A study of metabolic and inflammatory pathways throughout gestation

Christopher Chidiebere Onyiaodike
B.Sc. (Biomedical science)

Submitted in fulfilment of the requirements for the degree of

Doctor of Philosophy

Reproductive and Maternal Medicine

College of Medical, Veterinary and Life Sciences

University of Glasgow

February 2014

Abstract

The effect of metabolic and inflammatory parameters on pregnancy success in terms of implantation, metabolic adaptation to pregnancy and fetal programming is yet to be fully understood. This thesis explores the activity of metabolic and inflammatory pathways in pregnancy, highlighting their importance throughout gestation.

In a cell culture study, a model of *in vivo* blastocyst-uterine adhesion to study the effect of insulin during uterine implantation was explored. JAR spheroid-RL95-2 monolayer adhesion reached 98% by 24 hours in the absence of insulin. A low dose (0.03nM) of added insulin concentrations resulted in 26% adhesion, or 74% inhibition; a high level (0.24nM) inhibited the JAR spheroid-RL95-2 monolayer adhesion by 9%. Therefore insulin did not have a dose-dependent on JAR spheroid-RL95-2 monolayer adhesion in the cell culture model of implantation. Polymerase chain reaction (PCR) studies revealed laminin α1 RNA detection on JAR cells only, CD44 on RL95-2 cells only, no trophinin on both cell types, FBLN-1 and -2 on JAR and FBLN-1 on RL95-2 cells only and an insulin receptor in both cell types. Western blot and immunohistochemistry (IHC) studies showed laminin α1 detection and stains on the JAR cell extracellular matrix.

In a prospective human study, the metabolites of lipid and carbohydrate metabolism and inflammatory mediators very early (between day 0 and day 45) in gestation and their link to successful pregnancy in women undergoing natural cycle frozen embryo transfer (FET) in assisted conception, was investigated. Plasma triglyceride (TG), total cholesterol (TC), high-density lipoprotein cholesterol (HDL-C), glucose, c-reactive protein (CRP) and non-esterified fatty acid (NEFA) were measured on routine biochemistry; insulin, interleukin (IL)-6, plasminogen activated inhibitor (PAI)-1 and PAI-2 on ELISA; IL-8 (CXCL8), CCL2, CCL3, CCL4 and CCL11 on BioPlex; and human chronic gonadotrophin (hCG) on an Immulite system. For all 196 FET cycles, participants' demographics and plasma parameters of pregnant (n=36) and non-pregnant (n=106) women were explored. Neither obesity, the plasma parameters nor insulin resistance were predictive of successful pregnancy, but ICSI (predominately associated with male factor infertility) was. Overall, the hCG, insulin, rebound TG and HDL-C (except TC), homeostasis model assessment (HOMA), CRP and PAI-2 levels were higher, whereas CXCL8, CCL2, CCL11 and PAI-1 were significantly lower by day 45. Baseline obesity related to positive changes in plasma insulin, HDL-C and HOMA and negative changes in CXCL8, CCL3 and CCL4.

In a cross-sectional study in late pregnancy, offspring's reflection of parameters in women with preeclampsia (PE) (n=29) and intrauterine growth restriction (IUGR) (n=14), compared to BMI-matched healthy groups (n=87) and (n=42), respectively, was explored.

Fetal cord was found to be hyperlipidaemic, normoglycaemic and had reduced inflammatory response, while mothers who suffered PE had altered plasma TG, TC, NEFA, glucose, leptin and IL-10 compared to controls. IUGR babies were dyslipidaemic. The role of cholesterol transporters was assessed in PE (n=20) and IUGR (n=9) BMI-matched controls (n=20 and n=9) respectively. Among fifteen steroidogenic acute regulatory protein (STAR)-related lipid transfer domains, only STARD6 and STARD15 were not detected in the placenta via PCR. IHC studies were also explored on the placentae. The real-time PCR (RT-PCR) of messenger RNA of low-density lipoprotein receptor (LDLR), STARD3 and ATP-binding cassette A1 (ABCA1) without protein were higher in PE compared to controls. LDLR, STARD3 and ABCA1 localisation and detection were consistent to placental lipid (cholesterol) transport systems.

In summary, all this led to the conclusion of the importance of metabolic and inflammatory pathways in all stages of pregnancy in leading to pregnancy success; these pathways may influence implantation, adaptation to pregnancy and, potentially, fetal programming of offspring.

Table of Contents

List of Tables

List of Figures

List of Accompanying Materials

Abstracts

- Changes in plasma lipid metabolism are observed by day 10 of human pregnancy and pregnancy success is associated with higher levels of saturated fatty acids.
  Christopher Onyiaodike,[1] E. Ann Brown,[2] Helen Lyall,[1] Naveed Sattar,[2] Scott Nelson,[1] Heather Murray,[3] Barbara J Meyer,[4] Dilys J Freeman.[2]

  [1]School of Medicine, [2]Institute of Cardiovascular and Medical Sciences, [3]Robertson Centre for Biostatistics, University of Glasgow, Glasgow, UK, [4]School of Health Sciences, Metabolic Research Centre, University of Wollongong, NSW, Australia

- Maternal lipid and fatty acid changes in early pregnancy Barbara J Meyer[1], Christopher Onyiaodike,[2] E.Ann Brown,[2] A. Naveed Sattar,[2] Helen Lyall,[3] Robert Nibbs,[4] Scott Nelson,[3,5] Dilys J Freeman.[2]

  [1] School of Health Sciences, Metabolic Research Centre, Smart Foods Centre, University of Wollongong, Wollongong, NSW Australia
  [2] Institute of Cardiovascular and Medical Sciences, University of Glasgow, UK
  [3] Assisted Conception Service, Glasgow Royal Infirmary, UK
  [4] Infection, Immunity and Inflammation Medicine, University of Glasgow, UK
  [5] Medical Undergraduate School, University of Glasgow, UK

- Pregnancy Complicated by Preeclampsia is Associated with low Cord Plasma C-reactive protein and Tumor Necrosis Factor-alpha Levels. *Hypertension in Pregnancy, 27:513-711,* 2008 Pages 700-700 Christopher Onyiaodike, Vanessa MacKay, MD, PhD, Shahzya Huda, MD, Ann Brown, Dilys Freeman, PhD.

  Reproductive and Maternal Medicine, Faculty of Medicine, University of Glasgow, UK

- ABCA1, LDL receptor and STARD3 cholesterol transporter mRNA are upregulated in placentae from pre-eclamptic pregnancies*. Atherosclerosis*, Volume 199, Issue 1, July 2008, Pages 231-231 C.C. Onyiaodike[a], A. Young,[a] C. Lim,[a] A. Graham,[b] D. Freeman.[a]

  [a] Reproductive and Maternal Medicine, Faculty of Medicine, University of Glasgow, UK
  [b] Biological and Biomedical Science, Glasgow Caledonian University, Glasgow, UK

Oral presentations

- ABCA1, LDL receptor and STARD3 cholesterol transporter mRNA are upregulated in placentae from pre-eclamptic pregnancies. C. Onyiaodike, A. Young, C. Lim, A. Graham, D. Freeman.

  H·E·A·R·T 22nd Annual Conference at University of Hertfordshire, Hatfield, United Kingdom. Prize: (Young Investigator Award). 25-27th June, 2008: Oral.

- ABCA1, LDL receptor and STARD3 mRNA expression are upregulated in placenta from pre-eclamptic pregnancy. Onyiaodike C.C.,[1] Young A.,[1] Lim C.,[1] Graham A.,[2] Freeman D.J.,[1] [1]Reproductive and Maternal Medicine, Faculty of Medicine, University of Glasgow; [2]Biological and Biomedical Science, Glasgow Caledonia University, Glasgow, United Kingdom.

  Munro Kerr meeting 2008: Faculty of Medicine, University of Glasgow, United Kingdom. 30th April, 2008: Oral.

Posters

- Pregnancy complicated by preeclampsia is associated with low cord plasma C-reactive protein and tumour necrosis factor α levels. Christopher C. Onyiaodike, Vanessa A. Mackay, Shahzya S. Huda, E. Ann Brown, Dilys J. Freeman. Reproductive and Maternal Medicine, Faculty of Medicine, University of Glasgow, United Kingdom.

  Award: Robert travelling fellowship award 2008: Faculty of Medicine, University of Glasgow. *ISSHP World congress XVI* conference at Washington DC, United States. 21st-25th September, 2008: Poster.

- Placental ABCA1 and LDL receptor gene expression is upregulated in preeclampsia. Onyiaodike, C., LIM, A., YOUNG, A., GRAHAM, A.,* FREEMAN, D.J. Developmental Medicine, University of Glasgow, Royal Infirmary, *Biological and Biomedical Science, Glasgow Caledonia University, Glasgow, United Kingdom.

  Scottish Society for Experimental Medicine meeting 2007: Faculty of Medicine, Senate Rooms, Main Building, University of Glasgow, University Avenue, Glasgow, United Kingdom. 23rd November, 2007: Poster.

Preface

The importance of metabolic and inflammatory pathways in leading to pregnancy success, in terms of implantation, adaptation to pregnancy and fetal programming of offspring, is yet to be completely understood. This thesis explores the activity of metabolic and inflammatory pathways in pregnancy and highlights their importance in all stages of pregnancy. Prediction of pregnancy success, using very early metabolic and inflammatory parameters or obesity by the first 45 days of gestation, was made feasible. An *in vitro* model of implantation using cell culture was developed. This was in order to ascertain the effect of a metabolic parameter (insulin) during adhesion of JAR spheroids (representing the embryo) onto an RL95-2 monolayer (representing the endometrial epithelium), and the influence on key adhesion molecules involved during blastocyst/embryo-uterine endometrial epithelium adhesion. Very early changes in metabolic and inflammatory parameters were assessed in women undergoing natural cycle FET. In late pregnancy, offspring's cord blood reflection of their mother's parameters in healthy pregnancies as well as those with extreme pregnancies complicated by PE, were explored. Cases of IUGR were used as a control due to the absence of hypertension and endothelial cell dysfunction.

Only ICSI, usually associated with male factor infertility predicted pregnancy success by <7 weeks' gestation; none of the other parameters studied did so, and neither did obesity or insulin resistance. Insulin was not seen to play a significant role in JAR spheroid-RL95-2 monolayer adhesion, but insulin may perhaps influence laminin α1 adhesion molecule regulation during implantation. Very early changes in metabolic and inflammatory parameters were detectable by the first 45 days of gestation. Obesity played a role in some of the parameters changes in very early stage of pregnancy. Obesity and insulin resistance did not predict whether participants had pregnancy success or not. Fetal cord hyperlipidaemia was reflective of maternal levels in pre-eclamptic cases of pregnancy. This reflection in the fetus was suggested to be due to the upregulation of molecules involved in lipid transport, in particularly cholesterol, across the maternal-fetal interface. This is due to the upregulation of mRNA of LDLR, STARD3 and ABCA1, but not of protein, in the pre-eclamptic group. Reduced cord blood inflammatory parameters in PE cases, were suggested to be due to downregulation of fetal immune system response. A preliminary study using HUVEC from the umbilical cord as an index of endothelial cell function provided a platform for future studies. Overall, the findings of this thesis pinpoint the importance of metabolic and inflammatory pathways at all stages of pregnancy in leading to pregnancy success; these pathways may influence implantation, adaptation to pregnancy and, potentially, fetal programming of offspring.

This thesis is dedicated to my parents, Thomas and Janet Onyiaodike.

Acknowledgements

I extent my warmest thanks to Dr Dilys Jane Freeman, who oversaw all the studies presented in this thesis, for her support. l am very grateful to the subjects who gave their consent to participate in these studies. This includes those at the Assisted Conception Unit and the Princess Royal Maternity Unit at Glasgow Royal Infirmary (GRI), NHS Glasgow and Clyde, Scotland. I thank the technicians at the Reproductive and Maternal Medicine Section for all their training, support and supervision. I am very grateful to my advisors, Prof Maryann Lumsden and Prof Jane Norman for their support and encouragement throughout many difficult times, including when experiments did not seem to work. I also thank staff nurses and clinicians at the Assisted Conception Unit, GRI, for their dedication during the very early pregnancy study blood sample collection and for assistance in identifying patients qualified to take part in the study, of those undergoing natural cycle FET. Thanks to fellow researchers Vanessa MacKay, Francis Stewart and Shahzya Huda for their assistance in collecting some of the maternal and cord blood, placentae and umbilical cord samples used in the studies presented in the thesis. I appreciate the contributions and partial collaboration of Dr Annette Graham, Vascular Biology Group, Department of Biological and Biomedical Science, Glasgow Caledonian University, Glasgow. I gratefully acknowledge the financial assistance (studentship award) granted by the Graduate School, Faculty of Medicine, University of Glasgow, which went towards tuition. Finally, many thanks go to my family, particularly Samuel Chinedu Onyiaodike, and friends for their support and encouragement throughout the duration of my studies.

To all, thank you very much.

Author's Declaration

I declare that all sections of this thesis were solely written by me with full recognition of all the literature used, and none of the data generated have been published previously. Plasma lipid profiles (TG, TC and HDL-C), glucose and CRP were measured at a routine biochemistry laboratory at GRI, NHS Glasgow and Clyde. Non-esterified fatty acid (NEFA) was measured using a NEFA C test kit (Wako, Neuss Germany), according to the manufacturer's instructions. Insulin, PAI-1, PAI-2, CXCL8, CCL2, CCL3, CCL4, CCL11 and hCG were assayed by in-house measurements carried out by technical staff on behalf of the early pregnancy study grant. Irrespective of this support I was actively involved in and oversaw the measurement of the assays. I oversaw the daily participant recruitment and blood sampling on weekdays and weekends. I attended daily lunch-time meetings weekdays at the Assisted Conception Unit, where staff used the results of blood hormonal sampling tests to identify and schedule the patients' next hospital appointments. I liaised with weekend staff nurses to schedule the patients' next appointments for the collection of samples. In addition, I recalled the patients' hospital notes during and after ART procedures at the clinic and after delivery in hospitals throughout Scotland. Staff at the Assisted Conception Unit carried out the venepuncture, and a research team member, E. Ann Brown, assisted in blood sample collection, storage and sometimes attended lunch-time meetings during my other engagements. These included carrying out experimental studies and attending core postgraduate training courses, research meetings and conferences. Following the guidelines of the Well-being of Women Grant organisation, which provided the early pregnancy study grant, I used these samples to compile a complete dataset (which was subsequently utilised in a study on plasma and erythrocyte fatty acid composition, which is not part of the data presented in this thesis). I carried out all the data analysis of early pregnancy change in the metabolic and inflammatory parameters presented in the thesis. Placenta and cord tissue and biochemical data were part of archival collection by previous clinical research fellows for late stage pregnancy using mother-baby pair (PE, IUGR and Controls) used in our research team. I collected and received consent for the use of umbilical cord tissue for the preliminary work of clean HUVEC preparation and the selection of the best endogenous control gene for offspring endothelial cell study at birth. I solely undertook every other area of work for this thesis in terms of studies, data analyses and data presentation. Dr Dilys Jane Freeman, Reproductive and Maternal Medicine, Division of Developmental Medicine, Institute of Cardiovascular and Medical Science, School of Medicine, University of Glasgow, supervised the work done in this thesis.

Definitions/Abbreviations

Symbols

α	Alpha
~	Approximately
β	Beta
°C	Degree centigrade
IU	International unit
γ	Gamma
µ	Micro
%	Percent

Abbreviations (used in the text)

3βHSD1	3-beta hydroxysteroid dehydrogenase type 1
3βHSD2	3-beta hydroxysteroid dehydrogenase type 2
18S	Eukaryotic 18S ribosomal RNA
ABCA1	ATP-binding cassette transporter A1
ABCG1	ATP-binding cassette transporter G1
ACAT	Acyl-CoA:cholesterol acyltransferase
AGA	Appropriate-for-gestational age
Apo	Apolipoprotein
ART	Assisted reproductive technology
Akt	Protein kinase B
ATP	Adenosine triphosphate
β-Actin	Beta actin
Ca^{2+}	Calcium ion
cAMP	Cyclic adenosine monophosphate
CCL2	Monocyte chemotactic protein-1 (MCP-1)
CCL3	Macrophage inhibitory protein-1 alpha (MIP-1α)
CCL4	Macrophage inhibitory protein-1 beta (MIP-1β)
CCL11	Eotaxin
CD31	Endothelial cell adhesion molecule
CD44	Indian blood type
CI	Confidence interval
CRP	C-reactive protein
CVD	Cardiovascular disease

DEPCAT	Carstair's Score and Deprivation Categories
EC	Endothelial cells
EEC	Endometrial epithelial cells
eNOS	Endothelial nitric oxide synthase
ET-1	Endothelial-1
ER	Oestrogen receptor
ERK	Extracellular signal-regulated kinase
EVT	Extravillous trophoblast
FEC	Fetal endothelial cells
FSH	Follicle stimulating hormone
GAPDH	Glyceraldhyde 3 phosphate dehydrogenase
GIFT	Gamete intra-fallopian transfer
GnRH	Gonadotropin-releasing hormone
GR	Glucocorticoid receptor
GLUT	Glucose transporter
hCG	Human chorionic gonadotropin
HDL	High-density lipoprotein particle
HDL-C	High-density lipoprotein cholesterol
HIF-α1	Hypoxia inducible factor-alpha 1
HPA	Hypothalamic-pituitary-adrenal
hPL	Human placental lactogen
HRT	Hormone replacement therapy
HSL	Hormone sensitive lipase
HUVEC	Human umbilical cord vein endothelial cell
ICAM-1	Intracellular adhesion molecule-1
ICC	Immunocytochemistry
ICMART	International Committee for Monitoring Assisted Reproduction Technology
ICSI	Intra-cytoplasmic sperm injection
IDL	Intermediate-density lipoprotein particle
IDL-C	Intermediate-density lipoprotein cholesterol
IGF-1	Insulin-like growth factor-1
IGF-2	Insulin-like growth factor-2
IGFBP-1	Insulin-like growth factor binding protein-1
IHC	Immunohistochemical
IL-1	Interleukin-1
IL-1β	Interleukin-1 beta
IL-2	Interleukin-2
IL-4	Interleukin-4
IL-6	Interleukin-6

IL-8	Interleukin-8 (CXCL8)
IL-10	Interleukin-10
IL-11	Interleukin-11
IMM	Inner mitochondrial membrane
INF-γ	Interferon-gamma
IRS-1	Insulin receptor substrate-1
IRS-2	Insulin receptor substrate-2
IRS-3	Insulin receptor substrate-3
IRS-4	Insulin receptor substrate-4
IRS-5	Insulin receptor substrate-5
IRS-6	Insulin receptor substrate-6
IUGR	Intrauterine growth restriction
IUI	Intrauterine insemination
IVF	*In vitro* fertilization
JAR	Human chorionicarcinoma cell line
LCAT	Lecithin:cholesterol acyltransferase
LDL	Low-density lipoprotein particle
LDL-C	Low-density lipoprotein cholesterol
LGA	Large-for-gestational age
LH	Luteinizing hormone
LIF	Leukaemia inhibitory factor
LMP	Last menstrual period
LPL	Lipoprotein lipase
LXR-α	Liver X receptor-alpha
LXR-β	Liver X receptor-beta
MAPK	Mitogen-activated protein kinase
MMP	Matrix metalloproteinase
NEFA	Non-esterified fatty acid
NK cell	Natural killer cell
OMM	Outer mitochondrial membrane
P450c17	17α-hydroxylase/17,20 lyase
P450scc	Cholesterol side-chain cleavage enzyme
PA	Plasminogen activator
PAI-1	Plasminogen activator inhibitor-1
PAI-2	Plasminogen activator inhibitor-2
PAPP-A	Pregnancy-associated plasma protein-A
PCOS	Polycystic ovary syndrome
PE	Preeclampsia
PI3-K	Phosphatidylinositide 3-kinase

PIBF	Progesterone induced blocking factor
PIGF	Placental growth factor
PPIA	Peptidylprolyl isomerase A
PPAR	Peroxisome proliferator-activated receptor
RL95-2	Human endometrial epithelium cell line
RT	Room temperature
SAT	Subcutaneous adipose tissue
sFlt-1	Soluble Fms-like tyrosine kinase-1
SH2	Src homology-2 domain
slCAM-1	Serum intracellular adhesion molecule-1
SLOS	Smith Lemli Opitz syndrome
SMC	Smooth muscle cell
SIMD	Scottish Index Multiple Deprivation
SR-B1	Scavenger receptor class B member 1
SREBP	Sterol regulatory element binding protein
STAR	Steroidogenic acute regulatory protein
START	STAR-related lipid transfer
sVCAM-1	Serum vascular adhesion molecule-1
TBP	TATA box binding protein
TC	Total cholesterol
TG	Triglyceride
TGF-β	Transforming growth factor-beta
Th1	T helper cell 1
Th2	T helper cell 2
TIMP-1	Tissue inhibitory metalloproteinase-1
TNF-α	Tumour necrosis factor-alpha
tPA	Tissue-type plasminogen activator
T_{reg}	T regulatory cells
uPA	Urokinase-type plasminogen activator
VAT	Visceral adipose tissue
VCAM-1	Vascular cell adhesion molecule-1
VEGF	Vascular endothelial growth factor
VEGF-A	Vascular endothelial growth factor-A
VLDL	Very low density lipoprotein particle
VSMC	Vascular smooth muscle cell
VWF	Von Willebrand factor

1 Introduction

Successful reproduction in humans requires a spectrum of processes, from conception to the birth of a healthy infant, which are both biological and epidemiologically complex. The difficulties that arise during the course of the reproductive process define the complications of pregnancy as evident in epidemiological studies. With normal pregnancy, conception requires viable sperm to reach the ovum and fertilise it, and this must progress to implantation. The first weeks of gestation of normal development depend on the differentiation and migration of cells, events that must follow precise timing, leading to the formation of diverse organs and systems and subsequent fetal growth and development. A deviation from the optimal pathway at any point may perhaps result in the development of complications in pregnancy. In 2004, Hoozemans et al. noted that a pregnancy rate per cycle of approximately only 15% suggests that human reproduction is an inefficient process (Hoozemans et al. 2004). Of all fertilised oocytes, 40% are lost after fertilisation but before the end of gestation and, of those that survive, 7% of infants are born prematurely and 4% of newborns that go to term are born with birth anomalies.

Gynaecologists and reproductive scientists have encountered the increasing reproductive problem of obesity, evident by the growing number of women diagnosed with disorders of infertility and other significant sequelae (Sharpe and Franks 2002). The effect of environment and lifestyle on sperm count, oocyte development/ovulation, fertilisation and implantation are most likely to impact infertility. As the reproductive system and its hormonal control systems are established in fetal life, maternal factors that affect the 'fetal environment' may also influence the fetal vascular system. Because of its own physiological processes, the fetus adapts to its environment, and such adaptation may result in adverse effects. The increasing rate of maternal obesity provides a major challenge to obstetric practice. The high prevalence of (and increasing trend to) obesity in developed societies means that it is only a matter of time before real concern emerges over pregnancy in the developing world.

1.1 Maternal obesity and its adverse effects

In the UK alone, obesity has assumed epidemic proportions, and is one of the most important threats to reproductive success, with almost half of the women of childbearing age being overweight or obese. A report of body mass index (BMI) in the Scottish population of women attending antenatal care in 2002-04 (compared with 1990) shows 1 in 5 of this female population is classified as obese (Kanagalingam et al. 2005). In England, the proportion of women reported to be obese at start of pregnancy increased significantly from 7.6% to 15.6% ($P<0.01$) over 19 years between 1989 and 2007

(Heslehurst et al. 2010). In the United States, the mean maternal weight was observed to have increased by some 20% between 1980 and 1999, with an increase in maternal BMI greater than 29kg/m^2 from 16.3% to 36.4% in the same period (Lu et al. 2001). Another United States population-based study of the prevalence of obesity among women of childbearing age (20-44 years) in 2004 revealed that 24.5% were overweight (BMI 25-29kg/m^2) and 23% were obese (BMI ≥30kg/m^2) (Vahratian 2009). A Canadian based-study of women of childbearing age (19-44 years) highlighted that almost 72% of participants were overweight or obese, with average BMI of 29.7(7.9)kg/m^2 (Schaefer et al. 2011). Maternal obesity in pregnancy carries significant risk to the mother, and has long-term adverse effects on the offspring's health (Freeman 2010; Jarvie et al. 2010). A number of obesity-related effects with adverse outcomes for mother and child are listed in Table 1-1.

Table 1-1: **Consequences of increased maternal obesity**.

Pregnancy outcomes	№ of Subjects	Author
Menstrual disorders	726	(Wei et al. 2009)
Infertility (PCOS)	72	(Jungheim et al. 2009)
Preterm birth	187,290	(Smith et al. 2007)
Pre-eclamptic pregnancy	17,773	(Mostello et al. 2010)
PIH	6,534	(Jaques et al. 2010)
Fetal macrosomia	4,830	(Yogev and Langer 2008)
Neonatal death	187,290	(Smith et al. 2007)
Gestational diabetes	3,798	(Roman et al. 2011)
Intensive care admission	764,387	(Knight et al. 2010)
Iatrogenic	4,341	(Bergholt et al. 2007)
Congenital anomalies	974	(Anderson et al. 2005)
Stillbirth (fetal) death	40,932	(Tennant et al. 2011)
Miscarriage; pregnancy loss	4,932; 204	(Lashen et al. 2004; Landres et al. 2010)

PCOS indicates polycystic ovarian syndrome; PIH, pregnancy induced hypertension.

The adaptive response of obesity during pregnancy is described elsewhere in this chapter, but obesity significantly alters metabolic and inflammatory parameters in gestation. Various reports describe the change of parameters, in particular those involved in lipid and carbohydrate metabolism and inflammation throughout gestation (Catalano et

al. 1999; Redman et al. 1999; Stewart et al. 2007). The exacerbation of such parameters is linked to risk of poor pregnancy outcomes (Redman and Sargent 2004; Catalano 2010).

1.2 Implantation and placental development

Implantation is a unique process in mammals characterised as a pivotal step for pregnancy success. Successful implantation always predetermines the outcome of a pregnancy, and failure is primarily associated with numerous adverse pregnancy outcomes (Norwitz et al. 2001). However, for a successful implantation to occur, a functional blastocyst/embryo and a receptive uterus must be present. Studies on animal models classified the implantation process into three phases: apposition, adhesion and invasion (Bischof and Campana 1997; Enders and Lopata 1999; Simon et al. 1999). Each stage involves highly controlled orchestrated events, including paracrine dialogues and coordination of molecular activities. Onset of implantation is characterised by hatching of the free blastocyst from its adhesive coat the zona pellucida, as it drifts to the uterine cavity prior to apposition (Enders et al. 1986).

1.2.1 Timing of implantation

The timing of implantation depends on the relationship between events involved in implantation; this remains an ongoing area of study (Aboussahoud et al. 2010; Almog et al. 2010). Menstruation, occurring approximately on day 14 in a 28-day cycle, comprises one of four phases identified as follicular, ovulatory, luteal and menstrual phases. These phases are regulated by the endocrine system, the prime initial pathway in implantation. In the first place, a luteinising hormone (LH) surge precedes oocyte release by 24-36 hours, and is a definable standard clinical marker of ovulation. The oocyte has a life-span of around 24 hours from its burst from the ovary during ovulation, whereas some sperm can live in the right environment (such as the fallopian tube, uterus and cervix) for up to 5 days. However, most sperm die within 1-2 days after ejaculation, even in the right environment. The endometrium undergoes morphological and physiological changes that prepare the endometrial layer for implantation under the influence of progesterone. In the absence of fertilisation, the endometrial layer sloughs off as menstruation. But if viable sperm encounter an ovulated oocyte, usually in the ampulla of the oviduct, they surround it as they force their way through the cumulus mass. Shortly after fertilisation (approximately (~) 24-48 hours) (Red-Horse et al. 2004), when female and male pronuclei unite, the zygote rapidly divides to allow replication of maternal and paternal chromosomes and prepare the first cleavage. The first cleavage forms a compact mass of cells called the morula, as it moves along to the endometrial cavity. Further aggregation of cells ends in formation of the blastula, containing approximate 12 to 16 cells protected by

a non-adhesive coat called the zona pellucida. The blastocoel, a fluid-filled cell blastocyst, reaches the uterine cavity by day 3 to 4, and hatches out just before adhesion onto the uterine wall occurs. The hatched blastocyst constitutes two distinct cell populations known as an inner cell mass and an outer layer, the trophectoderm, which surrounds the inner cell mass (Adjaye et al. 2005; Cauffman et al. 2009). Consequently, while the inner cell mass differentiates into the fetus, the trophoblasts become responsible for the placenta formation. The blastocyst usually implants between day 6 to 10 post ovulation, LH+7 to LH+11, and/or day 20 to 24 post last menstrual period (LMP) (Aplin 1996; Wilcox et al. 1999; Lessey et al. 2000; van Mourik et al. 2009). This defined span of time is called the implantation window.

1.2.2 *In vitro* models of implantation

Implantation requires contact between the blastocyst and maternal uterine epithelium, a contact which is the defining step for successful implantation. Completing this step is paramount for achieving normal pregnancy outcome. Studies of embryonic implantation are extremely difficult because of poor accessibility and availability of embryonic tissues. Ethical concerns with regards to experimentation using primary human tissue during this period of life have necessitated the need to develop *in vitro* models to study the basic mechanism involved. For instance, the sub-cellular and molecular activities during implantation cannot be studied *in vivo*, and this is difficult even *ex vivo*. The *in vitro* model of blastocyst implantation is therefore based on assumptions that implantation of the blastocyst into the endometrium is a process that is very similar to tumour invasion of the host tissue. The cytotrophoblastic cells of a first trimester pregnancy retain almost all the properties of the trophectodermal cells of the blastocyst, and can be used as surrogates to study the implantation process *in vitro* (Bischof and Campana 1996).

1.2.3 Factors of importance for implantation

The uterus is mainly constituted by the epithelium and stromal cell components. Cellular changes transform the fibroblast-like endometrial stromal cell into the larger and rounded decidual cell, and cause the growth and development of the secretory gland and the emergence of pinopodes, i.e large apical protrusions, and microvilli on the luminal epithelium (Paria et al. 2002; Dunn et al. 2003). All of these changes modulate expression of major molecules involved in successful implantation, which include hormones, growth factors, cytokines/chemokines and adhesion molecules (Paria et al. 2002; Jabbour et al. 2009; Dekel et al. 2010). This thesis focuses on the hormonal and immune responses and adhesion molecules which are part of the complex process that requires interplay of these and many other systems during implantation (van Mourik et al. 2009).

1.2.3.1 Hormonal regulation

To date, human chorionic gonadotropin (hCG), a glycoprotein hormone, is used as an indicator of pregnancy. Its synthesis is found to start before day 7 of the blastocyst stage (Lopata and Hay 1989; Bansal et al. 2012) and the synthesis is later taken over by the placenta. Even so, primary and transformed trophoblast cells show a cytotrophoblastic phenotype producing hCG to a lesser extent than do syncytiotrophoblastic cells (White et al. 1988). In the same report, detectable hCG in urine at 2 weeks' gestation reaches a peak by 8 weeks, supporting corpus luteum recues between 8-10 weeks. As hCG has the ability to downregulate maternal inflammatory response against trophoblastic paternal antigen (Bansal et al. 2012), the corpus rescue is necessary until the functional placenta takes over hCG production. Consequently, the risk of compromised trophoblast invasion is reduced. Also, hCG clearly promotes angiogenic activity of extravillous trophoblasts (EVT), as its impairment has been shown to lead to poor placentation (Bansal et al. 2012).

The signals originating from the human hypothalamic-pituitary-ovarian axis end in the endometrium (Tabibzadeh 1998). Usually, there is intricate communication between the mother, placenta and fetus in order to maintain a balance supply of steroid hormone for optimal embryonic development. Preparation of the uterus for implantation and maintenance of pregnancy is regulated by progesterone. This crucial hormone is initially produced by the corpus luteum that forms immediately after ovulation. Cholesterol is a sterol that is a precursor to steroids in the steroidogenesis by its conversion into pregnenolone via the cholesterol side-chain cleavage enzyme (P450scc) (Voutilainen et al. 1986; Guibourdenche et al. 2009). In their report, Guibourdenche et al. mention that the pregnenolone is then converted into progesterone by the 3β-hydroxysteroid dehydrogenase type 1 (3βHSD1) localised in the mitochondria of steroidogenic tissues (but not placenta). There are five major classes of steroid hormones (oestrogen, progestin, androgen, glucocorticoid and mineralocorticoids), all use pregnenolone as a common precursor (Hu et al. 2010). The oestrogen and progesterone control the cellular and molecular mediators of endometrial receptivity, including growth factors, cytokines, adhesion molecules, pinopodes and homeobox genes (Kodaman and Taylor 2004). Oestrogen, naturally occurs in women as oestrone, oestradiol and oestriol (Guibourdenche et al. 2009), which induces rapid tissue proliferation, whereas the secretory pattern is controlled by progesterone, and both hormones are mediated via their receptors (Paria et al. 2002; Kodaman and Taylor 2004; Cha et al. 2012). The production of oestrogen and progesterone by the human placenta increases exponentially after week 6, following the involution of ovarian sex steroid production (Newbern and Freemark 2011). This importance of these steroid hormones cannot be overstated, as pregnancy cannot occur without them. Some of the functions carried out by oestrogen and

progesterone toward embryo implantation are summarised in Table 1-2 (Hoozemans et al. 2004).

Table 1-2: **A summary of oestrogen and progesterone actions**.

Oestrogen	Progesterone
Proliferative, follicular phase. Endometrial proliferation. Upregulation of progesterone receptors. Upregulation of VEGF, IGF-1, heparin binding epidermal growth factor and L-selectin.	Secretory, luteal phase. Morphological changes in endometrium. Pinopode formation. Upregulation of colony stimulating factor, IL-1, prostaglandins, VEGF, glycodelin A, IGF-2, heparin binding epidermal growth factor, fibronectin, mucin-1 and L-selectin. Downregulation of LIF and β-integrin. Downregulation of ER and oestrogen activity.

VEGF represents vascular endothelial growth factor; IGF-1, insulin-like growth factor-1; IGF-2, insulin-like growth factor-2; IL-1, interleukin-1; LIF, leukaemia inhibitory factor; ER, oestrogen receptor.

The pre-ovulatory elevation of oestrogen (oestradiol-17β) and progesterone stimulate the proliferation of uterine epithelial cells (Gude et al. 2004). The oestrogen receptor (ER) alpha, ER beta1 and ER beta2 mRNA expression in human luteal cells assist in the rescue and maintenance of the life-span of the corpus luteum (van den Driesche et al. 2008). In humans, the corpus luteum remains the source of progesterone for 4-5 weeks after implantation, at which time progesterone production is gradually taken over by the placenta to maintain pregnancy (Csapo and Pulkkinen 1978). The corpus luteum secreting progesterone is called the corpus luteum graviditatis. In the absence of fertilisation, and after placental sufficiency, the corpus luteum regresses and becomes corpus albican. The regression of corpus luteum is induced by prostaglandin F2 alpha (Wang et al. 2003). To date, the progesterone concentrations needed to maintain early pregnancy in humans are not known (Azuma et al. 1993). The presence in multiple species of progesterone increase at implantation indicates that the increase perhaps has a functional significance. Also after ovulation, numerous molecules in gap-junctions of the membrane of the ovary appear between the developing cumulus cells, including connexin 43 (Furger et al. 1996), and oocyte LH receptor expression is increased by activation of 3βHSD1 (Hamel et al. 2008). These molecules play a positive role in the synthesis of progesterone in particular. The 3βHSD1 is upregulated in follicles; this increase is involved in steroidogenesis, resulting in pregnancy success. The blastocyst/embryo, en route to the uterine cavity, must be prevented from adhering to areas where it will have a poor chance of implantation by the activity of a molecule such as mucin-1. This molecule lines the epithelial surface of the fallopian tube and uterus. Mucin-1 is a member of the mucin family of the highly glycosylated high-molecular weight integral membrane glycoproteins. Its anti-adhesion activity means that the highly adhesive blastocyst is

guided onto the proper site of the endometrium at the precise spot for adhesion. In humans, mucin-1 upregulation during the period of implantation happens under the influence of progesterone (Meseguer et al. 2001). This progesterone-dependent regulation of mucin-1 is thought to be partly responsible for determining endometrial receptivity (Horne et al. 2006).

1.2.3.2 Localised immune response

Immunological changes associated with pregnancy involve both local properties and broader systemic effects, which are described in the latter part of this chapter. Stanley Cohen first introduced the term lymphokine, or monokine, which is currently referred to as cytokine, a multifunctional protein ranging from 8-40kDa (Cohen et al. 1974). These proteins regulate peptides and glycoproteins, and are produced by virtually every cell in the body, as well as stimulating many different cell types. Redundancy generally exists within the cytokine families, and different cytokines often exert similar and overlapping functions on certain cells (Salamonsen et al. 2007). A specific type of cytokines, called chemokines, are small chemotactic molecules best known for leukocyte recruitment and activation. Approximately 50 chemokines and 20 receptors have been identified in humans (Zlotnik and Yoshie 2000). Chemokines are classified into four families based on their cysteine amino acid residue presence, namely CXC, CC, C (lymphotactin) and CX_3C (fractalkine). The variable 'X' represents amino acids involved in some group; a capital 'L' is added to identify the ligand, as distinct from the 'R' added to identify the receptor. The number of receptors is much less than the number of ligands (Salamonsen et al. 2007). Thus, there is promiscuous ligand-receptor binding and significant redundancy and overlap of functions.

Cytokines and chemokines are involved in achieving implantation success (Dimitriadis et al. 2010), placentation, cervical ripening and uterine activation of labour. The cytokines/chemokines are synthesised by several cell types at the maternal-fetal interface, such as leukocytes, endothelium, connective tissue cells and endometrial epithelium. Those produced by the endometrial epithelium may be secreted on the apical membrane of the uterine lumen, where they influence blastocyst development, migration and adhesion, or even basally, with effects on the transformation of underlying stroma (Salamonsen et al. 2007; van Mourik et al. 2009). None of this could be possible without polarity, important at several levels of embryonic implantation. At first, the polarity present within the oocyte and developing blastocyst is essential for optimal differentiation. Other levels of polarity are established when the blastocyst approaches the endometrium and adopts a specific orientation in association with the endometrium. In humans, the inner

cell mass of the blastocyst is directed toward the endometrium (Lee and DeMayo 2004) as the conceptus moves to the uterus.

While the mechanism by which the blastocyst orients itself as it migrates to the uterine cavity remains completely unknown, embryonic chemotaxis is among the hypotheses that have been put forward. This hypothesis involves a chemokine gradient in the endometrium (Dominguez et al. 2005), which is within the uterus, set up by cytokine activation, and thus guides the trophectoderm of blastocyst to the site of implantation. This interaction, called tethering, allows leukocytes to be drawn towards the endothelium, and this similar concept is proposed as being involved during blastocyst/embryo-endometrial epithelium interaction. A similar hypothesis has established a role for the chemokine in the attraction of leukocyte to the endometrial tissue during implantation (Red-Horse et al. 2001). At the precise site of the uterine wall where the blastocyst implants, the degradation of mucin-1 occurs. It is thought that mucin-1 at this spot is degraded by mediators from immune cells. This is because dendritic cells and macrophages secrete cytokine/chemokine that induces uterine stroma local degradation of mucin-1 (Dekel et al. 2010; Mor et al. 2011).

Next is the apposition phase, encompassing similar leukocyte-endothelial cell interactions, a process similar to that observed in the circulations as leukocyte extravasates across vascular subendothelial space. Then, is adhesion/attachment detailed below. This phase is a period by which the blastocyst interacts with endometrium, which the mechanism is yet fully understood, but, like the leukocyte-endothelium interaction, it relies on the dialogue of cytokine and chemokine parameters (Hannan and Salamonsen 2008).

Leukocytes do not usually cross blood vessels but they do so at sites where inflammatory responses occur through leukocyte chemotaxis. Studies on humans and animals have detected a number of chemokine receptors, such as CCR1, CCR2, CCR3, CCR5, CXCR1 and CXCR2, and the motif ligands they bind: monocyte chemotactic protein-1 (MCP-1), also known as CCL2, CCL3, CCL4, CCL7, CCL11, CCL14 and interleukin-8 (IL-8), also referred to as CXCL8. All of these are detectable in trophoblasts, epithelium and endometrium (Kayisli et al. 2002; Ulukus et al. 2005; Hannan et al. 2006; Dimitriadis et al. 2010; Chau et al. 2013). Their detectable presence in these tissues supports their potential role in blastocyst orientation. These chemokine receptors and motif ligands may also be required for effective leukocyte recruitment to support the trophoblast for decidualisation as the embryo implants. CCL2 and CXCL8 have been localised in the human uterus (Kayisli et al. 2002) and have an active role as chemoattractants of neutrophils, T cells, mast cells, natural killer (NK) cells and monocytes. In addition to this, there are nonimmune functions of these molecules in placentation, differentiation of

cytotrophoblast and uterine tissue remodelling, as well as removal of maternal-fetal debris and dead cells with the advance of pregnancy, which is well established (Li and Huang 2009; Hazan et al. 2010).

1.2.3.3 Adhesion molecules

Classical work dating from 1959 by Hertig et al. shows that inadequate adhesion of the embryo results in recurrent pregnancy loss (Hertig et al. 1959). This is an indication that adhesion molecules are necessary during the implantation window. The apical surface of the endometrial layer (epithelium) is in a non-adhesive setting compared to the basolateral surface. Adhesion occurs at the apical region of the trophectoderm of the blastocyst and the endometrium as a cell-to-cell interaction involving adhesion molecules' expression at the apical cell membrane. Cell adhesion molecules are composed of four members, termed integrin, cadherin, selectin and immunoglobulin (Achache and Revel 2006). The efforts to identify important adhesion molecules involved in embryo implantation have led to an increase *in vitro* studies (Heneweer et al. 2002; Sugihara et al. 2008).

A number of cell adhesion molecules have been implicated in the embryo-endometrial epithelium interaction. Trophinin (Fukuda et al. 1995), Indian blood type (CD44 molecule) (Campbell et al. 1995) and laminin α1 (Dziadek and Timpl 1985; Nissinen et al. 1991) are among the surface adhesion molecules expressed in human blastocysts and/or uterine epithelial basement membranes during embryonic implantation. Trophinin is an intrinsic plasma membrane glycoprotein containing 749 amino acids (Fukuda et al. 1995) that allows direct homophilic cell adhesion. Trophinin must interact with the cytoplasmic protein tastin, and both are bridged by the protein bystin forming a functional complex (Suzuki et al. 1998). CD44 expression in embryonic tissue occurs throughout preimplantation until the blastocyst stage highlights its potential function in embryo-endometrial epithelium adhesion (Campbell et al. 1995). CD44 is a highly polymorphic membrane glycoprotein, with a molecular weight of 80-200kDa, encoded by a single and highly conserved gene (Screaton et al. 1992). This adhesion molecule is a well-characterised member of the hyaluronic acid receptor family. Interaction of CD44 molecules with hyaluronate on many cell types (Aruffo et al. 1990) allows binding of cells to extracellular matrix ligands. CD44 is expressed on cellular surfaces of most vertebrate reproductive tissues, including the cervix (Tsubaki et al. 2005) and endometrial epithelium (Aplin et al. 1994).

A direct role for laminin α1 in the adhesion of the embryo to the endometrial epithelium was suggested when higher levels of IgG anti-laminin-1 autoantibodies were observed in women with recurrent miscarriage than in healthy pregnant and healthy non-pregnant

controls (Inagaki et al. 2001; Inagaki et al. 2003). Laminin-1 mRNA is present in JAR cells (Nissinen et al. 1991) and in mouse preimplantation embryos (Shim et al. 1996). Laminin-1 (also known as laminin-111) exists as part of at least 16 laminin isoforms, all recognised to have a role in cellular adhesion (Aumailley et al. 2005). These isoforms are comprised of different subunits: α1 to 5, β1 to 4 and γ1 to 3 (Miner and Yurchenco 2004; Miner 2008). Laminin α1 is a heavy chain, with molecular weight of 400kDa (Tiger et al. 1997), and its N-terminal domain interacts with β1 and β2 (also referred to as laminin γ1) chains. Laminin α1 is localised specifically at the basement membrane of the epithelium. Laminin α1 has a large, 100kDa C-terminal globular (G) domain (Sasaki et al. 1988) that functions as a recognition site for proteins such as integrin receptors which promote cell adhesion (Nomizu et al. 1995). Laminin α1 interacts with extracellular matrix components. One example is the fibulin (FBLN) protein family that comprises extracellular matrix proteins that serve to modulate cellular behaviour and the function of other proteins in the extracellular matrix (Argraves et al. 1990; de Vega et al. 2009). FBLN-1 has a molecular weight of 100kDa whereas that of FBLN-2 is 195kDa (de Vega et al. 2009). There are limited data showing that FBLN-1 (Timpl et al. 2000) and FBLN-2 (Utani et al. 1997) interact with laminin α1. FBLN-1 interacts with the C-terminal LG domain of laminin α1, while FBLN-2 binds to the N-terminal domain (Utani et al. 1997).

1.2.4 Trophoblastic invasion

The term invasiveness simply means the ability to cross anatomical barriers. The first barrier to implantation is the layer of endometrial epithelial cells; immediately beneath them is a specialised type of matrix known as the basement membrane, a thin continuous layer. This third and final phase of implantation is completed by invasive trophoblast cells penetrating the endometrial epithelium basement membrane into the endometrial stroma (Bischof and Campana 1997). Invasion permits the creation of the haemochorial placenta. Human trophoblasts are characterised by strong invasiveness which ensures adequate contact to maternal blood circulation. The invasive process is tightly regulated, as the embryo trophoblast cells invasion is limited to the decidualised endometrium up to the proximal third myometrium in normal pregnancy (Cartwright et al. 2010). As Cartwright et al. report, the importance of these events occurring in a regulated fashion is illustrated by complicated pregnancies associated with insufficient spiral arteries remodelling. Usually, the blastocyst is too large to squeeze through the endometrial layer, adhered trophoblasts uses the paracrine activity to induce apoptotic reaction, degrading underlying endometrial epithelium, as has been found in rodents (Galán et al. 2000). The basement membrane extracellular matrix then migrates into the decidual stroma. Eventually, the cytotrophoblast invades the entire endometrium, the uterine vasculature and into the inner third of the myometrium. The access to the uterine vessel creates the lacunae network comprising

the uteroplacental circulation that places the placental trophoblast in direct contact with maternal blood. This contact allows placenta to serve the transfer organ role for nutrients, gases, waste products, hormones and growth factors between mother and fetus.

The invasion is made possible by some protease/proteinase, such as serine protease, collagenase and metalloproteinase, which are capable of digesting the endometrial extracellular matrix, as is well reported. Examples of protease mobilised by the embryo as it embeds into/penetrates the maternal endometrium include matrix metalloproteinase (MMP) and plasminogen activator (PA) (Harvey et al. 1995; Nagaoka et al. 2003; Anacker et al. 2011). MMPs are a family of approximately 20 human zinc-dependent endopeptidases, collectively able to degrade all components of an extracellular matrix. MMP action is vital for the invasion of trophoblast cells; at the same time, it is regulated by the tissue inhibitors of metalloproteinase (TIMPs) that act to counterbalance excessive trophoblast invasion (Bischof and Campana 2000; Staun-Ram et al. 2009). Anacker et al. showed almost all MMPs, and all four TIMPs are expressed at the messenger ribonucleic acid (mRNA) level (Anacker et al. 2011) in uterine NK cells, decidual fibroblasts and trophoblasts, apart from MMP-20 and -25. PA is serine residue protease and is in two forms, tissue-type (t)PA and urokinase-type (u)PA. These proteins are secreted as a single chain with tPA but not uPA being catalytically active. These substrate-specific serine-proteases cleave the Arg-Val peptide bond and converts plasminogen to plasmin, which controls blood clotting by modulating fibrinolysis (Fay et al. 2007). Cleavage of the single chain form of uPA by plasmin leads to uPA becoming catalytically active. In the serine protease PA system (tPA or uPA) there exists a cognate receptor on the cell surface with two specific inhibitors, referred to as plasminogen activator inhibitors-1 (PAI-1) and -2 (PAI-2) (Ulisse et al. 2009). PAI-1, a mediator of the clotting process, is produced by vascular cells, whereas PAI-2 is derived from the placenta. Both PAI-1 and -2 have proteolytic implications for physiology, pathophysiology, uterine degradation, tissue remodelling and coagulatory processes. This broad spectrum of MMP expression in the maternal interface reflects its role in trophoblast invasion and uterine tissue remodelling. TIMP, PAI-1 and PAI-2 modulate PA activity, preventing excess invasion of the implanting embryo (Harvey et al. 1995; Ulisse et al. 2009).

The fetal growth depends on adequate transformation of uterine spiral arteries by a specific subset of EVT (Harris 2011). Since detailed knowledge of implantation in humans is lacking, studies in macaques show that the first invasion occurs during implantation via an invasive syncytiotrophoblast (Enders 2007). This multinucleated layer moves between epithelial cells and invades the upper layer of the decidual interstitium. It may already erode the first capillaries and glands. A similar pathway is thought to take place in humans (James et al. 2012). In a recent review, a widespread reorganisation of the

syncytiotrophoblast was evident in the first two weeks after implantation (Huppertz et al. 2013). The syncytiotrophoblast develops into a layer that completely surrounds the embryo, developing internal fluid-filled lacunae which are penetrated by the mononucleated cytotrophoblast in addition to embryo-derived mesenchymal cells. By the 5th week, the cytotrophoblast has penetrated through the syncytiotrophoblast, reaching the outer surface of this layer, the decidual tissue. At this point, trophoblastic cell columns develop and they proliferate the part facing the developing villous structure (anchoring villi) and the invasive part that faces the maternal tissues. The proliferating part comprises cells which are still in contact with the basement membrane of the anchoring villous. Once they lose contact with the basement membrane, simply by proliferative pressure, the cells start to differentiate and change their properties to those of an invasive phenotype (Kaufmann et al. 2003). Using the cell column as the proliferating source, EVT are now known to start to invade the maternal tissue, migrate through the decidual interstitium and reach the inner third of the myometrium within the next two weeks.

In normal pregnancy, EVT invade the decidua and inner third of myometrium. Invasion occurs via two pathways: the interstitial and endovascular EVT (Pijnenborg et al. 1980). The interstitial EVT invade through the decidua from anchoring villi and cytotrophoblast shell, the endovascular EVT arise from either direct intravascular invasion or transmural migration of interstitial EVT into spiral arteries. The invasive properties of EVT establish a route of nutrient supply from the mother to the placenta.

1.2.5 Human placentation

The placenta formation is named 'placentation' and completes the implantation window and establishes the means to support the fetus throughout gestation. The human placenta is described as haemochorial type I (Wildman 2008; Enders 2009), characterised by direct contact between the maternal blood (haemo) and epithelium of chorion (trophoblast). The uniqueness of human implantation has made animal model studies only vaguely applicable to humans. The placenta originates from the extra-embryonic membrane of the trophectoderm of the blastocyst after fertilisation, as the inner cell mass forms the conceptus and umbilical cord (Cauffman et al. 2009). In this stage of the early placenta, at the maternal-fetal interface, the human trophoblast differentiates along two pathways: the villous trophoblast pathway, involving the cytotrophoblastic cells that differentiate by fusion to form the syncytiotrophoblast, which covers the entire surface of villi and the EVT pathways (Huppertz 2008; Guibourdenche et al. 2009).

Villous trophoblasts are non-migratory. At about day 13 post conception the trabeculae begin to develop their first side branches, which may simply be syncytiotrophoblast

protrusion (syncytial sprouts) or may contain a core filled with cytotrophoblasts (Kaufmann et al. 2003). Cytotrophoblasts proliferate, differentiate and then fuse, forming an outer epithelial layer of chorionic villi: the syncytiotrophoblast (Gude et al. 2004). Evagination of the syncytiotrophoblast with the cytotrophoblast forms the primary villi. The next step happens when the fetal mesenchyme proliferate into the cytotrophoblast and form secondary villi; haematopoietic progenitor cells develop and start to differentiate (Huppertz 2008). These secondary villi later develop into fetal capillaries by 3 weeks' gestation, within which villous mesenchyme form tertiary villi (Gude et al. 2004). As gestation progresses, decidua on the uterine luminal pole regress (as well as the villi attached to them) resulting in placenta villi in the discoid region. Chorionic villi present on the surface of the entire chorion grow as pregnancy progresses. Usually, partial exchange occurs via the terminal villi projected into the intervillous space (Gude et al. 2004). The villi tips attach to the basal plate and form anchoring villi, resulting in an invasive cytotrophoblast cell column. Formation of these villi reflects a very basic stage of development of new villi, and the process occurs throughout gestation.

In the EVT pathway, trophoblasts migrate. This begins with the blastocyst firmly attached to the uterine epithelium; the polar trophoblast overlying the inner cell mass undergoes differential steps (described as the syncytial fusion of mononucleated cells) to form the first oligonucleated syncytiotrophoblast (Huppertz 2008). This syncytiotrophoblast displays invasive features as it penetrates the uterine epithelium, and, by the next few days, the embryo embeds itself into the decidual stroma, with the syncytiotrophoblast of the blastocyst forming a complete mantle surrounding the conceptus. The remaining mononucleated trophoblast (called a cytotrophoblast) is found in the second row, and does not make contact with the maternal tissue. The cytotrophoblast acts as a stem cell that rapidly divides, and later fuses with the syncytiotrophoblast (Pötgens et al. 2002), and this fusion forms a syncytium as pregnancy advances to term. Fluid-filled space occurs within the syncytiotrophoblast by day 8 post conception and soon coalesces to form larger lacunae. After this point, the remaining syncytiotrophoblastic mass within the lacunae, termed trabeculae, forms a villous tree (Huppertz 2008). From day 12 post conception, the cytotrophoblast penetrates the syncytiotrophoblastic mass of trabeculae until it reaches the maternal side of the placenta by the 15th day, marking the first time the cytotrophoblast establishes maternal contact. Only at 5 weeks' gestation does the cytotrophoblast leave the placenta proper and differentiate into the extravillous cytotrophoblast. Formation of the extravillous cytotrophoblast aids in the forming of the cytotrophoblastic shell at the maternal-fetal interface (Gude et al. 2004). Kaufmann et al. summarised that all trophoblast cells reside outside the placental villi under the EVT (Kaufmann et al. 2003). The endovascular trophoblasts invade the uterine spiral arteries, but the EVT – the interstitial trophoblasts – invade the decidua (Cartwright et al. 2010),

promoting the circumferential placental expansion by 8 weeks' gestation, as mucosa decidua become colonised. Maternal uterine decidua arterioles dilate in response to unresponsive maternal vasomotor regulation, promoting the maternal blood supply to the placenta by 30%, with maternal cardiac output increasing by 30-40% (Khong et al. 1986). The interstitial trophoblast is transformed into the multinucleated forming placental bed, the end-point of the extravillous or EVT pathways (Gude et al. 2004). Many factors assist in degradation of the uterine vascular bed. This includes the Hofbauer cells that support trophoblast cells in removing apoptotic cells and cellular debris, as well as assisting them in uterine vasculature remodelling and production of protease, which degrades the uterine extracellular matrix (Reister et al. 2001; Huppertz 2008; Hazan et al. 2010).

On the whole, by day 7-13 post ovulation, the growing trophoblast expands into masses of cytotrophoblasts and syncytiotrophoblasts, reaching the maternal vessel to form the lacunae networks (Tabibzadeh and Babaknia 1995). Jaffe et al. noted that not until 10-12 weeks' gestation does maternal blood supply, through spiral arteries into the intervillous space, begin (Jaffe et al. 1997). Placental development continues throughout gestation but begins to diminish from the 20th week (Pijnenborg et al. 1983).

1.2.6 Placental function

The placenta carries out several functions in order to ensure optimal fetal growth and development. Here this thesis focuses on hormonal and growth factor, which are vital for fetal growth and development.

1.2.6.1 Hormonal production

The function of the placenta is to facilitate the intricate communication between mother and fetus, which takes place in order to maintain a balanced supply of steroid hormone for optimal embryonic development. Placental hormones are essential for establishing and maintaining pregnancy, adaptation of the maternal organism to pregnancy and fetal growth. The EVT secrets a high amount of various hormones from the early placenta. An example is hCG, detectable as early as 48 hours after implantation from the cytotrophoblast (White et al. 1988). As pinpointed above, hCG prevents regression of corpus luteum for synthesis of progesterone after fertilisation. At 10-12 weeks' gestation, the syncytiotrophoblast is a major source of the polypeptide and steroid hormones which enter the maternal circulation, taking over the maternal metabolism to increase energy flux to the fetus (Guibourdenche et al. 2009). Placental progesterone synthesis, (as mentioned above, Section 1.2.3.1) is dependent on the delivery of cholesterol-rich lipoproteins from the maternal circulation and intracellular hydrolysis of cholesterol esterase to free

cholesterol for placental steroidogenesis. A detailed pathway for steroidogenesis is described in the latter part of this chapter. Briefly, the cholesterol is captured for steroidogenesis from a number of sources, including plasma low-density lipoprotein (LDL) and high-density lipoprotein (HDL), as shown in steroidogenic tissues (Grummer and Carroll 1988; Guibourdenche et al. 2009; Hu et al. 2010). Uptake of LDL cholesterol (LDL-C) carried by LDL occurs through apolipoprotein (apo) B-100 on the LDL and uptake of HDL cholesterol (HDL-C) through apo E on HDL particle. Both apolipoproteins (apo B and E) are recognised by the LDL receptor (LDLR) (Blasiole et al. 2008; Hu et al. 2010), which is present on surface of the placental syncytiotrophoblasts, by forming a lipoprotein-receptor complex via receptor-mediated endocytosis (Fuchs and Ellinger 2004). However, most of the cholesterol supplied by HDL mediates into cells leaving behind the HDL particle through a receptor called scavenger receptor class B member 1 (SR-B1), a process called selective pathway (Acton et al. 1996; Hu et al. 2010). Hu et al. pinpoint that the term selective cholesterol uptake (i.e. selective pathway) is used when cell surface bound cholesterol-rich lipoproteins (LDL or HDL, regardless of the lipoprotein composition), release cholesteryl ester without the parallel uptake and lysosomal degradation of the lipoprotein particle itself (Hu et al. 2010). Conversion of cholesterol to pregnenolone occurs in the inner mitochondrial membrane (IMM) producing progesterone, the prime source of other steroid hormones. The syncytiotrophoblast produced progesterone is useful in maintaining several functions, including uterine quiescence (Guibourdenche et al. 2009). Oestrogen enhances receptor mediated uptake of LDL-C for placental steroidogenesis and increases uteroplacental blood flow and endometrial prostaglandin production (Newbern and Freemark 2011). Other hormones, such as human placental lactogen (hPL), insulin, leptin and adiponectin, as well as relaxin, prolactin and oxytocin, are reportedly produced by the placenta. The hPL and prolactin increase high food intake by inducing central leptin resistance and promoting beta-cell expansion, which results in insulin resistance (Newbern and Freemark 2011). In their review, Pepe and Albrecht highlight that the placental hormones e.g. progesterone, are capable of stimulating erythropoiesis and consequently increasing red cell mass (Pepe and Albrecht 1995). This increase and subsequent change in blood viscosity, together with the hormonal effects on the vascular bed, have important regulatory effects on uterine and systemic vascular resistance.

1.2.6.2 Growth factor synthesis

Vasculogenesis is the process whereby new blood vessels are derived *de novo* from mesenchymal cells via haemangiogenic stem cell differentiation, whereas angiogenesis is the process that forms new blood vessels from already existing vessels (Charnock-Jones et al. 2004). Placental growth factor (PIGF) regulates placenta development (Maglione et

al. 1991), whilst vascular endothelial growth factor (VEGF) (also called VEGFα) is a potent angiogenic molecule that controls growth and the chemotactic activities of endothelial cells during vascular proliferation. These growth factors act to preserve normal vascular endothelial function. During gestation, the vascularisation of the placental villi occurs by day 21 post conception (Knoth 1968). Sequential steps of vasculogenesis and angiogenesis are observed within placental tissues (Demir et al. 2004). Both of these processes are regulated by angiogenic growth factors at each local villous and they aid in the establishment of fetal circulation, which starts with haemangiogenic stem cell induction through VEGF, forming prevascular networks in a stepwise manner. Cytotrophoblast, Hofbauer and differentiated perivascular cell (smooth muscle cell (SMC)) are all sources of growth factors. The study by Schiessl et al. on the expression of the VEFG family (VEGF-A, VEGF-C, VEGF-D) and their respective receptors (VEGF-R1, VEGF-R2 and VEGF-R3) and of the angiopioetin (Ang) family (Ang-1, Ang-2 and their receptor Tie-2) across the gestational placenta bed found lower VEGF-C, VEGF-R1, VEGF-R2, Ang-1, Ang-2 and Tie-2 expression on intramural EVT of the earlier placental bed (Schiessl et al. 2009). In their study, they conclude that this implies that VEGF and Ang families may play a role in spiral artery remodelling in normal pregnancy. So these factors are important, as the placenta is a key source of growth factors utilised for the developing fetus.

1.3 Assisted conception and the impact of obesity on infertility

Assisted reproductive technology (ART) has undoubtedly provided medical intervention options for couples with infertility disorder and a low chance of conception. These technologies, by definition, involve handling of human gametes and/or embryos *in vitro* (Zegers-Hochschild et al. 2009). A report by the International Committee for Monitoring Assisted Reproductive Technology (ICMART) highlights that ART accounted for approximately 219,000 to 246,000 offspring born worldwide in 2002 alone (de Mouzon et al. 2009). In 2003, an estimated 433,427 initiated cycles resulted in 232,980 babies born, a 10% increase in treatments from the previous year (Nygren et al. 2011); in 2004, a total of 954,743 cycles resulted in an estimated 237,809 births, a 2.3% increase in the number of reported cycles from 2003 (Sullivan et al. 2013). This is taking into account the missing or partial data from some reporting centres. To date, ART employs numerous variations of clinical practice to achieve pregnancy for infertile and subfertile patients.

1.3.1 *In vitro* fertilisation (IVF) and other procedures

IVF is an ART procedure where a human oocyte is fertilised *in vitro* outside the body; it was originally developed for patients with tubal dysfunction. Ovulation is induced using a

daily follicle stimulating hormone (FSH) determined by recipient age. Oocytes are retrieved after ovarian induction of follicular growth, and they are fertilised by mixing with high-quality fresh sperm that are allowed to fertilise oocytes before undergoing several cleavages to form a blastocyst/embryo *in vitro.* Implementation of the IVF-embryo transfer technique has contributed to high pregnancy rates through implementing a diverse range of modifications developed since the first birth, that of Louise Brown in 1978 (Steptoe and Edwards 1978). Gamete intra-fallopian transfer (GIFT), zygote intra-fallopian transfer (ZIFT), intrauterine insemination (IUI) and intra-cytoplasmic sperm injection (ICSI) are other procedures utilised in ART. There is also extended embryo culture, oocyte donation and embryo cryopreservation (Lieberman et al. 1992; Gelbaya et al. 2006). The GIFT infertility treatment employs a similar approach to IVF, with the replacement of both male and female gametes to allow fertilization to occur within the body. Conversely, ZIFT is a treatment option for infertility due to blockage of fallopian tubes that limits normal binding of sperm to the egg. Oocytes are removed from the ovary and *in vitro* fertilised before being replaced at pronuclei stages (zygote) in the oviduct, with laparoscopy, as described previously (Aslan et al. 2005). Aslan et al. also report that ZIFT did not demonstrate a difference in pregnancy success compared to a normal IVF-embryo transfer group. This finding is contradictory to others who observed higher pregnancy rates with ZIFT compared to IVF-embryo transfer (Levran et al. 1998; Levran et al. 2002). Overall, such a difference may occur due to the difference in sample collection patients and employed laboratory practice. In IUI, sperm is directly placed via the cervix into the uterus at the time of ovulation. This procedure was developed initially to treat women whose male partners suffer from low sperm count, premature ejaculation or poor sperm quality. A report by Kucuk shows that IUI improves pregnancy rates significantly, up to 23.5% (64/272) in women who had more ruptured follicles as seen on transvaginal ultrasonography compared to 8.8% (27/306) of those with an absence of detectable follicle rupture (Kucuk 2008). This is an indication that IUI improves conception rates in patients with appropriate ovarian follicular rupture because of oocyte accessibility by sperm.

ICSI is a technique that involves direct injection of sperm into the ovum with a micropipette penetrating the zona pellucida (Anifandis et al. 2010). This procedure improves pregnancy success with assisted conception, with a 56.6% pregnancy rate compared to that of 47.6% for other procedures in 2000, and rates of 75.9% recently reached in Latin America and 92.4% in the Middle East by 2002 (de Mouzon et al. 2009). A higher ICSI fertilisation rate was statistically linked to improved implantation rate (25.2% vs 17.8%), and remained significant compared to conventional insemination after adjustment for variables associated with implantation (Rosen et al. 2010). However, IVF and ICSI were found to have similar pregnancy and delivery rates (de Mouzon et al. 2009). Despite eliminating problems of low sperm count and poor quality, a new problem

of ICSI has emerged. This risk is the challenging dilemma transferring genetic anomalies that otherwise would not have been transmitted. This has led to preimplantation genetic diagnosis (PGD) an assessment tools adopted to identify embryo(s) at risk to avoid abnormal genetic transmission in couples before embryo transfer. PGD is widely reported in those suffering unexplained and recurrent miscarriage (Carp et al. 2004).

Despite the success of ART in overcoming infertility, the issue of aberrant embryo implantation remains a rate-limiting factor for successful IVF. Efforts to improve implantation success rates in ART have led to the practice of replacing more than one embryo. As a consequence, this led to multiple pregnancies attributable to successful multiple embryo transfer and survival to live birth. Multiple embryo transfer strategies are now recognised to be associated with the problem of high multiple pregnancy rates and deliveries (Giannini et al. 2004). Recognition and development of policies that aim to reduce the number of embryos transferred has assisted in reducing the multiple birth rate associated with assisted conception (Lieberman et al. 1992; de Mouzon et al. 2009).

1.3.2 Predicting pregnancy success

The efficacy of ART has resulted in its increased use, and this has led to ART becoming an emerging risk factor for several complications of pregnancy. Whether it is the ART procedure itself or factors associated with the condition of infertility that contribute to adverse outcomes is under study; reports are conflicting. Some suggest it is the specific procedure of ART which is responsible for risk (Shih et al. 2008), others conclude that laboratory procedures involved in IVF could not be responsible, but rather the risk factor is related to the health of the infertile women (Romundstad et al. 2008). The latter view is somewhat supported by studies in subfertility populations (Thomson et al. 2005; Jaques et al. 2010). For the embryos that survive in this practice, there is a higher risk of pregnancy loss than for those from natural conception, either because of the underlying problem for which ART intervention was needed or because of the assisted conception procedure by which pregnancy was achieved. Distinctly, aberrant implantation is identified as the rate-limiting factor in IVF/ICSI practice.

Thus, predicting pregnancy success is necessary for those undergoing assisted conception, but remains problematic. A major problem is the question of which sort of hormone and/or what amount to administer for optimal follicle growth. Maternal age plays a role in determining the protocol employed. Women of <36 years have 225IU and those of >36 years receive 300IU per day for follicle stimulation (Nelson et al. 2007). La Marca et al. observe that anti-Müllerian hormone levels have been shown to predict live birth rates, and measurement of this hormone could facilitate individualisation of the therapy

prior to the first ART cycle (La Marca et al. 2011). The degree to which obesity influences early pregnancy metabolic and inflammatory parameters and how this may impact on pregnancy success is still unclear. Obesity is a significant risk factor for altering metabolic metabolites and inflammatory mediators throughout gestation (Stewart et al. 2007). Maternal body fat may affect the parameters (including lipid and carbohydrate metabolites and inflammatory mediators, as well as hormones such as insulin and leptin) early in pregnancy. Exacerbation of this mechanism may provoke maternal insulin resistance and exaggerated lipid deposition in pregnancy. These changes may predetermine pregnancy success, because of their being linked with (so far unknown) changes in the metabolic and inflammatory parameters.

1.3.3 Evaluating natural cycle frozen embryo transfer (FET)

The treatments for assisted conception are continually advancing and improving. In a typical ART cycle, fresh cleavage stage embryos are transferred (Steptoe and Edwards 1978; Mettler et al. 1984). The merits of blastocyst/embryo(s) culture and cryopreservation and thawed cleavage stage embryo replacement (Nyboe Andersen et al. 2009) are evident by the increasing trend of ART. Because limitations arising from maternal age and parity are independently linked to abnormal pregnancy outcome (Bai et al. 2002), this suggests that individual factors determine the best ART approach for treatment. In natural cycle FET the embryo is transferred at a time relative to the endogenous LH surge depending on the embryonic stage of development, without artificial construction of the cycle with steroids (Al-Shawaf et al. 1993). The procedure is attractive and advantageous to women, as it eliminates the need for gonadotropin-releasing hormone (GnRH) agonist treatment with prior steroid administrations that suppress ovarian function and induce endometrial synchronisation. In women with a regular menstrual cycle (encompassing 212 natural cycles), using FET demonstrated a 14.1% implantation rate and 20 clinical pregnancies, while 205 women with pituitary-desensitised hormonal controlled cycles had a 13.5% implantation rate and 18 clinical pregnancies (Gelbaya et al. 2006). Nonetheless, the inherent problem with natural cycle protocol is that it is only feasible for women that have a regular menstrual cycle. Timing of the ovulation may pose difficulties to women with irregular menstrual cycles, resulting in high rates of cancellation. The workload associated with the unpredictability and number of natural cycle FETs per day can result in a centre being required to reduce the number of patients accepted. It also introduces a lack of certainty in the planned dates of embryo thaw and transfer. Usually, GnRH agonist treatment shows more complete pituitary suppression and effective cycle control than freeze-thawed embryo transfer in downregulated hormone replacement therapy (HRT), thus providing advantages compared to natural cycle FET. The centres need to be able to

organise staffing and workload systematically. For that reason, stress, fear and anxiety may be eliminated by patients having the ability to choose the embryo replacement date.

Whilst FET also lowers the number of multiple births (de Mouzon et al. 2009), it was found that FET clinical practices predetermined a higher proportion of pregnancy loss (14.5%) compared to (9%) fresh embryo transfer (Aflatoonian et al. 2010). In spite of the improve efficiency of the combination of the increases in pregnancy rates by fresh embryo transfer and the reduced cost (by reducing patients' waiting time at the clinic), the practice leads to a high risk of multiple births. Nygren et al. identified FET as having a 22.9% pregnancy rate versus a 17.1% death rate per transfer (Nygren et al. 2011). For ovarian failure therapy patients enrolled in a donation program, the advantage of frozen-thawed embryo transfer is the ability to eliminate the need for synchronisation between donor and recipient, as this is no longer necessary for successful replacement (Salatbaroux et al. 1988). Oocyte donor programmes of cryopreserved embryos allow flexibility in the timing of replacement procedures of thawed embryos (Bennun et al. 1989). Also, natural cycle FET overall allows for maintenance of normal pregnancy physiology close to that of natural pregnancy physiology in comparative HRT patients.

1.3.4 Insulin resistance links to infertility

The inadequate understanding of the outcomes of poor/improper implantation remains a challenge in tackling them. Some data suggest that the two greatest clinical predictors of poor outcomes of gonadotrophin ovulation induction in women with normogonadotrophic anovulatory infertility are obesity and insulin resistance (Ramsay et al. 2006). Encouraging weight loss maximises the chance of pregnancy success before starting to treat anovulatory subfertility. This implies a necessity to improve insulin resistance for optimal implantation. To date, access to data remains a problem in understanding the effect of metabolic and inflammatory parameters such as insulin. It is well known that insulin signals influence phosphorylation of several proteins and enzymes; they upregulate genes, synthesise lipids and glycogen and facilitate glucose translocation, as well as its actions being interfered by inflammation.

The mechanism by which insulin affects the human implantation window is unknown. Studies using animal models have demonstrated that insulin enhances blastocyst proliferation in mice, rats and rabbits (Harvey and Kaye 1990; De Hertogh et al. 1991; Herrler et al. 1998). In mouse models, insulin enhances embryo cleavage (Gardner and Kaye 1991) and stimulates cell numbers of blastocysts, resulting in an increase in inner cell mass (Harvey and Kaye 1990). The addition of insulin to preimplantation mammalian embryos leads to a physiological response similar to that in other insulin-responsive cells.

This suggests that insulin may have a direct role in the regulation of preimplantation embryo development. The underlying process in the metabolic disturbance of obesity which causes insulin resistance is complex. Being overweight is linked to reproductive dysfunction. In obese women, irregular menstrual cycle, low pregnancy rate and miscarriage are well documented (Lashen et al. 2004; Jungheim et al. 2009; Wei et al. 2009). In clinical situations, obese, polycystic ovary syndrome (PCOS) and diabetic women (conditions associated with higher insulin levels) are more likely to miscarry (Penney et al. 2003; Dokras et al. 2006; Jungheim et al. 2009; Beauharnais et al. 2012; Chang et al. 2013). All these conditions are indications of the negative effect of high insulin on the blastocyst/embryo stage at preimplantation (Cardozo et al. 2011). Obesity *per se* may impair fertility; there is a relationship between overweight/obesity and PCOS (Jungheim et al. 2009). Many women with PCOS have a BMI consistent with obesity (Norman et al. 2007). This syndrome is named due to the ovaries containing many small follicles; it is one of the commonest causes of infertility and is linked to failure of ovulation (Franks 1995).

1.4 Maternal adaptation to pregnancy

Human pregnancy physiology is besieged with extensive metabolic adaptation of different maternal systems. The changes mean that the mother's body has to work harder in order to meet the demand of the developing fetus and still be able to sustain maternal energy requirements. Maternal adaptation involves amplified biochemical parameters that influence this and other physiological changes. These are reflected in the level of hormones and metabolic and inflammatory parameters. Homeostatic complexes in mothers act to control and maintain internal environment insults arising due to such changes; however, breaches occur occasionally. This not only leads to inadequate or exaggerated metabolic and inflammatory parameters but also implicates upregulation and/or downregulation of the metabolic and inflammatory pathways. All this may create a vicious cycle throughout gestation, affecting several systems in mother and fetus.

1.4.1 The maternal metabolism and inflammation

Adaptation to pregnancy in humans involves major anatomic, physiological and metabolic changes in the mother which serve to support and provide for her nutritional and metabolic needs, as well as those of the growing fetus (Torgersen and Curran 2006). The exact time change in metabolic and inflammatory parameters in gestation is unknown. Since this change may predispose physiological adaptation by the late first trimester, this means that this change in parameters may perhaps happen during the first weeks of gestation. Normally, there is a shift in the homeostatic balance of pregnant mothers which

does not occur in their non-pregnant counterparts. This is mostly in response to the conceptus. When problems arise, there may be an inability of the maternal systems to cope. Over time in gestation, this inability may result in alterations in metabolic and inflammatory parameters, evident in extreme pregnancy cases.

1.4.1.1 Hormones

A number of hormones are vital at various time points throughout gestation in order to achieve pregnancy success. They include those involved in conception (hCG, FSH, LH, oestrogen and progesterone), implantation (hCG, oestrogen and progesterone), maternal adaptation (hPL, insulin, leptin and adiponectin, as well as oestrogen and progesterone) and, by term, those useful prior to and after delivery (relaxin, hPL, prolactin and oxytocin). As mentioned above, steroid hormone synthesis requires cholesterol conversion to pregnenolone in the IMM that first produces progesterone, used in turn for other steroid hormone synthesis (Miller and Auchus 2011). LDL has consistently been reported as stimulating ovarian steroidogenesis, by the provision of cholesterol as a steroidogenic substrate. HDL can also deliver cholesterol to support progesterone synthesis as observed in human granulosa-lutein cells (Ragoobir et al. 2002). A range of mechanisms is involved in utilisation of cholesterol content of lipoproteins for steroidogenesis. More than 95% of the mass of HDL cholesteryl ester entering the cell does so through the nonlysosomal (selective) pathway (Azhar et al. 1998). Here the cholesteryl esters released from HDL are taken up directly by cells without internalisation of apoprotein. Once inside, the cholesteryl esters are either hydrolysed and employed for steroidogenesis or stored as the cholesteryl ester until required.

As highlighted above, the cholesterol delivery to steroidogenic tissue for steroidogenesis occurs through LDL and HDL via receptor-mediated endocytosis and SR-B1 selective pathway (Grummer and Carroll 1988; Acton et al. 1996; Guibourdenche et al. 2009). The LDL-LDLR complex within the cell endosome (pH<6.5) draws upon a calcium-dependent mechanism (Zhao and Michaely 2009), dissociates and LDLR are recycled to the cell surface to bind additional lipoprotein. LDL-C and/or HDL-C reaches the steroidogenic tissues and the cholesterol is stored or utilised. Different cells may have a preference for the type of cholesterol employed for various purposes (Grummer and Carroll 1988). In their report, Grummer and Carroll assert that adrenal and ovarian tissues were shown to be able to use LDL-C for steroidogenesis. Ovarian tissue utilises LDL-C primarily for steroidogenesis, while fibroblasts use cholesterol primarily for membrane synthesis. Also, the rate of LDL-C uptake is slower for steroid-secreting cells than for fibroblasts, and preclustering of LDLR in coated pits appears unique to fibroblasts. Others report of HDL-C mediation into cells and tissues without HDL particle via the SR-B1 (Acton et al. 1996; Hu

et al. 2010). All of these unesterified cholesterols now inside the cells precede delivery to the cholesterol-poor outer mitochondrial membrane (OMM) appears to be transported by cholesterol transport proteins. Increasingly, data imply that steroidogenic acute regulatory proteins (STAR) facilitate the movement of cholesterol into the mitochondria (Miller 2007; Guibourdenche et al. 2009; Miller and Bose 2011). Steroidogenesis is initiated by the P450scc residing on the IMM, where cholesterol is converted to pregnenolone, the rate-limiting step of all steroid hormones syntheses (Hu et al. 2010; Miller and Auchus 2011).

Human pregnancy is characterised by a gestational rising of insulin resistance from pregnancy onset. Hyperglycaemia (Siegmund et al. 2008), a decline in insulin sensitivity, parallels the development of the feto-placental unit for transport of glucose to the fetus (Catalano et al. 1991). This decline is thought to be indicative of placental growth and fetus nutrient needs (Briana and Malamitsi-Puchner 2009). Pancreatic islets undergo major structural and functional changes during pregnancy to fulfil this increased demand for insulin, as shown in rat models (Weinhaus et al. 2007). In turn, increased insulin hormone secretion leading to gestational increase in insulin resistance occurs in order to maintain glucose tolerance (Catalano et al. 1993; Catalano et al. 1999; Stewart et al. 2007). Various components of the growth hormone axis (insulin-like growth factor binding protein-1 (IGFBP-1)), adipose tissue function (leptin and triglyceride (TG)) and placenta (leptin) also lead to insulin sensitivity during normal pregnancy (McIntyre et al. 2010).

The underlying mechanisms of insulin resistance are yet to be completely elucidated. A Catalano et al. report, utilised an oral glucose tolerance test and hyperinsulinaemic-euglycaemic clamp to study insulin sensitivity at conception, 12-14 weeks' and 34-36 weeks' gestation in non-obese women (control) and gestational diabetes women (Catalano et al. 1993). In both groups, there is a significant decrease in insulin sensitivity as gestation advances. In addition, women who developed gestational diabetes had even lower decreased insulin sensitivity than women in the control group. Usually, glucose tolerance is normal or slightly improved with peripheral sensitivity to insulin during early pregnancy (Catalano et al. 1993; Butte 2000). However, the precise mechanism is still unclear, since peripheral insulin sensitivity and hepatic glucose production are not different between past pregnancies. It is suggested that alterations in the hormonal influence of cortisol and progestin in lipogenesis and fat storage (Butte 2000) implicate insulin sensitivity. Placental hormones, including progesterone and cortisol, are proposed as the cause of decreased insulin sensitivity (Ryan and Enns 1988; Wada et al. 2010). Oestrogen also reduces insulin sensitivity in pregnancy (Huda et al. 2009), and possibly so do hPL and prolactin (Newbern and Freemark 2011).

1.4.1.2 Metabolism

The metabolic adaptations of pregnancy are orchestrated by hormones produced by the maternal pituitary gland and placenta (Newbern and Freemark 2011). Pregnancy is an intensive energy demand period. Lipid and carbohydrate metabolism play a vital role in meeting the high-energy demands of both mother and fetus. TG and cholesterol, the basic lipids, are products of the liver which are packaged into lipoprotein particles for transport in the bloodstream (Grummer and Carroll 1988). Excess amounts of both are exported from the liver into the blood in the form of very low-density lipoprotein (VLDL), when there are high amounts in the body. The TG in VLDL is hydrolysed by lipoprotein lipase (LPL) in the capillaries as free fatty acid called non-esterified fatty acid (NEFA), which are taken up and stored in the cells. The cholesterol-rich remnant, the intermediate-density lipoprotein (IDL), can be taken up by the liver as IDL cholesterol (IDL-C) for processing or can be converted into LDL by removing the TG.

A typical lipoprotein is surrounded by a shell of phospholipids and unesterified cholesterols. Usually, plasma chylomicron, VLDL and LDL (through their apo B content) are the main carriers of TG, phospholipids and cholesterols (Kane et al. 1980; Cardin et al. 1986). There are four forms of apo B which have been identified: apo B-26, apo B-48, apo B-74 and apo B-100. As noted above, apo B-48 is synthesised in the intestine as a major polypeptide constituent of chylomicron, whereas the larger form, apo B-100, is primarily synthesised in the liver and is the predominant protein in VLDL and LDL (Olofsson et al. 1983; Uchida et al. 1998). Uptake of LDL-C through apo B-100 is mediated by its binding to specific high affinity receptors on the cell membrane which direct the LDL-C to the proper cells (Goldstein and Brown 1977). The role of LDL is to transport cholesterol to peripheral tissue and regulate *de novo* cholesterol synthesis at the site. HDL has antiatherogenic properties and plays a role in picking up cholesterol released into the plasma from dying cells and from membranes undergoing turnover, via reverse cholesterol transport. The enzyme acyl-CoA:cholesterol acyltransferase (ACAT) in HDL esterifies the cholesterol into cholesteryl ester (Spector and Haynes 2007), which returns to the liver by HDL.

In pregnancy, maternal tissues rely mostly on TG as their energy source, whereas glucose is the primary energy fuel used by the fetal tissues, as it is readily available for transplacental transfer (Battaglia and Meschia 1978). Normal pregnancy is hyperlipidaemic due to steady gestational increase in plasma TG, total cholesterol (TC), VLDL cholesterol, IDL-C, LDL-C, HDL-C, lipoproteins particles, phospholipids and distribution of lipoproteins containing apo A-I, apo A-II and apo B (Mazurkiewicz et al. 1994; Martin et al. 1999; Winkler et al. 2000). All circulating lipids are typically increased

with TG rising to 200-400% (Lippi et al. 2007; Huda et al. 2009). These changes necessitate several changed physiological functions. Maternal metabolism during pregnancy adapts to benefit fetal growth and development, and can be divided into two phases. During the initial two thirds of gestation, the fetal energy demands are limited and maternal fat storage increases (Villar et al. 1992). All of these changes cause an increase in behavioural change, such as hyperphagia (Douglas et al. 2007) and increased lipogenesis (Ramos et al. 2003). Early in pregnancy, insulin sensitivity is typically normal, as is peripheral sensitivity to insulin and hepatic basal glucose production (Catalano et al. 1991).

The changed metabolic state (in addition to pregnancy-related endocrine changes involving progesterone, oestrogen and cortisol), favour lipogenesis and TG accumulation, as shown in an *in vitro* model (Ryan and Enns 1988). Usually, anabolism that occurs during the first stage of pregnancy changes to catabolism by the second stage, with a striking rise in the lipolysis rate and corresponding rises in maternal NEFA and glycerol (Catalano et al. 1998; Huda et al. 2009). In rat models, these changes are enhanced by increased hormone sensitive lipase (HSL) activity and mRNA level, and a decrease in LPL activity (Martin-Hidalgo et al. 1994). Overall TG (fat) levels increased by the activity of LPL in adipose tissue, which was found to be higher in the femoral region of women (whose rate of hydrolysis of stored TG was examined) than their abdominal region. This heightened activity was found until 8-11 weeks' gestation (Rebuffe-Scrive et al. 1985). Such oxidation of NEFA is necessary for energy metabolism in mothers. A general adipogenesis and lipogenesis pathway is described in Figure 1-1.

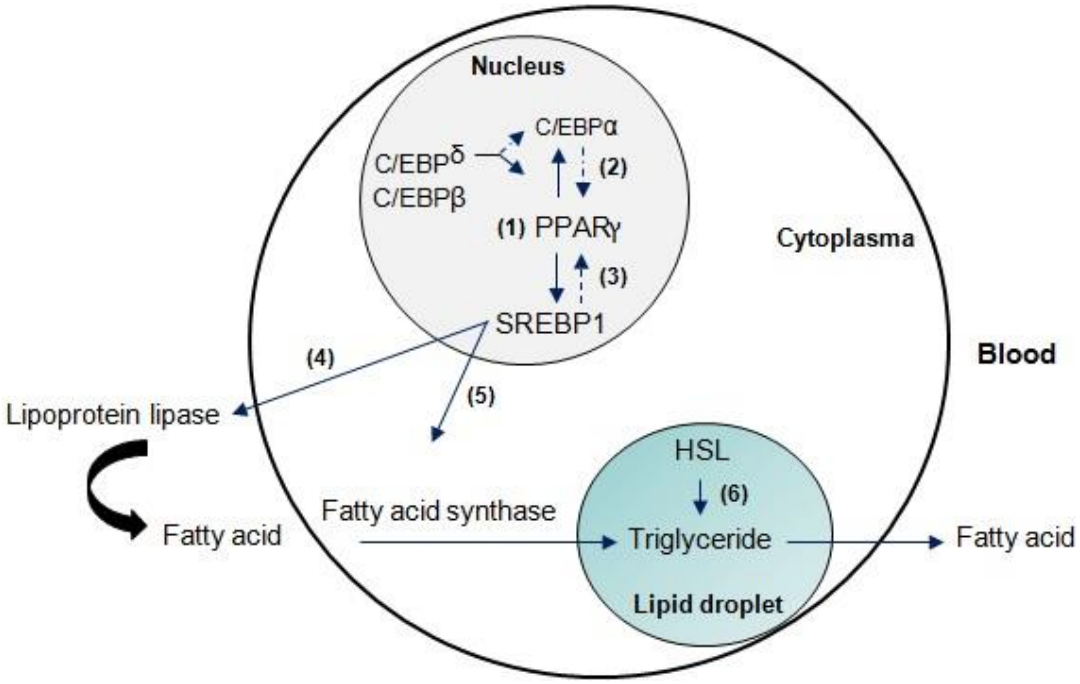


Figure 1-1: **Sequential regulation of adipogenesis and lipogenesis**. (1) CCAT/enhancer binding proteins (C/C/EBPδ and C/EBPβ) upregulate peroxisome proliferator-activated receptor gamma (PPAR)**γ** and C/EBPα; (2) (PPAR)**γ**2 upregulates C/EBPα and vice versa resulting in adipocyte differentiation; (3) PPAR**γ**2 upregulates lipogenic transcription factor sterol regulatory element binding protein (SREBP)1c; (4) SREBP1c upregulates extracellular lipolytic enzyme, lipoprotein lipase facilitating fatty acid uptake by adipocytes; (5) SREBP1c upregulates lipogenic enzyme, fatty acid synthase facilitating lipogenesis within adipocytes; (6) Intracellular lipolytic enzyme, hormone sensitive lipase (HSL) acts on TG to release fatty acid from adipocyte. Modified from Desai and Ross (Desai and Ross 2011).

The cholesterol is used by the placenta for steroidogenesis (Simpson et al. 1979) and membrane biosynthesis. The change in TC level reflects changes in lipoprotein fractions and is also found to rise as the pregnancy advances (Lippi et al. 2007). These data imply that the changes in lipid metabolism are necessary in either supplying energy for mother and conceptus or supplying building blocks (for processes including steroidogenesis, membrane formation and cellular fluidity) in early and mid-gestation as well as until term.

Carbohydrates are carbon-based molecules rich in hydroxyl groups. Their simplest form is monosaccharide (also called simple sugar), which serves not only as a fuel molecule but as a fundamental constituent of the living system (as does deoxyribose, a backbone of deoxyribonucleic acid). Glucose is hexose and is an essential energy source for virtually all forms of life. In pregnancy, glucose is the most abundant nutrient crossing the placenta. Its transfer is aided by glucose transporters such as glucose transporters 1 (GLUT1) and GLUT3, which are detected at the maternal-fetal interface as early as 7-13 weeks' gestation (Sato et al. 2002; Brown et al. 2011). This suggests that the glucose

taken up from maternal circulation may not only be needed for placental function but also for successful implantation by trophoblast invasion and proliferation, and that it has a support role of providing energy for maternal leukocytes (Korgun et al. 2005).

1.4.1.3 Systemic immune response

The maternal immune system is aware of the semiallogeneic conceptus and develops strategies to tolerate it. The maternal immune system undergoes profound transformation already at the very beginning of pregnancy (Zenclussen 2013). Such changes are essential to protect the fetus from a detrimental immune response. Usually, the maternal immune system becomes aware of the conceptus and paternal antigens rather than treating them as foreign, and thus actively protects them during pregnancy. This transient condition is explicit to the paternal antigen (Tafuri et al. 1995). As Tafuri et al. report, maternal T cells are aware of paternal alloantigens during pregnancy. After validation in mice models, it was suggested that maternal T cells acquire a transient state of tolerance specific to the paternal alloantigens. Also, a number of substances secreted, by the fetal trophoblast itself contribute to changes of adaptive immune response. The fetus is actively able to protect itself by generating tolerance to maternal antigens (Mold et al. 2008). In the report of Mold et al., it was shown that the human fetal immune system takes advantage of a mechanism that generates T regulatory cells (T_{reg}) that suppress fetal immune response. In the same report, it was clear that a considerable number of maternal cells cross the placenta to reside in fetal lymph nodes, where the development of $CD4^{+}CD25^{high}FoxP3^{+}$ T_{reg} suppresses fetal antimaternal immune response, which persists until adulthood. This means that the fetal immune system is already programmed to tolerate the mother and thus survive the pregnancy until term.

IL-4, IL-10 and leukaemia inhibitory factor (LIF) support the development and maintenance of the early fetus (Piccinni et al. 1998). It was found that decidual T cells had reduced IL-4, IL-10 and LIF in women with recurrent pregnancy loss compared to controls. LIF downregulates excessive leukocyte infiltration of the *in utero* tissue during implantation; maternal endometrium and decidua assist in generating the localised immuno-suppressive environment (Piccinni et al. 1998). This suppressive effect of some immune cells supports the paradigm of T helper cell (Th)1/Th2 balance, which postulates pregnancy as a Th2-mediated event. Progesterone suppresses maternal cell-mediated immunity to prevent rejection of the fetus, which expresses paternal antigens (Druckmann and Druckmann 2005). Druckmann and Druckmann highlight a significant factor, termed the progesterone induced blocking factor (PIBF) acting as immunomodulator protein. As Laskarin et al. report, PIBF mediates progesterone induced suppression of decidual lymphocytes cytotoxicity (Laskarin et al. 2002). The data were further supported by report

of patients presenting a Th1 phenotype in cases of miscarriage due to poor PIBF levels, versus a Th2 in normal pregnancies (Raghupathy 1997). Nonetheless, this known Th1/Th2 paradigm was disproved with the report of normal pregnancies in knockout mice for IL-10, and even in double-deficient mice (Svensson et al. 2001). After this disproof, it became apparent that the Th1/Th2 ratio is a marker predicting success or failure but not causing it.

Adaptive immune response in maternal pregnancy occurs from pregnancy onset, with features evident long after birth. Implantation, trophoblast invasion, uterine remodelling, placentation and decidualisation are all potential sources of shed debris. Such shed debris are perhaps implicated in eliciting maternal systemic response in normal pregnancy (Redman and Sargent 2003). This suggests that such debris may be due to syncytial apoptotic and necrotic events. These events are controlled to regulate eliciting maternal immune response while tolerating the fetus and paternal alloantigen. Pregnancy activities mean that the homeostatic switch from the non-pregnancy to pregnancy state occurs in order to maintain optimal homeostatic balance while protecting the developing fetus. These observable events categorise pregnancy as an adaptive or innate, acute or chronic immune response (Mor 2008; Li and Huang 2009) or even as a pro- or anti-inflammatory condition – depending on the stage of gestation. In the innate immune response, resident cells in the uterus (dendritic cells and macrophages), muscular tissue, mucous membrane and complement systems with basophils, NK cells, granulocyte and mast cells are all involved. The chronic immune response encompasses the leukocyte subpopulation of adaptive immune cell, T cells, B cells and plasma cells, and monocytes/macrophages (via antigen presentation). Depending on the response, this predetermined pathway is active throughout gestation. Data from ART shows evidence of a systemic maternal immune response early in gestation. Almagor et al. observed increased c-reactive protein (CRP) levels in women undergoing IVF as early as 14 days after embryo transfer (Almagor et al. 2004). In another group, there were raised concentrations of maternal CRP at 4 weeks' gestation (Sacks et al. 2004). By the late stages of pregnancy, there remained a significantly higher number of circulating maternal monocytes and granulocytes in healthy pregnancies compared to the non-pregnant groups (Sacks et al. 1998).

Specialised immune cells, including decidual leukocyte, infiltrate the pregnant uterus and are also useful for other nonimmune processes. These immune cells account for at least 15% of all cells in the decidualised uterine wall which are identifiable throughout gestation. The decidual leukocyte number is unique, being composed mainly (~70%) of an unusual type of NK cell ($CD45^{bright}/CD16^{-}$), accompanied by T cells (~15%) and macrophages (~15%) (Bulmer et al. 1991; Red-Horse et al. 2001). Red-Horse et al. highlight that chemokines are also useful in nonimmune function, including cytotrophoblast

differentiation (Red-Horse et al. 2001). Also, a specialised chemokine scavenger decoy (D6) receptor (that regulates chemokine bioavailability to leukocyte trafficking) is present in the uterine tissue of human (Madigan et al. 2010) and an animal model (Wessels et al. 2011). These D6 receptors are distributed among trophoblast-derived cells in the human placenta, decidua and gestational membrane throughout pregnancy (Madigan et al. 2010). Madigan et al. report that endogenous D6 can mediate a progressive chemokine scavenging with D6 ligand CCL2, which is specifically linked to human syncytiotrophoblast in the pregnancy term placenta in situ. Thus, binding of the decoy prevents signalling transmission and hence controls excessive inflammatory response mediators. These data highlight the regulation of immune response, preventing fetal allograft rejection and thus playing a protective role in ensuring the survival of the conceptus and developing fetus, throughout gestation.

1.4.2 Maternal metabolic and inflammatory changes

Evaluating a number of metabolic and inflammatory parameter changes in healthy pregnancy helps in understanding events in extreme cases. This is due to observations that women who suffer unhealthy pregnancies having altered metabolic and inflammatory parameters. These patients commonly incur long-term high cardiovascular risk. Insulin is recognised to play a fundamental role in maternal metabolic and inflammatory pathways during gestation. Saltiel et al. show that insulin regulates glucose metabolism with glycogen, protein and lipids as by-products (Saltiel and Kahn 2001). This regulation occurs when circulating glucose levels are high. Insulinaemia, dyslipidaemia and hypertension predispose women to increased risk of cardiovascular disease (Davidson 1995; Steinberger and Daniels 2003; Gaspard 2009). With regard to inflammatory pathways, a healthy pregnancy is associated with upregulated IL-6, CRP and a high leukocyte count, which all are definitive cardiovascular risks (Schmidt et al. 1999). Interaction between lipid or carbohydrates metabolites and orchestrated inflammatory response contributes to insulin resistance.

As described in detail in a later part of the chapter, maternal insulin resistance manifests in preeclampsia (PE) (Brewster et al. 2008), intrauterine growth restriction (IUGR) (Berends et al. 2008) and gestational diabetes (Uzelac et al. 2010). In short, the relationship between insulin secretion and sensitivity triggers insulin resistance due to aberrant compensatory ability of beta-cell insulin secretions, which initiates cardiovascular disturbances. Restoration of the endothelial function ameliorates insulin resistance and assists in recovering insulin sensitivity, alleviating endothelial dysfunction (Kim et al. 2006) and subsequent abnormal vascular conditions. In order to eliminate the devastating impact of maternal vascular disturbance, there is a new urgency to determine the best

early intervention time point during the peri-menopausal years. This determination should help prepare mothers who suffer extreme pregnancies with conditions including PE, IUGR and gestational diabetes to combat abnormal vascular condition. Emphasis on monitoring such patients, requiring them to attend routine screening for coronary heart disease and advising them on how to improve physical activity and lifestyle can provide better maternal health later in life.

1.4.3 Pregnancy as a stress test

Complicated pregnancies leading to health problems warrant attention in female reproductive medicine. Hypertension, dyslipidaemia, diabetes, obesity-related metabolic disorder and inflammatory disturbances are closely associated with the pathogenesis of cardiovascular disorder. A model proposed by Sattar and Greer, identified links between pregnancy maternal metabolic, inflammatory and vascular complications to risk of cardiovascular disorder later in life; this model is defined as a 'stress test' (Sattar and Greer 2002). Obesity or excessive weight gain predisposing women to insulin resistance may also exist as sub-clinical conditions in healthy women (Catalano et al. 1999; Innes et al. 2001; Dokras et al. 2006). Women exposed to a spectrum of altered metabolic and inflammatory response changes during pregnancy or previous sub-clinical status are at increased risk of them manifesting in subsequent gestation. Given that parameter alterations may facilitate the development of cardiovascular diseases which may re-emerge permanently later in life, there is a need to identify those women with these metabolic syndromes during pregnancy.

Shortly after conception, pregnant women develop hypercoagulability, raised inflammatory response and increased white cell counts (Sacks et al. 1998) as well as heightened cardiac output (Bosio et al. 1999). These changes can compromise maternal organs to become impaired during pregnancy, and it may be unlikely that they are subsequently fully repaired, in spite of such altered mediators receding postpartum. Assessment of women with a history of PE and IUGR and gestational diabetes shows exhibition of high risk of future cardiovascular disease, including dyslipidaemia, hypertension, obesity and insulin resistance, compared to women with uncomplicated pregnancies (Ray et al. 2005; Manten et al. 2007). Maternal death or admission due to ischemia heart disease are related to low birth weight, preterm birth and PE (Smith et al. 2001). Gestational diabetes that occurs either due to pancreatic beta-cell deficiency in increased insulin secretion or to pre-existing insulin resistance is related to increased risk of cardiovascular disease in women (Carr et al. 2006). Ordinarily, insulin resistance disappears after birth but reappears in subsequent pregnancies and women who developed gestational diabetes are likely to develop type II diabetes mellitus (Kim et al. 2002; Cheung and Byth 2003).

It therefore appears that suffering certain complications of pregnancy leads to individual susceptibility to future maternal cardiovascular diseases. This implies that such complications may serve as an effective diagnostic tool to monitor women with increased risk of maternal cardiovascular disease later in life. As put forward by Sattar and Greer, altered metabolic and inflammatory parameters manifesting in these metabolic pregnancy disorders could be considered as a stress-test outlook for long-term maternal health (Sattar and Greer 2002). This diagnostic tool may perhaps provide an opportunity for preventing the reoccurrence in women who have suffered complications of pregnancy, including PE, IUGR and gestational diabetes (Sattar and Greer 2002). Clinical advice to improve lifestyle, diet and physical activity could be provided for affected patients.

1.5 Impact of obesity on adaptive response and mechanistic links to poor implantation/placentation manifested as miscarriage, stillbirth, PE, IUGR and gestational diabetes

Obesity increases the demand of cell synthetic machinery in some secretory organ systems. This increase defines obesity as a chronic positive energy imbalance due to excess fat (TG) storage in adipose tissue that results in adipocyte hypertrophy and hyperplasia during adipogenesis in human (Heilbronn et al. 2004; Avram et al. 2007) and rat models (Faust et al. 1978). Obesity is associated with mechanical stress, excess lipid accumulation, abnormal intracellular energy fluxes and nutrient availability. This is perhaps due to the large number of adipocytes in obese subjects compared to lean subjects (Spalding et al. 2008). Spalding et al. report approximately 10% total fat cell renewal by ongoing adipogenesis and adipocyte death per annum at all adult ages and BMI levels. Another consequence of the adipose tissue acting as an energy storage factor is the production of soluble molecules, adipocytokines. It was found that expression of adipokine varies depending on the site of adipose deposit (Ouchi et al. 2011). The deposit of excess body fat (regional fat distribution) is a major determinant of degree of excess morbidity and mortality due to obesity (Lefebvre et al. 1998). At least three components of body fat are associated with obesity-related adverse health outcomes: the total amount of body fat (expressed as percentage of body weight), the amount of subcutaneous adipose tissue (SAT) or abdominal fat (upper body fat), and the amount of visceral adipose tissue (VAT) located in the abdominal cavity (Freedland 2004; Desai and Ross 2011). SAT is made of smaller, more insulin-sensitive adipocytes that act as a sink or buffer, avidly absorbing circulating fatty acids and TG in the postprandial period (Desai and Ross 2011). In contrast, excessive VAT increases secretion of fatty acid, adipokine and inflammatory molecules, which all contribute to insulin resistance, dyslipidaemia, glucose intolerance and hypertension. As knowledge of obesity-induced inflammation increases, evidence

relates obesity to chronic low-grade inflammation (Hotamisligil 2006; Ouchi et al. 2011) or a complex highly active metabolite source of various inflammatory mediators (Berg and Scherer 2005).

1.5.1 Impact of obesity in pregnancy

Adipose tissue metabolism plays an essential role in maternal energy homeostasis during gestation. Obesity is a significant risk factor for changes of metabolic and inflammatory parameters during pregnancy that affect adaptation. There are reports throughout gestation of the metabolic and inflammatory parameters revealing high levels of leptin, TG, CRP and IL-6, and low levels of HDL-C in obese mothers compared to women of normal BMI (Ramsay et al. 2002; Stewart et al. 2007; Madan et al. 2009). Stewart et al. found significantly higher levels of CRP in obese women by 13 to 14 weeks' gestation, compared to lean women (Stewart et al. 2007). This implies that the effect of obesity on inflammatory mediators is present early in pregnancy. Plasma TNF-α, PAI-1, IL-6, adiponectin (insulin sensitivity) and leptin (energy balance and appetite) production by fat cells are involved in inflammation in obese women (Huda et al. 2010). Obesity reduces placental villous proliferation and increases apoptosis; this raises susceptibility to complications of pregnancy (Higgins et al. 2013). Obesity exposes the placenta to the alterations of metabolic and inflammatory parameters in pregnancy. This is shown in the report by Challier et al., who observe a high accumulation of placental macrophage expression of proinflammatory cytokines that include IL-1, IL-6 and TNF-α in obese women compared to a lean group (Challier et al. 2008). Levels of resident $CD68^+$ and $CD4^+$ cells macrophage in the placentae of obese pregnant women are 2-3 times higher than in lean women. Other researchers found raised CRP and TNF-α levels in the amniotic fluid of obese pregnant women (Bugatto et al. 2010), suggesting a link to fetal production, as most inflammatory mediators usually do not cross the placenta (Aaltonen et al. 2005). Importantly, it appears that some inflammatory mediators, including IL-6 and TNF-α, are capable of stimulating the expression and secretion of leptin and adiponectin from adipose tissue and the placenta (Chen et al. 2006; Briana and Malamitsi-Puchner 2009). These findings highlight pregnancy obesity as implicative of insulin resistance (Steinberger and Daniels 2003; Catalano 2007) and oxidative stress, both of which induce endothelial dysfunction (Perticone et al. 2001). This also demonstrates that obesity effects are evident in all stages of gestation that affect adipose tissue and the placenta, resulting in the manifestation of a vicious cycle for metabolic and inflammatory parameter changes.

1.5.2 Mechanistics of poor implantation and placentation

Pregnancy loss, PE, IUGR and gestational diabetes (Gauster et al. 2012) are in part products of poor/improper implantation that in most cases, lead to abnormal placentation, whether through natural and spontaneous or assisted conception.

1.5.2.1 Poor implantation

Many identifiable factors affect implantation. The first is chromosomal abnormality, especially in couples who suffer pregnancy loss. In ART, early pregnancy loss was reportedly increased between patient in their mid thirties and late forties which links maternal age and chromosomal aberrations (Liu and Case 2011). Another report shows that abnormal embryos were significantly more frequent in patients that had recurrent miscarriage compared to controls (Rubio et al. 2003). In essence, abnormal chromosomes trigger poor implantation, as embryo(s) with inadequate numbers of chromosomes are arrested. As Fragouli et al. report, the rate of chromosomal aneuploidy was lower for the blastocyst (38.8%) compared to the (51%) embryo at early implantation, but chromosomal errors, including monosomy and complex aneuploidy, persisted at the final stage of preimplantation development (Fragouli et al. 2008). Many single gene defects or polygenic multifactorial syndromes, such as trisomy and translocation, lead to early pregnancy loss. In chromosomal translocation, the embryo may receive too much or too little genetic material, and this excess or lack readily results in pregnancy loss. As a result, aberrant embryos are likely to implant poorly and to be afterwards lost.

Studies have demonstrated that the anatomical factor can also result in poor implantation and complications of pregnancy. This is also evident in pregnancy loss and infertile women. An example is uterine anomalies, which can be acquired or congenital. Distorted uterine morphology may lead to inappropriate adhesion of the blastocyst onto the endometrium, resulting in poor implantation. A morphological study of uterine anomalies in women, with or without a history of recurrent miscarriage, reveals no difference in the relative frequency of various anomalies or depth of fundal distortion between the groups (Salim et al. 2003). In the same report, the arcuate and subseptate uteri were shorter and distortion was higher in those with recurrent pregnancy loss. Another study shows that arcuate uterus was the most common anomaly in the general and recurrent miscarriage population, while septate uterus was the most common anomaly in the infertile population (Saravelos et al. 2008).

Shoenfeld et al. note the prevalence of autoimmune disease and its association with a significant rise of the antiprothrombin antibody in infertile women than in control group

(odd ratio OR 5.15 (95% confidence interval CI 2.12, 12.74)); more prevalent in recurrent pregnancy loss than in controls 5.42 (2.4, 12.50) (Shoenfeld et al. 2006). This study also reveals that the antibody level was more closely linked to secondary abortion (miscarriage after live birth) than primary miscarriage (all pregnancies terminated in miscarriage). Others show that autoantibodies (antiphospholipid antibodies) directed to phospholipids, β2-glycoprotein I, cardiolipin and lupus anticoagulants molecules present in the trophoblast membrane lead to exposure of the external surface of trophoblasts during tissue remodelling, contributing to pregnancy loss (Chamley 1997; Zenclussen 2013). All these autoantibodies affect cell division during embryogenesis and trophoblast proliferations, resulting in comprised trophoblast invasion during implantation, and the conceptus is normally lost at some point, whether in early/late pregnancy or even in advanced gestation as stillbirth.

1.5.2.2 Poor placentation resulting from the above issues

PE, IUGR and gestational diabetes are all consequences arising from improper implantation. These have a relationship to placental dysfunction directly and/or indirectly. Usually, the developing fetus obtains oxygen and nutrients from the mother through placental transport. As the placenta is a distinctive organ that differentiates *per se*, it organises fetal growth throughout gestation. EVT invade the uterine endometrium (Lyall 2005), establishing sufficient blood flow from maternal circulation, as noted above. A common view, referred to as the 'poor placentation hypothesis', holds that EVT failing to invade the placental bed sufficiently partly predisposes women to complications of pregnancy. Placentation occurs around 6-18 weeks of pregnancy (Pijnenborg et al. 1980; Redman and Sargent 2003). The first wave of trophoblast invasion starts by the late first trimester, around 8 weeks' gestation, and the second phase takes place between 18 and 20 weeks' gestation (Hossain and Paidas 2007). The spiral arteries are transformed into large structureless conduits that can supply the hugely expanded blood flow of the third trimester placenta (Redman and Sargent 2003). The process of trophoblast invasion is usually completed by 20 to 22 weeks' gestation in normal pregnancy. In PE cases, it has been found that cytotrophoblast invasion of the uterine spiral arterioles is often incomplete by this time, and spiral arteries fail to lose their muscular elastic components (Brosens et al. 1972).

Two abnormalities affecting the spiral arteries, which are the end-arteries that supply the intervillous space, are that the arteries may be too small due to deficient placentation or obstructed because of acute atherosis, or both (Brosens et al. 1972). In PE and IUGR, endovascular trophoblasts do not invade the myometrial segment, and the physiological changes are restricted to decidual segments (Gerretsen et al. 1981; Pijnenborg et al.

2006). The inability of EVT to enable the muscular spiral arteries to invade the myometrial portion converts the arteries into 'low-resistance' capacitance vessels. Thus, acute atherosis is likely to develop in the myometrial spiral arteries, with a focal rather than generalised feature, and is often associated with thrombosis, possibly due to maternal systemic inflammatory response (Redman and Sargent 2003). This results in impairment of blood supply to the placenta, leading to infarction and, if widespread, resulting in impairment of fetal growth. As a result, placental reduced perfusion and placenta hypoxia are hallmarks of PE, resulting in turn in abnormally low flow and high resistance in pre-eclamptic placentae (Burton et al. 2009). In contrast, in excessive trophoblast invasion, the placenta may attach onto the myometrium (accreta), into the myometrium (increta) and completely through the myometrium (percreta) (Brahma et al. 2007).

There are reports that the placenta is partly responsible for releasing factors that cause endothelial dysfunction in maternal circulation called soluble fms-like tyrosine kinase-1 (sFlt-1) (Nagamatsu et al. 2004; Gilbert et al. 2008). sFlt-1 is a naturally occurring circulatory antagonist of VEGF-A and PIGF detected in maternal sera (Levine et al. 2004) and placenta (Maynard et al. 2003). Normally, the binding of sFlt-1 to the VEGF-A and PIGF blocks the action of both vascular and/or placental mediators. Higher sFlt-1 levels are found in PE (Chaiworapongsa et al. 2005), whereas PIGF and VEGF are deficient prior to and during clinical manifestation of PE, compared to healthy pregnancy (Reuvekamp et al. 1999; Livingston et al. 2000). These data suggest that PE promotes vasospasm coagulation and the increase of microvascular permeability, arising from the effect of inflammation and endothelial injury.

In gestational diabetes, aberrant villous vascularisation, disbalance of vasoactive molecules and enhance oxidative stress is apparent (Gauster et al. 2012). These changes affect the transplacental nutrient supply and impair fetal oxygenation. The combination of inflammation, placental ischemia and hypoxia from defected placental trophoblast invasion and poor remodelling of the uterine spiral arteries influences exaggerated metabolic and inflammatory response via the placenta (Benyo et al. 1997). In this way implantation and placentation may prompt complications of pregnancy such as pregnancy loss (early/late or stillbirth), PE, IUGR and gestational diabetes.

1.5.3 Manifested complications of pregnancy

Not all pregnancy complications are necessarily equal in regard to their aetiology and consequences. A number of complications that develop through the course of pregnancy can be detrimental to the mother’s and fetus’ health. Examples include pregnancy loss, whether early/late or stillbirth, placental previa, placental abruption, pregnancy-induced

hypertension, PE, IUGR and gestational diabetes. As stated above, most of these cases occur in part due to poor or excessive trophoblast invasion, which may be followed by systemic endothelial dysfunction and/or (in gestational diabetes patients) become glucose intolerance (Roberts et al. 1989; Metzger et al. 2007; Healy et al. 2010; Boyadzhieva et al. 2012; Gauster et al. 2012). The best approach to assessing pregnancy outcome is to monitor pregnancies from conception until birth. This period allows a higher chance of identifying the onset of complications at any point in time by pinpointing a specific incident throughout the course of gestation. Increasingly, research focus has been shifted to understanding metabolic and inflammatory pathways with regard to complications of pregnancy. Some such complications of pregnancy are highlighted in detail below.

1.5.3.1 Pregnancy loss

Pregnancy loss consists of early/late pregnancy loss and stillbirth. Miscarriage is the accepted formal term for early loss (before 12 weeks) of fetal viability or late loss (after 12 weeks) of pregnancy (Farquharson et al. 2005). Stillbirth is the intrauterine death of any conceptus at any time during gestation. The legal definitions vary, requiring the recording of fetal death at different gestational ages (16, 20, 22, 24 or 28 weeks) or birth weights (350, 400, 500 or 1000g). The United States has eight different definitions by combinations of gestational age and weight (Nguyen and Wilcox 2005; Mohangoo et al. 2013). Pregnancy loss is a common disorder in over 20% of pregnancies (Savitz et al. 2002), although it is notoriously difficult to detect with accuracy, especially in the early stages of gestation. Pregnancy loss is no doubt psychologically devastating to affected couples, causing immense grief, depression and emotional distress. It is accepted that couples having regular intercourse without contraception have a 25-30% chance of starting recognisable pregnancy in a single menstrual cycle (Savitz et al. 2002). In those that do not become pregnant in any one cycle, it is presumed that there has been either a failure of fertilisation or that fertilisation has occurred but the embryo has been lost before the first missed period. Of those pregnancies lost after 4 weeks' gestation, when clinical recognition is possible, many are unrecognised because the women were unaware of the pregnancy. A major mechanism underlying pregnancy loss is maternal inflammation. There is evidence in women who have recurrent pregnancy loss of having an increased altered cellular activation of peripheral blood $CD56^+$ NK cells (Emmer et al. 2000; King et al. 2010), which suggests the involvement of the innate immune system. NK cells produce signatures of Th1 cytokine, such as interferon-gamma (INF-γ), and the activation of such signature could explain the role of Th1 as a cause of recurrent pregnancy loss (Raghupathy 1997). As stated above, LIF involvement in embryo implantation allows a shift from Th1/Th2 response at the maternal-fetal interface that ensures pregnancy success. The decrease in production of LIF, IL-4 and IL-10 by decidual T cells was

observed in women with unexplained recurrent pregnancy loss compared to women with normal pregnancy (Piccinni et al. 1998). This observation demonstrates that poor antiinflammatory response to the suppression of proinflammatory response, increases the risk of pregnancy loss.

1.5.3.2 Pre-eclamptic pregnancy

PE is a multisystem disorder of pregnancy diagnosed after 20 weeks' gestation. It is characterised by the onset of hypertension, proteinuria and oedema. This disorder contributes to 40% of premature births and is linked to maternal and fetal morbidity and death (Chappell et al. 1999; Hendler et al. 2005). To date, the definitive treatment of PE remains to expedite delivery. PE prevalence varies, affecting 2-5% of pregnancies in the UK alone, and approximately 4 million pregnancies per annum worldwide. Although the underlying cause(s) and pathogenesis remain poorly understood, impaired trophoblast invasion of the placental spiral arteries is a recognisable feature of pre-eclamptic disorder (Brosens et al. 1972; Kadyrov et al. 2006) and implicative of abnormal placentation (Furuya et al. 2008). In PE, many homeostatic changes are exaggerated. This exaggeration is evident in the changes of metabolic and inflammatory parameters long having been shown to be disturbed in pre-eclamptic pregnancy. An example of the change of these parameters is the lipid adaptation indicated by a gestational (Enquobahrie et al. 2004; Baker et al. 2009) and cross-sectional report at term (Rodie et al. 2004; Adiga et al. 2007) of maternal plasma dyslipidaemia, including increased TG, TC and low HDL-C level in PE compared to controls. A classical pathological lesion (acute atherosis) seen in the pre-eclamptic placental bed potentially results from accumulated lipid-laden macrophages around the areas of fibrinoid necrosis of the spiral arteries (Brosens et al. 1972). Such lipid accumulation at the site of endothelial damage implies the possible role of lipid impact in vascular injury of PE. Huda et al. indicate that whatever the precise nature of the stimulating placental factor in PE, perhaps provokes the disturbance in lipid metabolism which contributes to vascular damage (Huda et al. 2009).

Women who later develop PE have increased maternal sera CRP early in gestation (Thilaganathan et al. 2010). Widespread activated immune cells are evidence of inflammatory response, as reported in pre-eclamptic pregnancy (Sacks et al. 1998; Redman and Sargent 2004; Thilaganathan et al. 2010). This response includes activated leukocyte exaggeration of the level of plasma TNF-α and CRP in women who have PE, compared to controls (Teran et al. 2001; Ramsay et al. 2004; Lockwood et al. 2008). Human decidual cells of PE were found to be involved in the raising of plasma IL-6 due to significantly higher IL-6 immunohistochemical (IHC) staining and mRNA levels, but this was not found in the control group (Lockwood et al. 2008). This systemic inflammatory

response is now linked to stimulating hypoxia and maternal endothelial activation and dysfunction in PE (Roberts et al. 1989; Ramsay et al. 2004; Royle et al. 2009; Lee et al. 2012). Adaptive response of vascular abnormality during pregnancy is assessed using both PAI-1 and -2. PE is related to increased PAI-1 levels in the blood, whereas it is related to reduced PAI-2 levels by term compared to normal pregnancy (Reith et al. 1993). To date, PAI-1/PAI-2 ratio is use to assess the index of placental function, with a low and high index implicating good and poor placental function respectively (Reith et al. 1993).

The idea that (in almost all cases) the effects of PE regress shortly after delivery of the placenta but persist if placental tissue is retained implicates the placenta as both necessary and sufficient for pre-eclamptic disorder. The placenta is involved in orchestrating oxidative stress, and a vast proinflammatory response is witnessed in PE (Redman and Sargent 2000; Cindrova-Davies 2009). Work on primary cytotrophoblast culture shows that lowering of percentage oxygen from 20% → 8% → 2%, promoted cytotrophoblast proliferation (Nagamatsu et al. 2004). In their report, the reduced oxygen tension resulted in the increase of sFlt-1 mRNA levels and sFlt-1 secretion, and diminished PIGF release (without detection of VEGF) in the culture media. Another study note a significantly higher placental TNF-α in PE that is positively correlated to lipid peroxide (Wang and Walsh 1996). The TNF-α also induce hypoxic stress through ischemia-injury on the endothelial cell (Hung et al. 2004), causing monocyte apoptosis and inhibiting trophoblast proliferation (Seki et al. 2007).

1.5.3.3 Intrauterine growth restriction

Data about IUGR is conflicting. Fetal growth provides a means of monitoring the fetal development *in utero* and can predict postnatal health and development. IUGR is a pathological condition of reduced growth velocity which compromises fetal well-being. IUGR pathology shares a number of similarities with PE, such as involvement of prothrombotic and inflammatory process, increased apoptosis and decreased cell growth; however, generally, there is an absence of endothelial damage. IUGR aetiology remains unknown and most of the available knowledge about the physiology and pathology of fetal growth is extrapolated from animal studies. Apart from the common factor of compromised nutrient supply across the placenta to the fetus, Sankara and Kyle highlight other causes resulting from early-onset insult to cellular hypoplasia, which are unlikely to be amendable through treatment (Sankaran and Kyle 2009). They also highlight that normal fetal growth is the result of a complex sets of activities involving maternal, placental and fetal factors. IUGR is related with reduced uterine blood flow or poor uteroplacental perfusion, both paramount for placental and fetal growth in IUGR (Khong et al. 1986). The disorder has

long been identified as one of the main causes of perinatal morbidity and mortality in both index and subsequent pregnancies (Ounsted et al. 1981; Rasmussen et al. 1999).

Offspring and maternal birthweight are inversely related with maternal cardiovascular disease. Established and novel cardiovascular risk factors, including vascular, metabolic and inflammatory function in women who delivered small-for-gestational age offsprings indicate altered lipids, including higher TG, cholesterol:HDL ratio, CRP, IL-6 and ICAM-1 compared to controls (Kanagalingam et al. 2009). A previous report of lipoprotein in IUGR cases and controls shows the cases women had significantly lower TC, LDL-C, IDL mass, $VLDL_2$ mass and total LDL mass than the control (Sattar et al. 1999). When Rodie et al. observed the offspring of women with IUGR, they found that increased fetal lipid (TG) which but no impact of maternal lipid (Rodie et al. 2004). Insulin resistance is a major metabolic milieu arising from altered placental leptin expression, which is thought to be paramount in the (IUGR) perinatal disorder (Tzschoppe et al. 2010). Debate on the maternal leptin role in IUGR, due to the varying levels compared to healthy women generally (Laivuori et al. 2006; Kyriakakou et al. 2008), implies that poor placenta perfusion leads to the development of IUGR. The fact that IUGR patients are normally lean implies that higher plasma leptin in some women drives increased placental leptin mRNA levels, reflecting the severity of placental dysfunction. IUGR women who are lean generally give birth to IUGR babies. IUGR babies are at increased risk of adult obesity. This is due to data from developmental programming studies that highlights the link between IUGR and the obesity epidemic (Desai and Ross 2011).

1.5.3.4 Gestational diabetes

Gestational diabetes is a disorder characterised by impaired glucose intolerance that appears from the second half of gestation. It affects 3-10% of pregnancies, with regard to the studied population, and as a result may be a natural phenomenon. The exact process underlying gestational diabetes remains unclear. The hallmark of the disorder is thought to be its interference with insulin action as it binds to the insulin receptor. As mentioned above, insulin resistance disappears after birth but reappears in subsequent pregnancies and women who have developed gestational diabetes are likely to develop type II diabetes mellitus (Kim et al. 2002; Cheung and Byth 2003). Gestational diabetes generally occurs in women with pre-existing insulin resistance and is influenced by maternal obesity (Lashen et al. 2004; Roman et al. 2011). The impact of obesity on gestational diabetes patients is related to neonatal hypoglycaemia (Roman et al. 2011).

The insulin resistance present in gestational diabetes is due to exaggerated maternal glucose levels, and has been found to be responsible for fetal macrosomia (Voldner et al.

2010). A singleton pregnancy of those suffering from gestational diabetes reveals first-trimester fasting glucose levels increasing from 1.0% in the lowest to 11.7% in the highest category (adjusted OR 11.92 (5.39, 26.37)) and large-for-gestational age (LGA) neonates and/or macrosomia increasing from 7.9% to 19.4% (2.82 (1.67, 4.76)) (Riskin-Mashiah et al. 2009). Apart from poor glucose tolerance in gestational diabetes mothers, there is also elevated plasma leptin, IL-6 and TNF-α, which are absent in controls (Ategbo et al. 2006). This partly implies inflammatory pathway involvement. Recent data highlights placental dysfunction as a factor in gestational diabetes. This is because the incidence of delayed villous maturation increases in pre-gestational diabetes patients compared to non-diabetic groups (Higgins et al. 2012). Aberrant villous vascularisation, an imbalance of vasoactive molecules and enhanced oxidative stress in gestational diabetes patients, is also reported (Daskalakis et al. 2008; Gauster et al. 2012). The consequences may be changed transplacental nutrient supply and impaired fetal oxygenation.

1.6 The offspring adaptive response

Adaptive response in the fetus is not completely understood. Fetal demand for growth and development assists in modulating maternal metabolic and inflammatory parameters to the fetus (Chandler-Laney et al. 2011). Because of difficulties in studying developing human fetuses, much current knowledge of human fetal adaptation has its origin in animal and cell culture models, although most is pertinent to humans. Less information has been obtained from primate models. In the available human data, obtained mostly at term, the offspring and mother differ profoundly.

1.6.1 The fetal metabolism and inflammation

1.6.1.1 Fetal hormones

The endocrine system of the fetus acts as a modulator of the classical physiology of organ system. One example is that of a basic function of the cardiovascular system to transport nutrient and waste products and perfuse the tissue with blood. However, the blood volume and osmolality is controlled by the action of the endocrine feedback process. Most peptide hormones circulate at a low level in the developing fetus and are not bound to transporters; thus are completely free from interactions with cell surface receptors. IGFs (IGF-1 and IGF-2) bind their receptors (Lelbach et al. 2005; Murphy et al. 2006), allowing fetal adjustment to their local levels; thereby modulating cellular growth and differentiation.

Placental hormones dominate the endocrine milieu in human gestation. The placenta has two steroidogenic pathways (Miller and Auchus 2011). First, as noted above, placenta

(like most steroidogenic tissues) can initiate steroidogenesis derived from cholesterols converted to pregnenolone and, then to progesterone; second, it can take C_{19} steroids (androgens) produced by the fetal adrenal, converting them to oestrogen. In primates, including humans, the placenta is a major source of oestrogen, since the biosynthesis depends upon an intact feto-placental unit (Ishimoto and Jaffe 2011). Human placenta lack the 17α-hydroxylase/17,20-lyase (P450c17) that catalyses both 17α-hydroxylase and 17,20-lyase activities (Voutilainen et al. 1986), whereas the enzyme is express in the adrenal and gonads (Miller and Auchus 2011). As Guibourdenche et al. report, the placenta is able to synthesise large amounts of oestrogen, as it receives a supply of oestrogen biosynthetic precursors from the maternal and fetal adrenal cortex (Guibourdenche et al. 2009). In the fetal zone, the fetal adrenal cortex have 3βHSD2 but lacks 3βHSD1 and 17βHSD1 (as in mothers) and cannot therefore convert either pregnenolone to progesterone or 17α-hydroxypregnenolone to 17α-hydroxypregesterone. Thus fetal (and maternal) adrenal cortex secretes dehydroepiandrosterone and dehydroepiandrosterone sulfate, as well as from fetal liver in response to the adrenocorticotropic hormone. These steroidogenic intermediates are taken up by the placenta utilising it 3βHSD1 in the oestrogen biosynthesis, bypassing the lack of P450c17 (Guibourdenche et al. 2009; Miller and Auchus 2011). The biosynthesised oestrogen is released into the fetal bloodstream and creates an endocrine environment that allows for fetal growth and development. Increased fetal oestrogen by the time of delivery is thought to arise because of the increase in myometrial contractility which initiates labour, perhaps allowing maternal oestrogen influx. An investigation of maternal and offspring insulin at birth reported on association between mother and baby (Chiesa et al. 2008); maternal insulin is related positively to babies' ponderal index and birthweight at birth.

1.6.1.2 Fetal metabolism

Appropriate fetal growth at gestation depends on genetic factors (such as the combination of material from the father and mother) as well as epigenetic factors, nutrient supply and general uterine environment, including the placenta (Huppertz et al. 2013). Nutrient supply across the maternal-fetal interface is of utmost importance for fetal growth and development. At first, nutrients and oxygen from the mother must reach the placenta before being further directed to the fetus. This is made possible by establishment of the EVT. The fetal metabolism is thought to be governed solely by the requirements of the fetus. Transplacental nutrient delivery promotes a rise in fetal insulin, which, together with a late-gestational surge in fetal cortisol levels, promotes fetal fat deposition and fetal growth (Murphy et al. 2006; Newbern and Freemark 2011). Amino acids, lipids and glucose are directed into the pathways for fetal cell growth, which are largely controlled by nutrient supply from the mother's circulation. This is due to the absence of some pathways

of essential amino acid homeostasis and glucose synthesis (requiring higher dependency on the mother) and low activity of the pathway for fatty acids in the fetus (Innis 2007; Tea et al. 2012). To date, it is well known that appropriate fetal growth is essentially determined by nutrient availability, which depends on nutrient transport and fetal metabolism. As a result, undernutrition *in utero* appears to be associated with persistent changes in the endocrine, metabolic and immune functions (Barker 1997; Kaser et al. 2001). Other changes, including cholesterol metabolism and insulin response to glucose, are affected by poor nutrition *in utero* (Barker 1997).

1.6.1.3 Fetal immune system

A concept postulated for some time, the existence and the need for a protective adaptive immune response to protect the fetus (Tafuri et al. 1995), led investigators to focus on the cells responsible for this state of active tolerance. As noted above, T_{reg} cells mediate to a large extent the state of active immune tolerance which prevents maternal lymphocytes from causing cytotoxic damage to the fetus (Aluvihare et al. 2004). Thus T_{reg} cells protect the fetus from immunological attack by the mother.

1.6.2 Offspring manifestation of the above in complicated cases

In this thesis, the emphasis has been on pregnancies that are problematic due to poor trophoblast invasion and placental function, including pregnancy loss, PE, IUGR and gestational diabetes. Using pre-eclamptic cases, for example, in both loss- and gain-of-function genetic mouse models, expression of adrenomedullin by fetal trophoblast cells is essential and sufficient to promote appropriate recruitment of uterine NK cells to the placenta (Li et al. 2013) and ultimately promote remodelling of maternal spiral arteries. Placental growth hormone levels in umbilical cord blood were higher in cases of PE (Mittal et al. 2007), an indicative of stressed placenta. Early pregnancy features intense secretory activity of placental cell types contributing to increased local and systemic inflammatory response mediators. Hofbauer cells, known as the placental resident macrophage, play a role in cytokine synthesis, as do syncytiotrophoblast and cytotrophoblast cells (Hauguel-de Mouzon and Guerre-Millo 2006). These molecules respond as part of a non-specific immune response to tissue injury and antigenic agents. As early as 1967, Brosens et al. proposed plaque build-up within the placental vascular bed as a likely indicator of excess circulatory immune cells (Brosens et al. 1967). As mentioned above, cord blood TG and TC levels were significantly higher in patients with PE compared to controls (Rodie et al. 2004). All of these data suggest that there is manifested impact on the fetal hormone, metabolism and immune system response on the offspring of pre-eclamptic women.

1.6.3 Placental role in communicating the above responses

The transport of metabolites and oxygen across the maternal-fetal interface is a prime function of the placenta, with exchanges of nutrients/gas and waste products between the mother and the developing fetus. The biochemical mechanisms involved in the transfer of maternal nutrients to the fetus include passive, facilitated and active diffusion (Economides et al. 1989), endocytosis or exocytosis (Sibley et al. 1997). Water, vitamins, amino acids such as L amino acid transporter (Roos et al. 2009), inorganic ions and minerals are all transported across the placenta to the fetus. Pregnant women use more fatty acid and less glucose as energy sources, particularly in the latter two thirds of pregnancy (Villar et al. 1992). This leaves more of the glucose available to nourish the growing fetus and means that the placenta plays a role in glucose transport across to the fetus (Korgun et al. 2005) by varied glucose transporter proteins.

A specific example is cholesterol that has a prominent role in steroidogenesis, cellular membrane biosynthesis, central nervous development, such as in the brain, myelin sheath formation and synthesis of bile acids, and has to be transferred to required targets. It is use for propagations and activations of the sonic hedgehog (SHH) signalling (Lewis et al. 2001) necessary for the function of the central nervous system. Napoli et al. showed that there is enhanced fetal atherosclerotic plaque lesion formation in the offspring of mothers with hypercholesterolaemia (Napoli et al. 1997). Work on the Smith Lemli Opitz syndrome (SLOS) highlights that maternal-derived cholesterol can be effluxed from trophoblast to fetal HDL and plasma (Jenkins et al. 2008). Jenkins et al. report that approximately 50% more effluxed HDL from SLOS patients via trophoblast cell lines were smaller in size, constituting low cholesterols to phospholipids than that of unaffected counterparts; still, plasma from SLOS fetus effluxed similar percentage as unaffected fetal plasma. These reports suggest that cholesterol placental transporters' involvement in the cholesterol transfer is inevitable. As detailed in Chapter 4 of this thesis, cholesterol transporters' upregulation is perhaps vital for cholesterol transport across the maternal-fetal interface. Some studies highlight that the cholesterol uptake involves upregulation of key cholesterol transporter molecules (Simpson et al. 1979; Bhattacharjee et al. 2010).

1.6.4 Role of maternal obesity/overnutrition

Maternal obesity is a risk factor for the mother as well as the fetus. Obesity raises the risk of offspring shoulder dystocia (Cedergren 2004), caesarean section (Bergholt et al. 2007), high postnatal high blood pressure and congenital anomalies such as heart defects (Mills et al. 2010) and neural tube defect (Shaw et al. 2000). The offspring of obese pregnant women consistently present related issues of high meconium aspiration, preterm birth,

instrumental birth, fetal distress, LGA and death (Sebire et al. 2001; Cedergren 2004; Yogev and Langer 2008; Chen et al. 2009). Data suggests a long-term consequence of obesity *in utero* is fetal programming (Catalano 2003). Obesity is associated with significant risk in altering metabolic metabolites, including less dense LDL, which indicates higher TG content in pregnancy (Meyer et al. 2013). Therefore, in pregnant women obesity exacerbates metabolic and inflammatory parameters. An Australian singleton women study of validated food-frequency between 18-24 weeks' and 36-40 weeks' gestation revealed a relation of food frequency to fetal adiposity and distribution (Blumfield et al. 2012). This study indicated that overnutrition also perhaps imposes on offspring metabolic milieu. Other studies have found that fetuses of obese women had altered cord IL-6 and leptin compared with those of lean women (Catalano et al. 2009). In addition, second trimester amniotic fluid was shown to have significantly higher TNF-α and CRP in obese and overweight women compared to lean women (Bugatto et al. 2010), suggestive of the impact of maternal obesity. When mothers and neonates were classified into three tertiles, constituting 309±25g in the first trimester, 478±40g in the second trimester and 529±39g at term neonates with fatness classified at term and second trimester had higher cord ET-1, C-peptide and leptin levels than neonates of the first trimester (Radaelli et al. 2006). These higher levels influence endothelial dysfunction and insulin resistance in the offspring. Overall, this means that maternal obesity perhaps sets up a vicious cycle in that changes in fetal metabolic and inflammatory parameters could increase the risk of *in utero* programming during fetal development.

1.7 Consequence of complicated metabolic and inflammatory environment

It has been shown that PE, IUGR and cardiovascular disease share similar metabolic disturbances and enhanced systemic inflammatory response (Bujold et al. 2003). As stated above, PE is linked with higher cord TC, TG and LDL-C levels than in controls (Rodie et al. 2004; Howlader et al. 2009). In the absence of maternal obesity, higher cord TNF-α levels were noted in cases of PE compared to a healthy pregnant group (Laskowska et al. 2006). There is also the finding that cord blood CD18, CD11b and CD11c neutrophils activation was significantly higher in women who had PE compared to infants from normotensive women (Saini et al. 2004). In cases of IUGR, despite the cord leptin and adiponectin not differing with respect to appropriate-for-gestational age (AGA) size, higher cord leptin was observed between IUGR and 1 day old offspring (Kyriakakou et al. 2008). These observable changes in metabolic and inflammatory parameters, as seen in PE and IUGR offspring are noticeably indicative of poor vascular function.

1.7.1 Endothelial function

A profound change of pregnancy is the impact on maternal vascular physiology. This is because of derived markers from endothelial cells during pregnancy (Anumba et al. 1999). Impaired production of such markers has been shown to be disturbed in some extreme pregnancies known to feature alteration of metabolic and inflammatory parameters. Endothelial cells are a key factor involved in the maintenance of vascular homeostasis, and are identified at the epicentre of vascular biology. Usually, intact endothelial cells have anti-adhesive and anti-coagulant features, which control the permeability and modulation of vasoconstrictor molecules to the vascular wall (Haller et al. 1996). This adds to the persistent role of normal endothelial activities that allows up- and/or downregulation of coagulation, vascular tone, vascular growth, fibrinolysis and interactions of leukocytes within the blood vessel wall (Avogaro and de Kreutzenberg 2005). Endothelium-leukocyte adhesion is mediated by classes of adhesion molecules: selectin, carbohydrates containing selectin ligands, integrins and immunoglobulin-like molecules (Hordijk 2006). Although the endothelium activation process is linked to adverse effects, it plays an important role. Cao et al. report that endothelial cell activation is paramount for trophoblast induction of inflammatory response in uterine endothelium, enhancing trophoblast invasion and transmigration (Cao et al. 2008). Leukocytes employ this mechanism for extravasation across subendothelial space during inflammatory response; it is also required for monocyte transformation into macrophage. A number of markers localised at and secreted by the endothelium are used to assess endothelial cell function and dysfunction as a measure of vascular tone are shown in Table 1-3.

Table 1-3: **Molecules derived from the endothelial cells**.

Molecule	Main source(s)	Biological function
CD31	Platelet, EC	EC/platelet-leukocyte interaction
eNOS	EC	Synthesis of nitric oxide
ET-1	EC, VSMC	Upregulate adhesion molecule expression
HIF-α1	EC	Regulator of oxygen homeostasis
ICAM-1	EC, leukocyte	Promote leukocyte adherence and migration
PAI-1	EC	Principal inhibitor of tPA
VCAM-1	EC, VSMC	Promote leukocyte adherence
vWF	Platelet, EC	Platelet adhesion

CD31 indicates platelet/endothelial cell adhesion molecule; EC, endothelial cell; eNOS, endothelial nitric oxide synthase; ET-1, endothelin-1; HIF-α1, hypoxia-inducible factor alpha 1; ICAM-1, intracellular adhesion molecule-1; VCAM-1, PAI-1, plasminogen activator inhibitor-1; vascular adhesion molecule-1; VSMC, vascular smooth muscle cell; vWF, von Willebrand factor

Examples of major endothelial cell markers include VCAM-1, ET-1, nitric oxide (NO) and ICAM-1 (Haller et al. 1997; Wang et al. 2003; Zhou et al. 2011). HIF-α1, vWF and CD31 are also reported (Friedman et al. 1995). These markers have been useful in accessing endothelial cell function but remain an area of study in vascular biology. PAI-1, a mediator of the clotting process, is produced by vascular cells and is also useful in assessment of endothelial (dys)function. The past three decades have provided substantial evidence that uterine environment perhaps influences fetal growth and development and offspring well-being. Mounting evidence suggests that nutritional milieu during critical stages of development early in life can program the fetus to develop metabolic syndromes in adult life (Khan et al. 2003; Alfaradhi and Ozanne 2011). Poor fetal nutrition between the second and late third trimester influence inappropriate fetal growth (Khan et al. 2003). Undernutrition and reduced utero-placental blood flow at the maternal-fetal interface have negative consequences on the fetus. Fetal organs and systems deprived of nutrition indicate the possibility of programming for future disease evident in adult life. Other studies now suggest that overnutrition causes an effect resulting in large birthweight and potential future risk of cardiovascular disorders, including obesity, type II diabetes and heart disease (Mcmillen and Robinson 2005; Desai and Ross 2011). Thus, the two ends of the nutritional spectrum may affect the developing fetus *in utero* as it depends on nutrient availability during pregnancy.

1.7.2 Fetal endothelial function

Endothelium lines and protects the integrity of the inside of the outermost hollow layer of vascular tissue used for circulation, including blood vessels. Blood circulation through the blood vessel wall is controlled by many vasodilators (e.g. NO) and vasoconstrictors that are derived from the endothelium (Celermajer 1997). This is important to ensure normal endothelial function and activity in order to maintain optimal vascular homeostasis. When there is deviation from the normal physiology of endothelium, the result is dysfunction of endothelial cells. Consequently the endothelium becomes activated, and this leads to vascular disturbances ranging from excessive vasospasm to vasoconstriction, increased thrombosis and abnormal vascular growth. This disturbance defines the pathogenesis of coronary vasoconstriction, atherosclerosis, hypertension and myocardial infarction. Although the pathophysiological mechanism and biological significance are yet unclear, epidemiological data identifies pregnancy-associated disorders (Srivatsa et al. 2001) as an important factor in early vascular tissue damage. It emerges that most complications of pregnancy linked to metabolic and inflammatory pathway disturbance in extreme cases (including PE, IUGR and gestational diabetes) affect fetal abnormal vascular health. Thus, endothelial function must be maintained in order to prevent the development of a dysfunctional state, as shown in Figure 1-2.

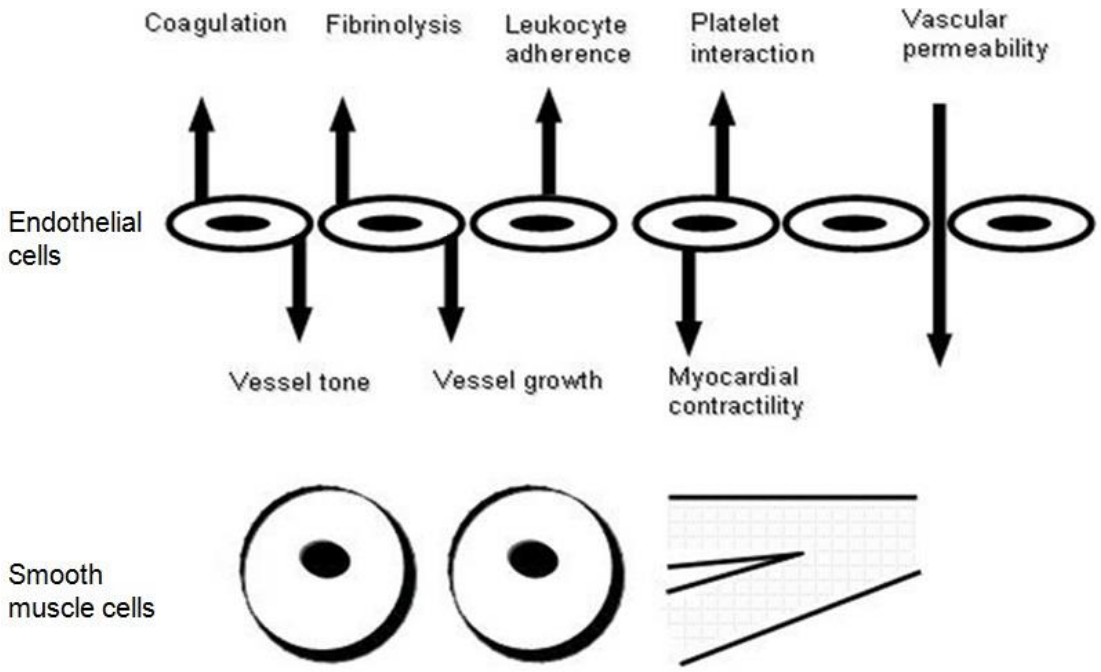


Figure 1-2: **Physiological role of normal endothelium.** Factors secreted into the lumen (upward arrows) include prostacyclin and t-PA, which influence coagulation. Cell surface adhesion molecules such as ICAM-1 and VCAM-1 regulate leukocyte adhesion. Factors secreted abluminally, towards the smooth muscle cell (downward arrow), may powerfully influence vascular tone and growth. Modified from Celermajer (Celermajer 1997).

1.7.3 Fetal programming

The origin of offspring disease later in life has occasioned intense debate but still lacks proof, since the initial proposal thirty years ago has not provided a reasonable interpretation of fetal programming. It is widely accepted that offspring who had disproportionate size *in utero* or in the first year of life are associated with risk factors of heart disease, hypertension, obesity and diabetes later in life. New attention to developmental biology has led to the formulation of the fetal origins hypothesis. Simply put, this hypothesis suggesting that conditions very early in development (even *in utero*) can leave lasting imprints on an organism's physiology, imprints that may affect susceptibility to disease with onsets occurring many decades later (Barker et al. 2002). These conditions impose direct damage on the internal memory that becomes deleterious, resulting in impaired cellular morphological and physiological 'setting' with underlying stimuli (Lucas 1994). While fetal programming is widely demonstrated across species and human populations, the adaptive significance of the effect and underlying mechanisms remain a matter of debate. Desai and Ross report that IUGR and maternal obesity/high-fat diet pregnancy indicates programmed adipocytes, given that intrinsic enhanced

lipogenesis and adipocyte proliferation contribute to the development of obesity (Desai and Ross 2011).

1.7.3.1 Undernutrition and overnutrition

Fetal tissues and organs go through a 'critical period' of development (Godfrey and Barker 2001). Insults during this 'critical period' of *in utero* growth and development or early life are not only detrimental to fetal growth but predetermine long-term offspring future health (Lucas 1991). Fetal adaptation, of for example an organ, caused by poor nutrition during the sensitive period of development may result in permanent changes to its function and structure in adult life (Fall et al. 1995). After the fetal origin hypothesis was first put forward, a great amount of data focussed on undernutrition or offspring low birthweight as prime implicative factors. This focus formed the basis of the 'thrift phenotype' hypothesis, proposing that poor fetal nutrition drives metabolic adaptation in order to maximise the chance of survival in a state of ongoing nutritional deprivation (Hales and Barker 1992). The fetus is forced to prioritise its limited nutrient supplies for essential organs, such as brain growth, at the expense of musculo-skeletal and abdominal organ growth. Undernutrition can alter maternal and fetal levels of hormones such as IGFs, insulin, growth hormone, leptin and certain placental hormones. Since most of these molecules cross the placenta, this implies that reflection of altered metabolites influences fetal endocrine glands (Fowden et al. 2005). This is important, as in the complex and dynamic process of growth and development *in utero,* interaction between the mother, placenta, fetus and macro- and micro-nutrients (involving endocrine signals) is one of the key early processes in uterine life (Warner and Ozanne 2010). All these fetal adaptive responses to poor nutrition result in metabolic, endocrine and cardiovascular abnormalities (Barker 2000; Mcmillen and Robinson 2005). A newly recognised primary cause of the obesity epidemic is the developmental programming effect of IUGR newborns exposed *in utero* to undernutrition (James et al. 2001), and normal or excessive weight newborns exposed to maternal obesity and high-fat diets (Ogden et al. 2007). This suggests that the impact of fetal programming can be attributed to under- and overnutrition (Desai and Ross 2011). Studies in human (Alfaradhi and Ozanne 2011; Blumfield et al. 2012) and animal models have shown that maternal overnutrition is reflected in high offspring birthweight (Howie et al. 2009; McCurdy et al. 2009). Consequently, changes made in fetal life or early in the offspring's life, because of under- or overnutrition perhaps affect morphological and physiological processes in the offspring in later life. An instance of these changes is the impact on offspring vascular architecture, resulting in poor vascular health of offspring that becomes evident in adult life. This under- or overnutrition may result in inappropriate weight acquisition and organ/system abnormality, including poor vascular health.

1.7.3.2 Uterine environment

The 'thrifty phenotype' hypothesis (Hales and Barker 1992), was extended to 'predictive adaptive response' hypothesis which proposes that the fetus makes adaptations *in utero* based on the predicted postnatal environment (Gluckman and Hanson 2004). Time points and processes that can be affected range from peri-conception, implantation, nutrient and oxygen supply and hormonal imbalance for the developing blastocyst, and improper distribution of cells between the trophoblast and inner cell mass (Fowden et al. 2005). Exposure to an adverse fetal and/or early postnatal life may enhance susceptibility to a number of chronic diseases. In this context, uterine/early environment may play a part in the causation of future disease (Phillips and Jones 2006). After birth, 'elastic' completion of organ/tissue development occurs *ex vivo*. This is apparent in activation of sweat glands, where the reduced activity of sweat glands means incomplete closure *in utero*, with the glands becoming functional only when activated after birth (Diamond 1991; Osmond and Barker 2000). An anecdote of a Japanese military soldier settling in an unfamiliar hotter climate, and shows this individual having more functioning sweat glands and cooling down faster. At birth, humans have a similar number of sweat glands, but none are functional. However, in the first three years, some of the glands become functional, depending on the exposure to temperature. The hotter the temperature, the greater the number of sweat glands programmed to function. By the end of three years programming is complete, and the number of the sweat glands is fixed. The actions of sweat glands encapsulate the essence of programming. In the 'critical period', the system is plastic and sensitive to the environment. This period is followed by loss of plasticity and fixed functional capacity (Barker 2000; Osmond and Barker 2000).

1.7.3.3 Epigenetic

Offspring birth weight has been identified as an indicator of risk of inheritable degenerative disease influenced by direct genetic modification of growth *in utero* (Waterland and Garza 1999). Examples of such identification are observed in the modification of gene expression of hypothalamic-pituitary-adrenal (HPA) axis and glucocorticoid receptor (GR) (Andrews and Matthews 2004; Weaver et al. 2004). In fetal animal models, epigenetic alterations of HPA axis activity and responsiveness are associated with disturbances in normal adult behaviour and stress responsiveness (Andrews and Matthews 2004; Oitzl et al. 2010). These alterations happen through insults inducing changes to the phenotype of the offspring in response to the prenatal environment. These changes take place at a specified time point, with permanent alteration of the HPA axis function which persists throughout the lifespan (Andrews and Matthews 2004). Rat models also show that increased maternal licking and grooming during the first week of life alters the methylation

pattern of the GR gene in the hippocampus of the offspring (Weaver et al. 2004). Changes persist to adulthood and alter the expression of the GR gene throughout life due to modification of the chromatin structure. Stable changes in gene transcription may result in altered functions of metabolic pathways and homeostatic control processes (Burdge et al. 2009). Altered gene function and cellular dysregulation manifest when the encoding gene is exposed to inappropriate environmental signals. Enhanced or diminished consequences of the epigenetic changes may readily occur in complications of pregnancy compared to healthy pregnancies. Pregnant rats fed with a protein-restricted diet showed stimulated changes in gene expression involved in energy balance and glucocorticoid activity (Burdge et al. 2009). Aberrant expression of transcription factors affect multiple pathways that worsen under prenatal undernutrition and nutrient homeostasis, such as glucocorticoid and PPAR activities (Bertram et al. 2001; Burdge et al. 2007; Burdge et al. 2009). Perhaps these effects involve transcription factor modification affecting activities of metabolic and vascular functions. Thus, a molecular mechanism for programming of genes *in utero* may relate to disease outcomes (Fowden et al. 2005).

1.7.4 Future offspring disorders

The hypothesis of fetal programming was proposed as a possible explanation of metabolic memory predisposing offspring to future metabolic syndromes (Heerwagen et al. 2010; Alfaradhi and Ozanne 2011). It has never been more urgent to understand the origin of obesity. A lack of detailed information on the underlying mechanism of fetal programming precludes firm statements determining the effect of obesity *in utero*. Studies showing that obese pregnant women are likely to have obese offspring suggest that the programming *in utero* of obesity is inevitable. Offspring born of obese mothers are likely to develop obesity by pre-school years, and obesity has been related to prepregnancy maternal BMI (Whitaker 2004). The childhood period appears to be a predictive factor for obesity in adulthood (Hofman et al. 2004; Boney et al. 2005). Being born with a higher birthweight increases the risk of obesity in adolescents as well as in adults (Garn et al. 1976; Lawlor et al. 2010). In another report, Catalano highlights that obese pregnancy is associated with metabolic anomalies in the intrauterine environment, leading to subsequent offspring future obesity (Catalano 2003). This suggests that the milieu of metabolism and inflammation parameters *in utero* potentially implicates the susceptibility of offspring to future vascular disorder, including obesity due to increased fetal exposure to maternal high free fatty acid, percentage of fat and hyperglycaemia (Ramsay et al. 2002; Armitage et al. 2008). On the whole, the inevitable consequence of all these condition, is an increase risk of future offsprings vascular disorders.

1.8 Hypotheses

There are several routes in which additional studies will be beneficial in order to gain insight into metabolic and inflammatory pathways over the course of pregnancy. These are summarised in the hypotheses below.

1. In Chapter 2, it is hypothesised that insulin plays an important role in the adhesion of the blastocyst to the uterine wall during human implantation by regulating the expression of adhesion molecules pivotal for implantation.

2. In Chapter 3, it is hypothesised that prepregnancy parameters of metabolic status and inflammatory mediators predict successful implantation. There are detectable changes in plasma metabolic and inflammatory parameters in the first 6 weeks of pregnancy. Also hypothesised is that obesity is associated with decreased pregnancy success and increased parameters of metabolism and inflammation evident over the first 6 weeks.

3. In Chapter 4, it is hypothesised that fetal cord metabolic and inflammatory parameters at birth reflect maternal parameters in the third trimester. It is also hypothesised that reflected offspring hyperlipidaemia, in particular cholesterol, is due to upregulated placental cholesterol transporter gene expression involved in cholesterol transfer across the maternal-fetal interface.

4. In Chapter 5, it is hypothesised that preparation of a clean HUVEC can be obtained at birth using a density gradient separation technique. It is also hypothesised that the established procedure would be useful in the selection and set up of a model of assessing disturbed offspring lipids, particularly TC in obese pregnancy, and, after an extreme case such as PE, for the impact on vascular health, using HUVEC as an index of endothelial cell function.

2 The effect of insulin on JAR/RL95-2 cell interaction: an *in vitro* model of blastocyst adhesion

2.1 Introduction

Implantation is a pivotal step for pregnancy success. Failure of the embryo to optimally implant has been linked to several adverse pregnancy outcomes in humans. These include early pregnancy loss and disorders of poor placentation such as PE, IUGR and gestational diabetes. Studies on animal models have classified implantation into three phases: apposition, adhesion and invasion. For implantation to occur, a functional blastocyst and receptive uterus are required. The process is initiated by apposition of the blastocyst trophectoderm to the endometrial epithelium, followed by adhesion. The blastocyst then penetrates the endometrial layer and the underlying basal lamina of the endometrium, and then trophoblast invasion occurs via stromal decidualisation to the ultimate destination: the maternal vasculature. Although all these steps are important, adhesion between the blastocyst and the maternal uterine wall defines an early rate-limiting step for successful implantation.

In humans, the embryonic implantation of the trophoblast onto maternal endometrial epithelium cannot be studied *in vivo*, and it is also difficult to study *ex vivo* (Hannan et al. 2010). Few laboratories worldwide have access to donated human embryos for research purposes, and this has limited the study of embryo-maternal uterine interaction during the implantation window. Endometrial curettage biopsies obtained at appropriate times of the menstrual cycle and first-trimester placenta after termination are available but less relevant. The uniqueness of the human implantation process means that no other mammal provides an appropriate animal model (Bischof and Campana 2000). This, along with the ethical concerns regarding experimentation using primary human tissues during this period of life, has necessitated the search for *in vitro* models using trophoblast and uterine cell lines.

Primary epithelial cells of the endometrium and trophoblasts are the closest representative cells relevant to the *in vivo* situation for *in vitro* studies. However, isolating sufficient primary epithelial and trophoblast cells from tissues for meaningful studies is difficult if not impossible. Also, there are extreme phenotypic changes of endometrial epithelial cells between the proliferative to the mid-secretory phase of the menstrual cycle and at the time when the endometrium becomes receptive for implantation *in vivo*. These differences can complicate the interpretation of experiments using primary cells. While one cell phenotype may be used to study one aspect of function, it may be an inappropriate choice to study

another (Hannan et al. 2010). This also means that epithelial cell lines are not necessarily representative of the *in vivo* state due to changes arising from cellular differentiation and loss of function. Indisputably, this suggests that the decision to choose an appropriate cell line for any particular experiment is not easy. Human choriocarcinoma (JAR cells) (John et al. 1993; Aboussahoud et al. 2010), and human endometrial carcinoma cells (RL95-2 cells) (John et al. 1993; Harduf et al. 2007), are among the cell lines that have been used extensively for implantation research. JAR cells can be grown and transformed into aggregates known as multi-cellular spheroids, which are a good model of the trophectoderm layer of the blastocyst. JAR cells existing as spheroids secrete human chorionic gonadotropin, progesterone and oestradiol into culture media in proportion to the spheroid size (White et al. 1988). These are key features of the *in vivo* blastocyst. RL95-2 cells serve as a model of receptive endometrial epithelium and are good for the investigation of apical adhesiveness (Harduf et al. 2007).

While previous studies have shown that embryo implantation requires the participation of various cytokines, growth factors and hormones, the effect of insulin during this process in humans has not been studied. Insulin is a pleiotropic hormone, capable of activating various intracellular pathways that lead to a cascade of multiple responses, including DNA synthesis, mRNA turnover, protein synthesis and degradation, and cell division. Insulin has a key role in cellular glucose metabolism and transport. Studies have demonstrated that the addition of insulin to the cell culture medium of preimplantation mammalian embryos leads to a physiological response similar to that in other insulin-responsive cells. For example, insulin enhances blastocyst proliferation in mouse, rat and rabbit models (Harvey and Kaye 1990; De Hertogh et al. 1991; Herrler et al. 1998). This suggests that insulin may have a direct role in the regulation of preimplantation embryo development. Some data suggest that clinical predictors of poor outcome from gonadotrophin ovulation induction in women with normogonadotrophic anovulatory infertility are obesity and insulin resistance (Ramsay et al. 2006). As weight loss increases, the chance of pregnancy success decreases. Before starting to treat anovulatory subfertility, insulin resistance must be improved; this suggests the importance of insulin. In obese women, irregular menstrual cycle, low pregnancy rate and miscarriage are well documented (Lashen et al. 2004; Jungheim et al. 2009; Wei et al. 2009). Obesity and PCOS are associated with increased insulin resistance, and affected women are less likely to become pregnant or are infertile (Jungheim et al. 2009), possibly due to insulin effects in pre- and peri-implantation.

Little is known about the molecular mechanisms that mediate blastocyst-endometrial epithelium adhesion. A current popular concept is that the implanting trophectoderm surface and the apical uterine wall bind opposing membranes directly through bridging molecules (Lopata 1996). *In vitro* studies suggest that the blastocyst-endometrial

epithelium interaction is regulated by the expression of key cellular adhesion molecules (Thie et al. 1998; Hans-Peter et al. 2000). Expression of adhesion molecules may result from a series of highly orchestrated events under hormonal and paracrine control. One proposal suggests that progesterone and oestradiol (Basak et al. 2002; Jha et al. 2006) regulate adhesion molecule expression at the endometrial epithelium (Figure 2-1).

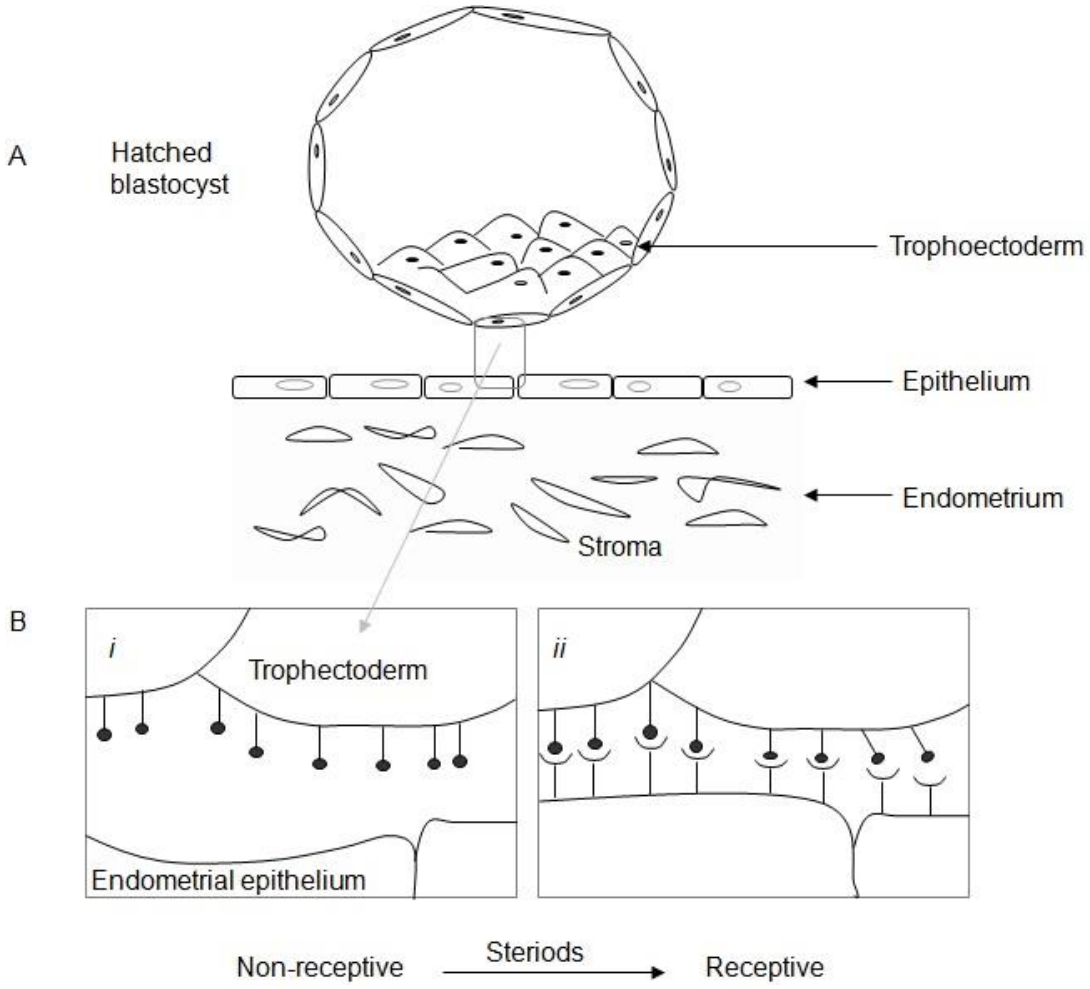


Figure 2-1: **Paracrine induction of endometrial receptivity for the blastocyst in humans**. A) The hatched blastocyst apposes to the epithelium of the endometrium, with the inner cell mass apposed proximally. The epithelium is converted by direct or indirect steroidal action from B) *i* a non-receptive state to B) *ii* a receptive state. One hypothesis predicts the appearance of adhesion molecules on the epithelium apical domain, which coincides with receptivity and attachment. Modified from the work of Aplin (Aplin 1997).

In Section 1.2.3.3, it is suggested that cell adhesion molecules are involved in the embryo-endometrial epithelium interaction. Trophinin (Fukuda et al. 1995), CD44 (Campbell et al. 1995) and laminin α1 (Dziadek and Timpl 1985; Nissinen et al. 1991) are among the surface adhesion molecules expressed in human blastocysts and/or uterine epithelial basement membranes. Laminin α1 interacts with other extracellular matrix components including FBLN-1 and FBLN-2, during embryonic development.

2.2 Hypothesis

The hypothesis explored in this chapter is that insulin plays an important role in the adhesion of the blastocyst to the uterine wall during human implantation by regulating the expression of adhesion molecules pivotal for implantation.

2.3 Aims

The aims of this chapter are to gain insight into the role of insulin during blastocyst-uterine adhesion, using an *in vitro* implantation model incorporating the binding of JAR spheroids to an RL95-2 monolayer, and to explore the effect of recombinant human insulin on key candidate adhesion molecule expression in the *in vitro* cell co-culture model.

2.4 Objectives

1. To establish and validate a human trophoblast cell (JAR cells) and human endometrial epithelial cell (RL95-2 cells) co-culture model of blastocyst-uterine adhesion.

2. To assess the effects of recombinant human insulin on JAR spheroid-RL95-2 monolayer adhesion in the *in vitro* culture model.

3. To determine expression in JAR cells and RL95-2 cells of trophinin, CD44, laminin α1, FBLN-1, FBLN-2 and insulin receptors, in order to select a relevant adhesion molecule to study.

4. To examine the effects of insulin on cell adhesion molecules and receptor expression in JAR and RL95-2 cells.

5. To investigate the involvement of the selected molecule as a cell adhesion molecule implicated in JAR spheroid-RL95-2 monolayer adhesion.

2.5 Materials and Methods

2.5.1 Media and buffers

Blocking solution (5%) was prepared by dissolving 5g non-fat milk (Marvel) in 100mL phosphate buffered saline (PBS) pH 7.3.

Citrate buffer 0.01M (pH 6.0) was prepared by dissolving 1.9g of citric acid in 1L of deionised water (DH_2O), adjusted to pH 6.0 with10mM NaOH.

3,3'-diaminobenzidine tetrahydrochloride (DAB) solution was made up by dissolving 1 tablet of DAB (Sigma) and 12µL H_2O_2 in a final volume of 15mL of 50mM Tris-HCl (pH 7.5).

Diethlpyrocarbonate (DEPC) nuclease-free DH_2O was prepared by treating DH_2O with 0.1% DEPC and incubating overnight at room temperature (RT) and then autoclaving (at least 15 min) to inactivate traces of DEPC.

DNA 0.1µg/µL ladder was prepared by diluting 10µL of 1000µg/mL 2-log DNA ladder (0.1-10.0kb) and 10µL 10X sample loading buffer (for PCR) in a volume of 80µL DH_2O.

Tris-HCl buffer 50mM (pH 7.5) was made by dissolving 6.1g Tris-HCl in a final volume of 1L of DH_2O and adjusting the pH to 7.5 with 10mM NaOH.

Solubilised membrane protein extraction solution was prepared by dissolving 10mM-0.744g EDTA, 10µg/mL aprotinin, 2mL 1% Triton X-100, 2mM 2-mercaptoethanol, 0.232g/mL $CaCl_2$ and 4 tablets of Protease Inhibitor Cocktail in a final volume of 200mL of 0.2M Tris-HCl (pH 7.5).

PBS (0.2M) was made by dissolving 1.2g NaH_2PO_4 and 9.0g NaCl in a final volume of 1L of DH_2O and bringing to pH 7.3 or 7.6 with 10mM NaOH.

PBSTween-20 (pH 7.3) buffer of 0.1% and 0.05% Tween-20 were made by adding 1000µL and 500µL Tween-20, respectively, to a total volume of 1L of 0.2M PBS (pH 7.3).

Protein extraction lysis buffer was made by dissolving 1 tablet Protease Inhibitor Cocktail in a final volume of 10mL CelLytic^{MT} MT mammalian tissue lysis/extraction reagent.

Protein loading sample buffer was prepared by adding 25µL 14.3M mercaptoethanol to a total volume of 475µL of Laemmli sample buffer (Bio-Rad Laboratories), making a final

composition of 62.5mM Tris-HCl (pH 6.8), 2% SDS, 20% 2-mercaptoethanol and 0.01% bromophenol blue.

A final 1X concentration of running buffer was prepared by diluting 40mL (20X) pre-mixed NuPAGE® MOPS SDS Running Buffer (formulation: 50mM MOPS, 50mM Tri-Base, 0.1% SDS, 1mM EDTA, pH 7.7) in a volume of 760mL DH_2O.

Sample loading buffer (10X) for PCR was made up by dissolving 0.1g SDS, 0.37g $Na_2EDTA.2H_2O$, 0.01g bromophenol blue, 0.01g xylene cyanole ff and 5mL glycerol in a total volume of 10mL DH_2O.

A 1.1M Tris-borate 0.1M EDTA buffer (TBE), 5X solution pH 8.3 was prepared by dissolving 136.25g tris-base, 69.7g boric acid and 18.75g EDTA in a final volume of 5L of DH_2O and then bringing to pH 8.3 using 5M NaOH. The final concentration of TBE components in 1X buffer was then made up by diluting 100mL of 5X stock in 400mL of DH_2O.

Transfer buffer was made by diluting 50mL 20X NuPAGE® Transfer buffer and 100mL 20% methanol in a final volume of 1L DH_2O.

Tris-HCl buffer (pH 7.5) 0.2M was made by dissolving 1.66g Tris-HCl in a final volume of 1L of DH_2O and adjusting the pH to 7.5 with 10mM NaOH.

2.5.2 JAR and RL95-2 cell culture

The human choriocarcinoma JAR cell line and the human endometrial carcinoma RL95-2 cell line were obtained from ATCC Manassas, VA, USA. The JAR cells were originally derived from a Caucasian fetal male (Pattillo RA et al. 1971), while the RL95-2 cells were originally derived from a moderately differentiated adeno-squamous carcinoma of the endometrium of a 65-year-old female Caucasian (Way et al. 1983). A safety cabinet flow-hood was used to prepare the growth media and handle cell line cultures in aseptic conditions. The media was allowed to reach 37°C in a water bath for 10 min before use. Frozen JAR cells were thawed in a 37°C water bath for 2 min, resuspended in 9mL RPMI and centrifuged (125*g*, 5 min) in a Jouan CR4-R centrifuge. The supernatant was decanted and cells were resuspended in 10mL complete growth media and transferred into 25cm^2 polystyrene culture flasks (Greiner Bio-one, Germany). Flasks were incubated at 37°C in a 5% CO_2 and 95% air. JAR complete growth media was made up of 1% Penicillin-Streptomycin (P-S) (100U/mL-100µg/mL) and 10% FBS in a volume of 500mL of formulated RPMI 1640 (ATCC: high glucose). The media was changed every two days

until 70-90% confluence was reached (one week). The JAR cells were collected and sub-cultured at a 1 in 5 dilution, i.e. approximately 6 x 10^6 cells/mL, and incubated in a 5% CO_2:95% air. The RL95-2 cells were similarly thawed, washed, pelleted, resuspended and transferred into 25cm^2 polystyrene culture flasks and subsequently seeded 1 in 4 dilution in culture flasks containing approximately 6 x 10^6 cells/mL and incubated in a 5% CO_2 and 95% air. The RL95-2 cells were maintained in DMEM/F12 (1:1) complete culture media containing 7.5% Na_2CO_3, 75g/L HEPES, 10mg/mL insulin, 1% P-S (100U/mL-100µg/mL) and 10% FBS DMEM/F12 (1:1). The media was changed three days per week until 70-90% confluence was reached. The JAR and RL95-2 cell subculture was carried out by dispersing the cells with 2mL and 5mL of 0.25% trypsin-EDTA respectively. The JAR and RL95-2 flasks were incubated at 37°C in a humidified condition (5% CO_2:95% air) for 2 min and 5 min respectively, to allow the cells to disperse. The JAR cells were resuspended in 5mL complete growth media. However, the RL95-2 cells were washed in 5mL serum-free media, pelleted at 125*g* in a Jouan CR4-R centrifuge for 5 min and then resuspended in 5mL of complete DMEM/F12 (1:1) growth medium. To estimate JAR and RL95-2 cell number and viability, cells were stained with 50% trypan blue and counted using a haemocytometer (see Section 2.5.3).

For storage, both the JAR and the RL95-2 cells were harvested at the end of passage 7, centrifuged (125*g*, 5 min) and then resuspended in a freezing medium in a final cell concentration between 3-5 x 10^6 cells/mL for JAR and 8-10 x 10^6 cells/mL of RL95-2 cells. The freezing medium was made up of 10mL 20% FBS, 10% DMSO and 100µL (100U/mL-100µg/mL) P-S, added to a volume of 6.9mL RPMI 1640 for JAR or DMEM/F12 (1:1) for RL95-2 cells. Then 1mL of the cell suspension was transferred into cryo-vials (Greiner bio-one) and immediately placed into a Mr Frosty Cryo 1°C Freezing Container (Thermo Fisher Scientific) that ensured a temperature decrease of 1°C per minute. The vials were left at -80°C for up to 24 hours and then stored in liquid nitrogen or at -150°C until required.

2.5.3 Assessment of cell growth rates and morphology

In order to identify the optimal point in the growth cycle at which to carry out experimental studies, so that cells could be used at the same passage number, cell growth rates after defrosting were assessed. Frozen JAR and RL95-2 cells (Section 2.5.2) were thawed. The JAR and RL95-2 cells were seeded in a 24-well plate (divided into 8 sections of 3 wells) at a starting density of 4 x 10^5 cells/mL in the appropriate growth media for up to 8 days. Media were changed every 48 hours by gentle aspiration with clean pipette tips. Cells were harvested after the addition of 1mL of 0.25% trypsin-EDTA. The JAR and RL95-2 cells were stained in 1:1 of trypan blue, placed in a haemocytometer and then

viewed under an Olympus Model CK2 inverted microscope (Olympus Japan) to count viable cells. The cell growth rate was examined by counting the total number of cells in triplicate wells per 24 hours to obtain the population doubling time: [0.0301 (Tx-Tzero) divided by logNx-logNzero], where 'T' is time and 'N' is the average number at the time; 'x', the average number at 'Tx', was then calculated. The JAR and RL95-2 cells were seeded at a density of 5 x 10^6 cells per 75cm^2 polystyrene culture flask and then cultured for 48 hours to examine their morphological appearance.

2.5.4 JAR spheroid and RL95-2 monolayer preparation

Flasks containing actively dividing JAR cells were harvested by incubating at 37°C in a solution of 0.25% trypsin-EDTA in a 5% CO_2:95% air for 2 min. The JAR cells were washed in serum-free RPMI media and then resuspended at a final concentration of 0.6 x 10^6 cells/6mL in complete JAR media in a 25mL autoclaved Erlenmeyer flask (VWR International). The Erlenmeyer flasks were incubated on a gyratory shaker (MTS 2/4 digital microtiter shaker) at 300 *rpm* at 37°C in humidified conditions of a 5% CO_2:95% air. The JAR cells formed multi-cellular spheroids which were harvested after 72 hours. The spheroids in the Erlenmeyer flasks were gently collected using wide bore pipette tips and then transferred into 1.5mL tubes. The spheroids were washed in serum-free RPMI media and allowed to settle, and the supernatant of the mixture were decanted by gentle aspiration. They were carefully resuspended in 100µL RPMI, and 50µL was gently collected and delivered onto a superfrost glass slide and viewed under a light microscope for quantitation.

The RL95-2 cells were thawed, resuspended in a clean culture flask and cultured for six days. They were then subcultured and incubated for an additional four days, at which point the cells were in the log phase of growth. These actively dividing cells were harvested (at 70-90% confluence). Clean sterile coverslips (12mm diameter Harvard Apparatus, Kent, UK) were gently placed into 24 multi-well plates and coated with 50µg/mL (70000-150000kDa) poly-D-lysine (Sigma), as described (Kouranova et al. 2008). The glass coverslips were gently shaken to allow equal coating in poly-D-lysine and then incubated at 37°C for 5 min in a humidified atmosphere of 5% CO_2 and 95% air. The coated glass coverslips were allowed to air dry under a laminar flow hood. The RL95-2 cells were plated at 0.2 x 10^5 cells/mL in complete growth media DMEM/F12 (1:1) into wells of 24-well plates containing the coverslips. The RL95-2 cell medium was changed twice, at 48 hours and 96 hours.

2.5.5 Adhesion assay

The cell adhesion assay was a modification of a previously reported method (John et al. 1993; Hans-Peter et al. 2000). After 96 hours, the RL95-2 medium in the coverslip cultures was replaced by the JAR (RPMI 1640) medium, and the RL95-2 cells were then incubated at 37°C for 1 hour in a humidified atmosphere of 5% CO_2:95% air. After 1 hour approximately 20 JAR spheroids in 50µL complete RPMI 1640 JAR medium were applied to the centre of each RL95-2 monolayer on a coverslip using a wide pipette tip. The cells were then incubated together at 37°C for up to 48 hours in a 5% CO_2:95% air. At the end of the co-culture period, the coverslips were carefully removed from the growth media with wide tip forceps and placed into a 10mL conical test tube (Greiner bio-one, CA, USA) containing 2mL serum-free RPMI 1640, with the surface covered with cells facing downwards. The conical test tubes were gently centrifuged (12*g* for 5 min) using a swing-out bucket rotor to remove the JAR spheroids that had not firmly adhered to the cell monolayer. The coverslips were then placed in a multi-well plate containing serum-free RPMI 1640. The multi-well plates were observed under an Olympus CK Japan Model CK2 microscope, and the adherent spheroids counted. Data were presented as spheroids bound as a percentage of the total spheroids applied.

2.5.6 Selecting a positive control for the adhesion assay

In order to control for specificity of spheroid binding in the adhesion assay, preparations of JAR and RL95-2 cell membranes were made and tested for their ability to inhibit JAR spheroid-RL95-2 monolayer binding. Cells were grown in a 75cm^2 culture flask and, at the actively dividing phase, were washed twice with 0.2M Tris (pH 7.5) solution and then scraped from the surface of the flask and incubated on wet ice for 15 min in a 2mL protein extraction solution (see Section 2.5.1). Cell debris was pelleted at 250*g* for 15 min in 15mL conical centrifuge tubes and then supernatants were pipetted into a clean 1.5mL eppendorf tube and centrifuged at 10000*g* for 15 min at 4°C. The supernatant containing total cell solublised extract was collected and its protein concentration estimated using the Bradford assay (Bradford 1976), as reported in Section 2.5.9. The prepared total cell solubilised extracts were utilised either after being freshly prepared or after having been frozen and stored at -80°C for at least 24 hours to establish whether competitive binding activity would be retained after freezing. Use of frozen total cell solubilised extracts would avoid the necessity for fresh extracts to be prepared for every adhesion assay.

Testing of controls for specificity of adhesion of JAR spheroids and RL95-2 monolayers were carried out following a previously reported protocol (Hans-Peter et al. 2000). In brief, JAR spheroids and RL95-2 monolayers were preincubated separately in a 0.2mg/mL (final

concentration) of solubilised cell extract for 1 hour in a culture medium (containing 10% FBS). Then, the same solubilised extract was added to a final concentration of 0.1mg/mL during the binding assay time point. Each solubilised cell extract was obtained from growing confluent JAR cells or RL95-2 cells in culture flasks. In the current study, JAR spheroids were preincubated for 1 hour in a culture medium containing a 0.2mg/mL final concentration of solublised extract prepared from confluent JAR cells added either freshly prepared or frozen. JAR spheroids were then washed in serum-free media before being placed in the medium with the same solubilised extract at a final concentration of 0.1mg/mL during the binding assay which took 48 hours at 37°C in a 5% CO_2 and 95% air. In a similar fashion, JAR spheroids were preincubated with solubilised extract from confluent RL95-2 cells either freshly prepared or frozen in a medium containing a 0.2mg/mL of final concentration for 1 hour. The JAR spheroids were washed and the same solubilised extract added in a 0.1mg/mL of final concentration of medium during binding assay. The spheroids binding to the RL95-2 monolayers were then quantitated as described above (Section 2.5.5).

It was established that the JAR cell solubilised extract at 0.2mg/mL with preincubation for 1 hour, with the further addition of 0.1mg/mL prior to the binding assay for 24 hours, maximally inhibited JAR spheroid-RL95-2 monolayer binding, whether freshly prepared or frozen. For convenience, the frozen JAR solubilised extract was selected as a positive control for inhibition of binding. A concentration range of frozen JAR cell solublised extracts at a concentration of 0.0002 to 200.0µg/mL were preincubated for 1 hour, followed by a wash in serum-free medium and the further addition of 0.0001 to 100.0µg/mL solubilised cell extracts during the binding assay, and then tested to assess the concentration-dependence of inhibition of binding in this cell culture model of implantation.

2.5.7 Concentration-dependence of insulin effects on JAR spheroid-RL95-2 monolayer binding

Human recombinant (rhuman) insulin Actrapid 100 IU/mL (Novo Nordisk A/S) was used to explore the effect of insulin on adhesion of JAR spheroids to RL95-2 monolayers. As there are no available data for localised embryo insulin concentration at implantation, circulating plasma levels of insulin in women (approximately 24mU/L (0.14nM)), and in the umbilical venous cord blood (~0.05nM) of healthy pregnancies, were used as a guide for selecting insulin concentration for experiments. Insulin concentration was based on the report of the correct conversion factor of 1mU/L (µU/mL) = 6pmol/L (Heinemann 2010). A concentration range of added insulin (0.03, 0.06, 0.12, 0.18 and 0.24nM) was used in the experiment. FBS preparations will contain some insulin, usually about 0.04nM insulin in

5% FBS. In my experiments 10% FBS (containing ~0.08nM insulin) were utilised in the current adhesion assay studies. In later experiments, rhuman insulin was added to the binding assay at low concentration (0.03nM) and at high concentration (0.24nM). The effect of insulin on cell morphology was assessed under an inverted microscope, as described above (Section 2.5.5).

2.5.8 RNA preparation and primer design for PCR

Culture flasks containing actively dividing JAR and RL95-2 cells were washed in sterile Dulbecco's PBS containing Ca^{2+}/Mg^{2+} and cells were harvested as described above. Cells were resuspended in 1mL TRIzol LS reagent (Life Technologies) and incubated at RT for 5 min to permit complete dissociation of nucleoprotein complexes. Chloroform (0.2mL) was added to the suspension, which was mixed vigorously and left to incubate at RT for 3 min. The upper aqueous phase was transferred to a clean sterile tube before 0.5mL isopropyl alcohol was added, followed by incubation at RT for 10 min and then centrifugation at 4700*g* for 10 min at 4°C. The supernatant was decanted and pellets washed in 1mL of 75% alcohol with gentle vortexing followed by centrifugation (250*g*, 5 min) at 4°C. The pelleted RNA was air dried and then resuspended in 40µL DH_2O and incubated at 65°C for 10 min. Total RNA concentrations were measured spectrophotometrically using a NanoDrop® ND-1000 spectrophotometer. RNA purity was assessed by using this A260/A280 ratio, which was 1.96 (1.88-2.03) for RNA prepared from JAR cells and 2.01 (2.00-2.03), for RNA prepared from RL95-2 cells. The JAR and RL95-2 cell RNA (0.1-1.0µg per reaction) was DNase treated using a DNA free™ kit (Ambion) according to the manufacturer's instructions. Briefly, 1µL DNase-1 was mixed with 5µL of (up to 5µg) RNA, 2.5µL 10X DNase buffer and 16.5µL DEPC. The mixture was vortexed gently and then incubated at 37°C for 30 min in an OMN-E thermalcycler. DNase inactivation reagent (2.5µL) was added, the tube mixed by flicking and then centrifuged (4700*g*) for 1 min. Aliquots of pre-treated RNA were collected and stored at -80°C until analysis.

DNase RNA was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystem). Complementary DNA was prepared by adding pre-treated RNA (5µL) and a cDNA reaction mix (5µL) made up of 1µL 10X reverse transcriptase buffer, 0.4µL 25X dNTPs, 1µL 10X random primers, 0.5µL multiscribe reverse transcriptase and 0.5µL Rnase inhibitor (superasein) 1U/µL dissolved in 1.6µL DEPC. A no multiscribe reverse transcriptase (NoRT) negative control was also prepared with the omission of multiscribe reverse transcriptase. The resulting mixture was incubated for 10 min at 25°C followed by 120 min at 37°C.

The presence of human trophinin, CD44, laminin α1, FBLN-1, FBLN-2 and insulin receptor mRNA in JAR and RL95-2 cells was investigated using specific PCR primers. β-Actin was employed as a control gene for gel loading. The sequences of the primers were designed using BLAST: Basic Local Alignment Search Tool, Pubmed. The sequences were as follows: trophinin (detects variants 2, 3 and 6) forward 5'-TCC-CTC-CAG-ATA-TAC-AGA-CTG-AGA-CCA-C-3' and reverse 5'-AGG-TAA-AGC-CTG-GCT-GAT-CTG-GGT-AAT-G-3', producing a 278-bp product; CD44 (detects variants 1, 2, 3, 4 and 5) forward 5'-ATT-GCA-GTC-AAC-AGT-CGA-AGA-AGG-TGT-G-3' and reverse 5'-ACG-GTT-GTT-TCT-TTC-CAA-GAT-AAT-GGT-G-3', producing a 262-bp product; laminin α1 forward 5'-TGC-CAT-TCT-CAA-TCT-TGC-CAG-3' and reverse 5'-TAA-GTC-CAG-AGT-GAT-TGT-GAC-3', producing a 265-bp product; FBLN-1 forward 5'-TGT-GAG-AGT-GGT-ATT-CAT-AAC-TGC-CTC-3' and reverse 5'-ACA-GCG-CGT-TCC-CTC-CTC-GTT-GAG-ATG-3', producing a 267-bp product; FBLN-2 (detects variants 1, 2 and 3) forward 5'-TCT-TCC-TAT-CCA-GGA-GGA-GAG-GGC-AGA-AG-3' and reverse 5'-AGA-ACT-TGC-GGC-TTC-CTG-GGC-ACT-TCT-CG-3', producing a 288-bp product; insulin receptor (detects variants 1 and 2) forward 5'-TTC-AGG-GTA-TCT-AAA-AAT-CCG-CCG-ATC-3' and reverse 5'-TTG-GTC-TTC-AGG-GCA-ATG TCG-TTT-CTC-3', producing a 294-bp product; and β-Actin forward 5'-TGT-GAT-GGT-GGG-AAT-GGG-TCA-G-3' and reverse 5'-TTT-GAT-GTG-ACG-CAC-GAT-TTC-C-3', producing a 514-bp product.

First Choice® human kidney total RNA served as a positive control for all the target genes. In the qualitative PCR assay, 1µg of cDNA was used as a template for PCR amplification in a total volume of 10µL PCR mix (5µL of 1X MegaMix~Double, 0.5µL 10µM forward and reverse primers and 4µL DEPC), making a final primer concentration of 1µM per assay. PCR was carried out as follows: denaturation at 94°C for 3 min x 1 cycle amplification at 94°C for 30 sec and 60°C for 30 sec, annealing at 57°C (trophinin, CD44, laminin α1 and β-Actin) and 56°C (FBLN-1, FBLN-2 and insulin receptor) for 1 min x 35 cycles, and extension at 72°C for 4 min x 1 cycle.

A sample loading buffer (1µL) was added to the PCR product which was then subjected to agarose gel electrophoresis on a 1.5% agarose gel in TBE (1X) pH 8.3 [100mL of 5X stock in a 400mL of DH_2O with a final concentration of 1µg/mL ethidium bromide]. A 0.1-10.0kb DNA ladder at 0.1µg/µL was also loaded. Electrophoresis was carried out for 1.20 hours at 100V. Bands were visualised using a UV transilluminator and the gel was photographed with a DS34 Polaroid-direct screen instant camera containing Polaroid black-and-white print film type 667 (Sigma).

2.5.9 Protein quantitation

Actively dividing cells were washed in sterile Dulbecco's PBS (Ca^{2+}/Mg^{2+}), scraped from the growth on the surface of the flask and pelleted at 250*g* for 10 min at 4°C. The supernatant was discarded and 2.5mL CelLytic™ MT mammalian tissue lysis reagent containing dissolved protease inhibitor cocktail (Roche) was added to the cell pellet and incubated on wet ice for 15 min. The suspension was centrifuged (5000*g*, 10 min) at 4°C and the supernatant pipetted into a clean sterile 1.5mL tube (10000*g*, 10 min). Protein lysate concentration of JAR cells, and the JAR and RL95-2 solubilised cell extracts (positive control) were measured using a Bradford assay. A 10µL aliquot (1:100 dilutions) of each cell homogenate was used for protein determination. A standard concentration curve of 0.1-1.4mg/mL was made using BSA as a standard protein. The Bradford reagent was left to reach RT. The Bradford reagent (1mL, Sigma) was added to the homogenate aliquot with mixing. Resulting suspensions were incubated at RT for 15 min before measuring absorbance on spectrometer at 595nM wavelength on a spectrometer. A standard curve of absorbance versus the protein concentration was prepared and the unknown protein concentration determined from the curve. The estimated concentration of the original sample was corrected for volume and dilution factor.

2.5.10 SDS electrophoresis and Western blotting

The JAR protein (70-130µg/µL) was then subjected to SDS-PAGE electrophoresis. The JAR protein was loaded onto gels in a 30µL final volume containing a 15µL protein loading sample buffer (Section 2.5.1). Proteins were separated using NUPAGE® Novex 4-12% Bis-Tris gels on an XCell Surelock™ Mini-Cell system (Life Technologies), according to the manufacturer's instructions. HiMark™ pre-stained high molecular weight protein standard (28-420kDa; LC5699, on a NuPAGE® 4-12% Bis-Tris/MOPS, Life Technologies) was used as a size marker. The inner chamber of the tank was filled with 200mL of 1% NuPAGE® MOPS SDS running buffer to which 100µL of 1mM dithiothreitol reagent had been added. Electrophoresis was carried out at 120V, 180mA and 40W for 4 hours. Separated proteins were electroblotted from the gel onto a nitrocellulose membrane filter at 25V, 180mA and 60W for 2 hours on an XCell™ blot Module (Life Technologies) according to the manufacturer's instructions. The transfer buffer was made by mixing 50mL 20X NuPAGE® Transfer buffer and 100mL 20% methanol to a final volume of 1L DH_2O.

Electroblotted membranes were blocked in a blocking solution (Section 2.5.1) for 1 hour at RT and then incubated with a primary antibody (1:600 anti-human laminin α1 antibody goat polyclonal; AF4187, R&D System) in 5% non-fat milk in a 0.05% PBSTween-20 (pH

7.3) buffer. Membranes were incubated overnight (16-18 hours) with mild agitation at 4°C. Membranes were washed in the 0.05% PBSTween-20 (pH 7.3) buffer for 5 x 10 min before incubating with the secondary antibody (1:1000 donkey anti-goat HRP IgG: SC-2020, Santa Cruz) made up in 5% non-fat milk in 0.05% PBSTween-20 containing 1:10000 β-Actin (C4) HRP mouse monoclonal antibody (SC-47778, Santa Cruz) at RT for 1 hour. The blot was washed stringently in 0.05% PBSTween-20 (pH 7.3) buffer for 5 x 10 min and the signal detected with 1:1 reagent A and B SuperSignal® West Pico Trial Kit (an enhancer chemiluminescent substrate for detection of HRP) (Thermo Scientific) after 5 min incubation at RT. Bands were detected by exposing the blots on an x-ray film (Fuji) and then developing on a Kodak OMAT 1000 Processor (Eastman Kodak, United States) according to the manufacturer's instructions. Laminin α1 molecular weight was measured by determining migration distance relative to dye front. Detected bands on the film were scanned as a pictorial record (Epson perfect 3200).

2.5.11 Immunocytochemistry

Cells were collected during the log growth phase and washed in sterile Dulbecco's PBS (Ca^{2+}/Mg^{2+}) (pH 7.6). Cells were resuspended in PBS before depositing on a Super-Frost Plus glass slide using a cytospin at 600*g* for 5 min, and cells were then fixed in 70% cold acetone for 10 min at RT. Acetone, like alcohol, is an organic solvent which fixes samples, removing lipids and dehydrating the cell as it precipitates the proteins on the cellular architecture. A standard Avidin Biotin Complex (ABC) method was utilised for cell staining as reported (Hsu et al. 1981). Paraffin-embedded positive control slides (breast tumour tissue) were heated to 60°C for 35 min and dewaxed in xylene for 2 x 10 min, rehydrated in an alcohol series (2 x 5 min at absolute (100%), 2 x 5 min at 95%, 1 x 5 min at 70%) and washed for 2 x 5 min in PBS. Peroxidase activity was quenched by immersing the slides in freshly prepared 0.5% H_2O_2 (5mL H_2O_2 and 300mL methanol) for 30 min. After 2 x 10 min PBS washes, retrieval of antigens in paraffin-embedded slides was performed by microwaving sections in a pressure cooker for 5 min in a 0.01M citrate buffer (Section 2.5.1). Paraffin slides were rinsed for 5 min in DH_2O and then 2 x 5 min in PBS pH 7.6, while acetone-fixed cell slides were rinsed for 5 min in PBS pH 7.6. A non-immune blocking reagent was added (20% rabbit serum and 20% human serum) in PBS pH 7.6, and incubated at RT for 30 min to block non-specific antibody binding. Slides were washed in DH_2O for 5 min, twice in PBS pH 7.6 for 10 min and then incubated with anti-human laminin α1 antibody of 2µg/mL concentration (AF4187, R&D System) overnight at 4°C in a humidified box. After two washes in PBS pH 7.6 for 5 min, the slides were incubated in biotinylated IgG H+L (30 min, 1:200, RT) (BA-5000, Vector laboratories). The 1° and 2° antibodies were diluted in 2% rabbit serum and 5% human serum in PBS pH 7.6. A negative control, which only contained 2% rabbit serum and 5% human serum was

used. After incubation with the 2° antibody, slides were washed twice in PBS (pH 7.6) for 5 min and incubated for 30 min at RT in Vectastain® standard ABC Kit Elite [two drops of reagent A and reagent B diluted in 5mL PBS, pH 7.6]. Staining was detected using 1mg/mL DAB solution (see Section 2.5.1) after 10 min incubation. Slides were washed in PBS (1 x 5 min) and then in DH_2O for 5 min before counterstaining in Harris stain for 15 sec. The paraffin slides were dehydrated in a series of alcohol concentrations and all slides were mounted in DPX for microscopy. Digital image capture was by Image-Pro Plus (version 6.2 MediaCybernetics) on a BX50 F-3 microscope (Olympus) equipped with X4, X10, X20, X40 and X100 lenses connected to a 3-CCD colour camera. ImageJ, a Java-based image processor (National Institute of Health Bethesda, Maryland, USA), was used for image processing.

2.5.12 Blocking of binding in laminin α1 JAR spheroid-RL95-2 monolayer adhesion assay

An adhesion assay was set up to investigate the effects of anti-human laminin α1 antibody (AF4187, R&D System) incubated with either JAR spheroids only or also with a JAR spheroid-RL95-2 monolayer on binding. In initial studies, JAR spheroids were preincubated (37°C for 1 hour) with anti-human laminin α1 antibody at final concentrations of 0.1, 0.2 and 0.4µg/mL and then the JAR cells were used in a JAR spheroid-RL95-2 monolayer binding assay. In another experiment, anti-human laminin α1 antibodies were added at final concentrations of 0.2, 1.0 and 4.0µg/mL to culture wells containing a co-culture of JAR spheroids with a RL95-2 monolayers and then incubated for 24 hours in 5% CO_2 and 95% air. All experiments were also repeated, as described above, but with replacing anti-human laminin α1 antibody with anti-GRP-78 (N-20) (SC-1050, Santa Cruz) as an irrelevant antibody (control). Replicate n=3 independent experiments were carried out.

2.5.13 Statistics

GraphPad Prism® 5 software (GraphPad, Inc; San Diego USA) was utilised for data analysis and presentation.

2.6 Results

2.6.1 Testing cell line growth rates to identify the optimal time period for experimental studies

Cultures of JAR and RL95-2 cell lines grew well up to the 7^{th} passage, and there was no observable transformational change from the original population. JAR and RL95-2 cells were able to grow successfully on a plastic surface and completely dispersed to a single cell suspension, as expected, with 0.25% trypsin-EDTA as visualised under a microscope.

Growth rates of JAR and RL95-2 cells were determined by initial seeding at a density of 4 x 10^5 cells per 2cm^2 well of a 24-well plate following growth over 7 days and 8 days respectively. Cell growth rates were assessed by daily cell counting. JAR and RL95-2 cells had a doubling time of 1.5 and 2.0 days respectively (Figure 2-2A and B). The maximal number of JAR cell was observed at day 4 and maximal growth rate appears to be between days 3 and 4 (Figure 2-2A). RL95-2 cell maximal number was observed at day 6 (Figure 2-2B) and maximal growth rate appears to be between days 4 and 5 (Figure 2-2B). Therefore, experiments were carried out after collecting JAR cells at day 4 and RL95-2 cells at day 6, approximately.

Cell surface morphology was assessed under a microscope in cells plated at a density of 5 x 10^6 cells per 75cm^2 culture flask. After 48 hours in culture, JAR cells appeared sparingly dispersed (Figure 2-2C) compared to RL95-2 cells, which showed a dense and clustered appearance (Figure 2-2D).

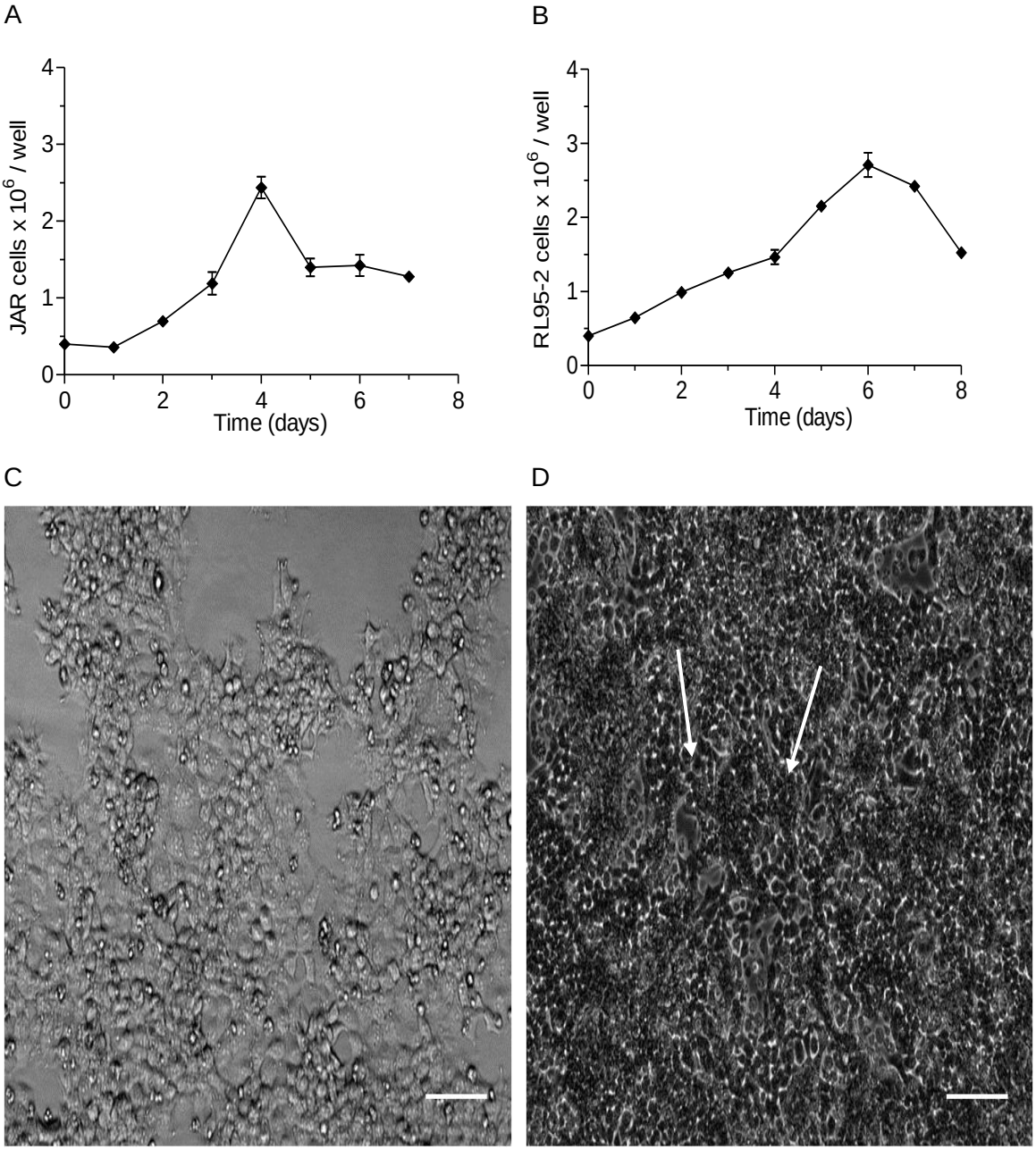


Figure 2-2: **Human choriocarcinoma JAR cell and human endometrial epithelium RL95-2 cell growth profiles and appearance in culture**. Cell proliferation was assessed by cell counting. Mean (SE) of n=3 independent experiments is presented. A) JAR and B) RL95-2 cells had a doubling time of 1.5 and 2 days respectively. The morphology of approximately 5 x 10^6 C) JAR cells and D) RL95-2 cells after 48 hours of incubation at X10 magnification (scale bar; 20µm).

2.6.2 JAR spheroid and RL95-2 monolayer preparations

In order to establish an adhesion assay, appropriate preparations of JAR spheroids and RL95-2 monolayers representing blastocysts and the endometrial epithelium, respectively, were made. Initial optimisation of the spheroid and RL95-2 monolayer interaction in cell culture was carried out. Preparations of multi-cellular spheroids appeared irregular in shape with rough edges, but were uniform in size (Figure 2-3A), a feature that was consistent throughout the experiments. Test cultures (n=21) of RL95-2 monolayers on coverslips confirmed that the monolayer stayed attached to the glass coverslips and remained intact during culture (Figure 2-3B).

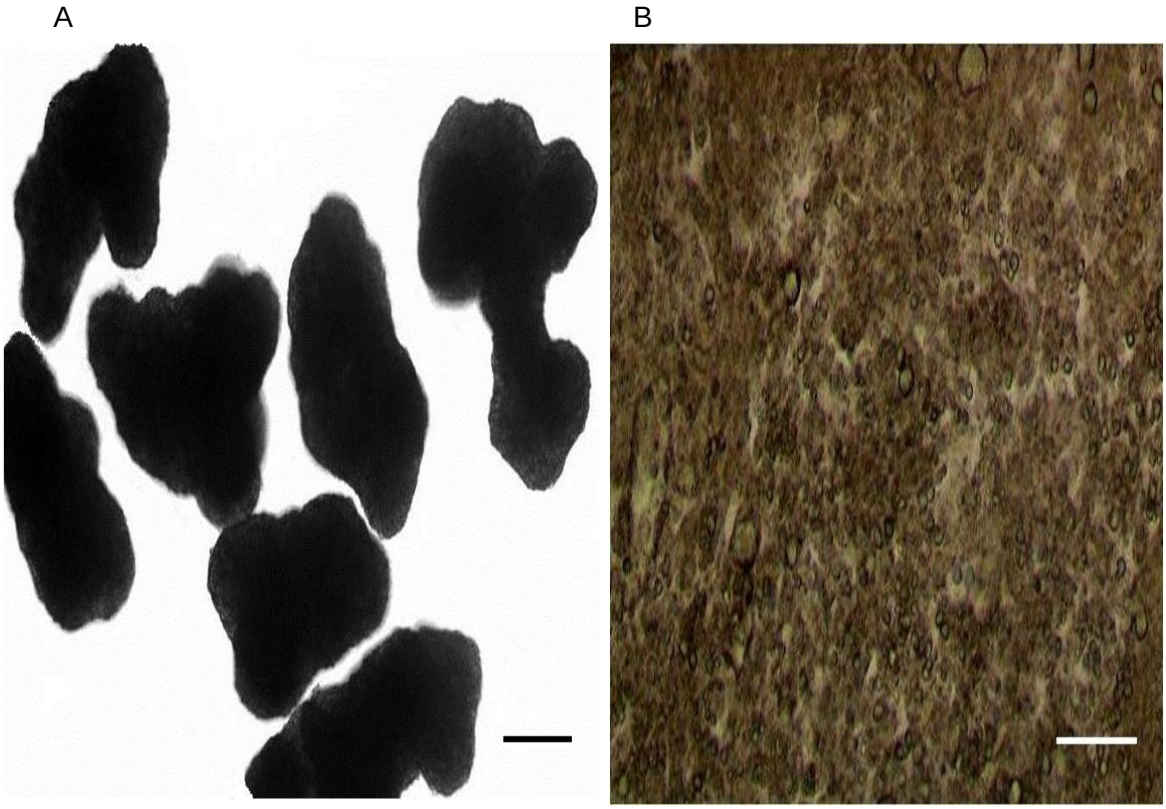


Figure 2-3: **JAR spheroids and a RL95-2 monolayer preparations used in the adhesion assay**. A) JAR spheroids at X10 magnification (20µm) (under phase contrast microscope). B) RL95-2 monolayer on glass coverslips after 96 hours (scale bar; 20µm).

It was important to ensure that the number of spheroids made in each preparation was reproducible. Spheroid preparations (50µL) were viewed under a phase contrast light microscope and the number of spheroids was quantitated. The number of spheroids achieved in six independent preparations is shown in Table 2-1. A mean of 20.5 (SD, 1.3) spheroids per 50µL preparation was recovered. The coefficient of variation (CV) for number of spheroids per preparation was 6.3%.

Table 2-1: **Reproducibility of spheroid number per preparation**.

Preparation	№ of spheroids	Mean per prep
A1	22	21.0
A2	20	
B1	18	18.5
B2	19	
C1	19	19.5
C2	20	
D1	23	22.0
D2	21	
E1	22	20.5
E2	19	
F1	21	21.5
F2	22	
Mean (SD)		20.5 (1.3)

2.6.3 Adhesion assay-time dependence

In order to determine the time dependence of JAR spheroid adhesion to the RL95-2 monolayer, the number of spheroids attached over 48 hours was assessed. Initial experiments showed that spheroids appear to detach after 24 hours (Figure 2-4A). In a shorter time course (Figure 2-4B), it was confirmed that maximal adhesion of around 98% was achieved by 24 hours. Thus, a 24 hour time point was selected for the adhesion assay.

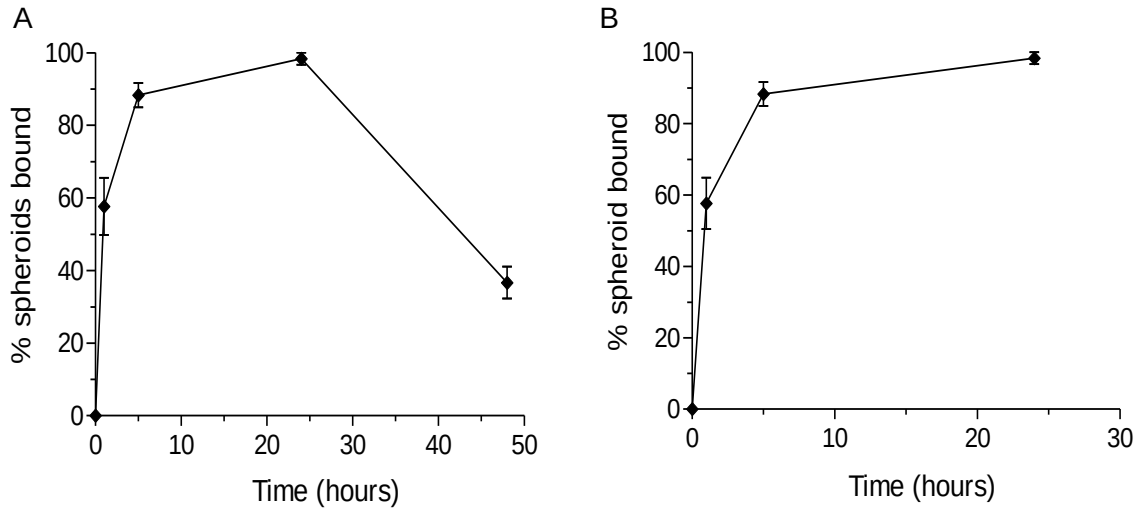


Figure 2-4: **Time course of adhesion of JAR spheroids onto RL95-2 monolayers**. A) 48 hour time course and B) 24 hour time course. n=3 independent experiments are presented. Mean (SD) shown.

2.6.4 Specificity of JAR spheroid adhesion to the RL95-2 monolayer

In order to determine whether adhesion between JAR spheroids and the RL95-2 monolayer was specific, solubilised protein extracts were prepared from each cell line, and their ability to block adhesion between JAR spheroid and RL95-2 monolayer was examined. The inhibition of adhesion between JAR spheroids and RL95-2 monolayers by these cell extracts was assessed in a time- and concentration-dependent fashion. In addition, the blocking activity of fresh and frozen preparations of solubilised total cell extracts was assessed in order to confirm whether frozen preparations might be conveniently used as a positive control in each of the binding assays. The experimental design for testing the inhibitory ability of solubilised JAR or RL95-2 cell extracts is shown in Figure 2-5.

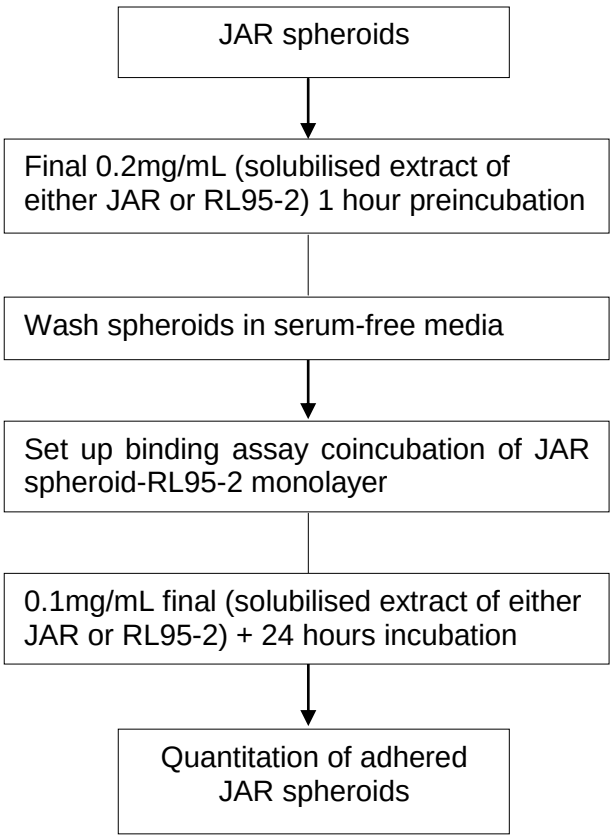


Figure 2-5: **Experimental design for testing inhibitory effects of JAR and RL95-2 cell solubilised extracts on the adhesion assay**.

Both fresh and freeze-thawed solubilised extracts at (0.2mg/mL) from either JAR or RL95-2 cells were exposed to JAR spheroids for a 1 hour preincubation period prior to initiating a binding assay. Incubation was then supplemented with further fresh and frozen-thawed solubilised extracts from either JAR or RL95-2 cells at 0.1mg/mL concentration immediately prior to the binding assay, according to previously established protocol (Hans-Peter et al. 2000). The binding assay incubation was for up to 48 hours (Figure 2-6). RL95-2 solubilised extracts fully inhibited the interaction between JAR spheroids and RL95-2 monolayers by 48 hours (Figure 2-6). A JAR cell solubilised extract showed maximal inhibition on spheroid binding by 24 hours. Therefore, it was selected as a positive control for the 24 hour adhesion assay. There was no difference between using frozen or fresh JAR solubilised extracts (Figure 2-6) so, for convenience; the frozen extract was used for adhesion assays as a positive control.

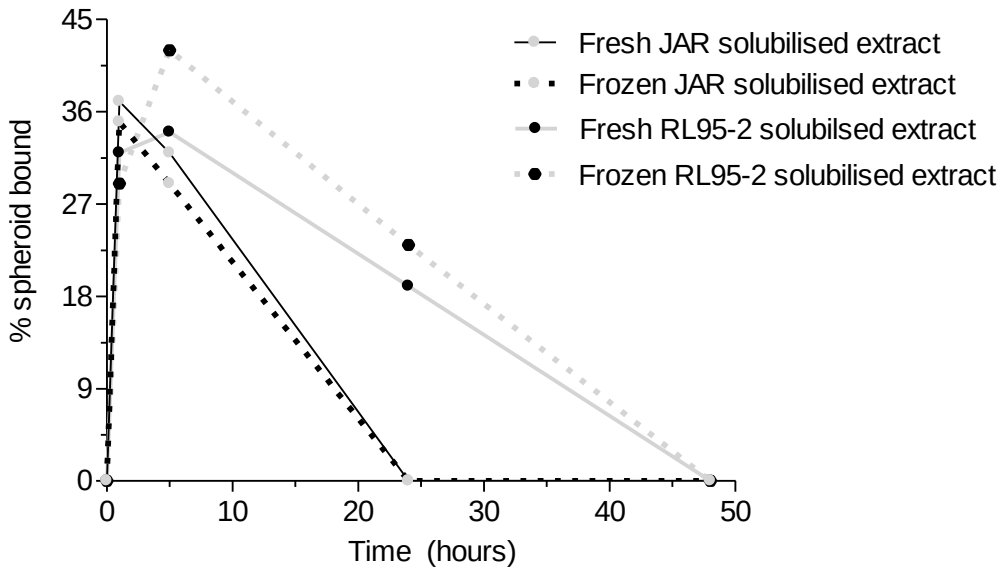
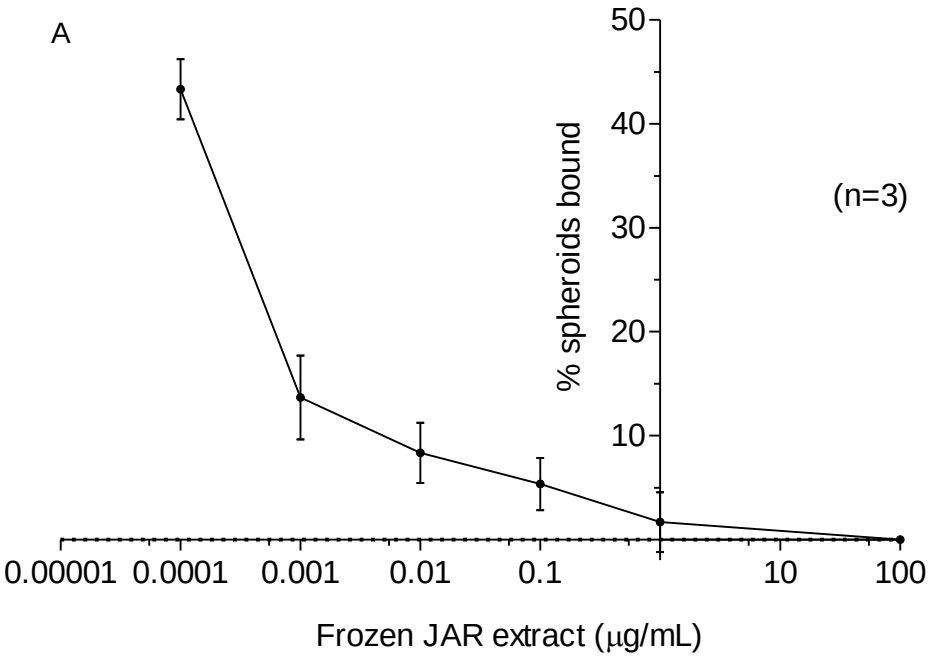


Figure 2-6: **Time course of inhibition of spheroid adhesion up to 48 hours by fresh and frozen JAR or RL95-2 cell solubilised extracts (n=1)**.

A concentration range of frozen JAR cell solublised extracts was used to identify the optimal concentration of extract for inhibition (Figure 2-7A). Maximal inhibition of adhesion was reached after preincubating the JAR spheroid(s) for 1 hour at 0.2mg/mL with 0.1mg/mL in the binding assay. Replicate experiments (n=5) were carried out to assess the reproducibility of inhibition by the positive control in the binding assay. JAR spheroid-RL95-2 monolayer binding was 99.2% of mean (SD, 2.9) (Figure 2-7B). In the presence of fresh JAR solubilised extracts, binding was 1.0% (2.2) and 0% (0) in the presence of frozen JAR extract. In the presence of fresh RL95-2 solubilised extracts, binding was 17.2% (6.8); and 21% (12.9) for frozen (Figure 2-7B).



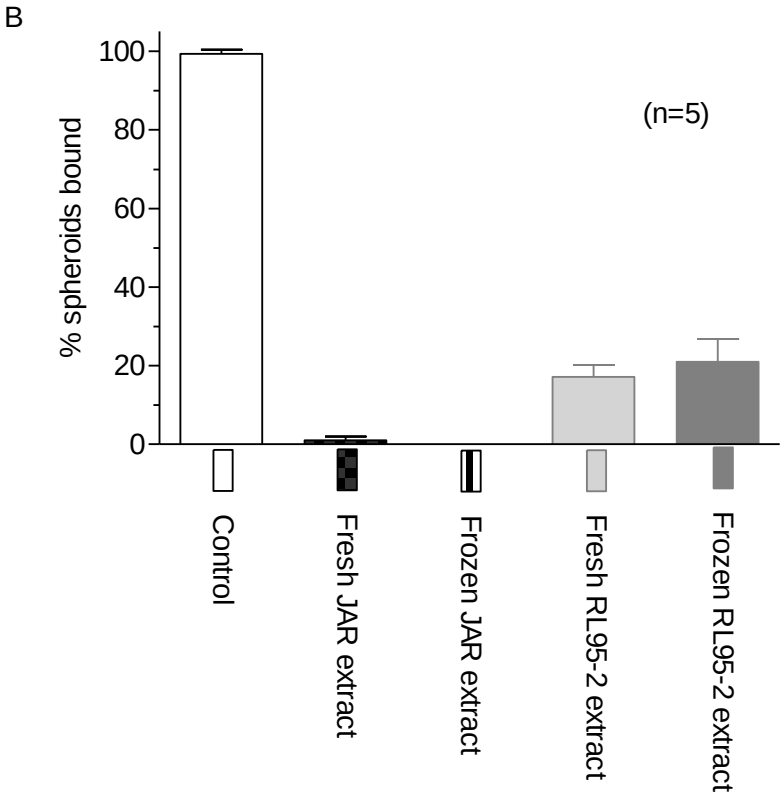


Figure 2-7: **Specificity of JAR-RL95-2 binding in the *in vitro* adhesion assay**. A) A concentration curve for frozen JAR solubilised extract 0.0002, 0.002, 0.02, 0.2, 2.0 and 200.0μg/mL for 1 hour preincubation, and 0.0001, 0.001, 0.01, 0.1, 1.0 and 100μg/mL respectively, for incubation in a 24 hour adhesion assay (n=3). B) Replicate adhesion assay experiments (n=5) carried out for assessment of maximal adhesion inhibition using 0.2mg/mL cell extract in a 1 hour preincubation and then 0.1mg/mL cell extract for a 24 hour incubation. The control was an assay carried out in the absence of cell solubilised extracts. Mean (SD) shown.

2.6.5 Insulin effects on JAR-RL952 monolayer adhesion assay

To examine the potential effects of insulin on the binding of JAR spheroids to RL95-2 monolayers, adhesion assays were carried out in the absence and presence of differing concentrations of added insulin (0-0.24nM) for 24 hours. Spheroid binding in the absence of insulin was defined as 100%. The effects of different concentrations of insulin on spheroid binding are shown in Figure 2-8A. It is interesting to note that in the absence of insulin there was 100% binding (by definition); however, low insulin concentrations (0.03nM) showed high inhibition, with only 26% binding, while high insulin levels (0.24nM) had low inhibition, with 91% binding (Figure 2-8A). These data do not support a concentration dependence of insulin inhibition of binding. This could be explained by experimental artefact, or potentially by activation of independent signalling pathways by low and high insulin loading to opposite effects on cell adhesion.

The assessment of the JAR spheroid-RL95-2 monolayer binding structural appearance (which includes the outward appearance, in a particular shape, size and pattern) was examined under a microscope. In the absence of insulin (Figure 2-8B), JAR spheroids showed no observable change, whereas at 0.24nM the JAR spheroids that bound to the RL95-2 monolayer appeared to grow bigger (Figure 2-8C). The increased size of these JAR spheroids may be due to increased division of cells constituted in the JAR spheroid aggregates.

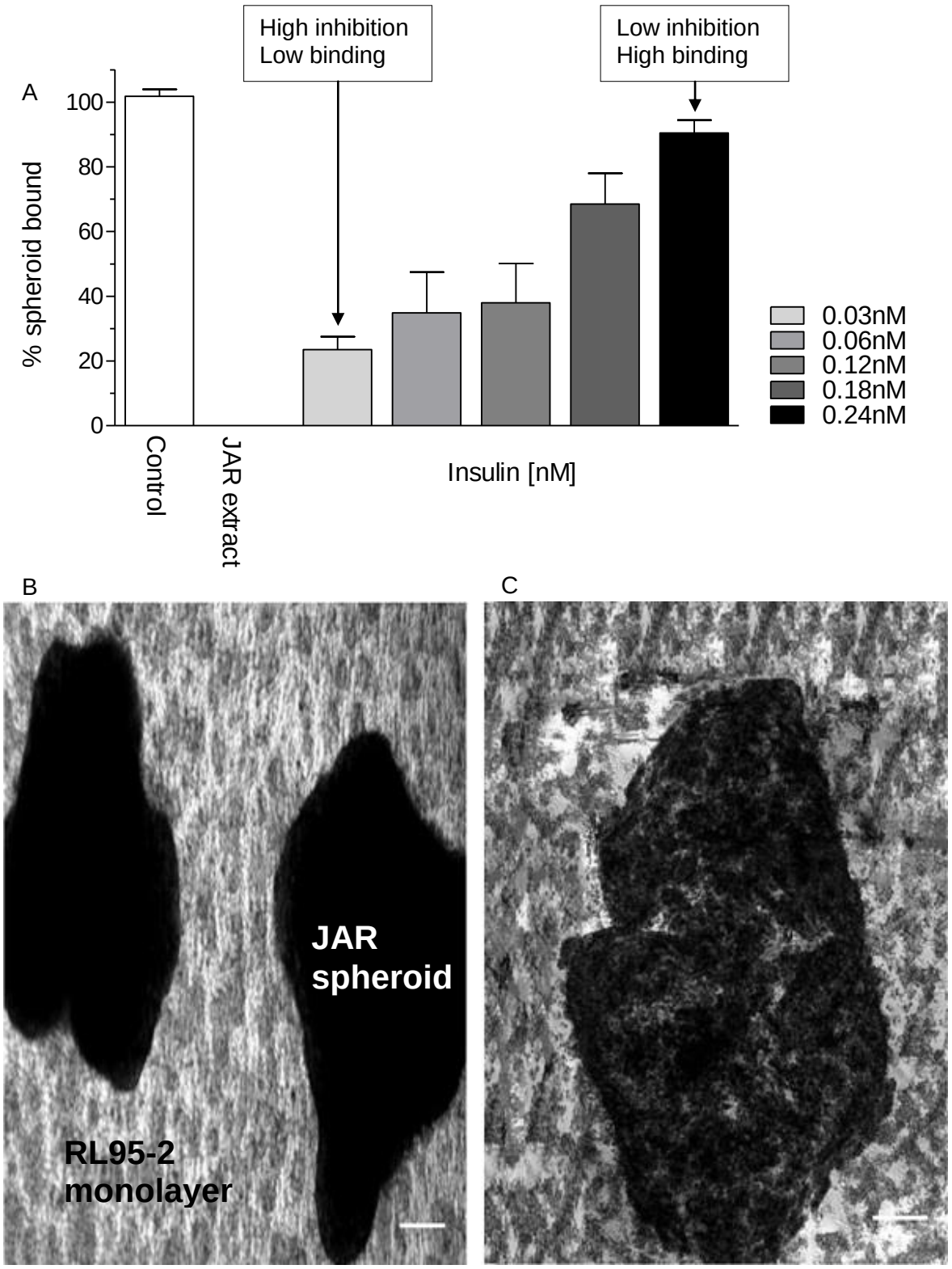


Figure 2-8: **Insulin effect on JAR spheroid adhesion to RL95-2 monolaye**r. A) A concentration range of insulin varying from 0-0.24nM was added to the binding assay. n=9 independent experiments presented. A low dose (0.03nM) showed high inhibition (low binding), while a high dose (0.24nM) revealed low inhibition (high binding). B) Absence of insulin doses showed no morphological changes of the size of JAR spheroids adhering to the RL95-2 monolayer, while C) the presence of insulin 0.24nM shows the spheroid to increase in size at X4 magnification (scale bar; 50µM). Mean (SE) shown.

2.6.6 Effects of low and high doses of insulin on JAR spheroid-RL95-2 monolayer binding

Due to the contrasting effects of low (0.03nM) and high (0.24nM) added insulin doses (Section 2.6.5) on JAR spheroid-RL95-2 monolayer binding, both insulin concentrations were selected for further studies. Adhesion assays were carried out where JAR spheroids only, RL95-2 monolayers only or both JAR spheroids and RL95-2 monolayers were exposed to low (0.03nM) or high (0.24nM) concentrations of insulin during the preincubation period but not during the binding assay, as illustrated below (Figure 2-9).

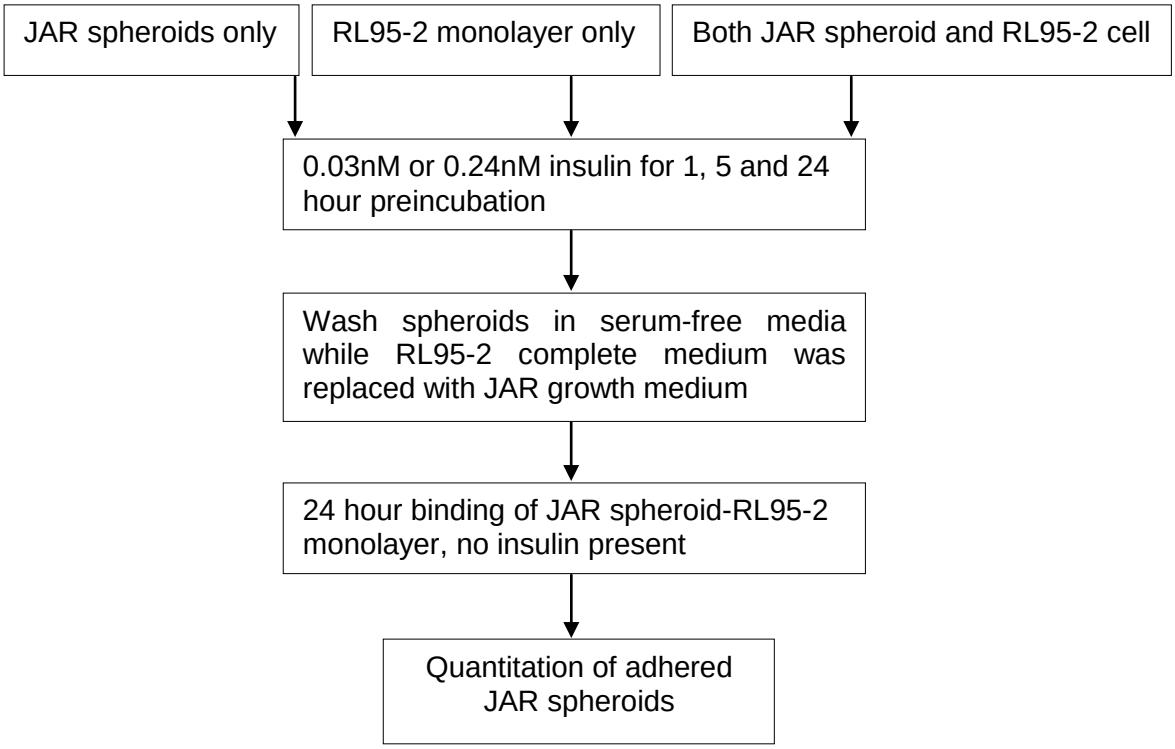


Figure 2-9: **Design for testing the effect of preincubation with low (0.03nM) and high (0.24nM) concentrations of insulin on JAR spheroid-RL95-2 monolayer binding**.

Consistent with previous experiments (Section 2.6.5), additional experiments where 0nM, 0.03nM (low) and 0.24nM (high) added insulin doses were present during a 24 hour binding assay with no insulin preincubation showed JAR spheroids binding to RL95-2 monolayers of 98%, 36% and 88% of spheroids adhered respectively (Figures 2-10A, B and C).

In Figure 2-10A, preincubating JAR spheroids only with 0.03nM insulin for 1 hour prior to binding assay resulted in 33% of the spheroids bound. At the fifth hour, 47% binding was observed after JAR spheroids were preincubated with 0.03nM insulin. Preincubating JAR spheroids with 0.03nM insulin resulted in 2% of spheroids bound after 24 hours prior to binding assay. However, the preincubation of JAR spheroids with 0.24nM after 1 hour resulted in 78% binding. The binding reached 46% after JAR spheroids were preincubated for 5 hours with 0.24nM insulin. After 24 hours preincubation with 0.24nM insulin, JAR spheroid binding reached 1%.

In Figure 2-10B, there was 38% adhesion after preincubating RL95-2 monolayer only with 0.03nM for 1 hour. Also, binding of 44% was reached on treatment of RL95-2 monolayer with 0.03nM insulin after 5 hours and 34% binding was reached after 24 hours preincubation of RL95-2 monolayer. However, the preincubation of RL95-2 monolayer with 0.24nM after 1 hour resulted in 62% binding. Preincubation of RL95-2 monolayer with 0.24nM insulin resulted in 61% of spheroids bound after 5 hours. By 24 hours preincubation of RL95-2 monolayer with 0.24nM insulin, 29% spheroid binding was noted.

In Figure 2-10C, after 1 hour, 40% binding was observed when both JAR spheroids and RL95-2 monolayers were preincubated with 0.03nM insulin separately before the binding assay. A binding rate of 26% was reached when both JAR spheroids and RL95-2 monolayers were preincubated with 0.03nM insulin after 5 hours. At 24 hours, 9% binding occurred in the presence of 0.03nM insulin preincubation of both JAR spheroids and RL95-2 monolayers. On the other hand the preincubation of both JAR spheroids and the RL95-2 monolayers with 0.24nM after 1 hour resulted in 71% binding. When both JAR spheroids and RL95-2 monolayers were exposed to 0.24nM insulin preincubation, by the fifth hour 50% spheroids binding was reached. In the presence of 0.24nM insulin preincubation of both JAR spheroids and the RL95-5 monolayer, there was 9% binding reported after 24 hours preincubation.

The observation of inhibition of binding at both doses of added insulin with long-term incubation, especially on JAR spheroid only, suggests that the transcriptional effect as protein synthesis or genomic response of insulin supposedly take a long time.

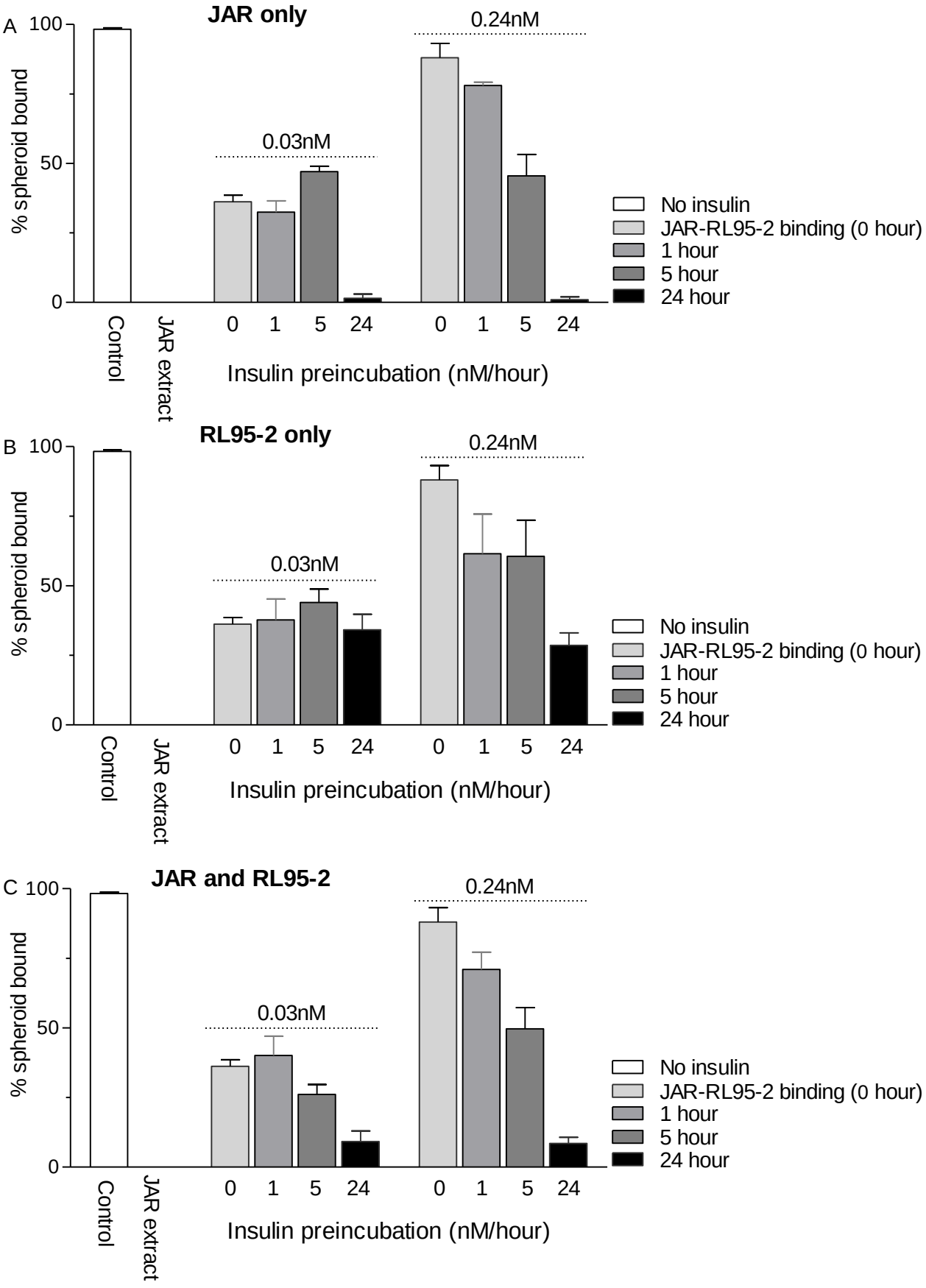


Figure 2-10: **Effect of preincubation in low and high doses of insulin**. A) JAR spheroids only, B) RL95-2 monolayers only or C) both together in the presence of 0.03nM and 0.24nM. The percentage of adhesion was assessed after 0 (no preincubation), 1, 5, and 24 hours preincubation in insulin, before a 24 hour JAR spheroid-RL95-2 monolayer binding. The absence of insulin (control) and JAR solubilised extract (negative control) are presented (in A, B and C). n=5 independent (duplicated) experiments presented. Mean (SE) shown.

2.6.7 Identifying molecules involved in JAR spheroid-RL95-2 monolayer binding

Potential adhesion molecules that may be involved in the binding of JAR spheroids and RL95-2 monolayers in the culture model were investigated. Several adhesion molecules previously found to be involved in blastocyst adhesion onto the uterine wall were assessed. First, it was tested whether JAR and RL95-2 cells expressed the adhesion molecules trophinin, CD44 and laminin α1. Expression of trophinin was not detected in JAR or RL95-2 cells (Figure 2-11A). CD44 mRNA expression was not detectable in JAR cells, but was present in RL95-2 cells, with a predicted product fragment of 262-bp (Figure 2-11B). Laminin α1 was expressed in JAR cells but not in RL95-2 cells, with a predicted fragment of 265-bp, as shown in Figure 2-11C. In these assays β-Actin was used as a loading control and kidney mRNA served as a positive control.

Laminin α1 was selected for further investigation to determine if it might be involved in the binding between JAR spheroids and the RL95-2 monolayer in the experimental model.

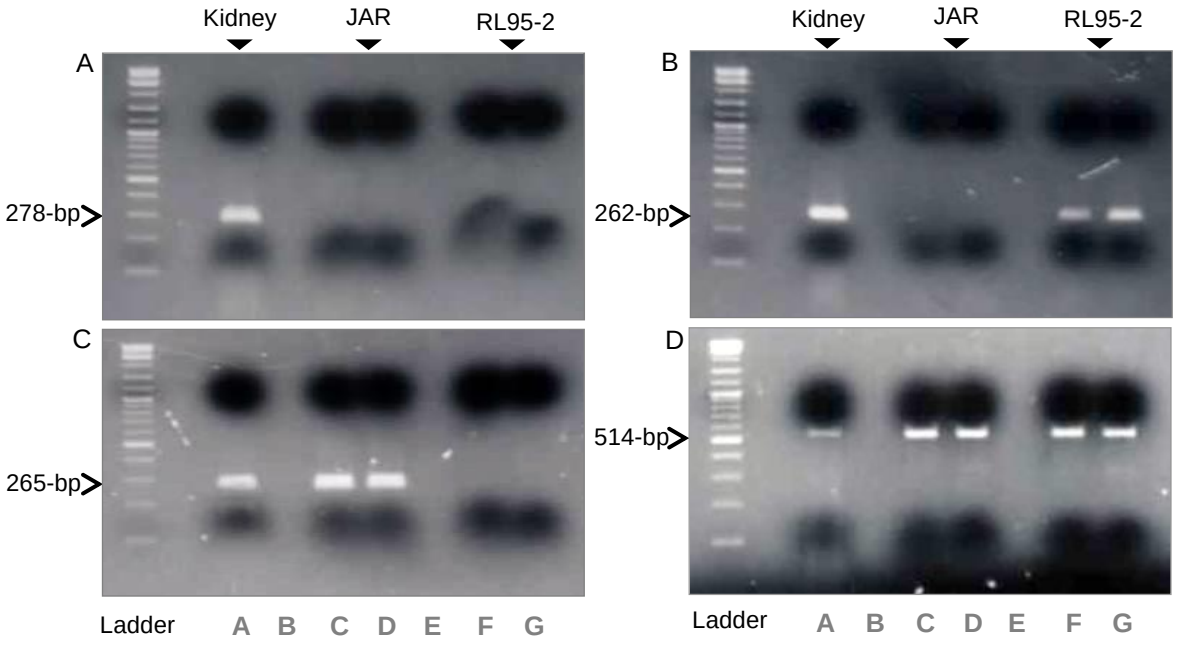


Figure 2-11: **Reverse transcription PCR assay of adhesion molecule mRNA expression**. A) Trophinin (variants 2, 3 and 6) isoforms, B) CD44 (variants 1, 2, 3, 4 and 5) isoforms and C) laminin α1 products were detected by ethidium bromide staining in an agarose gel. Messenger RNA loading was assessed using D) β-Actin as a control gene. Wells contained ladder (DNA ladder); A, kidney (positive control); B, negative control; C-D, JAR cells RNA; E, negative control and RL95-2 cells RNA; F-G.

2.6.8 Laminin α1 interacting protein and insulin receptor messenger RNA expression in JAR and RL95-2 cells

Since laminin α1 is proposed to play a role in mediating adhesion during blastocyst implantation, it was hypothesised that laminin α1 must interact with its interacting protein in order for adhesion to take place; thus, such protein would be expressed on the uterine epithelium during the receptive period. In the experimental adhesion model used in this chapter of the thesis, the presence of such molecules in JAR and RL95-2 cells was tested for. FBLN-1 was expressed in both JAR (lanes C-D) and RL95-2 cells (lanes F-G), with a predicted product fragment of 267-bp (Figure 2-12A). In contrast, FBLN-2 was only expressed in JAR cells (lanes C-D), with a predicted product of 288-bp, as seen in Figure 2-12B. Expression of an insulin receptor was also determined as insulin regulates cell adhesion between JAR and RL95-2 cells as it is proposed that insulin, perhaps regulate cell adhesion molecules. The semi-quantitative PCR studies indicated that insulin receptor mRNA was detected in both JAR (lanes C-D) and RL95-2 cells (lanes F-G), with a predicted product of 294-bp (Figure 2-12C). The mRNA gel loading was verified for JAR and RL95-2 cells using β-Actin primers producing a 514-bp product (Figure 2-12D).

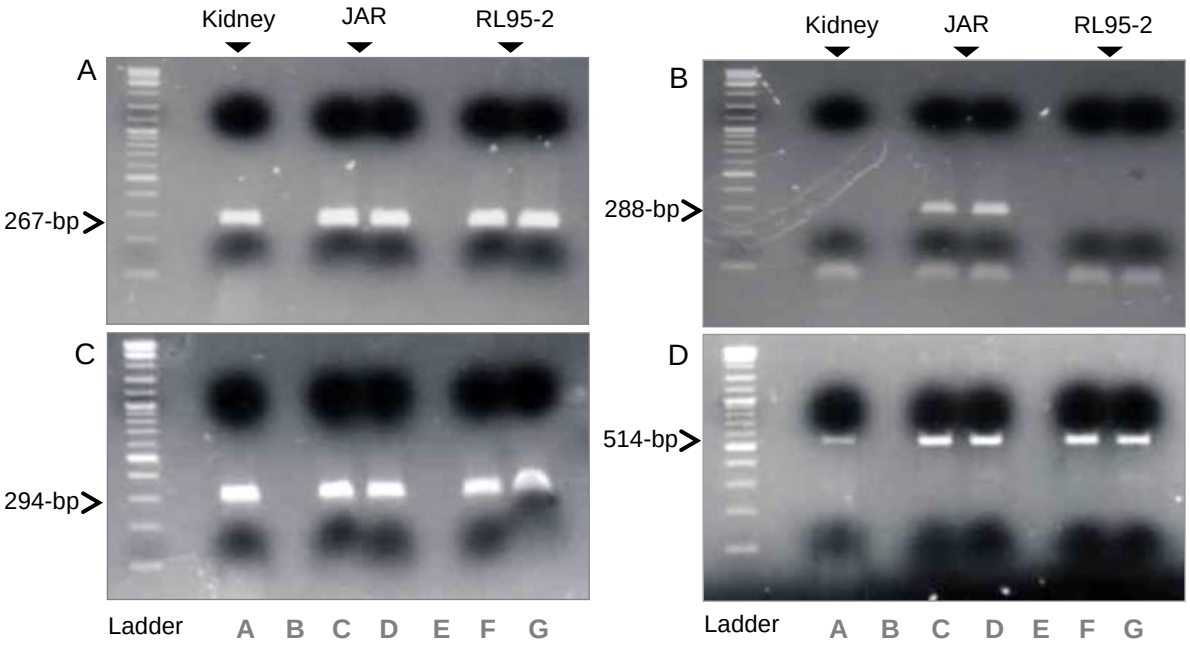


Figure 2-12: **Laminin α1 interacting molecule FBLN-1 & -2 and insulin receptor mRNA expressions**. RT-PCR product detected by ethidium bromide staining in agarose gel A) FBLN-1, B) FBLN-2 and C) insulin receptor. D) β-Actin was used as a loading control. Lane A, kidney (positive control); B, negative control; C-D, JAR cells; E, negative control, and F-G, RL95-2 cells.

2.6.9 Detection of laminin α1 protein expression on JAR cells

In order to validate protein expression of laminin α1 in the JAR cells, SDS-PAGE and Western blotting were carried out (Figure 2-13A). Laminin α1 protein expression was detected in JAR protein lysates of different concentrations (70-130μg/μL). Western blot analysis demonstrated the expression of laminin α1 detectable as a band of 400kDa. This confirms previous reports demonstrating laminin α1 expression in JAR cells (Tiger et al. 1997).

Immunohistochemistry (IHC) was performed to localise laminin α1 protein expression in JAR cells. Laminin α1 was localised in the intima but absent in the fibrinoid (Fib) areas of breast tumour tissue, which was the positive control tissue (Figure 2-13B). No detectable staining was observed in the negative control, as shown in Figure 2-13C. Intense laminin α1 staining was seen within the extracellular matrix of the basement membrane of JAR cells (Figure 2-13D). There was no laminin α1 expression in the negative control (Figure 2-13E).

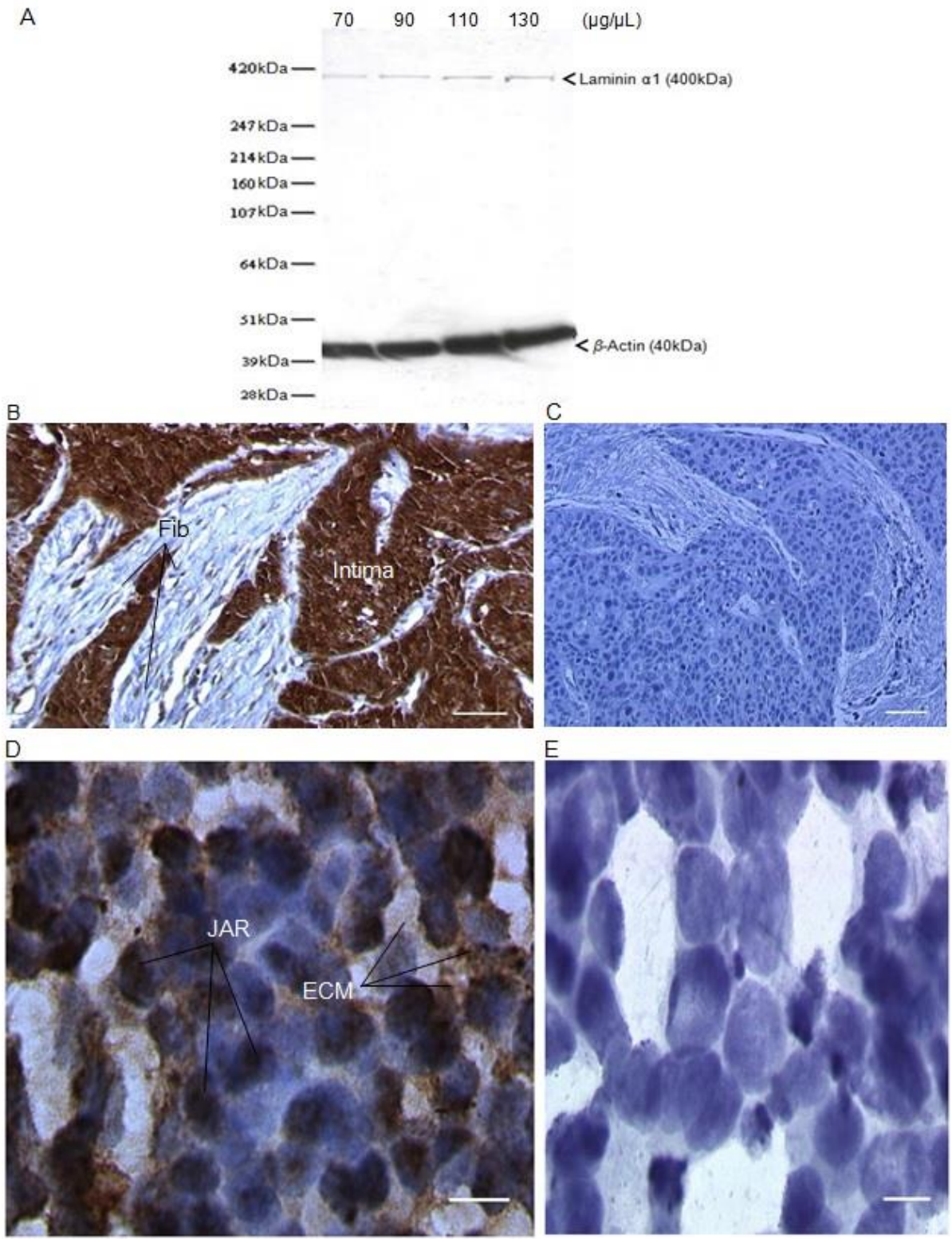


Figure 2-13: **Laminin α1 protein detections in JAR cells**. A) JAR protein lysates were loaded at 70, 90, 110 and 130µg/µL in each well, and laminin α1 (400kDa) was detected using (1:600 anti-human laminin α1 antibody goat polyclonal; AF4187, R&D System). β-Actin (40kDa) was used as a loading control. B) Immunohistochemistry indicates laminin α1 staining in intima with no staining in the fibrinoid (Fib) in the positive control tissue (breast tumour) (scale bar 10µm) with 20X magnification and C) negative control (10µm). D) Laminin α1 staining of JAR cells within the extracellular matrix (ECM) (2µm). E) Negative control (2µm) with 100X magnification.

2.6.10 Blocking laminin α1 on JAR cells using a specific anti-laminin α1 antibody

Having detected laminin α1 protein expression in JAR cells, further studies were undertaken to determine whether the anti-laminin α1 antibody could block binding of JAR spheroids to RL95-2 monolayers. The laminin α1 antibody concentration titre was determined after assessing its effect on JAR spheroid-RL95-2 monolayer binding after 24 hours. An irrelevant antibody, anti-GRP-78 (N-20) against GRP-78, a highly conserved protein, was used as a positive control. A series of laminin α1 antibody concentrations were incubated with JAR cells in a preincubation to allow the antibody to block any cell surface laminin α1 on JAR spheroids. JAR spheroids were preincubated with anti-laminin α1 (0.1, 0.2 and 0.4µg/mL) for 1 hour, and then a 24 hour JAR spheroid-RL95-2 monolayer binding assay was carried out (Figure 2-14A). In the absence of anti-laminin α1 or anti-GRP-78, there was 98.7% binding, while the addition of JAR solubilised extract showed 0% binding, as expected. After inclusion of 0.1, 0.2 and 0.4µg/mL anti-laminin α1 in the JAR spheroid preincubation, there was 18%, 34% and 43% binding, respectively, in the JAR spheroid-RL95-2 monolayer binding assay. In contrast, JAR spheroid binding after 1 hour preincubation with 0.1, 0.2 and 0.4µg/mL of irrelevant antibody anti-GRP-78, followed by a 24 hour JAR spheroid-RL95-2 monolayer binding assay, had a consistently higher percentage adhesion, at 63%, 62% and 56%, respectively. These data imply that anti-GRP-78 failed to provide complete inhibition of JAR spheroid adhesion.

When anti-laminin α1 (0.2, 1 and 4µg/mL) was included in the JAR spheroid-RL95-2 binding assay, spheroid binding was 27%, 46% and 55%, respectively (Figure 2-14B). Addition of an irrelevant antibody, anti-GRP-78, to the binding, using 0.2, 1 and 4µg/mL, showed binding of 63%, 61% and 63% respectively (Figure 2-14B). In the absence of antibodies, adhesion reached 98.7%, while in the presence of frozen JAR solubilised extract there was 0% binding of spheroids (Figure 2-14B). However, inhibition of the JAR spheroid adhesion induced by anti-laminin α1 and anti-GRP-78 lacked concentration-dependent response and specificity.

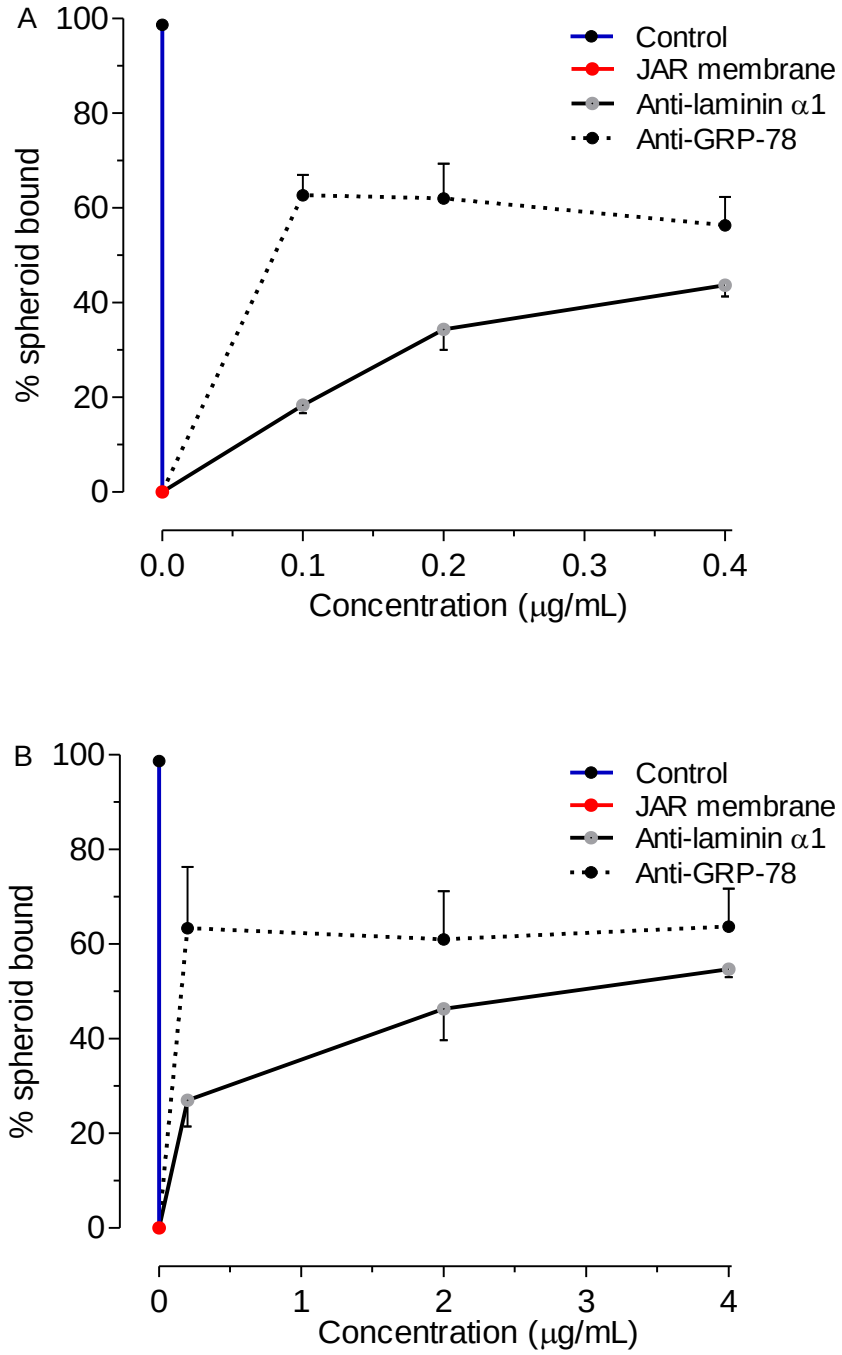


Figure 2-14: **Anti-laminin α1 effect on JAR spheroid-RL95-2 monolayer binding assay**. A) JAR spheroid preincubation for 1 hour with 0.1, 0.2 and 0.4μg/mL anti-laminin α1 and anti-GRP-78, followed by 24 hours JAR spheroid-RL95-2 monolayer binding assay. B) JAR spheroid-RL95-2 monolayer coincubation in a 24 hour binding with 0.2, 1 and 4μg/mL anti-laminin α1 or anti-GRP. All assays were carried out in duplicates with n=3 independent experiments; anti-laminin α1, represents anti-human laminin α1 antibody goat polyclonal; AF4187, R&D System. Mean (SE) shown.

2.7 Discussion

In many women, blastocyst/embryo-uterine adhesion and implantation does not happen, and this is a major cause of infertility. The aim of the present chapter is to explore the effect of insulin and adhesion molecules in the blastocyst-uterine wall interaction, using spheroids adhering to the RL95-2 monolayer as a cell culture model of implantation. In this model, trophoblast JAR spheroids binding to human uterine RL95-2 monolayers reached 98% by 24 hours. This is consistent with John et al. (John et al. 1993) while Hans-Peter et al. reported approximately 100% adhesion by 5 hours (Hans-Peter et al. 2000). This chapter has explored the effect of insulin in the cell adhesion model and found that added low insulin concentrations (0.03nM) inhibited spheroid binding by 74%, whereas high insulin (0.24nM) only inhibited it by 9%. The lack of a standard dose-response for insulin and anti-laminin α1 was difficult to interpret and is inconclusive at this stage of experimentation. However, these results suggest that insulin is important as a metabolic parameter, and laminin α1 may be an interesting candidate for an adhesion molecule, in the study of embryo adhesion during *in vitro* implantation.

The cell model of implantation has advantages and disadvantages. Human blastocyst/embryo implantation onto the maternal endometrial epithelium cannot be studied *in vivo* and is difficult to study *ex vivo*. Access to donated embryos for research purposes is limited to a few laboratories across the globe. The uniqueness of the human implantation process means that no other mammal provides an appropriate animal model (Bischof and Campana 2000). Ethical concerns regarding experimentation with primary human tissues during this period of life necessitate using *in vitro* models employing trophoblast and uterine cell lines. In this thesis the model used represents an important step of implantation (i.e. adhesion) and uses JAR spheroid-RL95-2 monolayer binding to provide a means to examine events during implantation in detail. It is difficult to assess how good this is at representing *in vivo* tissue. JAR cells derived from human choriocarcinoma (trophoblastic cells) (Pattillo RA et al. 1971; Rohde and Carson 1993) and RL95-2 cells from human endometrial epithelium (adeno-squamous carcinoma) (Way et al. 1983) provided at least the closest possible alternative to *in vivo* tissue. In order to provide robust data using this model, certain control experiments were undertaken. These included checking spheroid numbers for reproducibility, use of controls, keeping cell passage numbers the same and time-dependent assessment.

Embryo implantation requires the participation of various cytokines, growth factors and hormones. The effect of insulin during this process in humans has not been studied. Insulin is a pleiotropic hormone, capable of activating various intracellular pathways that lead to a cascade of multiple responses, including DNA synthesis, mRNA turnover,

protein synthesis and degradation, and cell division. Studies have demonstrated that insulin enhances blastocyst proliferation in mouse, rat and rabbit models (Harvey and Kaye 1990; De Hertogh et al. 1991; Herrler et al. 1998). In mouse models particularly, insulin enhances embryo cleavage (Gardner and Kaye 1991) and stimulates an increase in the number of blastocyst cells due to an increase in inner cell mass cell numbers (Harvey and Kaye 1990). As stated above, this suggests that insulin may have a direct role in the regulation of preimplantation embryo development. In a clinical situation of obesity, PCOS and diabetes women, which are usually associated with higher insulin level are likely to miscarry. This is an indication of the negative effect of high insulin on the blastocyst/embryo stage at preimplantation (Cardozo et al. 2011). In the experiment in this chapter, insulin receptor expression in both JAR cells and RL95-2 cells was detected. Results may imply a potential role for insulin signalling in the interaction of JAR spheroids and the RL95-2 monolayer.

Human pregnancy is characterised by a gestational increase in insulin resistance especially after mid-gestation. A report, utilising an oral glucose tolerance test and hyperinsulinaemic-euglycaemic clamps observed insulin levels of approximately 6µU/mL (0.04nM) prior to conception, 0.03nM at 12-14 and ~0.06nM at 34-36 weeks' gestation in healthy pregnant non-obese women (Catalano et al. 1993). This increased insulin hormone secretion, leading to a gestational increase in insulin resistance, occurs in order to maintain glucose tolerance. Another group reported a cord insulin level of 0.02nM at birth of healthy control mothers (Nelson et al. 2009). In the current experiments, it appeared that insulin may not affect binding (implantation), as previously proposed; an inconsistent dose response relationship between insulin and binding made these data hard to interpret. This observation, that insulin at low dosage inhibits spheroid binding more than insulin at high dosage, was unexpected, as it seemed reasonable that the higher dose would produce a more significant response. This unexpected result raises the possibility that the data obtained is due to an experimental artefact, possibly as a result of a number of limiting factors. The problem of unavailable data for localised embryo insulin concentration at implantation led to using circulating maternal and cord plasma levels of insulin in normal pregnancy as a guide for selecting insulin concentration for the experiment. John et al. also indicate that manipulation of the coverslip introduces shear forces, and that the centrifugal force based system of removing unbound spheroids may be problematic (John et al. 1993). There is a possibility of inadvertently detaching already bound spheroids. Also, bias may have contributed a false positive result due to the irregular edges of prepared JAR spheroids. As in previous studies (John et al. 1993; Grümmer et al. 1994; Hans-Peter et al. 2000), 25mL Erlenmeyer flasks were utilised in preparing JAR spheroids on a shaker. In all these observations, JAR cells formed multi-cellular spheroids which were harvested after 72 hours. Aboussahoud et al. prepare multi-

cellular spheroids from JAR cells using tissue culture Petri dishes (Aboussahoud et al. 2010). They observe spheroid formation, similar in size to human blastocysts, occurring after 24 hours of culturing of JAR cells on a shaker. A recent report by Wang et al., used an agarose-coated Petri dishes (cultured for 5 days) to transform a single JAR cells suspension into small spheroids, which were later transferred into an Erlenmeyer flask (cultured for a minimum of 5 days on a shaker) (Wang et al. 2012). Taking all these factors into account, there is the possibility that experimental artefact may perhaps have influenced the current data, even though the absence of comparable studies makes this only a possibility.

There is a possibility that added insulin have a toxic effect and may lead to cells beginning to die during the binding assay. Cell death would cause the breakdown of cell membranes, which may result in nonspecific binding of JAR spheroids to the RL95-2 monolayer, or merging of their cell membranes. Experiments to test cell toxicity after treatment with a range of insulin concentrations at both ends may help clarify the data. A number of parameters, including vital staining, cell growth, cytosolic enzyme release and cloning efficiency, may each be used as an end-point to measure toxicity. In the context of this experiment, viable JAR cell staining (using trypan blue) may have helped determine whether JAR cells die after exposure to low or high doses of insulin in culture well plates. In the absence of such staining, it was impossible to eliminate the factor of the death of JAR cells from the result. Therefore, this unexpected result (of increased binding with increased insulin) requires further elucidation. Rhee et al. find that when human-derived neuron stem cells are exposed to a high dose (4.3µM or 4300nM) compared to low dose (0.22µM or 220nM) of insulin, the result is severe cell death (Rhee et al. 2013). Nevertheless, both concentrations are outside the range of doses used in this present experiment. In spite of several factors that have perhaps contributed to the unexpected data, observations of both low and high insulin doses preincubation after 24 hours, before the 24 hour binding assay (Figure 2-10A), suggest a time-dependent response. Thus, it appears that inhibition of binding at both concentrations may occur with long incubation. It may be that the insulin was removed, i.e washed away too early from the preincubating system, and might require longer incubation.

An alternative explanation for this unusual data is that, in this system, insulin has different biological effects at different concentrations. At low doses, activation of the insulin receptor may be sufficient to trigger a definable pathway that leads to binding inhibition. Higher levels may involve activation of other pathways which counteract the initial inhibition and on balance lead to net relief of inhibition or activating an independent pathway that has the opposite effect. Consequently, low concentrations of insulin led to

poor adhesion of the spheroids, whereas high concentrations of insulin resulted in no or least inhibition (i.e. due to high spheroids binding).

The insulin receptor consists of two subunits: the α- and β-subunit. The signals are transferred after binding of insulin to the α-subunit, inducing a conformational change in the receptor to allow the β-subunit to become phosphorylated by adenosine triphosphate (ATP) binding (Hubbard et al. 1994). The phosphorylated β-subunit of the insulin receptor enhances autophosphorylation of a cytoplasmic protein tyrosine kinase (Murakami and Rosen 1991). After tyrosine phosphorylation, many downstream proteins, such as insulin receptor substrates 1-6 (IRS-1 to 6) (Hotamisligil 2006; Wang et al. 2009), are phosphorylated on the tyrosine residue. Puscheck et al. found that IRS-1 mRNA and protein were upregulated by insulin: in mouse peri-implantation embryos; in embryonic cell lines; and in cultured blastocysts (Puscheck et al. 1998). Phosphorylated IRS protein binds Src homology-2 domain (SH2) protein (Pronk et al. 1993). SH2 regulates enzymes such as p85 regulatory subunits including phosphatidylinositide 3-kinase (PI3-K) (Wilcox 2005). PI3-K controls gene and protein expression of enzymes involved in lipid and glycogen synthesis, glucose transport and cell division. Also, PI3-K activation leads to protein kinase B (Akt) phosphorylation, which regulates other molecules – particularly those that have proapoptotic and antiproliferative activities. Similar to the PI3-K pathway, the signalling of insulin activates the mitogen-activated protein kinase (MAPK), also called the extracellular signal-regulated kinase (ERK), pathway involved in intracellular insulin signal transduction (Kyriakis and Avruch 2001; White 2013).

The data obtained from this experiment is that low insulin levels (0.03nM) had a higher inhibitory effect on spheroid binding than high insulin levels (0.24nM), and that this difference could be explained by differences in the insulin pathway activation. Lathi et al. observe, in human decidualised endometrial stromal cells studies, that low insulin levels (0.1ng/mL or 0.02nM) cause activation of the PI3-K pathway and high insulin levels (1000ng/mL or 172nM) affect the MAPK pathway (Lathi et al. 2005), although 172nM levels were more extreme than those in this experiment. In the study of Lathi et al., the activation of the PI3-K and the MAPK pathways was assessed by the detection of phosphorylated Akt and ERK using Western blotting. Inhibitor studies of IGFBP-1 mRNA and protein expression revealed that at 0.02nM insulin acts through the PI3-K pathway, while at 172nM levels it activates the MAPK pathway in the inhibition of IGFBP-1 (Lathi et al. 2005). Lathi et al. find human endometrium to be a target for insulin activity (0.01-0.02nM) in regulation of IGFBP-1. These reports led to stipulation in this current experimental study that a low dose (0.03nM) of insulin may perhaps activate the PI3-K pathway, whereas a high dose (0.24nM) takes place via the MAPK pathway. The representative pathways suggested to be influenced by insulin doses, as proposed (Myers

et al. 1994; White 2003; Knight et al. 2006; White 2013) and recommended for future studies, is shown in Figure 2-15.

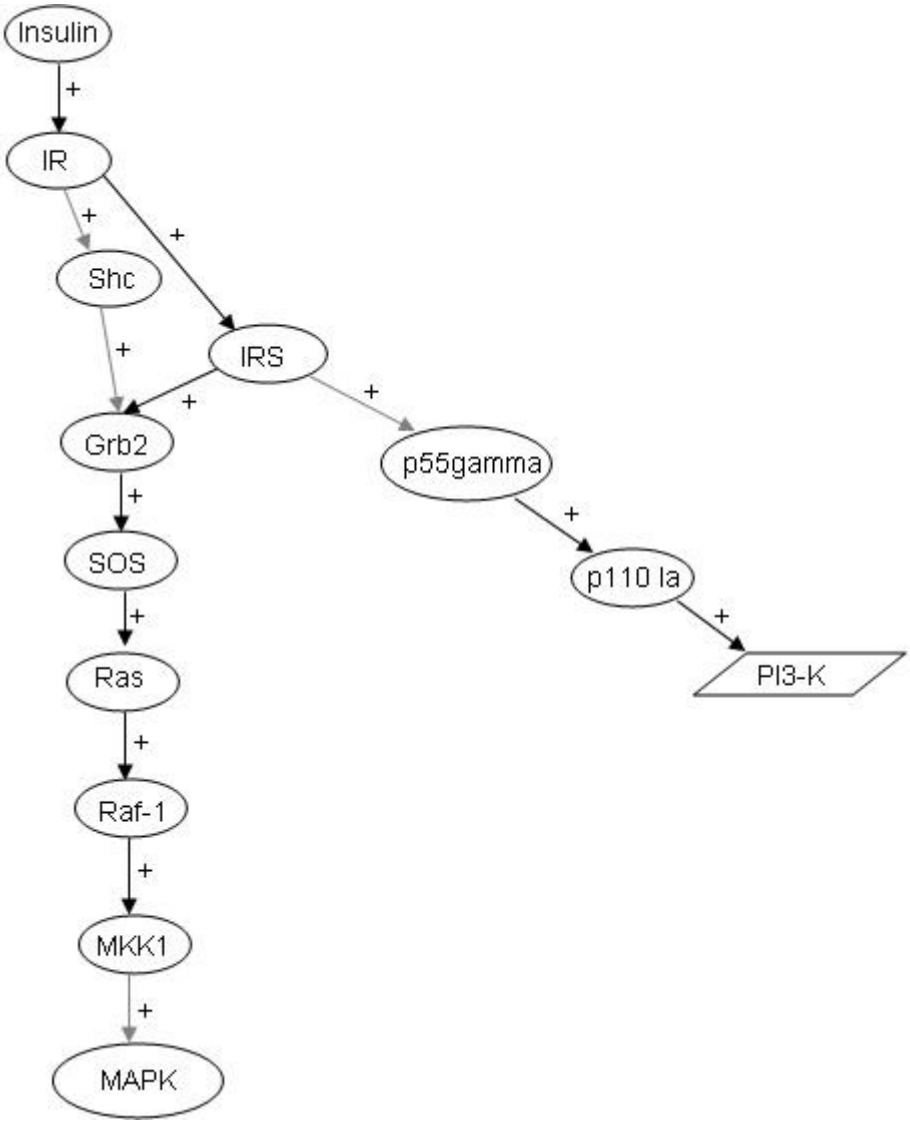


Figure 2-15: **Insulin response via the PI3-K and MAPK pathways**. Modified from White (White 2013).

Long-term transcriptional regulation effect is another potential effect of insulin in the *in vitro* model of implantation. The JAR spheroids exposed to high (0.24nM) levels of insulin appeared to approximately double in size (although this was not directly measured) compared to those that were not exposed to insulin. This is interesting as it suggests that the protein synthesis or genomic effect of insulin is perhaps a possibility. There may be an increase in JAR cell division in response to a high concentration of insulin, leading to increased cell numbers. Insulin was shown to increase the number of cells per embryo in mouse models (Gardner and Kaye 1991). The data from this current experimental study show that preincubation of insulin prior to binding assay, suggests a time-dependence relationship due to inhibition of binding at both concentrations with long incubation. Insulin

at different concentrations, becoming active in long incubation, may regulate gene expression of adhesion molecules. There is the possibility that such transcription regulation possibly occurs via different signalling pathways (see Figure 2-15).

Adhesion molecules are involved in the stability and migration of cells (Parsons et al. 2010). For most cells in most environments, movement starts by protrusion of the cell membrane and continues by the formation of new adhesion at the cell front that links the actin cytoskeleton to the substratum. This produces a traction force that moves the cell forwards and disassembles of adhesion at the rear of the cell. This adherence formation and disassembly is stimulated by Rho GTPase and drives the migration cycle (Parsons et al. 2010). The enzyme then regulates actin polymerisation and myosin activity and thus adhesion dynamics. Extracellular matrix and integrin subunits were involved in trophoblast cell adhesion to the endometrium (Nagaoka et al. 2003). In order to ascertain which adhesion molecules may be responsible for binding of JAR spheroids to RL95-2 monolayers, both cell types were assessed for expression of adhesion molecules and their interacting proteins. Trophinin was not detected in either JAR or RL95-2 cells, which suggested that trophinin is unlikely to be involved in the adhesion of the two cells. Trophinin has been detected in primary human endometrial epithelial cells, human embryonic carcinoma HT-H cells and human endometrial adenocarcinoma SNG-M cells (Fukuda et al. 1995; Fukuda and Sugihara 2007; Sugihara et al. 2008). Expression of CD44, a ubiquitously expressed hyaluronic acid receptor, was detected in RL95-2 cells but not in JAR cells. This suggests that CD44 is absent in the intercellular binding between both cells. CD44 expression has been reported in the preimplantation human embryo (Campbell et al. 1995). The expression of CD44 in RL95-2 cells alone perhaps indicates that RL95-2 monolayers interacts with JAR spheroids via hyaluronate, which allows binding of both types of cell to extracellular matrix ligands (Aruffo et al. 1990).

Laminin α1 mRNA and protein were found in JAR cells. Laminin α1 protein has previously been detected in JAR cells (Tiger et al. 1997). Laminin α1 was also localised in the extracellular matrix of JAR cells. Immunofluorescence, immunoprecipitation, SDS-PAGE and immunoblotting method would all be useful in characterising the extracellular matrix (Langhofer et al. 1993). Laminin α1, a subunit of laminin-1 (also called laminin-111, an isoform of the laminin family of extracellular matrix proteins), is commonly present in basement membranes (Patarroyo et al. 2002). There is a possibility that other subunits of the laminin-1 (laminin β1 and laminin γ1) chain compete for laminin α1 binding sites on JAR spheroids in adhering to RL95-2 monolayers. This study is unclear as to whether insulin supports synthesis of laminin α1, which influences binding. An *in vitro* model study showed a time-dependent insulin mediated increase in a laminin α2, laminin β1 and laminin γ1 chain protein (but not mRNA), likely identified as laminin-211 (Dekkers et al.

2009). Laminin α1 interacts with extracellular matrix molecules FBLN-1 and -2 (Utani et al. 1997). Both molecules were detected in JAR cells, while FBLN-1 was observed in RL95-2 cells only. In view of this, JAR spheroids have the potential to bind the RL95-2 monolayer via FBLN-1, which might be implicated *in vivo*. Data from this experiment where there was no specific block binding of spheroids to RL95-2 monolayers using a laminin α1 antibody, are difficult to interpret, as the non-relevant antibody (anti-GRP 78) also caused partial inhibition of binding. Absence of anti-laminin α1 antibody effect with increased concentrations may be suggestive of the ability of this antibody to activate other adhesion molecule(s) that otherwise may not have been active. Thus, this activation allows a higher number of JAR spheroids to bind on RL95-2 monolayers. A proposal of some important adhesion molecules, with or without insulin regulatory effect, is shown in Figure 2-16.

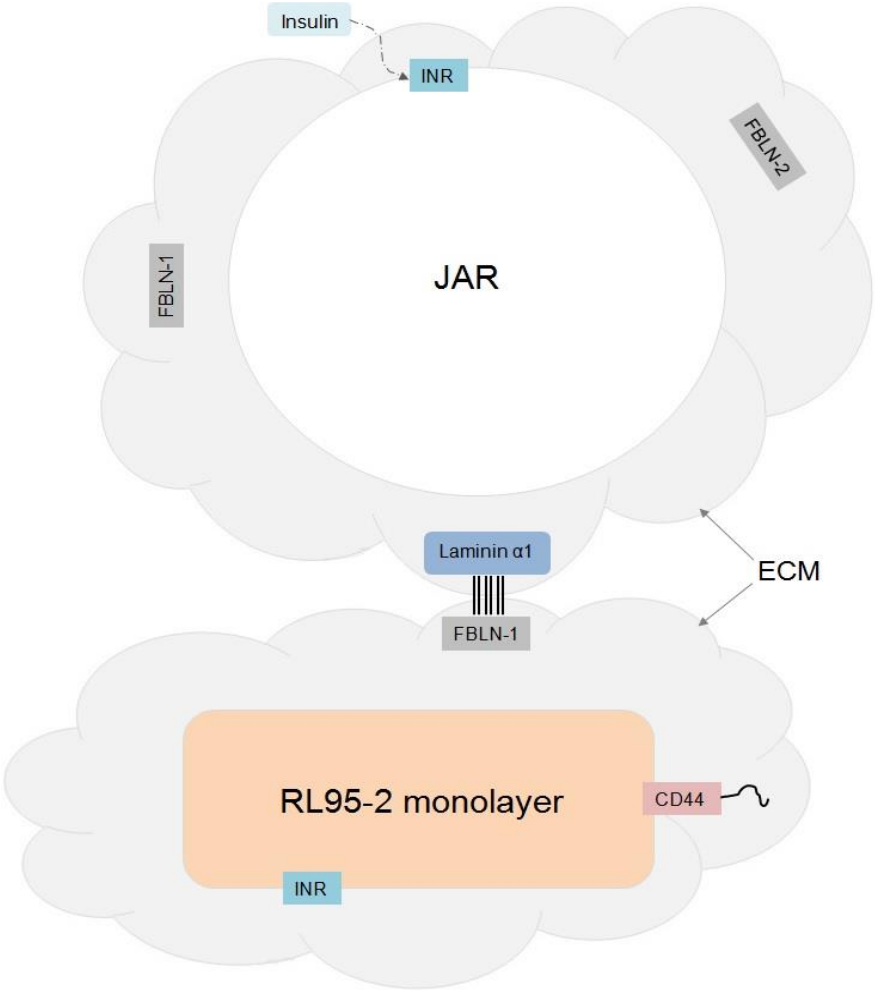


Figure 2-16: **Adhesion molecules during JAR spheroid-RL95-2 monolayer in cell culture model of implantation with or without insulin**. INR represents insulin receptor; ECM, extracellular matrix.

As well as the above proposal, there is also the possibility that other molecules may be implicated in JAR spheroid-RL95-2 monolayer binding. Since the N-terminal domain of laminin 1 is made of laminin α1, laminin β1 and laminin γ1 chains, there is the potential for cross binding in any of these chains to interact with common receptors as suggested above. There are also several G-domains, located in the COOH-domain of laminin 1, known to adhere with integrins and cellular adhesion molecule laminin α1 (Nomizu et al. 1995). Heparin sulfate proteoglycans (Rohde and Carson 1993) and integrins may also be involved (Fukuda et al. 1995; Wang and Dey 2006). Also, Rho GTPase protein regulator is found to enhance binding between JAR and RL95-2 cells (Heneweer et al. 2002). The anchorage of trophoblast cells (JAR cells) onto the maternal decidual RL95-2 cells during implantation has also been reported to be facilitated by fucosyltransferase VII (Zhang et al. 2009). Other anchoring molecules, such as integrin, including alpha v beta (3) and alpha (4) beta (1) (Quenby et al. 2007), cadherin and selectin, help facilitate adhesiveness between the embryo and endometrial epithelium. These data suggest a general consensus that these adhesion molecules may support binding of JAR spheroids to the RL95-2 monolayer.

A major limitation of the current study is that the implantation cell culture model lacks *in vivo* authenticity and does not fully represent the blastocyst-uterine interaction during implantation. The time-frame of adhesion interactions over a 24 hour time-frame only is much shorter than day 6 to 10 post ovulation, LH+7 to LH+11 and/or day 20 to 24 post LMP time windows *in vivo* (Aplin 1996; Wilcox et al. 1999; Lessey et al. 2000; van Mourik et al. 2009). Handling of coverslips has been reported as proving difficult and challenging for beginners and even for experienced hands, which may introduce an uncontrollable shear force however carefully they are handled (John et al. 1993; Li et al. 2002). There is the issue of the number of spheroids utilised *in vitro* being far more than the number *in vivo* (Aboussahoud et al. 2010). Also, because there is no available data on localised embryo-uterine insulin levels, this has led to the use of insulin data on circulating maternal and cord plasma levels at term as a guide; however, these levels may be quite different. Even when appropriate *in utero* insulin levels are identified, the pulsatile pattern of insulin secretion is likely to be affected sharply between meals *in vivo* (Polonsky et al. 1988). This suggests that insulin levels may not be the best metabolic parameter to be tested using the blastocyst-uterine *in vitro* model or that the current model requires further modification.

Ethical concerns with regard to experimentation with human tissue during the implantation period have necessitated the search for an *in vitro* model for elucidating the basic mechanisms involved during this period. This *in vitro* model may facilitate research into elucidating pregnancy complications in early pregnancy: pregnancy loss, PE, IUGR and gestational diabetes (Cha et al. 2012; Gauster et al. 2012). In this chapter, I found no

evidence that insulin played a role in binding, but insulin and adhesion molecule laminin α1 expression could be of importance during implantation, although the data analysis is at too early at a stage to draw firm conclusions. If low insulin can be shown conclusively to prevent spheroid adhesion, this finding might represent a difference between *in vitro* and *in vivo* studies as observed in women suffering from obesity, PCOS and diabetes, which are associated with hyperinsulinaemia and a likelihood of miscarriage (Penney et al. 2003; Dokras et al. 2006; Jungheim et al. 2009; Beauharnais et al. 2012; Chang et al. 2013). Even at this stage, these preliminary findings may suggest that there is a fundamentally different mechanism between *in vitro* and *in vivo* situations in terms of implantation.

It is recommended that future studies make improvements of this current model. It may be beneficial to use a different technique for preparing JAR spheroids. The use of tissue culture Petri dishes instead of only Erlenmeyer flasks must be explored (Aboussahoud et al. 2010). The use of Petri dishes may reduce spheroid preparation time from 72 hours to only 24 hours. It is recommended that other human choriocarcinoma cell lines (BeWo and Jeg-3), which can also be transform into spheroids (Grümmer et al. 1994) be investigated. As Grümmer et al. report, all these cell lines (JAR, BeWo and Jeg-3) spheroids were all proven to adhere human endometrial epithelium; each was invasive in a general invasion assay using embryonic chick heart fragment. Insulin doses ranges (assessments of toxicity) at high and low concentration are recommended. Also, a longer time (approximately 24 hours) for preincubation assay maybe advisable, as this resulted in noticeably greater effects. It appears that the insulin had begun to take effect by the 24th hour, as reported above. Investigation of the specific downstream signalling pathways' response to insulin dose, during JAR spheroids binding to the RL95-2 monolayer, can be explored through the PI3-K and MAPK pathways. Testing this model will require an approach which distinguishes the adhered JAR spheroids-RL95-2 monolayer. For instance, after counting the number of bound spheroids, 0.25% trypsin-EDTA can be added into the well containing bound spheroids and RL95-2 monolayers to disintegrate the cells and collect samples for further studies. This may require additional cell dispersal of JAR spheroids only and RL95-2 monolayer only as control samples. Methods, including immunofluorescence, immunoprecipitation, SDS-PAGE and immunoblotting, may all be useful in characterising the extracellular matrix involved in adhesion (Langhofer et al. 1993). Protein along the activated insulin response pathways (White 2013) can now be investigated. These methods may be useful to study other important mechanisms, such as those involved in the interaction between FBLN-1 and laminin α1 during *in vitro* implantation. Gene cloning procedures (Zhang et al. 2009) would also be useful to generate monoclonal anti-laminin α1 specific to laminin α1 in binding assay, if necessary.

In conclusion, difficulties in studying human embryo implantation *in vivo* have led to the continued search for an optimal *in vitro* cell culture model system due to the enormous problem of the uniqueness of human implantation and poor accessibility to experimental tissue and human embryos for research. This chapter proposes a cell culture model of implantation and also provides data on insulin effects on cell interaction and the role of laminin α1 in cell binding, which may be of interest to future research. Future studies into understanding human implantation and the effect of metabolic and inflammatory pathways are important. An optimal experimental model will assist in understanding the signalling pathways and transcriptional effects governing adhesion during implantation, and will provide future opportunities to design and implement interventions that may improve pregnancy success in natural and spontaneous or assisted conception.

3 Early pregnancy changes in parameters of metabolic status and inflammatory mediators

3.1 Introduction

Predicting pregnancy success for those undergoing assisted conception remains problematic in spite of the advances in the field. A major problem is selecting the type of hormone and/or amount administered for optimal ovarian follicle growth. Maternal age remains a key determinant of the protocol employed per person. An example is the report by Nelson et al., where women of <36 years were given 225IU FSH and those >36 years received 300IU per day (Nelson et al. 2007). La Marca et al. observed that anti-Müllerian hormone levels can predict live birth rates, and measurement of this hormone may facilitate individualisation of therapy prior to first ART cycle (La Marca et al. 2011). The degree to which obesity influences early pregnancy metabolic and inflammatory parameters, and how this may impact on pregnancy success, is yet unclear. Obesity is a significant risk factor for altering metabolic metabolites and inflammatory mediators throughout gestation (Stewart et al. 2007). Maternal body fat may affect metabolites (including lipid and carbohydrate metabolism) and inflammatory mediators, as well as hormones (such as insulin and leptin) early in pregnancy. Increase in these parameters, exacerbate maternal insulin resistance and lipid stores in pregnancy. Whether these changes may predetermine pregnancy success because of their links with changes in the metabolic and inflammatory parameters was unknown until now.

Usually, early first trimester pregnancy is referred to as the anabolic period; this is when the mother accumulates excess nutrients for preparation of the uterus for optimal implantation (Mattos et al. 2000). The anabolic state switches to the catabolic state in late gestation to provide fuel for fetal growth and development (Huda et al. 2009). This adaptation is characterised by a series of metabolic changes, such as those promoting adipose tissue accretion in early gestation and those leading to the development of insulin resistance in late pregnancy. The insulin resistance part of the adaptive response allows preferential diversion of glucose and lipids to the developing fetus (Aronne and Segal 2002). This response is thought to result from the combined effects of hPL, oestrogen, progesterone and cortisol, which act as counter-regulatory hormones to insulin (Wilcox 2005; Newbern and Freemark 2011). After fertilisation and over the course of a normal pregnancy, the mother must undergo these periods of hormonal, metabolic and inflammatory adaptation (Mor and Cardenas 2010; Newbern and Freemark 2011). In early gestation, insulin secretion increases while insulin sensitivity is unchanged, decreased or may even increase (Catalano et al. 1993; Catalano et al. 1999; Huda et al. 2009).

Overall, there are profound anatomical, physiological and biochemical adjustments in the mother (Torgersen and Curran 2006), which lead to several hemodynamic changes. There is increased cardiac output, reduced systemic vascular resistance by increased renal blood flow and raised glomerular infiltration rate by 6 weeks' gestation (Chapman et al. 1998). These physiological changes result in high sodium and water re-absorption and blood volume expansion. Enhanced storage of maternal nutrients, together with fetal nutrient demand, leads to accumulating body fat in mothers (Villar et al. 1992; Herrera 2000).

By late gestation, the maternal adipose tissue deposits decrease, while postprandial NEFA levels increase and insulin-mediated glucose disposal worsens by 40-60% compared with the prepregnancy period (Catalano et al. 1999). The ability of insulin to suppress whole-body lipolysis is also reduced during late pregnancy. Barbour et al. proposed that placental growth hormone may play an important role in accelerating the transition from lipid storage to lipolysis and insulin resistance during pregnancy (Barbour et al. 2007). This transition is accelerated in obese women, making them more insulin resistant, and is due to an increase in maternal hormones (e.g. hPL sensitivity). This increase results either from a reprogramming of maternal physiology to meet fetal needs or from a synergy with other obesity- or pregnancy-related factors. In any case, insulin resistance is often severe and disrupts the intrauterine milieu. As the natural interface between the mother and fetus, the placenta is the obligatory target of such disruption and is readily affected.

To date, there exist no robust reports of early metabolic and inflammatory pathway events, and their link to pregnancy success or implantation, whether with natural or assisted conception, is unclear. Most metabolic and inflammatory parameter changes have been studied in late gestation. Some data suggest that a number of parameters that lead to complicated perinatal outcome may be determined in the first trimester. This is because by the time adverse outcomes are evident during gestation, a number of metabolic and inflammatory parameters are detectable. It widely accepted that poor embryonic quality, implantation failure and aberrant placentation predetermine complications of pregnancy (Torry et al. 2004). These adverse states could have occurred before obstetric monitoring began. Examples of adverse outcomes include pregnancy loss whether early/late or stillbirth, PE, IUGR and gestational diabetes (Cha et al. 2012; Gauster et al. 2012).

The impact of metabolic and inflammatory adaptation is thought to occur at a defined period of gestation, resulting in complications of pregnancy. Welch et al. noted a correlation between placental perfusion and maternal serum PIGF levels evident as early

as 14 weeks' gestation (Welch et al. 2006). In another study, increased maternal CRP early in pregnancy is identified as a risk factor for preterm birth (Pitiphat et al. 2005). Women who suffered PE by 34 weeks' gestation were shown to have elevated sFlt-1 levels by 6-10 weeks (Chaiworapongsa et al. 2005). It has been found that those who develop PE had early (13 weeks' gestation) plasma changes in plasma lipid levels (10.4% and 13.6% higher LDL-C and TG) compared to normotensive women (Enquobahrie et al. 2004). It has also been shown that higher levels of circulating maternal placental proteins, such as pregnancy-associated plasma protein-A (PAPP-A) by the first 10 weeks of gestation, are associated with low birth weight at term (Smith et al. 2002). PAPP-A is a protease that binds IGFs (Laursen et al. 2001) to upregulate placental IGFs (Rutanen 2000). Reduced levels of PAPP-A at 8-12 weeks' gestation are associated with poor placental and fetal growth, leading to low birth weight (Smith et al. 2002). Plasma CRP levels of women who had an IUGR baby compared to the control group were already higher at first antenatal visit between 10 and 14 weeks' gestation (Tjoa et al. 2003). Singleton pregnancy first-trimester fasting glucose levels in women with gestational diabetes increase from 1.0% in the lowest to 11.7% in the highest category (adjusted OR 11.92 (5.39, 26.37)) (Riskin-Mashiah et al. 2009).

There have been no comprehensive studies of changes in metabolic and inflammatory parameters in early gestation. Data using assisted conception populations have begun to provide new insight into these early changes. Sack et al. observed increased maternal CRP at 4 weeks' gestation (Sacks et al. 2004). An increase in CRP levels was reported in women undergoing IVF as early as 14 days after embryo transfer (Almagor et al. 2004). This increase may be due to functional undertaking of such inflammatory mediators, which is paramount for implantation and placentation. Uterine cytokines and chemokines are known to modulate immune response during pregnancy, driving peripheral leukocytes into uterine tissue (Jabbour et al. 2009). Typically, since several cell types besides Th cells contribute to an overall Th1 or Th2 cytokine pattern, it was suggested that these response should instead be described as type 1 or type 2 (Raghupathy 2001). Also, as highlighted above (Section 1.4.1.3), a balance between these responses are paramount for successful pregnancy.

As studies show increasing evidence that fetal outcome is determined by the end of the first trimester, it is imperative that early events are investigated. The earliest detection of parameters associated with adverse outcome will allow a means of identifying when things may go wrong. Understanding early metabolic status and inflammatory mediator changes, and their relationship to pregnancy success, may facilitate developing effective diagnostic tests and suggest acceptable intervention strategies for identifying pregnancy risks early.

3.2 Hypotheses

In this chapter it is hypothesised that prepregnancy parameters of metabolic status and inflammatory mediators predict successful pregnancy; that there are detectable changes in plasma metabolic status and inflammatory mediators in the first 6 weeks of pregnancy. It is also hypothesised that obesity is associated with decreased pregnancy success, and increased parameters of metabolism and inflammation are evident over the first 6 weeks.

3.3 Aim

The aim of this chapter is to observe the changes in metabolic and inflammatory parameters in a longitudinal study of women undergoing natural cycle FET after detecting their LH surge over 6 weeks.

3.4 Objectives

1. To examine whether prepregnancy metabolic and inflammatory parameters predict pregnancy success in a population of women undergoing natural cycle FET.

2. To assess maternal plasma changes in parameters of lipid and carbohydrate metabolism, and of inflammation, in early pregnancy (<7 weeks' gestation).

3. To examine the impact of maternal obesity on metabolic and inflammatory parameter changes, using BMI and waist circumference.

3.5 Materials and Methods

3.5.1 Subject selection and recruitment

Women were recruited from the population of those attending the Assisted Conception Unit (ACU) at Glasgow Royal Infirmary (GRI), Scotland, after obtaining written informed consent. Ethical approval was granted in accordance with the guidelines of the Helsinki Declaration from the North Glasgow University Hospital Trust, National Health Service Greater Glasgow and Clyde Research Ethics Committee. Subjects were excluded if they had hepatitis, HIV, diabetes, chronic hypertension, connective tissue disorders or any long-term use of medication. None of the subjects were being treated with medications that interfere with metabolites of lipid and carbohydrate metabolism, inflammation or endothelial function.

Women received subcutaneous administration of Menopur® highly purified Menotropin or Gonal 450IU/0.75mL, Follitropin alpha or Organon 150-375IU for ovulation induction. When a minimum of three matured follicles of approximately 17-18mm in diameter were detected using ultrasonography, 10000IU of hCG (booster) was administered before oocyte retrieval, approximately 36-40 hours later for IVF and/or ICSI under general anaesthetic. Oocytes were exposed to spermatozoa for insemination, and ICSI was used in cases involving male infertility, as reported previously (Zhou et al. 2008). Oocytes exhibiting two pronuclei were cultured for embryonic development in Quinn's Advantage™ Sequential Culture Media, (Sega). At 18 hours post insemination, pronuclei were cultured in Quinn's Advantage Fertilisation Medium, at 43-44 hours post insemination in Quinn's Advantage Cleavage Medium and at 63-64 hours in Quinn's Advantage Blastocyst Medium. After culturing in the specified medium, blastocysts or embryos were frozen using Quinn's Advantage Embryo Freeze Kit.

Women undergoing natural cycle FET procedure with a regular menstrual cycle, with optimal timing of replacement determined by daily hormone analyses (as per the current clinic protocol) were recruited to the study. In the clinic in Glasgow, the BMI range is greater than 18kg/m^2 and lower than 30kg/m^2. This BMI range is similar across the board in Scotland. This group of women undergoing IVF were selected to eliminate the effects of ovarian stimulation, thus providing the most physiologically representation of natural pregnancy. Women were informed of the study when notifying the clinical nurse about the date of their LMP with a view to booking for FET, and a study information sheet was given to them. At day 10 after LMP, the women attended the ACU to commence daily hormonal sampling to detect LH surge and time of embryo replacements. At this point the subjects provided written informed consent. Embryos were replaced approximately ±3 days after

the LH surge. A hundred and ninety-six (196) women that underwent IVF with or without ICSI, of 34 years median age and ranging from 24 to 43 years, were recruited from September 2007 to July 2010 (34 months).

3.5.2 Sample and demographic data collection

Blood samples (approximate 20mL) were taken at different time points (7 visits) in a comfortable position, either lying or seated, by venepuncture from the antecubital fossa. Fasting blood samples were collected from women at all time points, apart from the FET day, when non-fasted samples were taken. After the day 10 LMP, a basal blood samples (baseline) were taken at the next routine clinic visits (of LH surge sampling). The date of LH surge was identified and taken as day 0 of gestation. The baseline sample was used as the sample of the day 0 of gestation. Additional samples were taken on the day of FET (≥ day 3 LH). Thereafter, samples were collected at 7, 10, 18, 29 and 45 days. Women who were unable to attend the seven appointments provided samples only on the routine clinic visits. This includes baseline, FET and day 18 in pregnant and non-pregnant groups, with an additional sample collected at day 45 for the pregnant women. The 20mL blood samples were collected using different anti-coagulants, as follows: 1.8mg/mL ethylene diaminetetraacetic acid (EDTA) (2 x 4mL blood), 18IU/mL heparin (1 x 6mL blood), 0.109mol/L citrate (1 x 3.5mL blood), and 2.5mg/mL fluoride oxalate (1 x 2mL blood) Vacuette® tubes (Greiner Bio-One), to accommodate all the required biochemistry analyses. Plasma was removed from the cell pellet by pipetting after being centrifuged (3000*g* for 10min). Each subject's plasma was split into 2mL cryo vial aliquots to avoid repeated freeze-thaw cycles, and were stored in a -80°C freezer within 2 hours.

Age, ethnicity, treatment options and the systolic and diastolic blood pressure at the time of IVF treatment, with or without ICSI, were recorded from patient notes. Height, weight and waist circumference were obtained at baseline and also on day 45. Maternal obesity was estimated by BMI and waist circumference. BMI measures body fat, whereas waist circumference is the best simple measure for intra-abdominal fat mass (Park et al. 2009). Previous reports indicate that waist circumference (≥80cm) is equivalent to BMI ≥25kg/m^2 (Lean et al. 1995). BMI, calculated by dividing weight in kilograms (kg)/height in meters square (m^2) (Keskin et al. 2005), at booking (baseline) as well as day 45 was categorised according to the WHO criteria with a BMI of ≥30kg/m^2 defined as obesity, a BMI of >25kg/m^2 defined as overweight, a BMI of 24.9-18.5kg/m^2 defined as healthy (recommended), and a BMI of ≤17.5kg/m^2 defined as underweight (lean). The waist circumference was measured at the narrowest part of the torso, located between the lower rib and the iliac crest, (while the participants were standing relaxed) at booking (baseline), and day 45 of gestation. The waist circumference measurement at the level of

the umbilicus was carried out twice to the nearest 0.5cm. If the difference between measurements was greater than 2cm, a third measurement was performed.

Menstrual period length, cycle length, smoking habits, reason for undergoing FET (including chemotherapy, endometriosis, genetic factor, male factor, PCOS, pelvic adhesion, PGD, tubal and unexplained), number of embryos transferred, number of previous pregnancies greater than or equal to 24 weeks and those less than 24 weeks were recorded. Scotland Index Multiple Deprivation (SIMD) version 2 (2009) was explored using the participant's postcode at the time of FET. SIMD is a tool used by the local authorities, the Scottish Government, the NHS and other government bodies in Scotland (Russell and Lough 2010). This version of SIMD combines 37 indicators across seven domains, namely: current income, employment, health, education, skill and training, housing, geographic access and crime. In SIMD categories, one represents most affluent and five most deprived.

3.5.3 Plasma lipids, glucose, CRP and NEFA assays

Plasma sample vials were allowed to thaw and reach RT. Plasma TG, TC, HDL-C, glucose and CRP were measured in an automated Roche/Hitachi MODULAR P (Roche) analyser by the Department of Biochemistry, GRI, Scotland. Lipid assays were carried out using the Standard Lipid Research Clinic Program (NIH) protocol (LRCP 1975). Plasma TG was measured by the glycerol-3-phosphate oxidase/phenol aminophenazone (GPO/PAP) technique on the automated analyser. Similarly, TC, HDL-C, glucose and CRP were measured using a CHOD/PAP kit, HDL cholesterol plus 3rd generation kit, glucose oxidase/PAP kit and Tin-quant CRP (Latex) high sensitivity immunoturbidimetric assay kit, respectively, all supplied by Roche Diagnostic, following the manufacturer's instructions. Non-esterified fatty acid (NEFA) was measured using a NEFA C test kit (Wako, Neuss Germany), according to the manufacturer's instructions, and was read at a 550nM wavelength.

3.5.4 Assays of plasma insulin, IL-6, PAI-1 and PAI-2

Human insulin, IL-6, PAI-1 and PAI-2 were quantitated by ELISA, according to the manufacturer’s instructions, using a plate reader (LKB Biochrom Pharmacia) at the specific wavelength. A standard curve of absorbance against the protein was carried out, and unknown protein determined from the curve before exporting to a new Microsoft Excel spreadsheet. Estimated concentration was calculated taking account of protein amount, volume and dilution factor. Plasma insulin (10-1113-01, Mercodia) was sensitive to 2mU/L and read at a 450nM wavelength. Homeostatic model assessment (HOMA) was

calculated using fasting insulin concentration (mU/L) x fasting glucose concentration (mmol/L) divided by 22.5, assuming the healthy subject had insulin resistance (Keskin et al. 2005).

The plasma lL-6 (Quantikine HS, R&D Systems) had a sensitivity of 0.02pg/mL and was determined at a 490nM wavelength. PAI-1 (TriniLIZE PAI-1 Antigen REF: T6003, Trinity Biotech) had a detection limit of 0.5ng/mL and was detected at 492nM. The PAI-2 (IMUBIND® PAI-2 ELISA; Stamford, USA) had a sensitivity of 50pg/mL and was read at 450nM.

3.5.5 Chemokine assays

Plasma chemokines (CXCL8, CCL2, CCL3, CCL4 and CCL11) were measured by a BioPlex Suspension Array System (also known as Luminex xMAP®) according to the manufacturer's instructions (Hannan et al. 2011). Plasma samples were thawed at RT and each assay determined using a MilliPlex® MAP kit (Bio-Rad). The multiplex assay has a broad sensitivity range of 0-32000pg/mL, which makes it feasible to eliminate the need for assay repeat measurement and sample dilutions. The plates were prewetted with a 200µL assay buffer (provided by the manufacturer) for 10 min and then aspirated using a vacuum manifold. The standards and plasma samples (25µL) were added to appropriate wells, followed by the addition of beads. Plates were incubated overnight (16-18 hours) with mild agitation at 4°C, the fluid removed by vacuum, and the wells washed twice with a wash buffer. Detection antibodies (25µL) were added to each well and incubated for 1 hour at RT, the fluorescent conjugate streptavidin-phycoerythrin added to each well, and plates incubated for 30 min at RT. The supernatant was then removed by vacuum and the wells were washed twice.

Data were collected and analysed using a BioPlex 200 instrument equipped with BioManager analysis software (Bio-Rad Hercules, CA). Test runs were performed before analyses of the full set of samples, to optimise the sample dilution such that each analyte was analysed within the functional range of its standard curve.

3.5.6 Plasma hCG assay

Human CG was measured on an IMMUNLITE®/IMMUNITE® 1000 system analyser (Siemens, USA), using a commercially available IMMUNLITE®/IMMUNITE® 1000 hCG kit (Siemens Medical Solution Diagnostic) according to the manufacturer's instructions. The hCG kit has a sensitivity of 1.1mIU/mL and levels were determined against quality control standards.

3.5.7 Statistical analysis

Statistical analysis was carried out using Minitab Vs16.2.2, and data were tested for normal distribution using dotplots; subsequently, the Ryan-Joiner test was applied. Where data were not normally distributed, logarithm (log) transformation was performed. Analysis of stability of baseline measure of metabolic and inflammatory parameters was carried out with repeated analysis of variance (ANOVA), due to the impracticality of collecting blood samples for each participant on exactly the same baseline day. Two-sample t-test measure was used to test continuous variables (including maternal age, BMI and birthweight), and Pearson's Chi-Square test was used to test categorical variables of demographic characteristics between women who became pregnant and those who did not. Demographics and metabolic and inflammatory parameters were explored for association in terms of predicting pregnancy success using logistic univariate and multivariate regression analysis. The OR and 95% CI were determined. The area under the receiver operator characteristic curve (c-statistic) was computed in order to give the measure of prediction using [0.5 (1 + Somer's D)], where Somer's D measures association between the response variable and predicted probabilities. A repeated measure ANOVA with General Linear Model (GLM) was also used to identify parameters that changed over time, in pregnant women only, by testing for differences at specific time points using a *post hoc* Tukey test. Pearson's correlation coefficient was used to determine the association between maternal obesity measures (BMI or waist circumference) and the changes in metabolic and inflammatory parameters. The data are given as mean and standard deviation (SD) unless otherwise stated, and statistical significance was defined as a P-value of less than 0.05.

3.6 Results

3.6.1 Recruitment of the natural cycle FET cohort

A total of 196 IVF cycles were included in the study, of which 35 withdrew (3 women withdrew consent, 7 were lost to follow up and 25 cycles were cancelled or constructed). A total of 38 women only became clinically pregnant, confirmed by fetal heartbeat during ultrasound scan at day 45 of gestation. The changes in treatment protocol at the clinic, which resulted in fewer women undergoing FET there, may have contributed to the number of total successful pregnancies over time. The majority of women who found out that they failed to become pregnant at day 18 (understandably) withdrew from the study at that point and gave no additional samples. After 161 IVF cycles, a total of 36 (rather than 38) clinically pregnant women at day 45 were compared with 106 (rather than 123) non-pregnant women at day 18. These were women who provided a sample after a single attempt. The sampling flowchart is shown in Figure 3-1.

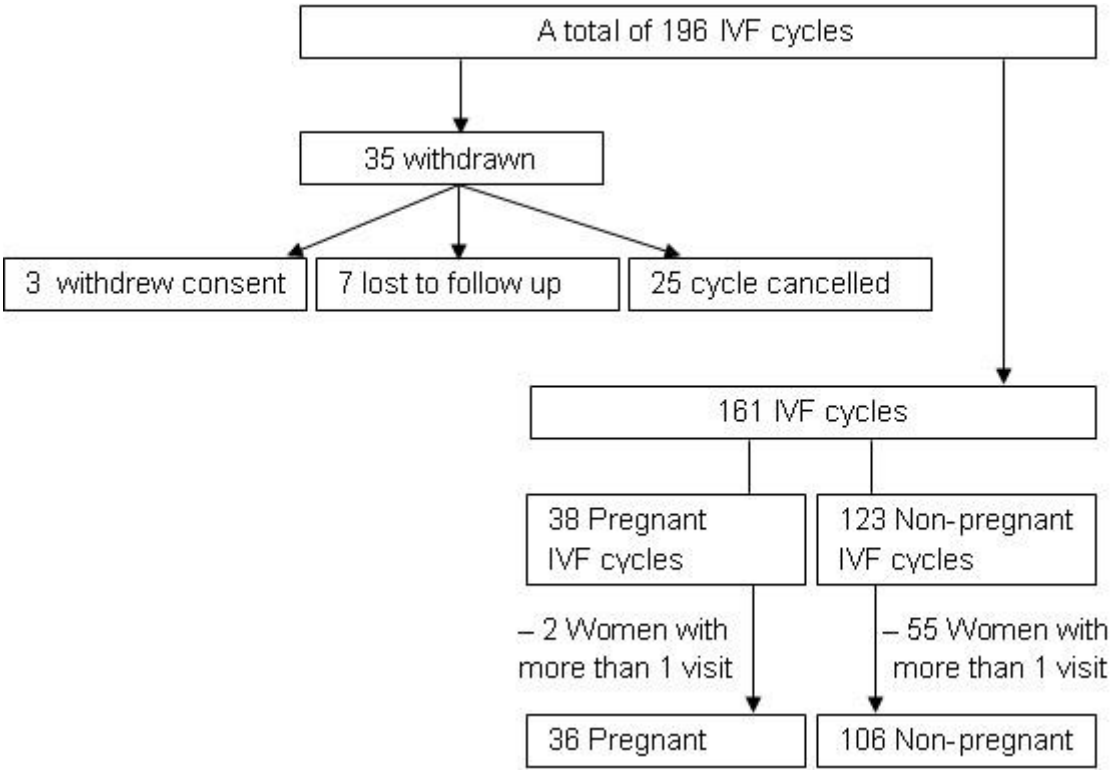


Figure 3-1: **A flow chart of blood collection of 196 IVF cycles**. This includes participants who withdrew (n=35) and the rest of the 161 IVF cycles, among them those who are pregnant (n=36) who had positive fetal heartbeat by 45 days of gestation, and non-pregnant (n=106) women at the clinic at outcome day 18 after a single attempt.

3.6.2 Demographic of pregnant and non-pregnant women

The clinical baseline features are shown in Table 3-1. Age and ethnicity were not different between pregnant and non-pregnant women. However, there is a trend toward higher ICSI vs IVF in women who became pregnant compared to the non-pregnant group (P=0.074). Systolic and diastolic blood pressure were measured during the time of treatment, and the values show no statistically significant difference between the pregnant women and those who failed to become pregnant. Height, weight, BMI, waist circumference, menstrual period length and cycle length were also not different among both groups of women. Smoking habits (including those who currently smoke, ex-smokers and those that never smoked) were not different between pregnant and non-pregnant women. There was no difference between the two groups in terms of women who were smoker. The reason for women undertaking FET, the number of embryos transferred, number of pregnancies greater than or equal to 24 weeks and those less than 24 weeks were not different between both groups. SIMD vs quintile, which measures socioeconomic deprivation or affluence in Scotland, was statistically not different.

Table 3-1: **Demographic variables of the participants by pregnancy outcome at day 45**.

Features	Pregnant (n=36)	Non-pregnant (n=106)	P-value
Age (years)	34.2 (4.8)	34.2 (5.1)	0.96
Ethnicity [n,(%)]			0.41
Black African	0.0 (0.0)	1.0 (1.0)	
Caucasian	36.0 (100.0)	100.0 (95.2)	
South East Asia	0.0 (0.0)	4.0 (3.8)	
Treatment [n,(%)]			0.074
IVF	13.0 (36.1)	54.0 (53.5)	
ICSI	23.0 (63.9)	47.0 (46.5)	
Systolic blood pressure (mmHg)	116.0 (13.0)	115.0 (11.8)	0.59
Diastolic blood pressure (mmHg)	65.0 (6.7)	66.0 (9.4)	0.36
Height (m)	1.6 (0.1)	1.6 (0.1)	0.66
Weight (kg)	71.2 (11.2)	68.5 (13.6)	0.24
BMI (kg/m^2)	26.5 (4.1)	25.6 (4.6)	0.31
Waist (cm)	88.9 (10.5)	85.6 (11.7)	0.13
Menstrual period length (days)	5.1 (1.5)	4.9 (0.9)	0.70
Cycle length (days)	28.2 (1.4)	28.4 (2.4)	0.70
Smoking [n,(%)]			0.26
Smoker	4.0 (11.4)	23.0 (21.9)	
Ex-smoker	0.0 (0.0)	2.0 (1.9)	
Never	31.0 (88.6)	80.0 (76.2)	
Current smoker [n,(%)]			0.17
Yes	4.0 (11.43)	23.0 (21.90)	
No	31.0 (88.57)	82.0 (78.10)	
Reasons for FET [n,(%)]			0.27
Chemotherapy	0.0 (0.0)	1.0 (1.0)	
Endometriosis	3.0 (8.3)	5.0 (4.8)	
Genetic factor	0.0 (0.0)	1.0 (1.0)	
Male factor	19.0 (52.8)	45.0 (42.9)	
PCOS	1.0 (2.8)	2.0 (1.9)	
Pelvic adhesion	1.0 (2.8)	0.0 (0.0)	
PGD	1.0 (2.8)	0.0 (0.0)	
Tubal	6.0 (16.7)	32.0 (30.5)	
Others/Unexplained	5.0 (13.9)	19.0 (18.1)	
№ of embryos transferred [n,(%)]			0.23
1	6.0 (16.7)	20.0 (19.4)	
2	29.0 (80.6)	83.0 (80.6)	
3	1.0 (2.8)	0.0 (0.0)	
№ of pregnancies ≥ 24 weeks [n,(%)]			0.62
0	35.0 (97.2)	101.0 (98.1)	
1	1.0 (2.8)	1.0 (1.0)	
2	0.0 (0.0)	1.0 (1.0)	
№ of pregnancies < 24 weeks [n,(%)]			0.77
0	27.0 (75.0)	72.0 (69.9)	
1	5.0 (13.9)	22.0 (21.4)	
2	3.0 (8.3)	5.0 (4.9)	
3	1.0 (2.8)	3.0 (2.9)	
4	0.0 (0.0)	1.0 (1.0)	
SIMD 2009 vs quintiles [n,(%)]			0.64
1	5.0 (13.9)	21.0 (19.8)	
2	9.0 (25.0)	20.0 (18.9)	
3	8.0 (22.2)	32.0 (30.2)	
4	5.0 (13.9)	15.0 (14.2)	
5	9.0 (25.0)	18.0 (17.0)	

Mean (SD) and [n,(%)], P-value from two-sample and Pearson's Chi-Square tests respectively.

3.6.3 Analysis of stability of baseline measure

It was considered impractical to collect blood samples for each participant on the exact day of LH surge (taken as day 0 of gestation) due to the difference in the time participants' hormone surged. However, it was imperative to examine the stability of the baseline measure of metabolic and inflammatory parameters. Thirteen (n=13) women selected at random provided additional blood samples between baseline and FET during their other subsequent clinic routine visits for the LH surge sampling. The data obtained are shown in Table 3-2. Plasma hCG, insulin, TG, TC, HDL-C, NEFA, glucose, HOMA, CRP, IL-6, CXCL8, CCL2, CCL3, CCL4 and CCL11 levels did not show a statistical difference from the blood sample collected on the day of the FET procedure, approximate day 3 after the LH surge from the baseline. The PAI-1 (P<0.001) and PAI-2 (P=0.039) nevertheless were significantly different from the baseline. Caution was taken with PAI-1 and PAI-2 data interpretation.

Table 3-2: **Baseline measure stability analysis**.

Parameters	P-value for day at baseline
Hormone	
hCG (mmol/L)	-
Insulin (mU/L)	0.22
Metabolism	
TG (mmol/L)	0.37
TC (mmol/L)	0.87
HDL-C (mmol/L)	0.90
NEFA (mmol/L)	0.25
Glucose (mmol/L)	0.10
HOMA	0.21
Inflammation	
CRP (mg/L)	0.19
IL-6 (pg/mL)	0.21
CXCL8 (pg/mL)	0.45
CCL2 (pg/mL)	0.88
CCL3 (pg/mL)	0.51
CCL4 (pg/mL)	0.37
CCL11 (pg/mL)	0.45
PAI-1 (ng/mL)	<0.001
PAI-2 (ng/mL)	0.039

P-value from a repeated ANOVA measure.

3.6.4 Maternal predictors of pregnancy success after IVF

In order to determine whether metabolic and inflammatory parameters at baseline could predict pregnancy success by day 45 of gestation, univariate and multivariate logistic regression analysis was carried out. The estimated OR for prediction of pregnancy success at 95% CI is shown in Table 3-3. None of the variables predicted pregnancy success by day 45 of gestation. Women treated with IVF were less likely to get pregnant than those treated with ICSI (OR 0.5 (95% CI 0.2, 1.1) P=0.076). There was a borderline difference of high IL-6 (1.6 (1.0, 2.6) P=0.074) by day 45 of gestation, a potential independent predictive feature of whether a woman became pregnant. Multivariate logistic regression for covariates with at least P=0.15 shows a trend toward lower IVF (0.4 (0.1, 1.0) P=0.056) predictive of pregnancy success compared with ICSI. However, the waist measurements, IL-6 and PAI-1 failed to show a statistical difference. Although not part of the data presented in this thesis, the results in the investigation of the plasma and erythrocyte fat composition, which used the samples and dataset of this chapter, showed the percentage of saturated fatty acid in erythrocyte as independently predictive of whether a woman became pregnant (unpublished data).

Table 3-3: **Models for prediction of pregnancy success by day 45 at baseline**.

Demographic / Parameters	Univariate			Multivariate		
	OR (95% CI)	P-value	c-statistic	OR (95% CI)	P-value	c-statistic
Age (years)	1.0 (0.9, 1.1)	0.96	0.52			0.68
Treatment (IVF vs ICSI)	0.5 (0.2, 1.1)	0.076	0.59	0.4 (0.1, 1.0)	0.056	
SBP (mmHg)	1.0 (1.0, 1.0)	0.57	0.53			
DBP (mmHg)	1.0 (0.9, 1.0)	0.43	0.54			
Height (m)	3.8 (1.0, 1.1)	0.65	0.53			
Weight (kg)	1.0 (1.0, 1.1)	0.28	0.57			
BMI (kg/m^2)	1.0 (1.0, 1.1)	0.34	0.57			
Waist (cm)	1.0 (1.0, 1.1)	0.15	0.59	1.0 (1.0, 1.1)	0.20	
Menstrual period length (days)	1.1 (0.7, 1.9)	0.61	0.49			
Cycle length (days)	1.0 (0.7, 1.3)	0.79	0.52			
№ of pregnancies < 24 weeks	0.9 (0.6, 1.5)	0.75	0.52			
Current smoker (Yes vs No)	0.5 (0.2, 1.4)	0.18	0.55			
Embryos transferred (1 vs 2 or 3)	1.4 (0.5, 3.9)	0.48	0.53			
SIMD 1-Most affluent vs 5-deprived	0.5 (0.1, 1.7)	0.25	0.59			
Hormone						
hCG (IU/L)	0.7 (0.1, 4.1)	0.69	0.51			
Insulin (mU/L)	1.0 (1.0, 1.1)	0.92	0.49			
Metabolism						
TG (mmol/L)	1.8 (0.80, 4.2)	0.16	0.59			
TC (mmol/L)	1.0 (0.6, 1.5)	0.74	0.54			
HDL-C (mmol/L)	0.6 (0.2, 1.5)	0.25	0.54			
NEFA (mmol/L)	0.6 (0.0, 24.4)	0.78	0.53			
Glucose (mmol/L)	1.1 (0.7, 2.0)	0.68	0.53			
HOMA	1.0 (0.8, 1.2)	0.93	0.49			
Inflammation						
CRP (mg/L)	1.0 (1.0, 1.1)	0.45	0.58			
IL-6 (pg/mL)	1.6 (1.0, 2.6)	0.074	0.63	1.2 (0.7, 2.3)	0.44	
CXCL8 (pg/mL)	1.0 (1.0, 1.0)	0.43	0.51			
CCL2 (pg/mL)	1.0 (1.0, 1.0)	0.78	0.47			
CCL3 (pg/mL)	1.0 (1.0, 1.0)	0.69	0.54			
CCL4 (pg/mL)	1.0 (1.0. 1.0)	0.86	0.49			
CCL11 (pg/mL)	1.0 (1.0, 1.0)	0.86	0.47			
PAI-1 (ng/mL)	1.0 (1.0, 1.1)	0.12	0.63	1.0 (1.0, 1.1)	0.18	
PAI-2 (ng/mL)	1.1 (0.8, 1.6)	0.61	0.65			

Univariate and multivariate individuals' data assessment. Univariates with at least P=0.15 were entered into a multivariate logistic regression model. OR (odds ratio) unit change, 95% CI associated with P-value and c-statistic shown.

3.6.5 Early pregnancy metabolic and inflammatory changes

The early changes of metabolic and inflammatory parameters in the pregnant women only were analysed and are presented as plasma concentrations in Table 3-4. As expected, hCG, which is specifically secreted by the implanting embryo and placenta, increased significantly over time. Plasma hCG increased from baseline (P=0.012) by day 29 and further (P<0.001) by day 45 of gestation (overall change P<0.001). The level of insulin increased significantly (654%, P=0.030) by day 45 of gestation, with an overall increment of log P=0.029.

Plasma lipids were affected by day 45 of gestation, with TG level decreasing by 20% (P=0.001) by day 18 but recovering by day 45 (overall log P<0.001). The plasma TC level declined to 51% (P<0.001) by day 18 but began to recover to 51% (P<0.001) by day 45 of gestation (overall P<0.001). Plasma HDL-C also decreased (10%) by day 18 (P=0.002) and then started to recover by day 29 to 10% (P=0.05) from the baseline, and rebounded by day 45 (overall P=0.001). Thus, by day 45 of gestation, TG and HDL-C, but not TC, had recovered. There was no observable difference in NEFA and glucose levels by day 45. HOMA measure increased up to 179% (P=0.012) at day 45, and significantly (log P=0.031) over time.

For the inflammatory response mediators measured, plasma CRP was significantly higher (263%, P=0.019) from baseline by day 45, with log P<0.001 overall. The level of IL-6, CCL3 and CCL4 showed no changes by the first 45 days of pregnancy. Plasma CXCL8 (256%, borderline; overall log P<0.031), CCL2 (6700%, P<0.001; overall P=0.001), CCL11 (2900%, P=0.002; overall P<0.001) and PAI-1 (1330%, P<0.001; overall P<0.001) were significantly reduced from baseline by day 45 in the pregnant women. PAI-2 increased significantly (1680%, P<0.001) by day 45 of gestation, with an overall increment of P<0.00.1.

Table 3-4: **Plasma concentrations of parameters over time from baseline to day 45 of gestation**.

Parameters	Baseline (n=36)	Day 10 (n=21)	Day 18 (n=35)	Day 29 (n=30)	Day 45 (n=35)	P-value
Hormone						
hCG (IU/L)	0.03 (0.03)	8.11 (2.33)δ	679.50 (91.20)δ	21086 (2465)δ	103026 (9846)δ	<0.001
Insulin (mU/L)	8.76 (1.37)	7.55 (0.82)	11.21 (1.66)	10.05 (1.41)	15.30 (2.91)δ	0.029*
Metabolism						
TG (mmol/L)	1.10 (0.08)	0.90 (0.06)	0.88 (0.03)δ	0.94 (0.07)	1.21 (0.07)δ	<0.001*
TC (mmol/L)	4.77 (0.13)	4.43 (0.11)	4.26 (0.12)δ	4.30 (0.11)δ	4.26 (0.11)δ	<0.001
HDL-C (mmol/L)	1.50 (0.05)	1.49 (0.06)	1.40 (0.04)δ	1.41 (0.04)	1.45 (0.05)	0.001
NEFA (mmol/L)	0.26 (0.03)	0.03 (0.04)δ	0.08 (0.03)δ	0.02 (0.03)δ	0.03 (0.03)δ	0.075
Glucose (mmol/L)	4.84 (0.07)	4.86 (0.09)δ	5.03 (0.11)δ	4.75 (0.10)δ	5.07 (0.11)δ	0.16
HOMA	1.90 (0.30)	1.64 (0.19)	2.60 (0.42)	2.14 (0.31)	3.69 (0.74)δ	0.031*
Inflammation						
CRP (mg/L)	2.48 (0.73)	2.60 (0.62)	3.34 (0.92)	2.68 (0.60)	5.12 (0.82)δ	<0.001*
IL-6 (pg/mL)	1.42 (0.16)	2.03 (0.34)δ	1.56 (0.26)δ	1.28 (0.16)δ	1.57 (0.15)δ	0.09*
CXCL8 (pg/mL)	40 (9)	36 (7)δ	45 (12)δ	47 (12)δ	38 (7)δ	0.031*
CCL2 (pg/mL)	263 (18)	202 (14)	248 (22)δ	222 (21)	196 (19)δ	0.001
CCL3 (pg/mL)	89 (17)	95 (21)δ	97 (19)δ	109 (21)δ	91 (17)δ	0.31
CCL4 (pg/mL)	271 (65)	293 (101)δ	248 (71)δ	331 (83)δ	285 (73)δ	0.38
CCL11 (pg/mL)	132 (14)	121 (14)δ	130 (19)δ	128 (21)	103 (14)δ	<0.001
PAI-1 (ng/mL)	27.30 (4.23)	18.93 (1.80)	23.75 (1.78)δ	18.91 (1.47)	14.03 (1.24)δ	0.001
PAI-2 (ng/mL)	1.30 (0.09)	1.10 (0.10)δ	1.12 (0.07)δ	1.34 (0.08)δ	18.06 (1.67)δ	<0.001

Mean (SE), P-value or (*****) P-value of log transformed variable, is from repeated measure ANOVA with GLM. Adjusted difference using *Post hoc* Tukey test at baseline, day 10, 18, 29 and 45 from baseline represented as (δ) is P<0.05.

A summary of the early changes of metabolic and inflammatory parameters over time in the women who became pregnant is provided in Figure 3-2.

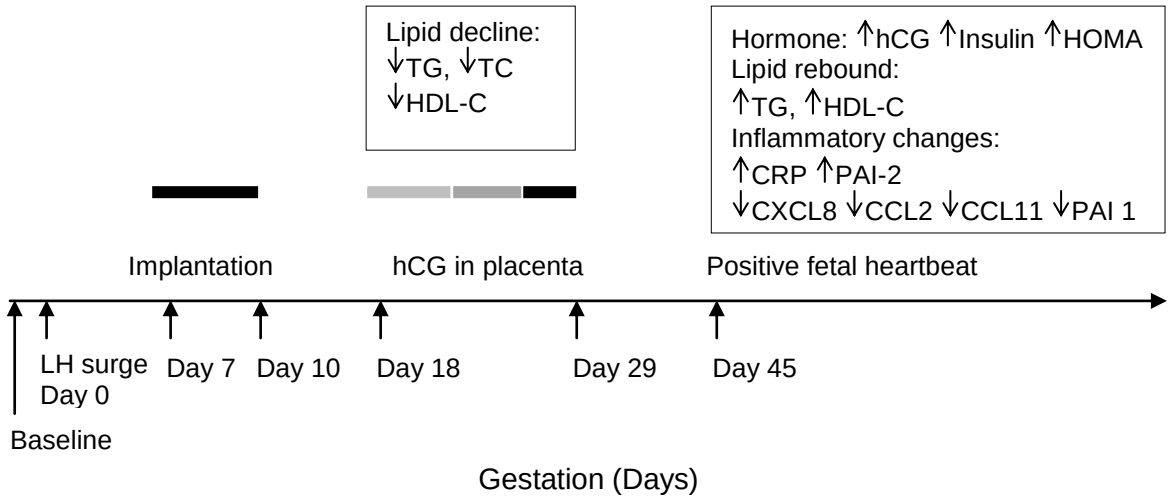


Figure 3-2: **Timeline summary of metabolic and inflammatory parameter changes in early pregnancy**. All the temporal changes were identified using repeated measure ANOVA, and those that were significantly different are shown. Significant changes between specific time points were investigated using *post hoc* Tukey test ($P<0.05$).

3.6.6 The impact of maternal obesity

The role of maternal obesity in early pregnancy changes of metabolic and inflammatory parameters was investigated by correlating baseline and changes in BMI and waist circumference with the change in parameters over the course of the study. Data are shown in Table 3-5. Maternal plasma change in insulin (r=0.37, P=0.040) and HOMA (r=0.39, P=0.028) correlated positively with baseline waist measurements of the women by day 45 of gestation. Plasma HDL-C over the course of pregnancy strongly correlated with maternal baseline BMI (r=0.53, P=0.001) and waist circumference (r=0.51, P=0.003) (see Figure 3-3). The assessment of inflammatory mediators revealed a relationship of the decreased plasma CXCL8 levels over time to the baseline waist (r=-0.42, P=0.017) and change in waist (r=0.42, P=0.021) measurements. Although decreased levels of CCL3 and CCL4 failed to become statistically significant from baseline up to day 45, the change in plasma CCL3 was associated with the baseline waist (r=-0.58, P=0.001) and change in waist (r=0.38, P=0.040) measurements. Over time, the change in plasma CCL4 related to baseline maternal waist circumference (r=-0.47, P=0.007). There was no observable association of maternal obesity measures (BMI or waist) with early changes in plasma TG, TC, glucose, CRP, IL-6, CCL2, CCL11, PAI-1 and PAI-2 levels in women that had pregnancy success.

Table 3-5: **Effect of obesity on metabolic and inflammatory parameters**.

Δ in parameters	Baseline BMI	Baseline Waist	Δ in BMI	Δ in Waist
Hormone				
Δ hCG (IU/L)	r=-0.07, P=0.70	r=0.06, P=0.75	r=-0.17, P=0.38	r=-0.10, P=0.60
Δ Insulin (mU/L)	r=0.14, P=0.42	r=0.37, **P**=0.040	r=0.35, P=0.062	r=-0.14, P=0.48
Metabolism				
Δ TG (mmol/L)	r=0.07, P=0.68	r=-0.16, P=0.39	r=0.06, P=0.76	r=-0.09, P=0.65
Δ TC (mmol/L)	r=0.32, P=0.066	r=0.26, P=0.16	r=0.18, P=0.33	r=-0.10, P=0.59
Δ HDL-C (mmol/L)	r=0.53, **P**=0.001	r=0.51, **P**=0.003	r=-0.08, P=0.67	r=0.16, P=0.40
Δ NEFA (mmol/L)	r=0.38, P=0.078	r=0.30, P=0.18	r=-0.33, P=0.17	r=-0.37, P=0.11
Δ Glucose (mmol/L)	r=-0.12, P=0.51	r=0.02, P=0.93	r=0.10, P=0.63	r=0.03, P=0.88
Δ HOMA	r=0.15, P=0.40	r=0.39, **P**=0.028	r=0.32, P=0.90	r=-0.12, P=0.55
Inflammation				
Δ CRP (mg/L)	r=0.27, P=0.12	r=0.18, P=0.32	r=0.05, P=0.79	r=-0.06, P=0.74
Δ IL-6 (pg/mL)	r=-0.17, P=0.35	r=0.08, P=0.68	r=0.27, P=0.15	r=-0.04, P=0.85
Δ CXCL8 (pg/mL)	r=-0.10, P=0.58	r=-0.42, **P**=0.017	r=0.09, P=0.64	r=0.42, **P**=0.021
Δ CCL2 (pg/mL)	r=-0.06, P=0.72	r=0.04, P=0.83	r=0.10, P=0.59	r=-0.07, P=0.71
Δ CCL3 (pg/mL)	r=-0.28, P=0.11	r=-0.58, **P**=0.001	r=0.08, P=0.70	r=0.38, **P**=0.040
Δ CCL4 (pg/mL)	r=-0.17, P=0.33	r=-0.47, **P**=0.007	r=0.12, P=0.52	r=0.27, P=0.14
Δ CCL11 (pg/mL)	r=-0.12, P=0.50	r=-0.21, P=0.24	r=0.01, P=0.95	r=0.10, P=0.63
Δ PAI-1 (ng/mL)	r=-0.15, P=0.41	r=-0.03, P=0.85	r=0.02, P=0.94	r=-0.03, P=0.87
Δ PAI-2 (ng/mL)	r=-0.21, P=0.24	r=-0.23, P=0.21	r=-0.10, P=0.64	r=0.04, P=0.84

Δ represents change, r represents coefficient of correlation and P represents P-value.

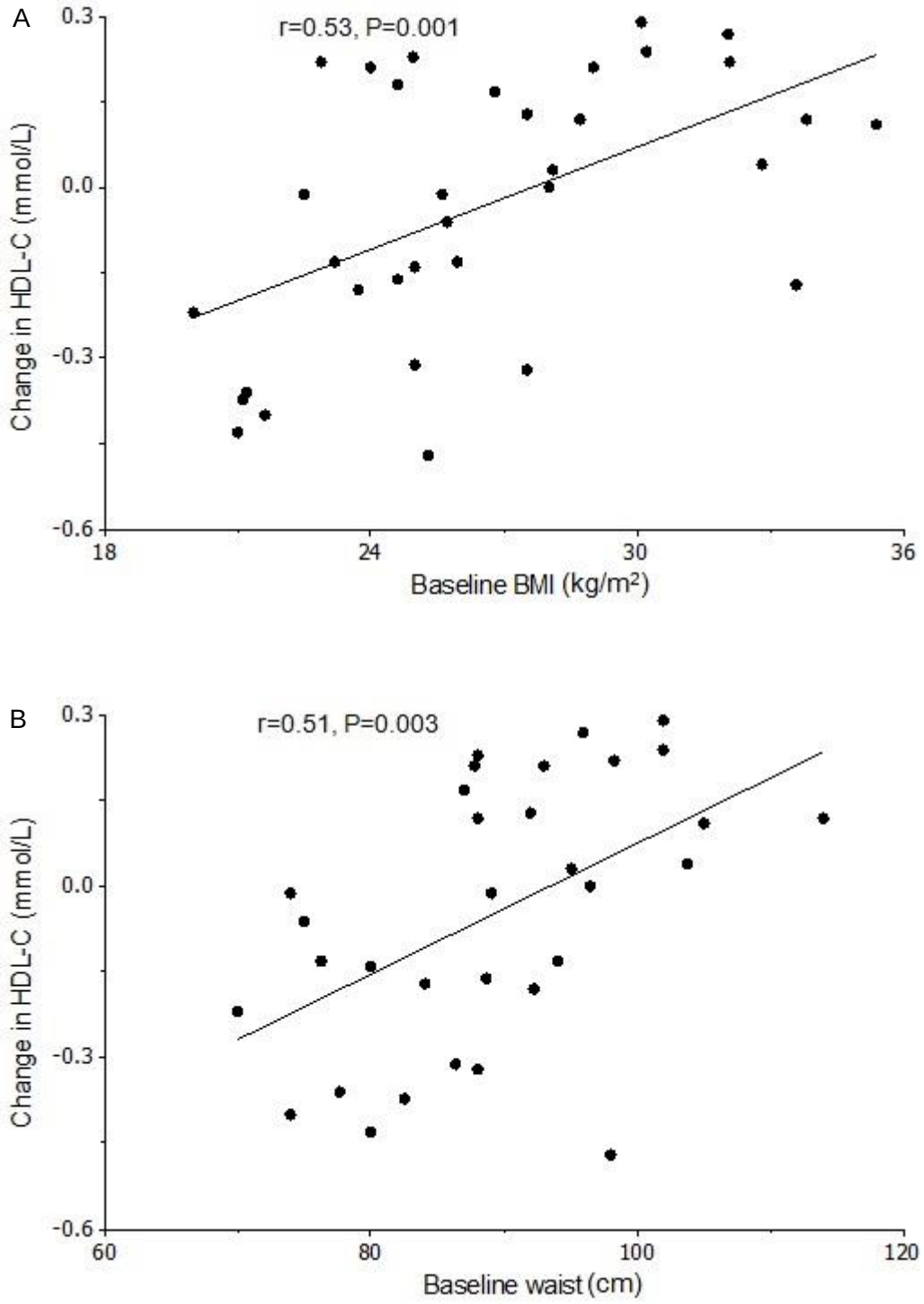


Figure 3-3: **Correlation of change in HDL-C level and baseline BMI (r=0.53, P=0.001) and waist (r=0.51, P=0.003) circumference in women with pregnancy success**.

3.7 Discussion

This cohort study is the first detailed report known to date of the very early changes in metabolic and inflammatory parameters in pregnant women. Use of the ICSI technique was a better predictor of pregnancy success than use of IVF, as shown by the univariate (OR 0.5 (CI 0.2, 1.1) P=0.076) and multivariate (0.4 (0.1, 1.0), P=0.056) analyses. However, none of the other measured parameters of metabolism, inflammation or obesity were associated with pregnancy success. Changes in many of the plasma parameters of metabolism and inflammation were observed in women who became pregnant. The plasma hCG level increased as expected. Overall, there was 654% higher insulin compared to baseline evident by day 45. Plasma lipids, including TG (20%) and HDL-C (10%), were significantly decreased by day 18 of gestation but had rebounded back to their former levels by day 45 of gestation. TC decreased by 51% by day 18 and remained at the lower levels by the end of the study period. HOMA, a measure of insulin resistance, showed increased by 179% by day 45. Plasma CRP (263%) and PAI-2 (1680%) levels were significantly higher, whereas CXCL8 (256%), CCL2 (6700%), CCL11 (2900%) and PAI-1 (1330%) levels were significantly lower by day 45. Baseline maternal obesity positively influenced the changes in plasma insulin, plasma HDL-C and HOMA while CXCL8, CCL3 and CCL4 levels were influenced negatively.

A variety of the participants' demographics and metabolic and inflammatory parameters were tested for their ability to predict pregnancy success. The only treatment technique employed which predicted success was ICSI, which is commonly associated with male factor infertility. Previous comparisons of implantation rate, according to a fertilisation protocol, found no difference in implantation rate of IVF compared to ICSI (Walls et al. 2012). The study detailed in this chapter showed that ICSI is predictive of pregnancy success at day 45 of gestation. ICSI has previously been shown to have higher fertilisation rates than IVF (Rosen et al. 2010; Fang et al. 2012). Lintsen et al. find that the most important predictors of pregnancy success rate in assisted conception are age and ICSI (Lintsen et al. 2007), supporting the findings of this current study. The ICSI treatment may increase the pregnancy rate by overcoming the difficulty of sperm penetrating the zona pellucida. In contrast, IVF women are more likely to have female factor infertility such as PCOS, inflammation, tubal blockage and endometriosis which will result in difficulties at additional stages of reproduction rather than just at fertilisation. However, caution is needed when interpreting these results, as ICSI may increase the risk (to an unknown extent) of genetic anomalies that otherwise would not have been transmitted. Thus in the current study, it seems feasible that male factor strongly predetermined pregnancy success.

Obesity is thought to be associated with decreased early pregnancy success. This is because women with a BMI higher than 36 kg/m^2 (Thum et al. 2007) have an increased risk of recurrent miscarriage (Metwally et al. 2010). Vilarino et al. found that BMI was not a good parameter for predicting a successful IVF (Vilarino et al. 2011). In the current study, maternal obesity at baseline, measured either by BMI or waist circumference, failed to predict pregnancy success. The obesity measured by BMI and waist circumference in the pregnant group (26.5 (4.1) vs 25.6 (4.6) kg/m^2, P=0.31) and (88.9 (10.5) vs 85.6 (11.7) cm, P=0.13) was not significant compared to that of the non-pregnant group. This is not surprising, as in the IVF clinic in Glasgow, the BMI range for women being treated is between greater than 18kg/m^2 and lower than 30kg/m^2. The lack of significance in the current study may perhaps indicate that the higher number of obese women may have been a better predictor. Thus, the issue that the sample size being under-powered may have affected the impact of the obesity measure on the results. There is also suggestion that the effect of obesity on pregnancy occurs after the first 45 days of gestation. Therefore, obesity failed to predict pregnancy success in early in gestation, despite influencing the early changes by increasing insulin, HDL-C and HOMA (insulin resistance) as well as significantly decreasing CXCL8, CCL3 and CCL4 levels. Overall, in this study, obesity does not influence pregnancy success. Nonetheless, it is important to highlight that this could be due to the study being under-powered and to the narrow BMI range of participants.

In those women who became pregnant, the plasma hCG (which is specifically secreted by the placenta) increased over the course of the study, as would be expected, with levels significantly higher than baseline by day 45. Urinary hCG, detected by 2 weeks' post implantation, reached a peak at around 8 weeks' gestation (Picciano 2003). This early hCG detection is classically used as a pregnancy test. An increase in hCG is necessary in order to rescue the corpus luteum and continue maintenance of progesterone secretion (Baird et al. 2003) before that function is taken over by the placenta. hCG is also synthesised as early as day 7 of gestation by the blastocyst (Lopata and Hay 1989; Bansal et al. 2012).

The increases in insulin, a hormone implicated in the metabolic milieu, and in HOMA (a measure of insulin resistance) that were evident by day 45 were of interest. Catalano et al. report insulin secretion increased from prepregnancy up to 12-14 weeks' gestation (Catalano et al. 1993). While increasing insulin was evident with advancing gestation, there was a subsequent decrease in insulin sensitivity (Catalano et al. 1991; Catalano et al. 1993). Results of the current study may indicate that the increase in insulin already evident by day 45 of gestation as part of the adaptive response early in gestation. The rising HOMA supports the view of increasing insulin resistance noticeable as early as day

45 of gestation. This occurrence at this very early (anabolic) stage of pregnancy is an indication that the maternal fat accumulation is supported by the action of insulin (Huda et al. 2009). The insulin resistance early in gestation may be due to changes in energy expenditure (Catalano et al. 1998). Therefore, considering the population of women studied here, it is clear that insulin resistance did not influence pregnancy success as anticipated, despite insulin resistance increasing with gestation.

That the early changes in plasma TG concentrations decreased by day 18 and that TG concentration rebounded by day 45 of gestation is revealing. In previous observations, there is a gestational rise in TG as early as week 8 of gestation (Ordovas et al. 1984; Lippi et al. 2007). A novelty of the current data is the first decrease in TG as early as day 18 of gestation, before beginning to increase as pregnancy advances. This decline in TG plasma concentration may result from either increased utilisation and/or reduced rates of synthesis in the liver and small intestine (Grummer and Carroll 1988). Early mobilisation of TG by day 7 was found to be a requirement for supplying energy to the implanting embryo in animal models (Ferguson and Leese 2006). It is possible that, likewise in humans, energy requirements lead to increased utilisation of TG, leading to reduced plasma levels. Perhaps pregnancy adaptation in this study (at this early gestation) may involve increased oxidation of stored TG in the adipose tissue. Indirect calorimetry would perhaps be beneficial to determining early variations in energy expenditure in the population studied. Another possible explanation for reduced TG may be temporary changes of homeostatic balance from the non-pregnant to the pregnant state in the mother, as the systemic balance tries to be restored. Perhaps these temporary changes are due to the presence of the conceptus and the maternal nutrient needs. The metabolic status, in addition to pregnancy-related endocrine changes (including progesterone, oestrogen and cortisol), favours lipogenesis and TG deposition (Ryan and Enns 1988; Huda et al. 2009). Thus, to maintain the homeostatic balance, increased utilisation of TG is necessary in pregnant women, with the result of reduced plasma TG levels in the circulation. Stable isotope tracer studies, examining the earliest changes in TG levels, may help validate the anabolic and catabolic processes. Most women, however, may decline to take part in such procedures in view of the associated risk to the developing fetus.

TC and HDL-C concentrations decreased by day 18, and HDL-C (but not TC levels) recovered by day 45 of gestation. There are reports of a gestational rise in TC and HDL-C as early as week 8 of gestation (Ordovas et al. 1984; Lippi et al. 2007). This rise also implies, as currently shown, that TC and HDL-C levels at first decreased as early as day 18 of gestation, before beginning to increase as pregnancy advanced. That TC concentrations were observed to fail to completely recover highlights a role for lipoproteins in early pregnancy adaptation. The early decrease in TC and HDL-C concentrations in

early pregnancy may be due to increased utilisation for steroidogenesis and cell membrane formation (Guibourdenche et al. 2009; Hu et al. 2010). As stated above, the majority of blood cholesterol (e.g. LDL-C and HDL-C) is transported by LDL and HDL, depending on the animal species (Grummer and Carroll 1988). In the steroidogenic tissues (such as corpus luteum), cholesterol is moved from cholesterol-poor OMM into IMM, where it is converted to pregnenolone, the precursor of all steroid hormones. The progesterone secreted by the corpus luteum acts to maintain pregnancy, until the placenta, which takes over the hormone synthesis, develops and becomes functional as pregnancy advances. The role of progesterone is to downregulate excess immune responses during trophoblast invasion (Paria et al. 2002). Also, the cholesterol not needed for steroid hormone synthesis is transported and utilised for cellular membrane biogenesis (Lange et al. 2004). Cholesterol is extensively involved in neurone formation (Chen et al. 2013; Yu and Lieberman 2013) and SHH signal activation and propagation, which is paramount for developing the central nervous system (Woollett 2005). Usually, the cholesterol used for cell membrane biosynthesis, play a role in the maintenance of fluidity in the circulatory system. Thus, the requirements of cholesterol for steroidogenesis and other functions (in the conceptus and mother) may explain the early decline of the TC and HDL-C parameters that begin to recover after day 18 gestation.

The data on systemic inflammation early in gestation presented in this chapter complement other studies' results. The earlier data has not really been changed, but the current study certainly makes the whole picture more detailed. An increase in CRP, which was noticeable from day 10 and significant by day 45 of gestation, was observed. These data support the concept of the development of a low grade inflammatory response as part of healthy adaptation to pregnancy. Other research has observed raised plasma CRP levels of similar magnitude by day 5-7 of gestation (Almagor et al. 2004) or by 4 weeks' gestation (Sacks et al. 2004) in successful pregnancies in women with assisted conception. This is a low grade inflammatory response, different from the acute phase response, as the CRP level remains below 5mg/L in the circulation (Pepys and Hirschfield 2003). This response is likely to occur because of the presence of the foreign conceptus, and tissue remodelling of the endometrial epithelium and decidua during implantation, with subsequent trophoblast invasion. This presence elicits the active immune response, required in order to carry out uterine remodelling with removal of cellular debris. CRP has been shown to upregulate MMP-1 secretion and mRNA expression, but not to upregulate TIMP-1 in U937 cells and human monocyte-derived macrophages (Williams et al. 2004). This upregulation may suggest an active role for CRP in promoting matrix degradation of the uterine wall, as the embryo implants and throughout placentation.

The low phase of the antiinflammatory state is driven by the actions of peripheral blood lymphocytes that secrete Th2 cytokines (and, to a lesser extent, Th1 cytokines) in pregnancy and prevent pathologic pregnancies (Marzi et al. 1996). In the study of Marzi et al., IL-2 and INF-γ decreased, accompanied by increased production of IL-4 and IL-10 in normal pregnancy compared to women with spontaneous abortion. This is an indication that the balance between pro- and antiinflammatory response is paramount and may have begun early in gestation. The trophoblast, the decidua, and the endometrium act as sources of Th2 cytokines, which are associated with suppression of cell-mediated immunity (Wegmann et al. 1993; Krasnow et al. 1996). It was observed that concentrations of CXCL8, CCL2 and CCL11 were significantly reduced by day 45 of gestation in the women that became pregnant. It is possible that these chemokines play an important part in chemotaxis of leukocytes to the uterine wall for tissue degeneration and remodelling. Consequently, chemotaxis of leukocytes (chemokine) contributes to the Th1/Th2 pathway switch, causing dampening of cell-mediated immune response. This is due to the higher expression of chemokines in the endometrium (Zhang et al. 2000; Ulukus et al. 2005). An additional factor leading to reduced levels of these chemokines is the need for dampening of the maternal inflammatory response, which possibly assists in preventing excessive embryo degradation. The reduced levels of these chemokines in turn reduce trophoblast invasion, which avoids compromised placenta and adverse outcomes. As widely reported, shallow and restricted trophoblast invasion may lead to PE and IUGR. In contrast, excessive trophoblast invasion may result in the placenta attaching on the myometrium (accreta) or into the myometrium (increta) or penetrating completely through the myometrium (percreta) (Brahma et al. 2007). Higher expression of CCL2 at 8-10 weeks' gestation in EVT (compared to that of the villous cytotrophoblast cells (Katsuhiko et al. 2010) in decidual endothelial cell culture of women that underwent elective pregnancy termination at 8-12 weeks of gestation), supports uterine NK cell recruitment in early gestation (Carlino et al. 2008). Madigan et al. find a strong expression of D6 in trophoblast-derived cells in human placenta, decidua and gestational membranes throughout pregnancy (Madigan et al. 2010). D6 binding to chemokines disrupts and prevents signalling transmission. In this report, Madigan et al. suggest that chemokine change probably happens to ensure fetal survival, by preventing fetal allograft rejection through suppressing excess immune response in maternal circulation. All these reports suggest that it is possible that the plasma CXCL8, CCL2 and CCL11 levels decreased due to their role for implantation success. Perhaps increased synthesis of these chemokines in uterine tissue is needed for the recruitment of uterine leukocytes, including neutrophils, monocytes (for tissue macrophages) and eosinophils possibly from the maternal circulation (Katsuhiko et al. 2010; Hannan et al. 2011; Chau et al. 2013). There is also a possibility that the action of D6 in uterine tissue results in its decreased level in the circulation. In spite of no observable change in plasma CCL3 and CCL4 by day 45, the

baseline change of obesity is associated with a decline in CXCL8, CCL3 and CCL4 levels. This suggests that downregulated activity of inflammation (systemic immune response) had begun to take effect by day 45, switching to the Th2 (type 2) cytokine productions at the maternal-fetal interface, in order to inhibit Th1 (type 1) and improve the chance of fetal survival (Wegmann et al. 1993; Madigan et al. 2010). This switch of the immune response from a Th1 type to a Th2 type allows the semi-allographic tissue of the placenta and fetus to be tolerated.

There is evidence that plasminogen activation inhibitors are pivotal for optimal implantation. PAI-1 and -2 proteins also play a role in dampening excessive degradative activity of the trophectoderm as the embryo embeds into the uterine endometrium (Harvey et al. 1995; Ulisse et al. 2009). This action protects the embryo against the maternal immune response. In the current study, a progressive decline of PAI-1 up to day 45 of gestation was found. It is most likely that during early pregnancy PAI-1 is involved extensively in dampening of immune response control. This is supported by the observation that PAI-1 is a more efficient inhibitor of plasminogen activator than PAI-2 (Jorgensen et al. 1987). Reith et al. highlight that by week 14 of gestation, PAI-1 levels are above the zero threshold (Reith et al. 1993). This implies that plasma PAI-1 concentration has to decline very early in gestation before recovering as gestation advances. Although PAI-2 possibly plays a role in immune control, it seems that the elevated PAI-2 observed by day 28 of gestation is an indication of placental formation, and may be involved in the protection of the developing embryo.

A summary of proposed very early changes of metabolic and inflammatory parameters by day 45 of gestation is shown in Figure 3-4.

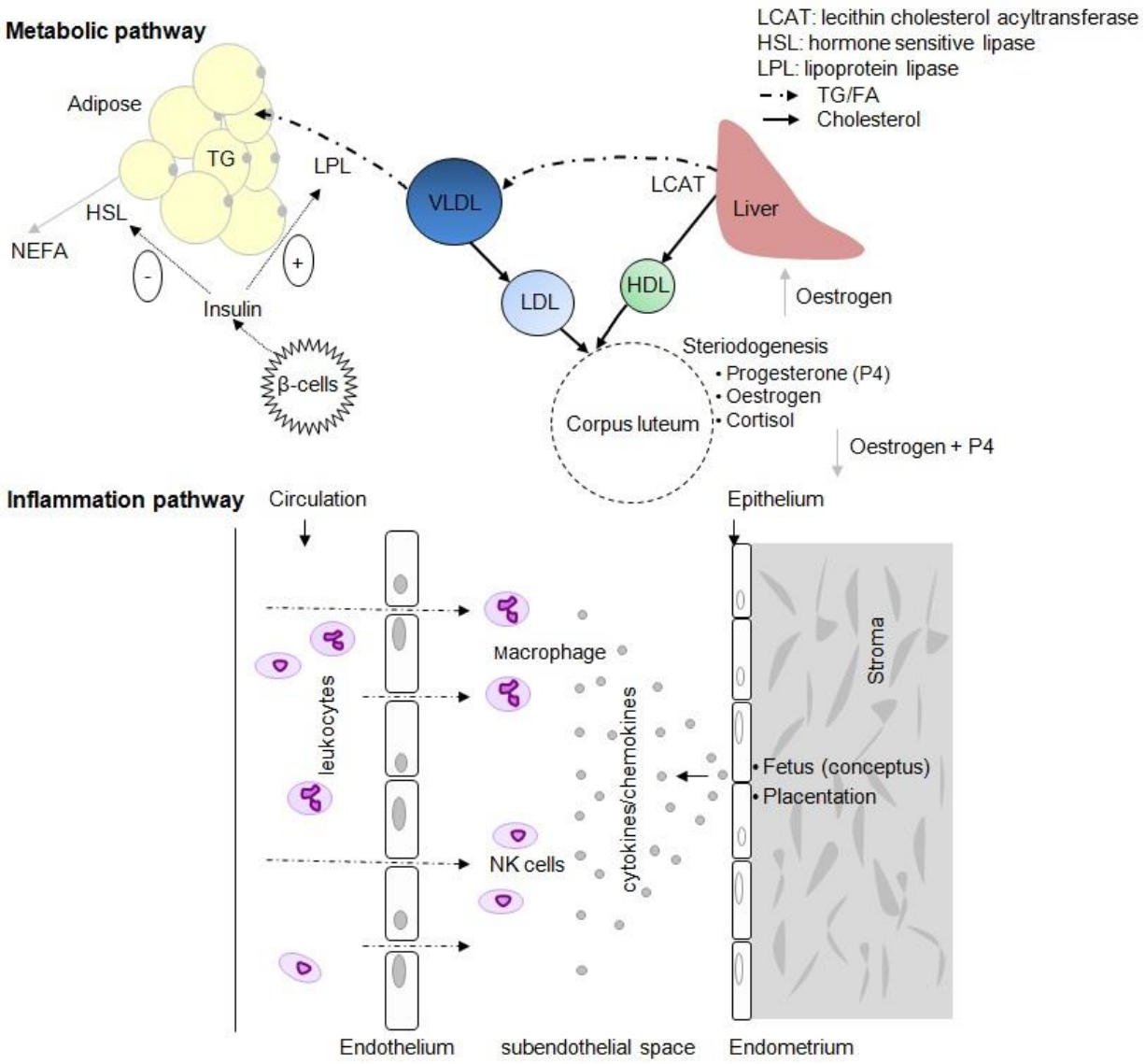


Figure 3-4: **Schematic representation of metabolic and inflammatory pathways involved in the very early changes of metabolic and inflammatory parameters in successful pregnancies**

The results presented in the current report have highlighted the need to have a fresh look at ways of improving pregnancy success with natural or assisted conception. Whether the changes are caused by simply being pregnant, diet or maternal obesity through fat deposit is difficult to deduce completely. It is most likely that a low-powered population could be blamed for the absence of prediction of pregnancy success by parameters or even by obesity. That ICSI was a predictor of pregnancy has been shown, and has been well established (Rosen et al. 2010; Komsky-Elbaz et al. 2013). However, the current data confirmed ICSI as a predictor of pregnancy success in assisted conception. Inevitably, not measuring the specific metabolic and inflammatory parameters which change early in gestation, and may perhaps predict pregnancy success, also adds to the weakness of the current data. Although not part of the data presented in this thesis, investigation results of

the plasma and erythrocyte fat composition using the same samples and dataset showed percentage of saturated fatty acid in erythrocyte as independently predictive of whether a woman became pregnant (unpublished data).

Among the strengths of the study in this chapter is the use of a population which had a natural menstrual cycle close to the normal physiology of pregnancy. Data obtained may therefore represent that which may be found in a natural conception population. Nonetheless, results are not necessarily transferable to other races and ethnicities, as recruited participants were mainly of Caucasian origin. Missing data, unavoidable for retrospective and prospective studies, also adds to the study's weakness. Longitudinal studies that collect data on a set of subjects repeatedly over time are subject to attrition. Also, some subjects provided only clinic sample visits, and vital data were not recorded in the patient's hospital notes. It would have been useful to carry out measurement of pregnancy hormones such as oestrogen, progesterone and hPL to assess whether any of these hormones had a temporal relationship with the very early metabolite status and inflammatory mediator changes. It is recommended that future studies utilise the cryo samples under study (stored at -80°C) so that other important parameters can be assayed later, such as measurement of TNF-α, leptin and adiponectin, which are adipose-derived adipocytokines (Ouchi et al. 2011). The role of the endocrine hormone in the change in some metabolic and inflammatory parameters may also be ascertained in future. Indirect calorimetry may have helped quantitate maternal energy in the studied population. With respect to lipids, temporal loss of homeostatic balance in the mother (as systemic balance tries to be restored as pregnancy advances) can also be validated, using stable isotope tracer studies.

In summary, the data show that ICSI was a better predictor of pregnancy success than other type of fertility treatment. This is usually because no other problems are associated with maternal environment when the cause is male factor. None of the measured parameters of metabolism, inflammation or obesity were associated with pregnancy success. However, very early changes were observed. These included higher hCG, insulin (654%) and HOMA (179%) by day 45; and decreased TG (20%), HDL-C (10%) and TC (51%) by day 18 of gestation. TG and HDL-C rebounded back to their former levels by day 45 but TC did not. Higher CRP (263%) and PAI-2 (1680%) levels were observed, whereas CXCL8 (256%), CCL2 (6700%), CCL11 (2900%) and PAI-1 (1330%) levels were lower by day 45. The baseline maternal obesity influenced the changes in plasma insulin, HDL-C and HOMA, as all of these increased, while CXCL8, CCL3 and CCL4 all decreased. Obesity and insulin resistance had no role in pregnancy success in this study. These data provide a new platform for future studies in understanding the importance of very early metabolic and inflammatory parameters in pregnancy.

4 Fetal cord metabolic and inflammatory parameters as a reflection of maternal parameters: a potential role of upregulated placental cholesterol transporters

4.1 Introduction

Fetal nutrient demand for growth and development contributes to the transfer of maternal metabolic and inflammatory parameters (Chandler-Laney et al. 2011). Assessing the metabolic and inflammatory pathways *in utero* remains problematic. This has contributed to the poor availability of data on the fetus; most data are obtained at term, where the offspring and adult differ profoundly. Data from normal pregnancy has shown umbilical cord blood lipid levels (including TG, cholesterol, LDL-C and HDL-C) and insulin levels to be lower than adult levels (Averna et al. 1991; Kaser et al. 2001; Tea et al. 2012). Tea et al. report that level of cord blood glucose exceeds that of the maternal plasma. Data about cord inflammatory mediators in gestation remain debatable. A study that used a sample of 261 umbilical cords blood found no difference in the levels of CRP, TNF-α and IL-1β between those babies that developed early neonatal sepsis with positive blood culture and those with non-infectious perinatal diseases and randomly selected controls (Santana et al. 2001). In other reports, newborns born earlier (preterm) than term counterparts had comparatively elevated IL-4, IL-6, IL-10, CRP, TNF-α, CCL2, CCL3, CCL4 and CXCL8 levels (Matoba et al. 2009; Mestan et al. 2009).

The biochemical mechanisms involved in the transfer of maternal nutrients to the fetus include passive, facilitated and active diffusion (Economides et al. 1989), as well as endocytosis and exocytosis (Sibley et al. 1997). These mechanisms have been proposed to be involved in the transfer of maternal-fetal metabolites. Data highlight the relationship between maternal and fetal lipids, particularly cholesterol in pregnancy. In the fetus, HDL is the major cholesterol carrier (Nagasaka et al. 2002). In their studies, Rodie et al. showed that maternal TC ($r=0.35$, $P=0.03$) was associated with fetal HDL-C level in healthy pregnant groups (Rodie et al. 2004). A report by Napoli et al. shows enhanced fetal atherosclerotic plaque lesion formation, containing cholesterol, in the fetuses of mothers with hypercholesterolaemia during pregnancy (Napoli et al. 1997). In SLOS although fetuses have very low plasma cholesterol levels (Jenkins et al. 2008) due to defective 7-dehydrocholesterol reductase that reduced ability to convert 7-dehydrocholesterol to cholesterol (Tint et al. 1994) they survive and thrive. This is because there is adequate cholesterol transport from the mother across the placenta. Jenkins et al. report increased placental cholesterol effluxed by HDL from SLOS patients in comparison to the unaffected controls (Jenkins et al. 2008). These data suggest a

significant route of cholesterol transport across the placenta to the fetal compartment, requiring the upregulation of important cholesterol transporters.

Insulin sensitivity involves unique and complex biological phenomena that provide support for fetal growth and development. It is affected not only by change in the maternal physiology and metabolism in gestation but also by placental metabolism. An end product of altered metabolic and inflammatory parameters is insulin resistance, a pathological state where insulin action is impaired in the target tissue (Courten et al. 1997; Yadav et al. 2013). Insulin resistance increases as pregnancy advances to term. Maternal-fetal medical research has catalogued the changes in metabolic and inflammatory parameters throughout gestation. In spite of this, much remains unknown about the implications for both maternal and, in particular, fetal health during pregnancy.

It is well documented that normal pregnancy is associated with a maternal gestational rise in cholesterol (Ordovas et al. 1984; Lippi et al. 2007) and CRP (Sacks et al. 2004), and a late increase in TG and IL-6 (Ramsay et al. 2004). Synthesis of Th2 cytokines, such as IL-4 and IL-10, is predominant in normal pregnancy via a shift in Th2/Th1 balance, and regulates homeostasis of the maternal immune response during pregnancy (Raghupathy 1997). These changes are part of the adaptive response associated with pregnancy physiology. In pre-eclamptic pregnancy, a well-studied extreme pregnancy outcome, there are further alterations in these plasma parameters. Available data from these babies born of pre-eclamptic pregnancies shows that fetal lipids, including TC and TG (Rodie et al. 2004; Catarino et al. 2008), are altered compared with controls. Bujold et al. report evidence of high numbers of umbilical cord blood NK cells in PE versus the control group (Bujold et al. 2003), whereas no difference in cord CRP level was observed in babies born of women suffering from PE compared to controls (Braekke et al. 2005). In pre-eclamptic mothers, there is markedly raised maternal cholesterolaemia, leptinaemia and systemic inflammatory mediators, and lower adiponectin (Sacks et al. 1998; Ramsay et al. 2004; Naruse et al. 2005). There is also evidence of activation of maternal plasma monocytes (Luppi and DeLoia 2006) and neutrophils (Sabatier et al. 2000), as well as elevated CRP (Tjoa et al. 2003; Deveci et al. 2009) and TNF-α (Johnson et al. 2002; Laskowska et al. 2006) in PE. Lipoproteins (Sattar et al. 1997), lipid peroxides (Hubel et al. 1996; Poranen et al. 1996) and fatty acids (Endresen et al. 1994) have been proposed as contributing to the inflammatory response in PE and subsequent maternal endothelial cell damage (Davidge et al. 1996).

With regards to cholesterol, it plays an important physiological role, especially as it is the starting substrate for steroidogenesis, membrane biosynthesis, cellular signalling and the maintenance of membrane fluidity (Hu et al. 2010). These roles highlight the importance

of cholesterol during gestation. Thus, for healthy pregnancy optimal transport mechanisms for cholesterol across the maternal-fetal interface, as illustrated in Figure 4-1, are required.

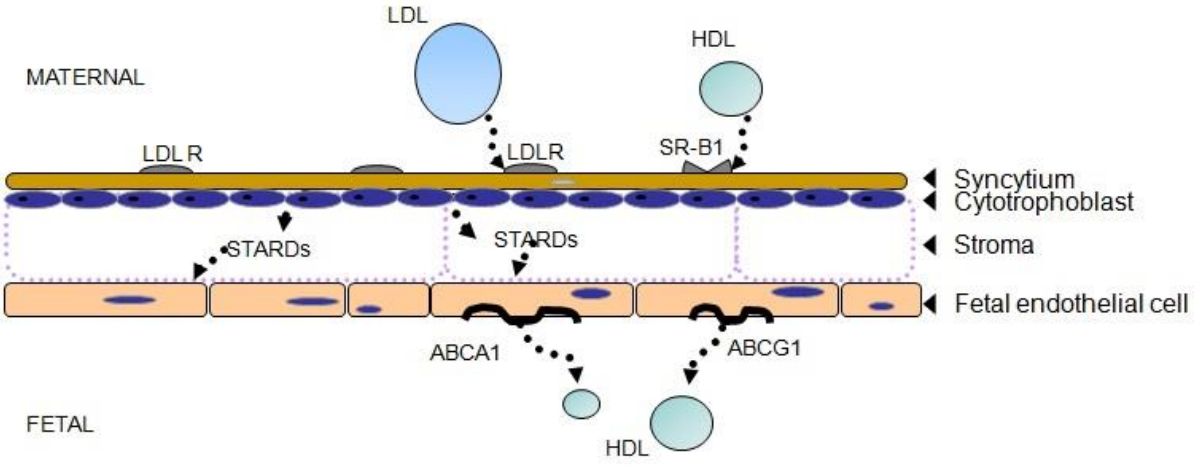


Figure 4-1: **Schematic pathway of cholesterol transport at the maternal-fetal interface of the chorionic core of human placental tissue**. LDL particle carrying LDL-C interacts initially with LDLR via receptor-mediated endocytosis on the syncytiotrophoblast. On entering LDL/LDLR complex dissociates in the endosome (a low pH) and free cholesterol is readily converted to cholesteryl ester by LCAT. Free cholesterol, by action of STAR-related lipid transfer domains mediators of intracellular lipid metabolism; is transferred cross the maternal-fetal interface and passed on to the placental/fetal endothelial cells, where it is effluxed via ATP-binding cassette transporter A1 (ABCA1) and ABCG1 to lipid-poor apo A-I, apo E or HDL (Stefulj et al. 2009) in the fetal compartment.

Maternal cholesterol destined for the fetus must cross the placental villous trophoblast, stroma and fetal endothelial cells. LDL, the most abundant cholesterol-carrying lipoprotein in human plasma, interacts with LDLR on the cell membrane via the apo B-100 component on LDL (Williams et al. 1992) and is internalised by receptor-mediated endocytosis (Brown and Goldstein 1986; Malassine et al. 1987). Often, but not always, the internalised LDL-LDLR complex is dissociated in endosomes where there is a low pH (Zhao and Michaely 2009), liberating LDL. This dissociation allows recycling of LDLR to the cell surface to bind more extracellular/maternal LDL to pass on to the fetus. The released LDL fuses with lysosomes, where the lipoprotein particle is degraded and cholesterol is salvaged for cellular use. Hydrolysis of free cholesterol is undertaken by lysosomal acid lipase (also called cholesteryl ester hydrolase and/or HSL) (Goldstein et al. 1975). At this point, cytoplasmic cholesterol becomes incorporated into vesicles, forming a cellular pool vital for plasma membrane homeostasis. HSL catalyses the hydrolysis of cholesteryl esters in steroidogenic tissue (Holm 2003; Manna et al. 2013), supplying cholesterol for lipoprotein transport which can be delivered to the fetus.

An intracellular protein carrier identified by Epstein et al. (Epstein and Orme-Johnson 1991) and purified by Clark et al. (Clark et al. 1994) was proposed to regulate intracellular cytosolic cholesterol homeostasis (Hao et al. 2002). STAR (Kallen et al. 1998), also called STARD1, is a hormone-sensitive 37kDa protein that contains an N-terminal mitochondrial signal sequence (Jefcoate 2002) which has a cholesterol transfer function (Artemenko et al. 2001). It has been demonstrated that STAR mediates cholesterol delivery to the inner mitochondrial membrane (Ferguson 1963; Garren et al. 1965; Strauss et al. 2003), the site of steroidogenesis within the cytochrome P450scc enzyme (Lin et al. 1995). This transfer of cholesterol, regardless of the source, from outer to inner mitochondria is the rate-limiting step in steroidogenesis (Jefcoate 2002). STARD1 protein is a member of a homologous protein family of 200 to 210 amino acid length proteins known as STAR-related lipid transfer (START) domains (Ponting and Aravind 1999). Fifteen distinct START domain proteins (STARD1-STARD15) have been reported in the human genome (Soccio et al. 2002; Soccio and Breslow 2003; Alpy and Tomasetto 2005) and are implicated in non-vesicular lipid transport, cell signalling and lipid metabolism (Alpy and Tomasetto 2005). STARD1 (Kallen et al. 1998), STARD3 (metastatic lymph node 64-MLN64) (Tomasetto et al. 1995), STARD4, STARD5 and STARD6 (Soccio et al. 2002) are specifically for intracellular cholesterol transfer.

HDL, in addition to mobilising cholesterol across LDLR, is also extensively involved in the removal of free cholesterol from peripheral tissue. The cholesterol becomes esterified by lecithin:cholesterol acyltransferase (LCAT) as well as ACAT into cholesteryl ester (Spector and Haynes 2007). Spector and Haynes note that LCAT functions in the plasma and forms cholesteryl ester in HDL by transferring polyunsaturated fatty acid from phosphatidylcholine to cholesterol. The ACAT function intracellularly, using fatty acid from acyl-CoA and forming cholesteryl ester enriched in monounsaturated fatty acid (Spector and Haynes 2007). Cholesterol efflux from cells to HDL can occur through the interaction of HDL with SR-B1 (Acton et al. 1996), a membrane-bound receptor detected in the placenta (Woollett 2005). SR-B1 has high selective affinity of HDL, leading to mobilisation of cholesteryl ester to the liver. However, a candidate protein ABCA1, reported as malfunctioning in Tangier disease patients (Bodzioch et al. 1999), has been shown to play a role in cellular cholesterol efflux from the cytoplasm. ABCA1 binds HDL-C via apo A-1 (Cavelier et al. 2006), a major protein constituent of HDL. In Tangier disease patients, nascent apo A-1 on HDL is unable to bind to the cell membrane and cholesterol efflux is inhibited (Young and Fielding 1999). This is due to a mutation in ABCA1 (Luciani et al. 1994; Rust et al. 1998). Consequently, Tangier disease homozygotes have a low HDL (Asztalos et al. 2001). Heterozygotes, on the other hand, exhibit reduced large α-1 and α-2 HDL particles (Brousseau et al. 2000; Asztalos et al. 2001) that result in only 50% normal cellular cholesterol efflux. Thus, mutation of ABCA1 results in accumulation of free

cholesterol and cholesteryl ester in various tissues, such as the reticuloendothelial system that clinically manifests as hepatosplenomegaly, hyperplastic orange tonsil, lymphadenopathy thymus and peripheral neuropathy.

Fatty acid translocase (also known as CD36) and apo E are other lipid transporters thought to be implicated at the maternal-fetal interface. CD36 is a multigand membrane-associated glycoprotein of 78 to 88kDa (Ge and Elghetany 2005; McDermott-Roe et al. 2008). Ge and Elghetany detected CD36 expression in monocyte, skeletal muscle, adipose tissue, the small intestine, the endothelial capillary and platelets. A defective CD36 gene impairs fatty acid metabolism (Rac et al. 2007) and this impairment is associated with a developing dyslipidaemic state. The CD36 ligands include oxLDLs (McDermott-Roe et al. 2008), collagens, thrombospondin, long-chain fatty acids, plasmodium falciparum parasite infected erythrocytes (Fonager et al. 2012) and β-amyloids. CD36 deficiency is linked to abnormal long-chain fatty acid uptake and metabolism (Yoshizumi et al. 2000). In CD36 $^{-/-}$ mice, there was reduced fatty acid and cholesterol uptake in the proximal but not the distal small intestine compared to wild-type (Nassir et al. 2007). Apo E plays a role in transport of lipoproteins via apo E receptor uptake (Kim et al. 1996). There is a suggestion that apo E plays a role in the maintenance of plasma cholesterol balance between mother and the developing fetus. A higher plasma apo E level was observed in the cord blood than that of normal adults (58.1 vs 35.8mg/mL) (Blum et al. 1985); another study showed almost similar levels in cord and adult sera (Nagasaka et al. 2002) but the character and metabolism of HDL in the fetus, notably of apo E-rich HDL, was to a great extent different from those in adults.

The transport of molecules, e.g. lipids such as cholesterol, across the maternal-fetal interface is driven by the transporting function of the placenta in addition to the fetal demand for optimal growth and development; the rate of this transport is driven by the activity of the regulatory proteins. ABCA1 is a recognised downstream target of the transcriptional regulation of liver x receptor-alpha (LXR-α). LXRs plays a major role in regulating the metabolism of cholesterol, as in the level of uptake and secretion with two LXR isoforms called LXR-α and LXR-β (Peet et al. 1998; Edwards et al. 2002). A physiological ligand of LXR is an oxysterol derivative of oxidised cholesterol from *de novo* synthesis and diet. On accumulation of cholesterol, LXR induces transcription of proteins involved in disposal of cholesterol from cells such as ABCA1 (Plosch et al. 2007). Studies in START domain proteins indicate that they are regulated by LXR targets. The expression of these proteins is stimulated by LXR reporter activity (Soccio et al. 2005), an indicator of the role of LXR in cholesterol transports. STAR genes are also found to be activated by LXR-α (Jefcoate 2006), and the stimulation of STAR expression by LXR-α

activation is explained by the identification of a new LXR-α response element in the STAR promoter (Cummins et al. 2006).

Therefore, to gain insight into events of metabolic and inflammatory pathways in late gestation, it was imperative to at least explore offsprings' at birth, and mothers by the end of gestation; and the processes involved in cholesterol transport across the maternal-fetal interface.

4.2 Hypotheses

In this chapter it is hypothesised that fetal cord metabolic and inflammatory parameters at birth reflect maternal parameters in the third trimester. It is also hypothesised that the reflected offspring hyperlipidaemia, in particular cholesterol, is due to upregulated placental cholesterol transporter gene expression involved in cholesterol transfer across the maternal-fetal interface.

4.3 Aims

This chapter aims to explore the relationship between offspring and maternal metabolic and inflammatory parameters at birth and at the end of the third trimester in mothers with healthy pregnancies and in extreme cases of PE. IUGR groups were used in order to determine the impact of poor placentation in the absence of hypertension and endothelial dysfunction, as seen in PE pregnancies. It also aims to gain insight into the effect of confounding factors of metabolic and inflammatory parameters, and to establish whether the reflected fetal lipidaemic state from the mother is due to lipid transfer molecules (in particular those involved in cholesterol transfer at the maternal-fetal interface).

4.4 Objectives

1. To examine the relationship between offspring and maternal metabolic and inflammatory parameters at birth and the end of the third trimester in healthy pregnancies and pregnancies complicated by PE, as an example of extreme metabolic and inflammatory perturbation. To do this we use an archival database of maternal third trimester and cord blood metabolic and inflammatory parameters in cases of PE (n=29) and IUGR (n=14), alongside BMI-matched controls (n=87 and n=42) respectively.

2. To explore the effect of confounding factors, including maternal smoking status, labour, gestation and/or mode of delivery and gestation at sampling.

3. To investigate protein and RNA detection and subsequent mRNA expression levels of molecules involved in the transport of maternal lipids across the maternal-fetal interface (placentae) of women with PE (n=20) and IUGR (n=9), alongside BMI-matched controls (n=20 and n=9) respectively.

4. To relate the metabolic and inflammatory parameters to placental gene expression of molecules involved in lipid transport in healthy pregnancy and PE.

4.5 Materials and Methods

4.5.1 Subjects

Subjects were recruited from a population of women receiving antenatal clinic care at the Princess Royal Maternity Unit, GRI, after receiving written informed consent. Ethical approval was granted in accordance with the guidelines of the Helsinki Declaration from the North Glasgow University Hospital Trust, National Health Service Glasgow and Clyde Research Ethic Committee, GRI, Scotland. Patients with suspected fetal anomalies likely to be contributory to reduced fetal growth were excluded from the study. None of the women were being treated with medication that interferes with parameters of lipid and carbohydrate metabolism, inflammation or endothelial function.

In this cross-sectional study, healthy pregnancy, pregnancy complicated by PE and pregnancy complicated by IUGR were used to investigate the relationship between offspring metabolic and inflammatory parameters at birth and maternal third trimester. An archival dataset of metabolites of lipid and carbohydrate metabolism and inflammatory response mediators in third trimester mother-baby paired plasma was analysed. The dataset was collected for previous studies investigating lipoprotein metabolism and adipocyte function in healthy and complicated pregnancies using ethical approvals *01OB007* and *06/S0704/14*. PE was defined according to the criteria of the International Society for the Study of Hypertension in Pregnancy, as diastolic blood pressure greater than 110mmHg on one occasion, or exceeding 90mmHg on repeated readings, with proteinuria of ≥0.3g/24 hour, or 2+ proteinuria on dipstick testing, in the absence of renal disease or infection (Rodie et al. 2004). IUGR was identified as having an estimated fetal birthweight less than the fifth (<5th) percentile for gestation with associated oligohydramnios (amniotic fluid index <5) and/or abnormal umbilical artery blood flow on a Doppler ultrasound (Rodie et al. 2004).

4.5.2 Study design and patients' demographics

One woman who had PE was matched with three healthy pregnant women (controls), i.e. one (PE woman) to three (control women). Thus, the total population size of women with PE (n=29) and BMI-matched controls (n=87), and, in a similar fashion, all cases of IUGR (n=14) and BMI-matched controls (n=42), were also explored. The BMI-matching was to account for the influence of maternal obesity. IUGR groups were assessed in order to determine the impact of the placental pathology in the absence of hypertension and endothelial dysfunction, as present in the PE pregnancies. Age and BMI were calculated (Section 3.5.2), and number of previous pregnancies greater than or equal to 24 weeks,

smoking status and Carstair's score and its deprivation category (DEPCAT), a measure of socioeconomic deprivation or affluence in Scotland (Morris and Carstairs 1991), were recorded. Confounding factors (such as smoking status, labouring mothers, gestation at sampling, gestation and/or mode of delivery) were examined in order to determine their impact on the metabolic metabolites and inflammatory response mediators. Fetal sex, placental weight and birthweight centile were also retrieved.

4.5.3 Blood and tissue collection

Blood samples (approximately 50mL) were taken from women in the third trimester of pregnancy by venepuncture. At delivery, the cord venous blood was carefully obtained using a 20mL syringe after clamping both ends of the cord with forceps. The maternal and cord blood were collected into recommended vials, depending on the metabolic and inflammatory parameters to assay. Vacuette® evacuated collection tubes (Greiner Bio-One) contained anticoagulants including 1.8mg/mL EDTA (8mL volume), 18IU/mL heparin (6mL volume), 0.109 mol/L citrate (3.5mL volume) and 2.5mg/mL fluoride oxalate (2mL volume). Blood samples were centrifuged at 3000*g* for 10 min, and the upper plasma layer was aliquoted into 2mL vials and stored at -80°C until assayed.

Gene expression of molecules involved in lipids transport was assessed in the placentae of women with PE (n=20) and IUGR (n=9), together with BMI-matched controls (n=20 and n=9) respectively. At time of delivery, full thickness placental tissue biopsies (approximately 1.5g) were taken at four predetermined areas of placenta distinct from umbilical cord insertion. Portions of umbilical cord (2cm) were also obtained. Half of each placental biopsies and all the umbilical cord biopsy were fixed in 10% buffered formalin (Adams Healthcare, England), embedded in paraffin and stored at RT. The other half placenta biopsies were snap-frozen in liquid nitrogen and then stored at -80°C for RNA and protein isolation.

A gestational series of paraffin-embedded placenta sections was available in the laboratory from an archival collection. The first trimester placentae (6-12 weeks' gestation) (n=2) were obtained from women undergoing suction termination of pregnancy, whereas the second trimester placentae (13-26 weeks' gestation) (n=2) were from those undergoing medical treatment related to their pregnancy. The third trimester placentae (27-40 weeks' gestation) (n=6) were from subjects that had normal vaginal delivery and caesarean sections of healthy pregnancies and cases with extremes of plasma lipid levels (PE and IUGR).

4.5.4 Assays of lipids, glucose, CRP and NEFA

Plasma TG, TC, HDL-C, glucose and CRP were measured in an automated Roche/Hitachi MODULAR P (Roche) analyser by the GRI Department of Biochemistry (see Section 3.5.3). As detailed above, lipid assays were carried out using the Standard Lipid Research Clinic Program (NIH) protocol at the GRI Department of Biochemistry, Centre for Disease Control and Prevention (Atlanta, GA) reference laboratory accredited by Clinical Pathology Accreditation UK (LRCP 1975). TG was measured by the glycerol-3-phosphate oxidase/phenol aminophenazone (GPO/PAP) technique on an automated analyser. TC, HDL-C, glucose and CRP were measured using a CHOD/PAP kit, HDL cholesterol plus 3rd generation kit, glucose oxidase/PAP kit and Tin-quant CRP (Latex) high sensitive immunoturbidimetric assay kit, respectively, which were supplied by Roche Diagnostics. NEFA was also assessed using a NEFA C test kit (Wako, Neuss Germany), according to the manufacturer's instructions, and was read at a 550nM wavelength.

4.5.5 Adipokines, endothelial cell function markers and inflammatory mediators assays

Human insulin, leptin, adiponectin, sICAM-1, sVCAM-1, IL-6, IL-10, TNF-α, PAI-1 and PAI-2 were quantitated by ELISA, according to manufacturer's instructions. Insulin ELISA (10-1113-01 Mercodia) was sensitive to 2mU/L, and detection was at 450nM absorbance. HOMA was calculated as noted above (Section 3.5.4). Plasma leptin and adiponectin (Quantikine, R&D Systems) were utilised with a minimum detectable dose of less than 7.8pg/mL and 0.08ng/mL, respectively. Soluble ICAM-1 and sVCAM-1 (R&D Systems) with less than 0.35ng/mL and less than 2.0ng/mL detection limits, respectively, were used for the quantitative determination of plasma sICAM-1 and sVCAM-1. The detection absorbance reading of products of leptin, adiponectin, sICAM-1 and sVCAM-1 was at 450nM. IL-6, IL-10 and TNF-α (Quantikine HS, R&D Systems) have detection limit sensitivity to 0.02pg/mL, less than 0.5pg/mL and 0.04pg/mL, respectively, with all products detected at an absorbance of 490nM. TNF-α/IL-6 ratio was used as an index of the Th1/Th2 balance (Freeman et al. 2004). Plasma PAI-1 was assayed by ELISA (TriniLIZE PAI-1 Antigen REF: T6003; Trinity Biotech), with a detection limit of 0.5ng/mL, and plasma PAI-2 using a PAI-2 ELISA (IMUBIND® Stamford, USA) was sensitive to 50pg/mL. PAI-1 and PAI-2 determination had an absorbance of 492nM and 450nM respectively. Placental function was assessed using PAI-1/PAI-2 ratio (Stewart et al. 2007).

4.5.6 Immunohistochemistry (IHC) assay

4.5.6.1 Processing of biopsies

Biopsies of umbilical cord and placental block which had previously been embedded were used in IHC studies. Paraffin-embedded first, second and third trimester placentae and umbilical cord tissue biopsies were cut into sagittal sections of 5µm thickness using a Leica RM 2135 rotary microtome (Leica Biosystems) and mounted on slides for later use.

4.5.6.2 ABC method

A standard ABC method was used for tissue staining, as described above (see Section 2.5.11). Briefly, the paraffin-embedded and positive control slides were incubated at 60°C for 35 min, dewaxed in xylene (2 x 10 min), rehydrated in alcohol series [absolute (2 x 10 min), 95% ethanol (2 x 5 min), 70% ethanol (5 min)] and then washed for 2 x 5 min in PBS, pH 7.6. Peroxidase activity was quenched by immersing slides in freshly prepared 0.5% H_2O_2 (5mL H_2O_2 in 300mL methanol) for 30 min. The rationale for choosing each positive control is because each tissue had been found to express the respective antigen during antibody staining optimisation. Antigenic retrieval procedures depended on the localising antibody and are detailed in Table 4-1.

Table 4-1: **Antibodies used for immunohistochemistry**.

Antigen 1°Ab Cat №, (diluting sera)	Antibody Type	Antigen Retrieval	Positive Control	1°Ab dilution
ABCA1 ABC1 (Y-15): SC 5490, Santa Cruz (2% rabbit/5% human sera)	Goat polyclonal	0.01M citrate buffer pH 6.0 (microwave)	Liver tissue	1:750
ABCG1 ABCG1 (E-20): SC 11150, Santa Cruz (2% rabbit/5% human sera)	Goat polyclonal	0.01M citrate buffer pH 6.0 (microwave)	Lung tissue	1:1250
LDLR LDLR (N-17): SC 11822, Santa Cruz (2% rabbit/5% human sera)	Goat polyclonal	None	Liver tissue	1:100
STARD3 MLN64 (D-20): SC 26062, Santa Cruz (2% rabbit/5% human sera)	Goat polyclonal	0.01M citrate buffer pH 6.0 (microwave)	Breast tissue	1:100
TNF-α TNF-α AF 210 NA, R&D System (2% rabbit/5% human sera in 0.1% Saponin)	Goat polyclonal	0.01M citrate buffer pH 6.0 (microwave)	Breast tissue	1:50

The detection of LDLR did not require antigen retrieval, whilst ABCA1, ABCG1, STARD3 and TNF-α were pre-treated in a 0.01M citrate buffer, pH 6.0, using a microwavable pressure cooker for 8 min. Paraffin slides were then washed in DH_2O for 5 min followed by washes of 2 x 10 min PBS, pH 7.6. A non-immune blocking reagent, 20% rabbit and 20% human sera in PBS, pH 7.6, was added for 30 min to block non-specific antibody binding. Slides were washed in DH_2O for 5 min, twice with PBS, pH 7.6, for 10 min and then incubated in a primary (1°) antibody overnight at 4°C (Table 4-1). After two washes in PBS, pH 7.6, for 5 min, slides were incubated in biotinylated anti-goat IgG H+L (30 min, 1:200, RT) (BA-5000, Vector laboratories). The 1° and 2° antibodies were diluted in 2% rabbit serum and 5% human serum PBS, pH 7.6. A negative control containing 2% rabbit serum and 5% human serum only was used. After incubation with the 2° antibody, slides were washed (2 x PBS, pH 7.6) for 5 min and incubated for 30 min at RT in a Vectastain® standard ABC Kit Elite from Vector laboratories, using two drops of reagent A and reagent B diluted in 5mL of PBS, pH 7.6. The staining was detected by adding 1mg/mL DAB solution (made by dissolving 1 tablet of DAB and 12µL H_2O_2 in a final volume of 15mL of

50mM Tris HCl, pH 7.5) and incubating for 10 min. Slides were washed in PBS for 5 min and DH_2O (5 min) before counterstaining in Harris stain for 15 sec. Paraffin-embedded slides were dehydrated via serial alcohol concentrations, and slides were mounted in DPX for microscopy. Digital image capture was by Image-Pro Plus (version 6.2 MediaCybernetics) on a BX50 F-3 microscope (Oympus) equipped with X4, X10, X20, X40 and X100 lenses connected to a 3-CCD colour camera. ImageJ was used for image processing.

4.5.7 Isolation of placental total RNA

Approximately 65mg of frozen placental tissue was mechanically disrupted, using a ceramic mortar and pestle, and the tissue was mixed with 2.5mL of Nucleic Acid Purification Lysis Solution (Applied Biosystem, UK). Samples were homogenised using a Polytron-aggregate homogeniser (PT 10-35; Kinematica) for 30 sec and incubated on wet ice for 30 min. RNA was isolated from the homogenate using an ABI 6100 Nucleic Acid Prepstation. Following the manufacturer's instructions, 600µL of homogenate was added to one well of the pre-filter tray and then pulled through the membrane for 180 sec at 80% vacuum. RNA Purification Wash Solution-1 (500µL) was added to the well and run for 180 sec at 80% vacuum. Similarly, RNA Purification Wash Solution-2 (400µL) was added and run for 180 sec at 80% vacuum. Then, absolute RNA wash (50µL) was added and run for 900 sec, followed by 600µL RNA Purification Solution-2, which was run for 600 sec. Samples were incubated for 120 sec and 300µL RNA Purification Wash Solution-2 was added and then the mixture was run for 120 sec at 60% vacuum, followed by an additional 300 sec incubation and run at 90% vacuum. Nucleic Acid Purification Elution Solution (100µL) was added into each well to elute RNA after 120 sec at 40% vacuum. The eluted RNA obtained with the ABI 6100 method was already DNased after the addition of the Nucleic Acid purification solution. Aliquots of DNase RNA were collected and stored at -80°C until analysis.

4.5.8 Standard PCR for detection of STARD mRNA expression

The DNase RNA was reverse transcribed with a High Capacity cDNA Archive Kit (Applied Biosystem), and cDNA was prepared as stated above (Section 2.5.8). Detection of placental mRNA expression of the 15 STARD proteins used PCR primers obtained from Eurogentec (S. A. Belgium). Primer sequences were generously provided as a gift by Dr Annette Graham, Vascular Biology Group, Department of Biological and Biomedical Science, Glasgow Caledonian University, Glasgow (Table 4-2).

Table 4-2: **Primers used for STARD proteins detection**. STARD proteins 1-15 forward (F) and reverse (R) primers.

STARD	Primer sequence	PCR cycle programme
STARD1	F. 5'-ACT-CAG-AGG-CGA-AGC-TTG-AG-3' R. 5'-CAG-CCC-TCT-TGG-TTG-CTA-AG-3'	94°C, 3 min x 1; 94°C, 30 sec; 60°C, 30 sec; 59°C, 1 min x 35; 72°C, 7 min x 1
STARD2	F. 5'-ATC-CGG-GTG-AAG-AAG-TAC-AA-3' R. 5'-CAC-CTC-AAA-TCC-CAG-CCT- AA-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 56°C, 1 min x 35; 72°C, 7 min x 1
STARD3	F. 5'-AGC-GAG-TGG-AAG-ACA-ACA-CC-3' R. 5'-AAA-CAT-CCA-CAG-GCC-AGA-CC-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 58°C, 1 min x 35; 72°C, 7 min x 1
STARD4	F. 5'-GGC-GAG-TTG-CTA-AGA-AAA-CG-3' R. 5'-AAA-GCT-GCA-GTG-AGC-TGT-GA-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 56°C, 1 min x 35; 72°C, 7 min x 1
STARD5	F. 5'-ACC-ATC-CTT-GTG-GTT-GCT-TC-3' R. 5'-AAC-AGG-CAG-ATG-GAG-TTT-GG-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 54°C, 1 min x 35; 72°C, 7 min x 1
STARD6	F. 5'-CAT-CAG-GCT-GGA-AAG-TGG-TT-3' R. 5'-TGA-AAT-CCA-CGT-CTT-GAT-GG-3'	94°C, 3 min x 1; 72°C, 45 sec; 60°C, 30 sec; 56°C, 1 min x 35; 72°C, 7 min x 1
STARD7	F. 5'-GCC-TAC-TGG-GAG-TGC-TCT-TG-3' R. 5'-CTG-AGA-GCT-CCA-AGG-GAG-TG-3'	94°C, 3 min x 1; 72°C, 30 sec; 60°C, 30 sec; 58°C, 1 min x 35; 72°C, 7 min x 1
STARD8	F. 5'-GGC-TGA-GGC-TGA-AGA-TGA-AG-3' R. 5'-AAC-TTG-GGC-ATT-GAC-CAG-AC-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 55°C, 1 min x 35; 72°C, 7 min x 1
STARD9	F. 5'-ACT-GGT-GTG-GGG-TTC-AGA-AG-3' R. 5'-AGA-AGT-TCC-CAA-GTC-GCT-CA-3'	94°C, 3 min x 1; 72°C, 30 sec; 60°C, 30 sec; 58°C, 1 min x 35; 72°C, 7 min x 1
STARD10	F. 5'-GAA-AGA-CTT-GGT-CCG-AGC-TG-3' R. 5'-TTC-CAC-TCG-GGG-TAC-TTG-AG-3'	94°C, 3 min x 1; 72°C, 30 sec; 60°C, 30 sec; 59°C, 1 min x 35; 72°C, 7 min x 1
STARD11	F. 5'-GGC-CAT-CTT-CAG-AAT-GGA-AA-3' R. 5'-AAC-CAG-TCA-CAG-CCA-AAA- CC-3'	94°C, 3 min x 1; 72°C, 30 sec; 60°C, 30 sec; 53°C, 1 min x 35; 72°C, 7 min x 1
STARD12	F. 5'-CCC-TCA-CTC-TGG-AAG-CAC- TC-3' R. 5'-CAC-AGG-CTC-CTT-TGG-GTA-AA-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 56°C, 1 min x 35; 72°C, 7 min x 1
STARD13	F. 5'-CTG-TAT-GCC-AGC-ACA-GGA-GA-3' R. 5'-GAG-AGG-AAC-GCC-AAA-GAC-AG-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 56°C, 1 min x 35; 72°C, 7 min x 1
STARD14	F. 5'-TGA-CCA-GCT-TGT-GTC-TCT-GG-3' R. 5'-GGT-TCA-CCT-TGG-CCT-TGA-TA-3'	94°C, 3 min x 1; 72°C, 30 sec; 60°C, 30 sec; 55°C, 1 min x 35; 72°C, 7 min x 1
STARD15	F. 5'-TGC-AAA-CCA-TCA-CGG-AAA- TA-3' R. 5'-ACG-TGC-TTT-TCA-ACC-CAA- AC-3'	94°C, 3 min x 1; 72°C, 1 min; 60°C, 30 sec; 51°C, 1 min x 35; 72°C, 7 min x 1

First Choice™ PCR-Ready human liver cDNA was used as a positive control for STARD product detection in the placentae, with a supplied control primer mix (containing targets housekeeping gene; AM3323, Applied Biosytem). The quality of RNA was verified for the liver (positive control) and the placenta using 18S primer of forward 5'-CAA-GTC-TGG-TGC-CAG-CAG-CCG-CGG-T-3' and reverse 5'-TCA-CCT-CTA-GCG-GCG-CAA-TAC-GAA-T-3', producing a 359-bp product. In the qualitative PCR assay, 1µg of cDNA served as a template in a total volume of 10µL PCR mix The resulting reaction mixture contained 5µL of 1X MegaMix~Double, 0.5µL each of 100µM STARD1-15 forward and reverse primer and 4µL DEPC. A sample loading buffer (10X) for PCR of 2µL was added before loading the PCR product onto an agarose gel (1.5%) along with 0.5µg/µL of 100bp DNA ladder (New England BioLab, UK), followed by electrophoresis for 1.20 hours at 100V (see Section 2.5.8). Bands were visualised using a UV transilluminator and the gel was photographed with a DS34 Polaroid direct screen instant camera containing Polaroid black-and-white print film type 667 (Sigma).

4.5.9 Quantitative Real-Time of placental mRNA expression

RT-PCR is the most sensitive technique for mRNA detection and quantitation currently available. RNA was reverse transcribed to cDNA using the High Capacity cDNA Reverse Transcription Kit (Applied Biosystems, Warrington, UK), following the manufacturer's instructions. A NoRT control was also prepared (Section 2.5.8).

Placental mRNA expression was quantitated using RT-PCR on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) with commercially available primer probe sets (Table 4-3). Briefly, 1.25µL of 20 x target assay or control assay mix was added to 12.5µL of 2 x Mastermix (Applied Biosystems), 10.25µL deionised distilled water and 1µL cDNA. The thermal cycler conditions were 50°C for 2 min and 95°C for 10 min, then 40 x 95°C for 15 sec and 60°C for 1 min. Following the manufacturers' instructions, quantitation of placental mRNA levels of each expressed gene target was carried out in duplicate. Data was analysed using the Sequence Detection system software, which calculates the threshold cycle (C_T) values. The placental gene targets were normalised by subtracting the C_T value of the endogenous control (18S rRNA) from the C_T value of the relevant target assay. The fold increase relative to control was obtained by using the formula $2^{-\Delta}C_T$.

Table 4-3: **Commercial primer probe sets**.

Primer probe set	Catalogue №
ATP-binding cassette sub-family A (ABC1), member 1	Hs00194045_m1
ATP-binding cassette sub-family G (WHITE), member 1	Hs00245154_m1
Apolipoprotein E (apo E)	Hs00000000_m1
CD36 molecule (thrombospondin receptor)	Hs00169627_m1
Eukaryotic 18S rRNA endogenous control (VIC/TAMRA)	PN 4310893E
Keratin 7	Hs00818825_m1
Platelet/Endothelial cell adhesion molecule (CD31 antigen)	Hs01055279_m1
Liver X receptor-alpha (LXR-α)	Hs00172885_m1
Low density lipoprotein receptor (LDLR)	Hs00181192_m1
Steroidogenic acute regulator 1 (STARD1)	Hs00264912_m1
START domain containing 3 (STARD3)	Hs00199052_m1
START domain containing 4 (STARD4)	Hs00287823_m1
START domain containing 5 (STARD5)	Hs00739050_m1
Tumour necrosis factor-alpha 1 (TNF-α1)	Hs00174128_m1

4.5.10 Protein quantitation

Placental protein was extracted from placenta tissue samples in order to confirm if mRNA expression resulted in changes of protein expression. Approximately 50mg of placental tissue was mechanically disrupted with a ceramic mortar and pestle, collected and resuspended with 2.5mg of 0.5mL lysis solution containing 1 tablet protease inhibitor cocktail (Roche) dissolved in a total volume of 10mL CelLytic^{MT} MT mammalian tissue lysis/extraction reagent (Sigma-Aldrich). Samples were homogenised using a Polytron-aggregate homogeniser for 30 sec and incubated on wet ice for 10 min. The placental tissue homogenates were centrifuged (5000*g*, 10 min) at 4°C. Then the supernatant was transferred into sterile 1.5mL tubes and centrifuged (10000*g,* 10 min) at 4°C. At this point aliquots of extracted proteins suspension were collected and stored at -80°C until analysis.

Protein concentration of the extracted placental protein suspension was determined using the Bradford procedure (Section 2.5.9). In brief, a 10µL aliquot (1:100) of placentae

homogenate was used for protein estimation. A standard concentration curve of 0.1-1.4mg/mL was made with BSA. The Bradford reagent (Sigma) was left to reach RT. One-mL of it was then added to the protein aliquot by mixing and incubated at RT for 15 min before reading the absorbance at 595nM on a spectrometer. A standard curve of absorbance versus the protein was plotted in Microsoft Excel, and the unknown protein concentrations determined from the curve. Protein concentrations were corrected for dilution factor.

4.5.11 SDS-PAGE and Western blots

As mentioned above (Section 2.5.10), placental protein homogenate extracts (2.5-10µg/µL) were subjected to SDS-PAGE electrophoresis. Placental proteins were loaded in a 30µL volume containing 50% (15µL) NuPAGE LDS sample buffer. Proteins were separated using NUPAGE® 4-12% Bio-Tris gels (Life Technology) on an XCell Surelock™ Mini-Cell system according to manufacturer's instructions. Pre-stained High Range Markers (44-200kDa molecular weight; SC 2362, Santa Cruz) were used as size markers. The inner chamber of the tank that enclosed the separating gel was filled with 200mL of 1% NuPAGE® MOP or MES SDS running buffer, with 100µL of 1mM dithiothreiol reagent added. Both LDLR and ABCA1 bands were detected after electrophoresis using a NuPAGE® MES SDS running buffer whereas STARD3 was detected after electrophoresis using a NuPAGE® MOP running buffer. Electrophoresis for LDLR and ABCA1 was performed at 100V, 170mA 40W for 1.40 hours, and for STARD3 at 200V, 180mA 40W for 45 min. Separated proteins were electroblotted from gels onto a nitrocellulose membrane filter at 25V, 180mA 60W for 2, 3 and 1.20 hours for LDLR, ABCA1 and STARD3 respectively on an XCell™ Blot Module (Life Technology). The electroblotted membrane was blocked in a blocking solution (5%) prepared by dissolving 5g non-fat milk in 100mL PBS (pH 7.3) for 1 hour, followed by incubation with a primary antibody overnight (12-18 hours) with mild agitation at 4°C (Table 4-4).

Table 4-4: **Primary and secondary antibodies' dilutions**.

Antigen (Catalogue №)	Protein loaded (µg/µL)	1°Ab dilution (ON) and diluents	Washes	2°Ab dilution factor
ABCA1 (Ab7360, Abcam)	10.0	1:2000 3% non-fat milk (0.05% PBST)	0.05% PBST (6x10 min)	1:5000 Goat polyclonal Rabbit IgG H&L (HRP):Ab6721 1:70000 β-Actin (C4) HRP:SC 47778
LDLR (Ab30532, Abcam)	2.5	1:10000 3% non-fat milk (PBS)	0.05% PBST (6x10 min)	1:6000 Goat polyclonal Rabbit IgG H&L (HRP):Ab6721 1:17000 β-Actin (C4) HRP:SC 47778
STARD3 (D-20: SC 26061, Santa Cruz)	10.0	1:1000 5% non-fat milk (0.1% PBST)	0.1% PBST (6x10 min)	1:1000 Donkey anti-goat HRP IgG:SC 2020 1:70000 β-Actin (C4) HRP:SC 47778

PBST represents PBSTween-20; ON, overnight.

The membranes were washed and incubated with a secondary antibody at RT for 1 hour (Table 4-4). Then blots were washed and signal detected (5 min incubation at RT) with 1:1 reagents A and B from the Supersignal® West Pico Trial Kit (an enhancer chemiluminescent substrate for detection of HRP; Thermo Scientific). Bands were detected by exposing the blots on an x-ray film (Fuji), 3 min for LDLR and ABCA1, and 10 min for STARD3, and then developed on the Kodak OMAT 1000 Processor (Eastman Kodak, USA) according to the manufacturer's instructions. LDLR, STARD3 and ABCA1 molecular weights were estimated by assessing the migration distance relative to dye front using molecular weight of pre-stained high range markers to provide a standard curve. Also, the detected bands on the films were scanned as a pictorial record (Epson Perfect 3200). A profile pixel density system was used to determine the volume intensity of each band, which represents the quantitation of (each) protein level using Bio-Rad's Quantity One version 4.6.2 I-D analysis software (Bio-Rad Laboratories) following the company's information manual. The volume intensity of LDLR, STARD3 and ABCA1 relative to β-Actin was recorded and analysed.

4.5.12 Statistical analysis

Data were tested for normal distribution before statistical analysis and, where data were not distributed normally, log transformation was performed, using Minitab Vs16.2.2 Statistical software. GraphPad Prism® 5 software (GraphPad, Inc; San Diego USA) was utilised for graphical presentation where appropriate. Two-sample t-test and Pearson's Chi-square test were used to compare demographic features for the continuous and categorical variables between PE and IUGR cases and their BMI-matched control groups, respectively. Significant variables identified in univariate analyses were then subjected to multivariate analyses using the General Linear Model in order to account for the contribution of potential covariate confounders (such as maternal smoking status, labouring mother, gestation and/or mode of delivery and gestation at sampling). Linear correlation using Pearson's correlation was also used to test for association between variables. Data were presented untransformed or transformed as mean and standard deviation (SD). P-values less than 0.05 were considered statistically significant.

4.6 Results

4.6.1 Subject characteristics

The clinical characteristics of the participants are shown in Table 4-5. Women with PE and IUGR were matched for age and BMI with controls. The number of previous pregnancies greater than or equal to 24 weeks gestation in women with PE and IUGR compared with controls was not statistically significant. A higher number of women with IUGR than controls smoked, as expected. DEPCAT was not different between the groups. As expected, higher maternal systolic blood pressure [135 (25) vs 117 (13) mmHg, P=0.021] and elevated diastolic blood pressure [82 (12) vs 70 (9) mmHg, P=0.002] was observed in women with PE compared to controls. Women with IUGR when compared to their respective controls, exhibited no difference in systolic blood pressure and diastolic blood pressure. There was a lower gestation at sampling in women with both PE [36 (3) vs 38 (2) weeks, P=0.001] and IUGR [36 (3) vs 38 (2) weeks, P=0.010] compared to their controls.

The offspring of women with PE and IUGR were delivered earlier compared to their respective control groups. There were observable differences in the proportion of women with different modes of delivery: 14 (48%) PE women had an emergency caesarean section, compared to only 4 (5%) women with healthy pregnancies. Conversely, 9 (38%) participants with PE laboured during delivery compared to 9 (16%) controls. However, 5 (36%) mothers with IUGR had an emergency caesarean section, compared to only 2 (5%) controls. In addition, 2 (14%) women with IUGR laboured during delivery, compared to 6 (22%) women in the normal pregnant group. Fetal sex ratio was not different between complicated pregnancy groups and their respective control groups. Placental weight was significantly lower in women with PE [457 (157) vs 716 (170) g, P<0.001], relative to the matched control group. The birthweight centile of offspring was also lower in women with PE [22 (27) vs 55 (32) centile, P<0.001], compared to the matched healthy pregnancy women. There was a similar lower mean placental weight in women with IUGR [297 (72) vs 690 (148) g, P<0.001], compared to their matched controls. Also, the birthweight centile of the babies was lower in mothers with IUGR [1 (2) vs 45 (30) centile, P<0.001], relative to the matched normal pregnant group.

Table 4-5: **Features of preeclampsia and IUGR pregnancies and their matched controls**.

Features	PE (n=29)	Control (n=87)	P-value	IUGR (n=14)	Control (n=42)	P-value
Age (years)	30.5 (6.3)	30.1 (5.3)	0.79	29.9 (4.8)	29.6 (5.1)	0.84
BMI (kg/m^2)	28.6 (6.7)	28.3 (6.4)	0.85	25.5 (5.3)	25.4 (4.9)	0.96
№ of pregnancies ≥ 24 weeks [n,(%)]			0.057			0.65
0	18 (62)	36 (41)		6 (43)	17 (41)	
1	5 (17)	36 (41)		4 (29)	17 (41)	
2	6 (22)	15 (17)		4 (29)	8 (19)	
Smoking status [n,(%)]			0.68			0.012
Non smoker	22 (79)	65 (75)		6 (43)	33 (79)	
Smoker	6 (21)	22 (25)		8 (57)	9 (21)	
DEPCAT [n, %]			0.47			0.51
Affluent (1-2)	1 (5)	10 (14)		1 (7)	5 (15)	
Intermediate (3-5)	11 (50)	32 (46)		7 (50)	11 (33)	
Deprived (6-7)	10 (46)	28 (40)		6 (43)	17 (52)	
Systolic BP (mmHg)	135 (25)	117 (13)	0.021	109 (10)	112 (13)	0.57
Diastolic BP (mmHg)	82 (12)	70 (9)	0.002	69 (7)	68 (9)	0.75
Gestation at sampling (weeks)	36 (3)	38 (2)	0.001	36 (3)	38 (2)	0.010
Gestation at delivery (days)	251 (21)	275 (10)	<0.001	253 (20)	276 (9)	0.001
Delivery mode [n, %]			<0.001			0.018
Assisted	2 (7)	6 (7)		0 (0)	5 (12)	
Elective caesarean section	7 (24)	55 (64)		7 (50)	24 (59)	
Emergency caesarean section	14 (48)	4 (5)		5 (36)	2 (5)	
Vaginal	6 (21)	21 (24)		2 (14)	10 (24)	
Labour [n, %]			0.035			0.54
Non-labour	15 (62.5)	47 (83.9)		12 (85.7)	21 (77.8)	
Labour	9 (37.5)	9 (16.1)		2 (14.3)	6 (22.2)	
Fetal sex [n, %]			0.99			0.89
Female	14 (48)	41 (48)		6 (46)	18 (44)	
Male	15 (52)	44 (52)		7 (54)	23 (56)	
Placental weight (g)	457 (157)	716 (170)	<0.001	297 (72)	689 (148)	<0.001
Birth weight centile	22 (27)	55 (32)	<0.001	1 (2)	45 (30)	<0.001

Mean (SD) and [n,(%)], P-value from two-sample and Pearson's Chi-Square tests respectively.

4.6.2 Fetal and maternal plasma metabolic and inflammatory parameters in pre-eclamptic women and controls

Metabolic and inflammatory parameters in cord blood at delivery were measured in extreme cases of PE and in healthy pregnancies; the data are reported in Table 4-6. Cord plasma insulin levels were not different between women with PE and their healthy pregnancy counterparts. Fetal leptin was lower in cases of PE [6.5 (6.1) ng/mL] compared to controls from the healthy BMI-matched pregnancies [12.9 (14.1) ng/mL, log P=0.005]. Equally, cord adiponectin was lower in the PE group [23.1 (14.1) mg/mL] than in the control group [34.3 (14.9) mg/mL, log P=0.004]. Multivariate analysis was carried out to examine the impact of confounding factors, including maternal smoking status, labour, gestation and/or mode of delivery and gestation at sampling. The lower cord log leptin in PE was influenced by gestational age at delivery (P<0.001), [28.7% adjusted sum of square (r^2), not significant]. In the same fashion, lower cord adiponectin in PE is dependent on labour (P=0.003) and gestation at delivery (P<0.001) but independent of smoking and mode of delivery (39.9%, unchanged). The cord plasma TG in PE was higher [0.6 (0.2) mmol/L] compared to control group cord plasma [0.5 (0.4) mmol/L, log P=0.001]. Higher cord log TG in the PE group was independent of gestation at delivery but influenced by maternal smoking status (P=0.033), mode of delivery (P=0.001) and labour (P=0.004), (37.4%, unchanged). In a similar fashion, PE was associated with significantly higher cord TC level [2.2 (0.6) mmol/L] compared to controls [1.6 (0.5) mmol/L, log P<0.001]. Higher cord log TC in PE cases was independent of maternal smoking status, labour, gestation and mode of delivery (18.7%, P=0.059). Cord HDL-C, NEFA, glucose and HOMA levels were not different between PE and control groups.

There was a trend toward lower fetal blood CRP in the PE group [0.1 (0.1) mg/mL] compared to the healthy pregnant group [0.3 (1.0) mg/L, log P=0.08]. However, after adjustment for confounding factors (including maternal smoking, gestation at delivery and mode of delivery), cord log CRP blood differed significantly (13.7%, P=0.001) between the PE group and controls. The difference in cord log CRP between the PE group and controls is influenced by mode of delivery (P<0.001) and labour (P=0.012) but independent of maternal smoking and gestation at delivery. Cord IL-6 and IL-10 levels were not different between the PE group and controls. Fetal TNF-α was lower in the PE group [1.9 (0.6) pg/mL] compared to babies from women that had a healthy pregnancy [2.3 (1.3) pg/mL, log P=0.026]. The fetal log TNF-α difference was independent of maternal smoking and gestation at delivery but dependent on labour (P=0.004) and mode of delivery (P=0.013), (39%, P=0.010). Umbilical cord blood TNF-α/IL-6 ratio was also lower in the PE group [0.5 (0.4)] compared to the healthy controls [0.9 (0.7), log P=0.040]. There was no difference in fetal sICAM-1 and sVCAM-1 levels between groups.

Maternal insulin level was not different between the PE group and controls. Leptin was significantly higher in women with PE [64.2 (39.0) ng/mL] compared to women with a healthy pregnancy [40.6 (30.0) ng/mL, log P=0.006]. In contrast, there was no difference in adiponectin level between the groups. Higher maternal TG was observed in the PE group [3.2 (0.9) mmol/L] compared to controls [2.7 (0.8) mmol/L, log P=0.002]. The difference in maternal log TG level between women with PE and controls is influenced partially by maternal smoking status (P=0.08) but independent of labour and gestation at sampling (11%, P=0.003). Women with PE had a trend towards higher TC levels compared to controls [6.6 (1.2) vs 6.2 (1.0) mmol/L, P=0.053)]. Also, the difference in maternal TC between women with PE and controls depended on maternal smoking status (P=0.019) but was independent of labour and gestation at sampling (8.0%, P=0.013). Maternal HDL-C was not different between PE and control groups. However, NEFA level was elevated in the PE group [0.6 (0.3) mmol/L] compared to [0.4 (0.2) mmol/L), P<0.001)] in women with healthy pregnancies. The difference in NEFA between women with PE and controls was dependent on gestation at sampling (P=0.055) but independent of smoking and labour (20.9%, P=0.002). Maternal glucose levels were higher in women with PE [6.1 (2.3) mmol/L] than healthy controls [5.0 (0.8) mmol/L, P=0.015]. Gestation at sampling (P=0.019) influenced glucose levels between women with PE and controls, independent of maternal smoking and labour (12.6%, P=0.031). HOMA level was not different between the two groups.

There were no noticeable differences in maternal inflammatory status (CRP and IL-6) in women with PE compared to controls. However, confounding factor assessment showed the difference in log IL-6 levels between women with PE and controls is influenced by labour (P=0.023) and gestation at sampling (P=0.008), (adjusted r^2 18.5%, P=0.039). Maternal IL-10 levels were higher in women with PE [2.6 (2.3) pg/mL] compared to women with a healthy pregnancy [1.4 (0.8) pg/mL, log P=0.014], although caution was taken during analysis, considering only 40% of the PE group and 50% of the control data were available. Plasma TNF-α and TNF-α/IL-6 ratio were not different between PE and control mothers. Maternal plasma sICAM-1 and sVCAM-1 did not differ between PE and control mothers. Maternal plasma PAI-1 level was elevated in the PE group [124 (30) ng/mL] compared to the control group [90 (40) ng/mL, P=0.001]. In contrast, PAI-2 level was lower in mothers with PE [217 (145) ng/mL] than those with a healthy pregnancy [432 (193) ng/mL, P<0.001]. An elevated PAI-1/PAI-2 ratio was observed in women with PE [1.8 (3.7)] compared to controls [0.3 (0.3), log P<0.001]. Caution was also taken with the results of PAI-1, PAI-2 and PAI-1/PAI-2, since approximately 50% of the data was unavailable.

Table 4-6: **Maternal and cord plasma metabolic and inflammatory parameters**.

Parameters	Cord blood			Maternal blood		
Hormone	PE (n=29)	Control (n=87)	P-value	PE (n=29)	Control (n=87)	P-value
Insulin (mU/L)	9.2 (13.6)	8.3 (12.2)	0.90*	25.8 (34.3)	23.6 (31.8)	0.87*
Leptin (ng/mL)	6.5 (6.1)	12.9 (14.1)	0.005*	64.2 (39.0)	40.6 (30.0)	0.006*
Adiponectin (µg/mL)	23.1 (14.1)	34.3 (14.9)	0.004*	10.4 (4.4)	8.8 (3.4)	0.082
Metabolism						
Triglyceride (mmol/L)	0.6 (0.2)	0.5 (0.4)	0.001*	3.2 (0.9)	2.7 (0.8)	0.002*
TC (mmol/L)	2.2 (0.6)	1.6 (0.5)	<0.001*	6.6 (1.2)	6.2 (1.0)	0.053
HDL-C (mmol/L)	0.8 (0.3)	0.8 (0.3)	0.62	1.7 (0.4)	1.7 (0.4)	0.73
NEFA (mmol/L)	0.1 (0.1)	0.1 (0.1)	0.85	0.6 (0.3)	0.4 (0.2)	0.001
Glucose (mmol/L)	4.5 (1.1)	4.3 (1.2)	0.36	6.1 (2.3)	5.0 (0.8)	0.020
HOMA	2.1 (3.1)	1.4 (1.6)	0.51*	10.0 (18.2)	6.0 (9.6)	0.52*
Inflammation						
CRP (mg/L)	0.1 (0.1)	0.3 (1.0)	0.082*	9.0 (12.8)	5.1 (3.9)	0.12
IL-6 (pg/mL)	6.1 (5.5)	6.3 (7.0)	0.30*	5.7 (6.4)	3.0 (1.9)	0.15*
IL-10 (pg/mL)	1.3 (0.5)	2.1 (2.5)	0.12*	2.6 (2.3)	1.4 (0.8)	0.014*
TNF-α (pg/mL)	1.9 (0.6)	2.3 (1.3)	0.026*	1.7 (0.8)	1.8 (1.0)	0.86
TNF-α/IL-6	0.5 (0.4)	0.9 (0.7)	0.040*	0.7 (0.6)	0.6 (0.5)	0.89*
sICAM-1 (ng/mL)	121 (44)	139 (41)	0.15	176 (29)	177 (47)	0.92
sVCAM-1 (ng/mL)	883 (233)	842 (306)	0.47*	436 (143)	390 (84)	0.24
PAI-1 (ng/mL)	-	-	-	124 (30)	90 (40)	0.001
PAI-2 (ng/mL)	-	-	-	217 (145)	432 (193)	<0.001
PAI-1/PAI-2	-	-	-	1.8 (3.7)	0.3 (0.3)	<0.001*

Mean (SD), P-value or (*) P-value of log transformed variable from two-sample t-test.

4.6.3 IUGR and healthy pregnancies

Parameters of metabolism and inflammation were assessed in IUGR and control pregnancies. Data are reported in Table 4-7. IUGR babies' insulin levels were not significantly different from the controls. Cord plasma leptin was significantly lower in IUGR pregnancies [4.3 (6.1) ng/mL] compared to healthy pregnancies [9.5 (8.8) ng/mL, log P=0.018], whereas cord plasma levels of adiponectin in IUGR babies did not differ significantly from the controls. Cases of IUGR showed an elevated cord plasma TG [0.6 (0.2) mmol/L] compared to healthy pregnancy controls [0.5 (0.3) mmol/L, log P=0.042]. A lower cord TC level was observed in the IUGR group [1.3 (0.4) mmol/L] compared to controls [1.6 (0.4) mmol/L, log P=0.011]. In addition, cord plasma HDL-C concentration was lower in the IUGR group [0.5 (0.2) mmol/L] than in the control group [0.8 (0.2) mmol/L, P=0.001]. Cord plasma levels of NEFA, glucose and HOMA in IUGR babies were not significantly different from the controls. Fetal plasma inflammatory mediators (CRP, IL-6, IL-10, TNF-α and TNF-α/IL-6) did not differ between IUGR babies and their matched controls. Fetal sICAM-1 levels were lower in IUGR pregnancies [111 (28) ng/mL] compared to healthy pregnancies [137 (36) ng/mL, P=0.032], although approximately 30% of IUGR and 50% of control data were missing, so analysis was done with caution. After adjustments for confounding factors (such as mode of delivery, smoking and gestation at delivery), the lower sICAM-1 was found to be independent of confounding factors. There was no difference in the levels of fetal cord sVCAM-1 between the IUGR and healthy groups.

Plasma insulin, leptin, adiponectin, TG, TC, HDL-C, NEFA, glucose and HOMA levels were not different between women who suffered from IUGR and healthy matched controls. Maternal plasma inflammatory mediators (CRP, IL-6, IL-10, TNF-α and TNF-α/IL-6) did not differ between the IUGR group and their matched controls. There is a trend towards higher maternal sICAM-1 in IUGR pregnancies [223 (59) ng/mL] compared to control pregnancies [179 (34) ng/mL, P=0.052]. Multivariate analysis demonstrates that the higher sICAM-1 is partly dependent on maternal smoking status. There was no difference in sVCAM-1 between women who had IUGR and the control group. There was no difference in maternal PAI-1 between the IUGR and the control group. However, PAI-2 levels were lower in the IUGR group [115 (78) ng/mL] than in the control group [432 (212) ng/mL, P<0.001]. Evidence of placental dysfunction was also given by the higher PAI-1/PAI-2 in the IUGR group [1.1 (0.7)] compared to the healthy pregnant group [0.3 (0.3), log P<0.001]. Caution should be taken as 30% of IUGR and 50% of control group data were missing for sICAM-1, sVCAM-1, PAI-1, PAI-2 and PAI-1/PAI-2.

Table 4-7: **Metabolic and inflammatory parameters in controls and IUGR pregnancies**.

Parameters	Cord blood			Maternal blood		
Hormone	IUGR (n=14)	Control (n=42)	P-value	IUGR (n=14)	Control (n=42)	P-value
Insulin (mU/L)	6.9 (10.4)	5.4 (4.3)	0.62	9.3 (4.4)	23.6 (38.7)	0.07*
Leptin (ng/mL)	4.3 (6.1)	9.5 (8.8)	0.018*	34.0 (27.1)	33.6 (19.9)	0.58*
Adiponectin (µg/mL)	33.6 (21.4)	35.9 (14.6)	0.74	8.5 (3.4)	9.2 (3.2)	0.51*
Metabolism						
Triglyceride (mmol/L)	0.6 (0.2)	0.5 (0.3)	0.042*	2.5 (1.2)	2.6 (0.9)	0.51*
TC (mmol/L)	1.3 (0.4)	1.6 (0.4)	0.011	6.0 (1.0)	6.4 (1.0)	0.14
HDL-C (mmol/L)	0.5 (0.2)	0.8 (0.2)	<0.001*	1.7 (0.5)	1.7 (0.4)	0.79
NEFA (mmol/L)	0.1 (0.1)	0.1 (0.1)	0.99	0.6 (0.6)	0.4 (0.2)	0.11*
Glucose (mmol/L)	4.5 (1.2)	4.3 (1.2)	0.68	5.3 (0.9)	5.0 (1.0)	0.27
HOMA	1.4 (2.0)	1.3 (1.7)	0.45*	2.3 (1.4)	6.2 (11.5)	0.14*
Inflammation						
CRP (mg/L)	0.1 (0.1)	0.4 (1.4)	0.34*	5.5 (4.4)	4.3 (3.0)	0.72*
IL-6 (pg/mL)	6.6 (5.7)	6.4 (7.2)	0.64*	3.0 (2.0)	2.7 (1.8)	0.95*
IL-10 (pg/mL)	1.2 (0.7)	2.8 (3.4)	0.071*	1.9 (2.0)	1.5 (1.0)	0.69*
TNF-α (pg/mL)	2.4 (0.8)	2.3 (1.4)	0.53*	2.1 (1.4)	1.7 (0.9)	0.43*
TNF-α/IL-6	0.8 (0.8)	0.7 (0.5)	0.90*	0.9 (0.5)	0.7 (0.6)	0.33*
sICAM-1 (ng/mL)	111 (28)	137 (36)	0.031	223 (59)	179 (34)	0.052
sVCAM-1 (ng/mL)	843 (229)	772 (155)	0.39	329 (82)	386 (79)	0.09
PAI-1 (ng/mL)	-	-	-	85 (300)	94 (38)	0.53
PAI-2 (ng/mL)	-	-	-	115 (78)	432 (212)	<0.001
PAI-1/PAI-2	-	-	-	1.1 (0.7)	0.3 (0.3)	<0.001*

Mean (SD), P-value or (*) P-value of log transformed variable from two-sample t-test.

4.6.4 Fetal TNF-α expression

The significantly lower cord plasma TNF-α in the babies of women with PE was thought to have been due to either lower placental synthesis of TNF-α or lower fetal endothelial cells TNF-α. To explore this, further placenta TNF-α mRNA expression was measured in placentae of women with PE (n=24) and those with healthy (n=49) pregnancies. Placental TNF-α mRNA expression had a trend towards higher levels in the PE group [18 (24) PE vs 11 (18) control TNF-α/18S ratio $x10^{-4}$, P=0.18]. Also, between women with IUGR (n=11) and controls (n=22), there was a trend towards higher placental TNF-α mRNA expression in the IUGR group compared to controls [13.2 (17.5) IUGR vs 9.2 (14.6) control; TNF-α/18S ratio $x10^{-4}$, P=0.52], but this was not significant.

Next, TNF-α protein expression in human umbilical cords from healthy and PE pregnancies were compared using IHC. The positive control, breast tumour tissue, showed TNF-α staining, whereas the no antibodies control (PBS only) showed no staining (Figure 4-2A and B). TNF-α was widely expressed in umbilical arterial endothelial cells (EC) and the SMC of the healthy pregnancies (Figure 4-2C). A similar distinct localisation was observed in the umbilical vein EC (as seen in Figure 4-2D). In the pre-eclamptic samples, TNF-α was also localised on the umbilical arterial EC, umbilical vein EC and SMC (Figure 4-2E and F). The comparison of TNF-α staining intensity implied that umbilical arterial EC, umbilical vein EC and SMC staining appears lower in pre-eclamptic samples compared to controls.

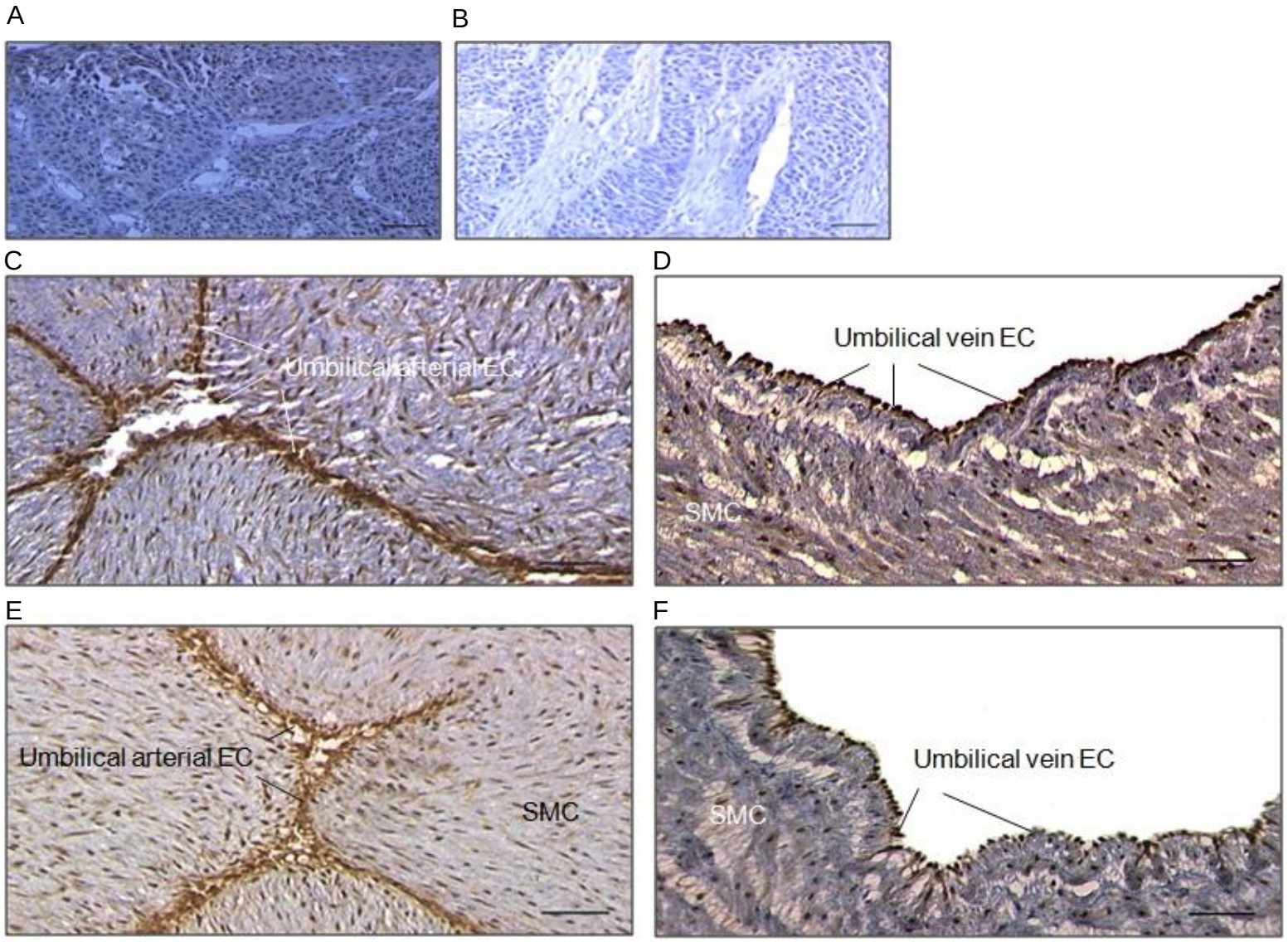


Figure 4-2: **Localisation of TNF-α in umbilical cord**. A) Breast tumour (positive control), B) PBS (negative control), C) stained umbilical arterial and D) vein endothelial cells in healthy pregnancy, and E) stained umbilical arterial and F) vein in PE at X20 magnification (scale bar; 10µm).

4.6.5 Placental START domain proteins mRNA expression

Since cord PE plasma TC levels were higher and IUGR TC and HDL-C levels were lower than controls, an analysis of cholesterol transporter expression in the placenta was carried out to establish whether changes in placental cholesterol transport systems may account for these differences. Emerging data has described a family of intracellular lipid transporters called the STAR-related lipid transfer domain. To identify which members of the START domain are expressed in the placenta, qualitative PCR assays were carried out in healthy term placentae.

Placental START domains mRNA expression data are summarised in Table 4-8. With the exception of STARD6 and STARD15, all other START domain proteins were expressed in placental tissue. STARD2, STARD5, STARD9, STARD12 and STARD13 had the highest expression levels; STARD3, STARD4, STARD7, STARD8, STARD10, STARD11 and STARD14 showed moderate expression. STARD1 had low expression in placentae.

Table 4-8: **START domains mRNA expression level in healthy placentae**. (•) represents faint expression, (••) moderate and (•••) strong; n=6 placentae studied.

START domain	Annealing temperature ^{o}C	Levels of expression
STARD1	59	•
STARD2	56	•••
STARD3	58	••
STARD4	56	••
STARD5	54	•••
STARD6	56	-
STARD7	58	••
STARD8	55	••
STARD9	58	•••
STARD10	59	••
STARD11	53	••
STARD12	56	•••
STARD13	56	•••
STARD14	55	••
STARD15	51	-

The START domain proteins which are responsible for intracellular cholesterol transport (including STARD1, STARD3, STARD4, STARD5 and STARD6) are presented in Figure 4-3. The quality of liver tissue (positive control) was investigated by a supplied control primer mix (see Section 4.5.8) employed during this entire process separating agarose gels (lane A). STARD1 mRNA, a predicted 410-bp fragment, was observed in the liver tissue and placenta (Figure 4-3A). In addition, there was faint detection of STARD1 in the liver (lane B) and the placenta (lanes E-J). STARD3 mRNA was also expressed in the placenta, producing a fragment 605-bp product (Figure 4-3B). The liver (lane B) and placenta (lanes E-J) show high staining of the STARD3. STARD4 mRNA was also expressed in all placentae producing a 803-bp product. The STARD4 detected product in the liver (lane B) and the placenta (lanes E-J) is presented in Figure 4-3C. Similarly, the STARD5 mRNA producing a 423-bp product was also detected in the liver (lane B) and the placenta (lanes E-J) (Figure 4-3D). The STARD6 was detected in the positive control (primer mix) (lane A) but not found in the liver and the placenta (Figure 4-3E). The quality of RNA was verified for the liver (positive control) and the placenta, using 18S primers producing a 359-bp product (Figure 4-3F).

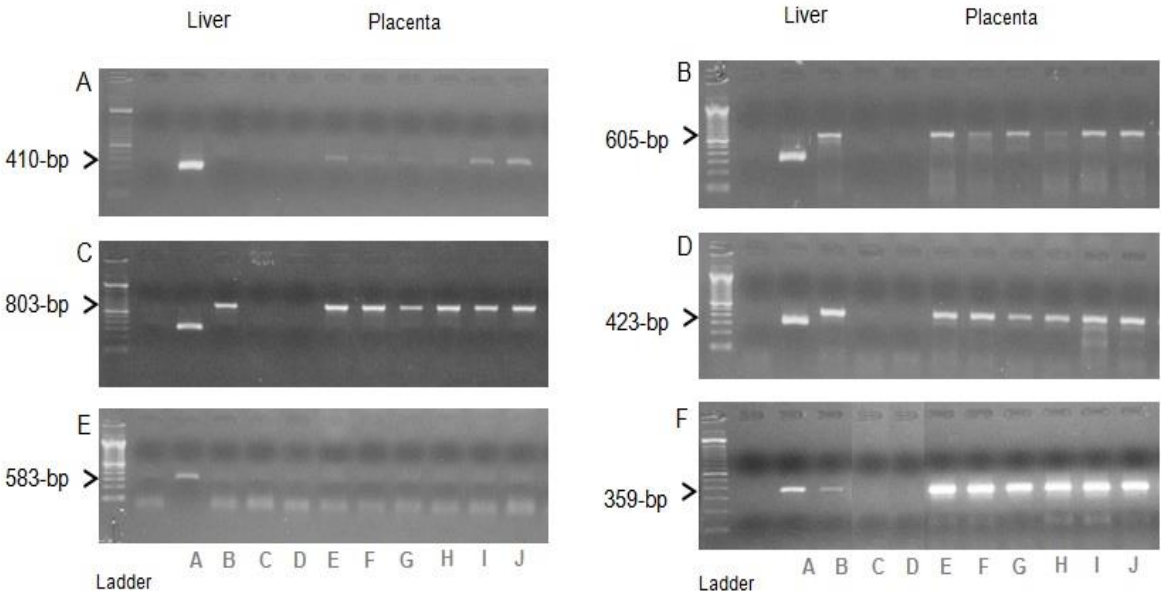


Figure 4-3: **Expression of START domain molecules in the placenta**. A) STARD1 revealed a predicted product of 410-bp, B) STARD3 (605-bp), C) STARD4 (803-bp), D) STARD5 (423-bp) and E) STARD6 (583-bp). Messenger RNA loading was assessed using F) 18S as a loading control gene. Lane A, supplied control primer mix (constitutive housekeeping genes); B, liver and START domain primer; C-D, negative control and E-J, placenta (n=6).

4.6.6 Immunolocalisation of LDLR, STARD3, ABCA1 and ABCG1 in healthy pregnancy, PE and IUGR placentae

IHC was carried out to localise the expression of the key cholesterol transporter molecules in placentae that could potentially play a role in the cholesterol transport between mother and fetus. The transporters localised were LDLR, STARD3, ABCA1 and ABCG1, after determining optimal antibody dilutions for IHC staining assay.

4.6.6.1 Localisation of LDLR in healthy, pre-eclamptic and IUGR placentae

There was no positive staining observed when the antibody was substituted with PBS (negative control), whereas staining was detected in the LDLR positive control tissue (human liver) (Figure 4-4A and B).

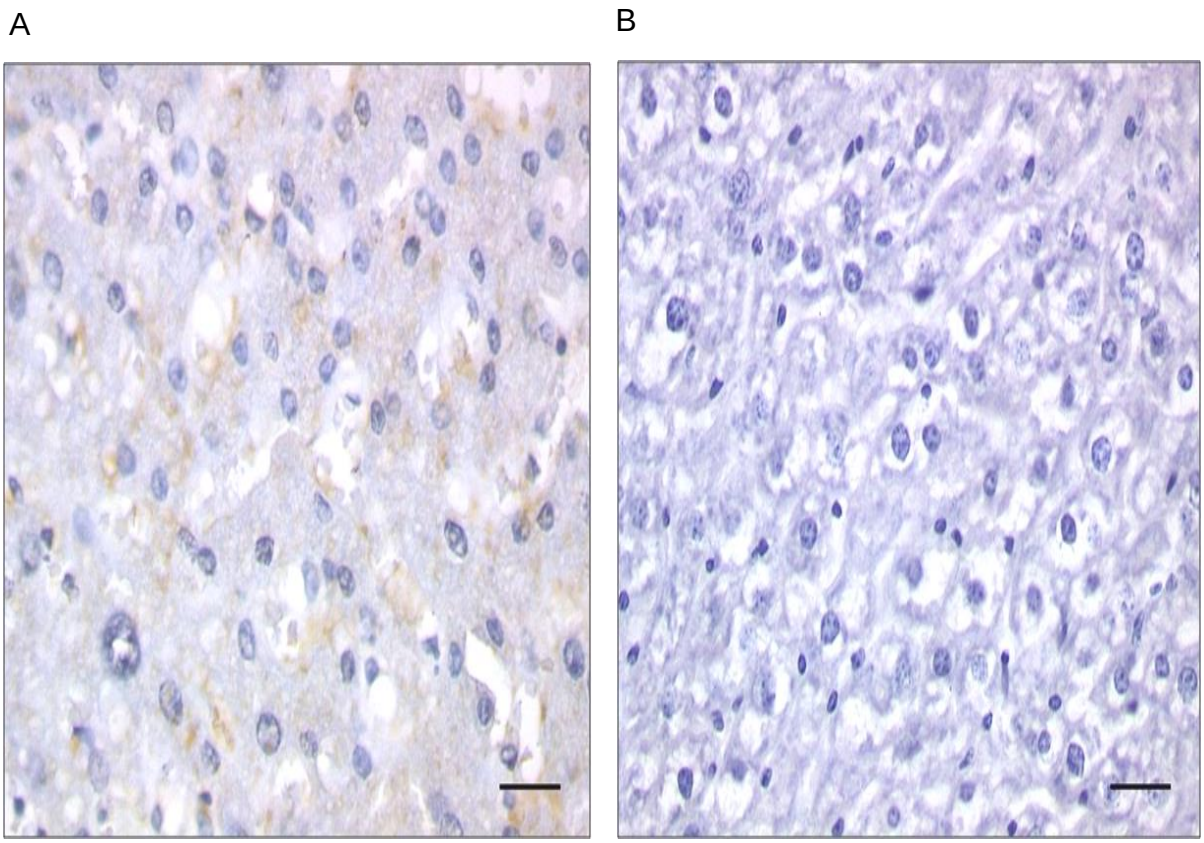


Figure 4-4: **LDLR staining in A) liver tissue (positive control) and B) PBS (negative control) at 40X magnification (scale bar**; **5µM)**.

IHC staining with anti-human LDLR polyclonal antibodies showed that the expression of LDLR in the healthy first and second trimester and term placentae was restricted to the syncytiotrophoblasts in the first and second trimester (Figure 4-5A and B) and in the syncytium at term (Figure 4-5C). Syncytiotrophoblast LDLR expression intensity progressively decreased between the first and second trimester. However, there appeared slightly reduced staining of LDLR on the syncytium of the third trimester/term healthy placental tissues (Figure 4-5C). LDLR was not localised in cytotrophoblast, stroma or fetal endothelial cells of the chorionic villi. In general, there was no staining observed in the stroma and fetal endothelial cells of the villi core. Staining was found in the fetal erythrocyte within the chorionic villi of the term placental tissue.

In the placentae of women with PE, there was faint staining of LDLR on the syncytial layer compared to the placentae of women with IUGR, which showed strong staining of LDLR on the syncytium (see Figure 4-6A and B). There was prominent LDLR staining in healthy term placental tissue compared to pre-eclamptic placental tissue. Both healthy term and IUGR placentae had strong LDLR staining on the syncytium and less intense staining on the fetal erythrocyte within the fetal blood vessels. The pattern of LDLR staining in placenta samples was consistent in all of the slides.

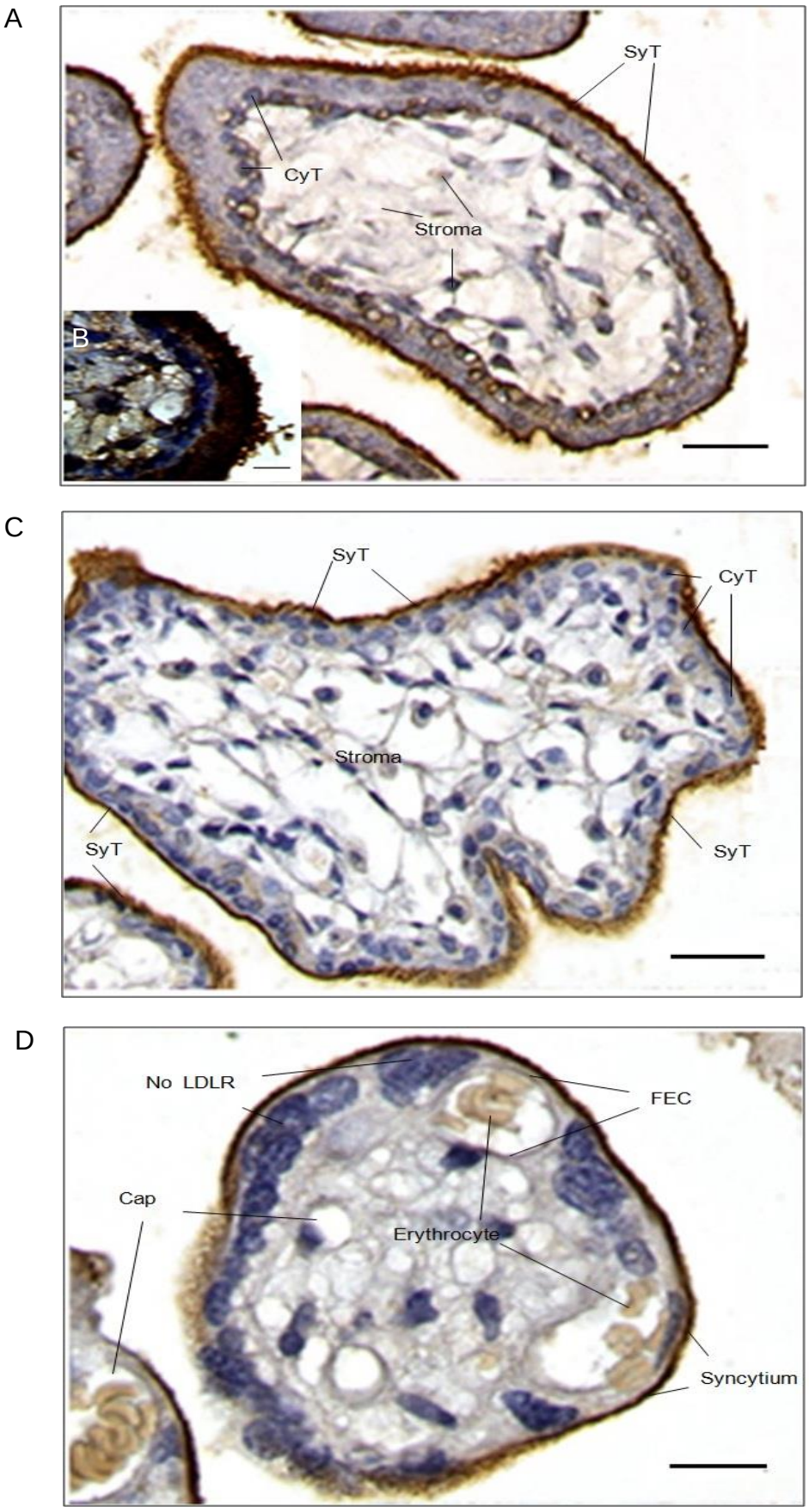
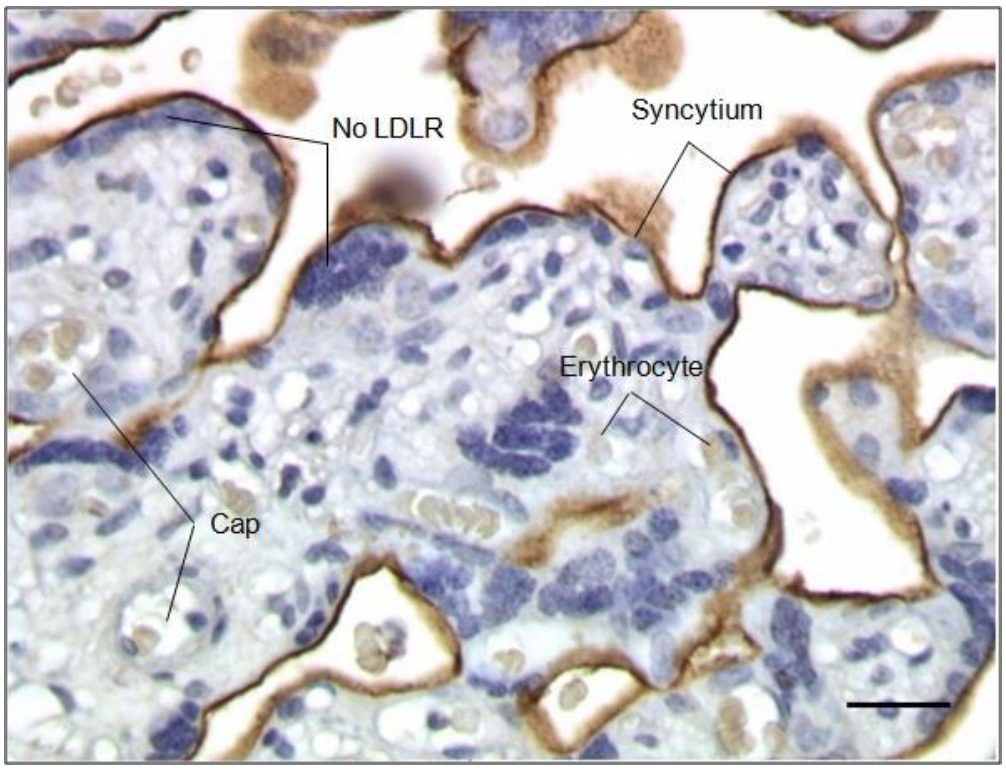


Figure 4-5: **LDLR staining in A) first trimester (scale bar 10µm) with B) insert (2µm), C) second trimester (10µm) and D) term (5µm) placentae**. Images were obtained at 20X magnification (scale bar; 10µm), 40X (5µM) or 100X (oil immersion) (2µM). SyT represents the syncytiotrophoblast and CyT the cytotrophoblast.

A



B

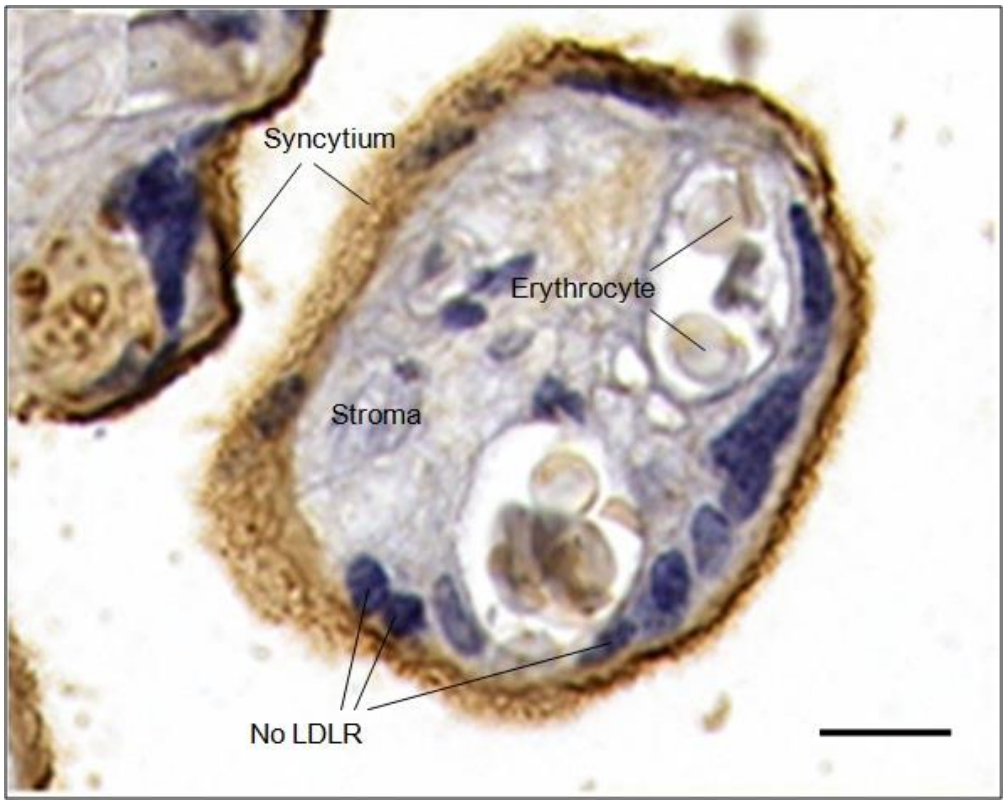


Figure 4-6: **LDLR staining in A) pre-eclamptic and B) IUGR placentae at X40 magnification (scale bar**; **5μM)**.

4.6.6.2 Localisation of STARD3 in healthy, pre-eclamptic and IUGR placentae

There was no positive STARD3 staining noticed when the antibody was replaced with PBS (negative control), whereas staining was seen in the positive control tissue (breast tumour) (Figure 4-7A and B).

A B

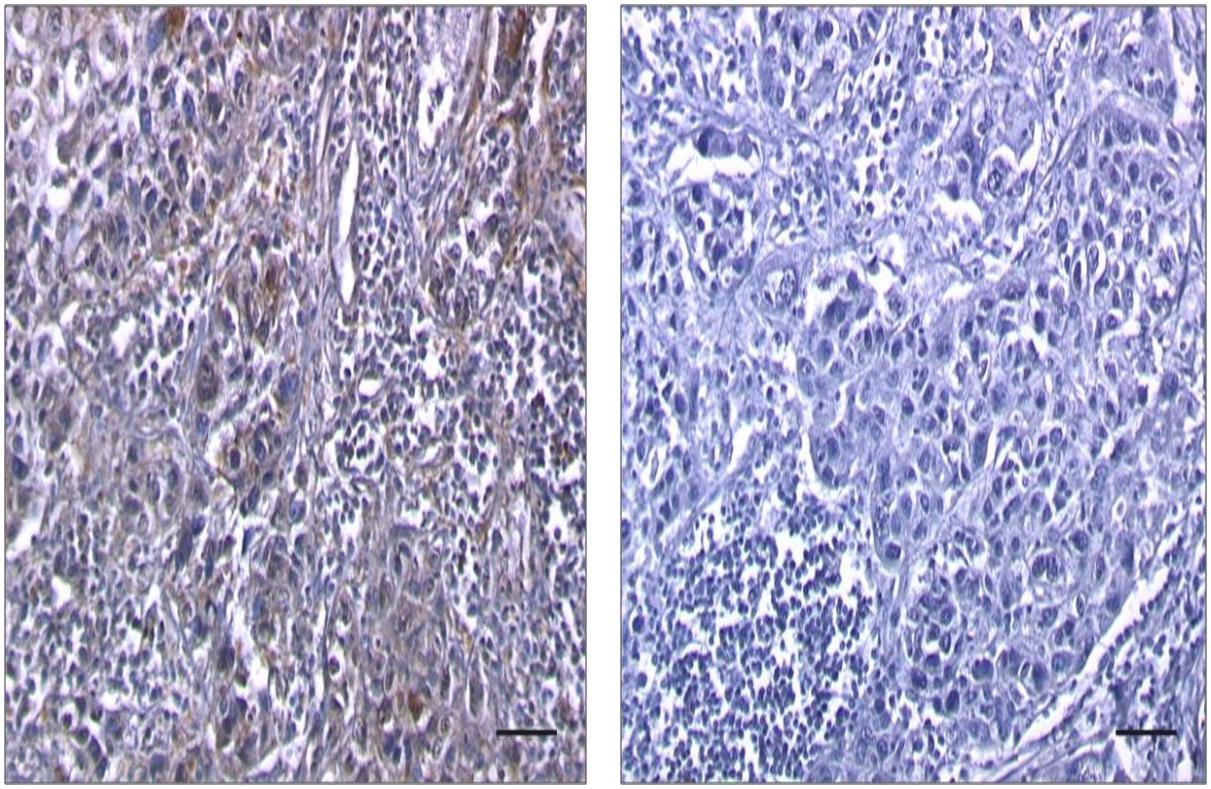


Figure 4-7: **STARD3 staining on A) breast tumour (positive control) and B) PBS (negative control) at 20X magnification (scale bar**; **10µM)**.

The IHC, using the anti-human STARD3 (MLN64) polyclonal antibody, showed expression of STARD3 in the first and second trimester and term placentae. STARD3 was widely localised in the villous and extravillous layer of the syncytiotrophoblast and the cytotrophoblast of the first and second trimester placental tissue (Figure 4-8A and B). STARD3 staining intensity was higher in trimester 2 compared to trimester 1. In addition, there was no strong observable STARD3 expression on the stroma of the placenta by term, but there was in trimesters 1 and 2. In term placental tissue, STARD3 was localised in the syncytium, stroma and the fetal endothelial cell of the chronic villi (Figure 4-8C). The difference between trimesters 2 and 3 is that STARD3 localisation intensity is higher in second trimester fetal endothelial cells, stroma, syncytiotrophoblast and cytotrophoblast compared to term placentae.

There was also STARD3 expression in PE and IUGR placentae. STARD3 staining was observed in the syncytial layer, stroma and fetal endothelial cells in pre-eclamptic samples (Figure 4-9A). STARD3 was expressed on the fetal erythrocyte. There was faint staining of STARD3 in the stroma. Similarly, IUGR placental tissue showed that STARD3 was localised in the syncytium, stroma and fetal endothelial cells in the chorionic villous (Figure 4-9B). Intensity of the STARD3 was also high in the syncytial layer and fetal endothelial cells compared to the expression in the stroma (as shown in Figure 4-9B). There appeared to be a greater intensity of STARD3 staining in the pre-eclamptic compared to the third trimester healthy placentae (Figures 4-9B and 4-8C). In the placentae of women with IUGR, there was STARD3 expression towards the stroma in the chorionic villous core compared to term placental tissue. STARD3 staining was higher in pregnancies complicated by IUGR compared to third trimester placenta samples. However, both PE and IUGR placenta STARD3 localisation revealed a slightly similar intensity of staining. There was a similar pattern of STARD3 staining in placenta samples that was consistent in all of the slides.

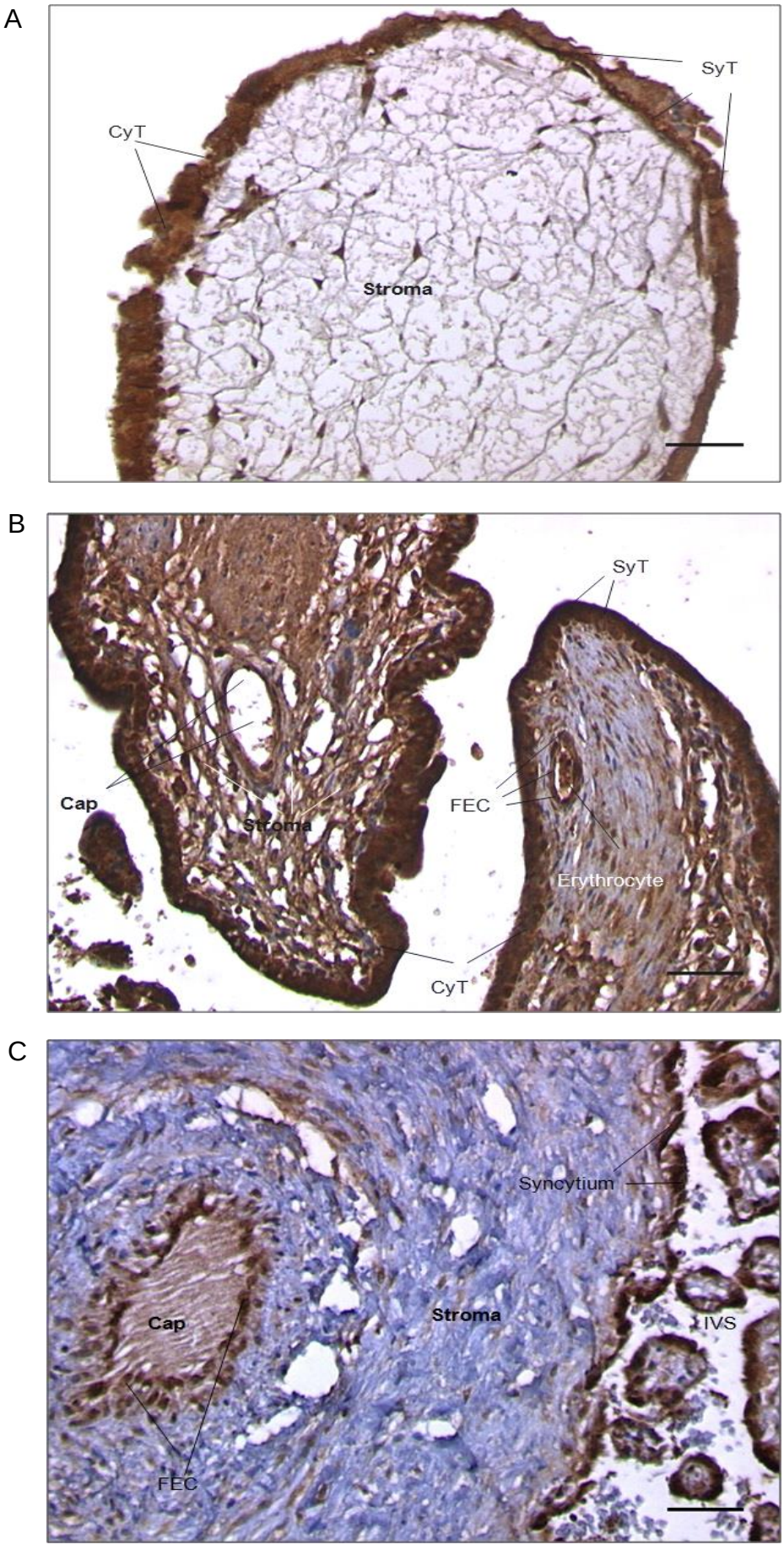
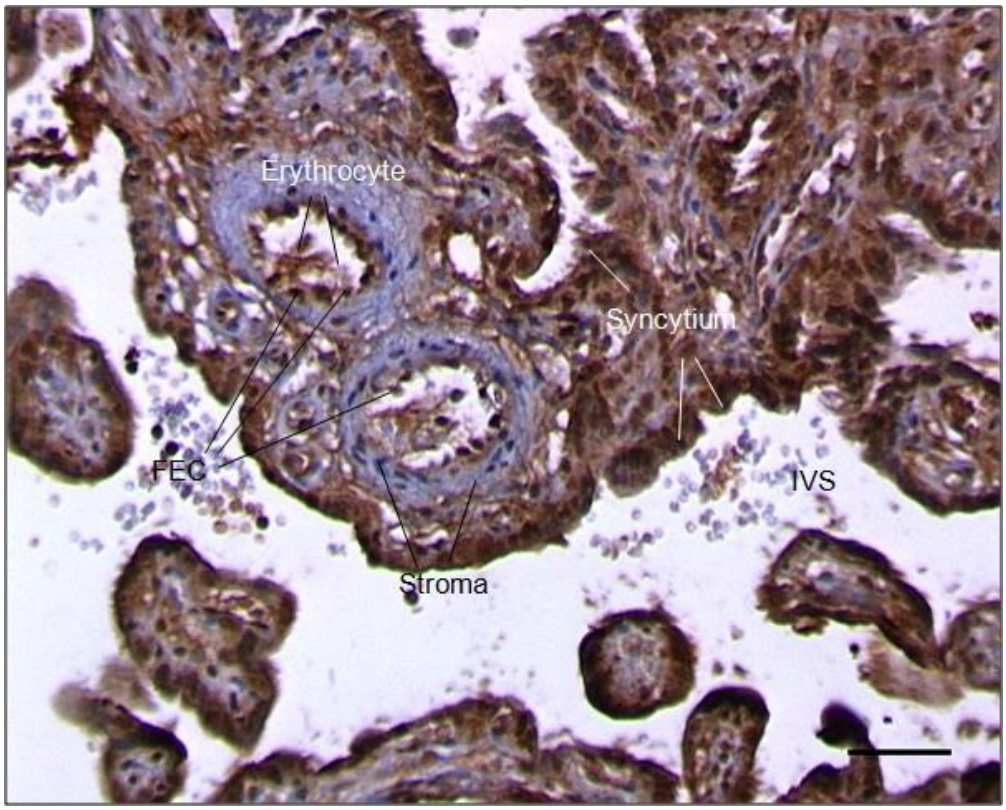


Figure 4-8: **STARD3 staining in A) first trimester, B) second trimester and C) term placentae at 20X magnification (scale bar**; **10µm)**. SyT represents the syncytiotrophoblast and CyT the cytotrophoblast.

A



B

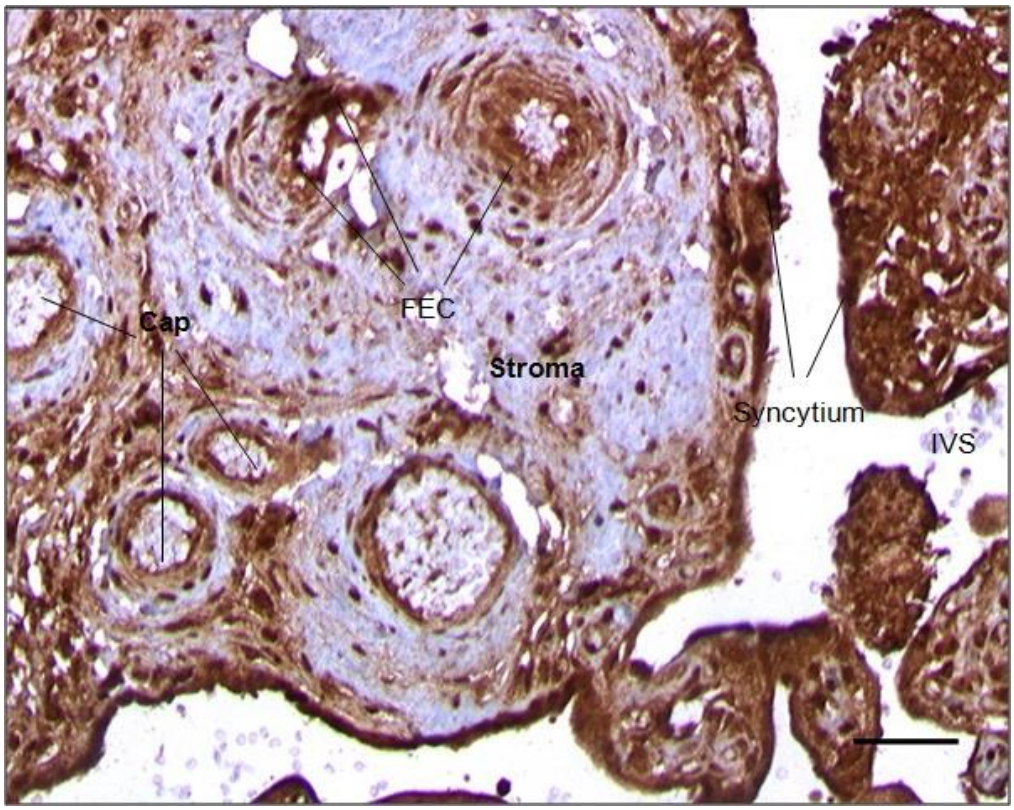


Figure 4-9: **STARD3 staining in A) pre-eclamptic and B) IUGR placentae at X20 magnification (scale bar**; **10μM**).

4.6.6.3 Localisation of ABCA1 in healthy, pre-eclamptic and IUGR placentae

No staining was observed in negative controls, but it was observable in the positive control (human liver) (Figure 4-10A and B).

A B

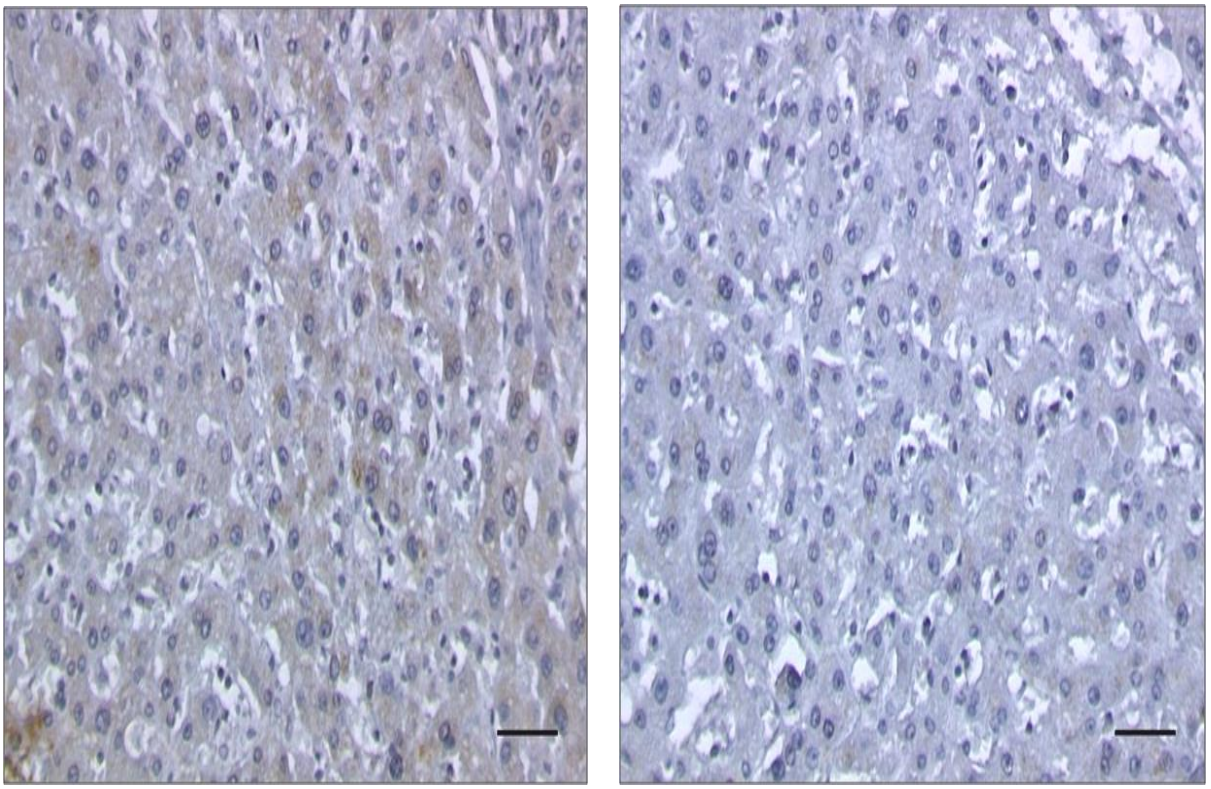


Figure 4-10: **ABCA1 staining on A) liver tissue (positive control) and B) PBS (negative control) at 20X magnification (scale bar**; **10µM)**.

The anti-human ABCA1 polyclonal antibody immunohistochemistry staining revealed that ABCA1 was expressed in the first and second trimester and term placental tissue (Figure 4-11A, B and C). Staining of ABCA1 was localised in the villous cytotrophoblast cells and the syncytiotrophoblasts of the first and second trimesters (Figure 4-11A and B). The second trimester placental tissue cytotrophoblasts and syncytiotrophoblasts showed a higher intensity of ABCA1 (Figure 4-11B) compared to first trimester (Figure 4-11A) placentae. In the term placenta, syncytium (Figure 4-11C) showed a less intense ABCA1 staining compared to the first and second trimester placental tissue (Figure 4-11A and B). A distinct staining was observed in the fetal endothelial cells of the chorionic villi, with no staining in the stroma of term placentae (as shown in Figure 4-11C). Essentially, there appeared to also be prominent ABCA1 staining in the fetal erythrocyte, as noticed in the fetal blood vessels of term placenta samples.

ABCA1 was also expressed in the pre-eclamptic and IUGR placentae (Figure 4-12A and B). In cases of PE there was extensive ABCA1 staining, including on the syncytial layer, the fetal endothelial cell and faintly on the stroma (Figure 4-12A), whilst staining was restricted to the syncytial layer and fetal endothelial cells in the IUGR placental tissue (Figure 4-12B). The staining intensity of ABCA1 was slightly higher in all the cell types, syncytial (trophoblasts) and fetal endothelial cells of the placentae of PE, compared to those of healthy term placentae (Figures 4-12A and 4-11C). In contrast, expression of ABCA1 in IUGR and term placentae appeared fairly similar levels of staining intensity (Figure 4-12B and 4-11C). The pattern of ABCA1 staining in placenta samples was consistent in all of the slides.

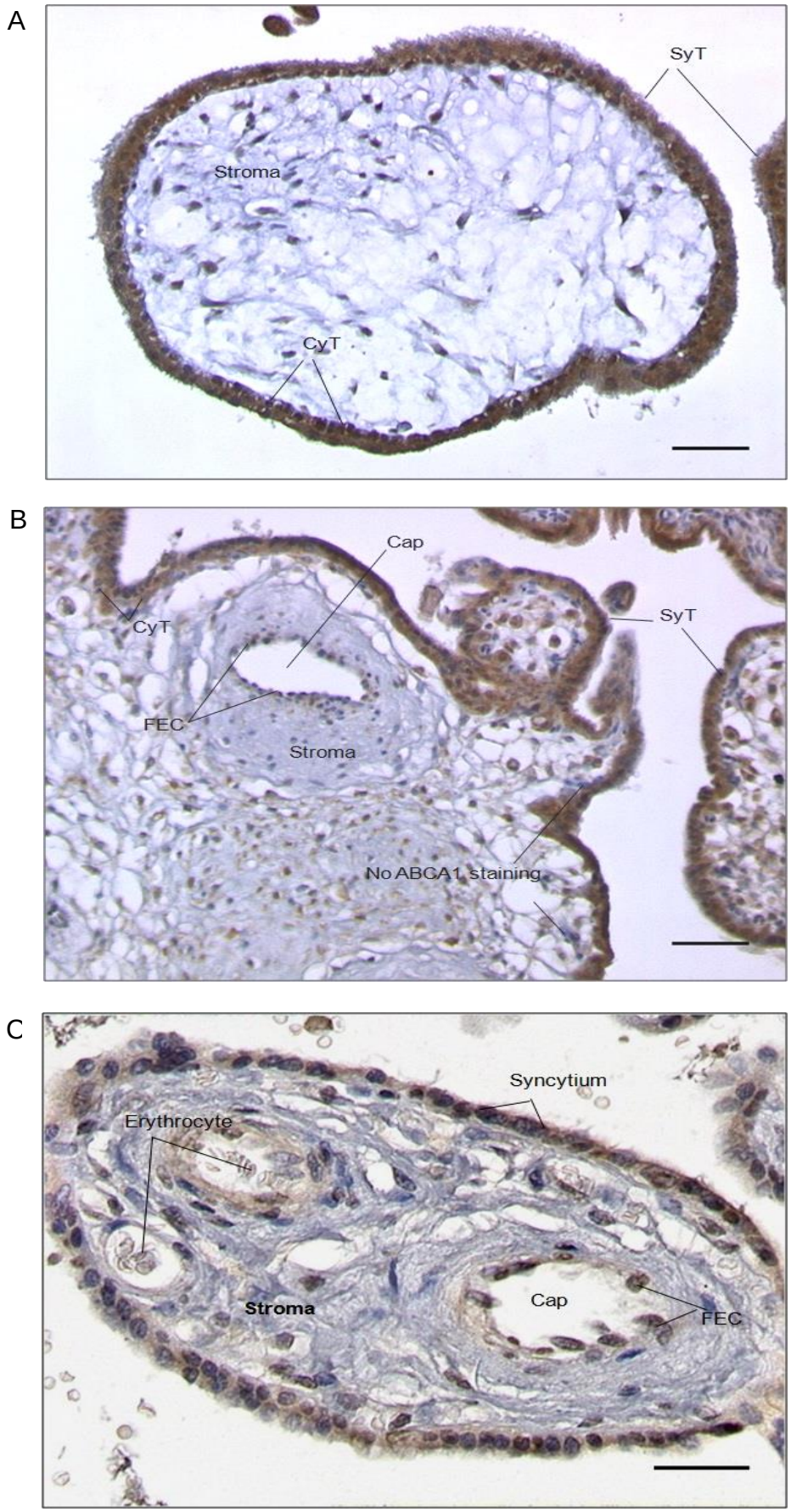
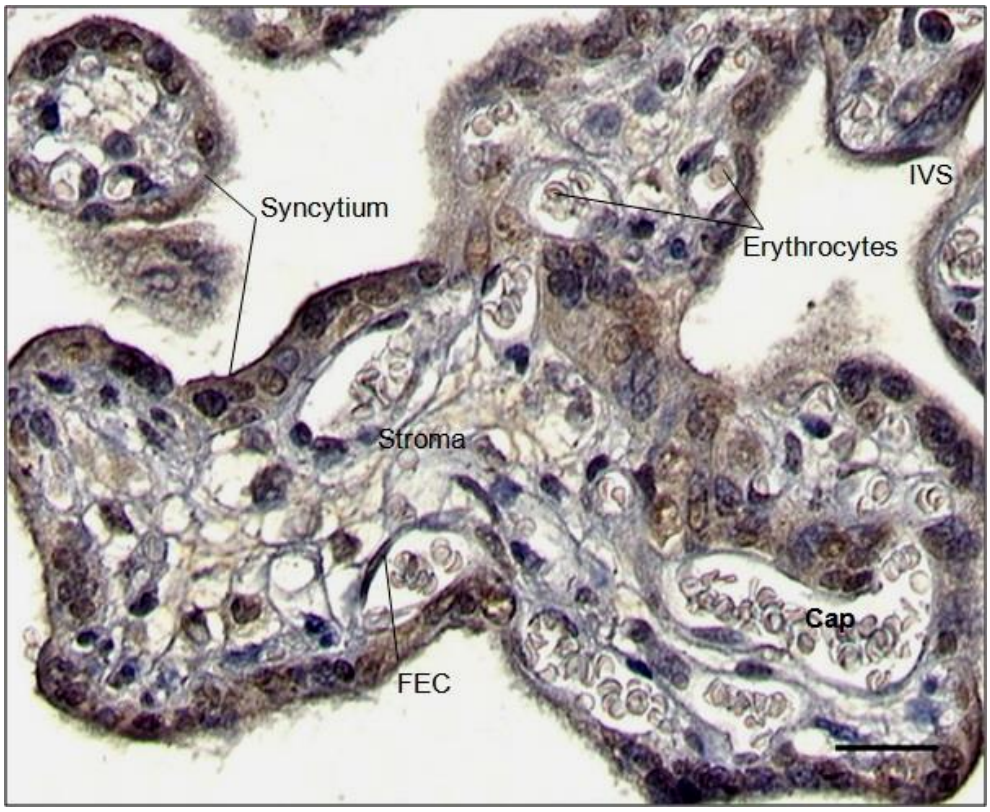


Figure 4-11: **ABCA1 staining in A) first (10μM), B) second (10μM) and C) third trimester (5μM) placentae**. Images were obtained at 20X magnification (scale bar; 10μm) and 40X (5μM). SyT represents the syncytiotrophoblast and CyT the cytotrophoblast.

A



B

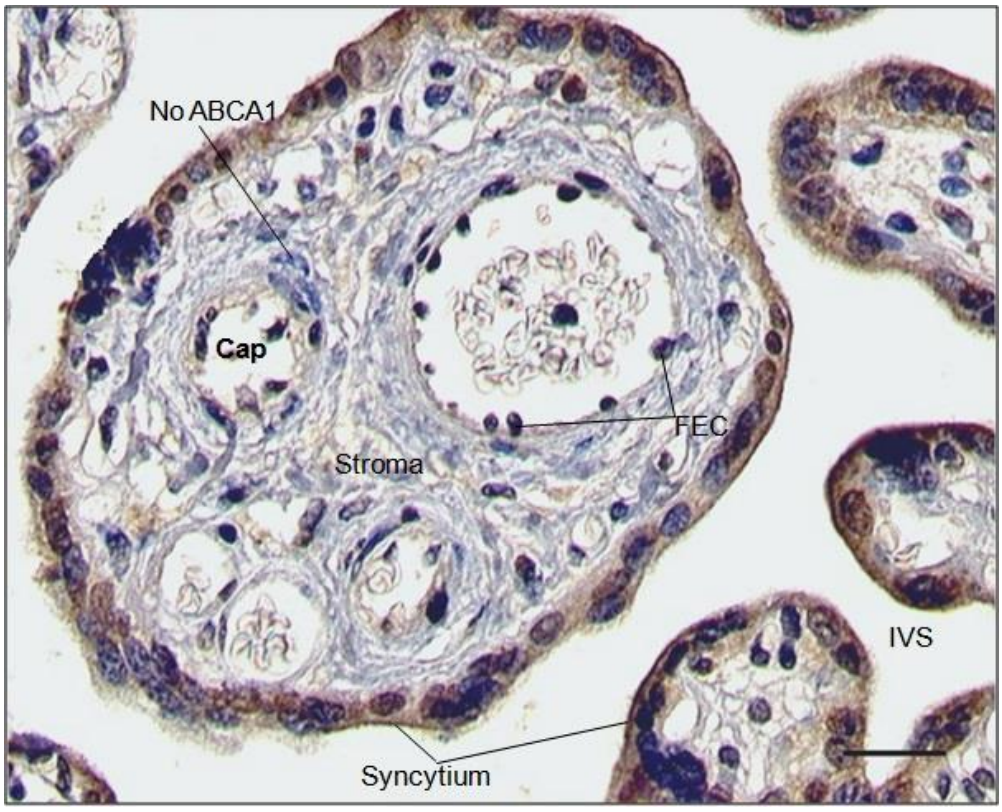


Figure 4-12: **ABCA1 staining in A) pre-eclamptic and B) IUGR placentae at X20 magnification (scale bar**; **10µM)**.

4.6.6.4 Localisation of ABCG1 in healthy, pre-eclamptic and IUGR placentae

There was also no positive staining seen when the antibody was replaced with PBS (negative control) compared to positive tissue (human lungs) (Figure 4-13A and B).

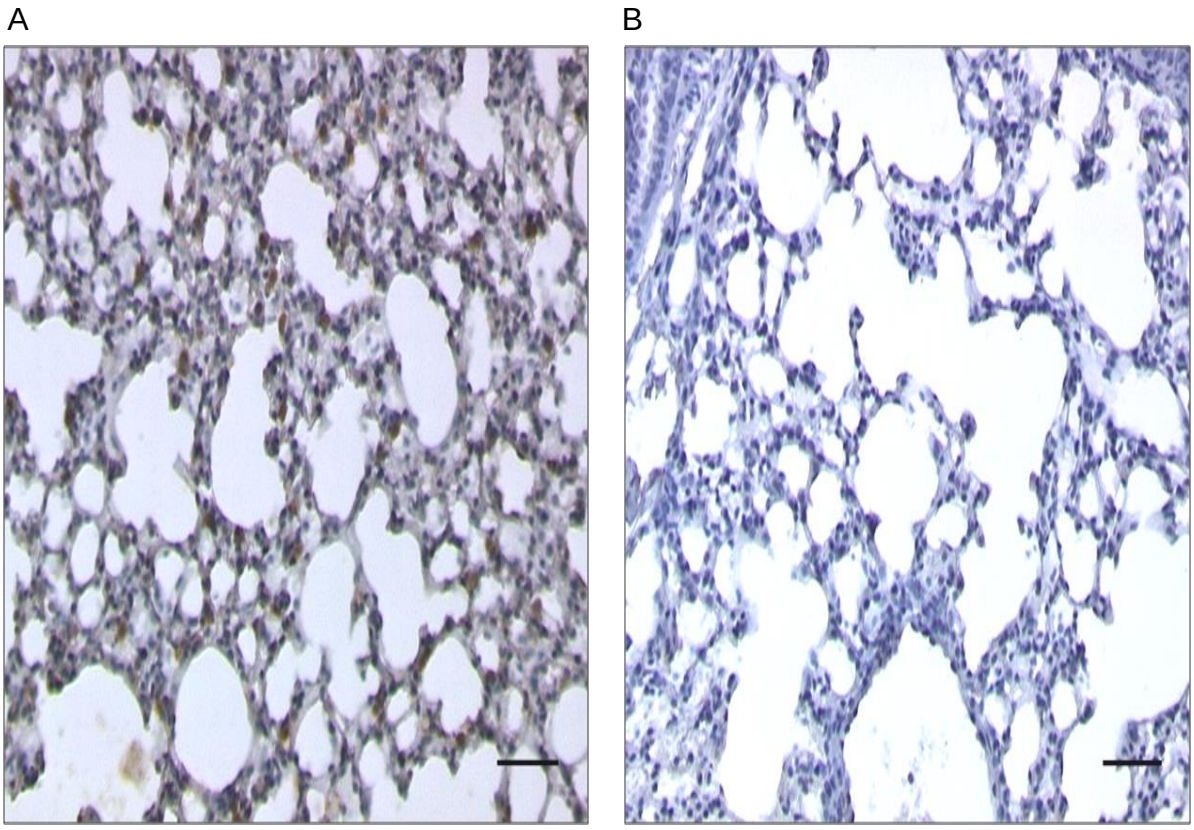


Figure 4-13: **ABCG1 staining on A) lung tissue (positive control) and B) PBS (negative control) at 20X magnification (scale bar**; **10µM)**.

IHC staining with anti-human ABCG1 polyclonal antibodies demonstrated expression of ABCG1 in trimester 1 and 2 and term placentae. Staining showed that ABCG1 was widely expressed in the syncytiotrophoblasts and cytotrophoblasts of the first trimester and second trimester placentae (Figure 4-14A and B). In the term placental sample tissue, ABCG1 was localised in the syncytial layer and the fetal endothelial cells in the chorionic core (Figure 4-14C). The staining intensity progressively increased throughout gestation (Figure 4-14A, B and C). A distinct expression of ABCG1 was observed in the syncytium, fetal endothelial cell and fetal erythrocyte in the term placental tissue whereas the stroma stained faintly (Figure 4-14C).

ABCG1 was expressed in PE and IUGR placentae (Figure 4-15A and B). In pre-eclamptic placentae, distinct ABCG1 staining was observed in the syncytium but less in the fetal endothelial cells (Figure 4-15A). Comparison between PE and term placentae showed slightly lower ABCG1 staining in stroma of the pre-eclamptic than in healthy term samples (Figures 4-15A and 4-14C). A similar ABCG1 staining of syncytial and fetal endothelial cells was observed in the IUGR placentae (Figure 4-15B). There was also no staining of the stroma with ABCG1 in the IUGR placentae (Figures 4-15A and B). Nevertheless, the intensity of ABCG1 staining was slightly higher in pre-eclamptic placentae compared to those of IUGR patients. Also, the pattern of ABCG1 staining in placenta samples was consistent in all of the slides.

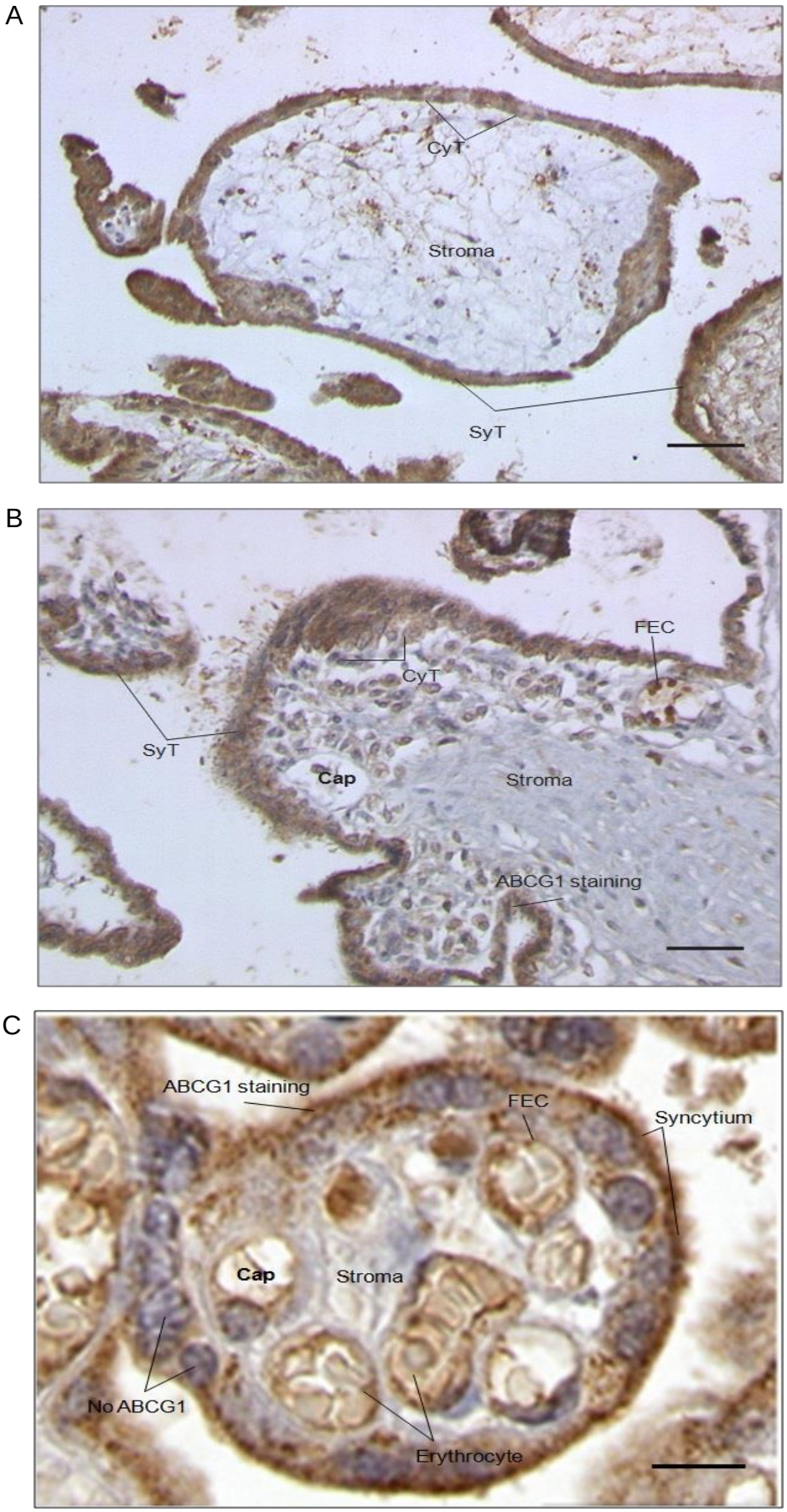
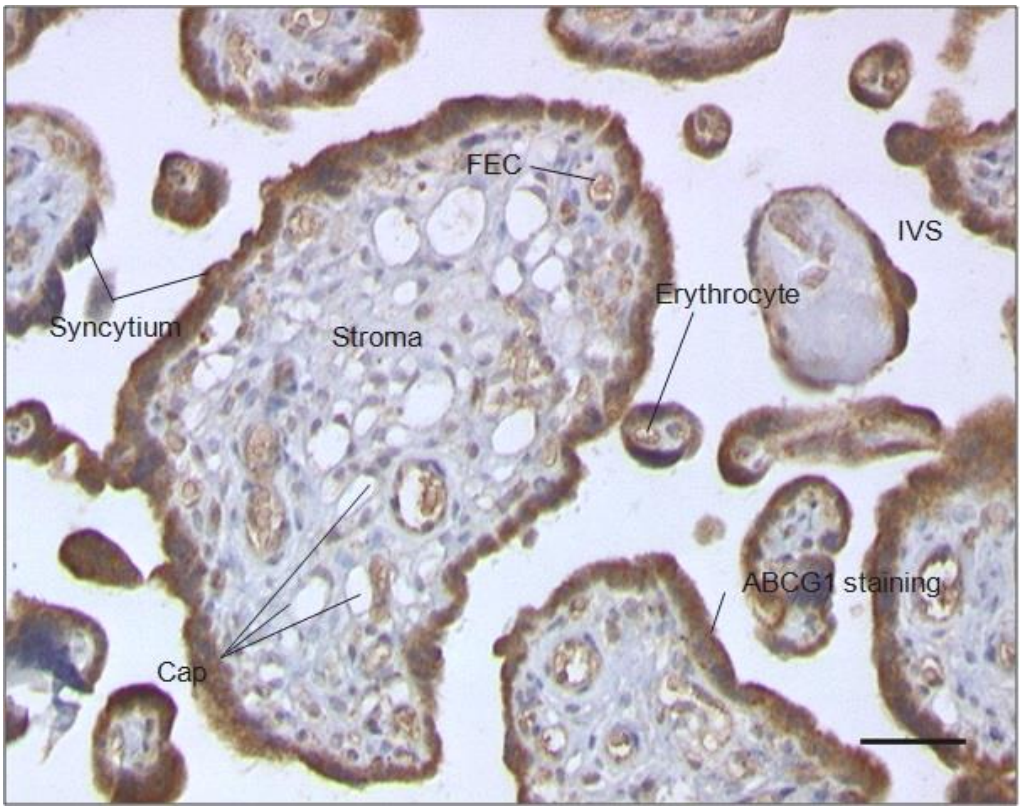


Figure 4-14: **ABCG1 staining in A) first (10μM), B) second (10μM) and C) third trimester (5μM) placentae**. Images were obtained at 20X magnification (scale bar; 10μm) and 40X (5μM). SyT represents the syncytiotrophoblast and CyT the cytotrophoblast.

A



B

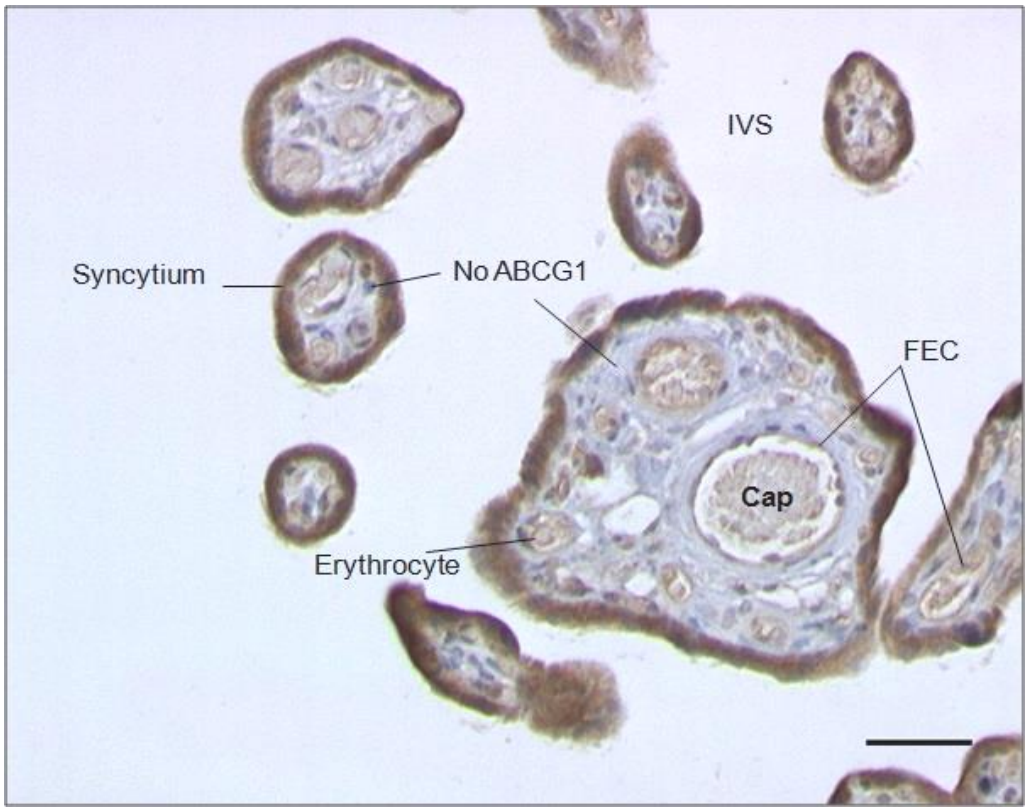


Figure 4-15: **ABCG1 staining in A) pre-eclamptic and B) IUGR placentae at X20 magnification (scale bar**; **10µM)**.

4.6.6.5 Placental mRNA expression of LDLR, STARD1, 3, 4, 5, ABCA1, ABCG1, CD36 and apo E in pre-eclamptic, IUGR and healthy pregnancies

Messenger RNA expression of a number of molecules potentially involved in placental cholesterol transport was assessed in healthy, PE and IUGR placentae. The lipid transporters assessed were LDLR, STARD1, STARD3, STARD4, STARD5, ABCA1, ABCG1, CD36 and apo E. The assay was performed in cases of PE (n=20) and BMI-matched controls (n=20). Quantitative expression data are shown in Figure 4-16. The LDLR mRNA expression profile in the placenta was higher in the PE group compared to the healthy group [424 (558) PE vs 139 (123) control; LDLR/18S ratio $x10^{-4}$, P=0.037]. The STARD3 mRNA expression was upregulated in the PE group [780 (516) PE vs 486 (368) control; STARD3/18S ratio $x10^{-4}$, P=0.046] compared to the control pregnancies. Placental expression of ABCA1 mRNA [354 (301) PE vs 107 (91) control; ABCA1/18S ratio $x10^{-4}$, P=0.002] was significantly higher in the PE group compared to healthy pregnancies. There was no observable difference in the STARD1, 4, 5, ABCG1 and apo E expression between the PE and control groups. However, there is a trend toward higher CD36 [3467 (2423) PE vs 2206 (1727) control; CD36/18S ratio $x10^{-4}$, P=0.067].

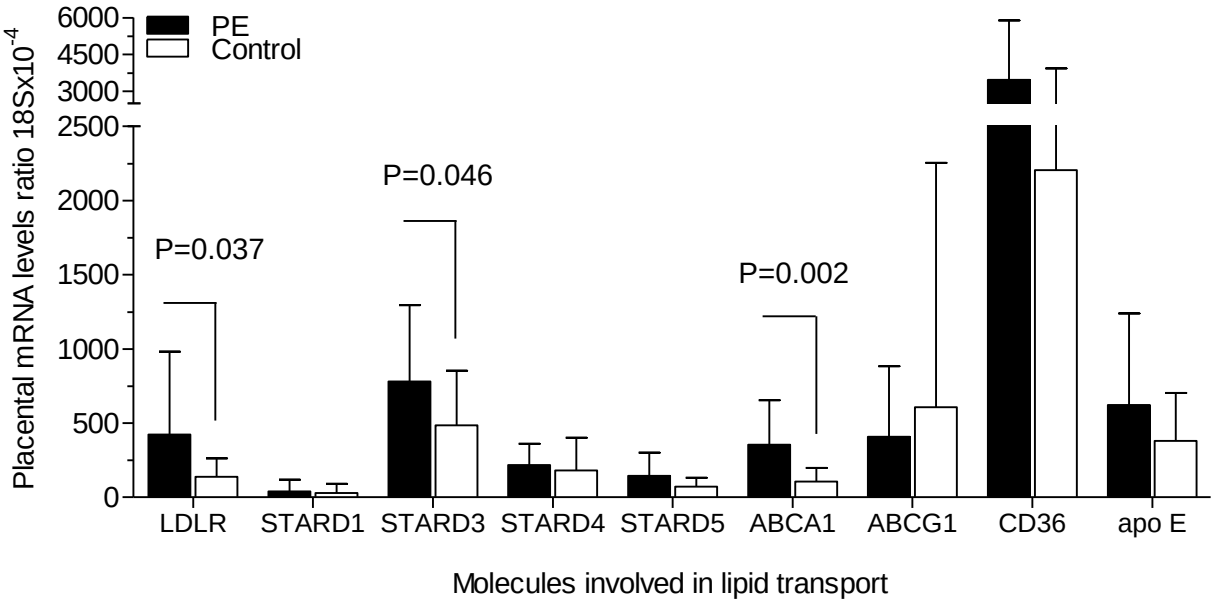


Figure 4-16: **Placental mRNA expression levels relative to 18S in women with PE (n=20) and BMI-matched controls (n=20)**. Mean (SD) shown.

Quantitation of mRNA expression of these transporters was also carried out in women with IUGR (n=9) and BMI-matched controls (n=9). There were no differences in the mRNA expression in lipid transporters in the women with IUGR compared to the BMI-matched controls, including LDLR, STARD1, 3, 4, 5, ABCA1, ABCG1, CD36 and apo E (Figure 4-17). However, there was the same pattern of higher LDLR, STARD3 and ABCA1 expression in IUGR pregnancies compared to healthy pregnancies, which may be indicative of the under-powered number.

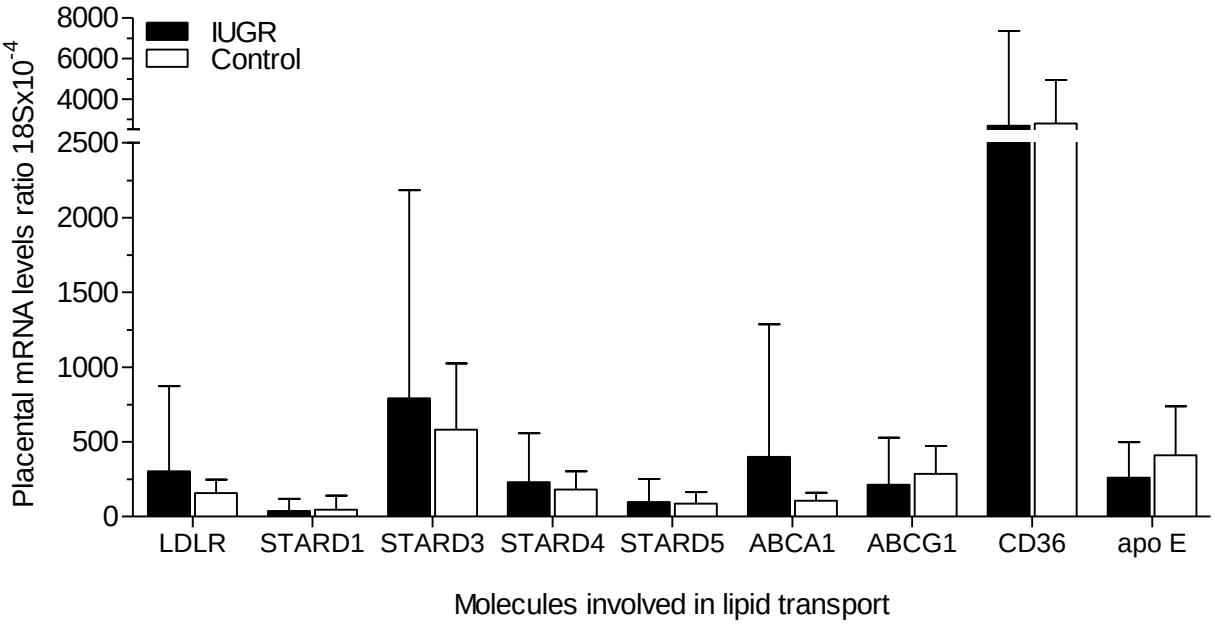


Figure 4-17: **Placental mRNA expression levels relative to 18S in IUGR pregnancies (n=9) and BMI-matched controls (n=9)**. Mean (SD) shown.

4.6.7 First and third trimester effect on placental levels

The expression of lipid transporters was compared between first trimester (n=6) and term (n=20) placentae. There was no difference in LDLR mRNA expression between first trimester and term placentae. However, there was a trend to lower LDLR mRNA expression in the third trimester. Comparison of STARD3 mRNA expression between first trimester and term placentae showed higher mRNA expression levels at term [mean (SD) 486 (368) term vs 252 (114) first trimester STARD3/18S ratio 10^{-4}, P=0.021] (Figure 4-18). There was no difference in ABCA1 mRNA expression between first trimester and term placentae.

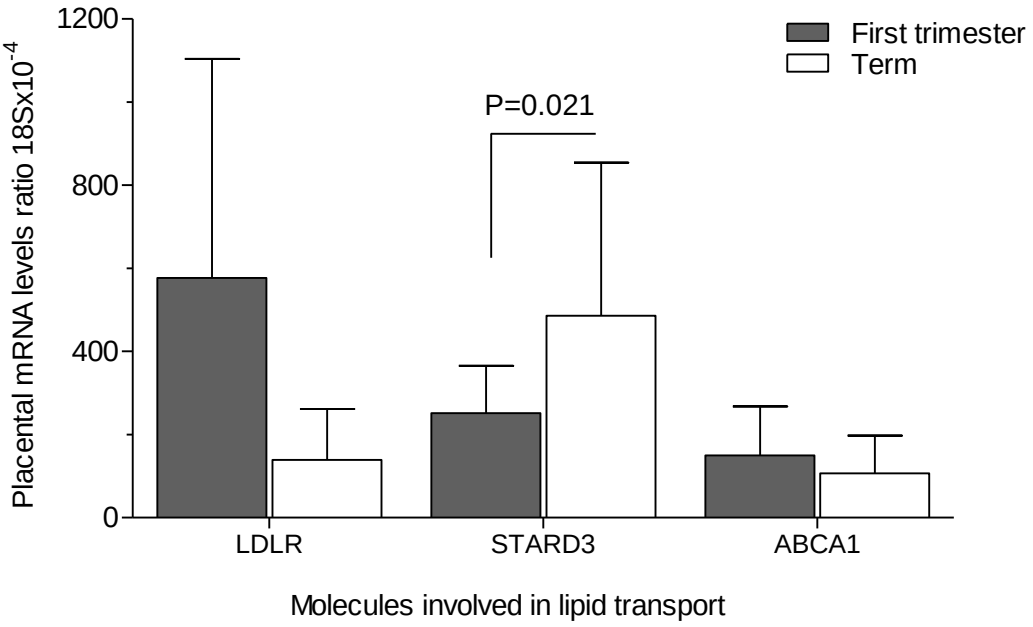


Figure 4-18: **Placental mRNA expression levels relative to 18S in first trimester (n=6) and third trimester placentae (n=20)**. Mean (SD) shown.

4.6.8 The role of fetal endothelial and trophoblast cells in placental transporters' expression level

Measurement of the endothelial cell adhesion molecule (CD31) and cytokeratin-7 (KRT7) was used to ascertain the presence of endothelial and trophoblast cells, respectively. This was in order to get an indication as to whether it was the transporter expression levels in endothelial cells or in trophoblasts that changed, by expressing the transporters relative to CD31 or KRT7. LDLR mRNA expression relative to CD31 was higher in pre-eclamptic placentae compared to control placentae [58 (12) PE vs 31 (5) control; LDLR/CD31 ratio, P=0.046] (Figure 4-19A). There was no difference in STARD3 expression relative to CD31 between PE and control groups. ABCA1 mRNA expression relative to CD31 was higher in the PE group compared to healthy pregnancies [61 (8) PE vs 22 (3) control ABCA1/CD31 ratio, P<0.001].

In contrast, LDLR had a borderline higher mRNA expression in the IUGR group compared to controls [44 (28) IUGR vs 23 (11) control; LDLR/CD31 ratio, P=0.056] (Figure 4-19B). There was higher STARD3 mRNA expression in IUGR pregnancies compared to healthy pregnancies [134 (60) IUGR vs 75 (44) control; STARD3/CD31 ratio, P=0.032]. However, no difference in ABCA1 expression relative to CD31 was observed between the IUGR group and controls.

These data would support the view that LDLR and ABCA1 expression located in endothelial cells is higher in PE samples than in controls. Similarly, STARD3 expression in endothelial cells may be higher in IUGR patients.

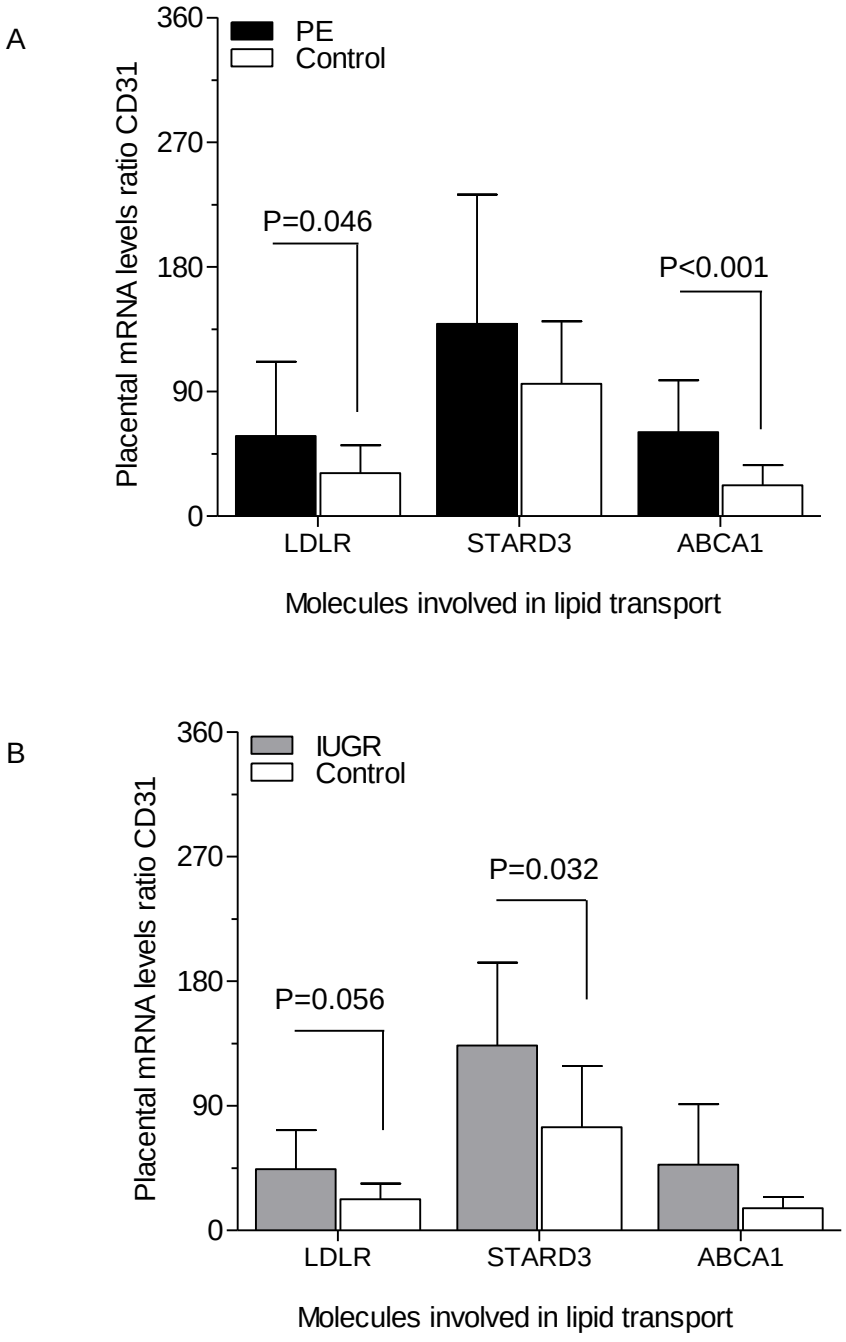


Figure 4-19: **Placental transporter mRNA levels expressed relative to an endothelial cell marker**. A) LDLR, STARD3 and ABCA1 mRNA expression relative to CD31 in PE cases (n=20) and BMI-matched controls (n=20); B) IUGR cases (n=9) and BMI-matched controls (n=9). Mean (SD) shown.

In order to ascertain whether it was the transporters' expression levels in trophoblasts that changed, expression of the transporters relative to KRT7 was carried out and showed that there was no difference in LDLR and STARD3 relative to KRT7 mRNA expression between PE cases and BMI-matched controls. In contrast, there was higher ABCA1 mRNA expression relative to KRT7 in women with PE compared to controls [94 (81) PE vs 50 (21) control; ABCA1/KRT7 ratio, P=0.029] (Figure 4-20A). LDLR, STARD3 and ABCA1 mRNA expression relative to KRT7 was not different between IUGR cases and controls (Figure 4-20B).

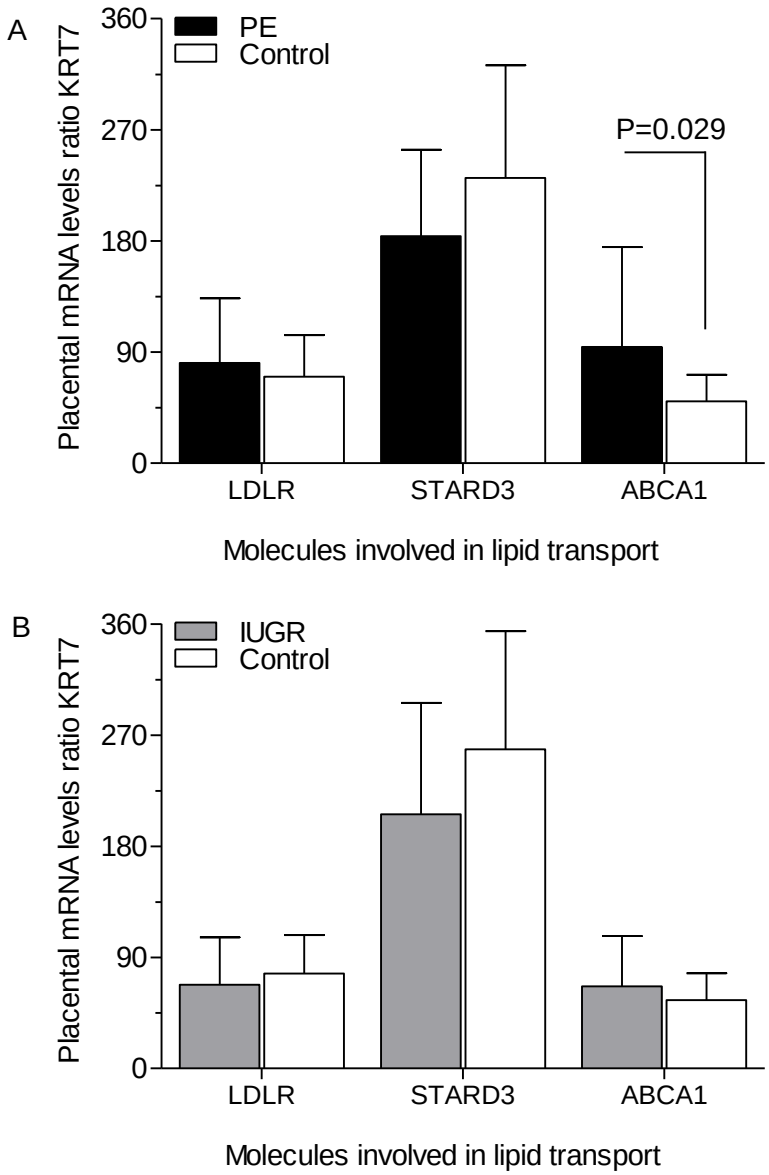


Figure 4-20: **Transporter expression expressed relative to a trophoblast marker**. A) LDLR, STARD3 and ABCA1 mRNA level relative to KRT7 in PE cases (n=20) and BMI-matched controls (n=20); B) IUGR cases (n=9) and BMI-matched controls (n=9). Mean (SD) shown.

4.6.9 ABCA1 relationship to maternal plasma cholesterol and potential ABCA1 and STARD proteins regulation by LXR-α

There was a need to examine the relationship between key cholesterol transporters since umbilical cord TC reflected maternal levels, with a trend towards higher levels. We investigated whether certain regulatory proteins may affect the activity of lipid transporter gene regulation. As noted above, ABCA1 is a recognised downstream target of the transcriptional regulation of LXR-α. LXRs regulate cholesterol uptake and secretion with two LXR isoforms known as LXR-α and LXR-β (Peet et al. 1998; Edwards et al. 2002). LXR-α broadly limits cholesterol accumulation by regulating the expression of genes involved in cholesterol efflux. LXR-α induces transcription of proteins involved in the disposal of cholesterol from cells by ABCA1 (Plosch et al. 2007).

Pearson correlation analysis showed a strong relationship between ABCA1 mRNA expression level and maternal plasma TC levels (r=0.58, P=0.012) in the PE group but not in the control group (Figure 4-21A). There was no difference in the placental LXR-α mRNA level [238 (214) PE vs 149 (137) control LXR-α/18S ratio $x10^{-4}$, P=0.13]; however, there was reasonably close correlation between ABCA1 mRNA levels and LXR-α mRNA expression (r=0.44, P=0.055) in the PE group (Figure 4-21B).

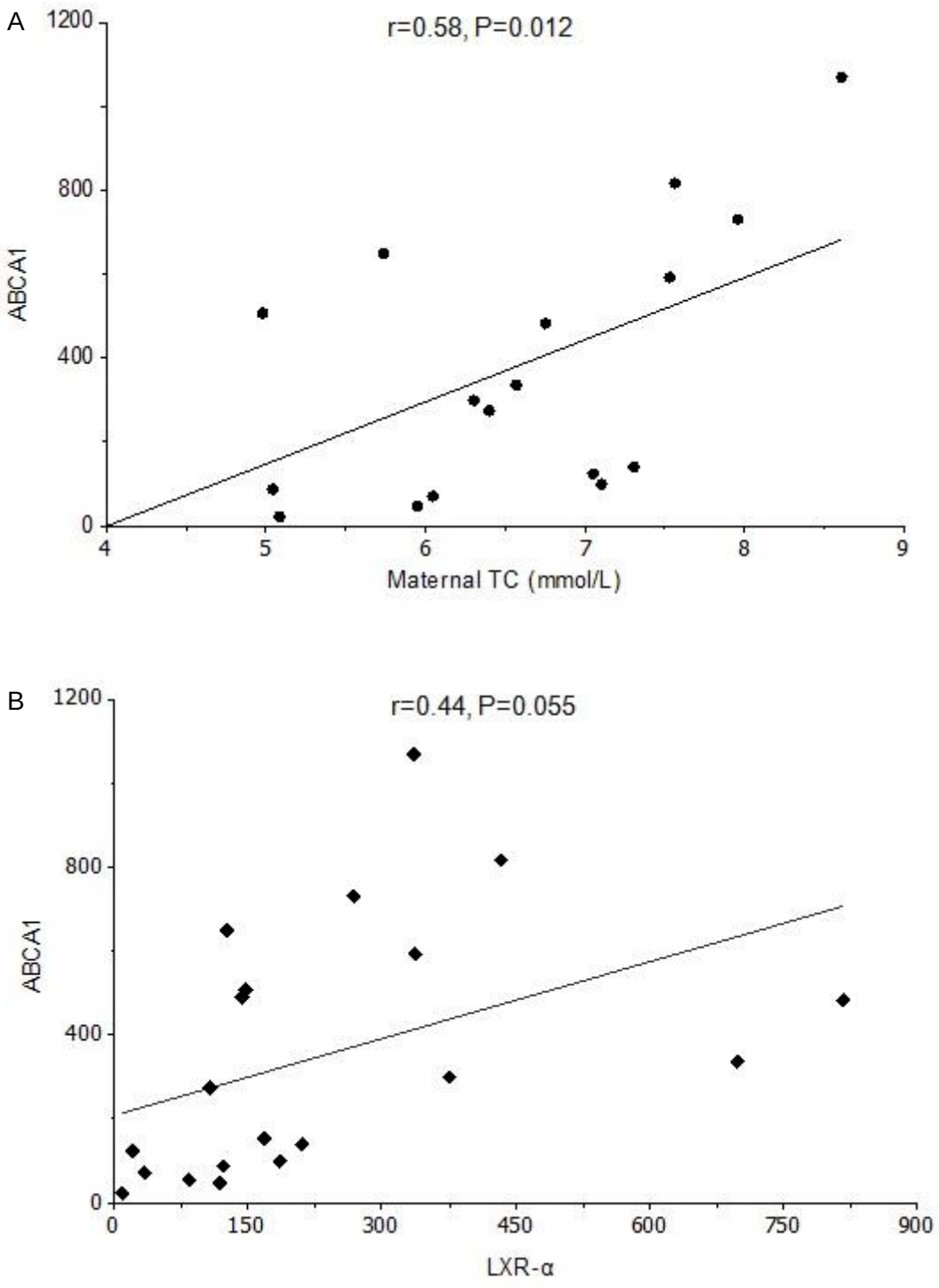


Figure 4-21: **ABCA1 mRNA level relationship to maternal cholesterol and LXR-α regulation**. A) Placental ABCA1 mRNA correlation with maternal TC in PE mothers (r=0.58, P=0.012). B) Relationship between ABCA1 and LXR-α mRNA expression in the PE group (r=0.44, P=0.055).

Also, START domain proteins are thought to be regulated by LXR targets, as their expression stimulates LXR reporter activity (Soccio et al. 2005), indicative of the role of LXR targets in cholesterol transports. The STAR gene is activated by LXR-α (Jefcoate 2006), and the stimulation of STAR expression by LXR-α activation is explained by the identification of a new LXR-α response element in the STAR promoter (Cummins et al. 2006). In this context, it was examined whether LXR-α may influence placental START domain proteins. It was observed that LXR-α correlated to STARD3 (r=0.71, P<0.001) (Figure 4-22A) and STARD4 (r=0.89, P<0.001) (Figure 4-22B).

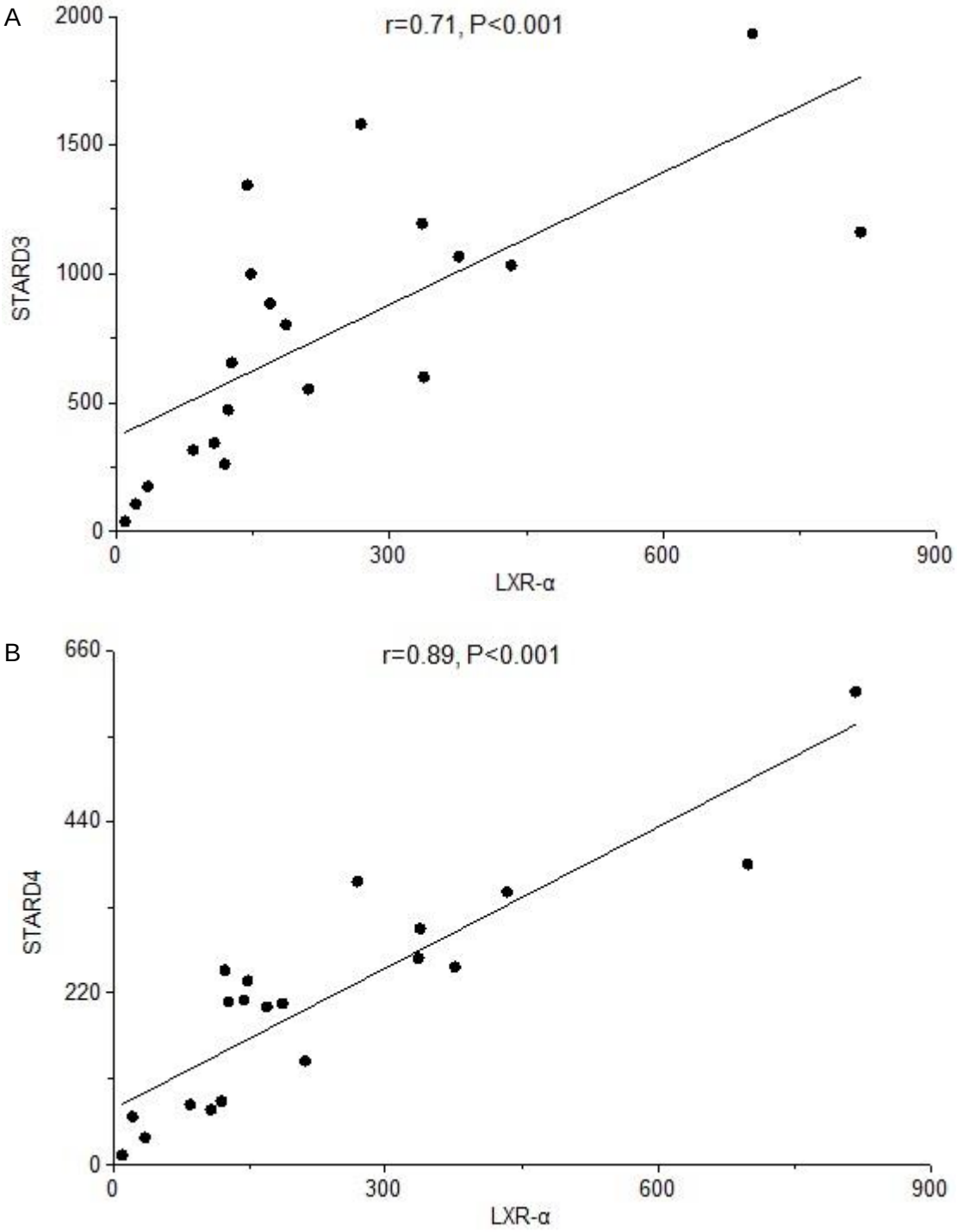


Figure 4-22: **Placental STARD3 and STARD4 mRNA level relationship to LXR-α regulation**. A) Placental STARD3 and B) STARD4 mRNA correction with LXR-α expression (r=0.71, P<0.001) and (r=0.89, P<0.001) in PE, respectively.

4.6.10 LDLR, STARD3 and ABCA1 placental protein expression

Having determined the placental mRNA expression levels of molecules involved in the cholesterol transport, it was necessary to test whether the differences in mRNA expression are reflected in differences in protein expression. Therefore, levels of placental protein expression were assessed for LDLR, STARD3 and ABCA1. Western blotting analysis was used to assess protein changes in placentae of pregnancies complicated by PE and BMI-matched control pregnancies. Similarly, protein expression of LDLR, STARD3 and ABCA1 was also assessed in IUGR placentae and BMI-matched controls.

Initially, the placental protein extractions were prepared. Equal amounts of protein were added to each lane for gel electrophoresis. As expected, placental expression of LDLR, STARD3 and ABCA1 were detected in all PE, IUGR and controls samples. The predicted band size, according to the manufacturer's instructions for LDLR, was 95kDa, compared to the expected 160kDa molecular size (Figure 4-23A). In this experiment, the estimated vs expected molecular weights for LDLR, STARD3 and ABCA1 were 95kDA vs 160kDa (Figure 4-23A), 53kDa vs 50kDa (Figure 4-23B) and 220kDa vs 220kDa (Figure 4-23C), respectively. Interestingly, there were no significant differences in LDLR, STARD3 and ABCA1 placental protein expression relative to β-Actin between pre-eclamptic placentae and BMI-matched healthy placentae. The lack of difference in mRNA expression of LDLR, STARD3 and ABCA1 in cases of IUGR and BMI-matched controls was confirmed by the absence of difference in the protein expression.

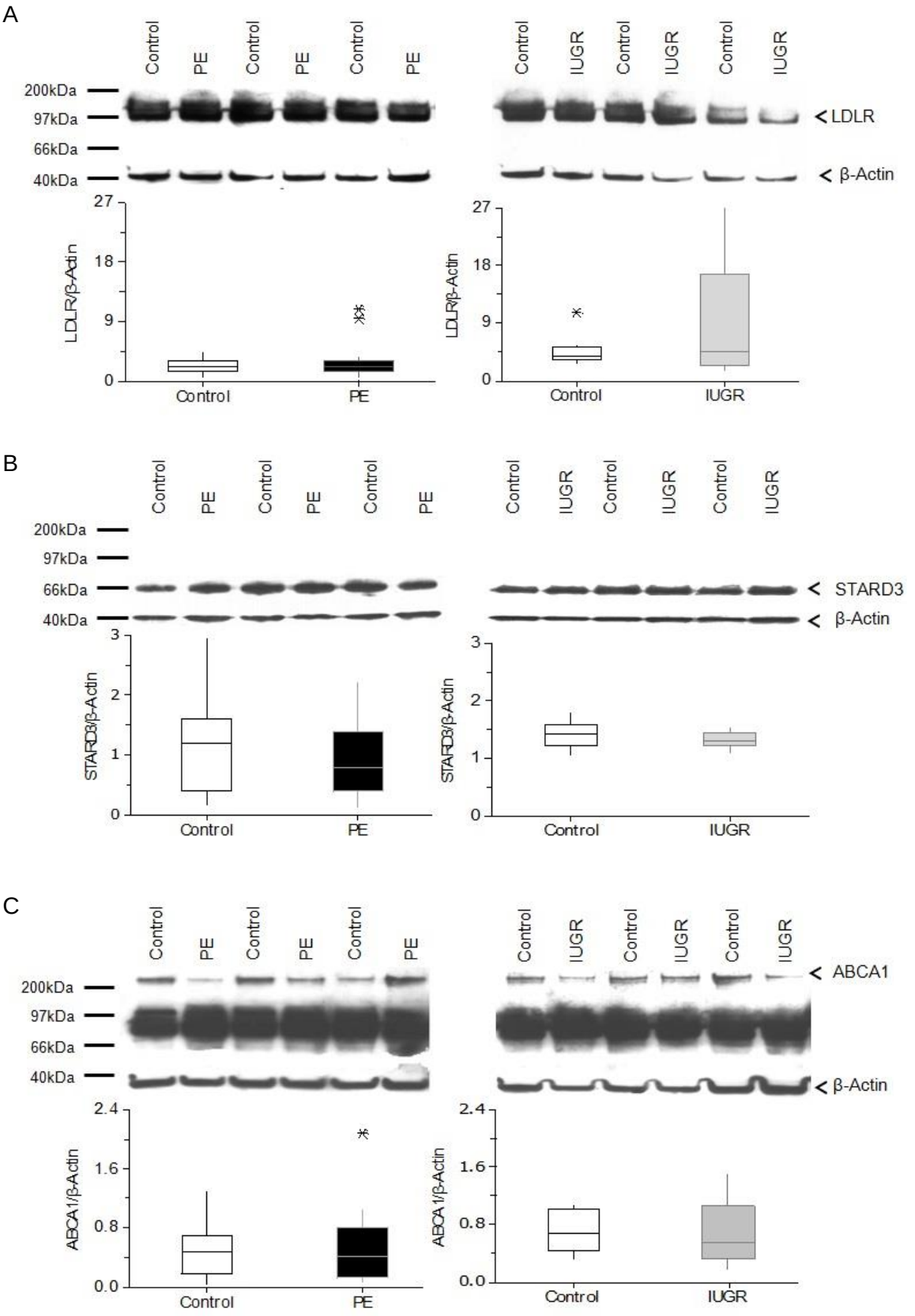


Figure 4-23: **Quantitation of placental protein levels by Western blot**. A) LDLR, B) STARD3 and C) ABCA1 relative to β-Actin in cases of PE (n=20) and BMI-matched controls (n=20); cases of IUGR (n=9) and BMI-matched controls (n=9). LDLR was detected at 95kDa, STARD3 at 53kDa and ABCA1 at 220kDa. Mean (SD) shown. Typical gels are shown for three cases and three BMI-matched control samples, and summary graphs for the relative density of the transporter to β-Actin are also provided.

4.7 Discussion

This chapter has aimed to explore the question of whether fetal cord metabolic and inflammatory parameters at birth reflected maternal parameters by the end of the third trimester. The offspring of pre-eclamptic mothers were associated with hyperlipidaemia, hypoleptinaemia, hypoadiponectinaemia, normoglycaemia and downregulated inflammatory response, independent of several confounding factors. There was dyslipidaemia, poor glucose control, hyperleptinaemia and proinflammatory status in PE mothers, compared to those who had a healthy pregnancy. Investigation of the molecules involved in lipid transfer (cholesterol) across the maternal-fetal interface highlights the role of LDLR, STARD3 and ABCA1, considering the detection of these molecules and their mRNA upregulation in the placentae of women with pre-eclamptic pregnancy. This detection and upregulation perhaps indicate that such placental lipid transporters may orchestrate the offspring's reflected maternal hyperlipidaemia at birth.

In the cord blood of pre-eclamptic mothers, there was significantly higher TG and TC, and lower leptin and adiponectin. The raised cord TG level was dependent on maternal smoking, mode of delivery and labour, but these confounding factors did not affect the fetal TC level in PE. Gestation at sampling affected the lower leptin and adiponectin in the studied group. In previous studies, increased venous umbilical cord blood TG and TC were observed in cases of PE (Rodie et al. 2004; Catarino et al. 2008), whereas other studies have reported no difference of cord TG and TC between these groups (Ophir et al. 2006). Leptin, unlike adiponectin, may have a role as a regulator of fetal growth and development during normal pregnancy (Hassink et al. 1997), as well as in pregnancies related to anomalous fetal growth (Tamura et al. 1998). Leptin and adiponectin are mostly produced by adipose tissue. The placenta is a target of leptin action as well as a source for leptin synthesis (Laivuori et al. 2006). Leptin is capable of stimulating placental amino acid transport (Jansson et al. 2003), which is beneficial to fetal growth. Unlike in the current study, Odegard et al. observe that umbilical cord leptin increased more strongly with gestational age in PE cases than in control subjects (Odegard et al. 2002). Although Odegard et al. had a larger sample size, they failed to consider an obesity measure (as was done in this current study), which perhaps may explain the discrepancies of the results. In the current study, lower cord blood leptin and adiponectin in the fetuses of mothers with PE suggests a fundamental compensatory ability in the fetus. Leptin contributes to insulin resistance and is thought to predispose fetal programming in the fetuses of obese mothers (Catalano et al. 2009). Thus, the reduced cord level of leptin in PE may suggest that the fetus is protected or existence of different pathway, unlike the fetuses of obese mothers. The cord blood adiponectin of cases of PE was noted to be lower than the controls; however, there is a reported initial trend towards lower levels,

which then rise with increasing gestational age, but not significantly different by term (Ogland et al. 2010). The fetal cord profiles of lower leptin and adiponectin may suggest a problem of placental insufficiency in cases of PE.

Aaltonen et al. find that most cytokines (such as TNF-α, IL-1β and IL-6) do not cross the placenta (Aaltonen et al. 2005). There is no consensus in the field regarding this particular finding, but this may be why maternal inflammatory mediators perhaps do not influence the changes in fetal circulation during gestation. The lower cord blood TNF-α in newborns of mothers that suffered PE, compared to normotensive counterparts, was not expected. Others have previously reported declined cord TNF-α in PE (Schiff et al. 1994; Kupferminc et al. 1999). Another group have found elevated cord TNF-α in PE instead (Laskowska et al. 2006). In contrast, Catarino et al found no change in cord TNF-α concentrations in PE cases in comparison with controls (Catarino et al. 2012). Interestingly, the impact of maternal obesity measure by BMI, was not considered in all these reports. In the current study, the lower TNF-α in cases of PE depended on labour and mode of delivery, which are factors that supposedly increase the inflammatory response. TNF-α is a non-glycosylated protein synthesised extensively by monocytes/resident macrophages, adipocyte and T cells (Weisberg et al. 2003; Cawthorn and Sethi 2008; Orsi and Tribe 2008). The lower cord TNF-α levels in PE in the current study are thought to be due to poor placental synthesis. Consequently a placental mRNA expression assay was undertaken, and it was found that there was a trend to higher placental TNF-α mRNA level in cases of PE. IHC studies were also carried out, and a lower HUVEC TNF-α staining in PE was observed. TNF-α staining of a number of PE and control cord tissue samples would had been useful, to semi-quantitate the stained HUVEC after two independent scorers scored blind. Nonetheless, the low cord TNF-α is possibly due to the low number of cord monocytes, since a large number transmigrate into the subendothelium. This transmigration is the mechanism monocytes use to transform into resident macrophages in absence of injury. Catarino et al. note that the total number of leukocytes as well as monocytes is significantly lower in cord blood from PE pregnancies (Catarino et al. 2012).

In the cord blood of women with PE, only the lipid profiles of TG and TC were reflective of the mothers' lipid profiles; other significant parameters (leptin, adiponectin and TNF-α) were observed to be lower. Also, after adjusting for the confounding factors, the borderline declined cord CRP level adjusted significantly, but was influenced by labour and mode of delivery. This observation (that, with the exception of lipid profiles in cord blood, most other profiles including inflammatory parameters decreased) is suggestive of a active metabolic pathway rather than inflammation in the fetuses of pre-eclamptic women. Thus, it hinted that downregulated inflammatory response perhaps resulted in the observed low inflammatory response. As a consequence, it was necessary to ascertain potential factors

which could have led to the declined cord blood inflammatory response of pre-eclamptic pregnancies. It is suggested that the reduction in cord inflammatory mediators is probably simply attributed to mothers suffering from pre-eclamptic disorder. In this context, pre-eclamptic condition perhaps triggers upregulated production of immune suppressor molecules in the fetuses. This may be due to a physiological process that suppresses fetal inflammatory response in order to protect/counteract the hostile enhanced maternal inflammatory response of PE, often present throughout gestation (Sacks et al. 1998; Catarino et al. 2012). This implies that, in general, fetuses have a lower or suppressed immune response, than as expected, irrespective of events in their mothers. It appeared that, among the parameters measured, the maternal third trimester TG related to fetal cord blood TG, since its increase in mothers led to the increase in fetuses. The maternal trend towards higher TC levels in cases of PE, was significantly higher in previous reports (Rodie et al. 2004; Catarino et al. 2008); this higher level possibly reflects the fetal hyperlipidaemia (hypercholesterolaemia). The data in this Chapter failed to show an association of maternal TC with either fetal TC or fetal HDL in the extreme pregnancies cases of PE, which was also absent in the previous report (Rodie et al. 2004). Nevertheless, as Pecks et al. observed, there was unchanged level of TC in mothers of PE cases (Pecks et al. 2012); however, only prepregnancy obesity compared to term, was considered. An explanation of disturbed lipid levels in both fetuses and mothers, perhaps was affected by lower peripheral lipoprotein metabolism in cases of PE (Huda et al. 2009). There is a suggestion of the overrepresentation of common mutations in the LPL gene in women with PE (Zhang et al. 2006). These mutations are linked with a reduction in LPL activity and dyslipidaemia in non-pregnant population. Therefore, the current data that fetal cord lipids, particularly TG and TC, are reflective of maternal lipid (TG and TC) levels, whereas the inflammatory pathway is downregulated in pre-eclamptic fetuses, is revealing.

Not surprisingly, perhaps, data of mothers with PE are consistent with the current literature (Catarino et al. 2008; Baker et al. 2009; Mackay et al. 2012). Maternal metabolic metabolites had higher TG, NEFA, glucose and leptin in cases of PE. Similar higher maternal levels of TG and NEFA (Mackay et al. 2012) and leptin (Aydin et al. 2008) in PE have been well reported. Maternal hyperglycaemia is a risk factor in predicting PE (Yogev et al. 2010), and the current study showed raised maternal glucose levels in PE cases at the end of gestation. It is difficult to control for gestation at sampling, and most previous studies have not done this. Despite adjustment, the gestation at sampling did not have a significant effect on maternal TG but influenced the raised level of NEFA and glucose. Smoking partly affected fasting TG, as reported in non-pregnant and premenopausal women compared to non-smokers (Willett et al. 1983). Maternal inflammatory mediators in PE cases showed higher IL-10, PAI-1 and lower PAI-2. Increased IL-10 in PE is also

observed (Freeman et al. 2004). Another group also note higher PAI-1 (Reith et al. 1993; Catarino et al. 2008) and lower PAI-2 (Reith et al. 1993) in women who had PE compared to those with healthy pregnancies. There is no influence of the confounding factors on changes in the maternal IL-10, PAI-1 and PAI-2 levels. Nonetheless, these data indicate of consistent metabolic milieu in PE cases.

Observations of cord blood pregnancies with IUGR showed higher TG when compared to controls. This result confirms a previous study (Rodie et al. 2004) which suggests that compromised lipid transport and fetal stress is to be blame. A significant decline in cord TC, HDL-C, leptin and sICAM-1 was also noticed in cases of IUGR. The confounding factors assessed did not play any role in the changes in the metabolic and inflammatory parameters in the IUGR group compared to controls. As anticipated, it was thought that reduction of these parameters occurred because of placental insufficiency (dysfunction) seen in cases of IUGR. Physiologically, there was no observed relationship between IUGR fetuses and mothers when compared to BMI-matched controls. As expected, in IUGR mothers, there were no changes in maternal metabolic or inflammatory parameters (except decreased PAI-2). Rodie et al. find no significant differences in the mean concentrations of maternal TG and TC between their IUGR and control groups (Rodie et al. 2004). The result is expected because, if anything, IUGR patients are mainly lean, having low BMI levels. Maternal plasma PAI-2 level decreased in the IUGR compared to the control group. A significant decrease in PAI-2 level was observed in the IUGR group, in comparison with women that had normal pregnancies (Gilabert et al. 1994). This result was also expected, as IUGR women have compromised placenta pathology, as the placenta is the primary source of PAI-2 (Harvey et al. 1995). Thus, the most significant difference between the babies and mothers of cases of IUGR was that fetal cord blood showed an increase in TG and a decrease in TC, HDL-C, leptin and sICAM-1. There was no change in metabolic and inflammatory parameters in the mothers except decreased PAI-2, suggestive of placental insufficiency. There was no observable impact of the confounding factors.

Whether the elevated cord TC levels in cases of PE were due to the maternal rise of these parameters, (which was absent in IUGR cases, which had lower TC and HDL-C levels than controls) requires further investigation. In terms of the consistent expression of lipid transporters at the maternal-fetal interface, the previous study data has not really changed. The current study makes the whole picture more detailed. As maternal TC relates to fetal HDL in healthy pregnancies, but is absent in extreme cases of PE and IUGR (Rodie et al. 2004), this suggests that molecules involved in lipid transport – in particular, cholesterol may be affected in the extreme cases. LDLR is well known to be involved in recognising LDL and HDL, via receptor mediated endocytosis (Simpson et al.

1979; Brown and Goldstein 1986; Grummer and Carroll 1988). Intracellular proteins, STAR domain protein (Miller and Bose 2011) and efflux protein ABCA1 and ABCG1 (Vaughan and Oram 2006; Adorni et al. 2007) were all investigated.

LDLR on the outer membrane of the placental villi only constituting syncytiotrophoblasts was localised (Fuchs and Ellinger 2004). Other studies have reported LDLR localisation on the syncytiotrophoblast (Simpson et al. 1979). In the experiment presented in this chapter, LDLR was localised throughout gestation. There was a progressive decline in LDLR expression as gestation advanced, as seen in first and second trimester and term placentae. LDLR stains were not noticed in the stroma and fetal endothelial cells. These data confirm the physiological role of LDLR in the transport of cholesterol-rich lipoproteins across the maternal-fetal interface in healthy pregnancies and adverse cases. Investigation of 15 STARD intracellular transporters in placenta detected all except STARD6 and STARD15. Those proteins involved in cholesterol transport, including STARD1, STARD3, STARD4 and STARD5, were selected for further study. STARD3 was extensively localised in the villous and extravillous layer of syncytiotrophoblasts, cytotrophoblasts, the stroma and the fetal endothelial cells in term placentae. STARD3 staining intensity increased by trimester 2 compared to trimester 1. By term, there was no strong observable expression on the stroma of the placenta, an indication of decline in staining. There was observable ABCA1 localisation on villous cytotrophoblast cells, confirming previous studies (Bhattacharjee et al. 2010), and on the syncytiotrophoblasts in the first and second trimester and syncytium. The second trimester placental tissue cytotrophoblasts and syncytiotrophoblasts showed greater intensity of ABCA1 compared to the first trimester, but became less intense in the term placenta and syncytium. This high staining in the second stage of pregnancy suggests that ABCA1 may have an important role in the transfer of cholesterol involved in the fetal steroidogenesis, cell membrane biosynthesis and neural system development (Lange et al. 2004; Guibourdenche et al. 2009; Chen et al. 2013). Another efflux protein, ABCG1 (Vaughan and Oram 2006), was demonstrated to be expressed in the trimester 1, trimester 2 and term placentae. ABCG1 was widely expressed in the syncytiotrophoblasts and cytotrophoblasts of the first trimester and second trimester placentae. ABCG1 was localised in the syncytial layer of the term placental sample tissue, and in the fetal endothelial cells in the chorionic core. Expression of ABCG1 staining was evident in the stroma of term placentae, and was prominent in the fetal erythrocyte; this staining was also observed in the fetal blood vessels of term placenta samples. Over time, ABCG1 intensity progressively increased. This demonstrates that the activity of the transporter increased as pregnancy advanced.

Analysis of cholesterol transporter gene expression in placentae was carried out to establish whether changes in placental cholesterol transport systems may account for the fetal cord being reflective of the maternal hypercholesterolaemia. There were upregulated placental mRNA levels of LDLR, STARD3 and ABCA1 in PE cases, compared to controls, but the protein expression did not change. Albrecht et al. note no difference in ABCA1 placental mRNA and protein expression between PE cases and controls (Albrecht et al. 2007). A similar pattern of higher mRNA levels of LDLR, STARD3 and ABCA1 expression was observed in IUGR pregnancies, which failed to differ significantly from healthy pregnancies. This may possibly be due to this study being under-powered. Wadsack et al. observed lower placental LDLR mRNA levels in IUGR cases, which showed hemodynamic changes in fetuses compared to AGA (controls) with higher (1.8-fold) protein expression (Wadsack et al. 2007). Nonetheless, consistency in the cholesterol transporter expression was demonstrated at the maternal-fetal interface. LDLR was prominently stained on the syncytial layer in healthy term placentae, compared to PE placentae. Both healthy term and IUGR placentae had strong LDLR staining on the syncytium and less on the fetal erythrocyte within the fetal blood vessels. Higher intensity of STARD3 was noted in PE placentae compared to third trimester healthy placentae. In cases of IUGR, there was STARD3 expression towards the stroma in the chorionic villous core, compared to term placental tissues. STARD3 staining was higher in pregnancies complicated by IUGR, compared to third trimester placenta samples. The intensity of ABCA1 was slightly higher in the syncytial layer, fetal endothelial cells and the stroma and especially concentrated in the syncytial and fetal endothelial cells of the PE compared to term placental tissues. In contrast, the expression of ABCA1 in IUGR and term placentae showed fairly similar levels of staining intensity. These staining data are consistent with the literature and Chapter 4's objective of detecting molecules involved in lipid transport at the expected locations/sites of the placentae (maternal-fetal interface) (Plosch et al. 2007; Stefulj et al. 2009). The data pinpointing upregulation of placental mRNA level but not protein of LDLR, STARD3 and ABCA1 in PE in cases, in comparison with controls, was enlightening. The inability of protein expression to confirm mRNA is perhaps due to the need for energy conservation for other activities. It may also imply that protein expressions are allowed to be expressed only when required. Data have highlights some cholesterol transport proteins such as ABCA1, as a 'labile protein' i.e. protein that must be synthesised continuously because it is degraded continuously (Remaley 2007), which is also observed in STARD1 (Epstein and Orme-Johnson 1991; Manna et al. 2009), but no report on LDLR and STARD3. This may have resulted in the lack of difference in LDLR, STARD3 and ABCA1 protein expressions in cases of PE. In assessment of the first and third trimester placenta mRNA levels, it appeared that STARD3 is higher at term, whereas LDLR and ABCA1 declined over time. An important point is that intracellular lipid transfer, such as via STARD3, is necessary for steroidogenesis (Guibourdenche et al. 2009), which

is essential even towards the end of gestation. This is supported by the putative role of STARD3 in cholesterol transport from the endosome (Strauss et al. 2002).

Transporters were expressed relative to 18S because 18S provides the best endogenous control, which allows for normalisation of placental gene expression. However, such endogenous control cannot distinguish between cell types. Thus, in order to determine whether it was the transporter expression levels in endothelial cells or in trophoblasts that changed, transporters were expressed relative to CD31 or KRT7, respectively. It was observed that in pre-eclamptic samples, the LDLR and ABCA1 mRNA were higher in the endothelial cells relative to CD31 compared to controls. In cases of IUGR, the LDLR and STARD3 were higher in endothelial cells relative to CD31 expression. In contrast, it was noted that a greater number of trophoblasts were involved in the ABCA1 expression relative to KRT7 in cases of PE, in comparison with the control group. Although the placental tissue transporters in women with IUGR were not significantly different; there was an observed trend of higher trophoblast involvement in ABCA1 mRNA levels relative to KRT7, compared to control placentae. These data implied that the upregulated transporters in the cases of PE, in particular LDLR and ABCA1 perhaps due to active involvement of trophoblasts and fetal endothelial cells.

The next step in analysis of this experimental data was to determine whether there is association between the metabolic and inflammatory parameters and placental transporters. In PE, LDLR mRNA expression correlated positively with fetal TC, HDL-C and leptin whereas IL-10 was negatively associated. This highlights that upregulated LDLR perhaps transports cholesterol across syncytiotrophoblasts (Simpson et al. 1979; Fuchs and Ellinger 2004). STARD3 levels were noted to be linked to increase fetal HDL-C and leptin in cord blood. ABCA1 level related to increased insulin and HOMA and decreased IL-10 concentration in the cord blood of women with PE. In contrast, in mothers with PE, LDLR levels were found to relate to increased maternal TG levels of PE, whereas STARD3 expression was associated with TG increase, insulin and HOMA in mothers with PE. Upregulated ABCA1 levels were positively linked to maternal insulin and HOMA (insulin resistance). Also, among the upregulated placental transporters (LDLR, STARD3 and ABCA1) mRNA levels in cases of PE, only that of ABCA1 related to the maternal TC in the plasma circulation. It was thought that a LXR-α expression may be responsible for upregulated ABCA1 mRNA expression in PE, compared to healthy controls. Thus, further analysis carried out, confirmed that the LXR-α mRNA was strongly positively related to the ABCA1 mRNA expression in the pre-eclamptic placentae. There was also unexpected positive correlation between LXR-α mRNA and the STARD3 mRNA level in placentae of women with PE.

These observations show that there is an association between the metabolic parameters and the placental transporters, particularly in the cholesterol transport pathway. It is suggested that these transporters may perhaps play a role in the transfer of maternal parameters to the fetus across the maternal-fetal interface. Consequently, this leads to the proposal that the fetal metabolic pathways are reflective of those in the mother. Rodie et al. suggest that fetal stress and compromised trophoblast invasion may been implicated, due to the observation of increased cord blood TC levels (Rodie et al. 2004). Overall, data from the current study highlights the concept that fetal lipid profiles (e.g. cholesterol) transported from the mother across the maternal-fetal interface perhaps occurs through the LDLR on syncytiotrophoblasts and/or syncytium, intracellularly transported via the STARD3 on stroma across to the fetal endothelial cells, where its effluxed into the fetal compartment by the ABCA1. This view is supported by report of Stefulj et al. that showed ABCA1 and ABCG1 efflux of lipid-poor apo A-I, in a term human placenta endothelial cells, with LXRs activators enhancing the cholesterol efflux to both acceptors (Stefulj et al. 2009).

PE is defined as a multisystem disorder of pregnancy diagnosed after 20 weeks of gestation, characterised by the onset of hypertension, proteinuria and oedema. To date, the definitive treatment of PE remains to expedite delivery. PE prevalence varies, affecting 2-5% of pregnancies in the UK alone. Underlying cause(s) and pathogenesis remain poorly understood. Impaired trophoblast invasion of the placental spiral arteries is a recognisable feature of pre-eclamptic disorder (Brosens et al. 1972; Kadyrov et al. 2006) and implicative of abnormal placentation (Furuya et al. 2008). IUGR is a pathological condition of reduced growth velocity which compromises fetal well-being. IUGR shares a number of similarities with PE pathology, with the exception of generalised endothelial damage. Experimental data analysed in this chapter and in other studies have shown that increased fetal lipid levels are due to the levels in mothers, which suggests that a trans-placental mechanism must be involved. In this study, IUGR was a useful control in determining factors involved in the transport of lipids, particularly cholesterol, across the maternal-fetal interface. This use of IUGR controls is an important strength of the current study. Use of paired data from the mother and baby means that much sampling bias is eliminated, and this allows assessment to be undertaken closest to the ongoing events between the mother and the fetus in pregnancy. There was broad BMI matching between the groups, i.e. BMI-matched controls. BMI-matched cases and controls help counteract bias posed by obesity, an identifiable risk factor that alters metabolic and inflammatory parameters. Very good measurement of parameters, including lipids, added to the strength of the assay. In contrast, weaknesses include an unreasonably small sample size, meaning that data may perhaps have been under-powered. The effect of smoking in IUGR cases may also have contributed to a false negative result that may otherwise not

have occurred. Differences in gestation at sampling possibly affected results, as it is impractical to collect blood, cord and placenta samples for all women on the same day of gestation. There is also the issue of mode of delivery employed, for instance caesarean section or emergency delivery. The many cell types in placentae present problems related to gene expression, whether mRNA or protein expression.

It is recommended that further studies undertake a differential isolation of placental cell types, such as syncytiotrophoblasts, cytotrophoblasts and fetal endothelial cells. This differentiation of cells would help identify the cell types involved during transport of lipids, particularly TC, across the maternal-fetal interface. Nonetheless, there is an indication that trophoblasts (syncytiotrophoblasts and cytotrophoblasts) and fetal endothelial cells may play major roles in the expression of upregulated transporters. Also, exploring certain immune repressor molecules in the fetuses of pre-eclamptic mothers in future (including HDL-C and IL-10) may help elucidate the downregulated fetal inflammatory response. Data have shown that monocyte TNF-α synthesis is reduced by HDL-C (Murphy et al. 2008) and IL-10 (de Waal Malefyt et al. 1991; Royle et al. 2009). However, it appears that the observed fetal downregulated inflammatory mediators are perhaps due to the changes in homeostatic balance by the action of the antiinflammatory pathway. This downregulation may serve to protect the fetus against a hostile environment of an extreme pregnancy outcome such as PE. The elucidation of mediators that suppresses inflammatory response in the offsprings of mothers with PE, a condition used as a model of an extreme pregnancy outcome, may perhaps be useful in understanding other inflammatory disorders affecting obstetric outcome.

In summary, it has been shown that fetal cords reflected maternal parameters of metabolism and inflammation in cases of PE, particularly hyperlipidaemia. Normoglycaemia, hypoleptinaemia, hypoadiponectinaemia and downregulated inflammatory mediators (mediators due to lower cord TNF-α, independent of key confounding factors) were also evident in the cord blood. There were altered maternal parameters in cases of PE (including hyperlipidaemia, insulin resistance, poor glucose tolerance, hyperleptinaemia and systemic inflammation) that are consistent with previous studies. Upregulation of LDLR, STARD3 and ABCA1 mRNA, but not proteins, is possibly suggestive of energy conservation. During lipid transport, particularly of cholesterol across the maternal-fetal interface, the localisation and upregulation of the transporters is perhaps implicated in fetal parameters reflecting those of the mother. Whether these changes affect fetal vascular health is recommended to be further explored in *in vivo* and *in vitro* studies in order to ascertain the status of fetal endothelial cell function and dysfunction at birth, in particular in obese pregnant women and after an extreme case e.g. PE.

5 Human Umbilical Vein Endothelial Cell Gene Expression as an Index of Offspring Endothelial Function

5.1 Introduction

A switch from a quiescent to an activated endothelium is a recognisable feature of vascular pathology. Endothelial activation refers to a specific change in endothelial phenotype, a change most often characterised by an increase in endothelial/leukocyte interaction and permeability, pivotal for inflammatory response in both physiological and pathological settings (Hordijk 2006; Alom-Ruiz et al. 2008). The normal endothelial cell plays a role in the maintenance of vascular homeostatic balance. It controls vascular tone, inflammation, thrombolysis and leukocyte proliferation and maintains low environment oxidative stress. Studies have described the aetiology of vascular disorders as evolving gradually over decades, from early childhood to adult life. However, debate remains over the implication of the uterine environment, particularly for future cardiovascular disease. There are proposals that lipotoxicity during the *in utero* period has long-term adverse effects on offspring's vascular health (Freeman 2010; Jarvie et al. 2010).

Data from non-human primates shows that both lean and obese mothers who chronically consume a high-fat diet present a three-fold increase in liver TGs in the fetal liver (McCurdy et al. 2009). Impaired tissue homeostasis attributed to changes in lipid utilisation or to lipid-induced change in intracellular signalling has been broadly termed lipotoxicity (Symons and Abel 2013). This lipotoxicity plays an important role in the pathogenesis of endothelial dysfunction and causes orchestration of altered inflammation and progression to atherosclerotic lesion.

The developmental origins of adult disease have been intensely debated; however, since its initial proposal (three decades ago), definitive evidence for programming in humans is still lacking. The pregnancy environment may impose direct damage on the developing fetus that becomes deleterious later on in life. An example of this type of damage is impaired cellular morphology, which may lead to a change in the physiological setting of a response to certain stimuli (Lucas 1994). Such disturbances in early physiological 'memory' could translate into a pathological state in adult life. This phenomenon is referred to as fetal programming, and its underlying mechanisms are yet to be fully established. Cooper et al. report that the susceptibility of offspring to future risk of developing PE is three-fold higher in those born of pre-eclamptic pregnancies compared to unexposed siblings (Cooper et al. 1988). Since both maternal and fetal dyslipidaemia are evident in PE (Rodie et al. 2004; Catarino et al. 2008), there is a high risk of lipid

peroxidation. This peroxidation generates free radical species, resulting in endothelial activation and subsequent high vascular leukocyte transmigration. Some data shows elevated malondialdehyde (a marker of lipid peroxidation) levels in the umbilical venous cord blood samples of pre-eclamptic pregnancies compared to normotensive pregnancy (Chamy et al. 2006; Negi et al. 2012).

Generation of the condition of lipotoxicity involves a number of steps. A definable step is the storage of the TG droplet in non-adipose tissue, referred to as ectopic fat accumulation (Jarvie et al. 2010; Snel et al. 2012). It is not the TG per se but the accumulation of intermediates of lipid metabolism in organs (such as the skeletal muscle, liver, pancreas, heart and circulatory system) which seem to disrupt the metabolic process and impair organ function. The placenta is another likely site of ectopic fat deposition in pregnancy, e.g. obese women (if they have exceeded their finite lipid accumulation capacity because of a high-fat diet) may induce lipotoxicity in their fetus (McCurdy et al. 2009). Palinski and Napoli find evidence from animal data suggesting that initiation of atherogenesis results from the uterine environment (Palinski and Napoli 2002).

An influx of leukocytes from the circulation into the subendothelial space has long been characterised as a hallmark of acute inflammation. Their transmigration penetrating through the capillaries occurs through several steps, including chemo-attraction, rolling and tight adhesion (Luscinskas et al. 1994) and transmigration. The stimuli involved (including cytokines/chemokines and oxidative substances) result in neutrophil, monocyte (McEvoy et al. 1997) and lymphocyte adherence to vascular endothelial cells. This transmigration is the mechanism monocytes employ to transform into resident macrophages in the absence of infection or injury. Free radical production by endothelial cells is usually at a low level under normal conditions, due to anti-oxidant defence mechanisms including catalase, glutathione peroxidase and superoxide dismustase (Wang and Walsh 1996; Lin et al. 2007). Free radicals attack molecules (including lipoproteins, polyunsaturated fatty acids and lipoxygenase), thus generating highly reactive lipid peroxidation products and lipid peroxides (Spanbroek et al. 2003; Belkner et al. 2005). Oxidized LDL (or minimally modified LDL) also activates endothelial cells and triggers binding of monocytes (Watson et al. 1997). It is now also apparent that oxidation of LDL lipids and of apo B-100 renders LDL pro-atherogenic (Miller et al. 2010). Oxidative modification of LDL-C promotes inflammation and monocyte and macrophage recruitment, resulting in foam cell formation producing the atherogenesis on endothelial cells (Steinberg 2009). This modification results in the cytotoxic condition of endothelial cell culture in a serum-free medium and also induces a wide variety of proinflammatory cytokines in macrophages as well as nitric oxide-induced vasodilatation. In most

instances, all these products react with cellular proteins, DNA and lipids, rendering them toxic and causing mutagenesis.

Studies in Chapter 4 showed that the fetal cord reflected maternal metabolic and inflammatory parameters, in particular TC of pre-eclamptic pregnancies. This was shown to be due to transporter molecules involved across the maternal-fetal interface in PE, as an example of an extreme pregnancy outcome in comparison with healthy pregnancy. It was suggested that perhaps uptake of lipids in the fetus may be implicated in abnormal vascular health at birth. When a target tissue is sampled, it is generally the case that different cell types in the tissue have different responses. The fundamental rationale for the use of microarray-based gene expression profiling to characterise biological samples is based in part on the principle that cells and tissues (and perturbation applied to them) can be characterised on the basis of their relative expression of genes and transcripts. The microarray, or ''gene chip'', technique measures relative expression levels of genes by determining the amount of messenger RNA present in one sample versus another, in order to identify the function of specific gene(s).

The selection of the best endogenous control gene is paramount for relative gene expression studies. An ideal endogenous control gene is expected, under different experimental conditions, to have similar RNA transcription levels in a variety of different tissues and cell types. The most common constitutively-expressed internal housekeeping genes routinely used in research include those involved/located in the glycolytic pathway, glyceraldhyde 3-phosphate dehydrogenase (GAPDH); protein folding, peptidylprolyl isomerase A (PPIA); eukaryotic 18S ribosomal RNA, 18S; transcription initiator, TATA-box binding protein (TBP) and cytoplasmic, β-Actin (Murthi et al. 2008; Li et al. 2009; Kastl et al. 2010).

Nevertheless, a number of potential future disorders, including obesity and cardiovascular disorder, have been proposed to arise after exposure of the fetus to an extreme uterine environment. That obesity is a significant risk factor in changes in metabolic and inflammatory parameters is well documented. It implies that the impact of obesity could contribute to a vicious cycle of the gestational metabolic and inflammatory pathways' effects, not only on the mother but, most importantly, on the developing fetus, perhaps becoming evident only in adult life. Understanding how such disturbances affect the fetus, in particular in terms of vascular health, should go a long way towards determining optimal intervention strategies for fetuses born of complicated pregnancies associated with altered metabolic and inflammatory parameters.

5.2 Hypotheses

In this chapter it is hypothesised that preparation of a clean HUVEC can be obtained at birth using a density gradient separation technique. It is also hypothesised that the established procedure would be useful in selecting and setting up a model of assessing the impact on vascular health of disturbed offspring lipids, particularly TC in the pregnancies of obese women, and cases of extreme complications, such as PE, using HUVEC as an index of endothelial cell function.

5.3 Aims

The aim of this chapter is explore the implications of changes in cord blood lipid metabolites on the offspring's vascular health, using HUVEC gene expression to assess the index of fetal endothelial function after establishing the best HUVEC preparation methodology, selecting the endogenous control genes and analysing a pool of endothelial (dys)function gene expression markers and transcripts in HUVEC of obese mothers or those with PE, in comparison with controls.

5.4 Objectives

1. To establish a simple and rapid methodology for the optimal isolation of fetal endothelial cells from umbilical cords at birth in normal pregnant women.

2. To evaluate a panel of control genes in order to select an endogenous control in order to normalise mRNA expression profiles of endothelial cell genes for RT-PCR experimental studies with HUVEC in healthy pregnancies.

3. To analyse the HUVEC subtracted libraries obtained from umbilical cords from healthy pregnancies, as well as those of obese mothers and women with PE, using microarray, in order to assess the effect of disturbed fetal metabolic parameters (particularly TC) on the expression of a wide range of endothelial (dys)function gene markers and transcripts.

5.5 Materials and Methods

5.5.1 Media and buffers

Solutions of crude collagenase (Clostridium histolyticum, type II, cat C6885, Sigma-Aldrich) were prepared using PBS (see Section 2.5.1), frozen quickly to aliquots (of 25mg/mL) and kept frozen at -20°C. Prior to usage, 24mL of PBS was added to 1mL of collagenase (25mg/mL) in order to produce a final concentration of 1mg/mL.

Red cell lysis solution X1 was made up by dissolving 8.32g ammonium chloride in 100mL DH_2O.

A complementary DNA reaction mix (8µL) was prepared using 1µL 10X reverse transcriptase buffer, 0.4µL 25X dNTP, 1µL 10X random primer, 0.5µL multiscribe reverse transcriptase and 0.5 µL Rnase inhibitor 1U/µL, dissolved in 4.6µL DEPC.

5.5.2 Recruitment and Study Design

A total of 27 participants were recruited from the healthy population of women attending the Princess Royal Maternity Unit, GRI, to undergo elective caesarean delivery. Women gave their written informed consent. Ethical approval was granted in accordance with the guidelines of the Helsinki Declaration from the North Glasgow University Hospital Trust, National Health Service Glasgow and Clyde research ethic committee, GRI, Scotland. Subjects were excluded if patients had hepatitis, HIV, diabetes, chronic hypertension, connective tissue disorders or any long-term use of medicine. Also, none of the subjects were being treated with medication that interferes with lipid and carbohydrate metabolism, inflammation or endothelial function.

5.5.3 Fresh umbilical cord collection

A preliminary study of umbilical cord sections (n=8) from an archival collection was stained for a specific endothelial cell marker, CD31, and SMC marker, myosin, using IHC (see Section 5.5.5). Fresh healthy umbilical cords (n=7) obtained at delivery were used to optimise the HUVEC isolation protocol in the preliminary study. In the subsequent study, umbilical cords from twenty subjects with healthy pregnancies were collected in a sterile container. In order to determine whether HUVEC could be isolated from frozen umbilical cords, the twenty umbilical cords were divided into two groups for fresh (n=10) and frozen (n=10) HUVEC. The fresh HUVEC were from umbilical cords isolated soon after birth (less than 20 min). The frozen HUVEC were from umbilical cords collected, washed with PBS (pH 7.6) and frozen (> 48 hours) at -80°C.

5.5.4 Isolation of HUVEC

The HUVEC isolation procedure was an adaptation of methods published previously (Maruyama 1963; Jaffe et al. 1973). A sterile technique was utilised in all manipulation of the cords. In all cases, the umbilical cord was handled with care to avoid too much bending during HUVEC isolation in order to minimise SMC contamination. The umbilical cord was inspected, its length was measured and then the cord vein was cannulated with 20G polyurethane intravenous cannula (BD Plastipak™, Drogheda) inserted from the baby end with the needle secured by clamping with forceps. A 20mL syringe (BD Plastipak™ Drogheda) was filled with PBS (pH 7.6), and cord blood was flushed out until it drained clear at the other end, with no blood contamination. The placental end was clamped with forceps. A 1mg/mL pre-warmed collagenase (clostridium histolyticum, type II, Sigma) solution was added to the vessel until a slight resistance was felt, and the other end was then clamped. The cords were incubated at 37°C for 15 min. Removal of the endothelial cells was facilitated by a gentle rubbing and massage of the cord before collection in a 50mL sterile polypropylene tube. Cells were collected by centrifugation at 250*g* for 1 min.

5.5.5 Optimal HUVEC separation

Isolated cells were stained in 1:1 of trypan blue concentration in order to determine the number and viability of untreated HUVEC on a haemocytometer, and then viewed under an Olympus Model CK2 inverted microscope (Olympus, Japan). A portion of untreated HUVEC (5 x 10^5 cells) was collected onto Super-Frost Plus slides in a cytospin (Thermo Shandon) at 120*g* for 6 min. Another portion of untreated HUVEC was preincubated with 10mL red cell lysis solution (X1) for 5 min to eliminate red cells contaminants, followed by washing in PBS, pH 7.6 (250*g* in for 1 min), after the washing, 5 x 10^5 cells were collected onto Super-Frost Plus slides on a cytospin (Thermo Shandon) at 120*g* for 6 min. Another portion of untreated HUVEC was resuspended in 3mL of PBS (pH 7.6) after isolation and transferred into 3mL of histopaque®-1077 (Sigma) in 15mL tubes, and centrifuged (400*g* in for 30 min) at RT. Histopaque®-1077 reagent separates cells according to their density gradient; thus, such separation using the reagent is referred to as a density gradient technique. A central band was observed in the tube and cells on the band were gently collected and washed twice in PBS (pH 7.6). After the washes, 5 x 10^5 cells were also placed in the cytospin onto the slides (Super-Frost Plus) (120*g* for 6 min). Cytospin slides were stored at -80°C until used for IHC staining assay.

After identifying histopaque as the best technique for isolating clean HUVEC samples from preliminary preparation, the histopaque method was utilised in the subsequent study

of fresh (n=10) and frozen (n=10) HUVEC samples. HUVEC aliquots from each preparation were briefly centrifuged (1000*g* for 30 sec), the supernatant was discarded and 1mL of TRIzol reagent (Invitrogen) was added to the cell pellet for RNA extraction. HUVECs in TRIzol were stored at -80°C until used for further study.

5.5.6 Immunohistochemical analysis

Paraffin-embedded healthy umbilical cord and/or isolated HUVEC (Sections 5.5.4 and 5.5.5) were utilised in IHC staining studies. Initially, sagittal serial sections of umbilical cord tissue biopsies of 5µm thickness were cut using a Leica RM 2135 rotary microtome. The standard Avidin Biotin Complex (ABC) method was used for IHC (Hsu et al. 1981), as stated above (see Section 2.5.11). Slides of paraffin-embedded umbilical cord or positive control sections were rehydrated in an alcohol series. HUVEC slides were placed in 70% cold acetone (VWR International) and left for 10 min. Peroxidase activity was quenched by immersing slides in freshly prepared 0.5% H_2O_2 containing 5mL H_2O_2 and 300mL methanol for 30 min, followed by washing for 2 x 10 min in PBS. Antigen retrieval was carried out in a 0.01M citrate buffer (pH 6.0). Slides for staining with CD31 antibodies were placed in a solution of the citrate buffer in a microwavable pressure cooker and microwaved for 8 min. The slides for staining with anti-myosin antibodies were placed in a beaker containing a solution of the citrate buffer that was microwaved for 45 min. Paraffin slides were rinsed in DH_2O for 5 min and washed for 2 x 5 min in PBS, pH 7.6, whereas acetone-fixed cell slides were rinsed for 5 min in PBS. A non-immune blocking reagent of PBS containing 20% horse and 20% human sera (Sigma) was added, and slides were incubated at RT for 30 min to block non-specific antibody binding. Slides were washed in DH_2O for 5 min and 2 x PBS (pH 7.6) for 10 min before incubating with monoclonal mouse anti-human CD31 at 1:500 dilution (M0823, DAKO, USA) or mouse monoclonal anti-myosin (mouse IgG1 isotype) at 1:250 dilution (M7786, Sigma-aldrich, USA) overnight at 4°C. After further washes (2 x PBS, pH 7.6, for 5 min), the slides were incubated in biotinylated IgG H+L (30 min, 1:200, RT) (BA-2000, Vector laboratories). The 1° and 2° antibodies were diluted in PBS containing 2% horse and 5% human sera, while a negative control contained 2% horse serum and 5% human serum only. After incubation with the 2° antibody, slides were washed twice in PBS (pH 7.6) for 5 min and incubated for 30 min at RT in a Vectastain® standard ABC Kit Elite [two drops of reagent A and reagent B diluted in 5mL PBS (pH 7.6)]. Staining was developed using 1mg/mL of DAB solution (Section 2.5.1) incubated for 10 min. The slides were washed in PBS for 5 min and then in 1 x DH_2O for 5 min before Harris stain counterstaining for 15 sec. Paraffin slides were dehydrated, fixed in a series of alcohol concentrations and then mounted in DPX for microscopy. Digital image capture was with the image analysis program Image-Pro Plus (version 6.2, MediaCybernetics) on a BX50 F-3 microscope (Olympus) equipped with X4,

X10, X20, X40 and X100 lenses connected to a 3-CCD colour camera, and images were processed using an ImageJ Java-based image processor (National Institute of Health Bethesda, Maryland, USA).

5.5.7 Selecting the best RT-PCR endogenous control gene for HUVEC

Isolated fresh and frozen HUVEC preparations that had been resuspended in 1mL TRIzol LS reagent (Life Technologies) (Section 5.5.5) were allowed to thaw and were then incubated at RT for 5 min to permit complete dissociation of nucleoprotein complexes. As mentioned above (see Section 2.5.8), chloroform (0.2mL) (VWR, International) was added to the suspension, which was mixed vigorously and left to incubate at RT for 3 min. The upper aqueous phase was transferred to a clean sterile tube before 0.5mL isopropyl alcohol was added; it was then incubated at RT for 10 min and then centrifuged (4700*g* for 10 min) at 4°C. The supernatant was decanted and the pellet washed in 1mL of 75% alcohol with gentle vortexing followed by centrifugation (250*g* for 5 min) at 4°C. The pelleted RNA was air dried, resuspended in 40µL DH_2O and incubated at 65°C for 10 min. Total RNA concentrations were measured spectrophotometrically on an I-NanoDrop® ND-1000 spectrophotometer. RNA purity was explored by using this A260/A280 ratio, and the average was 1.95 (1.69-2.20) for RNA prepared from fresh HUVEC and 1.70 (1.12-2.27) for RNA prepared from frozen HUVEC. RNA (1µg per reaction) was DNase treated using a DNA free kit (Ambion), following the manufacturer's instructions. DNase-1 (1µL) was mixed with 5µL of up to 5µg RNA, 2.5µL 10X DNase buffer and 16.5µL DEPC. The mixture was vortexed gently and then incubated at 37°C for 30 min. A DNase inactivation reagent (2.5µL) was added, and the tube was mixed by flicking and then centrifuged (4700*g*) for 1 min. Aliquots of DNase-treated RNA were collected and stored at -80°C until required.

DNase-treated RNA was reverse transcribed using a High Capacity cDNA Archive Kit (Applied Biosystem) (Section 2.5.8). Complementary DNA was prepared by adding DNase-treated RNA (2µL) to the cDNA reaction mix (8µL) as described above (Section 5.5.1). A 1µL [1µg per reaction of cDNA (as total input RNA)] sample was then amplified for quantitative RT-PCR. A NoRT negative control was also prepared (Section 2.5.8). The resulting suspension was incubated for 10 min at 25°C followed by 120 min at 37°C.

Several endogenous controls and target genes associated with endothelial function were assessed using qRT-PCR on an ABI PRISM® 7900HT Sequence Detection System (Applied Biosystems) with commercially available primer probe sets (Table 5-1).

Table 5-1: **Commercial primer probe sets for selecting a best endogenous control gene**.

Primer probe set	Catalogue №
Endogenous control gene	
Beta Actin (β-Actin)	4310881E - 0605021
Eukaryotic 18S rRNA endogenous control (VIC/TAMRA)	PN 4310893E
Glyceraldhyde 3-phosphate dehydrogenase (GAPDH)	PN 4310884E
Peptidylprolyl isomerase A (PPIA)	Hs99999904_m1
TATA-box binding protein (TBP)	Hs99999910_m1
Endothelial function gene	
Endothelial-1 (ET-1)	Hs00174961_m1
Hypoxia inducible factor-1 alpha (HIF-1α)	Hs00153153_m1
Intracellular adhesion molecule-1 (ICAM-1)	Hs00277001_m1
Vascular cell adhesion molecule-1 (VCAM-1)	Hs00174239_m1
Von Willebrand factor (vWF)	Hs00169795_m1

Following the manufacturers' instructions, selected endogenous control and endothelial function target genes were quantitated in quadruplicate using RNA from fresh and frozen HUVEC. The average C_T and standard deviation of C_T for the human endogenous control genes was assessed.

5.5.8 Statistics

Minitab (Vs16.2.2) and GraphPad Prism® 5 (GraphPad, Inc; San Diego USA) were utilised for data analysis and graphical presentation, respectively. Data were assessed for normal distribution before statistical analysis. Difference testing, for continuous variables between the fresh and frozen HUVEC preparations, was carried out using two-sample t-test. Pearson's correlation distribution was used to test for univariate associations between variables. Data is presented as mean (SD) unless otherwise stated, and P-values less than 0.05 were considered statistically significant.

5.6 Results

5.6.1 Identification of endothelial cells in human umbilical cord

First, it was imperative to ensure that highly purified preparations of HUVEC could be produced with no contaminating cells. A preliminary study of umbilical cord sections (n=8), stained for a specific endothelial cell marker, CD31, demonstrated expression of CD31 on the fetal endothelial cell (FEC) of placenta villi (positive control) (Figure 5-1A). In the negative control, substitution of the anti-human CD31 monoclonal antibodies with PBS only showed no observable staining, as expected (Figure 5-1B). The CD31 monoclonal antibody was localised on the umbilical artery EC (Figure 5-1C) and umbilical vein EC (Figure 5-1D) of the umbilical cord tissue.

Anti-human myosin monoclonal antibodies were employed to stain SMC in the umbilical cord tissue. Human myosin was detected on the positive control myometrium tissue (Figure 5-2A). When the anti-human myosin monoclonal antibody was substituted with PBS only (negative control), there was no staining in the myometrium (Figure 5-2B). Staining of SMC of the umbilical artery is shown in Figure 5-2C, and that of the umbilical vein is shown in Figure 5-2D.

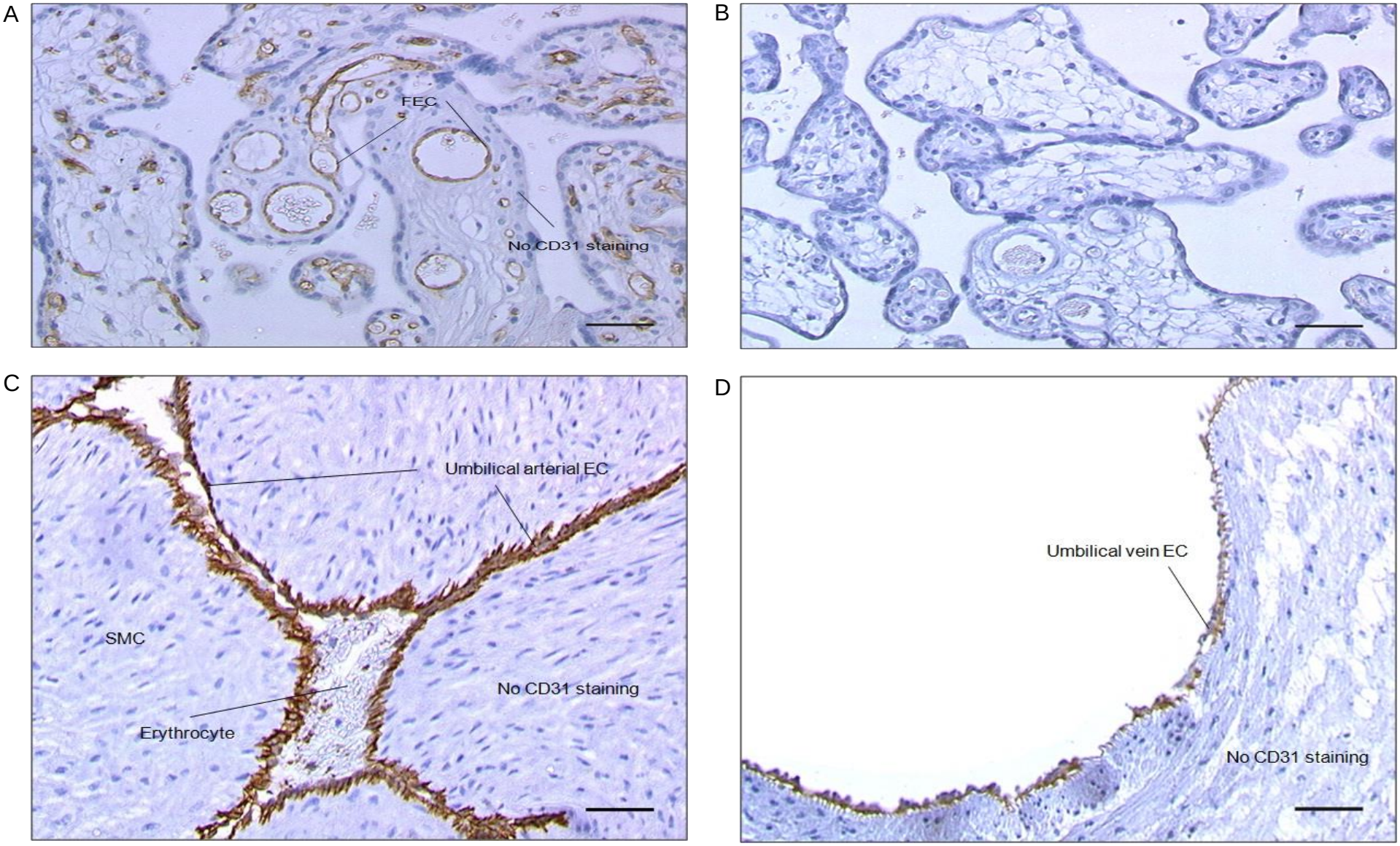


Figure 5-1: **Localisation of CD31 in healthy umbilical cord**. A) Placenta (positive control), B) Placenta (negative control), C) umbilical artery and D) umbilical vein at 20X magnification (scale bar; 10µM). EC represents endothelial cell.

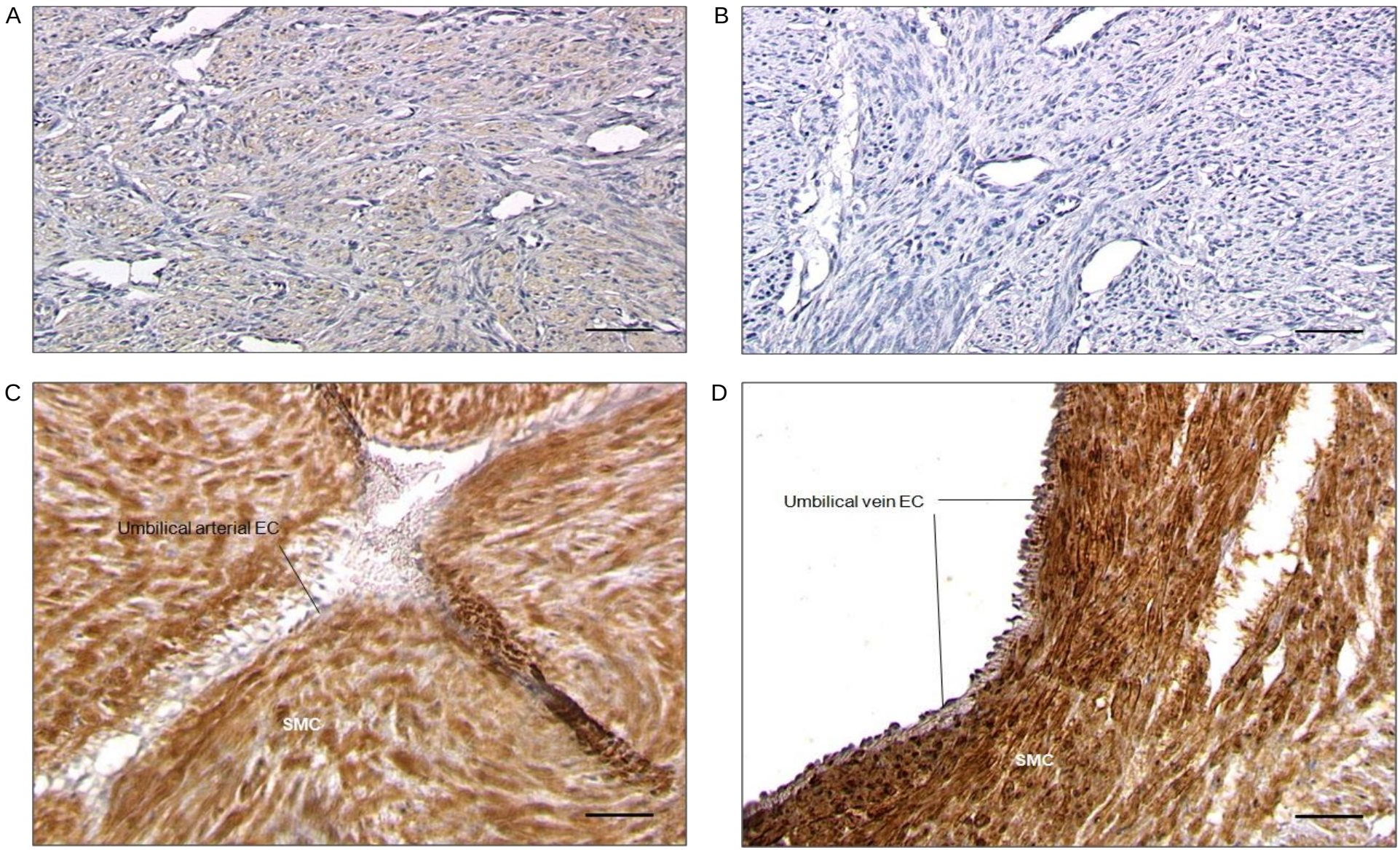


Figure 5-2: **Localisation of myosin in healthy umbilical cord**. A) Myometrium (positive control), B) Myometrium (negative control), C) umbilical artery and D) umbilical vein at 20X magnification (scale bar; 10µM). SMC represents smooth muscle cell.

5.6.2 Optimal separation protocol for HUVEC preparation

Once the discrete endothelial cells lining of the umbilical cord arteries and veins were identified, the vein endothelial cells were isolated from the umbilical cords as described in Sections 5.5.4 and 5.5.5. Several separation protocols were tried, and resulting preparations were tested for purity of HUVEC, using immunostaining by CD31 antibodies. In untreated HUVEC, there was contamination of red cells (Figure 5-3A). Since the untreated sample of HUVEC was contaminated with red cells, the untreated sample was then treated with a red cell lysis reagent in order to eliminate red cells. This step showed that HUVEC was also affected by the red cell lysis reagent resulting in the HUVEC membrane lyses; therefore, this step was not included in the protocol (Figure 5-3B). In contrast, the histopaque density separation technique produced highly purified preparations of HUVEC, as confirmed by immunostaining using anti-human CD31 monoclonal antibodies (Figure 5-3C). To verify whether the protocol produced a HUVEC preparation contaminated by SMC cells, myosin antibody staining was used to assess the purity of the HUVEC. When the histopaque separation protocol was employed, no SMC contamination was observed (Figure 5-3D). As expected, there was no observable staining when the CD31 or myosin antibody was substituted with PBS only as a negative control (Figure 5-3E).

These data suggest that the histopaque separation technique provides higher purity in HUVEC preparation compared to untreated HUVEC. Therefore, the protocol incorporating histopaque separation was adopted for isolating clean HUVEC preparation for the HUVEC study.

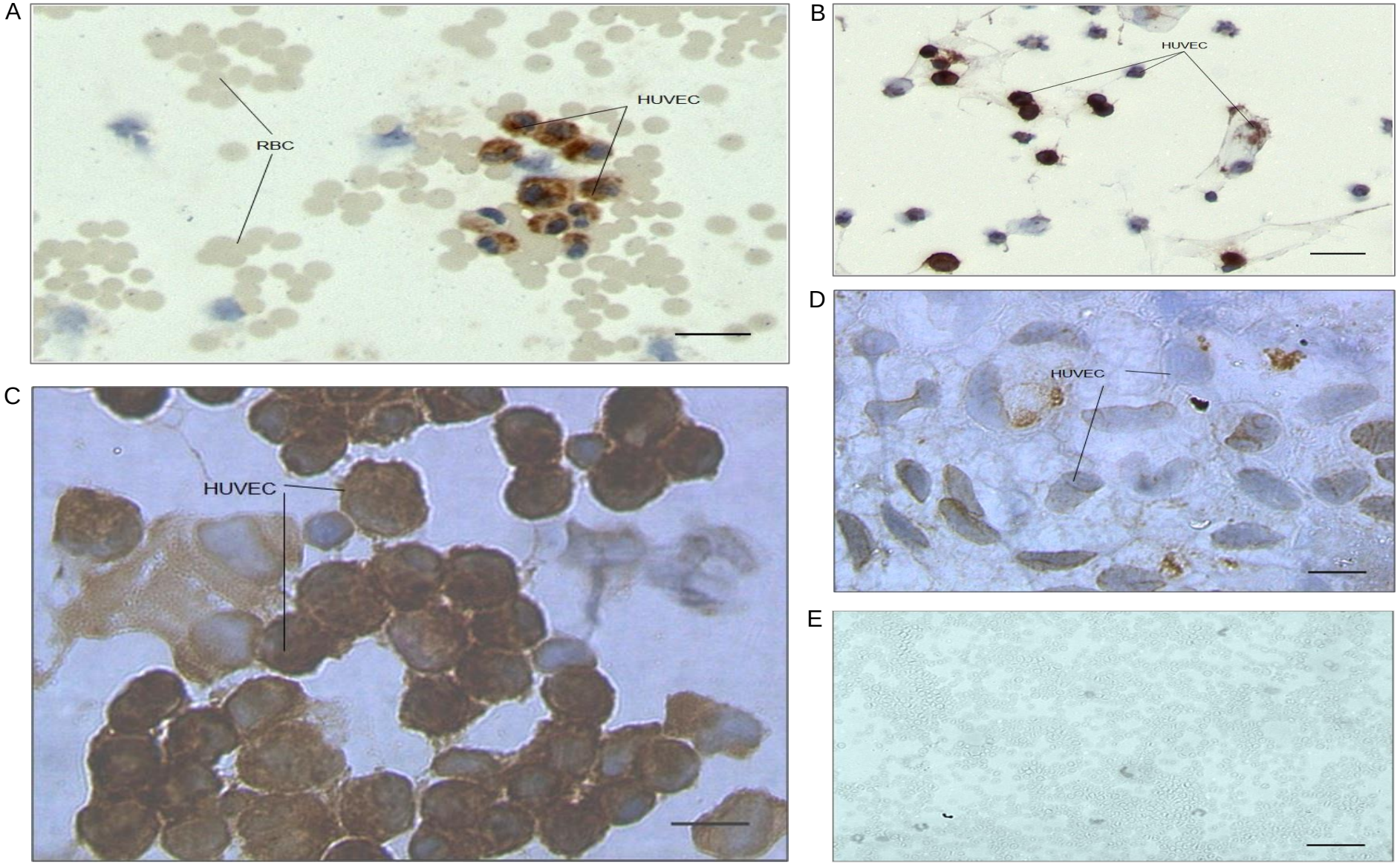


Figure 5-3: **Localisation of CD31 and myosin in healthy prepared HUVEC**. A) Untreated cells (5µM) stained in CD31, B) lysed cells (5µM) stained in CD31, C) histopaque separated HUVEC (2µM) stained in CD31, D) in myosin and E) when antibodies was replaced with PBS only (10µM) at 20X magnification (scale bar; 10µM), 40X magnification (5µM) and 100X magnification or oil immersion (2µM).

5.6.3 HUVEC RNA quantitation from fresh and frozen umbilical cord

Next, I investigated whether freezing the umbilical cord, which would allow for more convenient isolation of HUVEC, would affect HUVEC isolation and RNA expression. Twenty umbilical cords were obtained at birth from healthy pregnant women. Ten of the umbilical cords were used to isolate HUVEC immediately after birth while the other cords (n=10) were frozen immediately (for at least 48 hours), and HUVEC isolated after thawing. HUVEC numbers per umbilical cord from fresh cord (n=10) [0.09 (0.04) cells10^6/cord (cm)] were less variable than HUVEC numbers isolated from frozen cord (n=10) [0.05 (0.07) cells10^6/cord (cm)], although there was no statistical difference in the average number of cells isolated from either cord (P=0.21) (Figure 5-4A). RNA isolation from HUVEC isolated from fresh or frozen cords showed lower RNA concentration in fresh [3.4 (2.1) µg/10^6 cells] compared to frozen cord [39.5 (45.9) µg/10^6 cells, P=0.035] (Figure 5-4B).

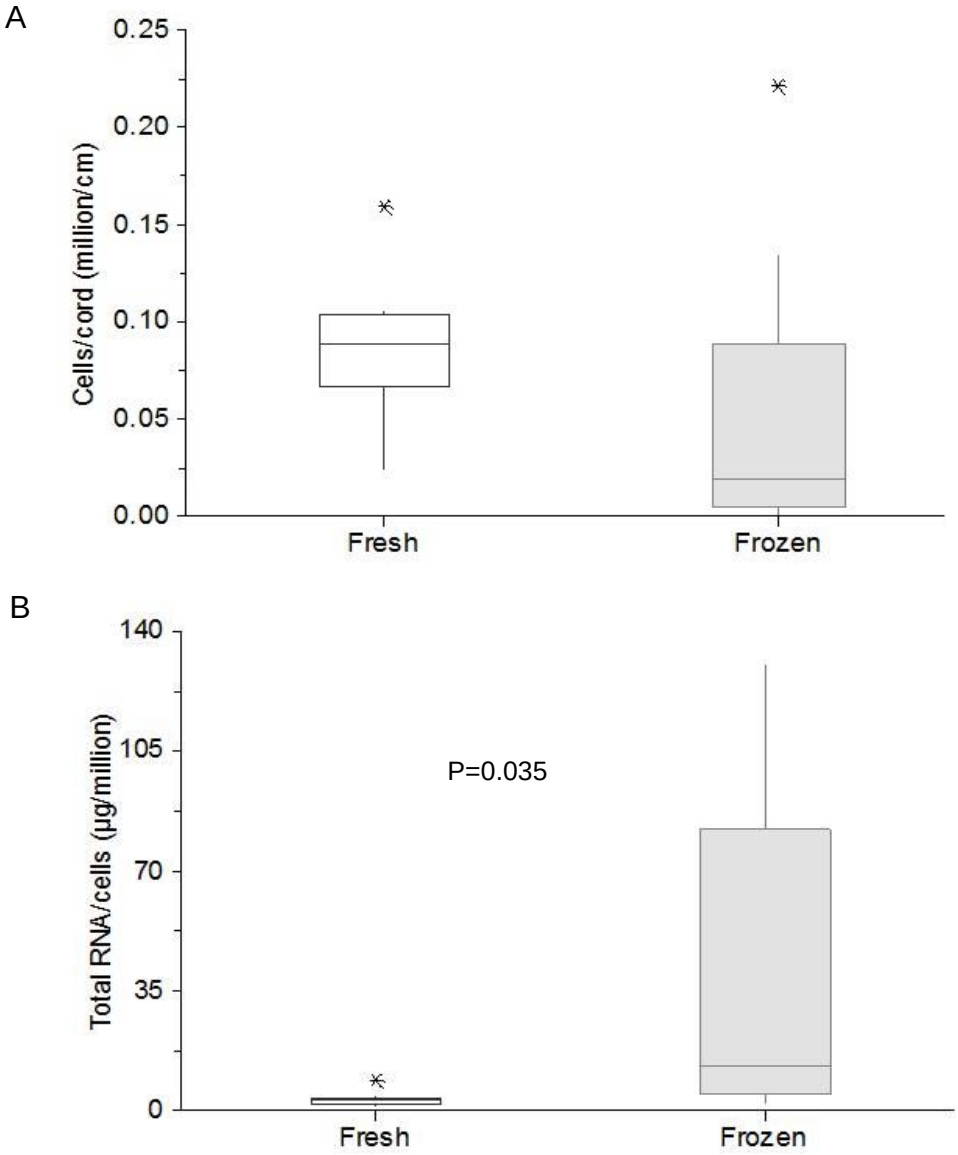


Figure 5-4: **HUVEC isolation and RNA verification**. A) Total cells per cord in fresh (n=10) and frozen (n=10) umbilical cords. B) Amount of RNA extracted from fresh and frozen samples, with less variation in fresh (P=0.035) compared to frozen umbilical cords.

5.6.4 Selection of a HUVEC endogenous control gene

In order to determine the best endogenous control gene for normalisation of endothelial cell gene expression in HUVEC, RNA was extracted from HUVEC isolated from fresh umbilical cord and gene expression levels of a panel of control genes were quantitated in quadruplicate. The average C_T for each endogenous control (Figure 5-5A) and endothelial function marker (Figure 5-5B) was determined and is shown below. Data revealed that the endogenous control 18S showed the highest expression, whilst the TBP had the lowest expression level in the fresh sample.

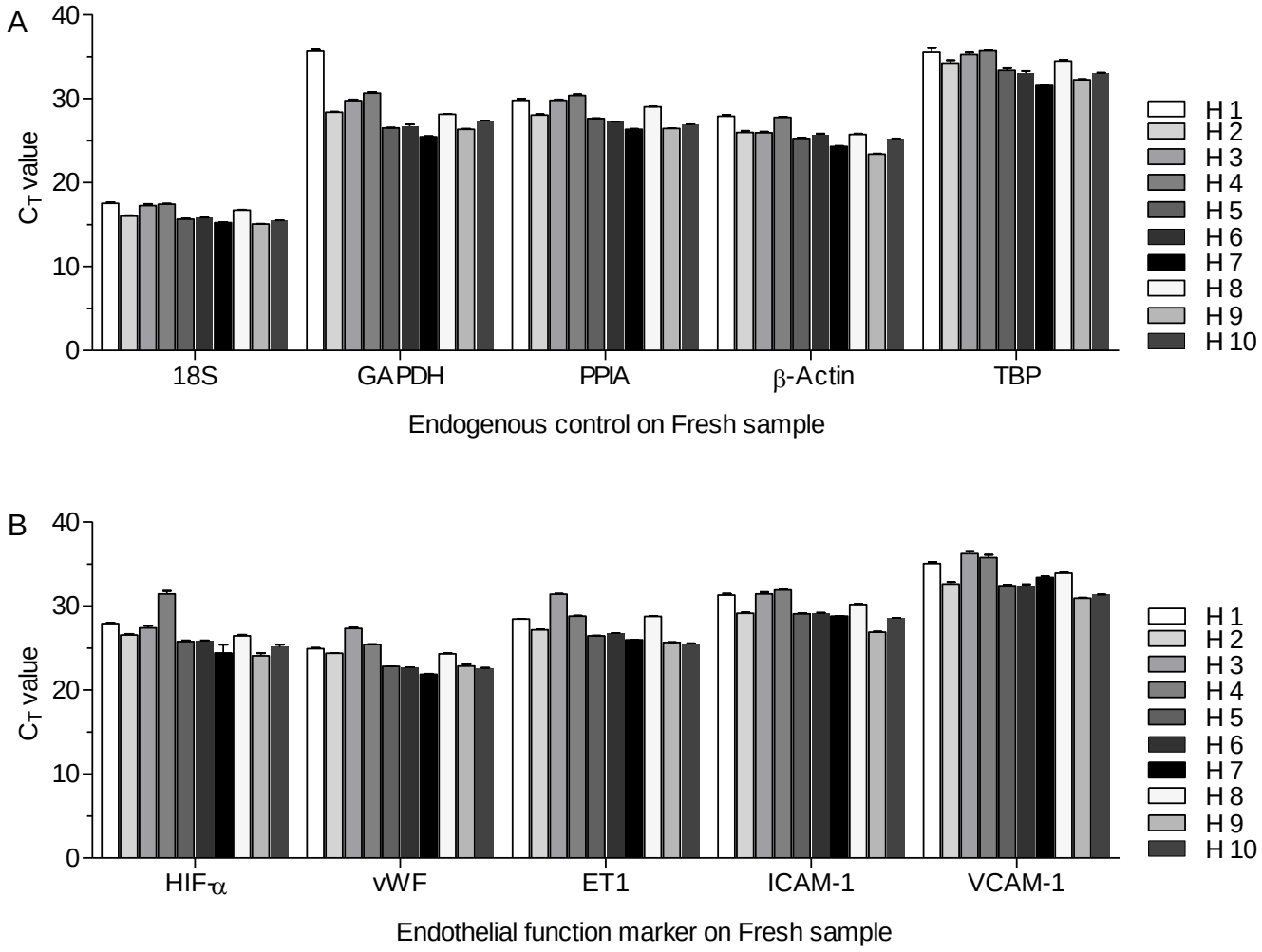


Figure 5-5: **Human endogenous control genes and endothelial function markers in fresh HUVEC**. Each bar represents a measured C_T value in an individual HUVEC preparation; error bars are inter-individual standard deviation and a H stands for HUVEC.

RNA expression of each endogenous control and endothelial function marker isolated from frozen (n=10) HUVEC samples were also quantitated in quadruplicate. The average C_T for each endogenous control panel (Figure 5-6A) and endothelial function markers (Figure 5-6B) are shown.

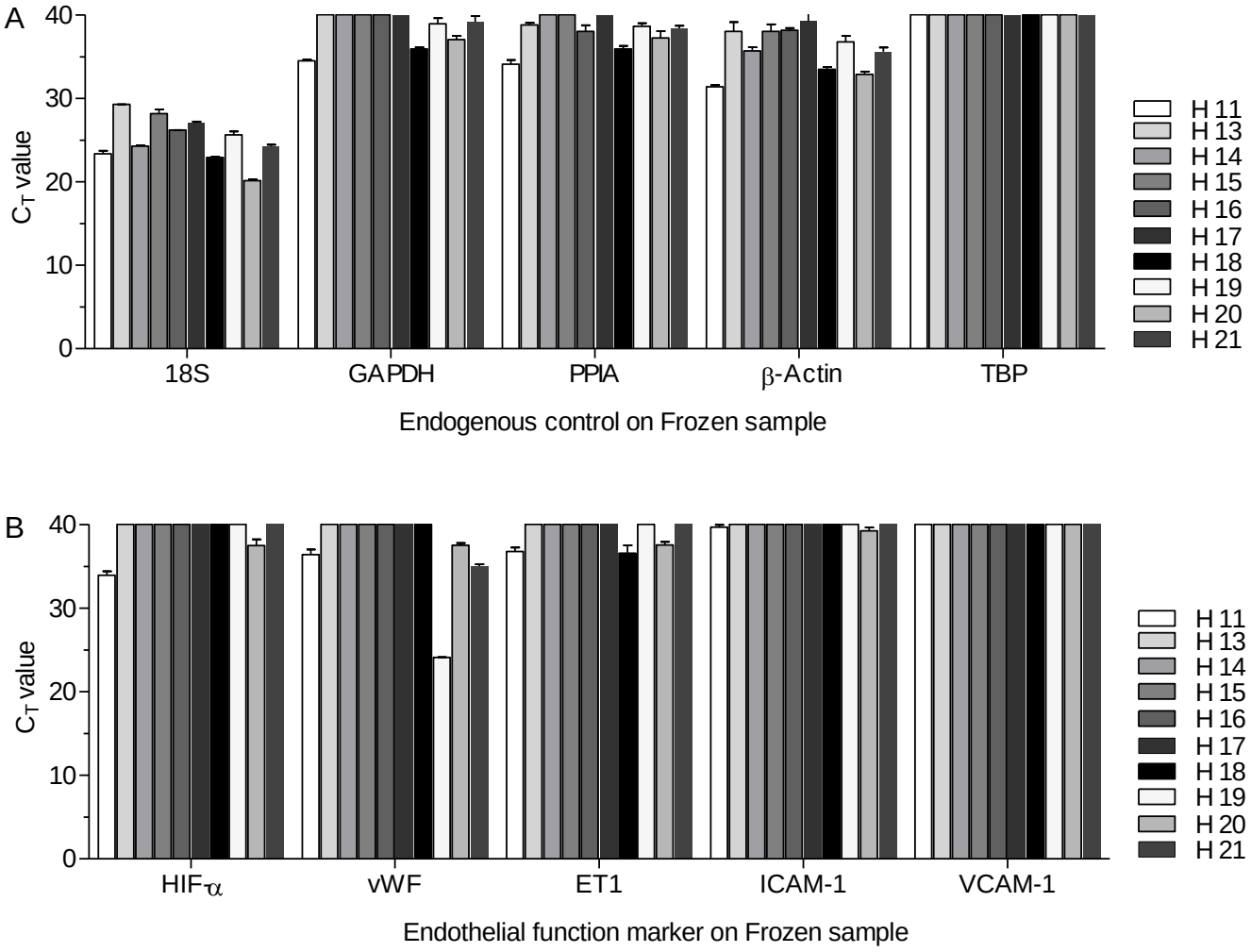


Figure 5-6: **Human endogenous genes control and endothelial function markers expression in frozen HUVEC**. Each bar represents individual measured C_T value; error bars are inter-individual standard deviation and a H stands for HUVEC.

5.6.5 The best endogenous control gene

Endogenous control gene targets were assessed for variability across the ten samples analysed. The best endogenous control gene is that showing the lowest inter-individual variability. The lowest standard deviation, and hence the least inter-individual variation, was for β-Actin (1.3), for TBP (1.4) and for PPIA (1.5). β-Actin appeared to be the best candidate to serve as the endogenous control gene in HUVEC under these conditions, as it had high expression and low inter-individual variability (Figure 5-7). Thus, β-Actin appeared as the best candidate for normalising gene expression studies involving HUVEC. The mRNA expression was extremely low from frozen preparations, and hence undetectable (C_T or greater).

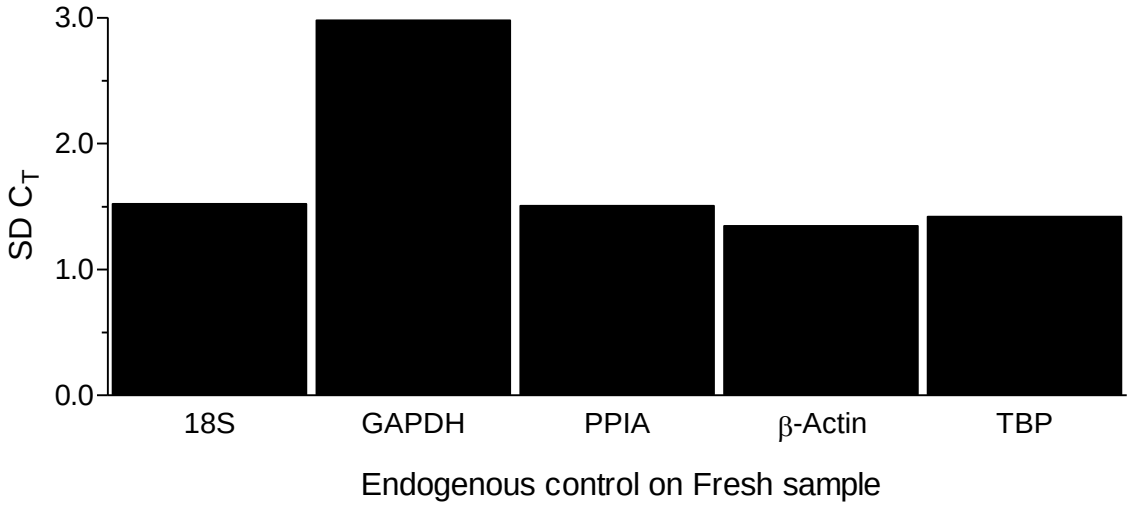


Figure 5-7: **Standard deviation C_T value of human endogenous control gene mRNA expression in fresh HUVEC**.

5.6.6 Endothelial cell marker expression relative to β-Actin

Having identified β-Actin as the best endogenous control gene for HUVEC gene expression studies, expression of endothelial cell function markers was then assessed relative to β-Actin. This was to identify whether the standard deviation of the endogenous control gene is more variable than that of the actin itself, or was similar. Observed data is shown in Table 5-2. HIF-1α showed less variability, while vWF revealed a high variation in the sample investigated (Figure 5-8A and B). A similar observation from the ET-1 (Figure 5-8C), ICAM-1 (Figure 5-8D) and VCAM-1 (Figure 5-8E) was apparent due to the variable expression on the HUVEC.

Table 5-2: **Standard deviation indicators of variability of endothelial function genes**.

Endothelial marker/β-Actin	Mean (SD)
HIF-α1	9.3 (2.7)
vWF	446.6 (252.9)
ET-1	36.3 (22.6)
ICAM-1	7.3 (3.0)
VCAM-1	0.6 (0.4)

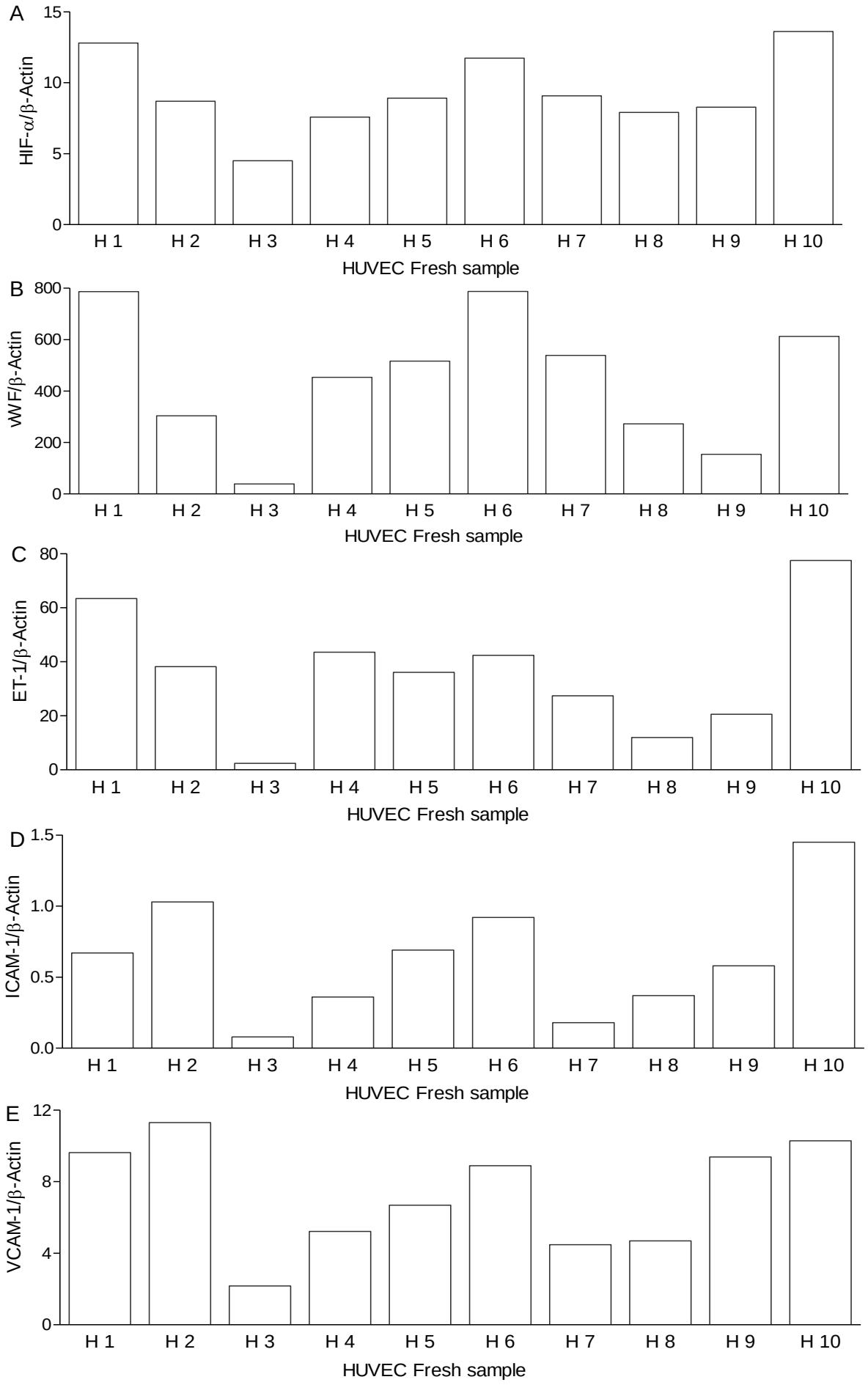


Figure 5-8: **HUVEC endothelial function marker gene expression in healthy pregnancies**. A) HIF-α, B) vWF, C) ET-1, D) ICAM-1 and E) VCAM-1 relative to β-Actin as an endogenous control in fresh HUVEC (n=10).

5.7 Discussion

Since offspring of pre-eclamptic women had reflected the lipid levels of the mothers (as shown in Chapter 4), there is a significant risk of this raised level of lipids causing endothelial damage. This chapter set out to establish a model for assessing offspring endothelial gene expression, and has optimised a protocol for preparing endothelial cells from umbilical cords. It has also established that β-Actin was the best candidate endogenous control gene for undertaking studies of mRNA gene expression in HUVEC as an index of offspring endothelial cell function at birth.

HUVEC preparation using the histopaque density gradient technique provided a relatively pure endothelial cell sample, as confirmed by immunohistochemical staining for CD31, a specific endothelial cell marker (van Beijnum et al. 2008). Lysing red blood cells during the isolation protocol is not recommended, as the lysis reagent also damaged the endothelial cells. Isolation of endothelial cells from fresh rather than frozen umbilical cord provided the best HUVEC and RNA preparations. Other studies have also found that optimal endothelial cell isolation is from fresh tissues (van Beijnum et al. 2008). Quantifying gene expression levels using RT-PCR has become standard for most laboratories. It is reasonably quick in quantitative analysis and has unparalleled sensitivity compared to traditional methods such as northern blot assay. Thus, it will be an invaluable tool in the study of offspring endothelial function at birth. In comparative C_T data analysis (Schmittgen and Livak 2008), gene expression requires normalisation to maintain consistency of expression between and/or within different samples and patients. Under the same experimental conditions, by definition, the control gene expression should not change. The control gene also corrects for variability in reverse transcription efficiency and variations in RNA extraction through preparation and pipetting.

β-Actin was found to be the most stable candidate internal control gene for assessing the mRNA level in HUVEC gene expression studies. In the experiments of this chapter, although 18S showed the lowest inter-individual variation across other endogenous control panels, β-Actin provided the least variability across n=10 analysed samples, determined by the lowest C_T standard deviation. Actins are a family of highly conserved cyto-skeletal protein in mammals (Strair et al. 1977). According to Vandekerckhove and Weber, there are different types of actin: striated muscle [α-skeletal and α-cardiac]; smooth muscle [α- and γ-SMA]; and cytoplasmic [β- and γ-CYA] (Vandekerckhove and Weber 1978). β-Actin, as part of the actin protein family, has important roles in diverse cellular functions, including cell structure, integrity, motility, phagocytosis and survival. β-Actin was also identified as the most stable endogenous gene with respect to other control genes in diabetic glomeruli and primary mesangial cells (Biederman et al. 2004). It is

notable that no one gene can serve completely as the best endogenous control under a given condition. For example, the membrane type 1-matrix metalloproteinase gene changed in response to matrigel stimulation, and there was also significant upregulation of β-Actin, whereas 18S demonstrated consistency and was not regulated by matrigel (Selvey et al. 2001). β-actin may not be the best candidate endogenous control for tissue undergoing morphological changes observed in different developmental stages. This is because developmental tissue changes over time due to concurrent change in muscular morphology. As HUVEC will be collected at birth, this is not a problem of any great magnitude. The endogenous gene of choice needs to be carefully monitored and selected based on specific experimental conditions, in order to provide the best relative comparison of the specific gene expression of a target gene. The current data also suggest that either TBP, PPIA or 18S could also serve as a good control gene for HUVEC due to their lower variability.

Limitations of the present study include a small sample size of participants, although it was just a pilot. Nevertheless, the use of the healthy pregnancy group only provides the best starting point for elucidating offspring endothelial function at birth. It is fair to stipulate that since obtaining clean preparation of HUVEC from umbilical cord tissue is highly desirable for such studies, exposure to the gradient force (400*g* for 30 min, RT) during the histopaque procedure could possibly alter HUVEC RNA expression of some target genes. It is recommended that effort should be made to ensuring that the procedure is undertaken under optimal condition that will minimise risk of gene alterations during the preparation steps. The histopaque procedure preserved cell viability and intactness, removed other cell contaminants, prevented cell distortion and eliminated the need for cumbersome columns for separation. It is hoped that HUVEC prepared in this way may retain a physiology and integrity close to the *in vivo* situation. It is also worth remarking that although the numbers of control genes assayed were few, the selection of β-Actin as the best control gene (from among 18S, GAPDH, PPIA and TBP) is the commonly use housekeeping genes in research. It is recommended that future studies, having identified β-Actin as the best endogenous control for normalising HUVEC gene studies, investigate implications of early lipid disturbance (*in utero* and *in vitro*) on HUVEC by assessing specific endothelial cell gene targets in obese, pre-eclamptic and IUGR pregnancies compared to controls. After umbilical cord collection and isolation of the HUVEC and RNA, assessment of endothelial cell (dys)functional markers (including HIF-α1, vWF, ET-1, ICAM-1, VCAM-1, CD31 and endothelial nitric oxides synthase) should be investigated. This list is by no means exhaustive. Due to the factor of limited time, this additional step was not taken for this current study. Microarray-based gene expression profiling in HUVEC can now be used to identify genes whose expression changes in response to

complications of pregnancy possibly due to the implication of fetal hyperlipidaemia *in utero.*

In summary, the present work demonstrates that high-quality HUVEC and RNA can be extracted from fresh umbilical cords at birth and a reliable control gene has been identified. Future studies using this methodology may be of use in elucidating potential physiologies and pathologies of early endothelial cells at birth. This could provide future insights into understanding the consequences of lipotoxicity in early offspring vascular health.

6 General discussion

The clinical context of the studies detailed in this thesis is that maternal obesity is a big problem. The obesity epidemic is one of the most important threats to reproductive success in the UK (Knight et al. 2010). As in many developed countries, the prevalence of obesity in the UK female population of reproductive age continues to increase significantly (Kanagalingam et al. 2005; Heslehurst et al. 2010). With this increasing trend in developed countries, it may be only a matter of time before this becomes evident in developing countries. There are numerous internal environmental impacts associated with this increasing trend, which looks set to affect female fertility worldwide. This problem, in addition to the trend (also most prevalent in developed countries, at this stage) of women delaying their first pregnancy is likely to result in an increased incidence of infertility. Crucially, maternal obesity in pregnancy poses a significantly high risk to the mother and has long-term adverse effects on her offspring's health whether in terms of infertility or subinfertility, or in various adverse pregnancy outcomes such as pregnancy loss (early/late or stillbirth), PE, IUGR or gestational diabetes (Jungheim et al. 2009; Mostello et al. 2010; Roman et al. 2011; Tennant et al. 2011). In the fetuses, obese pregnancy increases the pre- and antenatal risks. Examples include shoulder dystocia, stillbirth (Cedergren 2004; Roman et al. 2011), caesarean section (Bergholt et al. 2007) and congenital anomalies including neural tube defect (Shaw et al. 2000) and heart defects (Mills et al. 2010). There are consistent links between obese pregnant women and offspring high meconium aspiration, preterm birth, instrumental birth, fetal distress, LGA (macrosomia) and fetal death (Sebire et al. 2001; Cedergren 2004; Yogev and Langer 2008; Chen et al. 2009). Also, Catalano conceptualises the long-term consequence of obesity *in utero* as fetal programming (Catalano 2003).

As detailed in this thesis, maternal obesity affects the metabolic and inflammatory pathways by changing metabolic and inflammatory parameters throughout gestation (Stewart et al. 2007). There is exaggeration of such parameters in extreme pregnancies, which perhaps pinpoints the link between these altered parameters and the development of poor pregnancy outcomes that have been documented in this thesis. Also, data indicate that the changes of these parameters are similar to those of cardiovascular disorders, particularly in patients who suffer from coronary heart disease and heart failure (Belo et al. 2008). Cardiovascular disease is a major cause of mortality in developed countries, and the disease is projected to be problematic in future, including in developing countries. To date, cardiovascular disorders remain a leading cause of death for women internationally (Jacobs and Eckel 2005; Ray et al. 2005). A retrospective cohort study in Ontario, Canada, of 1.03 million women that were free from cardiovascular disease before first documented delivery, showed that 75,380 (7%) had maternal placental syndrome (Ray et

al. 2005). In this cohort, the incidence of cardiovascular disease was 500 per million in women that had placenta syndrome (including PE, placental abruption and placental infarction), compared to 200 per million in those that did not. A major factor in cardiovascular disorders is the increased risk factor of obesity, which is rising at an alarming rate. This increase implies that the menace of cardiovascular disease will continue to rise worldwide. As well as the risk factors already known to have some predictive value for this disease, there is a major conceptualisation of the mechanism of the uterine origin of adult disease: fetal programming. This concept, proposed three decades ago, still lacks definable proof. This lack of proof notwithstanding, the findings of this thesis suggest a possible direct link between the metabolic and inflammatory parameters in pregnancy and those evident in cardiovascular disturbance; potentially contributing to fetal programming.

In focussing on this link, this thesis has highlighted the difficulty (in most cases) in determining the precise timing of conception – a difficulty which may result in the loss of a viable fetus only being recognised some time after it has occurred. Worldwide, the onset of birth defects is rarely recognised, and fetal growth patterns are usually not measured through the course of pregnancy. When this information does become accessible, it is often incomplete with regard to timing and/or comprehensiveness. Several methodological practices have posed challenges and forced compromise in ascertaining the aetiology of mechanisms leading to adverse pregnancy outcomes, especially those that may have ramifications on offspring in adult life. This in part highlights the need to elucidate the metabolic and inflammatory pathways in pregnancy with the goal of gaining insight into offsprings' future disease. As a consequence, it was imperative to explore the importance of the metabolic and inflammatory parameters in all stages of pregnancy.

This exploration was conducted by focussing on these parameters at the implantation, in Chapter 2, by day 45 of gestation, in Chapter 3 and finally in later pregnancy, in Chapter 4. Prediction of pregnancy success was made feasible by use of the data presented in Chapter 3, which explored very early metabolic and inflammatory parameters by day 45 of gestation. It was noted that none of the parameters or obesity was predictive of pregnancy success, except for ICSI treatment technique, used predominately in cases of male factor infertility. In Chapter 2, a cell culture model of blastocyst/embryo-uterine wall adhesion during the implantation time period was developed. This was in order to ascertain whether insulin, an important metabolic parameter, affects adhesion of JAR spheroids (representing the blastocyst/embryo) onto an RL95-2 monolayer (representing the endometrial epithelium). Although the result is still inconclusive, findings support the view that insulin may be important in implantation. Laminin α1, an adhesion molecule useful at the basement membrane, was detected and is suggested to be important during JAR

spheroid-RL95-2 monolayer binding. Insulin may perhaps influence the regulation of adhesion molecule laminin α1, which possibly affects adhesion in the presence of insulin. In Chapter 3, the very early change in the metabolic and inflammatory parameters was assessed in women undergoing frozen embryo replacement in assisted conception. Women who participated were only those with a natural menstrual cycle. Observation that very early metabolic and inflammatory parameters changed over the course of the first 45 days of gestation, revealing detectable changes after implantation, was unique and fascinating. Obesity influenced the change of some of the very early parameters studied. However, obesity and insulin resistance did not predict pregnancy success. Therefore the data of this study did not support the hypothesis that obesity and insulin resistance are imperative as indicators of pregnancy success.

Chapter 4 investigated fetal cord metabolic and inflammatory parameters at birth, which were found to be reflective of the mother's parameters at the end of gestation. At this late stage of pregnancy, fetal TC was followed up after it was found that elevated fetal cord TC was correlated with an increased maternal TC level (Rodie et al. 2004). This reflected increase was proposed as possibly due to the upregulation of the key lipid transporters in the placenta of molecules involved in cholesterol transport. As a result, the gene expression of some key transporter molecules, particularly those involved in cholesterol transport, were examined and compared between groups. In the extreme cases of PE, upregulated placental mRNA levels of LDLR, STARD3 and ABCA1, but not the protein expression of the transporters was confirmed. There was a notable decrease in the cord blood TNF-α level in cases of PE, which is surprising in this well known maternal systemic inflammatory disorder (Sacks et al. 1998; Catarino et al. 2012). This decrease suggested a downregulated inflammatory response in the fetuses of the pre-eclamptic group. Further studies of placental mRNA expression, in order to determine whether it was due to poor placental TNF-α synthesis, were undertaken. The result showed a trend toward high mRNA TNF-α in pre-eclamptic placentae compared with controls. This finding resulted in the dismissal of poor placental synthesis as a factor for the reduced cord TNF-α level. It was also noted that there was a decline in the TNF-α localisation in the umbilical cord vein endothelial cells of the pre-eclamptic samples. Thus, it is evident that while there was elevated fetal hyperlipidaemia, (particularly cholesterolaemia, representing metabolic parameters), there were reduced cord TNF-α levels (representative of inflammatory parameters), indicative of downregulated inflammation in the fetuses of women with PE. To explore the effect of elevated cord lipidaemic (cholesterol) on fetal vascular health *in vivo*, there was a need to set up a useful model in Chapter 5, using HUVEC as an index of the endothelial function of the offspring at birth.

Therefore, overall, the studies presented in this thesis support the view that metabolic and inflammatory pathways are important in all stages of pregnancy, as shown in Figure 6-1.

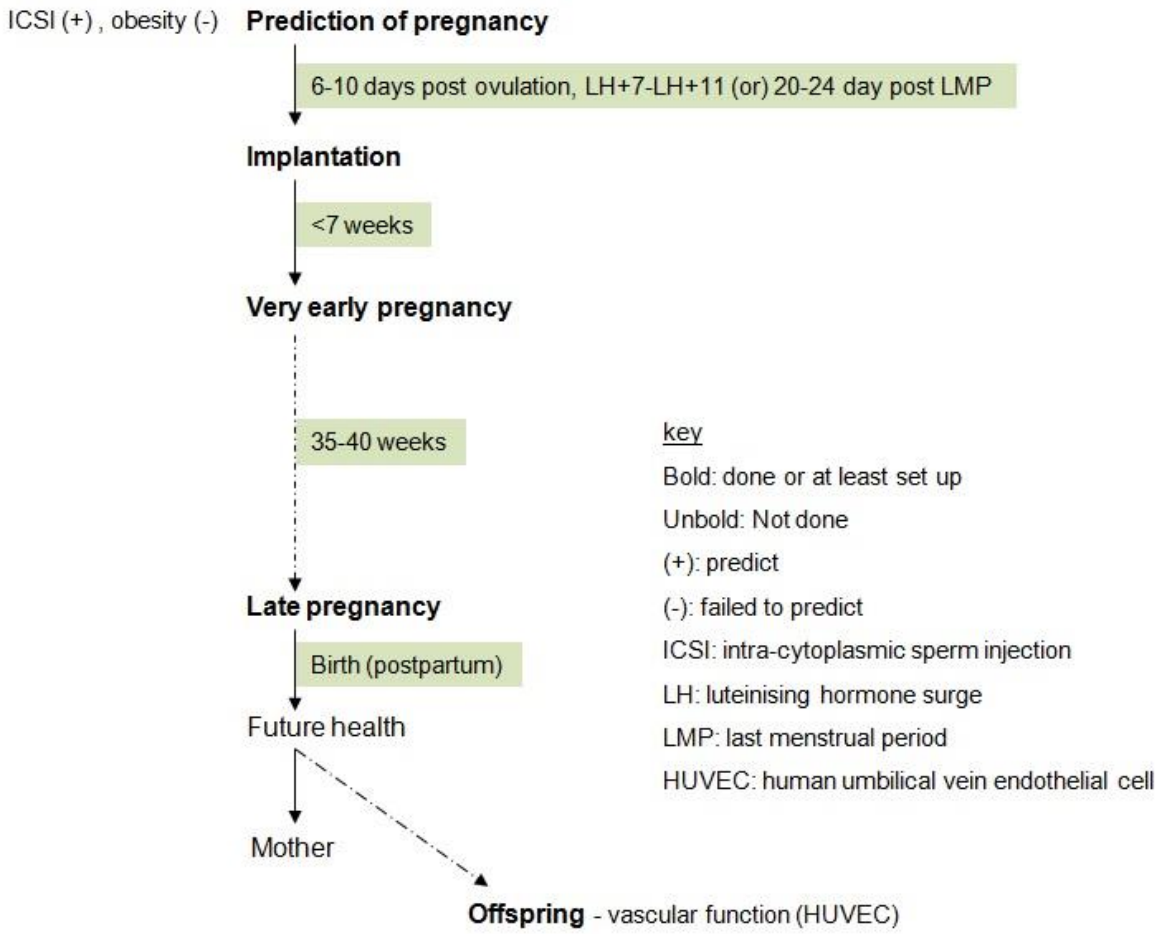


Figure 6-1: **Overview of the importance of the metabolic and inflammatory pathways in all stages of pregnancy**.

Chapter 2 notes that the aberration of implantation has remained an important problem in achieving optimal pregnancy, whether in natural and spontaneous or assisted conception. This problem has warranted the search for ways of predicting pregnancy success. In assisted conception particularly (IVF/ICSI practice), there is the observation that subfertility, as observed in populations attending for IVF, may be the factor leading to poor outcome after ART procedure (Thomson et al. 2005; Jaques et al. 2010). This is because (in the embryos that survive) there is a higher risk of pregnancy loss, either because of the underlying problem for which ART intervention was needed or because of the assisted conception procedure by which pregnancy was achieved. La Marca et al. observed that anti-Müllerian hormone levels were shown to predict live birth rates, and measurement of this hormone could facilitate individualisation of the therapy prior to the first ART cycle (La Marca et al. 2011). Also, maternal age is highlighted as a factor imperative in determining

the assisted conception protocol employed. As stated above, in their clinic in Glasgow, Scotland, Nelson et al. note that women of <36 years are administered 225IU FSH and those >36 years receive 300IU per day for follicles stimulation (Nelson et al. 2007). This is paramount, as follicle growth determines optimal oocytes retrieval for IVF/ICSI practice. Prior to undertaking the study of this thesis, it was unclear whether very early changes of metabolic and inflammatory parameters may predict pregnancy success. Also, the role of obesity as a predictor of early pregnancy success was not completely clear. It was thought that maternal body fat affected the very early pregnancy change in metabolites of lipid and carbohydrate metabolism and inflammatory mediators, as well as hormones such as insulin and leptin. Prepregnancy obesity was considered to perhaps exacerbate the early changes of the parameters and, as a consequence, to perhaps play a role in predetermining the pathway resulting in maternal insulin resistance and lipid deposition occurrence as pregnancy advanced.

However, this thesis indicated that none of the parameters measured, or obesity, predicted pregnancy success. Interestingly, assessment of erythrocytes and plasma saturated fatty acid, using the sample and dataset in Chapter 3, revealed that the percentage of saturated fatty acid in erythrocytes was independently predictive of whether a woman became pregnant or not (unpublished data). The failure of any of the parameters measured in this thesis to predict pregnancy success early in gestation was unexpected. This failure may suggest that early metabolic and inflammatory parameters become implicative of pregnancy success after the first trimester, and most noticeably in the second trimester. It is anticipated that by this time point (second trimester), for instance, accumulated TG (fat) essentially for energy oxidation has begun to be metabolised (Herrera 2000; Yadav et al. 2013). This period is the catabolic stage, important for subsequent active growth and development of the fetus. Whether prepregnancy obesity or accumulated fat early during the anabolic phase of pregnancy (Huda et al. 2009), predisposes women to these early changes in parameters is very difficult to ascertain at this point. Obesity may have been unable to predict pregnancy success because of the recommended BMI range (of greater than 18 kg/m^2 but lower than $30kg/m^2$) in the IVF clinic at which treatment is performed at GRI, Glasgow, Scotland. This BMI range is similar across the board in Scotland. Nevertheless, BMI does not appear to be a good predictor of a successful IVF (Vilarino et al. 2011). Thum et al. highlighted that pregnancy losses are most prevalent in women with BMIs above 36 kg/m^2 (Thum et al. 2007). This may perhaps mean that the ability of obesity to predict pregnancy success went undetected because this study was under-powered. Overall, obesity does not have a role in pregnancy success, at least in this study.

That ICSI is a predictor of pregnancy has been well reported (de Mouzon et al. 2009; Rosen et al. 2010; Komsky-Elbaz et al. 2013), and this thesis supports these previous findings. In ART, ICSI improves pregnancy success of assisted conception, with a 56.6% pregnancy rate compared to that of 47.6% for other procedures (in 2000), and rates recently reaching 75.9% in Latin America, and 92.4% in the Middle East by 2002 (de Mouzon et al. 2009). The ICSI-increased fertilisation rate was associated with an improved implantation rate (25.2% vs 17.8%); this rate remained significant compared to conventional insemination after adjustment for variables associated with implantation (Rosen et al. 2010). The observation that ICSI predicted pregnancy success in the present study was reassuring. However, there is uncertainty concerning the safety of ICSI. It is imperative that ICSI should be used cautiously and judiciously (Avendano and Oehninger 2011). Avendano and Oehninger noted that there are still unanswered questions regarding the safety of ICSI. Their report highlights the issue of DNA fragmentation apparent in infertile human spermatozoa, raising the probability that sperm with normal morphology with DNA fragmentation could be mistakenly selected to fertilise oocytes during the ICSI technique. Consequently this DNA fragmentation (chromosomal anomalies and/or DNA damage) may result in poor-quality embryos (Aitken and De Iuliis 2007; Avendano and Oehninger 2011).

ICSI treatment increases the pregnancy rate by overcoming the difficulty of sperm penetrating the zona pellucida. In this thesis, the data suggest that ICSI overwhelmingly predetermined whether there is pregnancy success (implantation). To a great extent this predetermination represents male factor infertility (possibly due to low sperm count and/or poor quality sperms), compared to female factor infertility (including PCOS, inflammation, tubal blockage and endometriosis), where women will be more likely to have other biological barriers to successful implantation, placentation and adaptation to pregnancy. However, caution is needed when interpreting these results, as there are potential downsides to ICSI which are presently unclear. This is because ICSI increases the risk (to an unknown extent) of genetic anomalies that otherwise would not have been transmitted.

Therefore, the need to identify predictors of pregnancy success, particularly in assisted conception, warrants further assessment of very early changes in metabolic and inflammatory parameters not examined in this thesis. The lack of predictive ability of any of the parameters or obesity, and the problematic effects of ICSI, suggests the need to have a fresh look at other factors which may play a role as predictors of improved pregnancy success with natural or assisted conception. The search for a predictor of pregnancy success was a specific aim in the general scope of this thesis, which was to elucidate the effect of these parameters, and obesity, on the development of the fetus. Notwithstanding the lack of predictive precision in that specific objective, there remained

the need to clarify the importance of metabolic and inflammatory parameters at all stages of pregnancy. This clarification proceeded by focussing on implantation and then early and late pregnancy.

Chapter 2 focussed on implantation, which has long been identified as an important stage of pregnancy. Until now, the effect of insulin during implantation remain unknown. In mouse models, insulin enhances embryo cleavages (Gardner and Kaye 1991) and stimulates the blastocyst cell number by increasing the inner cell mass cell numbers (Harvey and Kaye 1990) of preimplantation blastocysts. This implies that insulin may have a direct role in the regulation of preimplantation embryo development. *In vivo,* obese, PCOS and diabetic women are likely to miscarry (Mills et al. 1988; Penney et al. 2003; Cardozo et al. 2011; Beauharnais et al. 2012). These conditions are primarily associated with hyperinsulinaemia. In ART insulin resistance does not affect maturation, fertilisation, cleavage rates, or the number of good-quality embryos and blastocysts, but significantly decreases implantation and clinical pregnancy rate (Chang et al. 2013). Hertig et al. show in their classical work that improper adhesion of the embryo to the uterine wall results in recurrent pregnancy loss (Hertig et al. 1959), which is indicative of the necessity of adhesion molecules during the implantation window. The direct role of laminin α1 in the adhesion of the embryo to the endometrial epithelium was supported by the finding that higher levels of IgG anti-laminin-1 autoantibodies are observed in women with recurrent miscarriage than in healthy pregnant and healthy non-pregnant controls (Inagaki et al. 2001; Inagaki et al. 2003). Difficulty in studying implantation means that a cell culture model of implantation had to be developed. As noted above, human blastocyst/embryo implantation onto the maternal uterine endometrial epithelium cannot be studied *in vivo* and is difficult to study *ex vivo* (Hannan et al. 2010). Access to donated embryos for research purposes is limited to a few laboratories across the globe. The uniqueness of the human implantation process means that no other mammal provides an appropriate animal model (Bischof and Campana 2000). There are ethical concerns regarding experimentation with primary human tissues during this period of life, which necessitates using *in vitro* models employing trophoblast and uterine cell lines (John et al. 1993; Grümmer et al. 1994; Hans-Peter et al. 2000; Aboussahoud et al. 2010).

Data presented in this thesis indicated that in the absence of the insulin, the developed model appeared optimal for an *in vitro* implantation system. In the presence of insulin the model appeared to be less optimal than expected. However, the study of insulin's effect and the role of the laminin α1 adhesion molecule was suggestive of their importance; which is interesting even though the results obtained are at too early a stage to draw conclusions. It appears that insulin did not have observable consistent effect on the adhesion of JAR spheroids to RL95-2 monolayers. Nonetheless, whether insulin regulates

adhesion molecule expression of laminin α1 during the period of cell-culture model implantation is difficult to deduce at this point. This limitation of this study may be due to the technique performed, in particular the inability to determine the conclusive insulin level in the uterine implantation environment. It is recommended that for future studies this cell culture model of implantation should be improved. This is important, as an improved model may provide a valuable platform to study and understand the effects of other metabolic and inflammatory parameters during human embryonic implantation. On the other hand, it may be that assessment of other parameters than insulin may perhaps yield a better result. Also, investigation of other human choriocarcinoma cell lines (BeWo and Jeg-3) that can also be transform into spheroids, like as the JAR (Grümmer et al. 1994) is recommended. These views should be considered as part of the future recommendations with regards to the cell culture model of implantation. Overall, it appears that insulin may be important during implantation, possibly by affecting adhesion molecule regulation, e.g. laminin α1. Laminin α1 elastase-generated fragments stimulate macrophage uPA and MMP-9 expression (Khan et al. 2002), indicating the additional role of this expression for successful implantation. This model may perhaps facilitate the development of effective and acceptable intervention strategies to help improve pregnancy success. All things considered, results showed that metabolic and inflammatory parameters are important at the implantation stage of pregnancy. The clarification now proceeds with the early and late stages, in Chapter 3 and 4 respectively.

As stated in Chapter 3, the lack of comprehensive robust reports of very early pregnancy metabolic and inflammatory pathways, and the increasing evidence that fetal outcome is determined by the end of the first trimester, led to the investigation of the early time point of gestation. It was generally assumed that the earliest detection of parameters associated with adverse outcome would allow a means of identifying pregnancies that were at risk early, by developing effective diagnostic tests.

The data in Chapter 3 showed evidence of very early changes in metabolic and inflammatory parameters. Pregnant women that undertook a successful natural cycle FET showed a rebound in TG and HDL-C parameters; that did not include TC, as the TC level failed to recover by the first 45 days. Energy demand possibly accounted for the declined in the TG level. Perhaps progesterone critical at this early pregnancy time point may have played a vital role in the metabolism of TG (Mattos et al. 2000), as shown in animal models. Nevertheless, the decreased TC and HDL-C levels as early as day 18 of gestation probably suggested the utilisation of such parameters for steroidogenesis and cell membrane biogenesis (Grummer and Carroll 1988; Lange et al. 2004; Guibourdenche et al. 2009). There were also higher levels of some parameters (hCG, insulin, HOMA, CRP and PAI-2) and lower levels of others (CXCL8, CCL2, CCL11 and PAI-1) in those

who became pregnant in comparison with those that did not. The rise in insulin secretion stimulates lipogenesis and reduces fatty acid oxidation, as well as promoting maternal fat storage (Newbern and Freemark 2011). Data on CXCL8, CCL2 and CCL11 levels show a decrease at this time point, suggesting a role in the implantation success. This could be due to increased synthesis of these chemokines in uterine tissue as well as an increase need for the recruitment of uterine leukocytes, such as neutrophils, monocytes (for resident macrophages) and eosinophils possibly from the maternal circulation (Katsuhiko et al. 2010; Hannan et al. 2011; Chau et al. 2013). In another instance, the significant decrease in early pregnancy of PAI-1 levels by day 45, and the increase in PAI-2 over time, was surprising. At this early stage of gestation, it appears that the reduced PAI-1 level was due to the need to inhibit excessive trophoblast invasion, whereas the increased PAI-2 resulted from it being synthesised from the developing embryo and newly-formed placenta. This surprising result contrasts late pregnancy PAI-1 and 2 parameters reported in Chapter 4. At this late gestation stage PAI-1 was found to be higher in extreme cases (PE) (Reith et al. 1993; Catarino et al. 2008), whilst PAI-2 was lower (Reith et al. 1993), compared to healthy pregnancy. In their report, Reith et al. implied that significantly higher PAI-1 in the PE cases, may be due to hypertension or renal damage which is not specific to pregnancy or a reflection of altered placental function, whereas the lower PAI-2 is probably a result of decreased placental mass or function by end of gestation. This observation helps define a fundamental difference between very early and late stages of gestation. It was of interest that none of the very early parameters assessed in this thesis, or obesity, were independently predictive of pregnancy success by the first 6 weeks of gestation, despite obesity playing a role in the early changes in some of the parameters. Overall, it was only the male factor infertility that predicted whether the study participants had pregnancy success or not, rather than obesity and insulin resistance as originally anticipated.

Most importantly, in Chapter 3, it was not possible to use natural conception for assessment of very early pregnancy parameters. This is due to the impracticality of pinpointing exactly when women became pregnant. As a result, the parameters during very early pregnancy was explored using assisted conception patients as models. This provided the opportunity to monitor when women became pregnant. Participating women were those undergoing natural cycle FET. This use of women with a natural menstrual cycle allows for the maintenance of normal pregnancy physiology, similar to that of natural conception. The data obtained is from the first collected samples at this early period of pregnancy and the large amount of samples stored should provide a platform for many very early parameter studies in future. The study in this chapter is prospective and robustly designed; however, numerous difficulties were encountered; the most difficult being that this early pregnancy study took two and a half years. Recruitment for blood

collection from participants was undertaken prospectively over seven visits. The impracticality of participants having an LH surge on the exact same day meant that it was necessary to attend daily meetings where the individual blood hormonal samples were evaluated; and the LH surge was determined throughout the study. This means that the collection of such prospective data took considerable effort; an enormous amount of time was spent collecting samples, attending daily meetings and retrieving of data recorded in the patients' hospital notes. As it was a prospective study, the assayed values of some parameters may perhaps have been under- or overestimated for the women who took part. This is perhaps due to different emotional stress or dietary intake, even though most of the women fasted. There is also the issue of direct effect of the embryo, implanted conceptus and/or newly-forming placenta influencing the change of these parameters produced at the very early stage of gestation.

In regard to the data obtained from this study in Chapter 3, there are several directions in which further studies may be beneficial in gaining insight into the metabolic and inflammatory pathways over the course of pregnancy. It is recommended that future studies utilise the cryo samples under study (stored at -80°C) so that other important parameters can be assayed later, including measurement of TNF-α, leptin and adiponectin, which are adipose-derived adipocytokines (Ouchi et al. 2011). The role of the endocrine hormone in the change in some metabolic and inflammatory parameters can also be ascertained in future. Examples of these endocrine hormones include hPL, progesterone, oestrogen and cortisol. Whether these hormones may relate to the very early changes in metabolic and inflammatory parameters will be of interest. These data showed that metabolic and inflammatory parameter changes are important in early pregnancy. The clarification now proceeds with the late stage, as reported in Chapter 4.

In Chapter 4, healthy pregnancy was compared to pre-eclamptic pregnancy, which was used as a model of an extreme case for parameters assessment in the late stage of pregnancy. The studies are well documented (Redman and Sargent 2003; Thilaganathan et al. 2010); however, the most relevant for this thesis is the focus on evaluating effects in offspring in PE and control groups. IUGR groups were used as a control in order to determine the impact of poor placentation in the absence of hypertension and endothelial dysfunction, which are major characteristics of PE. A common feature of PE and IUGR pregnancies is the placental pathology, and the difference in their pathologies may be due to changes in the underlying metabolic and inflammatory parameters occurring only in PE.

As reported in Chapter 4, it is observed that fetal cord hypercholesterolaemia is significantly reflective of maternal hypercholesterolaemia as previously reported (Rodie et al. 2004). As a consequence, it is suggested that placental transporters are implicated in

the transfer of cholesterol across the maternal-fetal interface. Molecules involved in lipid transport (cholesterol, in particular) showed LDLR, STARD3 and ABCA1 mRNA upregulation in the placentae of PE cases. There is consistency between these lipid data and that of previous research in terms of the concept of lipid (cholesterol) transporters and localisation of LDLR on syncytiotrophoblasts and syncytium, STARD3 on stroma and ABCA1 on fetal endothelial cells in cholesterol transport. LDLR is an important receptor recognising lipoprotein rich-cholesterol carriers LDL and HDL through their apo B and apo E recognition sites, as highlighted in this thesis. Thus, as LDLR is localised in the syncytiotrophoblasts, this implies that cholesterol carriers, on reaching the cells, interact with an intracellular protein called the STARDs protein (STARD3) (Guibourdenche et al. 2009; Hu et al. 2010) – which may be involved in the intracellular transport of lipids, e.g. cholesterol. The STARD3 may help transport the cellular cholesterol toward the fetal compartment via the fetal endothelial cells, where the lipoprotein becomes effluxed into fetal circulation. Fetal endothelial cells contain acceptors proteins such as ABCA1 and ABCG1, as observed in the term human placental endothelial cells (Stefulj et al. 2009), effluxing cholesterol into fetal circulation. The efflux of cholesterol into the fetal compartment perhaps happens mainly as the HDL-C effluxed is accepted by the carrier HDL particle in fetal circulation. In this way, the maternal cholesterol is readily transported into the fetal cord, and subsequently fetal circulation, leading to the fetus reflecting the maternal level of this metabolic parameter (cholesterol). Observation between offspring and mothers (in extreme and healthy pregnancies) show evidence of fetal immuno-protection in the offspring of mothers with PE, since the mother usually presented a varied array of inflammatory response; this response was also unexpected.

For the study in this chapter, maternal samples were collected prior to the end of the third trimester in healthy and extreme cases for the assessment of metabolic and inflammatory parameters at late stages of gestation. However, samples could not be collected from the babies by conventional venepuncture procedure as carried out on the mothers. Thus, cord blood samples were collected and utilised instead. Also, even though the parameters of metabolic and inflammatory changes were BMI matched, the effect of under- or overnutrition cannot be overemphasised. Findings indicated that poor nutrient supply may have implicated the reduced parameters reaching the fetal circulation rather than overnutrition or obesity. Data from this study suggest that the placental pathologies of PE, in addition to fetal stress and need for nutrients for growth and development, possibly drive the upregulation of mRNA of LDLR, STARD3 and ABCA1, but not protein expression. This observation that upregulation of LDLR, STARD3 and ABCA1 mRNA levels failed to translate into protein possibly suggests that pharmacological targeting of such transporters in the metabolic pathway may not provide optimal protection of fetal vascular health (lipotoxicity) due to fetal hyperlipidaemia in pre-eclamptic cases. There

was also the problem of the inability to distinguish the extent to which the parameters are affected by early pregnancy or even by extreme pregnancy state (PE) at late gestation. Nonetheless, this study helped highlight directions in which further studies may be beneficial in gaining insight into the metabolic and inflammatory pathways over the course of pregnancy. The pathway involved in the transport of cholesterol across the maternal-fetal interface was determined. Together, these data showed that metabolic and inflammatory parameter changes are important in the late stage, which concludes the clarification of their importance at all stages of pregnancy.

To continue the exploration of the general scope of this thesis, the effect of metabolic and inflammatory parameter changes on the developing fetus, Chapter 5 focussed on fetal hyperlipidaemia (e.g. increased cholesterol) as a reflection of maternal lipid levels. Cholesterol accumulation, through sterol-laden macrophage 'foam cells' in the vascular wall, is a pivotal early event in the formation of atherosclerotic lesions. This is supported by Napoli et al., who observed enhancement of fetal atherosclerotic plaque lesion formation in the fetuses (6.2 ± 1.3 months) of mothers with hypercholesterolaemia (Napoli et al. 1997). Another report by Palinski and Napoli, find evidence from animal study suggesting that initiation of atherogenesis results from the uterine environment (Palinski and Napoli 2002).

Data in Chapter 4, suggesting that some cholesterol transporters may be pivotal in the transfer of maternal cholesterol, imply a possible effect on fetal vascular health at least toward the end of gestation. Additional data in late gestation in extreme cases indicate that fetal cord blood cells may be a vital source of antiinflammatory mediators, which may lead to downregulation of fetal inflammatory parameters observed in PE but not in IUGR pregnancies. As discussed in Chapter 4, this finding about the fetuses of pre-eclamptic mothers is unexpected, as these mothers are associated with altered systemic inflammatory response (Sacks et al. 1998; Catarino et al. 2012). Fetal programming of adult disease advances *in utero* is increasingly being proposed to be influenced by maternal obesity, which increases the risk of fetal lipotoxicity (Freeman 2010; Jarvie et al. 2010). This suggests that lipotoxicity *in utero*, as a potential cause of fetal programming, contributes to the early origin of future poor vascular health. Therefore, the studies presented in this thesis suggest that metabolic rather than inflammatory pathways are perhaps involved to play a key role in early programming of fetal vascular abnormality in the uterine environment. Notwithstanding this suggestion, these studies have reinforced the importance of the metabolic and inflammatory pathways in all stages of pregnancy. Specifically, elucidating the period of lipid switch from the useful (e.g. fetal membrane biosynthesis) to the harmful state (fetal plaque), towards an understanding of potential vascular risk, is now imperative. For the study reported in Chapter 5, studying fetal

vascular condition at birth, the umbilical cord was used to provide HUVEC samples, which could be explored as an index of fetal endothelial cell function at birth. A recommended direction for further studies may be gaining insight into the metabolic and inflammatory pathways over the course of pregnancy (especially at late gestation), by investigating the implication of fetal metabolic pathways on the fetus. How the metabolic parameters (lipids) may affect fetal vascular health is yet to be completely understood. Whether this abnormal state plays a role in fetal programming is a question that studies in fetal vascular endothelial (dys)function may provide further answers to in this exciting area of research: the impact of fetal metabolic pathway (lipids) in metabolic disorder of pregnancy. Although it is not completely clear to what extent, this thesis data suggest that there is a strong possibility that indication of fetal hyperlipidaemia (raised TC levels, by term in cases of PE) may provide insight into understanding the origin of offspring vascular disease. A preliminary *in vitro* study using HUVEC as an index of endothelial cell function, to assess the impact of fetal metabolic parameters (lipids, such as cholesterol), is recommended for future studies. Such study is necessary despite systemic review by Huxley et al. suggesting that impaired fetal growth does not have an effect on the blood cholesterol levels that would have an impact on vascular disorder risk (Huxley et al. 2004). However, Huxley et al. did not assess the direct blood cholesterol impact on epigenetic factors on the vascular system, as Chapter 5 proposed to explore.

It is clear that this thesis has provided a platform for future investigations of the implication of fetal metabolic pathways on the cardiovascular system, a link to fetal programming. However, several questions remain unanswered. It would be valuable to have a better understanding of the underlying epigenetic predisposition, physiology and mechanisms linking maternal and feto-placental interaction, and how these affect fetal growth and development, in terms of the implication of parameters of metabolic and inflammatory pathways. These questions remain complex and controversial and are far from being completely understood. The absence of evidence of maternal obesity as a predictor of early pregnancy success, detailed in this thesis, may then warrant focus of future studies on late gestation. Overall, it appears that the metabolic pathway is explicitly more important than the inflammatory pathway, particularly with regard to the link with adversity in offsprings as pregnancy advances. The only major pitfall of inflammation is its harmful effect on the conceptus and developing fetus early in gestation, which may potentially lead to pregnancy loss; for example, the Th1 (type1) cytokine or response may damage the placenta directly or indirectly via the (abnormal) activation of cytokine cell types (Raghupathy 1997). In the same report, Raghupathy points out the possibility of fetal expulsion due to uterine contraction or necrosis of implanted embryos, or even the thrombosis of the blood vessels supplying the conceptus by TNF-α mediator effects.

Thus, as documented in the studies of this thesis, it appears that as pregnancy advances lipids (particularly cholesterol) are important for steroidogenesis, as shown in steroidogenic tissue (Grummer and Carroll 1988; Guibourdenche et al. 2009; Hu et al. 2010). Also, cholesterol is involved in cell membrane biogenesis and neural system development (Lange et al. 2004; Chen et al. 2013). Together, these data indicate that cholesterol is vital during *in utero* development. Usually, fetal cholesterol sources include *de novo* synthesis and exogenous sources (Woollett 2001; Jenkins et al. 2008), which is necessary in order to meet these gestational cholesterol requirements. Nevertheless, it may be possible that as pregnancy advances, cholesterol becomes detrimental to the mother or developing fetus. Consequently, determining at what point the gestational increase in lipid mobilisation begins to adversely affect pregnancy is necessary. The effect on the placenta of ectopic lipid accumulation (lipotoxicity) and the loss of placental function caused by other factors are yet to be completely determined. Identifying molecular pathways involved in diverse mechanisms that control fetal vascular function, pathways supposedly evident in fetal endothelial cells (as an index of endothelial cell function *in utero*), has never been more urgent.

The problem is that the trend of more and more women around the world continuing to put off childbearing to an advanced age will continue to have as its inevitable consequence, a rise in infertility, caused by their eggs dying off and those that survive being of poor quality (Janny and Menezo 1996; Silber and Barbey 2012). Female fertility and education remain major concerns, with the rising proportion of first births occurring at 30 years of age and older amongst women with the highest level of education, with education in itself and female career pursuit blamed for the delay of childbearing (Heck et al. 1997; Shevell et al. 2005). Expectedly, this postponing of childbearing may increase the chance of complications of pregnancy, such as those linked to poor implantation, including compromised trophoblast invasion, whether through natural and spontaneous or assisted conception (Shevell et al. 2005; Daskalakis et al. 2008; Shih et al. 2008; Mostello et al. 2010; Cha et al. 2012; Gauster et al. 2012). Examples include: PE, IUGR and gestational diabetes; all these conditions are now known to be linked to increased risk of cardiovascular disorder and diabetes in women (that suffer from these complicated pregnancies) after their childbearing years (Sattar and Greer 2002). In the offspring, the long-term consequences of being born with any of these complications have not been fully addressed. However, what is clear is that complications of PE, IUGR and gestational diabetes are associated with a variety of altered metabolic and inflammatory parameters. This thesis suggests the view that the metabolic pathway (lipids) in the fetuses born of extreme cases (PE) may play a key role in the fetal programming of poor fetal vascular health that may become evident in adult life.

The past decades have witnessed extensive studies on metabolic and inflammatory response in several pregnancy outcomes, particularly disorders associated with poor implantation and placental perfusion. Obesity remains a risk factor for metabolic and inflammatory parameters, and is rapidly rising at an alarming rate worldwide. It has never been as urgent as now for the implications of metabolic and inflammatory pathways to be addressed and understood. Devising effective policy and practice to combat childhood obesity is a major priority for many health professionals and governments. There is irrevocable evidence that departures from optimal growth *in utero*, whether from under- or overnutrition, increase the relative risk of adult obesity. This thesis essentially provides a basis for the fetal programming hypothesis and presents a challenge to elucidate the mechanisms by which gene-nutrient interaction during early life (embryo and fetal) development sets the stage for adult susceptibility to multiple metabolic and inflammatory abnormalities.

In summary, this thesis has shown the potential importance of metabolic and inflammatory parameters in all stages of pregnancy. There is a need to continue the search for potential predictors of pregnancy success. Although there was an absence of a predictor (except from ICSI treatment) in the data from the studies in this thesis, changes of parameters were shown to often depend on obesity. Interestingly, obesity and insulin resistance did not play a role in the achieving pregnancy success. The *in vitro* model of implantation developed, may help determine whether genotypic and phenotypic composition of the embryo is affected by various metabolic and inflammatory parameters, and how this effect may play a part during the implantation window. The results emphasise the need for understanding ways of improving implantation, whether in natural and spontaneous or assisted conception, in order to have a better chance of live birth. Insulin failed to show an observable effect on binding, but may perhaps have an effect on enhancing adhesion of JAR spheroids to RL95-2 monolayers in the *in vitro* model of implantation, by regulating laminin α1 adhesion molecules during binding. These was evidence, through the observation of very early hyperinsulinaemia, insulin resistance, dyslipidaemia, normoglycaemia and inflammatory haemostatic balance (high and low inflammatory parameters), that each active pathway may promote pregnant success in women by day 45 of gestation. Understanding the process of how early metabolic and inflammatory parameter changes may be implicated in the development of complications of pregnancy is exciting. The idea that embryo quality or presence, as well as diet and/or weight gain, may possibly be important factors during implantation and the early stages of maternal adaptation as pregnancy advances, is of great interest. At the late stage of gestation, fetal parameters, in particular of lipids (TC) in the PE model, were reflective of maternal parameters. Dyslipidaemia, poor glucose tolerance and inflammation present in pre-eclamptic pregnant mothers are consistent with well-known facts. It appears that immuno-

protective pathways are present in the offspring's circulation, possibly part of the blood cells, where they may serve an immune suppressive role. The localisation of key lipid transporters, especially cholesterol, at specific areas of placental villi confirms consistent pathways of cholesterol transfer across the maternal-fetal interface. The observed molecules involved in lipid transport (in particular, cholesterol) may have been responsible for fetal lipid profiles reflecting that of their mothers, despite the lack of change in the protein levels. This implicates the metabolic pathway as consequently providing a link to the orchestration of maternal lipotoxicity in the offspring which could be enhanced in the pregnancies of obese women and extreme cases such as PE. It is now paramount that the potential consequences for offspring vascular health are explored in future studies.

Therefore, on the whole, these data highlight that metabolic and inflammatory pathways are of importance in all stages of pregnancy. Metabolic and inflammatory homeostasis throughout gestation is paramount for pregnancy success involving nutrition supply, fat accumulation, placental growth and fetal uptake and utilisation. These processes are in concert with each other in meeting the demand of the developing conceptus, fetus and mother in all stages of gestation. Dysregulation of these events at any one time may be detrimental, not only resulting in the possibility of adverse pregnancy outcome but also in the risk of fetal programming of future adult disease. In either case, the findings of this thesis pinpoint the importance of metabolic and inflammatory pathways in all stages of pregnancy in leading to pregnancy success, in terms of implantation, adaptation to pregnancy and potentially on fetal programming of offspring.

Appendix: 1 Suppliers

Chemicals and reagents

Most of the chemicals and reagents were obtained from Sigma-Aldrich UK and Life Technologies UK unless otherwise stated here or above.

Chapter 2

Agarose, aprotinin, Bradford reagent, bromophenol blue, celLytic™ MT (mammalian tissue lysis/extraction reagent), 3,3'-diaminobenzidine tetrahydrochloride (DAB), dimethyl sulfoxide, diethlpyrocarbonate (DEPC), ethidium bromide, glycerol, Harris stain, HEPES buffer, histopaque 1077, hydrogen peroxide (H_2O_2) and human serum were obtained from Sigma-Aldrich. Sigma-Aldrich also supplied insulin solution (human), Kodak® BioMax™ light film, lauryl sulfate (sodium dodecyl sulphate [SDS]), 2-mercaptoethanol, polaroid black-and-white print film, poly-D-lysine hydrobromide (molecular weight 70000-150000), protein standard (micro standard: 1mg BSA/ml in 0.15M NaCl) and rabbit serum. Sodium bicarbonate (Na_2CO_3) solution (7.5%) by volume, tris-base, tris-HCl, 0.25% trypsin-EDTA solution cell culture tested, trypan blue solution cell culture tested, trypsin, Triton X-100 and xylene cyanole ff were also from Sigma. Acetone, ammonium chloride (NH_4Cl), boric acid, calcium chloride ($CaCl_2$), citric acid, DPX mountant for microscopy, ethanol, ethylenediaminetetra-acetic acid (EDTA), glycine, hydrochloric acid (HCl), isopropanol, methanol, potassium chloride, sodium chloride (NaCl), sulphuric acid and xylene were supplied by VWR International. Fisher Scientific provided chloroform and sodium hydroxide (NaOH), while complete Mini protease inhibitor cocktail tablets were from Roche Diagnostics. Dithiothreiol was supplied by Melford, DNA free™ (Ambion) and 2-log DNA ladder 0.1-10.0 kb were from New England BioLabs. Human recombinant insulin Actrapid 100 IU/mL was obtained from Novo Nordisk A/S. GIBCO Invitrogen Life Technologies provided Dulbecco's modified Eagle medium (DMEM)/Ham's F12, fetal bovine serum (FBS), HiMark™ pre-stained high molecular weight protein standard, NuPAGE® Novex 4-12% Bis-Tris gels, NUPAGE® MOPS SDS running buffer, NUPAGE® transfer buffer, Dulbecco's phosphate buffered saline (DPBS) Ca^+/Mg^+, (calcium/magnesium ion free DPBS) penicillin-streptomycin (P-S) and TRIzol reagent. DNA primers for trophinin, CD44, laminin α1, insulin receptor, FBLN1 and FBLN2 mRNA detection were from Life Technologies. First Choice® human kidney total RNA and nucleic acid purification lysis solution were obtained from Applied Biosystems. Formulated RPMI 1640 was supplied by the ATCC, Manassas, USA, while Laemmli sample buffer and Tween-20 were from Bio-Rad Laboratories. MegaMix~Double (2MMD-5) (Cambio, Cambridge, UK), sodium dihydrogen phosphate (NaH_2PO_4) (Merck, Chemical Germany) and SuperSignal® West Pico (an enhanced Chemiluminescent

Substrate for detection of horseradish peroxidase [HRP] were from Thermo Scientific. Vectastain® standard ABC Kit was supplied by Vector Laboratories.

Chapter 3

Glycerol-3-phosphate oxidase/phenol aminophenazone (GPO/PAP), CHOD/PAP kit, HDL cholesterol plus 3rd generation kit, glucose oxidase/PAP kit and Tin-quant CRP (Latex) high sensitivity immunoturbidimetric assay kit, all supplied by Roche Diagnostic. NEFA C test kit was from Wako, Neuss Germany. Human insulin (10-1113-01, Mercodia), Uppsala, Sweden whereas IL-6 (Quantikine HS, R&D Systems), PAI-1 (TriniLIZE PAI-1 Antigen REF: T6003, Trinity Biotech) and PAI-2 (IMUBIND® PAI-2 ELISA; Stamford, USA). Plasma chemokines (CXCL8, CCL2, CCL3, CCL4 and CCL11) were obtained from MilliPlex® MAP kit (Bio-Rad). Human CG was measured on an IMMUNLITE®/IMMUNITE® 1000 system analyser; Siemens, USA and IMMUNLITE®/IMMUNITE® 1000 hCG kit (Siemens Medical Solution Diagnostic).

Chapter 4

Bradford, ethidium bromide, celLytic MT^{TM} mammalian tissue lysis reagent, glycerol, hydrogen peroxide (H_2O_2), trypan blue, EDTA, NaOH, isopropanol, Harris stain and SDS were obtained from Sigma-Aldrich. Dithiothreiol was from GIBCO Invitrogen. Acetone, chloroform, citric acid, ethanol, hydrochloric acid, methanol, potassium chloride, NaCl and xylene were from VWR International. Tween-20 was from Bio-Rad laboratories and 100 bp DNA ladder was supplied by New England BioLab® Inc. ABI 6100 Nucleic Acid Prepstation, First Choice™ PCR-Ready human Liver cDNA and control primer mix (primer target is a constitutive housekeeping gene), Nucleic Acid Purification, Primer Probe Target Mix and Nucleic Acid Purification Elution Solution, universal Taqman mix and RNA Purification wash solution-1 and -2 were from Applied Biosystem. 10% Formalin buffered solution was obtained from Adams Healthcare, England. The primers sequence of 18S was from TAGN.

Chapter 5

Collagenase from Clostridium Histolyticum-type II, Histopaque 1077, haematoxylin, SDS, TRIzol reagent, H_2O_2, isopropanol and NaOH were supplied by Sigma-Aldrich. Acetone, chloroform, citric acid, ethanol, hydrochloric acid, methanol, potassium/NaCl and xylene were from VWR International. Universal Taqman Mix was from Applied Biosystem. Formalin buffered solution was from Adams healthcare, England. A platelet endothelial cell adhesion molecule (PECAM) CD31 antibody was obtained from DAKO and myosin antibodies from Sigma-Aldrich.

Appendix: 2 Consents

Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Early pregnancy metabolic and inflammatory changes research study

Name of Researcher: Mr Christopher Onyiaodike
Research nurse

Please initial box

1. I confirm that I have read and understand the information sheet dated 21st Sept 2007 (version 3) for the above study and have had the opportunity to ask questions. ☐
2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐
3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the Department of Obstetrics and Gynaecology, University of Glasgow or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐
4. I agree for the Consultants in the ACS Unit to be informed that I am in the study. ☐
5. I agree that samples from this study will be stored in the Division of Developmental Medicine for up to 20 years and may be used in other related research pending ethical approval for its use. ☐
6. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
----------	----------	----------
Name of Person taking consent (if different from researcher)	Date	Signature
----------	----------	----------
Researcher	Date	Signature
----------	----------	----------

1 for patient; 1 for researcher; 1 to be kept with hospital notes

Centre Number:
Study Number:
Patient Identification Number for this trial:

CONSENT FORM

Title of Project: **Factors which affect cardiovascular risk in pregnancy – Study 2**

Name of Researcher: Mr Christopher Onyiaodike

Please initial box

1. I confirm that I have read and understand the information sheet dated 8th December 2006 (version 4) for the above study and have had the opportunity to ask questions. ☐

2. I understand that my participation is voluntary and that I am free to withdraw at any time, without giving any reason, without my medical care or legal rights being affected. ☐

3. I understand that sections of any of my medical notes may be looked at by responsible individuals from the Department of Obstetrics and Gynaecology, University of Glasgow or from regulatory authorities where it is relevant to my taking part in research. I give permission for these individuals to have access to my records. ☐

4. I agree to take part in the above study. ☐

Name of Patient	Date	Signature
---	---	---
Name of Person taking consent (if different from researcher)	Date	Signature
---	---	---
Researcher	Date	Signature
---	---	---

1 for patient; 1 for researcher; 1 to be kept with hospital notes

List of References

Aaltonen, R., T. Heikkinen, et al. (2005). "Transfer of Proinflammatory Cytokines Across Term Placenta." Obstet Gynaecol **106**(4): 802-807.

Aboussahoud, W., C. Bruce, et al. (2010). "Activation of Toll-like receptor 5 decreases the attachment of human trophoblast cells to endometrial cells in vitro." Hum Reprod **25**(9): 2217-2228.

Achache, H. and A. Revel (2006). "Endometrial receptivity markers, the journey to successful embryo implantation." Hum Reprod Update **12**(6): 731-746.

Acton, S., A. Rigotti, et al. (1996). "Identification of Scavenger Receptor SR-BI as a High Density Lipoprotein Receptor." Science **271**(5248): 518-520.

Adiga, U., V. D'Souza, et al. (2007). "Antioxidant activity and lipid peroxidation in preeclampsia." J Chin Med Assoc **70**(10): 435-438.

Adjaye, J., J. Huntriss, et al. (2005). "Primary differentiation in the human blastocyst: comparative molecular portraits of inner cell mass and trophectoderm cells." Stem Cells **23**(10): 1514-1525.

Adorni, M. P., F. Zimetti, et al. (2007). "The roles of different pathways in the release of cholesterol from macrophages." J Lipid Res **48**(11): 2453-2462.

Aflatoonian, A., F. Mansoori Moghaddam, et al. (2010). "Comparison of early pregnancy and neonatal outcomes after frozen and fresh embryo transfer in ART cycles." J Assist Reprod Genet **27**(12): 695-700.

Aitken, R. J. and G. N. De Iuliis (2007). "Origins and consequences of DNA damage in male germ cells." Reprod Biomed Online **14**(6): 727-733.

Al-Shawaf, T., D. Yang, et al. (1993). "Infertility: Ultrasonic monitoring during replacement of frozen/thawed embryos in natural and hormone replacement cycles." Hum Reprod **8**(12): 2068-2074.

Albrecht, C., S. Soumian, et al. (2007). "Placental ABCA1 Expression is Reduced in Primary Antiphospholipid Syndrome Compared to Pre-eclampsia and Controls." Placenta **28**(7): 701-708.

Alfaradhi, M. Z. and S. E. Ozanne (2011). "Developmental programming in response to maternal overnutrition." Front Genet **2**: 27.

Almagor, M., A. Hazav, et al. (2004). "The levels of C-reactive protein in women treated by IVF." Hum Reprod **19**(1): 104-106.

Almog, B., E. Shalom-Paz, et al. (2010). "Promoting implantation by local injury to the endometrium." Fertil Steril **94**(6): 2026-2029.

Alom-Ruiz, S. P., N. Anilkumar, et al. (2008). "Reactive oxygen species and endothelial activation." Antioxid Redox Signal **10**(6): 1089-1100.

Alpy, F. and C. Tomasetto (2005). "Give lipids a START: the StAR-related lipid transfer (START) domain in mammals." J Cell Sci **118**(13): 2791-2801.

Aluvihare, V. R., M. Kallikourdis, et al. (2004). "Regulatory T cells mediate maternal tolerance to the fetus." Nat Immunol **5**(3): 266-271.

Anacker, J., S. E. Segerer, et al. (2011). "Human decidua and invasive trophoblasts are rich sources of nearly all human matrix metalloproteinases." Mol Hum Reprod **17**(10): 637-652.

Anderson, J. L., D. K. Waller, et al. (2005). "Maternal obesity, gestational diabetes, and central nervous system birth defects." Epidemiology **16**(1): 87-92.

Andrews, M. H. and S. G. Matthews (2004). "Programming of the hypothalamo-pituitary-adrenal axis: serotonergic involvement." Stress **7**(1): 15-27.

Anifandis, G., K. Dafopoulos, et al. (2010). "Effect of the Position of the Polar Body During ICSI on Fertilization Rate and Embryo Development." Reprod Sci **17**(9): 849-853.

Anumba, D. O. C., S. C. Robson, et al. (1999). "Nitric oxide activity in the peripheral vasculature during normotensive and preeclamptic pregnancy." Am J Physiol Heart Circ Physiol **277**(2): H848-H854.

Aplin, J. D. (1996). "The cell biology of human implantation." Placenta **17**(5-6): 269-275.

Aplin, J. D. (1997). "Adhesion molecules in implantation." Rev Reprod **2**(2): 84-93.

Aplin, J. D., M. W. Seif, et al. (1994). "The endometrial cell surface and implantation. Expression of the polymorphic mucin MUC-1 and adhesion molecules during the endometrial cycle." Ann N Y Acad Sci **734**: 103-121.

Argraves, W. S., H. Tran, et al. (1990). "Fibulin is an extracellular matrix and plasma glycoprotein with repeated domain structure." J Cell Biol **111**(6): 3155-3164.

Armitage, J. A., L. Poston, et al. (2008). "Developmental origins of obesity and the metabolic syndrome: the role of maternal obesity." Front Horm Res **36**: 73-84.

Aronne, L. J. and K. R. Segal (2002). "Adiposity and Fat Distribution Outcome Measures: Assessment and Clinical Implications." Obes Res **10**(S11): 14S-21S.

Artemenko, I. P., D. Zhao, et al. (2001). "Mitochondrial Processing of Newly Synthesized Steroidogenic Acute Regulatory Protein (StAR), but Not Total StAR, Mediates Cholesterol Transfer to Cytochrome P450 Side Chain Cleavage Enzyme in Adrenal Cells." J Biol Chem **276**(49): 46583-46596.

Aruffo, A., I. Stamenkovic, et al. (1990). "CD44 is the principal cell surface receptor for hyaluronate." Cell **61**(7): 1303-1313.

Aslan, D., S. E. Elizur, et al. (2005). "Comparison of zygote intrafallopian tube transfer and transcervical uterine embryo transfer in patients with repeated implantation failure." Eur J Obstet Gynaecol Reprod Biol **122**(2): 191-194.

Asztalos, B. F., M. E. Brousseau, et al. (2001). "Subpopulations of high density lipoproteins in homozygous and heterozygous Tangier disease." Atherosclerosis **156**(1): 217-225.

Ategbo, J. M., O. Grissa, et al. (2006). "Modulation of Adipokines and Cytokines in Gestational Diabetes and Macrosomia." J Clin Endocrinol Metab **91**(10): 4137-4143.

Aumailley, M., L. Bruckner-Tuderman, et al. (2005). "A simplified laminin nomenclature." Matrix Biol **24**(5): 326-332.

Avendano, C. and S. Oehninger (2011). "DNA fragmentation in morphologically normal spermatozoa: how much should we be concerned in the ICSI era?" J Androl **32**(4): 356-363.

Averna, M. R., C. M. Barbagallo, et al. (1991). "Lipids, lipoproteins and apolipoproteins AI, AII, B, CII, CIII and E in newborns." Biol Neonate **60**(3-4): 187-192.

Avogaro, A. and S. V. de Kreutzenberg (2005). "Mechanisms of endothelial dysfunction in obesity." Clinica Chimica Acta **360**(1–2): 9-26.

Avram, M. M., A. S. Avram, et al. (2007). "Subcutaneous fat in normal and diseased states: 3. Adipogenesis: From stem cell to fat cell." J Am Acad Dermatol **56**(3): 472-492.

Aydin, S., S. P. Guzel, et al. (2008). "Serum leptin and ghrelin concentrations of maternal serum, arterial and venous cord blood in healthy and preeclamptic pregnant women." J Physiol Biochem **64**(1): 51-59.

Azhar, S., L. Tsai, et al. (1998). "Human granulosa cells use high density lipoprotein cholesterol for steroidogenesis." J Clin Endocrinol Metab **83**(3): 983-991.

Azuma, K., I. Calderon, et al. (1993). "Is the luteo-placental shift a myth? Analysis of low progesterone levels in successful art pregnancies." J Clin Endocrinol Metab **77**(1): 195-198.

Bai, J., F. W. S. Wong, et al. (2002). "Parity and pregnancy outcomes." Am J Obstet Gynaecol **186**(2): 274-278.

Baird, D. D., C. R. Weinberg, et al. (2003). "Rescue of the Corpus Luteum in Human Pregnancy." Biol Reprod **68**(2): 448-456.

Baker, A. M., R. L. Klein, et al. (2009). "Maternal serum dyslipidemia occurs early in pregnancy in women with mild but not severe preeclampsia." Am J Obstet Gynaecol **201**(3): 293.e291-293.e294.

Bansal, A. S., S. A. Bora, et al. (2012). "Mechanism of human chorionic gonadotrophin-mediated immunomodulation in pregnancy." Expert Rev Clin Immunol **8**(8): 747-753.

Barbour, L. A., C. E. McCurdy, et al. (2007). "Cellular Mechanisms for Insulin Resistance in Normal Pregnancy and Gestational Diabetes." Diabetes Care **30**(Suppl 2): S112-S119.

Barker, D. J. (1997). "Fetal nutrition and CVD in later life." Br Med Bull **53**(1): 96-108.

Barker, D. J. (2000). "In utero programming of cardiovascular disease." Theriogenology **53**(2): 555-574.

Barker, D. J., J. G. Eriksson, et al. (2002). "Fetal origins of adult disease: strength of effects and biological basis." Int J Epidemiol **31**(6): 1235-1239.

Basak, S., R. Dhar, et al. (2002). "Steroids modulate the expression of α4 integrin in mouse blastocysts and uterus during implantation." Biol Reprod **66**(6): 1784-1789.

Battaglia, F. C. and G. Meschia (1978). "Principal substrates of fetal metabolism." Physiol Rev **58**(2): 499-527.

Beauharnais, C. C., D. J. Roberts, et al. (2012). "High rate of placental infarcts in type 2 compared with type 1 diabetes." J Clin Endocrinol Metab **97**(7): E1160-1164.

Belkner, J., P. Chaitidis, et al. (2005). "Expression of 12/15-Lipoxygenase Attenuates Intracellular Lipid Deposition During In Vitro Foam Cell Formation." Arterioscler Thromb Vasc Biol **25**(4): 797-802.

Belo, L., A. Santos-Silva, et al. (2008). "Similarities between pre-eclampsia and atherosclerosis: a protective effect of physical exercise?" Curr Med Chem **15**(22): 2223-2229.

Bennun, I., Y. Ghetler, et al. (1989). "Egg Donation In An Invitro Fertilization Program - An Alternative Approach To Cycle Synchronization And Timing Of Embryo Transfer." Fertil Steril **52**(4): 683-687.

Benyo, D. F., T. M. Miles, et al. (1997). "Hypoxia stimulates cytokine production by villous explants from the human placenta." J Clin Endocrinol Metab **82**(5): 1582-1588.

Berends, A. L., C. J. M. de Groot, et al. (2008). "Shared Constitutional Risks for Maternal Vascular-Related Pregnancy Complications and Future Cardiovascular Disease." Hypertension **51**(4): 1034-1041.

Berg, A. H. and P. E. Scherer (2005). "Adipose Tissue, Inflammation, and Cardiovascular Disease." Circ Res **96**(9): 939-949.

Bergholt, T., L. K. Lim, et al. (2007). "Maternal body mass index in the first trimester and risk of cesarean delivery in nulliparous women in spontaneous labor." Am J Obstet Gynaecol **196**(2): 163.e161-163.e165.

Bertram, C., A. R. Trowern, et al. (2001). "The Maternal Diet during Pregnancy Programs Altered Expression of the Glucocorticoid Receptor and Type 2 11 β-Hydroxysteroid Dehydrogenase: Potential Molecular Mechanisms Underlying the Programming of Hypertension in Utero." Endocrinology **142**(7): 2841-2853.

Bhattacharjee, J., F. Ietta, et al. (2010). "Expression and localization of ATP binding cassette transporter A1 (ABCA1) in first trimester and term human placenta." Placenta **31**(5): 423-430.

Biederman, J., J. Yee, et al. (2004). "Validation of internal control genes for gene expression analysis in diabetic glomerulosclerosis." Kidney Int **66**(6): 2308-2314.

Bischof, P. and A. Campana (1996). "A model for implantation of the human blastocyst and early placentation." Hum Reprod Update **2**(3): 262-270.

Bischof, P. and A. Campana (1997). "Trophoblast differentiation and invasion: its significance for human embryo implantation." Early pregnancy **3**(2): 81-95.

Bischof, P. and A. Campana (2000). "Molecular mediators of implantation." Best Pract Res Clin Obstet Gynaecol **14**(5): 801-814.

Blasiole, D. A., A. T. Oler, et al. (2008). "Regulation of ApoB secretion by the low density lipoprotein receptor requires exit from the endoplasmic reticulum and interaction with ApoE or ApoB." J Biol Chem **283**(17): 11374-11381.

Blum, C. B., P. A. Davis, et al. (1985). "Elevated levels of apolipoprotein E in the high density lipoproteins of human cord blood plasma." J Lipid Res **26**(6): 755-760.

Blumfield, M. L., A. J. Hure, et al. (2012). "Dietary balance during pregnancy is associated with fetal adiposity and fat distribution." Am J Clin Nutr **96**(5): 1032-1041.

Bodzioch, M., E. Orso, et al. (1999). "The gene encoding ATP-binding cassette transporter 1 is mutated in Tangier disease." Nat Genet **22**(4): 347-351.

Boney, C. M., A. Verma, et al. (2005). "Metabolic syndrome in childhood: association with birth weight, maternal obesity, and gestational diabetes mellitus." Pediatrics **115**(3): e290-296.

Bosio, P. M., P. J. McKenna, et al. (1999). "Maternal Central Hemodynamics in Hypertensive Disorders of Pregnancy." Obstet Gynaecol **94**(6): 978-984.

Boyadzhieva, M. V., I. Atanasova, et al. (2012). "Comparative analysis of current diagnostic criteria for gestational diabetes mellitus." Obstetric Medicine: The Medicine of Pregnancy **5**(2): 71-77.

Bradford, M. M. (1976). "A Rapid and Sensitive Method for the Quantitation of Microgram Quantities of Protein Utilizing the Principle of Protein-Dye binding." Anal Biochem **72**(1-2): 248-254.

Braekke, K., M. R. Holthe, et al. (2005). "Calprotectin, a marker of inflammation, is elevated in the maternal but not in the fetal circulation in preeclampsia." Am J Obstet Gynaecol **193**(1): 227-233.

Brahma, P. K., R. Lieberman, et al. (2007). "Placenta percreta following first trimester miscarriage." Int J Gynaecol Obstet **99**(2): 140-141.

Brewster, J. A., N. M. Orsi, et al. (2008). "Gestational effects on host inflammatory response in normal and pre-eclamptic pregnancies." Eur J Obstet Gynaecol Reprod Biol **140**(1): 21-26.

Briana, D. D. and A. Malamitsi-Puchner (2009). "Reviews: adipocytokines in normal and complicated pregnancies." Reprod Sci **16**(10): 921-937.

Brosens, I., W. B. Robertson, et al. (1967). "The Physiological Response of The Vessels of The Placental Bed to Normal Pregnancy." J Pathol Bacteriol **93**(2): 569-579.

Brosens, I. A., W. B. Robertson, et al. (1972). "The role of the spiral arteries in the pathogenesis of preeclampsia." Obstet Gynaecol Annu **1**: 177-191.

Brousseau, M. E., G. P. Eberhart, et al. (2000). "Cellular cholesterol efflux in heterozygotes for Tangier disease is markedly reduced and correlates with HDL-C concentration and particle size." J Lipid Res **41**(7): 1125-1135.

Brown, K., D. S. Heller, et al. (2011). "Glucose transporter 3 (GLUT3) protein expression in human placenta across gestation." Placenta **32**(12): 1041-1049.

Brown, M. S. and J. L. Goldstein (1986). "A receptor-mediated pathway for cholesterol homeostasis." Science **232**(4746): 34-47.

Bugatto, F., A. Fernandez-Deudero, et al. (2010). "Second-trimester amniotic fluid proinflammatory cytokine levels in normal and overweight women." Obstet Gynaecol **115**(1): 127-133.

Bujold, E., T. Chaiworapongsa, et al. (2003). "Neonates born to pre-eclamptic mothers have a higher percentage of natural killer cells (CD3-/CD56+16+) in umbilical cord blood than those without pre-eclampsia." J Matern Fetal Neonatal Med **14**(5): 305-312.

Bulmer, J. N., L. Morrison, et al. (1991). "Granulated lymphocytes in human endometrium: histochemical and immunohistochemical studies." Hum Reprod **6**(6): 791-798.

Burdge, G. C., M. A. Hanson, et al. (2007). "Epigenetic regulation of transcription: a mechanism for inducing variations in phenotype (fetal programming) by differences in nutrition during early life?" Br J Nutr **97**(6): 1036-1046.

Burdge, G. C., K. A. Lillycrop, et al. (2009). "Nutrition in early life, and risk of cancer and metabolic disease: alternative endings in an epigenetic tale?" Br J Nutr **101**(5): 619-630.

Burton, G. J., A. W. Woods, et al. (2009). "Rheological and physiological consequences of conversion of the maternal spiral arteries for uteroplacental blood flow during human pregnancy." Placenta **30**(6): 473-482.

Butte, N. F. (2000). "Carbohydrate and lipid metabolism in pregnancy: normal compared with gestational diabetes mellitus." Am J Clin Nutr **71**(5 Suppl): 1256S-1261S.

Campbell, S., H. R. Swann, et al. (1995). "Fertilization and early embryology: CD44 is expressed throughout pre-implantation human embryo development." Hum Reprod **10**(2): 425-430.

Cao, T. C., T. L. Thirkill, et al. (2008). "Trophoblasts and shear stress induce an asymmetric distribution of icam-1 in uterine endothelial cells." Am J Reprod Immunol **59**(2): 167-181.

Cardin, A. D., C. A. Price, et al. (1986). "Structural organization of apolipoprotein B-100 of human plasma low density lipoproteins. Comparison to B-48 of chylomicrons and very low density lipoproteins." J Biol Chem **261**(35): 16744-16748.

Cardozo, E., M. E. Pavone, et al. (2011). "Metabolic syndrome and oocyte quality." Trends Endocrinol Metab **22**(3): 103-109.

Carlino, C., H. Stabile, et al. (2008). "Recruitment of circulating NK cells through decidual tissues: a possible mechanism controlling NK cell accumulation in the uterus during early pregnancy." Blood **111**(6): 3108-3115.

Carp, H. J. A., M. Dirnfeld, et al. (2004). "ART in recurrent miscarriage: preimplantation genetic diagnosis/screening or surrogacy?" Hum Reprod **19**(7): 1502-1505.

Carr, D. B., K. M. Utzschneider, et al. (2006). "Gestational Diabetes Mellitus Increases the Risk of Cardiovascular Disease in Women With a Family History of Type 2 Diabetes." Diabetes Care **29**(9): 2078-2083.

Cartwright, J. E., R. Fraser, et al. (2010). "Remodelling at the maternal–fetal interface: relevance to human pregnancy disorders." Reproduction **140**(6): 803-813.

Catalano, P. M. (2003). "Obesity and pregnancy--the propagation of a viscous cycle?" J Clin Endocrinol Metab **88**(8): 3505-3506.

Catalano, P. M. (2007). "Management of obesity in pregnancy." Obstet Gynaecol **109**(2 Pt 1): 419-433.

Catalano, P. M. (2010). "Obesity, insulin resistance, and pregnancy outcome." Reproduction **140**(3): 365-371.

Catalano, P. M., L. Huston, et al. (1999). "Longitudinal changes in glucose metabolism during pregnancy in obese women with normal glucose tolerance and gestational diabetes mellitus." Am J Obstet Gynaecol **180**(4): 903-916.

Catalano, P. M., L. Presley, et al. (2009). "Fetuses of obese mothers develop insulin resistance in utero." Diabetes Care **32**(6): 1076-1080.

Catalano, P. M., N. M. Roman-Drago, et al. (1998). "Longitudinal changes in body composition and energy balance in lean women with normal and abnormal glucose tolerance during pregnancy." Am J Obstet Gynaecol **179**(1): 156-165.

Catalano, P. M., E. D. Tyzbir, et al. (1991). "Longitudinal changes in insulin release and insulin resistance in nonobese pregnant women." Am J Obstet Gynaecol **165**(6 Pt 1): 1667-1672.

Catalano, P. M., E. D. Tyzbir, et al. (1993). "Carbohydrate metabolism during pregnancy in control subjects and women with gestational diabetes." Am J Physiol **264**(1 Pt 1): E60-67.

Catarino, C., I. Rebelo, et al. (2008). "Fetal lipoprotein changes in pre-eclampsia." Acta Obstet Gynaecol Scand **87**(6): 628-634.

Catarino, C., I. Rebelo, et al. (2008). "Relationship between maternal and cord blood hemostatic disturbances in preeclamptic pregnancies." Thrombosis Research **123**(2): 219-224.

Catarino, C., A. Santos-Silva, et al. (2012). "Inflammatory disturbances in preeclampsia: relationship between maternal and umbilical cord blood." J Pregnancy **2012**: 684384.

Cauffman, G., M. De Rycke, et al. (2009). "Markers that define stemness in ESC are unable to identify the totipotent cells in human preimplantation embryos." Hum Reprod **24**(1): 63-70.

Cavelier, C., L. Rohrer, et al. (2006). "ATP-Binding Cassette Transporter A1 Modulates Apolipoprotein A-I Transcytosis Through Aortic Endothelial Cells." Circ Res **99**(10): 1060-1066.

Cawthorn, W. P. and J. K. Sethi (2008). "TNF-α and adipocyte biology." FEBS Letters **582**(1): 117-131.

Cedergren, M. I. (2004). "Maternal morbid obesity and the risk of adverse pregnancy outcome." Obstet Gynaecol **103**(2): 219-224.

Celermajer, D. S. (1997). "Endothelial dysfunction: does it matter? Is it reversible?" J Am Coll Cardiol **30**(2): 325-333.

Cha, J., X. Sun, et al. (2012). "Mechanisms of implantation: strategies for successful pregnancy." Nat Med **18**(12): 1754-1767.

Chaiworapongsa, T., R. Romero, et al. (2005). "Plasma soluble vascular endothelial growth factor receptor-1 concentration is elevated prior to the clinical diagnosis of pre-eclampsia." J Matern Fetal Neonatal Med **17**(1): 3-18.

Challier, J. C., S. Basu, et al. (2008). "Obesity in Pregnancy Stimulates Macrophage Accumulation and Inflammation in the Placenta." Placenta **29**(3): 274-281.

Chamley, L. W. (1997). "Antiphospholipid antibodies or not? The role of beta 2 glycoprotein 1 in autoantibody-mediated pregnancy loss." J Reprod Immunol **36**(1-2): 123-142.

Chamy, V. M., J. Lepe, et al. (2006). "Oxidative stress is closely related to clinical severity of pre-eclampsia." Biol Res **39**(2): 229-236.

Chandler-Laney, P. C., N. C. Bush, et al. (2011). "Maternal glucose concentration during pregnancy predicts fat and lean mass of prepubertal offspring." Diabetes Care **34**(3): 741-745.

Chang, E. M., J. E. Han, et al. (2013). "Insulin resistance does not affect early embryo development but lowers implantation rate in in vitro maturation-in vitro fertilization-embryo transfer cycle." Clin Endocrinol (Oxf) **79**(1): 93-99.

Chapman, A. B., W. T. Abraham, et al. (1998). "Temporal relationships between hormonal and hemodynamic changes in early human pregnancy." Kidney Int **54**(6): 2056-2063.

Chappell, L. C., P. T. Seed, et al. (1999). "Effect of antioxidants on the occurrence of pre-eclampsia in women at increased risk: a randomised trial." Lancet **354**(9181): 810-816.

Charnock-Jones, D. S., P. Kaufmann, et al. (2004). "Aspects of Human Fetoplacental Vasculogenesis and Angiogenesis. I. Molecular Regulation." Placenta **25**(2-3): 103-113.

Chau, S. E., P. Murthi, et al. (2013). "Control of extravillous trophoblast function by the eotaxins CCL11, CCL24 and CCL26." Hum Reprod **28**(6): 1497-1507.

Chen, A., S. A. Feresu, et al. (2009). "Maternal obesity and the risk of infant death in the United States." Epidemiology **20**(1): 74-81.

Chen, J., B. Tan, et al. (2006). "Secretion of adiponectin by human placenta: differential modulation of adiponectin and its receptors by cytokines." Diabetologia **49**(6): 1292-1302.

Chen, J., X. Zhang, et al. (2013). "Cholesterol efflux is differentially regulated in neurons and astrocytes: implications for brain cholesterol homeostasis." Biochim Biophys Acta **1831**(2): 263-275.

Cheung, N. W. and K. Byth (2003). "Population health significance of gestational diabetes." Diabetes Care **26**(7): 2005-2009.

Chiesa, C., J. F. Osborn, et al. (2008). "Ghrelin, leptin, IGF-1, IGFBP-3, and insulin concentrations at birth: is there a relationship with fetal growth and neonatal anthropometry?" Clin Chem **54**(3): 550-558.

Cindrova-Davies, T. (2009). "Gabor than award lecture 2008: Preeclampsia–From placental oxidative stress to maternal endothelial dysfunction." Placenta **30**: 55-65.

Clark, B. J., J. Wells, et al. (1994). "The purification, cloning, and expression of a novel luteinizing hormone-induced mitochondrial protein in MA-10 mouse Leydig tumor cells. Characterization of the steroidogenic acute regulatory protein (StAR)." J Biol Chem **269**(45): 28314-28322.

Cohen, S., P. E. Bigazzi, et al. (1974). "Similarities of T cell function in cell-mediated immunity and antibody production." Cell Immunol **12**(1): 150-159.

Cooper, D. W., J. A. Hill, et al. (1988). "Genetic control of susceptibility to eclampsia and miscarriage." BJOG **95**(7): 644-653.

Courten, M. d., P. Zimmet, et al. (1997). "Hyperleptinaemia: the Missing Link in the Metabolic Syndrome?" Diabetic Medicine **14**(3): 200-208.

Csapo, A. I. and M. Pulkkinen (1978). "Indispensability of the human corpus luteum in the maintenance of early pregnancy. Luteectomy evidence." Obstet Gynaecol Surv **33**(2): 69-81.

Cummins, C. L., D. H. Volle, et al. (2006). "Liver X receptors regulate adrenal cholesterol balance." J Clin Invest **116**(7): 1902-1912.

Daskalakis, G., S. Marinopoulos, et al. (2008). "Placental pathology in women with gestational diabetes." Acta Obstet Gynaecol Scand **87**(4): 403-407.

Davidge, S. T., A. P. Signorella, et al. (1996). "Distinct factors in plasma of preeclamptic women increase endothelial NO or prostacyclin." Hyperten **28**(5): 758-764.
Davidson, M. B. (1995). "Clinical implications of insulin resistance syndromes." Am J Med **99**(4): 420-426.
De Hertogh, R., I. Vanderheyden, et al. (1991). "Stimulatory and inhibitory effects of glucose and insulin on rat blastocyst development in vitro." Diabetes **40**(5): 641-647.
de Mouzon, J., P. Lancaster, et al. (2009). "World Collaborative Report on Assisted Reproductive Technology, 2002." Hum Reprod **24**(9): 2310-2320.
de Vega, S., T. Iwamoto, et al. (2009). "Fibulins: Multiple roles in matrix structures and tissue functions." Cell Mol Life Sci **66**(11): 1890-1902.
de Waal Malefyt, R., J. Abrams, et al. (1991). "Interleukin 10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes." J Exp Med **174**(5): 1209-1220.
Dekel, N., Y. Gnainsky, et al. (2010). "Inflammation and implantation." Am J Reprod Immunol **63**(1): 17-21.
Dekkers, B. G. J., D. Schaafsma, et al. (2009). "Insulin-Induced Laminin Expression Promotes a Hypercontractile Airway Smooth Muscle Phenotype." Am J Respir Cell Mol Biol **41**(4): 494-504.
Demir, R., U. A. Kayisli, et al. (2004). "Sequential Expression of VEGF and its Receptors in Human Placental Villi During Very Early Pregnancy: Differences Between Placental Vasculogenesis and Angiogenesis." Placenta **25**(6): 560-572.
Desai, M. and M. G. Ross (2011). "Fetal programming of adipose tissue: effects of intrauterine growth restriction and maternal obesity/high-fat diet." Semin Reprod Med **29**(3): 237-245.
Deveci, K., E. Sogut, et al. (2009). "Pregnancy-associated plasma protein-A and C-reactive protein levels in pre-eclamptic and normotensive pregnant women at third trimester." J Obstet Gynaecol Res **35**(1): 94-98.
Diamond, J. (1991). "Pearl Harbor and the Emperor's physiologists." Nat Hist(12): 2-7.
Dimitriadis, E., G. Nie, et al. (2010). "Local regulation of implantation at the human fetal-maternal interface." Int J Dev Biol **54**(2-3): 313-322.
Dokras, A., D. H. Jagasia, et al. (2006). "Obesity and insulin resistance but not hyperandrogenism mediates vascular dysfunction in women with polycystic ovary syndrome." Fertil Steril **86**(6): 1702-1709.
Dominguez, F., M. Yanez-Mo, et al. (2005). "Embryonic implantation and leukocyte transendothelial migration: different processes with similar players?" Faseb J **19**(9): 1056-1060.
Douglas, A. J., L. E. Johnstone, et al. (2007). "Neuroendocrine mechanisms of change in food intake during pregnancy: a potential role for brain oxytocin." Physiol Behav **91**(4): 352-365.
Druckmann, R. and M. A. Druckmann (2005). "Progesterone and the immunology of pregnancy." J Steroid Biochem Mol Biol **97**(5): 389-396.
Dunn, C. L., R. W. Kelly, et al. (2003). "Decidualization of the human endometrial stromal cell: an enigmatic transformation." Reprod Biomed Online **7**(2): 151-161.
Dziadek, M. and R. Timpl (1985). "Expression Of Nidogen And Laminin In Basement-Membranes During Mouse Embryogenesis And In Teratocarcinoma Cells." Dev Biol **111**(2): 372-382.
Economides, D. L., K. H. Nicolaides, et al. (1989). "Plasma amino acids in appropriate- and small-for-gestational-age fetuses." Am J Obstet Gynaecol **161**(5): 1219-1227.
Edwards, P. A., M. A. Kennedy, et al. (2002). "LXRs;: Oxysterol-activated nuclear receptors that regulate genes controlling lipid homeostasis." Vascul Pharmacol **38**(4): 249-256.
Emmer, P. M., W. L. D. M. Nelen, et al. (2000). "Peripheral natural killer cytotoxicity and CD56posCD16pos cells increase during early pregnancy in women with a history of recurrent spontaneous abortion." Hum Reprod **15**(5): 1163-1169.
Enders, A. C. (2007). "Implantation in the macaque: expansion of the implantation site during the first week of implantation." Placenta **28**(8-9): 794-802.

Enders, A. C. (2009). "Reasons for diversity of placental structure." Placenta **30**(S1): 15-18.

Enders, A. C. and A. Lopata (1999). "Implantation in the marmoset monkey: expansion of the early implantation site." Anat Rec **256**(3): 279-299.

Enders, A. C., S. Schlafke, et al. (1986). "Differentiation Of The Embryonic Disk, Amnion, And Yolk-Sac In The Rhesus-Monkey." Am J Anat **177**(2): 161-185.

Endresen, M. J., E. Tosti, et al. (1994). "Effects of Free Fatty-Acids Found Increased in Women Who Develop Preeclampsia on the Ability of Endothelial-Cells to Produce Prostacyclin, Cgmp and Inhibit Platelet-Aggregation." Scand J Clin Lab Invest **54**(7): 549-557.

Enquobahrie, D. A., M. A. Williams, et al. (2004). "Maternal plasma lipid concentrations in early pregnancy and risk of preeclampsia." Am J Hypertens **17**(7): 574-581.

Epstein, L. F. and N. R. Orme-Johnson (1991). "Regulation of steroid hormone biosynthesis. Identification of precursors of a phosphoprotein targeted to the mitochondrion in stimulated rat adrenal cortex cells." J Biol Chem **266**(29): 19739-19745.

Fall, C. H., M. Vijayakumar, et al. (1995). "Weight in infancy and prevalence of coronary heart disease in adult life." BMJ **310**(6971): 17-19.

Fang, C., J. Tang, et al. (2012). "Comparison of IVF outcomes using conventional insemination and ICSI in ovarian cycles in which only one or two oocytes are obtained." J Gynaecol Obstet Biol Reprod (Paris) **41**(7): 650-656.

Farquharson, R. G., E. Jauniaux, et al. (2005). "Updated and revised nomenclature for description of early pregnancy events." Hum Reprod **20**(11): 3008-3011.

Faust, I., P. Johnson, et al. (1978). "Diet-induced adipocyte number increase in adult rats: a new model of obesity." Am J Physiol Gastrointest Liver Physiol **235**(3): G279-G286.

Fay, W. P., N. Garg, et al. (2007). "Vascular functions of the plasminogen activation system." Arterioscler Thromb Vasc Biol **27**(6): 1231-1237.

Ferguson, E. M. and H. J. Leese (2006). "A potential role for triglyceride as an energy source during bovine oocyte maturation and early embryo development." Mol Reprod Dev **73**(9): 1195-1201.

Ferguson, J. J. (1963). "Protein Synthesis and Adrenocorticotropin Responsiveness." J Biol Chem **238**(8): 2754-2759.

Fonager, J., E. M. Pasini, et al. (2012). "Reduced CD36-dependent tissue sequestration of Plasmodium-infected erythrocytes is detrimental to malaria parasite growth in vivo." J Exp Med **209**(1): 93-107.

Fowden, A. L., D. A. Giussani, et al. (2005). "Endocrine and metabolic programming during intrauterine development." Early Hum Dev **81**(9): 723-734.

Fragouli, E., M. Lenzi, et al. (2008). "Comprehensive molecular cytogenetic analysis of the human blastocyst stage." Hum Reprod **23**(11): 2596-2608.

Franks, S. (1995). "Polycystic ovary syndrome." N Engl J Med **333**(13): 853-861.

Freedland, E. S. (2004). "Role of a critical visceral adipose tissue threshold (CVATT) in metabolic syndrome: implications for controlling dietary carbohydrates: a review." Nutr Metab (Lond) **1**(1): 12.

Freeman, D. J. (2010). "Effects of maternal obesity on fetal growth and body composition: implications for programming and future health." Semin Fetal Neonatal Med **15**(2): 113-118.

Freeman, D. J., F. McManus, et al. (2004). "Short- and long-term changes in plasma inflammatory markers associated with preeclampsia." Hyperten **44**(5): 708-714.

Friedman, S. A., E. Schiff, et al. (1995). "Biochemical corroboration of endothelial involvement in severe preeclampsia." Am J Obstet Gynaecol **172**(1, P1): 202-203.

Fuchs, R. and I. Ellinger (2004). "Endocytic and transcytotic processes in villous syncytiotrophoblast: role in nutrient transport to the human fetus." Traffic **5**(10): 725-738.

Fukuda, M. N., T. Sato, et al. (1995). "Trophinin and tastin, a novel cell adhesion molecule complex with potential involvement in embryo implantation." Genes Dev **9**(10): 1199-1210.

Fukuda, M. N. and K. Sugihara (2007). "Signal transduction in human embryo implantation." Cell Cycle **6**(10): 1153-1156.

Furger, C., L. Cronier, et al. (1996). "Human granulosa cells in culture exhibit functional cyclic AMP-regulated gap junctions." Mol Hum Reprod **2**(8): 541-548.

Furuya, M., J. Ishida, et al. (2008). "Pathophysiology of placentation abnormalities in pregnancy-induced hypertension." Vasc Health Risk Manag **4**(6): 1301-1313.

Galán, A., J. E. O'Connor, et al. (2000). "The Human Blastocyst Regulates Endometrial Epithelial Apoptosis in Embryonic Adhesion." Biol Reprod **63**(2): 430-439.

Gardner, H. G. and P. L. Kaye (1991). "Insulin increases cell numbers and morphological development in mouse pre-implantation embryos in vitro." Reprod Fertil Dev **3**(1): 79-91.

Garn, S. M., D. C. Clark, et al. (1976). "Trends in Fatness and the Origins of Obesity." Pediatrics **57**(4): 443-456.

Garren, L. D., R. L. Ney, et al. (1965). "Studies on the role of protein synthesis in the regulation of corticosterone production by adrenocorticotropic hormone in vivo." Proc Natl Acad Sci USA **53**(6): 1443-1450.

Gaspard, U. (2009). "Hyperinsulinaemia, a key factor of the metabolic syndrome in postmenopausal women." Maturitas **62**(4): 362-365.

Gauster, M., G. Desoye, et al. (2012). "The Placenta and Gestational Diabetes Mellitus." Current Diabetes Reports **12**(1): 16-23.

Ge, Y. and M. T. Elghetany (2005). "CD36: a multiligand molecule." Lab Hematol **11**(1): 31-37.

Gelbaya, T. A., L. G. Nardo, et al. (2006). "Cryopreserved-thawed embryo transfer in natural or down-regulated hormonally controlled cycles: a retrospective study." Fertil Steril **85**(3): 603-609.

Gerretsen, G., H. J. Huisjes, et al. (1981). "Morphological changes of the spiral arteries in the placental bed in relation to pre-eclampsia and fetal growth retardation." BJOG **88**(9): 876-881.

Giannini, P., C. Piscitelli, et al. (2004). "Number of embryos transferred and implantation." Ann N Y Acad Sci **1034**: 278-283.

Gilabert, J., A. Estelles, et al. (1994). "Evaluation of plasminogen activators and plasminogen activator inhibitors in plasma and amniotic fluid in pregnancies complicated with intrauterine fetal growth retardation." Gynaecol Obstet Invest **38**(3): 157-162.

Gilbert, J. S., M. J. Ryan, et al. (2008). "Pathophysiology of hypertension during preeclampsia: linking placental ischemia with endothelial dysfunction." Am J Physiol Heart Circ Physiol **294**(2): H541-H550.

Gluckman, P. D. and M. A. Hanson (2004). "Developmental origins of disease paradigm: a mechanistic and evolutionary perspective." Pediatr Res **56**(3): 311-317.

Godfrey, K. M. and D. J. Barker (2001). "Fetal programming and adult health." Public Health Nutr **4**(2B): 611-624.

Goldstein, J. L. and M. S. Brown (1977). "The low-density lipoprotein pathway and its relation to atherosclerosis." Annu Rev Biochem **46**: 897-930.

Goldstein, J. L., S. E. Dana, et al. (1975). "Role of lysosomal acid lipase in the metabolism of plasma low density lipoprotein. Observations in cultured fibroblasts from a patient with cholesteryl ester storage disease." J Biol Chem **250**(21): 8487-8495.

Grümmer, R., H. P. Hohn, et al. (1994). "Adhesion and invasion of three human choriocarcinoma cell lines into human endometrium in a three-dimensional organ culture system." Placenta **15**(4): 411-429.

Grummer, R. R. and D. J. Carroll (1988). "A review of lipoprotein cholesterol metabolism: importance to ovarian function." J Anim Sci **66**(12): 3160-3173.

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

Guibourdenche, J., T. Fournier, et al. (2009). "Development and hormonal functions of the human placenta." Folia Histochem Cytobiol **47**(5): S35-40.

Hales, C. N. and D. J. Barker (1992). "Type 2 (non-insulin-dependent) diabetes mellitus: the thrifty phenotype hypothesis." Diabetologia **35**(7): 595-601.

Haller, H., J. Eichhorn, et al. (1996). "Circulating leukocyte integrin expression in Wegener's granulomatosis." J Am Soc Nephrol **7**(1): 40-48.
Haller, H., E.-M. Ziegler, et al. (1997). "Endothelial Adhesion Molecules and Leukocyte Integrins in Preeclamptic Patients." Hypertension **29**(1): 291-296.
Hamel, M., I. Dufort, et al. (2008). "Identification of differentially expressed markers in human follicular cells associated with competent oocytes." Hum Reprod **23**(5): 1118-1127.
Hannan, N. J., R. L. Jones, et al. (2006). "The Chemokines, CX3CL1, CCL14, and CCL4, Promote Human Trophoblast Migration at the Feto-Maternal Interface." Biol Reprod **74**(5): 896-904.
Hannan, N. J., P. Paiva, et al. (2010). "Models for study of human embryo implantation: choice of cell lines?" Biol Reprod **82**(2): 235-245.
Hannan, N. J., P. Paiva, et al. (2011). "Analysis of Fertility-Related Soluble Mediators in Human Uterine Fluid Identifies VEGF as a Key Regulator of Embryo Implantation." Endocrinology **152**(12): 4948-4956.
Hannan, N. J. and L. A. Salamonsen (2008). "CX3CL1 and CCL14 Regulate Extracellular Matrix and Adhesion Molecules in the Trophoblast: Potential Roles in Human Embryo Implantation." Biol Reprod **79**(1): 58-65.
Hans-Peter, H., L. Manuela, et al. (2000). "Adhesion of trophoblast to uterine epithelium as related to the state of trophoblast differentiation: In vitro studies using cell lines." Mol Reprod Dev **57**(2): 135-145.
Hao, M., S. X. Lin, et al. (2002). "Vesicular and non-vesicular sterol transport in living cells. The endocytic recycling compartment is a major sterol storage organelle." J Biol Chem **277**(1): 609-617.
Harduf, H., S. Goldman, et al. (2007). "Human uterine epithelial RL95-2 and HEC-1A cell-line adhesiveness: the role of plexin B1." Fertil Steril **87**(6): 1419-1427.
Harris, L. K. (2011). "IFPA Gabor Than Award lecture: Transformation of the spiral arteries in human pregnancy: key events in the remodelling timeline." Placenta **32 Suppl 2**: S154-158.
Harvey, M. B. and P. L. Kaye (1990). "Insulin increases the cell number of the inner cell mass and stimulates morphological development of mouse blastocysts in vitro." Development **110**(3): 963-967.
Harvey, M. B., K. J. Leco, et al. (1995). "Roles of growth factors during peri-implantation development." Hum Reprod **10**(3): 712-718.
Hassink, S. G., E. de Lancey, et al. (1997). "Placental leptin: an important new growth factor in intrauterine and neonatal development?" Pediatrics **100**(1): E1-6.
Hauguel-de Mouzon, S. and M. Guerre-Millo (2006). "The Placenta Cytokine Network and Inflammatory Signals." Placenta **27**(8): 794-798.
Hazan, A. D., S. D. Smith, et al. (2010). "Vascular-leukocyte interactions: mechanisms of human decidual spiral artery remodeling in vitro." Am J Pathol **177**(2): 1017-1030.
Healy, D. L., S. Breheny, et al. (2010). "Prevalence and risk factors for obstetric haemorrhage in 6730 singleton births after assisted reproductive technology in Victoria Australia." Hum Reprod **25**(1): 265-274.
Heck, K. E., K. C. Schoendorf, et al. (1997). "Delayed Childbearing by Education Level in the United States, 1969–1994." Matern Child Healt J **1**(2): 81-88.
Heerwagen, M. J., M. R. Miller, et al. (2010). "Maternal obesity and fetal metabolic programming: a fertile epigenetic soil." Am J Physiol Regul Integr Comp Physiol **299**(3): R711-722.
Heilbronn, L., S. R. Smith, et al. (2004). "Failure of fat cell proliferation, mitochondrial function and fat oxidation results in ectopic fat storage, insulin resistance and type II diabetes mellitus." Int J Obes Relat Metab Disord **28**(S4): S12-S21.
Heinemann, L. (2010). "Insulin assay standardization: leading to measures of insulin sensitivity and secretion for practical clinical care: response to Staten et al." Diabetes Care **33**(6): e83; author reply e84.
Hendler, I., R. L. Goldenberg, et al. (2005). "The Preterm Prediction study: Association between maternal body mass index and spontaneous and indicated preterm birth." Am J Obstet Gynaecol **192**(3): 882-886.

Heneweer, C., L. H. Kruse, et al. (2002). "Adhesiveness of human uterine epithelial RL95-2 cells to trophoblast: Rho protein regulation." Mol Hum Reprod **8**(11): 1014-1022.

Herrera, E. (2000). "Metabolic adaptations in pregnancy and their implications for the availability of substrates to the fetus." Eur J Clin Nutr **54**(Suppl 1): S47-S51.

Herrler, A., C. A. Krusche, et al. (1998). "Insulin and insulin-like growth factor-I promote rabbit blastocyst development and prevent apoptosis." Biol Reprod **59**(6): 1302-1310.

Hertig, A. T., J. Rock, et al. (1959). "Thirty-four fertilized human ova, good, bad and indifferent, recovered from 210 women of known fertility; a study of biologic wastage in early human pregnancy." Pediatrics **23**(1 Part 2): 202-211.

Heslehurst, N., J. Rankin, et al. (2010). "A nationally representative study of maternal obesity in England, UK: trends in incidence and demographic inequalities in 619 323 births, 1989-2007." Int J Obes (Lond) **34**(3): 420-428.

Higgins, L., T. A. Mills, et al. (2013). "Maternal obesity and its effect on placental cell turnover." J Matern Fetal Neonatal Med **26**(8): 783-788.

Higgins, M. F., N. M. Russell, et al. (2012). "Clinical and ultrasound features of placental maturation in pre-gestational diabetic pregnancy." Early Hum Dev **88**(10): 817-821.

Hofman, A., V. W. V. Jaddoe, et al. (2004). "Growth, development and health from early fetal life until young adulthood: the Generation R Study." Paediatr Perinat Epidemiol **18**(1): 61-72.

Holm, C. (2003). "Molecular mechanisms regulating hormone-sensitive lipase and lipolysis." Biochem Soc Trans **31**(Pt 6): 1120-1124.

Hoozemans, D. A., R. Schats, et al. (2004). "Human embryo implantation: current knowledge and clinical implications in assisted reproductive technology." Reprod Biomed Online **9**(6): 692-715.

Hordijk, P. L. (2006). "Endothelial signalling events during leukocyte transmigration." FEBS J **273**(19): 4408-4415.

Horne, A. W., E.-N. Lalani, et al. (2006). "The effects of sex steroid hormones and interleukin-1-beta on MUC1 expression in endometrial epithelial cell lines." Reproduction **131**(4): 733-742.

Hossain, N. and M. J. Paidas (2007). "Adverse Pregnancy Outcome, the Uteroplacental Interface, and Preventive Strategies." Semin Perinatol **31**(4): 208-212.

Hotamisligil, G. S. (2006). "Inflammation and metabolic disorders." Nature **444**(7121): 860-867.

Howie, G. J., D. M. Sloboda, et al. (2009). "Maternal nutritional history predicts obesity in adult offspring independent of postnatal diet." J Physiol **587**(Pt 4): 905-915.

Howlader, M. Z. H., S. Parveen, et al. (2009). "Oxidative Stress and Antioxidant Status in Neonates Born to Pre-eclamptic Mother." J Trop Pediatr **55**(6): 363-367.

Hsu, S. M., L. Raine, et al. (1981). "Use of avidin-biotin-peroxidase complex (ABC) in immunoperoxidase techniques: a comparison between ABC and unlabeled antibody (PAP) procedures." J Histochem Cytochem **29**(4): 577-580.

Hu, J., Z. Zhang, et al. (2010). "Cellular cholesterol delivery, intracellular processing and utilization for biosynthesis of steroid hormones." Nutr Metab (Lond) **7**: 47.

Hubbard, S. R., L. Wei, et al. (1994). "Crystal structure of the tyrosine kinase domain of the human insulin receptor." Nature **372**(6508): 746-754.

Hubel, C. A., M. K. McLaughlin, et al. (1996). "Fasting serum triglycerides, FFAs, and malondialdehyde are increased in preeclampsia, are positively correlated, and decrease within 48 hours post partum." Am J Obstet Gynaecol **174**(3): 975-982.

Huda, S. S., L. E. Brodie, et al. (2010). "Obesity in pregnancy: prevalence and metabolic consequences." Semin Fetal Neonatal Med **15**(2): 70-76.

Huda, S. S., N. Sattar, et al. (2009). "Lipoprotein metabolism and vascular complications in pregnancy." Clin Lipidol **4**(1): 91-102.

Hung, T.-H., D. S. Charnock-Jones, et al. (2004). "Secretion of tumor necrosis factor-α from human placental tissues induced by hypoxia-reoxygenation causes endothelial cell activation in vitro: A potential mediator of the inflammatory response in preeclampsia." Am J Pathol **164**(3): 1049-1061.

Huppertz, B. (2008). "The anatomy of the normal placenta." J Clin Pathol **61**(12): 1296-1302.
Huppertz, B., G. Weiss, et al. (2013). "Trophoblast invasion and oxygenation of the placenta: measurements versus presumptions." J Reprod Immunol.
Huxley, R., C. G. Owen, et al. (2004). "Birth weight and subsequent cholesterol levels: exploration of the "fetal origins" hypothesis." Jama **292**(22): 2755-2764.
Inagaki, J., E. Matsuura, et al. (2001). "IgG Anti-laminin-1 Autoantibody and Recurrent Miscarriages." Am J Reprod Immunol **45**(4): 232-238.
Inagaki, J., M. Sugiura-Ogasawara, et al. (2003). "An association of IgG anti-laminin-1 autoantibodies with endometriosis in infertile patients." Hum Reprod **18**(3): 544-549.
Innes, K. E., J. H. Wimsatt, et al. (2001). "Relative glucose tolerance and subsequent development of hypertension in pregnancy." Obstet Gynaecol **97**(6): 905-910.
Innis, S. M. (2007). "Dietary (n-3) fatty acids and brain development." J Nutr **137**(4): 855-859.
Ishimoto, H. and R. B. Jaffe (2011). "Development and function of the human fetal adrenal cortex: a key component in the feto-placental unit." Endocr Rev **32**(3): 317-355.
Jabbour, H. N., K. J. Sales, et al. (2009). "Inflammatory pathways in female reproductive health and disease." Reproduction **138**(6): 903-919.
Jacobs, A. K. and R. H. Eckel (2005). "Evaluating and managing cardiovascular disease in women: understanding a woman's heart." Circulation **111**(4): 383-384.
Jaffe, E. A., R. L. Nachman, et al. (1973). "Culture of human endothelial cells derived from umbilical veins. Identification by morphologic and immunologic criteria." J Clin Invest **52**(11): 2745-2756.
Jaffe, R., E. Jauniaux, et al. (1997). "Maternal circulation in the first-trimester human placenta--Myth or reality?" Am J Obstet Gynaecol **176**(3): 695-705.
James, J. L., A. M. Carter, et al. (2012). "Human placentation from nidation to 5 weeks of gestation. Part I: What do we know about formative placental development following implantation?" Placenta **33**(5): 327-334.
James, P. T., R. Leach, et al. (2001). "The worldwide obesity epidemic." Obes Res **9 Suppl 4**: 228S-233S.
Janny, L. and Y. J. R. Menezo (1996). "Maternal age effect on early human embryonic development and blastocyst formation." Mol Reprod Dev **45**(1): 31-37.
Jansson, N., S. L. Greenwood, et al. (2003). "Leptin stimulates the activity of the system A amino acid transporter in human placental villous fragments." J Clin Endocrinol Metab **88**(3): 1205-1211.
Jaques, A. M., D. J. Amor, et al. (2010). "Adverse obstetric and perinatal outcomes in subfertile women conceiving without assisted reproductive technologies." Fertil Steril **94**(7): 2674-2679.
Jarvie, E., S. Hauguel-de-Mouzon, et al. (2010). "Lipotoxicity in obese pregnancy and its potential role in adverse pregnancy outcome and obesity in the offspring." Clin Sci **119**(3): 123-129.
Jefcoate, C. (2002). "High-flux mitochondrial cholesterol trafficking, a specialized function of the adrenal cortex." J Clin Invest **110**(7): 881-890.
Jefcoate, C. R. (2006). "Liver X receptor opens a new gateway to StAR and to steroid hormones." J Clin Invest **116**(7): 1832-1835.
Jenkins, K. T., L. S. Merkens, et al. (2008). "Enhanced placental cholesterol efflux by fetal HDL in Smith-Lemli-Opitz syndrome." Mol Genet Metab **94**(2): 240-247.
Jha, R. K., S. Titus, et al. (2006). "Profiling of E-cadherin, β-catenin and Ca^{2+} in embryo-uterine interactions at implantation." FEBS Letters **580**(24): 5653-5660.
John, N. J., M. Linke, et al. (1993). "Quantitation of Human Choriocarcinoma Spheroid Attachment to Uterine Epithelial-Cell Monolayers." In Vitro Cell Dev Biol Animal **29A**(6): 461-468.
Johnson, M. R., N. Anim-Nyame, et al. (2002). "Does endothelial cell activation occur with intrauterine growth restriction?" BJOG **109**(7): 836-839.
Jorgensen, M., M. Philips, et al. (1987). "Plasminogen activator inhibitor-1 is the primary inhibitor of tissue-type plasminogen activator in pregnancy plasma." Thromb Haemost **58**(3): 872-878.

Jungheim, E. S., S. E. Lanzendorf, et al. (2009). "Morbid obesity is associated with lower clinical pregnancy rates after in vitro fertilization in women with polycystic ovary syndrome." Fertil Steril **92**(1): 256-261.

Kadyrov, M., J. C. P. Kingdom, et al. (2006). "Divergent trophoblast invasion and apoptosis in placental bed spiral arteries from pregnancies complicated by maternal anemia and early-onset preeclampsia/intrauterine growth restriction." Am J Obstet Gynaecol **194**(2): 557-563.

Kallen, C. B., J. T. Billheimer, et al. (1998). "Steroidogenic acute regulatory protein (StAR) is a sterol transfer protein." J Biol Chem **273**(41): 26285-26288.

Kanagalingam, M. G., N. G. Forouhi, et al. (2005). "Changes in booking body mass index over a decade: retrospective analysis from a Glasgow Maternity Hospital." BJOG **112**(10): 1431-1433.

Kanagalingam, M. G., S. M. Nelson, et al. (2009). "Vascular dysfunction and alteration of novel and classic cardiovascular risk factors in mothers of growth restricted offspring." Atherosclerosis **205**(1): 244-250.

Kane, J. P., D. A. Hardman, et al. (1980). "Heterogeneity of apolipoprotein B: isolation of a new species from human chylomicrons." Proc Natl Acad Sci USA **77**(5): 2465-2469.

Kaser, S., C. F. Ebenbichler, et al. (2001). "Lipoprotein profile and cholesteryl ester transfer protein in neonates." Metabolism **50**(6): 723-728.

Kastl, L., I. Brown, et al. (2010). "Effects of decitabine on the expression of selected endogenous control genes in human breast cancer cells." Mol Cell Probes **24**(2): 87-92.

Katsuhiko, N., A. I. Barbara, et al. (2010). "Secretion of cytokines by villous cytotrophoblast and extravillous trophoblast in the first trimester of human pregnancy." J Reprod Immunol **86**(2): 148-150.

Kaufmann, P., S. Black, et al. (2003). "Endovascular trophoblast invasion: implications for the pathogenesis of intrauterine growth retardation and preeclampsia." Biol Reprod **69**(1): 1-7.

Kayisli, U. A., N. G. Mahutte, et al. (2002). "Uterine chemokines in reproductive physiology and pathology." Am J Reprod Immunol **47**(4): 213-221.

Keskin, M., S. Kurtoglu, et al. (2005). "Homeostasis Model Assessment Is More Reliable Than the Fasting Glucose/Insulin Ratio and Quantitative Insulin Sensitivity Check Index for Assessing Insulin Resistance Among Obese Children and Adolescents." Pediatrics **115**(4): e500-503.

Khan, I. Y., L. Lakasing, et al. (2003). "Fetal programming for adult disease: where next?" J Matern Fetal Neonatal Med **13**(5): 292-299.

Khan, K. M. F., G. W. Laurie, et al. (2002). "Exposure of cryptic domains in the α1-chain of laminin-1 by elastase stimulates macrophages urokinase and marix metalloproteinase-9 expression." J Biol Chem **277**(16): 13778-13786.

Khong, T. Y., F. De Wolf, et al. (1986). "Inadequate maternal vascular response to placentation in pregnancies complicated by pre-eclampsia and by small-for-gestational age infants." BJOG **93**(10): 1049-1059.

Kim, C., K. M. Newton, et al. (2002). "Gestational diabetes and the incidence of type 2 diabetes: a systematic review." Diabetes Care **25**(10): 1862-1868.

Kim, D. H., H. Iijima, et al. (1996). "Human apolipoprotein E receptor 2. A novel lipoprotein receptor of the low density lipoprotein receptor family predominantly expressed in brain." J Biol Chem **271**(14): 8373-8380.

Kim, J.-a., M. Montagnani, et al. (2006). "Reciprocal Relationships Between Insulin Resistance and Endothelial Dysfunction: Molecular and Pathophysiological Mechanisms." Circulation **113**(15): 1888-1904.

King, K., S. Smith, et al. (2010). "Detailed analysis of peripheral blood natural killer (NK) cells in women with recurrent miscarriage." Hum Reprod **25**(1): 52-58.

Knight, M., J. J. Kurinczuk, et al. (2010). "Extreme obesity in pregnancy in the United Kingdom." Obstet Gynaecol **115**(5): 989-997.

Knight, Z. A., B. Gonzalez, et al. (2006). "A pharmacological map of the PI3-K family defines a role for p110alpha in insulin signaling." Cell **125**(4): 733-747.

Knoth, M. (1968). "Ultrastructure of chorionic villi from a four-somite human embryo." J Ultrastruct Res **25**(5-6): 423-440.

Kodaman, P. H. and H. S. Taylor (2004). "Hormonal regulation of implantation." Obstet Gynaecol Clin North Am **31**(4): 745-766, ix.

Komsky-Elbaz, A., A. Raziel, et al. (2013). "Conventional IVF versus ICSI in sibling oocytes from couples with endometriosis and normozoospermic semen." J Assist Reprod Genet **30**(2): 251-257.

Korgun, E. T., C. Celik-Ozenci, et al. (2005). "Do glucose transporters have other roles in addition to placental glucose transport during early pregnancy?" Histochem Cell Biol **123**(6): 621-629.

Kouranova, E. V., B. W. Strassle, et al. (2008). "Hyperpolarization-activated cyclic nucleotide-gated channel mRNA and protein expression in large versus small diameter dorsal root ganglion neurons: Correlation with hyperpolarization-activated current gating." Neuroscience **153**(4): 1008-1019.

Krasnow, J. S., D. J. Tollerud, et al. (1996). "Implantation and early pregnancy: Endometrial Th2 cytokine expression throughout the menstrual cycle and early pregnancy." Hum Reprod **11**(8): 1747-1754.

Kucuk, T. (2008). "Intrauterine insemination: is the timing correct?" J Assist Reprod Genet **25**(8): 427-430.

Kupferminc, M. J., A. M. Peaceman, et al. (1999). "Tumor necrosis factor-alpha is decreased in the umbilical cord plasma of patients with severe preeclampsia." Am J Perinatol **16**(5): 203-208.

Kyriakakou, M., A. Malamitsi-Puchner, et al. (2008). "Leptin and adiponectin concentrations in intrauterine growth restricted and appropriate for gestational age fetuses, neonates, and their mothers." Eur J Endocrinol **158**(3): 343-348.

Kyriakis, J. M. and J. Avruch (2001). "Mammalian Mitogen-Activated Protein Kinase Signal Transduction Pathways Activated by Stress and Inflammation." Physiol Rev **81**(2): 807-869.

La Marca, A., S. M. Nelson, et al. (2011). "Anti-Mullerian hormone-based prediction model for a live birth in assisted reproduction." Reprod Biomed Online **22**(4): 341-349.

Laivuori, H., M. J. Gallaher, et al. (2006). "Relationships between maternal plasma leptin, placental leptin mRNA and protein in normal pregnancy, preeclampsia and IUGR without pre-eclampsia." Mol Hum Reprod **12**(9): 551-556.

Landres, I. V., A. A. Milki, et al. (2010). "Karyotype of miscarriages in relation to maternal weight." Hum Reprod **25**(5): 1123-1126.

Lange, Y., J. Ye, et al. (2004). "How cholesterol homeostasis is regulated by plasma membrane cholesterol in excess of phospholipids." Proc Natl Acad Sci U S A **101**(32): 11664-11667.

Langhofer, M., S. B. Hopkinson, et al. (1993). "The matrix secreted by 804G cells contains laminin-related components that participate in hemidesmosome assembly in vitro." J Cell Sci **105 (Pt 3)**: 753-764.

Lashen, H., K. Fear, et al. (2004). "Obesity is associated with increased risk of first trimester and recurrent miscarriage: matched case-control study." Hum Reprod **19**(7): 1644-1646.

Laskarin, G., V. S. Tokmadzic, et al. (2002). "Progesterone induced blocking factor (PIBF) mediates progesterone induced suppression of decidual lymphocyte cytotoxicity." Am J Reprod Immunol **48**(4): 201-209.

Laskowska, M., B. Leszczynska-Gorzelak, et al. (2006). "Evaluation of maternal and umbilical serum TNFα levels in preeclamptic pregnancies in the intrauterine normal and growth-restricted fetus." J Matern Fetal Neonatal Med **19**(6): 347-351.

Lathi, R. B., A. P. Hess, et al. (2005). "Dose-dependent insulin regulation of insulin-like growth factor binding protein-1 in human endometrial stromal cells is mediated by distinct signaling pathways." J Clin Endocrinol Metab **90**(3): 1599-1606.

Laursen, L. S., M. T. Overgaard, et al. (2001). "Pregnancy-associated plasma protein-A (PAPP-A) cleaves insulin-like growth factor binding protein (IGFBP)-5 independent of IGF: implications for the mechanism of IGFBP-4 proteolysis by PAPP-A." FEBS Letters **504**(1-2): 36-40.

Lawlor, D. A., A. Fraser, et al. (2010). "Association of existing diabetes, gestational diabetes and glycosuria in pregnancy with macrosomia and offspring body mass index, waist and fat mass in later childhood: findings from a prospective pregnancy cohort." Diabetologia **53**(1): 89-97.

Lean, M. E. J., T. S. Han, et al. (1995). "Waist circumference as a measure for indicating need for weight management." BMJ **311**(6998): 158-161.

Lee, K. Y. and F. J. DeMayo (2004). "Animal models of implantation." Reproduction **128**(6): 679-695.

Lee, S. M., R. Romero, et al. (2012). "Systemic inflammatory stimulation by microparticles derived from hypoxic trophoblast as a model for inflammatory response in preeclampsia." Am J Obstet Gynaecol **207**(4): 337.e331-337.e338.

Lefebvre, A. M., M. Laville, et al. (1998). "Depot-specific differences in adipose tissue gene expression in lean and obese subjects." Diabetes **47**(1): 98-103.

Lelbach, A., G. Muzes, et al. (2005). "The insulin-like growth factor system: IGFs, IGF-binding proteins and IGFBP-proteases." Acta Physiol Hung **92**(2): 97-107.

Lessey, B. A., A. J. Castelbaum, et al. (2000). "Use of integrins to date the endometrium." Fertil Steril **73**(4): 779-787.

Levine, R. J., S. E. Maynard, et al. (2004). "Circulating Angiogenic Factors and the Risk of Preeclampsia." N Engl J Med **350**(7): 672-683.

Levran, D., J. Farhi, et al. (2002). "Prospective evaluation of blastocyst stage transfer vs. zygote intrafallopian tube transfer in patients with repeated implantation failure." Fertil Steril **77**(5): 971-977.

Levran, D., S. Mashiach, et al. (1998). "Zygote intrafallopian transfer may improve pregnancy rate in patients with repeated failure of implantation." Fertil Steril **69**(1): 26-30.

Lewis, P. M., M. P. Dunn, et al. (2001). "Cholesterol modification of sonic hedgehog is required for long-range signaling activity and effective modulation of signaling by Ptc1." Cell **105**(5): 599-612.

Li, H. Y., S. P. Chang, et al. (2002). "Establishment of an efficient method to quantify embryo attachment to endometrial epithelial cell monolayers." In Vitro Cell Dev Biol Anim **38**(9): 505-511.

Li, M. and S. J. Huang (2009). "Innate immunity, coagulation and placenta-related adverse pregnancy outcomes." Thromb Res **124**(6): 656-662.

Li, M., N. M. Schwerbrock, et al. (2013). "Fetal-derived adrenomedullin mediates the innate immune milieu of the placenta." J Clin Invest **123**(6): 2408-2420.

Li, Y.-L., F. Ye, et al. (2009). "Identification of suitable reference genes for gene expression studies of human serous ovarian cancer by real-time polymerase chain reaction." Anal Biochem **394**(1): 110-116.

Lieberman, B., S. Troup, et al. (1992). "Cryopreservation of embryos and pregnancy rates after IVF." Lancet **340**(8811): 116-116.

Lin, D., T. Sugawara, et al. (1995). "Role of steroidogenic acute regulatory protein in adrenal and gonadal steroidogenesis." Science **267**(5205): 1828-1831.

Lin, S.-J., S.-K. Shyue, et al. (2007). "Superoxide dismutase and catalase inhibit oxidized low-density lipoprotein-induced human aortic smooth muscle cell proliferation: Role of cell-cycle regulation, mitogen-activated protein kinases, and transcription factors." Atherosclerosis **190**(1): 124-134.

Lintsen, A. M., M. J. Eijkemans, et al. (2007). "Predicting ongoing pregnancy chances after IVF and ICSI: a national prospective study." Hum Reprod **22**(9): 2455-2462.

Lippi, G., A. Albiero, et al. (2007). "Lipid and lipoprotein profile in physiological pregnancy." Clin Lab **53**(3-4): 173-177.

Liu, K. and A. Case (2011). "Advanced reproductive age and fertility." J Obstet Gynaecol Can **33**(11): 1165-1175.

Livingston, J. C., R. Chin, et al. (2000). "Reductions of vascular endothelial growth factor and placental growth factor concentrations in severe preeclampsia." Am J Obstet Gynaecol **183**(6): 1554-1557.

Lockwood, C. J., C. F. Yen, et al. (2008). "Preeclampsia-related inflammatory cytokines regulate interleukin-6 expression in human decidual cells." Am J Pathol **172**(6): 1571-1579.

Lopata, A. (1996). "Blastocyst-endometrial interaction: an appraisal of some old and new ideas." Mol Hum Reprod **2**(7): 519-525.

Lopata, A. and D. L. Hay (1989). "The potential of early human embryos to form blastocysts, hatch from their zona and secrete HCG in culture." Hum Reprod **4**(Suppl 8): 87-94.

LRCP (1975). "Manual of laboratory operations, vol 1.Lipid and lipoprotein analysis. Bethesda, MD: ." DHEW Pub (NIH); 75 Lipids Research Clinic Program.

Lu, G. C., D. J. Rouse, et al. (2001). "The effect of the increasing prevalence of maternal obesity on perinatal morbidity." Am J Obstet Gynaecol **185**(4): 845-849.

Lucas, A. (1991). "Programming by early nutrition in man." Ciba Found Symp **156**: 38-50; discussion 50-35.

Lucas, A. (1994). "Role of nutritional programming in determining adult morbidity." Arch Dis Child **71**(4): 288-290.

Luciani, M. F., F. Denizot, et al. (1994). "Cloning of Two Novel ABC Transporters Mapping on Human Chromosome 9." Genomics **21**(1): 150-159.

Luppi, P. and J. A. DeLoia (2006). "Monocytes of preeclamptic women spontaneously synthesize pro-inflammatory cytokines." Clin Immunol **118**(2-3): 268-275.

Luscinskas, F. W., G. S. Kansas, et al. (1994). "Monocyte rolling, arrest and spreading on IL-4-activated vascular endothelium under flow is mediated via sequential action of L-selectin, β 1-integrins, and β 2-integrins." J Cell Biol **125**(6): 1417-1427.

Lyall, F. (2005). "Priming and remodelling of human placental bed spiral arteries during pregnancy – A Review." Placenta **26, Suppl**(0): S31-S36.

Mackay, V. A., S. S. Huda, et al. (2012). "Preeclampsia Is Associated With Compromised Maternal Synthesis of Long-Chain Polyunsaturated Fatty Acids, Leading to Offspring Deficiency." Hypertension **60**(4): 1078-1085.

Madan, J. C., J. M. Davis, et al. (2009). "Maternal obesity and markers of inflammation in pregnancy." Cytokine **47**(1): 61-64.

Madigan, J., D. J. Freeman, et al. (2010). "Chemokine Scavenger D6 Is Expressed by Trophoblasts and Aids the Survival of Mouse Embryos Transferred into Allogeneic Recipients." J Immunol **184**(6): 3202-3212.

Maglione, D., V. Guerriero, et al. (1991). "Isolation of a Human Placenta cDNA Coding for a Protein Related to the Vascular-Permeability Factor." Proc Natl Acad Sci USA **88**(20): 9267-9271.

Malassine, A., C. Besse, et al. (1987). "Ultrastructural visualization of the internalization of low density lipoprotein by human placental cells." Histochemistry **87**(5): 457-464.

Manna, P. R., J. Cohen-Tannoudji, et al. (2013). "Mechanisms of action of hormone-sensitive lipase in mouse Leydig cells: its role in the regulation of the steroidogenic acute regulatory protein." J Biol Chem **288**(12): 8505-8518.

Manna, P. R., M. T. Dyson, et al. (2009). "Regulation of the steroidogenic acute regulatory protein gene expression: present and future perspectives." Mol Hum Reprod **15**(6): 321-333.

Manten, G. T., M. J. Sikkema, et al. (2007). "Risk factors for cardiovascular disease in women with a history of pregnancy complicated by preeclampsia or intrauterine growth restriction." Hypertens Pregnancy **26**(1): 39-50.

Martin-Hidalgo, A., C. Holm, et al. (1994). "Lipoprotein lipase and hormone-sensitive lipase activity and mRNA in rat adipose tissue during pregnancy." Am J Physiol **266**(6 Pt 1): E930-935.

Martin, U., C. Davies, et al. (1999). "Is normal pregnancy atherogenic?" Clin Sci **96**(4): 421-425.

Maruyama, Y. (1963). "The human endothelial cell in tissue culture." Z Zellforsch Mikrosk Anat **60**: 69-79.

Marzi, M., A. Vigano, et al. (1996). "Characterization of type 1 and type 2 cytokine production profile in physiologic and pathologic human pregnancy." Clin Exp Immunol **106**(1): 127-133.

Matoba, N., Y. Yu, et al. (2009). "Differential Patterns of 27 Cord Blood Immune Biomarkers Across Gestational Age." Pediatrics **123**(5): 1320-1328.

Mattos, R., C. Staples, et al. (2000). "Effects of dietary fatty acids on reproduction in ruminants." Rev Reprod **5**(1): 38-45.

Maynard, S. E., J.-Y. Min, et al. (2003). "Excess placental soluble fms-like tyrosine kinase 1 (sFlt1) may contribute to endothelial dysfunction, hypertension, and proteinuria in preeclampsia." J Clin Invest **111**(5): 649-658.

Mazurkiewicz, J. C., G. F. Watts, et al. (1994). "Serum lipids, lipoproteins and apolipoproteins in pregnant non-diabetic patients." J Clin Pathol **47**(8): 728-731.

McCurdy, C. E., J. M. Bishop, et al. (2009). "Maternal high-fat diet triggers lipotoxicity in the fetal livers of nonhuman primates." J Clin Invest **119**(2): 323-335.

McDermott-Roe, C., J. Martin, et al. (2008). "CD36 N-terminal cytoplasmic domain is not required for the internalization of oxidized low-density lipoprotein." Biosci Rep **28**(3): 145-151.

McEvoy, L. M., H. L. Sun, et al. (1997). "Novel vascular molecule involved in monocyte adhesion to aortic endothelium in models of atherogenesis." J Exp Med **185**(12): 2069-2077.

McIntyre, H. D., A. M. Chang, et al. (2010). "Hormonal and Metabolic Factors Associated With Variations in Insulin Sensitivity in Human Pregnancy." Diabetes Care **33**(2): 356-360.

Mcmillen, I. C. and J. S. Robinson (2005). "Developmental Origins of the Metabolic Syndrome: Prediction, Plasticity, and Programming." Physiol Rev **85**(2): 571-633.

Meseguer, M., J. D. Aplin, et al. (2001). "Human Endometrial Mucin MUC1 Is Up-Regulated by Progesterone and Down-Regulated In Vitro by the Human Blastocyst." Biol Reprod **64**(2): 590-601.

Mestan, K., Y. Yu, et al. (2009). "Cord blood biomarkers of the fetal inflammatory response." J Matern Fetal Neonatal Med **22**(5): 379-387.

Mettler, L., H. W. Michelmann, et al. (1984). "In vitro fertilization and embryo replacement at the Department of Obstetrics and Gynecology, University of Kiel, F.R.G." J In Vitro Fert Embryo Transf **1**(4): 250-262.

Metwally, M., S. H. Saravelos, et al. (2010). "Body mass index and risk of miscarriage in women with recurrent miscarriage." Fertil Steril **94**(1): 290-295.

Metzger, B. E., T. A. Buchanan, et al. (2007). "Summary and Recommendations of the Fifth International Workshop-Conference on Gestational Diabetes Mellitus." Diabetes Care **30**(Supplement 2): S251-S260.

Meyer, B. J., F. M. Stewart, et al. (2013). "Maternal obesity is associated with the formation of small dense LDL and hypoadiponectinemia in the third trimester." J Clin Endocrinol Metab **98**(2): 643-652.

Miller, W. L. (2007). "StAR Search—What We Know about How the Steroidogenic Acute Regulatory Protein Mediates Mitochondrial Cholesterol Import." Mol Endocrinol **21**(3): 589-601.

Miller, W. L. and R. J. Auchus (2011). "The molecular biology, biochemistry, and physiology of human steroidogenesis and Its disorders." Endocr Rev **32**(1): 81-151.

Miller, W. L. and H. S. Bose (2011). "Early steps in steroidogenesis: intracellular cholesterol trafficking." J Lipid Res **52**(12): 2111-2135.

Miller, Y. I., S. H. Choi, et al. (2010). "Lipoprotein modification and macrophage uptake: role of pathologic cholesterol transport in atherogenesis." Subcell Biochem **51**: 229-251.

Mills, J. L., J. L. Simpson, et al. (1988). "Incidence of spontaneous abortion among normal women and insulin-dependent diabetic women whose pregnancies were identified within 21 days of conception." N Engl J Med **319**(25): 1617-1623.

Mills, J. L., J. Troendle, et al. (2010). "Maternal obesity and congenital heart defects: a population-based study." Am J Clin Nutr **91**(6): 1543-1549.

Miner, J. H. (2008). "Laminins and their roles in mammals." Microsc Res Tech **71**(5): 349-356.

Miner, J. H. and P. D. Yurchenco (2004). "Laminin functions in tissue morphogenesis." Annu Rev Cell Dev Biol **20**: 255-284.

Mittal, P., J. Espinoza, et al. (2007). "Placental growth hormone is increased in the maternal and fetal serum of patients with preeclampsia." J Matern Fetal Neonatal Med **20**(9): 651-659.

Mohangoo, A. D., B. Blondel, et al. (2013). "International comparisons of fetal and neonatal mortality rates in high-income countries: should exclusion thresholds be based on birth weight or gestational age?" PLoS One **8**(5): e64869.

Mold, J. E., J. Michaelsson, et al. (2008). "Maternal alloantigens promote the development of tolerogenic fetal regulatory T cells in utero." Science **322**(5907): 1562-1565.

Mor, G. (2008). "Inflammation and Pregnancy: the role of toll-like receptors in trophoblast-immune interaction." Ann N Y Acad Sci **1127**(1): 121-128.

Mor, G. and I. Cardenas (2010). "The immune system in pregnancy: a unique complexity." Am J Reprod Immunol **63**(6): 425-433.

Mor, G., I. Cardenas, et al. (2011). "Inflammation and pregnancy: the role of the immune system at the implantation site." Ann N Y Acad Sci **1221**(1): 80-87.

Morris, R. and V. Carstairs (1991). "Which deprivation? A comparison of selected deprivation indexes." J Public Health Med **13**(4): 318-326.

Mostello, D., J. Jen Chang, et al. (2010). "Recurrent preeclampsia: the effect of weight change between pregnancies." Obstet Gynaecol **116**(3): 667-672.

Murakami, M. S. and O. M. Rosen (1991). "The role of insulin receptor autophosphorylation in signal transduction." J Biol Chem **266**(33): 22653-22660.

Murphy, A. J., K. J. Woollard, et al. (2008). "High-density lipoprotein reduces the human monocyte inflammatory response." Arterioscler Thromb Vasc Biol **28**(11): 2071-2077.

Murphy, V. E., R. Smith, et al. (2006). "Endocrine regulation of human fetal growth: the role of the mother, placenta, and fetus." Endocr Rev **27**(2): 141-169.

Murthi, P., E. Fitzpatrick, et al. (2008). "GAPDH, 18S rRNA and YWHAZ are Suitable Endogenous Reference Genes for Relative Gene Expression Studies in Placental Tissues from Human Idiopathic Fetal Growth Restriction." Placenta **29**(9): 798-801.

Myers, M. G., Jr., L. M. Wang, et al. (1994). "Role of IRS-1-GRB-2 complexes in insulin signaling." Mol Cell Biol **14**(6): 3577-3587.

Nagamatsu, T., T. Fujii, et al. (2004). "Cytotrophoblasts up-regulate soluble fms-like tyrosine kinase-1 expression under reduced oxygen: an implication for the placental vascular development and the pathophysiology of preeclampsia." Endocrinology **145**(11): 4838-4845.

Nagaoka, K., H. Nojima, et al. (2003). "Regulation of Blastocyst Migration, Apposition, and Initial Adhesion by a Chemokine, Interferon γ-inducible Protein 10 kDa (IP-10), during Early Gestation." J Biol Chem **278**(31): 29048-29056.

Nagasaka, H., H. Chiba, et al. (2002). "Unique character and metabolism of high density lipoprotein (HDL) in fetus." Atherosclerosis **161**(1): 215-223.

Napoli, C., F. P. D'Armiento, et al. (1997). "Fatty streak formation occurs in human fetal aortas and is greatly enhanced by maternal hypercholesterolemia. Intimal accumulation of low density lipoprotein and its oxidation precede monocyte recruitment into early atherosclerotic lesions." J Clin Invest **100**(11): 2680-2690.

Naruse, K., M. Yamasaki, et al. (2005). "Peripheral blood concentrations of adiponectin, an adipocyte-specific plasma protein, in normal pregnancy and preeclampsia." J Reprod Immunol **65**(1): 65-75.

Nassir, F., B. Wilson, et al. (2007). "CD36 is important for fatty acid and cholesterol uptake by the proximal but not distal intestine." J Biol Chem **282**(27): 19493-19501.

Negi, R., D. Pande, et al. (2012). "Trace elements and antioxidant enzymes associated with oxidative stress in the pre-eclamptic/eclamptic mothers during fetal circulation." Clin Nutr **31**(6): 946-950.

Nelson, S. M., P. M. Coan, et al. (2009). "Placental structure in type 1 diabetes: relation to fetal insulin, leptin, and IGF-I." Diabetes **58**(11): 2634-2641.

Nelson, S. M., R. W. Yates, et al. (2007). "Serum anti-Müllerian hormone and FSH: prediction of live birth and extremes of response in stimulated cycles—implications for individualization of therapy." Hum Reprod **22**(9): 2414-2421.

Newbern, D. and M. Freemark (2011). "Placental hormones and the control of maternal metabolism and fetal growth." Curr Opin Endocrinol Diabetes Obes **18**(6): 409-416.

Nguyen, R. H. and A. J. Wilcox (2005). "Terms in reproductive and perinatal epidemiology: 2. Perinatal terms." J Epidemiol Community Health **59**(12): 1019-1021.

Nissinen, M., R. Vuolteenaho, et al. (1991). "Primary Structure Of The Human Laminin A-Chain - Limited Expression In Human Tissues." Biochem J **276**: 369-379.

Nomizu, M., W. H. Kim, et al. (1995). "Identification of cell binding sites in the laminin alpha 1 chain carboxyl-terminal globular domain by systematic screening of synthetic peptides." J Biol Chem **270**(35): 20583-20590.

Norman, R. J., D. Dewailly, et al. (2007). "Polycystic ovary syndrome." Lancet **370**(9588): 685-697.

Norwitz, E. R., D. J. Schust, et al. (2001). "Implantation and the survival of early pregnancy." N Engl J Med **345**(19): 1400-1408.

Nyboe Andersen, A., V. Goossens, et al. (2009). "Assisted reproductive technology and intrauterine inseminations in Europe, 2005: results generated from European registers by ESHRE: ESHRE. The European IVF Monitoring Programme (EIM), for the ESHRE." Hum Reprod **24**(6): 1267-1287.

Nygren, K. G., E. Sullivan, et al. (2011). "International Committee for Monitoring Assisted Reproductive Technology (ICMART) world report: assisted reproductive technology 2003." Fertil Steril **95**(7): 2209-2222 (e2201-2217).

Odegard, R. A., L. J. Vatten, et al. (2002). "Umbilical cord plasma leptin is increased in preeclampsia." Am J Obstet Gynaecol **186**(3): 427-432.

Ogden, C. L., S. Z. Yanovski, et al. (2007). "The epidemiology of obesity." Gastroenterology **132**(6): 2087-2102.

Ogland, B., P. R. Romundstad, et al. (2010). "Preeclampsia and adiponectin in cord blood." Horm Res Paediatr **74**(2): 92-97.

Oitzl, M. S., D. L. Champagne, et al. (2010). "Brain development under stress: Hypotheses of glucocorticoid actions revisited." Neurosci Biobehav Rev **34**(6): 853-866.

Olofsson, S. O., P. Elias, et al. (1983). "Human liver RNA-programmed in vitro synthesis of a polypeptide related to human apolipoprotein B." FEBS Lett **156**(1): 63-66.

Ophir, E., G. Dourleshter, et al. (2006). "Newborns of pre-eclamptic women: a biochemical difference present in utero." Acta Obstet Gynaecol Scand **85**(10): 1172-1178.

Ordovas, J. M., M. Pocovi, et al. (1984). "Plasma lipids and cholesterol esterification rate during pregnancy." Obstet Gynaecol **63**(1): 20-25.

Orsi, N. M. and R. M. Tribe (2008). "Cytokine networks and the regulation of uterine function in pregnancy and parturition." J Neuroendocrinol **20**(4): 462-469.

Osmond, C. and D. J. Barker (2000). "Fetal, infant, and childhood growth are predictors of coronary heart disease, diabetes, and hypertension in adult men and women." Environ Health Perspect **108 Suppl 3**: 545-553.

Ouchi, N., J. L. Parker, et al. (2011). "Adipokines in inflammation and metabolic disease." Nat Rev Immunol **11**(2): 85-97.

Ounsted, M., V. Moar, et al. (1981). "Perinatal morbidity and mortality in small-for-dates babies: The relative importance of some maternal factors." Early Hum Dev **5**(4): 367-375.

Palinski, W. and C. Napoli (2002). "The fetal origins of atherosclerosis: maternal hypercholesterolemia, and cholesterol-lowering or antioxidant treatment during pregnancy influence in utero programming and postnatal susceptibility to atherogenesis." Faseb J **16**(11): 1348-1360.

Paria, B. C., J. Reese, et al. (2002). "Deciphering the cross-talk of implantation: advances and challenges." Science **296**(5576): 2185-2188.

Park, S. H., S. J. Choi, et al. (2009). "Waist circumference and waist-to-height ratio as predictors of CVD risk in Korean adults." Circ J **73**(9): 1643-1650.

Parsons, J. T., A. R. Horwitz, et al. (2010). "Cell adhesion: integrating cytoskeletal dynamics and cellular tension." Nat Rev Mol Cell Biol **11**(9): 633-643.

Patarroyo, M., K. Tryggvason, et al. (2002). "Laminin isoforms in tumor invasion, angiogenesis and metastasis." Semin Cancer Biol **12**(3): 197-207.

Pattillo RA, Ruckert A, et al. (1971). "The Jar cell line – continuous human multihormone production and controls." In Vitro **6**: 398-399.

Pecks, U., R. Caspers, et al. (2012). "The evaluation of the oxidative state of low-density lipoproteins in intrauterine growth restriction and preeclampsia." Hypertens Pregnancy **31**(1): 156-165.

Peet, D. J., S. D. Turley, et al. (1998). "Cholesterol and Bile Acid Metabolism Are Impaired in Mice Lacking the Nuclear Oxysterol Receptor LXR±." Cell **93**(5): 693-704.

Penney, G. C., G. Mair, et al. (2003). "Outcomes of pregnancies in women with type 1 diabetes in Scotland: a national population-based study." BJOG **110**(3): 315-318.

Pepe, G. J. and E. D. Albrecht (1995). "Actions of placental and fetal adrenal steroid hormones in primate pregnancy." Endocr Rev **16**(5): 608-648.

Pepys, M. B. and G. M. Hirschfield (2003). "C-reactive protein: a critical update." J Clin Invest **111**(12): 1805-1812.

Perticone, F., R. Ceravolo, et al. (2001). "Obesity and body fat distribution induce endothelial dysfunction by oxidative stress: protective effect of vitamin C." Diabetes **50**(1): 159-165.

Phillips, D. I. and A. Jones (2006). "Fetal programming of autonomic and HPA function: do people who were small babies have enhanced stress responses?" J Physiol **572**(Pt 1): 45-50.

Picciano, M. F. (2003). "Pregnancy and lactation: physiological adjustments, nutritional requirements and the role of dietary supplements." J Nutr **133**(6): 1997S-2002S.

Piccinni, M.-P., L. Beloni, et al. (1998). "Defective production of both leukemia inhibitory factor and type 2 T-helper cytokines by decidual T cells in unexplained recurrent abortions." Nat Med **4**(9): 1020-1024.

Pijnenborg, R., J. M. Bland, et al. (1983). "Uteroplacental arterial changes related to interstitial trophoblast migration in early human pregnancy." Placenta **4**(4): 397-413.

Pijnenborg, R., G. Dixon, et al. (1980). "Trophoblastic invasion of human decidua from 8 to 18 weeks of pregnancy." Placenta **1**(1): 3-19.

Pijnenborg, R., L. Vercruysse, et al. (2006). "The uterine spiral arteries in human pregnancy: facts and controversies." Placenta **27**(9-10): 939-958.

Pitiphat, W., M. W. Gillman, et al. (2005). "Plasma C-reactive protein in early pregnancy and preterm delivery." Am J Epidemiol **162**(11): 1108-1113.

Plosch, T., E. M. E. van Straten, et al. (2007). "Cholesterol Transport by the Placenta: Placental Liver X Receptor Activity as a Modulator of Fetal Cholesterol Metabolism?" Placenta **28**(7): 604-610.

Polonsky, K. S., B. D. Given, et al. (1988). "Twenty-four-hour profiles and pulsatile patterns of insulin secretion in normal and obese subjects." J Clin Invest **81**(2): 442-448.

Ponting, C. P. and L. Aravind (1999). "START: a lipid-binding domain in StAR, HD-ZIP and signalling proteins." Trends Biochem Sci **24**(4): 130-132.

Poranen, A. K., U. Ekblad, et al. (1996). "Lipid peroxidation and antioxidants in normal and pre-eclamptic pregnancies." Placenta **17**(7): 401-405.

Pötgens, A. J. G., U. Schmitz, et al. (2002). "Mechanisms of Syncytial Fusion: A Review." Placenta **23**(Supplement 1): S107-S113.

Pronk, G. J., J. McGlade, et al. (1993). "Insulin-induced phosphorylation of the 46- and 52-kDa Shc proteins." J Biol Chem **268**(8): 5748-5753.

Puscheck, E. E., E. Pergament, et al. (1998). "Insulin receptor substrate-1 is expressed at high levels in all cells of the peri-implantation mouse embryo." Mol Reprod Dev **49**(4): 386-393.

Quenby, S., M. Anim-Somuah, et al. (2007). "Different types of recurrent miscarriage are associated with varying patterns of adhesion molecule expression in endometrium." Reprod Biomed Online **14**(2): 224-234.

Rac, M. E., K. Safranow, et al. (2007). "Molecular basis of human CD36 gene mutations." Mol Med **13**(5-6): 288-296.

Radaelli, T., J. Uvena-Celebrezze, et al. (2006). "Maternal Interleukin-6: Marker of Fetal Growth and Adiposity." J Soc Gynaecol Investig **13**(1): 53-57.

Raghupathy, R. (1997). "Th 1-type immunity is incompatible with successful pregnancy." Immunol Today **18**(10): 478-482.

Raghupathy, R. (2001). "Pregnancy: success and failure within the Th1/Th2/Th3 paradigm." Semin Immunol **13**(4): 219-227.

Ragoobir, J., D. R. Abayasekara, et al. (2002). "Stimulation of progesterone production in human granulosa-lutein cells by lipoproteins: evidence for cholesterol-independent actions of high-density lipoproteins." J Endocrinol **173**(1): 103-111.

Ramos, M. P., M. D. Crespo-Solans, et al. (2003). "Fat accumulation in the rat during early pregnancy is modulated by enhanced insulin responsiveness." Am J Physiol Endocrinol Metab **285**(2): E318-328.

Ramsay, J. E., W. R. Ferrell, et al. (2002). "Maternal obesity is associated with dysregulation of metabolic, vascular, and inflammatory pathways." J Clin Endocrinol Metab **87**(9): 4231-4237.

Ramsay, J. E., W. R. Ferrell, et al. (2004). "Divergent metabolic and vascular phenotypes in pre-eclampsia and intrauterine growth restriction: relevance of adiposity." J Hypertens **22**(11): 2177-2183.

Ramsay, J. E., I. Greer, et al. (2006). "ABC of obesity. Obesity and reproduction." BMJ **333**(7579): 1159-1162.

Rasmussen, Irgens, et al. (1999). "A history of placental dysfunction and risk of placental abruption." Paediatr Perinat Epidemiol **13**(1): 9-21.

Ray, J. G., M. J. Vermeulen, et al. (2005). "Cardiovascular health after maternal placental syndromes (CHAMPS): population-based retrospective cohort study." Lancet **366**(9499): 1797-1803.

Rebuffe-Scrive, M., L. Enk, et al. (1985). "Fat cell metabolism in different regions in women. Effect of menstrual cycle, pregnancy, and lactation." J Clin Invest **75**(6): 1973-1976.

Red-Horse, K., P. M. Drake, et al. (2001). "Chemokine ligand and receptor expression in the pregnant uterus: reciprocal patterns in complementary cell subsets suggest functional roles." Am J Pathol **159**(6): 2199-2213.

Red-Horse, K., Y. Zhou, et al. (2004). "Trophoblast differentiation during embryo implantation and formation of the maternal-fetal interface." J Clin Invest **114**(6): 744-754.

Redman, C. W. and I. L. Sargent (2003). "Preeclampsia, the placenta and the maternal systemic inflammatory response - a review." Placenta **24 Suppl A**: S21-27.

Redman, C. W. G., G. P. Sacks, et al. (1999). "Preeclampsia: An excessive maternal inflammatory response to pregnancy." Am J Obstet Gynaecol **180**(2): 499-506.

Redman, C. W. G. and I. L. Sargent (2000). "Placental Debris, Oxidative Stress and Pre-eclampsia." Placenta **21**(7): 597-602.

Redman, C. W. G. and I. L. Sargent (2004). "Preeclampsia and the systemic inflammatory response." Semin Nephrol **24**(6): 565-570.

Reister, F., H.-G. Frank, et al. (2001). "Macrophage-Induced Apoptosis Limits Endovascular Trophoblast Invasion in the Uterine Wall of Preeclamptic Women." Lab Invest **81**(8): 1143-1152.

Reith, A., N. A. Booth, et al. (1993). "Plasminogen activator inhibitors (PAI-1 and PAI-2) in normal pregnancies, pre-eclampsia and hydatidiform mole." BJOG **100**(4): 370-374.

Remaley, A. T. (2007). "Old drug, new tricks: the unexpected effect of doxazosin on high-density lipoprotein." Circ Res **101**(2): 116-118.

Reuvekamp, A., F. V. Velsing-Aarts, et al. (1999). "Selective deficit of angiogenic growth factors characterises pregnancies complicated by pre-eclampsia." BJOG **106**(10): 1019-1022.

Rhee, Y. H., M. Choi, et al. (2013). "Insulin concentration is critical in culturing human neural stem cells and neurons." Cell Death Dis **4**: e766.

Riskin-Mashiah, S., G. Younes, et al. (2009). "First-Trimester Fasting Hyperglycemia and Adverse Pregnancy Outcomes." Diabetes Care **32**(9): 1639-1643.

Roberts, J. M., R. N. Taylor, et al. (1989). "Preeclampsia - an Endothelial-Cell Disorder." Am J Obstet Gynaecol **161**(5): 1200-1204.

Rodie, V. A., M. J. Caslake, et al. (2004). "Fetal cord plasma lipoprotein status in uncomplicated human pregnancies and in pregnancies complicated by pre-eclampsia and intrauterine growth restriction." Atherosclerosis **176**(1): 181-187.

Rohde, L. H. and D. D. Carson (1993). "Heparin-like glycosaminoglycans participate in binding of a human trophoblastic cell line (JAR) to a human uterine epithelial cell line (RL95)." J Cell Physiol **155**(1): 185-196.

Roman, A. S., A. Rebarber, et al. (2011). "The effect of maternal obesity on pregnancy outcomes in women with gestational diabetes." J Matern Fetal Neonatal Med **24**(5): 723-727.

Romundstad, L. B., P. R. Romundstad, et al. (2008). "Effects of technology or maternal factors on perinatal outcome after assisted fertilisation: a population-based cohort study." Lancet **372**(9640): 737-743.

Roos, S., Y. Kanai, et al. (2009). "Regulation of placental amino acid transporter activity by mammalian target of rapamycin." Am J Physiol Cell Physiol **296**(1): C142-150.

Rosen, M. P., S. Shen, et al. (2010). "Fertilization rate is an independent predictor of implantation rate." Fertil Steril **94**(4): 1328-1333.

Royle, C., S. Lim, et al. (2009). "Effect of hypoxia and exogenous IL-10 on the pro-inflammatory cytokine TNF-[alpha] and the anti-angiogenic molecule soluble Flt-1 in placental villous explants." Cytokine **47**(1): 56-60.

Rubio, C., C. Simón, et al. (2003). "Chromosomal abnormalities and embryo development in recurrent miscarriage couples." Hum Reprod **18**(1): 182-188.

Russell, M. and M. Lough (2010). "Deprived areas: deprived of training?" Br J Gen Pract **60**(580): 846-848.

Rust, S., M. Walter, et al. (1998). "Assignment of Tangier disease to chromosome 9q31 by a graphical linkage exclusion strategy." Nat Genet **20**(1): 96-98.

Rutanen, E. M. (2000). "Insulin-like growth factors in obstetrics." Curr Opin Obstet Gynaecol **12**(3): 163-168.

Ryan, E. A. and L. Enns (1988). "Role of gestational hormones in the induction of insulin resistance." J Clin Endocrinol Metab **67**(2): 341-347.

Sabatier, F., F. Bretelle, et al. (2000). "Neutrophil activation in preeclampsia and isolated intrauterine growth restriction." Am J Obstet Gynaecol **183**(6): 1558-1563.

Sacks, G. P., L. Seyani, et al. (2004). "Maternal C-reactive protein levels are raised at 4 weeks gestation." Hum Reprod **19**(4): 1025-1030.

Sacks, G. P., K. Studena, et al. (1998). "Normal pregnancy and preeclampsia both produce inflammatory changes in peripheral blood leukocytes akin to those of sepsis." Am J Obstet Gynaecol **179**(1): 80-86.

Saini, H., B. L. Puppala, et al. (2004). "Upregulation of Neutrophil Surface Adhesion Molecules in Infants of Pre-eclamptic Women." J Perinatol **24**(4): 208-212.

Salamonsen, L. A., N. J. Hannan, et al. (2007). "Cytokines and chemokines during human embryo implantation: roles in implantation and early placentation." Semin Reprod Med **25**(6): 437-444.

Salatbaroux, J., D. Cornet, et al. (1988). "Pregnancies After Replacement Of Frozen-Thawed Embryos In A Donation Program." Fertil Steril **49**(5): 817-821.

Salim, R., L. Regan, et al. (2003). "A comparative study of the morphology of congenital uterine anomalies in women with and without a history of recurrent first trimester miscarriage." Hum Reprod **18**(1): 162-166.

Saltiel, A. R. and C. R. Kahn (2001). "Insulin signalling and the regulation of glucose and lipid metabolism." Nature **414**(6865): 799-806.

Sankaran, S. and P. M. Kyle (2009). "Aetiology and pathogenesis of IUGR." Best Pract Res Clin Obstet Gynaecol **23**(6): 765-777.

Santana, C., M. C. Guindeo, et al. (2001). "Cord blood levels of cytokines as predictors of early neonatal sepsis." Acta Pædiatrica **90**(10): 1176-1181.

Saravelos, S. H., K. A. Cocksedge, et al. (2008). "Prevalence and diagnosis of congenital uterine anomalies in women with reproductive failure: a critical appraisal." Hum Reprod Update **14**(5): 415-429.

Sasaki, M., H. K. Kleinman, et al. (1988). "Laminin, a multidomain protein. The A chain has a unique globular domain and homology with the basement membrane proteoglycan and the laminin B chains." J Biol Chem **263**(32): 16536-16544.

Sato, M., Y. Nakamura, et al. (2002). "Immunolocalization of glucose transporter 1 and 3 in the placenta: application to cytodiagnosis of Papanicolaou smear." Diagn Cytopathol **26**(6): 373-379.

Sattar, N., A. Bendomir, et al. (1997). "Lipoprotein subfraction concentrations in preeclampsia: Pathogenic parallels to atherosclerosis." Obstet Gynaecol **89**(3): 403-408.

Sattar, N. and I. A. Greer (2002). "Pregnancy complications and maternal cardiovascular risk: opportunities for intervention and screening?" BMJ **325**(7356): 157-160.

Sattar, N., I. A. Greer, et al. (1999). "Lipid and lipoprotein concentrations in pregnancies complicated by intrauterine growth restriction." J Clin Endocrinol Metab **84**(1): 128-130.

Savitz, D. A., I. Hertz-Picciotto, et al. (2002). "Epidemiologic measures of the course and outcome of pregnancy." Epidemiol Rev **24**(2): 91-101.

Schaefer, S. E., E. Erber, et al. (2011). "Sources of food affect dietary adequacy of Inuit women of childbearing age in Arctic Canada." J Health Popul Nutr **29**(5): 454-464.

Schiessl, B., B. A. Innes, et al. (2009). "Localization of Angiogenic Growth Factors and Their Receptors in the Human Placental Bed Throughout Normal Human Pregnancy." Placenta **30**(1): 79-87.

Schiff, E., S. A. Friedman, et al. (1994). "Tumor necrosis factor-alpha in pregnancies associated with preeclampsia or small-for-gestational-age newborns." Am J Obstet Gynaecol **170**(5 Pt 1): 1224-1229.

Schmidt, M. I., B. B. Duncan, et al. (1999). "Markers of inflammation and prediction of diabetes mellitus in adults (Atherosclerosis Risk in Communities study): a cohort study." Lancet **353**(9165): 1649-1652.

Schmittgen, T. D. and K. J. Livak (2008). "Analyzing real-time PCR data by the comparative CT method." Nat Protocols **3**(6): 1101-1108.

Screaton, G. R., M. V. Bell, et al. (1992). "Genomic structure of DNA encoding the lymphocyte homing receptor CD44 reveals at least 12 alternatively spliced exons." Proc Natl Acad Sci USA **89**(24): 12160-12164.

Sebire, N. J., M. Jolly, et al. (2001). "Maternal obesity and pregnancy outcome: a study of 287,213 pregnancies in London." Int J Obes Relat Metab Disord **25**(8): 1175-1182.

Seki, H., K. Matuoka, et al. (2007). "TNF-alpha from monocyte of patients with pre-eclampsia-induced apoptosis in human trophoblast cell line." J Obstet Gynaecol Res **33**(4): 408-416.

Selvey, S., E. W. Thompson, et al. (2001). "β-Actin - an unsuitable internal control for RT-PCR." Mol Cell Probes **15**(5): 307-311.

Sharpe, R. M. and S. Franks (2002). "Environment, lifestyle and infertility: an inter-generational issue." Nat Cell Biol **4 Suppl**: s33-40.

Shaw, G. M., K. Todoroff, et al. (2000). "Spina bifida phenotypes in infants or fetuses of obese mothers." Teratology **61**(5): 376-381.

Shevell, T., F. D. Malone, et al. (2005). "Assisted reproductive technology and pregnancy outcome." Obstet Gynaecol **106**(5 Pt 1): 1039-1045.

Shih, W., D. D. Rushford, et al. (2008). "Factors affecting low birthweight after assisted reproduction technology: difference between transfer of fresh and cryopreserved embryos suggests an adverse effect of oocyte collection." Hum Reprod **23**(7): 1644-1653.

Shim, C., H. B. Kwon, et al. (1996). "Differential expression of laminin chain-specific mRNA transcripts during mouse preimplantation embryo development." Mol Reprod Dev **44**(1): 44-55.

Shoenfeld, Y., H. J. A. Carp, et al. (2006). "Autoantibodies and Prediction of Reproductive Failure." Am J Reprod Immunol **56**(5-6): 337-344.

Sibley, C., J. Glazier, et al. (1997). "Placental transporter activity and expression in relation to fetal growth." Exp Physiol **82**(2): 389-402.

Siegmund, T., N. T. Rad, et al. (2008). "Longitudinal changes in the continuous glucose profile measured by the CGMS in healthy pregnant women and determination of cut-off values." Eur J Obstet Gynaecol Reprod Biol **139**(1): 46-52.

Silber, S. J. and N. Barbey (2012). "Scientific molecular basis for treatment of reproductive failure in the human: An insight into the future." Biochim Biophys Acta Mol Bas Dis **1822**(12): 1981-1996.

Simon, C., J. C. Martin, et al. (1999). "Embryonic regulation in implantation." Semin Reprod Endocrinol **17**(3): 267-274.

Simpson, E. R., D. W. Bilheimer, et al. (1979). "Uptake and degradation of plasma lipoproteins by human choriocarcinoma cells in culture." Endocrinol **104**(1): 8-16.

Smith, G. C., E. J. Stenhouse, et al. (2002). "Early-pregnancy origins of low birth weight." Nature **417**(6892): 916.

Smith, G. C. S., J. P. Pell, et al. (2001). "Pregnancy complications and maternal risk of ischaemic heart disease: a retrospective cohort study of 129 290 births." Lancet **357**(9273): 2002-2006.

Smith, G. C. S., I. Shah, et al. (2007). "Maternal Obesity in Early Pregnancy and Risk of Spontaneous and Elective Preterm Deliveries: A Retrospective Cohort Study." Am J Public Health **97**(1): 157-162.

Snel, M., J. T. Jonker, et al. (2012). "Ectopic fat and insulin resistance: pathophysiology and effect of diet and lifestyle interventions." Int J Endocrinol **2012**: 983814.

Soccio, R. E., R. M. Adams, et al. (2005). "Differential gene regulation of StarD4 and StarD5 cholesterol transfer proteins. Activation of StarD4 by sterol regulatory element-binding protein-2 and StarD5 by endoplasmic reticulum stress." J Biol Chem **280**(19): 19410-19418.

Soccio, R. E., R. M. Adams, et al. (2002). "The cholesterol-regulated StarD4 gene encodes a StAR-related lipid transfer protein with two closely related homologues, StarD5 and StarD6." Proc Natl Acad Sci USA **99**(10): 6943-6948.

Soccio, R. E. and J. L. Breslow (2003). "StAR-related lipid transfer (START) proteins: mediators of intracellular lipid metabolism." J Biol Chem **278**(25): 22183-22186.

Spalding, K. L., E. Arner, et al. (2008). "Dynamics of fat cell turnover in humans." Nature **453**(7196): 783-787.

Spanbroek, R., R. Gräbner, et al. (2003). "Expanding expression of the 5-lipoxygenase pathway within the arterial wall during human atherogenesis." Proc Natl Acad Sci USA **100**(3): 1238-1243.

Spector, A. A. and W. G. Haynes (2007). "LDL cholesteryl oleate: a biomarker for atherosclerosis?" Arterioscler Thromb Vasc Biol **27**(6): 1228-1230.

Srivatsa, B., S. Srivatsa, et al. (2001). "Microchimerism of presumed fetal origin in thyroid specimens from women: a case-control study." Lancet **358**(9298): 2034-2038.

Staun-Ram, E., S. Goldman, et al. (2009). "p53 Mediates Epidermal Growth Factor (EGF) Induction of MMP-2 Transcription and Trophoblast Invasion." Placenta **30**(12): 1029-1036.

Stefulj, J., U. Panzenboeck, et al. (2009). "Human endothelial cells of the placental barrier efficiently deliver cholesterol to the fetal circulation via ABCA1 and ABCG1." Circ Res **104**(5): 600-608.

Steinberg, D. (2009). "The LDL modification hypothesis of atherogenesis: an update." J Lipid Res **50**(Suppl): S376-S381.

Steinberger, J. and S. R. Daniels (2003). "Obesity, insulin resistance, diabetes, and cardiovascular risk in children: an American Heart Association scientific statement from the atherosclerosis, hypertension, and obesity in the young committee (Council on CVD in the young) and the diabetes committee (Council on nutrition, physical activity, and metabolism)." Circulation **107**(10): 1448-1453.

Steptoe, P. C. and R. G. Edwards (1978). "Birth after the reimplantation of a human embryo." Lancet **2**(8085): 366-366.

Stewart, F., V. A. Rodie, et al. (2007). "Longitudinal assessment of erythrocyte Fatty Acid composition throughout pregnancy and post partum." Lipids **42**(4): 335-344.

Stewart, F. M., D. J. Freeman, et al. (2007). "Longitudinal assessment of maternal endothelial function and markers of inflammation and placental function throughout pregnancy in lean and obese mothers." J Clin Endocrinol Metab **92**(3): 969-975.

Strair, R. K., A. I. Skoultchi, et al. (1977). "A characterization of globin mRNA sequences in the nucleus of duck immature red blood cells." Cell **12**(1): 133-141.

Strauss, J. F., 3rd, P. Liu, et al. (2002). "Sterols and intracellular vesicular trafficking: lessons from the study of NPC1." Steroids **67**(12): 947-951.

Strauss, J. F., T. Kishida, et al. (2003). "START domain proteins and the intracellular trafficking of cholesterol in steroidogenic cells." Mol Cell Endocrinol **202**(1-2): 59-65.

Sugihara, K., M. Kabir-Salmani, et al. (2008). "Induction of trophinin in human endometrial surface epithelia by CGβ and IL-1β." FEBS Letters **582**(2): 197-202.

Sullivan, E. A., F. Zegers-Hochschild, et al. (2013). "International Committee for Monitoring Assisted Reproductive Technologies (ICMART) world report: assisted reproductive technology 2004." Hum Reprod **28**(5): 1375-1390.

Suzuki, N., J. Zara, et al. (1998). "A cytoplasmic protein, bystin, interacts with trophinin, tastin, and cytokeratin and may be involved in trophinin-mediated cell adhesion between trophoblast and endometrial epithelial cells." Proc Natl Acad Sci USA **95**(9): 5027-5032.

Svensson, L., M. Arvola, et al. (2001). "The Th2 cytokines IL-4 and IL-10 are not crucial for the completion of allogeneic pregnancy in mice."J Reprod Immunol **51**(1): 3-7.

Symons, J. D. and E. D. Abel (2013). "Lipotoxicity contributes to endothelial dysfunction: a focus on the contribution from ceramide." Rev Endocr Metab Disord **14**(1): 59-68.

Tabibzadeh, S. (1998). "Molecular control of the implantation window." Hum Reprod Update **4**(5): 465-471.

Tabibzadeh, S. and A. Babaknia (1995). "The signals and molecular pathways involved in implantation, a symbiotic interaction between blastocyst and endometrium involving adhesion and tissue invasion." Mol Hum Reprod **1**(4): 179-202.

Tafuri, A., J. Alferink, et al. (1995). "T cell awareness of paternal alloantigens during pregnancy." Science **270**(5236): 630-633.

Tamura, T., R. L. Goldenberg, et al. (1998). "Serum leptin concentrations during pregnancy and their relationship to fetal growth." Obstet Gynaecol **91**(3): 389-395.

Tea, I., G. Le Gall, et al. (2012). "^{1}H-NMR-Based Metabolic Profiling of Maternal and Umbilical Cord Blood Indicates Altered Materno-Foetal Nutrient Exchange in Preterm Infants." PLoS ONE **7**(1): e29947.

Tennant, P. W. G., J. Rankin, et al. (2011). "Maternal body mass index and the risk of fetal and infant death: a cohort study from the North of England." Hum Reprod **26**(6): 1501-1511.

Teran, E., C. Escudero, et al. (2001). "Elevated C-reactive protein and pro-inflammatory cytokines in Andean women with pre-eclampsia." Int J Gynaecol Obstet **75**(3): 243-249.

Thie, M., R. Rospel, et al. (1998). "Interactions between trophoblast and uterine epithelium: monitoring of adhesive forces." Hum Reprod **13**(11): 3211-3219.

Thilaganathan, B., B. Wormald, et al. (2010). "Early-pregnancy multiple serum markers and second-trimester uterine artery Doppler in predicting preeclampsia." Obstet Gynaecol **115**(6): 1233-1238.

Thomson, F., S. Shanbhag, et al. (2005). "Obstetric outcome in women with subfertility." BJOG **112**(5): 632-637.

Thum, M. Y., A. El-Sheikhah, et al. (2007). "The influence of body mass index to in-vitro fertilisation treatment outcome, risk of miscarriage and pregnancy outcome." J Obstet Gynaecol **27**(7): 699-702.

Tiger, C.-F., M.-F. Champliaud, et al. (1997). "Presence of Laminin α5 Chain and Lack of Laminin α1 Chain during Human Muscle Development and in Muscular Dystrophies." J Biol Chem **272**(45): 28590-28595.

Timpl, R., D. Tisi, et al. (2000). "Structure and function of laminin LG modules." Matrix Biology **19**(4): 309-317.

Tint, G. S., M. Irons, et al. (1994). "Defective cholesterol biosynthesis associated with the Smith-Lemli-Opitz syndrome." N Engl J Med **330**(2): 107-113.

Tjoa, M. L., J. M. G. van Vugt, et al. (2003). "Elevated C-reactive protein levels during first trimester of pregnancy are indicative of preeclampsia and intrauterine growth restriction." J Reprod Immunol **59**(1): 29-37.

Tomasetto, C., C. Régnier, et al. (1995). "Identification of Four Novel Human Genes Amplified and Overexpressed in Breast Carcinoma and Localized to the q11-q21.3 Region of Chromosome 17." Genomics **28**(3): 367-376.

Torgersen, K. L. and C. A. Curran (2006). "A systematic approach to the physiologic adaptations of pregnancy." Crit Care Nurs Q **29**(1): 2-19.

Torry, D. S., M. Hinrichs, et al. (2004). "Determinants of Placental Vascularity." Am J Reprod Immunol **51**(4): 257-268.

Tsubaki, H., M. Ogawa, et al. (2005). "Expression of CD44 mRNA induced by interleukin-1[beta] in human cultured uterine cervical fibroblasts." Eur J Obstet Gynaecol Reprod Biol **122**(2): 156-161.

Tzschoppe, A. A., E. Struwe, et al. (2010). "Differences in gene expression dependent on sampling site in placental tissue of fetuses with intrauterine growth restriction." Placenta **31**(3): 178-185.

Uchida, Y., Y. Kurano, et al. (1998). "Establishment of monoclonal antibody against human Apo B-48 and measurement of Apo B-48 in serum by ELISA method." J Clin Lab Anal **12**(5): 289-292.

Ulisse, S., E. Baldini, et al. (2009). "The urokinase plasminogen activator system: a target for anti-cancer therapy." Curr Cancer Drug Targets **9**(1): 32-71.

Ulukus, E. C., M. Ulukus, et al. (2005). "Expression of interleukin-8 and monocyte chemotactic protein-1 in adenomyosis." Hum Reprod **20**(10): 2958-2963.

Utani, A., M. Nomizu, et al. (1997). "Fibulin-2 binds to the short arms of laminin-5 and laminin-1 via conserved amino acid sequences." J Biol Chem **272**(5): 2814-2820.

Uzelac, P. S., X. Li, et al. (2010). "Dysregulation of Leptin and Testosterone Production and Their Receptor Expression in the Human Placenta with Gestational Diabetes Mellitus." Placenta **31**(7): 581-588.

Vahratian, A. (2009). "Prevalence of overweight and obesity among women of childbearing age: results from the 2002 National Survey of Family Growth." Matern Child Health J **13**(2): 268-273.

van Beijnum, J. R., M. Rousch, et al. (2008). "Isolation of endothelial cells from fresh tissues." Nat Protocols **3**(6): 1085-1091.

van den Driesche, S., V. M. Smith, et al. (2008). "Expression and regulation of oestrogen receptors in the human corpus luteum." Reproduction **135**(4): 509-517.

van Mourik, M. S., N. S. Macklon, et al. (2009). "Embryonic implantation: cytokines, adhesion molecules, and immune cells in establishing an implantation environment." J Leukoc Biol **85**(1): 4-19.

Vandekerckhove, J. and K. Weber (1978). "At least six different actins are expressed in a higher mammal: An analysis based on the amino acid sequence of the amino-terminal tryptic peptide." J Mol Biol **126**(4): 783-802.

Vaughan, A. M. and J. F. Oram (2006). "ABCA1 and ABCG1 or ABCG4 act sequentially to remove cellular cholesterol and generate cholesterol-rich HDL." J Lipid Res **47**(11): 2433-2443.

Vilarino, F. L., D. M. Christofolini, et al. (2011). "Body mass index and fertility: is there a correlation with human reproduction outcomes?" Gynaecol Endocrinol **27**(4): 232-236.

Villar, J., M. Cogswell, et al. (1992). "Effect of fat and fat-free mass deposition during pregnancy on birth weight." Am J Obstet Gynaecol **167**(5): 1344-1352.

Voldner, N., E. Qvigstad, et al. (2010). "Increased risk of macrosomia among overweight women with high gestational rise in fasting glucose." J Matern Fetal Neonatal Med **23**(1): 74-81.

Voutilainen, R., J. Tapanainen, et al. (1986). "Hormonal regulation of P450scc (20,22-desmolase) and P450c17 (17 alpha-hydroxylase/17,20-lyase) in cultured human granulosa cells." J Clin Endocrinol Metab **63**(1): 202-207.

Wada, T., S. Hori, et al. (2010). "Progesterone inhibits glucose uptake by affecting diverse steps of insulin signaling in 3T3-L1 adipocytes." Am J Physiol Endocrinol Metab **298**(4): E881-E888.

Wadsack, C., S. Tabano, et al. (2007). "Intrauterine growth restriction is associated with alterations in placental lipoprotein receptors and maternal lipoprotein composition." Am J Physiol Endocrinol Metab **292**(2): E476-484.

Walls, M., S. Junk, et al. (2012). "IVF versus ICSI for the fertilization of in-vitro matured human oocytes." Reprod Biomed Online **25**(6): 603-607.

Wang, H. and S. K. Dey (2006). "Roadmap to embryo implantation: clues from mouse models." Nat Rev Genet **7**(3): 185-199.

Wang, H., F. Pilla, et al. (2012). "A novel model of human implantation: 3D endometrium-like culture system to study attachment of human trophoblast (Jar) cell spheroids." Mol Hum Reprod **18**(1): 33-43.

Wang, X., N. Yang, et al. (2009). "Interruption of Growth Hormone Signaling via SHC and ERK in 3T3-F442A Preadipocytes upon Knockdown of Insulin Receptor Substrate-1." Mol Endocrinol **23**(4): 486-496.

Wang, Y. and S. W. Walsh (1996). "Antioxidant Activities and mRNA Expression of Superoxide Dismutase, Catalase, and Glutathione Peroxidase in Normal and Preeclamptic Placentas." J Soc Gynaecol Investig **3**(4): 179-184.

Wang, Y. and S. W. Walsh (1996). "TNFα concentrations and mRNA expression are increased in preeclamptic placentas." J Reprod Immunol **32**(2): 157-169.

Wang, Y., Y. Zhang, et al. (2003). "Protease Chymotrypsin Mediates the Endothelial Expression of P- and E-selectin, but not ICAM and VCAM, Induced by Placental Trophoblasts from Pre-eclamptic Pregnancies." Placenta **24**(8-9): 851-861.

Wang, Z., K. Tamura, et al. (2003). "Prostaglandin F2alpha-induced functional regression of the corpus luteum and apoptosis in rodents." J Pharmacol Sci **92**(1): 19-27.

Warner, M. J. and S. E. Ozanne (2010). "Mechanisms involved in the developmental programming of adulthood disease." Biochem J **427**(3): 333-347.

Waterland, R. A. and C. Garza (1999). "Potential mechanisms of metabolic imprinting that lead to chronic disease." Am J Clin Nutr **69**(2): 179-197.

Watson, A. D., N. Leitinger, et al. (1997). "Structural Identification by Mass Spectrometry of Oxidized Phospholipids in Minimally Oxidized Low Density Lipoprotein That Induce Monocyte/Endothelial Interactions and Evidence for Their Presence in Vivo." J Biol Chem **272**(21): 13597-13607.

Way, D. L., D. S. Grosso, et al. (1983). "Characterization of a new human endometrial carcinoma (RL95-2) established in tissue culture." In Vitro **19**(3 Pt 1): 147-158.

Weaver, I. C. G., N. Cervoni, et al. (2004). "Epigenetic programming by maternal behavior." Nature Neurosci **7**(8): 847-854.

Wegmann, T. G., H. Lin, et al. (1993). "Bidirectional cytokine interactions in the maternal-fetal relationship: is successful pregnancy a TH2 phenomenon?" Immunol Today **14**(7): 353-356.

Wei, S., M. D. Schmidt, et al. (2009). "Obesity and Menstrual Irregularity: Associations With SHBG, Testosterone, and Insulin." Obesity **17**(5): 1070-1076.

Weinhaus, A. J., L. E. Stout, et al. (2007). "Regulation of glucokinase in pancreatic islets by prolactin: a mechanism for increasing glucose-stimulated insulin secretion during pregnancy." J Endocrinol **193**(3): 367-381.

Weisberg, S. P., D. McCann, et al. (2003). "Obesity is associated with macrophage accumulation in adipose tissue." J Clin Invest **112**(12): 1796-1808.

Welch, P. C., K. S. Amankwah, et al. (2006). "Correlations of placental perfusion and PlGF protein expression in early human pregnancy." Am J Obstet Gynaecol **194**(6): 1625-1629.

Wessels, J. M., N. F. Linton, et al. (2011). "Expression of chemokine decoy receptors and their ligands at the porcine maternal-fetal interface." Immunol Cell Biol **89**(2): 304-313.

Whitaker, R. C. (2004). "Predicting Preschooler Obesity at Birth: The Role of Maternal Obesity in Early Pregnancy." Pediatrics **114**(1): e29-36.

White, M. F. (2003). "Insulin signaling in health and disease." Science **302**(5651): 1710-1711.

White, M. F. (2013). "Insulin Signaling Pathway." Sci Signal Connections Map in the Database of Cell Signaling, as seen **13** August 2013: http://stke.sciencemag.org/cgi/cm/stkecm;CMP_12069.

White, T. E., R. A. Saltzman, et al. (1988). "Human choriocarcinoma (JAr) cells grown as multicellular spheroids." Placenta **9**(6): 583-598.

Wilcox, A. J., D. D. Baird, et al. (1999). "Time of Implantation of the Conceptus and Loss of Pregnancy." N Engl J Med **340**(23): 1796-1799.

Wilcox, G. (2005). "Insulin and insulin resistance." Clin Biochem Rev **26**(2): 19-39.

Wildman, D. E. (2008). "Sources for Comparative Studies of Placentation. II. Genomic Resources." Placenta **29**(2): 144-147.

Willett, W., C. H. Hennekens, et al. (1983). "Effects of cigarette smoking on fasting triglyceride, total cholesterol, and HDL-cholesterol in women." Am Heart J **105**(3): 417-421.

Williams, K. J., G. M. Fless, et al. (1992). "Mechanisms by which lipoprotein lipase alters cellular metabolism of lipoprotein(a), low density lipoprotein, and nascent lipoproteins. Roles for low density lipoprotein receptors and heparan sulfate proteoglycans." J Biol Chem **267**(19): 13284-13292.

Williams, T. N., C. X. Zhang, et al. (2004). "C-reactive protein stimulates MMP-1 expression in U937 histiocytes through FcγRII and extracellular signal-regulated kinase pathway: an implication of CRP involvement in plaque destabilization." Arterioscler Thromb Vasc Biol **24**(1): 61-66.

Winkler, K., B. Wetzka, et al. (2000). "Low Density Lipoprotein (LDL) Subfractions during Pregnancy: Accumulation of Buoyant LDL with Advancing Gestation." J Clin Endocrinol Metab **85**(12): 4543-4550.

Woollett, L. A. (2001). "The origins and roles of cholesterol and fatty acids in the fetus." Curr Opin Lipidol **12**(3): 305-312.

Woollett, L. A. (2005). "Maternal cholesterol in fetal development: transport of cholesterol from the maternal to the fetal circulation." Am J Clin Nutr **82**(6): 1155-1161.

Yadav, A., M. A. Kataria, et al. (2013). "Role of leptin and adiponectin in insulin resistance." Clin Chim Acta **417**: 80-84.

Yogev, Chen, et al. (2010). "Hyperglycemia and Adverse Pregnancy Outcome (HAPO) study: preeclampsia." Am J Obstet Gynaecol **202**(3): 255 e251-257.

Yogev, Y. and O. Langer (2008). "Pregnancy outcome in obese and morbidly obese gestational diabetic women." Eur J Obstet Gynaecol Reprod Biol **137**(1): 21-26.

Yoshizumi, T., S. Nozaki, et al. (2000). "Pharmacokinetics and Metabolism of 123I-BMIPP Fatty Acid Analog in Healthy and CD36-Deficient Subjects." J Nucl Med **41**(7): 1134-1138.

Young, S. G. and C. J. Fielding (1999). "The ABCs of cholesterol efflux." Nat Genet **22**(4): 316-318.

Yu, T. and A. P. Lieberman (2013). "Npc1 acting in neurons and glia is essential for the formation and maintenance of CNS myelin." PLoS Genet **9**(4): e1003462.

Zegers-Hochschild, F., G. D. Adamson, et al. (2009). "International Committee for Monitoring Assisted Reproductive Technology (ICMART) and the World Health Organization (WHO) revised glossary of ART terminology, 2009." Fertil Steril **92**(5): 1520-1524.

Zenclussen, A. C. (2013). "Adaptive immune responses during pregnancy." Am J Reprod Immunol **69**(4): 291-303.

Zhang, C., M. A. Austin, et al. (2006). "Functional variants of the lipoprotein lipase gene and the risk of preeclampsia among non-Hispanic Caucasian women." Clin Genet **69**(1): 33-39.

Zhang, J., L. J. Lathbury, et al. (2000). "Expression of the Chemokine Eotaxin and Its Receptor, CCR3, in Human Endometrium." Biol Reprod **62**(2): 404-411.

Zhang, Y., S. Liu, et al. (2009). "Overexpression of fucosyltransferase VII (FUT7) promotes embryo adhesion and implantation." Fertil Steril **91**(3): 908-914.

Zhao, Z. and P. Michaely (2009). "The Role of Calcium in Lipoprotein Release by the Low-Density Lipoprotein Receptor." Biochemistry **48**(30): 7313-7324.

Zhou, C. C., R. A. Irani, et al. (2011). "Autoantibody-Mediated IL-6–Dependent Endothelin-1 Elevation Underlies Pathogenesis in a Mouse Model of Preeclampsia." J Immunol **186**(10): 6024-6034.

Zhou, L., R. Li, et al. (2008). "Local injury to the endometrium in controlled ovarian hyperstimulation cycles improves implantation rates." Fertil Steril **89**(5): 1166-1176.

Zlotnik, A. and O. Yoshie (2000). "Chemokines: A New Classification System and Their Role in Immunity." Immunity **12**(2): 121-127.