chance. For this reason many of the observations that were observed here are likely to have been due to chance. One way to address this would have been to only accept a much higher P value as statistically significant which would have reduced the likelihood of false positives. However this may also have increased the possibility of false negatives and given that the purpose of this exploratory study was to identify targets for future research it was decided not to take this approach. In recognition of the multiple comparisons issue, however, this chapter has focused on changes which showed a cluster of relationships with maternal environment or those that had a particularly strong correlation. While care needs to be taken not to over-interpret these data, they have produced a number of interesting observations where the expressions of multiple genes are associated with a maternal factor, such as smoking and parity, which deserve future research.

As discussed above one of the issues with this study is the possibility of confounding factors which mean that when an association is observed, for example between smoking and placental gene expression, it could be that it is not the smoking but a factor related to smoking which is the real issue, for instance smokers' diets may be different from non-smokers'. It should always be remembered that as correlation is not causation this study was carried out to highlight areas that are promising for future research.

As mRNA levels do not always correlate to protein expression the changes in mRNA levels observed in this study do not demonstrate changes in the activity of the proteins they encode. What this means is that there were changes in the activity of the proteins which regulate the expression and stability of these mRNA species. These regulatory proteins will be signalling molecules in second messenger pathways, such as the mTOR pathway, transcription factors (both for the genes themselves and microRNAs which regulate their stability) and proteins which directly affect mRNA stability. The activity of these proteins will change in response to stimuli such as hormones or nutrient levels and will not just be affecting the genes that have been studied in this thesis but all the

genes affected by a particular pathway or transcription factor. So while this study does not demonstrate that maternal environment is associated with the changes in activity of its target protein, it does demonstrate that maternal environment is associated with changes in the regulatory environment within the placenta. It is the fact that maternal environment is affecting the placenta that is the real purpose of this study.

With a sample size of 102, the numbers in the study exceeded that recommended by our initial power calculations. However, wherever subgroup analyses were required, for instance when it was demonstrated that male and female placentas were responding differently to a maternal factor, the overall number was diluted. The numbers were further diluted when there was an imbalance between the groups, for instance of the total of 102 in the study there were only 6 mothers of female babies who smoked during pregnancy. In these cases additional care needs to be taken when interpreting the data.

## **5.6: Implications**

This study provides further evidence consistent with the hypothesis that maternal environment does affect the placenta. Alterations in placental amino acid metabolic enzyme and transporter mRNA levels may be one of the mechanisms through which maternal environment affects placental amino acid transfer to the fetus. As amino acid transporter and metabolic enzymes are subject to hormonal regulation, the placenta may respond to maternal environment and fetal metabolic demand via hormonal signals by altering mRNA expression.

## **5.7: Future work**

This study has identified several interesting areas for future work, including the effects of maternal smoking and parity on the placenta. More studies are required to test these observations using larger numbers so that issues of sex differences can be better addressed. In addition to confirming the mRNA data reported in this thesis, further studies should investigate how these changes in gene expression were influencing placental function.

Where these studies confirmed the original observations more mechanistic studies could be undertaken, for instance studying the effect of toxins from cigarette smoke on trophoblast in cell culture.

The long-term aim of future studies arising from the results in this thesis would be to confirm the original observations and to demonstrate a causal link between the maternal factor (or associated confounding variable) and the changes in the placenta. Beyond this the aim would be to determine how this affects fetal growth and development.

## **5.8: Summary**

In conclusion, this study demonstrates relationships between maternal factors and placental gene expression which suggest that maternal factors may influence placental function. In addition, the relationships with maternal smoking and exercise are particularly interesting as they represent potentially modifiable lifestyle factors. As mRNA levels may not necessarily reflect protein function these studies do suggest that maternal signals are acting on the placenta to regulate gene expression. Both amino acid transporters and metabolic genes function as parts of complex systems and may also be regulated and altered at protein level and their decreased or increased mRNA expression cannot be assumed to reflect increased or decreased placental transfer as a whole (Jadhao *et al.*, 2004). However, this study provides a basis for further studies as maternal signals through hormones, such as the glucocorticoids, may regulate amino acid or metabolic signals such as the levels of specific nutrients (Sengers *et al.*, 2010).

## **Chapter 6**

### **General discussion**



## 6.1: Background

Glucose and amino acids are important substrates for fetal growth but the factors involved in their transfer across the placenta are not well understood. This thesis focused on investigating the transport and metabolism of glucose and amino acids in the normal term human placenta and investigating whether placental mRNA levels for genes involved in amino acid transfer were associated with maternal and fetal factors.

The studies in Chapter 3 suggest that there is a high capacity for glucose transfer across the placenta which is not limited by transporters on the MVM and the BM. However, as the glucose gradient across the placenta is maintained, it is likely that other factors such as placental diffusive barrier, blood flow, paracellular diffusion and placental metabolism offer a greater barrier to glucose transfer than the transporters. Alterations in these factors may therefore limit glucose transfer to the fetus and lead to poor fetal growth.

Having established the mechanisms which may be involved in placental glucose transfer and metabolism, studies in Chapter 4 were carried out to investigate placental amino acid transfer and metabolism. These studies have demonstrated that the placenta utilises nitrogen from glutamate and leucine to form other amino acids and that glutamate is central to nitrogen exchange between amino acids. The studies in Chapter 4 have also demonstrated that the carbon skeleton from glutamate is used for amino acid synthesis, particularly glutamine and also for energy production. Together with the transfer of glutamate, aspartate and proline, the finding that there is activity for the novel amino acid transporter system N which may transport glutamine, indicates that there are other aspects of amino acid transport in the human placenta that are not fully understood.

As previous studies have shown that placental function is subject to maternal and fetal signals, an exploratory study was carried out to investigate whether placental mRNA for metabolic enzymes and amino acid transporters shown to be active in the placenta, are associated with maternal and fetal factors (Roos *et al.*, 2009). Studies in Chapter 5 suggest that maternal factors such as

smoking, parity, strenuous exercise and body composition are associated with altered placental mRNA expression. However there were relatively few associations between placental mRNA levels and fetal growth parameters. While the data in Chapter 5 do not show a causal relationship, they have highlighted factors which may affect placental function and are therefore an important basis for future studies.

This final chapter will discuss the findings of this thesis, in relation to the role of placental transport and metabolism of glucose and amino acids and how these may determine fetal growth and whether the placenta may be influenced by maternal and fetal factors. It will go on to discuss the limitations of this study together with possible future studies that may be undertaken to improve fetal growth.

## **6.2: Transfer and metabolism of glucose across the human placenta**

In contrast to previous studies this study suggests that membrane transporters are not rate-limiting for glucose transfer across the human placenta. This does not imply that glucose transporters provide no limitation to transport but that in relation to other factors the barrier offered by the transporters is minimal. Altered placental glucose transfer is implicated in IUGR and fetal macrosomia. To understand mechanisms involved in these pregnancy pathologies previous studies have concentrated on the regulation of the glucose transporters (Cleal & Lewis, 2008). This study, however, suggests that, in order to understand mechanisms underlying altered placental glucose transfer in IUGR and fetal macrosomia, the focus for future studies should be on factors such as the novel idea of placental diffusive resistance (identified in this study) together with placenta metabolism, placental blood flow and paracellular diffusion rather than membrane transporters alone.

The differences between maternal and fetal glucose concentrations demonstrate that even though transfer is not transporter-limited there is still a diffusion barrier. This diffusion barrier may represent the time taken for glucose to diffuse across the placental barrier (approximately 8  $\mu\text{m}$ ) or diffuse through the cell junctions of the fetal capillary endothelium. These observations suggest

that placental structure (diffusion distance, endothelial cell arrangement) may play a greater role in limiting glucose transfer to the fetus than might have been imagined.

While the effect of placental blood flow on glucose transfer was not investigated in this study the evidence that glucose transfer is not transporter-limited suggests that blood flow may well be a limiting factor and that glucose transfer may be flow-limited. This is consistent with a previous study which found alterations in placental glucose transfer in response to changes in blood flow (Jansson *et al.*, 2002b; Osmond *et al.*, 2000). Future experiments need to address this in more depth.

Changing placental glucose consumption rate would also be one way of protecting the fetus from low or high maternal glucose levels. The findings of this study, in terms of glucose consumption and export of lactate, suggest that placental glucose metabolism is not altered by maternal or fetal glucose levels. Recent studies of pregnant mothers living at high altitude do however indicate that in some circumstances the placenta is able to alter its metabolic rate, in this case by lowering oxidative metabolism of glucose to preserve oxygen required by the fetus (Illsley, 1987). Targeting glucose metabolism may therefore be a better way to modulate glucose supply to the fetus.

Paracellular diffusion is another factor thought to play a minor role in transferring glucose across the placenta. This study has demonstrated a high capacity for unidirectional glucose transfer across the placenta. While net diffusion in the perfused placenta was in the fetal to maternal direction it is difficult to translate these findings into understanding net transfer *in vivo*. The main reason for this is that the amount of conductive diffusion, as determined by the capillary-intervillous space pressure difference is not known *in vivo*. This does however suggest that altering fetal capillary pressure may provide a mechanism by which paracellular flux can be regulated. The fact that paracellular diffusion is altered in GDM may indicate that placental paracellular routes are subject to regulation in pathological pregnancies (Zamudio *et al.*, 2010). Further work is required to better understand the role of paracellular routes and their regulation in determining nutrient transfer to the fetus, whether

this is altered in pathological pregnancies and whether it could be a target for interventions.

### **6.2.1: Implications of glucose transfer for fetal growth**

The findings that there is a high capacity for glucose transfer across the placenta and that glucose transporters are not rate-limiting have implications on the current views on the role of glucose transporters in both IUGR and fetal macrosomia. First, that any attempt to target glucose transporters in order to improve fetal growth may not be successful in doing so and second, that, although glucose transporters are altered at concentrations above 15 mmol/L, this may not be sufficient to limit glucose transfer to the fetus in GDM where glucose levels are below 15 mmol/L (Osmond *et al.*, 2000). However a focus towards the factors proposed to be limiting for glucose transfer in this study may be important for future studies aiming to help improve fetal growth. Placental diffusive barrier, blood flow and paracellular diffusion are specifically of interest as they are dependent on factors such as placental structure and size. Small placental volume has been shown to be associated with low birth weight, which is associated with increased risk of chronic disease in adulthood (Hahn *et al.*, 1998). It is possible that placental diffusive resistance, blood flow and paracellular diffusion, are altered in parallel with a reduced/increased placental volume and structure and that it is these factors that lead to altered glucose transfer and subsequently IUGR and fetal macrosomia.

### **6.3: Placental amino acid metabolism**

Studies in Chapter 4 demonstrated that there is significant inter-conversion and metabolism of amino acids in the normal term human placenta and that some of the carbon from glutamate is catabolised for energy production. It has also provided novel insights into factors that may be associated with both amino acid and glucose metabolism such as pyruvate recycling.

The important role of glutamate as a central molecule for the transfer of nitrogen has been highlighted in this study, with the <sup>15</sup>N from leucine being first incorporated into glutamate and then transferred to other amino acids. Interestingly within the placenta there was no evidence for the incorporation of

nitrogen into serine or glycine. This may suggest that the pathways for the incorporation of ammonia from glutamate into these amino acids are not active within the human placenta. Alternatively it may be that glutamate was mainly catabolised by aminotransferase reactions rather than by glutamate dehydrogenase to produce ammonia. As the amine and amide forms of nitrogen of glutamine could not be distinguished and ammonia was not measured in this study it is not possible to draw conclusions from these observations. The fate of the 80% nitrogen that could not be accounted for in this study also remains to be established.

It has previously been suggested that both BCAAs and glutamate may be utilised by the human placenta to produce non-essential amino acids which provide the rate-limiting non-essential nitrogen to the fetus (Kinare *et al.*, 2000). The findings that the nitrogen from leucine and glutamate is used for glutamine, aspartate and alanine synthesis in the human placenta is consistent with these suggestions. Future studies should investigate the extent to which non-essential nitrogen available to the fetus derives from placental glutamate and leucine metabolism

In contrast to what happens in the ovine placenta, in the studies in this thesis very small quantities of BCKAs were transferred to the fetal circulation and maternal circulation and very small amounts were measured in the placenta (Kalhan & Parimi, 2006). In addition, in the human placenta the rate of BCKA transfer to maternal circulation is higher than that to the fetal circulation and this is in contrast to the ovine placenta (Smeaton *et al.*, 1989). The lower levels of BCKAs in the human placenta may suggest that the human placenta catabolises BCKAs for energy. As BCAAs and BCKAs do not seem to be spared by placental metabolism, it may be that there is a higher demand for non-essential amino acids than for the essential amino acids.

The fact that some of the glutamate taken up by the placenta is metabolised for energy may also suggest a link to overall placental metabolism. Although quantitatively smaller than glucose the provision of intermediary metabolites at different points in the Krebs's cycle may be metabolically important for placental energy metabolism. This may be similar to the gut which, like the placenta, is an

epithelial organ and predominantly metabolises dietary glutamate for energy and spares glucose from complete oxidation for its use in other biosynthetic pathways (Smeaton *et al.*, 1989). As the metabolism of glutamate in Krebs's cycle involving glutamate dehydrogenase is regulated by the redox state of the cell, it is likely glutamate metabolism via the Krebs's cycle may be affected by hypoxia or conditions in which the NADH to NAD<sup>+</sup> ratio is altered (Burrin & Stoll, 2009; Riedijk *et al.*, 2007). Further studies are required to investigate how placental redox state may affect placental amino acid metabolism and whether this may have implications for amino acid delivery to the fetus.

In most tissues, the metabolism of amino acids plays an important role in regulating glucose availability and in some cases glycolysis may be abolished to preserve glucose with the cell relying on amino acid metabolism for energy (Riedijk *et al.*, 2007; Owen *et al.*, 2002). While results in this thesis suggest that pyruvate may have been formed from glutamate, there is no evidence for glucose and alanine synthesis from glutamate. This may be due to the preferential utilisation of carbon from glutamate for energy, than for glucose and alanine synthesis. As lactate was not measured in the <sup>13</sup>C-glutamate studies, future studies should determine whether lactate was synthesised from glutamate. This will help to determine whether lactate released in the glucose experiments was derived from amino acids rather than from glucose alone. The ability for the placenta to switch from glucose utilisation to amino acid utilisation for energy production may be an important pathway for regulating energy and substrate availability. Future studies need to establish whether the redirection of the carbon towards the Krebs's cycle rather than towards glucose synthesis is a regulated pathway and how this may determine overall substrate availability in the placenta and fetal circulation.

In contrast to previous suggestions, this study has shown that glutamate, aspartate and proline are transferred across the placenta and that there is an activity for the novel amino acid transporter system N. Overall these observations indicate that the mechanisms of placental amino acid transport across the placenta are not fully understood. Further studies are required to confirm the presence of the system N transporter and exchanger (s) responsible

for glutamate and aspartate transfer to the fetus. As system N is responsible for transferring major substrates that contain an amide nitrogen (glutamine, asparagine and histidine), it is likely that alterations in this transporter may lead to poor fetal growth. Understanding how system N and glutamate and aspartate exchanger are regulated will help in determining whether these transporters are altered and associated with IUGR and macrosomia.

Evidence for metabolic compartmentalisation within the placenta provided in Chapter 4 was intriguing as there are no obvious cellular compartments within the placenta in which this could take place. If this compartmentalised metabolism is occurring within the syncytiotrophoblast the localisation of the metabolically active compartment may determine its regulation. If the compartment is on the fetal side of the placenta or the endothelium it may suggest regulation by the fetal factors whereas if it is within the syncytiotrophoblast it would be more available to regulation by maternal signals. Metabolic compartmentalisation may be important for the regulation of amino acid transfer between the mother and the fetus.

### **6.3.1: Implications of placental amino acid metabolism for fetal growth**

The ability for the placenta to metabolise amino acids has implications for both maternal and fetal substrate availability and fetal growth. This is because placental glutamate and leucine uptake strongly correlate with the conversion of these substrates to other metabolites within the placenta rather than their transfer to the fetus (Appendix 3). Additionally, as amino acids produced in the placenta are transferred to both the maternal and the fetal circulation, placental metabolism will change the quantity and composition of amino acids in both circulations and may also provide the fetus with the amino acids it requires for its metabolic demand. Another implication of placental amino acid metabolism is that it will affect the already complex mechanisms of amino acid transport across the placenta where an amino acid from the fetus, e.g. glutamate may be metabolised in the placenta to form for example, leucine, which then exchanges for glutamine in the fetal circulation, in turn the glutamine will exchange for another amino acid such as threonine. As a result, if the metabolism of one

amino acid is altered in whatever process, not only will it affect the composition and quantity of amino acids transferred to the fetus, it will also have an effect on the pathways associated with each of the amino acids. It is also possible that the placenta will determine the partitioning of amino acids and their metabolic products between the mother and the fetus, depending on the demand.

## **6.4: Maternal influences on the placenta and placental gene expression**

This thesis has provided evidence that there are associations between maternal factors and the expression of placental genes involved in nitrogen transfer and metabolism. These findings suggest that maternal factors such as smoking, strenuous exercise and parity may be important factors that may influence placental gene expression. Another important finding in this study was that there are mRNA sex differences in the genes that are stably expressed in the placenta, in that they are higher in the placentas from males than those from females. Future studies should therefore take into account these differences as they may obscure otherwise important findings. Very few associations were observed between placental genes and fetal growth parameters. This may suggest that the placenta is capable of normalising maternal effects to ensure that fetal growth is not affected. Further studies are required to verify this at protein level and determine whether the placenta does indeed normalise maternal effects, such that the fetus is not affected.

### **6.4.1: Implications of placental gene expression studies**

Overall mRNA studies have provided further evidence that maternal environment does influence placental gene expression and to a lesser extent fetal growth parameters. This raises questions as to how the determinants of fetal growth are regulated within the placenta. This study has also provided an important basis for future studies that may aim to enhance placental amino acid transfer across the placenta to enhance fetal growth.

The possibility of making predictions from these data and testing them using the SWS datasets was considered as a way of testing and making further use of the placental gene expression data set. In particular, the 27 cases in which

placental gene expression was related to both a maternal factor and a fetal factor could have been investigated further. However, due to the possibility of other confounding factors, this study could not demonstrate whether there was a causal relationship between maternal factors, placental mRNA levels and fetal growth parameters.

#### **6.4.2: Implications of glucose and amino acid metabolism on fetal growth**

The metabolism of glucose and amino acids are closely inter-related. Although there was no evidence for the incorporation of  $^{13}\text{C}$  into glucose there was evidence for pyruvate production and recycling and it is possible that some of this pyruvate may have been used for lactate production rather than for glucose. However the transfer of  $^{13}\text{C}$  to lactate in the U- $^{13}\text{C}$ -glutamate studies was not determined. It is important to determine whether lactate and pyruvate are produced from amino acids and glucose in the human placenta. Lactate concentrations have been shown to be high in the maternal and fetal circulations of IUGR fetuses and lactate release to the fetal venous circulation is reduced in the placentas of insulin treated GDM mothers (Amaral *et al.*, 2011;Olstad *et al.*, 2007b). This indicates that the pathways for lactate metabolism are altered in IUGR and GDM pregnancy pathologies and that this may be subject to hormonal regulation. Interestingly, increased lactate concentrations which lead to lactic acidosis are associated with altered glycolytic pathways in glycogen storage disease which in combination with lactate, also shows other symptoms similar to those observed in IUGR and preeclampsia (hypoglycaemia, hypertriglyceridemia and hyperurecaemia) (Osmond *et al.*, 2000;Marconi *et al.*, 2006).

Future studies should therefore investigate the levels of these substrates in the fetal and maternal compartments and determine how placental metabolism may affect their availability. The extent of inter-relation of placental metabolic pathways may specifically be investigated using metabolomic studies which give a snapshot of cellular metabolic profiles.

As glutamine has previously been shown to be a good substrate for lactate production especially in rapidly growing cells, such as tumor cells, it is also

important to establish to what extent glutamine transferred to the fetus contributes to lactate synthesis (Marconi *et al.*, 2006;Cohen *et al.*, 1985). The availability of the glutamine-glutamate cycle may be an important pathway to prevent glutamine catabolism in the fetal tissue to lactate, which may result in lactic acidosis.

Future studies should therefore establish whether lactate and pyruvate are synthesised from glutamine or glutamate in the human placenta and whether the extent to which their synthesis occurs is different in normal, IUGR and macrosomic fetuses. This may provide a link through which low or high amino acid concentrations are associated with fetal IUGR or macrosomia respectively (DeBerardinis *et al.*, 2007). It is also possible that other gluconeogenic amino acids may contribute to the production of lactate and pyruvate, so future studies should aim to produce a profile of amino acid carbon metabolites in the human placenta.

## **6.5: Future studies**

The results in this thesis have highlighted important aspects of placental glucose and amino acid transfer that require further investigation, which may in future provide important strategies for improving fetal growth. This section will outline the possible factors that require further investigation.

### **6.5.1: Placental glucose transfer follow up studies**

As data in this thesis suggest that placental glucose transfer is not limited by membrane transporters, future studies should concentrate on factors such as diffusive resistance, the regulation of paracellular diffusion and placental metabolism and blood flow.

In most pathological conditions such as IUGR and fetal macrosomia, placental structure and morphology may be altered and this may lead to altered placental diffusive properties. There is evidence for altered placental shape, structure and morphology in GDM and IUGR (Cetin *et al.*, 1992;Cetin *et al.*, 2005) (Tewari *et al.*, 2011). Future studies should therefore investigate the effects of altered placental structure and morphology on placental glucose transfer and whether these are responsible for altered glucose transfer and fetal hypoglycaemia.

Placental glucose transfer could be investigated in an isolated human placenta or using maternal stable isotope tracer studies and the results could be correlated to placental structural and morphological observations from immunohistochemistry studies.

Placental blood flow has also been shown to be altered in placentas from GDM mothers and abnormal utero-placental blood flow is associated with IUGR (Schwartz *et al.*, 2010; Verma *et al.*, 2008). Future studies should therefore investigate whether placental glucose transfer is altered in parallel to altered blood flow in these pregnancy pathologies. This could be done by correlating utero-placental blood flow measurements taken during pregnancy when IUGR and fetal macrosomia are suspected, with measurements of placental glucose transfer in a placental perfusion model. Placental vasculature could also be correlated with placental glucose transfer capacities in these pregnancy conditions.

As discussed in chapter four, placental glucose metabolism may also be influenced by placental metabolism of amino acids such as leucine and glutamate. As there are contradictory data on whether placental glucose consumption determines the capacity of glucose transfer to the fetus, future studies should aim to give clarity on this issue, and investigate other factors related to glucose metabolism such as amino acids.

Placental paracellular diffusion of L-glucose is altered in placentas from GDM mothers and there is evidence for altered paracellular routes in the human placenta in GDM (Omar *et al.*, 1998; Yagel *et al.*, 1999). Future studies could investigate whether the fibrinoid-filled denudations and the trans-trophoblastic channels are responsible for glucose transfer and whether their alteration observed in GDM is associated with altered paracellular glucose transfer. In addition, further studies are required to determine factors associated with the distribution of these paracellular routes in normal and pathological pregnancies and whether they are subject to maternal or fetal signal regulation.

### 6.5.2: Placental amino acid transfer follow up studies

The methods used in this study could not distinguish the amine and amide nitrogens of glutamine. Further studies using Nuclear Magnetic Resonance (NMR) or Liquid chromatography-Mass-spectrometry (LC-MS) should establish what proportion of the label was going to amino nitrogen and what to amide nitrogen. The distinction of the amine and amide nitrogen will enable the determination of whether there are other sources of ammonia in the human placenta and what they are. It may be that these sources of ammonia are also used for the synthesis of serine and glycine in the placenta. Future studies could use NMR to identify positions of both nitrogen and carbon labelling. As LC-MS can analyse samples that are not derivatised and not volatile it can be used for the detection of almost all amino acids. Future studies could therefore use LC-MS for the detection of amino acids that could not be measured in this study (arginine and cysteine) and ammonia as this may explain the 80% nitrogen that was unaccounted for.

Technology that is now becoming more widely available would allow future studies to undertake full metabolomics profiles in perfusion studies measuring the changes in the levels of hundreds of metabolites in response to changes in specific amino acids. These would allow a more comprehensive understanding of placental metabolism especially if combined with tracer studies as used here.

While it was shown in this study that proline was transferred to the fetus as deduced from its enrichment in the fetal vein samples, its concentrations could not be determined as the derivatisation of amino acids using OPA only allows the detection of primary amines (Frank *et al.*, 1994; Osmond *et al.*, 2000). Future studies could also use an alternative derivatising reagent such as 9-fluorenylmethyl chloroformate (MFOC-Cl) which can be used for the derivatisation of both primary and secondary amines (Furst & Kuhn K, 1999).

This study only investigated the metabolism of glutamate and leucine in the human placenta, however there are yet more studies required in order to determine the extent to which each amino acid contributes to metabolism during pregnancy. There are unresolved issues relating to the origins of glycine and serine in the human placenta as glycine is not transferred to the fetus. Glycine

is one of the amino acids shown to be rate-limiting for the provision of non-essential nitrogen during growth, future studies should investigate whether glycine is synthesised from serine or other substrates in the fetal circulation (Furst & Kuhn K, 1999). This could be investigated by introducing labelled serine into the maternal circulation a few hours prior to the child's birth, and measuring placental and neonatal plasma glycine enrichments and concentrations soon after birth.

### **6.5.3: Placental gene expression follow up studies**

Gene expression data have also highlighted important maternal factors that could affect placental function. Future studies should investigate these factors at protein level and use large cohort studies to determine whether maternal factors such as smoking and strenuous exercise could be modified to improve fetal growth. It is also possible that placenta amino acid metabolic and transporter genes are subject to hormonal regulation and therefore future studies should investigate whether this is the case.

Although in all the studies perfusions and tissue collection was carried out within 30 minutes of placental delivery it is also possible that prolonged labour may have affected placental integrity. Future studies should therefore investigate whether prolonged labour affects placental nutrient metabolism and transfer as well as mRNA integrity and if so, these factors should be put accounted for.

## **6.6: Summary**

Placental transfer of glucose and amino acids to the fetus is thought to be altered in IUGR and fetal macrosomia. IUGR and fetal macrosomia are associated with increased risk of maternal and fetal complications at birth and chronic disease in adulthood. In order to improve fetal growth outcomes, it is crucial to understand how important substrates such as glucose and amino acids are transferred to the fetus. Although a number of studies have investigated the transfer of these substrates across the placenta, the mechanisms involved are still poorly understood. This thesis was therefore carried out to investigate mechanisms involved in the transfer of glucose and

amino acids across the human placenta and determine whether placental mRNA expression of genes involved in amino acid transfer is associated with maternal and fetal factors.

This thesis contributes to our understanding of the transfer and metabolism of glucose and amino acids in the human placenta. It has been demonstrated in this thesis that placental transfer of glucose and amino acids involves mechanisms that may be determined by several factors other than transporters alone. In addition to membrane transporters this study suggests that future studies should also concentrate on placental diffusive barrier, blood flow, paracellular diffusion and metabolism as important determinants of placental glucose transfer. If all these factors play a role in glucose transfer, the mechanisms involved in altered glucose transfer may be much more complex than is currently known. This is because factors such as placental diffusion and blood flow may depend on other factors such as placental morphology, structure and vasculature. As well as being determined by blood flow (oxygen availability) and all the factors affecting blood flow, placental metabolism may also be affected by the energy status of the cells and circulating hormones. While the role of placental amino acid metabolism in determining glucose availability is not clear from studies in this thesis, there is emerging evidence that glucose may be produced from amino acids but that this may depend on substrate availability.

The observation that amino acids from both the maternal and the fetal arterial circulation are metabolised in the human placenta, suggests that placental metabolism may be an important determinant of amino acid transfer to the fetus. It is also likely that placental metabolism may change the composition and quantity of amino acids being transferred to the fetus. The knowledge of which amino acids are metabolised by the human placenta and transferred to the fetus may enable future studies to supply these amino acids in order to modify the concentration and composition of certain substrates.

The demonstration that placental amino acid metabolism can generate amino acids that are transferred to the fetus raises the question as to whether transporters and metabolic pathways act in a coordinated way. Incorporating

amino acid transport mechanisms with metabolic pathways may be an important step in determining how amino acid delivery to the fetus is regulated. The fact that most amino acids synthesised in the placenta are transferred to the maternal circulation highlights the complexity of the mechanisms involved in placental amino acid transfer as previously described in a mathematically modelled placenta (Jackson, 1991). Due to the complexity of amino acid transfer across the human placenta, future studies need to incorporate experimental data of metabolism and transport with mathematical modelling data. This thesis has also provided an important basis for future studies on maternal influences on the placenta.

The findings of this thesis have implications for our understanding of mechanisms involved in altered fetal growth. Future interventions to combat IUGR and fetal macrosomia and subsequently chronic disease in adulthood should aim to target factors such as placental diffusive barrier, blood flow and metabolism rather than transport alone.



# **Appendices**



## Appendix 1: Reference list

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## Appendix 2: Glucose tracer buffers

### Glucose for maternal arterial perfusion (plus Creatinine)

For 200 mmol/L stock, 9g of glucose was dissolved in 100ml of water, 50ml of solution B and 2.5ml ( 200 mmol/L ) Creatinine was added to the glucose solution in a conical flask and this was made up to 250ml with distilled water. The stock solution (200 mmol/L) was used to make a series of maternal glucose concentrations (see Table).

### Glucose for fetal arterial perfusion (minus Creatinine)

A 50ml batch of 200 mmol/L glucose to be perfused on the fetal side was prepared without Creatinine. This was done by dissolving 1.8g of glucose in 20ml of water, 10ml of solution B was added to the glucose solution in a conical flask and this was made up to 50ml with distilled water. The stock solution (200 mmol/L) was used to make 3 mmol/L glucose to be perfused on the fetal side (see Table 1).

### Tracer buffers

1. **A** -3 L EBB 0 mmol/L glucose
2. **B** -5 L 1 x EBB + 4  $\mu\text{Ci}$   $^{14}\text{C}$ -L-glucose + 40  $\mu\text{Ci}$   $^3\text{H}$ -3-o-mehtyl glucose + 2.2 mmol/L creatinine
3. **C** -20ml/ experiment 2 mol/l glucose stock solution in 1 x EBB

Make 200 ml and aliquot (90 g of 2M glucose (MW 180.16) in 250ml)

### To make

1 L 0 mmol/L glucose EBB to start up an as reserve

3.9L maternal tracer buffer = 3250ml + tracers and 650 mixed with glucose

Make Total	buffer	glucose
A1 - 1.5 L	0 mmol/L glucose EBB	NO GLUCOSE 7.5 ml EBB
A2 – 500ml	3 mmol/L glucose EBB	0.75 ml 2M glucose+1.75 ml EBB
A3 - 1L	6 mmol/L glucose EBB	3 ml 2 M glucose+2 ml EBB
B1 - 1L	0 mmol/L glucose EBB + tracers	NO GLUCOSE + 5 ml EBB
B2 - 1L	3 mmol/L glucose EBB + tracers	1.5ml2 M glucose+3. 5mlEBB

<b>B3</b> - 1L	6 mmol/L glucose EBB + tracers	3 ml 2 M glucose + 2ml EBB
<b>B4</b> - 1L	9 mmol/L glucose EBB + tracers	4.5 ml 2 M glucose + 0.5ml EBB
<b>B5</b> -500ml	12 mmol/L glucose EBB + tracers	3 ml 2 M glucose NO EBB

1 L 0 mmol/L glucose EBB to start up an as reserve

3L fetal tracer buffer = 3000ml + tracers and 650 mixed with glucose

<b>Make Total</b>	<b>buffer</b>	<b>glucose</b>
<b>A1</b> - 1.5 L	0 mmol/L glucose EBB	NO GLUCOSE 7.5 ml EBB
<b>A2</b> - 1.5 L	3 mmol/L glucose EBB + tracers	0.75 ml 2M glucose+1.75 ml EBB
<b>A3</b> – 500ml	6 mmol/L glucose EBB + tracers	3 ml 2 M glucose+2 ml EBB
<b>A4</b> - 1L	9 mmol/L glucose EBB + tracers	4.5 ml 2 M glucose + 0.5ml EBB
<b>A5</b> -500ml	20 mmol/L glucose EBB + tracers	5ml glucose no EBB
<b>B1</b> - 1L	0 mmol/L glucose EBB	NO GLUCOSE + 5 ml EBB
<b>B2</b> - 1L	3 mmol/L glucose EBB	1.5ml 2 M glucose+3. 5ml EBB
<b>B3</b> - 1L	6 mmol/L glucose EBB	3 ml 2 M glucose + 2ml EBB
<b>B4</b> - 1L	9 mmol/L glucose EBB	4.5 ml 2 M glucose + 0.5ml EBB
<b>B5</b> -500ml	12 mmol/L glucose EBB	3 ml 2 M glucose NO EBB
<b>B6</b> -500ml	15 mmol/L glucose EBB	3.75 ml 2 M glucose +.25ml EBB

## Fetal side glucose tracer experiential plan

Time	Action	Maternal	Fetal ( recovery > 95%)
0	Start fetal perfusion	<b>B1</b> EBB 0 mmol/L glucose	No flow
<b>5 and 10 min</b>	Check fetal recovery is > 95%	<b>B1</b> EBB 0 mmol/L glucose	No flow
<b>15 min</b>	If fetal recovery > 95% then <b>start maternal perfusion</b> for a 15 min washout	<b>B1</b> EBB 0 mmol/L glucose	<b>A1</b> <sup>-14</sup> ml/min EBB 0 mmol/L glucose
<b>30min</b>	<b>Add tracer and to maternal circuit</b> <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine <b>Sample every 2 min 0, +2, +4, +6, +8, +10, +12, +14, +16, +18, +20</b>	<b>B1</b> EBB 0 mmol/L glucose	<b>A2</b> <sup>-14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>50 min</b>	Change maternal to 3 mmol/L ol/L ol/L <b>Sample at +5, +10, +15 and +18</b>	<b>B2</b> EBB 3 mmol/L glucose	<b>A2</b> <sup>-14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>1 hr 10 min</b>	Change maternal to 6 mmol/L ol/L ol/L <b>Sample at +5, +10, +15 and +18</b>	<b>B3</b> EBB 6 mmol/L glucose	<b>A2</b> <sup>-14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>1 hr 30 min</b>	Change maternal to 9 mmol/L Sample at +5, +10, +15 and +18	<b>B4</b> EBB 9 mmol/L glucose	<b>A2</b> <sup>-14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>1 hr 50 min</b>	Change <b>fetal</b> to 3 mmol/L Sample at +5, +10, +15 and +18	<b>B4</b> EBB 9 mmol/L glucose	<b>A3</b> <sup>-14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>2 hr 10 min</b>	Change maternal to 6 mmol/L Sample at +5, +10, +15 and +18	<b>B3</b> EBB 6 mmol/L glucose	<b>A3</b> <sup>-14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>2 hr 30 min</b>	Change maternal to 3 mmol/L Sample at +5, +10, +15 and +18	<b>B2</b> EBB 3 mmol/L glucose	<b>A3</b> <sup>-14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>2 hr 50 min</b>	Change maternal to 0 mmol/L Sample at +5, +10, +15 and +18	<b>B1</b> EBB 0 mmol/L glucose	<b>A3</b> <sup>-14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>3 hr 10 min</b>	Change <b>fetal</b> to 6 mmol/L Sample at +5, +10, +15 and +18	<b>B1</b> EBB 0 mmol/L glucose	<b>B1</b> <sup>-14</sup> ml/min EBB 0 mmol/L glucose + <sup>14</sup> C-L-Glucose 3H-3-0-Methyl glucose 1.8 mmol/L creatinine
<b>3 hr 30 min</b>	Change maternal to 3 mmol/L Sample at +5, +10, +15 and +18	<b>B2</b> EBB 3 mmol/L glucose	<b>A4</b> <sup>-14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>3 hr 50 min</b>	Change maternal to 6 mmol/L Sample at +5, +10, +15 and +18	<b>B3</b> EBB 6 mmol/L glucose	<b>A4</b> <sup>-14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>4 hr 10 min</b>	Change maternal to 9 mmol/L Sample at +5, +10, +15 and +18	<b>A3</b> EBB 6 mmol/L glucose	<b>A4</b> <sup>-14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>4 hr 30 min</b>	Change maternal to 12 mmol/L Sample at +5, +10, +15 and +18	<b>A3</b> EBB 6 mmol/L glucose	<b>A4</b> <sup>-14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>4 hr 50 min</b>	Change maternal to 12 mmol/L Sample at +5, +10, +15 and +18	<b>B4</b> EBB 9 mmol/L glucose	<b>A4</b> <sup>-14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>5 hr 10 min</b>	Change maternal to 12 mmol/L Sample at +5, +10, +15 and +18	<b>B5</b> EBB12 mmol/L glucose	<b>A4</b> <sup>-14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
	Change maternal to 12 mmol/L Sample at +5, +10, +15 and +18	<b>B5</b> EBB15 mmol/L glucose	<b>A5</b> <sup>-14</sup> ml/min EBB 20 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
<b>5 hr 30 min</b>	<b>END</b>	<b>END</b>	<b>END</b>

**Maternal side glucose tracer experiential plan**

Time	Action	Fetal	Maternal
0	Start fetal perfusion	A1 - 6ml/min EBB 0 mmol/L glucose	No flow
5 and 10 min	Check fetal recovery is > 95%	A1 - 6ml/min EBB 0 mmol/L glucose	No flow
15 min	If fetal recovery > 95% then <b>start maternal perfusion</b> for a 15 min washout	A1 - 6ml/min EBB 0 mmol/L glucose	A1 - <sup>14</sup> ml/min EBB 0 mmol/L glucose
30min	<b>Add tracer and to maternal circuit</b> <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine <b>Sample every 2 min 0, +2, +4, +6, +8, +10, +12, +14, +16, +18, +20</b>	A1 EBB 0 mmol/L glucose	B1 - <sup>14</sup> ml/min EBB 0 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
50 min	Change maternal to 3 mmol/L <b>Sample at +5, +10, +15 and +18</b>	A1 EBB 0 mmol/L glucose	B2 - <sup>14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
1 hr 10 min	Change maternal to 6 mmol/L <b>Sample at +5, +10, +15 and +18</b>	A1 EBB 0 mmol/L glucose	B3 - <sup>14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-Glucose 3H-3-O-Methyl glucose 1.8 mmol/L creatinine
1 hr 30 min	Change maternal to 9 mmol/L Sample at +5, +10, +15 and +18	A1 EBB 0 mmol/L glucose	B4 - <sup>14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
1 hr 50 min	Change <b>fetal</b> to 3 mmol/L Sample at +5, +10, +15 and +18	A2 EBB 3 mmol/L glucose	B5 - <sup>14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
2 hr 10 min	Change maternal to 6 mmol/L Sample at +5, +10, +15 and +18	A2 EBB 3 mmol/L glucose	B3 - <sup>14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
2 hr 30 min	Change maternal to 3 mmol/L Sample at +5, +10, +15 and +18	A2 EBB 3 mmol/L glucose	B2- <sup>14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
2 hr 50 min	Change maternal to 0 mmol/L Sample at +5, +10, +15 and +18	A2 EBB 3 mmol/L glucose	B1 - <sup>14</sup> ml/min EBB 0 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
3 hr 10 min	Change <b>fetal</b> to 6 mmol/L Sample at +5, +10, +15 and +18	A3 EBB 6 mmol/L glucose	B1 - <sup>14</sup> ml/min EBB 0 mmol/L glucose + <sup>14</sup> C-L-Glucose 3H-3-O-Methyl glucose 1.8 mmol/L creatinine
3 hr 30 min	Change maternal to 3 mmol/L Sample at +5, +10, +15 and +18	A3 EBB 6 mmol/L glucose	B2 - <sup>14</sup> ml/min EBB 3 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
3 hr 50 min	Change maternal to 6 mmol/L Sample at +5, +10, +15 and +18	A3 EBB 6 mmol/L glucose	B3 - <sup>14</sup> ml/min EBB 6 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
4 hr 10 min	Change maternal to 9 mmol/L Sample at +5, +10, +15 and +18	A3 EBB 6 mmol/L glucose	B4- <sup>14</sup> ml/min EBB 9 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
4 hr 30 min	Change maternal to 12 mmol/L Sample at +5, +10, +15 and +18	A3 EBB 6 mmol/L glucose	B5 - <sup>14</sup> ml/min EBB 12 mmol/L glucose + <sup>14</sup> C-L-glucose + <sup>3</sup> H-3-o-methyl glucose + 1.8 mmol/L creatinine
4 hr 50 min	<b>END</b>	<b>END</b>	<b>END</b>

## Appendix 3: Supplementary material for amino acid metabolism experiments

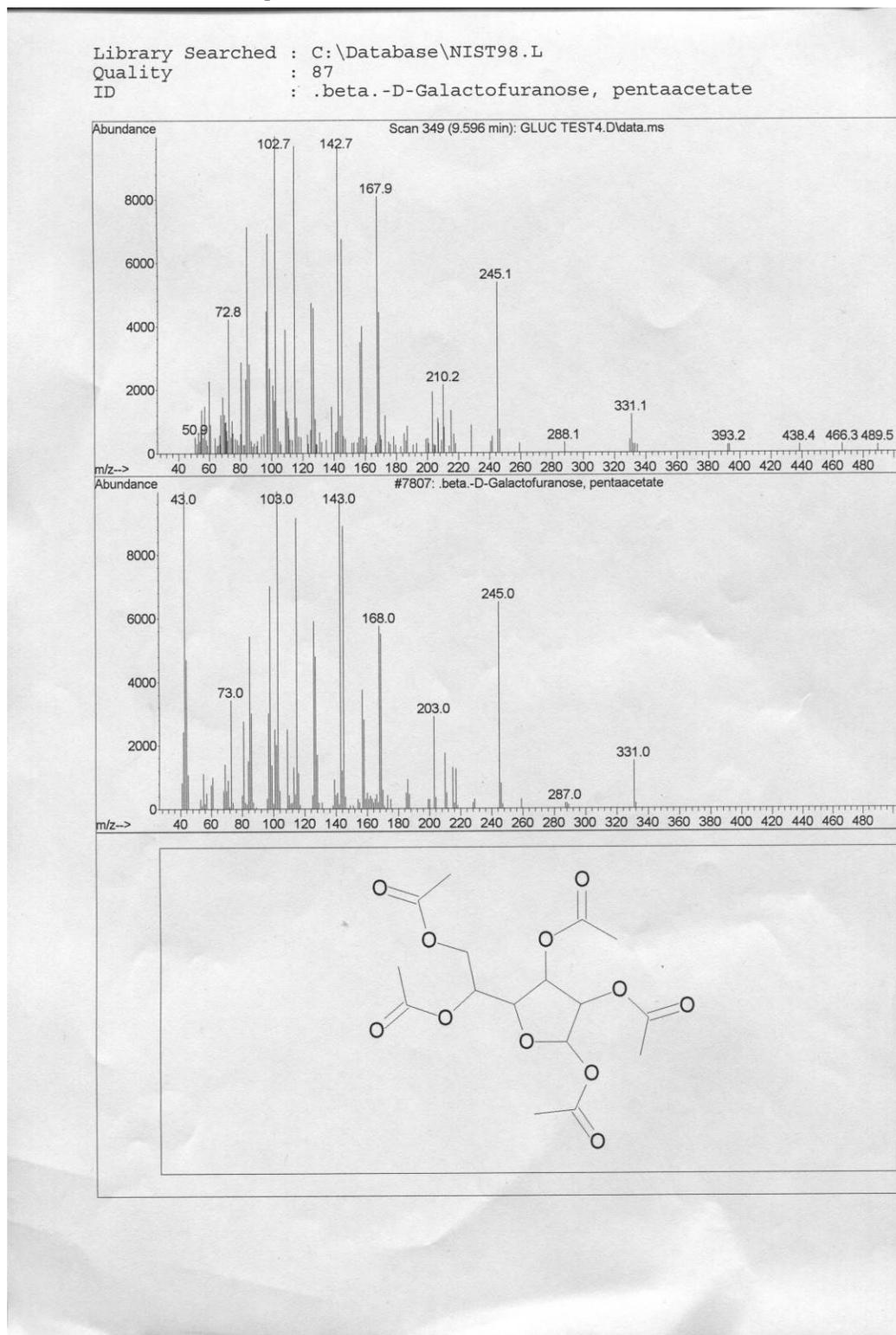


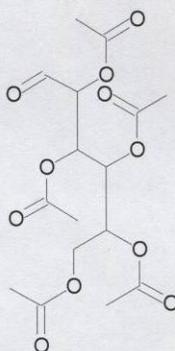
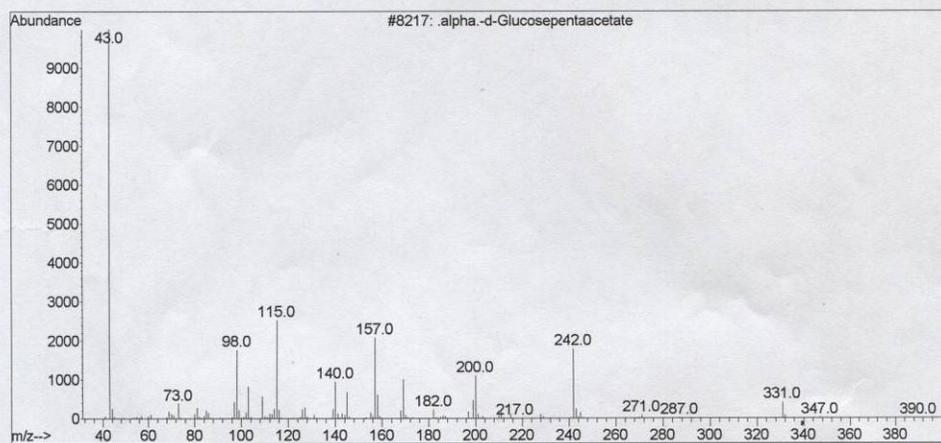
Figure I: Mass-spectrum finger print ions of beta-D-galactofuranose-pentaacetate.

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.alpha.-d-Glucosepentaacetate

Entry Number 8217 from C:\DATABASE\nist98.L  
CAS 1000126-09-9  
Melting Point -300  
Boiling Point -300  
Retention Index 0  
Mol Formula C<sub>16</sub>H<sub>22</sub>O<sub>11</sub>  
Mol Weight 390.116  
Company ID NIST 1998

Miscellaneous Information

NIST MS# 126099, Seq# M8217, CAS number = 10<sup>9</sup> + NIST MS#



C:\DATABASE\nist98.L

Wed Sep 29 14:40:13 2010

Page 1

Figure II: Mass-spectrum finger print ions of alpha D-glucose-pentaacetate.

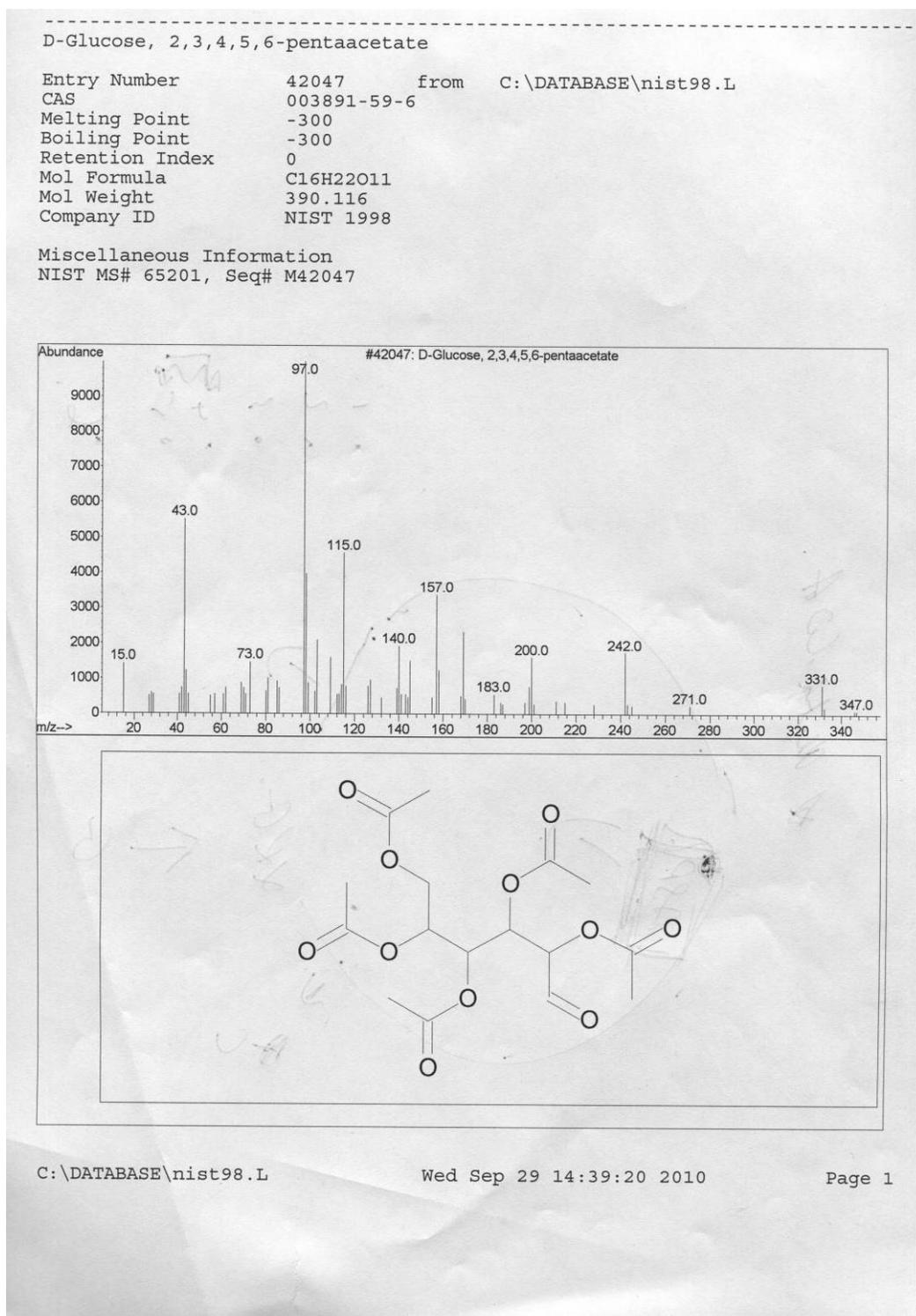
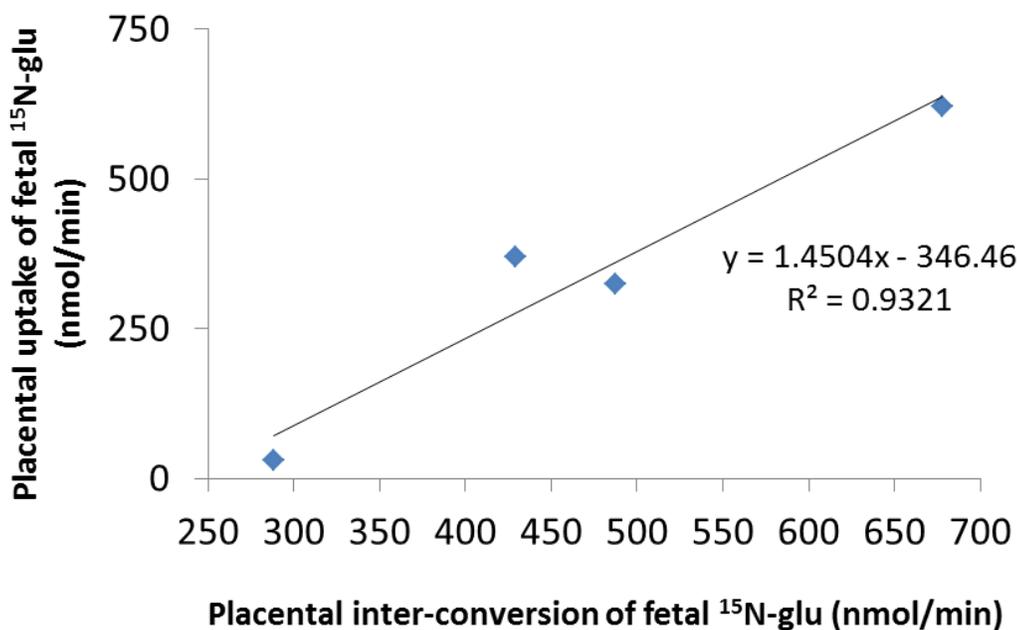
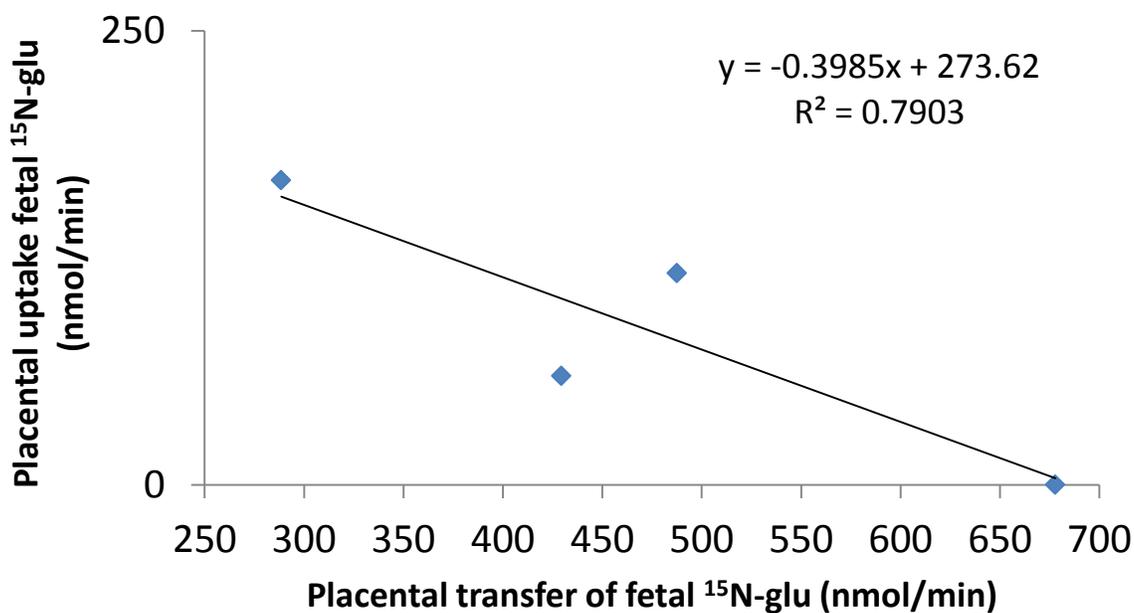


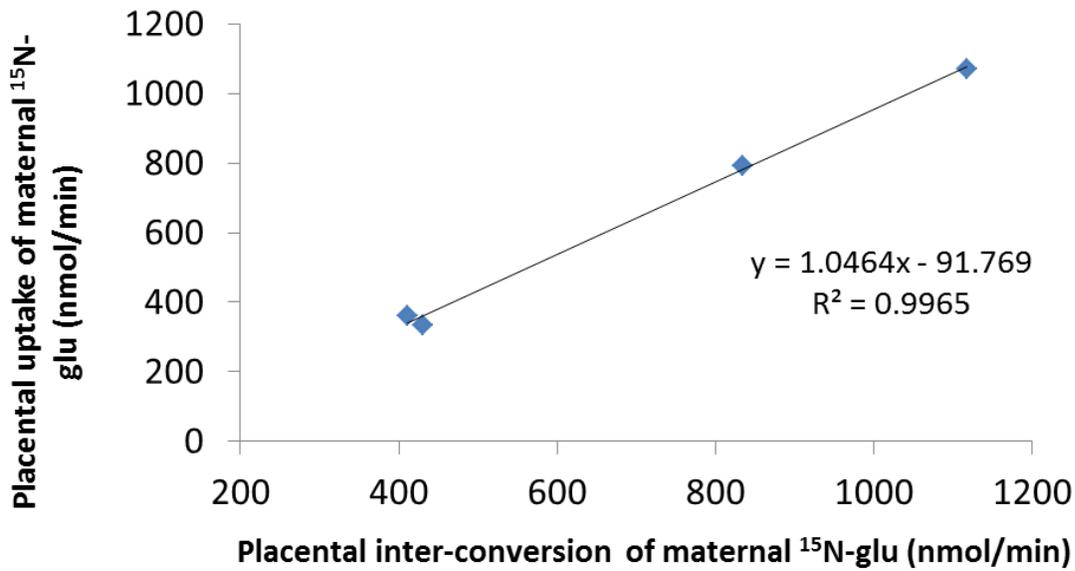
Figure III: Mass-spectrum finger print ions of D-glucose, 2, 3, 4, 5, 6-pentaacetate.



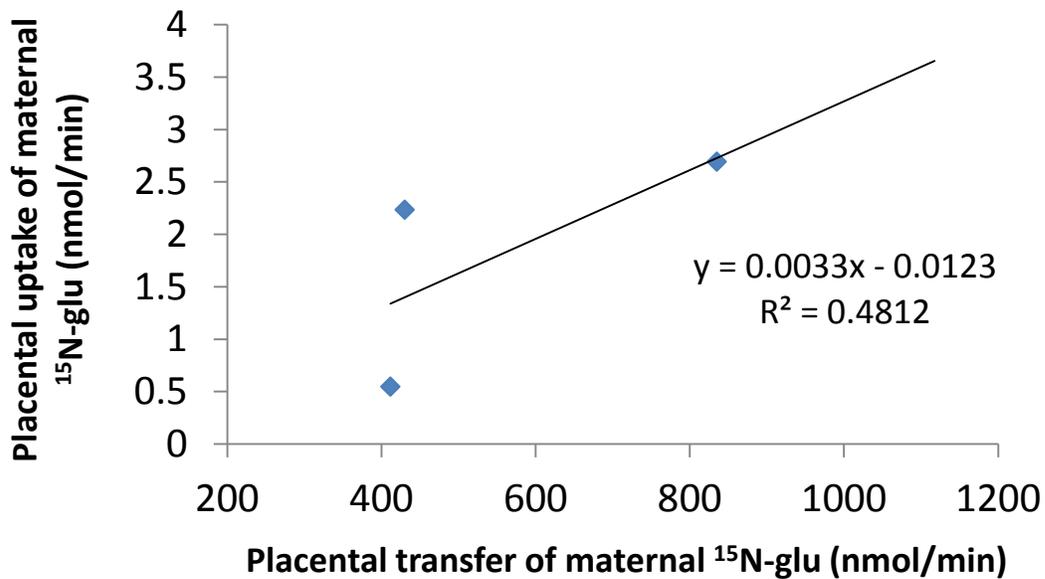
**Figure IV: Placental uptake vs. inter-conversion of <sup>15</sup>N-glutamate from the fetal arterial circulation** showing a positive correlation between placental uptake of <sup>15</sup>N-glutamate from the fetal circulation and <sup>15</sup>N-glutamate consumption by the placenta.



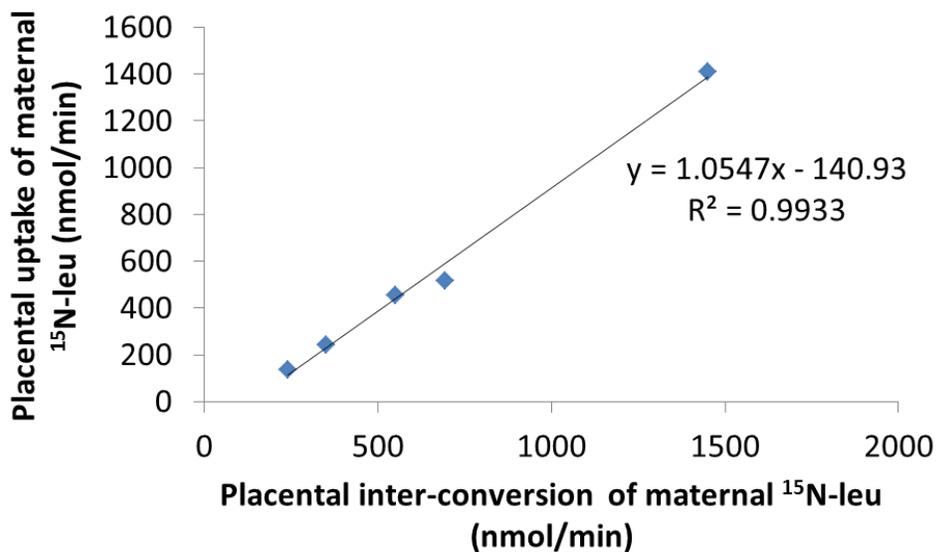
**Figure V: Placental uptake vs. transfer of <sup>15</sup>N-glutamate from the fetal arterial circulation** showing a positive correlation between placental uptake of <sup>15</sup>N-glutamate from the fetal circulation and the transfer of <sup>15</sup>N-glutamate to the maternal venous circulation.



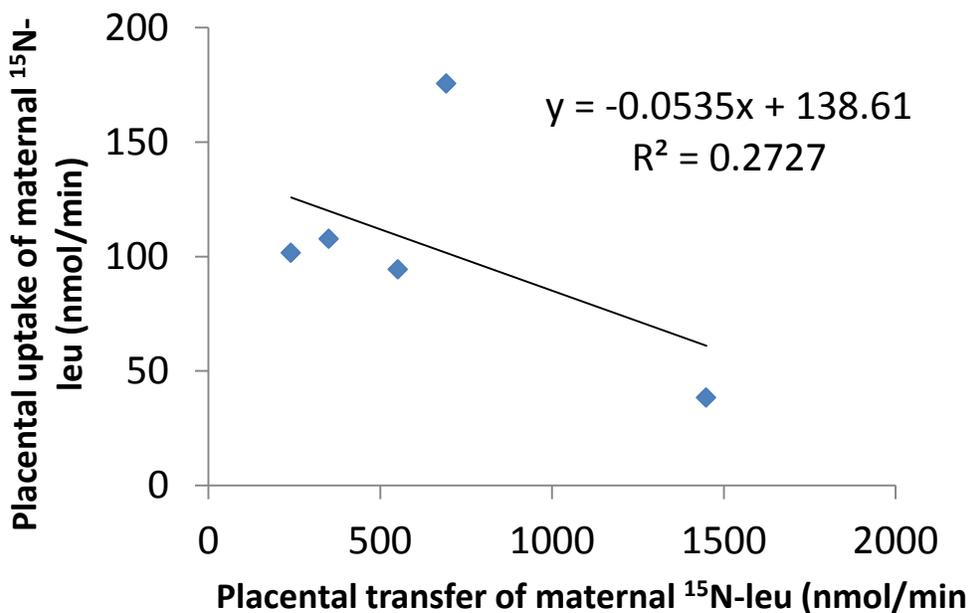
**Figure VI: Placental uptake vs. inter-conversion of <sup>15</sup>N-glutamate from the maternal arterial circulation** showing a positive correlation between placental uptake of <sup>15</sup>N-glutamate from the maternal circulation and <sup>15</sup>N-glutamate consumption by the placenta.



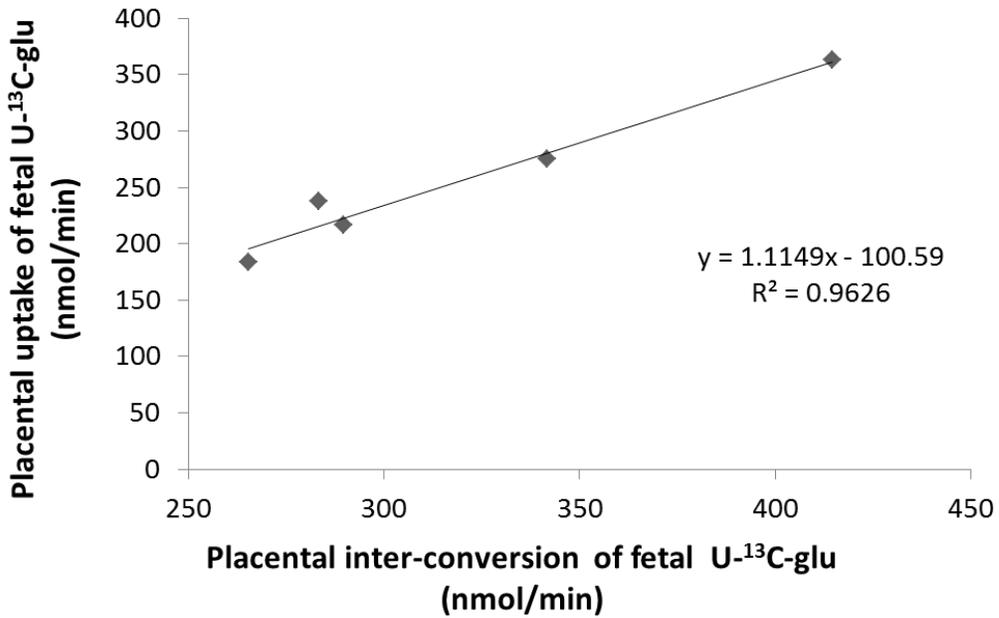
**Figure VII: Placental uptake vs. transfer of <sup>15</sup>N-glutamate from the maternal arterial circulation** showing a positive correlation between placental uptake of <sup>15</sup>N-glutamate from the maternal circulation and the transfer of <sup>15</sup>N-glutamate to the fetal venous circulation.



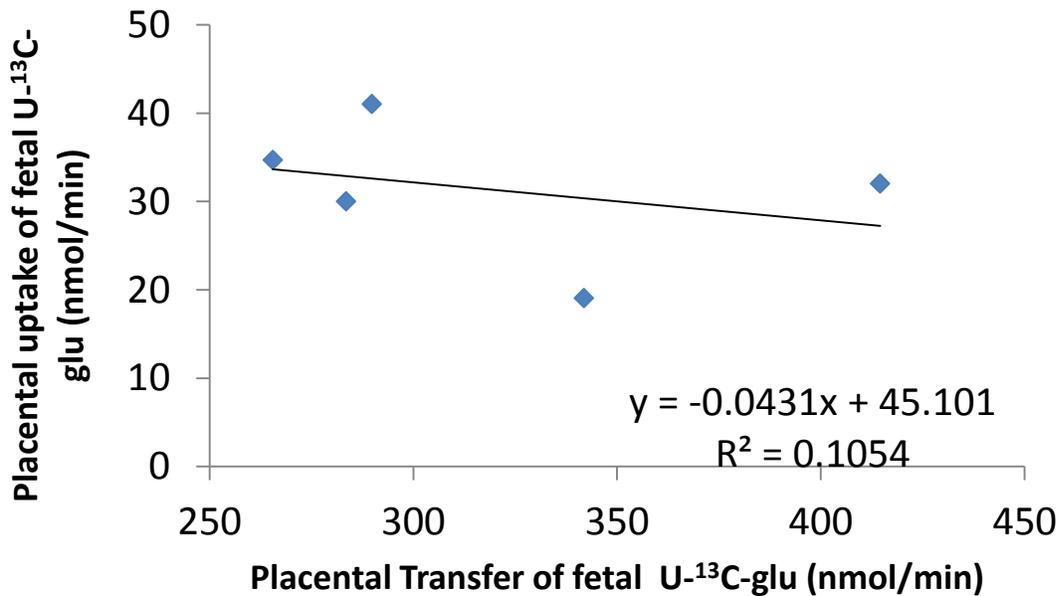
**Figure VIII: Placental uptake vs. inter-conversion of <sup>15</sup>N-leucine from the maternal arterial circulation** showing a positive correlation between placental uptake of <sup>15</sup>N-leucine from the maternal circulation and <sup>15</sup>N-leucine consumption by the placenta.



**Figure IX: Placental uptake vs. transfer of <sup>15</sup>N-leucine from the maternal arterial circulation** showing a positive correlation between placental uptake of <sup>15</sup>N-leucine from the maternal circulation and the transfer of <sup>15</sup>N-leucine to the fetal venous circulation.



**Figure X: Placental uptake vs. inter-conversion of U-<sup>13</sup>C-glutamate from the fetal arterial circulation** showing a positive correlation between placental uptake of U-<sup>13</sup>C-glutamate from the fetal circulation and U-<sup>13</sup>C-glutamate consumption by the placenta.



**Figure XI: Placental uptake vs. transfer of U-<sup>13</sup>C-glutamate from the fetal arterial circulation** showing a positive correlation between placental uptake of U-<sup>13</sup>C-glutamate from the fetal circulation and the transfer of U-<sup>13</sup>C-glutamate to the maternal venous circulation.

## Appendix 4: Supplementary materials for gene expression data

**Table I: Amino acid concentrations in the placental tissue.** Data is expressed as mean  $\pm$  SEM

Amino acids	Initial (Creatinine perfusion) ( $\mu\text{mol/L}$ ) Mean ( $\pm$ SEM) n = 6	$^{15}\text{N}$ -leu Maternal perf ( $\mu\text{mol/L}$ ) Mean ( $\pm$ SEM) n = 5	$^{15}\text{N}$ -glu maternal perf ( $\mu\text{mol/L}$ ) Mean ( $\pm$ SEM) n = 4	$^{13}\text{C}$ -glu fetal perf ( $\mu\text{mol/L}$ ) Mean ( $\pm$ SEM) n = 4	$^{15}\text{N}$ -glu fetal perf ( $\mu\text{mol/L}$ ) Mean ( $\pm$ SEM) n = 4
glu	4775.1 (534)	4005.0 (811)	5438.0 (1181)	5447.7 (340)	4900.1 (794)
tau	3394.6 (462)	2693.0 (617)	2493.0 (643)	3321.2 (214)	2945.8 (782)
gly	2113.6 (271)	1527.0 (326)	1429.0 (238)	1541.0 (64)	1649.8 (363)
asp	1634.8 (224)	1424.0 (331)	2344.0 (747)	2089.1 (408)	2014.5 (447)
ala	1372.5 (214)	887.0 (205)	824.0 (114)	842.1 (47)	1004.3 (187)
ser	1154.0 (173)	860.0 (171)	783.0 (97)	977.3 (59)	957.6 (269)
leu	560.2 (71)	496.0 (102)	401.0 (37)	365.4 (42)	438.7 (118)
lys	774.1 (152)	478.0 (104)	459.0 (36)	221.0 (111)	492.4 (87)
thr	728.0 (102)	371.0 (69)	380.0 (36)	486.2 (16)	508.7 (132)
arg	490.7 (73)	341.0 (62)	319.0 (29)	456.9 (28)	460.4 (101)
val	499.0 (73)	335.0 (72)	328.0 (33)	308.6 (13)	378.7 (90)
gln	472.9 (137)	216.0 (40)	366.0 (53)	591.6 (57)	398.8 (198)
tyr	196.4 (31)	166.0 (33)	158.0 (21)	160.7 (8)	168.4 (65)
phe	237.7 (33)	155.0 (33)	147.0 (17)	190.3 (23)	190.4 (54)
ile	226.8 (29)	129.0 (26)	118.0 (16)	172.5 (21)	172.5 (46)
asn	188.1 (27)	125.0 (24)	155.0 (22)	224.7 (17)	153.7 (73)
met	105.8 (16)	100.0 (23)	96.0 (14)	81.4 (8)	91.1 (31)
his	183.7 (40)	99.0 (23)	111.0 (11)	150.8 (10)	110.4 (42)

**Table II: Relationship between placental mRNA levels and maternal lifestyle (adjusted for sex) at birth**

	LAT 1**		LAT 2**		ASCT 1**		ASCT 2**		y <sup>+</sup> LAT 1**	
	Mean (SD)	P	Mean (SD)	P						
Parity										
0	0.01 (0.96)		-0.06 (0.97)		0.06 (0.93)		-0.32 (0.93)		-0.15 (0.91)	
1+	-0.03 (0.93)	0.830	0.04 (0.95)	0.607	-0.04 (0.95)	0.594	0.27 (0.88)	0.001	0.13 (0.98)	0.143
Pre pregnancy smoking status										
No	0.01 (0.97)		-0.14 (0.96)		0.01 (0.91)		-0.08 (0.94)		-0.12 (0.92)	
Yes	-0.07 (0.88)	0.699	0.41 (0.82)	0.011	0.02 (1.03)	0.965	0.27 (0.93)	0.098	0.36 (1.00)	0.027
In pregnancy smoking status										
No	0.05 (0.97)		-0.03 (1.02)		0.01 (0.91)		-0.07 (0.90)		-0.05 (0.93)	
Yes	-0.23 (0.90)	0.317	0.30 (0.55)	0.250	0.10 (1.05)	0.743	0.17 (0.99)	0.359	0.16 (0.92)	0.434
Dietary prudence										
No	0.02 (0.95)		0.03 (0.98)		0.03 (0.88)		-0.05 (0.93)		-0.12 (0.94)	
Yes	-0.08 (0.94)	0.608	-0.09 (0.91)	0.565	-0.03 (1.06)	0.756	0.14 (0.98)	0.351	0.29 (0.96)	0.046
Depression GHQ										
No	0.14 (1.05)		0.03 (0.97)		0.01 (0.93)		0.14 (0.91)		0.20 (0.83)	
Yes	-0.05 (0.73)	0.497	0.13 (1.10)	0.732	-0.03 (1.06)	0.900	0.05 (1.06)	0.759	0.07 (1.14)	0.630
Strenuous exercise										
No	-0.07 (0.86)		-0.22 (0.94)		-0.24 (1.02)		0.01 (0.67)		-0.21 (0.86)	
Yes	0.03 (0.99)	0.612	0.13 (0.94)	0.078	0.17 (0.85)	0.031	0.01 (1.09)	0.976	0.14 (1.00)	0.073

\*Log transformed \*\*Fisher-Yates transformed

**Table III: Relationship between placental mRNA levels and maternal lifestyle (adjusted for sex) at birth**

	<i>Y<sup>+</sup>LAT 2**</i>		<i>4F2HC**</i>		<i>BCATc**</i>		<i>BCATm**</i>		<i>GLUD1/2**</i>		<i>GLS1**</i>	
	Mean (SD)	P	Mean (SD)	P	Mean (SD)	P	Mean (SD)	P	Mean (SD)	P	Mean (SD)	P
Parity												
0	-0.09 (1.01)		-0.15 (0.90)		-0.24 (0.98)	0.018	0.15 (0.91)		0.23 (1.01)		-0.02 (0.95)	
1+	0.10 (0.81)	0.298	0.11 (0.99)	0.168	0.21 (0.90)		-0.12 (0.99)	0.156	-0.19 (0.89)	0.027	0.03 (0.95)	0.796
Pre pregnancy smoking status												
No	-0.10 (0.89)		-0.09 (0.90)		-0.06 (0.95)		-0.08 (0.94)		0.07 (0.92)		0.01 (0.94)	
Yes	0.35 (0.89)	0.026	0.24 (1.08)	0.128	0.19 (0.98)	0.266	0.26 (0.98)	0.121	-0.23 (1.06)	0.159	0.00 (0.99)	0.971
In pregnancy smoking status												
No	-0.05 (0.90)		0.01 (0.91)		-0.08 (0.92)		-0.06 (0.95)		0.00 (0.97)		0.05 (0.91)	
Yes	0.33 (1.06)	0.162	0.23 (1.08)	0.417	0.38 (0.85)	0.090	0.32 (1.03)	0.170	-0.09 (0.98)	0.739	-0.36 (0.78)	0.116
Dietary prudence												
No	0.02 (0.91)		0.00 (0.98)		-0.05 (0.90)		0.00 (0.99)		0.03 (0.97)		-0.07 (0.97)	
Yes	-0.01 (0.90)	0.880	-0.02 (0.92)	0.900	0.13 (1.09)	0.393	0.01 (0.89)	0.954	-0.07 (0.96)	0.628	0.17 (0.87)	0.241
Depression GHQ												
No	0.08 (0.81)		0.08 (0.94)		0.16 (0.88)		0.01 (1.04)		-0.12 (1.02)		0.17 (0.80)	
Yes	0.42 (0.77)	0.141	0.23 (0.99)	0.580	0.22 (1.31)	0.829	0.28 (0.68)	0.326	-0.39 (0.73)	0.336	0.12 (1.13)	0.851
Strenuous exercise												
No	-0.09 (0.76)		-0.11 (1.01)		-0.05 (0.85)		-0.28 (0.91)		0.07 (0.91)		-0.27 (0.77)	
Yes	0.08 (0.99)	0.367	0.06 (0.92)	0.388	0.04 (1.02)	0.636	0.18 (0.95)	0.017	-0.05 (1.00)	0.527	0.18 (1.01)	0.019

\*\*Fisher-Yates transformed

**Table IV: Relationship between placental mRNA levels and maternal lifestyle (adjusted for sex) at birth**

	GLUL**		ALT2**		AST2**		AST1**		EAAT2**		EAAT3**	
	Mean (SD)	P										
Parity												
0	0.13 (0.96)		0.20 (0.91)		-0.26 (0.98)		0.10 (0.86)		0.02 (1.00)		-0.21 (1.00)	
1+	-0.09 (0.92)	0.226	-0.16 (0.98)	0.057	0.22 (0.90)	0.011	-0.10 (0.96)	0.278	-0.00 (0.90)	0.896	0.19 (0.84)	0.030
Pre pregnancy smoking status												
No	0.07 (0.88)		0.02 (0.96)		-0.13 (0.94)		0.10 (0.89)		-0.02 (0.96)		-0.07 (0.90)	
Yes	-0.18 (1.09)	0.246	-0.06 (1.01)	0.702	0.39 (0.95)	0.018	-0.33 (0.94)	0.038	0.08 (0.90)	0.657	0.23 (1.00)	0.163
In pregnancy smoking status												
No	0.05 (0.89)		-0.02 (0.95)		-0.05 (0.95)		0.10 (0.84)		-0.01 (1.00)		-0.08 (0.93)	
Yes	-0.32 (1.29)	0.184	-0.13 (1.05)	0.704	0.36 (1.04)	0.143	-0.34 (1.00)	0.087	0.34 (0.43)	0.196	0.43 (0.92)	0.060
Dietary prudence												
No	-0.07 (0.94)		0.05 (0.97)		-0.01 (0.93)		0.06 (0.97)		0.00 (0.98)		-0.11 (0.94)	
Yes	0.19 (0.93)	0.209	-0.12 (0.96)	0.396	0.04 (1.04)	0.810	-0.19 (0.79)	0.207	0.03 (0.87)	0.907	0.27 (0.88)	0.057
Depression GHQ												
No	0.13 (0.94)		-0.06 (1.01)		0.16 (0.90)		-0.04 (0.92)		0.08 (0.95)		0.08 (0.91)	
Yes	-0.46 (1.10)	0.040	0.38 (0.82)	0.118	0.17 (1.00)	0.944	-0.01 (1.03)	0.904	0.02 (0.99)	0.822	0.14 (0.92)	0.820
Strenuous exercise												
No	-0.22 (0.86)		-0.16 (0.99)		0.09 (0.87)		0.03 (0.87)		0.13 (0.98)		-0.17 (0.82)	
Yes	0.15 (0.96)	0.049	0.10 (0.94)	0.195	-0.05 (1.02)	0.453	-0.04 (0.96)	0.730	-0.07 (0.92)	0.312	0.12 (0.99)	0.126

\*\*Fisher-Yates transformed

**Table V: Relationship between placental mRNA levels at birth and maternal lifestyle (maternal walking speed and qualification level) (mRNA levels adjusted for sex at birth)**

	Normal speed or slower (n = 55)		Faster than normal (n = 47)		P	O levels or lower (n = 37)		A levels or higher (n = 64)		P
	Mean	SD	Mean	SD		Mean	SD	Mean	SD	
<i>LAT 1</i> **	0.04	0.97	-0.06	0.91	0.607	-0.01	0.87	-0.00	1.00	0.965
<i>LAT 2</i> **	0.11	1.02	-0.14	0.87	0.180	0.16	0.84	-0.10	1.01	0.188
<i>ASCT 1</i> **	-0.01	1.04	0.04	0.80	0.799	0.01	0.94	-0.02	0.92	0.881
<i>ASCT 2</i> **	0.17	0.92	-0.18	0.95	0.067	0.05	1.09	0.00	0.86	0.799
<i>y<sup>+</sup>LAT 1</i> **	0.15	0.89	-0.16	1.02	0.109	0.11	1.07	-0.05	0.90	0.424
<i>y<sup>+</sup>LAT 2</i> **	0.10	0.86	-0.09	0.95	0.285	0.07	0.96	-0.01	0.88	0.673
<i>4F2HC</i> **	0.10	1.00	-0.12	0.89	0.251	0.14	0.79	-0.07	1.02	0.291
<i>BCAT<sub>c</sub></i> **	0.09	1.05	-0.10	0.84	0.309	0.05	0.99	-0.01	0.95	0.757
<i>BCAT<sub>m</sub></i> **	0.15	0.98	-0.17	0.91	0.096	0.02	0.96	-0.01	0.96	0.876
<i>GLUD1/2</i> **	-0.11	1.05	0.12	0.84	0.243	-0.10	0.89	0.03	1.00	0.529
<i>GLS1</i> **	0.17	0.95	-0.19	0.91	0.054	-0.02	1.05	0.02	0.90	0.842
<i>GLUL</i> **	-0.01	0.95	0.03	0.94	0.868	-0.06	0.96	0.01	0.89	0.719
<i>ALT2</i> **	0.08	1.02	-0.09	0.90	0.398	-0.03	0.90	0.03	1.01	0.775
<i>AST2</i> **	0.21	0.88	-0.24	1.01	0.019	0.13	0.88	-0.05	1.00	0.369
<i>AST1</i> **	-0.06	0.86	0.05	0.99	0.543	-0.11	0.96	0.05	0.90	0.413
<i>EAAT2</i> **	-0.02	0.93	0.04	0.97	0.748	-0.19	0.75	0.12	1.03	0.121
<i>EAAT3</i> **	0.19	0.92	-0.20	0.91	0.036	0.19	0.90	-0.10	0.94	0.130

\*Log transformed \*\*Fisher-Yates transformed

**Table VI: Relationship between placental mRNA levels at birth and maternal lifestyle (maternal social class) (adjusted for sex)**

	I/II (n = 47)	III/IV (n = 43)	V (n = 11)	
	Mean(SD)	Mean(SD)	Mean(SD)	P-value
<i>LAT 1</i> **	-0.06 (0.90)	0.09 (1.03)	-0.27 (0.74)	0.502
<i>LAT 2</i> **	-0.19 (1.00)	0.21 (0.94)	0.02 (0.72)	0.143
<i>ASCT 1</i> **	-0.12 (0.99)	0.20 (0.87)	-0.17 (0.89)	0.205
<i>ASCT 2</i> **	0.03 (0.89)	0.08 (1.01)	-0.35 (0.92)	0.402
<i>y</i> <sup>+</sup> <i>LAT 1</i> **	0.03 (0.97)	0.03 (0.97)	-0.13 (0.92)	0.874
<i>y</i> <sup>+</sup> <i>LAT 2</i> **	-0.09 (0.91)	0.24 (0.84)	-0.37 (1.01)	0.075
<i>4F2HC</i> **	-0.27 (0.87)	0.23 (1.04)	0.29 (0.66)	0.026
<i>BCATc</i> **	-0.03 (0.92)	0.07 (1.05)	-0.12 (0.82)	0.798
<i>BCATm</i> **	0.03 (0.84)	0.00 (1.09)	-0.05 (0.97)	0.974
<i>GLUD1/2</i> **	0.04 (1.06)	-0.10 (0.86)	0.19 (0.99)	0.603
<i>GLS1</i> **	0.08 (0.97)	-0.02 (0.81)	-0.27 (1.35)	0.532
<i>GLUL</i> **	0.05 (0.88)	0.03 (1.01)	-0.31 (0.94)	0.522
<i>ALT2</i> **	-0.10 (1.05)	0.16 (0.89)	-0.23 (0.85)	0.302
<i>AST2</i> **	-0.04 (1.02)	0.09 (0.87)	-0.06 (1.13)	0.791
<i>AST1</i> **	-0.02 (0.88)	-0.02 (1.02)	0.04 (0.78)	0.977
<i>EAAT2</i> **	-0.13 (0.99)	0.16 (0.93)	-0.06 (0.72)	0.322
<i>EAAT3</i> **	-0.07 (0.93)	0.11 (0.95)	0.10 (0.83)	0.644

\*Log transformed \*\*Fisher-Yates transformed

**Table VII: Relationship between placental mRNA levels and parental body composition (adjusted for sex at birth)**

	Maternal birth weight (g) (n = 88)		Paternal birth weight (g) (n = 47)		Maternal height (cm) (n = 101)		Maternal body mass index (n = 101)	
	r	P	r	P	r	P	r	P
<i>LAT 1</i> **	-0.063	0.562	0.136	0.361	-0.181	0.070	-0.056	0.581
<i>LAT 2</i> **	-0.110	0.312	0.123	0.411	-0.086	0.393	0.010	0.924
<i>ASCT 1</i> **	0.090	0.407	-0.077	0.605	-0.181	0.070	-0.270	0.006
<i>ASCT 2</i> **	-0.074	0.494	-0.084	0.576	-0.083	0.410	-0.025	0.805
<i>y</i> <sup>+</sup> <i>LAT 1</i> **	-0.079	0.465	-0.074	0.620	-0.124	0.217	-0.023	0.820
<i>y</i> <sup>+</sup> <i>LAT 2</i> **	-0.035	0.745	-0.069	0.643	-0.077	0.445	-0.202	0.043
<i>4F2HC</i> **	-0.070	0.517	0.023	0.879	-0.076	0.452	0.047	0.640
<i>BCAT<sub>c</sub></i> **	-0.068	0.529	0.071	0.636	-0.200	0.045	-0.105	0.296
<i>BCAT<sub>m</sub></i> **	-0.270	0.011	0.178	0.233	-0.152	0.128	-0.090	0.372
<i>GLUD1/2</i> **	0.035	0.749	0.062	0.679	0.098	0.332	0.068	0.500
<i>GLS1</i> **	-0.041	0.702	-0.401	0.005	-0.113	0.261	0.213	0.033
<i>GLUL</i> **	0.150	0.164	-0.073	0.625	0.051	0.614	-0.050	0.620
<i>ALT2</i> **	-0.110	0.308	-0.212	0.153	-0.045	0.655	-0.053	0.598
<i>AST2</i> **	-0.108	0.319	-0.122	0.413	-0.105	0.294	0.004	0.971
<i>AST1</i> **	-0.024	0.822	0.278	0.058	0.245	0.014	0.115	0.254
<i>EAAT2</i> **	0.131	0.224	0.016	0.913	-0.123	0.222	-0.181	0.070
<i>EAAT3</i> **	-0.051	0.638	-0.307	0.036	-0.182	0.069	0.062	0.537

\*Log transformed \*\*Fisher-Yates transformed

**Table VIII: Relationship between placental mRNA levels and parental body composition (adjusted for sex at birth)**

	Pre pregnant sum of skin fold thickness (n = 100) *		Pre pregnant triceps skin fold thickness (n = 100) *		Derived fat mass (Kg) (n = 101) *		Pre pregnant calf circumference (n = 100) *	
	r	P	r	P	r	P	r	P
<i>LAT 1</i> **	-0.054	0.595	-0.175	0.082	-0.075	0.459	-0.039	0.697
<i>LAT 2</i> **	0.041	0.688	-0.091	0.372	0.000	0.998	-0.032	0.750
<i>ASCT 1</i> **	-0.204	0.042	-0.057	0.573	-0.266	0.007	-0.342	0.000
<i>ASCT 2</i> **	0.011	0.911	0.034	0.740	-0.021	0.838	-0.019	0.851
<i>y<sup>+</sup>LAT 1</i> **	-0.069	0.493	0.050	0.622	-0.060	0.553	-0.055	0.584
<i>y<sup>+</sup>LAT 2</i> **	-0.171	0.089	0.016	0.871	-0.184	0.065	-0.143	0.155
<i>4F2HC</i> **	-0.027	0.793	-0.003	0.977	-0.000	0.997	0.000	0.999
<i>BCATc</i> **	-0.082	0.416	-0.048	0.634	-0.122	0.225	-0.127	0.209
<i>BCATm</i> **	0.002	0.988	-0.012	0.908	-0.052	0.607	-0.119	0.238
<i>GLUD1/2</i> **	0.026	0.801	0.027	0.788	0.045	0.656	0.062	0.541
<i>GLS1</i> **	0.158	0.116	0.146	0.148	0.159	0.113	0.164	0.102
<i>GLUL</i> **	0.005	0.960	-0.124	0.220	-0.026	0.798	-0.042	0.676
<i>ALT2</i> **	-0.101	0.317	-0.049	0.626	-0.079	0.433	-0.025	0.807
<i>AST2</i> **	-0.046	0.646	-0.058	0.565	-0.040	0.693	0.008	0.938
<i>AST1</i> **	0.121	0.231	0.054	0.591	0.168	0.093	0.193	0.054
<i>EAAT2</i> **	-0.130	0.198	-0.127	0.207	-0.168	0.094	-0.175	0.082
<i>EAAT3</i> **	0.024	0.810	0.032	0.754	0.017	0.863	-0.048	0.638

\*Log transformed \*\*Fisher-Yates transformed

**Table IX: Relationship between placental mRNA levels and parental body composition (adjusted for sex at birth)**

	Pre pregnant mid upper arm circumference (n = 101) *		11 week gestation: Mid upper arm circumference (n = 76) *		34 week gestation: Mid-upper arm circumference (cm) (n = 95)	
	r	P	r	P	r	P
<i>LAT 1</i> **	-0.064	0.523	-0.033	0.778	-0.064	0.537
<i>LAT 2</i> **	-0.018	0.859	0.165	0.157	0.060	0.564
<i>ASCT 1</i> **	-0.255	0.010	-0.231	0.045	-0.267	0.009
<i>ASCT 2</i> **	-0.014	0.893	0.074	0.524	0.087	0.404
<i>y<sup>+</sup>LAT 1</i> **	-0.097	0.334	0.016	0.888	-0.010	0.920
<i>y<sup>+</sup>LAT 2</i> **	-0.140	0.162	-0.148	0.202	-0.080	0.442
<i>4F2HC</i> **	0.032	0.752	0.100	0.392	0.056	0.593
<i>BCATc</i> **	-0.126	0.208	-0.147	0.205	-0.144	0.165
<i>BCATm</i> **	-0.086	0.393	-0.019	0.871	0.004	0.973
<i>GLUD1/2</i> **	0.039	0.697	-0.015	0.900	0.024	0.817
<i>GLS1</i> **	0.110	0.274	0.163	0.159	0.162	0.117
<i>GLUL</i> **	-0.081	0.421	-0.101	0.387	-0.117	0.260
<i>ALT2</i> **	-0.070	0.488	-0.056	0.634	-0.058	0.575
<i>AST2</i> **	-0.072	0.476	-0.006	0.962	0.014	0.890
<i>AST1</i> **	0.182	0.069	0.084	0.472	0.127	0.219
<i>EAAT2</i> **	-0.153	0.128	-0.176	0.128	-0.159	0.125
<i>EAAT3</i> **	0.034	0.736	0.103	0.378	0.051	0.626

\*Log transformed \*\*Fisher-Yates transformed

**Table X: Relationship between placental mRNA levels and parental body composition (adjusted for sex at birth)**

	Pre pregnancy: Arm muscle area (n = 101) *		11 week gestation: Arm muscle area (n = 76) *		34 week gestation: Arm muscle area (n = 95) *	
	r	P	r	P	r	P
<i>LAT 1</i> **	-0.141	0.160	-0.068	0.559	-0.075	0.468
<i>LAT 2</i> **	-0.129	0.201	0.108	0.358	0.016	0.875
<i>ASCT 1</i> **	-0.209	0.036	-0.230	0.046	-0.211	0.040
<i>ASCT 2</i> **	-0.023	0.816	0.063	0.590	0.072	0.485
<i>y<sup>+</sup>LAT 1</i> **	-0.120	0.233	0.054	0.640	0.007	0.948
<i>y<sup>+</sup>LAT 2</i> **	0.009	0.929	-0.102	0.380	-0.084	0.419
<i>4F2HC</i> **	0.071	0.478	0.110	0.343	0.061	0.555
<i>BCATc</i> **	-0.136	0.175	-0.139	0.230	-0.128	0.217
<i>BCATm</i> **	-0.143	0.153	-0.081	0.489	-0.044	0.673
<i>GLUD1/2</i> **	0.001	0.992	-0.036	0.757	0.019	0.852
<i>GLS1</i> **	0.028	0.782	0.097	0.404	0.082	0.431
<i>GLUL</i> **	-0.189	0.058	-0.121	0.298	-0.121	0.244
<i>ALT2</i> **	-0.066	0.510	-0.087	0.457	0.013	0.903
<i>AST2</i> **	-0.063	0.533	0.027	0.816	0.000	0.998
<i>AST1</i> **	0.181	0.071	0.073	0.534	0.053	0.608
<i>EAAT2</i> **	-0.101	0.314	-0.187	0.106	-0.140	0.175
<i>EAAT3</i> **	0.020	0.840	0.124	0.287	0.059	0.569

\*Log transformed \*\*Fisher-Yates transformed

**Table XI: Relationship between placental mRNA levels and fetal growth parameters (both adjusted for sex at birth)**

	19 wk scan: Abdominal Circumference (n = 58) ***		34 wk scan: Abdominal Circumference (n = 59) ***		19-34 wk scan: Abdominal Circumference growth rate (n = 58) ***	
	r	P	r	P	r	P
<i>LAT 1</i> **	0.182	0.170	-0.064	0.631	-0.178	0.181
<i>LAT 2</i> **	0.002	0.990	0.032	0.814	-0.022	0.874
<i>ASCT 1</i> **	0.013	0.926	0.240	0.067	0.290	0.027
<i>ASCT 2</i> **	-0.003	0.980	0.074	0.577	0.116	0.387
<i>y<sup>+</sup>LAT 1</i> **	-0.005	0.968	0.221	0.093	0.241	0.069
<i>y<sup>+</sup>LAT 2</i> **	0.067	0.6158	0.179	0.174	0.099	0.459
<i>4F2HC</i> **	0.165	0.216	0.130	0.326	-0.020	0.881
<i>BCAT<sub>c</sub></i> **	0.075	0.575	0.181	0.169	0.145	0.278
<i>BCAT<sub>m</sub></i> **	0.107	0.426	-0.026	0.843	-0.099	0.461
<i>GLUD1/2</i> **	-0.041	0.761	-0.113	0.393	-0.053	0.691
<i>GLS1</i> **	0.003	0.985	0.042	0.749	0.110	0.413
<i>GLUL</i> **	-0.220	0.097	0.198	0.132	0.390	0.002
<i>ALT2</i> **	-0.004	0.975	-0.137	0.302	-0.084	0.529
<i>AST2</i> **	0.153	0.252	0.163	0.218	0.134	0.316
<i>AST1</i> **	0.053	0.692	0.109	0.412	-0.004	0.979
<i>EAAT2</i> **	-0.011	0.933	0.001	0.996	-0.011	0.933
<i>EAAT3</i> **	-0.015	0.910	0.016	0.906	0.103	0.440

\*Log transformed \*\*Fisher-Yates transformed \*\*\* Royston z-score

**Table XII: Relationship between placental mRNA levels at birth and fetal growth parameters (both adjusted for sex at birth)**

	19 wk scan: Head Circumference (n = 58) ***		34 wk scan: Head Circumference (n = 59) ***		19-34 wk scan: Head Circumference (n = 58) ***	
	r	P	r	P	r	P
<i>LAT 1**</i>	0.021	0.875	-0.173	0.190	-0.238	0.071
<i>LAT 2**</i>	0.028	0.838	-0.195	0.143	-0.272	0.040
<i>ASCT 1**</i>	0.050	0.712	0.061	0.647	0.032	0.811
<i>ASCT 2**</i>	0.116	0.388	0.119	0.369	0.077	0.565
<i>y<sup>+</sup>LAT 1**</i>	-0.040	0.764	0.067	0.613	0.056	0.674
<i>y<sup>+</sup>LAT 2**</i>	0.284	0.031	0.194	0.141	0.023	0.861
<i>4F2HC**</i>	0.132	0.324	0.040	0.761	-0.035	0.793
<i>BCAT<sub>c</sub>**</i>	0.010	0.939	0.091	0.491	0.053	0.691
<i>BCAT<sub>m</sub>**</i>	0.077	0.565	0.069	0.601	0.050	0.712
<i>GLUD1/2**</i>	-0.036	0.787	-0.141	0.287	-0.121	0.367
<i>GLS1**</i>	0.005	0.970	-0.129	0.330	-0.187	0.160
<i>GLUL**</i>	-0.059	0.660	-0.106	0.426	-0.086	0.522
<i>ALT2**</i>	0.104	0.439	-0.036	0.785	-0.089	0.509
<i>AST2**</i>	0.106	0.429	0.085	0.520	0.021	0.875
<i>AST1**</i>	0.078	0.562	0.045	0.737	-0.047	0.728
<i>EAAT2**</i>	0.055	0.684	0.088	0.505	0.085	0.526
<i>EAAT3**</i>	-0.053	0.693	-0.135	0.308	-0.100	0.457

\*Log transformed \*\*Fisher-Yates transformed \*\*\* Royston z-score

**Table XIII: Relationship between placental mRNA levels and fetal growth parameters (both adjusted for sex at birth)**

	19 wk scan: Femur length (n = 58) ***		34 wk scan: Femur length (n = 59) ***		19-34 wk scan: Femur length (n = 58) ***		11 wk scan: Crown rump length (n = 51) ***	
	r	P	r	P	r	P	r	P
<i>LAT 1</i> **	-0.083	0.534	-0.123	0.352	-0.082	0.541	-0.077	0.591
<i>LAT 2</i> **	0.034	0.802	-0.046	0.729	-0.070	0.606	-0.151	0.294
<i>ASCT 1</i> **	0.069	0.605	-0.061	0.644	-0.093	0.486	0.029	0.839
<i>ASCT 2</i> **	-0.055	0.683	-0.164	0.215	-0.137	0.304	0.083	0.561
<i>y<sup>+</sup>LAT 1</i> **	-0.023	0.865	-0.166	0.210	-0.165	0.215	-0.019	0.897
<i>y<sup>+</sup>LAT 2</i> **	0.120	0.368	-0.201	0.126	-0.272	0.039	0.239	0.091
<i>4F2HC</i> **	0.093	0.488	0.139	0.292	0.088	0.510	0.075	0.602
<i>BCAT<sub>c</sub></i> **	-0.070	0.603	-0.128	0.334	-0.104	0.438	0.030	0.835
<i>BCAT<sub>m</sub></i> **	0.019	0.887	-0.002	0.986	-0.012	0.926	-0.106	0.459
<i>GLUD1/2</i> **	0.013	0.922	-0.033	0.805	-0.034	0.801	-0.025	0.861
<i>GLS1</i> **	-0.092	0.491	-0.126	0.340	-0.082	0.541	-0.041	0.778
<i>GLUL</i> **	-0.029	0.831	-0.288	0.027	-0.266	0.043	-0.118	0.411
<i>ALT2</i> **	-0.052	0.697	-0.135	0.306	-0.105	0.431	0.005	0.970
<i>AST2</i> **	-0.002	0.986	-0.040	0.763	-0.040	0.764	0.193	0.175
<i>AST1</i> **	0.116	0.385	0.171	0.196	0.105	0.433	0.087	0.545
<i>EAAT2</i> **	-0.039	0.773	-0.057	0.670	-0.036	0.787	0.063	0.663
<i>EAAT3</i> **	-0.070	0.602	-0.192	0.145	-0.155	0.246	-0.018	0.898

\*Log transformed \*\*Fisher-Yates transformed \*\*\* Royston z-score

**Table XIV: Relationship between placental mRNA levels and neonatal parameters** (adjusted for gestational age and sex)

	Birth weight (n = 102)		Placental weight (n = 101)		Placental/birth weight ratio (n = 101)	
	r	P	r	P	r	P
<i>LAT 1</i> **	-0.153	0.125	-0.190	0.058	-0.117	0.242
<i>LAT 2</i> **	-0.094	0.348	-0.223	0.026	-0.229	0.022
<i>ASCT 1</i> **	0.056	0.573	0.147	0.143	0.121	0.229
<i>ASCT 2</i> **	0.007	0.942	-0.008	0.940	-0.004	0.967
<i>y<sup>+</sup>LAT 1</i> **	0.144	0.148	0.113	0.262	0.041	0.684
<i>y<sup>+</sup>LAT 2</i> **	0.026	0.795	0.023	0.818	0.027	0.787
<i>4F2HC</i> **	-0.079	0.428	-0.169	0.091	-0.137	0.171
<i>BCAT<sub>c</sub></i> **	0.060	0.552	0.027	0.790	-0.000	0.998
<i>BCAT<sub>m</sub></i> **	-0.109	0.276	-0.093	0.356	-0.040	0.691
<i>GLUD1/2</i> **	-0.029	0.774	-0.053	0.598	-0.047	0.640
<i>GLS1</i> **	0.074	0.458	0.104	0.302	0.093	0.355
<i>GLUL</i> **	0.053	0.593	-0.016	0.876	-0.093	0.354
<i>ALT2</i> **	-0.199	0.045	-0.242	0.015	-0.155	0.121
<i>AST2</i> **	-0.090	0.369	0.018	0.861	0.109	0.279
<i>AST1</i> **	-0.032	0.748	-0.069	0.495	-0.054	0.589
<i>EAAT2</i> **	0.052	0.601	0.160	0.110	0.155	0.122
<i>EAAT3</i> **	0.023	0.820	0.114	0.258	0.130	0.194

\* Log-transformed \*\*Fisher-Yates transformed

**Table XV: Relationship between placental mRNA levels and neonatal parameters** (mRNA adjusted for sex and neonatal parameters adjusted for gestational age and sex)

	Head circumference(cm)(n = 102)		Abdominal circumference(cm)(n = 102)		Crown-heel length(cm)(n = 102)	
	r	P	r	P	r	P
<i>LAT 1</i> **	-0.063	0.530	-0.124	0.214	-0.104	0.297
<i>LAT 2</i> **	-0.132	0.189	-0.041	0.687	-0.134	0.182
<i>ASCT 1</i> **	-0.015	0.883	0.066	0.509	-0.019	0.852
<i>ASCT 2</i> **	0.028	0.776	0.021	0.836	-0.057	0.570
<i>y<sup>+</sup>LAT 1</i> **	0.208	0.036	0.187	0.060	0.056	0.576
<i>y<sup>+</sup>LAT 2</i> **	0.120	0.230	-0.009	0.932	-0.028	0.777
<i>4F2HC</i> **	-0.066	0.507	-0.064	0.523	-0.013	0.894
<i>BCAT<sub>c</sub></i> **	0.132	0.187	0.089	0.376	0.003	0.975
<i>BCAT<sub>m</sub></i> **	0.059	0.554	-0.107	0.283	-0.092	0.359
<i>GLUD1/2</i> **	-0.105	0.294	-0.091	0.364	0.015	0.883
<i>GLS1</i> **	0.107	0.284	0.125	0.211	-0.019	0.850
<i>GLUL</i> **	-0.057	0.570	0.065	0.515	-0.102	0.308
<i>ALT2</i> **	-0.026	0.796	-0.250	0.011	-0.082	0.411
<i>AST2</i> **	0.017	0.867	-0.070	0.482	-0.032	0.753
<i>AST1</i> **	-0.097	0.330	-0.012	0.902	0.136	0.172
<i>EAAT2</i> **	0.094	0.348	0.064	0.521	-0.032	0.749
<i>EAAT3</i> **	0.019	0.847	0.023	0.816	-0.081	0.419

\* Log-transformed \*\*Fisher-Yates transformed

**Table XVI: Relationship between placental mRNA levels at birth and neonatal parameters** (mRNA adjusted for sex and neonatal parameters adjusted for gestational age and sex)

	Neonatal ponderal index (n = 102)		Subscapular skin fold thickness(mm) (n = 102)		Mid-upper arm circumference (cm) (n = 102)	
	r	P	r	P	r	P
<i>LAT 1</i> **	-0.104	0.296	-0.294	0.003	-0.055	0.582
<i>LAT 2</i> **	0.022	0.827	-0.077	0.443	-0.123	0.221
<i>ASCT 1</i> **	0.119	0.234	0.078	0.435	0.001	0.993
<i>ASCT 2</i> **	0.075	0.452	-0.035	0.726	-0.027	0.791
<i>y<sup>+</sup>LAT 1</i> **	0.139	0.162	0.055	0.582	0.071	0.481
<i>y<sup>+</sup>LAT 2</i> **	0.070	0.485	-0.069	0.489	-0.048	0.630
<i>4F2HC</i> **	-0.105	0.295	-0.139	0.162	-0.109	0.273
<i>BCAT<sub>c</sub></i> **	0.071	0.476	-0.030	0.767	-0.004	0.965
<i>BCAT<sub>m</sub></i> **	-0.032	0.750	-0.156	0.118	-0.158	0.113
<i>GLUD1/2</i> **	-0.069	0.492	0.039	0.696	0.031	0.759
<i>GLS1</i> **	0.112	0.263	0.105	0.295	0.118	0.237
<i>GLUL</i> **	0.188	0.059	0.085	0.394	0.085	0.393
<i>ALT2</i> **	-0.188	0.058	-0.330	0.001	-0.143	0.151
<i>AST2</i> **	-0.087	0.387	-0.139	0.165	-0.145	0.147
<i>AST1</i> **	-0.196	0.049	-0.028	0.781	-0.030	0.767
<i>EAAT2</i> **	0.107	0.286	0.019	0.847	0.063	0.531
<i>EAAT3</i> **	0.129	0.196	0.105	0.294	-0.004	0.968

\* Log-transformed \*\*Fisher-Yates transformed

**Table XVII: Relationship between placental mRNA levels and neonatal parameters** (mRNA adjusted for sex and neonatal parameters adjusted for gestational age, sex and age at DXA)

	Neonatal bone mineral content (g) (n = 102)		Neonatal lean mass (g) (n = 102)		Neonatal fat mass (g) (n = 102)	
	r	P	r	P	r	P
<i>LAT 1</i> **	-0.083	0.409	-0.076	0.447	-0.093	0.352
<i>LAT 2</i> **	-0.049	0.625	-0.074	0.464	-0.136	0.174
<i>ASCT 1</i> **	0.066	0.513	0.013	0.894	0.066	0.510
<i>ASCT 2</i> **	-0.110	0.273	-0.002	0.984	-0.004	0.967
<i>y<sup>+</sup>LAT 1</i> **	-0.038	0.701	0.108	0.279	0.061	0.540
<i>y<sup>+</sup>LAT 2</i> **	-0.126	0.207	0.079	0.433	-0.075	0.455
<i>4F2HC</i> **	-0.152	0.128	-0.016	0.870	-0.194	0.050
<i>BCAT<sub>c</sub></i> **	-0.061	0.542	-0.024	0.807	-0.002	0.988
<i>BCAT<sub>m</sub></i> **	-0.166	0.096	-0.093	0.354	-0.121	0.226
<i>GLUD1/2</i> *	0.149	0.135	-0.071	0.481	0.074	0.461
<i>GLS1</i> **	0.016	0.872	0.026	0.797	0.016	0.870
<i>GLUL</i> **	0.119	0.234	-0.039	0.701	0.189	0.057
<i>ALT2</i> **	-0.070	0.485	-0.137	0.171	-0.178	0.074
<i>AST2</i> **	-0.160	0.107	-0.057	0.572	-0.057	0.565
<i>AST1</i> **	-0.088	0.380	0.002	0.986	-0.011	0.915
<i>EAAT2</i> **	0.058	0.566	0.031	0.757	0.050	0.619
<i>EAAT3</i> **	-0.052	0.605	-0.031	0.754	-0.009	0.927

- Log-transformed \*\*Fisher-Yates transformed

**Table XVIII: Relationship between placental mRNA levels and 4 year parameters (mRNA adjusted for sex)**

	4 year height (n = 56)		4 year weight (kg) (n = 56)		Total BMC (g) (n = 102)		Total lean (g) (n = 102)		Total fat (g) (n = 102)	
	r	P	r	P	r	P	r	P	r	P
<i>LAT 1**</i>	-0.189	0.163	-0.253	0.060	-0.096	0.545	-0.221	0.160	-0.039	0.807
<i>LAT 2**</i>	0.091	0.509	0.019	0.893	0.241	0.125	0.179	0.257	0.173	0.273
<i>ASCT 1**</i>	0.111	0.416	0.169	0.214	-0.069	0.666	0.127	0.424	0.069	0.664
<i>ASCT 2**</i>	-0.003	0.984	0.022	0.874	-0.180	0.255	0.052	0.745	-0.161	0.310
<i>y<sup>+</sup>LAT 1**</i>	-0.071	0.602	0.105	0.439	-0.133	0.401	0.088	0.579	-0.119	0.453
<i>y<sup>+</sup>LAT 2**</i>	0.005	0.972	0.023	0.866	-0.201	0.202	0.092	0.564	-0.162	0.306
<i>4F2HC**</i>	-0.015	0.911	0.026	0.847	0.093	0.558	0.039	0.808	-0.034	0.829
<i>BCAT<sub>c</sub>**</i>	0.004	0.977	0.041	0.766	-0.117	0.461	0.037	0.815	-0.081	0.610
<i>BCAT<sub>m</sub>**</i>	0.100	0.462	0.085	0.533	0.063	0.693	0.055	0.728	0.200	0.205
<i>GLUD1/2**</i>	0.102	0.454	0.021	0.880	0.292	0.061	0.075	0.636	0.050	0.754
<i>GLS1**</i>	-0.090	0.509	0.024	0.860	-0.184	0.244	-0.031	0.844	-0.199	0.207
<i>GLUL**</i>	0.167	0.219	0.166	0.221	0.062	0.695	0.218	0.165	-0.089	0.576
<i>ALT2**</i>	0.151	0.266	0.032	0.814	0.035	0.827	0.115	0.467	-0.178	0.259
<i>AST2**</i>	-0.166	0.222	-0.119	0.383	-0.359	0.019	-0.247	0.115	-0.042	0.792
<i>AST1**</i>	0.062	0.648	0.064	0.637	0.120	0.449	-0.051	0.750	0.210	0.181
<i>EAAT2**</i>	-0.018	0.897	0.068	0.616	0.067	0.672	0.077	0.630	0.051	0.748
<i>EAAT3**</i>	-0.205	0.129	-0.061	0.654	-0.452	0.003	-0.155	0.326	-0.167	0.291

• Log-transformed \*\*Fisher-Yates transformed

**Table XIX: Additional table for relationship between placental mRNA levels and 4 year parameters (both adjusted for sex at birth)**

	4 year height (n = 56)		4 year weight (kg) (n = 56)		Total BMC (g) (n = 102)		Total lean (g) (n = 102)		Total fat (g) (n = 102)	
	r	P	r	P	r	P	r	P	r	P
<i>LAT 1**</i>	-0.190	0.161	-0.253	0.059	-0.084	0.596	-0.213	0.175	-0.068	0.670
<i>LAT 2**</i>	0.096	0.487	0.021	0.880	0.257	0.101	0.220	0.161	0.153	0.332
<i>ASCT 1**</i>	0.112	0.410	0.170	0.211	-0.066	0.679	0.143	0.365	0.066	0.679
<i>ASCT 2**</i>	-0.010	0.939	0.018	0.895	-0.211	0.179	-0.003	0.985	-0.106	0.503
<i>y<sup>+</sup>LAT 1**</i>	-0.081	0.551	0.101	0.460	-0.185	0.241	-0.003	0.985	-0.020	0.901
<i>y<sup>+</sup>LAT 2**</i>	0.005	0.972	0.023	0.866	-0.213	0.175	0.078	0.623	-0.149	0.348
<i>4F2HC**</i>	-0.011	0.936	0.028	0.835	0.107	0.500	0.067	0.673	-0.064	0.688
<i>BCATc**</i>	-0.002	0.988	0.038	0.782	-0.128	0.421	0.021	0.893	-0.065	0.681
<i>BCATm**</i>	0.097	0.478	0.084	0.540	0.067	0.675	0.066	0.678	0.204	0.195
<i>GLUD1/2*</i>	0.103	0.450	0.021	0.877	0.298	0.055	0.087	0.584	0.046	0.772
*										
<i>GLS1**</i>	-0.092	0.501	0.023	0.865	-0.206	0.192	-0.073	0.646	-0.168	0.289
<i>GLUL**</i>	0.164	0.226	0.165	0.223	0.047	0.768	0.204	0.195	-0.060	0.705
<i>ALT2**</i>	0.146	0.283	0.030	0.828	-0.001	0.996	0.054	0.732	-0.113	0.477
<i>AST2**</i>	-0.157	0.246	-0.115	0.398	-0.355	0.021	-0.251	0.109	-0.062	0.698
<i>AST1**</i>	0.071	0.605	0.068	0.616	0.160	0.310	0.022	0.891	0.140	0.377
<i>EAAT2**</i>	-0.009	0.948	0.073	0.594	0.087	0.586	0.119	0.454	0.015	0.925
<i>EAAT3**</i>	-0.205	0.129	-0.061	0.653	-0.468	0.002	-0.190	0.228	-0.152	0.337

• Log-transformed \*\*Fisher-Yates transformed

TABLES OF ASSOCIATIONS WHICH MUST BE REPORTED SEPERATELY FOR MALES AND FEMALES DUE TO SIGNIFICANCE OF INTERACTIONS.

**Table XX: The relationships between placental genes and pre-pregnancy smoking in male and female placentas.**

	Pre pregnancy smoking status		P
	No	Yes	
	Mean/SD	Mean/SD	
<b>Males (n = 53)</b>			
<i>y<sup>+</sup>LAT 1**</i>	-0.123 (0.944)	-0.070 (1.107)	0.863
<b>Females (n = 49)</b>			
<i>y<sup>+</sup>LAT 1**</i>	-0.119 (0.892)	0.849 (0.738)	0.001

**Table XXI: The relationships between placental genes and smoking in pregnancy in male and female placentas.**

	In pregnancy smoking status		P
	No	Yes	
	Mean/SD	Mean/SD	
<b>Males (n = 53)</b>			
<i>y<sup>+</sup>LAT 2**</i>	-0.305 (0.892)	-0.439 (0.980)	0.702
<i>ALT2**</i>	0.061 (0.983)	-0.625 (1.003)	0.076
<b>Females (n = 49)</b>			
<i>y<sup>+</sup>LAT 2**</i>	0.193 (0.915)	1.240 (0.942)	0.013
<i>ALT2**</i>	-0.119 (0.913)	0.533 (0.790)	0.107

**Table XXII: The relationships between placental genes and parity in male and female placentas.**

	Parity		P
	0	1+	
	Mean/SD	Mean/SD	
<b>Males (n = 53)</b>			
<i>LAT2</i> **	-0.139 (1.030)	0.361 (0.793)	0.051
<i>ALT2</i> **	0.399 (0.760)	-0.340 (1.079)	0.007
<b>Females (n = 49)</b>			
<i>LAT2</i> **	0.017 (0.871)	-0.321 (1.081)	0.242
<i>ALT2</i> **	0.004 (1.013)	0.037 (0.849)	0.902

**Table XXIII: The relationships between placental genes and strenuous exercise in male and female placentas.**

	Strenuous exercise		P
	No	Yes	
	Mean/SD	Mean/SD	
<b>Males (n = 53)</b>			
<i>GLS1</i> **	-0.650 (0.728)	0.167 (0.963)	0.001
<b>Females (n = 49)</b>			
<i>GLS1</i> **	0.190 (0.780)	0.208 (1.031)	0.949

**Table XXIV: The relationships between placental genes and maternal diet in male and female placentas.**

	EP: Fisher-Yates normalised PC1			EP: Fisher-Yates normalised PC2			Initial: Fisher-Yates normalised PC2		
	count	r	P	count	r	P	count	r	P
<b>MALES</b>									
<i>LAT2</i> **	-	-	-	39	-0.182	0.269	-	-	-
<i>ASCT2</i> **	-	-	-	-	-	-	53	0.222	0.109
<i>BCATm</i> **	-	-	-	39	-0.249	0.126	-	-	-
<i>AST1</i> **	-	-	-	-	-	-	53	-0.142	0.311
<i>EAA2</i> **	-	-	-	-	-	-	53	0.273	0.048
<b>FEMALES</b>									
<i>LAT2</i> **	-	-	-	39	0.294	0.070	-	-	-
<i>ASCT2</i> **	-	-	-	-	-	-	49	-0.251	0.081
<i>BCATm</i> **	-	-	-	40	0.253	0.115	-	-	-
<i>AST1</i> **	-	-	-	-	-	-	49	0.289	0.044
<i>EAA2</i> **	-	-	-	-	-	-	49	-0.148	0.310

**Table XXV: The relationships between placental genes and maternal body composition in male and female placentas**

	Pre pregnant mid-upper arm circumference (cm)			Maternal birth weight (g)			Pre pregnant calf circumference (cm)			Maternal height (cm)			Father's birth weight (g)		
	count	r	P	count	r	P	count	r	P	count	r	P	count	r	P
<b>MALES</b>															
<i>LAT 2**</i>	-	-	-	44	-0.453	0.002	-	-	-	-	-	-	-	-	-
<i>ASCT 1**</i>	-	-	-	44	-0.277	0.069	-	-	-	-	-	-	-	-	-
<i>4F2HC**</i>	-	-	-	-	-	-	53	0.191	0.171	-	-	-	-	-	-
<i>BCATm**</i>	-	-	-	-	-	-	-	-	-	53	-0.372	0.006	-	-	-
<i>GLUL**</i>	-	-	-	-	-	-	-	-	-	-	-	-	19	0.318	0.184
<b>FEMALES</b>															
<i>LAT 2**</i>	-	-	-	43	0.169	0.278	-	-	-	-	-	-	-	-	-
<i>ASCT 1**</i>	-	-	-	44	0.354	0.018	-	-	-	-	-	-	-	-	-
<i>4F2HC**</i>	-	-	-	-	-	-	47	-0.224	0.130	-	-	-	-	-	-
<i>BCATm**</i>	-	-	-	-	-	-	-	-	-	48	0.070	0.635	-	-	-
<i>GLUL**</i>	-	-	-	-	-	-	-	-	-	-	-	-	28	-0.451	0.016

**Table XXVI: The relationships between placental genes and fetal head circumference in male and female placentas**

	19 week scan: Royston AC z-score			19 week scan: Royston HC z-score			19 week scan: Royston FL z-score		
	count	r	P	count	r	P	count	r	P
<b>MALES</b>									
<i>BCATm**</i>	-	-	-	-	-	-	29	-0.347	0.065
<b>FEMALES</b>									
<i>BCATm**</i>	-	-	-	-	-	-	29	0.307	0.105

**Table XXVII: The relationships between placental genes and neonatal growth parameters in male and female placentas**

	4 year height			4 year weight (kg)			Total lean (g)		
	count	r	P	count	r	P	count	r	P
<b>MALES</b>									
<i>AST1</i> **	27	-0.402	0.038	27	-0.429	0.025	20	-0.432	0.057
<i>EAAT2</i> **	-	-	-	27	0.464	0.015	-	-	-
<i>ASCT 2</i> **	-	-	-	-	-	-	20	0.423	0.063
<i>GLUL</i> **	-	-	-	-	-	-	20	0.572	0.008
<b>FEMALES</b>									
<i>AST1</i> **	29	0.289	0.128	29	0.274	0.150	22	0.338	0.124
<i>EAAT2</i> **	-	-	-	29	-0.153	0.429	-	-	-
<i>ASCT 2</i> **	-	-	-	-	-	-	22	-0.320	0.147
<i>GLUL</i> **	-	-	-	-	-	-	22	-0.173	0.442

