TITLE: AN INVESTIGATION OF GLUCOCORTICOID AND SEROTONERGIC SYSTEMS IN HUMAN PLACENTA

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I. Abstract

Every week of human gestation is important for optimal fetal development, particularly the last two weeks of gestation (gestational weeks 39 and 40) during which fetal organs and organ systems undergo maximum growth. Infants born at full term are generally healthier and have a lower risk of developing various health complications post-partum and in adult life compared to those born early-term (gestational weeks 37 and 38). The risk factors of early-term births are not discovered yet; however, emerging research suggests that alterations in the placental structure and function may contribute to suboptimal placental and fetal development resulting in premature births, with infants born with lower than normal birth weight. Glucocorticoid hormones (cortisol and corticosterone) maintain pregnancy and fetal growth; nevertheless, fetal exposure to high maternal glucocorticoids (GCs) during pregnancy is associated with structural and functional changes in the placenta, intrauterine fetal growth restriction and premature births. The levels of the GCs that are transferred from the mother to the fetus through the placenta are under the stringent control of placental 11β-HSD (11β-hydroxysteroid dehydrogenase) enzymes, 11β-HSD1 and 11β-HSD2. The expression and activity of 11β-HSD enzymes in the placenta vary throughout gestation and may be affected by GC levels. The reports on the activity and expression of placental 11β-HSD enzymes late in human gestation are quite contradictory. In the human placenta, GC signaling is mediated via diverse glucocorticoid receptors (GRs). The GR isoforms are generated via alternative splicing and alternative translation initiation, with data on GR isoform expression in the human placenta being limited and suggesting that GR expression in the human placenta vary in relation to subcellular localization, fetal sex and gestational age. There is evidence that besides nuclear and cytoplasmic localization, GRs may reside in the mitochondria where they may play roles in cell life and death. Whether GRs are present in the mitochondria of human trophoblast cells and whether mitochondrial GRs play roles in

trophoblast cell death is unclear. Emerging research suggests that GCs play crucial roles in human pregnancy and may alter placental serotonin (5-HT) system, the dysfunction of which is reported in pregnancy complications and is associated with neurodevelopmental and behavioral disorders in human. Thus the main aims of this doctoral study were to investigate the GC and serotonin systems in the human placenta to find out whether early-term births are associated with alterations in the GC and/or serotonin system function and whether these changes (if any) are related to cortisol concentrations. A better understanding of the impact of GCs on the serotonin system in the human placenta and the effect of GC and serotonin interaction on maternal health, placental function and fetal development may reduce gestational complications and improve perinatal outcomes leading to healthier Australian and global population.

The first study (Chapter 3) aimed to examine a) fetal characteristics including gestational age, birthweight and placental weight in relation to umbilical cord cortisol levels and b) placental 11β-HSD1 and 11β-HSD2 enzymes. There was a significant correlation between cord blood cortisol levels and fetal gestational age in weeks, with high cord cortisol levels associated with advanced weeks of human gestation ($p = 0.005$), a relationship previously reported in human gestation. Placental and fetal weight was unrelated to cortisol levels. The enzymes, 11β-HSD1 and 11β-HSD2, that control cortisol bioavailability in the placenta and fetus were detected in the placenta, with the 11β-HSD1 enzyme expressed differentially in relation to fetal sex and gestational age. In the full-term group, placentae from the female fetuses had significantly higher 11β-HSD1 protein levels compared to the male fetus placentae ($p < 0.01$). Contrary to the full-term group, in the early-term group placentae from the female fetuses had lower 11β-HSD1 protein expression than male fetus placentae ($p < 0.01$), possibly indicating that the expression of the placental 11β-HSD1 enzyme is sex- and gestational age-specific. Although there was no significant correlation of

cord cortisol levels with placental 11β-HSD1 and 11β-HSD2 enzyme expression, placental 11β-HSD1 enzyme expression tended to increase with gestational age, whereas 11β-HSD2 enzyme levels were unchanged between 37 and 40 weeks of pregnancy. Because very little is known about 11β-HSD1 enzyme levels in the human placenta, the defined role of the 11β-HSD1 enzyme in controlling fetal cortisol exposure to maternal GCs remains obscure.

The second study (Chapter 4) explored the expression of GR isoforms in the placenta with respect to subcellular localization (nuclear versus cytoplasm), fetal sex and gestational age. The isoforms detected were as follows: GRα-A, GRα-C, GRα-D1, GRα D2/D3, GRβ and unknown 68 kDa and 38 kDa. The GR protein isotypes varied depending on subcellular localization, fetal sex and gestational age. In the full-term group, male fetus placentae had higher GRα-A and GRβ levels in the nucleus than in the cytoplasm ($p < 0.05$), whereas female fetus placentae had lower GRα-D1 levels in the nuclear compartment compared to the cytoplasm ($p < 0.05$). In the early-term group, male and female placentae had higher expression of GRα-A and GRα-D1 in the nucleus than in the cytoplasm ($p < 0.05$); however, female fetus placentae had also much higher nuclear GRα-C, unknown 68 kDa and 38 kDa protein levels compared to the cytoplasmic levels ($p < 0.05$).

The GR profile in the placenta was also fetal sex- and gestational age-specific (Chapter 4). In the placentae of early-term male fetuses, the cytoplasmic levels of GRβ and nuclear levels of GRα-A and unknown 38 kDa protein were higher compared to the placentae of full-term male fetuses. The co-expression of GRβ and GRα-A has been proposed to play a crucial role in controlling growth-related genes in the placentae and male fetuses between gestational weeks of 37 and 38. In the placentae of early-term female fetuses the nuclear levels of GRα-A, GRβ, GRα-C, GRα-D1, GRα-D2/D3 unknown 68 kDa protein were significantly higher compared to the placentae of full-term female fetuses. We hypothesized that simultaneous expression of GRα-A with GRβ, GRα-C, GRα-D1, GRα-D2/D3 and

unknown 68 kDA may regulate the growth of the fetus and the placenta between 37 and 38 gestational weeks in the women pregnant with a female fetus.

The third study (Chapter 5) sought to characterize the GRs in the mitochondria of placental cells and to examine the expression of caspase – 3 and caspase – 6 in the placental tissues as biomarkers of placental cell death; c) to explore the relationship between cord cortisol levels and placental apoptosis and d) to determine whether mitochondrial GRs contribute to placental cell death. This study for the first time detected mitochondrial GRs in the human placenta and assessed the GR isoforms in relation to fetal sex, gestational age, cortisol levels and placental cell death to find out if early-term birth were related to changes in the mitochondrial GR expression. Three bands with molecular weights of 68-69 kDa, 50-51 kDa and 38 kDa were detected in the mitochondria, potentially representing unknown 68 kDa, GRα D2/D3 and unknown 38 kDa proteins. The proteins did not significantly differ in relation to fetal sex, gestational age or cord cortisol levels ($p > 0.05$). Placental apoptosis is a normal physiological process in pregnancy; however, when it is exaggerated it may lead to placental dysfunction. Thus, the pro-apoptotic markers, caspase -3 and -6, were examined in placental extracts to find out if early-term births were related to increased placental apoptosis. Both pro-apoptotic proteins were detected in the human placenta. The results of this study revealed a significant upregulation in the expression of caspase-3 in the placentae of the early-term male fetuses compared to the placentae of full-term male counterparts ($p < 0.05$), with the increased caspase – 3 levels being unrelated to cord cortisol levels ($p > 0.05$), suggesting that other factors besides GCs may play roles in the regulation of caspase -3 expression in the human placenta. Whether the up-regulated expression of caspase – 3 indicates an increased placental cell death or is a physiologically normal process associated with placental cell differentiation or cell-cycle progression is not clear. There was no correlation between mitochondrial GRs and caspase – 3 expression, implying that other

mitochondrial GR-independent mechanisms may regulate caspase -3 activity in the early-term male fetus placentae.

Finally, studies in Chapter 6 aimed a) to examine the components of serotonin system such as serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors in placental tissues; b) to explore 5-HT_{1A} and 5-HT_{2A} receptors in placental blood vessels and c) to investigate the correlations of cord cortisol levels with placental tissue serotonin, SERT, TPH1, MAO-A, 5-HT_{1A} receptor and 5-HT_{2A} receptor levels. Serotonin, SERT, TPH1, MAO-A, 5-HT_{1A} receptors and 5-HT_{2A} receptors were detected in placental samples independent of fetal gestational age or sex. Although the levels of these factors were unrelated to the sex or gestational age, the presence of serotonin system components in the human placenta indicates on an important role of serotonin in fetal and placental development. 5-HT_{2A} receptors, but not 5-HT_{1A} receptors, were discovered in the placental veins. Of all the factors of serotonin system examined in relation to cord cortisol levels, only SERT expression was inversely related to cord cortisol levels, with high levels of cortisol associated with low levels of SERT ($p < 0.05$). It has been proposed that GC-induced down-regulation in SERT expression may result in an increase in the extracellular levels of placental serotonin that through the interaction with 5-HT_{2A} receptors in the placental veins alter fetoplacental blood flow and induce labor.

In conclusion, we observed significant sex-specific differences between the early-term and full-term placentae. Further studies are required to determine how these changes affect fetal development and long-term health of the infants.

II. Statement of Originality

This work has not previously been submitted for a degree or diploma in any university. To the best of my knowledge and belief, the thesis contains no material previously published or written by another person except where due reference is made in the thesis itself.

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VI. Acknowledgements

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List of Abbreviations

11b-HSD1	11b-Hydroxysteroid dehydrogenase type 1
11b-HSD1	11b-Hydroxysteroid dehydrogenase type 2
5-HT	5-hydroxytryptamine, serotonin
AAAD	Aromatic acid decarboxylase
ACTH	Adrenocorticotropic hormone
AF1	Activation function domain 1
AF2	Activation function domain 2
AP1	Activated protein 1
ATP	Adenosine triphosphate
AUG	Adenine-uracil-guanine
BCA	Bicinchoninic acid
BMI	Body mass index
BSA	Bovine serum albumin
cAMP	Cyclic adenosine monophosphate
CBG	Cortisol binding globulin
CBP	CREB-binding protein
CDK	Cyclin-dependent kinase
CNS	Central nervous system
COX-IV	Cyclooxygenase - 4
CREB	Cyclic-AMP response element binding protein
CRH	Corticotropin-releasing hormone
CV	Cardiovascular
DBD	DNA-binding domain
DEX	Dexamethasone
DNA	Deoxyribonucleic acid
DTT	Dithiothreitol
EDTA	Ethylenediaminetetraacetic acid
EGTA	Ethylene glycol-bis(2-aminoethyl ether)
GC	Glucocorticoid
GCM1	Glial cell missing homolog 1
GDM	Gestational diabetes mellitus
GR	Glucocorticoid receptor
GRE	Glucocorticoid response element
GRIP-1	Glutamate-receptor-interacting protein 1
GSK-3	Glycogen synthase kinase-3
HeLa	Homo sapiens cervical carcinoma cell line
Hep G2	Homo sapiens hepatocellular carcinoma cell line
Hep-2	Homo sapiens HeLa contaminant carcinoma cell line
HEPES	4-(2-hydroxyethyl) piperazine-1-ethanesulfonic acid
hGR	Human glucocorticoid receptor
HPA	Hypothalamic-pituitary-adrenal axis

HR	Hinge region
Hsp	Heat shock protein
IL	interleukin
IUGR	Intrauterine growth restriction
JNK	c-Jun N-terminal kinase
KCl	Potassium chloride
LBD	Ligand-binding domain
LBW	Low birth weight
MAO-A	Monoamine oxidase
MARK	Mitogen-activated protein kinase
MgCl2	Magnesium chloride
MR	Mineralocorticoid receptor
mRNA	Messenger ribonucleic acid
mRNA	Messenger ribonucleic acid
mtDNA	Mitochondrial DNA
mtGR	Mitochondrial GR
NaF	Sodium fluoride
NCoR	Nuclear co-receptor
NF-kB	Nuclear factor kB
NR3C1	Nuclear receptor superfamily 3, group C, member 1
NTD	N-terminal domain
OXPHOS	Oxidative phosphorylation
PBS	Phosphate-buffered saline
p-GR	Phosphorylated glucocorticoid receptor
PMSF	Phenylmethanesulfonyl fluoride
PVDF	Polyvinylidene difluoride
RNA	Ribonucleic acid
ROS	Reactive oxygen species
rRNA	Ribosomal RNA
SaOS-2	Osteogenic sarcoma human cell line
SCL2A1	Salute carrier family 2, facilitated glucose transporter, member 1
SERT	Serotonin transporter
SGA	Small-for-gestational-age
SMRT	Silencing mediator of retinoid and thyroid hormone receptor
SRC-1	Steroid receptor cofactor-1
STAT	Signal transducers and activators related protein
SUMO	Small ubiquitin-related modifier
TBS	Tris-buffered saline
TIF-2	Transcriptional intermediary factor - 2
TPH	Tryptophan hydroxylase
tRNA	Transfer RNA
uM	Micromolar concentration
VIP	Vasoactive intestinal peptide

List of Publications and Conferences

Svetlana Bivol, Suzzanne J. Owen and Roselyn B. Rose'Meyer, Glucocorticoid-induced changes in glucocorticoid receptor mRNA and protein expression in the human placenta as a potential factor for altering fetal growth and development. *Reproduction, Fertility and Development*, 2016, **29**: p. 845-854.

Conferences Attended

Jul, 2017	Queensland Perinatal Consortium (QPaCT) conference, "Hot topics in perinatal research"
Aug, 2016	Australian and New Zealand Placental Research Association satellite meeting, Gold Coast, Australia, "The placenta – life support for healthy development"
Dec, 2016	Queensland Perinatal Consortium (QPaCT) conference, "Placental adaptation to maternal environment"

CHAPTER 1: Background

1.1Placenta and intrauterine fetal development

Intrauterine fetal growth is of great interest in antenatal care because aberrant fetal development during gestation is associated with adverse pregnancy outcomes, with long-term consequences manifested later in adult life. Fetal growth is a complex process affected by various factors, including maternal characteristics and placental function [1-3]. The human placenta is the least investigated organ. This unique, autonomous and transient organ has multiple vital roles. It supplies oxygen, nutrients, salts, and water to the growing fetus via the umbilical vein and removes carbon dioxide and waste products from the fetal circulation through the umbilical arteries. The human placenta is an endocrine organ, synthesizing not only a wide range of hormones and hormone receptors but also neurotransmitters, transporters, amino acids, growth factors and proteins to establish and maintain the pregnancy, immune function, maternal and fetal well-being. These heterogeneous roles of the placenta are performed by the syncytiotrophoblast cells, arising from the fusion of the cytotrophoblast cells [4, 5]. Given the importance of optimal placental function during gestation, it is not surprising that abnormal placental development has dramatic effects on both, the mother and fetus.

Recent investigations demonstrate the importance of placental development in long-term health and disease for both mother and fetus, with alterations in placental structure and function associated with maternal hypertension, preeclampsia, and insulin resistance [6-8]. The aberrant placental development also affects the fetus, causing prematurity and neurodevelopmental abnormalities [9, 10]. Different perturbations in the maternal compartment alter the structure and function of the placenta and lead to placental dysfunction and developmental programming. These factors include altered maternal nutrition, reduced uteroplacental blood flow, and glucocorticoid (GC) overexposure during critical periods in

human development [11]. The following sections focused on the relationship between overexposure of the developing fetus to GCs and the developmental origins of disease.

1.2 Glucocorticoid excess and fetal programming

1.2.1 Cortisol metabolism, circadian rhythm, and regulation

Cortisol and corticosterone are essential GC hormones synthesized primarily in zona fasciculata of the adrenal gland. In humans, corticosterone levels in the circulation are 10-20 times lower compared to the concentrations of the principal human GC - cortisol [12]. Although cortisol regulates numerous physiological processes, including (but not limited to) metabolism, stress response, vascular responsiveness, embryogenesis, reproduction and programmed cell death [13-18], the biological roles of corticosterone in humans are yet to be identified.

Most (95%) of GCs in the plasma are bound to carrier proteins (corticosteroid-binding globulin (CBG) or albumin) [19-21]. Tissue cortisol availability is controlled by 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1) and 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) enzymes, with 11β-HSD1 upregulating the circulating levels of GCs by converting biologically inactive cortisone into its active form, cortisol, and 11β-HSD2 diminishing cortisol concentrations by metabolizing cortisol into its inert form, cortisone. The synthesis of the enzymes is tissue-specific [22-27], with both enzymes expressed in the human placenta to regulate fetal development and the pathways associated with labor [28].

Plasma cortisol secretion varies with time of day obeying a strict circadian rhythm with hormone concentrations greatest in the early morning hours, moderate during the day and lowest during late afternoon and evening [29]. The circadian variation in cortisol production is essential not only for alerting the body in the morning and reducing its activity prior to sleep but also for controlling body temperature, renal function and cardiovascular

activity [29]. Cortisol synthesis and release is under the stringent control of the hypothalamic-pituitary-adrenal axis (HPA) neuroendocrine axis, which constitutes the interaction hormones such as corticotropin-releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol [30]. The HPA axis is activated by physiological or psychological stress factors, either acute or chronic. The activated HPA axis initiates a stress response inducing an array of physiologic and behavioral responses that aim to re-establish the disturbed internal balance by triggering the secretion of hypothalamic CRH [31]. CRH activates the release of ACTH from the anterior pituitary gland resulting in increased circulating concentrations of GCs synthesized by the adrenal gland. Once released into the systemic circulation, GCs (the final hormonal effectors of HPA axis) shut down the activated HPA-axis and restore the innate body equilibrium by suppressing the production of both CRH and ACTH in the brain [32]. The physiological and psychological stress factors that activate the HPA axis and stimulate cortisol synthesis include pathogens, hypoglycemia, disease, fever, trauma, surgery, fear, pain, physical exertion and extreme temperatures [18, 33].

Although, adaptive stress responses aim to maximize the chances of the individual for survival when chronically stimulated by excessive stressors the adaptive stress responses may turn maladaptive, disrupt normal HPA axis activity and impair crucial physiological functions. The adverse effects of long-term excessive GC exposure are hypertension, dyslipidemia, obesity, Cushing's syndrome, type II diabetes, osteoporosis, immune dysfunction, neuronal death as well as anxiety, depression and metabolic syndrome [13, 34-39]. Disruption of the normal activity of the HPA axis and associated cortisol excess is of particular importance during gestation because fetal concentrations of cortisol are directly related to maternal cortisol concentrations [40].

1.2.2 Glucocorticoids, placenta and fetal development

In addition to regulating essential biological functions and ameliorating an array of human pathologies, GCs play an important role in mediating fetal growth and development. Although physiological levels of GCs are beneficial for both mother and fetus, repeated and/or prolonged intrauterine exposure to excess GCs in early life has adverse transient and persistent effects on the developing fetus, with male and female fetuses responding differentially to the environmental insults, including overexposure to GCs.

Each human pregnancy is associated with hypercortisolemia, with cortisol concentrations rising two- to fourfold compared with pre-pregnancy values [41]**.** Total and free cortisol concentrations in a mother increase markedly over the course of normal gestation, especially in the second and third trimesters [42]. An increase in plasma cortisol is essential for the development and maturation of multiple fetal organs, such as thyroid, heart, lung, gastrointestinal tract, liver, kidney, and brain, as well as hematopoietic and lymphatic systems [43-45]. Moreover, pregnancy cortisol is essential for the growth and development of the placenta where it controls multiple biochemical processes and signaling pathways by altering the expression of numerous ion channels, clotting factors, gap and tight junction proteins, growth factors, cytoskeleton and binding proteins, enzymes, receptors and transporters involved in growth [46]. An increase in cortisol levels is not only due to hyperactivation of the maternal HPA axis but also to increased synthesis and release of CRH and ACTH by an important feto-maternal organ, the placenta [47, 48]. The upregulation of cortisol levels by the placenta is mediated through increased production of steroid hormones such as progesterone and estrogen, required for the maintenance of the human pregnancy [49]. The former is associated with CBGs increasing biologically active cortisol in the blood [50]**;** the latter elevates plasma GC levels by increasing the half-life of cortisol [51].

The transport of GCs from the mother to the fetus is strictly regulated by placental 11β-HSD enzymes, which control placental GC metabolism by catalyzing the interconversion of hormonally active cortisol and inert cortisone [24]. Under normal conditions, fetal GC concentrations are much lower than maternal levels [52]. The gradient is achieved and maintained by the physiological 'barrier' to maternal GCs, namely 11β-HSD2, which inactivates 80%-90% of maternal GCs by breaking GCs down into their 11-keto metabolites during passage through the placenta [53-55]. However, maternal physiological and psychological factors such as undernutrition, sepsis, viral infection, inflammation, iron deficiency, uncontrolled asthma, depression, stress, obesity, advancing age, alcohol intake, smoking and GC medications may substantially augment maternal levels of GCs [56-64], saturate [65] or downregulate 11β-HSD2 activity [66, 67] and lead to increased maternofetal GC transfer and altered placental and fetal maturation. In addition to endogenous (originating from within the mother) sources of GCs, maternal GC levels can be also elevated by exogenous or synthetic GC drugs including dexamethasone (DEX), prednisone and betamethasone used to treat a variety of pregnancy and fetal complications. In contrast to endogenous GCs that are good substrates for 11β-HSD2 enzymes and inactivated during the passage through the placenta, exogenous GCs poorly interact with the 11β-HSD2 enzymes and therefore freely cross the placenta and increase fetal levels of GCs. Although the placental barrier to GCs is achieved predominantly by the presence of 11β-HSD2, other proteins present in the placenta may also limit the transfer of maternal GCs to the fetus, including the multidrug resistance P-glycoprotein, a membrane-bound protein mediating the efflux of GCs out of placental cells, thus minimizing the growth inhibitory actions of GCs on the fetus and placenta [68, 69]. Although GCs are maturational, environmental and programming signals that optimize the offspring's viability and survival by promoting cellular differentiation and maturation of various tissues and organs, sustained fetal exposure

to excess maternal GCs is associated with maldevelopment of the fetus and an elevated risk of cardiovascular, neuroendocrine and psychiatric disorders in adulthood.

1.2.3 Effects of glucocorticoid excess on fetal and placental development

Data from experimental studies conducted in animals and humans support the concept that GC excess during the critical period of fetal development (early in gestation) has short-term and lifelong impacts on the growing fetus with overexposure to elevated maternal GCs adversely affecting both the fetus and placenta.

High GC concentrations during sensitive windows in development inhibit fetal cell division and differentiation, resulting in a reduced fetal brain development and intrauterine fetal growth, with babies born preterm with lower than normal birth weight [70-72]. Fetal exposure to high maternal GCs during pregnancy has also persistent effects on the offspring. Some of the consequences in adult life associated with intrauterine growth restriction (IUGR) and low birth weight (LBW) include hypertension, Type 2 diabetes, dyslipidaemia, insulin resistance, obesity, heart disease, stroke, renal failure, altered immune and gonadal responses, as well as anxiety, depression, psychoses and cognitive and behavioural problems [65, 73-80]. These long-term adverse effects on the well-being of the offspring are thought to be attributed to changes in the regulation of the offspring's HPA axis, with evidence from animal and human studies showing that pharmacological or physiological GC overexposure permanently alters the set point of the fetal HPA axis, predisposing the individuals to diseases later in life [78].

In addition to the inhibition of *in utero* fetal growth, the antenatal (occurring at the time of pregnancy) GC overload changes placental phenotype and size, with GC-treated placentae being friable and pale and weighing less compared with untreated controls. The changed placental phenotype resulted from inhibition of cell division and growth and

increased apoptosis (or programmed cell death) of the placental cells [81]. An interesting observation was reported by Newnham *et al.* (1999) who demonstrated that while maternal administration of a synthetic GC betamethasone caused IUGR, administration of repeated doses of betamethasone directly into the fetal circulation did not restrict fetal growth nor affected the placental to birth weight ratio [82], implying that pregnancy GC overexposure mediates adverse effects on fetal development in part by changing placental structure and therefore function. This notion was supported by several other studies that provided evidence that high doses of GCs administered to the mother during pregnancy led to marked changes in gene expression profile in the placenta including down-regulation of genes involved in cell division, growth, differentiation, protein biosynthesis and placental vascular development and up-regulation of genes involved in apoptosis [81, 83, 84]. Overall 1212 genes were down-regulated and 1382 genes were up-regulated in the placenta after treatment with GCs [81].

The activity of 11β-HSD2, which limits fetal and placental exposure to maternal GCs, is also suppressed by GCs [85, 86] allowing inappropriately high levels of GCs to transfer from the maternal to fetal compartments and inhibit fetal growth. This notion is supported by several investigations demonstrating an association between reduced 11β-HSD2 expression and/or activity and restricted fetal growth [87, 88]. Thus GC-induced morphological and functional changes in the placenta could be a plausible mechanism by which GC adversely affect optimal placental function and fetal growth and result in fetal programming of adult life disease. This GC-induced inhibition of cell growth promoting genes and stimulation of genes mediating apoptosis in the placenta may explain the association between high GC levels and reduced placental and fetal weight.

Furthermore, human male and female fetuses use different mechanisms to survive environmental adversities, including pregnancy complications (e.g., maternal asthma, pre-eclampsia, preterm delivery, etc.), stress and exposure to excess GCs. Research suggests that

in an adverse intrauterine environment the female fetus reduces growth to survive any further environmental challenges, while the male fetus continues to grow normally, placing itself at risk of compromise in the presence of a second adverse environmental event (e.g., reduced oxygen or nutrient supply) [89, 90]. Although these gender-specific adaptations to a poor *in utero* environment may seem to benefit the developing fetus for a short period of time, the negative consequences of these changes in the placental and fetal development in response to the suboptimal intrauterine environment are manifested later in life.

1.2.4 Gender-specific placental responses to excess GCs

Sex-specific effects of excess GCs during pregnancy on placental development have well been described in human and animal studies. In humans, GC treatment adversely affects placental vasculogenesis, with placentae from the male fetuses exhibiting a reduced fetal capillary length [91]. Glucocorticoid exposure alters the placental pro-oxidant : anti-oxidant balance sex-specifically, with emerging evidence linking antenatal GC treatment with an increased production of reactive oxygen species and decreased antioxidant levels in the placentae from male fetuses [92]. Animal studies likewise demonstrate gender-specific placental responses to GC treatment. In rodents, exposure to the synthetic GC dexamethasone (DEX) significantly reduces the expression of genes associated with nutrient delivery and fetal growth in the placentae from male fetuses only. Dexamethasone-treated spiny mice placentae of male fetuses exhibit significantly lower expression of glucose transporter *SCL2A1* (solute carrier family 2, (facilitated glucose transporter) member 1) compared to the placentae of female fetuses [93]. There is also a difference between male and female placentae in the expression of genes regulating syncytiotrophoblast differentiation and villous morphogenesis including glial cell missing homolog 1 (*GCM1*), the expression of which is significantly downregulated in the male fetus placentae but upregulated in the placentae of

female fetuses after GC treatment [93]. Overall, these data may imply that GC excess affects embryonic survival and fetal growth through the placenta in a gender-specific manner.

Moreover, there is a gender difference in placental cortisol metabolism, with female fetuses showing increased GC inactivation compared with male fetuses. Term female fetus placentae exhibit higher 11β-HSD2 activity relative to the placentas of male fetuses, suggesting augmented cortisol to cortisone conversion [94, 95]. Because a reduction in the activity of placental 11β-HSD2 is associated with increased fetal cortisol [59], reduced 11β-HSD2 activity in the placentae from male fetuses may result in greater male fetus exposure to maternal GCs and more prominent *in utero* alterations in physiological development than in female fetuses. These GC-induced sex-specific alterations in placental function may explain why males are 20% more likely to experience poorer survival rates than females, at least in the context of pregnancies complicated by preterm birth and IUGR [89, 96, 97].

The link between gestational GC excess and impaired fetal maturation is not well understood; however, GC-induced changes in the expression of specific transcription factors in the placenta, including glucocorticoid receptors (GRs), may explain this relationship.

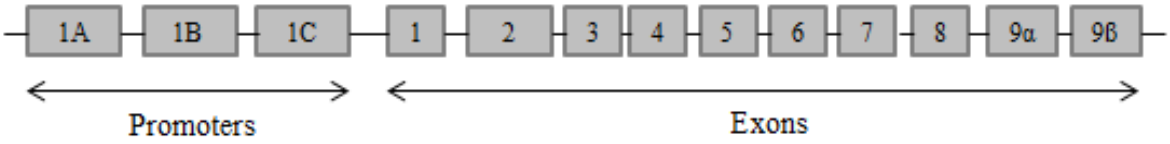
1.3 Glucocorticoid receptor (GR)

1.3.1 Human glucocorticoid receptor gene and domain structure of GR

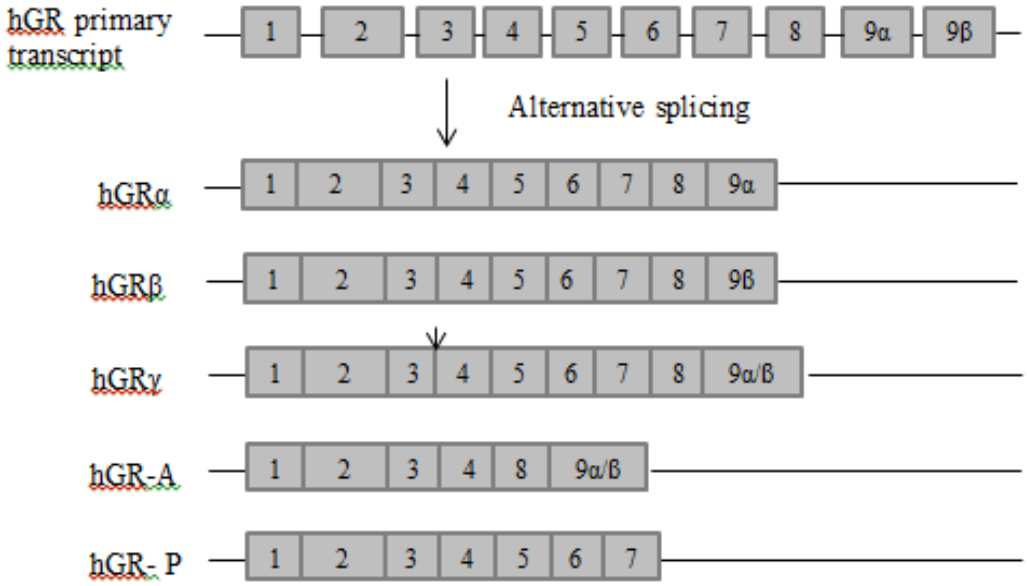
To discuss the GRs in the human placenta and understand how changes in placental GR expression affect placental and fetal development, the following sections explain the current understanding of the GR. The human GR protein, like other members of the nuclear receptor superfamily of transcription factor proteins, consists of three major functional domains: the N-terminal domain (NTD), the central DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD) [98] (Figure 1.1 (*a*)). The protein is encoded by the *NR3C1* (nuclear receptor subfamily 3, group C, member 1) gene, which consists of 10 exons including exons 1 → 8, 9α and 9β [99] (Figure 1.1 (b)). Exon 1 contains three different transcription initiation sites regulated by promoters 1A, 1B and 1C [100]. Alternative splicing of exon 1A containing three different splice donor sites gives rise to three untranslated exon 1A splice variants including exon 1A1, 1A2 and 1A3 [101]. The untranslated exon 1 can be spliced into 11 different promoter variants expressed in a tissue-specific manner to control the expression of GR protein [102]. Each of the first exon variants fuses to the same splice acceptor site in exon 2. Exon 2 encodes the NTD, exon 3 and 4 encode the DBD and exons 5-9 encode the hinge region (HR) and LBD. Furthermore, exons 2-9 represent the coding region of the GR protein and can generate different isoforms of the GR via alternative splicing or alternative translation initiation [103-106].

(*a*). hGR protein structure

(*b*). hGR gene structure



(*c*). hGR mRNA transcripts generated by alternative splicing



(*d*). hGR protein isoforms generated by alternative translation initiation

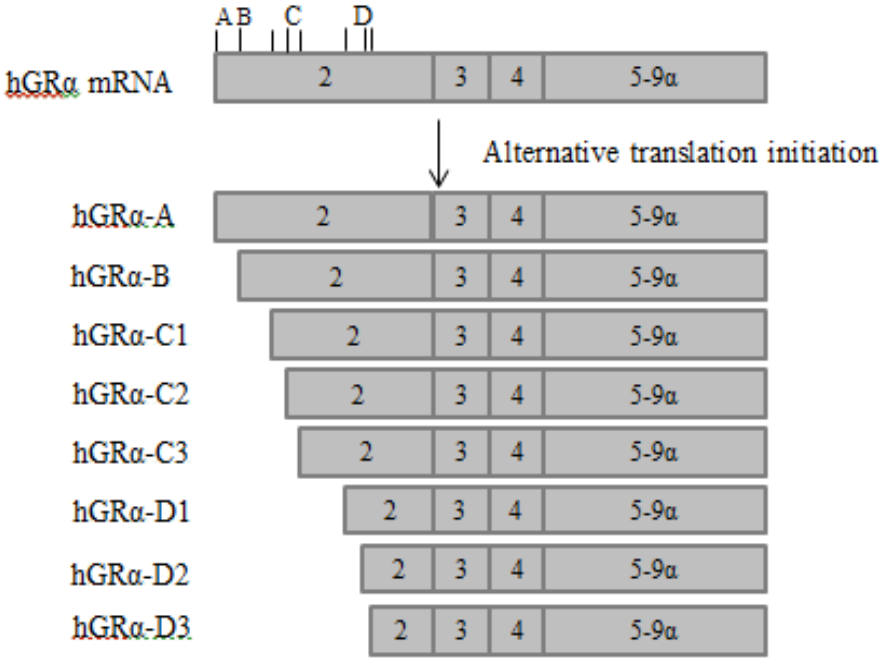


Figure 1. 1: Generation of multiple human glucocorticoid receptor (hGR) splice variants and protein isoforms from a single gene. (*a*) Structure of the hGR protein. hGR contains three functional domains: the N-terminal domain (NTD), the DNA-binding domain (DBD) and the C-terminal ligand-binding domain (LBD). The DBD and LBD are joined by a hinge region (HR). (*b*) Organization of the *hGR* gene. The *hGR* gene consists of a total of 10 exons (1 – 8, 9α and 9β) with promoters 1A, 1B and 1C located upstream of the *hGR* coding sequence. (*c*) hGR mRNA transcripts generated via alternative splicing. Alternative splicing in exon 9 of the hGR pre-mRNA generates two mRNAs coding for hGRα and hGRβ. Alternative splicing of other exons can also result: in hGRγ mRNA containing an arginine codon between exons 3 and 4 (↓); hGR-A mRNA in which exons 5-7 are missing; and hGR-P mRNA lacking exons 8 and 9. (*d*) hGR protein isoforms generated via alternative translation initiation. hGR mRNA can be translated from multiple translation initiation sites (vertical lines indicated as capital A, B, C and D), generating eight isoforms of hGRα with progressively shorter NTDs, namely hGRα-A, -B, -C1, -C2, -C3, -D1, -D2 and –D3 [99, 105].

1.3.2 Alternative splicing of human GR (hGR) pre-mRNA

The GRs display a remarkable heterogeneity as a result of two mechanisms, including alternative splicing of protein-coding exons and alternative translation initiation [107]. Alternative splicing of hGR pre-mRNA results in hGR transcripts such as hGRα, hGRβ, hGRγ, hGRA and hGRP, coding for hGRα, hGRβ, hGRγ, hGRA and hGRP proteins, respectively (Figure 1.1 (*c*)). The isoforms differ in terms of their relative intracellular abundance, location, GC binding and effect on gene expression.

Alternative splicing of exon 9 generates two mRNAs encoding hGRα and hGRβ proteins [99]. The proteins are identical up to amino acid 727 (encoded by exons 2-8) after which they diverge. The hGRα or predominant isoform is 777 amino acids long and is generated when exon 8 is joined to exon 9α. In hGRβ, the 50 C – terminal amino acids of hGRα (encoded by exon 9α) have been replaced by 15 distinct amino acids (encoded by exon 9β) resulting in a protein of 742 amino acids long. hGRα is highly expressed in almost every cell and tissue and resides in the cytoplasm [108]. Upon binding GCs, the activated hGRα translocates to the nucleus of cells to modulate the expression of GR-dependent genes. In contrast with hGRα, the relative intracellular levels of hGRβ are much lower than of the hGRα [108]. hGRβ isoform is primarily localized in the nucleus, it does not bind GCs and is transcriptionally inactive [109]. Although hGRβ does not directly regulate GC-responsive genes, it acts as a dominant inhibitor of hGRα-mediated transcriptional activity. When co-expressed, hGRβ modulates the transcriptional potential of hGRα through the following mechanisms including constitutively induced histone deacetylation, direct interaction with hGRα to form transcriptionally inactive hGRα - hGRβ heterodimer or by competing with hGRα for binding to the specific GREs and transcriptional co-regulators [107, 110-112].

In addition to hGRα and hGRβ transcripts, alternative splicing of other protein-coding exons results in hGR splice variants such as hGRγ, hGRA and hGRP encoding hGRγ, hGRA and hGRP proteins, respectively. hGRγ splice variant is formed when an additional arginine codon is inserted between exons 3 and 4 [113]. Similar to GRα, the gamma isoform of the GR also binds to GCs and DNA influencing the transcriptional activity of GC-responsive genes though it shows a transcriptional profile different from GRα on the genes commonly regulated by these receptors [107]. Despite the fact that hGRγ is widely expressed in various tissues at relatively high levels (4-8% of total GR), an insertion of an amino acid arginine in the region separating the two zinc-fingers in the DBD of the receptor, diminishes the transactivation potential of the hGRγ by 50% compared to the more abundant isoform hGRα [113, 114]. Moreover, in the hGRA exons 5, 6 and 7 are lacking whereas in the hGRP exons 8 and 9 are missing. Although little is known regarding the hGRγ, hGRA and hGRP splice variants, an increased expression of these transcripts has been associated with GC resistance and multiple pathological conditions including myeloma and childhood leukemia [115-118].

1.3.3 Alternative translation initiation of hGR mRNA generates N-terminal isoforms of hGRα

It has been reported that hGRα mRNA can be translated from eight alternative translation initiation sites within exon 2 ($AUG^{1, 27, 86, 90, 98, 316, 331}$ and ^{336}), generating eight alternative N-terminal isoforms of hGRα such as GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2 and GRα-D3 [105] (Figure 1.1 (*d*)). These isoforms control common and specific genes and differ in terms of their subcellular localization and transcriptional regulation in response to GC treatment.

Several mechanisms are utilized to generate the N-terminal GRα isoforms including leaky ribosomal scanning and ribosomal shunting. The classic hGRα (hGRα-A) is produced via conventional linear scanning of the ribosomes resulting in a peptide of 777 amino acids.

The hGRα-B is translated by leaky ribosomal scanning past the first AUG codon to start translation at the second AUG codon at the position 27. Three hGRα-C isoforms are generated via both leaky scanning and ribosomal shunting to start alternative translation from the translation initiation sites located at the amino acids 86, 90 and 98 producing hGRα-C1, hGRα-C2 and hGRα-C3 respectively [19]. The hGRα-D transcripts are translated by ribosomal shunting only (translation initiation sites: AUG^{316} for hGRα-D1, AUG^{331} for hGRα-D2 and AUG^{336} for hGRα-D3) [105].

Each N-terminal GR isoforms control common and unique sets of genes and show distinct cytoplasmic-to-nucleus patterns and transcriptional activities in response to GCs with the D isoforms localized primarily to the nucleus independent of GC treatment and displaying the lowest transcriptional activity [105]. In contrast to hGRα-D isoforms, the hGRα-A, -B and -C isoforms reside in the cytoplasm and translocate from the cytoplasm to the nucleus upon GC binding. Glucocorticoids increase transcriptional activity of each hGRα isoform in a dose-dependent manner with hGRα-C isoform displaying the highest transcriptional activity and hGRα-A and hGRα-B isoforms exhibiting intermediate levels of transcriptional activities *in vitro* [105]. Out of more than 2000 of genes the expression of which is modulated by GRs, 180 genes are commonly controlled by all the hGRα isoforms [105].

1.3.4 Classic mechanism of GC actions – role of GRα in gene transcription

In order to determine the molecular pathways by which GCs overexposure induces pathological changes in a cell, it is important to understand the mode of GC action. After being synthesized and released from the adrenal gland, GC hormones are transported through the blood to target tissues by carrier proteins. Being highly lipophilic in nature, GCs freely cross the cell membrane and interact with mineralocorticoid and glucocorticoid receptors (MR and GR, respectively), the cytoplasmic ligand-dependent transcription factors. Free or

unoccupied MR and GR are held in the cytoplasm by associated proteins which hold the inactive receptors in a state of high affinity for their cognate ligands. Upon ligand binding, the activated MR and GR translocate to the nucleus to modulate the expression of mineralocorticoid- and GC-dependent genes (respectively), though some evidence suggests that MR and GR might directly interact with each other in the regulation of transcriptional initiation in cells that express both receptors [119]. Of interest to this study is the role of GR in regulating gene transcription. Thus, the following sections describe the mechanisms by which the most studied isoform of the GR, the hGRα, influences the transcriptional activity of target genes.

Prior to ligand binding, the hGRα (94 kDa protein), resides in the cytoplasm as a multi-protein complex consisted of several Hsp90 and immunophilins, cytoplasmic chaperones as well as protein kinases which ensure cytoplasmic retention of hGRα [120-123]. Following ligand binding, the activated receptor undergoes a conformational change, sheds most of the associated proteins and translocates to the nucleus where it dimerizes and modulates the transcription of target genes. In the nucleus, the hGRα binds to either positive or negative glucocorticoid response elements (GREs) within the promoter region of target genes resulting in up-regulation or down-regulation of gene expression, respectively. The recognition site of GREs to which the activated GR binds is a palindromic sequence of 15 base pairs – GGTACANNTGTTCT [124]. Several positive GREs have been identified in the promoters of genes mediating anti-inflammatory responses of GCs including lipocortin-1, IL-10, IL-1 receptor antagonist and mitogen-activated protein kinase (MAPK) [125]. The genes containing negative GREs include bovine prolactin, human osteocalcin, type 1 vasoactive intestinal polypeptide (VIP) receptor, human corticotropic-releasing hormone (CRH) and neuronal serotonin receptor 5-HT1A [126-130]. Although binding of hGRα to the negative GREs results in inhibition of transcription of specific genes, the main negative effect

on gene expression is mediated via protein-protein interaction and involves physical association of hGRα with other transcription factors in the nucleus including activator protein (AP1), nuclear factor κB (NF-κB), Sma Mad-related protein (Smad), cAMP response element-binding protein as well as signal transducers and activators of transcription (STAT) involved in many aspects of growth, survival and cell differentiation [16, 131-135]. Furthermore, the DNA-bound GR modulates transcription of target genes by recruiting a variety of co-factors including co-activator proteins and co-repressors. In the first instance, the interaction of the GR with co-activators CBP, SRC-1, TIF-2, p300/CBP co-integrator protein or GRIP-1 results in transcriptional up-regulation of GR-dependent genes through chromatin remodeling [136-138]. The second instance of GR-mediated modulation of transcriptional activity involves binding of the GR to the nuclear receptor co-repressor (NCoR) or silencing mediator of retinoid and thyroid hormone receptor (SMRT) which leads to transcriptional down-regulation of the GR-responsive genes [139, 140].

1.3.5 Modes of regulation of GR activity

Multiple post-translational mechanisms are utilized to modify the function of the hGR including phosphorylation, ubiquitination, SUMOylation, acetylation, and methylation. These mechanisms play role in the receptor's subcellular distribution, degradation, signaling pathways, transcriptional potential and interaction with other proteins.

The hGR can be phosphorylated at five specific serine sites within AF-1 region of the N-terminal domain including at serines 113, 141, 203, 211 and 226 (S113, S141, S203, S211 and S226) [141]. Serines 203, 211 and 226 are three major phosphorylation sites involved in transcriptional regulation. The phosphorylation sites are usually followed by a proline recognized by mitogen protein kinases (MAPKs), glycogen synthase kinase-3 (GSK-3), c-Jun N-terminal kinases (JNK) and cyclin-dependent kinases (CDKs), mediating the

phosphorylation of the receptor [142, 143]. Only phosphorylated GRs are able to bind ligands and are protected from being degraded [144]. The phosphorylation status of individual serine residues modifies the GR turnover, subcellular localization, cofactor interaction, strength and duration of receptor signaling or target gene activity. While, in the absence of a ligand, cytoplasmic GRs are phosphorylated at either S203 or S211, in the presence of a ligand, GRs are hyperphosphorylated either at both S203 and S211 or at S203 or S211 alone. Interestingly, GRs that are phosphorylated at both S203 and S211 or at S203 alone remain in the cytoplasm whereas GRs that are phosphorylated only at S211 translocate from the cytoplasm to the nucleus and affect target-gene activity [144, 145].

Glucocorticoid signaling pathways can be also regulated by ubiquitination and SUMOylation. Ubiquitination controls the degradation rates of the GR and involves covalent attachment of multiple ubiquitin molecules at lysine residues within the NTD of the GR thus marking the GR for degradation by a multi-protein complex, proteasome [146, 147]. SUMOylation is another post-translational process similar to ubiquitination but involves ligation of a small ubiquitin-related modifier (SUMO) at lysine sites in the GR. Three SUMO attachment sites have been identified: two in the N-terminal transactivation region and one in the LBD [148]. In contrast to ubiquitination which results in proteasome-mediated target protein turnover, SUMOylation regulates protein-protein and protein-DNA interactions as well as subcellular localization, stability and transcriptional activity of target proteins [149, 150].

In addition to phosphorylation, ubiquitination and SUMOylation, protein function is influenced by acetylation and methylation. As described above, the GR signaling depends on GR interaction with various proteins including chaperones, co-regulators and transcription factors. The actions of these proteins can be modified by acetylation involving an addition of an acetyl group to lysine residues within target proteins. Early studies suggest that Hsp90

chaperone activity is regulated by acetylation resulting in a GR that is defective in ligand binding, nuclear translocation and transcriptional potential [151, 152]. Likewise, GR itself is acetylated within its hinge region by the circadian rhythm-related transcription factor Clock [153] leading to Clock-induce repression of the GR actions in target tissues. GR signaling is also modulated through methylation of the GR co-activators including p300 leading to inhibition of the interaction between p300 and GRIP-1 [154] and alteration in the activity of GR-dependent genes. While post-translational modes of regulation of the GR signaling pathways have been recognized, precisely how these post-translational mechanisms affect the GR activity, stability, subcellular localization and interaction of each isoform of the GR with other proteins has received little attention to date.

1.3.6 Glucocorticoid effect on GR mRNA and protein expression

Multiple studies in cultured human and animal cells have revealed that the GR mRNA abundance and protein levels are affected by treatment with endogenous or exogenous GCs [155-159]. Although transient exposure to elevated natural or synthetic GCs is associated with a temporal reduction in GR mRNA and protein expression in a time- and dose-dependent manner, persistent aberrantly high GC levels lead to permanent alterations in intracellular distribution of GR mRNA and protein [158], which may subsequently impair essential processes required for proper cellular functioning and development. Studies conducted in several cell types have demonstrated that long-term treatment with GCs results in downregulation of the GR [158, 160, 161]. Downregulation of the GR is dependent on the concentration of the GC as well as on the duration of GC treatment. For example, treatment of HeLa S3 cells with medium containing 1 nM of a synthetic GC, dexamethasone (DEX), results in a 40% reduction in GR protein levels, whereas a treatment with 1 μM of DEX leads to a pronounced 70% GR downregulation [158]. A relatively short-term treatment (24

or 48 hours) of HeLa S3 cells with 1 μM DEX downregulates both GR mRNA and protein, with the expression of the GR protein more affected than that of the GR transcript. Although 2 weeks treatment of HeLa S3 cells with DEX shows maximal downregulation of receptor protein and mRNA, chronically DEX-treated (for up to 2 years) HeLa S3 cells show no detectable GR protein or mRNA [162]. It worth noting, that the down-regulation of GR mRNA and protein that occurred after treatment with DEX for 2 weeks was completely reversible upon DEX removal, while in chronically treated cells the levels of both GR mRNA and protein did not return to normal levels when DEX treatment ceased [162]. This finding may indicate that chronic exposure to high GCs may lead to a population of cells with a reduced GR abundance or even completely lacking GRs and therefore insensitive to GC signals.

Thus, there is evidence that GCs induce downregulation of the GR mRNA and protein levels, but the isoforms of the GR affected following treatment with GCs *in vitro* have not been identified in these investigations. Despite the fact that numerous studies in cultured cells from various species demonstrated GC-induced reduction in the GRs at both the mRNA and protein levels, research on the effect of GCs on GR levels in human placental cells and tissues is limited and suggests that the expression of GRs is dependent on cell type, GC treatment and fetal gender, with female fetuses becoming hypersensitive to changes in GC levels whereas male fetuses develop GC resistance when exposed to prolonged elevated GCs levels [163]. The mechanism by which male fetuses develop GC insensitivity in the environment of GC excess in unknown, but changes in ratios among GR isoforms could be a contributing factor of gender-specific differences in the fetal-placental response to cortisol.

1.4 Glucocorticoid receptors in human placenta

Human placental cells are GC-responsive cells expressing GRs and MRs [164-168]. Although GRs are expressed in most fetal tissues, fetal membranes and in the human placenta from the early embryonic stages [169], the expression of MRs is more limited and present only during the later stages of fetal development, at least in animal models [170]. Because of the limited data on the role of MRs in the human placenta, the following sections focus primarily on GRs in the placentas of human fetuses and the effects of GC exposure on the expression of these receptors.

In the context of pregnancy, research reveals that the transcription and regulation of placental GR are cell-type specific and depends on GC treatment and gender. Lee *et al.* (2005) examined the expression of total and phosphorylated (p-) GR in human smooth muscle cells, fibroblasts and trophoblasts in the placentas obtained from uncomplicated full-term pregnancies [167]. Lee *et al.* (2005) found that cultured placental fibroblasts expressed relatively high levels of both total GR and p-GR compared with placental trophoblasts and smooth muscle cells [167]. Moreover, short-term (2 hours) treatment with 100 nM DEX resulted in an increased phosphorylation of GR and subsequent GR transactivation in placental fibroblasts, whereas long-term (96 hours) DEX exposure led to a nearly 90% reduction in the GR levels independent of changes in GR mRNA abundance [167], suggesting downregulation in GR protein expression in fibroblasts. A reduction in GR protein levels could be the result of ligand-induced homologous downregulation of the GR, a mechanism though which cells regulate GC responsiveness [171, 172]. Although the mechanism of homologous downregulation is not well understood, downregulation at the GR mRNA and protein levels has been proposed, including repression of the activity of the GR promoter, decreased stability of the GR mRNA and increased proteasome-mediated degradation of GR [166, 171, 173-175].

Furthermore, Johnson *et al*. (2008) investigated the expression of GR mRNA transcripts in human placenta in relation to fetal sex and GC treatment and found that women delivering a female fetus had significantly lower GR-1A3 mRNA levels compared with women carrying a male fetus, implying that the use of 1A3 promoter of the untranslated exon 1 of hGR may play a role in modifying GC responsiveness and explain differential responses of placentas associated with fetal gender. Johnson *et al*. (2008) also reported a significant decrease in GR-1B and -1C promoter-derived transcripts, as well as mRNA levels of GR-α and GRP after treatment with GCs [176]. These findings may imply that differential promoter use could be the factor determining the composition and proportion of individual GR isoforms and regulating the way placental tissues respond to changes in GC levels during pregnancy. Because research suggests that GRP may have been involved in upregulation of GRα-mediated transcriptional activity [177], a significant reduction in GRP mRNA may decrease the responsiveness of the placenta to GCs and increase the risk of adverse fetal outcomes.

Moreover, a recent study by Saif *et al*. (2014) examined whether the human placenta expresses different forms of the GR and whether the expression of the placental GR isoforms was affected by maternal asthma, fetal sex and birthweight. Twelve protein bands corresponding to various GR isoforms have been identified in placental trophoblasts, including bands at 94, 91, 81, 74, 69, 68, 65, 60, 55, 50, 48 and 38 kDa. The bands at 94, 91, 81, 74, 65 and 50-55 kDa correspond to known isoforms of the GR, namely GRα-A, GRα-B, GRα-C, GRP, GRA and GRα D1-D3 respectively [163]. The 69, 68, 60, 48 and 38 kDa proteins represent putative GRs that have not been reported previously, although this finding warrants further investigation to confirm the results. Interestingly, the isoforms of the GR differed in terms of subcellular localization, the gender of the fetus, fetal weight at birth and the presence and absence of maternal asthma. Both males and females had greater expression

of GRα in the nucleus than in the cytoplasm, but in male fetus placentae, there was a greater expression of GRP and GRA in the nucleus than in the cytoplasm compared with placentas from female fetuses [163]. Although there was no relationship between cord blood cortisol levels and the expression of the GR isoforms in female placentae, an increase in cortisol in umbilical cord blood was associated with augmented expression and translocation of GRβ to the nucleus in male placentas and in placentas of small-for-gestational-age (SGA) infants [163]. Because GRβ exerts a dominant negative effect on GRα function [110, 178, 179], GC-induced localization of the β isoform of the GR to the nucleus may have contributed to the modulation of GC responsiveness in male and SGA fetuses. These data may indicate that augmented expression and localization of GR-β, GRA and GRP to the nucleus could be the factors that modify GRα-mediated specific biological responses and actions of GCs on target tissues in male fetuses.

Finally, the importance of the GRs during fetal development has been demonstrated in rodents, with GR-null mice dying within a few hours after birth due to severe respiratory failure [180]. Similarly, transgenic mice with a reduction in tissue levels of GRs exhibited significant neuroendocrine, metabolic and immunological abnormalities [181]. These data reveal that the development of a healthy fetus is dependent on the level of expression of GRs. Because GC excess affects the relative abundance and subcellular localization of the GRs, the intracellular transcription factors controlling the expression of 10%-20% of genes in the human genome [182], GC-induced changes in GR levels in the placenta would also result in modification in the expression of a great number of placental genes crucial for sustaining fetal life. Thus GC-mediated changes in the ratios and intracellular localization of the GR isoforms in the placenta could be a plausible mechanism by which GC excess mediates adverse effects on the placenta and developing fetus.

1.5 Mitochondrial glucocorticoid receptors

1.5.1 Mitochondrial structure and function

Much effort has been devoted to clarifying the subcellular distribution of the GR; however, the findings of these studies are contradictory, possibly due to differences in experimental conditions, cell types and stages of cell cycle. Some researchers believe that GR resides predominantly in the cytoplasm and translocates to the nucleus upon ligand binding to influence the transcription of nuclear genes [183]. Other investigators showed a prevailing nuclear localization of the GR or both, a nuclear and cytoplasmic distribution [184]. Mitochondrial research also revealed that the GR continuously shuttles between the cytoplasm and the nucleus [185, 186] or cytoplasm and mitochondria as confirmed by *in vivo* studies on the intracellular distribution of the GR in rat liver [187, 188] and *in vitro* investigations in reticulocyte lysates [189].

GRs, through which GC hormones regulate mitochondrial function, have been detected in mitochondria of animal and human cells including rat liver [189], Muller cells of salamander retina [190], rat brain [191], hepatocarcinoma HepG2 and SaOS-2 osteosarcoma cells [192], C6 glioma cells [193], HeLa and Hep-2 cells [194]. The mechanisms by which GRs are imported into mitochondria and their role in modulation of mitochondrial processes are poorly understood; however, the presence of the GRs in the mitochondria and of the nucleotide sequences in the mitochondrial genome with a high similarity to known nuclear GREs suggested a direct action of the GR on the mitochondrial gene transcription [195].

Mitochondria are essential membrane-bound intracellular structures found in virtually all eukaryotic cells. Mitochondria have outer and inner membranes encapsulating mitochondrial DNA (mtDNA) which is expressed and replicated by nuclear-encoded transcription factors imported into the organelles from the cytoplasm. In humans, maternally-

inherited mtDNA consists of 37 genes encoding 13 proteins of the mitochondrial respiratory chain and 24 RNAs, including two ribosomal RNAs (rRNAs) and 22 transfer RNAs (tRNAs) required for the translation of mitochondrial proteins [196, 197]. These fascinating organelles generate most of the energy of the cell in the form of adenosine triphosphate (ATP) via oxidative phosphorylation (OXPHOS), carry out metabolism, nucleotide biosynthesis, cell differentiation, calcium homeostasis, generate and release reactive oxygen species (ROS), control cellular redox status and transcription initiation, coordinate ribosomal biosynthesis and coupling of transcription to protein translation [196, 198, 199]. The molecular pathways underlying the GR-regulated mitochondrial transcription are poorly understood; however, a direct binding of the mitochondrially localized GR to the mtDNA and activation of the nuclear-encoded mitochondrial transcription factors which translocate to mitochondria to induce the transcription of mtDNA and biosynthesis of OXPHOS enzymes have been proposed [195, 200]. In addition to remodeling their structure and dynamics as a sensor of their quality control, mitochondria are also known for their role in regulating bioenergetic efficiency, energy expenditure and programmed cell death or apoptosis [201, 202]. These mitochondrial-regulated processes are controlled by steroid hormones, including GCs possibly by the way of their cognate mitochondrial GRs (mtGRs) which translocate from the cytoplasm into the mitochondria following GC treatment [203]. Interestingly, GR translocation to the mitochondria correlates with the sensitivity of cells to GC-induced apoptosis [204].

1.5.2 Mitochondria-induced apoptosis – role of glucocorticoid receptors

Apoptosis, or programmed cell death, is a highly coordinated process during which a dying or damaged cell is engulfed by the neighboring cells of the immune system. Apoptosis is characterized by distinctive morphological and molecular changes such as chromatin

condensation, nuclear fragmentation and plasma membrane blebbing, with the formation of apoptotic bodies [205]. It plays a central role in maintaining normal cellular and embryonic development and homeostasis of multicellular organisms with defects in apoptosis linked to autoimmune disorders, neurodegenerative disease, ischaemic damage and many types of cancer [206-208]. In the context of pregnancy, placental apoptosis is an essential feature of the normal development of placental cells. As the normal gestation advances, apoptosis of trophoblast cells increases. However, in pregnancies complicated by IUGR (one of the pregnancy complications associated with GC excess) trophoblast cell death is notably exaggerated impairing trophoblast function [209].

Apoptosis can be induced via an intrinsic [210] or extrinsic pathway [211], with the former signaling pathway initiated by events in the mitochondria. The apoptotic pathways in mitochondria can be triggered by multiple intracellular stress conditions, including but not limited to DNA damage, cytosolic Ca^{+2} overload, oxidative stress, accumulation of unfolded proteins in the endoplasmic reticulum, excitotoxicity [212] as well as GC treatment. It is worth noting that GC-induced apoptosis is associated with the GR translocation to the mitochondria, but not to the nucleus [204, 213]. The mechanisms by which GCs induce apoptosis remain to be elucidated; however, several biochemical changes occurring following GC treatment have been characterized and include the release of cytochrome C from the mitochondria into the cytoplasm, formation of apoptosome and activation of caspases (cysteinyl aspartate-specific proteinases) [214, 215]. There two known types of caspases: the initiator caspases and the effector or executioner caspases. The former include caspases-2, -8, -9, -10 and the later comprise caspases - 3, - 6 and - 7 [216, 217]. The initiator caspases activate the effector caspases that in turn initiate cell destruction (Refer to Figure 1.2 for the intrinsic pathway of apoptosis).

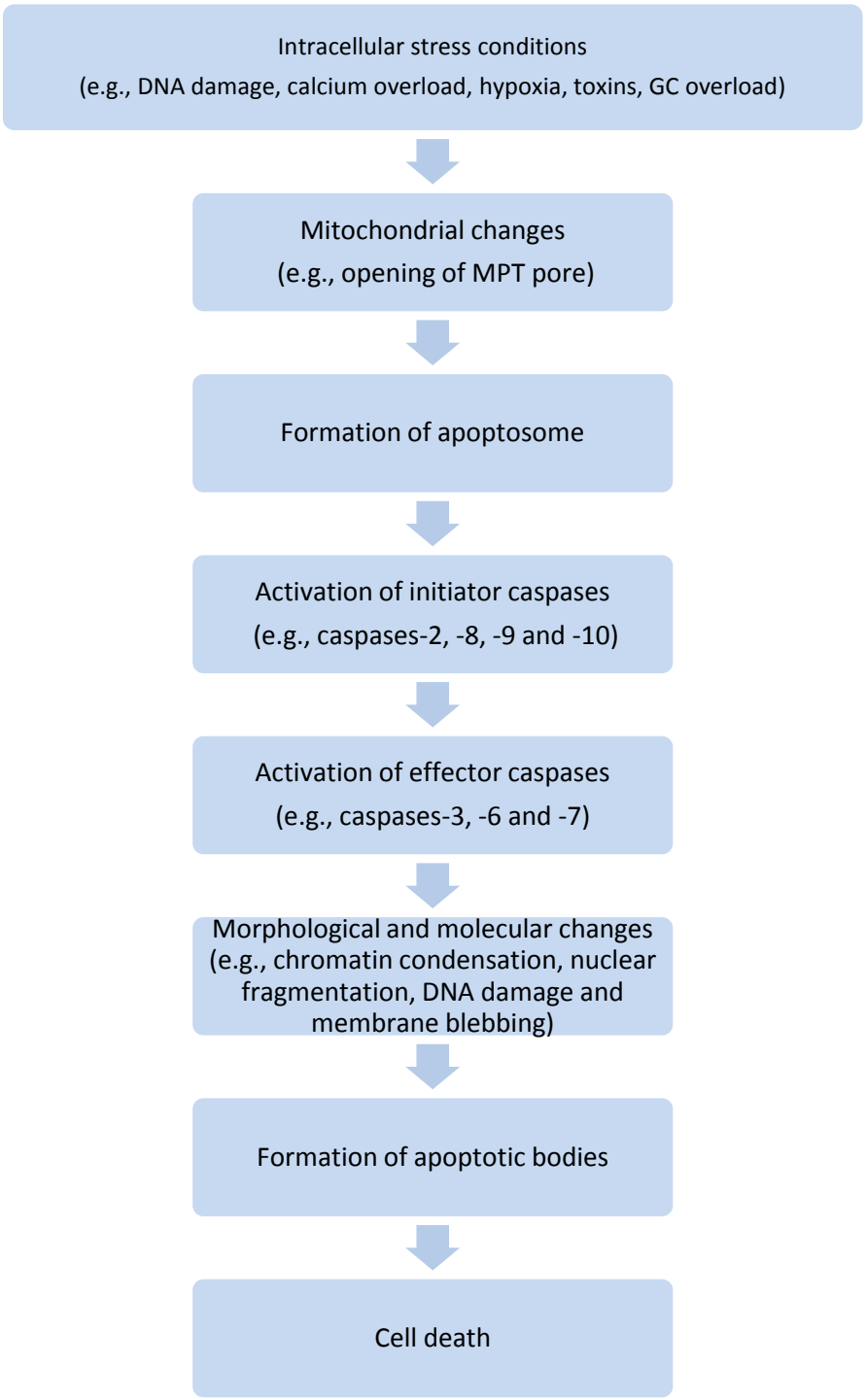


Figure 1. 2: Simplified diagram of the intrinsic pathway of apoptosis. Intracellular stress stimuli damage mitochondria leading to the formation of mitochondrial permeability transition (MPT) pore, release of cytochrome C from the mitochondria into the cytosol and formation of a large protein structure called apoptosome. The apoptosome recruits the initiator caspases (caspases-2, -8, -9 and -10) that activate the effector caspases (caspases-3, -6 and -7) leading to morphological and molecular changes followed by the formation of apoptotic bodies and subsequently cell death

It has been suggested that the effector caspases - 3 and - 6 are upregulated following treatment with GCs [218-220], with caspase-3 being the key effector caspase in GC-mediated apoptosis [221, 222]. The GR receptors, through which GCs mediate their intracellular apoptotic signaling, are GRα-A, -B, -C and -D, with the GRα-C isoform being more efficient in inducing apoptosis compared with other GR isoforms [223]. The current knowledge on the GRs in mitochondria and their potential roles is limited, with no investigations up to date examining whether GRs are present in the mitochondria of human trophoblast cells and whether the expression of mitochondrial GRs varies depending on gestational age, fetal gender and GC levels. Because mitochondria regulate life and death, possibly through the GRs, it would be interesting to evaluate and compare the expression of caspases -3 and -6 along with mitochondrial GRs in the placentas of full-term and early-term babies a) to find out whether there are any differences in the expression of mitochondrial GRs, caspases – 3 and - 6, and b) to determine if the differences are related to cortisol concentrations, fetal sex and age at birth.

1.6 Serotonin system

1.6.1 Serotonin metabolism and regulation

There is convincing evidence that GCs control serotonergic system including serotonin synthesis and degradation, and therefore signaling. To understand how GCs dysregulate the serotonin-mediated transmission at the molecular levels, it is important to firstly review serotonin metabolism and regulation.

Serotonin (5-hydroxytryptamine, 5-HT) is synthesized from the essential amino acid tryptophan obtained only through food and supplements (eg., grains, meat and dairy products). The name reflects the discovery of serotonin in the serum and its ability to increase blood vessel tone [224]. The monoamine is metabolized in both the central nervous system

(CNS) and periphery by the enzymes tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (AAAD) [225] (Figure 1.3). In the brain, serotonergic neurons are found in the raphe nuclei of the brainstem and project to most brain areas releasing serotonin widely throughout the brain [226]. In the periphery, 95% of serotonin is synthesized and stored in the gut within enterochromaffin cells [227], with insignificant levels of serotonin released into the bloodstream and stored in platelets [228]. Once synthesized and released into the circulation serotonin interacts with its membrane-bound receptors to exert physiological responses. There are currently 7 distinct serotonin receptor families (5-HT1 - 5-HT7) classified further into 16 serotonin receptor subtypes according to their pharmacological and structural properties, including 5-HT_{1A}, 5-HT_{1B}, 5-HT_{1D}, 5-HT_{1E}, 5-HT_{1F}, 5-HT_{2A}, 5-HT_{2B}, 5-HT_{2C}, 5-HT_{3A}, 5-HT_{3B}, 5-HT_{3C}, 5-HT_{4}, 5-HT_{5A}, 5-HT_{5B}, 5-HT_{6}, 5-HT_{7}. The 5-HT_{3} receptors are ligand-gated ion channels and to date only 5-HT_{3} receptors form a homomer of 5 subunits, with the 5-HT_{3A} subunits creating heteromeric complexes with 5-HT_{3B}, 5-HT_{3C}, 5-HT_{3D} or 5-HT_{3C} subunits [229]. The receptors are found in the CNS as well as in the periphery including gut, CV and reproductive system, blood vessels and placenta [229].

In the CNS, serotonin controls mood, behavior, appetite, pain sensitivity, body temperature, sleep, learning and hormone release [227]. Outside of the CNS, serotonin plays a variety of roles including control of CV responses, vomiting, peristalsis, immune signaling, platelet function as well as vasoconstriction and vasodilation of blood vessels [227]. Whereas the vasoconstrictive effect of serotonin is mediated via 5-HT_{1B} or 5-HT_{2A} receptors, the vasodilatory action of 5-HT is mediated through the interaction with 5-HT_{1A} or 5-HT_{2B} receptors [229, 230]. The action of serotonin is terminated by serotonin transporter (SERT), a membrane-bound protein responsible for the active transport of serotonin from the extracellular space back into the cells. Once serotonin is back in the cell, it is either reloaded into cellular vesicles or degraded by the mitochondrial outer membrane enzyme monoamine oxidase A (MAO-A), with the generation of reactive oxygen species as a product of its catalytic activity.

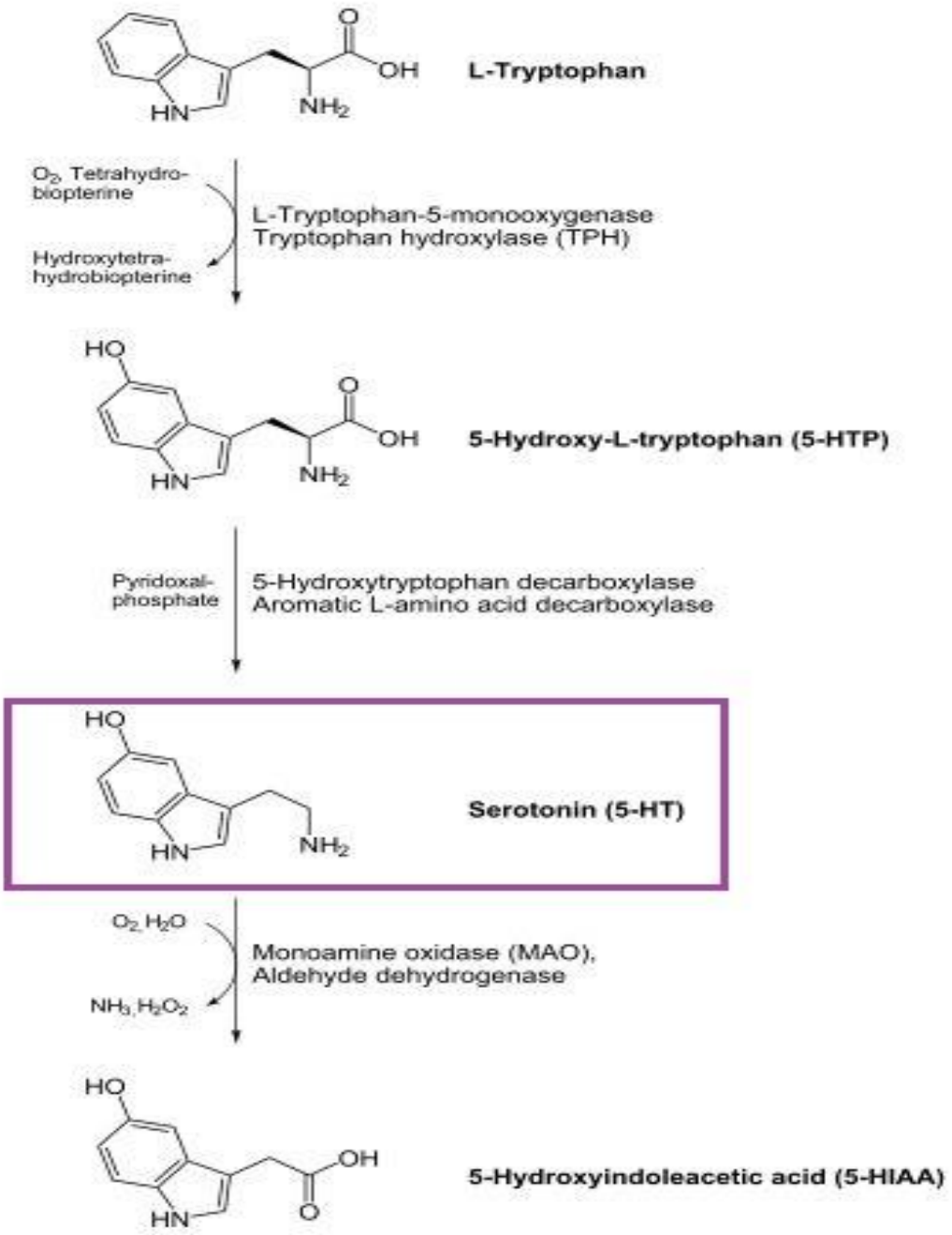


Figure 1. 3: Serotonin synthesis and degradation pathway. Serotonin is synthesized from L-tryptophan. L-tryptophan-5-monooxygenase tryptophan hydroxylase (TPH) catalyzes the conversion of L-tryptophan to 5-hydroxy-L-tryptophan (5-HTP) which is subsequently decarboxylated to serotonin by 5-hydroxytryptophan decarboxylase aromatic L-amino acid decarboxylase. Monoamine oxidase (MAO) and aldehyde dehydrogenase degrade serotonin into 5-hydroxyindole acetic acid (5HIAA).

1.6.2 Serotonin and pregnancy – effects on fetal and placental development and long-term consequences

In addition to regulation of a wide range of cellular processes including cell differentiation, cell proliferation, gene transcription, apoptosis and cell survival, serotonin acts as a developmental signal in early embryogenesis and controls fetal development and pregnancy outcome [231, 232]. Normal human pregnancy is accompanied by high levels of 5-HT [233]. In mice, serotonin and its receptors appear early during development and affect fetal neural crest migration, neuronal connectivity, craniofacial and limb development, gastrointestinal, CV and humoral morphogenesis [234]. Fetal serotonin is either of maternal or placental origin, with placental serotonin synthesized from the maternal tryptophan being the major determinant of fetal brain development, at least in murine models [235]. In the fetal brain, serotonin is responsible for cell migration, proliferation and maturation, axon growth and wiring [236]. In human placental trophoblast cells, it acts as a potent mitogen increasing cell viability, affecting cell cycle progression, stimulating placental cell proliferation and placentation via 5-HT_{2A} receptors [237, 238]. In addition to having a mitogenic effect on placental cells via the placental 5-HT_{2A} receptors, serotonin may play roles in pregnancy maintenance and regulation of placental and fetal development via 5-HT_{1A} receptors [239]. Although serotonin-induced activation of 5-HT_{1B} and 5-HT_{2A} receptors results in vasoconstriction of blood vessels, whereas stimulation of 5-HT_{1A} and 5-HT_{2B} receptors by serotonin is associated with vasodilation blood vessels [229, 230, 240, 241], the serotonin receptor subtype in placental blood vessels remains to be identified.

Given the pivotal role serotonin plays in the placental and fetal development, several studies have investigated the molecular mechanisms linking the aberrant serotonergic signaling with pregnancy complications, altered placental function and long-term developmental and behavioral abnormalities in the offspring. Viau *et al.* (2009) reported a

significant decrease in the expression of both 5-HT_{2A} receptors and SERT in human placental tissues from pregnancies complicated by gestational diabetes mellitus (GDM) in comparison with non-GDM women, implying that 5-HT_{2A} receptors and SERT may be implicated in the etiology of this pregnancy complication [242]. Likewise, hyperserotonergic conditions with altered serotonin metabolism have been suggested to play role in the pathophysiology of preeclampsia, the most common complication of pregnancy and the major cause of perinatal morbidity and mortality [243]. Recent data support the notion that disruption of serotonin signaling, and a concomitant reduction in serotonin levels during critical periods in fetal development (first trimester in mice and early second trimester in humans), negatively modulate the developmental processes including wiring of major axon pathways, cell division and laminar organization of the neocortex [244-247]. Moreover, in animal models, disruption of serotonin system function during the critical period of fetal brain development leads to behavioral abnormalities throughout the life, while in humans perturbations in serotonin signaling are associated with behavioral and neuropsychiatric conditions such as anxiety, depression, aggression and Autism Spectrum Disorders [248-252]. Although serotonin is a modulator of placental and fetal development, at high concentrations during embryogenesis it may cause congenital abnormalities or kill the fetus, possibly by constricting the uteroplacental blood vessels and reducing the supply of nutrients to the developing embryo [253]. Thus, there is evidence that dysfunction of serotonin metabolism during development (too much serotonin or not enough), is associated with pregnancy complications with negative effects on both, the mother and fetus. Because the placenta is an essential source of serotonin (an important regulator of embryogenesis), it is important to identify maternal conditions/factors that alter placental serotonin availability during gestation. It must be noted, that GCs can play role in the modulation of serotonin-mediated pathways.

1.6.3 Modulation of serotonin signaling by glucocorticoids

Although GCs control a large number of human genes (10%-20%), little is currently known about the GC-mediated control of serotonergic signaling. Accumulating evidence points out that MAO-A, located on the outer membrane of the mitochondria and responsible for the degradation of serotonin, is one of the major target genes for GCs. In human skeletal muscle myocytes, GC treatment upregulates MAO-A catalytic activity along with MAO-A mRNA expression and protein synthesis [254]. Likewise, in human cell lines, MAO-A gene expression significantly increases in a DNA-dependent and DNA-independent manner upon treatment with GCs [255]. An interesting finding has been also reported by Li *et al.* (2006) who investigated a correlation between GCs and serotonin in the liver and intra-abdominal adipose tissue of rats after DEX treatment. In *Li et al.* (2006) investigation GC treatment upregulated the expression of 5-HT_{2A} receptors and enzymes involved in serotonin synthesis, including TPH1 and AAAD. Moreover, an increase in the expression of serotonin synthesizing enzymes was accompanied by an increase in serotonin levels in a dose- and time-dependent manner [256]. Up to date, there is no consensus on the effect of GCs on the expression of SERT, the primary mechanism for the termination of the serotonin action after its synthesis and release into the circulation. Several studies documented that synthetic GCs increase the expression of SERT thus elevating the uptake of serotonin into the cells and attenuating its biological actions [257, 258]. By contrast, other stated decreased serotonin reuptake as a result of reduced SERT levels as seen in a mouse model of prenatal stress and associated GC excess [259]. Although, research over the last decade provided significant information on GC-induced changes in serotonergic signaling, a clearly defined pathway by which GC modulates serotonin system in the human placenta has not been investigated.

1.7 Summary

Understanding healthy placental and fetal development is crucial in order to detect abnormal developmental trajectories. Current knowledge on the effect GC-excess on pregnancy outcomes derives largely from studies with premature infants (born before 37 completed weeks of pregnancies), demonstrating that preterm babies have lower than normal birth weight and altered placental structure and function. However, research over the past several years shows that every week of gestation matters for the health of newborns, particular the last two weeks of pregnancy during which there is the maximal development of fetal organs and organ systems [46, 260]. Whether there is a difference in placental structure and function in early-term infants (born between 37 weeks 0 days and 38 weeks 6 days) compared with full-term infants (born between 39 weeks 0 days and 40 weeks 6 days), or whether this difference is attributed to cortisol overload, has not been investigated. Due to gaps in knowledge on the relationship between GC-induced alteration in the placental phenotype and adverse pregnancy outcomes, this doctoral thesis will investigate glucocorticoid and serotonergic systems in the placentas from early-term and full-term infants to determine the plausible molecular mechanisms by which GCs alter placental and fetal development. Since the placenta is not often investigated, a better understanding of it would prevent GC excess associated placental dysfunction, pregnancy complications and fetal prematurity with a structural and functional abnormality in fetal organs and organ systems; and improve pregnancy outcomes and lifelong health of the future generations.

General Aims of the Project

- Current knowledge on the distribution of GRs in different subcellular compartments and the effect of fetal sex on GR ratios in the human placenta is limited, with no studies so far examining mitochondrial GRs. Therefore, GR profile will be a) identified in mitochondrial, cytoplasmic and nuclear fractions and b) evaluated in relation to fetal gender.
- Serotonin and serotonin system components in the placenta including SERT, TPH1, MAO-A, 5-HT_{1A} and 5-HT_{2A} receptors play crucial role during pregnancy, with alterations in serotonin signalling associated with pregnancy complications and abnormal fetal development. However, data on the distribution of the components of the serotonin system in human placenta are scarce. The expression of the components of serotonin system will be explored in the human placenta to determine whether early-term births are related to significant changes in the expression of these factors.
- The importance of serotonin regulation and signalling in the placenta and fetus has been acknowledged; however, to date there have been no investigations conducted to measure serotonin levels in the placental tissues. Therefore, for the first time serotonin levels will be measured in human placental extracts.
- Serotonin modulates vasodilation and vasoconstriction of blood vessels via 5-HT_{1A} and $5HT_{2A}$, respectively; however, the presence of these receptors in placental veins that regulate the nutrient and oxygen transfer from the mother to fetus have not been investigated. The protein expression of 5-HT_{1A} and $5HT_{2A}$ receptors will be examined in placental veins.
- Serotonin bioavailability in the placenta is dependent on TPH1 and MAO-A enzymes involved in serotonin synthesis and degradation, respectively. The protein expression of TPH1 and MAO-A enzymes will be evaluated in placental tissues to determine

whether early-term births are associated with abnormal expression of serotonin metabolizing enzymes.

CHAPTER 2: Methodology

This observational, cross-sectional study was approved by Human Research Ethics Committee of Griffith University (MSC 05/15 HREC) and Queensland Prince Charles Hospital (HREC 14/QPCH/246). Twenty four pregnant women were recruited from the Gold Coast University Hospital (Maternal and Fetal Unit) to take part in this study. An informed written consent was obtained from each participant prior to the child delivery to donate their placentas and cord blood samples following birth. The confidentiality of all patient records was maintained.

2.1 Maternal and neonatal characteristics

The study group consisted of 24 women: 12 women who gave birth to early-term babies (born between 37 weeks 0 days and 38 weeks and 6 days of gestation) and 12 women who delivered full-term babies (born between 39 weeks 0 days and 40 weeks and 6 days of gestation). The participants who had mental health issues or pregnancy complications were excluded from the study including those who had gestational diabetes, hypertension or preeclampsia. The recruited healthy women donated their cord blood samples and placentas following vaginal or cesarean delivery. Information on maternal and neonatal characteristics was obtained from medical records, including data on maternal age and body mass index (BMI), fetal sex, birth weight, mode of delivery, gestational age and placental weight.

2.2 Placenta and placental blood vessel collection

The placental tissues and veins were collected within 45 minutes of delivery. The umbilical cord venous blood was collected immediately after delivery. Placental cotyledons and placental veins were isolated from multiple randomly selected areas (to account for intra-placental variability of proteins), washed of blood in cold phosphate-buffered saline (PBS) and then snap-frozen in liquid nitrogen. The samples were stored at -80°C until further

analysis. The blood samples were collected from the umbilical vein by midwives in ethylenediaminetetraacetic acid (EDTA) containing tubes for cortisol level evaluation.

2.3 Cord blood cortisol collection and cortisol measurement

The quantitative measurement of cortisol was performed using a portable high-performance Immunoassay-Analysis-System *i*-CHROMA™ (Boditech, Med Inc, Gangwon-doo, Korea) with the analytical sensitivity of the device equal to 4.8 nmol/L. *i*-CHROMA™ cortisol kit contained a test device, an ID chip and a detection buffer pre-dispensed into tubes. The test device contained a test strip in which BSA-labelled cortisol and streptavidin have been immobilized. The detection buffer contained fluorescently labeled anti-cortisol antibody, biotin-labeled BSA, gelatine and sodium azide. All the reagents were allowed to attain room temperature prior the test.

Briefly, 50 µL of whole blood was transferred from the EDTA tube into a tube containing detection buffer and then mixed gently by inverting the tube. After transferring 75 µL of the sample mixture into the test device, the test device was left on the bench for 10 minutes at room temperature. After 10 minutes the test device was inserted into the i-CHROMA™ and cortisol levels were measured and recorded in nmol/L. A quality control test was performed regularly as a part of good testing practice according to the manufacturer's instructions. The quality control test assured the accuracy of test results and confirmed the validity of *i*-CHROMA™ cortisol assay.

2.4 Preparation of cytoplasmic and nuclear protein extracts

Placental tissues were crushed in liquid nitrogen using mortar and pestle and then homogenized in complete cytosolic and nuclear fractionation buffers mixed with protease and phosphatase inhibitors. Saif *et al*. (2014) protocol was used to prepare cytoplasmic and nuclear fractions. The cytosolic buffer (pH 7.9) included 10 mM HEPES, 10 mM KCl, 1.5

mM $MgCl_2$, 340 mM Sucrose, 0.5 mM dithiothreitol (DTT), 10% glycerol, 0.1% of Triton X-100, 10 μM leupeptin, 5 μM pepstatin A and 1 mM phenylmethanesulfonyl fluoride (PMSF). The nuclear buffer (pH 7.9) contained 20 mM HEPES, 420 mM NaCl, 1.5 mM $MgCl_2$, 0.2 mM ethylenediaminetetraacetic acid (EDTA), 0.5 mM DTT, 25% glycerol, 10 μM leupeptin, 5 μM pepstatin A and 1 mM PMSF. Briefly, 200 mg of crushed frozen tissue was homogenized in 800 μL of cytosolic buffer. The homogenized samples were centrifuged at 8000 rpm for 6 minutes at 4°C in a centrifuge. The supernatants were collected and stored at -80°C as a cytosolic fraction until further analysis. The pellets were lysed in 500 μL of nuclear buffer for μminutes at 4°C on a spinning wheel. In 30 minutes the samples were sonicated for 10 seconds at 20% amplitude, then centrifuged for 8 minutes at 13000 rpm. The supernatants were collected as nuclear fractions. The protein concentration in each sample was measured via a BCA assay (Pierce™ BCA Protein Assay Kit).

2.5 Preparation of mitochondrial protein extracts

Mitochondrial extracts were prepared by homogenizing crushed frozen tissues in Mitochondrial Isolation Buffer mixed with protease and phosphatase inhibitors. The Mitochondrial Isolation Buffer (pH 7.4) contained 20 mM HEPES, 220 mM Mannitol, 70 mM sucrose, 1 mM EDTA, 1 mM PMSF, 10 μM Leupeptin, 3 mM Benzamidine, 5 μM Pepstatin A, 1 mM NaO and 0.1% Triton X-100. Trophoblast mitochondria were isolated using differential centrifugation and density gradient centrifugation. Briefly, 250 mg of placental tissue was homogenized in 800 μL of Isolation Buffer and left on ice for 15 minutes. In 15 minutes the samples were centrifuged at 720 g for 5 minutes at 4°C to pellet nuclei, unbroken cells and cell debris. The supernatant was transferred to a clean tube, then spun again at 10000 g for 30 minutes in a cold centrifuge to pellet mitochondria. The mitochondrial pellet was surface washed several times with 1 mL of Isolation Buffer and then

treated with proteinase K (Sigma Aldrich, Castle Hill, NSW) at a final concentration of 200 mg of proteinase K per 1 mL of Isolation Buffer. The treatment with proteinase K removed cytoplasmic and nuclear protein constituents, assuring the purity of mitochondrial fraction without compromising the integrity of mitochondria. Furthermore, to activate proteinase K, the samples were placed in a water bath set at 37°C for 30 minutes. Following 30 minutes incubation, the proteinase K in the samples was inactivated by adding the Isolation Buffer containing 5 mM PMSF. After proteinase K inactivation, the samples were centrifuged at 10000 g for 30 minutes to obtain mitochondrial pellets that were lysed in Mitochondrial Lysis Buffer containing 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM sodium fluoride, 40 mM β-glycerophosphate, 20 mM sodium tetrapyrophosphate, 10 µM leupeptin, 5 µM pepstatin A, 3 mM benzamidine, 1 mM PMSF, 0.1% Triton-X 100 and 1 mM sodium orthovanadate. The lysed mitochondrial pellets were sonicated twice at 30% amplitude for 10 seconds and stored in a -80°C freezer until BCA protein analysis.

2.6 BCA protein quantification

The prepared lysates have been diluted to approximately of 1 µg of protein per 1 µL. The dilutions used were as follows: 1:20 for cytoplasmic and nuclear fractions and 1:10 for mitochondrial lysates. The BCA Assay Kit was used for the colorimetric detection and quantification of total protein in tissue and blood vessel extracts. The samples were prepared according to the manufacturer's protocol with the absorbance of the samples red at 562 nm using the microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland). The protein concentrations were determined from the standard curve using bovine serum albumin (BSA) as a standard. After protein quantification, aliquots of 20 µg/µL were prepared in appropriate volumes of Kinexus Buffer (pH 7.2) for the Western immunoblotting. The reagents in the

Kinexus Buffer included: 20 mM MOPS, 2 mM EGTA, 5 mM EDTA, 30 mM NaF, 40 mM β-glycerophosphate, 20 mM NaPP. The prepared aliquots were stored in a - 80°C freezer.

2.7 Purity of subcellular fractions

The purity of the cytoplasmic, nuclear and mitochondrial fractions was validated to provide information on the biochemical purity of these subcellular fractions. To demonstrate that the isolated extracts were of a reasonable purity, the cytoplasmic, nuclear and mitochondrial fractions were assessed by Western blotting for β-actin, lamin A/C and COXIV, respectively.

2.8 Antibody optimization

To achieve a robust and specific signal, the quality of the antibodies listed in Table 2.10 was tested, with the concentrations of each primary and secondary antibody optimized prior to the Western immunoblotting.

2.9 Specificity control – a blocking peptide assay

The GR antibody (Bethyl Laboratories, cat. No A303-491A) recognizes all the isoforms of the GR. To confirm the antibody specificity and eliminate non-specific antibody binding, the blocking peptide (Bethyl Laboratories, cat. No BP303-491A) corresponding to the GR antibody was used according to the manufacturer's instructions. Shortly, the GR antibody was pre-incubated with its blocking peptide at a concentration of 2 times higher than of the GR antibody preventing the subsequent target protein binding. The control GR antibody (GR antibody alone, not blocked with the blocking peptide) was used in the Western blotting side-by-side with the neutralized GR antibody (GR antibody mixed with the blocking peptide) to detect and compare the strengths of the signals. The protein abundance was assessed using the Odyssey CLx Infrared Imaging System (Millennium Science, Mulgrave,

Australia). The absence of the signals on the membranes incubated with the blocked GR antibody confirmed the specificity of the GR antibody.

2.10 Western blotting

The Western blotting protocol was optimized for each antibody including the running time and voltage for the gel electrophoresis and protein transfer. The incubation duration and temperature (e.g., room temperature versus 4°C) for the blocking step and for the incubations step with primary and secondary antibody have also been optimized.

Polyacrylamide gel electrophoresis and protein transfer

The prepared samples were thawed on ice and mixed with 2 x Loading Dye and β-mercaptoethanol to a final volume of 30 µl. The protein aliquots were left for 5 minutes in a heating block set at 95°C to denature proteins. Gel electrophoresis was performed by loading 28 µl of each sample into a hand-cast 15- well 7.5% Tris-glycine gel. The proteins were separated by running the gel at 125 V for 1 hour 20 minutes, then transferred to polyvinylidene difluoride (PVDV) membrane at 70 V for 1 hour 15 minutes. After blocking non-specific binding with the Odyssey Blocking Buffer (Li-Cor Biosciences) for 2 hours at room temperature on a rocker platform, the membranes were incubated with primary antibodies for 18 hours at 4°C with gentle shaking. The membranes were washed 4 times for 5 minutes with gentle shaking at room temperature in Tris-buffered saline (TBS) containing 0.1% Tween 20 and then incubated with respective fluorescently-labeled secondary antibodies for 1 hour 20 minutes in the dark. After 80 minutes, the membranes were washed again 4 times for 5 minutes in TBS + 0.1% Tween 20 protected from light. The target proteins were detected using the Odyssey CLx Infrared Imaging System (Millennium Science, Mulgrave, Australia) (see Table 2.10 for the list of antibodies).

Table 2. 1: Table of Antibodies

Antibody Name	*Animal Source*	*Manufacture*	*Catalogue Number*	*Dilution*	*Corresponding Secondary Antibody*
GR-α	Rabbit	Bethyl Laboratories	A303-491A	1:1000	Goat anti-rabbit 800CW at 1:5000
SLC6A4/5HTT/SERT	Goat	Thermo Fisher	PA5-18374	1:3000	Donkey anti-goat 800CW at 1:30000
GR-β	Rabbit	Abcam	Ab3581	1:3000	Goat anti-rabbit 800CW at 1:30000
MAO-A	Rabbit	Abcam	Ab137841	1:500	Goat anti-rabbit 800CW at 1:30000
TPH1	Rabbit	Abcam	Ab52954	1:1000	Goat anti-rabbit 800CW at 1:30000
5-HT1A	Rabbit	Abcam	Ab85615	1:2000	Goat anti-rabbit 800CW at 1:40000
5HT2A	Rabbit	Abcam	Ab66049	1:800	Goat anti-rabbit 800CW at 30000
11β-HSD1	Rabbit	Abcam	Ab39364	1:1000	Goat anti-rabbit 800CW at 1:30000
11β-HSD2	Rabbit	Abcam	Ab80317	1:1000	Goat anti-rabbit 800CW at 1:15000
Caspase 3	Rabbit	Abcam	Ab32042	1:1000	Goat anti-rabbit 800CW at 1:30000
Caspase 6	Rabbit	Abcam	Ab2326	1:500	Goat anti-rabbit 800CW at 1:15000
Cofilin	Mouse	Abcam	Ab54532	1:1000	Donkey anti-mouse 680RD at 1:30000
Cox	Mouse	Abcam	Ab33985	1:1000	Donkey anti-mouse 680RD at 1:15000
Lamin A+C	Mouse	Abcam	Ab8984	1:1000	Donkey anti-mouse 680RD at 1:60000
β-actin	Mouse	Cell signaling	3700	1:4000	Donkey anti-mouse 680RD at 1:30000

Notes: Secondary antibodies purchased from Millenium Science included IRDye 800CW Donkey anti-goat IgG (H+L) (cat. No 926-32214), IRDye 680RD Donkey anti-mouse IgG (H+L) (cat. No 925-68072), IRDye 800CW Goat anti-rabbit IgG (H+L) (cat. No 926-32211).

2.11 Serotonin ELISA

Serotonin ELISA kit (Abcam, cat. No ab133053) has been used to measure placental tissue serotonin levels according to the manufacturer's instructions with the absorbance of the samples red at 405 nm using the microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland). The kit contained 20 x wash buffer concentrate, assay buffer, goat anti-rabbit IgG microplate (12 x 8 wells), plate sealer, pNpp substrate, serotonin alkaline phosphatase conjugate, serotonin antibody, serotonin standard and stop solution. Undiluted cytoplasmic fractions were used to evaluate serotonin in the placental lysates (Refer to section 2.4 for the preparation of placental extracts). Serotonin concentrations in the samples were determined in duplicate. The sensitivity of the serotonin ELISA assay was 0.293 ng/mg and cross-reactivity with other serotonergic compounds < 0.1 %. Results expressed in ng/mg of placental tissue.

2.12 Statistical analysis

Assessment of variable type and level of measurement and normality check for each variable of interest was conducted to choose an appropriate statistical test. Normally distributed data were presented as mean (SEM). The parametric Student t-test, Pearson correlation and One-way ANOVA with Bonferroni correction were used to analyze normally distributed data. The variables that were not normally distributed were presented as median (range) and tested with the non-parametric Mann-Whitney test, Spearman's rank-order correlation and Kruskal-Wallis tests. The Statistical Package for the Social Sciences (SPSS version 22) and GraphPad Prism version 7 were used for statistical analysis. The level of significance was set at $p < 0.05$ (two-tailed).

CHAPTER 3: Cortisol metabolism in the placenta

3.1 Introduction

Glucocorticoids play a major regulatory role in human pregnancy. Pregnancy and an associated progressive rise in maternal and fetal cortisol levels is a normal physiological process. The rise in GC levels near term is a result of hyperactivation of maternal and fetal HPA axes and an increased synthesis of cortisol by placental trophoblast cells [47, 48]. An increase in endogenous GC production in the last few weeks of gestation is required to maintain pregnancy and facilitate the successful transition of the fetus from intrauterine to extrauterine life [46, 261]. Placental cortisol metabolism is modulated mainly by 11β-HSD1 and 11β-HSD2 enzymes expressed in the placental tissues and fetal membranes, with a 11β-HSD1 enzyme acting predominantly as a reductase to convert inactive cortisone to cortisol, and 11β-HSD2 enzyme serving as an inactivator of GCs through its oxidative activity.

During most of the human pregnancy, fetal GC levels are substantially lower than in the mother. This gradient is achieved by a placental 11β-HSD2 enzyme that acts as "GC barrier," inactivating most maternally derived GCs during the passage through the placenta. However, the activity and abundance of the 11β-HSD2 enzyme can change in response to various factors, including GCs, with 11β-HSD2 enzyme activity [85] and expression [86] significantly reduced following GC treatment, at least in animal models. In the context of pregnancy, a reduction in placental 11β-HSD2 enzyme activity would mean higher placental and fetal exposure to maternal GCs and greater adverse effects on the placenta and fetus. GCs are known to alter placental phenotype, with GC-exposed placentae weighing less than non-treated controls [81]; and inhibit intrauterine fetal growth, resulting in babies born before term with lower than normal birth weight [46, 55, 71, 78, 262].

Furthermore, it has been suggested that the activity and expression of 11β-HSD enzymes in the human placenta markedly change throughout gestation as demonstrated by

multiple investigations over the past decades. However, the findings of these studies are quite contradictory. Arcuri *et al.* (1998) detected 11β-HSD2 mRNA and protein in first trimester trophoblasts. While 11β-HSD2 enzyme was highly expressed in the trophoblast cells (suggesting important roles of the 11β-HSD2 enzyme in controlling the exposure of the embryo to active GCs during the sensitive period of early development), the 11β-HSD1 enzyme was undetectable [263]. Giannopoulos *et al*. (1982) found that activity of 11β-HSD enzymes changes with gestational age, with 11β-HSD2 activity down-regulated and 11β-HSD1 activity up-regulated towards term [264]. Likewise, Clifton *et al*. (2003) reported that enzymatic activity of 11β-HSD2 is significantly reduced and the gene expression of 11β-HSD1 is increased during the final few weeks of human pregnancy, allowing greater cortisol transfer from the mother to the fetus to facilitate structural development and functional maturation of fetal organs and initiation of parturition [28]. Contrary to these observations, it has been shown that 11β-HSD2 enzyme activity [72] and mRNA abundance [265] increase with gestational age.

While the key role of 11β-HSD2 enzyme in controlling fetal growth and determining neonatal and adult well-being has been demonstrated in previous studies in which attenuated expression and activity of 11β-HSD2 enzyme has been linked to IUGR [72], low birth weight [59, 266] and increased occurrence of cardiovascular and endocrine disorders in adult life [267], the causal relationship has yet to be established. Although placental 11β-HSD2 enzyme is better studied and understood, the function of the 11β-HSD1 enzyme in the human placenta remains elusive. It worth noting, that cortisol metabolism mediated by 11β-HSD enzymes in the placenta depends on fetal gender, with placentae from women pregnant with a female fetus showing greater 11β-HSD2 enzyme activity compared with placentae of women pregnant with a male fetus [94, 95], suggesting that female fetuses are more protected than male fetuses from high maternal cortisol by higher placental 11β-HSD2 activity. The exact

mechanisms by which GC alter fetal development still remain poorly understood; however GC-induced changes in the placental function, including the alteration in the expression of 11β-HSD enzymes, could be one of the mechanisms by which GCs control fetal physiology and length of gestation.

Due to inconsistencies in the reported results regarding the relative abundance of cortisol metabolizing enzymes in the placenta, this study aims to determine whether there are any significant changes in 11β-HSD1 and 11β-HSD2 protein expression in the human placentae from early-term (born between 37 weeks 0 days and 38 weeks 6 days) and full-term (born between 39 weeks 0 days and 40 weeks 6 days) infants. Hence, there is little information on the association between the expression of cortisol metabolizing enzymes and cortisol levels in the placenta; the project will determine if there is a relationship between 11β-HSD1 and 11β-HSD2 protein levels and placental cortisol concentrations. We will also investigate the effect of cord blood cortisol on the length of gestation. Because a reduction in 11β-HSD2 enzyme expression is associated with IUGR and LBW we will find out if fetal weight at birth is related to 11β-HSD2 enzyme expression. Since GCs overexposure has an inhibitory effect on placental and fetal growth, we will examine the relationship between cord blood cortisol and placental and fetal weight.

3.2 Specific Aims

i. Examine maternal characteristics including age and BMI

ii. Examine fetal characteristics such as: gender, gestational age, mode of delivery, birth weight and placental weight

iii. Measure cortisol in the umbilical vein blood

iv. Evaluate 11β-HSD1 and 11β-HSD2 protein expression in the placentae from early-term and full-term infants in a gender-specific manner

v. Investigate relationship between 11β-HSD2 expression and fetal weight

vi. Examine 11β-HSD1 and 11β-HSD2 protein expression, gestational age, placental weight and fetal birth weight in relation to cord cortisol

3.3 Materials and Methods

3.3.1 Maternal and neonatal characteristics

The study group consisted of 24 women: 12 women who gave birth to early-term babies (born between 37 weeks 0 days and 38 weeks and 6 days of gestation) and 12 women who delivered full-term babies (born between 39 weeks 0 days and 40 weeks and 6 days of gestation). The participants who had mental health issues or pregnancy complications were excluded from the study including those who had gestational diabetes, hypertension or preeclampsia. The recruited healthy women donated their cord blood samples and placentas following vaginal or cesarean delivery. Information on maternal and neonatal characteristics was obtained from medical records including data on maternal age and body mass index (BMI), fetal sex, birth weight, mode of delivery, gestational age and placental weight.

3.3.2 Placental tissue collection

Placental tissues were collected within 45 minutes of delivery for protein detection and quantification. The tissues were extracted from multiple randomly selected areas and washed of blood. Please refer to Chapter 2 (Methodology), section 2.2 for more information.

3.3.3 Cord blood cortisol collection and cortisol measurement

The umbilical cord venous blood was collected immediately after delivery. The blood samples were collected by midwives in EDTA - containing tubes for cortisol level estimation via portable high-performance Immunoassay-Analysis-System *i*-CHROMA™ (Boditech, Med Inc, Gangwon-do, Korea). For details on cortisol level measurement in blood samples please refer to Chapter 2, section 2.3.

3.3.4 Preparation of cytoplasmic protein extracts

Collected placental tissues were crushed in liquid nitrogen using mortar and pestle and homogenized in cytoplasmic buffer containing protease and phosphatase inhibitors. Saif *et al*. (2014) protocol was used to prepare cytoplasmic protein extracts with protein concentration in each sample measured via a BCA assay (Pierce^{TM} BCA Protein Assay Kit). Please refer to Chapter 2 sections 2.4 for details on the preparation of cytoplasmic protein extracts.

3.3.5 BCA protein quantification

The prepared lysates have been diluted to approximately of 1 µg of protein per 1 µL. One in twenty dilutions was used for protein evaluation with BCA Assay Kit used to detect and quantify proteins in tissue extracts. Please refer to Chapter 2 section 2.6 for details.

3.3.6 Purity of subcellular fractions

The purity of the cytoplasmic fractions was validated. To demonstrate that the isolated extracts were of a reasonable purity the cytoplasmic fractions were assessed for β-actin by Western blotting.

3.3.7 Antibody optimization

To achieve a robust and specific signal, the quality of the antibodies listed in Table 2.10 (Chapter 2) was tested, with the concentrations of each primary and secondary antibody optimized prior to the Western immunoblotting.

3.3.8 Western blotting

Running time and voltage for the gel electrophoresis and protein transfer was optimized for each antibody used in Western blotting. Likewise, the incubation duration and temperature (e.g., room temperature versus 4°C) for the blocking step and for the incubation step with primary and secondary antibody were tested and optimized.

Polyacrylamide gel electrophoresis and protein transfer

Gel electrophoresis was performed by loading 28 µL of each sample into a hand-cast 15-well 7.5% Tris-glycine gel. Each sample contained 20 µg of proteins. The proteins were separated by running the gel at 125 V for 1 hour 20 minutes, with details of the Western blotting conditions found in Chapter 2 section 2.10. The target proteins were detected using the Odyssey CLx Infrared Imaging System (Millennium Science, Mulgrave, Australia) (Please refer to Table 2.10 in Chapter 2 for the list of antibodies).

3.3.9 Statistical analysis

For each variable of interest, variable type and level of measurement were assessed and normality check performed to choose an appropriate statistical test. The parametric Pearson correlation and One-way ANOVA with Bonferroni correction were used to analyze normally distributed data. The Statistical Package for the Social Sciences (SPSS version 22) and GraphPad Prism version 7 were used for statistical analysis, with the level of significance set at $p < 0.05$ (two-tailed).

3.4 Results

3.4.1 Participants

Twenty four pregnant women with no health issues took part in this study. Participants consented on donating their placentae and umbilical cord blood samples for the investigation carried out in accordance with Human Research Ethics Committee of Griffith University (MSC 05/15 HREC) and Queensland Prince Charles Hospital (HREC 14/QPCH/246). The study cohort consisted of 12 women who delivered full-term neonates (6 males, 6 females) and 12 women who delivered early-term neonates (6 males, 6 females). The samples were classified into 4 groups to assess the variables of interest in a gender-specific manner. The groups were as follows: full-term male group (FM), full-term female group (FF), early-term male group (EM) and early-term female group (EF). The maternal and neonatal characteristics were examined to find out if there were significant differences among groups in regard to maternal and neonatal data. There were no significant differences among groups in maternal characteristics including maternal age and BMI (one-way ANOVA, $p > 0.05$) or neonatal characteristics of birth weight at delivery, cord cortisol and placental weight (one-way ANOVA, $p > 0.05$). The neonatal data between term controls and early-term pregnancies significantly differed in relation to gestational age (one-way ANOVA, $p < 0.0002$). Maternal and neonatal data are shown in Table 3.1.

Table 3. 1: Maternal and neonatal characteristics

	Full-term group (n=12)		Early-term group (n=12)		One-way Anova
	Male (n=6)	Female (n=6)	Male (n=6)	Female (n=6)	p-value
Maternal characteristics					
Age (years)	30.0 (1.4)	29.5 (1.1)	31.0 (2.3)	27.1 (1.8)	0.54
BMI (kg/m^2)	22.4 (1.4**)**	25.7 (1.3)	24.4 (2.7)	34.9 (4.2)	0.09
Neonatal characteristics					
Birth weight (g)	3284 (171)	3402 (131)	3377 (166)	3063 (136)	0.48
Placental weight (g)	606 (27)	644 (39)	681 (40)	627 (30)	0.55
Gestational age (wks)	39.3 (0.3)	39.6 (0.2)	38.3 (0.1)	38.2 (0.2)	0.0002
Mode of delivery	Vaginal (n=4)	Vaginal (n=4)	Vaginal (n=5)	Vaginal (n=6)	-
	Cesarean (n=2)	Cesarean (n=2)	Cesarean (n=1)	Cesarean (n=0)	-
Cord cortisol (nmol/L)	426 (50)	385 (56)	302 (35)	320 (47)	0.33

Data expressed as mean (SEM); SEM = standard error of the mean; BMI = body mass index

3.4.2 Relationships between cord blood cortisol and fetal weight at delivery, placental weight and gestational age

As GC exposure restricts both placental and fetal growth and may lead to the birth of the child before term, the relationship between cord cortisol levels and neonatal characteristics, including birth weight, placental weight and gestational age was evaluated.

There were no significant correlations observed between cord cortisol and fetal weight at birth (Pearson r = 0.2, p = 0.44, Figure 3.1 (a)) and placental weight (Pearson r = -0.1, p = 0.51, Figure 3.1 (b)). However, there was a significant positive correlation between blood cortisol and gestational age (Pearson r = 0.5 p = 0.005, Figure 3.1 (c)).

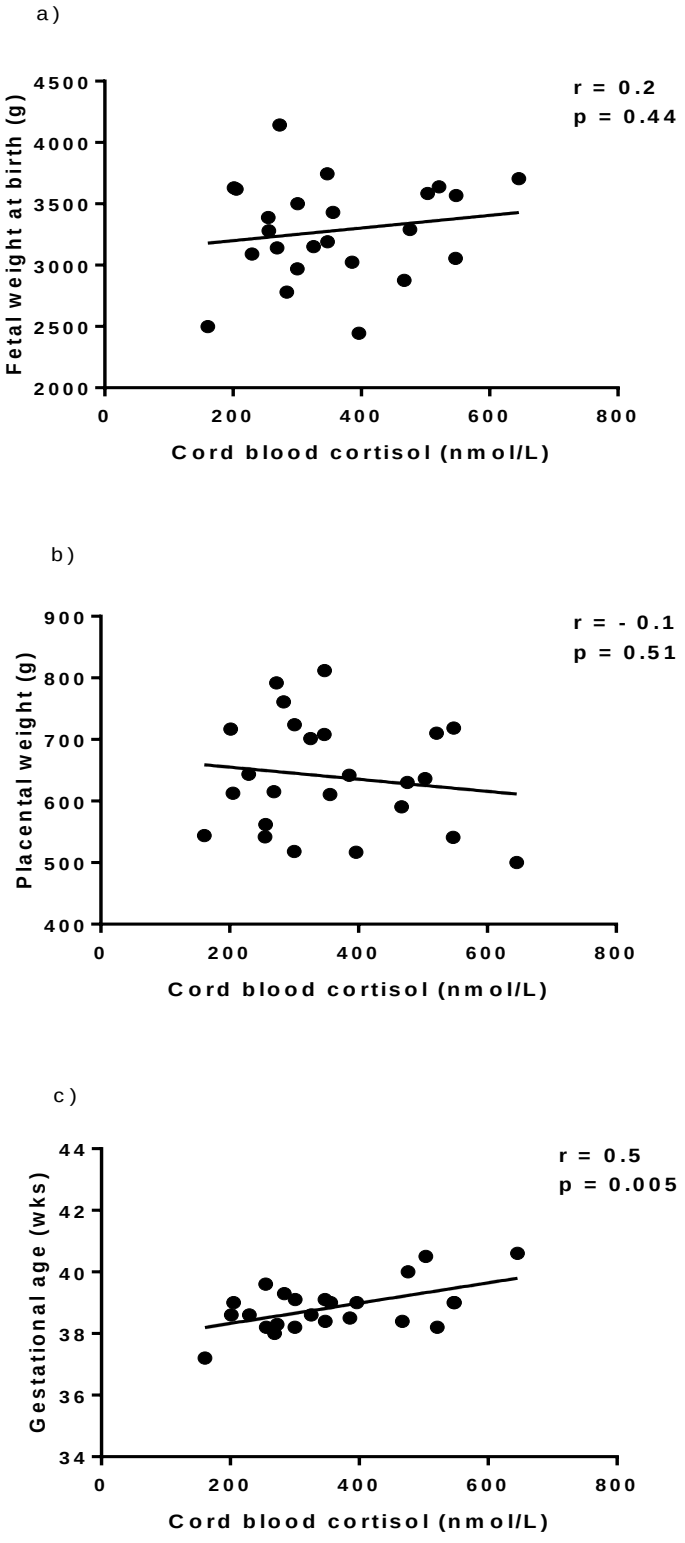


Figure 3. 1: Scatterplots exhibiting relationships of cord blood cortisol with neonatal characteristics. Correlation of cord blood cortisol with birth weight (a), placental weight (b) and gestational age (c).

3.4.3 11β-HSD1 and 11β-HSD2 protein expression in relation to fetal gender

To date, there are no studies that explored if there is a gender difference in the expression of cortisol metabolizing enzymes in the human placentae from early-term and full-term infants. Therefore, sex differences in 11β-HSD1 and 11β-HSD2 expression were examined in the study groups.

Comparison analysis of 11β-HSD1 expression in the study groups revealed that there were significant differences in 11β-HSD1 expression between groups in relation to fetal sex. In the full-term group, female fetus placentae had significantly higher 11β-HSD1 expression compared to the male fetus placentae (one-way ANOVA, $p < 0.01$, Figure 3.2 (a)). In the early-term group, 11β-HSD1 enzyme expression was significantly lower in the placentae of female fetuses compared to the placentae of male counterparts (one-way ANOVA, $p < 0.01$, Figure 3.2 (b)). There were no significant differences in protein expression of the placental 11β-HSD2 enzyme in relation to fetal sex (one-way ANOVA, $p > 0.05$).

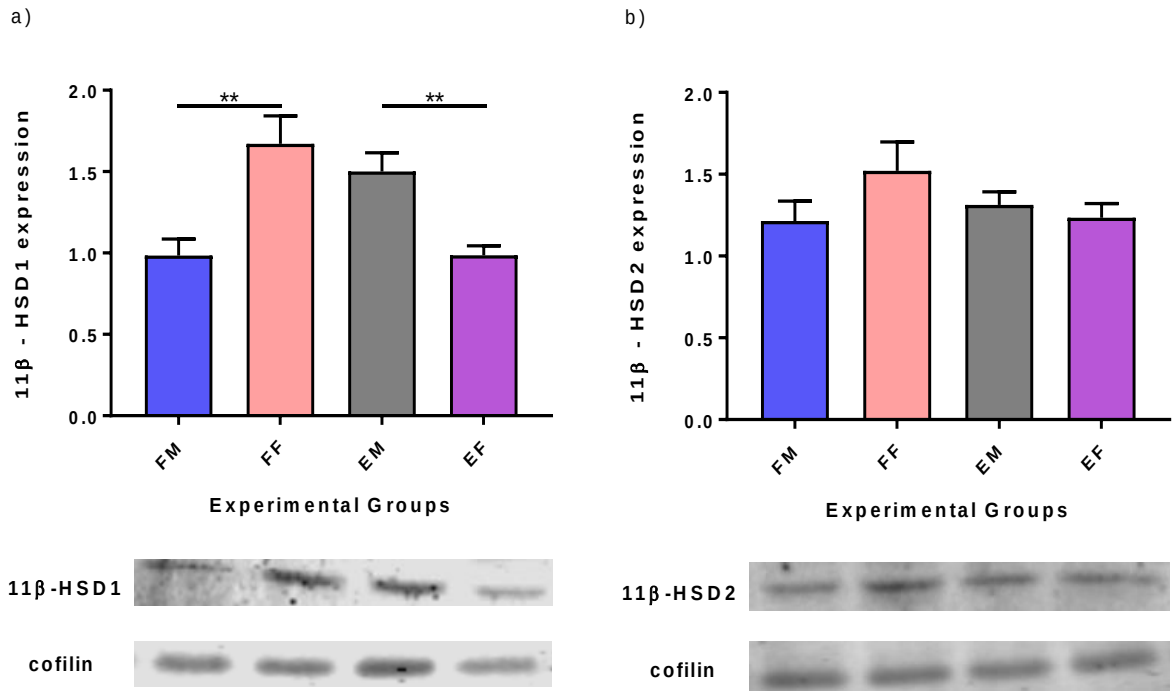


Figure 3. 2: Cortisol metabolizing enzymes in the human placentae in relation to fetal sex. Top: densitometry quantification of 11β-HSD1 (a), and 11β-HSD2 (b) protein expression normalized to cofilin. Bottom: representative Western blot images. Cofilin is shown as a loading control. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). One-way ANOVA followed by Tukey-Kramer test was performed for data analysis with values considered significant when the p-value was less than 0.05. ** P-value < 0.01. Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female).

3.4.4 Relationships between placental 11β-HSD1 and 11β-HSD2 protein expression and cord blood cortisol

Maturation of fetal organs and organ systems in the last few weeks of gestation is regulated by cortisol. Since cortisol levels in the fetus are mainly dependent on placental 11β-HSD enzymes, with 11β-HSD1 converting cortisone to cortisol and 11β-HSD2 inactivating cortisol to cortisone, the expression these enzymes was examined in the placenta. Since GCs may affect the expression the enzymes, the relationships of placental 11β-HSD1 and 11β-HSD2 expression with cord blood cortisol were tested.

There were no significant correlations between cord blood cortisol and protein expression of 11β-HSD1 (Pearson r = 0.01, p = 0.91, Figure 3.3 (a)) or 11β-HSD2 (Pearson r = - 0.21, p = 0.31, Figure 3.3 (b)) enzyme.

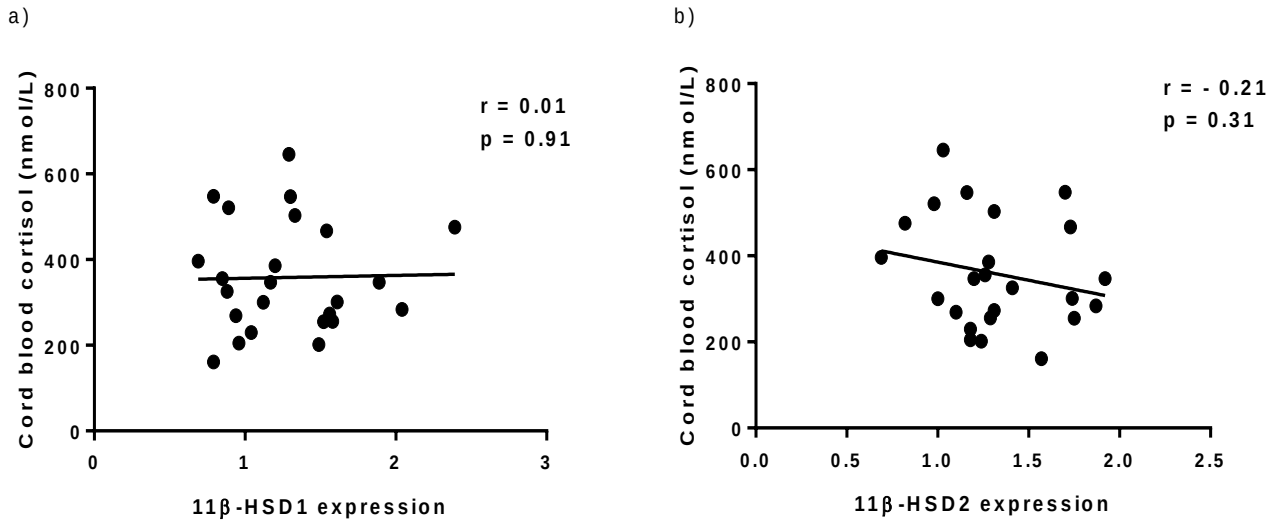


Figure 3. 3: Scatterplots exhibiting relationships of placental 11β-HSD1 and 11β-HSD2 expression with cord blood cortisol. Correlations of cord blood cortisol with 11β-HSD1 (a) and 11β-HSD2 (b) expression. Pearson correlation test was used to analyze the data.

3.4.5 Relationships between gestational age and placental 11β-HSD1 and 11β-HSD2

Previous investigations on the expression and activity of placental 11β-HSD enzymes during gestation have reported contradictory results. Some studies indicated that the activity and expression of the placental 11β-HSD1 enzyme are upregulated towards term, whereas the activity of 11β-HSD2 is downregulated to promote maturation of fetal organs. However, other investigations failed to demonstrate these results. This study evaluated the protein expression of placental 11β-HSD enzymes to clarify the controversies in the reported findings on the expression of the enzymes towards term.

Overall, there was an increase in the protein expression of placental 11β-HSD1 enzyme towards term; however, the relationship between placental 11β-HSD1 expression and gestational age was not significant (Pearson's r = 0.32, p = 0.12, Figure 3.4 (a)). Protein expression of placental 11β-HSD2 enzyme did not significantly change with gestational age (Pearson's r = - 0.03, p = 0.86, Figure 3.4 (b)).

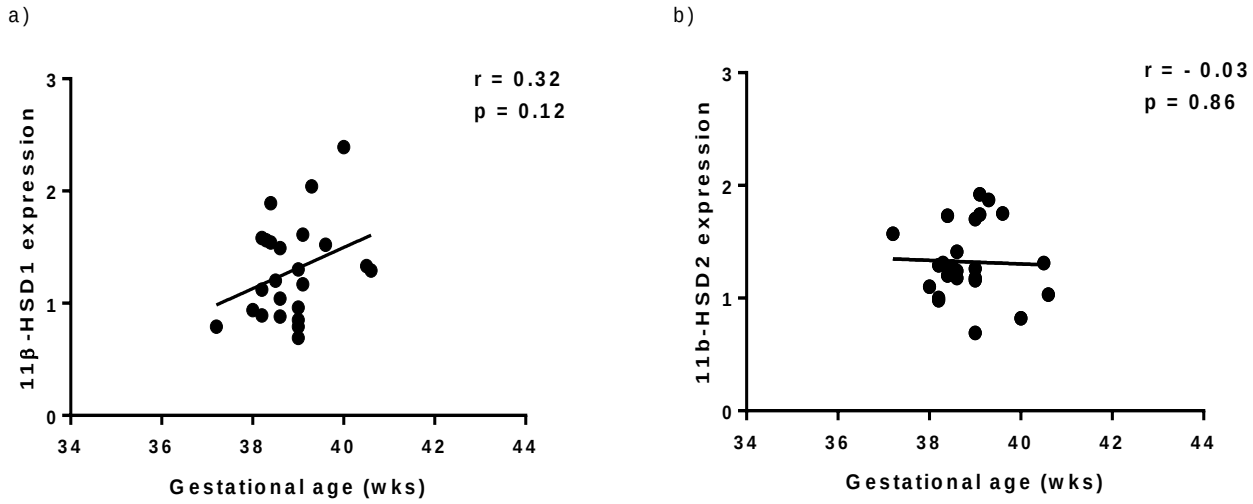


Figure 3. 4: Scatterplots exhibiting relationships of gestational age with placental cortisol metabolizing enzymes. Correlations of gestational age with 11β-HSD1 (a) and 11β-HSD2 (b) expression. Pearson correlation test was used to analyze the data.

3.4.6 Placental 11β-HSD1 and 11β-HSD2 protein expression and fetal weight at birth

Impaired activity of 11β-HSD2 or reduced expression in the placenta is common in IUGR, a condition frequently associated with low birth weight. But it is still unclear if there is a relationship between 11β-HSD2 expression and weight of the infants born between 37 and 39 weeks of gestation. Little attention has also been paid to find out whether birth weight is determined by the expression of 11β-HSD1 in the human placenta. To address these questions, the correlations between placental 11β-HSD1 and 11β-HSD2 expression and birth weight were examined. Fetal birth weight was not correlated with 11β-HSD1 (Pearson's r = 0.12, p = 0.57, Figure 3.5 (a)) or 11β-HSD2 (Pearson's r = 0.08, p = 0.68, Figure 3.5 (b)) expression.

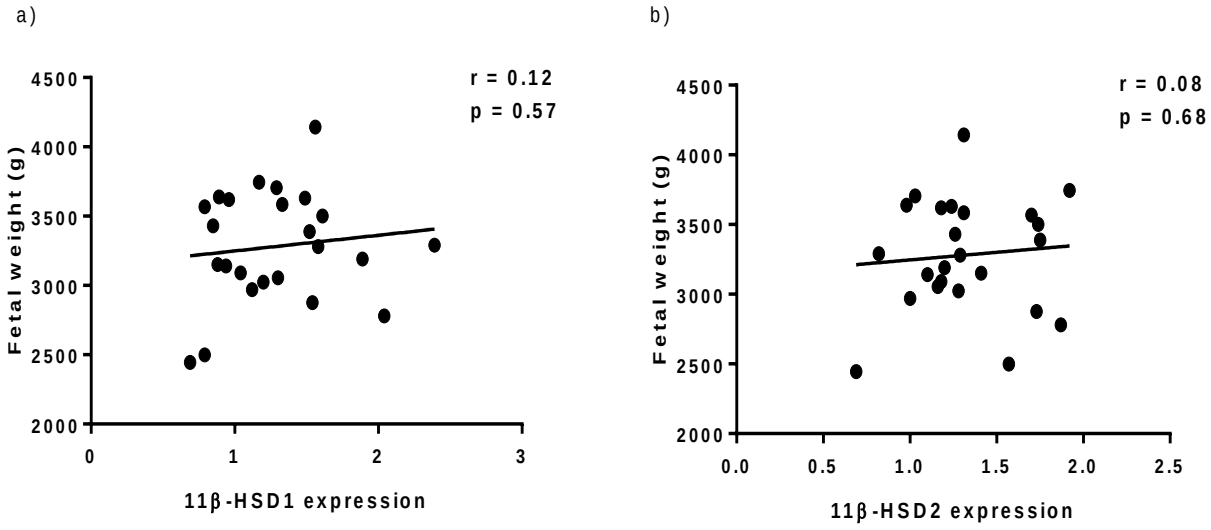


Figure 3. 5: Scatterplots of 11β-HSD1 and 11β-HSD2 expression versus fetal weight at birth. Pearson correlation coefficients (r) of fetal weight at birth versus 11β-HSD1 (a) and 11β-HSD2 (b) enzyme expression were determined to analyze the data.

3.5 Discussion

Every week of gestation is crucial for fetal development, particularly the last two weeks (weeks 38 and 39 of gestation) during which fetal adrenal glands, lungs, liver, kidneys and gut undergo major maturational changes to take over the functions of the placenta and prepare the fetus for extra-uterine life [46]. The maturational trajectories of the fetus are dependent on cortisol, with synthetic GCs routinely administered to pregnant women to improve neonatal viability in threatened preterm labor. However, GC treatment/exposure is not without side effects. Our knowledge on the adverse effects of GCs on the placenta and fetus comes primarily from studies on small-for-gestational-age infants, with reduced placental and fetal size and premature births (birth at < 37 gestational weeks) reported as a common complication of GC exposure and risk factors associated with the onset of various diseases later in life. However, recent evidence indicates that early-term deliveries (births at 37-38 gestational weeks) are associated with a significant increase in neonatal morbidity, with infants born at 37 to 38 weeks having higher rates of respiratory distress syndrome, pneumonia, hypothermia, hypoglycaemia, asthma and gastrointestinal disorders compared to full-term infants (born at 39-40 gestational weeks) [268, 269]. The factors that affect the lengths of the human gestation are yet to be identified. However, it has been suggested that GC overload could be a contributing factor that shortens the length of gestation. We for the first time compared placental weight, fetal birthweight and gestational age in early-term infants and full-term controls in a gender-specific manner in relation to cord blood cortisol to find out whether early-term births are related to cord blood cortisol levels.

Maternal and neonatal characteristics according to fetal sex and gestational age

The results of the comparison studies showed that early-term group did not significantly differ from the full-term group in relation to the neonatal characteristics of

placental weight, fetal birth weight and cortisol levels. Cortisol levels in the early-term group were lower compared to the full-term group, however, the difference in cortisol levels between the groups was insignificant. Although the early-term neonatal data did not significantly differ from full-term neonatal results, the full-term female neonates had slightly higher placental weight and birth weight and lower cortisol levels than full-term male newborns. On the contrary, in the early-term group, the early-term male infants had higher placental weight and birth weight and lower cortisol levels relative to the early-term female infants. As cortisol exposure may inhibit placental and fetal growth, this result may indicate that full-term males and early-term females had lower placental weight and birth weight due to being exposed to higher cortisol levels compared to full-term female and early-term male counterparts, respectively. The work published by Mericq *et al*. (2009) supports our hypothesis. In human term placentae (37-40 weeks of gestation), small-for-gestational-age (SGA) newborns have higher cortisol levels and lower placental weight and birth weight in comparison with average-for-gestational age counterparts [270]. Further longitudinal investigations with detailed examinations of human pregnancies from the very start of gestation until parturition will clarify the relationship between cortisol levels during gestation and placental weight, fetal birth weight and disease later in life.

11β-HSD1 and 11β-HSD2 protein expression in relation to fetal gender

Placental 11β-HSD1 and 11β-HSD2 enzymes are known to play a major role in controlling cortisol concentrations in the placenta and fetus, but the activity and expression of the enzymes towards the end of the human gestation are not well understood. Too much or not enough cortisol transferred from the mother to the fetus through the placenta may lead to suboptimal fetal growth and impact the life-long health of the offspring, with females having higher survival rates, particularly in pregnancies complicated with premature births and

IUGR [96, 97]. Because placental cortisol metabolizing enzymes may indirectly affect fetal development, the relative levels of placental 11β-HSD1 and 11β-HSD2 enzymes were examined in relation to fetal sex and gestational age.

Both 11β-HSD1 and 11β-HSD2 enzymes were detected in the human placentae from full-term and early-term infants. This project reports a significant difference in the expression of 11β-HSD1 enzyme between the groups in relation to fetal sex. Full-term female fetus placentae had higher 11β-HSD1 enzyme levels compared to the full-term male fetus placentae. On the contrary, in the early-term group male fetus placentae had higher 11β-HSD1 protein expression compared to the female fetus placentae. Surprisingly, in the placentae of full-term female infants and early-term male infants the expression of the 11β-HSD1 enzyme was higher and cortisol levels lower than in the placentae of full-term male newborns and early-term female newborns, respectively. The opposite relationship between cortisol levels and the expression and activity of the 11β-HSD1 enzyme was also observed in the study conducted by Mericq *et al.* (2009) in which cord cortisol levels were inversely correlated to 11β-HSD1 enzyme expression and activity [270]. We propose that alteration in 11β-HSD1 expression and activity in the human placenta could be a possible mechanism of regulation of cortisol concentrations in the fetal compartment. Although the 11β-HSD2 enzyme was also detectable in all placental samples independent of fetal sex or gestational age, the difference in the relative levels of between the groups did not reach statistical significance.

Correlational studies

The correlational studies on the relationship of cortisol levels with placental and fetal weight did not detect significant correlations, with placental and fetal weight being unrelated to cortisol levels. The neonatal characteristic of gestational age was positively correlated with

cortisol levels, with overall cortisol concentrations increased significantly with advancing gestation. An increase in fetal cortisol levels towards term is a well-established event and it occurs in all species studied so far including sheep, pig, horse, rat, guinea-pig and humans [46]. Our results on the upregulation in cortisol levels in the last few weeks of gestation are consistent with the other studies [28, 46], with cortisol levels rising at term to control the maturational changes in fetal respiratory, cardiovascular and neural systems. Since early-term births in our cohort were not due to cortisol overload at the end of gestation, we hypothesized that in the early-term infants GC overexposure occurred during critical periods of fetal development (first trimester of pregnancy). This hypothesis is supported by Sandman *et al.* (2006) who demonstrated that levels of cortisol early in pregnancy predict the gestational age at term. Sandman et al. (2006) reported that women who delivered preterm infants had higher cortisol concentrations at 15 weeks of gestation than women who delivered infants at term [271]. Interestingly, the upregulation of cortisol levels early in pregnancy was associated with the surge in placental CRH levels at 31 weeks of gestation, with CRH levels at 31 weeks being the best predictor of preterm birth and gestational length [271]. Furthermore, excessive levels of CRH increase the risk of earlier onset of spontaneous labor [272-274]. Since upregulated cortisol levels during sensitive windows in fetal development are associated with heightened levels of placental CRH, cortisol overload early in life may regulate the placental clock that controls a cascade of physiological events leading to parturition through upregulation of placental CRH levels late in gestation.

We next examined the overall relationship of cortisol metabolizing enzymes in the placenta with cord cortisol, birth weight and gestational age to understand which factors are important for normal fetal growth. There was no significant relationship between cord cortisol and placental 11β-HSD1 expression. High cord cortisol levels were associated with low placental 11β-HSD2 expression; however, the strength of the relationship was weak and

did not reach statistical significance. Protein levels of placental 11β-HSD1 tended to increase with gestational age, but not significantly, whereas placental 11β-HSD2 abundance was similar between 37 and 40 gestational weeks. Likewise, there was no relationship of birth weight with placental 11β-HSD1 or 11β-HSD2 protein expression. Given that little is known concerning placental 11β-HSD1 and 11β-HSD2 enzymes in human gestation, the precise role 11β-HSD1 and 11β-HSD2 in controlling fetal cortisol exposure and particular birth weight remains elusive.

3.6 Summary

In the present study, we investigated whether there is a gender difference in placental weight, fetal birth weight, gestational age and cord cortisol levels in full-term and early-term neonates. We further evaluated the expression cortisol metabolizing enzymes, 11β-HSD1 and 11β-HSD2, in the human placentae of early-term and full-term newborns to find out if fetal sex regulates the expression of the enzymes. To identify the factors that determine normal fetal development we examined the relationship between the expression of 11β-HSD1 and 11β-HSD2 and gestational age, birth weight and cord cortisol. To our knowledge, no study has used human placentae of full-term and early-term infants to evaluate sex differences in the expression of placental 11β-HSD1 and 11β-HSD2 enzymes.

No differences were observed between sexes or between groups in neonatal characteristics including placental weight, fetal birth weight and cord cortisol levels. Cord cortisol levels significantly increased with gestational age. Full-term male and early-term female infants had high cord cortisol levels; however, increased cortisol concentration in these groups was not accompanied by increased placental 11β-HSD1 enzyme expression. On the contrary, placentae of full-term male and early-term female fetuses exhibited significantly lower 11β-HSD1 protein expression than placentae from full-term female and early-term male counterparts, respectively. Unexpectedly, high cortisol levels in full-term male infants

and early-term female infants coincided with lower placental weight and birth weight. A downregulation in placental 11β-HSD1 enzyme activity and expression in response to high cord cortisol has been suggested as a possible mechanism of fetal protection from high maternal cortisol concentrations. The scarcity of information on the effect of gender on the distribution of cortisol metabolizing enzymes during various stages of human gestation and the effect of cortisol on 11β-HSD1 and 11β-HSD2 enzyme levels makes interpretation of the results challenging. Currently, researchers do not know as to what is considered normal in terms of cortisol concentrations during pregnancy. More studies are required to determine maternal cortisol levels optimal for the placental development and intrauterine fetal growth in a gender-specific manner. Finally, cord blood cortisol, fetal birth weight and gestational age were not correlated with placental 11β-HSD1 or 11β-HSD2 enzyme levels. Complex regulatory mechanisms control fetal development and necessitate further investigations to identify all the determinants of fetal growth.

CHAPTER 4: Glucocorticoid Receptors in the Human Placenta

4.1 Introduction

Glucocorticoids play a pivotal role in a variety of physiological processes, including human reproduction with cortisol levels rising towards term to facilitate fetal growth and parturition. However long-term fetal exposure to maternal stress or elevated GCs is associated with altered placental structure and function and restricted fetal growth, with infants born before term with lower normal birth weight - the indicator for placental insufficiency and a risk factor for disease in adult life. Research suggests that human male and female fetuses respond differentially when exposed to high maternal GC concentrations. Female fetuses become hypersensitive to changes in GC levels, whereas male fetuses develop GC resistance when exposed to prolonged elevated GCs levels [163]. The mechanisms that control GC responses in the placenta in a sexually dimorphic pattern are yet to be established; however variation in GR expression and subcellular localization may determine sex differences in response to GCs. To understand how changes in GRs ratios and intracellular location affect GC signaling and fetal growth in a gender-specific manner, the following sections review the mechanisms responsible for the heterogeneity of GRs.

Glucocorticoid receptors, through which GCs mediate their molecular actions, display a remarkable diversity as a result of alternative splicing and alternative translation initiation. Alternative splicing of GR pre-mRNA creates GR transcripts including GRα, GRβ, GRγ, GRA and GRP, coding for GRα, GRβ, GRγ, GRA and GRP proteins, respectively [107]. GRα mRNA can be translated from eight alternative translation initiation sites, resulting in GRα-A, GRα-B, GRα-C1, GRα-C2, GRα-C3, GRα-D1, GRα-D2 and GRα-D3 [105]. The GR isoforms vary in relation to their intracellular levels, localization, gene regulation and functional profiles. GRα isoforms are the most abundant isoforms and constitute 90 % of all GRs. GRα-A, -B and -C proteins reside primarily in the cytoplasm and translocate to the

nucleus following ligand binding to stimulate or repress the expression of target genes. On the contrary, GRα-D isoforms have the lowest transcriptional activity and are localized to the nucleus independent of ligand binding [105]. GRβ does not bind GCs, it is transcriptionally inactive and resides constitutively in the nucleus of the cells [108]. When co-expressed with GRα, GRβ acts as a negative inhibitor of GRα-mediated transcriptional activity. It is known to alter GRα signaling indirectly by binding to the GRα GREs or directly interacting with GRα to form transcriptionally inactive GRα – GRβ heterodimers. GRγ constitutes approximately 10 % of total GRs, it binds to the GCs and shows reduced transcriptional activity compared to GRα [114]. GRγ has been recently detected in the mitochondria of human cells where it plays a pivotal role in mitochondrial function by regulating the ATP generation [275]. Little is known about the non-hormone binding GRA and GRP isoforms, though the expression of these isoforms is associated with multiple pathologies and GC resistance [115-118].

Although the GRs receptors have been detected and extensively investigated in cultured cell of animal and human origins, the current knowledge on the distribution of GRs in various subcellular compartments and the effect of sex on the regulation of GC responses and GR ratios in the human placenta is limited and comes primarily from the studies conducted by the Australian researchers - Professor Vicky Clifton and Dr Zarqa Saif. In 2008 Johnson *et al.* provided the most complete picture of the GR exon 1 mRNA transcripts and GR mRNA splice variants in the human placentae at term and demonstrated that GR-1C and GR-1B promoters generate the majority of the GRs including GRα, GRP and GRγ [176]. According to Johnson *et al.* (2008), placental GRP mRNA transcripts and GR-1A3 differ in relation to the mode of delivery and fetal sex, respectively. While GRP mRNA expression decreases with labor independent of fetal sex, the expression of GR mRNA transcripts from the untranslated exon 1A3 are gender-dependent, with the placentae from women carrying a

female fetus having significantly lower GR-1A3 mRNA expression compared to the placentae from women carrying a male fetus [176]. A differential GR promoter use and alternative splicing have been suggested as mediators of differential responses to cortisol observed in male and female fetus placentae.

Furthermore, in 2014 Saif *et al*. were the first who demonstrated that the expression GR protein isoforms in the placentae of term infants differed in relation to the cellular localization, fetal sex, growth restriction and maternal asthma. Both male and female fetus placentae had higher GRα-A protein levels in the nucleus than in the cytoplasm. However, male fetus placentae showed higher expression of GRP and GRA in the nucleus than in the cytoplasm [163]. Interestingly, in the placentae of male fetuses and SGA infants, cord blood cortisol was positively correlated with nuclear GRβ levels. Because an increased expression of GRβ is associated with GC resistance, cortisol-induced translocation of GRβ from the cytoplasm to the nucleus (along with increased nuclear expression of GRP and GRA) in the male fetus placentae could be the mechanisms explaining why male fetuses develop GC resistance when exposed to high maternal GCs [163]. Moreover, placentae of asthmatic women pregnant with a female fetus exhibited a reduced nuclear expression of GRβ along with an increased nuclear expression of GRα-C and GRα-D3. Together these findings may indicate that female fetus placentae are more sensitive to cortisol compared to male fetus placentae due to the interaction of GRα-A with GRα-C and GRα-D3 in the nucleus, at least in the pregnancies complicated by maternal asthma [163]. These results prompted Saif and associates to conduct a subsequent investigation of GR isoforms in placentae of preterm pregnancies in relation to fetal sex, birth weight and betamethasone treatment to find out whether alterations in GR expression may contribute to the pathophysiology of preterm birth [276]. GRα, GRβ, GRα-C, GRP, GRA and GRαD1–D3 were detected in preterm placentae with GRα-C expression being higher in preterm placentae compared to the full-term

placentae and GRα-D2 expression being greater in the placentae of preterm males than in preterm females [276]. While GR expression varied in terms of gestational age and fetal sex, GR levels did change significantly in relation to betamethasone exposure. Saif *et al.*, 2015 compared GR isoform expression in the placentae of preterm births (24-36 gestational weeks) with full-term births (> 37 gestational weeks). In their study researchers combined the placentae of early-term infants (born between 37 and 38 gestational weeks) and full-term infants (born between 39 and 40 gestational weeks) and classified the group as a full-term group. They did not investigate the GR expression in their full-term group in relation to gestational age. Since GCs may control the length of gestation via their cognate GRs, we for the first time examined whether changes in the GR patterns in the early-term group placentae is a possible contributor to the early-term births.

4.2 Specific Aims

i. Fractionate placental lysates to obtain cytoplasmic and nuclear fractions
ii. Examine GR isoform expression in relation to gestational age, fetal sex and subcellular localization

4.3 Materials and methods

This observational, cross-sectional study was approved by Human Research Ethics Committee of Griffith University (MSC 05/15 HREC) and Queensland Prince Charles Hospital (HREC 14/QPCH/246). Twenty four pregnant women were recruited from the Gold Coast University Hospital (Maternal and Fetal Unit) to take part in this study. An informed written consent was obtained from each participant prior to the child delivery to donate their placentas and cord blood samples following birth. The confidentiality of all patient records was maintained.

4.3.1 Maternal and neonatal characteristics

The study group consisted of 24 women: 12 women who gave birth to early-term babies (born between 37 weeks 0 days and 38 weeks and 6 days of gestation) and 12 women who delivered full-term babies (born between 39 weeks 0 days and 40 weeks and 6 days of gestation). The participants who had mental health issues or pregnancy complications were excluded from the study including those who had gestational diabetes, hypertension or preeclampsia. The recruited healthy women donated their cord blood samples and placentas following vaginal or cesarean delivery. Information on maternal and neonatal characteristics was obtained from medical records including data on maternal age and body mass index (BMI), fetal sex, birth weight, mode of delivery, gestational age and placental weight.

4.3.2 Placental tissue collection

Placental tissues were collected within 45 minutes of delivery for protein detection and quantification. The tissues were extracted from multiple randomly selected cotyledons, washed of blood in PBS and snap-frozen in liquid nitrogen. Please refer to Chapter 2, section 2.2 for details.

4.3.3 Preparation of cytoplasmic and nuclear protein extracts

Frozen placental tissues were crushed in liquid nitrogen using mortar and pestle and then homogenized in cold cytoplasmic and nuclear fractionation buffers containing protease and phosphatase inhibitors. Saif *et al*. (2014) protocol was used to extract cytoplasmic and nuclear proteins. The BCA assay (Pierce^{TM} BCA Protein Assay Kit) was used to measure the protein concentration in the samples. Please refer to Chapter 2, section 2.4 for the preparation of cytoplasmic and nuclear protein extracts.

4.3.4 BCA protein quantification

The prepared lysates have been diluted to approximately of 1 µg of protein per 1 µL. One in twenty dilutions was used for protein evaluation with BCA Assay Kit used to detect and quantify proteins in tissue extracts. Following protein quantification, aliquots of 20 µg/µL were prepared in appropriate volumes of Kinexus Buffer (pH 7.2) for the Western blotting. The prepared aliquots were stored in a - 80°C freezer. Please refer to Chapter 2 section 2.6 for details.

4.3.5 Antibody optimization

To achieve a robust and specific signal, the quality of the antibodies listed in Table 2.10 (Chapter 2) was tested, with the concentrations of each primary and secondary antibody optimized prior to the Western immunoblotting. To generate the most reliable results, it is paramount in the Western blotting to initially identify and optimize the following conditions: a) the detection limit of the target protein and b) the linear range of detection of primary and secondary antibodies. The amount of protein loaded must be above the level of detection as loading insufficient amount of proteins will make the detection and quantification of target proteins difficult. Likewise, loading too much protein leads to signal saturation and may alter data analysis and interpretation. As signal saturation can alter data, the optimal sample concentration and linear range of detection for each primary and secondary antibody was

determined. The optimal conditions must be determined for both, the target protein and its corresponding loading control. Please refer to Appendix, Supplementary figures 1 and 2 for the data on the detection limit of target proteins and linear range of detection of primary and secondary antibodies for GR and its loading control β – actin.

4.3.6 Specificity control – a blocking peptide assay

The GR antibody (Bethyl Laboratories, cat. No A303-491A) recognizes all the isoforms of the GR. To confirm the antibody specificity and eliminate non-specific antibody binding, the blocking peptide (Bethyl Laboratories, cat. No BP303-491A) corresponding to the GR antibody was used according to the manufacturer's instructions. Please refer to Chapter 2 section 2.9 for the specific details of the assay (Supplementary figure 3 in the Appendix demonstrates the Western blotting results of the blocking peptide assay).

4.3.7 Purity of subcellular fractions

The purity of the cytoplasmic and nuclear fractions was validated. To demonstrate that the isolated extracts were of a reasonable purity, the cytoplasmic and nuclear fractions were assessed for β-actin and lamin A/C, respectively. Please refer to Supplementary figure 4 in the Appendix for Western blotting results on the purity of subcellular fractions.

4.3.8 Western blotting

All the steps of the Westing blotting have been optimized including the running time and voltage for the gel electrophoresis and protein transfer. The incubation duration and temperature (e.g., room temperature versus 4°C) for the blocking step and for the incubation step with primary and secondary antibody were tested and optimized.

Polyacrylamide gel electrophoresis and protein transfer

Gel electrophoresis was performed by loading 28 µL of each sample into a hand-cast 15-well 7.5% Tris-glycine gel. Each sample contained 20 µg of proteins. The proteins were separated by running the gel at 125 V for 1 hour 20 minutes, with details of the Western blotting conditions found in Chapter 2 section 2.10. The target proteins were detected using the Odyssey CLx Infrared Imaging System (Millennium Science, Mulgrave, Australia) (Please refer to Table 2.10 in Chapter 2 for the list of antibodies).

4.3.9 Statistical analysis

For each target variable, variable type and level of measurement were identified and normality check concluded to choose an appropriate statistical method. The non-parametric Kruskal-Wallis test and Wilcoxon test were used to analyse the variables. The Statistical Package for the Social Sciences (SPSS version 22) and GraphPad Prism version 7 were used for statistical analysis, with the level of significance set at $p < 0.05$ (two-tailed).

4.4 Results

Seven bands were identified in placental samples including the bands at 95, 91, 81, 55, 50-51, 68-69 and 38 kDa. The bands corresponded to the known GRα-A, GRβ, GRα-C, GRα-D1, GRα-D2/D3 and unknown 68-69 kDa and 38 kDa proteins, respectively. These GR isoforms were detected in all placentae independent of subcellular localization, fetal sex or gestational age. GRγ, GRA and GRP were not detected in the placental extracts (For the Western blotting images refer to Appendix, Supplementary figures 2 and 3).

4.4.1 Effect of fetal sex and gestational age on GR subcellular localization

In the full-term group, there were no significant differences in the expression of GR isoforms between male and female term placentae except for the expression of GRα-A, GRα-D1 and GRβ. While placentae of term males had a significantly higher expression of GRα-A and GRβ in the nucleus than in the cytoplasm (Wilcoxon test, $p < 0.05$), the full-term female placentae exhibited significantly lower expression GRα-D1in the nucleus than in the cytoplasm (Wilcoxon test, $p < 0.05$, Table 4.1). In the early-term group, both male and female fetus placentae had higher expression of GRα-A and GRα-D1 in the nucleus than in the cytoplasm (Wilcoxon test, $p < 0.05$). The expression of GRα-C and unknown 68 kDa and 38 kDa was also significantly upregulated in the nuclear fractions of placentae from early-term females (Wilcoxon test, $p < 0.05$, Table 4.1).

Table 4. 1: Effect of fetal sex and gestational age on GR subcellular localization

GR subtype	Fraction	Full-term		Early-term	
		Male	Female	Male	Female
GRα-A	C	1.68 (2.34)	1.91 (1.57)	2.19 (5.5)	1.81 (2.49)
	N	**13.3 (23.4)[a]**	3.91 (15.6)	**40.8 (36.0)[a]**	**51.7 (37.4)[a]**
GRα-C	C	0.863 (1.45)	1. 52 (2.63)	1.56 (1.83)	0.742 (0.80)
	N	1.93 (1.44)	0.704 (0.72)	2.78 (2.64)	**5.39 (5.29)[a]**
68-69 kDa	C	0.726 (0.33)	0.824 (1.17)	0.999 (0.36)	0.925 (0.27)
	N	0.988 (3.09)	0.561 (0.46)	1.33 (1.95)	**3.49 (1.95)[a]**
GRα-D1	C	0.369 (0.69)	0.929 (0.46)	0.646 (0.67)	0.613 (0.83)
	N	0.920 (1.03)	**0.499 (0.24)[a]**	**1.69 (1.39)[a]**	**3.70 (2.52)[a]**
GRα-D2/D3	C	0.844 (0.54)	0.827 (0.19)	0. 991 (0.52)	0.945 (0.29)
	N	1.14 (1.28)	0.462 (0.40)	0.837 (0.66)	1.17 (1.42)
38 kDa	C	0.825 (0.70)	0.896 (0.49)	1.41 (1.93)	0.979 (1.03)
	N	1.89 (3.88)	2.64 (4.21)	12.5 (14.0)	**6.78 (8.44)[a]**
GRβ	C	0.206 (0.17)	0.474 (0.91)	1.03 (0.96)	1.05 (0.73)
	N	**0.519 (0.65)[a]**	0.247 (0.28)	1.09 (0.99)	1.38 (0.70)

Data presented as median (IQR), N = 6 per group; IQR = interquartile range; C = cytoplasmic fraction; N = nuclear fraction.

[a] p-value < 0.05 for the comparison between nuclear and cytoplasmic fractions

4.4.2 Effect of fetal sex and gestational age on GR expression

Cytoplasmic GR profile in the placental samples did not significantly differ in relation to fetal sex, nor did it vary in relation to gestational age (Wilcoxon test, $p > 0.05$, Figure 4.1, top), except for the cytoplasmic GRβ, the expression of which was significantly higher in the early-term male fetus placentae compared to the full-term male fetus counterparts (Wilcoxon test, $p < 0.01$, Figure 4.3 (a)). There were significant sex differences in the nuclear GR profiles in relations to gestational age. While early-term female fetus placentae exhibited significantly higher nuclear expression of GRα-A, GRα-C, GRα-D1, GRα-D2/D3, unknown 68 kDa protein (Wilcoxon text, $p < 0.05$, Figure 4.1, bottom) and GRβ (Wilcoxon test, $p < 0.01$, Figure 4.3 (b)) compared to the full-term female placentae, the placentae of early-term male fetuses had higher expression of nuclear GRα-A (Wilcoxon text, $p < 0.05$, Figure 4.1) and unknown 38 kDa protein than the placentae of full-term males (Wilcoxon text, $p < 0.05$, Figure 4.1). Representative Western blots of GR isoforms are demonstrated in Figures 4.2 and 4.4.

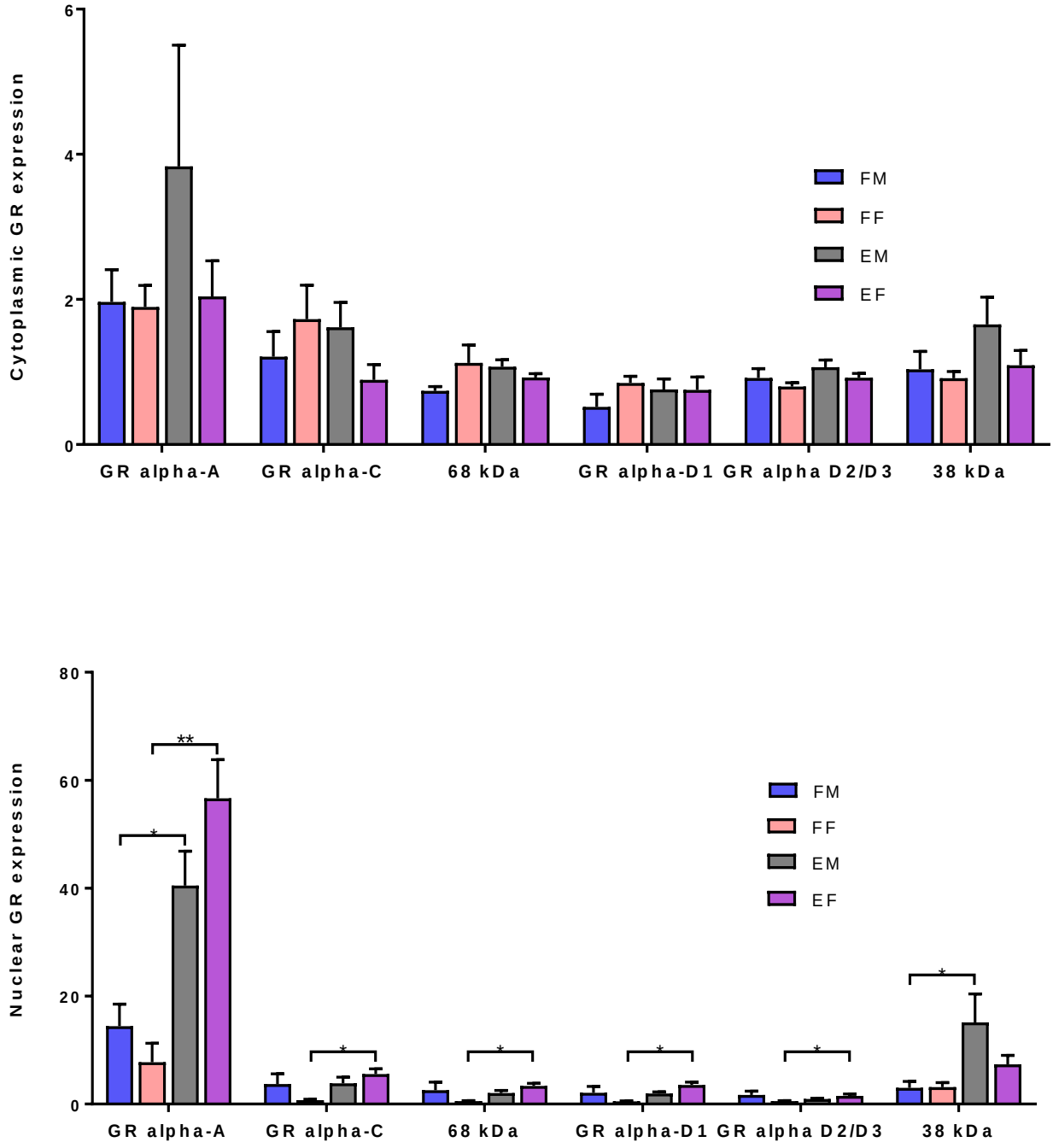
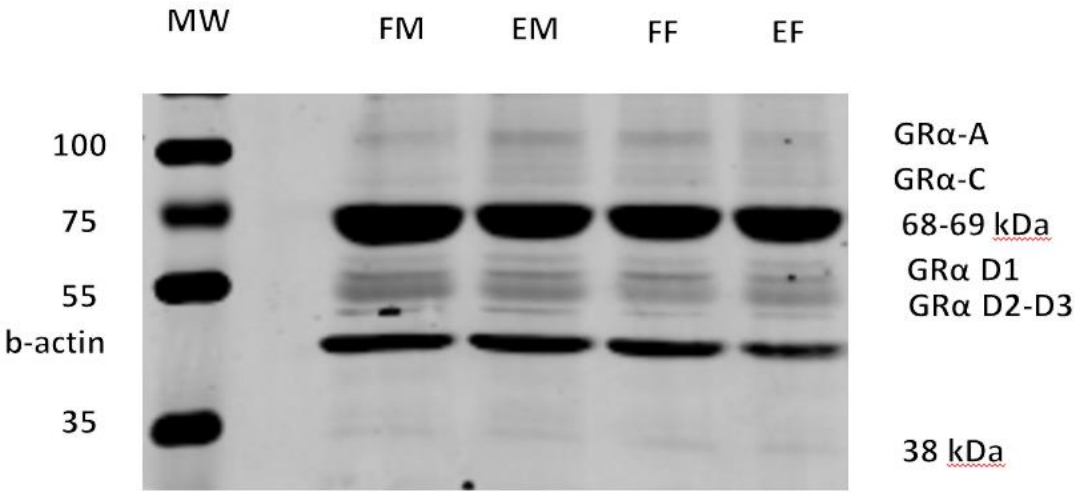


Figure 4. 1: GR profiles in relation to subcellular localization, fetal sex and gestational age. Top: Cytoplasmic GR isoform expression relative to b-actin. Bottom: Nuclear GR isoform expression relative to lamin A/C. Kruskal-Wallis test was performed for data analysis with values considered significant when the p-value was less than 0.05. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female). * P-value < 0.05, ** P-value < 0.01.

Cytoplasmic GR profile



Nuclear GR profile

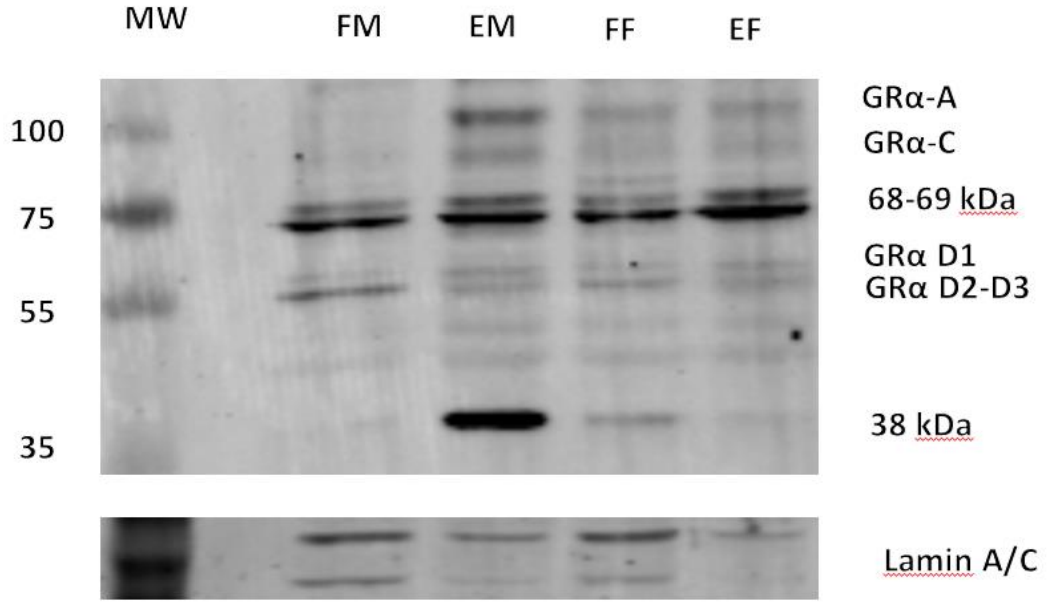


Figure 4. 2: Representative Western blotting data of placental glucocorticoid receptor (GR) isoforms. Cytoplasmic and nuclear protein extracts from full-term and early-term deliveries were used to detect GRs. Top: cytoplasmic GRs. Blots have been probed with β – actin as a loading control; Bottom: nuclear GRs. Blots have been probed lamin A/C as a loading control. Groups were classified into FM (full-term male), EM (early-term male), FF (full-term female) and EF (early-term female). *Significant changes **have been detected** in the expression of nuclear GRs.*

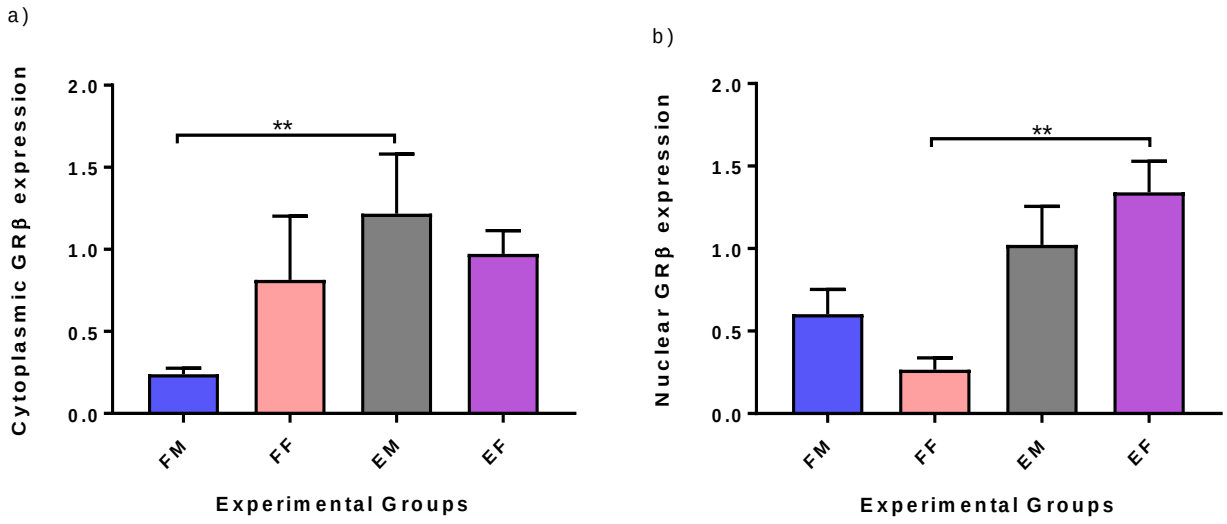


Figure 4. 3: GRβ expression in relation to subcellular localization, fetal sex and gestational age. a) Cytoplasmic GRβ expression relative to b-actin; b) Nuclear GRβ expression relative to lamin A/C. Kruskal-Wallis test was performed for data analysis with values considered significant when the p-value was less than 0.05. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female). ** P-value < 0.01.

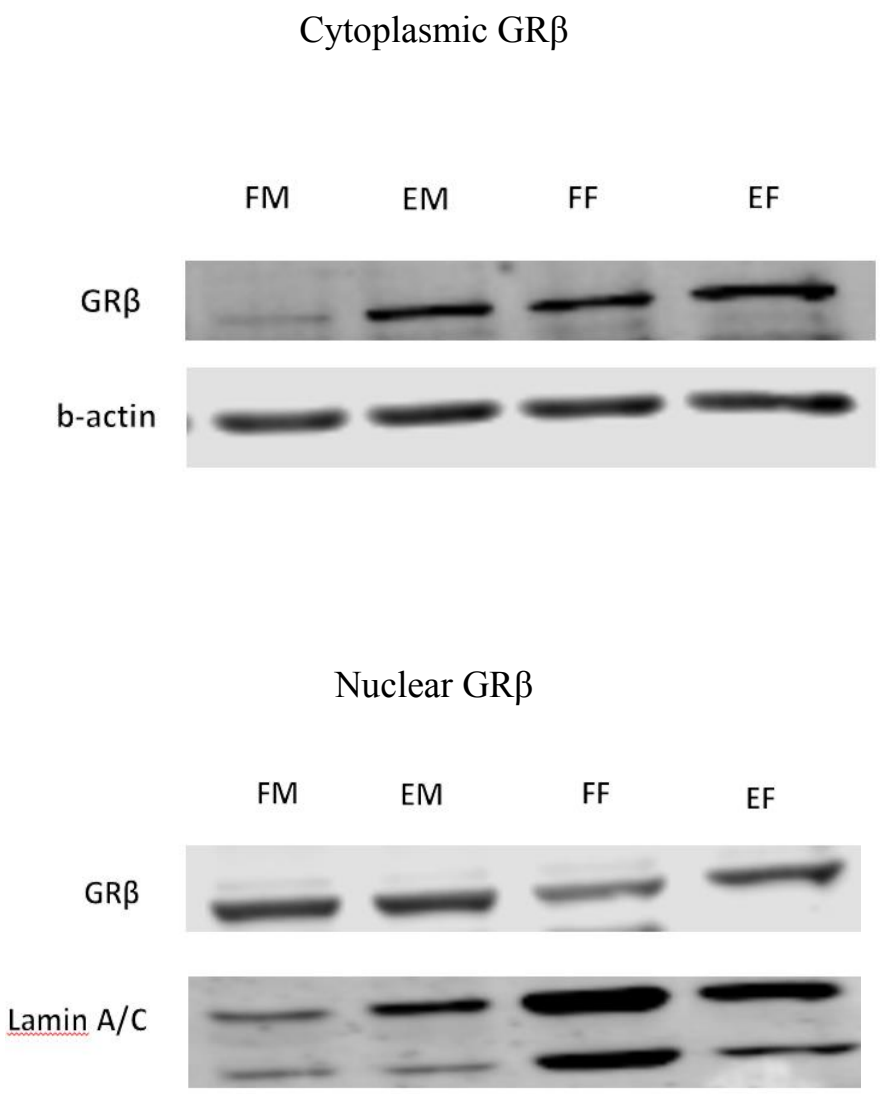


Figure 4. 4: Representative Western blotting data of placental glucocorticoid receptor β (GRβ). Cytoplasmic and nuclear protein extracts of placentae from full-term and early-term deliveries were used to detect GRβ. Top: cytoplasmic GRβ. Blots have been probed with β – actin as a loading control; Bottom: nuclear GRβ. Blots have been probed with lamin A/C as a loading control. Groups were classified into FM (full-term male), EM (early-term male), FF (full-term female) and EF (early-term female). *Significant changes **have been detected** in the expression of cytoplasmic and nuclear GRβ.*

4.5 Discussion

This study for the first time investigated whether the GR expression pattern in the placentae of early-term infants is different compared to the placentae of full-term infants. GRα-A, GRα-C, GRα-D1, GRα D2/D3, GRβ and unknown 68 kDa and 38 kDa have been identified in both cytoplasmic and nuclear fractions in all placentae. These isoforms were also detected in the studies examining the GR profiles in the human placentae in relation to fetal sex, fetal birth weight, maternal health and gestational age [163, 176, 276]. Contrary to Saif *et al*. (2014 and 2015), we did not detect GRγ, GRA and GRP proteins, possibly due to differences in the experimental protocols and/or variations in the sensitivities of assays used to detect target proteins [163, 276], with plausible causes of variations discussed below.

It is well-acknowledged, that gradient gels provide excellent separation of protein isoforms that differ in molecular size by one or two kilodaltons. Currently, in proteomic research these high performance polyacrylamide gels are considered the best gels for the investigation of protein isoforms. Although, the gradient gels are significantly better compared to the other gels when it comes to exploring protein isoforms, they are quite costly and not always affordable. Contrary to Saif et al. (2015) investigation in which gradient gels were used to provide optimal GR protein isoform separation, the current study used hand-cast gels. As hand-cast gels are not as effective as gradient gels in resolving proteins isoforms that differ from each other by one or two kilodaltons, the difference in the quality of the selected gels could have led to the discrepancies in the findings on the presence of GR isoforms in human placental samples in our study with Saif et al.'s (2015) investigation. Also, the GR isoforms vary in relation to their intracellular levels, with GRγ, GRA and GRP being the least abundant. To detect and quantify these proteins in human placental samples using Western blotting, high concentrations of proteins need to be used. In this study, high protein concentrations in Western blotting led to signal saturation (Supplementary figures 1 and 2)

and made GR data analysis using LICOR imager impossible. Due to signal saturation, only 20 µg of proteins were used in Western blotting as apposed to 60 µg in Saif et al.'s (2015) research. Overall, these differences in methodology selected could explain the variability in the results reported in this investigation compared to the reports of the previous investigations.

Furthermore, in accordance with other studies [163, 276], in our investigation, the GR profiles in the human placentae varied in relation to subcellular location, fetal sex and gestational age. GRα-A and unknown 38 kDa protein were higher in the nucleus than in the cytoplasm in all placentae independent of fetal sex or gestational age. Full-term male fetus placentae had a significantly higher expression of both GRα-A and GRβ in the nucleus than in the cytoplasm, whereas the placentae of full-term female had significantly lower GRα-D1 in the nuclear fraction compared to the cytoplasmic fraction. Interestingly, the full-term male infants had the highest umbilical cord cortisol levels compared to the infants of other experimental groups, indicating that full-term male fetuses have been exposed to elevated maternal GCs. Cortisol levels are significantly upregulated towards the end of pregnancy to facilitate maturation of fetal organs and initiate labor; however, it is still unclear as to what cortisol levels at the end of gestation are considered normal or optimal for the fetal development. An increased nuclear expression of GRβ in our full-term male fetus placentae could be a possible mechanism by which the placenta altered or blocked at least some of the GRα-A-mediated responses in the nucleus and protected the fetuses from the adverse effects of high maternal GCs so that the full-term male fetuses continued to grow normally in high GC environment during the final two weeks of gestation. More experimental investigations with larger sample sizes are needed to find out the normal range of cortisol concentrations throughout gestation and the effects of concomitant expression of GRα-A and GRβ in the nucleus on fetal growth.

The early-term fetus placentae also varied in relation to the GR profiles in the nuclear and cytoplasmic fractions. Both early-term male and female placentae had higher expression of GRα-A and GRα-D1 in the nucleus than in the cytoplasm; however, the early-term female fetus placentae had also upregulated nuclear GRα-C, unknown 68 kDa and 38 kDa proteins. It has been suggested that female fetus reduces growth when exposed to high GC levels from the maternal compartment and the sensitivity of female fetus to high maternal cortisol is mediated through the interaction of GRα-A with GRα-C or GRα-D [163]. Therefore, the upregulated expression of GRα-A, GRα-C and GRα-D isoforms in the nucleus in the placentae of the early-term females could be a pathway by which GRα-A interacted with GRα-C and/or GRα-D isoform to alter the expression of placental genes responsible for fetal growth. This hypothesis is supported by our finding with early-term female infants having the lowest birth weight compared to the infants of the other groups; however, further investigations are required to confirm these findings. Moreover, the potential functions of unknown 68 kDa and 38 kDa proteins and the effect of GCs on the subcellular localization of these proteins in the human placenta are yet to be identified, however an increased expression of cytoplasmic and nuclear 38 kDa protein in both full-term and early-term guinea pig placentae treated with GCs has been recently reported [277]. The finding of Saif *et al*. (2016) study and our result on the upregulated nuclear expression of the unknown 38 kDa protein may indicate that unknown 38 kDa may play important roles in mediating intracellular GR signaling in response to GCs and influencing fetal and placental growth.

Furthermore, the GR profile varied in a gender-specific manner in relation to gestational age. In the early-term male fetus placentae, the cytoplasmic expression of GRβ and nuclear expression of GRα-A and unknown 38 kDa protein were higher than in the placentae of full-term male fetuses. The effect of the nuclear co-expression of GRα-A and unknown 38 kDa protein on GR signalling, and a clearly defined pathway by which the co-

expression of GRα-A with unknown 38 kDa protein affects *in utero* fetal growth are still misunderstood and further research is required; however, the co-expression of nuclear GRα-A and unknown 38 kDa in the placentae of women carrying a male fetus might be important to control the expression of the placental genes responsible for the development of the placenta and the fetus between 37 and 38 weeks of the human gestation. Furthermore, in the early-term female fetuses placentae the nuclear levels of GRα-A, GRα-C, GRα-D1, GRα-D2/D3, unknown 68 kDa and GRβ were higher compared to their full-term female counterparts. This result may imply that the co-expression of GRα-A with other GRα isoforms and unknown 68 kDA may regulate the development of the fetus and the placenta between 37 and 38 gestational weeks in the women pregnant with a female fetus. As GRβ is an inhibitor of GRα transcriptional activities, a concomitant increase in the expression of the GRβ with GRα isoforms in the nucleus of the early-term female fetus placentae may indicate that some of the GRα-mediated responses have been either directly blocked through the interaction of GRβ with GRα isoforms or indirectly inhibited via interaction of GRβ with GRα GREs. Future studies may answer the question as to which of the nuclear GRα isoforms are inhibited through the interaction with GRβ in the human placentae. The results of the previous studies suggested that GRα-A expression is negatively correlated to the placental weight [163] and may interact with GRα-C and other isoforms to control placental growth [276], therefore the upregulated expression in of the nuclear GRα-A and GRα-C in the placentae of the early-term infants could be a possible mechanism by which cortisol controls placental development during 37 and 38 gestational weeks.

4.6 Summary

This study examined for the very first time GR profile in early-term infants (born between 37 and 38 gestational weeks) and full-term infants (born between 37 and 40 gestational weeks) in relation to subcellular localization and fetal sex. The GR patterns differed in relation to intracellular location, fetal sex and gestational age. The isoforms detected in cytoplasmic and nuclear fractions were as follow: GRα-A, GRα-C, GRα-D1, GRα D2/D3, GRβ and unknown 68 kDa and 38 kDa. GRα-A and unknown 38 kDa protein were higher in the nucleus than in the cytoplasm in all placentae independent of fetal sex or gestational age. Full-term male placentae had higher expression of both GRα-A and GRβ in the nucleus than in the cytoplasm, whereas full-term female placentae had a significantly lower nuclear expression of GRα-D1 compared to the cytoplasmic GRα-D1 levels, indicating a gender difference in GR expression in various intracellular fractions. Early-term male and female placentae had higher expression of GRα-A and GRα-D1 in the nucleus than in the cytoplasm. Besides the increased expression of nuclear GRα-A and GRα-D1, the early-term female fetus placentae had also upregulated nuclear GRα-C, unknown 68 kDa and 38 kDa proteins. Although early-term male infants and early-term female newborns were of the same gestational age, the early-term males weighed more compared to the early-term females. We speculated that the upregulated expression of GRα-A, GRα-C and GRα-D isoforms in the nucleus in the placentae of the early-term females could be a mechanism by which GRα-A interacted with GRα-C and/or GRα-D isoform to alter placental gene expression responsible for fetal growth which in turn resulted in a reduced weight of the female infants born early. It is unclear regarding the functions of unknown 68 kDa and 38 kDa proteins and the effect of GCs on the subcellular localization of these proteins in the human placenta, but the upregulation of the nuclear 38 kDa protein may play roles in GR intracellular signaling in response to GCs and fetal and placental development.

Moreover, GR profile varied in a gender-specific manner in relation to gestational age. In the early-term male fetus placentae, the cytoplasmic expression of GRβ and nuclear expression of GRα-A and unknown 38 kDa protein were higher than in the placentae of full-term male fetuses. The co-expression of nuclear GRα-A and unknown 38 kDa in the placentae of women carrying a male fetus might be essential in controlling the expression of growth-related genes in the placenta and fetus between 37 and 38 weeks of the human gestation. In the early-term female fetuses placentae the nuclear expression of GRα-A, GRα-C, GRα-D1, GRα-D2/D3, unknown 68 kDa and GRβ were higher compared to the full-term female fetus placentae. We hypothesized that the co-expression of GRα-A with other GRα isoforms and unknown 68 kDA may regulate the development of the fetus and the placenta between 37 and 38 gestational weeks in the women pregnant with a female fetus. Increased levels of nuclear GRα and GRβ in the early-term female fetus placentae may indicate GRβ-mediated inhibition of GRα signaling. The co-expression in of the nuclear GRα-A and GRα-C in the placentae of the early-term infants could be a way by which cortisol regulates placental and fetal growth during 37 and 38 gestational weeks.

Overall these data confirmed the findings of the other studies and provided evidence on complicated interaction between multiple GR isoforms in regulating fetal and placental development, with placenta responding differentially to cortisol and GR expression being dependent on fetal sex, gestational age and GC levels. Research on the roles of cortisol and GRs in placental and fetal development and the contribution of the alteration in GR expression in various subcellular fractions to programming of adult diseases is still an enigma. A better understanding of how GCs induce alterations in the placental structure and function and adversely affect *in utero* fetal development may improve the pregnancy outcomes and long-term wellbeing of future generations.

CHAPTER 5: Mitochondrial GRs

5.1 Introduction

Glucocorticoids regulate a plethora of cellular functions through GRs, the intracellular transcription factor that resides mainly in the cytoplasm and translocates from the cytoplasm to the nucleus following ligand binding to activate or inhibit nuclear gene transcription. In addition to the regulation of the transcription of nuclear genes by their cognate receptors, GCs may also modulate the mitochondrial gene transcription by mechanisms that remain unclear.

The GRs have been detected in the mitochondria of Muller cells of salamander retina [190], rat brain [191], C6 glioma cells [193], HeLa and Hep-2 cells [194]. GRs were observed in the mitochondria of human hepatocarcinoma HepG2 and osteosarcoma SaOS-2 cells using various detection techniques including Western blotting [192]. Two main bands were identified corresponding to the proteins with a molecular size of 95 kDa and 90 kDa, as well as a few smaller molecular weight bands at 70 kDa and 50 kDa were reported which could potentially represent GR isoforms [192]. Various molecular weight GR species have also been observed in rat liver mitochondria following GC treatment [189]. The functions of the mitochondrial GRs are not well understood, but the similarities of the nucleotide sequences in the mitochondrial genome with nuclear GREs may suggest direct roles of the GRs in the regulation of mitochondrial function and gene transcription [195]. The GR isoforms coordinating mitochondrial functions in humans are yet to be specified; though GRγ that comprises 10 % of all GR transcripts, has been recently detected in the mitochondria of human cells where it has been proposed to regulate the ATP production [275]. Besides energy production, mitochondria regulate a wide range of functions including but not limited to the cellular metabolism, calcium homeostasis, transcription, protein synthesis, development and cellular life and death [196, 198, 199, 202, 278].

In the context of gestation, placental apoptosis maintains normal embryonic and placental development, and increases with placental growth and advancing gestation [209]. However, trophoblast apoptosis is exaggerated in pregnancy complications including pre-eclampsia [209]. Placental pathology and widespread placental apoptosis may impair syncytiotrophoblast functions and lead to diminished nutrient transport to the fetus as demonstrated in IUGR [209]. A variety of stimuli can initiate placental apoptosis, including GC treatment. A common feature of mitochondria-mediated apoptosis is a release of cytochrome c from the mitochondria into the cytoplasm, leading to the activation of intracellular caspases that mediate the death cascade. Apoptosis induced by GC is associated with the translocation of GRs from the cytoplasm to the mitochondria (but not to the nucleus) [204, 213], and an up-regulation of effector caspases including caspases – 3 and – 6 [218-220]. Notably, caspase-3 is the main effector caspase in GC-mediated apoptosis as seen in previous investigations [221, 222]. The GR receptors through which GCs induced apoptotic events are GRα-A, -B, -C and -D as demonstrated by Western blotting using the whole cell extracts from COS-1 and U-2 cells with GRα-D isoforms being the least potent and GRα-C being the most efficient inducer of the apoptotic events [223]. The GR isoforms in placental mitochondria and their potential roles in modifying mitochondrial functions are not well elucidated, with no studies up to date investigating whether GRs are present in the mitochondria of human trophoblast cells and whether the expression of mitochondrial GRs varies depending on fetal sex and gestational age. Since, mitochondria control cell survival and death, possibly through the GRs, it would be interesting to detect and compare the expression mitochondrial GRs as well as cytoplasmic caspases -3 and -6 in the placentae of full-term and early-term infants a) to find out whether there are any differences in the expression of mitochondrial GRs and cytoplasmic caspases - 3 and - 6, b) to determine if the

differences are related to cortisol concentrations, fetal sex or gestational age, and c) to identify the GR isoforms associated with placental apoptosis.

5.2 Specific Aims

i. Separate placental lysates to obtain cytoplasmic and mitochondrial fractions
ii. Extract mitochondrial GRs and compare the GR isoform expression in relation to fetal sex and age at birth
iii. Examine cytoplasmic expression of caspases – 3 and – 6 in relation to gestational age, fetal sex and cortisol levels
iv. Identify the GR isoforms related to placental apoptosis

5.3 Materials and methods

This observational, cross-sectional study was approved by Human Research Ethics Committee of Griffith University (MSC 05/15 HREC) and Queensland Prince Charles Hospital (HREC 14/QPCH/246). Twenty four pregnant women were recruited from the Gold Coast University Hospital (Maternal and Fetal Unit) to take part in this study. An informed written consent was obtained from each participant prior to the child delivery to donate their placentas and cord blood samples following birth. The confidentiality of all patient records was maintained.

5.3.1 Maternal and neonatal characteristics

The study group consisted of 24 women: 12 women who gave birth to early-term babies (born between 37 weeks 0 days and 38 weeks and 6 days of gestation) and 12 women who delivered full-term babies (born between 39 weeks 0 days and 40 weeks and 6 days of gestation). The participants who had mental health issues or pregnancy complications were excluded from the study including those who had gestational diabetes, hypertension or preeclampsia. The recruited healthy women donated their cord blood samples and placentas following vaginal or cesarean delivery. Information on maternal and neonatal characteristics was obtained from medical records including data on maternal age and body mass index (BMI), fetal sex, birth weight, mode of delivery, gestational age and placental weight.

5.3.*2 Placenta and placental blood vessel collection*

The placental tissues and veins were collected within 45 minutes of delivery. Placental cotyledons and placental veins were isolated from multiple randomly selected areas to account for intra-placental variability of proteins. Please refer to Chapter 2 (Methodology), section 2.2 for details.

5.3.3 Cord blood cortisol collection and cortisol measurement

The umbilical cord venous blood was collected immediately after delivery. The quantitative measurement of cortisol was performed using a portable high-performance Immunoassay-Analysis-System *i*-CHROMA™ (Boditech, Med Inc, Gangwon-doo, Korea) with the analytical sensitivity of the device equal to 4.8 nmol/L. Please refer to Chapter 2, section 2.3 for details.

5.3.4 Preparation of cytoplasmic protein extracts

Placental tissues were crushed in liquid nitrogen using mortar and pestle and then homogenized in complete cytosolic fractionation buffer mixed with protease and phosphatase inhibitors with Saif *et al*. (2014) protocol used to prepare cytoplasmic fractions. Please refer to Chapter 2, section 2.4 for details.

5.3.5 Preparation of mitochondrial protein extracts

Mitochondrial extracts were prepared by homogenizing crushed frozen tissues in Mitochondrial Isolation Buffer containing protease and phosphatase inhibitors. The protein concentration in each sample was measured via a BCA assay (Pierce™ BCA Protein Assay Kit). Please refer to Chapter 2, section 2.5 for details.

5.3.6 BCA protein quantification

The prepared lysates have been diluted to approximately of 1 µg of protein per 1 µL with. The dilutions used were as follows: 1:20 for cytoplasmic and 1:10 for mitochondrial lysates. The BCA Assay Kit was used for the colorimetric detection and quantification of total protein in tissue extracts. After protein quantification, aliquots of 20 µg/µL were

prepared in appropriate volumes of Kinexus Buffer (pH 7.2) for the Western immunoblotting and stored in a - 80°C freezer. Please refer to Chapter 2, section 2.6 for details.

5.3.7 Purity of subcellular fractions

The purity of the cytoplasmic and mitochondrial fractions was validated. To demonstrate that the isolated extracts were of a reasonable purity, cytoplasmic and mitochondrial fractions were assessed for β – actin and COX IV, respectively. Please refer to Supplementary figure 4 in the Appendix for Western blotting results on the purity of subcellular fractions.

5.3.8 Antibody optimization

To achieve a robust and specific signal, the quality of the antibodies listed in Table 2.10 (Chapter 2) was tested, with the concentrations of each primary and secondary antibody optimized prior to the Western immunoblotting.

5.3.9 Western blotting

The Western blotting protocol was optimized for each antibody including the running time and voltage for the gel electrophoresis and protein transfer. The incubation duration and temperature (e.g., room temperature versus 4°C) for the blocking step and for the incubations step with primary and secondary antibody have also been optimized. Please refer to Chapter 2, section 2.10 for details on Western blotting protocol. Chapter 2 Table 2.10 provides the list of antibodies.

5.3.10 Statistical analysis

Assessment of variable type and level of measurement and normality check for each variable of interest was conducted to choose an appropriate statistical test. Normally distributed data were presented as mean (SEM). The parametric Student t-test, Pearson correlation and One-way ANOVA with Bonferroni correction were used to analyze normally

distributed data. Non-parametric Spearman's rank-order correlation and Kruskal-Wallis tests were used for data analysis. The Statistical Package for the Social Sciences (SPSS version 22) and GraphPad Prism version 7 were used for statistical analysis. The level of significance was set at $p < 0.05$ (two-tailed).

5.4 Results

5.4.1 Glucocorticoid receptor profile in mitochondria of human trophoblast cells

The GRs have been identified in the mitochondria of various cells of animal and human origin. However, currently, there are no studies that examined whether GRs are present in the mitochondria of the human trophoblast cells and whether the expression of mitochondrial GRs varies depending on fetal sex and gestational age. Mitochondrial GR levels were evaluated in the human placenta to discover if the expression of the GRs is fetal sex- or gestational age-specific.

Three bands were detected in the mitochondria of the trophoblast cells, including the bands at 68-69, 50-51 and 38 kDa. These bands correspond to unknown 68-69 kDa, GRα D2/D3 and unknown 38 kDa proteins, respectively. Data suggest that unknown 68 kDa, GRα D2/D3 and unknown 38 kDa proteins did not significantly differ in relation to fetal sex (Kruskal-Wallis test, $p > 0.05$) or gestational age (Kruskal-Wallis test, $p > 0.05$) (Figure 5.1). Refer to Figure 5.2 for Western blotting images.

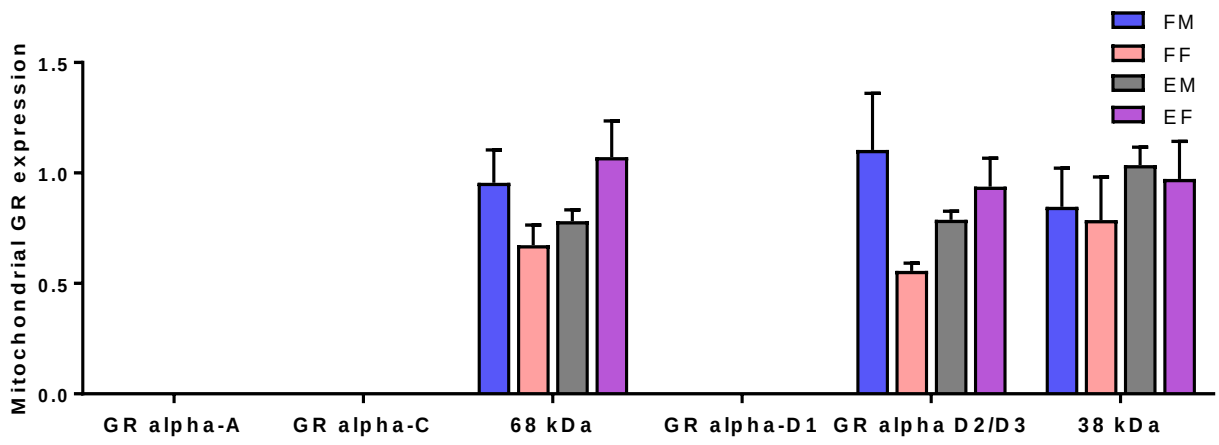


Figure 5. 1: Mitochondrial GR profile in relation to fetal sex and gestational age. Relative expressions of GRα D2/D3 and unknown 38 and 68 kDa were assessed in mitochondria of trophoblast cells. COX IV was used as a loading control. Kruskal-Wallis test was performed for data analysis with values considered significant when the p-value was less than 0.05. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female).

Mitochondrial GR profile

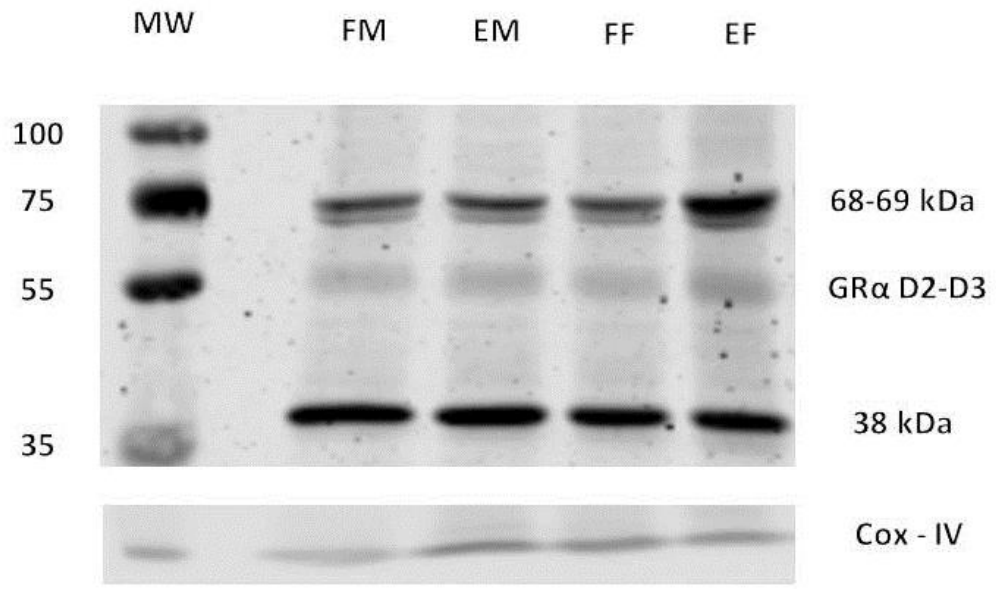


Figure 5. 2: Representative Western blotting of placental mitochondrial glucocorticoid receptor (GR) isoforms from full-term and early-term deliveries. Mitochondrial protein extracts of placentae from full-term and early-term deliveries were used to detect GRs. Blots have been probed with COX IV as a loading control. Groups were classified into FM (full-term male), EM (early-term male), FF (full-term female), EF (early-term female). *Significant changes have not been detected.*

5.4.2 Placental apoptosis

Placental apoptosis during pregnancy is a normal physiological process required for proper embryonic and placental development. However, when exaggerated it may impair placental and fetal development leading to the inhibition of fetal growth *in utero*. The relative expressions of pro-apoptotic markers, caspases – 3 and – 6, were measured in placental trophoblast cells to find out if early-term births are associated with exaggerated placental apoptosis. Caspase – 3 and caspase – 6 levels were also evaluated in relation to fetal sex.

The expressions of caspases – 3 and – 6 were examined as biomarkers of cell death. Caspase – 3 expression did not differ in relation to fetal sex (Kruskal-Wallis test, $p > 0.05$, Figure 5.3 (a)), but it was significantly different in relation to fetal age at birth, with the early-term male fetus placentae exhibiting significantly higher caspase – 3 levels compared to the placentae of full-term male foetuses (Kruskal-Wallis test, $p < 0.05$, Figure 5.3 (a)). Caspase – 6 levels did not vary in regard to fetal sex (Kruskal-Wallis test, $p > 0.05$, Figure 5.3 (b)) or gestational age (Kruskal-Wallis test, $p > 0.05$, Figure 5.3 (b)). Refer to Figure 5.4 for Western blotting images.

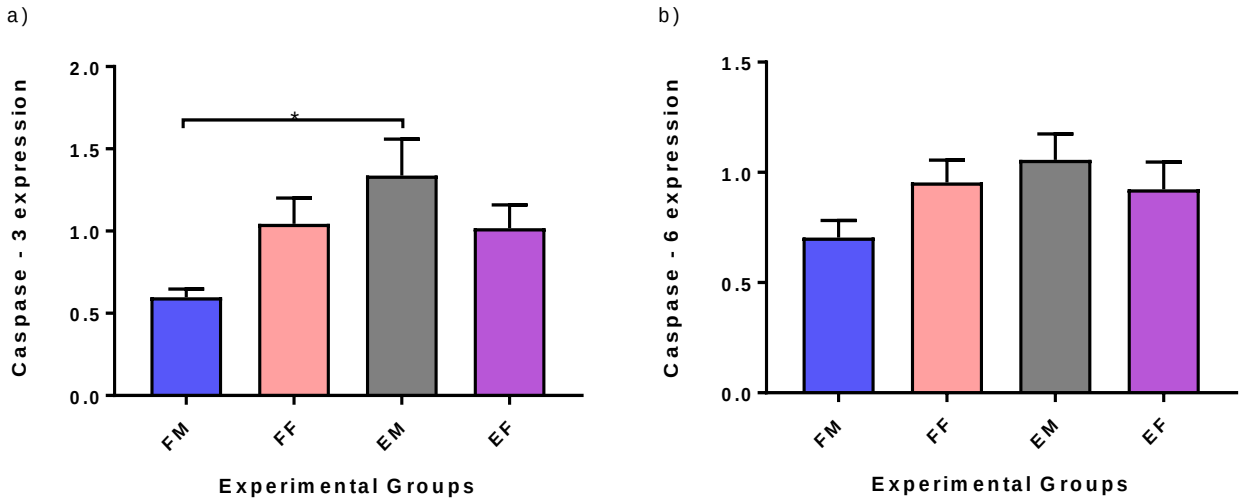
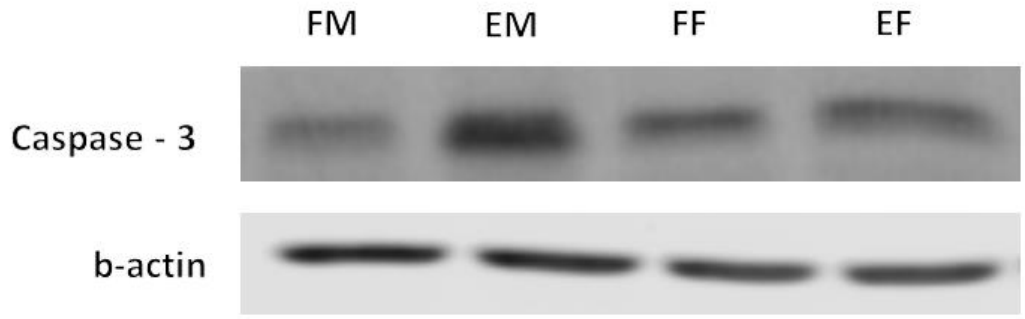


Figure 5. 3: Expression of pro-apoptotic caspases in the human placentae in relation to fetal sex and gestational age. Caspase – 3 (a) and caspase – 6 (b) expressions relative to β-actin. Kruskal-Wallis test was performed for data analysis, with values considered significant when the p-value was less than 0.05. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female). * P-value < 0.05.

Cytoplasmic caspase – 3



Cytoplasmic caspase – 6

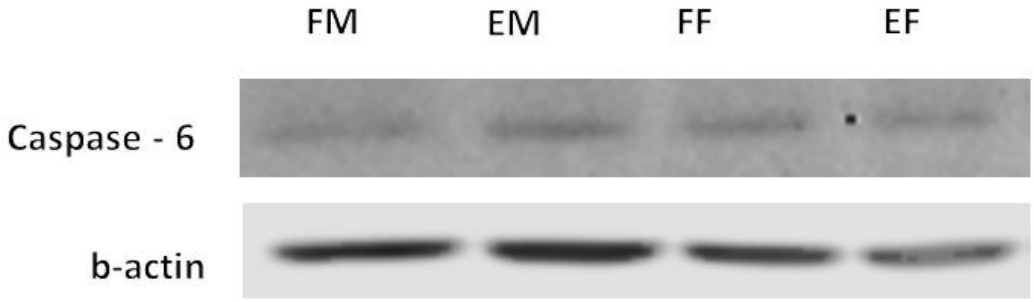


Figure 5. 4: Representative Western blot of placental caspase – 3 (top) and caspase – 6 (bottom) from full-term and early-term deliveries. Cytoplasmic protein extracts of placentae from full-term and early-term deliveries were used to detect caspase – 3 and caspase – 6. Blots have been probed with β – actin as a loading control. Groups were classified into FM (full-term male), EM (early-term male), FF (full-term female) and EF (early-term female). *Significant changes **have been detected** in the expression of caspase – 3.*

5.4.3 Relationship between cortisol levels and caspase – 3 expression

Research suggests that GC treatment is associated with caspase – 3 upregulation followed by apoptosis. The correlation between cortisol levels and expression of caspase – 3 was examined to determine if caspase – 3 upregulation in the early-term males is related to cortisol levels.

Caspase – 3 expression in the placentae of early-term males did not correlated with cortisol levels (Spearman r = 0.08, p > 0.05, Figure 5.5).

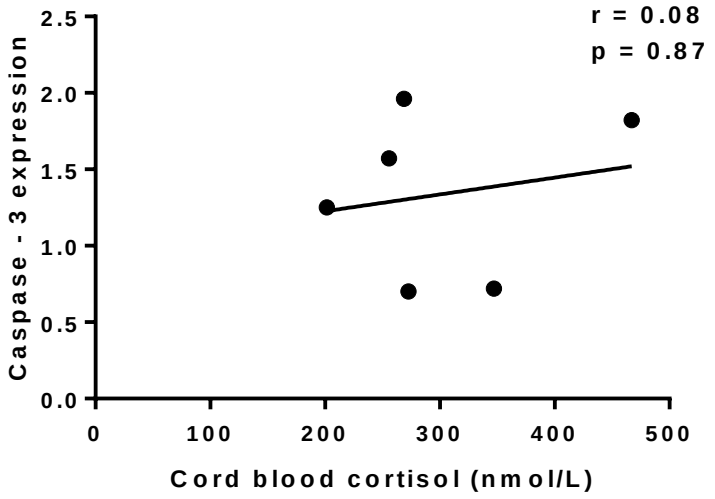


Figure 5. 5: Scatterplot exhibiting the relationship of cord blood cortisol with caspase – 3 expression in early-term male placentae. Spearman correlation test was used to analyze the data.

5.4.4 Relationship between placental apoptosis and mitochondrial GRs

Caspase – 3 levels were significantly up-regulated in the placentae of early-term males. Since GC-induced apoptosis is associated with the translocation of GRs into mitochondria, the relationships of cytoplasmic caspase – 3 with mitochondrial GRs in the placentae of early-term males were investigated.

There were no significant relationships between mitochondrial GRs and caspase – 3 levels in the placentae of early-term males (Spearman test results, with the corresponding p – values are presented in Figure 5.6).

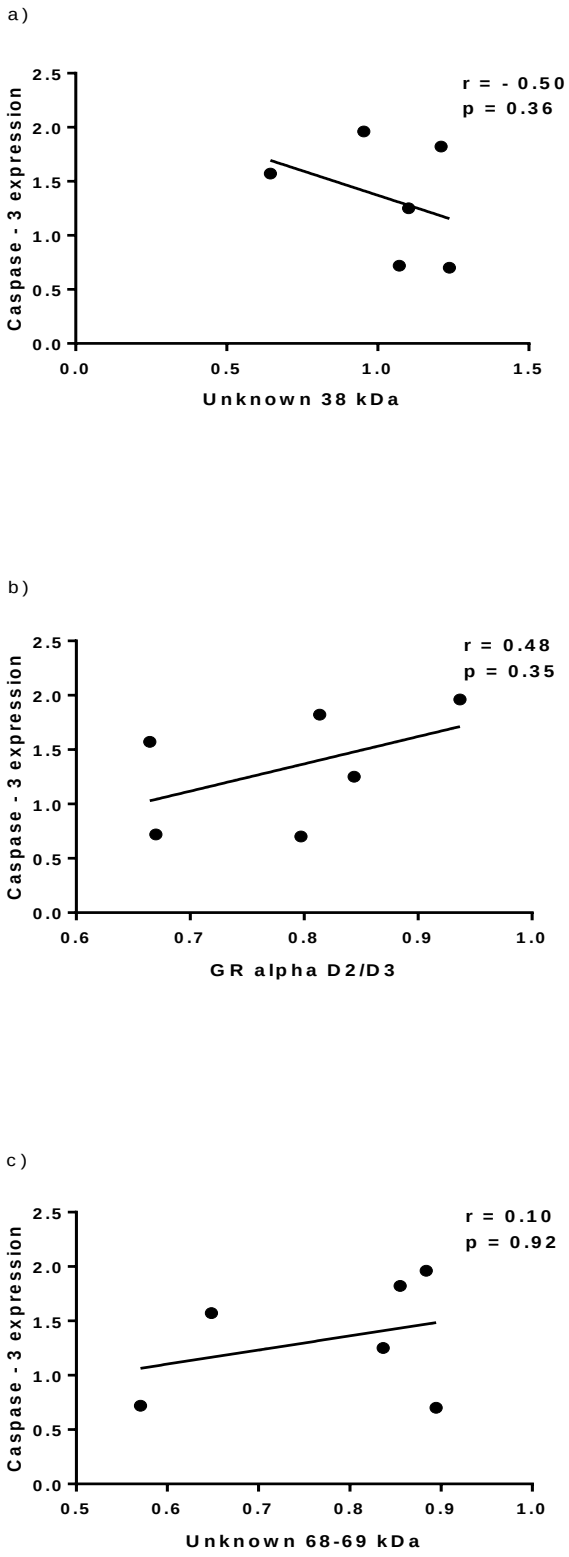


Figure 5. 6: Scatterplots exhibiting relationships of cytoplasmic caspase – 3 with mitochondrial GRs in the placentae of early-term males. Correlation of caspase – 3 with unknown 38 kDa (a), GRα D2/D3 (b) and unknown 68-69 kDa (c) proteins. Spearman correlation test was used to analyze the data.

5.5 Discussion

The GRs have been detected in mitochondria of various animal and human cells, but not in the trophoblast cells. Whether GRs are present in the mitochondria of human placental cells and whether GR expression varies in relation to fetal sex and gestational age it is still unclear. The current study provides an insight into mitochondrial GRs as potential determinants of mitochondrial function during the last few weeks of human gestation. This is the first study to examine mitochondrial GRs in human trophoblast cells in relation to fetal sex, gestational age and placental apoptosis.

GR profile in mitochondria of human trophoblast cells

Three molecular bands of 68-69, 50-51 and 38 kDa were detected via Western blotting technique. The observed bands correspond to unknown 68-69 kDa, GRα D2/D3 and unknown 68 kDa proteins. Despite there were no significant sex differences in the expression of these mitochondrial proteins in relation to gestational age, some trends were apparent. In early-term male fetus placentae, the mitochondrial expression of unknown 68 kDa and GRα D2/D3 tended to be lower than in the full-term male fetus placentae. Conversely, in early-term female fetus placentae, the expression of these proteins tended to be higher compared to the placentae of full-term females. The detection of these small molecular weight proteins in the mitochondria of human trophoblast cells is consistent with previous findings in other cell types [189-191] and with the investigation conducted by Solakidi et al. (2007) who discovered small molecular weight GRs in human HepG2 and SaSO-2 mitochondrial extracts by Western blotting. Contrary to the earlier investigations on the presence of GRs in mitochondria [189, 191, 275], we failed to demonstrate the large GR polypeptides with a molecular weight of 95 or 90 kDa. The GR proteins with large molecular size detected in previous studies could correspond to GRα-A, GRα-B, GRγ or a phosphorylated form of the

GR. The inconsistencies in the reported results in regard to the size of the GRs detected in mitochondria could reflect the variations in the experimental methods, protein detection techniques or cell types used for the investigations and necessitate more research to specify the isoforms of the GR present in mitochondria. One of the explanations of the inconsistencies in the listed findings could be attributed to the differences in the types of cells selected for the experimentations. For instance, Morgan et al. (2016) detected high molecular weight GRα and GRγ in mitochondria of adenocarcinomic human alveolar basal epithelial cells (A459 cell line) by proteomic analysis [275]. Likewise, in murine models, large GR polypeptides have been observed in liver and brain mitochondria via immunoblotting [189, 191]. The current investigation implemented Western blotting and failed to detect high molecular weight GR proteins with molecular sizes ranging from 75 kDa to 95 kDa. As the expression of GR isoforms is cell-type specific, it is quite possible that these GRs are either not present in mitochondria of human placental cells or are expressed at significantly lower levels than in the mitochondria of rodent hepatic and brain cells. In the future, high levels of protein extracts should be used in Western blotting to examine the presence of large GR proteins in human placental cells mitochondria. Alternative methods of protein detection, including immunohistochemistry and proteomic analysis, may provide a better understanding of GR profile in the mitochondria of placental trophoblast cells.

Placental apoptosis

Although GRs in mitochondria have been discovered nearly 30 years ago [279] and have been consistently detected in mitochondria in multiple subsequent studies, the roles of mitochondrial GRs in modulating a broad spectrum of mitochondrial functions including apoptosis is still poorly understood. The equilibrium between cell proliferation and death during development is of a pivotal importance since altered trophoblast apoptosis is related to fetal growth restriction, abnormal placentation and syncytial loss [209]. To find out if early-

term births are associated with exaggerated placental apoptosis, caspase – 3 and caspase – 6 expressions were examined as biomarkers of placental cell death.

Caspases – 3 and – 6 were detected in all placental samples independent of fetal sex or age. Placental expression of caspase – 6 did not significantly differ in relation to fetal sex or gestational age. While there were no significant differences in the expression of placental caspase – 3 in relation to fetal sex, caspase – 3 levels significantly varied in relation to gestational age, with the placentae of early-term male fetuses exhibiting higher levels of pro-apoptotic caspase – 3 compared to the placentae of full-term male counterparts. Caspase -3 upregulation in the early-term male placentae was not associated with the syncytial loss as demonstrated by normal placental weight of the early-term male fetuses. Growing evidence now indicates that caspases exert important functions beyond cell death. In the human placentae, caspases may play roles in syncytiotrophoblast maintenance with caspases – 3, - 8 and – 9 mediating the remodeling of the sub-membranous cytoskeleton during trophoblast fusion and are essential for differentiation and syncytial fusion of cytotrophoblasts [280]. In primary T-cells caspase – 3 and caspase – 8 activation is a prerequisite for cell division and cell-cycle progression with inactivation of caspase activity leading to reduce cell-cycle progression [281]. The non-apoptotic functions of caspase – 3 in the fetal development have been confirmed in mice, with caspase – $3^{-/-}$ being smaller at birth than their littermates [282]. Based on these findings we may suggest that the presence of caspases – 3 and – 6 in the placentae of early-term and full-term infants do not necessarily reflect placental dysfunction, but perhaps indicate cytotrophoblasts differentiation, syncytial fusion and/or trophoblast cell-cycle progression and is essential for fetal growth. Clearly, much remains to be found out regarding the dual role of caspases in cell death and proliferation. Future studies may provide new insights into the exact contribution of placental caspases in placental and fetal development.

Relationship between cord blood cortisol levels and apoptosis in early-term male fetus placentae

Glucocorticoid-induced apoptosis is characterized by several biochemical changes, including activation of caspase – 3 [218-220]. To further test whether apoptosis in the placentae of early-term male fetuses is related to cortisol concentrations, the correlation between cord blood cortisol and caspase – 3 expression was examined. Caspase – 3 levels in the early-term male fetus placentae were unrelated to cord blood cortisol levels, indicating that the increased caspase – 3 expression was not in response to cortisol upregulation. As mentioned in the section above, the upregulated expression of caspase – 3 could be a normal process required for the differentiation, proliferation and cell-cycle progression of human trophoblast cells and optimal fetal development; however this notion has to be confirmed by further investigations because caspase upregulation could also be related to placental hypoxia [283], reactive oxygen species or calcium overload [284].

Relationship between mitochondrial GRs and apoptosis in early-term male fetus placentae

It has been proposed that of all GRα isoforms, GRα-C3 is the most potent inducer of apoptosis, whereas GRα-D3 is the least potent activator of apoptotic events, with cells expressing GRα-D3 being resistant to GC-induced cell death [223]. The GRα isoforms reported to play role in GC-induced apoptosis in Bender *et al.* (2013) study have been examined in whole cell lysates, so it is unclear whether cytoplasmic, nuclear or mitochondrial GRα isoforms or a combination of these receptors play role in the induction of apoptosis. The detailed pathways by which GCs induce apoptosis are not clear; however, GC-induced translocation of the GRs from the cytoplasm to the mitochondria has been suggested [204, 213]. To find out which mitochondrial GR isoform is associated with up-regulated expression

of caspase – 3 in early-term male fetus placentae, the correlations between mitochondrial GR isoforms and caspase – 3 were investigated.

Western blot data analysis suggests that there is no relationship between caspase – 3 and GRα D2/D3, unknown 38 or 68 kDa mitochondrial proteins in the placentae of early-term male fetuses. The basis of caspase – 3 upregulation in this group is unclear. Although GCs may regulate apoptosis via GRs, other mechanisms (mitochondrial GR-independent) could be involved in caspase – 3 activation in the early-term male fetus placentae. Future studies with larger sample groups may shed light on the potential signaling pathways upregulating caspase activities in the human placenta and the effects of altered caspase expression on pregnancy outcomes.

5.6 Summary

This investigation was the first investigation to detect several GR species in the mitochondria of human trophoblast cells, including proteins with molecular weights of 38 kDa, 50-51 kDa and 68 kDa, corresponding to unknown 38, GRα D2/D3 and unknown 68 kDa proteins, respectively. The GRs in the mitochondria did not differ significantly in relation to fetal sex or age at birth. Nevertheless, in the placentae of early-term male fetuses, the mitochondrial expression of unknown 68 kDa and GRα D2/D3 tended to be lower than those of the full-term male fetus placentae, while in early-term female fetus placentae the expression of these proteins tended to be higher compared to the placentae of full-term females. Whether mitochondrial GR profile is fetal sex– or gestational age-specific remains to be established in future studies. Furthermore, small molecular weight GR proteins have been also detected in the mitochondria of animal and human cells in previous studies. In contrast to earlier investigations, we did not identify high molecular weight GR polypeptides including proteins with the molecular weight of 90 or 95 kDa, possibly due to variations in the methodological protocols or cell types selected for the experiments.

Placental apoptosis was examined as a possible contributor to early-term births. While caspase – 6 expression was not related to fetal sex or gestational age, caspase – 3 levels varied in regard to fetal gestational age, with the early-term male fetus placentae having higher levels of caspase – 3 compared to the full-term male fetus placentae. An increase in the expression of caspase – 3 could reflect the differentiation, proliferation and/or cell-cycle progression of human trophoblast cells, although more studies are needed to rule out caspase – 3 mediated placental dysfunction as a result of placental hypoxia, calcium overload or reactive oxygen species. Upregulated caspase – 3 levels in the early-term group were not related to cord blood cortisol levels, nor to mitochondrial GRs, implying that mitochondrial

GR-independent pathways could regulate caspase – 3 in the placentae of the early-term male group.

Since GRs have been detected in the mitochondria of human trophoblast cells, more research is needed to confirm the results of this study and find out the exact role of each GR isoform in the regulation of mitochondrial functions. Because early-term male fetus placentae had a significantly higher expression of caspase – 3 compared to the full-term male fetus counterparts, it is paramount to further examine the relationship between caspase activity in the placenta and fetal development. Future investigations may clarify whether early-term births are associated with placental dysfunction as a result of the upregulation of caspase activities in the human placentae.

CHAPTER 6: Placental Serotonin System – Effects of Glucocorticoids

6.1 Introduction

During gestation serotonin (either of maternal or placental origin) controls the development of both, placenta and fetus. Accordingly, abnormalities in serotonin synthesis, degradation or signaling transduction are associated with pregnancy complications and abnormal *in utero* fetal development that have long-lasting impacts on the offspring.

Serotonin is produced in the CNS and in the periphery, including but not limited to GI tract and placenta. It is synthesized by tryptophan hydroxylase (TPH) and aromatic amino acid decarboxylase (AAAD) from tryptophan and degraded by monoamine oxidase A (MAO-A), an enzyme that is located on the mitochondrial outer membrane. In the placenta, serotonin intracellular transduction is terminated via serotonin transporter or SERT, the plasma membrane-bound protein controlling extracellular levels of serotonin in the trophoblast cells. Serotonin signaling is mediated via various types of serotonin receptors, with tissue and blood vessel 5-HT_{1A} and 5-HT_{2A} receptors being of interest to the present study.

In the placental tissues, serotonin stimulates trophoblast cell viability, cell cycle progression, proliferation and placentation via 5-HT_{2A} receptors [237, 238], and maintains pregnancy and placental and fetal growth via 5-HT_{1A} receptors [239]. In the blood vessels, serotonin is known to constrict bronchial, uterine and urinary vascular smooth muscles via 5-HT_{1B} or 5-HT_{2A} receptors, and mediates vasorelaxation of pig pulmonary arteries and rat jugular veins via 5-HT_{1A} or 5-HT_{2B} receptors present in the walls of the blood vessels [229, 230, 240, 241]; however the subtype of serotonin receptors present in the placental blood vessels of human is yet to be found out. In the fetus, serotonin plays crucial roles during early stages of embryogenesis and regulates neural crest migration, neuronal connectivity, craniofacial and limb development as well as gastrointestinal, CV and humoral

morphogenesis [234]. Thus, dysfunction in the serotonin system signaling has been linked to pregnancy complications, aberrant placental function and fetal development, with adverse consequences manifested in adulthood, including abnormal development and behaviour in children [248-252].

Evidence supports the hypothesis that altered serotonergic signaling is associated with pregnancy complications including gestational diabetes mellitus (GDM) and pre-eclampsia. A substantial reduction in the 5-HT_{2A} receptors and SERT mRNA and protein levels has been detected in villous trophoblast cells in the placentae of pregnancies complicated by GDM, indicating that aberrant 5-HT_{2A} and SERT expression may play role in the pathogenesis of gestational diabetes in human [242]. Similarly, in pre-eclampsia (the major cause of maternal and prenatal mortality and morbidity), a hyper serotonergic placental state has been associated with altered serotonin metabolism [243]. Previous data have revealed that decreased serotonin levels during sensitive developmental period adversely affect the connectivity of major axons, cell division and neocortex laminar organization [244-247], whereas increased serotonin levels may lead to congenital abnormalities or kill the developing embryo [253]. In animal models, dysfunction in the serotonin signalling during the critical period of fetal brain development is related to behavioural abnormalities; in human, perturbations in serotonin transduction is linked to neurodevelopmental and behavioural disorders [248-252]. Moreover, the impact of serotonin, SERT and TPH1 on placenta has been recently explored in SERT $^{-/-}$ and TPH1$^{-/-}$ mice. Whereas in SERT $^{-/-}$ mice plasma serotonin levels were two times higher compared with wild-type (WT) mice, in TPH1$^{-/-}$ mice serotonin levels were undetectable [285]. Although levels of serotonin in TPH1$^{-/-}$ mice were insignificant, both SERT $^{-/-}$ and TPH1$^{-/-}$ mice had increased levels of placental apoptosis with SERT $^{-/-}$ mice showing greater placental damage compared to TPH1$^{-/-}$ mice.

These findings demonstrated the importance of serotonin regulation and ongoing signaling in placental and fetal growth.

There is now sufficient evidence to indicate that GCs may regulate or alter serotonin signaling. In human skeletal muscle cells, GC excess significantly increases MAO-A enzymatic activity and mRNA and protein expression. The upregulation in the expression of MAO-A is accompanied by the production of ROS in these cells and may play role in the pathogenesis of GC-induced muscle loss [254]. Similar effects of GCs on MAO-A expression has been demonstrated in human neuroblastoma SK-N-BE(2)-C and glioblastoma 1242-MG cells where DEX treatment significantly increased MAO-A gene expression [255]. In rat liver and fat tissue, DEX treatment upregulated levels of serotonin in a dose- and time-dependent manner as well as the expression of 5-HT_{2A} receptors, TPH1 and AAAD [256]. Whether the expression of SERT is affected following treatment with GCs is not clear. Several investigations have shown DEX-induce upregulation of SERT expression in human B-lymphoblastoid cells and in rat brainstem [257, 258], whereas other demonstrated downregulation in SERT levels as a result of prenatal stress and concomitant GC excess [259]. Whether GC hormones affect serotonin system in the human placenta and whether GC-induced alterations in serotonin function results in early-term births is unknown. Therefore, this study investigates the role of serotonin in the human placenta to determine whether early-term births are associated with the alteration in the serotonin function or metabolism in the human placenta and whether fetal sex has an effect on the components of the serotonin system.

6.2 Specific Aims

i. Examine if there are differences in serotonin levels

ii. Evaluate SERT, TPH1, MAO-A, 5-HT_{1A} and 5-HT_{2A} receptor protein expression in early-term and full-term placentae

iii. Examine whether there are differences in 5-HT_{1A} and 5-HT_{2A} receptor expression in the placental veins

iv. Determine if differences (if any) in the expression of the components of the serotonergic system are related to cord blood cortisol levels

6.3 Material and methods

This observational, cross-sectional study was approved by Human Research Ethics Committee of Griffith University (MSC 05/15 HREC) and Queensland Prince Charles Hospital (HREC 14/QPCH/246). Twenty four pregnant women were recruited from the Gold Coast University Hospital (Maternal and Fetal Unit) to take part in this study. An informed written consent was obtained from each participant prior to the child delivery to donate their placentas and cord blood samples following birth. The confidentiality of all patient records was maintained.

6.3.1 Maternal and neonatal characteristics

The study group consisted of 24 women: 12 women who gave birth to early-term babies (born between 37 weeks 0 days and 38 weeks and 6 days of gestation) and 12 women who delivered full-term babies (born between 39 weeks 0 days and 40 weeks and 6 days of gestation). The participants who had mental health issues or pregnancy complications were excluded from the study including those who had gestational diabetes, hypertension or preeclampsia. The recruited healthy women donated their cord blood samples and placentas following vaginal or cesarean delivery. Information on maternal and neonatal characteristics was obtained from medical records including data on maternal age and body mass index (BMI), fetal sex, birth weight, mode of delivery, gestational age and placental weight.

6.3.2 Placenta and placental blood vessel collection

The placental tissues and veins were collected within 45 minutes of delivery. The umbilical cord venous blood was collected immediately after delivery in EDTA-containing tubes for cortisol level evaluation. Placental cotyledons and placental veins were isolated from multiple randomly selected areas to account for intra-placental variability of proteins. Please refer to Chapter 2, section 2.2 for details.

6.3.3 Cord blood cortisol collection and cortisol measurement

The quantitative measurement of cortisol was performed using a portable high-performance Immunoassay-Analysis-System *i*-CHROMA™ (Boditech, Med Inc, Gangwon-doo, Korea). Cortisol levels in each sample were measured following the manufacturer's instructions. Please refer to Chapter 2, section 2.3 for details.

6.3.4 Preparation of cytoplasmic protein extracts

Placental tissues were crushed in liquid nitrogen using mortar and pestle and then homogenized in complete cytosolic and nuclear fractionation buffers mixed with protease and phosphatase inhibitors. Saif *et al*. (2014) protocol was used to prepare cytoplasmic and nuclear fractions. Prepared samples were stored at -80°C as a cytosolic fraction until further analysis. Please refer to Chapter 2, section 2.4 for details on the preparation of cytoplasmic protein extracts. The protein concentration in each sample was measured via a BCA assay (Pierce™ BCA Protein Assay Kit).

6.3.5 Preparation of mitochondrial protein extracts

Mitochondrial extracts were prepared by homogenizing crushed frozen tissues in Mitochondrial Isolation Buffer containing protease and phosphatase inhibitors. Please refer to Chapter 2, section 2.5 for details on the preparation of mitochondrial protein exptracts.

6.3.6 BCA protein quantification

The prepared lysates have been diluted to approximately of 1 µg of protein per 1 µL. The dilutions used were as follows: 1:20 for cytoplasmic and 1:10 for mitochondrial lysates. The BCA Assay Kit was used for the colorimetric detection and quantification of total protein in tissue and blood vessel extracts. After protein quantification, aliquots of 20 µg/µL were prepared in appropriate volumes of Kinexus Buffer (pH 7.2) for the Western immunoblotting.

The prepared aliquots were stored in a - 80°C freezer. Please refer to Chapter 2, section 2.6 for details.

6.3.7 Purity of subcellular fractions

The purity of the cytoplasmic and mitochondrial fractions was validated to provide information on the biochemical purity of these subcellular fractions. To demonstrate that the isolated extracts were of a reasonable purity, the cytoplasmic and mitochondrial fractions were assessed for β-actin and COXIV, respectively. Please refer to Supplementary figure 4 in the Appendix for the Western blotting results on the purity of subcellular fractions.

6.3.8 Antibody optimization

To achieve a robust and specific signal, the quality of the antibodies listed in Table 2.10 (Chapter 2) was tested, with the concentrations of each primary and secondary antibody optimized prior to the Western immunoblotting.

6.3.9 Western blotting

The Western blotting protocol was optimized for each antibody including the running time and voltage for the gel electrophoresis and protein transfer. The incubation duration and temperature (e.g., room temperature versus 4°C) for the blocking step and for the incubations step with primary and secondary antibody have also been optimized.

Polyacrylamide gel electrophoresis and protein transfer

The prepared samples were thawed on ice and mixed with 2 x Loading Dye and β-mercaptoethanol to a final volume of 30 µl. The protein aliquots were left for 5 minutes in a heating block set at 95°C to denature proteins. Gel electrophoresis was performed by loading 28 µl of each sample into a hand-cast 15- well 7.5% Tris-glycine gel. The proteins were separated by running the gel at 125 V for 1 hour 20 minutes, then transferred to polyvinylidene difluoride (PVDV) membrane at 70 V for 1 hour 15 minutes. The target

proteins were detected using the Odyssey CLx Infrared Imaging System (Millennium Science, Mulgrave, Australia) (see Table 2.10 in Chapter 2for the list of antibodies). Please refer to Chapter 2, section 2.10 for details on Western blotting protocol. Chapter 2 Table 2.10 provides the list of antibodies.

6.3.10 Serotonin ELISA

Serotonin ELISA kit (Abcam, cat. No ab133053) has been used to measure placental tissue serotonin levels according to the manufacturer's instructions, with the absorbance of the samples red at 405 nm using the microplate reader (Tecan infinite M200 Pro, Mannedorf, Switzerland). The sensitivity of the serotonin ELISA assay was 0.293 ng/mg and cross-reactivity with other serotonergic compounds < 0.1 %. Results expressed in ng/mg of placental tissue. Please refer to Chapter 2, section 2.11 for details on the protocol.

6.3.11 Statistical analysis

Assessment of variable type and level of measurement and normality check for each variable of interest was conducted to choose an appropriate statistical test. The non-parametri Spearman's rank-order correlation and Kruskal-Wallis tests were used to analyse data. The Statistical Package for the Social Sciences (SPSS version 22) and GraphPad Prism version 7 were used for statistical analysis. The level of significance was set at $p < 0.05$ (two-tailed).

6.4 Results

6.4.1 Examination of serotonin levels in placental tissues in relation to fetal sex and gestational age

To find out if early-term births were due to abnormal serotonin bioavailability in the placenta, serotonin levels were evaluated in all placental tissues. Serotonin concentration in the tissues was examined in relation to fetal sex and gestational age.

Placental tissue serotonin levels did not differ in relation to fetal sex (Kruskal-Wallis test, $p > 0.05$, Figure 6.1) or gestational age (Kruskal-Wallis test, $p > 0.05$, Figure 6.1).

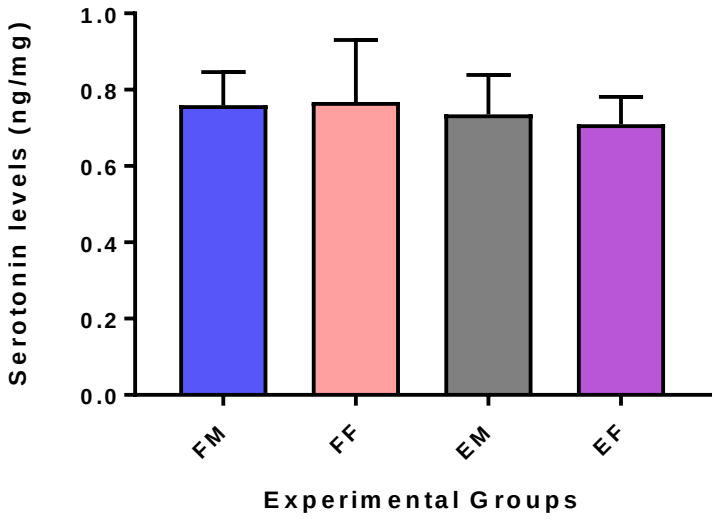


Figure 6. 1: Serotonin levels in placental tissues in relation to fetal sex and gestational age. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female). Kruskal-Wallis test was performed for data analysis with values considered significant when the p-value was less than 0.05.

6.4.2 Examination of components of serotonin system in placental tissues in relation to fetal sex and gestational age

To test if early-term births were due to alterations in the serotonin signal transduction, the components of serotonin system were evaluated in human placental tissues, including: 1) SERT – the transporter that terminates the action of serotonin in the placental tissues; 2) TPH1 and MAO-A - the enzymes involved in serotonin synthesis and degradation, respectively; and 3) 5-HT_{1A} and 5-HT_{2A} receptors through which serotonin mediates its signalling and controls placental and fetal development. The relative expression of these factors was examined in relation to fetal sex and gestational age.

Placental tissue SERT, TPH1, MAO-A, 5-HT_{1A} and 5-HT_{2A} receptor levels did not differ in relation to fetal sex (Kruskal-Wallis test, $p > 0.05$, Figure 6.2) or gestational age (Kruskal-Wallis test, $p > 0.05$, Figure 6.2). Refer to Figure 6.3 for Western blotting images.

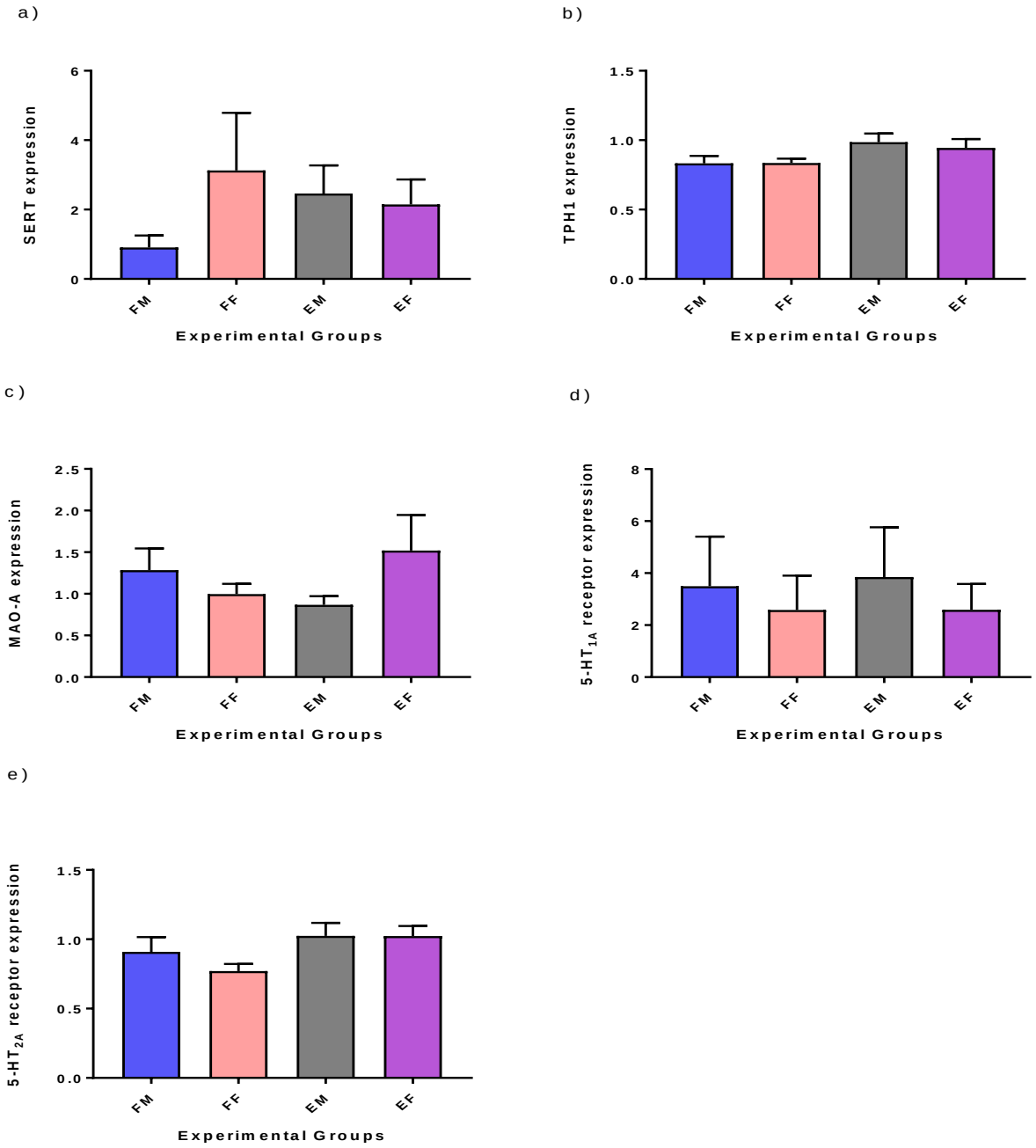


Figure 6. 2: Examination of components of the serotonin system in human placental tissues in relation to fetal sex and gestational age. Shown are levels of a) Serotonin transporter (SERT); b) Tryptophan hydroxylase 1 (TPH1); c) Monoamine oxidase A (MAO-A); d) 5-HT_{1A} receptor and e) 5-HT_{2A} receptor. Protein expression of SERT, TPH1, 5-HT_{1A} and 5-HT_{2A} were normalized to those of β-actin to account for differences in protein loading and/or transfer, whereas MAO-A protein levels were normalized to COX IV. Kruskal-Wallis test was performed for data analysis with values considered significant when the p-value was less than 0.05. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female).

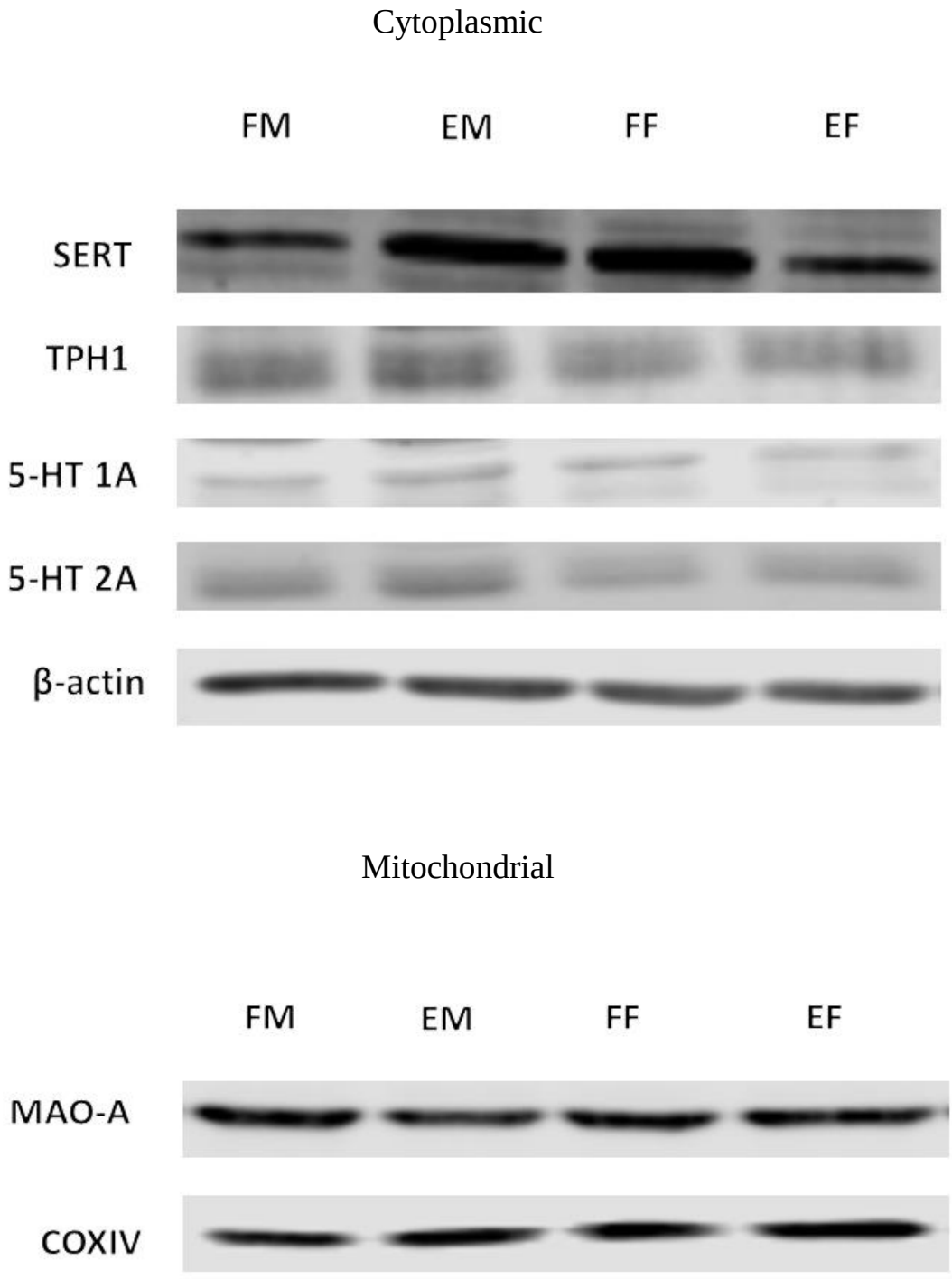


Figure 6. 3: Representative Western blotting of placental components of serotonin system from full-term and early-term deliveries. Top: cytoplasmic protein extracts of placentae from full-term and early-term deliveries were used to detect SERT, TPH1, 5-HT_{1A} and $5HT_{2A}$. Blots have been probed with β – actin as a loading control. Bottom: mitochondrial protein extracts of placentae from full-term and early-term pregnancies were used to detect MAO-A. Blots have been probed with COX IV as a loading control. Groups were classified into FM (full-term male), EM (early-term male), FF (full-term female) and EF (early-term female). *Significant changes have not been detected.*

6.4.3 Examination of 5-HT_{1A} and 5-HT_{2A} receptors in placental blood vessels in relation to fetal sex and gestational age

To sustain optimal fetal growth nutrients, hormones, growth factors and oxygen are transferred from the mother to the fetus via placental veins. Serotonin is known as a potent vasoconstrictor that controls the vascular tone through the interaction with various types of 5-HT receptors. Of interest to the present study were 5-HT_{1A} and 5-HT_{2A} receptors in the placental veins. The activation of these receptors by serotonin has an opposite effect on the blood vessel vasculature. Whereas the activation of 5-HT_{1A} receptors in the blood vessel by serotonin is known to have a vasodilatory effect, serotonin-induced stimulation of 5-HT_{2A} receptors constricts the blood vessel. To our knowledge, serotonin receptors have not been extensively examined in the placental blood vessels of the human.

The protein levels of 5-HT_{1A} and 5-HT_{2A} receptors in placental veins were examined in relation to fetal sex and gestational age. The 5-HT_{1A} receptors were not detected in the placental blood vessels. The 5-HT_{2A} receptor protein expression did not differ in relation to fetal sex (Kruskal-Wallis test, $p > 0.05$, Figure 6.4) or gestational age (Kruskal-Wallis test, $p > 0.05$, Figure 6.4). Refer to Figure 6.5 for Western blotting images.

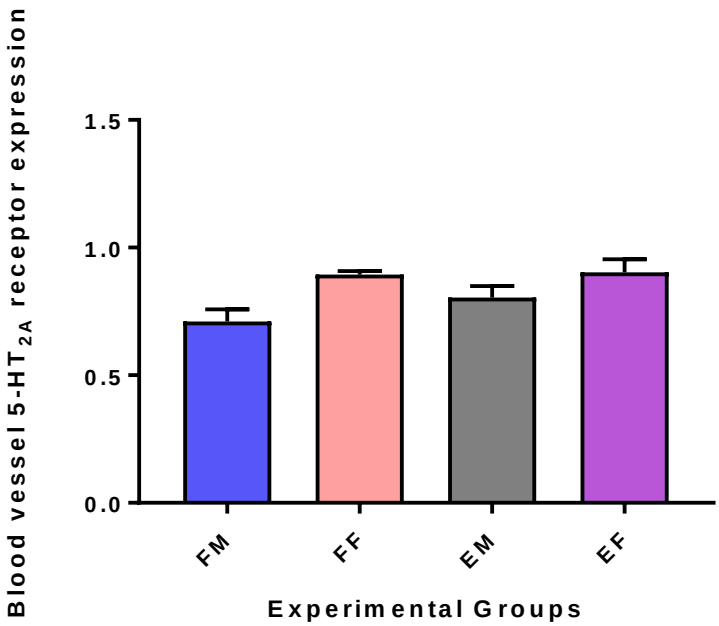


Figure 6. 4: Expression of 5-HT_{2A} receptors in placental veins in relation to fetal sex and gestational age. Protein expression of 5-HT_{2A} was normalized to those of β-actin to account for differences in protein loading and/or transfer. Kruskal-Wallis test was performed for data analysis with values considered significant when the p-value was less than 0.05. Data represent the mean protein ± SEM of three independent experiments (n = 6 per group). Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female).

Blood vessel

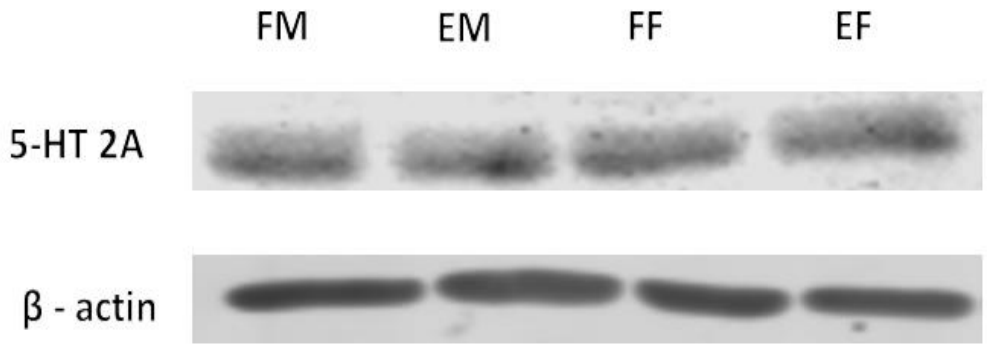


Figure 6. 5: Representative Western blot of placental 5-HT 2A from full-term and early-term deliveries. Cytoplasmic blood vessel protein extracts of placentae from full-term and early-term pregnancies were used to detect 5-HT_{2A}. Blots have been probed with β – actin as a loading control. Groups were classified into FM (full-term male), EM (early-term male), FF (full-term female) and EF (early-term female). *Significant changes have not been detected.*

6.4.4 Examination of the effect of cortisol on the components of the serotonin system

Research suggests that GCs may affect serotonin signaling by altering the expression of the components of the serotonergic system, including the levels of serotonin, SERT, TPH1, MAO-A and 5-HT_{2A}. Whether the expression of these factors in the human placenta is affected by cortisol is not known. Therefore, the levels of serotonin, SERT, TPH1, MAO-A as well as the expression of 5-HT_{1A} and 5-HT_{2A} receptors were evaluated in the placental tissue samples in relation to the umbilical cord blood cortisol levels.

Umbilical cord cortisol levels were significantly correlated to SERT expression. There was a negative correlation between cord cortisol levels and SERT expression – the higher was the levels of cortisol in the cord blood the lower was the expression of SERT (Spearman r = - 0.45, p = 0.02). The levels of serotonin, TPH1, MAO-A as well as the expression of 5-HT_{1A} and 5-HT_{2A} receptors was not related to cortisol levels (Spearman correlation coefficients and corresponding p-values are presented in Figure 6.6).

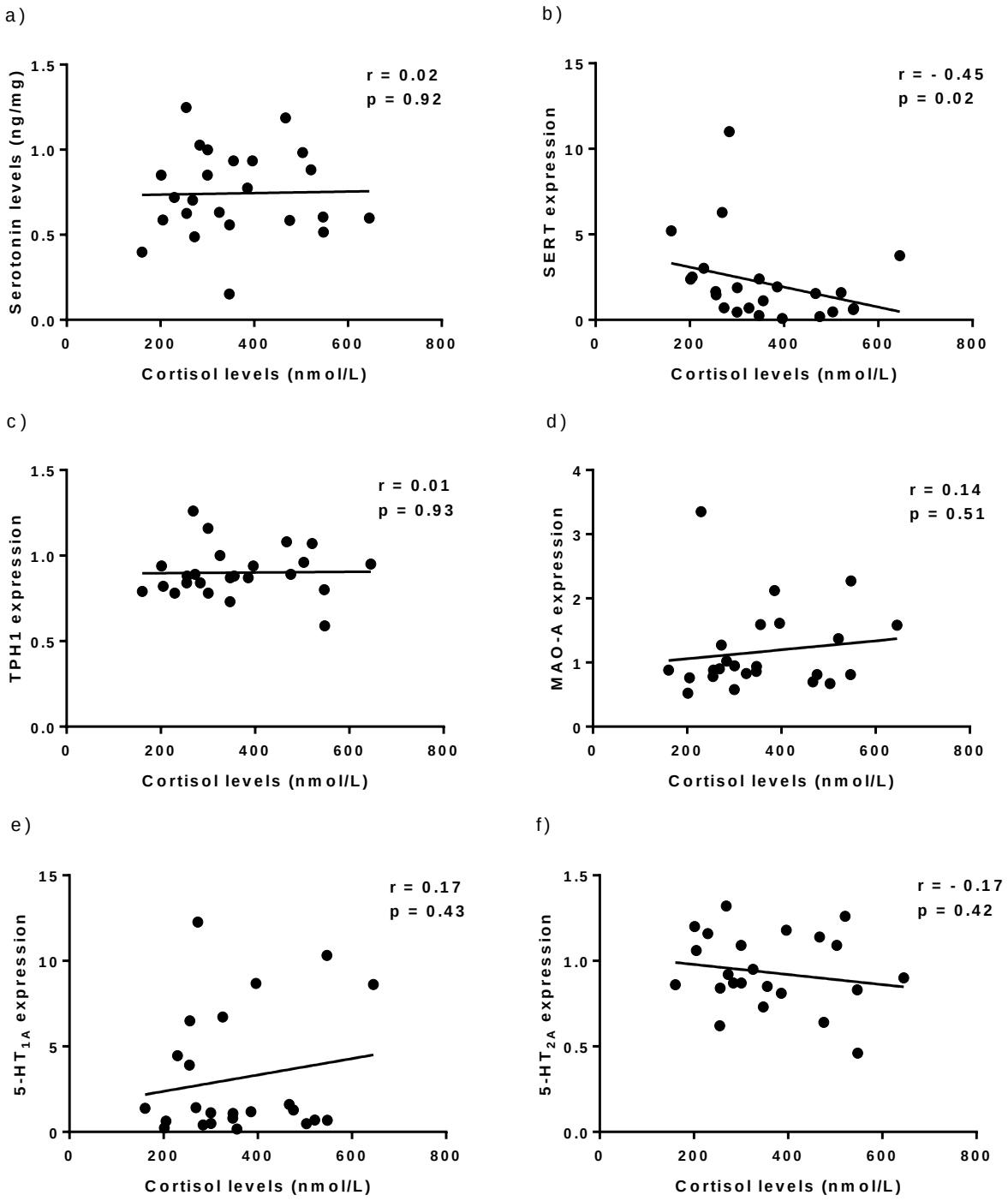


Figure 6. 6: Scatterplots exhibiting relationships of cord blood cortisol levels and the components of the serotonin system. Correlations of cord blood cortisol with serotonin (a); SERT (b); TPH1 (c); MAO-A (d); 5-HT_{1A} (e) and 5-HT_{2A} (f). Spearman correlation test was used to analyze the data. *A significant correlation* was detected between cord cortisol levels and SERT expression.

6.5 Discussion

Serotonin controls a diverse range of physiological functions including pregnancy [227]. It has been found to sustain pregnancy, placental and fetal development via 5-HT_{1A} receptors [239] and trophoblast cell viability and proliferation and 5-HT_{2A} receptors [237, 238]. Serotonin regulates vascular tone via a vast range of serotonin receptors. In the CNS and in the periphery, serotonin induces vasoconstriction via 5-HT_{1B} and 5-HT_{2A} receptors and dilates blood vessels through the interaction with 5-HT_{1A} and 5-HT_{2B} receptors found in the walls of the blood vessels [229, 230, 240, 241], but serotonin receptor subtype through which serotonin modulates the vascular tone in the human placenta has not been studied extensively. Emerging studies suggest that disturbances in serotonin system functioning may complicate pregnancy and adversely affect maternal health [242] and fetal development [244-247]. Too much or not enough serotonin during *in utero* fetal development may damage the growing fetus and deleteriously impact the wellbeing of the offspring. To our knowledge, there are no studies that examined whether early-term births are related to changes in serotonin metabolism or function. In this study, we therefore, investigated the components of the serotonin system to find out if early-term deliveries are due to abnormalities in the function of serotonin and whether alterations (if any) in the expression of the components of serotonin system are related to cortisol.

Examination of components of serotonin system in placental tissues in relation to fetal sex and gestational age

The serotonin factors examined included placental tissue serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors. These components of the serotonin system were tested in relation to gestational age and fetal sex. In this study, we present novel data showing no significant changes in 5-HT levels, nor in protein expression of SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors in relation to fetal sex or gestational age. The presence of

serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors have been previously reported in human placenta and choriocarcinoma cells [237-239, 242, 286-288]. Huang *et al.* (1998) used immunohistochemistry and in situ hybridisation to find out if serotonin and its receptors are present in the human placentae of 6-10, 16, 24 and 40 weeks of pregnancy and demonstrated that fetal white blood cells, syncytiotrophoblasts and cytotrophoblasts were serotonin immunoreactive, with 5-HT_{1A} receptor mRNA detected in the cytoplasm of cell lysates [239]. While 5-HT_{1A} receptor mRNA levels did not vary in relation to gestational age, immunoreactivity to serotonin decreased progressively with gestational age, suggesting that serotonin play may be more important for placental and fetal development during early stages of pregnancy in regulating placental growth and embryogenesis. Laurent *et al*. (2017) detected TPH1 mRNA and protein in syncytiotrophoblasts, trophoblasts, fetal capillaries and decidual cells in the placentae of first and third trimester of pregnancy using PCR and Western blotting and demonstrated that human trophoblast cells are functional endocrine cells that synthesize serotonin in the placenta [287]. Furthermore, Carrasco *et al*. (2000) examined serotonin, SERT and MAO-A in normotensive pregnancies and pregnancies complicated by pre-eclampsia and showed elevated serotonin levels in placentae of pre-eclamptic pregnancies is not due to the down-regulation of SERT cell surface expression, but is a result of the reduction in MAO-A activity and expression [288]. Likewise, Viau et al. (2009) examined SERT and 5-HT_{2A} receptors in villous trophoblast cells from normal pregnancies and pregnancies complicated with gestational diabetes mellitus and found out a significant decrease in SERT and 5-HT_{2A} mRNA and protein levels in the placentae of GDM pregnancies [242]. Overall the results of the previous investigations have demonstrated the important role of the components of the serotonin system in fetal and placental development and may suggest that alterations in the regulation of serotonin signaling are implicated in the etiology of some pregnancy complications.

The resent study confirmed previous findings and demonstrated that serotonin, SERT, TPH1, MAO-A, 5-HT_{1A} and 5-HT_{2A} receptors are synthesized/expression in the human placenta and may play role in regulating placental and fetal development. The lack of significant changes in the levels of the components of the serotonin system in early-term and full-term groups suggests that early-term births are not related to the alterations in serotonin function, at least in this study cohort; however further investigation with a larger sample size is necessary to confirm this observation.

Examination of 5-HT_{1A} and 5-HT_{2A} receptors in placental blood vessels in relation to fetal sex and gestational age

To our knowledge, this study was the first study that examined serotonin receptors in the veins of the human placenta; specifically, vasodilatory 5-HT_{1A} and vasoconstrictive 5-HT_{2A} receptors. Here we demonstrate the presence of 5-HT_{2A} receptors, but not of the 5-HT_{1A} receptors, in the placental veins with no significant difference in the expression of 5-HT_{2A} receptors in relation to fetal sex or gestational age. Similar observation was reported by Huang *et al.* (1998) who demonstrated a strong serotonin receptor immunoreactivity to serotonin receptors class 2 (5-HT_2), but no 5-HT_{1A} mRNA-hybridised signals detected in the capillaries of human placental villi [239], implying that 5-HT_2 receptors are the main class of serotonin receptors present in the villous endothelium of the capillaries. Even though serotonin class 2 receptors have been detected in the placental capillaries, the subtype of serotonin receptors (e.g., 5-HT_{2A}, 5-HT_{2B} or 5-HT_{2C}) has not been specified in the investigation conducted by Huang and associates (1998). Unfortunately, most of the data on serotonin receptor class and the effect of serotonin in human umbilical cord blood vessels and chorionic arteries and veins comes from the studies conducted a few decades ago, with no investigations carried out recently. The effect of serotonin on vascular tone and serotonin receptor subtypes that mediate serotonin responses in human placental blood vessels have not

been fully understood. Nevertheless, a strong vasoconstrictive effect of serotonin on human isolated placental chorionic arteries and veins has been shown by Reviriego & Marin (1989), with low concentrations of serotonin constricting both types of the blood vessels via 5-HT_1 receptors, whereas high levels of serotonin mediating the vasoconstriction through the interaction with 5-HT_2 receptors [289]. Nearly five decades ago Koren *et al.* (1965) postulated that late in pregnancy serotonin, released from uterine and placental stores, may cause the contraction in placental vasculature leading to the induction of labour [290]. In this study, we detected serotonin in the placental tissues and vasoconstrictive 5-HT_{2A} receptors in the placental veins independent of gestational age. It is possible, that late in gestation serotonin, released from the placental tissues, acting on 5-HT_{2A} receptors in the blood vessels may lead to the constriction of the fetoplacental blood flow and induce labour in this study cohort; however more research is required to prove this assumption and examine the effect of the interaction of serotonin with blood vessel 5-HT_{2A} receptors in the human placenta on fetal growth and pregnancy outcome.

Examination of the effect of cortisol on the components of the serotonin system

Research suggests that cortisol may play role in controlling the magnitude of serotonin signaling by modifying the levels of serotonin and the expression of serotonin transporter and enzymes involved in serotonin synthesis and degradation, with reported findings being quite contradictory in their nature. In this study, the correlation of cortisol with serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors was examined to find out the effect of cortisol on the levels of these factors. Of all the factors examined, only SERT expression was related to cord cortisol levels. Importantly, SERT protein levels were found to be significantly correlated to cord cortisol levels, with higher cortisol levels associated with lower SERT expression. Most of the data on the effect of GCs on SERT expression comes from animal and human studies on the association between stress and serotonergic

dysfunction in the CNS, with no investigations so far examining the relationship between GC level and SERT expression in the human placenta. Yet, in animal models, GC excess following prenatal maternal stress early in pregnancy reduces SERT levels, with male mice showing a significant reduction in SERT expression in several brain tissues [259]. An inhibitory effect of GCs on SERT expression has been also reported in rats where DEX administration led to a decrease in SERT levels in a few brain areas of aged animals [291]. Contradictory to this findings, in human immortalized B-lymphoblastoid cells synthetic glucocorticoid DEX increased the expression of SERT [257].

In the current chapter, we demonstrated that cord cortisol levels are inversely correlated to SERT expression, with high cortisol levels associated with lower SERT protein expression. In terms of placental and fetal development, cortisol-induced downregulation of SERT expression may consequently result in higher bioavailability of serotonin in the placental and fetal tissues. An increase in levels of serotonin in the placenta, due to cortisol-mediated reduction in SERT expression, may be required for serotonin-stimulated vasoconstriction of placental vascular beds via 5-HT_{2A} receptors and an induction of labor late in gestation.

6.6 Summary

To our knowledge, this study for the first time evaluated the serotonergic system in the placentae of full-term and early-term infants to find out if early-term births are related to alterations in serotonin signaling. The factors examined included serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors, with 5-HT_{1A} and 5-HT_{2A} receptors tested in both, placental tissues and veins. Placental tissue serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors were investigated in relation to fetal sex, gestational age and cord cortisol levels.

Serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors were detected in all placental tissues, with no significant differences in protein levels of these serotonin system components observed in relation to fetal sex or gestational age. The 5-HT_{2A} receptor, but not the 5-HT_{1A} receptor, has been detected in the placental veins independent of fetal sex or age at birth. Although, the above listed serotonergic factors did not differ in regard to fetal sex or gestational age, the presence of serotonin, SERT, TPH1, MAO-A and 5-HT_{1A} and 5-HT_{2A} receptors (through each serotonin mediates its signalling), may indicate that serotonin system plays an important role in the development of human placenta and fetus. Of all the components that control serotonin levels and function tested in relation to fetal cord cortisol levels, only SERT expression was negatively correlated to cord cortisol levels, with high cortisol levels being correlated to low SERT levels. A negative relationship between cord cortisol levels and SERT expression may imply that late in human gestation cortisol inhibits the expression of SERT in placental trophoblasts to upregulate the extracellular levels of serotonin, which in turn interacts with 5-HT_{2A} receptors in the placental blood vessels to constrict the vascular tone and stimulate labor. Certainly, more research is required to determine serotonin receptor subtypes present in the blood vessels of the human placenta and the outcome of the interaction of serotonin with its receptors on the vascular tone and fetal

development. Nevertheless, serotonin system components have been detected in the human placenta where they may control the growth of the fetus, placenta and pregnancy outcome.

CHAPTER 7: General Discussion, Conclusions and Future Directions

This doctoral project sought to examine GC and serotonin systems in the human placenta to explore if there are fundamental differences in placental protein expression and determine whether these changes (if any) are associated with umbilical cord cortisol levels and full-term and early-term births.

Glucocorticoids regulate crucial physiological processes and pregnancy in humans [13-18]; nevertheless, long-term exposure to elevated cortisol levels is linked to metabolic, cardiovascular and psychological disorders [13, 34-39]. Gestational cortisol concentrations increase progressively in the mother and fetus in all species [46] to maintain pregnancy and fetal and placental development, with placental 11β-HSD1 and 11β-HSD2 enzymes regulating the levels of cortisol in the placenta and fetus [28]. However, placental and fetal exposure to high maternal GCs may alter placental structure and function [81, 83, 84] and adversely affect fetal growth. Glucocorticoid-induced structural and functional changes in the placenta may result in IUGR and infants born before term, with lower than normal birth weight [70-72], with the IUGR and LBW frequently associated with a variety of metabolic, cardiac and neurodegenerative health issues later in life [65, 73-80]. While GCs may adversely affect the growth of the placenta and fetus and shorten the length of the gestation, it remains unclear as to whether infants born early-term (gestational weeks 37 and 38) are due to cortisol-induced changes in the placental structure and function, including the expression of 11β-HSD1 and 11β-HSD2 enzymes in human placenta.

In this study cohort, umbilical cord cortisol levels at delivery did not differ in relation to fetal sex or gestational age. Since early-term births were not related to cord cortisol concentrations late in pregnancy, we proposed that early-term infants could have been exposed to cortisol overload early in pregnancy during sensitive periods of embryogenesis (second trimester), with Sandman *et al.* (2006) finding supporting this hypothesis on the

association of fetal cortisol exposure early in gestation and preterm births [271]. The examination of the relationship of umbilical cord cortisol levels with fetal weight, placental weight and gestational age revealed a significant correlation between cord cortisol levels and gestational age, with advanced weeks of gestation associated with high cortisol levels. This result corroborates with other investigations in which an increase in cortisol concentrations was observed towards term and is suggested to play important roles in the regulation of the maturational trajectories of respiratory, cardiac and CNS in the fetus during the last weeks of human gestation [28, 46]. Furthermore, both enzymes 11β-HSD1 and 11β-HSD2 were expressed in the placental tissues, indicating that the enzymes are required to control cortisol availability in the placenta and fetus during last weeks of pregnancy. The 11β-HSD1 enzyme activates cortisone into cortisol, whereas 11β-HSD2 enzyme inactivates cortisol into cortisone in a tissue-specific manner [22-27]. The expression of the least studied 11β-HSD1 enzyme in the human placenta differed in regard to fetal sex and gestational age. While the full-term female fetus placentae exhibited a higher expression of 11β-HSD1 enzyme compared to the full-term male fetus placentae, the early-term female fetus placentae had lower 11β-HSD1 enzyme expression compared to the early-term male fetus placentae, suggesting that 11β-HSD1 enzyme may be involved in the regulation of cortisol signalling and indirectly influencing placental and fetal development in a gender- and gestational age-specific manner. Surprisingly, placentae of fetuses exposed to high cortisol concentrations transferred from the mother to the fetuses through the umbilical vein had lower 11β-HSD1 enzyme expression compared with the placentae of fetuses that were exposed to low cortisol levels. Mericq *et al.* (2009) also reported an inverse relationship between 11β-HSD1 enzyme expression and fetal cortisol concentrations in human placentae of term pregnancies, with lower 11β-HSD1 enzyme expression and activity and higher fetal cortisol levels reported in the chorionic plate in the placentae of small-for-gestational-age infants [270]. Whether a

reduction in 11β-HSD1 enzyme expression and activity is a possible compensatory mechanism to protect the fetuses against adverse effects of the long-term exposure to high maternal GCs remain to be determined. Further investigations on the activity of placental 11β-HSD enzymes measured by radiometric conversion assay are required to define cortisone to cortisol interconversion rates during last weeks of human gestation and expand our understanding on the role of the enzymes in human gestation. As far as placental and fetal development are concerned, the exact cortisol concentrations that are considered normal during first, second and third trimesters of pregnancy have not been established and future research may shed light on the optimal cortisol levels for normal placental and *in utero* fetal development. Since GC signaling is mediated through the interaction with GR, we next evaluated the GRs in the placental tissues to find out if early-term births are related to changes in the expression of the GRs in the placenta, with the effect of cortisol and fetal sex on the expression of the GR isoforms assessed.

Glucocorticoids control growth and development via GRs, with various GR isoforms generated as a result of alternative splicing [107] and alternative translation initiation [105]. In human placenta, GR isoform ratios and levels are dependent on the subcellular localization, fetal sex, gestational age and cortisol levels. For instance, in term placentae of male fetuses, the expression of GRP and GRA in the nucleus is higher than in the cytoplasm and cord blood cortisol levels are positively correlated with nuclear GRβ levels in the placentae of full-term male fetuses and SGA infants [163]. In pregnancies complicated by maternal asthma, female fetus placentae exhibit a reduced nuclear expression of GRβ along with an increased nuclear expression of GRα-C and GRα-D3 [163]. In the placentae of preterm infants, the GRα-C expression is higher compared to placentae of term pregnancies and GRα-D2 expression is sex-specific, with GRα-D2 levels being higher in the placentae of preterm males than those of preterm female fetuses [276]. In accordance with former studies,

the GR isoforms observed in the human placenta were as follows: GRα-A, GRβ, GRα-C, GRα-D1, GRα-D2/D3 and unknown 68-69 kDa and 38 kDa [163, 176, 276]. In our study, the GR subtype did vary in relation to intracellular location, fetal sex and gestational age.

In full-term placentae, significant differences were detected in the levels of GRα-A, GRα-D1 and GRβ in the nuclear versus cytoplasmic fraction, with the male fetus placentae having higher GRα-A and GRβ levels in the nucleus than in the cytoplasm and female fetus placentae exhibiting lower GRα-D1 levels in the nuclear compartment compared to the cytoplasm. It worth noting, that out of all experimental groups, the full-term male group had the highest cortisol concentration in the placenta and the presence of GRβ in the nuclear extracts may indicate that some of the GRα-regulated functions could have been blocked by its inhibitor, the GRβ, to maintain optimal fetal development before parturition.

Similar differences in GR isoform expression in relation to subcellular fraction were observed in the early-term group, with male fetus placentae having significantly higher GRα-A and GRα-D1 protein levels in the nucleus than in the cytoplasm. In addition to the upregulated expression of GRα-A and GRα-D1 in the nucleus compared to the cytoplasm, female fetus placentae had also significantly higher GRα-C, unknown 68 kDa and 38 kDa expression in nuclear than in the cytoplasmic compartment. Research suggests, that female fetus becomes hypersensitive and reduces growth when exposed to high maternal cortisol levels, possibly due to the interaction of GRα-A with the GRα-C or GRα-D isoform [163]. We postulated that in early-term female placentae, the GRα-A interacted with GRα-C and/or GRα-D isoform in the nucleus to alter the expression of genes responsible for fetal growth, which in turn may result in a reduced weight of the female infants born early.

Furthermore, GR profile in the placenta was fetal sex- and gestational age-specific. In the placentae of early-term male fetuses the cytoplasmic levels of GRβ and nuclear levels of GRα-A and unknown 38 kDa protein were higher compared to the placentae of full-term

male fetuses, with the co-expression of both proteins being essential in controlling growth-related genes in the placentae and male fetuses between 37 and 38 weeks of the human gestation. In the placentae of early-term female fetuses the nuclear levels of GRα-A, GRβ, GRα-C, GRα-D1, GRα-D2/D3 unknown 68 kDa protein were significantly higher compared to the placentae of full-term female fetuses. We postulated that concomitant expression of GRα-A with GRβ, GRα-C, GRα-D1, GRα-D2/D3 and unknown 68 kDA may play role in controlling the development of the fetus and the placenta between 37 and 38 gestational weeks in the women pregnant with female fetuses. In contrast to Saif *et al.* (2014 and 2015) reports, the GRγ, GRA and GRP isoforms were not detected in early-term or full-term placentae possibly because of the variations in experimental methods or detection techniques used to test the proteins. To date, the exact roles of GR isoforms and especially unknown 38 kDa and 68 kDa proteins, in the human placenta and fetal development are not identified yet. Nonetheless, the presence of these proteins in the placental samples points towards possible essential functions in human reproduction. Overall, our results related to the GR profile in the human placenta confirm previous findings on the variations in the GR isoform ratios in different subcellular fractions and point out that distinct GR isoforms play role in the regulation of fetal development in a gender- and gestational age-specific manner. In the future, immunohistochemical staining of placental tissues and proteomic analysis of GRs in trophoblasts will clarify GR profile in human placenta.

Moreover, besides the cytoplasm and nucleus, the GRs have been detected in mitochondria of cells of animal and human origin [190, 194], where they may control the regulation of mitochondrial functions, including apoptosis by the mechanisms that are still undiscovered. Notwithstanding, GCs may play role in the initiation of apoptosis through the activation of the effector caspase-3 and caspase-6 [218-220]. The GR receptors that may play roles in GC-induced apoptotic events are GRα-A, -B, -C and -D [221, 222]. As far as the

pregnancy and placenta are concerned, trophoblast apoptosis is common in pregnancy and regulates the growth of the embryo and placenta. However, when upregulated, placental apoptosis is associated with placental dysfunction and is observed in pregnancy complications, including pre-eclampsia and IUGR [209]. Placental changes at the molecular level that contribute to placental apoptosis, shorten the length of the human gestation and induce the parturition before term are not clear. To date, there are no investigations conducted to examine the GR isoforms in mitochondria of human trophoblast cells and their potential role in placental apoptosis and fetal development. To fill the gaps in the knowledge, we further examined the GRs in the mitochondria and their role in placental cell death in fetal sex- and gestational age-specific manner.

Using Western blot technique we detected three bands at 68-69 kDa, 50-51 kDa and 38 kDa that potentially correspond to unknown 68-69 kDa, GRα D2/D3 and unknown 38 kDa proteins, respectively. This result is consistent previous investigations on GRs detected in various cells [189-191], including human HepG2 and SaSO-2, where small molecular weight proteins have been observed in the mitochondrial lysates by Western blotting [189, 191, 275]. Multiple bands have been also detected by the Western blot technique in rat liver mitochondria treated with GCs, including the bands with molecular sizes of 95 kDa, 90 kDa, 70 kDa and 50 kDa, representing potential GRs [192]. Unlike former investigations [189, 191, 275], large GR proteins were not observed in the mitochondria of placental trophoblast cells in this study. In this investigation, the discovered GR proteins in the mitochondria of trophoblast cells, including unknown 68-69 kDa, GRα D2/D3 and unknown 38 kDa proteins, did not significantly differ in relation to fetal sex or gestational age. Since early-term births were not related to changes in the expression of mitochondrial GRs in the trophoblast cells, we next tested the apoptotic markers in the placental cells, including caspase-3 and -6.

Both pro-apoptotic caspases were detected in the placental samples. The results of the examination of effector caspases in the placenta revealed significant differences in the levels of caspase-3 in relation to fetal gestational age. Caspase-3 expression was much higher in the early-term male placentae compared to the full-term male counterparts. The up-regulated expression of placental caspase-3 in the early-term male group was not related to syncytial loss (as shown by normal placental weight of the early-term infants) and could imply trophoblast cell differentiation, fusion and cell-cycle progression. This hypothesis is supported by finding on caspase-3 and caspase-8 involvement in cell division and cell-cycle progression as demonstrated in primary T-cells [281]. Evidence on important non-apoptotic functions of caspases in human placenta also comes from the study conducted by Gauster et al. (2010) where caspases – 3, - 8 and – 9 have been suggested to play roles in remodeling sub-membranous cytoskeleton during trophoblast fusion and facilitate syncytial fusion of cytotrophoblasts. Whether the augmented expression of caspase -3 in the placentae of the early-term group represent placental dysfunction or it is a normal process reflecting placental trophoblast proliferation remains unclear and more investigations are required.

Furthermore, research suggests that translocation of GR from the cytoplasm to mitochondria [204, 213] and upregulation in the expression of caspase-3 [218-220] are associated with GC-induced apoptosis. Therefore, we next analyzed the relationships of caspase-3 expression with cord blood cortisol levels and mitochondrial GRs expression to establish if upregulated caspase-3 levels correlated with cord cortisol levels and whether mitochondrial GRs play role in the up-regulation of caspase-3 expression in the early-term male group. We have shown no relationships of caspase-3 expression with cord cortisol levels or mitochondrial GR proteins. Since increased caspase-3 levels in the placentae of early-term males are not related to cord cortisol levels, future studies should examine other potential stimuli of apoptosis in the placenta, including hypoxia [283], calcium overload and

ROS [284], to eliminate placental dysfunction as a possible contributor to early-term births. Since the expression of cytokeratin 18 neo-epitope is a specific marker of caspase -3 induced cell death in human placenta, future studies need to explore cytokeratin 18 neo-peptide in trophoblasts to correlate trophoblast apoptosis with caspase -3 activation [292]. Because there were no correlations between caspase-3 expression and mitochondrial GR proteins, it is possible that mitochondria- or mitochondrial GR-independent mechanisms could be involved in the heightened caspase-3 expression in early-term male fetus placentae. Further studies are necessary to show how cortisol mediates distinct GR-mediated pathways in mitochondria to control placental cell death and growth, the length of human gestation and fetal development.

Much has been learned about GCs and how GCs control a plethora of physiological systems in humans, including serotonin system. In human skeletal muscle cells, GC treatment upregulates MAO-A protein and mRNA levels and enzymatic activity resulting in an increase in ROS production and muscle loss [254]. In rodents, GCs increase serotonin levels and the expression of 5-HT_{2A} receptors, TPH1 and AAAD in liver and fat tissues [256]. When it comes to placental research, much remains to be learned about GC-regulated serotonin system in the human placenta. Since serotonin regulates embryogenesis and serotonin system dysfunction has been observed in pregnancy complications (GDM and pre-eclampsia) [242, 243] and may contribute to congenital abnormalities [253], therefore we next explored the impact of cortisol on the components of serotonin system to find out if early-term births could be related to alterations in serotonin system function.

In accordance with other studies, all the components of serotonin have been detected in the human placenta, including serotonin, TPH1, MAO-A, SERT and 5-HT_{1A} and 5-HT_{2A} receptors [239, 242, 287, 288]. Although these factors did not significantly differ in relation to fetal sex or gestational age, the presence of serotonin and serotonin system components in the trophoblast cells indicates a role for serotonin in the development of the placenta and

fetus late in human gestation. The potential functions of serotonin in the placenta include placentation, trophoblast cell proliferation [237, 238] and pregnancy maintenance [239], in which 5-HT_{1A} and 5-HT_{2A} receptors may play significant roles.

Moreover, earlier reports suggest that serotonin may regulate vascular tone via various types of receptors [229, 230, 240, 241]. Serotonin may dilate the blood vessels via 5-HT_{1A} receptors and constrict them via 5-HT_{2A} receptors, but to date, the serotonin receptor subtype through which serotonin may regulate the vascular tone in the human placenta has not been explored well. We for the first time detected 5-HT_{2A} receptors, but not 5-HT_{1A} receptors in the placental veins. A similar observation was documented by Huang and colleagues (1998) who showed the presence of 5-HT_2 mRNA, but not 5-HT_1 mRNA in the endothelium of human villous capillaries [239]. Koren and associates (1965) hypothesized that uterine and placental serotonin may stimulate parturition late in gestation through the contraction of placental blood vessel [290]. Clearly, much remains to be examined and more research is needed to further explore this hypothesis and the impact of the interaction of serotonin with 5-HT_{2A} receptors in the placenta on the length of human gestation and fetal development. However, it is quite plausible that late in human pregnancy 5-HT_{2A} receptors are required to mediate the constriction of fetoplacental blood flow and stimulate the start of the parturition in response to serotonin synthesized and released from the placental tissues.

Finally, GCs may affect the magnitude of serotonin transduction by altering the expression or levels of the components of the serotonin system. To test this assumption in the placental tissues, we next examined the components the serotonin system in relation to cord cortisol levels. Of all the factors tested, only SERT levels were inversely related to cord cortisol levels. The results of previous investigations on the impact of GCs on SERT expression in animal and human tissues are inconclusive, with GC-induced upregulation [257, 258] and downregulation [259] in SERT expression discussed in the literature. In this

study sample, high cord cortisol levels were associated with low SERT expression in the placenta. We suggest that cortisol-induced downregulation of SERT expression and concomitant upregulated serotonin extracellular levels in the placenta may play role in the stimulation of labor late in pregnancy.

There were a few limitations that warrant some cautions in interpreting our findings with respect to the GC and serotonin system function in the human placenta. First, small sample size limited the statistical power. As the study cohort represented a very specific subset of the population, it was pretty challenging to recruit pregnant women and collect placental tissues and blood vessels within 45 minutes after childbirth for cortisol measurement and sample processing. Second, in our study, umbilical cortisol levels had been measured at birth and did not represent the levels of cortisol during entire pregnancy. Future directions for the placental research may include regular assessment of maternal cortisol levels from the very start of pregnancy until childbirth in larger cohorts. Certainly, long-term measurement of cortisol levels can be challenging; however, advances in research over the past years have led to the development of a reliable non-invasive method for cortisol extraction and analysis using saliva or hair samples.

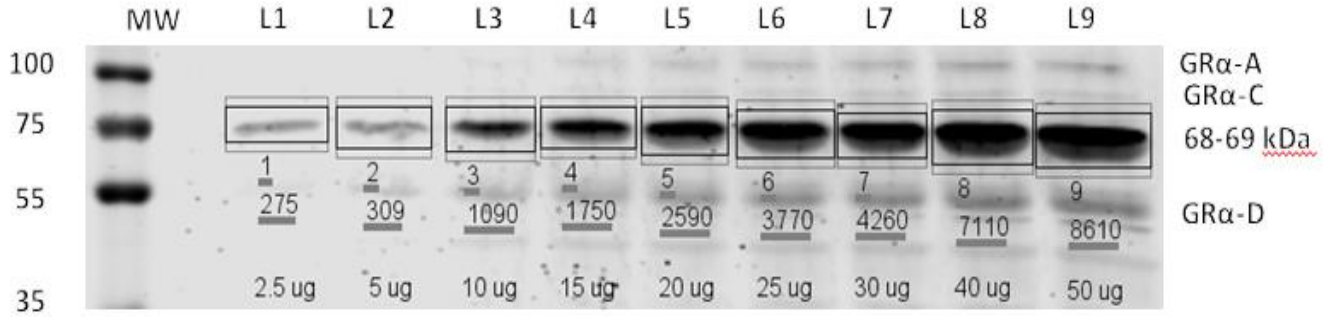
Future studies can potentially use maternal scalp hair to evaluate maternal cortisol levels at different points of time during pregnancy or at the end of pregnancy to assess cortisol levels in the mother retrospectively. Clinicians, placental researchers and experts from diverse technological research areas should develop and implement innovative non-invasive technologies to safely assess pregnant women and the developmental trajectories of the placenta across human pregnancy to prevent placental dysfunction disorders. A combined evaluation of maternal GC levels and non-invasive assessment (e.g., ultrasound) of the placenta and fetal organs throughout gestation will uncover the impact of cortisol overload on placental and fetal development. Histology of human placental samples demonstrating all

placental aspects through the gestation are necessary to provide valuable information and help clinicians and researchers to distinguish between normal and abnormal placental development.

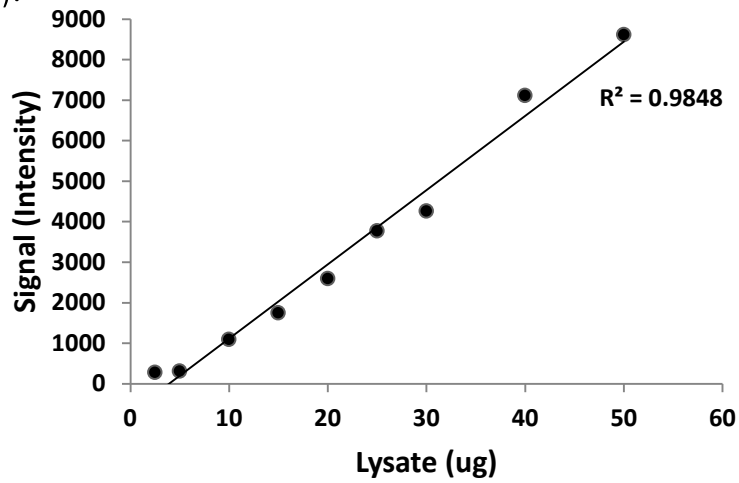
Much remains to be discovered when it comes to the factors adversely affecting the placenta and fetus. More investigations are needed to examine the molecular pathways by which GCs through the interaction with their cognate cytoplasmic, nuclear and mitochondrial receptors control maternal health, placental function and the timely onset of labor and birth. Nevertheless, advances in our understanding of the placental structure and function and synergistic action of GC and serotonin systems in the human placenta will potentially result in improved pregnancy outcomes and reduce the mortality rates and economic burden of adult illnesses associated with births of the infants before term.

APPENDIX

A).

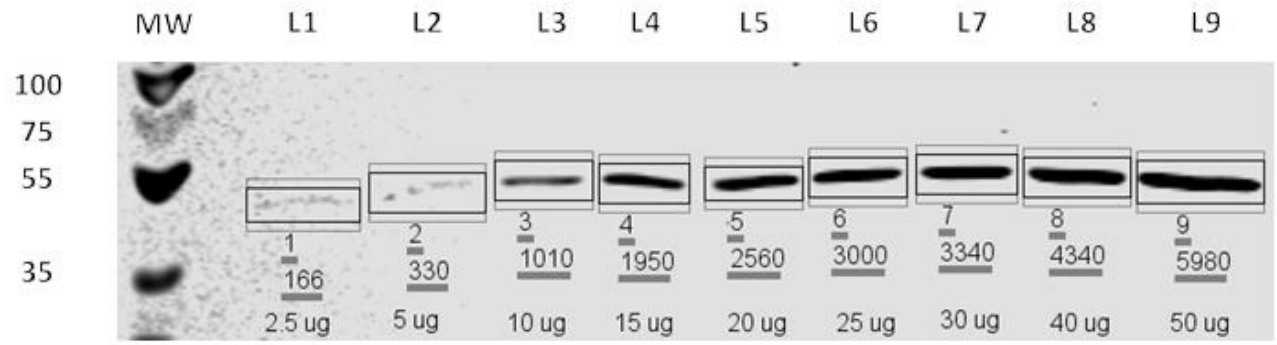


B).

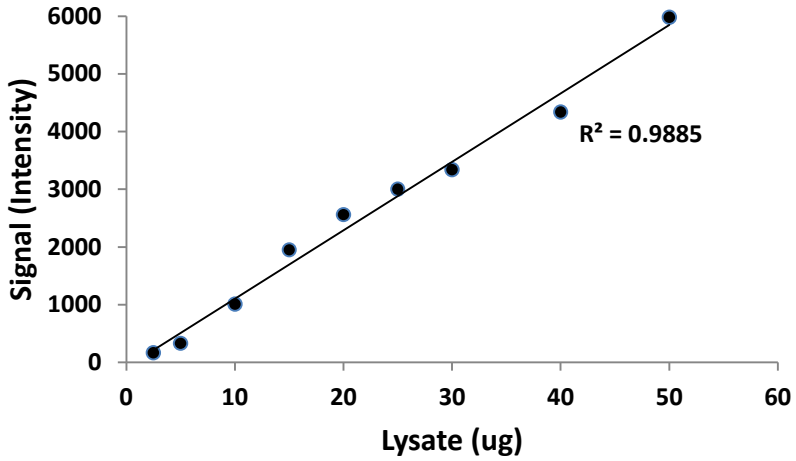


Supplementary figure 1: Optimization of protein concentration and antibody validation to determine linear range of detection for 68-69 kDa protein. The 68-69 kDa protein has been shown as an example. A). Representative Western blotting data of GR profile in placental cytoplasmic extracts. To determine the detection limit (lowest concentration of the analyte that can be reliably detected by instrumentation), various protein concentrations were prepared and loaded into the wells, ranging from 2.5 µg to 50 µg (Lanes 1-9). The 68-69 kDa protein was detected at the lowest concentration used – 2.5 µg of proteins per well (Lane 1). Not all GR isoforms were detected by the primary and secondary antibodies at low concentrations (2.5 µg – 15 µg of proteins per well, Lanes 1-4). Loading high concentrations of proteins per well (25 µg -50 µg, Lanes 6-9) led to signal saturation and unspecific bands. Primary antibody: rabbit anti-GR (Bethyl Laboratories, cat. # A303-491A), 1:1000 dilution; Secondary antibody: goat anti-rabbit 800CW, 1:5000 dilution; B). Determination of linear range of detection of antibodies. When protein concentration increased from 10 µg/well to 20 µg/well, the signal intensity increased proportionally from 1090 to 2590. The concentration of 20 µg/well was above the detection limit and within linear range of detection and therefore selected as the optimal concentration to detect and analyse the cytoplasmic GR profile.

A).

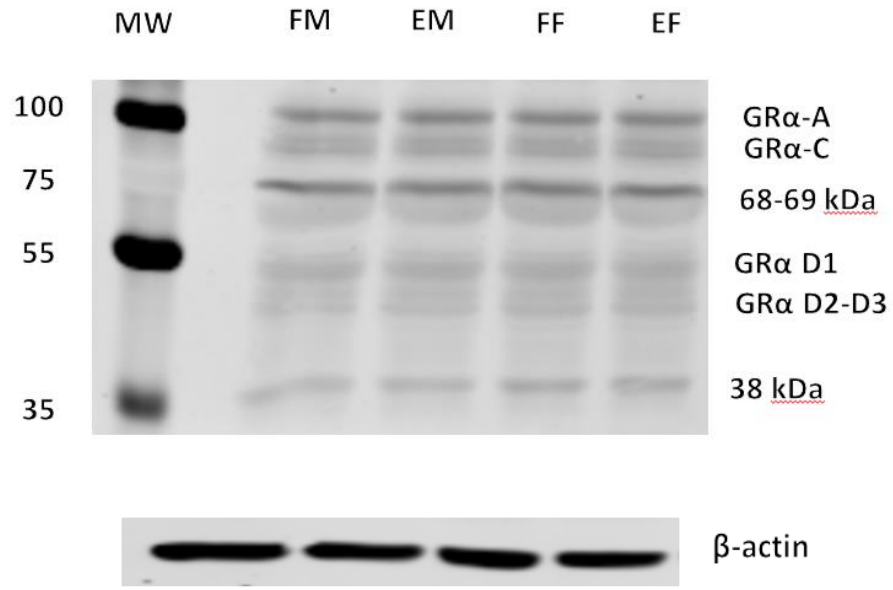


B).

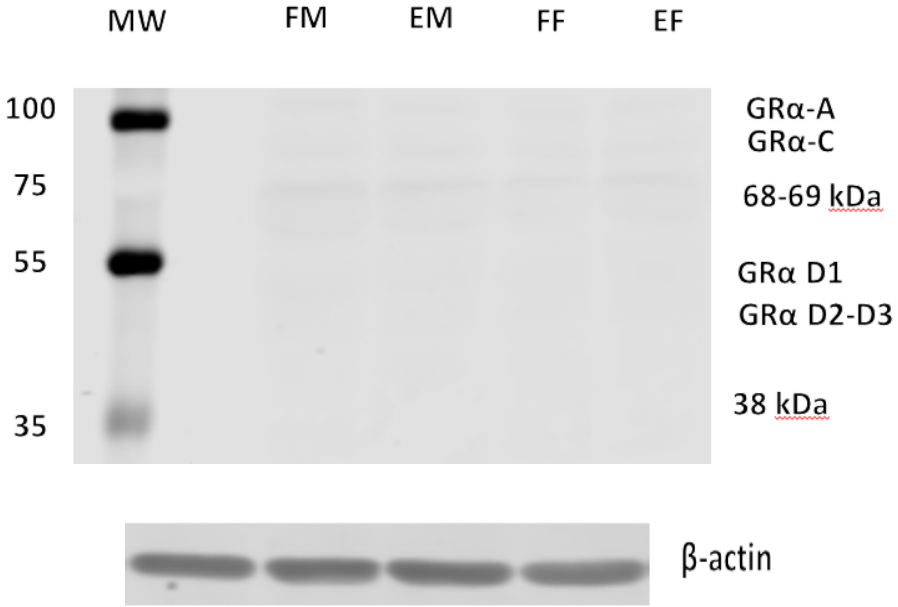


Supplementary figure 2: Optimization of protein concentration and antibody validation to determine linear range of detection for β - actin. β – actin was used as a loading control to normalize GR profile data (Supplementary figure 1). A). Representative Western blotting data of β - actin in placental cytoplasmic extracts. To determine the detection limit (lowest concentration of the analyte that can be reliably detected by instrumentation), various protein concentrations were prepared and loaded into the wells, ranging from 2.5 µg to 50 µg (Lanes 1 – 9). β - actin was detected at the lowest concentration used – 2.5 µg of proteins per well (Lane 1). Loading higher concentrations of proteins per well (25 µg -50 µg, Lanes 6-9) led to signal saturation and made statistical analysis impossible. Primary antibody: mouse anti β-actin, 1:4000 dilution; Secondary antibody: donkey anti-mouse 680RD, 1:30000 dilution; B). Determination of linear range of detection of antibodies. When protein concentration increased from 10 µg/well to 20 µg/well, the signal intensity increased proportionally from 1010 to 2560. The concentration of 20 µg/well was above the detection limit and within linear range of detection and therefore selected as the optimal concentration to detect β-actin in placental samples and normalize GR data.

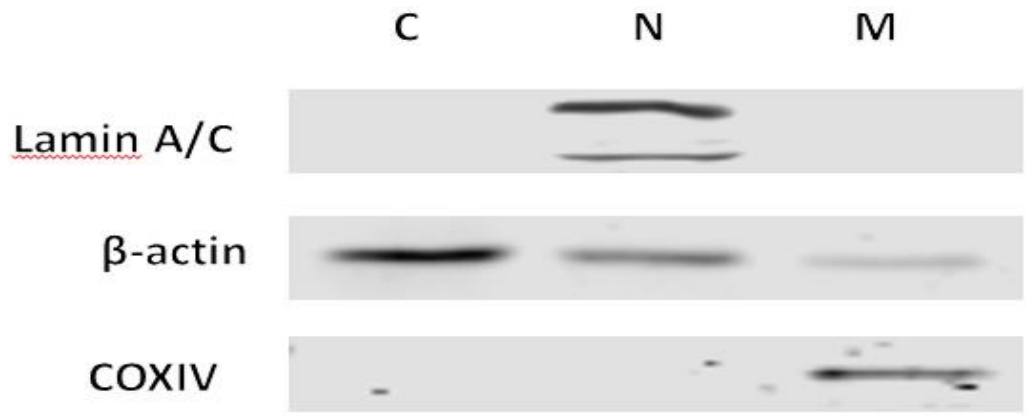
Anti-GR antibody



Anti-GR antibody + blocking peptide



Supplementary figure 3: Representative Western blotting data of blocking peptide assay. Specificity of the anti-GR antibody was confirmed using anti-GR antibody blocking peptide. The cytoplasmic placental extracts were used for this test. Top: Signals obtained with the anti-GR antibody; Bottom: signals obtained with anti-GR antibody incubated with its blocking peptide. Loading control: β - actin. Groups were classified into FM (full-term male), FF (full-term female), EM (early-term male) and EF (early-term female).



Supplementary figure 4: Western blot analysis of Lamin A/C, β – actin and COXIV in cytoplasmic, nuclear and mitochondrial fractions. Representative immunoblot showing distribution of Lamin A/C, β-actin and COXIV in subcellular fractions (C=cytoplasmic, N=nuclear, M=mitochondrial). The purity of subcellular fractions was confirmed by Western blotting using specific protein markers: Lamin A/C (nuclei), β-actin (cytoplasm) and COXIV (mitochondria). Fractionated placental samples containing 20 µg of proteins were added to each well. To validate fraction purity, each fraction was added side-by-side on the same blot and probed separately against each of the three primary antibodies: Lamin A/C, β-actin and COX IV (refer to Chapter 2, Table 2.1 for antibody list).

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