A role for *Delta-like homologue 1* (*DLK1*) in a secretory placental cell population and implications for fetal growth

Dr Muriel Meso
BM BSc MRCPCH

Submitted in partial fulfilment of the requirements of the Degree of Doctor of Medicine

Centre for Endocrinology
William Harvey Research Institute
Barts and the London School of Medicine and Dentistry
Queen Mary University of London

Abstract

Intrauterine growth restriction (IUGR) affects between 5-10% of pregnancies carrying with it an increased risk of mortality and morbidity. Several serum analytes have been studied in the hope of finding a relevant marker linked to IUGR, but none of them have proven to be sufficiently accurate to be used in routine clinical practice as a single predictive marker.

IUGR is likely to be caused by both genetic and environmental factors. Studies in rodents have identified imprinted genes as some of the major genetic modulators of intrauterine growth. In addition, misregulation of imprinted gene dosage in humans is associated with paediatric growth disorders.

One such imprinted gene is *Delta-like homologue 1* (DLK1)*. DLK1* is expressed predominantly from the paternally inherited chromosome, while the maternally inherited copy remains silent. Genetic aberrations affecting this imprinted gene cluster, as see in both humans and mouse models, result in distinct phenotypes affecting growth.

A small cohort of participants was recruited from the Royal London Hospital, Fetal Medicine Unit for this study. Maternal serum *DLK1* levels were measured in this preliminary cohort. *DLK1* serum levels rose during gestation with levels falling after delivery. Furthermore, *DLK1* was found to be predictive of birth weight and has a positive correlation with fetal growth parameters.

This analysis was extended to a new cohort previously recruited at the Kuopio University Hospital, Finland. *DLK1* serum levels were seen to be significantly lower in IUGR pregnancies when compared to normal pregnancies.

Using immunohistochemistry methods I was able to detect robust expression of DLK1 in the fetal endothelium and trophoblast cells. Histology studies further identified the presence of multinucleated trophoblastic giant cells, a sign of maternal vascular malperfusion, as a finding in pregnancies associated with low *DLK1* levels.

These findings allow a better understanding of *DLK1.* Further investigations are warranted to enable elucidation of a mode of action for this protein.

Statement of Originality

I, Muriel Martha Meso, confirm that the research included within this thesis is my own work or that where it has been carried out in collaboration with, or supported by others, that this is duly acknowledged below and my contribution indicated. Previously published material is also acknowledged below.

I attest that I have exercised reasonable care to ensure that the work is original, and does not to the best of my knowledge break any UK law, infringe any third party's copyright or other Intellectual Property Right, or contain any confidential material.

I accept that the College has the right to use plagiarism detection software to check the electronic version of the thesis.

I confirm that this thesis has not been previously submitted for the award of a degree by this or any other university.

Signature:

Date: 29/08/2017

Acknowledgements

I would like to thank my supervisors Leo Dunkel, Marika Charalambous and Helen Storr for their encouragement and support during this period. Their commitment and keen attention to detail have allowed me to develop in this field.

I am grateful to the participants in this study and their families as without them I would not have been able to carry out this research project. In addition Mr Aquilina, Ms Rebecca Allen and Ms Shemoon Marleen (Fetal Medicine Unit) have made an invaluable contribution by carrying out antenatal scans and providing a platform for participant identification for the study. I am indebted to Dr Irene Scheimberg and her team (Core Pathology, Royal London Hospital, Whitechapel) who helped prepare the placenta for immunohistochemistry studies. I am extremely grateful to Dr Tanja Kuiri-Hänninen (Kuopio, Finland) for sharing clinical information and arranging for sample transfer from Finland.

I have been privileged to work at the William Harvey Research Institute, an internationally recognized research facility with world-renowned academics. I am grateful to my friends and colleagues in the lab for their support and advice and I would especially like to thank Harshini Katugampola.

This work would not have been possible without the support from my funding bodies, the Joan Adams Research Training Fellowship/ grant code MEA9926B and William Harvey Research Limited/ grant code TMTL1B3R.

Thank you to my family for their constant reassurance and untiring support for me. I would like to especially thank my husband, Lanre, for his unfailing support and for continuing to inspire me daily. Last but my no means least, I am eternally grateful to God, ever present, ever faithful.

Table of Contents

Table of Figures

Table of Tables

List of Abbreviations

AC	Abdominal circumference
ANG	Angiopoietin
ANOVA	Analysis of variance
AS	Angelman syndrome
BAT	Brown adipose tissue
BM	Basement membrane
BMI	Body mass Index
BPD	Bipareital diameter
BWS	Beckwith-Weidermann Syndrome
CD31	Cluster of differentiation 31
CK7	Cytokeratin 7
CpG	Cytosine , Guanine dinucleotide
CT	Cytotrophoblast
DAB	3,3'-diaminobenzidine
DF	Decidual fibroblasts
DIO3	Iodothyronine deiodinase 3
DLK1	Delta-like homologue 1
DMR	Differentially methylated region
DNA	Deoxyribonucleic acid
dNTP	Deoxynucleotide
DSL	Delta, Serrate. Lag2
EB	Elution buffer
eCTB	Endovascular cytotrophoblast
EDTA	Ethylenediametetraacetic acid
EFW	Estimated fetal weight

EGF	Epidermal Growth Factor
ELISA	Enzyme Linked-Immunosorbent Assay
EM	Extraembryonic mesoderm
ERK	Extracellular Signal-regulated Kinase -1
EVT	Extravillous trophoblasts
FA1	Fetal antigen 1
FATP	Fatty acid transport protein
FE	Fetal endothelial
FL	Femoral length
FMU	Fetal Medicine Unit
GA	Gestational Age
GH	Growth Hormone
GLUT	Glucose transporter
GTL2	Gene trap locus 2
HCG	Human Chorionic Gonadotropin
HDBR	Human Development Biology Resource
HPGH	Human placental growth hormone
HPL	Human placental lactogen
HRP	Horseradish Peroxidase
ICM	Inner cell mass
ICR	Imprinting control region
iCTB	Interstitial cytotrophoblast
IGF	Insulin-Like Growth Factor
IgG	Gamma immunoglobulin
IHC	Immunohistochemistry
IRDye	Infrared Flourescent Dye
IUGR	Intrauterine growth restriction

IVF	
LH	Placental growth factor
MAC	Preadipocyte factor 1
MAPK	Prader Willi Syndrome
MEG	Royal College of Obstetricians and Gynaecologists
mFA1	Resistance Index
miRNA	Royal London Hospital
MOPS	Ribonucleic acid
MRC	Ribonuclease
mRNA	Relative risk
MTGC	Russell – Silver syndrome
matUPD	Retrotransposon Gaglike 1
MVM	Sodium dodecyl sulphate polyacrylamide gel electrophoresis
NaCl	Small for gestational age
NaN3	Statistical Package for the Social Science
ncRNA	Syncytiotrophoblast
NREC	Signal transducer
OFC	And activator of transcription 3
OFD	Tumour necrosis factor alpha converting enzyme
OR	Tris-acetate – EDTA buffer
PAPP-A	Thermus aquaticus
patUPD	3,3’,3,5,5’ – tetramethylbenzidine
PBS	Transient neonatal diabetes mellitus
PCR	Tissue necrotic factor
PIGF	Temple syndrome
PREF	Uterine Natural Killer
PWS	Ultrasound Scan

RCOG	Royal College of Obstetrics and Gynaecology
RI	Resistance Index
RLH	Royal London Hospital
RNA	Ribonucleic Acid
RNase	Ribonuclease
RR	Relative Risk
RSS	Russell – Silver Syndrome
RTL1	Retrotransposon Gaglike 1
SDS-PAGE	Sodium docedyl sulphate polyacrylamide gel electrophoresis
SGA	Small for gestational age
SPSS	Statistical Package for the Social Science
ST	Syncytiotrophoblast
STAT3	Signal transducer and Activator of transcription 3
TACE	Tumour necrosis factor alpha converting enzyme
TAD	Tris- acetate factor alpha converting enzyme
TAE	Tris acetate – EDTA buffer
TAQ	Thermus aquaticus
TMB	3,3',3,5,5'- tetramethylbenzidine
TNDM	Transient neonatal diabetes mellitus
TNF	Tissue necrotic factor
TS	Temple syndrome
uNK	Uterine Natural Killer
USS	Ultrasound Scan
UTR	Untranslated region
UV	Uterine Vessel
vCTB	Villous cytotrophoblast
VEGFA	Vascular endothelial growth factor

WAT	White adipose tissue
WW II	World War 2

Chapter 1: Introduction

1.1 Mechanism of Fetal growth

Fetal growth is a complex process regulated by fetal, maternal and placental factors. The drive for fetal growth is under the control of the fetal genome, which exerts a significant demand for maternal nutrients through the placenta. The right balance needs to be achieved between this fetal demand and maternal nutrient requirements; in order to avoid pregnancy related complications, which can have long standing effects[1]. The placenta plays an important role in maintaining this balance by adapting to fetal signals of nutrient demand, in addition to maternal signals of nutrient availability, thereby allowing for appropriate allocation of nutrients[2].

1.1.1 Factors regulating fetal growth

A functioning placenta is paramount for fetal growth. Several factors influence transport across the placenta including utero-placental and umbilical blood flows, the area available for exchange, placental metabolism and the expression of specific transporter proteins in the placental barrier. The transfer of highly permeable molecules such as oxygen and carbon dioxide is especially influenced by reduced blood flow[3]. For other substrates, the placenta possesses both passive and active transport mechanisms. Endocrine hormones, which can be secreted by the fetus, the placenta, or be of a maternal source also regulate fetal growth. In addition gene regulation and expression in both the placenta and fetus can influence the process of development in utero.

1.1.1.1 Nutrient transporters

Fetal growth is largely determined by nutrient supply, which is mainly dependent upon placental nutrient transport. In human pregnancies complicated by either IUGR or fetal overgrowth, placental nutrient transporters are found to be specifically regulated suggesting an important role for nutrient transfer in fetal growth [4–6]. Glucose is a primary substrate for fetal growth and is transported across the placenta via GLUT1 (glucose transporter 1) found in both the microvillus and basal membranes of the syncytiotrophoblast (ST) layer. GLUT1 is more abundant on the microvillus membrane (MVM), which is maternal facing (section 1.2) when compared to the fetal facing basal membrane (BM). This allows for transfer of Glucose by facilitated diffusion. GLUT1 expression increases over the latter half of gestation concurrent with an increased rate of growth in the third trimester[4]. IGF1 has recently been shown to stimulate glucose transport capacity by increasing the translocation of GLUT1 to the trophoblast plasma membrane[7]. Net glucose transfer is therefore highly dependent on maternal-fetal concentration gradients[8] . Other nutrients such as calcium are transported by primary active

transport[9]. A number of nutrients are transported across the placenta by secondary active transport, utilising energy provided by ion gradients[9]. Changes in energy availability or ion gradients can consequently influence net transfer of substrates transported by active mechanisms.

The primary barrier limiting nutrient transfer across the human placenta is the ST (Figure 4). The ST plasma membranes express numerous nutrient transporters, which may be regulated by fetal, maternal and placental signals. Most nutrient transporters are more abundant on the MVM compared to BM thereby favouring transport of nutrients in the fetal-maternal direction[10]. Gene expression changes in placental nutrient transporter systems have been seen in pathology such as IUGR, pre-eclampsia, chronic hypoxia and diabetes[11]. This highlights the importance of the nutrient transporters in maintaining a normal pregnancy.

1.1.1.2 Placental vasculature

The importance of the role played by the placental vasculature in fetal growth can be appreciated in the impaired uterine and umbilical artery blood flows seen in IUGR as described in section 1.1.5. The development of new capillaries from pre-existing ones (angiogenesis) requires a well-controlled balance between pro-angiogenic and anti-angiogenic factors. The main pro-angiogenic factors are: vascular endothelial growth factor A (VEGFA), which is induced by the hypoxia-inducible factor 1 (HIF1), placental growth factor (PlGF), which is activated directly by oxygen concentrations; and angiopoietins ANG1 and ANG2[11,12]. The main anti-angiogenic factors are the soluble, truncated form of the Flt1 receptor (sFlt1) and the soluble endoglin (sEng), a truncated form of endoglin (CD105)[13]. Further evidence for the role played by angiogenic factors in placentation is seen in pathology. Altered concentrations of VEGFA and its receptors have been documented in human placental fetal capillaries from pregnancies complicated by type 1 and type 2 diabetes [14]. In addition, placentas from women with type 1 and type 2 diabetes have increased fetal capillary volume and length compared with controls[15]. Furthermore, increased fetal capillary volume has been documented in human placentas in cases of exposure to chronic hypobaric hypoxia at high altitudes, as well as in the context of maternal iron-deficient anaemia[16,17]. Hypoxia and anaemia have also been shown to reduce the thickness of the interhaemal barrier[16,17]. Factors affecting perfusion and nutrient exchange will inevitably influence fetal growth. The process of angiogenesis, including the intricate control from various factors working in synchrony, is another example of the placenta adapting in response to the intrauterine milieu. Aspects that interfere with the balance between pro and anti angiogenic factors will impact placental function and therefore the trajectory of fetal growth.

1.1.1.3 Endocrine control of fetal growth

As we have seen from the descriptions above, the regulation of fetal growth is complex and multifactorial. We have seen how fetal growth can be influenced by nutrient transport and placental vasculature. In addition, fetal growth is also under endocrine control. The main hormonal drivers of intrauterine growth are insulin, insulin-like growth factors (IGF) and thyroid hormones[18]. Hormones can act on fetal growth directly (via genes) and indirectly through changes in placental growth, fetal metabolism or production of growth factors and other hormones by the feto-placental tissues. The source of these hormones could be: from the endocrine glands of the fetus (fetal pancreas, thyroid, pituitary and adrenal glands); from the uteroplacental tissues (mainly the ST); from the mother by transplacental diffusion; and from circulating precursor molecules by metabolism in the fetal or placental tissues.

Insulin controls cell number as it has direct mitogenic effects on cellular development. It leads to glucose uptake and consumption by body tissues and decreases protein breakdown. Insulin also stimulates production of IGF-I while fetal insulin specifically acts as a signal of nutrient availability for growth[18]. Insulin concentration in utero rises between early and mid gestation, thereafter remaining stable until term. The insulin – like growth factor (IGF) system is the main established endocrine regulator of fetal growth [19]. IGF-I is synthesised in utero with the presence of IGF-I mRNA noted from as early as 12-14 weeks gestation in humans[20]. There is a progressive increase in IGF-I and IGF-II during gestation. IGF-I is positively regulated by glucose supply to the fetus. Furthermore, fetal plasma IGFs are derived from a range of feto-placental tissues throughout gestation[21]. IGF-I has mitogenic properties resulting in induction of somatic cell growth and proliferation and has further function in regulating transport of glucose and amino acids cross the placenta[22]. Thyroid hormones present in the fetal circulation are mainly derived from the fetus but can also be of maternal or placental origin[23].Hypothyroxinaemia in the fetus leads to decreased oxygen consumption and oxidation of glucose thereby resulting in decreased fetal energy supply for growth.

Growth hormone is the main regulator of postnatal growth but doesn't appear to affect intrauterine growth[24,25]. Other hormones that indirectly influence fetal growth mainly through their effect on implantation and placentation include oestrogen (oestradiol), progesterone, human placental lactogen (HPL), Human placental growth hormone (HPGH), leptin, adiponectin and resistin[26]. Leptin in particular has been shown to stimulate system A amino acid activity[27]. Amino acids are an important nutrient for fetal growth and development. Placental system A amino acid transporter

activity is significantly lower in pregnancies complicated by IUGR[28,29]. Circulating leptin levels during pregnancy are elevated 2 to 3 fold above that observed in non-pregnant women and in addition leptin receptors are present in the placenta[30]. These suggest an important role for leptin in fetal growth. We can see that fetal growth is controlled by a number of closely related factors, some of which are dependent on each other. It is important for us to understand the events that influence the functioning of these factors.

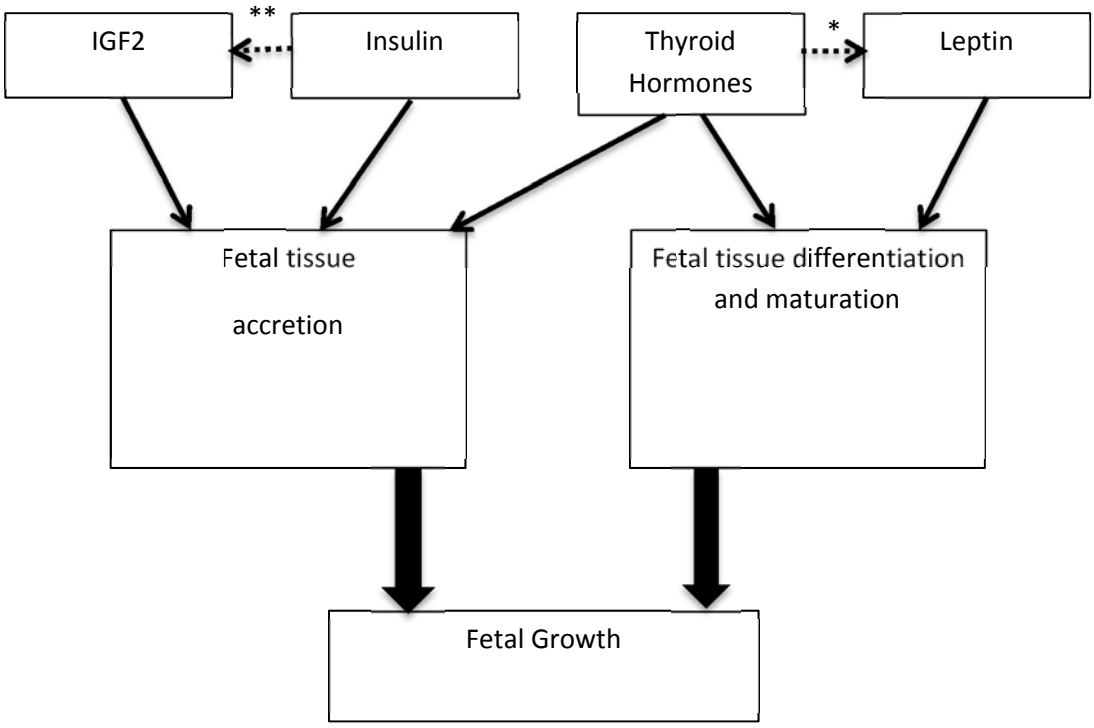


Figure 1: **Hormonal influence of fetal growth**

IGF2 and Insulin both have anabolic actions on fetal growth leading to fetal tissue accretion. Thyroid hormones influence growth, metabolism and differentiation of fetal tissue. These actions in combination lead to overall fetal growth. * Thyroid hormones may regulate leptin gene expression. ** Insulin mainly acts directly but has some indirect actions via the IFG receptors.

1.1.1.4 Clinical Presentation of IUGR

Intrauterine growth restriction (IUGR) refers to the decline of the growth rate in utero, which may or may not result in a baby born small for gestational age (SGA) (Figure 2). Similarly a baby may be born

SGA with no reduction in the rate of growth: this would therefore not be as a result of IUGR. SGA is defined as a birth weight and / or length that is 2 standard deviations or less below the population mean; therefore 2.5% of all new-borns will meet the definition (Figure 2). The focus of this thesis will be on placenta mediated growth restriction.

IUGR is associated with increased fetal and neonatal mortality and morbidity both in the short term (prematurity, cerebral palsy, intrauterine fetal death, neonatal death) and more long term (obesity, hypertension, type 2 diabetes)[31–35]. In the western world, the most common reason for IUGR is uteroplacental insufficiency[36]. Chromosomal abnormalities (5-20%), some fetal malformations (1-2%), fetal infection (5-10%) and multiple gestation (3%) can be associated with a reduction in growth velocity of varying severity[37]. IUGR can be either symmetrical or asymmetrical. In symmetrical growth restriction, there is an equivalent reduction in growth velocity of both the fetal head circumference and abdominal circumference. In asymmetric growth restriction, there is a differential reduction in growth velocity of the fetal head in comparison to the abdominal circumference, favouring head sparing. Symmetry or asymmetry is thought to depend on the timing and origin of fetal growth restriction. SGA accounts for approximately 10% of pregnancies and only a proportion of these are secondary to IUGR.

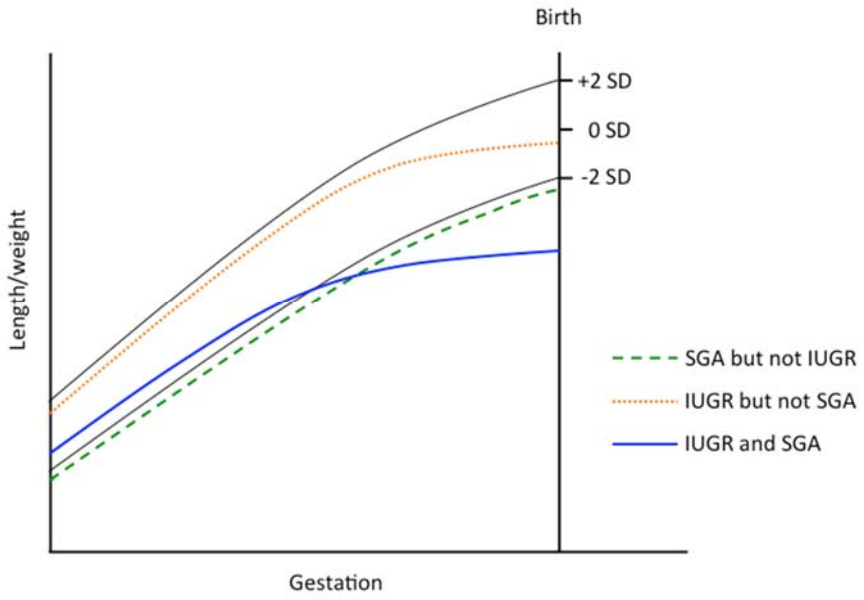


Figure 2: **Distinction between IUGR and SGA**

This graph shows the difference between IUGR and SGA. SGA IUGR implies a reduction in growth velocity in utero. This can result in a fetus that is not SGA (red dotted line) or one that is SGA (blue solid line). In addition it is possible to be SGA with IUGR (green dotted line). IUGR –Intrauterine growth restriction; SGA - Small for gestational age

The diagnosis of IUGR is made after an insult has occurred. Early recognition of risk factors for IUGR in pregnant women would allow for closer surveillance during gestation. Clinical risk factors however show a different impact for each individual case and are therefore not relevant as general screening tools. The current strategies employed in the evaluation of IUGR are covered below.

1.2 The human placenta

1.2.1 Normal placental development

The placenta is a transient organ found in eutherian mammals with a role in providing nutrients for the developing fetus in addition to offering support and protection. The placenta exchanges a wide range of nutrients, endocrine signals, cytokines and growth factors between the mother and the fetus, thereby regulating intrauterine development. It is important to understand the normal development of this vital organ including its structure and function as it closely influences the growing fetus and resulting neonate. In an attempt to appreciate normal placental development, the anatomy of this organ will be described and the process of placental development covered below.

Pregnancy is a complex process involving germ cell differentiation, gametogenesis, ovulation, fertilisation, implantation, decidualisation, placentation and parturition. Progress at each stage of human pregnancy is dependent upon the success of the preceding steps and requires careful coordination[38]. Fertilisation involves the formation of a diploid zygote from a haploid sperm and a haploid egg or ova. The zygote undergoes cell division resulting in differentiation into a blastocyst.

Interaction between the blastocyst and maternal uterine luminal epithelium initiates the process of implantation, a process by which blood vessels of the embryo are brought into functional communication with the maternal circulation leading to the establishment of a functional placenta and pregnancy[39]. The trophectoderm forms the wall of the blastocyst whilst the mesoderm or inner cell mass forms the extra embryonic mesoderm (Figure 3). The trophectoderm differentiates into trophoblasts, which form the epithelial covering of the placenta. The inner cell mass (ICM) further differentiates into two distinct lineages, the epiblast and the primitive endoderm[40]. The epiblast gives rise to the fetus, the allantois, which forms the placental vasculature and the umbilicus, while

the primitive endoderm and trophectoderm give rise to the fetal membranes and the placenta respectively[40]. The uterus undergoes developmental changes during the pre-implantation period, mediated by hormonal changes and signalling pathways[41]. These processes allow for the successful implantation of the blastocyst. Immediately after implantation, trophectodermal cells give rise to diverse trophoblast cell types. These include the lytic syncytiotrophoblast (ST) and the cytotrophoblast (CT) cells. CT are mononucleated cells that are able to proliferate and differentiate into other trophoblast subtypes, functioning as a precursor pool for the ST, which form the syncytial layer, and extravillous trophoblasts (EVT)[42]. CT may also acquire invasive properties, forming the EVT. These trophoblasts are able to invade and remodel maternal tissues and uterine spiral artery, reducing the resistance against blood flow that irrigates the fetus[43,44]. The syncytial layer has no proliferative capacity and is in intimate contact with maternal blood, participating in fetal nourishment, gas exchange and also playing important roles in other placental functions, mainly in protein biosynthesis (Figure 3).

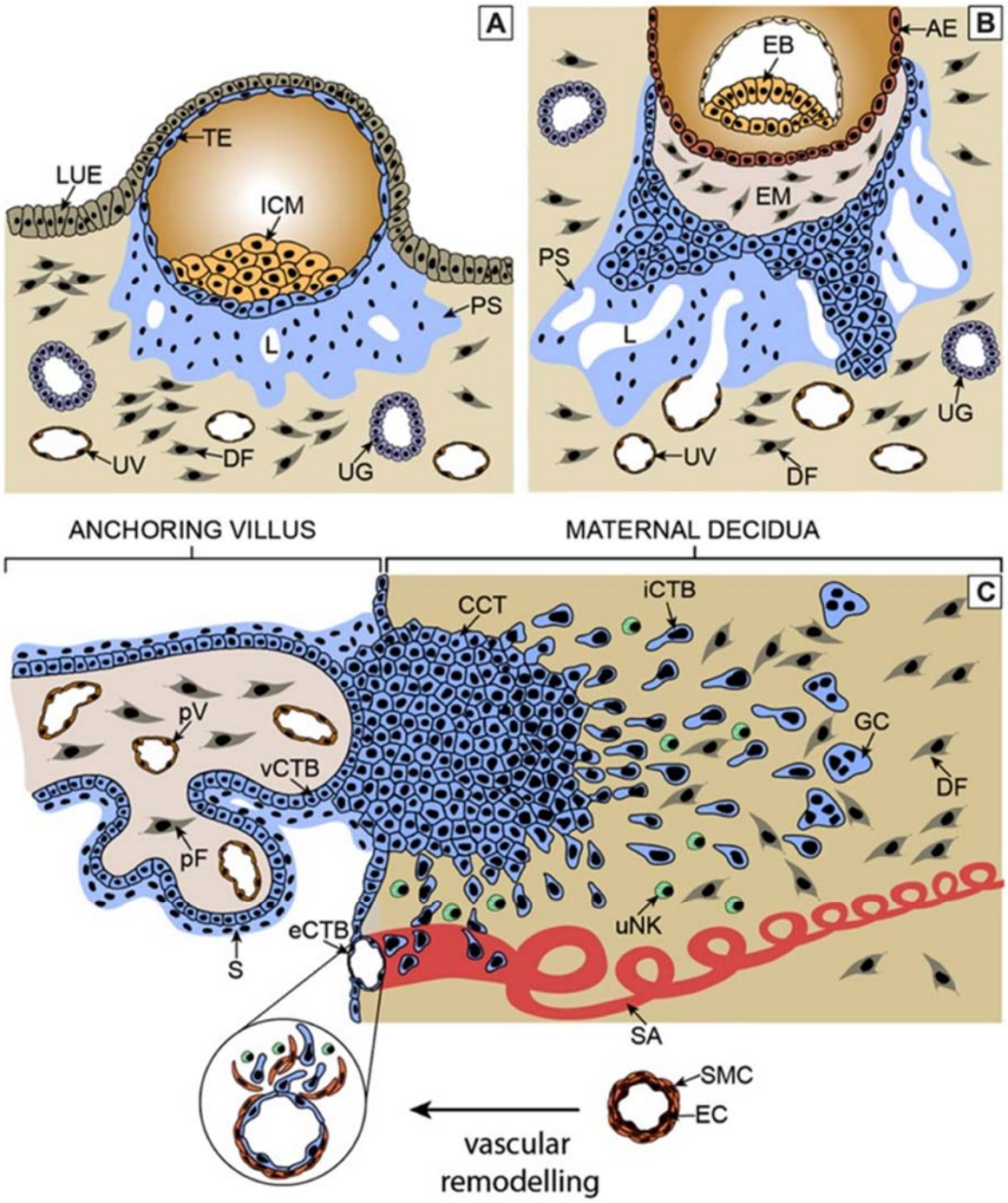


Figure 3: **Schematic showing placental development**

A – Trophectoderm gives rise to the primitive syncytium after implantation. B– Proliferative CTB form primary villi. C – Tertiary villi are formed by migration of extraembryonic mesodermal cells. Endovascular extravillous trophoblasts are involved with immune function and remodelling of spiral arteries. AE, amniotic epithelium; CCT, cell column trophoblast; DF, decidual fibroblast; EB, embryoblast; EM, extraembryonic mesoderm; eCTB, endovascular cytotrophoblast; GC, giant cell; ICM, inner cell mass, iCTB, interstitial cytotrophoblast; LUE, luminal uterine epithelium; L, lacunae, pF placental fibroblast; PS, primitive syncytium; TE, trophectoderm; UG, uterine gland; uNK, uterine NK cell, UV, uterine vessel; vCTB, villous cytotrophoblast. Used with permission from the authors[44].

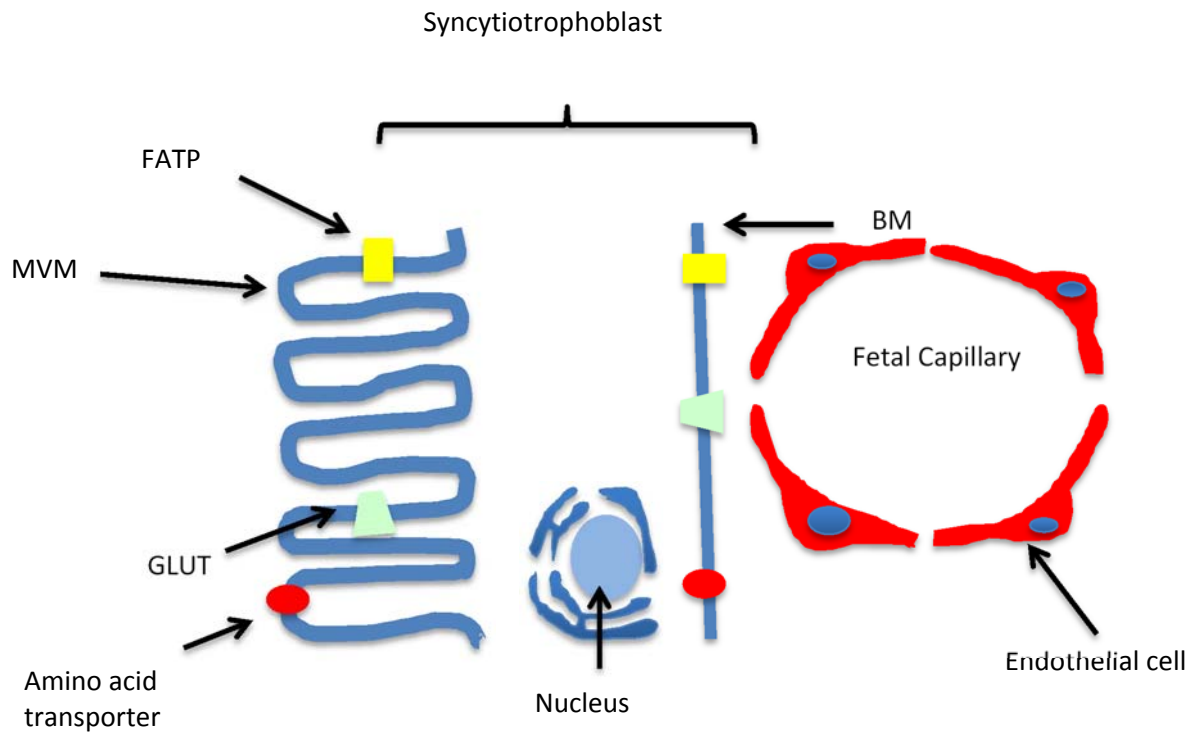


Figure 4: **Nutrient transporters in the syncytiotrophoblast**

Nutrient transporters are seen on both the maternal circulation facing microvillous membrane (MVM) and the fetal circulation facing basement membrane (BM). Maternal blood pools in the intervillous space and bathe the MVM. Transporters for the transfer of amino acids, glucose (GLUT), and fatty acids (FATPs) are expressed in both plasma membranes of the syncytiotrophoblast.

Macroscopically the mature placenta consists of a discoid organ weighing approximately 500 grams at term[40]. The surfaces consist of a chorionic plate that faces the fetus and to which the umbilical cord is attached and the basal plate that abuts the maternal endometrium. Between these plates is the intervillous space into which 30 - 40 branched fetal villous trees project[45]. Each villous tree arises from a stem villous in the chorionic plate and branches to create a globular lobule 1 - 3 cm in diameter[45]. The centre of the lobule is located over the opening of a maternal spiral artery through the basal plate. Each lobule therefore represents an independent maternal- fetal exchange unit [45,46](Figure 5).

The final branches of the villus tree are the terminal villi. These are richly vascularised by fetal capillaries. The capillaries display local dilations or sinusoids, which bring the endometrium within close proximity to the trophoblasts. This area is thinned, thus reducing the diffusion distance between the maternal and fetal circulations to as little as 2-3 µm. Terminal villi are formed primarily from 20 weeks gestation onwards and further elaboration of the villous tree continues to term[45].

The epithelial cover of the villous tree is the ST, a multinucleated syncytium presenting no intercellular clefts to the intervillous space. Because of its location, the ST is involved in syntheses and secretion of steroid and peptide hormones, protection against foreign organisms and active transport. An understanding of the placental morphology allows us to appreciate the various functions of the placenta covered below.

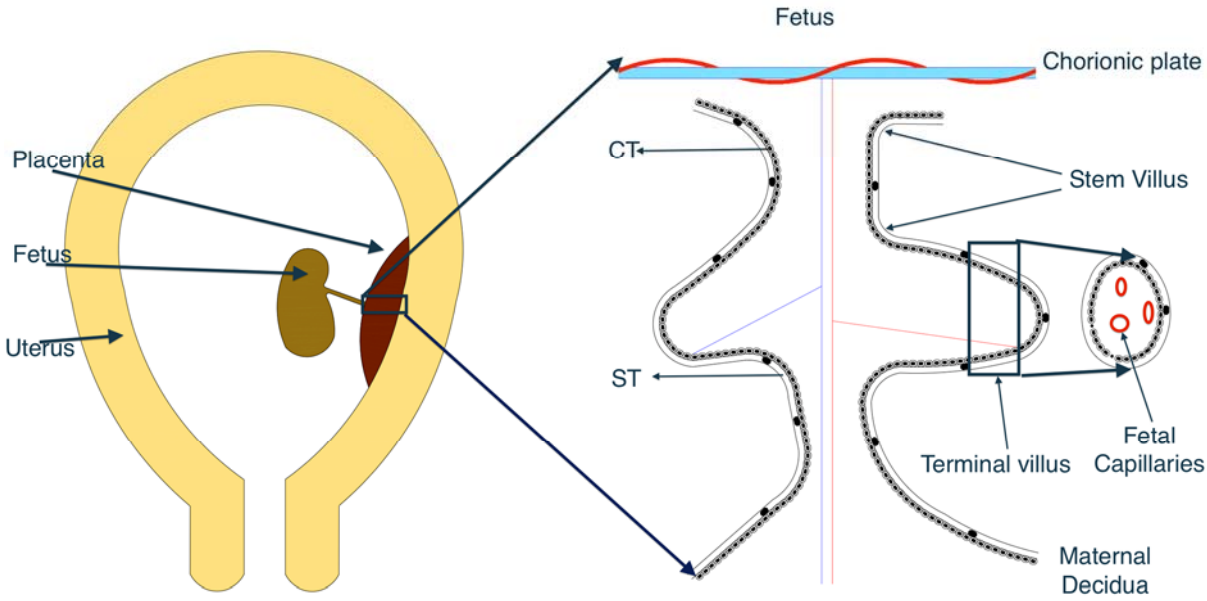


Figure 5: **Depiction of the fetoplacental unit**

The macroscopic appearance is shown on the left with a zoomed in section of the intervillous space featuring the chorionic plate in contact with the fetus, stem and terminal villi and the basal plate in contact with the uterine myometrium. The trophoblast layer surrounding the villi are depicted with the fetal capillaries situated within the villi. CT, cytotrophoblast, ST, syncytiotrophblast

1.2.2 Placental function

The window of implantation is a limited time span dependent on blastocyst competency and the receptive state of the uterus. Knowledge obtained from transgenic mouse models has helped to better understand the molecular basis of uterine receptivity, implantation and decidualisation in humans[47]. Oestrogen and progesterone coordinate uterine functions through multiple paracrine, juxtacrine and autocrine factors in a spatiotemporal manner. This is achieved through mediators such as cytokines. Furthermore sets of key regulatory transcription factors control the switch between trophoblast proliferation and EVT differentiation[43]. Key signalling pathways involved in trophoblast motility include growth factors such as Human Chorionic Gonadotropin (HCG), Endodermal Growth Factor (EGF) and Insulin-Like Growth Factor 2 (IGF2), which activate MAPK kinase and phosphoinositide 3-kinase signalling. Further investigations in human primary cultures as well as in trophoblast cell lines have provided evidence for an autocrine role of canonical Wnt signalling in human trophoblast proliferation and invasion[48].

One of the major functions of the human placenta is the capacity to synthesise important hormones and other mediators, crucial for gestational success. Placental-related hormones play important roles during several gestational events. The ST layer is the main source of placental hormones. Other trophoblast phenotypes however, may also produce some placental hormones and affect the gestational course. The hormones produced by the EVT are thought to contribute to vascular and uterine tissue remodelling and regulate EVT migration and invasion[26].

Human chorionic gonadotropin (HCG) is one of the most important hormones in pregnancy. From day 8 after fertilisation, HCG is detectable in maternal serum and its levels peak in week 10 of gestation, thereafter decreasing slowly until the end of pregnancy[49]. The HCG receptor is expressed in the CT, ST and EVT. HCG mediated effects are essential for the maintenance of early pregnancy. HCG also promotes in-vitro human CT differentiation into ST, by activating the LH-HCG receptor and consequently Protein Kinase A (PKA) pathway[50]. Progesterone is mainly produced from the corpus luteum during the first few weeks of pregnancy. At 6-8 weeks, after HCG concentration decreases, the placenta is the main source of progesterone due to the formation of the syncytial layer. Progesterone has important functions in menstrual bleeding but additionally in placentation and preparation of the mammary gland for lactation. Oestrogens play several roles during pregnancy including in angiogenesis. Adipocyte derived signalling proteins including leptin, adiponectin and resistin have roles to play in nutrient transport, immune modulation and placentation[27,51,52].

The functions of the placenta are closely orchestrated by regulatory factors, which may be influenced by the intrauterine milieu. In this way the placenta carries out its functions efficiently while maintaining pregnancy to term. As part of the placenta's function it may undergo adjustments in order to keep the intrauterine environment optimum for fetal growth and development. Below we address some of these adaptations that take place during gestation.

1.2.3 Placental adaptations

Placental adaptation is required in humans in order to maintain pregnancy while adjusting to changes during gestation. Changes in supply capacity of the placenta can be triggered by intrinsic (genetic and developmental) or extrinsic (environmental) factors that regulate intrauterine development[11]. As the placenta is the main interface between the mother and fetus, it receives and integrates a wide range of fetal and maternal signals that ultimately determine its plasticity. Placental adaptations occur as gestational age advances. Fetal growth increases exponentially with fetal weight increasing most dramatically during the last half of gestation. On the other hand utero-placental growth slows down or ceases during the last half of gestation whilst placental transport capacity keeps pace with the continually increasing demands of the fetus[53]. This increase in placental function can be primarily attributed to increases in vascularity and blood flow together with associated morphological changes. In addition, in both humans and mice there is a progressive thinning of the trophoblast barrier layer with gestational age thus decreasing diffusion distance for passive exchange[54]. Furthermore there is a continual increase in fetal capillary surface area[54,55]. Fetal signalling of demand for maternal nutrients is an important regulator of placental transport capacity, most prominently in late gestation as shown by animal studies[56,57].

Further evidence for placental plasticity is seen in the adaptive changes noted with pathology in this organ. Morphological adaptations seen in the placenta include changes to placental vasculature as seen in Doppler flow changes with fetal growth restriction[58], changes to interhaemal barrier thickness as seen in alteration of microvillous membrane (MVM) thickness in human pregnancies at high altitudes[16,17,59] and changes to cellular composition as seen in mouse studies with both maternal diet manipulation and knockout models[60,61]. These changes result in modulation of nutrient transport within the placenta. Functional adaptations include alterations to nutrient transport channels or changes in placental hormone secretion.

The underlying molecular mechanisms involved in the placental adaptive response are not well understood, however recent work suggests that nutrient sensing pathways and epigenetic mechanisms may play significant roles in these processes[11]. Changes in gene expression associated

with placental adaptive responses can be attributed, at least in part, to epigenetic alterations. Epigenetics refers to the study of molecular modifications that influence gene activity and chromosome structure without a change to DNA sequence. These modifications are heritable and result in a gene expression state that persists through over cell generations[62]. One of the best-studied epigenetic modifications is DNA methylation, which involves the addition of a methyl group at 5' position of a cytosine adjacent to a guanine (CpG dinucleotide)[63]. Recent genome-wide studies of DNA methylation in the human placenta have revealed large-scale differences in DNA methylation levels through the three pregnancy trimesters, with an overall progressive increase in average methylation being observed with increasing gestational age[64]. Work has shown an association between patterns of DNA methylation and infant growth resulting in the identification of 22 critical loci that are highly predictive of IUGR[65]. DNA methylation profiles were obtained on 206 term human placenta samples. This work was carried out to add to existing work on imprinted genes and therefore did not identify DLK1 or IGF2. With this work it was not possible to determine whether altered profiles of DNA methylation are in response to the intrauterine environment or whether the changes were causative for IUGR. In addition there was an overrepresentation of CpG island associated loci. Gene regulatory methylation events may occur in regions outside CpG islands. These data together suggest that methylation status in the human term placenta may function both as a marker of intrauterine environment and also play a critical functional role in fetal development and growth. The placenta therefore undergoes adaptive changes to ensure optimal fetal growth and development within the constraints of prevailing intrauterine conditions.

In addition to the control of transcription at DNA level (DNA methylation), epigenetic regulation controls transcription at protein level (histone modification) and at RNA level (non-coding RNAs). The nucleosome is a protein complex, consisting of two copies of four core histones (H2A, H2B, H3 and H4) around which the DNA is wrapped. The N-terminal of nucleosomal histones undergoes epigenetic modifications including methylation, acetylation, phosphorylation and ubiquitinylation. These modifications are accomplished by a range of enzymes. Proteins can recognise and bind to these specific modifications and exert an effect on gene activity. Majority of the transcripts from the transcribed mammalian genome consists mainly of non-coding (nc) RNAs. These nc RNAs can act in *cis* (same chromosome) or in *trans* (different chromosome/ mature RNAs in cytoplasm). This method of epigenetic regulation is seen later in the section about DLK1.

1.2.4 Abnormalities in placental development

Pregnancy is programmed as a dynamic process and any major aberration in its normal course will

either terminate pregnancy at the time of insult or perpetuate defects throughout pregnancy. These defects are reflected in aberrations in decidualisation, placentation and intrauterine embryonic growth, manifesting as intrauterine growth restriction (IUGR), preeclampsia, miscarriages and/or preterm birth.

Several knockout mouse models helped to define propagation of early defects during the course of pregnancy. Mutant mice have been seen to have abnormalities such as defective uterine receptivity or deferred implantation. This deferred implantation results in embryo overcrowding, conjoined placenta, placental insufficiency, fetal growth restriction, fetal resorption and reduced litter size[47]. Further studies have shown that suboptimal decidualisation can lead to premature decidual senescence, resulting in preterm birth with neonatal death or in abnormal guidance of placentation, which can lead to shallow invasion. A number of genes have been identified as being essential for the placentation process in these knockout models with expression in human myometrium suggesting conservation of the genes of interest[47].

1.2.4.1 Consequences of abnormal placentation in human pregnancies

Placental adaptation has been mentioned previously as part of the physiological adjustments required by the placenta in normal pregnancy. The nature of the placental changes associated with adaptive responses depends on a variety of factors, which include the severity of the environmental challenges, the timing and duration of the challenge and possibly the sex of the offspring. Adaptive responses may, however, be blunted or compromised if there is a need to activate severe stress responses aimed at safeguarding maternal-fetal survival. Preeclampsia and fetal growth restriction are disorders that are rooted in defects of early placental development [66]. These defects include poor trophoblast uterine invasion, impaired transformation of the uterine spiral arteries to high capacity and low impedance vessels, and abnormalities in the development of chorionic villi.

Defective uterine receptivity, implantation and / or decidualisation can lead to infertility. Furthermore, implantation outside the window of receptivity can lead to misguided embryo placement, resulting in placenta praevia, ectopic placentation (placenta accreta) or placental insufficiency resulting in intrauterine growth restriction and /or preeclampsia[43,67]. Implantation beyond the normal window can also give rise to spontaneous abortion, miscarriage and recurrent pregnancy loss[68]. The underlying mechanisms and pathways involved in the process of placentation are poorly understood. A better understanding of this process could lead to more definitive management of fetal growth restriction and other gestational conditions associated with aberrant placentation, during pregnancy.

1.2.5 Clinical impact of placental pathology

As seen in the previous sections, the process of placentation involves carefully orchestrated events that are required to take place at the appropriate time in order to achieve normal gestation. Variations in the process can lead to pathology, which inevitably can affect the pregnancy and the resulting neonate. These can present in a variety of ways during pregnancy. However due to the nature of pregnancy, the methods currently available tend to detect pathology late in gestation once an insult has occurred. This is true for IUGR where interventions usually take place once a reduced fundal height has been detected during antenatal follow up. This is usually in the third trimester for most pregnancies.

1.2.5.1 Current antenatal evaluation of IUGR

Clinical examination is unreliable in detecting SGA fetuses on its own. Diagnosis of a SGA fetus usually relies on ultrasound measurement of fetal abdominal circumference or estimation of fetal weight[69]. A number of surveillance tests are available, including cardiotocography, Doppler and ultrasound to assess biophysical activity. At booking, those identified as being at risk are offered appropriate surveillance (Table 1). Those identified to have major risk factors are referred for serial ultrasound measurement of fetal size and umbilical Doppler flow assessment from 26-28 weeks of pregnancy [69](Table 1). In addition those with three or more minor risk factors are referred for uterine artery Doppler at 20-24 weeks gestation[69]. Several biochemical markers have been investigated as screening tests for SGA. Systematic reviews found low predictive accuracy for alpha-fetoprotein, elevated hCG and inhibin A, low unconjugated estriol and the combined triple test to predict a SGA fetus[70,71]. Low levels of first trimester PAPP-A and /or hCG were associated with an increased frequency of adverse obstetrical outcome including a SGA infant[72].

Early ultrasound features of IUGR include a reduction in growth velocity and oligohydramnios. The Doppler ultrasound sampling of uterine arteries is a useful non-invasive method for the assessment of the interaction between the fetal and maternal haemodynamic compartment. Doppler studies typical of uteroplacental insufficiency include uterine artery notches, absent or reversed end-diastolic flow in the umbilical artery and fetal arterial redistribution. Similar features are seen in fetal abnormality or infection, but usually the uterine Doppler values and amniotic fluid volume are normal. Women with an abnormal uterine artery Doppler at 20 – 24 weeks (PI > 95th centile) and/ or notching should be referred for serial ultrasound measurement of fetal size and assessment of wellbeing with umbilical artery Doppler commencing at 26-28 weeks of pregnancy. Additionally serological screening for congenital infections and karyotyping (especially in the presence of

structural anomalies) is carried out in cases of severe SGA (AC and EFW < 3rd centile). The combined approach using clinical information, serum markers, and biophysical parameters such as ultrasound, showed increased predictive relevance[73].

Risk category	Definition of risk
Maternal RF	
Age	Maternal age ≥ 35
	Maternal age ≥40
Parity	Nulliparity
BMI	BMI <20
	BMI 25 – 29.9
	BMI ≥30
Maternal substance exposure	Smoker
	Smoker 1-10 cigarettes/day
	Smoker ≥ 11 cigarettes/ day
IVF	**Cocaine**
Exercise	IVF singleton pregnancy
Diet	**Daily vigorous exercise**
	Low fruit intake pre pregnancy
Previous Pregnancy History	
Previous SGA	**Previous SGA baby**
Previous still birth	**Previous still birth**
Previous pre-eclampsia	Pre-eclampsia
Pregnancy interval	Pregnancy interval < 6 months
	Pregnancy interval ≥ 60 months
Maternal Medical History	
SGA	**Maternal SGA**
Hypertension	**Chronic hypertension**
Diabetes	**Diabetes with vascular disease**
Renal disease	**Renal impairment**
APLS	**Antiphospholipid syndrome**
Paternal Medical History	
SGA	**Paternal SGA**

Table 1: **Antenatal risk factors for intrauterine growth restriction**

Table detailing risk factors for SGA available from history at booking (usually at ≤ 12 weeks gestation). Major risk factors are shown in bold. APLS – Antiphospholipid syndrome; RF – Risk factors; SGA – Small for gestational age. (RCOG Green-top Guideline No. 31)

The main focus of research is towards a first trimester diagnosis, as the optimal period during which a prophylactic treatment is effective in optimising placentation is before 16 weeks gestation. A biomarker in early pregnancy with a strong positive predictive value for IUGR could potentially reduced the incidence if IUGR thereby reducing complications in the resulting neonate.

1.3 Genomic imprinting

The placenta is a plastic organ that responds to fetal signals in addition to changes in the intrauterine environment. These adaptive changes result in alteration to the morphology and function of the placenta. These events are thought to be effected by a change in gene expression, which can be attributed, at least in part, to epigenetic alterations[65,74].

Genomic imprinting is an epigenetic process by which a gene is differentially expressed according to the parental origin[75]. Imprinted genes, which are abundantly expressed in the placenta, are often found in clusters with gene expression being regulated by imprinting control regions (ICR). DNA methylation is the best characterised epigenetic modification and involves the addition of a methyl group to cytosines that precede a guanosine in the DNA sequence (the CpG dinucleotide)[76]. All ICRs identified are differentially methylated regions (DMR) on the two parental chromosomes. These differential methylation marks are acquired in the developing gametes and are maintained in the developing embryo throughout life[77]. In addition to allele-specific methylation, non-coding (nc) RNAs also regulate imprinting. They are able to regulate expression of genes on the same chromosome (when acting in cis), on different chromosomes (when acting in trans) or in mature mRNA in the cytoplasm[78]. These epigenetic marks acquired during development result in fetal programming and have an effect in later life.

1.3.1 Developmental origin of Health and Disease

Thorough comprehension of the processes occurring in utero during gestation is important, as the quality of life of a growing fetus in utero is likely to be a major determinant of quality of life in adulthood. Epidemiological studies suggest that suboptimal growth during the fetal period predisposes to specific diseases later in life including adult onset hypertension, type 2 DM and coronary heart disease[79]. This is known as the 'thrifty phenotype' hypothesis and refers to the fetal response to inadequate nutrient supply and consists of decreased muscle mass, insulin resistance, decreased capillary network and an increased stress response[80]. In this setting, subsequent abundance of nutritional supplements may predispose an individual to the development of the metabolic syndrome which consists of centripetal fat accumulation, insulin resistance, glucose intolerance, hyperlipidaemia and hypertension. Many studies worldwide have confirmed the initial epidemiological evidence. These include a follow up study carried out on a population in Hertfordshire born between 1911 and 1930 (5585 women and 10,141 men) with weight at birth and one year of age recorded. Standard mortality ratio was seen to fall with increasing birth weight in both sexes[81]. Further studies have shown an association with low birth weight and syndrome X (type

2 DM, hypertension and hyperlipidaemia). In this study the risk for syndrome X was 10 times greater in cases of lower birthweight[82]. Interestingly, a genome - wide association study (GWAS) has linked the DLK1 locus, an imprinted gene, with Type 1 Diabetes Mellitus (T1D)[83]. A newly identified locus with the strongest association with T1D susceptibility occurred in an imprinting region on chromosome 14q32.2 marked by SNP rs 941576 A>G. This region contains paternally derived (DLK1, RTL1, DIO3) and maternally derived (MEG3, MEG8) as covered in section 1.4 below. Of the paternally expressed genes, only DLK1 has a strong functional candidacy. It is most strongly expressed in the human heart, pancreatic islet cells, pituitary tissue, ovaries, placenta and testes. In addition it encodes a membrane bound protein, which can be cleaved to form fetal antigen 1 (FA1). FA1 is involved in the differentiation of many cell types including pancreatic beta cells. FA1 is also involved in haematopoiesis including differentiation and function of B-lymphocytes and has been shown to increase expression of pro-inflammatory cytokines in human bone marrow mesenchymal stem cells and promote B cell proliferation in human peripheral blood. In this way, variation in the expression of DLK1 could alter susceptibility to T1D, which is caused by autoimmune destruction of insulin producing beta cells in the pancreas.

Additionally, a retrospective study compared birth weight to the risk of non-fatal cardiovascular disease with a finding of RR 1.49 (95% CI 1.05 – 2.10) for BW < 2268 grams. This is in comparison to RR of 1 for BW > 3175 – 3836 grams; 0.96 (0.8 – 1.15) for BW > 3856 – 4356 and 0.68 (0.46 – 1) for BW > 4356 grams[84]. The biological basis for suggesting developmental plasticity as an influence on the risk of disease derives from numerous studies in animals in which dietary, endocrine or physical challenges induce persistent changes in cardiovascular and metabolic function in the offspring. Embryos of pregnant rats fed a low-protein diet during the preimplantation period showed altered development in multiple organ systems and in addition the offspring had reduced birth weight, relatively increased postnatal growth and adult onset hypertension[85] Further studies on rats involving the administration of a low-protein diet resulted in a reduction in the number of nephrons and hypertension[86]. Furthermore, in a rat model of nutritional imbalance, the offspring of rats fed an imbalanced diet during pregnancy later had elevated blood pressure, reduced nephron number and increased responses to salt loading as well as reduced vasodilator function in systemic arteries[87].

Exposure to under nutrition in early gestation, as seen in studies of the Dutch Hunger Winter, a severe wartime famine at the end of WW II affecting the western part of The Netherlands, has been associated with subtle changes in methylation at three DMRs in different imprinted clusters in blood samples of affected when compared to unaffected siblings[62,88]. In animal studies, manipulation of the maternal diet during pregnancy leads to a persistent shift in average DNA methylation levels of

specific genes in offspring resulting in permanent changes in coat colour or tail shape[89,90]. The findings in the human and animal studies support the theory that intrauterine disturbances in early gestation may cause changes in later life, mediated by epigenetic modification during development. Early gestation is a sensitive period with carefully orchestrated events controlled at various levels. A better understanding of these events and the factors that influence outcome is necessary in order to reduce the risk of long-term sequelae.

1.3.2 Imprinting and fetal growth

Both animal knock out studies and known human syndromes with growth phenotypes, secondary to imprinting defects, together support a role for imprinted genes in fetal growth. Several uniparental disomies (UPD) have been identified in humans, with specific phenotypes and they include Prader-Willi (PWS) and Angelman syndrome (AS)[91], Beckwith-Wiedermann syndrome (BWS), Silver-Russell syndrome (RSR)[92,93] and transient neonatal diabetes mellitus (TNDM)[93], maternal and paternal UPD14 syndromes[94] and psuedohypoparathyroidism[95]. Insulin growth factor 2 (*Igf2*), one of the first imprinted genes to be identified, is expressed from the paternally inherited copy, resulting in placental stunting and fetal growth retardation on deletion and fetal growth enhancement on overexpression[96,97]. Subsequent studies confirmed that overexpression of *Igf2* via loss of imprinting through deletion of imprinting controlling sequences can cause placentomegaly as well as fetal overgrowth[98]. In the human overgrowth syndrome BWS, human *IGF2* was found to undergo loss of imprinting in a subset of patients further supporting this finding. Knockout of the maternally expressed, paternally imprinted *Grb10* results in mice that are 30% heavier than wild type[99], further supporting a role for imprinted genes in fetal growth.

Knockout or transgenic mouse models for most imprinted genes result in growth defects. For instance deletion of the *Peg1/Mest* paternal allele causes fetal and placental growth retardation in mice[100]. In addition deletion of the paternal allele of *Peg3* results in fetal growth retardation and placental stunting[101]. Additionally the maternally expressed/paternally silenced genes Phlda2, Slc22a1, Cdkn1c, Kcnq1 and Ascl2 are clustered on an imprinting domain on mouse chromosome 7[78]. Paternal deletion of a cis acting differentially methylated DNA element, Kvdmr1, causes coordinate loss of imprinting and overexpression of all these genes and the resulting conceptus show IUGR[78]. Furthermore placental overgrowth is seen after gene deletion and growth retardation after loss of imprinting. Specific genes such as H19/ IGF2 have been studied in the context of growth restriction, demonstrating differential methylation of the imprinting control regions in placentas from growth-restricted infants[65]. A number of other imprinted loci such as PHLDA2, IKL2, NNAT, CCDC86, PEG10,

PLAGL1, DFKR24, ZNF331 and CDKALI have been shown to demonstrate differential expression between growth restricted and non-restricted infant placentas[102–104].

The role of imprinting genes in non-syndromic human IUGR is poorly understood. A number of studies have attempted to explore expression of imprinted genes in IUGR compared to normal pregnancies. One study[105]employed microarrays to interrogate differential expression of both imprinted and non-imprinted genes between 14 IUGR and 15 non-IUGR placenta. 7% of the differentially expressed genes were imprinted, making up 22% of all the imprinted genes. Another study observed that 17% of 52 imprinted genes were significantly differentially expressed in cases of IUGR compared to normal pregnancies[104].

It appears that overall however; imprinted genes are dysregulated in IUGR and therefore have an important role to play in intrauterine growth. One such gene is Delta-Like homologue 1 or DLK1. This is an imprinted gene, paternally expressed with the maternal allele remaining silent. This gene is described further in section 1.4. Further support for the role for imprinted genes and IUGR is noted in a recent GWAS[106]. GWAS data for birth weight was combined in 153, 781 individuals representing multiple ancestries from 37 studies across three components including the UK Biobank. 60 loci associated with birth weight at genome wide significance ($P < 5 \times 10^{-8}$) were identified. 53 of these loci were novel. Previously reported loci included CCNLI-LEKRI, HMGA2, CDKALI, ADCY5, ADRBI, LCORL and 5q11.2. The novel loci identified included INS-IGF2, IGF2BP3, IFF1R, PLAC1, IGF1 and RB1. DLK1 association signals approached significance with $P = 5.6 \times 10^{-8}$. The group felt that despite the large sample size, analyses were underpowered and much larger samples are required for definitive analysis.

1.4 DLK1

1.4.1 Background

DLK1 is expressed in an imprinting cluster on the long arm of human chromosome 14 (14q32.2)[107]. This domain contains the protein coding genes *DLK1, RTL1* and *DIO3* expressed from the paternal allele and the imprinted maternal allele expressed genes *GTL2/MEG3, MEG8* and antisense *RTL1*[108,109] which are non-coding RNAs. *DLK1* also known as Pref-1 was cloned and characterised during experiments using 3T3-L1, an adipocyte cell line[110]. Here it was found to regulate adipocyte differentiation.

Imprinting expression is controlled by a primary imprinting control region (ICR), intergenic differentially methylated region (IG-DMR)[111,112] located between *DLK1* and *GTL2/MEG3* (Figure 6),

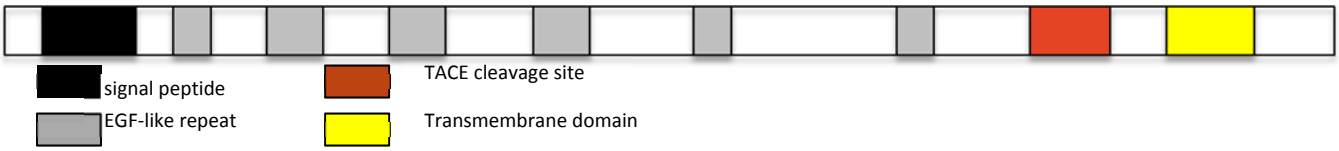
which is usually only methylated on the paternal allele[112]. The imprint on IG-DMR is acquired during gametogenesis on the male germ line and subsequently influences methylation on the paternal allele of a somatic DMR within the *GTL2/MEG3*. The unmethylated IG-DMR on the maternal allele is associated with expression of *GTL2/MEG3* and *RTL1* which represses expression of *DLK1* and *RTL1* in cis[113].

Figure 6: **Imprinting cluster on human chromosome 14**

Paternally expressed genes are shown in blue whilst the maternally expressed genes are shown in red. Two DMRs are shown; with methylation of the germ line IG-DMR and the MEG3 DMR. Used with permission from the authors.[113]

1.4.1.1 DLK1 structure

DLK1/PREF1 as characterised by Smas *et al* consists of a 1589 bp transcript[110]. It has also been referred to as zona glomerulosa specific mRNA[114] and Fetal antigen 1(FA1)[115] depending on the context in which it was isolated. The cell surface transmembrane protein has a predicted structure which consists of six tandem EGF-like repeats in an extracellular domain[110]. These begin shortly after the signal sequence and encompass 60% of the protein (Figure 7). A juxtamembrane region precedes the transmembrane domain and in addition the short cytoplasmic domain is rich in serine, threonine and proline and contains several potential phosphorylation sites[110]. The DLK1 transcript undergoes alternate splicing, with four major isoforms of the transcript detected[116] (Figure 7). The longest form (PREF-1A) is the most abundant whilst in-frame juxtamembrane deletions result in three other transcripts (PREF - 1B, C and D)[116] Figure 7. The tandem nature of the repeats shows similarities to the EGF-like repeats of the Drosophila cell fate determining proteins Notch and Delta[110]. DLK1 exists as both a transmembrane and soluble form[117] with cleavage of the full-length protein resulting in a soluble product of 50 kDa [116]. This cleavage is carried out by a protease, TNF-α converting enzyme (TACE), in the juxtamembrane domain[118]. The soluble form of DLK1 shares functions with the membrane bound full-length molecule[117]. Based on these data we can postulate that DLK1 achieves its actions in both a juxtacrine and paracrine manner in utero.



PREF 1A

PREF 1B

PREF 1C

PREF 1D

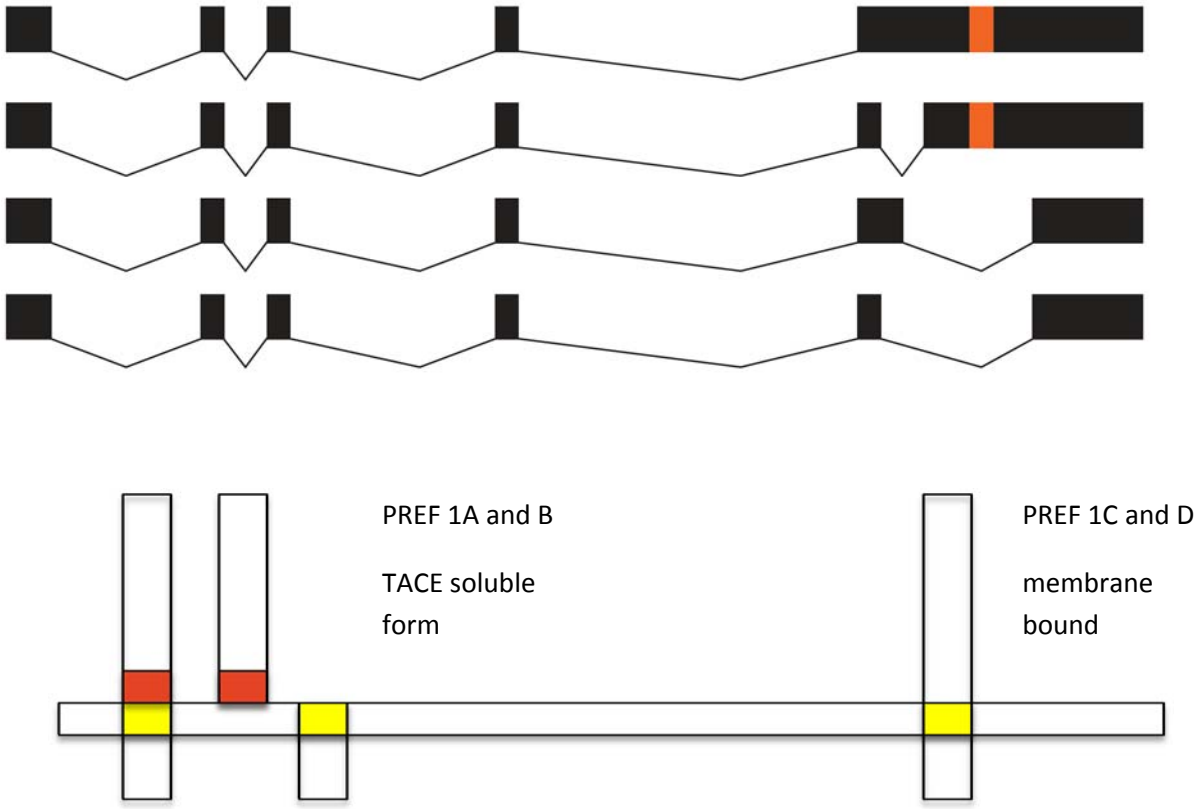


Figure 7: **DLK1 structure**

Isoforms of DLK1 showing soluble, cleavable forms of the molecule (PREF 1A and B) along with the membrane bound forms (PREF 1C and D). The full-length molecule is seen at the top of the diagram with the signal peptide, EGF-like repeats, TACE cleavable site and transmembrane domain depicted. Used with permission from M. Charalambous.

1.4.1.2 DLK1 and growth

Knockout studies in mice have highlighted the importance of DLK1 in growth and development.[119,120].DLK1 null mice display pre- and postnatal growth retardation, eyelid and skeletal abnormalities such as asymmetrical junction of ribs to sternum and fusion of ribs[119]. In addition, DLK1 null mice were seen to have abnormal placenta including placenta hypoplasia and abnormal vasculature[120–122]. Conditional deletion in mouse pancreatic beta cells, pituitary somatotropes and fetal endothelial cells of the placenta labyrinth did not cause pathology in knock out mouse models however[120]. This suggests that DLK1 has tissue specific functions during development.

In humans, maternal uniparental disomy of chromosome 14 (mUPD14)[123], paternal deletions[94] and loss of methylation at the IG-DMR[124] result in a phenotype of low birth weight, hypotonia, early puberty and markedly short adult stature. This constellation of abnormalities constitutes a condition known as Temple syndrome (TS)[124]and is associated with dysregulation of expression of genes at the imprinted locus on chromosome 14. mUPD is the most widely recognised cause of TS and results in loss of expression of all paternally expressed genes and overexpression of maternally expressed genes within this imprinting domain[123,125–132]. Imprinting in this cluster is controlled by an IGDMR, which is located between DLK1 and GTL2/ MEG3 and is normally methylated only on the paternal allele. The function of the unmethylated IGDMR on the maternal allele is to regulate DLK1 and RTL1 expression in cis. In addition to mUPD (which accounts for 70-80% of cases), dysregulation of expression of genes at this imprinted locus can arise from: copy number change (10%); mutation of expressed coding genes (<2%) and due to epigenetic error (12%)[113]. The study of rare TS patients with copy number changes enabled *Kagami et al*[133]to confine the region of interest to a 108kB paternal deletion involving DLK1 and GTL2/ MEG3. Two patients had many features of TS but stature was more severely affected in a third reported case with a larger deletion (411 kB), which included RTL1 (but not DIO3). Silencing mutations have not been reported in humans, but in the mouse null mutations of DLK1 and RTL1 inherited from the male have TS features. Furthermore, paternal uniparental disomy (pUPD) causes thoracic skeletal anomalies, polyhydramnios, placentomegaly and limited survival. The hypothesised cause is overexpression of paternally expressed RTL1, due to the absent regulatory effects of maternal RTL1as[134]. The 14q32-imprinted region is dosage sensitive with deletions of different critical regions causing mUPD14 and pUPD14 like phenotypes.

In a mouse model, hypomethylation of the IG-DMR resulted in reduced expression of DLK1[135] therefore all mechanisms are predicted to result in reduced expression from the paternal allele. In addition, the abnormalities seen in pUPD14 highlight the importance of DLK1 in intrauterine

development[94,136].

1.4.1.3 DLK1 during intrauterine development

DLK1 has an important role to play in embryonic development and expression is pronounced in the embryonic period in both mice and humans, thereafter decreasing during fetal development[137,138]. In the human placenta, expression is seen in the mesenchymal fibroblasts adjacent to fetal endothelial cells[138]. DLK1 was mainly seen in early gestation apart from in human fetal chondroblasts and human endothelial cells where expression is seen all through gestation[138]. These findings suggest a role for DLK1 in cell differentiation, which mainly takes place in early gestation.

A group in Denmark, Floridon *et al*, measured maternal serum DLK1 levels in pregnant women and demonstrated a rise in DLK1 concentrations from 17 weeks gestation of pregnancy, with a peak in late gestation at approximately 36 weeks[138]. Prior to 17 weeks gestation, levels were comparable to males and the non-pregnant female population[138]. This pattern has been replicated in mouse studies[139]. As the weight of the fetus increases most dramatically during the last half of gestation[140], the peak in DLK1 suggests that higher levels may be required during this period of rapid growth and placental adaptation.

1.4.2 Current knowledge with regards to DLK1 function

DLK1 has been shown to be involved in the proliferation and differentiation of various precursor cells[110,141]. Experiments using pre adipocyte cell lines (3T3-L1) have demonstrated a role in the inhibition of adipocyte differentiation[110,142]. DLK1 has been found to play an important role in lipid metabolism and recent mouse knock out models have demonstrated an increase in lumps of white adipose tissue (WAT) and abnormal body composition in virgin dlk1 null mice[143]. WAT is associated with increased leptin, which is an important in utero hormone with a function in fetal growth by influencing nutrient transporters in the placemt[144]. DLK1 has also been identified as a regulatory factor for human mesenchymal stem cell differentiation to osteoblasts and adipocytes[145–147]. Further functions have been suggested in haematopoiesis, neuronal cell regeneration and in thermoregulation in brown adipose tissue (BAT). More recently DLK1 has been found to have a function in differentiation of pancreatic ductal cells into insulin producing pancreatic β cells[148]. DLK1 is thought to exert most of its actions in these tissues by activating the mitogen-activated protein kinases (MAPKs) signalling pathways and has been found to increase phosphorylation of ERK in adipose tissue[149]. Understanding the mechanisms of action of DLK1 in other tissues will allow us to

begin to elucidate its role in intrauterine growth. This knowledge may lead to the recognition of therapeutic targets for intervention.

Current work using genetically modified mice that lack a functional copy of Dlk1 has been carried out to determine the source of maternal DLK1 in pregnancy. This confirmed that the maternal DLK1 levels are elevated during pregnancy as previously shown by other groups including *Bachmann et al*. Serial measurement of maternal plasma DLK1 in crosses of mice where the mother, the conceptus or both were unable to express Dlk1 were conducted. DLK1 was detected at high levels in maternal plasma only if the conceptus retained the ability to express Dlk1. This suggests that the conceptus is the source of elevated maternal plasma DLK1 in pregnancy. Additional models of Dlk1 dosage manipulation were utilised to distinguish whether the placenta or embryo was the source of maternal plasma DLK1. Their data showed that the source of circulating maternal plasma DLK1 is the embryo, not the placenta[143]. This remains to be confirmed in the human fetoplacental unit.

1.5 Thesis Rationale

I was initially involved in the design of a clinical study for a laboratory project already underway within our group. This was a project evaluating fetal adrenal gland development[150]. The design of the project involved the recruitment of participants from the fetal medicine unit. The case group was selected to include a group of pregnant women with placental dysfunction and a control group of women with uncomplicated pregnancies. A systematic review and meta-analysis[151]examined the diagnostic accuracy of first trimester (12 weeks gestation) uterine artery Doppler measurements in predicting pre-eclampsia. This revealed that a uterine artery pulsatility index (PI) greater than 1.96 in the 1st trimester suggested an increased risk of pre-eclampsia. The specificity for predicting early onset pre-eclampsia using this screening method was high. First trimester uterine artery Doppler is therefore a useful tool to stratify women by risk status and target them for appropriate clinical management and identify higher risk individuals for research purposes. Pre-eclampsia is a condition associated with placental dysfunction, and therefore presumed placental insufficiency, which is associated with IUGR.

A research study carried out in the antenatal clinic at the Royal London Hospital from 2011 revealed that 20% of women scanned at 12 weeks have PI measurements greater than 1.96. Out of this group, 35% of those with a PI greater than 1.96 go on to develop pre-eclampsia. The remaining 65% of women screened have uncomplicated normal pregnancies. This work has since been published.[152]

This screening method, carried out as part of an on-going research study in the Fetal Medicine Unit, was used as a selection tool to enrol women in my study. The finding of a PI greater than 1.96 at 12

weeks was predicted to provide both a cohort of women with normal pregnancies and a cohort that go on to develop placental dysfunction. The advantage of using this starting point for selection and enrolment is that both cohorts will receive exactly the same follow up during the course of the study. In addition this screening method was thought to provide a group of participants with a common mechanism for IUGR. I therefore decided to take advantage of this unique cohort, to assess DLK1 as a potential biomarker for IUGR as it was going to be possible to collect measurements associated with fetal growth parameters prospectively in addition to maternal parameters.

The main cause of intrauterine growth restriction is placental insufficiency. The mechanism behind this pathology is poorly understood. The process appears to involve placental adaptations in response to both fetal and maternal signals. I decided to focus on DLK1 in this work as evidence from animal knockout studies[119,153] and human Temple syndrome[123] suggest an important role for this protein in intrauterine growth. Furthermore, DLK1 is widely expressed during the embryonic period in humans and in addition expression has been seen in trophoblast cells within the labyrinth in mouse placenta[143]. My work therefore involved interrogating the function of DLK1 in utero by comparing maternal serum levels to fetal growth parameters. In addition, as uteroplacental insufficiency is an important cause of fetal growth restriction, I embarked on placental studies to further attempt to understand what occurs in the placenta in cases of abnormal DLK1 production.

In the first instance I carried out an analysis of maternal serum DLK1 levels in a cohort of women recruited prospectively from our Fetal Medicine Unit (FMU) at the Royal London Hospital as described above. These women also underwent serial ultrasound scans for fetal growth parameters along with other ultrasonic assessments of fetal growth. As DLK1 was shown to increase insulin pancreatic β cells[148] , I hypothesise that DLK1 will effect some of its function on fetal growth through insulin either directly or by influencing the bioavailability of IGF and leptin. As insulin is found to peak in the third trimester during pregnancy, and has been shown to increase nutrient transporters in the placenta [6,154], these effects are likely to result in an overall increase in fetal size due to mass accumulation and tissue growth, and may be characterised by an increase in abdominal circumference clinically. This is the most sensitive measure of fetal growth by ultrasound assessment[155]. Maternal serum DLK1 levels in the third trimester should therefore be predictive of abdominal circumference.

In addition, animal knock out studies have shown that dlk1 null mice have reduced size due to a reduction in skeletal length[143]. With this in mind, in addition to the finding in human embryos of

DLK1 immunostaining in resting chondrocytes within the epiphyseal disc[138] , I hypothesise that maternal serum DLK1 levels will be predictive of long bone growth in utero, demonstrated by femoral length growth.

Further experiments were carried out on placentas collected from this cohort of women at delivery. The placental histomorphometrics were carried and compared to clinical information collected from the cohort and maternal serum DLK1 levels. As DLK1 has been found to be expressed in the trophoblast layer of cells in animal studies[143], I hypothesise that immunostaining of the placenta will reveal DLK1 positive cytotrophoblast and syncytiotrophoblast group of cells and that DLK1 will have a role in trophoblast invasion. Cases with low DLK1 levels will therefore show histological evidence of maternal vascular malperfusion of the placenta bed such as distal villous hypoplasia, accelerated maturation of the villi, decidual arteriopathy and the presence of multinucleated trophoblastic giant cells in the maternal decidua. Furthermore, as trophoblast cells within the placenta are secretory cells with an endocrine function, serum DLK1 levels will affect placental function as will be indicated by a positive relationship between serum DLK1 levels and placental weight/ volume. I further hypothesise that DLK1 regulates placental growth.

Given what we know about fetal growth and the role of DLK1 in this process, this research was carried out in an attempt to ascertain whether maternal serum DLK1 could be used to predict adverse outcomes in pregnancy with particular reference to IUGR. Taking into consideration what we know about placental development, fetal growth and the role of DLK1 in growth, this project was focused to evaluate a possible role for DLK1 as a biomarker for intrauterine growth, especially when severe. The development of a biomarker, particularly in early pregnancy can provide a non-invasive screening tool for pregnancies that need closer surveillance in the way of more frequent ultrasound scans. In addition once the mechanism of DLK1 in intrauterine growth is better understood, it may be possible to intervene therapeutically in cases where abnormal DLK1 levels and growth have been identified.

Chapter 2 Materials and methods

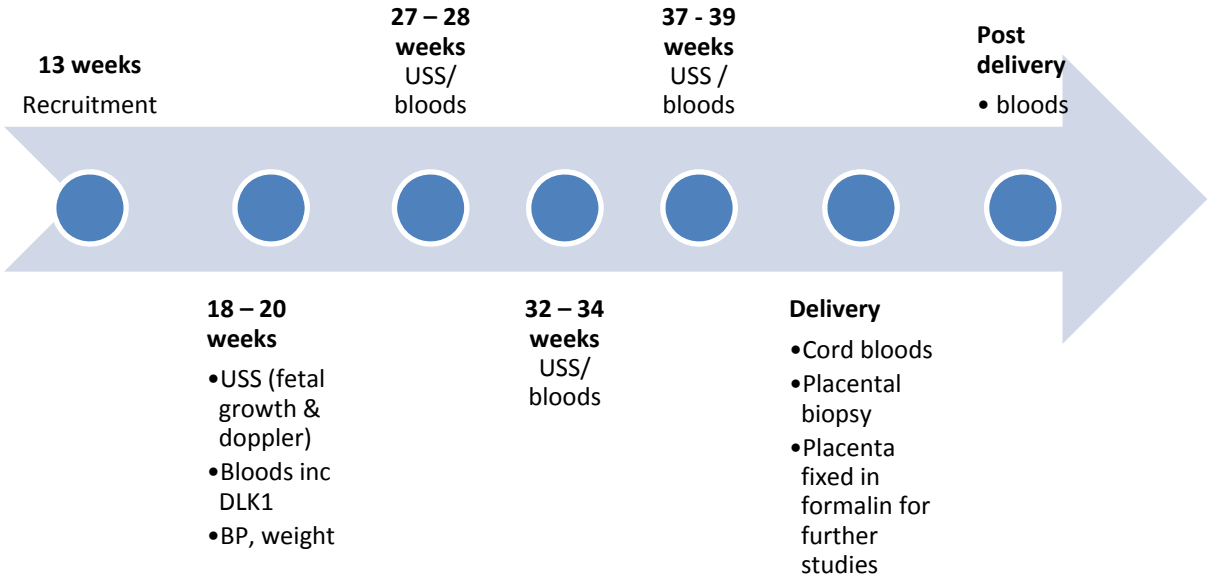
2.1 Circulating DLK1 concentrations during pregnancy

2.1.1 Description of the clinical cohort

Following full ethical approval of the study protocol obtained from the Brighton and Sussex NREC, recruitment of participants attending routine antenatal scans (12 weeks ultrasound) between February 2013 and April 2014 at the Fetal Medicine Unit (FMU) occurred. This pilot study involved prospective follow up of participants antenatally leading to a cohort of normal pregnancies and those complicated by IUGR secondary to placental insufficiency (Figure 8). Inclusion criteria are shown in table 2. Subjects were recruited from the following cohort of patients: Pregnant women who present to the antenatal clinic at the Royal London Hospital and have already consented to have their routine 12 week scan performed by a senior obstetric doctor as part of a study examining Doppler measurements. Patients who were found to have PI measurements greater than 1.96 (carried out as part of an on-going obstetric study) on their 12 week dating scan were identified as eligible for this study by the doctors carrying out the scan. Either our obstetric collaborators or myself provided written information about my study during this 12-week antenatal appointment once the patient gave verbal consent. Written informed consent from those patients who agreed to participate in the study was obtained once again either by myself or our research nurse at the time of the anomaly scans between 18-22 weeks. Data was collected and added to a database created on a secure research laptop, which was stored in a locked drawer in a locked office at the Centre for Endocrinology. These participants were followed up on 4 separate occasions antenatally as seen in the flow diagram (Figure 6) with two further instances of contact at delivery and 24 to 48 hours following delivery. At each visit, participants underwent blood sampling for DLK1 levels and metabolic markers. Further serum was taken for storage. In addition, the participants underwent an ultrasound scan where fetal growth parameters (abdominal circumference, bipareital diameter and femoral length) and umbilical artery Doppler were carried out. Maternal clinical parameters were measured at each visit including blood pressure and weight and clinical information including medication history, underlying illness and pregnancy complications were obtained. At delivery, the placenta was obtained on average after twenty minutes after delivery for further macroscopic and microscopic analysis. Further serum samples were obtained 24 – 48 hours after delivery. Any complications arising during labour or thereafter were noted. The serum obtained was analysed for DLK1 levels using Enzyme- Linked Immunosorbent Assay methods. The primary endpoint for this

study was the correlation of DLK1 serum levels with fetal growth. Secondary endpoints included the association of DLK1 levels with neonatal and pregnancy outcome as DLK1 was postulated to act as a biomarker to signify intrauterine pathology.

Figure 8: **Recruitment and prospective follow up of participants**



Flow diagram showing recruitment and prospective follow up of participants. USS, Ultrasound Scan

Table summarising inclusion and exclusion criteria for study
Inclusion criteria: • Women found to have PI greater than 1.96 on ultrasound at 12 weeks gestation. • Singleton pregnancies
Exclusion criteria: • Other coexistent maternal medical conditions (chronic hypertension, diabetes, thyroid disease, adrenal disease, renal disease). • Congenital abnormalities of fetus or chromosomal anomalies. • Maternal exposure to psychotropic medication. • Heavy smoking or alcohol abuse in pregnancy. • Multiple pregnancies. • Maternal infection with TORCH infections (Toxoplasmosis, rubella, CMV, syphilis), or human immunodeficiency virus.

Table 2: **Inclusion and exclusion criteria for study**.

PI - pulsatility index; TORCH – Toxoplasmosis, Other (syphilis, varicella zoster, parvovirus B19), Rubella, Cytomegalovirus and Herpes.

Recruitment resulted in a cohort of women from an ethnically diverse background. 40 suitable patients were initially recruited for the study whilst attending for anomaly scans at the Fetal Medicine Unit of the Royal London Hospital. 33 of these patients completed the study. Of the patients failing to complete the study, three were lost to follow up (one delivered abroad whilst 2

delivered in other hospitals), whereas 4 decided to discontinue the study, as they felt unable to attend for the additional visits required outside their routine care. Our patient cohort is comparable to the general population (Table 2). The average age in our cohort is comparable to that of pregnant women in the UK and Wales. The average booking BMI in our study group reflects the rising BMI seen in pregnant women in the UK with maternal obesity accounting for 15.6% of pregnancies in England[156]. Our final cohort consisted of 17 (51.5%) recruits with a normal BMI (range 19.39 – 24.46), 13 (39.3%) overweight (BMI range 25.2 – 29.17) and 3 (0.1 %) obese recruits (BMI range 33.55 – 41.79). Most of the cohort delivered at term. There were however two preterm deliveries at 26.9 and 33.6 weeks gestation. With the former being spontaneous and the later secondary to an induction of labour for IUGR. Four of the neonates in the cohort had pregnancies complicated by IUGR with birth weight SDS ranging between -3.3 and -2 (Figure 8).

Table showing maternal characteristics for pregnancies complicated by IUGR

Maternal age	BMI	Ethnicity	Parity	Complications	BW in grams (SDS)	BL in cm (SDS)	GA
28	28.1	Caucasian	G3, P1*	Poor previous obstetric history. Commenced on aspirin	2420 (-2.4)	44.8 (-4.6)	39
28	26.84	Bengali	G4, P1 *	IOL for absent EDF	1720(-3.3)	44.5 (-1.98)	37
23	20	Bengali	G2, P0*	IOL for absent EDF	1340 (-2)	40.5 (-1.72)	33
33	20.2	Caucasian	G1, P0	SVD	1860 (-2.88)		37

Table 3: **Pregnancies complicated by IUGR in the RLH cohort**.

3 of the 4 pregnancies were complicated by multiple miscarriages - asterix. * - 2 miscarriages. G - Gravida, P - Parity; IOL - Induction of labour; EDF - End Diastolic Flow; SVD – Spontaneous Vaginal Delivery

2.1 Participant demographics

Characteristic	Mean ± SD	Range	N
Age, years	26.8 ± 6.1	17.0 - 37.0	33
BMI	25.4 ± 5.3	18.8 - 41.8	33
Gravidity	1	1.0 - 4.0	33
Parity	0	0.0 - 2.0	33
Gestational age, weeks			
Visit 1	20.35 ± 0.68	19.29 - 22.57	31
Visit 2	28.07 ± 0.75	26.29 - 29.86	32
Visit 3	34.42 ± 0.75	33.00 - 36.14	28
Visit 4	38.09 ± 0.62	36.00 - 39.71	23
Gestation at delivery, weeks	39.33 ± 2.83	26.86 - 42.14	31
Birth weight, grams	3006 ± 650	950 - 3680	31
Birth weight SDS	-0.698 ± 0.913	-3.30 - 0.840	31
Maternal ethnicity			
White European			10
South East Asian			10
Far East Asian			4
Afro Caribbean			3
Mixed			3
Black African			2
Other			1

Table 4: **Royal London Hospital participant characteristics**

Table showing participant characteristics in the Royal London Hospital cohort. Mean and std dev are shown with a range of values between the minimum and maximum. The number of participants is shown in the last column.

2.2 Measurement of Fetal growth and assessments for signs of fetal growth restriction

2.2.1 Measurement of fetal growth parameters

Two FMU fellows, independently, using the Voluson 730 and E8 systems (Voluson Expert; Milawaukee, WI) performed fetal Ultrasound Scans (USS). BPD, HC, AC and FL in addition to assessment for signs of fetal growth restriction were carried out at four different time points during gestation. USS assessments also included estimation of gestational age and fetal weight. The methodology used is described below.

2.2.1.1 Measurement of BPD and HC

The BPD is the maximum diameter of the transverse section of the fetal skull at a level of the parietal eminences. Measurements were made using thalamic views. This view includes a rugby-football-shaped skull, rounded at the back (occiput) and more pointed at the front (synciput), a short midline equidistant from the proximal and distal skull echoes, the cavum septum pellucidum bisecting the midline one- third of the distance from the synciput to the occiput, the thalami and the basal cisterns. A brief description of BPD and HC measurements using thalamic views are to follow below.

To begin with, a longitudinal section of the fetus is obtained. The transducer is then rotated through 90° to obtain a transverse section of the fetal head ensuring that the midline is centrally placed. A section of the anterior horns is obtained and on making a slight rotation of the probe towards the fetal neck, the basal cisterns are imaged. This is then followed by a sliding movement of the probe downwards, towards the fetal body, allowing the lower body of the cavum to be just visible in addition to the optimal view of the thalami. The BPD is then measured on the frozen image. Callipers are placed on the outer border of both the proximal and distal parietal bones, at the widest diameter. The HC is measured from the same view as the BPD. The two-diameter method is used which involves the measurement of the BPD and Occipito-frontal diameter (OFD). The machine's software then calculates the HC using the formula πd derived from the formula for the circumference of a circle (2πr)

$$HC = 3.14 (BPD + OFD) / 2$$

2.2.1.2 Measurement of AC

The AC was measured using specified landmarks. A longitudinal view of the fetus showing the heart and the fetal bladder was obtained after which the transducer was moved laterally until the spine was visualised. The transducer was then rotated through 90° at the level of the fetal stomach to

obtain a cross-section. The circumference of the abdomen was measured using a two-diameter method. The anteroposterior diameter (APAD) was measured from the fetal spine to the anterior abdominal wall. The transverse abdominal diameter (TAD) was then measured across the widest part of the abdominal circumference section at 90° to the APAD. The machine's software was then used to calculate the AC using the formula πd, derived from 2πr as per the HC above:

$$AC = 3.14 (TAD + APAD)/2$$

2.2.1.3 Measurement of FL

The femur can be measured from 12 weeks to term. Measurement of the FL was undertaken typically after measuring the AC. The probe was moved caudally from the AC section until the iliac bones were visualised. At this point, a cross section of one or both the femurs was seen. The upper femur was selected for measurement. Whist keeping the anterior femur in view, the probe was then rotated slowly until the full length of the femur was obtained. The measurement of the femur was made from the centre of the 'U' shape at each end of the bone to obtain the length of the metaphysis. Measurements were obtained from three separate images of the same femur. All three measurements above were then plotted on growth charts in order to ascertain that the measurements were within the normal range for gestation. Both BPD and FL were used to estimate gestational age independently, with the help of dating tables. In addition, estimated fetal weight was derived from the BPD, HC, FL and AC using software on the machine, which is based on the Shepard and Hadlock equations.

2.2.2 Assessment for features of fetal growth restriction

Ultrasound features of uteroplacental insufficiency including placental abnormality (lakes, calcification, jelly-like consistency) and reduction in amniotic fluid volume were assessed. These assessments were made alongside Doppler assessments. Methodology for this is described below.

2.2.2.1 Doppler flow assessments

Uteroplacental waveforms were acquired from the uterine artery by means of colour, pulsed Doppler ultrasound. To begin with colour flow imaging was used to identify the bifurcation of the common iliac artery in longitudinal section. The probe was moved medially angled slightly towards the symphysis pubis to reveal the uterine artery just medial to the bifurcation, as it ascends towards the uterus. On obtaining an optimal waveform, the maximum frequency follower was turned on and the image frozen once the automatic calculations were displayed. Both the pulsatility index (PI) and resistance index (RI) were calculated automatically by the machine. PI and RI represent an index of

impedance to flow. High resistance patterns were described as bilateral notches with mean RI > 0.55 or unilateral notch with mean RI>0.65. Doppler findings typical of uteroplacental insufficiency include: uterine notches, absent / reversed end-diastolic flow in the umbilical artery and arterial redistribution.

Umbilical artery waveforms were also obtained. These waveforms reflect downstream placental vascular resistance. Colour flow imaging was used to identify the umbilical cord at the placental insertion. Once the waveform from the umbilical arteries was seen, the maximum frequency follower was switched on and the image frozen once the automatic calculations were displayed. The PI was derived from the average of three waveforms. In addition, the frequency in end diastole was recorded as either normal, absent or reversed. In the case of loss of end-diastolic frequencies, the signal was rechecked from another site.

2.1.2 Collection and storage of serum samples

Approximately 5 mls of blood was obtained by venepuncture from the participants for analysis of DLK1 levels. Samples were taken into a yellow top serum separating tube. These samples were then spun within 3 minutes of venepuncture. The spinning was carried out using a cold centrifuge, at a temperature of 4°C and a speed of 3000rpm for a total of 10 minutes. Once separation of serum from the plasma was obtained, serum was collected into microcentrifuge tubes in triplicate and stored transiently in a -20°C freezer at the hospital prior to transfer in dry ice to the Centre for Endocrinology lab, where they were stored at -80°C.

2.1.3 ELISA assay for DLK1 levels

Stored serum samples were analysed for DLK1 levels. This was carried out using a sandwich Enzyme Linked-Immunosorbent Assay (ELISA) for quantitative determination of human DLK1 in biological fluids from Adipogen®. Sample aliquots were brought to room temperature and diluted (1 in 10) using ELISA buffer. 100 µl of each sample was then added to a pre-coated 96 well plate as per the manufacturer's instructions. Each sample was analysed in duplicate. The assay procedure was carried out according to the supplier's instructions. Each run included a standard curve:

Standard 0	0ng/ml
Standard 1	0.469ng/ml
Standard 2	0.938ng/ml

Standard 3	1.875ng/ml
Standard 4	3.75ng/ml
Standard 5	7.5ng/ml
Standard 6	15ng/ml
Standard 7	30ng/ml

Each standard was added to the wells in duplicate. 10X ELISA buffer was diluted using deionised water at a dilution of 1:10 prior to use. 10X Washer buffer was also diluted using deionised water at 1:10 to obtain a 1X solution. DLK1 is recognized by the addition of a purified polyclonal antibody specific for DLK1 (Detection Antibody). After removal of excess polyclonal antibody, HRP conjugated anti-IgG (HRP) is added. Following a final washing, peroxidase activity is quantified using the substrate 3, 3', 5, 5'-tetramethylbenzidine (TMB). The intensity of the colour reaction is measured at 450 nm after acidification and is directly proportional to the concentration of DLK1 in the samples.

2.2 Placental studies

2.2.1 Sampling from the placenta at delivery

Table 3 shows the features of the placentas collected from the RLH cohort. We were able to obtain placenta from 25 out of the 33 participants. Out of the 8 missed placentas, 4 participants delivered in hospitals out of area whilst the other 4 delivered in emergency situations with no time to inform the research team of delivery. Most of the neonates were delivered at term as can be seen from the table. Majority of the umbilical cords were seen to have 3 vessels with only 1 out of the 25 placentas obtained demonstrating a 2-vessel cord. In addition, 60% of the placentas were found to have a peripheral cord insertion; 24% marginal and 16% exhibited a central insertion. There was no significant difference seen in maternal DLK1 serum levels with the varying cord insertions at 18 – 21; 26-29; 32-34 and 37-39 weeks gestation. See Appendix

N = 25	Mean ± std dev	Range
GA	39.63± 1.732	33.57 – 42.14
Neonatal weight (grams)	3074 ± 589.7	1340 – 3680
Placental weight (grams)	426.6 ± 122.8	168 – 762
Feto - placental ratio	7.645±2.359	3.56 – 16.83
Placental length (mm)	189.3±64.26	130 - 480
Placental width (mm)	152.8 ±21.95	110 – 192
Placental depth (mm)	26.48 ± 5.108	20 – 40
Cord length (mm)	377.3±152.5	15 -750
Cord diameter (mm)	15.92±16.22	3 – 90

Table 5: **Placental and neonatal characteristics**

Table showing neonatal and placental characteristics from the cohort. GA – Gestational Age.

In the first instance, on attendance to the delivery, cord blood was collected from both the umbilical artery and umbilical vein. These blood samples were then spun in a cold centrifuge as described above prior to storage transiently in a -20°C freezer at the Royal London Hospital if out of hours for no more than 24hours, or collection in dry ice prior to transfer to a -80° C freezer at the Centre for Endocrinology, QMUL. Placental biopsies were then obtained from five peripheral regions (P1 – 5) and one central (C)(Figure 7) using a 5mm punch biopsy (Schucho Miltex Biopsy Punch). At each region, two biopsy samples were obtained. One for DNA and the other was placed in RNA later (R0901 Sigma RNAlater ®) for future RNA extraction. In addition umbilical cord samples were obtained for both DNA and RNA extraction. Umbilical cord samples were collected as representations of fetal tissue as we know that both the fetus and umbilical cord arise from the ICM developmentally. These samples were also subsequently stored at -80°C.

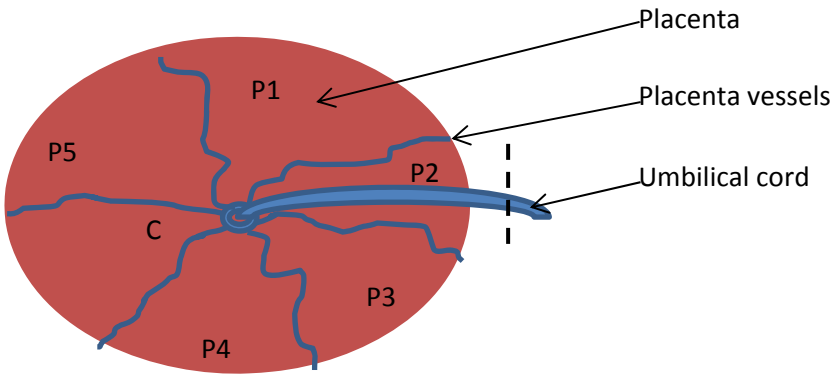


Figure 9: **Obtaining placental biopsy tissue at delivery**

Diagram showing areas of placental biopsy (P1-5; C) and umbilical cord sampling. Dashed line represents section of umbilical cord obtained for sampling. P1 to 5, Peripheral; C Central. Note that the central biopsy was obtained 2 cm from the cord insertion to allow for histological examination of the cord insertion.

2.2.2 Morphological characterisation of placenta

After the biopsy samples were obtained, the placenta was stored in 4% formaldehyde for 24 hours prior to fixing in paraffin. The placenta was then characterised and sampled as per the Amsterdam Placental Workshop Group Consensus Statement[157]. In the first instance placental weight was obtained and included the placenta trimmed of extraplacental membranes umbilical cord. Prior biopsy sampling was noted along with any disruption of the basal plate. The placenta was then measured in three dimensions: the maximal linear dimension (length), the greatest dimension of the axis perpendicular to this linear measurement (width), and the mural minimal and maximal thickness. This was then followed by description of the cord which included the average diameter of the cord, length, site of insertion in relation to the centre /margin of the placenta (distance between insertion and the nearest placental margin), the presence of strictures, and whether cord appears to be hypocoiled or hypercoiled. Insertion is further described as central, marginal (<1cm from the nearest margin),velamentous (insertion into the fetal membranes) and peripheral (<3cm from the nearest margin).The membranes were then described including a description of the colour/opacity and completeness.

Subsequent to the description, the cord, membranes and placental disk were sampled. Four blocks were obtained as a minimum: 1 block included a roll of the extraplacental membranes from the rupture edge to the placental margin (including part of the marginal parenchyma) and 2 sections from the umbilical cord, one from the fetal end and another approximately 5 cm from the placenta insertion end; and three other blocks containing the full thickness section of normal appearing placenta parenchyma. The full- thickness samples were taken from within the central two-thirds of

the disc and include one adjacent to the insertion site (Figure 7). A further block was obtained if there was pathology noted on macroscopic inspection. Each block had an ID number automatically assigned to it.

In the first instance, H&E staining of the blocks above was carried out as described in section 2.2.4. The histology was reported both by myself and perinatal pathologist from the Royal London Hospital, Dr Irene Scheimberg. We were both blinded to clinical details and identity of the participant whilst reporting. The slides were reviewed systematically as shown in table 4. Examples of H&E staining and from the cohort are shown in Figure 10 - 12.

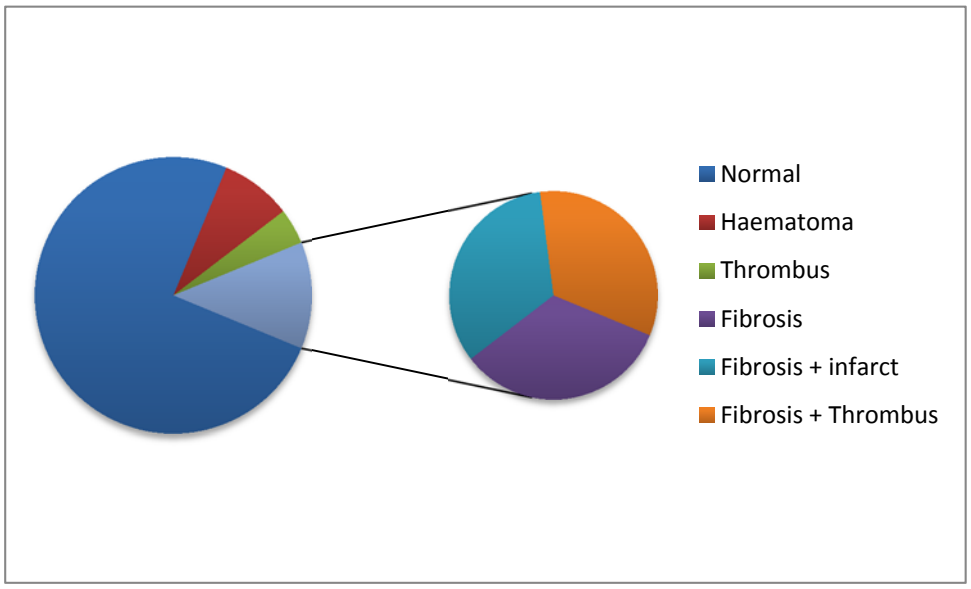


Figure 10: **Macroscopic findings on examination of placenta**

72% of placentas were normal. Haematoma was seen in 2 (8%) whilst 1 placenta was seen to have a thrombus with no other changes.3 (12%) were seen to have focal fibrosis. Of these 1 showed fibrosis with no other changes, 1 showed fibrosis in addition to an infarct whilst one showed fibrosis in the presence of a thrombus.

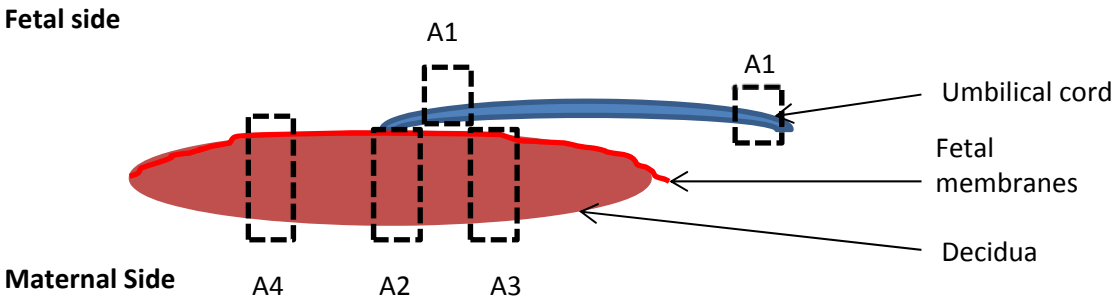


Figure 11: **Placental sampling**

Diagram showing transverse section through placenta. A1= cord + membranes; A2 = cord insertion; A3 = peripheral section; A4 = random peripheral section.

Compartment	Features
Villi	Maturation (accelerated/ delayed/ normal) Distal villous hypoplasia – present/ absent Avascular villi - present/ absent
Decidua	Decidual arteriopathy - present / absent
	- acute atherosis - fibrinoid necrosis - absence of spiral artery remodelling
	Persistence of endovascular trophoblast Presence of MTGC
Fetal Membranes	Infection/ inflammation (see grading)
Other	Thrombosis – present/ absent/ location Hemorrhage – present / absent/ location Intramural fibrin – present / absent/location

Table 6: **Methodology for placental histology characterisation**

Table showing features reviewed when carrying out histological staging. The villi, decidua and fetal membranes were examined. Other abnormalities outside these areas were also noted. Grading of infection/ inflammation is shown in table 2.4. MTGC – multinucleated trophoblastic giant cells.

Staging and Grading of the Maternal and Fetal Inflammatory Responses in Ascending Intrauterine Infection	
Maternal Inflammatory Response	
Stage 1 – acute subchorionitis or chorionitis	Grade 1 – not severe as defined
Stage 2 – aute chorioamnionitis: PMN leukocytes extend into fibrous chorion and /or amnion	Grade 2 – severe: confluent PMN leukocytes or with microabscesses
Stage 3 – necrotizing chorioamnionitis: karyorrhexis of PMN leukocytes, amniocyte necrosis, and/or amnion basement membrane hypereosinophilia	
Fetal Inflammatory Response	
Stage 1 – chorionic vasculitis or umbilical phlebitis Stage 2 – involvement of the umbilical vein and one or more umbilical arteries Stage 3 – necrotizing funisitis	Grade 1 – not severe as defined Grade 2 – severe: near – confluent intramural PMN leukocytes with attenuation of vascular smooth muscle

Table 7: **Histological staging and grading of placenta**

Table showing criteria used for grading and staging inflammation. It is divided into maternal and fetal inflammatory response. PMN – Polymorphonuclear. Adapted from Khong *et al*, Arch Pathol Lab Med:**140**, 2016[157].

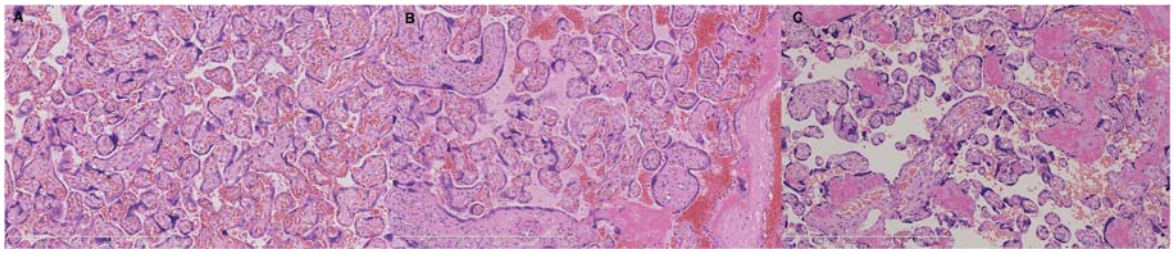


Figure 12: **Maturation of placental villi**

Picture showing instances of appropriate maturation - A (at 39 weeks gestation). Terminal villi are seen as typical of term placenta; delayed maturation B - (centre at 40 weeks gestation). Although placenta is term, note the presence of intermediate villi and a paucity of terminal villi; and accelerated maturation - C (right at 33 +3 weeks gestation). Note the presence of small terminal villi despite a gestational age of 33 weeks.

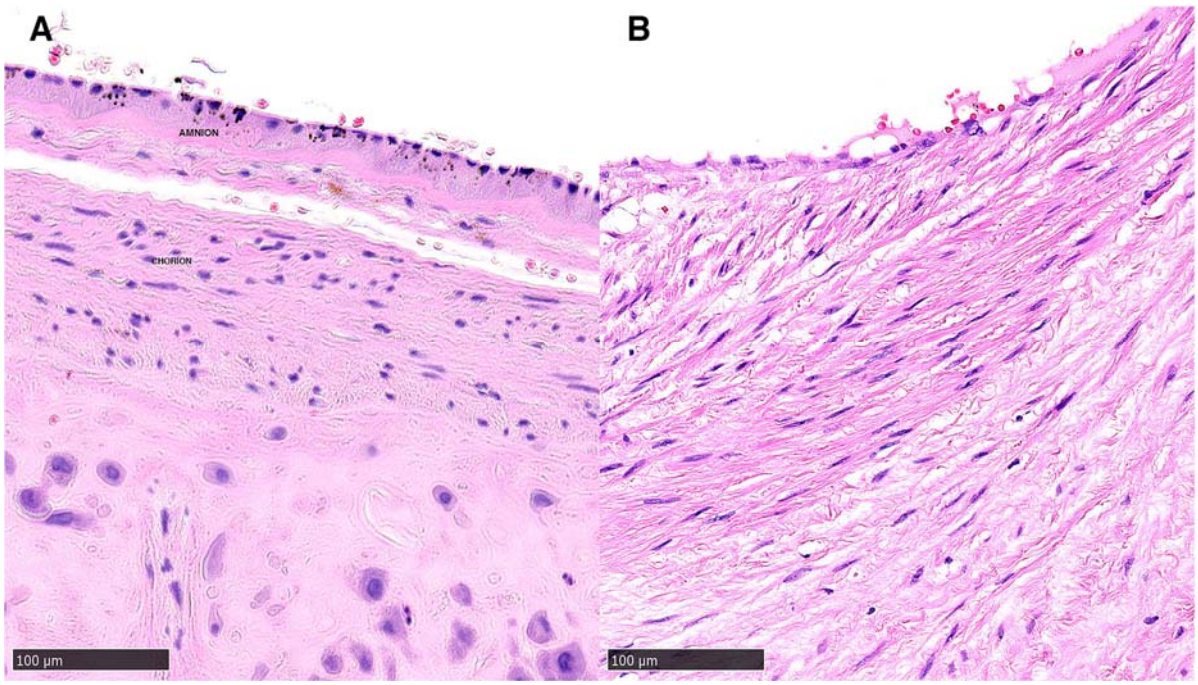


Figure 13: **Chorioamnionitis compared to normal placenta**

An example of normal fetal membranes is shown on the left (A). The amnion and chorion are depicted and are well defined with a normal morphology of cells. On the right (B) we see an infiltration of inflammatory cells in the both the chorion and amnion as seen in chorioamnionitis with loss of the clear depiction of the amnion and chorion.

.

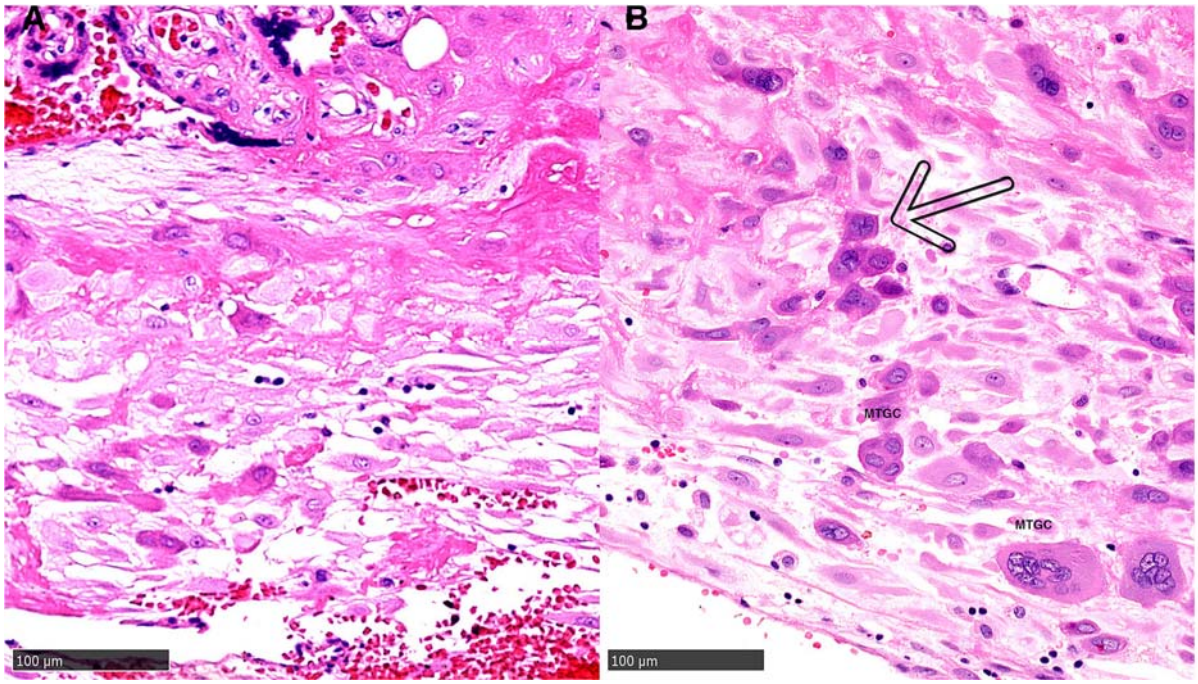


Figure 14: **Normal decidua compared to one with MTGC**

Figure showing H&E of the maternal decidua. Normal decidua is seen on the left (A) with extravillous trophoblasts and decidual stromal cells. On the right (B) we see clusters of MTGC depicted by a large black arrow and labels. MTGC – multinucleated trophoblastic giant cells

2.2.3 Early gestation tissue

Early gestation umbilical cord and placental samples were obtained from the Medical Research Council (MRC) / Wellcome Trust funded HDBR (London), with appropriate maternal consent and approval from the local National Health Authority Ethics Committee. The Brighton and Sussex REC approved the experimental protocol for this research. The samples were transported from the clinic to the HDBR resource, staged, dissected, fixed in formalin and embedded in paraffin prior to preparation in histology cassettes. The age of the fetal samples were estimated following the guidelines of Hern (Hern 1984) and the embryos were staged using the Carnegie Staging classification system (O'Rahilly & Müller 2010). A snip of tissue from each sample was taken to perform cytogenetic analysis to determine the mitotic karyotype. All samples had a normal male or female karyotype. Samples were then transported to our lab in the cassettes where slides were prepared for immunohistochemical studies.

2.2.4 Immunohistochemistry studies

Immunohistochemistry was carried out on the placental slides using the EnVision FLEX ™ system from Dako. As paraffin is insoluble in water, most staining methods require it to be removed. The slides underwent dewaxing and deparaffinisation in xylene twice and alcohol twice (95%, 70% ethanol) respectively prior to rehydration by washing in tap water for 5 minutes. Antigen retrieval was carried out using a High pH target retrieval solution (50X) after dilution to 1X. Retrieval was carried out by placing slides in target retrieval solution into a water bath, heated to 99°C over 40 minutes. This results in heat induced epitope retrieval. The slides were then cooled in the high pH solution at room temperature over a period of 20 minutes. A PAP pen was then used to draw around the specimen on the slides before carrying out washes using Washer Buffer (20X) after dilution to 1X. Three washes were carried out each lasting 3 minutes each prior to addition of EnVision™ FLEX Peroxidase – Blocking Reagent for 5minutes. This is a phosphate buffer containing hydrogen peroxide, 15mmol/L NaN_3 and detergent. This was followed by a further 3 washes in Washer Buffer lasting 3 minutes each. Antibody was then diluted in Dako Antibody Diluent and incubated on slides for 40 minutes followed by a further three washes in Washer Buffer. EnVision™ FLEX + Rabbit LINKER/ Mouse LINKER (depending on the primary antibody) was then added to the slides and allowed to incubate for 20 minutes. The LINKER results in signal amplification of primary antibodies. After three washes in Washer Buffer, EnVision™ FLEX HRP (Horseradish peroxidase) reagent was added to the slides and allowed to incubate for 30 minutes. This reagent consists of a dextran

backbone to which a large number of HRP molecules and secondary antibody molecules have been coupled. Following three washes using Washer Buffer, EnVision™ FLEX DAB Chromogen was then used for visualisation. DAB, a concentrated diaminobenzidine solution, needs dilution in EnVision™ FLEX Substrate Buffer, containing hydrogen peroxide, before use. Dab + Substrate Buffer was added to the slides for 5 minutes before washing in tap water for a further 5 minutes. The substrate system produces a crisp brown end product at the site of the target antigen. Haematoxylin was used for counterstaining. The slides were dipped in haematoxylin for three minutes prior to rehydration in tap water. Washes were then carried out in alcohol and xylene prior to covering the slides with glass slips using DPX mounting medium (Lamb laboratories) to preserve staining. Visualisation and imaging was carried out using the NanoZoomer-2.0 HT Digital Slide Scanner (Hamamatsu) and Panoramic Viewer (3DHistech) software, and the Leica DMR Light microscope, Leica DC200 digital camera, and Leica DC Viewer software.

Antibodies used for immunohistochemistry

Ab number	Antigen	Species	Company	Dilution	Catalogue
1° ab110636	DLK1	Rabbit	Abcam	1 in 200	
1° ab28364	CD31	Rabbit	Abcam	1 in 100	
1°	Cytokeratin 7 Clone OV-TL 12/30	Mouse	Dako	1 in 100	M7018

Table 8: **Primary antibodies used in immunohistochemistry methods**

List of primary antibodies used for IHC studies along with dilutions.

2.2.5 Haematoxylin and Eosin staining

Slides were dipped for 3 minutes in haematoxylin (Lamb Laboratories) and thereafter left under running water for 3 minutes. They were then dipped in ethanol 3 times (95%, 70% and 50%), followed by another 2 minutes under running water. This was followed by dip in Eosin for 30 seconds (Lamb Laboratories), and four dips in alcohol (100% X2 ethanol, 95% and 70%) and washes in xylene. Excess xylene was wiped from the back and edges of the slides, and they were covered with glass slips, using DPX mounting media (Lamb Laboratories) to preserve the staining.

2.2.6 Staging of immunohistochemistry studies

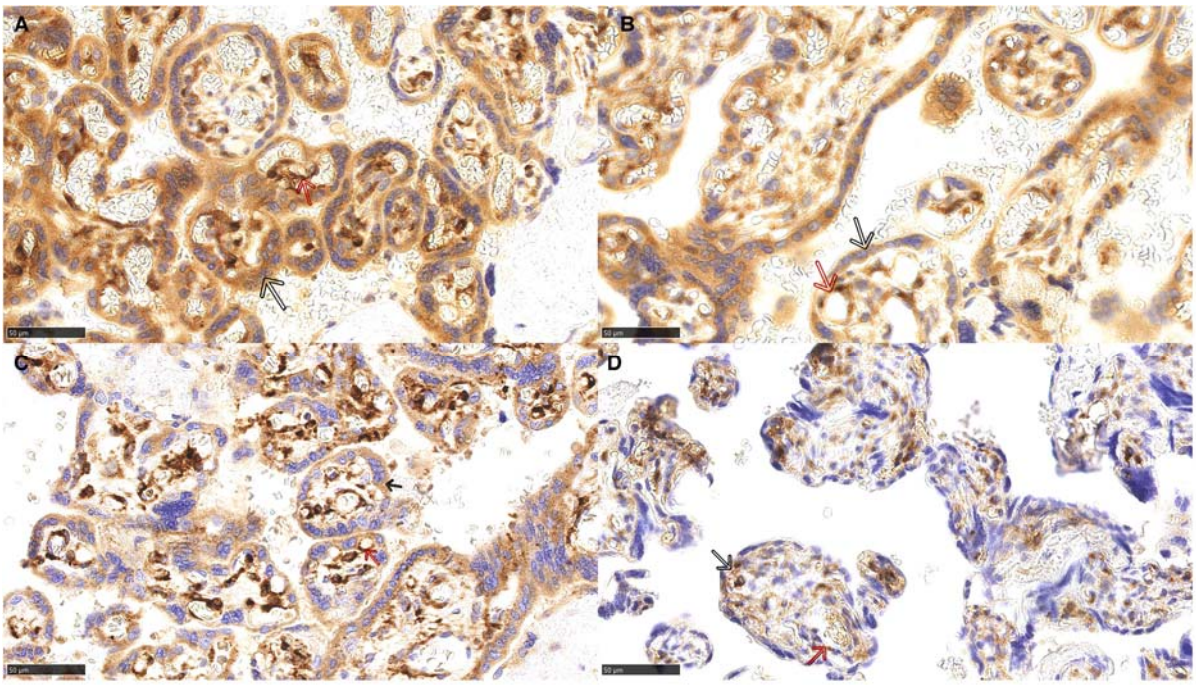


Figure 15: **Staging and grading of DLK1 staining in placental villi**

Picture showing examples of intensity of DLK1 staining in trophoblasts and fetal endothelial cells. A: a+++ in FE and Tr; B: a +++ in FE and b++ in Tr; C: a+++ in FE and a ++ in Tr; D: c+ in both FE and Tr. FE (fetal endothelial); Tr (Trophoblast). Trophoblasts are indicated by a black arrow and fetal endothelial cells by a red arrow.

Reporting was carried out independently, prior to comparing findings using criteria in table 6. In addition we were blinded to clinical information.

Criteria	Feature recorded
Location	Fetal Endothelial Cells
	Trophoblast Cells
	Decidua
Pattern of staining	A – Diffuse
	B – Moderate
	C – Focal
Intensity	+++ Strong
	++ Moderate
	+ Weak

Table 9: **Staging and grading of staining intensity in immunohistochemistry methods**
Table highlighting criteria used to stage immunohistochemistry.

2.2.6 Finnish Cohort

In view of the results obtained from the pilot data, we proposed to extend our analysis to a larger cohort of participants. These participants were recruited between 2006 and 2007 with longitudinal follow up thereafter, at Kuopio University Hospital (Kuopio, Finland) and from local maternity units in Kuopio, for other studies including a study on minipuberty[158]. These patients were stratified into normal pregnancies, those at risk of IUGR secondary to placental insufficiency and those at risk of imminent premature delivery. 173 participants were initially recruited with 113 completing the study. 91 participants were included in our study after exclusion of twin and other multiple deliveries (Table 7). This cohort is larger and consists of a more homogenous group of participants. SGA was defined as sex and gestational age specific birth height < -2SD, according to the new Finnish birth size reference[159]. Within the selected cohort for analysis in this group, the SGA group had features of fetal growth restriction caused by pathology in utero and therefore IUGR. We are grateful to have received serum samples serially collected from this cohort along with clinical information, antenatal ultrasound scan data and placental blocks from our Finnish collaborators.

Serum samples received were taken in the second and third trimester, at delivery and on day one post delivery. In addition participants ELISA assays and immunohistochemical studies were carried out on the Finnish samples as detailed above for the RLH cohort.

In view of the findings in our pilot study locally, advantage was taken of existing serum samples that had been collected from a cohort of participants antenatally for a study on mini puberty as described in the previous chapter..

These investigations were carried out to strengthen findings from the pilot studies. From my IHC I hope to show that DLK1 is expressed in trophoblast cells, which are known to have a function in secreting endocrine hormones. In this way DLK1 will influence placental function and fetal growth by regulating hormones with important functions in growth.

Characteristic	Mean/ Median ±std dev (95% CI)/ Range	N
Age, years	31.4 ± 5.28 (29.9 – 32.41)	69
Weight, Kg	65.18 ± 12.51 (62.15 – 68.21)	68
Height, cm	165.7 ± 6.12 (164.3 – 167.2)	69
BMI	23.99 ± 4.66 (22.85 – 25.12)	67
Weight increase	12.36 ± (11.09 – 13.62)	58
Gravidity	2 (1 – 13)	69
Parity	1 (0 – 10)	68
Average gestational age at 2nd trimester visit	21.46 ± 2.87 (20.15 – 22.76)	21
Average gestational age at 3rd trimester visit	31.6 ± 4.32 (30.49 – 32.7)	61
Gestational age at birth, weeks	36.97 ± 4.63 (35.85 – 38.08)	69
Birth weight, grams	2815 ± 1051 (2562 – 3067)	69
Birth weight SDS	-0.82 ± 1.39 (-1.15 - -0.49)	69
SGA		18
AGA		50
Birth length, mm	46.41 ± 5.64 (45.06 – 47.77)	69
Birth length SDS	-0.95 ± 1.59 (-1.34 - -0.57)	69
SGA		15
AGA		53
Occipitofrontal Circumference (cm)	32.85 ± 4.1 (31.87 – 33.83)	68
Occipitofrontal Circumference SDS	-0.7 ± 1.44 (-1.05 - -0.34)	64
Sex		Male 37 Female 32

Table 10: **Participant characteristics for the Finnish cohort**

Table showing participant characteristics within the Finnish cohort along with details of resulting neonates

2.3 Genetic studies on embryonic representative tissue to assess DLK1 expression in IUGR when compared to normal pregnancies

2.3.1 DNA extraction from umbilical cord

Frozen umbilical cord sections were brought to room temperature. After removal of the outer maternal sheath (Figure 14), 200mg of tissue was collected from the internal Wharton jelly containing the umbilical vessels. This sample, representative of fetal tissue, was used for genomic DNA extraction. (Figure 8)

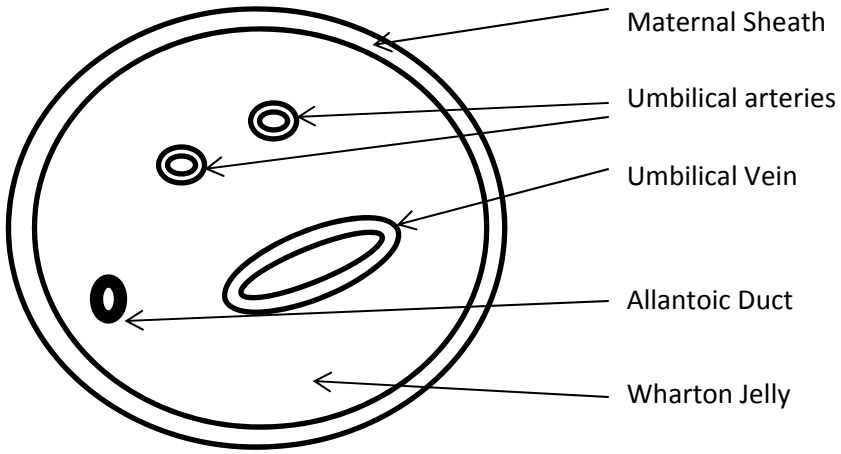


Figure 16: **Transverse section through umbilical cord**

The outer maternal sheath was removed prior to using the rest of the umbilical cord, representative of embryonic tissue, for extraction of genomic DNA.

DNA was extracted from umbilical cord tissue using chloroform: isomylalcohol. In the first instance 50mls of buffer was made up containing 2.5 ml of 50mM Tris pH 8.0, 2.5ml of 100mM EDTA, 1 ml 100mM NaCl, 5ml 1% SDS and 39.5ml MilliQ water. 200 mg of umbilical cord tissue was obtained as detailed above. This was placed into a 1.5ml microcentrifuge tube after which 525µl of buffer was added. This was followed by the addition of 35 µl of 10mg/ml Proteinase K (molecular grade, Sigma-Aldrich) followed by incubation at 55 °C overnight. Proteinase K is used to dissociate the tissue to allow DNA to become available. In addition it releases DNA from histones. On day 2, Digestion of RNA was carried out by addition of 2 µl of 0.2mg/ml RNase A and incubating at 37°C for 1 hour. Polysaccharide removal was then carried out by the addition of 200 µl 5M NaCl and gently mixing. This was followed by the addition of 700 µl of chloroform: isoamylalcohol and mixing gently on an orbital shaker for 2 hours. The samples were then centrifuged at 10000rpm for 10 minutes. The

upper aqueous layer was removed into a new tube and the organic and inter-phases discarded. 700 µl of isopropanol was added to the mixture prior to mixing gently by inverting the tube. The precipitated DNA was visible at this stage. Further centrifugation was carried out at 10000rpm for 10 minutes. This resulted in the formation of a DNA pellet. The pellet was washed for 1 hour at 4°C with 300 µl 70% Ethanol. After removal of ethanol, 200 µl of TE buffer (1ml of 1M Tris pH 8.0, 40 µl of 0.5M EDTA, 99 ml MilliQ Water) was added. DNA was then resuspended by mixing on a roller overnight.

2.3.2 PCR studies of DLK1

Each 20µl reaction contained 1µl DNA, 0.5 µl dNTPs (10mM A+C+G+T), 1 µl primers (10 µM Forward + Reverse) see table, 2 µl 10X PCR buffer, 0.2 µl TAQ DNA Polymerase (Thermus aquaticus) and 15.3 µl distilled water.

Standard PCR program:			
Cycle	**Temperature**	**Time**	
1	94°C	5 minutes	Strand separation = denaturation of the hydrogen bonds within the double stranded DNA
40 cycles	94°C	30 seconds	Further strand separation
	59°C	30 seconds	Annealing - allows the primers to bind to a section of the single stands of DNA for which they have been specifically designed. This temperature may vary slightly depending on the T_m (melting temperature) of the primers
	72 °C	1 minute	Extension - Taq DNA polymerase binds to the primers and uses dNTPs as building blocks to enzymatically synthesise a complementary strand of DNA. The newly formed DNA contains one strand of parental DNA, and one newly synthesised strand. This process is repeated 35-40 times to amplify the piece of DNA between the forward and reverse primer sequences
1	72 °C	10 minutes	Further elongation
1	10 °C	∞	Preservation

Table 11: **PCR programme for DLK1 gene product**

Table showing the PCR programme used. DLK1 primers for the 5 exons of the molecule were constructed and used for the reaction.

Primer	Tm (°C)	Annealing Temperature (°C)	Sequence	Product Length (bp)
Human DLK1 Exon 1 F	59	62	AAGTGTTTCGGTGTTCCTGC	900
Human DLK1 Exon 1 R	59	62	CCTCTGGCGCCACTTCTG	
Human DLK1 Exon 2 F	59	59	GCTGTTGGTGCCCTCGAG	385
Human DLK1 Exon 2 R	59	59	CTCGCCCCACCAGTTTTCT	
Human DLK1 Exon 3 F	62	62	TCAGGTGGCTGGTGGTACT	511
Human DLK1 Exon 3 R	64.8	62	CGGCCCAGCTGTATGTGTAT	
Human DLK1 Exon 4 F	62	62	AGGGAATCTCAGCTAGGCCAC	657
Human DLK1 Exon 4 R	62.2	62	GACTGTCACTCACACGCATC	
Human DLK1 Exon 5 F	59	62	GTTGTAGCCTAGCCCCTGAG	972
Human DLK1 Exon 5 R	59	62	CCGCGTATAGTAAGCTCTGC	

Table 12: **DLK1 primers used for PCR reaction**

Table showing the nucleotide sequence for the 5 primers. Both forward (F) and reverse (R) primers are shown.

2.3.3 Gel Electrophoresis

After the DNA has been amplified, samples were combined with 5µl of loading dye (Fermentas) and run on a 1% agarose gel. The loading dye adds weight to the DNA to contain it within the wells of the gel. Higher molecular weights are separated more easily on lower percentage gels. The gel, containing GelRed ™ Nucleic Acid Gel Stain (1:20 dilution), was placed in an electrophoresis chamber

containing 1X TAE buffer (Tris-acetate-EDTA buffer; National Diagnostics), and an electrical current passed through it. DNA was visualised using a transilluminator.

2.3.4 DNA Gel Extraction

Gel extraction was carried out using Qiaquick® Gel extraction Kit from Qiagen. DNA fragments were excised from the agarose gel using a clean sharp scalpel. The gel slices were then weighed in a falcon tube. 3 volumes of buffer QG, a buffer containing a pH indicator allowing easy determination of the optimal pH for DNA binding, to 1 volume of gel was added to the gel strips. This was then incubated at 50°C for 10 minutes. A Qiaquick spin column was placed into a collection tube after which the mixture was added to the column, 750 µl at a time, to bind DNA after which the mixture is spun in a centrifuge for 1 minute and the flow-through discarded. Nucleic acids adsorb to the silica membrane in the high salt conditions provided by the buffer. A further 500 µl of buffer QG was added to the spin column and the sample centrifuged once more for 1 minute. 750 µl of Buffer PE (after addition of ethanol) is then used to wash the mixture. The sample was then left to stand for five minutes before spinning in a centrifuge for 1 minute. The spin column provided was centrifuged one more time at a speed of 13000rpm for 1 minute to get rid of excess wash buffer. The column was then placed into a new 1.5ml microcentrifuge tube. To elute DNA, 30 µl of buffer EB (10 mM Tris·Cl, pH 8.5) was added to the Qiaquick membrane and the sample allowed to stand at room temperature for 1 minute prior to centrifugation for 1 minute. The purified DNA was then analysed on a gel prior to sending the DNA samples to the Genome Centre for sequencing.

2.4 DLK1 protein expression in placentas

2.4.1 Protein extraction for Western blotting to detect DLK1 in placental tissue

Placental biopsy samples were dissected on ice to prevent degradation by proteases. The tissue was then placed in an Eppendorf tube. 300µL of cold lysis buffer (Sigma-Aldrich) was added to a 5mg piece of placental tissue followed by homogenisation of the mixture. Subsequent to homogenisation, the solution was then placed in a cold centrifuge (4°C) for 20 minutes at 12,000 rpm. The tubes were then removed from the centrifuge and placed on ice. Supernatant was collected and added to an equal volume of 2x Laemmli buffer (Sigma-Aldrich), containing SDS and β-ME, then boiled for 10 minutes to denature proteins prior to gel loading.

2.4.2 Western blotting

A pre-cast gel (SDS PAGE precast NuPage ®BisTris gels 4-12%, 10% or 12%) (Invitrogen) was briefly rinsed with distilled water and inserted into the gel tank. The tank was filled with 1x MOPS running buffer (50x 3-(N-morpholino)propanesulfonic acid (50nM MOPS, 50nM Tris Base, 0.1% SDS, 1mM EDTA pH 7.7)) to cover the gel and the comb removed. The protein ladder (Kaleidoscope pre-stained Protein Marker (Biorad) was loaded into the first well of the gel (10 µl for 10 well gel, 8 µl for 15 well gels). Samples prepared in 2x Laemmli buffer were centrifuged for 1 minute at 13 000 rpm and loaded into adjacent wells, typically between 10-20 µl, depending on the protein content of the lysate. The gel was run for one hour at 120V to begin with prior to increasing voltage to a maximum of 160V.

2.4.3 Semi-dry transfer of proteins from gel to membrane

A square segment of 0.1 µm pore nitrocellulose membrane was cut to the dimensions of the gel using extra thick filter paper (Biorad) as a template. The membrane was soaked in transfer buffer (0.9% glycine (w/v), 0.242% Tris (w/v), 20% methanol (v/v)) for 10 minutes prior to the transfer procedure. A sandwich of filter paper-membrane-gel-filter paper was assembled in the transfer cell. First, filter paper was briefly soaked in transfer buffer and placed on the transfer cell, pressed with a roller to remove excess buffer. Next the membrane was placed on top of the filter paper and then the gel was soaked and placed on top of the membrane. Bubbles were removed by gently pressing the gel from the centre outwards. Finally the second filter paper was soaked and placed on top of the gel. The sandwich was pressed again with the roller to remove excess buffer. The transfer cell (Trans-Blot SD semi-dry transfer cell) (Biorad) was set to 15V for 40 minutes for one blot or 50 minutes for two blots. After transfer the gel was discarded and the membrane subjected to Ponceau-S staining to determine the efficiency of the transfer.

2.4.4 Ponceau-S staining

Ponceau-S (0.2% Ponceau-S and 1% acetic acid) (Sigma-Aldrich) is a dye that reversibly stains protein bands without any adverse effects on the proteins. Staining membranes with Ponceau-S reveals any regions where the transfer has failed (seen as bubbles) as well as the amount of protein loaded per lane and the efficiency of the transfer. Membranes were placed in small containers, covered with Ponceau-S and left on a shaker for about two minutes. Ponceau-S was removed and the membrane

washed with PBS-tween (1x PBS +0.1% (v/v) Tween20) (Sigma-Aldrich). After one wash, protein bands became visible, and Ponceau-S staining was then removed with further washes in PBS-tween.

2.4.5 Immunoblotting

A blocking buffer was prepared by dissolving 5% non-fat dried powdered milk (Asda) in PBS-tween (0.1%), and mixing by inversion. Nitrocellulose membranes were transferred to 50 ml falcon tubes with 10 ml blocking buffer for one hour to prevent non-specific binding of the antibody. Tubes were rotated on a roller. After blocking, membranes were incubated in milk containing relevant dilutions of primary antibodies (Table 2) overnight at 4^{o}C.

The following day, membranes were subjected to 3x 10 minute washes in PBS-tween, incubated in milk containing fluorophore-conjugated secondary antibodies (Table 11) (goat anti-rabbit 800 1:5000, goat anti-mouse 680 1:20 000) (Odyssey) for one hour and wash steps were repeated. Bands were detected using an Odyssey scanner at wavelengths 800 and 680.

Table 2 Antibodies used for western blotting

Ab	Antigen/Fluorophore	Species	Company	Dilution
1°	α-tubulin	Mouse monoclonal	Sigma-Aldrich (T6199)	1 in 10 000
1°	DLK1	Rabbit monoclonal	Abcam (ab 110636)	1 in 200
2°	IRDye®680LT	Goat anti-mouse	Li-cor (925-68020)	1 in 20 000
2°	IRDye®800CW	Goat anti-rabbit	Li-cor (925-32211)	1 in 5000

Table 13: **Primary and secondary antibodies used for western blot experiments**

Table showing primary and secondary antibodies used for western blot experiments

2.3 Data Analysis

Multiple regression analysis was used to determine the significant independent contributors in the prediction of birth weight, femoral length, abdominal circumference, BPD respectively and maternal serum DLK1, maternal weight, maternal age, parity and ethnic origin [Caucasian, Afro-Caribbean, South Asian (Indian, Pakistani, Bangladeshi), Far East Asian (Chinese and the Orient), South East Asian (Philippines, Malaysia), Mixed]. The association between maternal serum DLK1 and the incidence of IUGR was assesses by comparing the relative risk at DLK1 levels below the 5th centile, at four different time points in pregnancy. Odds ratios (OR) for IUGR were calculated. Multiple regression analysis was further used to determine the independent contributors in the prediction of estimated fetal weight from the following variables: maternal serum DLK1, maternal age, maternal

weight, smoking status, parity) in the second and third trimester, in the Finnish cohort. Significance of Statistical significance was determined by 1-way analysis and 2-way analysis of variance (Friedman ANOVA). A *p* value under 0.05 ($p<0.05$) was taken as statistically significant. This was used to analyse the statistical difference between the IUGR and normal pregnancy DLK1 levels within the Finnish cohort and in addition the statistical difference in DLK1 levels in normal compared to abnormal placental histology findings in the RLH cohort. Multiple correlation analysis was run to compare DLK1 serum levels with placental parameters. Statistical analysis was performed with SPSS Statistics for MAC (v. 24, IBM), Microsoft Excel for MAC and GraphPad PRISM for MAC (v.7, Software MacKiev) software

Chapter 3 Results: Serum DLK1 levels during pregnancy

3.1 Aims of study

DLK1 has been shown to be present in human embryonic tissue [138]and reduced levels as seen in animal knockout models[119] and human Temple syndrome[123]result in IUGR. In addition, DLK1 levels have been shown to be reduced in IUGR in humans when compared to normal pregnancies[143]. With this in mind, my aims and objectives include:

- To investigate whether maternal serum DLK1 can be predictive of fetal growth parameters such as abdominal circumference, bipareital diameter and femoral length at four time points in pregnancy.
- To investigate the gestation at which DLK1 can be predictive of birth weight and moreover assess the relationship between DLK1 and estimated fetal weight during pregnancy.
- To assess whether maternal serum DLK1 levels can be used to evaluate the relative risk for IUGR. In this way I aim to ascertain the important period of action for DLK1 and therefore establish a rationale for interpreting maternal DLK1 levels depending on timing of sampling, as a biomarker for intrauterine growth.

3.2. DLK1 levels during pregnancy compared to fetal growth parameters

In the first instance, DLK1 levels were measured in serum by ELISA as detailed in chapter 2. These results showed a steady rise in concentrations during gestation with a sharp fall after delivery (Figure 15). This is in keeping with previous studies in both humans[138]and animals [139]. Serum DLK1 levels were then compared to in utero growth parameters. A multiple regression analysis was carried out to predict birth weight and fetal growth parameters based on DLK1 levels(Table 12). Additional variables taken into account include maternal age and weight at booking, ethnicity and parity. Serum DLK1 levels at four time points were taken into consideration and compared to birth weight and in addition fetal growth parameters measured at the same time as the sampling. Fetal growth parameters considered included femoral length (FL), abdominal circumference (AC) and bipareital diameter (BPD). Serum DLK1 levels taken at 32-34 weeks gestation were found to be highly significant of birth weight and abdominal circumference ($F = 19.905$, $P < 0.0001$) and $F = 17.252$, $P<0.0001$) respectively. In addition serum levels at 26 weeks gestation were also predictive of birth weight and in addition of femoral length ($F = 5.902$, $P = 0.02$ at 26 – 28 weeks; $F = 6.495$, $P = 0.017$ at 32-34 weeks). DLK1 was not predictive of BPD at any gestation

Further analysis was carried out to assess relative risk of IUGR when maternal serum DLK1 levels were low. Analysis of all maternal serum DLK1 levels was carried out and serum levels in the 5th and 10th percentile were used as a cut off for low DLK1 levels (Table 13). This was run for all four-time points but however samples from 26 – 29 weeks gestation were found to be predictive of IUGR. Using the 5th percentile as a cut off (9.69 nag/l) revealed OR 14.7(95% CI 3.85 - 55.2). The CI (Confidence interval) is wide but this is likely de to the small sample size. These results would therefore need to be confirmed in a larger cohort with a larger proportion of IUGR pregnancies. They however highlight the potential for maternal serum DLK1 levels as biomarkers for IUGR.

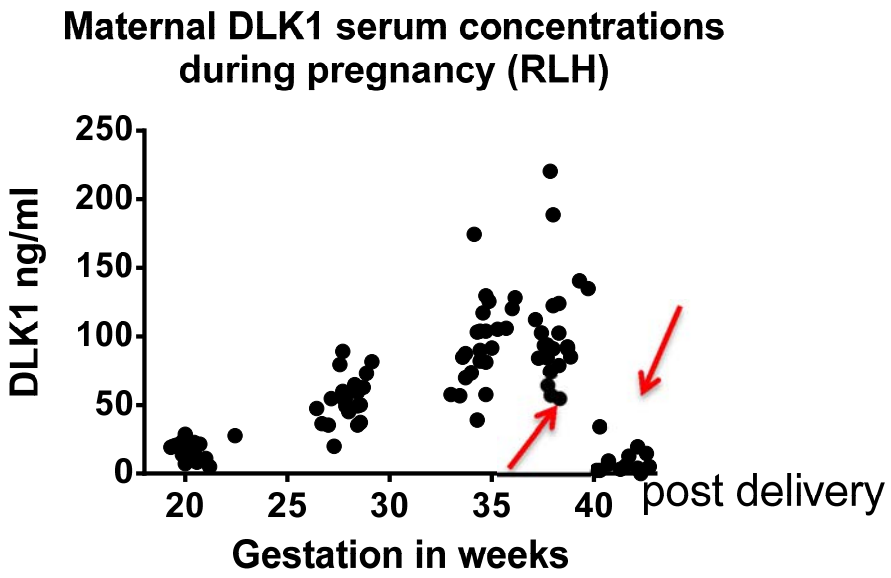


Figure 17: Maternal serum DLK1 levels during pregnancy

Graph showing maternal serum DLK1 levels taken at four time points during pregnancy with a further sample taken after delivery. Levels rise particularly between the 28th and 34th week of pregnancy. Samples taken 24 – 48 hours after delivery show a drop in DLK1 to pre pregnancy levels. Post delivery levels are depicted by the red arrows. The earliest post delivery sample obtained was at 37 weeks (first arrow).

Gestation in weeks	Statistical Analysis	Birth weight SDS	Femoral length	Abdominal circumference	Biparietal diameter
18 – 21	R^2	0.154	0.267	0.002	0.131
	F ratio	0.633	1.989	0.095	0.096 (0.983)
	P value	0.433	0.17	0.822	0.747
27 – 29	R^2	0.147	0.179	0.073	0.506 (2.84)
	F ratio	**4.642**	**5.902**	2.118	2.06 (0.118)
	P value	**0.04**	**0.02**	0.157	0.203
32 – 34	R^2	0.424	0.440	0.390	0.512
	F ratio	**19.905**	**6.495**	**17.252**	2.13(0.108)
	P value	**<0.0001**	**0.017**	**<0.0001**	0.95
37 – 39	R	0.175	0.370	0.242	0.559 (3.37)
	F ratio	**4.867**	3.644	1.936	2.28 (0.97)
	P value	**0.038**	0.069	0.243	0.06

Table 14: **Predictability of DLK1 for birth weight and other fetal growth parameters.**

Multiple regression analysis run to compare DLK1 serum levels taken at four times points in pregnancy to different fetal growth parameters and birth weight. Maternal age, weight, parity and ethnicity were included and they were shown not to be predictors of the fetal growth parameters or birth weight. Significant P values are shown in bold. These results are from the RLH cohort.

DLK1 cut off	Gestational age in weeks	OR (95% CI)
5th centile	26 – 28	14.5 (3.81 – 55.2)
10th centile	26 – 28	4.5 (0.56 – 36.133)

Table 15: **Relative risk for intrauterine growth restriction based on maternal serum DLK1 levels**

Table showing relative risk, shown as odds ratio of developing IUGR with DLK1 levels in the 5th centile or below or for DLK1 levels in the 10th centile and below, at between 26 to 28 weeks gestation. Confidence intervals are shown in brackets. OR – Odds ratio

3.2.3 Finnish Data

In the first instance the DLK1 ELISA was carried out as described in the previous chapter. We see from Figure 16 that there is a significant difference in serum levels between IUGR and AGA pregnancies in the third trimester. This is seen with both birth length and birth weight. The difference between the two groups was not significant in the second trimester (Table 14).

Demographics of the two groups are shown in table 14. There was no significant difference in most of the two groups' demographics. Interestingly there was a significant difference in gestational age at birth. This is likely because in most cases of severe IUGR neonates will be delivered early to avoid further complications. There was a highly significant difference seen in BW and BL between the two groups.

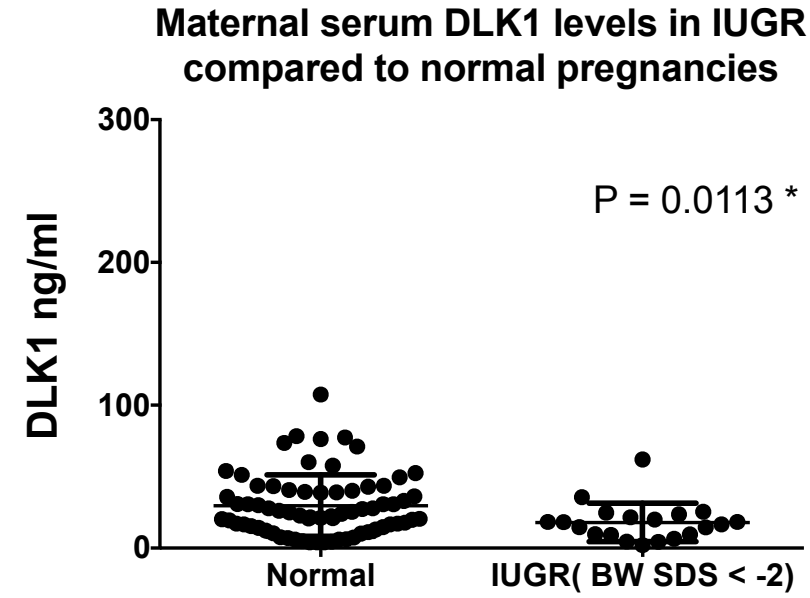
A multiple correlation analysis was carried out to assess whether DLK1 is an independent variable when levels were compared to EFW. The analysis was carried out both in the second and third trimester. Variables such as maternal age, weight, smoking status and parity were taken into consideration when running the analysis. We see that there are significant R-values for both trimesters suggesting that DLK1 levels taken during pregnancy can predict EFW. The other variables (maternal age, weight, smoking status and parity) were found not to have an effect on EFW.

Normal			IUGR			
Gestation in weeks **Mean (Range)**	**DLK1 levels ng/ml** **Range**	**Mean ± SD**	**Gestation in weeks** **Median (Range)**	**DLK1 levels ng/ml** **Range**	**Mean ± SD**	**P value**
Trimester 2 19.82 (15.3 – 23.9) N = 9	3.93 – 30.73	8.36 ±8.65	21.66 (19.1 – 23.9) N = 5	1.99 – 20.81	9.34	P = 0.9
Trimester 3 30.97 (23 – 40.1) N = 47	5.87 – 107.6	35.5± 22.36	30.99 (24.9 – 38.3) N = 16	9.05 – 62.07	21.61 ± 12.73	**P = 0.0113 ****

Table 16: **DLK1 levels in normal compared to IUGR pregnancies in the Finnish**

Analysis of DLK1 levels in normal and IUGR pregnancies in both the second and third trimester within the Finnish cohort. There is a significant difference between the two groups in the third trimester (p values in bold) but not in the second. IUGR - intrauterine growth restriction.

3.2.7 DLK1 and IUGR



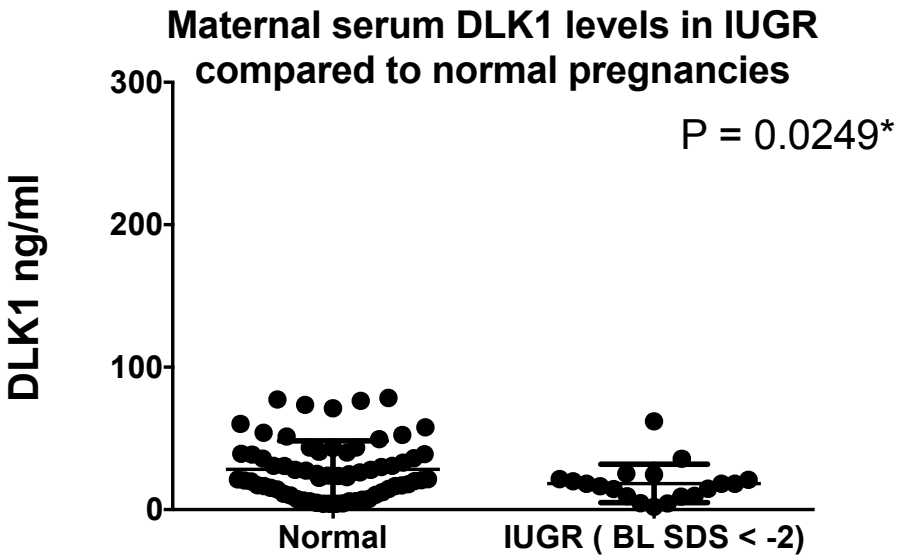


Figure 18: **Birth length and birth weight with serum DLK1 levels in IUGR compared to normal pregnancies** Comparisons are made with both birth weight (top graph) and birth length (bottom). There is a significant difference in DLK1 levels between the IUGR and normal pregnancy groups. BL – birth length; BW – birth weight. Results from the Finnish cohort

Characteristics	IUGR N = 19	Normal N = 50	P value
Age, yrs.	31.26 (□□□□□□□□□□□□□□□)	31.09 (18.95 - 40.91)	0.91
BMI	25.81 (19.38 – 36.05)	23.37 (18.14 - 38.48)	0.12
Gravidity	2 (1-7)	2.86 (1 - 13)	0.1
Gestational age at delivery	35.88 (25.1 – 41.1)	37.38 (24.7 - 42.1)	**0.03 ***
Birth weight (grams)	2031 (550 – 3370)	3113 (690 - 4750)	**< 0.0001 ****
Birth weight SDS	-2.55 (-4.8 - -0.9)	-0.162 (-1.7 - 1.9)	
Birth length	42.62 (30 – 48)	47.85 (31 - 54)	**< 0.0001 ****
Birth length SDS	-2.96 (-4.9 - -1.5)	-0.18 (-1.7 - 2.3)	
Female neonate	7 (37%)	25 (50%)	> 0.9999
Male neonate	12 (63%)	25 (50%)	> 0.9999

Table 17: **Characteristics of IUGR compared to normal birth weight neonates in the Finnish cohort**

Comparison of demographics of the IUGR and Normal groups. Apart from a significant difference in size and a difference in gestational age at birth there was no other significant difference in the characteristics between the IUGR and the normal pregnancy groups. The median is shown with the range in brackets/ number and percentage in brackets as appropriate. This data is from the Finnish cohort

3.2.8 Maternal serum DLK1 levels compared to Estimated Fetal Weight

Variable	Second trimester N = 10	Third trimester N = 34
DLK1 R^2 (95% CI) P value	**0.7161 (0.46 – 0.96)** **0.002****	**0.45 (0.43 – 0.82)** **<0.0001******
Maternal age R^2 (95% CI) P value	0.01813 (-0.70 – 0.54) 0.7108	0.0849 (-0.57 – 0.05) 0.0945
Maternal weight R^2 (95% CI) P value	0.124 (-0.36 – 0.8) 0.3182	0.0637 (-0.10 – 0.54) 0.1565
Maternal Smoking Status R^2 (95% CI) P value	0.0012 (-0.65 – 0.61) 0.9243	

Table 18: **Correlation between DLK1 levels and estimated fetal weight in the second and third trimester within the Finnish cohort.**
Table showing the result of a multiple correlation analysis. A significant correlation is seen when maternal serum DLK1 levels in the second and third trimester are compared to estimated fetal weight. There is no significant correlation between estimated fetal weight and maternal age, weight or smoking status. Of note there were no smokers in the third trimester

3.3 Discussion

These studies confirm previous knowledge about maternal serum *DLK1* levels in pregnancy. I show further that there is a relationship between fetal growth parameters and maternal serum *DLK1* levels, which is seen as an independent variable from as early as 26 weeks gestation onwards. In my aims I set out to explore whether *DLK1* serum levels could give an indication of growth at specific time points in pregnancy. Importantly serum levels from 26 weeks gestation onwards can give an idea of the fetal growth trajectory and thereby identify at risk pregnancies. Interestingly maternal serums *DLK1* levels taken at 32 to 34 weeks gestation are highly predictive of both birth weight and abdominal circumference. This is an important period for the fetus in utero as it is a time of rapid growth. During this period there is a rise in a number of placental hormones including insulin and leptin levels. *DLK1* may interact with some of these placental hormones to effect its mode of action. *DLK1* has been shown to stimulate an increase in insulin producing pancreatic β cells[160] and this can be supported by the peak in insulin levels in late gestation which is similar to the timing of peak *DLK1* levels in gestation. This time point in gestation may involve complex signal mechanisms and interplay between placental and fetal hormones in order to ensure sufficient nutrients for growth. DLK1 levels taken at this gestation can be used to assess the efficiency of this process and detect pathology.

Reduced femoral length can be seen in IUGR. In addition however the effects of *DLK1* on endochondral ossification[161] may contribute to the relationship between DLK1 serum levels and fetal femoral length. Further works including functional studies are required to clarify this proposed mechanism of action. I hypothesised that DLK1 would have a role in regulating long bone growth as seen by serial measurements of femoral length. This has been proven by the data. There is a significant regression (F5.902, P0.022) from as early as 26 weeks gestation. Serial results were useful in determining time points at which DLK1 effects would be most significant. DLK1 is likely to have tissue specific functions that vary depending on the gestational age.

My final aim was to investigate whether DLK1 levels can be used to assess the risk of developing IUGR. Ideally a larger cohort is required to make such an assessment but in any case the result suggest the need for further investigation. When the 5th centile for DLK1 levels at 26 weeks gestation is used as a cut off, RR for IUGR as 14.5 (95% CI, 3.8 – 55.2). This work provides important information about aspects of fetal growth that are likely to be regulated by DLK1. The significant difference seen in maternal DLK1 levels between IUGR and AGA pregnancies within the Finnish cohort confirms further that DLK1 has an important role to play in fetal growth. This larger cohort was important in elucidating the significant difference between the two groups. In addition, I was able to show with this cohort that DLK1 levels were also predictive of EFW. The limitations to using samples not collected prospectively however means that blood sampling was not always taken as the same time as other measures such as ultrasonography for instance, thereby leaving fewer results available for use when performing analysis. In addition unlike the RLH cohort, sampling was not always carried out at a similar gestation each time. There is a need for a larger study carried out prospectively, to allow further interrogation of DLK1 actions in utero. Below is a proposed mechanism for he actions of DLK1 (Figure 17). Other modes of actions are likely, especially via the placenta. DLK1 is likely to exert its effect either directly on the fetus or on the placenta, at different time points in pregnancy. I went on to carry out placenta studies, as the placenta is an important component of the feto-maternal unit.

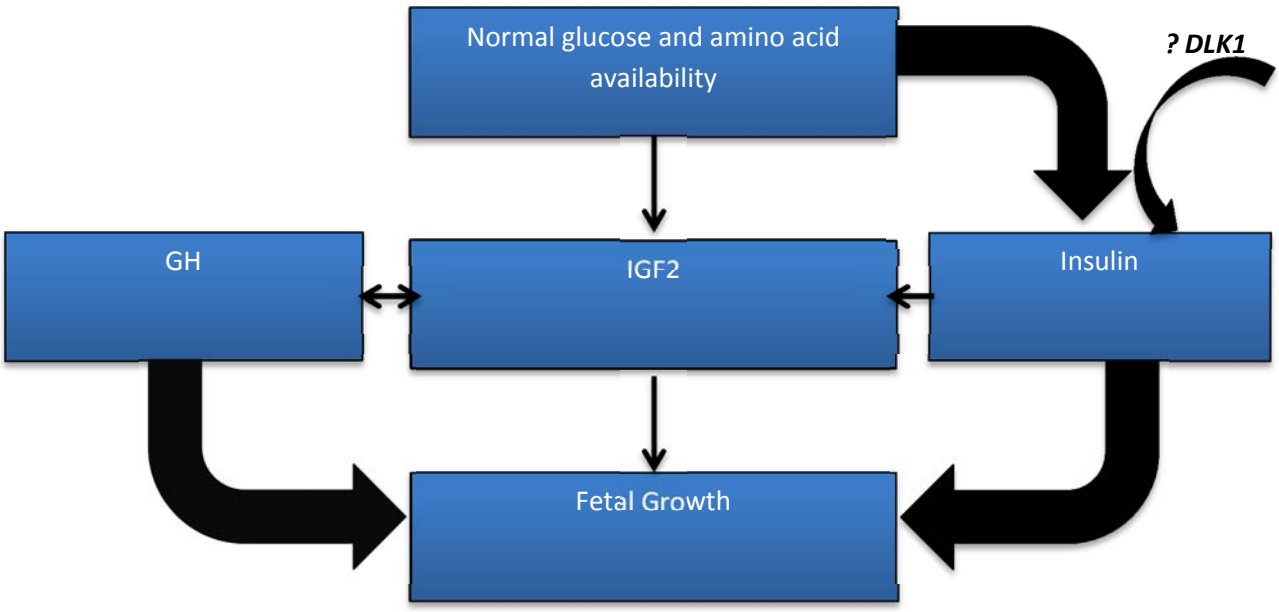


Figure 19: **Proposed mechanism for DLK1 mode of action**

Flow diagram showing factors influencing fetal growth and a proposed mechanism for *DLK1* mode of action. GH – growth hormone. IGF2 – Insulin – like Growth Factor-2

Chapter 4 Results: Placental studies

4.1 Aims of study

In the previous chapter I have shown that DLK1 plays an important role in fetal growth, especially in the third trimester. To further evaluate the functions of DLK1, my aims are:

- To assess the correlation of maternal DLK1 levels at various stages of pregnancy with the placental size and other placental parameters as these placental parameters are surrogate markers for placental function.
- To investigate cells in the placenta that are likely to produce DLK1 by performing immunohistochemistry experiments. The trophoblast layer of cells in the human placenta contains receptors for a number of hormones including leptin, insulin and IGF. DLK1 expression in the trophoblast layer of these cells may suggest a role in trophoblast invasion in addition to other regulatory functions in the placenta during pregnancy.
- To assess whether DLK1 levels are associated with abnormal placental histology findings, particularly those suggestive of maternal vascular malperfusion of the vascular bed.

4.2 Relationship between serum DLK1 levels and placental parameters

Gestational Age (weeks)	**Placental weights**	**Placental Volume**	**Cord Diameter**	**Feto: placental Ratio**
18 – 21	R = 0.92 P = 0.67 N = 24	R = 0.24 P =0.25 N = 24	R = -0.14 P = 0.51 N = 23	R = 0.21 P = 0.34 N = 24
26 - 29	R = 0.26 P = 0.21 N = 25	R = 0.42 **P = 0.04 *** N = 25	R = -0.19 P = 0.38 N = 24	R = -0.10 P = 0.62 N = 25
32 – 34	R = 0.42 **P = 0.035 *** N = 25	R = 0.42 **P = 0.04 *** N = 25	R = -0.17 P = 0.44 N = 24	R = -0.20 P = 0.33 N = 25
37 - 39	**R = 0.70** **P = 0.0006 ***** N = 20	R = 0.53 **P = 0.02 *** N = 20	R = -0.41 P = 0.08 N = 19	R = -0.34 P = 0.14 N = 20

Table 19: **Comparison between DLK1 and placental parameters in the Royal London Hospital cohort**

Table comparing DLK1 maternal serum levels taken at 4 time points to placental weight, volume, cord diameter and feto: placental ratio. Significant p values are highlighted in bold.

To achieve my first aim, a correlation analyses were carried out to access whether there was a significant correlation between placental characteristics and maternal serum *DLK1* levels. There was a positive correlation seen between maternal serum *DLK1* levels and placental weights from 32 weeks gestation onwards. This correlation was seen to be strongly significant (P = 0.0006) at 37 – 39 weeks gestation. Further correlation was seen when serum *DLK1* levels were compared to placental volume. There appears to be a correlation with *DLK1* levels from 26 weeks gestation onwards. These findings suggest a role for *DLK1* in regulating placental function. There was no correlation seen when either cord diameter or feto: placental ratio was compared to serum *DLK1* levels (Table 16)

4.3 Relationship between gross anatomy of placenta and DLK1 levels

Table 20:

Gestational Age (weeks)	Analysis	Cord insertion	Thrombus	Fibrosis	Haematoma
18 – 21	R^2 = 0.291 F = 1.2 N = 24	P = 0.7	P = 0.877	P = 0.705	P = 0.287
26 – 29	R^2 = 0.291 F = 0.77 N = 24	P = 0.845	P = 0.129	P = 0.970	P = 0.822
32-34	R^2 = 0.430 F = 1.414 N = 24	P = 0.472	P = 0.247	P = 0.560	P = 0.134
37-39	R^2 = 0.401 F = 0.838 N = 23	P = 0.66	P = 0.227	P = 0.197	P = 0.686

Comparison between DLK1 maternal serum levels at four time points and placental gross morphology
There was no significant difference seen when DLK1 levels compared with cord position and either the absence or presence of thrombus, fibrosis or haematoma.

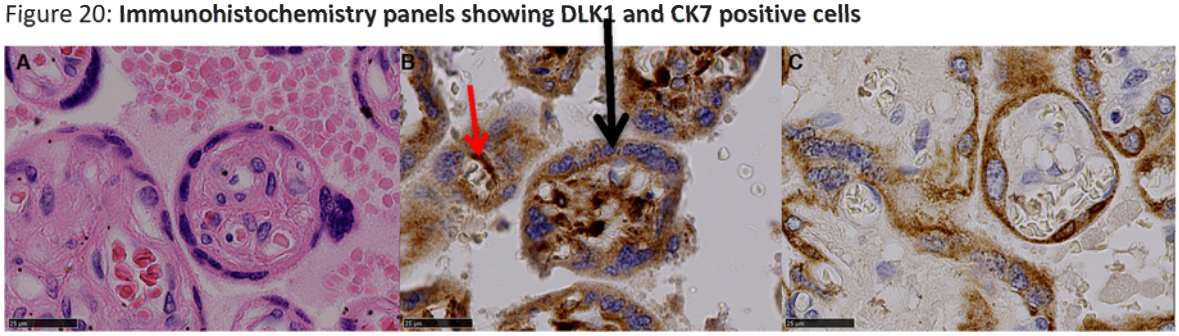
A multiple regression analysis was carried out to assess whether there was a relationship between DLK1 levels at four time points during gestation and gross placental morphology. Cord insertion (central, marginal or peripheral), and the presence or absence of thrombus, fibrosis or haematoma was taken into consideration. There was no significant difference in the DLK1 levels when these variables were taken into consideration. In addition maternal age, parity and ethnicity were taken into consideration at each gestation and these were also found not to influence DLK1 levels and were therefore not confounding factors.

4.2.1 Localisation of *DLK1* expression in human placenta

Having assessed the macroscopic appearance of the placenta, immunohistochemical studies were carried out, to fulfil my second aim. In the first instance, *DLK1* immunostaining was carried out on placenta from our cohort as detailed in chapter 2. The results of this staining can be seen in figure 18. DLK1 is seen as brown staining in the villi (panel B). Staining appears cytoplasmic in the trophoblast layer (black arrow) and dot-like in the fetal endothelial cells (red arrow). An H&E panel is shown for comparison (Panel A). CK7 staining is seen in panel C. CK 7 is a marker for trophoblast cells and confirms the trophoblast staining we see with *DLK1*. This suggests that DLK1 may have a role in regulating secretion of hormones, in addition to regulating other trophoblast functions such as invasion of the myometrium. Staining is shown in the EVT (Figure 19), which displays a pattern suggestive of *DLK1* secretion by these cells.

Western blot analysis carried out in Figures 19 shows the isolation of a 50-kDa protein, which is the soluble form of DLK1. This soluble isoform of DLK1 is likely the more abundant one in the placenta. This may explain some of the functions of DLK1. Further stimulation results possible with placental explants or cell lines may clarify this.

Figure 20: **Immunohistochemistry panels showing DLK1 and CK7 positive cells**



Immunohistochemistry panels showing H& E panel of the villi (A); DLK1 positive cells are seen as the fetal endothelial cells and trophoblast (B); CK7 is seen as positive in trophoblasts,(C)

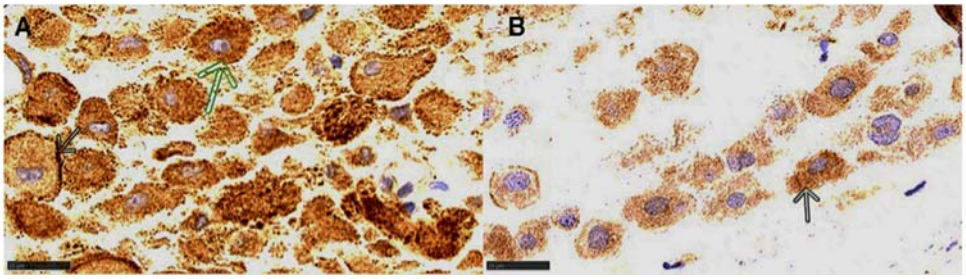


Figure 21: **Extravillous trophoblasts in normal (A) compared to IUGR placenta (B).**

This is from an IUGR placental decidua. Dot like staining is seen in the cytoplasm – black arrow. Dot like staining is seen in the cytoplasm (green arrow) with some membrane staining (black arrow).

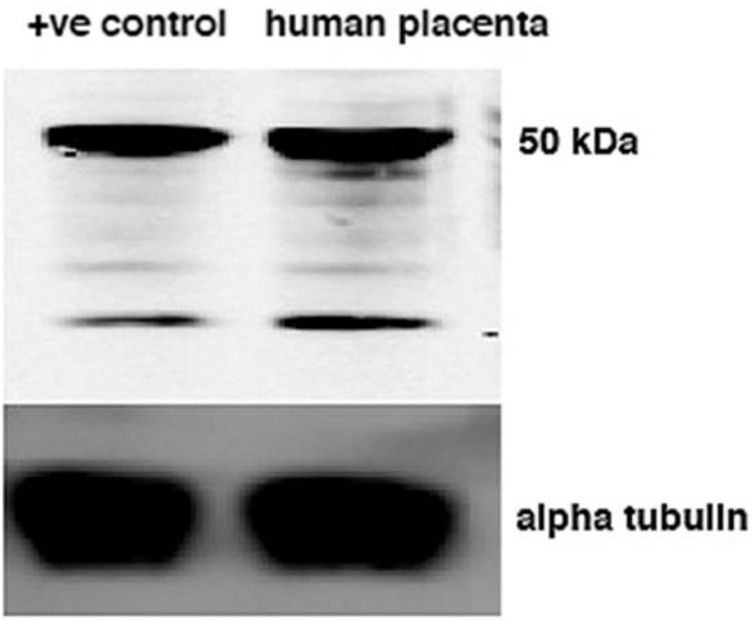


Figure 22: **DLK1 protein expression in the placenta**

DLK1 shown here as a 50kDa protein. Mouse pituitary lysates were used as a positive control.

4.2.2 Intensity of DLK1 immunostaining in comparison to serum levels

Following the initial immunostaining with *DLK1*, comparisons were made between the intensity of staining in the different cell compartments and maternal serum *DLK1* levels, as described in the methods section. There was however no significant difference seen when serum DLK1 levels were compared to intensity of staining. Analysis was carried out using one-way ANOVA.

Further comparisons were made between intensity of staining in fetal endothelial cells as compared to trophoblast cells (Figure 20). Here we see that there is a significant difference in the intensity of staining seen in the two groups of cells. Overall fetal endothelial cells are seen to exhibit a higher intensity of staining. This may provide a clue to the source of *DLK1* being the fetus. Studies have shown that certainly in mice, the source of *Dlk1* is the fetus[143]. Further studies are required on human placenta however as the mouse placenta is different morphologically and may therefore differ in function.

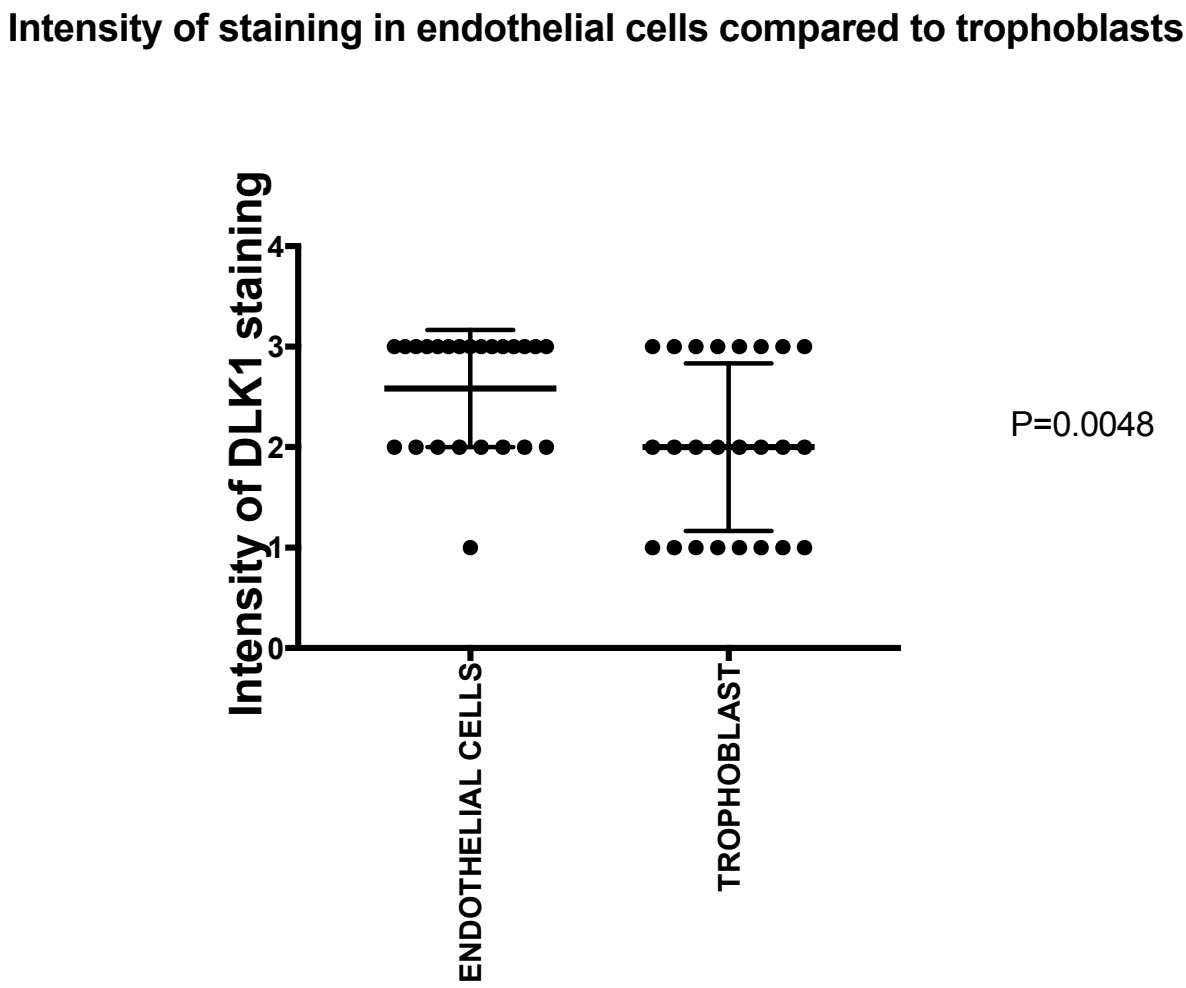


Figure 23: **Staining of DLK1 in fetal endothelial cells compared to trophoblasts**

Graph showing a comparison in DLK1 intensity between fetal endothelial and trophoblast cells. A significant difference is seen in the intensity of staining between the two cell types.

4.2.3 Comparison of histomorphometric findings to serum DLK1 levels

To fulfil my final aim, set criteria were used to review H&E slides of the placenta as described in the methods section. After reviewing the histology, findings were compared to *DLK1* levels at four different time points. There was no significant difference in *DLK1* levels in cases of abnormal villous maturation or signs of inflammation. When findings were compared in cases where clusters of MTGC were seen compared to the normal decidua, at 18-21 weeks there was a significant difference in *DLK1* levels between the two groups (P = 0.0051). A further significant difference is seen at 31-35 weeks gestation (P = 0.0191). There was no significant difference seen in serum levels at either 26-29 weeks or 37-39 weeks gestation.

These suggest that DLK1 has an important function to play in trophoblastic invasion. Other abnormalities of placental bed malperfusion were seen in one of the case with sever IUGR (BD SWS -3.3) and low DLK1 levels all through gestation. There were findings of avascular villi with accelerated maturation. A larger IUGR cohort would allow for evaluation of the predictability of DLK1 in cases of poor placentation/ trophoblastic invasion.

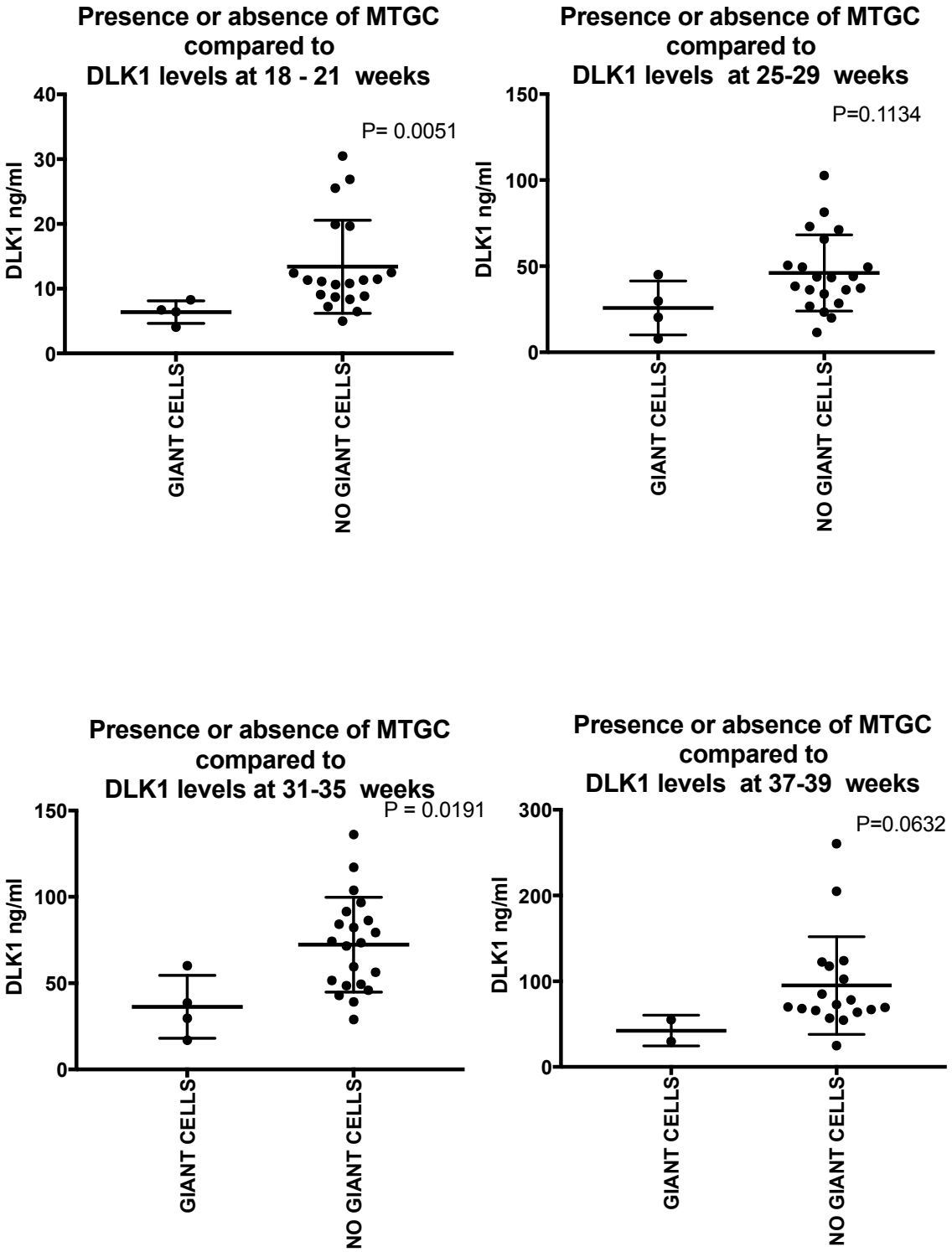


Figure 24: **Comparison of DLK1 serum levels in cases of MTGC compared with normal maternal decidua**

Graphs showing comparisons of maternal serum DLK1 levels between participants exhibiting MTGC in the decidua and those in which MTGC are absent. A significant difference is seen at 18-21 weeks and 31-35 weeks. MTGC – multinucleated trophoblastic giant cells.

4.3 Discussion

The placenta comprises different 3D spaces (cells, syncytium, vascular and extracellular spaces) whose volume reflects compartmentalisation of function at different levels of organisation[162]. From the results we can see in the first instance that maternal serum DLK1 levels in late gestation correlate both with placental weight and volume. The correlation seen between DLK1 levels at 37-39 weeks gestation and placental weight is particularly strong. Placental weight is known to relate to fetal growth with maternal nutrition being one of the factors known to influence placental weight. In my work this correlation is only seen with DLK1 serum levels taken from 37 weeks gestation onwards. Serum levels taken at this gestation can be used to predict placental function in nutrient transfer. We know that nutrient transfer capacity of the placenta depends on its size, morphology, blood flow and availability of nutrient transporters[2]. Despite the close correlation known between placental size and fetal growth, the mediation of the effect of placental weight on associations between prenatal factors is only partial. The pattern of fetal growth is a major determinant of the subsequent health of the infant. *Sferruzi-Peri et al* have showed that in undernourished mice fetal growth is maintained until late pregnancy, despite reduced placental weight, through adaptive up-regulation of placental nutrient transfer[163]. In my work correlation is seen between serum DLK1 levels from 26 weeks gestation onwards and placental volume. This would suggest a further influence of *DLK1* on placental function. Animal studies have shown cases of placental hypoplasia in cases of mUPD12 [164]. *DLK1* is likely to promote villi growth and development, which may be reflected in the placental volume. In addition DLK1 serum levels were found to be associated with fetal growth (EFW) and birth weight in the previous chapter. This effect is most likely due to the influence of DLK1 on placental function. DLK1 may have a role to play in the functioning of nutrient transporters, in addition to other modes on action during gestation. Due to the association of DLK1 serum levels early in gestation with placental volume and not weight until late gestation, DLK1 may have a role to play in some of the adaptive responses of the placenta in optimising nutrient delivery to the fetus. Placental weight is but one of several physical parameters that may predict outcomes, including placental thickness, width, length and cord placement position. These parameters are shown in the appendix.

My immunohistochemistry studies showed DLK1 staining in fetal endothelial and trophoblast cells. The staining in the fetal endothelial cells appears to be more intense when compared to that in trophoblast cells. This may mean, as previously suggested by *Floridon et al*[138], that the source of DLK1 is the fetus and that DLK1 is transported from the fetal endothelial cells to the trophoblasts ,

via the polarised membrane (BM) as above prior to entering the maternal circulation via the MVM. Further experiments including *In Situ* hybridisation studies are required in order to ascertain mRNA expression of *DLK1*.

From the histological studies we see that a strongly significant difference was seen when serum *DLK1* levels at 18-21 weeks were taken into consideration in comparing cases in which MTGC were seen as opposed to those with normal extravillous trophoblasts in the maternal decidua. MTGC are seen at the site of implantation in the myometrium as a normal occurrence, in early gestation[165]. They are fused extravillous trophoblast cells that have lost the ability to invade. MTGC presence is inversely related to gestation with numbers peaking at the turn of the second and third trimester with a decline in numbers after that. The presence of MTGC at term is associated with abnormal implantation and poor placental perfusion. These results would imply that a serum DLK1 level taken as early as 18 weeks gestation can give an indication of how efficient the process of implantation has been in addition to elucidating the effectiveness of placental perfusion. DLK1 may have a role to play in the invasive processes seen during placentation and may influence placental perfusion and ultimately fetal growth. Placental insufficiency was a feature in all our cases of IUGR as detected by Doppler studies antenatally.

DLK1 may therefore play a role in the process of implantation with serum levels at 18 weeks gestation being able to give an indication as to whether invasion of the myometrium is appropriate or shallow, resulting in poor placental function ,by regulating signalling pathways involved in trophoblast invasion. Furthermore, in view of the correlation seen with placental weight and volume, there is likely to be a further role in nutrient transport within the placenta later in gestation. Finally from the previous chapter we see an association between DLK1 levels and FL suggesting a role for *DLK1* in longitudinal growth. These functions need to be explored with further experiments including functional studies and *In Situ* hybridisation. We can however begin to appreciate that the effects of *DLK1* in utero are far reaching and therefore measuring circulating levels of this protein can provide us with information about the intrauterine conditions, depending on timing of the samplings. Further studies required are discussed in the next chapter.

Chapter 5: General Discussion

5.1 DLK1 levels rise in late gestation

I embarked on this work to demonstrate that DLK1 has an important role to play in intrauterine growth and development in man, and that this role may vary depending on the stage of pregnancy. In late gestation, the rise in *DLK1* levels is also seen to coincide with a time for rapid growth for the fetus. It appears that *DLK1* has functions both in early and late gestation. Early gestation functions appear to focus on placentation whilst in late gestation; *DLK1* may regulate nutrient transport and in addition interact with placental hormones to allow for adaptation at this gestation. Although *DLK1* levels were found to be undetectable before 17 weeks gestation[138], serum levels may not be completely reflective of amounts being secreted by the fetus and or placenta. It would be useful to measure urine levels at this stage and calculate creatinine clearance of DLK1 in order to better understand its function in early gestation. Renal clearance tends to increase in early gestation as an adaptive process to the changes occurring to the vasculature and increased circulating volume of blood. Creatinine clearance would therefore give us a more accurate measure of *DLK1* concentration.

5.2 DLK1 regulates femoral length and abdominal circumference growth particularly in late gestation

I have shown a relationship between DLK1 and fetal growth parameters, specifically abdominal circumference and femoral length. In chapter 3 we saw that there was an association between DLK1 serum levels and FL from as early as 26 weeks gestation. Floridon *et al* have shown that a small number of resting chondrocytes within epiphyseal discs of the human embryo remain positive for DLK1 throughout gestation when immunohistochemistry studies were carried out[138]. We know that the epiphyses of long bones are an important site of endochondral ossification where a cartilage cast is invaded by mesenchymal progenitors, which leads to the appearance of a chondrocyte-enriched growth plate that allows for longitudinal bone growth. The secreted DLK1 may have a role in osteogenesis via the MAPK pathway whilst the membrane bound *DLK1* may have a direct influence on ossification. Both processes result in the growth of long bones, of which the femur is an example, and may explain the positive correlation seen between serum *DLK1* levels and FL. Knockout studies in animals have shown skeletal abnormalities in *Dlk1* null mice further supporting a role for *DLK1* in skeletal development[119]. In addition patients with TS are seen to have abnormal skeletal development. Phenotypes of TS described, which suggest abnormal musculoskeletal development, include scoliosis, small hands, frontal bossing with a broad forehead and hyperextensible joints[124].

These patients have also been described as having delayed motor milestones particularly walking which may however also be influenced by muscle weakness rather than solely by skeletal development. This may mean that in cases where *DLK1* levels are low during pregnancy, the resulting neonates may require follow up to ensure normal bone health as we know that apart from locomotion and organ protection, skeletal functions include glucose and adipose metabolism in addition to phosphate renal clearance and maintenance of the haematopoietic niche. In addition a better understanding of *DLK1* mechanisms of action may help prevent potential skeletal abnormalities in pregnancies identified to have low *DLK1* levels.

The significant correlation seen between maternal serum *DLK1* levels and abdominal circumference from 32 weeks gestation onwards is likely to be reflective of placental adaptations in late gestation. As mentioned previously in chapter 1, placental growth slows during the last half of gestation but the placenta adapts in order to provide for the growing fetus. One of the ways in which the placenta adapts is by a change in morphology. It is likely that nutrient transporters, particularly GLUT1, are up regulated by DLK1 leading to an increase in abdominal circumference. In addition, serum levels of one of the placental hormones, leptin, is seen to rise during gestation with a peak in late second to early third trimester, thereby remaining stable until labour[166]. We can see from our results, in chapter 3, that the correlation between abdominal circumference and DLK1 is highly significant ($P = 0.0004$) at 32-34 weeks, compared to 37-39 weeks ($P = 0.025$). The earlier gestation probably corresponds to the leptin peak. DLK1 is likely to have a role in regulating leptin secretion by the placenta in order to optimise fetal growth in mid to late gestation. The protein has been shown to regulate leptin secretion in adipocytes cells[110] and may therefore regulate placental leptin. This proposed mechanism of action requires further work with respect to functional studies. Neonates born as a result of IUGR particularly if secondary to placental insufficiency are seen to have reduced abdominal circumferences. An appreciation of the relationship between DLK1 and abdominal circumference, which is likely to involve leptin, may allow a reduction in some of the complications seen in neonates with IUGR. Abdominal circumference is seen as the most sensitive measure of fetal growth and this correlation with *DLK1* may signify the overall influence of DLK1 on fetal growth, making it a potential biomarker for IUGR.

5.3 DLK1 is predictive of birth weight from 26 weeks gestation onwards

I have in addition shown that *DLK1* can predict birth weight from 26 weeks gestation onwards from multiple regression analyses. *DLK1* is likely to exert its effect directly, on the growing fetus, in addition to indirectly, through actions on the placenta (Figure 22). The impact of DLK1 on fetal growth is likely to be secondary to interplay between a numbers of factors. The effects of DLK1 are

likely to vary through gestation as the rate of growth and the needs of the fetus fluctuate. Further studies including immunohistochemical experiments with co-localisation within the placenta of DLK1 and other placental hormones will offer an explanation for the changes seen in the various fetal growth parameters in relation to DLK1 maternal serum levels.

5.4 DLK1 regulates placental function

I then went on to carry out placental studies, which have also yielded interesting results. We know from animal knock out studies that the *Dlk1* null mice are seen to have abnormal placentas, which are hypoplastic and also exhibit abnormal vasculature. In the first instance we saw a correlation between maternal serum *DLK1* levels from 32 weeks gestation onwards and placental weight at term. This correlation from 32 weeks gestation onwards is also likely to be secondary to adaptive changes by the placenta as described above, such as an up regulation of nutrient transporters. Glucose is the principle energy substrate for the placenta and fetus. Placenta glucose transport is mediated primarily by GLUT1, found at both the intervillous space facing MVM and at the fetal facing BM and by GLUT3 found on the MVM only. It would be useful to look at the distribution of nutrient transporters at different stages during gestation and compare this to maternal serum DLK1 levels. DLK1 may affect nutrient transport by directly binding to the transporters or may produce posttranscriptional changes thus influencing the function of nutrient transporters in that way. There appears to be a highly significant correlation between maternal serum *DLK1* serum levels from 37 weeks gestation onwards and placental weight, supporting the suggestion that *DLK1* has a role in the functioning of the placenta. The difficulties of obtaining human placenta before term makes looking at the direct influence of maternal serum *DLK1* on placental weight at earlier gestations challenging. Our initial results however suggest conceptions for further avenues of investigation. In addition if *DLK1* levels are seen to be low, it may trigger further assessment of the placenta for example Doppler flow measurements to access placental efficiency. Maternal serum levels would identify those pregnancies that may need closer surveillance. Furthermore, we have shown a positive correlation between maternal serum DLK1 levels and placental volume at term. This correlation is seen with maternal serum levels from as early as 26 weeks gestation. This earlier correlation compared to that seen with placental weight suggests that other physical parameters of the placenta other than weight can predict function. The placenta volume is reflective of the surface area of the placenta, which is important for the function of trophoblasts in the villi. In addition the depth of the placenta for instance can provide evidence of the efficiency of implantation and

development of placental vasculature. It would be useful to carry out further studies with serial placental volume estimation by ultrasonography.

5.5 DLK1 is secreted by fetal endothelial and extravillous trophoblastic cells

Immunohistochemistry studies revealed *DLK1* staining in a secretory population of placental cells. In addition, demonstration of the protein in the placenta via western blots further confirms the presence of *DLK1* in these tissues. The identification of the protein as a 50-kDa protein suggests that the *DLK1* found in maternal serum is the soluble isoform. The dot-like staining seen suggests secretion and extracellular transport of *DLK1* by the fetal endothelial cells and EVT. In addition, the cytoplasmic staining seen may suggest posttranslational influences by DLK1 in trophoblast cells. There is a flow of substances from the fetus (fetal endothelial cells) through the BM on the ST and MVM before being transported into the maternal circulation, which is in direct contact with the MVM. The converse is true with transport of substances occurring from the maternal circulation to the fetus. The staining seen in the EVT also suggests that these cells are involved in secretion and extracellular transport of DLK1. DLK1 may have a role to play in trophoblast invasion.

5.6 Low DLK1 levels are associated with signs of maternal vascular malperfusion in the placenta

This is supported by the significant difference seen in maternal serum levels associated with placentas showing an increase in MTGC in the decidua when compared to placentas with normal decidual histology. MTGC are seen in normal placental development during implantation. The number of MTGC are however inversely proportional to gestation. Numbers are high during the first trimester but reduce as gestation advances. As the placenta continues to provide for the needs of the growing and developing fetus, the EVT need to continue their functions of invasion and remodelling of the spiral arteries in order to reduce the resistance of the maternal vessels. The MTGC are fused EVT that have lost the ability to invade. A higher number seen at term suggests poor invasion by the EVT. This in turn would give rise to lack of remodelling of the uterine blood vessels, which can affect blood flow to the fetus. We have seen how DLK1 has an effect on placental weight and volume, which indirectly suggests an influence on placental function. An increased number of MTGC in late gestation can result in poor Doppler flow studies as seen in IUGR. This was certainly the case in our participants with IUGR and low maternal DLK1 serum levels. It would be useful to analyse serum DLK1 levels in the first trimester at a point when interventions can be taken to improve vascularisation. Currently aspirin is commenced in high-risk pregnancies as dictated by previous

obstetric history and co existing medical conditions. The IUGR fetus needs early diagnosis and management in order to minimise neonatal and perinatal mortality. Presence of MTGC at term can be compared to serum DLK1 levels at this early gestation in order to assess the predictive value of DLK1 in abnormal invasion at this gestation. Our cohort underwent blood sampling at 12 weeks as part of the follow-up carried out by our obstetric collaborators. In the first instance these samples can be obtained and ELISA studies carried out for DLK1. A significant difference between the IUGR and AGA groups can for the first time provide an early marker for abnormal implantation.

5.7 Limitations of study

Gestation has been shown to influence *DLK1* concentration. A small cohort such as the RLH cohort means that the influence of gestation, however small may limit experiments. Further work is warranted particularly prospective studies, as these allow for more accurate and focused data collection. A larger cohort may therefore reinforce some of my findings. In addition, some of the immunohistochemistry studies may have more weight with a larger cohort and further allow us to associate pathology with *DLK1* levels.

5.8 Proposed mechanism of action of DLK1

A proposed mechanism of DLK1 action is seen below (Figure 22). Overall this work has shown important and novel associations between DLK1 and intrauterine growth. The prospective nature of our data collection allowing for serial measurements of parameters gives this work strength. Further work however needs to be carried out to understand the mode of action of this molecule. In particular, the identification of sequence or epigenetic variants may allow for genetic screening in cases where DLK1 levels are found to be low. If epigenetic variants are found, larger studies including pregnancies complicated by smoking, alcohol and other substance ingested during pregnancy can be reviewed to assess whether there is an effect on DLK1 expression at a genetic level in these cases.

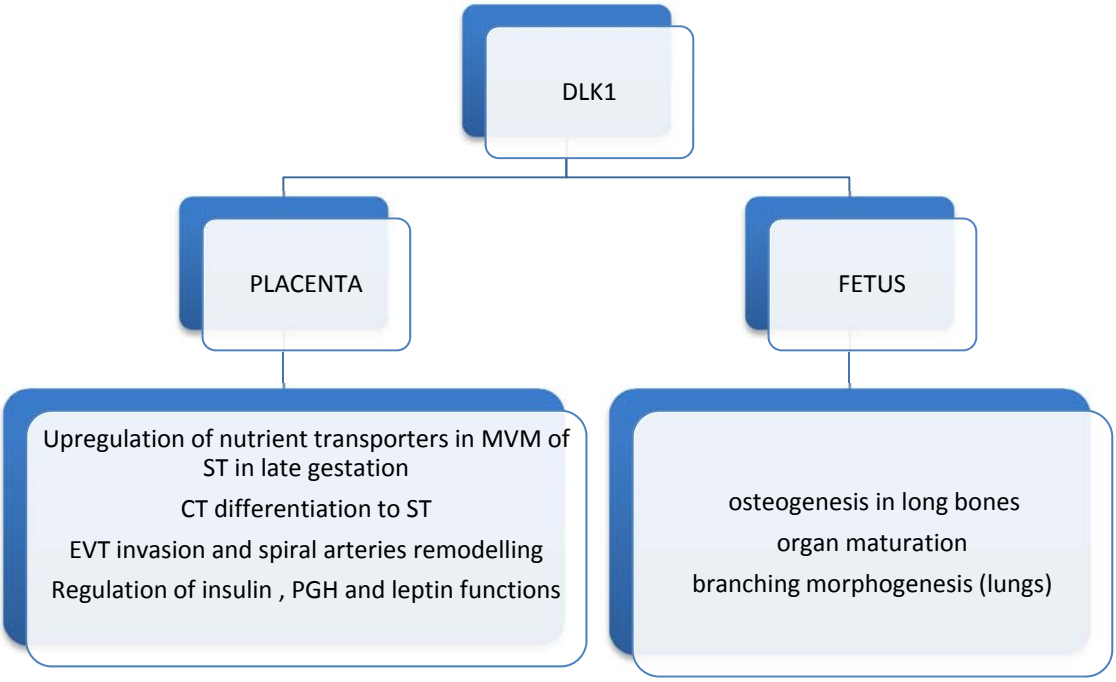


Figure 25: **Proposed mechanism for DLK1 mode of action**

Proposed mechanism of action of DLK1 in intrauterine growth. Flow diagram showing the proposed mechanism of action of DLK1 in influencing fetal growth either directly on the fetus or indirectly via effects on the placenta.

Chapter 6: Further studies informed by the current results

6.1 Introduction

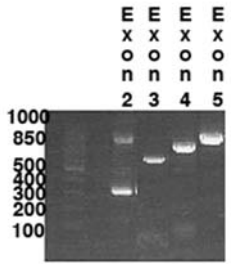
The exact mechanism of action of *DLK1* protein is not well understood. We have shown DLK1 staining in the placenta specifically in fetal endothelial cells and trophoblast cells. It is however not clear whether *DLK1* is synthesised by the placenta or the fetus, in human pregnancies. Given the staining pattern we have seen in the trophoblast group of cells however there may be an element of synthesis both within the placenta and the fetus. This is likely to change during gestation however and may also be under the control of various fetal/ placental signals. An understanding of the source of DLK1 will allow a better appreciation of the proposed mechanism of action, which can further provide a target for intervention or allow for closer and more accurate monitoring during pregnancy. In addition, some developmental studies to detect DLK1 staining and relate this to serum samples taken at the same time can further elucidate the function of this protein. Work on nutrient transporters within the placenta in addition to metabolic studies would be helpful in understanding the actions of DLK1 in the placenta. Below we describe proposed further work to support current findings.

6.2 Are there genetic or epigenetic variants seen in cases of low DLK1 levels?

Given my initial results, it would be useful to assess whether the changes in DLK1 levels during gestation and with pathology were associated with changes in the genome. *DLK1* primers were designed and optimised as described in chapter 2. It was possible to optimise primers for 4 out of the 5 exons of DLK1 (Exon 2 – 5) (Figure 23). Fetal DNA was extracted from umbilical cords of our participants but good signals were only yielded on sequencing PCR products from exon 4 however. The experiment was abandoned due to time constraints but it would however be helpful to pursue this further with a view to designing robust primers for the experiments.

Further work is required to assess the presence sequence variants in the *DLK1* coding region that commonly associate with low maternal serum DLK1 and if the levels of DNA methylation at the imprinting control region or other well described regulatory regions correlate with alterations of DLK1 in maternal serum. Our group have extensive experience of epigenetic profiling within this region in the mouse, and robust assays for measuring methylation at the chromosome 14 imprinting control regions in the human have been published. These studies would provide an additional level of mechanistic information about variation in *DLK1* dosage, as well as potentially identifying epigenetic/genetic markers for IUGR.

Figure 26: **DLK1 PCR products for Exon 2 – 5**



Bands corresponding to the size of the primers seen with exon 2,3 4 and 5 respectively. Ladder is seen on the left with corresponding sizes.

Differential expression of a number of imprinted genes including DLK1 has been shown in cases of IUGR[102,104]. Gene expression at the DLK1-DIO3 imprinting cluster is regulated at multiple levels. These regulatory mechanisms can take place both during transcription and post transcription. Transcription generates RNA strands ranging from long to short non-coding RNAs (ncRNA). These include microRNAs, piwi interacting RNAs, small nucleolar RNAs and long intergenic RNAs. MicroRNAS function as guide molecules in post transcriptional gene regulation by base pairing with target mRNAs, usually in the 3' UTR[167]. Certain non-coding microRNAs have been identified to be perturbed in both human and animal studies[167–170]. The DLK1-DIO3 genomic region hosts one of the largest microRNA clusters in the genome[171]. Many of these microRNAs are differentially expressed in severe pathological processes and cancer[168,172–175]. The microRNAs can be studied by obtaining RNA from tissue samples from our cohort stored in RNA later. Further expression in this cluster can be regulated at the two DMRs as mentioned in chapter 1. Methylation studies to detect the presence of differential methylation in the DMRs between our two groups within our cohort may provide useful information. For both experiments, it would be useful to match cases and controls, rather than performing these experiments on the whole cohort. It would be useful to choose abnormal cases with low DLK1 levels and IUGR with a finding of MTGC clusters in the maternal decidua. Controls will be of matched gestation. Testing if there are sequence variants in the DLK1 coding region that commonly associate with low maternal serum DLK1 and if the levels of DNA methylation at the imprinting control region or other well described regulatory regions correlate with alterations of DLK1 in maternal serum would provide further information. These studies will provide an additional level of mechanistic information about variation in DLK1 dosage, as well as potentially identifying epigenetic/genetic markers for IUGR.

6.3 Which cell types/ compartments are involved in the synthesis of DLK1?

Immunohistochemistry studies have shown DLK1 staining in the fetal endothelial cells and trophoblast layers. Positive staining in the trophoblast layer of cells appeared to include the CT, ST and EVT. When staining is looked at in more detail we see that the staining in the fetal endothelial cells is cytoplasmic and dot like. This appearance is suggestive of the peri-nuclear Golgi zone and would indicate an active DLK1 synthesis within these cell types. In the trophoblast cells DLK1 staining is also cytoplasmic but seen to be uniform. This may suggest an important regulatory function. *In situ* studies would be useful in clarifying sites of mRNA expression and comparing these with areas of localisation by immunohistochemistry studies. A midi prep is available allowing for construction of a DLK1 labelled probe, which can then be used to identify mRNA expression within placental cells.

6.4 Which cells express DLK1 in early gestation and how does this change during pregnancy?

I would be useful to carry out equally robust immunochemistry studies on early gestation placenta, in order to better understand events at these gestations. *In situ* studies on these placentas would be useful in ascertaining mRNA expression of *DLK1* through gestation. It would be useful to obtain tissue from 18, 28 and 34 weeks gestation in addition to some earlier gestation tissue, in order to compare DLK1 expression to our already existing serial data collected from our cohort at these gestations.

6.5 How does DLK1 carry out its function?

DLK1 appears to have an important function in intrauterine growth. Whereas DLK1's impact on tissue and organism development is substantial, the mechanism behind its underlying function is poorly understood. Clarifying the mode of action of this molecule would be valuable particularly if this molecule proves useful as a biomarker in pregnancy. DLK1 function is likely to be dependent on cell type. The effects in pregnancy as suggested by the results seen in chapter 3 and 4 are likely to be achieved through action on the placenta and also directly on fetal cells. The most characteristic placental cells are the trophoblasts. These cells have different phenotypes and as described earlier have roles in hormone secretion, nutrient transport in addition to maternal decidua invasion. In the first instance, co-localisation of DLK1 with nutrient transporters (e.g. GLUT1 and system A amino acid transporters) and in addition insulin, leptin and placental growth hormone may help elucidate a mode of action for DLK1 in intrauterine growth. This can be achieved by immunohistochemistry studies on placental histology slides. Secondly, stimulation studies can be carried out using human

placental explants, isolated primary human CT or cell lines such a choriocarcinoma derived BeWo (mimic in vivo functions of CT) or HTR8/SVneo (transformed EVT cell line)[176]. The respective cell lines can undergo treatment with varying concentrations of DLK1 (using the different isoforms including full length, soluble and intracellular forms), after which the amount of protein (western blots) and quantity (mRNA via RT-PCR) can be assessed. Further experiments can be carried out to assess the influence of DLK1 on the endocrine functions of the placenta. We know that the CT, ST and EVT secrete various placental hormones with important functions in fetal growth, maternal spiral artery remodelling and trophoblast cell invasion. Abnormalities in these processes can lead to IUGR. Cell lines can be treated with varying concentrations of DLK1 after which enzyme immunoassays can be used to measure production of the important placental hormones such as Leptin, the IGFs and Insulin. Furthermore, cells in both explant cultures and cell cultures from the HTR8/SVneo cell line can be used to assess a role for DLK1 in trophoblast invasion using cell invasion and migration assays[176].

These experiments can be further supported by maternal serum insulin, IGF1 and leptin levels in order to further access the influence of these hormones if any, on the function of DLK1. Further potential modes of action of DLK1 are supported by my immunohistochemistry findings. In Figure 23 DLK1 staining is seen in the BM of the ST. In addition there is uniform cytoplasmic staining. Dot-like staining is seen in the fetal endothelial cells.

DLK1 is highly homologous to NOTCH ligands and participates in multiple developmental processes in which also NOTCH receptors and ligands are known to play pivotal roles[177]. The five known canonical activating NOTCH ligands were previously thought to interact with their receptors via a DSL domain. Emerging evidence suggests however that non-canonical ligands such as DLK1 also exist[178,179]. These studies suggest that NOTCH is responsible for DLK1 function during development but DLK1 may also work through NOTCH – independent mechanisms depending on the tissue or cell type. They also suggest that DLK1 may interact with itself to produce effects. Once a proposed mechanism of action is arrived at from studies on cell cultures of both primary cells and cell lines as mentioned above, the signalling pathways thought to produce DLK1 effects can be interrogated further.

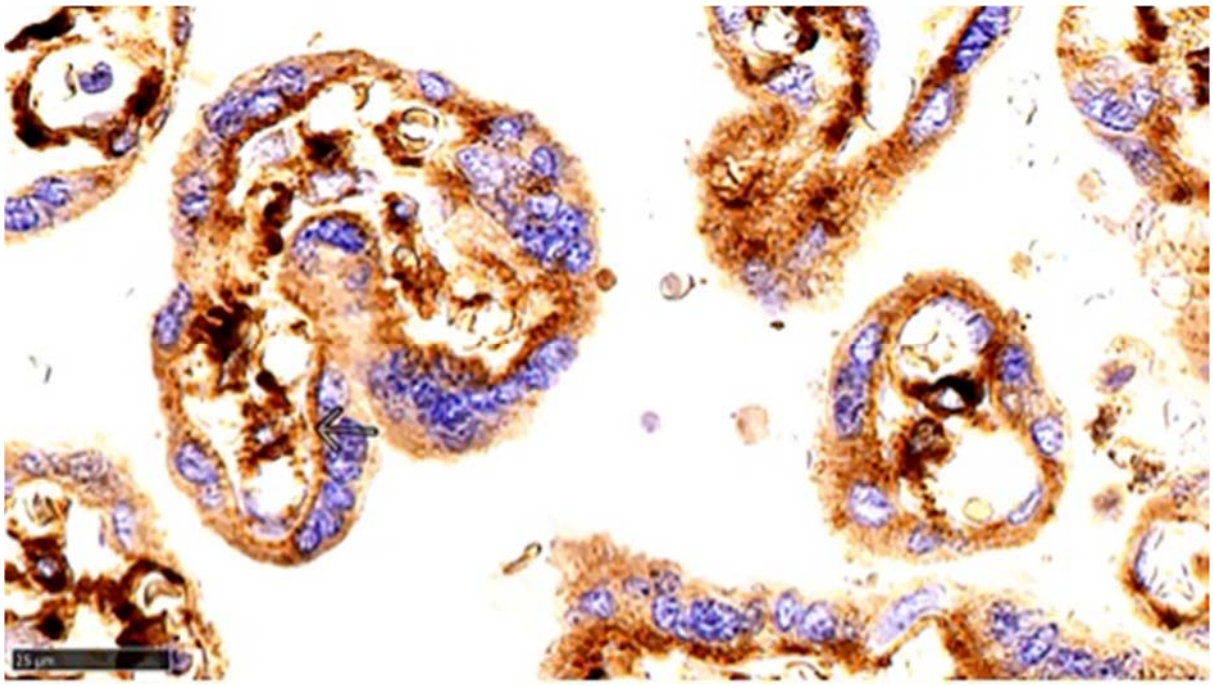


Figure 27: **DLK1 staining in term villi**

Dot-like staining is seen on the BM of the ST (black arrow) with additional uniform cytoplasmic staining of the ST. ST - syncytiotrophoblast

Chapter 7: Conclusions

I have shown that DLK1 has an important role to play in fetal growth. Emerging work particularly animal studies have confirmed this with more recent studies suggesting that the source of DLK1 in pregnancy is the fetus, at least in rodents as shown by *Cleaton et al*[143]. The influence on growth may be through DLK1's effect on intrauterine nutrient supply and signalling. Rather than just be the effect of one protein, it is likely that the effects on growth are secondary to an interaction between DLK1 and IGF2, amongst other hormones. Further exploration of the association between DLK1 and autoimmunity is warranted particularly due to the identified link with type 1 DM[83]. There have been suggestions that there is an association between genomic imprinting and autoimmune disease, which may explain the role of DLK1 in this setting. Either way DLK1 is an important molecule that appears to have various roles and therefore further work is warranted to improve understanding of this multifaceted molecule. An understanding of its role particularly in utero may result in prevention or better management of intra uterine pathology that we know can have far reaching effects well into adult life.

References

1. Barker DJP. Developmental origins of adult health and disease. *J Epidemiol Community Health*. 2004;58(2):114-115. doi:10.1136/JECH.58.2.114.

2. Fowden AL, Ward JW, Wooding FPB, Forhead AJ, Constancia M. Programming placental nutrient transport capacity. *J Physiol*. 2006;572(Pt 1):5-15. doi:10.1113/jphysiol.2005.104141.

3. Carter AM. Evolution of factors affecting placental oxygen transfer. *Placenta*. 2009;30 Suppl A:S19-25. doi:10.1016/j.placenta.2008.11.006.

4. Jansson T, Ylvén K, Wennergren M, Powell TL. Glucose Transport and System A Activity in Syncytiotrophoblast Microvillous and Basal Plasma Membranes in Intrauterine Growth Restriction. *Placenta*. 2002;23(5):392-399. doi:10.1053/plac.2002.0826.

5. Glazier JD, Cetin I, Perugino G, et al. Association between the Activity of the System A Amino Acid Transporter in the Microvillous Plasma Membrane of the Human Placenta and Severity of Fetal Compromise in Intrauterine Growth Restriction. *Pediatr Res*. 1997;42(4):514-519. doi:10.1203/00006450-199710000-00016.

6. Jansson T, Ekstrand Y, Björn C, Wennergren M, Powell TL. Alterations in the activity of placental amino acid transporters in pregnancies complicated by diabetes. *Diabetes*. 2002;51(7):2214-2219. http://www.ncbi.nlm.nih.gov/pubmed/12086952. Accessed August 20, 2017.

7. Baumann MU, Schneider H, Malek A, et al. Regulation of human trophoblast GLUT1 glucose transporter by insulin-like growth factor I (IGF-I). *PLoS One*. 2014;9(8):e106037. doi:10.1371/journal.pone.0106037.

8. Baumann MU, Deborde S, Illsley NP. Placental glucose transfer and fetal growth. *Endocrine*. 2002;19(1):13-22. doi:10.1385/ENDO:19:1:13.

9. Lager S, Powell TL. Regulation of nutrient transport across the placenta. *J Pregnancy*. 2012;2012:179827. doi:10.1155/2012/179827.

10. Sibley CP. Understanding placental nutrient transfer--why bother? New biomarkers of fetal growth. *J Physiol*. 2009;587(Pt 14):3431-3440. doi:10.1113/jphysiol.2009.172403.

11. Sandovici I, Hoelle K, Angiolini E, Constância M. Placental adaptations to the maternal-fetal environment: implications for fetal growth and developmental programming. *Reprod Biomed Online*. 2012;25(1):68-89. doi:10.1016/j.rbmo.2012.03.017.

12. Lobo SE, Leonel LCPC, Miranda CMFC, et al. The Placenta as an Organ and a Source of Stem Cells and Extracellular Matrix: A Review. *Cells Tissues Organs*. April 2016. doi:10.1159/000443636.

13. Agarwal I, Karumanchi SA. Preeclampsia and the anti-angiogenic state. *Pregnancy Hypertens An Int J Women's Cardiovasc Heal*. 2011;1(1):17-21. doi:10.1016/j.preghy.2010.10.007.

14. LEACH L. Placental Vascular Dysfunction in Diabetic Pregnancies: Intimations of Fetal Cardiovascular Disease? *Microcirculation*. 2011;18(4):263-269. doi:10.1111/j.1549-8719.2011.00091.x.

15. Higgins M, Felle P, Mooney EE, et al. Stereology of the placenta in type 1 and type 2 diabetes. *Placenta*. 2011;32(8):564-569. doi:10.1016/j.placenta.2011.04.015.

16. Reshetnikova OS, Burton GJ, Milovanov AP. Effects of hypobaric hypoxia on the fetoplacental unit: the morphometric diffusing capacity of the villous membrane at high altitude. *Am J Obstet Gynecol*. 1994;171(6):1560-1565. http://www.ncbi.nlm.nih.gov/pubmed/7802068. Accessed August 9, 2017.

17. Reshetnikova OS, Burton GJ, Teleshova O V. Placental histomorphometry and morphometric diffusing capacity of the villous membrane in pregnancies complicated by maternal iron-deficiency anemia. *Am J Obstet Gynecol*. 1995;173(3 Pt 1):724-727. http://www.ncbi.nlm.nih.gov/pubmed/7573233. Accessed August 9, 2017.

18. Fowden AL, Forhead AJ. Endocrine mechanisms of intrauterine programming. *Reproduction*. 2004;127(5):515-526. doi:10.1530/rep.1.00033.

19. Gluckman, PD; Milner R. *Regulation of Intrauterine Growth. In: Pediatrics and Perinatology. The Scientific Basis, Ed 2*. London: Arnold; New York: Oxford University Press; 1996.

20. Kao PC, Matheny AP, Lang CA. Insulin-like growth factor-I comparisons in healthy twin children. *J Clin Endocrinol Metab*. 1994;78(2):310-312. doi:10.1210/jcem.78.2.8106617.

21. Fowden AL. The insulin-like growth factors and feto-placental growth. *Placenta*. 24(8-9):803-812. http://www.ncbi.nlm.nih.gov/pubmed/13129676. Accessed August 20, 2017.

22. Strange KS, Wilkinson D, Edin G, Emerman JT. Mitogenic Properties of Insulin-Like Growth Factors I and II, Insulin-like Growth Factor Binding Protein-3 and Epidermal Growth Factor on Human Breast Stromal Cells in Primary Culture. *Breast Cancer Res Treat*. 2004;84(2):77-84. doi:10.1023/B:BREA.0000018384.64326.dd.

23. Kilby MD, Barber K, Hobbs E, Franklyn JA. CURRENT TOPIC Thyroid Hormone Action in the Placenta. doi:10.1016/j.placenta.2004.08.004.

24. FRANKENNE F, CLOSSET J, GOMEZ F, SCIPPO ML, SMAL J, HENNEN G. The Physiology of Growth Hormones (GHs) in Pregnant Women and Partial Characterization of the Placental GH Variant*. *J Clin Endocrinol Metab*. 1988;66(6):1171-1180. doi:10.1210/jcem-66-6-1171.

25. Eriksson L. Growth hormone in human pregnancy. Maternal 24-hour serum profiles

and experimental effects of continuous GH secretion. *Acta Obstet Gynecol Scand Suppl*. 1989;147:1-38. http://www.ncbi.nlm.nih.gov/pubmed/2728860. Accessed August 27, 2017.

26. Costa MA. The endocrine function of human placenta: an overview. *Reprod Biomed Online*. 2016;32(1):14-43. doi:10.1016/j.rbmo.2015.10.005.

27. von Versen-Höynck F, Rajakumar A, Parrott MS, Powers RW. Leptin affects system A amino acid transport activity in the human placenta: evidence for STAT3 dependent mechanisms. *Placenta*. 2009;30(4):361-367. doi:10.1016/j.placenta.2009.01.004.

28. Brett, Elizabeth Kendra; Ferraro, Zachary Michael; Yockell-Lelievre, Julien; Guslin, Andrée; Adamo KB. Maternal–Fetal Nutrient Transport in Pregnancy Pathologies: The Role of the Placenta. *Int J Mol Sci*. 2014;15(19):16153-16185.

29. Briffa, Jessica; O'Dowd, Rachael; Moritz, Karen M; Romano, Tania; Jedwab, Lisa R; McAInch, Andrew J; Hryciw, Deanna H; Wlodek ME. Uteroplacental insufficiency reduces rat plasma leptin concentrations and alters placental leptin transporters: ameliorated with enhanced milk intake and nutrition. *J Physiol*. 2017;595(11):3389-3407.

30. Laivuori, H; Gallaher, M.J; Collura, L; Crombleholme, W.R; Markovic, N; Rajakumar, A; Hubel, C.A; Roberts, J.M; Powers RW. Relationships between maternal plasma leptin, placental leptin mRNA and protein in normal pregnancy, pre-eclampsia and intrauterine growth restriction without pre-eclampsia. *Mol Hum Reprod*. 2006;12(9):551-556. https://academic.oup.com/molehr/article/12/9/551/1033468.

31. Hackett GA, Campbell S, Gamsu H, Cohen-Overbeek T, Pearce JM. Doppler studies in the growth retarded fetus and prediction of neonatal necrotising enterocolitis, haemorrhage, and neonatal morbidity. *Br Med J (Clin Res Ed)*. 1987;294(6563):13-16. http://www.ncbi.nlm.nih.gov/pubmed/3101778. Accessed August 27, 2017.

32. Soothill PW, Nicolaides KH, Campbell S. Prenatal asphyxia, hyperlacticaemia, hypoglycaemia, and erythroblastosis in growth retarded fetuses. *Br Med J*. 1987;294. https://www.ncbi.nlm.nih.gov/pmc/articles/PMC1246217/pdf/bmjcred00017-0009.pdf. Accessed August 27, 2017.

33. Ounsted M, Moar V, Scott WA. Perinatal morbidity and mortality in small-for-dates babies: the relative importance of some maternal factors. *Early Hum Dev*. 1981;5(4):367-375. http://www.ncbi.nlm.nih.gov/pubmed/7197208. Accessed August 27, 2017.

34. Ross MG, Beall MH. Adult sequelae of intrauterine growth restriction. *Semin Perinatol*. 2008;32(3):213-218. doi:10.1053/j.semperi.2007.11.005.

35. Saenger P, Czernichow P, Hughes I, Reiter EO. Small for Gestational Age: Short Stature and Beyond. *Endocr Rev*. 2007;28(2):219-251. doi:10.1210/er.2006-0039.

36. Henriksen T, Clausen T. Acta Obstetricia et Gynecologica Scandinavica The fetal origins hypothesis: placental insufficiency and inheritance versus maternal

malnutrition in well-nourished populations. *Acta Obs Gynecol Scand Obs Gynecol Scand C Acta Obs Gynecol Scand*. 2002;81(81):112-114. http://onlinelibrary.wiley.com/store/10.1034/j.1600-0412.2002.810204.x/asset/j.1600-0412.2002.810204.x.pdf?v=1&t=j6ufswui&s=3b54df968c3055ccd6898160e136e08d6309e11f. Accessed August 27, 2017.

37. Hendrix N, Berghella V, Piercy WN, al. et. Non-placental causes of intrauterine growth restriction. *Semin Perinatol*. 2008;32(3):161-165. doi:10.1053/j.semperi.2008.02.004.

38. Dey SK. How we are born. *J Clin Invest*. 2010;120(4):952-955. doi:10.1172/JCI42380.

39. Carson DD, Bagchi I, Dey SK, et al. Embryo Implantation. *Dev Biol*. 2000;223(2):217-237. doi:10.1006/dbio.2000.9767.

40. Burton GJ, Fowden AL. The placenta: a multifaceted, transient organ. *Philos Trans R Soc Lond B Biol Sci*. 2015;370(1663):20140066. doi:10.1098/rstb.2014.0066.

41. Atwood CS, Meethal SV. The Spatiotemporal Hormonal Orchestration of Human Folliculogenesis, Early Embryogenesis and Blastocyst Implantation. *Mol Cell Endocrinol*. April 2016. doi:10.1016/j.mce.2016.03.039.

42. Lunghi L, Ferretti ME, Medici S, et al. Control of human trophoblast function. *Reprod Biol Endocrinol*. 2007;5(1):6. doi:10.1186/1477-7827-5-6.

43. Lunghi L, Ferretti ME, Medici S, et al. Control of human trophoblast function. *Reprod Biol Endocrinol*. 2007;5(1):6. doi:10.1186/1477-7827-5-6.

44. Knöfler M, Pollheimer J. Human placental trophoblast invasion and differentiation: a particular focus on Wnt signaling. *Front Genet*. 2013;4:190. doi:10.3389/fgene.2013.00190.

45. Burton, Graham J; Kaufmann, P; Huppertz B. *Anatomy and Genesis of the Placenta. In Knobil and Neill's Physiology of Reproduction*. 3rd ed. (Neill J, ed.). Amsterdam: Academic Press; 2006.

46. Benirschke, K; Burton GBR. *Pathology of the Human Placenta*. 6th ed. Heidelberg: Springer International Publishing; 2012.

47. Wang H, Dey SK. Roadmap to embryo implantation: clues from mouse models. *Nat Rev Genet*. 2006;7(3):185-199. doi:10.1038/nrg1808.

48. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med*. 2012;18(12):1754-1767. doi:10.1038/nm.3012.

49. Cole LA. Immunoassay of human chorionic gonadotropin, its free subunits, and metabolites. *Clin Chem*. 1997;43(12). http://clinchem.aaccjnls.org/content/43/12/2233.long. Accessed July 13, 2017.

50. Pidoux G, Gerbaud P, Tsatsaris V, et al. Biochemical characterization and modulation

of LH/CG-receptor during human trophoblast differentiation. *J Cell Physiol*. 2007;212(1):26-35. doi:10.1002/jcp.20995.

51. Lappas M, Permezel M, Rice GE. Leptin and Adiponectin Stimulate the Release of Proinflammatory Cytokines and Prostaglandins from Human Placenta and Maternal Adipose Tissue via Nuclear Factor-κB, Peroxisomal Proliferator-Activated Receptor-γ and Extracellularly Regulated Kinase 1/2. *Endocrinology*. 2005;146(8):3334-3342. doi:10.1210/en.2005-0406.

52. Jones HN, Jansson T, Powell TL. Full-length adiponectin attenuates insulin signaling and inhibits insulin-stimulated amino Acid transport in human primary trophoblast cells. *Diabetes*. 2010;59(5):1161-1170. doi:10.2337/db09-0824.

53. Fowden AL, Forhead AJ, Coan PM, Burton GJ. The Placenta and Intrauterine Programming. *J Neuroendocrinol*. 2008;20(4):439-450. doi:10.1111/j.1365-2826.2008.01663.x.

54. Mayhew TM. A stereological perspective on placental morphology in normal and complicated pregnancies. *J Anat*. 2009;215(1):77-90. doi:10.1111/j.1469-7580.2008.00994.x.

55. Coan PM, Ferguson-Smith AC, Burton GJ. Developmental Dynamics of the Definitive Mouse Placenta Assessed by Stereology1. *Biol Reprod*. 2004;70(6):1806-1813. doi:10.1095/biolreprod.103.024166.

56. Reik W, Constancia M, Fowden A, et al. Regulation of supply and demand for maternal nutrients in mammals by imprinted genes. *J Physiol*. 2003;547(1):35-44. doi:10.1113/jphysiol.2002.033274.

57. Constância M, Angiolini E, Sandovici I, et al. Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems. *Proc Natl Acad Sci U S A*. 2005;102(52):19219-19224. doi:10.1073/pnas.0504468103.

58. Pardi G, Cetin I, Marconi AM, et al. Diagnostic Value of Blood Sampling in Fetuses with Growth Retardation. *N Engl J Med*. 1993;328(10):692-696. doi:10.1056/NEJM199303113281004.

59. Burton GJ, Reshetnikova OS, Milovanov AP, Teleshova O V. Stereological evaluation of vascular adaptations in human placental villi to differing forms of hypoxic stress. *Placenta*. 1996;17(1):49-55. http://www.ncbi.nlm.nih.gov/pubmed/8710813. Accessed August 9, 2017.

60. Lopez MF, Dikkes P, Zurakowski D, Villa-Komaroff L. Insulin-like growth factor II affects the appearance and glycogen content of glycogen cells in the murine placenta. *Endocrinology*. 1996;137(5):2100-2108. doi:10.1210/endo.137.5.8612553.

61. Coan PM, Vaughan OR, McCarthy J, et al. Dietary composition programmes placental phenotype in mice. *J Physiol*. 2011;589(Pt 14):3659-3670. doi:10.1113/jphysiol.2011.208629.

62. Tobi EW, Lumey LH, Talens RP, et al. DNA methylation differences after exposure to prenatal famine are common and timing- and sex-specific. *Hum Mol Genet*. 2009;18(21):4046-4053. doi:10.1093/hmg/ddp353.

63. Bird AP. CpG-rich islands and the function of DNA methylation. *Nature*. 1986;321(6067):209-213. doi:10.1038/321209a0.

64. Novakovic B, Yuen RK, Gordon L, et al. Evidence for widespread changes in promoter methylation profile in human placenta in response to increasing gestational age and environmental/stochastic factors. *BMC Genomics*. 2011;12(1):529. doi:10.1186/1471-2164-12-529.

65. Banister CE, Koestler DC, Maccani MA, Padbury JF, Houseman EA, Marsit CJ. Infant growth restriction is associated with distinct patterns of DNA methylation in human placentas. *Epigenetics*. 2011;6(7):920-927. doi:10.4161/epi.6.7.16079.

66. Chaddha V, Viero S, Huppertz B, Kingdom J, Kaufmann P, Kingdom J. Developmental biology of the placenta and the origins of placental insufficiency. *Semin Fetal Neonatal Med*. 2004;9(5):357-369. doi:10.1016/j.siny.2004.03.006.

67. Cha J, Sun X, Dey SK. Mechanisms of implantation: strategies for successful pregnancy. *Nat Med*. 2012;18(12):1754-1767. doi:10.1038/nm.3012.

68. Wilcox AJ, Baird DD, Weinberg CR. Time of Implantation of the Conceptus and Loss of Pregnancy. *N Engl J Med*. 1999;340(23):1796-1799. doi:10.1056/NEJM199906103402304.

69. The Investigation and Management of the Small–for–Gestational–Age Fetus. 2013. https://www.rcog.org.uk/globalassets/documents/guidelines/gtg_31.pdf. Accessed August 27, 2017.

70. Gagnon A, Wilson RD, Audibert F, et al. Obstetrical complications associated with abnormal maternal serum markers analytes. *J Obstet Gynaecol Can*. 2008;30(10):918-949. http://www.ncbi.nlm.nih.gov/pubmed/19038077. Accessed August 27, 2017.

71. Morris RK, Cnossen JS, Langejans M, et al. Serum screening with Down's syndrome markers to predict pre-eclampsia and small for gestational age: Systematic review and meta-analysis. *BMC Pregnancy Childbirth*. 2008;8(1):33. doi:10.1186/1471-2393-8-33.

72. Spencer K, Cowans NJ, Avgidou K, Molina F, Nicolaides KH. First-trimester biochemical markers of aneuploidy and the prediction of small-for-gestational age fetuses. *Ultrasound Obstet Gynecol*. 2008;31(1):15-19. doi:10.1002/uog.5165.

73. Karagiannis G, Akolekar R, Sarquis R, Wright D, Nicolaides KH. Prediction of small-for-gestation neonates from biophysical and biochemical markers at 11-13 weeks. *Fetal Diagn Ther*. 2011;29(2):148-154. doi:10.1159/000321694.

74. Nelissen ECM, van Montfoort APA, Dumoulin JCM, Evers JLH. Epigenetics and the placenta. *Hum Reprod Update*. 17(3):397-417. doi:10.1093/humupd/dmq052.

75. Reik W, Walter J. Genomic imprinting: parental influence on the genome. *Nat Rev Genet*. 2001;2(1):21-32. doi:10.1038/35047554.

76. Herman JG, Baylin SB. Gene Silencing in Cancer in Association with Promoter Hypermethylation. *N Engl J Med*. 2003;349(21):2042-2054. doi:10.1056/NEJMra023075.

77. Radford EJ, Ferrón SR, Ferguson-Smith AC. Genomic imprinting as an adaptative model of developmental plasticity. *FEBS Lett*. 2011;585(13):2059-2066. doi:10.1016/j.febslet.2011.05.063.

78. Koerner M V, Pauler FM, Huang R, Barlow DP. The function of non-coding RNAs in genomic imprinting. *Development*. 2009;136(11):1771-1783. doi:10.1242/dev.030403.

79. Barker DJ, Osmond C. Infant mortality, childhood nutrition, and ischaemic heart disease in England and Wales. *Lancet (London, England)*. 1986;1(8489):1077-1081. http://www.ncbi.nlm.nih.gov/pubmed/2871345. Accessed July 20, 2017.

80. Hales CN. The thrifty phenotype hypothesis. *Br Med Bull*. 2001;60(1):5-20. doi:10.1093/bmb/60.1.5.

81. Osmond C, Barker DJ, Winter PD, Fall CH, Simmonds SJ. Early growth and death from cardiovascular disease in women. *BMJ*. 1993;307(6918):1519-1524. http://www.ncbi.nlm.nih.gov/pubmed/8274920. Accessed August 21, 2017.

82. Barker DJ, Hales CN, Fall CH, Osmond C, Phipps K, Clark PM. Type 2 (non-insulin-dependent) diabetes mellitus, hypertension and hyperlipidaemia (syndrome X): relation to reduced fetal growth. *Diabetologia*. 1993;36(1):62-67. http://www.ncbi.nlm.nih.gov/pubmed/8436255. Accessed April 22, 2014.

83. Wallace C, Smyth DJ, Maisuria-Armer M, Walker NM, Todd JA, Clayton DG. The imprinted DLK1-MEG3 gene region on chromosome 14q32.2 alters susceptibility to type 1 diabetes. *Nat Genet*. 2010;42(1):68-71. doi:10.1038/ng.493.

84. Rich-Edwards JW, Stampfer MJ, Manson JE, et al. Birth weight and risk of cardiovascular disease in a cohort of women followed up since 1976. *BMJ*. 1997;315(7105):396-400. http://www.ncbi.nlm.nih.gov/pubmed/9277603. Accessed August 21, 2017.

85. Kwong WY, Wild AE, Roberts P, Willis AC, Fleming TP. Maternal undernutrition during the preimplantation period of rat development causes blastocyst abnormalities and programming of postnatal hypertension. *Development*. 2000;127(19):4195-4202. http://www.ncbi.nlm.nih.gov/pubmed/10976051. Accessed August 21, 2017.

86. Langley-Evans SC, Welham SJ, Jackson AA. Fetal exposure to a maternal low protein diet impairs nephrogenesis and promotes hypertension in the rat. *Life Sci*. 1999;64(11):965-974. http://www.ncbi.nlm.nih.gov/pubmed/10201645. Accessed August 21, 2017.

87. Woods LL, Ingelfinger JR, Nyengaard JR, Rasch R. Maternal Protein Restriction Suppresses the Newborn Renin-Angiotensin System and Programs Adult Hypertension in Rats. *Pediatr Res*. 2001;49(4):460-467. doi:10.1203/00006450-200104000-00005.

88. Heijmans BT, Tobi EW, Stein AD, et al. Persistent epigenetic differences associated with prenatal exposure to famine in humans. *Proc Natl Acad Sci U S A*. 2008;105(44):17046-17049. doi:10.1073/pnas.0806560105.

89. Waterland RA, Dolinoy DC, Lin J-R, Smith CA, Shi X, Tahiliani KG. Maternal methyl supplements increase offspring DNA methylation atAxin fused. *genesis*. 2006;44(9):401-406. doi:10.1002/dvg.20230.

90. Waterland RA, Jirtle RL. Transposable elements: targets for early nutritional effects on epigenetic gene regulation. *Mol Cell Biol*. 2003;23(15):5293-5300. http://www.ncbi.nlm.nih.gov/pubmed/12861015. Accessed July 20, 2017.

91. Horsthemke B, Wagstaff J. Mechanisms of imprinting of the Prader-Willi/Angelman region. *Am J Med Genet Part A*. 2008;146A(16):2041-2052. doi:10.1002/ajmg.a.32364.

92. Abu-Amero S, Monk D, Frost J, Preece M, Stanier P, Moore GE. The genetic aetiology of Silver-Russell syndrome. *J Med Genet*. 2008;45(4):193-199. doi:10.1136/jmg.2007.053017.

93. Temple IK. Imprinting in human disease with special reference to transient neonatal diabetes and Beckwith-Wiedemann syndrome. *Endocr Dev*. 2007;12:113-123. doi:10.1159/0000109638.

94. Ogata T, Kagami M, Ferguson-Smith AC. Molecular mechanisms regulating phenotypic outcome in paternal and maternal uniparental disomy for chromosome 14. *Epigenetics*. 3(4):181-187. http://www.ncbi.nlm.nih.gov/pubmed/18698157. Accessed June 27, 2014.

95. Plagge A, Kelsey G, Germain-Lee EL. Physiological functions of the imprinted Gnas locus and its protein variants Galpha(s) and XLalpha(s) in human and mouse. *J Endocrinol*. 2008;196(2):193-214. doi:10.1677/JOE-07-0544.

96. DeChiara TM, Robertson EJ, Efstratiadis A. Parental imprinting of the mouse insulin-like growth factor II gene. *Cell*. 1991;64(4):849-859. http://www.ncbi.nlm.nih.gov/pubmed/1997210. Accessed August 21, 2017.

97. Ferguson-Smith AC, Cattanach BM, Barton SC, Beechey C V., Surani MA. Embryological and molecular investigations of parental imprinting on mouse chromosome 7. *Nature*. 1991;351(6328):667-670. doi:10.1038/351667a0.

98. Eggenschwiler J, Ludwig T, Fisher P, Leighton PA, Tilghman SM, Efstratiadis A. Mouse mutant embryos overexpressing IGF-II exhibit phenotypic features of the Beckwith-Wiedemann and Simpson-Golabi-Behmel syndromes. *Genes Dev*. 1997;11(23):3128-3142. http://www.ncbi.nlm.nih.gov/pubmed/9389646. Accessed August 21, 2017.

99. Charalambous M, Smith FM, Bennett WR, Crew TE, Mackenzie F, Ward A. Disruption of the imprinted Grb10 gene leads to disproportionate overgrowth by an Igf2-independent mechanism. *Proc Natl Acad Sci U S A*. 2003;100(14):8292-8297. doi:10.1073/pnas.1532175100.

100. Takahashi M, Kamei Y, Ezaki O. Mest/Peg1 imprinted gene enlarges adipocytes and is a marker of adipocyte size. *AJP Endocrinol Metab*. 2004;288(1):E117-E124. doi:10.1152/ajpendo.00244.2004.

101. Li L, Keverne EB, Aparicio SA, Ishino F, Barton SC, Surani MA. Regulation of maternal behavior and offspring growth by paternally expressed Peg3. *Science*. 1999;284(5412):330-333. http://www.ncbi.nlm.nih.gov/pubmed/10195900. Accessed August 21, 2017.

102. McMinn J, Wei M, Schupf N, et al. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta*. 2006;27(6-7):540-549. doi:10.1016/j.placenta.2005.07.004.

103. Kappil MA, Green BB, Armstrong DA, et al. Placental Expression Profile of Imprinted Genes Impacts Birth Weight. *Epigenetics*. July 2015. doi:10.1080/15592294.2015.1073881.

104. Diplas AI, Lambertini L, Lee M-J, et al. Differential expression of imprinted genes in normal and IUGR human placentas. *Epigenetics*. 2009;4(4):235-240. http://www.ncbi.nlm.nih.gov/pubmed/19483473. Accessed April 22, 2014.

105. McMinn J, Wei M, Schupf N, et al. Unbalanced placental expression of imprinted genes in human intrauterine growth restriction. *Placenta*. 2006;27(6-7):540-549. doi:10.1016/j.placenta.2005.07.004.

106. Horikoshi M, Beaumont RN, Day FR, et al. Genome-wide associations for birth weight and correlations with adult disease. *Nature*. 2016;538(7624):248-252. doi:10.1038/nature19806.

107. Wylie AA, Murphy SK, Orton TC, Jirtle RL. Novel imprinted DLK1/GTL2 domain on human chromosome 14 contains motifs that mimic those implicated in IGF2/H19 regulation. *Genome Res*. 2000;10(11):1711-1718. doi:10.1101/GR.161600.

108. Charlier C, Segers K, Wagenaar D, et al. Human-ovine comparative sequencing of a 250-kb imprinted domain encompassing the callipyge (clpg) locus and identification of six imprinted transcripts: DLK1, DAT, GTL2, PEG11, antiPEG11, and MEG8. *Genome Res*. 2001;11(5):850-862. doi:10.1101/gr.172701.

109. Irving MD, Buiting K, Kanber D, et al. Segmental paternal uniparental disomy (patUPD) of 14q32 with abnormal methylation elicits the characteristic features of complete patUPD14. *Am J Med Genet A*. 2010;152A(8):1942-1950. doi:10.1002/ajmg.a.33449.

110. Smas CM, Sul HS. Pref-1, a protein containing EGF-like repeats, inhibits adipocyte differentiation. *Cell*. 1993;73(4):725-734. doi:10.1016/0092-8674(93)90252-L.

111. Lin S-P, Youngson N, Takada S, et al. Asymmetric regulation of imprinting on the maternal and paternal chromosomes at the Dlk1-Gtl2 imprinted cluster on mouse chromosome 12. *Nat Genet*. 2003;35(1):97-102. doi:10.1038/ng1233.

112. Geuns E, De Temmerman N, Hilven P, Van Steirteghem A, Liebaers I, De Rycke M. Methylation analysis of the intergenic differentially methylated region of DLK1-GTL2 in human. *Eur J Hum Genet*. 2007;15(3):352-361. doi:10.1038/sj.ejhg.5201759.

113. Ioannides Y, Lokulo-Sodipe K, Mackay DJG, Davies JH, Temple IK. Temple syndrome: improving the recognition of an underdiagnosed chromosome 14 imprinting disorder: an analysis of 51 published cases. *J Med Genet*. June 2014:jmedgenet-2014-102396-. doi:10.1136/jmedgenet-2014-102396.

114. Okamoto M, Takemori H, Halder SK, Hatano O. Zona glomerulosa-specific factor: cloning and function. *Steroids*. 1997;62(1):73-76. http://www.ncbi.nlm.nih.gov/pubmed/9029718. Accessed August 22, 2017.

115. Jensen CH, Krogh TN, Højrup P, et al. Protein structure of fetal antigen 1 (FA1). A novel circulating human epidermal-growth-factor-like protein expressed in neuroendocrine tumors and its relation to the gene products of dlk and pG2. *Eur J Biochem*. 1994;225(1):83-92. http://www.ncbi.nlm.nih.gov/pubmed/7925474. Accessed April 22, 2014.

116. Smas CM, Chen L, Sul HS. Cleavage of membrane-associated pref-1 generates a soluble inhibitor of adipocyte differentiation. *Mol Cell Biol*. 1997;17(2):977-988. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=231823&tool=pmcentrez&rendertype=abstract. Accessed April 23, 2014.

117. Smas CM, Green D, Sul HS. Structural characterization and alternate splicing of the gene encoding the preadipocyte EGF-like protein Pref-1. *Biochemistry*. 1994;33(31):9257-9265. doi:10.1021/bi00197a029.

118. Wang Y, Sul HS. Ectodomain shedding of preadipocyte factor 1 (Pref-1) by tumor necrosis factor alpha converting enzyme (TACE) and inhibition of adipocyte differentiation. *Mol Cell Biol*. 2006;26(14):5421-5435. doi:10.1128/MCB.02437-05.

119. Moon YS, Smas CM, Lee K, et al. Mice lacking paternally expressed Pref-1/Dlk1 display growth retardation and accelerated adiposity. *Mol Cell Biol*. 2002;22(15):5585-5592. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=133956&tool=pmcentrez&rendertype=abstract. Accessed April 22, 2014.

120. Appelbe OK, Yevtodiyenko A, Muniz-Talavera H, Schmidt J V. Conditional deletions refine the embryonic requirement for Dlk1. *Mech Dev*. 2013;130(2-3):143-159. doi:10.1016/j.mod.2012.09.010.

121. Schuster-Gossler K, Simon-Chazottes D, Guénet J-L, Zachgo J, Gossler A. Gtl2 lacZ , an insertional mutation on mouse Chromosome 12 with parental origin-dependent phenotype. *Mamm Genome*. 1996;7(1):20-24. doi:10.1007/s003359900006.

122. Steshina EY, Carr MS, Glick EA, Yevtodiyenko A, Appelbe OK, Schmidt J V. Loss of

imprinting at the Dlk1-Gtl2 locus caused by insertional mutagenesis in the Gtl2 5' region. *BMC Genet*. 2006;7:44. doi:10.1186/1471-2156-7-44.

123. Temple IK, Cockwell A, Hassold T, Pettay D, Jacobs P. Maternal uniparental disomy for chromosome 14. *J Med Genet*. 1991;28(8):511-514. http://www.pubmedcentral.nih.gov/articlerender.fcgi?artid=1016977&tool=pmcentrez&rendertype=abstract. Accessed April 22, 2014.

124. Temple IK, Shrubb V, Lever M, Bullman H, Mackay DJG. Isolated imprinting mutation of the DLK1/GTL2 locus associated with a clinical presentation of maternal uniparental disomy of chromosome 14. *J Med Genet*. 2007;44(10):637-640. doi:10.1136/jmg.2007.050807.

125. Fokstuen S, Ginsburg C, Zachmann M, Schinzel A. Maternal uniparental disomy 14 as a cause of intrauterine growth retardation and early onset of puberty. *J Pediatr*. 1999;134(6):689-695. doi:10.1016/S0022-3476(99)70282-9.

126. Pentao L, Lewis RA, Ledbetter DH, Patel PI, Lupski JR. Maternal uniparental isodisomy of chromosome 14: association with autosomal recessive rod monochromacy. *Am J Hum Genet*. 1992;50(4):690-699. http://www.ncbi.nlm.nih.gov/pubmed/1347967. Accessed August 22, 2017.

127. Antonarakis SE, Blouin JL, Maher J, Avramopoulos D, Thomas G, Talbot CC. Maternal uniparental disomy for human chromosome 14, due to loss of a chromosome 14 from somatic cells with t(13;14) trisomy 14. *Am J Hum Genet*. 1993;52(6):1145-1152. http://www.ncbi.nlm.nih.gov/pubmed/8503447. Accessed August 22, 2017.

128. Healey S, Powell F, Battersby M, Chenevix-Trench G, McGill J. Distinct phenotype in maternal uniparental disomy of chromosome 14. *Am J Med Genet*. 1994;51(2):147-149. doi:10.1002/ajmg.1320510213.

129. Papenhausen PR, Mueller OT, Johnson VP, Sutcliffe M, Diamond TM, Kousseff BG. Uniparental isodisomy of chromosome 14 in two cases: An abnormal child and a normal adult. *Am J Med Genet*. 1995;59(3):271-275. doi:10.1002/ajmg.1320590302.

130. Tomkins DJ, Roux A-F, Waye J, Freeman VCP, Cox DW, Whelan DT. Maternal Uniparental Isodisomy of Human Chromosome 14 Associated with a Paternal t(13q14q) and Precocious Puberty. *Eur J Hum Genet* . 1996;4:153-159. doi:10.1159/000472189.

131. Splitt MP, Goodship JA. Another case of maternal uniparental disomy chromosome 14 syndrome. *Am J Med Genet*. 1997;72(2):239-240. http://www.ncbi.nlm.nih.gov/pubmed/9382150. Accessed August 22, 2017.

132. Miyoshi O, Hayashi S, Fujimoto M, Tomita H, Sohda M, Niikawa N. Maternal uniparental disomy for chromosome 14 in a boy with intrauterine growth retardation. *J Hum Genet*. 1998;43(2):138-142. doi:10.1007/s100380050056.

133. Kagami M, Sekita Y, Nishimura G, et al. Deletions and epimutations affecting the human 14q32.2 imprinted region in individuals with paternal and maternal upd(14)-

like phenotypes. *Nat Genet*. 2008;40(2):237-242. doi:10.1038/ng.2007.56.

134. Rosenfeld JA, Fox JE, Descartes M, et al. Clinical features associated with copy number variations of the 14q32 imprinted gene cluster. *Am J Med Genet A*. 2015;167A(2):345-353. http://www.ncbi.nlm.nih.gov/pubmed/25756153. Accessed November 19, 2018.

135. Schmidt J V., Matteson PG, Jones BK, Guan X-J, Tilghman SM. The Dlk1 and Gtl2 genes are linked and reciprocally imprinted. *Genes & Dev*. 2000;14(16):1997-2002. doi:10.1101/gad.14.16.1997.

136. Kagami M, Nishimura G, Okuyama T, et al. Segmental and full paternal isodisomy for chromosome 14 in three patients: narrowing the critical region and implication for the clinical features. *Am J Med Genet A*. 2005;138A(2):127-132. doi:10.1002/ajmg.a.30941.

137. Yevtodiyenko A, Schmidt J V. Dlk1 expression marks developing endothelium and sites of branching morphogenesis in the mouse embryo and placenta. *Dev Dyn*. 2006;235(4):1115-1123. doi:10.1002/dvdy.20705.

138. Floridon C, Jensen CH, Thorsen P, et al. Does fetal antigen 1 (FA1) identify cells with regenerative, endocrine and neuroendocrine potentials? A study of FA1 in embryonic, fetal, and placental tissue and in maternal circulation. *Differentiation*. 2000;66(1):49-59. doi:10.1046/j.1432-0436.2000.066001049.x.

139. Bachmann E, Krogh TN, Højrup P, Skjødt K, Teisner B. Mouse fetal antigen 1 (mFA1), the circulating gene product of mdlk, pref-1 and SCP-1: isolation, characterization and biology. *J Reprod Fertil*. 1996;107(2):279-285. http://www.ncbi.nlm.nih.gov/pubmed/8882295. Accessed November 3, 2014.

140. Reynolds LP, Redmer DA. Utero-placental vascular development and placental function. *J Anim Sci*. 1995;73(6):1839-1851. http://www.ncbi.nlm.nih.gov/pubmed/7545661. Accessed July 1, 2015.

141. Tanimizu N, Nishikawa M, Saito H, Tsujimura T, Miyajima A. Isolation of hepatoblasts based on the expression of Dlk/Pref-1. *J Cell Sci*. 2003;116(Pt 9):1775-1786. http://www.ncbi.nlm.nih.gov/pubmed/12665558. Accessed July 21, 2017.

142. Smas CM, Sul HS. Molecular mechanisms of adipocyte differentiation and inhibitory action of pref-1. *Crit Rev Eukaryot Gene Expr*. 1997;7(4):281-298. http://www.ncbi.nlm.nih.gov/pubmed/9607168. Accessed April 23, 2014.

143. Cleaton MAM, Dent CL, Howard M, et al. Fetus-derived DLK1 is required for maternal metabolic adaptations to pregnancy and is associated with fetal growth restriction. *Nat Genet*. 2016;48(12):1473-1480. doi:10.1038/ng.3699.

144. Jansson N, Greenwood SL, Johansson BR, Powell TL, Jansson T. Leptin Stimulates the Activity of the System A Amino Acid Transporter in Human Placental Villous Fragments. *J Clin Endocrinol Metab*. 2003;88(3):1205-1211. doi:10.1210/jc.2002-021332.

145. Abdallah BM, Jensen CH, Gutierrez G, Leslie RGQ, Jensen TG, Kassem M. Regulation of human skeletal stem cells differentiation by Dlk1/Pref-1. *J Bone Miner Res*. 2004;19(5):841-852. doi:10.1359/JBMR.040118.

146. Abdallah BM, Ding M, Jensen CH, et al. Dlk1/FA1 is a novel endocrine regulator of bone and fat mass and its serum level is modulated by growth hormone. *Endocrinology*. 2007;148(7):3111-3121. doi:10.1210/en.2007-0171.

147. Wang Y, Sul HS. Pref-1 Regulates Mesenchymal Cell Commitment and Differentiation through Sox9. *Cell Metab*. 2009;9(3):287-302. doi:10.1016/j.cmet.2009.01.013.

148. Rhee M, Lee S-H, Kim J-W, et al. Preadipocyte factor 1 induces pancreatic ductal cell differentiation into insulin-producing cells. *Sci Rep*. 2016;6:23960. doi:10.1038/srep23960.

149. Kim K-A, Kim J-H, Wang Y, Sul HS. Pref-1 (Preadipocyte Factor 1) Activates the MEK/Extracellular Signal-Regulated Kinase Pathway To Inhibit Adipocyte Differentiation. *Mol Cell Biol*. 2007;27(6):2294-2308. doi:10.1128/MCB.02207-06.

150. Katugampola H, King PJ, Chatterjee S, et al. Kisspeptin Is a Novel Regulator of Human Fetal Adrenocortical Development and Function: A Finding With Important Implications for the Human Fetoplacental Unit. *J Clin Endocrinol Metab*. 2017;102(9):3349-3359. doi:10.1210/jc.2017-00763.

151. Velauthar L, Plana MN, Kalidindi M, et al. First-trimester uterine artery Doppler and adverse pregnancy outcome: a meta-analysis involving 55 974 women. *Ultrasound Obstet Gynecol*. 2014;43(5):500-507. doi:10.1002/uog.13275.

152. Allen R, Aquilina J. Prospective observational study to determine the accuracy of first-trimester serum biomarkers and uterine artery Dopplers in combination with maternal characteristics and arteriography for the prediction of women at risk of preeclampsia and other adverse pregnancy outcomes. *J Matern Neonatal Med*. 2018;31(21):2789-2806. doi:10.1080/14767058.2017.1355903.

153. Appelbe OK, Yevtodiyenko A, Muniz-Talavera H, Schmidt J V. Conditional deletions refine the embryonic requirement for Dlk1. *Mech Dev*. 2013;130(2-3):143-159. doi:10.1016/j.mod.2012.09.010.

154. Ericsson A, Hamark B, Jansson N, Johansson BR, Powell TL, Jansson T. Hormonal regulation of glucose and system A amino acid transport in first trimester placental villous fragments. *Am J Physiol - Regul Integr Comp Physiol*. 2005;288(3). http://ajpregu.physiology.org/content/288/3/R656. Accessed August 24, 2017.

155. Rad S, Beauchamp S, Morales C, Mirocha J, Esakoff TF. Defining fetal growth restriction: abdominal circumference as an alternative criterion. *J Matern Neonatal Med*. August 2017:1-6. doi:10.1080/14767058.2017.1364723.

156. Public Health England. Maternal obesity :: Public Health England Obesity Knowledge and Intelligence team. http://www.noo.org.uk/NOO_about_obesity/maternal_obesity_2015. Published

2016.

157. Khong TY, Mooney EE, Ariel I, et al. Sampling and Definitions of Placental Lesions: Amsterdam Placental Workshop Group Consensus Statement. *Arch Pathol Lab Med*. 2016;140(7):698-713. doi:10.5858/arpa.2015-0225-CC.

158. Kilpeläinen L, Ivaska KK, Kuiri-Hänninen T, et al. Urinary osteocalcin and serum pro-C-type natriuretic peptide predict linear catch-up growth in infants. *J Bone Miner Res*. 2012;27(7):1528-1535. doi:10.1002/jbmr.1596.

159. Sankilampi U, Hannila M-L, Saari A, Gissler M, Dunkel L. New population-based references for birth weight, length, and head circumference in singletons and twins from 23 to 43 gestation weeks. *Ann Med*. 2013;45(5-6):446-454. doi:10.3109/07853890.2013.803739.

160. Rhee M, Lee S-H, Kim J-W, et al. Preadipocyte factor 1 induces pancreatic ductal cell differentiation into insulin-producing cells. *Sci Rep*. 2016;6(1):23960. doi:10.1038/srep23960.

161. Taipaleenmäki H, Harkness L, Chen L, et al. The Crosstalk Between Transforming Growth Factor-β1 and Delta Like-1 Mediates Early Chondrogenesis During Embryonic Endochondral Ossification. *Stem Cells*. 2012;30(2):304-313. doi:10.1002/stem.792.

162. Mayhew TM. Morphomics: An integral part of systems biology of the human placenta. *Placenta*. 2015;36(4):329-340. doi:10.1016/j.placenta.2015.01.001.

163. Sferruzzi-Perri AN, Vaughan OR, Coan PM, et al. Placental-specific Igf2 deficiency alters developmental adaptations to undernutrition in mice. *Endocrinology*. 2011;152(8):3202-3212. doi:10.1210/en.2011-0240.

164. Georgiades P, Watkins M, Surani MA, Ferguson-Smith AC. Parental origin-specific developmental defects in mice with uniparental disomy for chromosome 12. *Development*. 2000;127(21):4719-4728. http://www.ncbi.nlm.nih.gov/pubmed/11023874. Accessed June 30, 2017.

165. Stanek J, Biesiada J. Sensitivity and specificity of finding of multinucleate trophoblastic giant cells in decidua in placentas from high-risk pregnancies. *Hum Pathol*. 2012;43(2):261-268. doi:10.1016/j.humpath.2011.03.012.

166. Costa MA. The endocrine function of human placenta: an overview. *Reprod Biomed Online*. 2016;32(1):14-43. doi:10.1016/j.rbmo.2015.10.005.

167. Kim VN, Han J, Siomi MC. Biogenesis of small RNAs in animals. *Nat Rev Mol Cell Biol*. 2009;10(2):126-139. doi:10.1038/nrm2632.

168. Gupta RA, Shah N, Wang KC, et al. Long non-coding RNA HOTAIR reprograms chromatin state to promote cancer metastasis. *Nature*. 2010;464(7291):1071-1076. doi:10.1038/nature08975.

169. Guttman M, Amit I, Garber M, et al. Chromatin signature reveals over a thousand

highly conserved large non-coding RNAs in mammals. *Nature*. 2009;458(7235):223-227. doi:10.1038/nature07672.

170. Surani MA, Barton SC, Norris ML. Development of reconstituted mouse eggs suggests imprinting of the genome during gametogenesis. *Nature*. 308(5959):548-550. http://www.ncbi.nlm.nih.gov/pubmed/6709062. Accessed July 7, 2014.

171. Benetatos L, Hatzimichael E, Londin E, et al. The microRNAs within the DLK1-DIO3 genomic region: involvement in disease pathogenesis. *Cell Mol Life Sci*. 2013;70(5):795-814. doi:10.1007/s00018-012-1080-8.

172. Hamano R, Miyata H, Yamasaki M, et al. Overexpression of miR-200c Induces Chemoresistance in Esophageal Cancers Mediated Through Activation of the Akt Signaling Pathway. *Clin Cancer Res*. 2011;17(9). http://clincancerres.aacrjournals.org/content/17/9/3029. Accessed June 29, 2017.

173. Lu J, Getz G, Miska EA, et al. MicroRNA expression profiles classify human cancers. *Nature*. 2005;435(7043):834-838. doi:10.1038/nature03702.

174. Chira P, Vareli K, Sainis I, Papandreou C, Briasoulis E. Alterations of MicroRNAs in Solid Cancers and Their Prognostic Value. *Cancers (Basel)*. 2010;2(2):1328-1353. doi:10.3390/cancers2021328.

175. Calin GA, Sevignani C, Dumitru CD, et al. Human microRNA genes are frequently located at fragile sites and genomic regions involved in cancers. *Proc Natl Acad Sci*. 2004;101(9):2999-3004. doi:10.1073/pnas.0307323101.

176. Chang WL, Yang Q, Zhang H, et al. Role of placenta-specific protein 1 in trophoblast invasion and migration. *Reproduction*. 2014;148(4):343-352. doi:10.1530/REP-14-0052.

177. Falix FA, Aronson DC, Lamers WH, Gaemers IC. Possible roles of DLK1 in the Notch pathway during development and disease. *Biochim Biophys Acta*. 2012;1822(6):988-995. doi:10.1016/j.bbadis.2012.02.003.

178. Traustad?ttir G ?sta, Jensen CH, Thomassen M, et al. Evidence of non-canonical NOTCH signaling: Delta-like 1 homolog (DLK1) directly interacts with the NOTCH1 receptor in mammals. *Cell Signal*. 2016;28(4):246-254. doi:10.1016/j.cellsig.2016.01.003.

179. Traustad?ttir G ?sta, Jensen CH, Garcia Ramirez JJ, Beck HC, Sheikh SP, Andersen DC. The non-canonical NOTCH1 ligand Delta-like 1 homolog (DLK1) self interacts in mammals. *Int J Biol Macromol*. 2017;97:460-467. doi:10.1016/j.ijbiomac.2017.01.067.

APPENDIX 1

Patient demographics

Maternal age	Maternal weight (kg)	Ethnicity	Gravidity/ Parity	Height (m)	BMI	GA at birth	Birth weight (grams)	Birth length (cm)
24	67	N.African	G1, P0	1.53	28.6	40.71	3000	
29	62.2	Caucasian	G1,P0	1.7	21.52	42.14	3680	51.5
34	56	Chinese	G1,P0	1.6	21.87	39.43	2610	42
28	65	Caribbean	G4, P1	1.63	24.46	41.29	3230	51
29	59.9	Bengali	G2,P1	1.54	25.26	39.14	2980	
28	***72***	***Caucasian***	***G3,P1***	***1.6***	***28.1***	***39.29***	***2420***	***44.8***
32	59	Caucasian	G1,P0	1.66	21.41	39	3200	
34	64.2	Caucasian	G1,P0	1.68	22.75	40.86	3640	49.5
30	44.5	Caribbean	G3,P1	1.54	18.76	39.29	2680	48.6
20	64	Caribbean	G1,P0	1.76	20.66	40	3220	
35	103	Caucasian	G1,P0	1.57	41.79	40.14	3250	51
30	76	Indian	G1,P1	1.63	28.6	38.29	3040	50.4
25	74.4	Bengali	G2,P0	1.65	27.33	40.86	3000	50.5
31	80.6	Pakistani	G2,P1	1.55	33.55	39.29	3120	49.7
37	71	Caucasian	G1,P0	1.66	25.77	40.57	3280	50.02
29	71	Bengali	G3,P2	1.56	29.17	41.71	3120	52
32	58.4	Chinese	G3,P1	1.66	21.19	39.57	3515	51.9
31	65.5	A. American	G1,P0	1.68	23.21	26.86	950	
31	62	Indian	G1,P0	1.57	25.15	39.14	3360	47.5

25	65	Caucasian	G1,P0	1.67	23.31	41	3620	52.4
33	***52.4***	***Caucasian***	***G1,P0***	***1.66***	***20.2***	***37***	***1860***	
28	***62***	***Bengali***	***G4,P2***	***1.52***	***26.84***	***37.14***	***1720***	***44.5***
32	58	Chinese/ Caucasian	G2,P1	1.65	21.3	39.9	3500	49
24	73	Caucasian	G1,P0	1.62	27.82	39.86	3500	49
30	69	African	G3,P2	1.68	24.45	39.29	3540	51.4
25	76.4	Caucasian	G1,P0	1.74	25.2	39.26	3040	52.3
17	51.5	Bengali	G1,P0	1.57	20.89	42	3640	51
39	66	Phillipina	G2,P1	1.56	27.12	41.14	3580	50.5
24	52	N. Africa	G1,P0	1.65	19.1	39	2900	
23	59	Bengali	G2,P0	1.5	26.22	40.43	3425	51.8
37	***109***	***Bengali***	***G2,P0***	***1.63***	***41.03***	***33.57***	***1340***	***40.5***
35	47.8	Malaysian	G1,P0	1.57	19.39	38.43	2640	47.6
26	65.8	Bengali	G2,P0	1.54	27.74	39.71	3060	51.2

Table showing patient demographics. IUGR patients are shown in bold italics.

APPENDIX 2

Antenatal parameters

Gestational age (weeks)	AC (mm)	FL (mm)	BPD (mm)	DLK1 ng/l
20.23 ±0.58	147.7 ±7.57	32.09±1.87	48.75±2.54	12.66±6.83
27.95±0.75	227.4±10.31	51.89±2.47	72.89±2.98	43.17±21.35
34.29±0.94	285.1±17.49	64.17±3.14	87.11±4.58	66.91±27.74
37.86±1.21	324.2±14.25	70.85±3	92.84±3.73	87.08±51.39

Table showing antenatal parameters. Mean (±std dev). AC – abdominal circumference, FL – Femoral length, BPD – Bipareital Diameter

APPENDIX 3

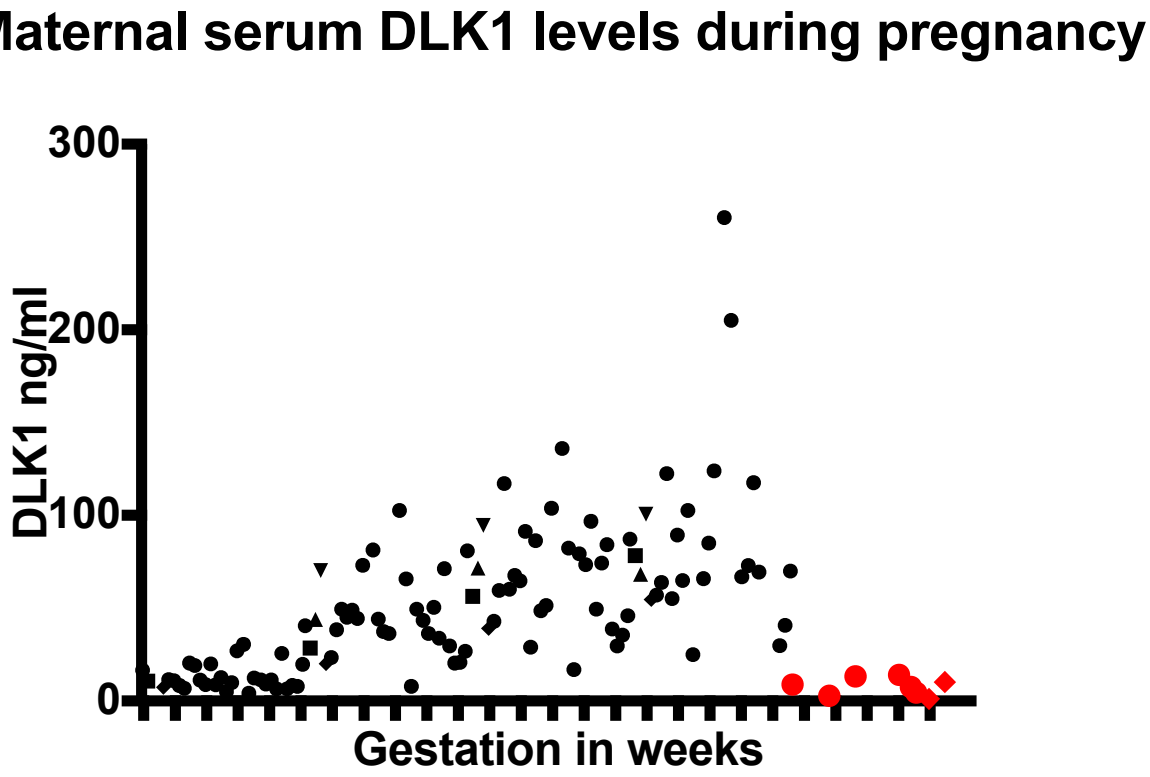
Placental characteristics

Parameters	Mean/ N
Placental weight	426.6 ± 122.8
Feto: placental ratio	7.64±2.36
Placental length	189.3±64.26
Placental width	152.8±21.95
Placental depth	26.48±5.11
Cord insertion Central: Peripheral: Marginal:	 N= 5 N = 15 N = 5
No of vessels in cord 2 vessels: 3 vessels:	 1 24
Cord diameter	15.28±16.2

Placental characteristic are show in the table above. Mean ± std deviation or N values are shown depending on parameter.

APPENDIX 4

Scatter showing individual participants and DLK1 levels during pregnancy



Post delivery samples are shown in red.

APPENDIX 5

Presentations carried out as a result of this work

Oral Communication

1. Meso M, Katugampola H, Allen R, Marlene S, Aquilina J, Storr H, Dunkel L, Charalambous M. A role for Delta-*like homologue 1* (DLK1) in a secretory placental population and implications for fetal growth. **53rd Annual European Society for Endocrinology Meeting, Dublin September 2014**
2. Meso M, Katugampola H, Allen R, Marlene S, Aquilina J, Storr H, Dunkel L, Charalambous M. A role for *Delta-like homologue 1* (DLK1) in a secretory placental population and implications for fetal growth. **42nd meeting of the British Society of Paediatrics and Endocrinology, Winchester November 2014**