CONVERGENCE OF MTOR AND GLUCOCORTICOID RECEPTOR SIGNALLING IN THE HUMAN PLACENTA: EFFECTS OF PRE-TERM LABOUR, NUTRITION AND MATERNAL STRESS

A thesis submitted for the degree of Doctor of Philosophy by

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Abstract

A vital factor for foetal development is the nutrient transport at placental level. This is because any disturbances in the maternal compartments, for example due to maternal stress or nutritional status, which will affect foetal development, will involve the foetal-placental barrier. Moreover, numerous studies have linked other factors such as preterm labour as the leading cause of perinatal morbidity and mortality in the developed world. To this date, despite a numerous epidemiological and clinical studies that identify potential risk factors for the mother as well as the foetus, there is no comprehensive analysis at all these levels taken from the same cohort of patients. Our working hypothesis is that for a successful pregnancy certain events at nutritional, biochemical, genetic and molecular level could be tightly linked. Therefore, in this study we followed a "holistic" approach investigating how maternal stress, nutrition, placental mTOR and glucocorticoid receptor (GR) signalling can influence pregnancy outcome. We have decided to map in detail the components of these two signalling pathways as they appear to cross-talk as well as been implicated in stress responses.

The largest part of the questionnaire was focused on the nutritional status with questions targeting the maternal dietary habits before, as well as during, pregnancy. The collection of data took place at the Department of Obstetrics and Gynecology, University of Crete Medical School. With regards to this profile, key findings included the significant reduction in the intake of alcohol, caffeine-containing and sugar-containing refreshments, whereas passive smoking during pregnancy stayed the same. Another major finding of this part of the study was the effects of maternal stress on foetal weight and how pregnancy planning was implicated in this complex relation. In our cohort, women with negative attitudes during pregnancy gave birth to infants with significantly lower birth weights (2.5Kg) than those women showing positive or neutral attitudes towards their pregnancy (2.9Kg). We then assessed how maternal stress might affect this signalling cascade using two placental models (BeWo and JEG-3 cell lines) mimicking a stress milieu *in vitro*. Treatment of these cell lines with cortisol (100nM and 1000nM) significantly downregulated Deptor and upregulated GAS5 at mRNA level.

In an attempt to dissect further a potential gene-environment interaction, we have assessed how 4 well-characterised polymorphisms (Tht*III* 1, *Bcl* I, ER22/23EK, N363S) of the GR gene might affect foetal and placental weight. We have demonstrated that only the maternal Tht*III* 1 polymorphism was suggestive of a nature-nurture interaction since only in Tht*III* 1

(CC), maternal stress attitude predicts foetal weight-reduction, but not in Tht*III* 1 (GC) independent of confounders such as BMI, pregnancy planning or fast food eating during pregnancy. This is the first time that a gene-environment interaction between a common GR polymorphism and foetal weight was noted. Finally, one of the most important findings of our study came from the preclinical studies using placental tissues. Quantitative PCR revealed that the major transcripts in the human placenta are GRα, GAS5 (decoy for GR DNA binding) and Deptor. We have shown for first time that there are marked differences in the relative mRNA abundance of these components between term and preterm labour as well as colocalisation of GRα with GAS5.

With regards to placental regulation these data conclusively demonstrate that: a) there is evidence of gene-environment interaction between maternal stress, pregnancy planning, glucocorticoid receptor polymorphisms and foetal weight and b) potential cross-talk of mTOR and glucocorticoid signalling. We propose that measuring maternal stress levels in addition to circulating cortisol and mapping for known GR polymorphisms could become a useful non-invasive tool of diagnostic and prognostic value, with implications for preterm labour.

PUBLICATIONS

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ORAL PRESENTATIONS

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ABSTRACT PRESENTATIONS

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Mparmpakas, **D**., Zachariades, E., Goumenou, A., Karteris, E., Gidron, Y. "*Correlation of a Glucocorticoid Recaptor (GR) polymorphism but not GR slice variant expression with maternal attitudes towards pregnancy and fetal weight*" Congress on Steroid Research, 27th-29th March 2011, Chicago, USA.

Mparmpakas, **D**., Zachariades,E., Goumenou, A., Karteris, E. "*Maternal stress regulates placental mTOR signalling components*"**.** British Ecological Society, Biochemical Society, and the Society for Experimental Biology. 4th - 7th January 2011, London, U.K.

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Mparmpakas, D., Zachariades, E., Harvey, A., Goumenou, A., Karteris,. E. "*Placental DEPTOR: Another twist in mTOR signalling during pregnancy*"**.** Biochemical Society, 11th – 12th November 2010, London, U.K.

Mparmpakas, D., Foster,H.,Harvey,A., Karteris, E. "*Expression of m-TOR signalling components in a human placental cell line (BeWo)*". *Biochemical Society, 15th - 16th September 2008, Oxford, U.K.*

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Abbreviations

ACTH	- Adrenocorticotropic Hormone
AF-1	- Activation- Function-1
AF-2	- Activation- Function-2
Apgar score	- **A**ppearance, **P**ulse, **G**rimace, **A**ctivity, **R**espiration
AR	- Androgen Receptor
BeWo CT	- BeWo Cytotrophoblasts
BeWo ST	- BeWo Syncytiotrophoblasts
BMI	- Body Mass Index
bp	- Base Pairs
BSA	- Bovine Serum Albumin
cAMP	- Cyclic Adenosine Monophosphate
cDNA	- Complementary Deoxyribonucleic Acid
CRH	- Corticotropin-Releasing Hormone
Ct	- Cycle Threshold
DBD	- DNA binding domain
Deptor	- DEPDC6, DEP-containing protein 6
dH_2O	- Deionized Water
DHT	- Dihydrotestosterone
DMEM	- Dulbecco's Modified Eagle Medium
DMSO	- Dimethyl Sulfoxide
DNA	- Deoxyribonucleic Acid
DNase	- Deoxyribonuclease
dNTP	- Deoxyribonucleotide Triphosphates
ER	- Estrogen Receptor
EtOH	- Ethanol
FBS	- Fetal Bovine Serum
GAPDH	- Glyceraldehyde 3-Phosphate Dehydrogenase
GAS5	- Growth Arrest Specific 5
GC	- Glucocorticoids
GR	- Glucocorticoid Receptor

hCG - Human Chorionic Gonadotropin

HPA - Hypothalamic-Pituitary-Adrenal axis

hPL - Human Placental Lactogen

HSP 70 - Heat Shock Protein 70

HSP 90 - Heat Shock Protein 90

LBD - Ligand binding domain

MedDiet - Mediterranean Diet

MEM - Minimun Essential Medium

MR - Mineralocorticoid Receptor

mRNA - Messenger Ribonucleic Acid

mTOR - Mammalian Target of Rapamycin

MW - MolecularWeight

NLS - Nuclear Localization Signal

PBS - Phosphate Buffered Saline

PCR - Polymerase Chain Reaction

PR - Progesterone Receptor

Raptor - Regulatory-associated protein of mTOR

Rictor - Rapamycin-insensitive companion of mTOR

SD - Standard Deviation

SEM - Standard Error of Mean

Tween 20 - Polyoxyethylene Sorbitan Monolaurat

UV - Ultraviolet

Units

g - Grams

M - Molar

mg - Milligrams

ml - Millilitres

mm - Milimeters

mM - Milimolar

nm - Nanometer

^{o}C	- Degree centigrade
µg	- Microgram
µl	- Microlitre
µM	- Micromolar
v/v	- Volume / Volume
w/v	- Weight / Volume

Table of Contents

List of Figures

List of tables

CHAPTER 1

INTRODUCTION

1.1. Nutrition and Mediterranean Diet

Up to very recent years in the history of human nutrition, people were heavily relying on the food produced in the local environment for their nutrition. The biological and cultural resilience of humans is demonstrated by the fact that populations have survived and developed in highly different climates and ecological backgrounds with respectively changeable dietary patterns. Even though the survival through the reproductive age was achieved by the different populations living in very wide range of environments, the dietary patterns adhered to in these environments were not always in support of the populations' long-term health and well-being. The options of the human population in selecting their foods to eat have dramatically increased by the transplantation of numerous plant species across the continents and by the modern developments of transportation and food preservation technologies. Consequently, the implications of these revised options on health outcomes have become of a great interest (Willett, 2006).

According to the Seven Countries Study that started in the late 1950s led by Ancel Keys and his colleagues (Keys, 1980), the first evidence were presented to the scientific community showing the several important health benefits of the Mediterranean diet in a number of diseases compared with other dietary models examined (Keys, 1980). In this study seven countries in four regions of the world (United States, Northern Europe, Southern Europe, and Japan) were included. Specifically, one cohort was in the United States, two cohorts were in Finland, one in the Netherlands, three in Italy, five in the former country of Yugoslavia, two in Greece (Crete and Corfu), and two in Japan (Keys, 1980).

The Mediterranean diet (MedDiet) could be defined as a dietary pattern originally found in the Mediterranean area where the prominent regional characteristic was the high olive cultivation, in late 1950 and early 1960's, when the consequences of the Second World War

had been overcome, but the culture of fast-food had not yet reach the area (Fung *et al*., 2009; Kushi *et al*., 1995[A]; Kushi *et al*., 1995[B]). A large body of evidence has shown that the traditional MedDiet meets several important criteria for a healthy diet (Trichopoulou, 2004). The main characteristics of the MedDiet as seen in pyramid Fig. 1.1 are the high ratio of monounsaturated to saturated dietary lipids (mainly olive oil), the moderate consumption of alcohol, the high consumption of legumes, the high consumption of non-refined cereals including bread, the high consumption of fruits, the high consumption of vegetables, the low consumption of meat and meat products and the moderate consumption of milk and dairy products (Trichopoulou, 2009; Trichopoulou, 2004).

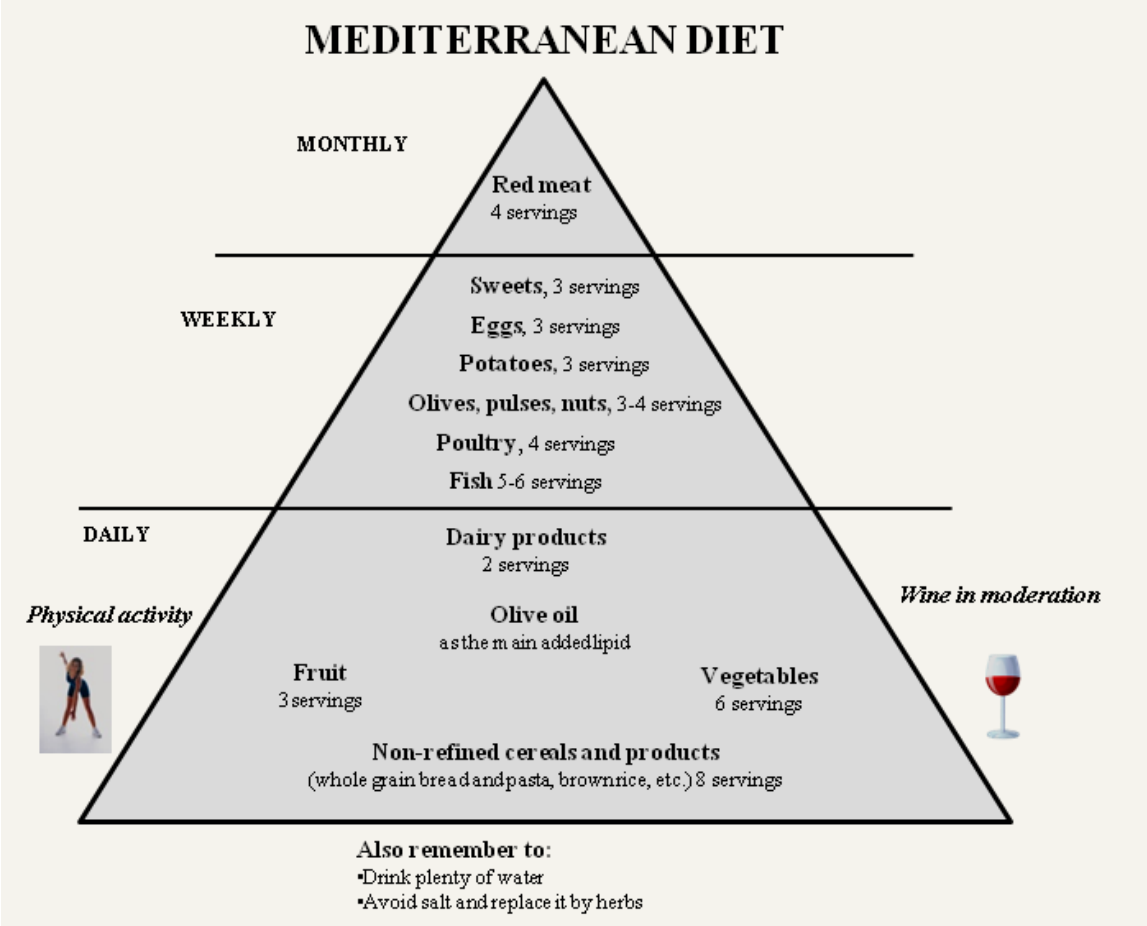


Figure: 1.1: Food Pyramid (Adapted from: Supreme Scientific Health Council, Hellenic Ministry of Health).

The beneficial effects of the MedDiet have been corroborated by a number of studies performed in large cohorts in different parts of the world. For example, a large study of 74.607 participants where the main objective was to examine if the adherence to a modified MedDiet, in which unsaturated fats were substituted for monounsaturated lipids, was associated with a longer life expectancy among older people showed that indeed an adherence to a modification of this diet was related with an increased survival among older people (Trichopoulou *et al*., 2005). In another study by Martínez-González where 13.380 Spanish university graduates without diabetes were followed up for a median of 4.4 years, it was shown that adhering to a MedDiet reduced the risk of developing type II diabetes among healthy individuals (Martínez-González *et al*., 2008).

The effects of the MedDiet on the status of metabolic syndrome have also been examined (Salas-Salvadó *et al*., 2008). Metabolic syndrome or "Syndrome X" is the combination of abdominal obesity, hypercholesterolemia, hypertension and hyperglycemia linked by an underlying resistance to insulin (Reaven, 1998). The National Health Nutritional Survey conducted from 1988-1994 showed that 47 million people in the U.S.A had metabolic syndrome, and in the last decade it was anticipated that the prevalence would be one in four adults in the U.S.A. The pernicious health impact of metabolic syndrome qualifies it the foremost public health problem that the Western societies are experiencing (Ford *et al*., 2002). In a study of 1224 participants were that were recruited from the PREDIMED (Prevención con Dieta Mediterránea), the authors concluded that the metabolic syndrome could be managed by adhering to a traditional MedDiet enriched with nuts (Salas-Salvadó *et al*., 2008).

1.2. Nutrition and Pregnancy

Pregnancy is associated with major physiological and psychological changes, and adaptations to these changes are crucial for normal foetal development (Lazinksi *et al*., 2008; Lobel *et al*., 2008). Maternal nutrition during pregnancy is an important factor which influences the health of both, mother and foetus (Mouratidou *et al*., 2006). A healthy balanced diet should supply the quantity and quality of nutrients to ensure the optimal health of the mother and the offspring. Pregnant women are in need of more energy and nutrients to help meet the increasing demands of the developing foetus (Scholl, 2008).

The nutritional needs of pregnant women have long been addressed (Laraia *et al*., 2004). The growth of the foetus is affected by the nutritional status of the woman before, as well as during, pregnancy; this also affects the course of the pregnancy and enhances the risk of obesity for both mother and infant (Scholl, 2008; Siega-Riz *et al*., 2001; Shaw *et al*., 1995). In addition, the risk of developing gestational diabetes mellitus (GDM) is increased by an unbalanced nutrient supply where the carbohydrates of the diet are substitute with fats (Saldana *et al*., 2004). If a diet of low glycaemic index is followed this can lead to low birth weight, where a diet with a high-fat and low-carbohydrate content can double the risk of a small-for-gestational-age baby (Scholl *et al*., 2004). Macronutrient components of the diet such as, proteins, fats and carbohydrates, have been associated to lower birth weight and lower placental weight which include high intakes of carbohydrates and low intakes of protein in the diet (Godfrey *et al*., 1996). In contrast, where a very high protein to carbohydrate diet is followed, blood pressure has been found to be raised later on in adult life (Shiell *et al*., 2001). A higher placental size and birth weight has been associated with high maternal intakes of iron and folate late in pregnancy (Godfrey *et al*., 1996). As a result, a suitable eating pattern is important not only throughout the childbearing years but also during pregnancy in order to ensure a healthy pregnancy and offspring development (Pick *et al*.,

2005; Siega-Riz *et al*., 2001). Recent studies focusing on healthy diet during pregnancy have compared Northern European diets with the Mediterranean-type diet, which has been shown to have possible benefits, including the prevention of premature birth (Mikkelsen *et al*., 2008). The results obtained from the Danish National Birth Cohort showed that pregnant women adhering to a diet rich in red and processed meat and high-fat dairy products, were associated with an increased risk of small-for-gestational-age infants (Knudsen *et al*., 2008). A recent study, in which the participants adhered to the highest quality diet in the first trimester of pregnancy, had a significantly lower risk of delivering a growth restricted baby (Rodriguez-Bernal *et al*., 2010). A poor *in utero* environment triggered by either maternal dietary or placental insufficiency may result in "programming" foetal susceptibility for the development of cardiovascular metabolic diseases later in adult life. This is also known as the *thrifty phenotype* or Barker's hypothesis (Prentice, 2005; Godfrey, 2002; Barker, 1997). Inadequacy of the maternal nutrient supply to meet the foetal demands can obstruct the normal foetal growth leading to severe consequences on the health in adult life (Constancia *et al*., 2005). On the other hand, an abundance of maternal nutrients being available exceeding the requirements of the foetus can lead to a superimposition of the deviation of the normal growth pattern thus leading to disease outcomes later in life (Godfrey & Barker, 2001). Foetal programming depicts *in utero* events "whereby a stimulus or insult at a critical, sensitive period of early life has permanent effects on structure, physiology and metabolism" (Godfrey & Barker, 2001).

Two key studies have investigated the relationship between decreased maternal food intake and the long-term consequences of prenatal exposure to famine conditions. These studies were the Dutch famine (Rooij *et al*., 2010; Schulz, 2010) and the study of the Siege of Leningrad (Stanner & Yudkin, 2001). The main evidence from the Dutch Hunger Winter study was that exposures *in utero* resulting in long lasting consequences for adult health do

not always shows an altered weight at birth. Even though a reduced infant weight at birth is easily accepted as a proxy for intrauterine deprivation, it is not the sole cause of the development of later in life diseases. The adverse foetal environment which is followed by an abundance of food in adulthood may result in the development of adult chronic disease (Schulz, 2010). This is in support with the Siege of Leningrad study where no associations were detected between intrauterine starvation and a number of pathologies such as glucose intolerance, dyslipidaemia, hypertension or cardiovascular disease in adult life. These studies add to the understanding of the mechanisms of the 'thrifty phenotype' and also sustain the importance of the catch-up growth during early childhood which was the result of the famine in the Netherlands but not in Leningrad (Stanner & Yudkin, 2001). The evidence from the studies of the Dutch Hunger Winter (Rooij *et al*., 2010) and the Leningrad Siege (Stanner & Yudkin, 2001) emphasize the importance of the pre- and early postnatal environment on the growth and development, and also the timing of the insult or deviation from the normal nutrition, as important as the insult itself in determining (a) the affected organ system(s) and (b) the onset and the severity of disease outcome (Godfrey & Barker, 2001).

Strong associations between low weight at birth and the increased risk of developing any metabolic disorders are well studied. Cardiovascular disease (Godfrey & Barker, 2001), type 2 diabetes (Hales *et al.,* 1991), central adiposity (Laitinen *et al*., 2004), abnormal lipid metabolism and hypertension (Phillips, 1998) and increased risk of death from ischemic heart disease (Eriksson *et al*., 1999; Forsen *et al*., 1999), have all been constantly linked to birth weight. Adverse consequences in the offspring have also been observed by maternal overnutrition before and during pregnancy. This has been suggested to lead to long-term effects on offspring's adiposity (Gale *et al*., 2007). Maternal diet can exert differential effects depending on the gestational trimester and development stage. Undernutrition early in development results in decreased demands for nutrients to avoid the adverse consequences of

growth restriction in late gestation. This may also result in the 'brain-sparing' reflex observed later in gestation, when nutrients fuel brain development at the expense of other organs and body segments. For example, offspring's thinness at birth, can be due to early influences during pregnancy when placental development still takes place (Godfrey, 2002; Barker, 1997). A careful distinction should be drawn between maternal and foetal nutrition, which describes the net supply of metabolic substrates delivered to the foetus. A "supply line" at the end of which the foetus grows, links the maternal diet at one end with the uptake from the foetal tissue at the other end (Bloomfield & Harding, 1998). This "supply line" includes the maternal nutrient uptake, the maternal metabolism and endocrine milieu, the uterine and umbilical blood flows as well as the placental transfer and metabolism. Fairly large changes in the maternal diet may have only a small impact on the foetal nutrition if the capacity of the foetal supply line is such to allow a large boundary of safety for optimum foetal growth. On the contrary, some of the common clinical causes of impaired foetal growth such as maternal hypertension being associated with reduced uterine blood flow or placental infarctions that comprise the placental transfer capacity, may severely limit the nutrient supply reaching the foetus but without a corresponding change in maternal nutrition (Harding, 2001).

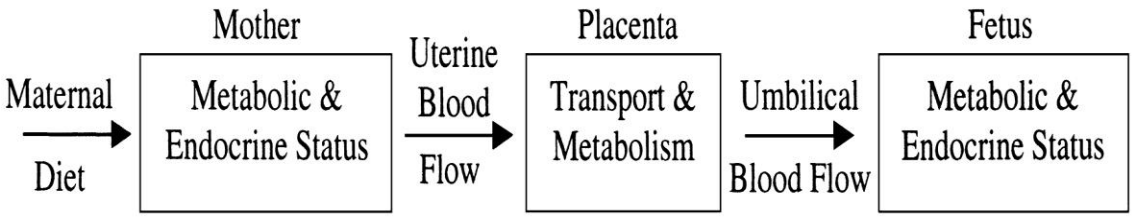


Figure 1.2: The foetal "supply line" along with the factors which mediate the differences between the maternal and foetal nutrition (Harding, 2001).

An important component of the foetal supply line is the placenta having multiple roles (Jansson & Powell, 2007). The capacity of the placenta to transport nutrients from the maternal to the foetal circulation is the most important placental influence on the nutrition of the foetus. Placental surface area and availability of specific nutrient transporters on the membranes are key factors that influence the transfer capacity of the placenta where these factors may be influenced in turn by the maternal nutritional environment (Das *et al*., 2004; Bassett *et al*., 1996). The foetal nutrition is also influenced by the placenta's metabolic demands for nutrients. As shown in sheep, the placenta consumes 60% of the glucose and oxygen that is taken up from the uterine circulation in the late gestation (Owens *et al*., 1989). In a scenario where the uterine glucose levels decrease, the placenta consumes an increasing amount of foetal glucose in order to maintain its own metabolic needs (Gu *et al*., 1987). Equally, it has been shown that the foetus exports amino acids back to the placenta when the maternal supply is limited (Owens *et al*., 1989). Furthermore, the placenta influences foetal nutrition by producing hormones which in turn influence the foetal and maternal nutritional supply. For example, placental lactogen and growth hormone are produced and contribute to the maternal insulin resistance thus increasing the availability of glucose and other nutrients in the maternal circulation being available for transfer to the foetus (Bauer *et al*., 1998).

1.3. The physiology of the human placenta

The human placenta is a unique transient organ that separates foetal and maternal blood. Two cell types are the main cellular components of the chorionic villi: the trophoblast and the mesenchymal cells. The trophoblast is evident approximately 4 days after fertilisation as the outermost layer of the blastocyst. From the early stage of gestation, it differentiates into two cell layers: the outer differentiated syncytiotrophoblast and the inner proliferative

cytotrophoblast (Benirschke, 1998). Syncytiotrophoblast and cytotrophoblast form the primary villi which become secondary villi. This transformation occurs when the mesenchymal cells, derived from the extraembryonic mesenchyma, begin to invade the villi and tertiary villi during the formation of the foetal blood vessels. In the tertiary villi, the villous trophoblast consists of the highly differentiated syncytiotrophoblast (surface layer) and the cytotrophoblast (basal layer) (Petraglia *et al*., 1996). The cytotrophoblasts are the germinal cells and the syncytia, the secretory cells, are derived from the cytotrophoblasts. Each cytotrophoblast is characterised as a single cell with well-demarcated borders and a single distinct nucleus. Among the cytotrophoblasts there are frequent mitoses (Ramsey, 1982). However, these characteristics are lacking in the syncytium, in which the cytoplasm is amorphous, lacking cell borders and the nuclei are multiple and diverse in shape and size (Cunningham *et al*., 1993). The remaining trophoblasts are used for the development of other parts of the placenta such as the: chorion laeve, marginal zone, chorionic plate, basal plate including cell columns, septa, and cell islands. They are named extravillous trophoblast (Petraglia,1996).

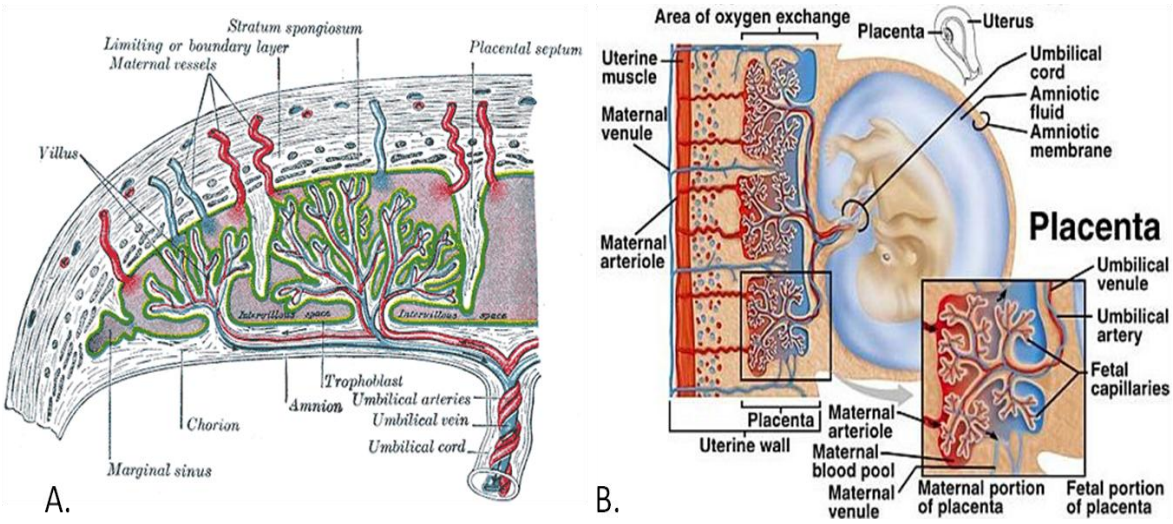


Figure 1.3: Panel (A): Diagrammatic representation of the anatomy of human placenta (www.theodora.com/anatomy/images39.gif), Panel (B): Proposed placental functions (The Mc Graw-Hill Companies, Inc.)

A large number of studies show that syncytiotrophoblasts express the majority of pituitary-like hormones such as human chorionic gonadotropin (hCG), human placental lactogen (hPL) (Kato & Braunstein, 1989), corticotropin-releasing hormone (CRH) (Frim *et al*., 1988), adrenocorticotropic hormone (ACTH) (Petraglia *et al*., 1987), as well as neurohormones/ neuropeptides, cytokines, and growth factors which have been preferentially localised in the cytotrophoblast (Wood, 1980; Hunt, 1989). In addition, near term, the human placenta is involved in steroidogenesis, and vast amounts of estrogens are produced almost exclusively by syncytiotrophoblast cells (Cunningham *et al*., 1993).

1.4. Maternal exercise during pregnancy

Physical activity is highly recommended during pregnancy by physicians and the American College of Obstetricians and Gynaecologists (ACOG): in the absence of any medical or obstetric complications, moderate exercise on a daily basis of 30 minutes or more is recommended for most pregnant women (ACOG Committee Obstetric Practice, 2002). Although the common sense as well as a small number of observational studies and controlled trials are in support of this recommendation, a recent review by Kramer has concluded that the available evidence of the possible benefit or harm to mother or foetus regarding the recommendations of aerobic exercise during pregnancy are highly insufficient. The authors concluded that the available studies are very small, the experimental design is possibly flawed, and an inconsistence in methodology is observed which renders these studies unable to support this recommendation (Kramer, 2004). The benefits of regular aerobic exercise during pregnancy have become of an interest to the American College of Obstetricians and Gynaecologists and an attempt has been made to redirect the focus on the variety of theoretical risks that exercise poses to the mother and the foetus (Snyder & Pendergraph, 2004). The positive benefits of regular exercise during pregnancy on women as reported by Kramer, include improvement of overall body image and steady maintenance of their physical fitness (Kramer, 2004). In the currently available studies where the parameters studied included mode of delivery, length of labour, growth parameters, preterm birth, and Apgar scores, no other benefits or risks on the health of the mother or the foetus were supported (Kramer, 2004). However, despite the many limitations of the current studies, some evidence has emerged suggesting that smaller babies were born to women who exercised vigorously during their pregnancy, resulting in neonatal fat mass restriction. Also, it has been suggested that these leaner babies could have a lower risk of becoming obese as children and as adults (Magann *et al*., 2002).

Excess weight gain during pregnancy and the postpartum inability to return to a healthy weight have been shown to be associated with increased rates of maternal obesity eight to ten years later (Rooney & Schauberger, 2002). Many women find appealing enough the enhanced body image resulting from exercise and along with the perceived benefits of physical fitness continue to exercise during pregnancy. Yet, very little has been published regarding the acceptability of exercise during pregnancy in women of diverse cultural backgrounds. Given that in some population subgroups there is a high prevalence of obesity and overweight (U.S Department of Health and Human Services: office of the surgeon general, 2001) an extra encouragement to exercise during pregnancy may be desirable. Therefore, for the successful implementation of the ACOG guidelines a cultural acceptance of the idea of exercising during pregnancy will be a necessity, thus an understanding of the attitude of women from diverse backgrounds towards exercise during pregnancy is crucial (Snyder & Pendergraph, 2004). Moderate aerobic exercise is a safe, affordable way for women to improve their sense of well-being during pregnancy and this is further enhanced by the ACOG's statement that potential risks are rare in properly screened pregnant women who avoid extreme environmental conditions and activities that can lead to abdominal trauma (Snyder & Pendergraph, 2004). However the specific benefits of regular exercise for the mother and the foetus have yet to be confirmed by further studies but shifting away from the view that aerobic exercise poses potential hazard for the health of pregnant women is still of major benefit (Snyder & Pendergraph, 2004). Given the current epidemic of obesity and the associated increasing incidence rates of metabolic syndrome and type II diabetes, it would therefore appear that well-designed, large-scale prospective clinical trials examining the effects of exercise during pregnancy as well as long-term follow-up of the exercising women and their infants would help to elucidate the real benefits versus the risks of exercise during

pregnancy and allow future recommendations to be based on evidence rather than expert opinion (Snyder & Pendergraph, 2004).

1.5. Maternal Stress and Foetal Outcome

Stress is defined as "a state of physiological or psychological strain caused by adverse stimuli physical, mental, or emotional, internal or external, that tend to disturb the functioning of an organism and which the organism naturally desires to avoid" (Dorland, 2007). Widely accepted as being a complex construct, stress, is divided into several stages: 1) the stressors which are the event leading to the clinical features associated with stress, 2) the mediators which can involve the appraisal, of as well as coping with, the stressor, 3) the moderators where this can include the social support being available as well as the personality of the individual and finally, 4) the stress response itself which includes innervations of the hypothalamic-pituitary-adrenal (HPA) axis and the sympathetic nervous system (Gidron & Ronson, 2008; Thanker & Sood, 2008; Smith & Vale, 2006; Weinman & Kaptein, 2004). The physiological response to stress is usually where the HPA axis is activated above the basal level, as well as stimulation of the sympathetic nervous system to induce the synthesis and secretion of glucocorticoids (GC) (such as cortisol) and catecholamines (such as adrenalin) (Ogden, 2007). A stressor can include a stimulus received from the individual, a situation or a circumstance experienced by an individual and could potentially induce a stress response (Weinman & Kaptein, 2004). Perceived stressors are subjected to appraisal which is connected to the personality of the individual, to their past experiences as well as to the physical and psychological state of one being in (Weinman & Kaptein, 2004). Stressors can vary from being acute (short termed) to chronic (constant) and also to chronic intermittent (long lasting but not constant). Mediators will determine and moderate the level of the stress

response by acting as "buffers" against the negative health consequences induced by the stressor. Psycho-social factors, for example the ability of coping and the social support being available can have a great effect on the outcome (Weinman & Kaptein, 2004; Olstad *et al*, 2001). A stress reaction elicits responses on more than one level and more than one (i.e. emotional, cognitive or behavioural) of the responses can be felt at any one time (Weinman & Kaptein, 2004).

Pregnancy is associated with major physiological changes and maternal adaptation to these changes is crucial for a normal development of the foetus (Lazinksi *et al*., 2008; Lobel *et al*., 2008). Emotional stress is considered to be a response to the stressor, therefore during pregnancy; the developed emotional stress may interfere with the necessary adaptation and lead to a dysregulation of the two major stress response systems: the HPA axis and the sympathetic nervous system (Lazinski *et al*, 2008). Perceived life-event stress including depression and anxiety predicted lower birth weight, decreased Apgar scores, smaller head circumference, and small for gestational age babies (Marcus & Heringhausen, 2009). It has been widely documented, that stress during pregnancy correlates with pre-term birth as well as lower birth weight (Bolten *et al*., 2010; Dole *et al*., 2003). Also in association with smoking, it has been shown that pregnancy-specific stress aid directly to pre-term delivery and indirectly to low birth weight (Lobel *et al*, 2008). By examining these effects it is evident that pregnancy-specific stress may be a more powerful contributor to problematic birth outcomes than general stress (Lobel *et al*, 2008). Despite the origin of the stressor, the physiological responses to it are the innervations of the HPA axis and that of the sympathetic nervous system (Gidron & Ronson, 2008). The responses following the perception of a stressor can be seen in Fig. 1.4.

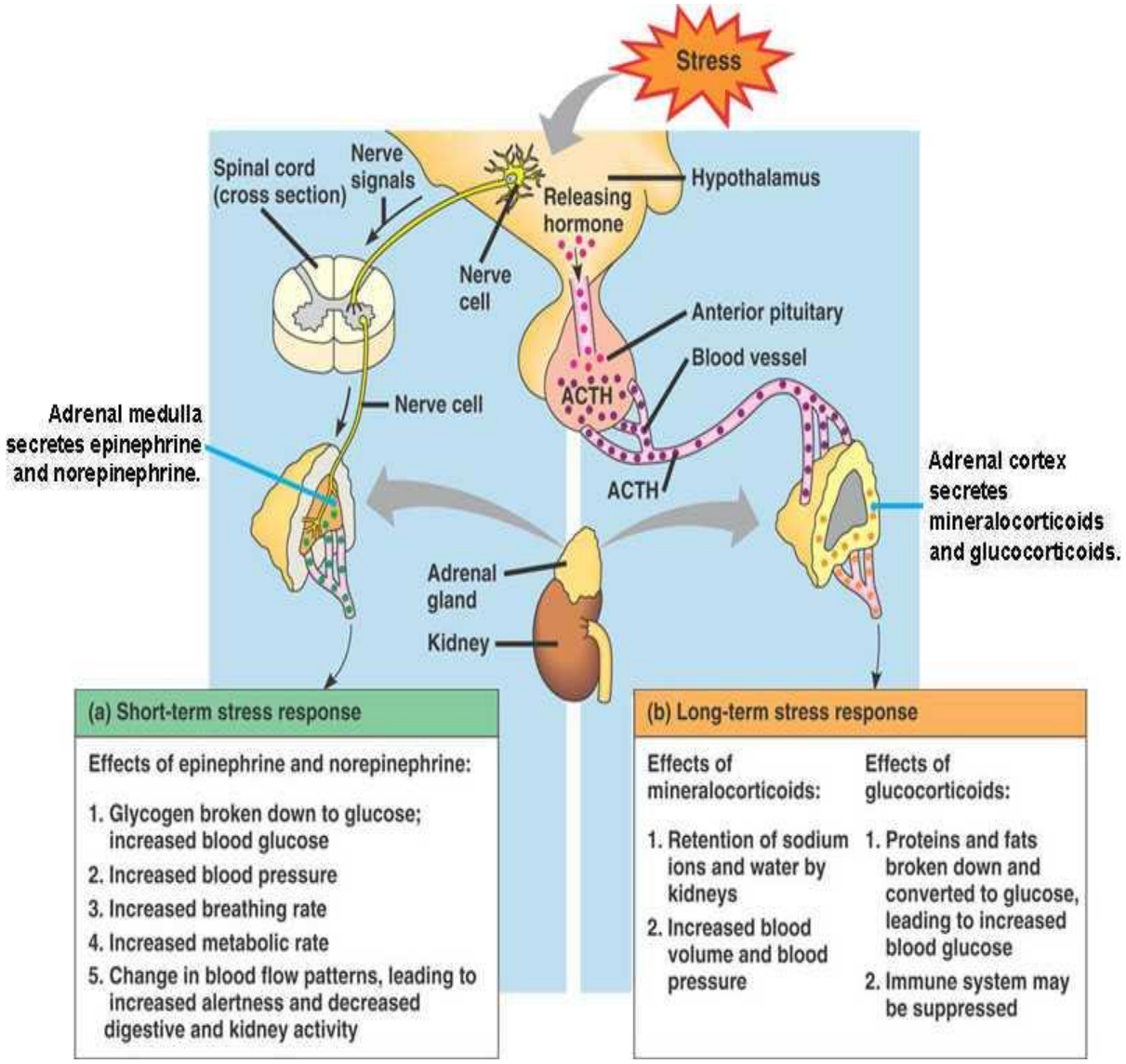


Figure 1.4: Diagram illustrating the parts of the adrenals (blue lines) including what they secrete and how stress is perceived and the following responses (Barot *et al*., 2005)**.**

It is of great importance to note that with an exogenous stimuli like chronic and repeated stress, dysregulation of the HPA axis is seen leading to an altered cortisol secretion, thereby affecting the end-organ function (Guilliams & Edwards, 2010; Nussey & Whitehead, 2001).

1.6. Hypothalamic-Pituitary-Adrenal (HPA) Axis

Neurosecretory cells that are present in the periventricular nucleus of the hypothalamus secrete corticotropin-releasing hormone (CRH). This peptide moves to the anterior pituitary where it initiates a signal transduction cascade stimulating the synthesis and the secretion of the adrenocorticotropic hormone (ACTH) via arginine vasopressin which is also being released in the hypothalamus (Nussey & Whitehead, 2001). ACTH acts on the adrenal glands and specifically the adrenal cortex, where it increases the transport of cholesterol to the inner mitochondria membranes and consequently increases the rate of synthesis of cortisol (Nussey & Whitehead, 2001). The synthesis and secretion of cortisol is highly regulated by the presence/absence of ACTH. In the adrenal cortex, ACTH acts to stimulate GC synthesis via a G protein-coupled receptor (GPCR) (Nussey & Whitehead, 2001) and cortisol is then released in a pulsatile fashion (Nussey & Whitehead, 2001; Sapolsky *et al*, 2000). The bioavailability of cortisol and glucocorticoids are regulated by a negative feedback loop (Fig. 1.5) which controls the circulating concentrations of cortisol and the other adrenal glucocorticoids. Neurons in the hypothalamus are able to detect these concentrations and therefore glucocorticoids act back on the hypothalamus and pituitary by suppressing CRH and ACTH production in a negative feedback cycle thus modulating/co-ordinating the synthesis and secretion of ACTH, and subsequently cortisol (Miller *et al*., 2007; Nussey & Whitehead, 2001). This feedback mechanism can be temporarily interrupted when stress is introduced. The exogenous stress stimulus encourages increases in CRH and therefore ACTH, consequently increasing cortisol concentrations. The level of cortisol, can be restored via the feedback mechanism (Nussey & Whitehead, 2001; Laudat *et al*, 1988).

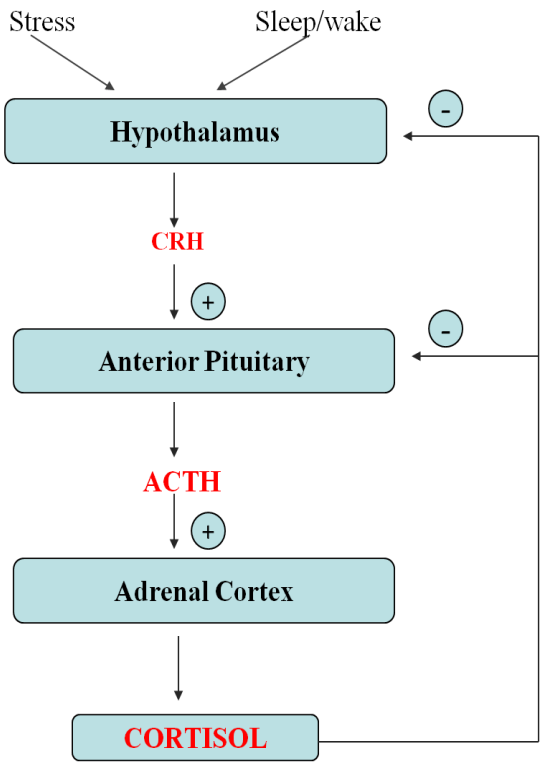


Figure 1.5: The hypothalamic-pituitary-adrenal (HPA) axis and its regulation. Stress as well as sleep/wake cycles can induce the secretion of corticotrophin releasing hormone (CRH) in the hypothalamus. CRH is perceived by the pituitary gland which secretes adrenocorticotropic hormone (ACTH). ACTH is perceived by the adrenal cortices which secrete cortisol. Cortisol reacts in the body in a number of different ways and also provides a negative feedback loop to the hypothalamus which adjusts its secretion of CRH accordingly (Nussey & Whitehead, 2001, Porterfield, 2001).

The light/dark or sleep/wake cycles affect the levels of ACTH that are being released and consequently the levels of circulating cortisol, which can be described to follows a circadian rhythm (Weerth *et al*, 2003**;** de Bosscher *et al*, 2003). Fig.1.6 below shows the levels of (nmol/l) that are being secreted per hours of the day. Cortisol's secretions peaks in the morning, at around the awaking hour (8 am) after which a gradually decline through the waking hours is noticed, achieving a daily minimum during the half of the sleep cycle (Czeisler *et al*., 1976). When the body is stressed or when specific foods are eaten this can

lead to episodic spikes of cortisol to occur. On the other hand, cortisol levels are at the lowest after midnight and up to 4:00 am. The cortisol levels in the morning are indicative of the peak of the output of this hormone whereas the levels of cortisol at noon show the adaptability of the body to this hormone as well as its usage. The levels in the afternoon are mainly associated with the regulation of sugar than with the function of the adrenal glands. The baseline function of the adrenals is indicated by the levels of this hormone in the evening hours (Lam & Lam, 2010).

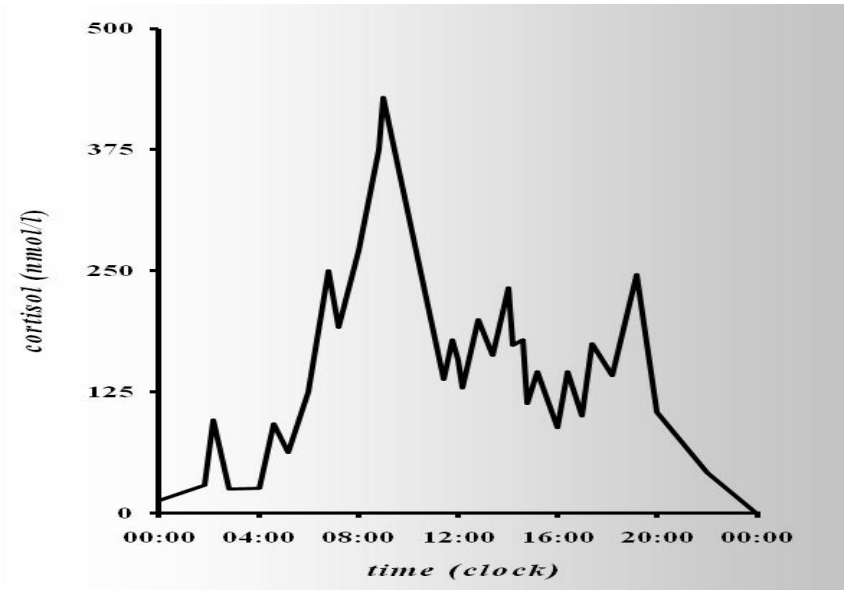


Figure 1.6: Secretory pattern of cortisol over a 24-hour period (Nussey & Whitehead, 2001)

1.7. Biological effect of Glucocorticoids / Cortisol

An uncontrollable biological response to a stressor can exert harmful effects to the body. Glucocorticoids are able to protect the body against disruption induced by stress by regulating and sustaining gluconeogenesis, metabolism and blood pressure (de Bosscher *et al*, 2003; Fujiwara *et al*, 1996). Cortisol and indeed all glucocorticoids are able to affect lipid metabolism (de Bosscher et al, 2003; Christiansen *et al*, 2007). Steroid hormones including cortisol readily cross the blood/brain barrier affecting the neural pathways (Reichardt & Schutz, 1998) where in response to various stimuli, including stress, they are able to coordinate metabolic, endocrine, immune and nervous system responses (Tronche *et al*, 1999). Glucocorticoids are also considered to have an effect on brain function by modulating emotional behaviour, cognitive functions and addictive states (Flanagan-Cato & Fluharty, 1997; Tronche *et al*, 1999). More specifically as it can be seen from Table 1.1, glucocorticoids can also exert effects on the cardiovascular system controlling the fluid volume, elevating blood pressure, heart rate and cardiac output with blood being diverted to muscles via a series of constrictions and dilation of blood vessels. It has also been shown to affect immunity and inflammation, as cortisol influences cells participating in the body's immune reaction and particularly white blood cells (Lam & Lam, 2010). In addition, cortisol is reported to be a powerful anti-inflammatory agent. Cortisol increases the blood sugar levels in the body thus providing it with the needed energy to physically escape a potential threat or injury. Moreover, cortisol can affect metabolic events, neurobiological processes as well as reproductive physiology (Scott, 2008; Sapolsky *et al*, 2000).

Effects of Cortisol
• Decrease the effects of insulin on blood glucose
• Increases block glucose
• Increases fat and cholesterol in the blood
• Increases breakdown of proteins into amino acids.
• Decreases inflammation, immune system function, and healing.
• Decreases memory due to inhibition and /or death of brain cells. Therefore, overtime and problem solving skills are reduced.
• Increases the number of receptors for epinephrine and norepinephrine, leading to blood pressure and heart rate rises.

Table 1.1: Summary of the effects of cortisol on human physiology

Glucocorticoids are administered in humans during the late stages of pregnancy in order to treat maternal asthma and decrease neonatal respiratory morbidity. However, growth retardation and reduced birth weight are reported as side effects of this treatment (Reinisch *et al*., 1978)**.** The programming of the hypothalamo–pituitary–adrenal (HPA) axis has been hypothesized to be regulated by prenatal glucocorticoids or stress mediators as the excess of glucocorticoids may be associated with hypertension and glucose intolerance (Clark, 1998). Animal studies have shown (Vallee *et al*., 1996) that offspring of prenatally stressed rats had increased basal blood glucose levels, were eating less and weighed less in comparison to the control group. Another study (Lesage *et al*., 2004) reproduced these findings; that increase in maternal stress resulted in reduced body, adrenal and pancreas weights as well as decreased plasma corticosterone and glucose levels in foetuses at term. Male rats of age (24 months old) exhibited hyperglycaemia, glucose intolerance and decreased basal levels of leptin. In addition, the intake of food following fasting was increased (Lesage *et al*., 2004). Therefore,

an *in utero* environment with excess glucocorticoids provoked a long-lasting disturbance in the feeding behaviour and led to metabolic dysfunction, in the offspring (Lesage *et al*., 2004).

1.8. The Nuclear Receptor superfamily

1.8.1. Glucocorticoid Receptor

Cortisol exerts its actions by binding and activating glucocorticoid receptors (Rhen & Cidlowski, 2005; De Bosscher *et al.*, 2003). The glucocorticoid receptor (GR) is a phosphoprotein and a transcription factor, which is able to positively and negatively regulate the expression of different genes. The GR is a member of the steroid hormone receptors subgroup that belongs to the super-family of nuclear receptors. The nuclear receptors are members of a large superfamily of DNA-binding transcription factors which are believed to be evolutionary maintained and derived from a common ancestor (Owen & Zelent, 2000; Escriva *et al*., 1997). Nuclear receptors regulate a range of programs involved in growth, differentiation, metabolism, reproduction and morhogenesis (Lu *et al.*, 2006; Germain *et al*, 2006; Aranda & Pascual, 2001). The nuclear receptors are of major importance for intercellular signalling as they converge intracellular and extracellular signals on the regulation of genetic programs (Chambon, 2005). As they are transcription factors they respond through association with a variety of hormonal and metabolic signals. They integrate diverse signalling pathways as they relate to targets of post-translational modifications and also are able to regulate the activities of other signalling cascades (Chambon *et al*, 2005).

The steroid hormone receptors subgroup of the nuclear receptor superfamily includes the estrogen receptor (ER), the cortisol binding glucocorticoid receptor (GR), the aldosterone binding mineralocorticoid receptor (MR), the progesterone receptor (PR) and the androgen receptor (AR). In addition, the steroid hormone receptors subgroup contains three orphan

receptors closely related to ER (Kallen *et al*., 2004; De Bosscher et al, 2003; Escriva *et al*., 2000; Thornton & DeSalle, 2000; McKay & Cidlowski, 1999; Nuclear Receptor Nomenclature Committee, 1999; Stein & Yang, 1995). All steroid hormone receptors consist of a variable N-terminal domain (A/B domain), a conserved domain that binds DNA (DBD or C domain), a hinge domain (D domain), a ligand –binding domain (LBD) also known as E domain which is conserved among the nuclear receptors (Fig. 1.7) (Bain *et al*., 2007; Germain *et al*., 2006; Hirata *et al*, 2003; Aranda & Pascual, 2003).

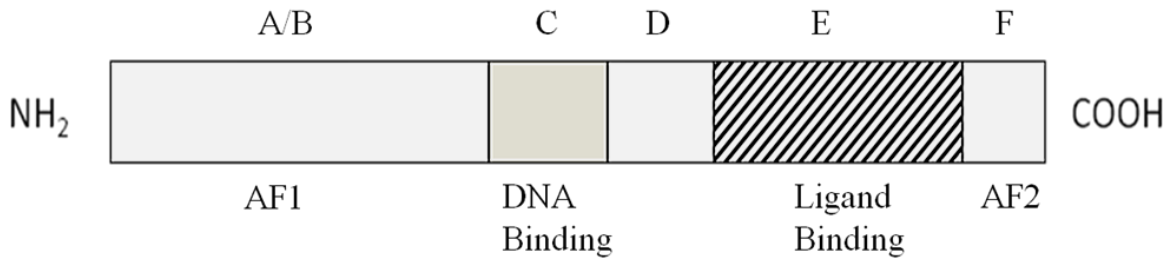


Figure 1.7: Illustration of the distinct domains that are common in nuclear receptors.

The amino terminus contains an autonomus transcriptional activation function known as activation- function-1 (AF-1) which contributes to the integral ligand- independent activation by the receptor. It can also function in synergy with activation function-2 (AF-2). The N-terminal amino acid domain is unique to each steroid hormone receptor and is of variable sequence and length. The DNA binding domain (DBD) which is highly conserved among the group binds to DNA and confers sequence-specific DNA recognition. The D domain, is situated between the DBD and ligand binding domain (LBD), functions as a flexible hinge and contains the nuclear localization signal (NLS). The LBD is key to receptor-ligand interactions. All ligand-binding domains fold into a highly homologous three-layered structure with a small ligand-binding pocket in the centre. Upon ligand binding, the NLS is exposed and assists the translocation of the receptor to the nucleus. This domain also contains AF-2 which is a ligand-dependent transcriptional activation function necessary for the

recruitment of transcriptional co-activators. The function of the carboxy terminus (F domain) remains unknown and it has been shown to have an extremely variable sequence (Nagpal *et al*., 1993; Aranda & Pascual, 2001).

1.9. Activation of the Glucocorticoid Receptor by Cortisol

As mentioned, cortisol exerts its biological effects via binding to its receptor (Rhen & Cidlowski, 2005; De Bosscher et al, 2003). In the absence of hormone, the glucocorticoid receptor (GR) is in the cytosol and it is complexed with a variety of proteins including heat shock protein 90 (hsp90), the heat shock protein 70 (hsp70) and the protein FKBP52 (FK506-binding protein 52) which retain it in an inactive state (Pratt *et al*., 2006; De Bosscher *et al*, 2003). Cortisol diffuses through the cell membrane into the cytoplasm and binds to the glucocorticoid receptor resulting in the release of the heat shock proteins by changing the stereochemical configuration of the receptor (Buckingham, 2006).

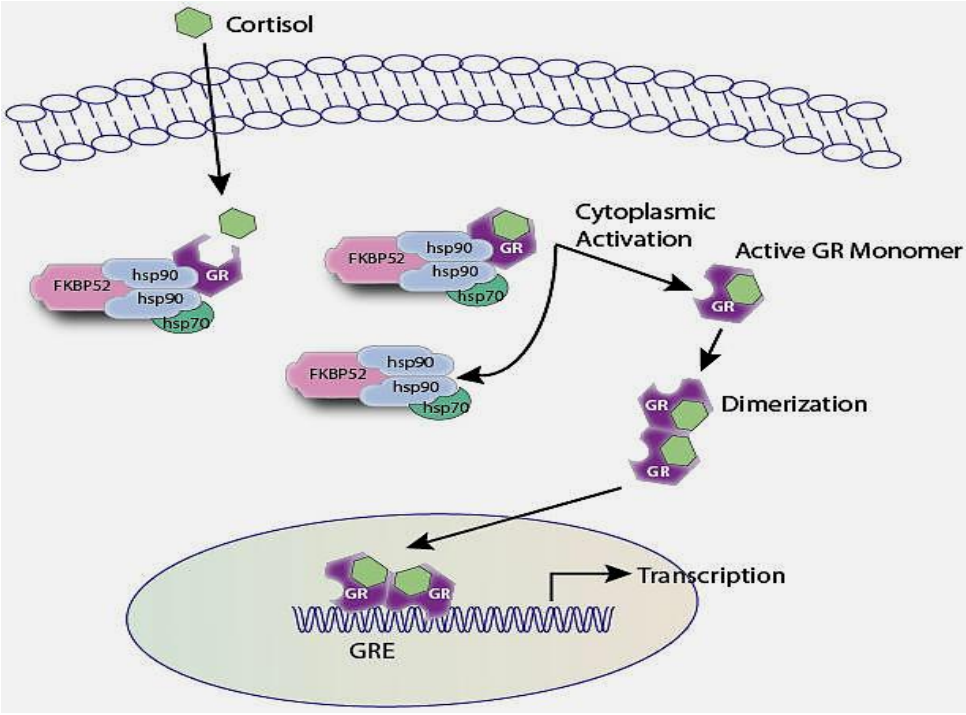


Figure 1.8: Activation of GR by cortisol (panomics.com)

As can be seen in Fig. 1.8, ligand binding activates and changes the conformation of the GR which undergoes phosphorylation (Davies *et al.*, 2002; Dittmar *et al.*, 1997). The phosphorylation of the receptor facilitates translocation of the ligand-receptor complex from the cytoplasm into the nucleus. Once in the nucleus it forms a homo- or heterodimer with another ligand – receptor complex (Nussey & Whitehead, 2001). The zinc fingers that are present in the DNA-binding domain (DBD) of the dimerized receptors are able to interact with specific grooves in the DNA helix that contain a consensus sequence, known as the glucocorticoid response element (GRE) (Nussey & Whitehead, 2001). The GRE (5'-GGTACA*nnn*TGTTCT-3', where *n* is any nucleotide) is found in the promoter regions of the glucocorticoid responsive genes (Beato, 1989). The GR activates transcription of target genes by interaction with the basal transcription machinery (Newton, 2000; McKay & Cidlowski, 1999).

1.10. Glucocorticoid Receptor splice variants

Govindan and co-workers in 1985 were the first to isolate the human glucocorticoid receptor from the breast cancer cell line MCF-7 (Govindan *et al*, 1985). Following the cloning of the human GR another group determined the structure of the hGR gene (Encio & Detera-Wadleigh, 1991). It was shown that the gene is of a minimum size of 80kilobases (kb) within chromosome 5 and it contains eight coding exons (Zong *et al*, 1990, Encio & Detera-Wadleigh, 1991). The first report regarding the transcripts resulting from alternative splicing of the GR gene came from Hollenberg and his colleagues (Hollenberg *et al*, 1985). These were the highly conserved GRα and GRβ isoforms that differ only at the carboxy-terminus (Dahia *et al*., 1997). More specifically, GRα isoform is encoded by exons 2, 3, 4, 5, 6, 7, 8 and 9α of the GR gene (Hollenberg *et al*, 1985; Tissing *et al*, 2003) whereas GRβ isoform is

encoded by the same exons but there is alternative splicing of exon 9β instead of 9α and this gives rise to 15 unique C-terminal amino acids (Tissing *et al*, 2003). From the two isoforms only GRα shows to be the functional receptor in terms of ligand binding (Encio & Detera-Wadleigh, 1991; Rivers *et al*, 1999) whereas GRβ is unable to bind GC and is transcriptionally inactive (Rivers *et al*, 1999). GRβ forms heterodimers with ligand-bound GRα either in the cytoplasm with subsequent translocation into the nucleaus or in the nucleus to exhibit the dominant negative effect over the GRα isoform (Dahia *et al*, 1997). In the past years it was shown that expression of GRβ at mRNA or protein level was low in almost every human tissue and it was found to be complexed with heat shock proteins, leading to the assumption that GRβ has an active role in determining the sensitivity of target tissues to GC (Bamberger *et al*, 1995). Rivers *et al*, described a new splice variant of the GR gene termed GRγ where exon 4 is alternatively spliced to exon 3 and has three basepairs of the intron region (Rivers *et al*, 1999). This results to an addition of another amino acid -arginine- in the DNA binding domain of the receptor (Rivers *et al*, 1999). The insertion of this amino acid at this site has been shown to decrease the transcriptional activation of GRγ to 48% that of GRα (Ray *et al*, 1996). The cloning of another variant was reported by Moalli and his team members. This splice variant, GR-P, is encoded by exons 2-7 and a part of intron 7 whereas exons 8 and 9 are missing (de Lange *et al*., 2010; Moalli *et al*, 1993). As a result, GR-P lacks the ligand binding domain and is unable to bind to glucocorticoid (GC) (Tissing *et al*, 2003). A recent study has demonstrated the presence of GR-P mRNA in tumour cells isolated from patients with haematological malignancies, further the activity of GRα was increased upon transfection of cells with GR-P. This effect though was found to be cell type specific suggesting that specific cell factors and even the ratio of the receptors mRNA influences the responsiveness of the tissues to glucocorticoids (de Lange *et al*., 2010).

Up to now, the cloning of four splice variants of the GR gene have been reported formed by alternative splicing, these being GRα, GRβ, GRγ and GR-P. The diagram (Fig.1.9) illustrates the GR gene as well as the four splice variants (Tissing *et al*., 2003). A number of studies have reported the expression of different GR splice variants in different cell and tissue types (Pujols *et al*., 2002; Honda *et al*., 2000; Oakley *et al*., 1999; de Castro *et al.*, 1996). Specifically the expression of GRα mRNA was found to be expressed in cells of the brain, skeletal, muscle, lung, kidney, liver, heart, nasal mucosa and in the colon. Also in inflammatory cells such as macrophages, eosinophils, neutrophils. GRβ mRNA was detected in cells of the liver, skeletal muscle, kidney, lung, brain, nasal mucosa, heart and in the inflammatory cells eosinophils, macrophages, neutrophils , although in concentrations at least 400 times lower than GRα mRNA (Pujols *et al*., 2002). Regarding the expression of GR in reproductive tissues, the expression of GR has been reported in the ovary (Alsop *et al*., 2009; Rae *et al*., 2005) and studies have confirmed the expression of GR in the rat testis (Weber *et al*., 2000) and in the testis of the amphibian *Bufo arenarum* (Denari & Ceballos, 2006).

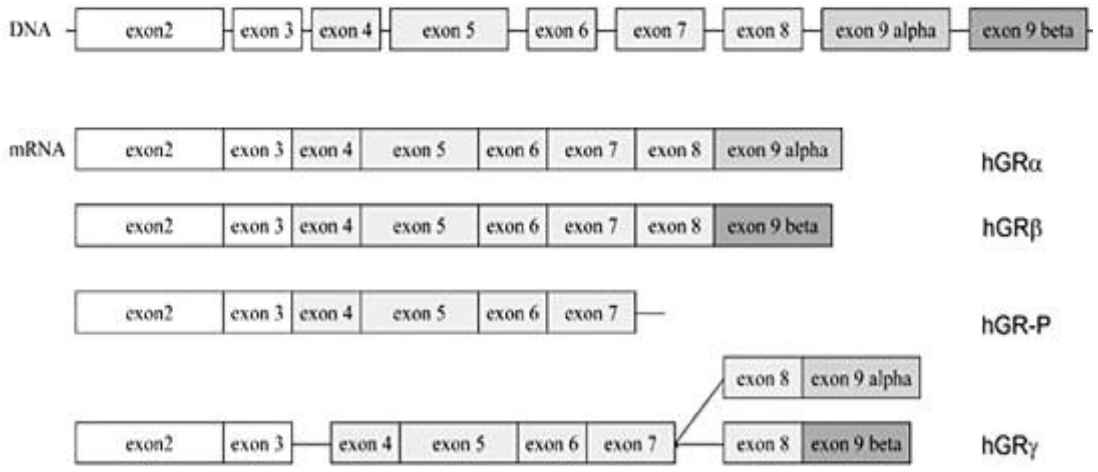


Figure 1.9: A schematic representation of the GR gene and the GR splice variants (Tissing *et al*, 2003).

1.11. Glucocorticoid Receptor Polymorphisms

A number of polymorphisms have been described in the gene coding for the GR although it is still unclear whether the variability in the glucocorticoid responses observed are due to the polymorphisms or to other factors (van Rossum & Lamberts, 2004). Only few of these polymorphisms are functionally relevant and these are: the *ThtIII* 1, the ER22/23EK, the N363S, the *Bcl*I and the GR-9β polymorphisms (Fig.1.10). Studies have shown that at least three polymorphisms are associated with altered glucocorticoid sensitivity and also changes in body composition and metabolic parameters (Manenschijn *et al*, 2009; van Rossum & Lamberts, 2004). Apart from the increased sensitivity to gluocorticoids, a tendency towards a lower bone mineral density along with increased body mass index and increased abdominal obesity has been observed and associated with the GR polymorphisms (Ukkola *et al.*, 2001; Rosmond *et al.*, 2000; Buemann *et al.*, 1997;). A study has reported the relative resistance of a polymorphism (ER22/23EK) to glucocorticoids and in addition to this, lower total cholesterol and low-density lipoprotein cholesterol levels as well as lower fasting insulin concentrations and a better insulin sensitivity was observed (van Rossum & Lamberts, 2004).

GR polymorphisms have also been associated with lower risk of dementia and white matter lesions in elderly (van Rossum & Lamberts, 2004). These polymorphisms have been associated with altered sensitivity to glucocorticoids and also altered cortisol levels and with certain other diseases including autoimmune, cardiovascular as well as differences in body composition and metabolic parameters and may contribute considerably to the observed variability in gluococorticoid sensitivity (Manenschijn *et al*, 2009).

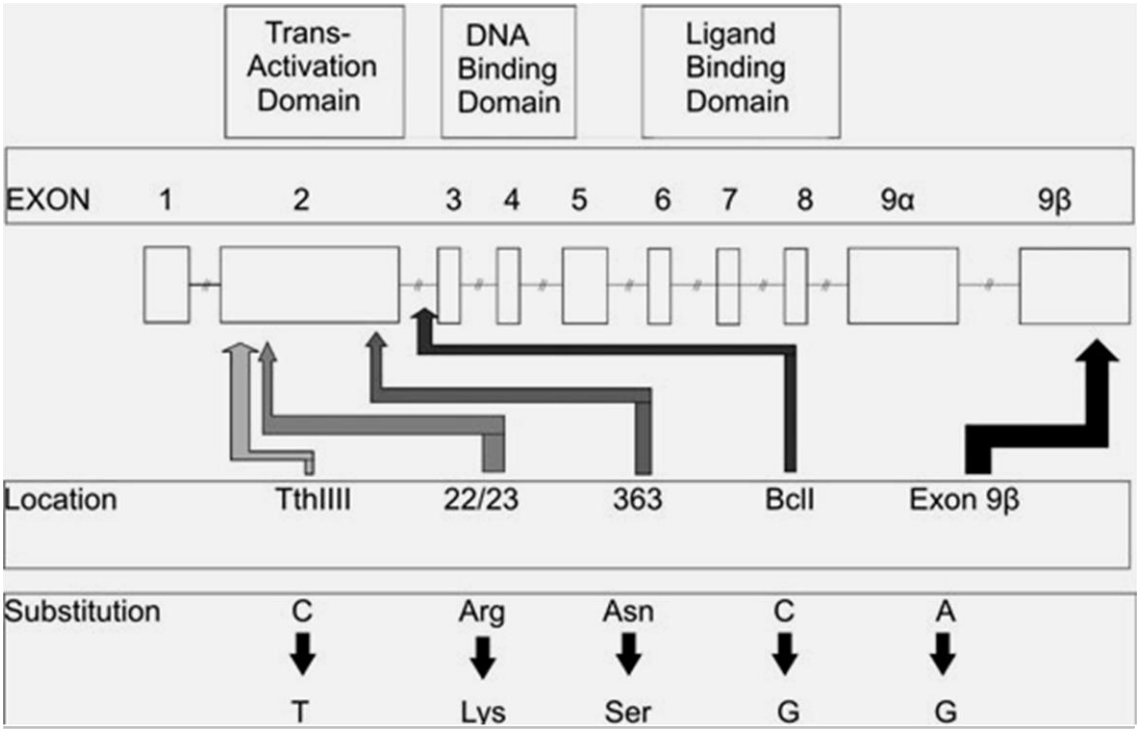


Figure 1.10: A presentation of the GR gene and the polymorphisms associated with it (Manenschijn *et al*, 2009).

The *ThtIII* 1 polymorphism has been described as a restriction fragment length polymorphism (RFLP) that is caused by a C→ T substitution 3807bp upstream the mRNA start site on the GR gene (van Rossum *et al*, 2003). By RFLP we refer to the type of polymorphism which results from a variation in the DNA sequence that is recognized by restriction enzymes. The functionality of this polymorphism has been questioned as the linkage between it, and the ER22/23EK polymorphism shows that carriers of the ER22/23EK carry a *ThtIII* 1 minor allele and the sensitivity of those individuals to GCs is decreased. Whereas, carriers of the *ThtIII* 1 allele without been carriers of the ER22/23EK polymorphism showed no alternations in sensitivity to GCs (Manenschijn *et al*, 2009; Van Rossum *et al*, 2003). The ER22/23EK polymorphism of the GR gene has been located in the transactivation domain of the gene. A single nucleotide polymorphism in codons 22 and 23 alters the sequence at DNA level resulting in the protein glutamic acid – lysine to be translated instead of glutamic acid-

arginine. The N363S polymorphism has been reported in codon 363 of exon 2 resulting in an AAT→ AGT nucleotide change. The result of this substitution is a change of the amino acid serine to asparagine. The *Bcl* I polymorphism has a nucleotide alteration between a C and a G, is found 646 nucleotides downstream from exon 2 resulting in fragments of 2.2kb and 3.9kb. Most frequently occurring is the C allele and thus considered to be the wild-type allele (Manenschijn *et al*, 2009; Van Rossum *et al*, 2003). The 9β polymorphism has an A to G substitution which has been reported in an "ATTTA motif" in the 3'UTR of exon 9β. This substitution alters the stability of the mRNA, and subsequently translational events (Manenschijn *et al*, 2009). The table 1.2 summarizes the sensitivity of the carriers to GC's and the main phenotypes that accompany these polymorphisms.

Polymorphisms	**Sensitivity to GC's**	**Phenotypes**	**Substitution**
ThtIII 1	Not linked	Associated with depression / carriers at reduced risk of bipolar disorder, lower volume of hippocampus.	C → T
ER22/23EK	Resistance (minor)	Increased risk of depression, decreased risk of dementia, healthy metabolic profile, and balanced body composition.	Arg → Lys
N363S	Hypersensitivity	Association with increased BMI (controversy in field), elevated cholesterol levels.	Asn → Ser
Bcl I	Hypersensitivity	Deposition of fat in abdominal area, less lean mass, increased risk of depression.	C → G
9β	Not known?	Balanced body composition, increased risk of autoimmune diseases, increases risk of myocardial infarction.	A→G

Table 1.2: Association of all polymorphisms with sensitivity to GC's and phenotypes (Manenschijn *et al.,* 2009)**.**

1.12. Signalling of the mammalian Target of Rapamycin (mTOR)

mTOR is a highly conserved member of the phosphatidylinositol 3-kinase (PI3K)-related superfamily in which a lipid kinase homology sector functions as a 289kDa Ser/Thr kinase (Wen *et al*, 2005). mTOR consists of 2549 amino acids (Sabatini *et al*., 1994) which are organized in several structural domains as can be seen in Figure 1.11. The mTOR pathway is responsible for basic cellular functions such as cell metabolism and proliferation, growth as well as cell cycle progression, checks for DNA damage, and telomere length maintenance (Laplante & Sabatini, 2009; Petroulakis *et al*, 2006). This signal transduction cascade has also been under intense investigation for its participation in apoptosis and cell survival (Petroulakis *et al*, 2006), as it also contributes to pathologies including immunodeficiency and cancer (Bjornsti & Houghton, 2004).

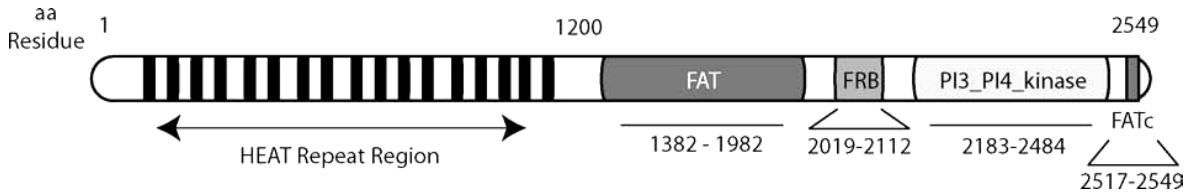


Figure 1.11: The basic structure of mTOR (Kristof, 2010)

The N- terminus of mTOR consists of 20 sequential HEAT [*H*untington, *E*longation factor 3 (eEF3), *A* subunit of type 2A protein phosphatase (PP2A) and *T*OR] repeated areas (Kristof, 2010; Petroulakis *et al*, 2006). These HEAT repeats are associated with protein-protein interactions and consist of two α helices, with both hydrophobic and hydrophilic residues. The C- terminus consists of a kinase domain with sequence similarity to that of the catalytic domain of PI3K. In addition there is an FKPB12-rapamycin binding (FRB) domain, a negative regulatory domain (NRD) and a relatively large FAT [(FRAP, Ataxia telangiectasia

mutated (ATM), Transformation/transcription domain associated protein (TRAP)] as well as a FAT c- terminal (FATc) domain (Jacinto & Lorberg, 2008; Bosotti *et al*, 2000). The two latter domains are essential for mTOR activity whereas a deletion of an amino acid can potentially diminish the activity of mTOR (Peterson *et al*, 2000; Takahaski *et al*, 2000). Additionally, the interaction of FAT and FATc domains are also thought to eventually expose the catalytic domain (Takahashi *et al*, 2000).

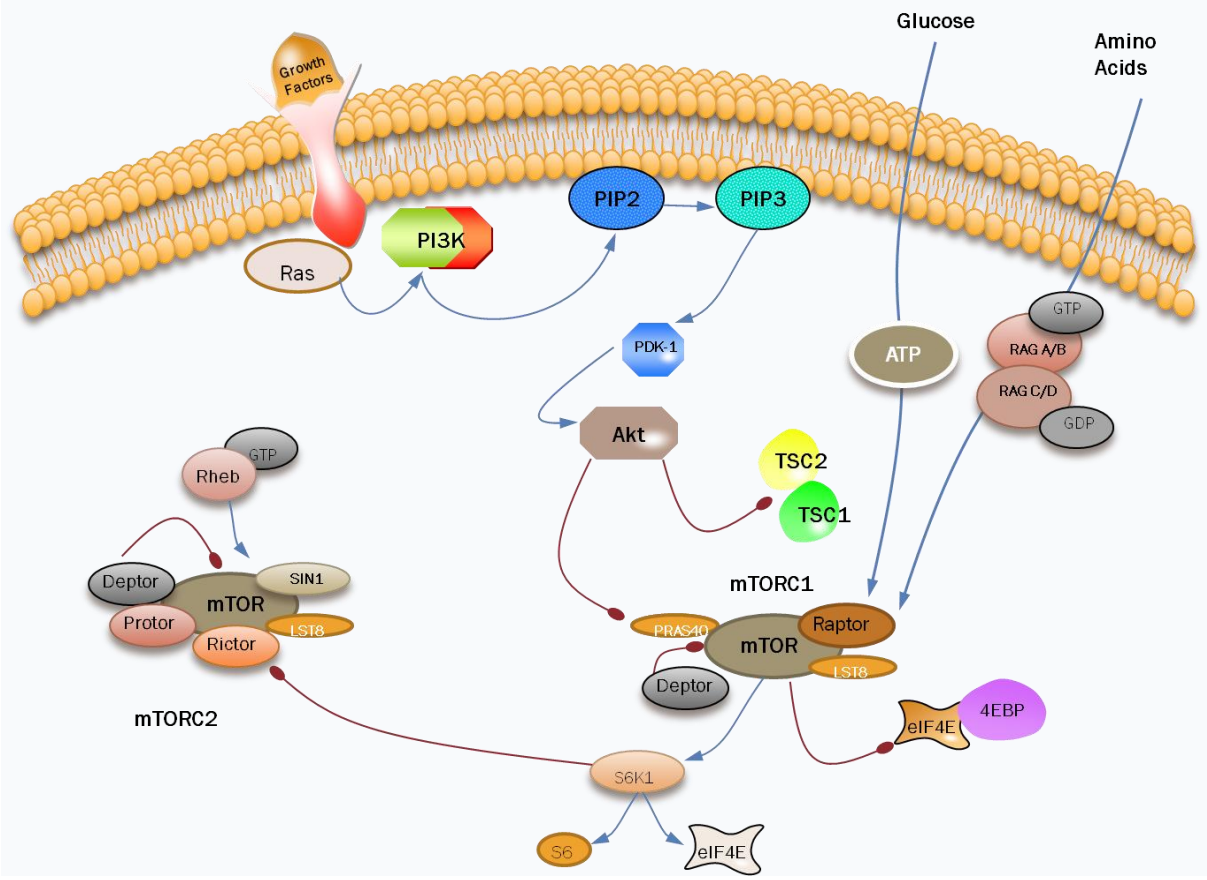


Figure 1.12: Summary of the mTOR signalling pathway (Adapted from Qiagen)

Growth factors acting as stimuli result in the PI3K phosphorylation of the lipid phosphatidylinositol-4, 5-biphosphate at the 3'-OH site producing phosphatidylinositol-3, 4,

5-triphosphate, which is the second lipid messenger as can be seen from Fig.1.12 (Petroulakis *et al*, 2006). The newly produced triphosphate recruits its downstream effector Akt, at the plasma membrane which becomes phosphorylated at two sites respectively, at Ser473 and Thr308 resulting in its activation. This leads to the phosphorylation of the product of the tuberous sclerosis complex- 2 gene (TSC-2) and inhibits the tuberous sclerosis complex (TSC) which comprises of the heterodimer TSC-1 and TSC-2 (Laplante & Sabatini, 2009; Petroulakis *et al*, 2006). The TSC-1 product which is known as hamartin and TSC-2 product which is known as tuberin, act as negative regulators of mTORC-1 which is prevented via phosphorylation (Gao *et al*, 2002). Phosphorylation of tuberin through Akt/PKB leads to its dissociation from hamartin and the subsequent loss of GTPase activating protein action (Tee *et al*, 2005). Tuberin and hamartin heterodimers function as GTPase activating proteins inactivating Ras homolog enriched in brain (Rheb), which has a key role in the activation of mTORC-1. Therefore, the regulation of mTOR as a growth factor, involves the activation of PI3 kinase signalling which results in the phosphorylation of tuberin (Laplante & Sabatini, 2009). The signalling pathway of mTOR with its associated components is illustrated in Fig.1.12 whereas the rest of the main molecules that are included in the signalling cascade will be further explained below.

In mammals there are two separate TOR complexes present: mTORC-1 and mTORC-2. The first complex consists of: mTOR which is the catalytic subunit of the complex; regulatory-associated protein of mTOR (Raptor); mammalian lethal with Sec13 protein 8 (mLST8); [also known as GβL (G-protein β-like)]; proline rich AKT substrate 40 kDa (PRAS40); and DEP-domain-containing mTOR-interacting protein (Deptor) (Peterson *et al*., 2009; Abraham, 2002). The precise function of most of the mTOR-interacting proteins participating in mTORC-1 is not fully elucidated. Recently it has been proposed that Raptor may affect the activity of mTORC-1 by regulating the assembly of the complex and also by recruiting

substrates for mTOR (Hara *et al*., 2002; Kim *et al*., 2002). The mLST8's role in mTORC-1 function remains elusive, as a deletion of this protein has not been shown to affect the activity of mTORC-1 *in vivo* (Guertin *et al*., 2006). PRAS40, as well as Deptor, has been characterized by several studies as distinct negative regulators of mTORC-1 (Peterson *et a*l., 2009; Sancak *et al*., 2007; Vander Haar *et al*., 2007). PRAS40 and Deptor are recruited to the complex when the activity of mTORC-1 is reduced, and promote the inhibition of mTORC-1. It has been suggested that PRAS40 regulates the kinase activity of mTORC-1 by functioning as a direct inhibitor of substrate binding (Wang *et al*., 2007). Upon activation, mTORC-1 directly phosphorylates PRAS40 and Deptor and as a result, their physical interaction with mTORC-1 is reduced and this activates mTORC-1 signalling (Peterson *et al*., 2009; Wang *et al*., 2007).

The mTORC-2 comprises of six different proteins, which some of them are common to mTORC-1: mTOR, rapamycin-insensitive companion of mTOR (Rictor), mammalian stress-activated protein kinase interacting protein (mSIN1), protein observed with Rictor-1 (Protor-1), mLST8, and Deptor. Evidence has shown that Rictor and mSIN1 function in order to stabilize each other, thus establishing the structural foundation of mTORC-2 (Frias *et al*., 2006; Jacinto *et al.*, 2006). Rictor has also been shown to interact with Protor-1, but the physiological function of this interaction is under investigation (Thedieck *et al*., 2007; Woo *et al*., 2007). mLST8 has been shown to be essential for the orderly functioning of mTORC-2, as deletion of this protein severely impairs the stability as well as the activity of the complex (Guertin *et al*., 2006). Deptor has a similar role as in mTORC-2 to its role in mTORC-1 and negatively regulates mTORC-2 activity (Peterson *et al*., 2009). So far, Deptor is the only endogenous inhibitor of mTORC-2 that has been characterised (Laplante & Sabatini, 2009). The use of the bacterial microlide rapamycin has significantly enhanced the knowledge of mTORC-1 function. When rapamycin enters the cell binds to FK506-binding

protein of 12 kDa (FKBP12) and then it interacts with the FKBP12-rapamycin binding domain (FRB) of mTOR, thereby inhibiting the functions mTORC-1 (Guertin & Sabatini, 2007). FKBP12-rapamycin does not physically interact with or inhibit mTORC-2 (Jacinto *et al*., 2004; Sarbassov *et al*., 2004). Following these observations, mTORC-1 and mTORC-2 have been characterized as rapamycin-sensitive and rapamycin-insensitive complexes respectively. However, Sabrassov and his colleagues have shown this may not be entirely accurate, as chronic treatment with rapamycin can, in some cases, inhibit the activity of mTORC-2 by blocking its assembly (Sarbassov *et al*., 2006).

1.13. Key components of mTOR Complexes

1.13.1. Raptor (Regulatory-associated protein of mTOR)

Raptor is an essential subunit of mTORC-1. Is a conserved 150-kDa protein able to bind the downstream effectors of mTOR, ribosomal protein S6 kinase 1(S6K1) and eukaryotic translation initiation factor 4E-binding protein 1 (4E-BP1). Raptor homologs contain a unique as well as conserved region in the amino- terminal followed by three HEAT repeats and seven WD-40 repeats in the C-terminal. The HEAT repeats present in the mTOR which are situated in the N-terminal are responsible for the efficient interaction with Raptor (Hara *et al*., 2002; Kim *et al*, 2002). Mutations that occur in the conserved N-terminal of Raptor or within the HEAT repeats interfere with the ability of Raptor to bind to mTOR suggesting the presence of multiple contact points between the two molecules (Kim *et al*, 2002). Knockdown experiments of Raptor in mammalian cells underpinned the importance of this gene for mTOR activity (Kim *et al*, 2002). Opposing the positive regulation of mTOR by Raptor, one report indicates that, upon nutrient deprivation the association of Raptor–mTOR becomes stabilized in a manner that inhibits the activity of mTOR kinase (Kim *et al*, 2002).

Raptor appears to be an adaptor protein, recruiting mTOR substrates by binding the downstream effectors of mTOR, S6K1 and 4E-BP1. Moreover, Raptor is necessary for the *in vitro* phosphorylation of 4E-BP1 by mTOR as well as for the efficient phosphorylation of S6K1 (Beugnet *et al*, 2003; Choi *et al*, 2003; Nojima *et al*, 2003; Schalm *et al*, 2003). The interaction of Raptor with S6K1 and 4E-BP1 is facilitated by a 5 amino acid motif termed TOR signaling (TOS) which is present in the N-terminus of both S6K1 and 4E-BP1 (Schalm & Blenis, 2002). Mutations that are present in the TOS motif distinctly inhibit mTOR-mediated phosphorylation of 4E-BP1 (Beugnet *et al*, 2003; Choi *et al*, 2003; Nojima *et al*, 2003; Schalm *et al*, 2003). It has been reported that rapamycin is able to disrupt the mTOR–Raptor interaction (Kim *et al*, 2002; Oshiro *et al*, 2004) thereby preventing the phosphorylation of S6K and 4E-BP by mTOR. Taken together, these data support a model whereby a change in the configuration of the mTOR-Raptor complex, which is sustained by nutrient conditions such as amino acid availability, affects the ability of mTOR to interact with and phosphorylate its substrates. In a state where amino acids are absent, the mTOR–mLST8–Raptor complex prevents mTOR from binding to its substrates and prevents the access of mTOR (or mTOR-associated kinases) to its substrates. On the other hand, in the presence of amino acids, a conformational change promotes the efficient interaction between Raptor and mTOR substrates and increased accessibility of the substrates to mTOR and its associated kinases (Oshiro *et al*., 2004; Kim *et al*., 2002).

1.13.2. Rictor (Rapamycin-insensitive companion of mTOR)

Rictor has a predicted molecular weight of 190 kDa and it was the first subunit identified that is unique to mTORC-2 (Jacinto *et al*., 2004; Sarbassov *et al*., 2004). It shares homology with AVO3 in yeast and is the defining member of the rapamycin-insensitive complex. The amino

terminal region has some domain structures that are relatively conserved among species, but the functions of these domains are still unknown. It has been suggested that these domains may mediate substrate binding and play an important role for the assembly of mTORC-2. Rapamycin does not block the interaction between Rictor and mTOR nor does the level of nutrients, where these are conditions known to regulate the activity of mTORC-1. Therefore, it is not a surprise that knocking down Rictor by RNA based interference (RNAi) in cultured cells does not change the phosphorylation status of S6K1 and 4EBP1. This therefore suggests that mTORC-2 has different physiological functions from mTORC-1. The physiological importance of Rictor is evident in knockout studies, where mice die due to defects in their vascular development (Guertin *et al*., 2006; Shiota *et al*., 2006; Yang *et al*., 2006).

1.13.3. Deptor (DEPDC6, DEP-containing protein 6)

Another key modulator of the mTOR pathway, termed Deptor has been recently described. Deptor binds to both mTORC-1 and mTORC-2 complexes and this was evident from co-immunoprecipitation experiments (Peterson *et al*, 2009). Its precise function is not fully elucidated, but Peterson *et al*. have shown in a series of elegant experiments that knocking down Deptor leads to activation of signalling through mTORC-1 and mTORC-2. This is demonstrated both by the observation that there is a change in the phosphorylation status of S6K1 and Akt/PKB when Deptor levels are decreased RNAi and by the increased *in vitro* activity against these substrates of mTOR complexes from cells with decreased levels of Deptor (Proud *et al*, 2009). Loss-of-function data presented by Peterson shows that Deptor is able to inhibit both mTOR complexes pathways (Peterson *et al*., 2009). Conversely, with mTORC-1 inhibition, the overexpression of Deptor eases mTORC-1 mediated inhibition of PI3K, thus causing the activation of PI3K and also the mTORC-2-dependent outputs, such as

Akt. Also when Deptor is greatly overexpressed following the inhibition of mTORC-1, and the unexpected activation of the PI3K/mTORC-2/Akt pathway it has been shown that this indirect mode of PI3K activation is important for the viability of a subset of myeloma cells which normally lack PI3K-activating mutations. Therefore Deptor functions as an endogenous inhibitor of mTOR where its deregulated overexpression is able to promote the survival of myeloma cancer cells (Peterson *et al*., 2009). Deptor interacts with mTOR via its PDZ domain, but so far there is no information regarding the function of the tandem DEP domains that the Deptor protein also contains. In other proteins though, the DEP domains mediate protein-protein interactions (Ballon *et al*, 2006; Yu *et al*, 2007). Based on the current evidence, Deptor protein shows preference for mTOR regulation and it has been proposed that in vertebrates it is likely to be involved in regulating other outputs of the mTOR signalling network besides the growth and survival pathways that have been already examined. The mTOR complexes and Deptor have the potential to negatively regulate each other, therefore suggesting the existence of a loop where loss of Deptor leads to an increase in the activity of mTOR activity, which in turn reduces further the expression of Deptor.

1.14. Mammalian Target of Rapamycin (mTOR) and the human placenta

A vital factor for foetal development is nutrient transport at the placental level. This is because any disturbances in the maternal compartments, for example due to maternal stress or nutritional status, which will affect foetal development, will involve the feto-placental barrier. Interestingly, mTOR is a highly conserved Serine/Threonine protein kinase that functions as an ATP and amino acid sensor to balance nutrient availability and cell growth (Dennis *et al*., 2001, Brown *et al*., 1994). Since foetal growth is critically dependent on placental nutrient transport, placental mTOR signalling plays an important role in the regulation of foetal growth (Roos *et al*., 2009). Recent data suggest that mTOR functions as an important placental growth signalling sensor, linking maternal nutrient and growth factor concentrations to amino acid transport (Roos *et al*., 2007; Wen *et al*., 2005). As mTOR acts as a nutrient sensor, several studies have investigated the role of mTOR in the placenta where it was shown to be critical for early growth and proliferation as a potential deletion of this gene has led to lethal phenotype (Gangloff *et al*., 2004; Murakami *et al*., 2004). Moreover, in placentas from intrauterine growth restriction (IUGR) pregnancies, the protein expression of placental phospho-S6K1 (Thr 389), a measure of the activity of the mTORC-1 complex, was significantly reduced (Roos *et al*., 2007). In a hyperthermia (HT)-induced stress model in sheep, a range of changes were apparent regarding the mTOR pathway: there was upregulation of the phosphorylation status of placental mTOR and Akt and decrease of P70S6K (Arroyo *et al*., 2009). Collectively these data indicate that there is an intact mTOR signalling pathway at placental level that can act as a molecular switch depending on nutrient availability and stress conditions (Arroyo *et al*., 2009).

1.15. Growth Arrest Specific 5 (GAS5)

1.15.1. A convergent of mTOR and GR signalling

The numbers of noncoding RNAs (ncRNAs) posing regulatory functions are rapidly increasing (Mattick, 2005). NcRNAs are shown to affect mRNA transcription, degradation, and translation thus influencing the abundance of proteins and also can influence the nuclear translocation of proteins thus influencing not only protein abundance but localization as well (Barrandon *et al.,* 2008; Mattick, 2005). The growth arrest specific-5 (GAS5), a single-stranded ncRNA is such a molecule, named so as it accumulates in growth-arrested cells (Schneider *et al*., 1988). The gene encoding for this ncRNA, GAS5 is a member of the 5′-terminal oligopyrimidine (5′TOP) class genes which are characterized by the presence of an oligopyrimidine upstream its tract sequence (Smith & Steitz, 1998; Coccia *et al*., 1992). This gene is composed of 12 exons, from which two alternatively spliced transcripts originate, that differ by the presence or absence of exon 7 (Coccia *et al*., 1992). GAS5's introns encode small nucleolar RNAs (snoRNAs) where its exons contain a small open reading frame that has been shown unable to encode a functional protein (Raho *et al*., 2000; Muller *et al*., 1998).

Cell growth arrest caused by serum starvation or by treating with protein translation inhibitors results to the translation of 5′TOP RNAs and to the inhibition of their degradation (Amaldi & Pierandrei-Amaldi, 1997), thus resulting in the accumulation of spliced GAS5 RNA in the cell (Smith & Steitz, 1998). The functions of GAS5 non coding cRNA are still unknown; though, the multiple small noncoding nucleolar RNAs (snoRNAs) that are expressed from its intronic sequences are involved in the biosynthesis of ribosomal RNA (Smith & Steitz, 1998). As previously mentioned inhibition of mTOR results in inhibition of protein synthesis, partly by reduced the phosphorylation of 4E-BP1 (Fingar *et al*., 2004; Brunn *et al*., 1997) and S6 kinase1 (Tee & Blenis, 2005; Fingar *et al*., 2004). Additionaly,

inhibition of mTOR results in the disproportionate reduction of the translation of several groups of RNAs, including the 5'TOP transcripts, as well as mRNAs that encode for several key regulators of the cell-cycle (Rosenwald *et al*., 1993). The 5' oligopyrimidine tract of the 5'TOP RNAs is mainly shown to be on mRNAs that encode for ribosomal and other proteins involved in translation, thus the inhibition of mTOR specifically inhibits the protein synthesis by directly blocking the production of the protein synthesis machinery *per se* (Meyuhas, 2000). Since GAS5 is a 5'TOP RNA its translation might be controlled by the mTOR pathway. Even though GAS5 does not appear to encode a functional protein (Raho *et al*., 2000; Muller *et al*., 1998) it may still have some functional effects by interactions with steroid receptors that inhibit the receptors action (Kino *et al*., 2010). The cellular behaviour is regulated among others by the availability of nutrients, which control the cell growth. The nutrient status dictates the activity as well as the abundance of several transcription factors, thus altering the transcriptional profile of certain set of genes, including those that encode for proteins involved in the energy metabolism and the stress responses at cell level as well as those that encode proteins that are involved in the immune responses (Lopez-Maury *et al*., 2008; Sellick & Reece, 2005). The glucocorticoids regulate the body's metabolic activities; modulate the body's immune function as well as influencing the cell survival by altering the cell's sensitivity to apoptosis in response to either external, internal stress or both (Chrousos & Kino, 2005; Frankfurt & Rosen, 2004; Chrousos, 2001; Wen *et al*., 1997). It has been shown that GAS5 non coding RNA interacts with the DBD of the ligand-activated glucocorticoid receptor (GR) through a decoy RNA "glucocorticoid response element" (GRE) and in this manner suppresses GR-induced transcriptional activity of endogenous glucocorticoid-responsive genes by inhibiting binding of GRs to target genes' GREs (Kino *et al*., 2010).

1.16. Aims and objectives

Our working hypothesis is that for a successful pregnancy certain events at nutritional, biochemical, genetic and molecular could be tightly linked. Despite a plethora of studies that identify potential risk factors for the mother as well as the foetus, there is no comprehensive analysis at all these levels taken from the same cohort of patients. Therefore, in this study we followed a "holistic" approach investigating how maternal stress, nutrition, placental mTOR and glucocorticoid receptor (GR) signalling can influence pregnancy outcome.

More specifically we have decided to study in a cohort of Mediterranean patients, their exercise, immune, nutritional and stress profiles before and during pregnancy in an attempt to assess how these might influence foetal outcome.

We have then decided to map key mTOR components (mTOR, Deptor, Rictor and Raptor) and all known GR splice variants (including GAS5) at placental level and assessed how maternal stress might affect this signalling cascade using clinical data and two distinct placental models (BeWo and JEG-3 cell lines) mimicking a stress milieu *in vitro*. In an attempt to dissect further a potential gene-environment interaction, we have assessed (in a matched cohort of mothers and babies) how 4 well-characterised polymorphisms of the GR gene might affect foetal and placental weight.

Finally, we have used the fusigenic BeWo cells to mimic fully syncytialised cells in an attempt to study how an inducer of syncytialisation (i.e. forskolin) may affect the expression of mTOR/GR signalling components.

CHAPTER 2

MATERIALS & METHODS

2.1. Solutions

2.1.1. General solutions and Buffers

- Elution buffer: Molecular grade H_2O.
- Wash buffer 1: 1 M Tris (pH 7).
- DNase I containing buffer: Amplification Grade DNase I - 10mM Tris-HCl (pH 7.5), 10mM CaCl2, 10mM MgCl2 and 10X Reaction Buffer- 200mM Tris-HCl (pH 8.3), 20mM MgCl2 per 1ml.
- Stop buffer: 50mM EDTA per 1ml.
- 1x TBE buffer: 90mM Tris, 90mM Boric acid, 2 mM EDTA.
- 10x loading buffer : 20% Ficoll 400 (Fisher Scientific, Cat.No 26873-85-8), 0.1 M Na_2EDTA (pH 8), 1% SDS, 0.25% Bromophenol blue, 0.25% xylene cyanol.
- 5X first-strand buffer : 250mM Tris-HCl (pH 8.3), 375mM KCl, 15mM $MgCl_2$.
- 2x SSC : 300mM Sodium Chloride, 30mM Sodium Citrate (pH 7).
- DEPC treated H_2O: 0.1% Diethylpyrocarbonate (DEPC) per 1000ml distilled water
- Hybridization Mix: 50% Formamide, 2x SSC, 200ng/µl sheared, denatured, salmon sperm DNA, 5x Denhardt, 50mM NaH_2PO_4/Na_2HPO_4 (pH 7), 1mM EDTA.

2.2. Cell culture

The cell lines (BeWo & JEG-3) were obtained from the European Collection of Cell Cultures (ECACC, Salisbury, UK), (Appendix I). BeWo cells were cultured using Ham F12 (Gibco) media containing 10% heat-inactivated fetal bovine serum (FBS) (Gibco), and 0.5% penicillin-streptomycin (Gibco). Following the supplier's instructions in order to prevent glucose exhaustion in the BeWo cell culture fresh medium was supplied at maximum every 48 hours. The medium used for culturing JEG-3 cells was Minimum Essential Medium (MEM) (Sigma Aldrich) supplemented with 10% heat-inactivated fetal bovine serum (FBS) (Gibco), and 0.5% penicillin-streptomycin (Gibco), 0.5% L-glutamine (Gibco), 0.5% sodium pyruvate (Gibco) and 1% (v/v) Non Essential Amino Acids (Gibco). JEG-3 cell cultures were supplied with fresh medium approximately every 72 hours. Both cell lines were maintained in 75cm^2 non treated culture flasks (Nunc) under standard tissue culture conditions of 5% CO_2 in air at 37˚C. When reaching a confluent level the cells were sub-cultured by splitting in a 1:3 ratio into 19ml per culture flask of specific media for each cell line. Firstly, the cells were rinsed with 2.5ml of 1x Phosphate-Buffered Saline (PBS) (Gibco). To detach the cells, 2.5ml of 0.25% Trypsin (Gibco) was used for a few minutes at 37˚C and the culture flask was gently tapped to ensure full detachment of the cells. The activity of trypsin was neutralised by the addition of tissue culture media. The cells were centrifuged for 5 minutes at 1200 rpm. The supernatant was removed and the cells were gently resuspended in fresh medium and added to new culture flasks to a total volume of 20ml and the flasks were returned to the incubator.

2.2.1. Syncytialisation

The syncytialisation process was performed using the cAMP inducer, Forskolin (Sigma, Cat. No. F3917). Forskolin was dissolved in Dimethyl Sulfoxide **(**DMSO) (Sigma, Cat. No.

D8418) at a stock concentration of 0.1M. The syncytialisation process was achieved by treating BeWo cells with 50μM and 100μM of forskolin over a period of 72 hours. Forskolin treatment was renewed every 24 hours. During the time-dependent treatments, the cells were maintained in 75cm^2 non treated culture flasks (Nunc) under standard culture conditions of 5% CO_2 at 37˚C.

2.3. Cell treatments

For treatment purposes BeWo and JEG-3 cells were grown in 6-well multi dishes and were maintained in cell culture as previously described. Once a satisfactory confluence (~70%) level was achieved the media was aseptically removed and the cells were rinsed with 1x PBS. A known volume of phenol red free media containing only 100μg/ml of Penicillin and 100μg/ml of Streptomycin (Gibco) was added to the cells 24 hours before start of the treatments. BeWo and JEG-3 cells were treated with three different concentrations (10nM, 100nM and 1000nM) of cortisol (Sigma, Cat. No. H0888) for 24 hours.

2.4. Placental Tissue Collection

Tissue from human placentae were obtained from women at term (>37 weeks) and pre-term (<37 weeks) undergoing either normal vaginal delivery or elective caesarean section. All samples were collected from pregnancies without any known pathological problems. Immediately after delivery, the maternal and foetal surfaces were dissected off and the foetal membranes were peeled gently away from the placenta. Placental tissues were obtained from the maternal side of the placentas at maximum of 30 minutes after delivery. The tissue samples taken were approximately 0.2-0.5cm^3 in size and were taken from the centre of the

cotyledons evenly across the placenta. The tissues were dissected to remove any visible connective tissue and calcium deposits. Depending on the experimental analysis, some of the tissues were transferred to 10 volumes of RNAlater solution (Invitrogen). Placental tissue sections were also embedded in paraffin by the histology services of the University Hospital of Crete were. Ethical approval was obtained from the local ethical committees and also informed consent was obtained from all the patients.

2.5. RNA Extraction

GenElute^{TM} Mammalian Total RNA miniprep kit (Sigma-Aldrich, Cat. No. RTN70) was used to extract total RNA from mammalian cells and tissues. The procedures are explained below:

2.5.1. RNA Extraction from cells

Before adding the Lysis Solution/2-ME (2-mercaptoethanol) mixture to the 6-well plates or T-75 flasks, the lysis solution was prepared accordingly; for each 1ml of lysis solution 10μl of 2-ME were added. Since BeWo and JEG-3 are adherent cells, they could be lysed directly in the culture vessel. The medium was removed and the cells were washed once with PBS prior to adding 1ml of Lysis/2-ME solution in every 75cm^2 culture flask or 500μl in each well of a 6-well multidish. The vessels were then rocked and tapped for a few seconds to completely cover the cells. The culture vessels were allowed to sit for 1 to 2 minutes with the Lysis Solution/2-ME mixture covering the cells. The rocking and tapping was repeated and the culture vessels were tilted to collect the lysates. The lysed cells were then pipetted into a filtration column and centrifuged at 16.000 rpm for 2 minutes. This step removes cellular debris and shears DNA. Equal volume (500μl) of 70% ehtnanol solution was added to each column containing the filtered lysate. The mixture was vortexed to ensure a thorough mix.

Since the total volume of the lysate/ethanol mixture per tube was 1ml, the RNA was bound to the column in two steps. 700µl of the lysate/ethanol mixture was pipetted into a binding column and centrifuged at 16.000 rpm for 15 seconds. The flow-through liquid was discarded and the remaining lysate/ethanol mixture was added to the column and centrifuged at 16.000 rpm for 15 seconds. 500µl of Wash Solution 1 were pipetted into the columns and centrifuged at 16.000 rpm for 15 seconds. The binding columns were transferred into a fresh collection tub. The flow-through liquid was discarded. 500µl of the ethanol containing Wash Solution 2 was added into each column and centrifuged at 16.000 rpm for 15 seconds. The flow-through liquid was again discarded but the collection tube was retained. A second 500µl volume of the ethanol containing Wash Solution 2 was added into each column and centrifuged at 16.000 rpm for 2 minutes to dry the binding columns. Since the binding column must be free of ethanol before eluting the RNA, the columns were centrifuged for an additional 1 minute at 16.000 rpm. After the additional drying step, the binding columns were transferred to fresh collection tubes and 40µl of the elution solution were added into each binding column and centrifuged at 16.000 rpm for 1 minute. The purified RNA was ready to be stored at -80°C until use. Prior to transferring the RNA samples to -80°C, the samples were DNase treated to ensure removal of any DNA from the RNA preparations. Deoxyribonuclease I Amplification Grade (AMP-D1, Sigma, USA) was used by adding 4µl of 10x Reaction Buffer and 4µl of Amplification Grade DNase I per sample tube and incubated at room temperature for 15 minutes. DNase was inactivated by adding 4µl of Stop solution to each tube and heating at 70˚C for 10 minutes using a pre-warmed water bath. RNA samples were then transferred to -80˚C where they were stored until further usage.

2.5.2. RNA Extraction from placental tissue

After collecting our placental tissue samples as previously described, TissueLyser II (Qiagen) was used to homogenise the tissue before extracting RNA form our samples. The frozen

samples were left to thaw on ice. Once thawed, the placental tissues were transferred into sterile 2ml eppendorf tubes containing the lysis mixture. A sterile stainless steel bead was added to each tube to facilitate cell disruption as samples were vigorously vortexed for 2 minutes. Proteinase K (Sigma-Aldrich, Cat. No. P4850) was added directly to each sample after homogenization to ensure efficient cell disruption and incubated at 55°C for 10 minutes. Next, the lysate was filtered and the same steps as above (section 2.3.1) were followed. The quantity and quality of the extracted RNA was measured by analyzing 1µl of RNA sample on a Nanodrop-1000 Spectrophotometer (Fisher Scientific). As a blank reading, 1µl of elution solution was used. Further, to ensure no RNA degradation, 2µl of 10x loading buffer [(20% Ficoll 400/ FisherScientific, Cat.No 26873-85-8) – (0.1 M Na2EDTA pH.8, 1% SDS, 0.25% Bromophenol blue, 0.25% xylene cyanol / Sigma, Cat.No 220-167-5)] were added to 5µl of RNA sample and each sample was electrophoresed on a 1% agarose gel (see below section 2.11 for explanation of the technique) at 80V for 45 minutes and subsequently visuallised under UV . Purified RNA was stored at -80 ºC until further use.

2.6. Complimentary DNA (cDNA) synthesis

Prior to constructing the complimentary DNA the quantity and quality of the previously extracted RNA was measured. This was performed by analyzing 1µl of RNA sample on a Nanodrop-1000 Spectrophotometer (Fisher Scientific). As a blank reading, 1µl of elution solution was used. The concentration of RNA used for cDNA synthesis was standardized at 500ng/µl for each sample and the corresponding volume of molecular grade H_2O was added to make it up to 10µl. In a nuclease-free microcentrifuge tube 1µl of random primers, 1µl of dNTP Mix (10mM each) and 5µl of molecular grade H_2O were added and mixed thoroughly. In a clearly labelled nuclease-free microcentrifuge tubes the corresponding volumes of

molecular grade H_2O and RNA were added for each sample. 7µl of the previously prepared mixture were added in each tube and heated using a heat block at 65°C for 5 minutes. The samples were briefly centrifuged and 4µl of 5x First-strand Buffer (250mM Tris-HCL, pH 8.3 at room temperature; 375mM KCL; 15mM MgCl2), and 2µl of 0.1M DTT, 1µl of molecular grade H_2O were added in each sample tube. The contents were gently mixed and incubated for 2 minutes at 25°C. 1µl of SuperScript™ II RT (Invitrogen) was added to each tube and mixed up and down using a pipette. The samples were then incubated for 50 minutes at 42°C and then at 70°C for a further 15 minutes to inactivate the reaction. The newly synthesised cDNA was used as a template for the polymerase chain reaction (PCR).

2.7. Primer design

The primers design began at: www.ncbi.nlm.nih.gov where gene sequences were searched for the genes of interest (listed below) sequences by reference number available from publishing. This target sequence was then inserted into Invitrogens own primer design tool, OligoPerfect^{TM} Designer at: www.invitrogen.com/content.cfm?pageid=9716 was used for the primer design, synthesizing a primer set of sense and antisense primer . The maximum primer size required was 25bp, the minimum product should be 200-500bp, and as extremes were preferably avoided, the percentage of GC (around 50%) content and temperature of the primer (around 60 ^{o}C) was required to be the same for each one in the pair to prevented problems with annealing during PCR. Preparation of the primers to be used from the main stock (1µg/µl) was performed prior to commencing the RT-PCR. A 1:10 dilution of each primer was carried out by adding 10µl of the primer stock solution and 90µl of molecular grade H_2O in 1.5ml eppendorf tubes yielding a 100ng/µl working concentration per primer. The primers used in this study are listed in Table 2.1 below:

Primer	**Sequence**		**Size (bp)**
mTOR	sense	5’- TGCCAACTATCTTCGGAACC –3’	135
	anti-sense	5’- GCTCGCTTCACCTCAAATTC–3’	
Deptor	sense	5’-CACCATGTGTGTGATGAGCA-3’	202
	anti-sense	5’-TGAAGGTGCGCTCATACTTG-3’	
Rictor	sense	5’-GGAAGCCTGTTGATGGTGAT-3’	117
	anti-sense	5’-GGCAGCCTGTTTTATGGTGt-3’	
Raptor	sense	5’-ACTGATGGAGTCCGAAATGC-3’	170
	anti-sense	5’-TCATCCGATCCTTCATCCTC-3’	
GRα	sense	5’- CTATGCATGAAGTGGTTGAAAA-3’	96
	anti-sense	5’-TTTCAGCTAACATCTCGGG-3’	
GRβ	sense	5’-GAAGGAAACTCCAGCCAGAA-3’	81
	anti-sense	5’-CCACATAACATTTTCATGCATAGA-3’	
GRγ	sense	5’-TTCAAAAGAGCAGTGGAAGGTA-3’	264
	anti-sense	5’-GGTAGGGGTGAGTTGTGGTAACG-3’	
GR-P	sense	5’-GCTGTGTTTTGCTCCTGATCTGA-3’	194
	anti-sense	5’-TGACATAAGGTGAAAAGGTGTTCTACC-3’	
GAS5	sense	5’-CAGTGTGGCTCTGGATAGCA-3’	168
	anti-sense	5’-TTAAGCTGGTCCAGGCAAGT-3’	
PCNA	sense	5’-GCCGAGATCTCAGCCATATT-3’	452
	anti-sense	5’-ATGTACTTAGAGGTACAAAT-3’	
β-Actin	sense	5’- AAGAGAGGCATCCTCACCCT –3’	216
	anti-sense	5’- TACATGGCTGGGGTGTTGAA –3’	

Table 2.1: Primer Sequences with adjacent base pair (bp)

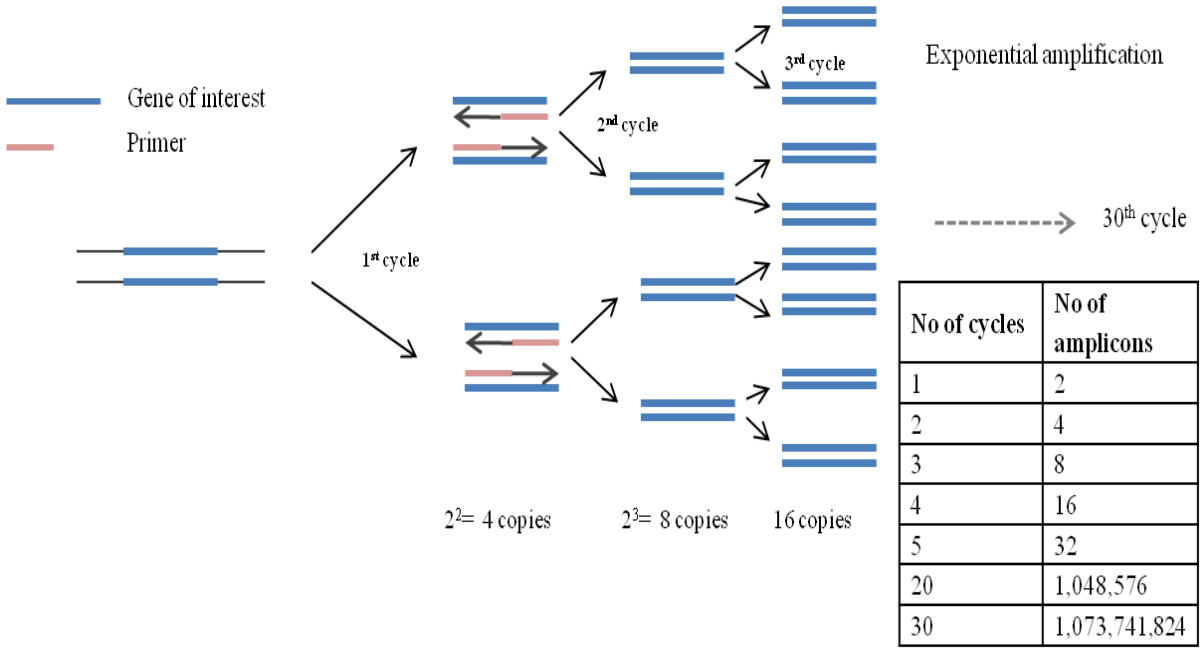
2.8. Reverse – Trancriptase Polymerase Chain Reaction (RT-PCR)

Polymerase chain reaction (PCR) is a technique by which the amplification of a specific DNA sequence is achieved where the ends of the sequence are known. The primers, which are designed to complement the DNA region of interest, bind to the DNA and the enzyme DNA polymerase extends them in the presence of dNTPs. Under optimised conditions the reaction synthesises a new complementary DNA strand. By the end of the number of cycles performed, the quantity of new DNA synthesised increases exponentially (Fig 2.1). The following table (Table 2.2.) shows the reagents along with the volumes and the concentrations used for each sample.

Reagent	Concentration	Quantity
10 X PCR Buffer	200mM Tris-HCL [pH 8.4], 500mM KCL	5µl
dNTP Mix	10mM	1µl
MgCl2	50mM	1.5µl
Forward Primer	10µM	1µl
Reverse Primer	10µM	1µl
cDNA		1µl
Taq DNA polymerase	5 U/µl	0.5µl
Sterile, distilled water		39µl

Table 2.2: Quantities and concentrations required of the reagents used for each gene for PCR**.**

In each clearly labelled tube, the corresponding volume of each reagent was added. 49µl of the master mix were pipetted into 100µl eppendorf tubes; this step was repeated for each master mix. One µl of either the cDNA of interest or distilled water (used as a negative control) was added to make the final volume up 50µl in each 100µl tube. The samples were then placed into the thermal cycler (GeneAmp PCR system 2400, Perkin Elmer). The components were mixed thoroughly before starting the PCR amplification procedure. The samples were initially denatured at 94ºC for 4 minutes followed by 30 cycles at 94ºC for 30 seconds, primer specific annealing temperature (58˚C - 60˚C) for 30 seconds and 72ºC for 1 minute, the final cycle was followed by 72ºC extension for 1 minute.



No of cycles	No of amplicons
1	2
2	4
3	8
4	16
5	32
20	1,048,576
30	1,073,741,824

Figure 2.1: Overview of gene amplification using PCR technology. Insert table depicts the number of specific amplicons generated over 30 cycles of amplification

2.9. Quantitative-PCR (Q-PCR)

Expression of the genes of interest were assessed by quantitative PCR (Q-PCR) on an ABI 7400 instrument (Applied Biosystems). The 7900HT instrument features a Peltier-based, interchangeable sample block module based on the technology established in the GeneAmp® PCR System 9700 thermal cycler. The sample block module houses an internal Peltier heating/cooling unit. The sample block module is made of aluminum to provide an optimal thermal transfer rate between the block and the reaction plate. SYBR® Green-PCR reaction mixture (Sigma-Aldrich, UK) was used following the manufacturer's instructions and the primers described in Table 2.1. A final reaction volume of 25µl included 2x JumpStartTaq ReadyMix that contained 1.25 units *Taq* DNA polymerase, 10mM Tris-HCl, 50mM KCl, 3.5mM $MgCl_2$, 0.2mM dNTP, and stabilizers were used along with 1µl of each specific forward and reverse primers (0.1µg/µl; Table 2.1), 1x reference dye and cDNA. The reaction mixture was thoroughly mixed and 24µl were aliquoted in each of the 96 wells of the plate (MicroAmp™ Fast Optical 96-Wells Reaction Plate, Applied Biosystems, Cat.No. 4314320). 1µl of cDNA was added and the plate was sealed using an optical adhesive film (MicroAmp® Optical Adhesive Film, Applied Biosystems, Cat.No. 4314320). The plate was centrifuged for 1 minute at 1000 rpm to collect the reactions to the bottom of the wells and then loaded onto the Sequence Detection System. All reactions were performed in triplicates and control reactions (i.e. no cDNA input) were also added in each 96-well plate. The conditions used for Q-PCR reaction were the following:

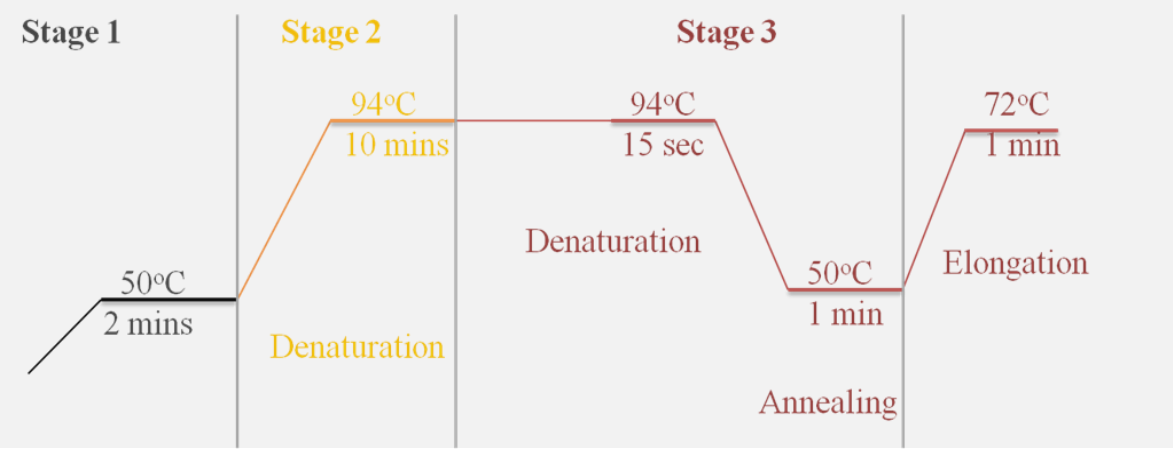


Figure 2.2: Real time PCR cycle conditions.

The DNA levels were expressed as a "relative quantification" (RQ) value, using the "Delta Ct method" for comparing relative expression results between treatments in Q-PCR. The following equations (*User Bulletin #2*, ABI PRISM 7700 Sequence Detection System) were used to analyse the results obtained from the Q-PCR:

For cell samples: $\Delta Ct = Ct_{\text{(gene of interest)}} - Ct_{\text{(housekeeping gene)}}$

$\Delta\Delta Ct = \Delta Ct_{\text{(samples)}} - \Delta Ct_{\text{(calibrator)}}$

Relative Quantity (RQ) = $2^{-\Delta\Delta Ct} \pm (2^{-\Delta\Delta SD_{(+)}} - 2^{-\Delta\Delta SD_{(-)}})$

For placental samples: $\Delta Ct = Ct_{\text{(gene of interest)}} - Ct_{\text{(housekeeping gene)}}$

Arbitrary Value : $2^{-\Delta Ct}$

2.10. Genetic Studies

Our genetic studies were performed on a cohort of matched mothers (No. 81) and infants (No. 87) of which 6 were twins, from whom blood and serum samples were obtained.

Medical records of the mothers were evaluated and the subjects had no known pathologies or receiving any medications. The subjects were genotyped for the *Bcl* I, *N363S*, *ER22/EK23* and *Tth111* 1 polymorphisms using the Restriction Fragment Length Polymorphism (RFLP) method.

2.10.1. *Bcl* I Polymorphism

Genomic DNA was used to amplify the *Bcl* I RFLP C to G mutation which is 646 bp downstream of exon 2 by designing specific intron 2 primers. Phusion® Blood Direct PCR Kit (New England Biolabs) was used according to the manufacturer's instructions. The following primers were used: 5'-AAGCTTAACAATGGCCAT-3' and 5'-TGCTGCCTTATTTGTAAATTCGT-3'. The PCR conditions used where as following: forty cycles were performed consisting of an initial denaturing step at 98°C for 5 sec, annealing at 50°C for 5 sec and elongation at 72°C for 15 sec. To confirm the presence of the *Bcl* I polymorphism, 17µl of the PCR product were digested with 2µl of 10x Restriction Endonuclease Buffer and 15 U of the restriction enzyme *Bcl* I (New England Biolabs, UK, Cat. No. R0160S) for 1.5 hours at 50°C. The resulted digested fragments were separated on a 2% agarose gel to determine the genotypes. From the digestion of the PCR products, the following fragment sizes were obtained: 117 and 222 bp in the case of CC homozygotes, an additional band of 335 bp for CG heterozygous individuals, and a single band of 335 bp for GG larger allele homozygotes.

2.10.2. *N363S* Polymorphism

The genotyping was performed on genomic DNA using Phusion® Blood Direct PCR Kit (New England Biolabs) according to the manufacturer's instructions along with specific PCR primers, 5'-AGTACCTCTGGAGGACAGAT-3' and 5'-GTCCATTCTTAAGAAACAGG-3' The samples were amplified for an initial 5 minutes at 94°C followed by 35 cycles of 94°C

for 30 sec, annealing at 50°C for 30 sec and elongation at 72°C for 30 sec. The restriction enzyme *Tsp509I* (10 U) and 2μl of buffer (New England Biolabs, UK, Cat. No. R0576S) were used to digest the PCR products (17μl) at 65°C for 4 hours. The resulted digested fragments were separated on a 2.5% agarose gel to determine the genotypes. From the digestion of the PCR products, the following fragment sizes were obtained: 113 bp, 95 bp and 9 bp in the case of the wild type and 122 bp and 95 bp in the case of homozygotes mutants.

2.10.3. *ER22/23EK* Polymorphism

The subjects were genotyped for the *ER22/23EK* polymorphism by PCR with restriction fragment length polymorphism (RFLP) analysis. Genomic DNA was used to amplify with the use of Phusion® Blood Direct PCR Kit (New England Biolabs) the region of the polymorphism by using specific PCR primers : 5'- GATTCGGAGTTAACTAAAAG-3' and 5'-CTACCCTTTACTGGACCCTA-3'. The samples were amplified for an initial 5 minutes at 95°C followed by 35 cycles of 95°C for 30 sec, annealing at 58°C for 30 sec and elongation at 72°C for 30 sec 16.8μl of the PCR product were incubated with 2μl of 1x NEBuffer 4 and 0.2μl of 100x BSA and 5U of the restriction endonuclease *MnI I* (New England Biolabs, UK, Cat. No. R0163S) was used to digest the PCR products at 37°C for 4 hours. The resulted digested fragments were separated on a 2,5% agarose gel to determine the genotypes. From the digestion of the PCR products, the following fragment sizes were obtained: 163 and 149 bp in the case of the wild type, and 163 bp and 184 bp for the heterozygotes.

2.10.4. Tth*III* 1 Polymorphism

The last polymorphism which the subjects were genotyped for was the *TthIII* 1 polymorphism. PCR with restriction fragment length polymorphism (RFLP) analysis was performed where genomic DNA was used as the template to amplify with the use of

Phusion® Blood Direct PCR Kit (New England Biolabs, UK) the region of the polymorphism by using specific PCR primers: 5'-GGCCACAACAATAACCCAGT-3' and 5'-CCTATGACACGTATTTTGTGAAAGT-3'. The samples were amplified for an initial 5 minutes at 95°C followed by 35 cycles of 95°C for 30 sec, annealing at 61°C for 30 sec and elongation at 72°C for 30 sec. The restriction endonuclease *TthIII 1* (4 U) (New England Biolabs, UK, Cat. No. R0185S) was used to digest the PCR products at 65°C for 4 hours. The resulted digested fragments were separated on a 2.5% agarose gel to determine the genotypes. From the digestion of the PCR products, the following fragment sizes were obtained: 167 (C allele) the T allele consisted of 337 bp and the CT allele of 337 bp and 167 bp.

2.11. Agarose gel electrophoresis

The gels were prepared by dissolving 2g of agarose (Fisher, Cat.No. BP1356) in 100ml of 1 x TBE buffer in an autoclaved conical flask and boiled until clear. Once agarose had cooled, 5µl of ethidium bromide (Sigma, Cat. No. E1510) were added in order to stain the DNA. Swirled gently ensuring that the ethidium bromide has dissolved then the gel was allowed to set for approximately 20 minutes. In each PCR product, 5µl of 10x loading dye were added and vortexed well. 20µl of the PCR products were loaded per well. 1Kb DNA ladder (Invitrogen, Cat.No. 10787-018) or low molecular weight DNA Ladder (New England Biolabs, Cat. No. N3233S) was loaded once all of the samples had been loaded into the gel. Then, the gel was electrophoresed for a suitable length of time at 80 volts. To visualise the DNA, the gels were placed on a short wave, ultra-violet transilluminator, and photographed with an Alpha Imager 2200 (Alpha Innotech MultiImage™ Light Cabinet).

2.12. Indirect Immunofluorescene Analysis

Immunofluorescence technique allows the visualisation of the antigens of interest by using specific antibodies that are tagged to a fluoresent dye. The distribution of the antigen is then visualised with the use of a fluorescent microscope. In our study, indirect immunofluorescence was used to determine the presence/distribution of mTOR, Deptor, Rictor, Raptor, and GRα in placental cell lines (JEG-3 and BeWo) as well as in placental tissues.

2.12.1. Indirect Immunofluorescene on cells

The cells (BeWo and JEG-3) were grown on sterile 0.13mm - 0.17mm glass coverslips in 6-well multidishes to a sufficient confluence. Cells were firstly rinsed with 1x PBS three times before fixing by using 4% Paraformalhehyde (pfa) and by incubating for 10 minutes at room temperature. After the 10 minute incubation three washes with 1x PBS followed. The cells were then permeabilised using 0.2% PBS Tween 20 solution, incubated at room temperature for 20 minutes and followed by three washes in 1x PBS solution containing 100mM Glycine. Following, cells were incubated with 10% specific species serum (Jackson ImmunoResearch) in 1x PBS for 1 hour at room temperature in order to minimize any non-specific binding of IgG. The blocking serum used for each primary antibody was chosen based on the species in which the secondary antibody was raised in. Antibodies were diluted in 1.5% of specific species serum dilute in 1x PBS. Primary antibodies used in this study are listed in Table 2.3 along with their dilutions. The coverslips with the primary antibody were incubated at 4°C overnight. Next, the coverslips were washed three times with 1x PBS and incubated with a respective secondary antibody (Table 2.3) for 1 hour at room temperature. Cells were then washed with 1x PBS and rinsed in distilled water before mounting in Vectashield® Mounting Medium containing 4',6-diamidino-2-phenylindole **(**DAPI). Negative controls were prepared by omitting the primary antibody. The slides were viewed and images were captured using a

Plan Apo Neofluor 63X NA 1.25 oil objective (Zeiss) on a Zeiss Axiovert 200M microscope and AxioVision software was used to view the images. The data was derived after examining three independent coverslips for each sample along with the negative control.

Primary Antibodies			Secondary Antibodies	
Antibody	**Dilution**	**Species**	**Antibody**	**Dilution**
mTOR	1:50	Rabbit	Donkey anti-rabbit	1:400
Deptor	1:50	Rabbit	Donkey anti-rabbit	1:250
Rictor	1:50	Rabbit	Donkey anti-rabbit	1:400
Raptor	1:50	Rabbit	Donkey anti-rabbit	1:75
GRα	1:50	Rabbit	Donkey anti-rabbit	1:400

Table 2.3: List of primary and secondary antibodies used with their respective dilutions.

2.12.2. Indirect Immunofluorescence on paraffin-embedded placental tissues.

The paraffin embedded placental tissue samples that were used were heated in an oven at 65^{o}C for 1 hour. Then the slides were dewaxed using Histo-Clear (Fisher Scientific, Cat.No. HIS-010-010S) a non-hazardous substitute of xylene. The sections were then dehydrated in 100% ethanol, twice for 5 minutes each time. The sections were rehydrated through a graded series of ethanol in dH_2O over 20 minutes (95%, 70%, 50%, and 30%). The slides were rinsed in dH_2O once, and then in 1x TBS tween 20. The endogenous peroxidise activity was blocked by incubating the sections with 3% hydrogen peroxide for 10 minutes at room

temperature. After these steps the previously stated methodology in section 2.12.1 was followed starting from the blocking step and onwards. The primary and secondary antibodies used are listed in table 2.3. The slides were viewed and imaged using a Plan Apo Neofluor 63X NA 1.25 oil objective (Zeiss) on a Zeiss Axiovert 200M microscope and viewed using AxioVision software. The data was derived after examining three independent coverslips for each sample along with the negative control.

2.13. Construction of Questionnaire

A questionnaire was constructed (Appendix II), comprising of 59 questions which were related to the following specific subject areas and variables: a) BMI, b) immune problems, c) exercise, d) nutrition, e) stress, and f) medical history. Specific questions were used to acquire anthropometrical data which included gestational age, weight of the mother before conception as well as the weight of the mother at the time and self-reported height. The sources from where the questions were adapted are included in (Appendix III). Behavioural data that was collected included smoking, drinking habits, physical activity and was based on the Paffenbarger *et al*. (1978) measure, also multi-mineral/multivitamin supplement usage before and during pregnancy was assessed.

There were specific questions related to the stress profile, which included: whether the pregnancy was planned or not planned; how stressed was each woman during the pregnancy, with responses ranging from 1 to 4 (1= low, 2= medium, 3= high, 4= very high). This stress questionnaire was based on a study by Wang *et al*. (2004) where women with dysmenorrhoea were asked to describe their stress in preceding cycles as ‘low’, ‘medium’ or ‘high’.

2.14. RNA Fluorescent in situ hybridization (FISH)

The slides of the paraffin embedded placental tissue samples were placed in a coplin jar and deparaffinised for 30 minutes at 37^{o}C using histo-clear (Fisher Scientific, Cat.No. HIS-010-010S). Following, the tissue sections were rehydrated by placing the slides in ethanol solution of different concentrations (100%, 90%, 80%, 70%, 50%, 30%) for 3 minutes. A brief wash in 1x PBS solution was performed after this. Pepsin (0.01% in 0.01M HCl) was used to treat the tissue for 5 minutes at 37^{o}C, and a brief rinse in DEPC treated H_2O followed. Then the tissue samples were dehydrated by placing them in ethanol solutions (70%, 90%, 100%) for 3 minutes. A specific Alexa 488-conjugated GAS5 hybridization probe (GTGCTATCCAGAGCCACACTGCATCTGCACCCAGCACCATACCTCACAG) was utilised as previously described (Kino *et al*., 2010) and followed by an overnight incubation at 37^{o}C in a humidified chamber. After this incubation, three 10 minute washes were followed in 2xSSC at 37^{o}C. The slides were then briefly rinsed in DEPC treated H_2O and mounted in Vectashield® Mounting Medium containing DAPI prior to examining the emitted fluorescent signal under a Zeiss axiovert 200 M microscope viewed using AxioVision software.

2.15. Enzyme-linked immunosorbent assay (ELISA)

A cortisol enzyme immunoassay kit (Enzo Life Sciences, Cat.No. 900-071) was used for the quantitative determination of plasma cortisol levels collected from the patients participating in the study. Maternal nonfasting blood samples were collected into EDTA tubes between 09.00 and 10.00 hr and centrifuged immediately at 2500 rpm for 10 min. The resulted plasma was separated and snap frozen in dry ice and stored at −20^{o}C until further use. The maternal bloods were collected on the same day that the questionnaire has been administered. Foetal

blood samples were obtained from the umbilical artery at the time of delivery. Identical preparations to maternal samples took place regarding plasma isolation. The assay's sensitivity is 56.72 pg/ml with a range between 156-10.000 pg/ml (Appendix IV). The inter-assay coefficient of variation for cortisol was 7.8% at 969 pg/ml and the intra-assay coefficient of variation for cortisol was 6.6 % at 1.088 pg/ml. These results were expressed as *% B/B0* i.e. (B/Bo)* 'x'100 where B equals A405nm at 'x' pg/ml standard concentration and B0 equals A405nm at 0pg/ml standard concentration. The principle of this assay lies in the use of a monoclonal antibody directed to cortisol that binds to cortisol present in samples. Following incubation, a substrate is then added which produces a colorimetric reaction resulting in a yellow colour being generated that is read on a microplate reader.Prior to commencing the reagents were brought to room temperature for at least 30 minutes. Following the manufacturer's instructions all standards and samples were run in duplicate. 100µl of standard diluents (assay buffer) were pipetted into the non-specific binding (NSB) and the maximum binding (Bo) wells. In the appropriate wells 100µl of the standards were added and 100µl of the samples was added. Into the NSB wells 50µl of the assay buffer was added. 50µl of the conjugate were added in each well except the wells indicated to measure total activity and the blank wells. 50µl of antibody were then added to the wells except the total activity, the blank wells and the NSB wells. The plate was incubated at room temperature on a plate shaker for 2 hours at 500 rpm. The wells were emptied from their contents and washed by adding 400µl of wash solution in every well. The washes were repeated twice so that a total of three washes were performed. After the final wash, the content of the wells was aspirated, and the plate was firmly tapped on a lint free paper towel thus removing any remaining wash buffer. Following, 5µl of conjugated was added to the total activity wells. 200µl of the p-nitrophenyl phosphate (pNpp) substrate in buffer was added to each well and incubated at room temperature for 1hour without any shaking.

Following, 50µl of stop solution was added to every well, in order to stop the reaction and the plate was immediately read. The plate reader was blanked against the blank wells and the optical density was read at 405 nm.

2.16. Statistical Analysis

Data were expressed as mean± SEM. For the quantitative PCR, the following equations were used: ΔCt= Ct (gene of interest)-Ct (house keeping gene), ΔΔCt= ΔCt (sample) - ΔCt (calibrator), Relative Quantity (RQ) $=2^{-\Delta\Delta Ct}$. RQ value was set up as 1 for the untreated (no supplement) BeWo and JEG-3 cells. We have also calculated the gene expression levels as RQ values, using the untreated control for each cell line as a calibrator. We did so, since RQ values provide a more accurate comparison between the initial amounts of template in each sample, without requiring an exact copy number for analysis. Statistical analysis of the ΔCt and RQ value was performed using student‘s t-test. For the correlation studies, a two-tailed test using SPSS (Version 18) was used. A *P* value of ≤ 0.05 was regarded as significant. The ΔCt values from clinical samples were log transformed in order to acquire a normal distribution.

CHAPTER 3

QUESTIONNAIRE DATA: ANALYSIS OF DATA OF THE STUDY

3.1. Introduction

A considerable part of this research is dependent on the analysis of a questionnaire (Appendix II) which was constructed to help obtain information in specific subject areas that would further help understanding the experimental part of the project. The questionnaire had five main parts giving us the ability to extract information on the general profile of the subjects such as age and BMI [weight (kg)/height (m) ^{2}], exercise and the immune profile, as well as their nutritional habits, their stress levels and some information about their medical history. Anthropometrical data which included the gestational age, the weight of the mother before the time of conception as well as the weight and height of the mother at the time of the survey were acquired through a series of specific questions. Also behavioural data was collected that included the maternal attitudes towards pregnancy, smoking and drinking habits as well as the consumption of multi-mineral/multivitamin supplements before and during pregnancy. The largest part of the questionnaire was focused on the nutritional status with questions targeting the maternal dietary habits before, as well as during, pregnancy. The collection of data took place at the Department of Obstetrics and Gynecology of the Medical School at the University Hospital of Crete. Questionnaires were handed out to the female patients at the labour ward of the hospital. A face-to-face interview was conducted with each woman, with each interview lasting at least 30min.

3.2. Results

3.2.1. General profile of the participants of the study

A summary of the general profile is presented in Table 3.1 and the analysis of the questionnaire data is presented as percentage for each question. These include the age of the prospective mother, which was sub-grouped per decade and as it can be seen, a high

percentage of perspective mothers was found in the 3rd decade (21-30 years old), where as the lowest percentage was observed in the 2nd decade (below 20). The self-reported information on the weight and height of the subjects was used to calculate their Body Mass Index [BMI; weight (kg)/height (m)²] before conception. This information was further sub-grouped according to the BMI weight status categories. The majority of the participants were appeared to be of normal BMI. Information of the weight of the mother as a newborn, as well as if she was born at term or pre-term, was also collected and the majority of the participants were born in the normal for gestational age range and after the 37th week of gestation which is considered to be at term. The maternal body shape was also recorded to help us understand the type of the participants adiposity, for example if a participant reported have an apple shape the fat is stored around the abdominal region, where the pear shape women tend to store the majority of the fat in the lower body, hips and buttocks and lastly the proportionate shape shows an equal fat distribution. As seen in the table 3.1 the majority of the participants reported to have a pear shape body type. Information on the duration of the pregnancy as well as the weight of the foetus was also recorded. Surprisingly, a slightly higher percentage of the infants were born prematurely. Lastly, the participants were asked if they had any iron deficiency induced anaemia and the majority reported not to have. This question was asked in this cohort of patients due to higher prevelance of β-Thalassaemia in the Eastern Medittareanean region.

GENERAL PROFILE	
Age (years)	**%**
• < 20	7.7
• 21 - 30	56.7
• 31 - 40	35.6
BMI	**%**
• Underweight (< 18.5)	13.6
• Normal (18.5 - 24.9)	64.1
• Overweight (25 - 29.9)	12.6
• Obese (> 30)	9.7
Weight of mother as newborn (gr)	**%**
• Small (< 2500)	5.1
• Normal (2500 – 3800)	85.6
• Large (> 3800)	9.3
Born prematurely	**%**
• Yes	7.1
• No	92.9
Body shape (B.P) mostly matches yours	**%**
• Apple shape	8.7
• Pear shape	56.3
• Proportionate shape	35.0
Duration of pregnancy (weeks)	**%**
• Term (< 37)	43.2
• Pre- term (> 37)	56.8
Foetal weight (gr)	**%**
• Small (< 2500)	25.3
• Normal (2500 – 3800)	72.8
• Large (> 3800)	1.9
β-thalassaemia	**%**
• Yes	18.3
• No	74.5

Table 3.1: Details of the general profile of the participants in our study

3.2.2. Immune Profile of the participants of the study

The second profile that was analysed was related to the immune status of the participants, and consisted of seven questions in total as it is described in Table 3.2. The participants were asked of the number of colds and infections they contracted during their pregnancy and according to the replies obtained, certain subgroups were set. The majority of the participants did not contract any colds or infections during their pregnancy. As to the difficulty the participants had to recover from an infection the majority responded that they did not find it hard to "fight" an infection. Regarding the proneness to thrush or cystitis, the majority of the participating subjects reported not to be prone to these gynaecological conditions. Further, the majority had reported not to have taken any antibiotics during the last month prior to the interview. In addition the majority of the women reported not to have any inflammatory diseases or allergies. A significant inverse correlation has been noted between difficulty to "fight" an infection and number of colds (r = -0.289, P = 0.003) as well as number of infections (r = -0.446, P < 0.0001) during pregnancy. An inverse correlation was also observed between pregnancy days and number of infections during pregnancy (r = -0.212, P = 0.004). Interestingly, the use of antibiotics also inversely correlated with difficulty to get rid of a cold (r = -0.422, P < 0.0001) and how prone these women were to cystitis infections (r = -0.389, P < 0.0001).

IMMUNE PROFILE	
How many colds did you get during pregnancy?	**%**
• 0	68.9
• 1	6.8
• 2	21.4
• 3	1.9
• 4	1.0
How many infections did you get during pregnancy?	**%**
• 0	77.9
• 1	10.6
• 2	9.6
• 3	1.9
Do you find it hard to shift an infection (e.g cold)?	**%**
• Yes	36.5
• No	63.5
Are you prone to thrush or cystitis?	**%**
• Yes	16.3
• No	83.7
How often did you take antibiotics in the last month?	**%**
• None	71.1
• Once	21.2
• Twice	4.8
• More than 3 times	2.9
Do you have an inflammatory disease (e.g arthritis)?	**%**
• Yes	6.8
• No	93.2
Do you suffer from hay fever, allergies?	**%**
• Yes	2.9
• No	97.1

Table 3.2: Details of the immune profile of the participants in our study

3.2.3. Exercise Profile of the participants of the study

The third profile that was incorporated and analysed in our questionnaire was the exercise profile of the women that were taking part in our research (Table 3.3). This profile aimed to estimate the level of activity of the women before and during their pregnancy. The majority of the women reported to embark on light activity; this consisted of 2-4 flights of stairs that they climbed on a daily basis. This pattern was consistent before as well as during pregnancy. As far as their walking habits were concerned, an almost even percentage of women reported to light as well as moderate walking activity before pregnancy whereas this the moderate activity was lower during pregnancy and the light walking activity (consisting of the equivalent of 2-4 city blocks) was the highest category. With regards to the participation to light sports (e.g. dancing, gardening or walking) a good proportion of women appeared to be inactive before pregnancy. The inactivity was increased to 60% during pregnancy, with a simultaneous drop of the high activity to only 2%. When the participants were asked the number of hours per week they would participate in any strenuous sports such as running or cycling or tennis, again the majority reported to be inactive both before and during their pregnancy.

EXERCISE PROFILE		
	B. P.	**D. P.**
How many flights of stairs do you climb every day (10 steps one flight)?	**%**	**%**
• Inactive (0-1 flight of stairs)	23.1	31.7
• Light (2-4 flights of stairs)	47.1	47.1
• Moderate (5-7 flights of stairs)	29.8	21.2
• Active (8+ flights of stairs)	0.0	0.0
How many city blocks do you walk each day (1 block=130 metres)?	**%**	**%**
• Inactive (0-1 city blocks)	12.5	23.1
• Light (2-4 city blocks)	34.6	42.3
• Moderate (5-7 city blocks)	31.7	22.1
• Active (8+ city blocks)	21.2	12.5
How many hours/week do you participate in any light sports (e.g. dancing, gardening, walking)?	**%**	**%**
• Inactive (0 - 1,5) hours/week	41.3	59.6
• Light (1,6 - 2,5) hours/week	22.1	28.8
• Moderate (2,6 - 3,5) hours/week	28.8	8.7
• Active (3,6 +) hours/week	7.7	1.9
How many hours/week do you participate in any strenuous sports (e.g. running, cycling, swimming, tennis)?	**%**	**%**
• Inactive (0 - 1,5) hours/week	76.9	93.3
• Light (1,6 - 2,5) hours/week	8.7	6.7
• Moderate (2,6 - 3,5) hours/week	12.5	0.0
• Active (3,6 +) hours/week	1.9	0.0

Table 3.3: Details of the exercise profile of the participants in our study. (B.P : before pregnancy; DP: during pregnancy).

3.2.4. Nutrition Profile of the participants of the study

The fourth profile (Table 3.4) that was assessed in this study was the nutritional profile which was also the most important profile of the study and hence the numbers of questions were more in comparison to the previous profiles. As the aim of the analysis of this profile was to understand the nutritional habits and detect any changes in them, the questions were asked on two levels, before and during pregnancy. The vast majority of the participants were not vegetarian and the percentage that reported this remained the same as during pregnancy. A higher percentage of women reported to consume full-fat dairy products more often compared to those that reported to rarely use full-fat dairy products before pregnancy. When women were asked how many of their weekly meals included foods that are high in lipids and more specifically in saturated lipids (pies, pastries, fried foods) the majority reported to excessively (5 times a week or more) consume these kind of rich in lipids foods before pregnancy whereas percentage was lowered significantly during pregnancy where the majority reported light consumption of these foods. A high percentage of women reported to moderately consume vegetables on a daily basis before pregnancy and this was maintained during pregnancy as well. This was also consistent with the responses obtained regarding the daily consumption of fruits, before as well as during, pregnancy where the majority reported to moderately consume fruits in both states. Excess consumption was recorded for cereals both before as well as during pregnancy. Also the majority of the participants reported to eat iron rich foods (e.g. lean red meat, chicken, green leafy vegetables) on a daily basis both before pregnancy but percentage of pregnant women consuming more iron rich foods on a daily basis was significantly increased compared to pre-pregnancy. This was also consistent when the participants were asked about the servings of cheese, milk, yoghurt or calcium enriched milk they would consume every day where the percentage was increased during pregnancy. More than 1 litre of water / sugar free drinks were consumed by the participants

both before as well as during pregnancy. A high percentage of women reported to consume 2-3 cups of coffee or other caffeine containing beverages before pregnancy however during pregnancy the majority of women reduced their daily consumption to 1 cup or fewer. Again the percentage of women drinking 2-3 sugary drinks on a daily basis was reduced during pregnancy to 1 or fewer. The majority of participants reported not to consume any alcoholic beverages before or during pregnancy. Lastly, the majority of the participants reported to change their fast food eating habits during pregnancy.

NUTRITION PROFILE		
Are you vegetarian?	**B.P. %**	**D.P. %**
• Yes	7.8	7.8
• No	92.2	92.2
How often do you buy full-fat dairy products?	**%**	**%**
• Often	63.1	N/A
• Rarely	36.9	N/A
How many meals per week would include any of the following: pies, pastries, fried foods? *	**%**	**%**
• None (0)	1.0	1.0
• Light consumption (1-2)	33.0	46.1
• Moderate consumption (3-4)	31.1	40.2
• Excess consumption (5 or more)	35.0	12.7
How many servings of vegetables/legumes do you have each day?	**%**	**%**
• None (0)	3.9	2.9
• Light consumption (1-2)	38.8	29.4
• Moderate consumption (3-4)	52.4	60.8
• Excess consumption (5 or more)	4.9	6.9
How many servings of fruit do you have each day?	**%**	**%**
• None (0)	1.9	1.0
• Light consumption (1-2)	32.1	17.6
• Moderate consumption (3-4)	50.5	65.7
• Excess consumption (5 or more)	15.5	15.7
How many servings of cereals do you have each day?	**%**	**%**
• None (0)	2.9	2.0
• Light consumption (1-2)	1.9	1.0
• Moderate consumption (3-4)	33.1	32.6
• Excess consumption (5 or more)	62.1	64.4
Do you eat iron rich foods (e.g. lean red meat, chicken, green leafy vegetables) every day?* **	**%**	**%**
• Yes	66.7	94.1
• No	33.3	5.9
Do you eat 2 or more servings of cheese, milk, yoghurt or calcium enriched milk every day? *	**%**	**%**
• Yes	65.0	85.3
• No	35.0	14.7

How much water/sugar-free drinks do you drink each day?	**%**	**%**
• Less than ½ litre	5.8	3.0
• ½ - 1 litre	8.8	4.0
• More than 1 litre	85.4	93.0
How many cups of coffee, black tea or caffeine containing beverages do you drink each day? ***	**%**	**%**
• 4-6	1.0	0.0
• 3-4	27.2	2.0
• 2-3	43.6	12.7
• 1 or fewer	28.2	85.3
How much soda, sugary drinks do you normally have each day? ***	**%**	**%**
• More than 3	36.9	2.0
• 2-3	38.8	29.4
• 1 or fewer	24.3	68.6
How many alcoholic beverages do you consume on a weekly basis? ***	**%**	**%**
• More than 5	0.0	0.0
• 3-4	14.6	1.0
• 2 or fewer	23.3	5.2
• None	62.1	93.8
How many times a week do you eat fast food? **	**%**	**%**
• Never	36.9	58.8
• 1-2 times	42.8	35.3
• 3-4 times	18.4	5.9
• 5 or more times	1.9	0.0

Table 3.4: Details of the nutritional profile of the participants in our study. (B.P : before pregnancy; DP: during pregnancy). Red stars indicate the significant responses presented further below in graph format, when $P < 0.05$ *, $P < 0.001$ **, $P < 0.0001$ ***.

3.2.5. Stress Profile of the participants of the study

The last profile analysed was the stress profile of the women aiming to understand their stress levels as well as their attitude and feelings towards their pregnancy (Table 3.5). The majority of the women reported that they did not plan their pregnancy but it should be noted that the percentages of the planned and non-planned pregnancy were respectively very close to each other. The majority of the participants (65.1%) rated their stress level to vary from low to medium. Moreover, almost half of the participants reported to have a positive attitude towards their pregnancy describing it using positive words such as happy, ok and satisfied. Lastly, regarding the sleeping pattern of the subjects, one third reported to sleep 10 hours or more every night.

STRESS PROFILE	
Is this a planned pregnancy?	**%**
• Yes	43.7
• No	56.3
How would you rate your current stress level?	**%**
• Low	27.2
• Medium	37.9
• High	29.1
• Very high	5.8
What word(s) describe how you feel about being pregnant?	**%**
• Happy - ok - satisfied	50.5
• Tired - Sad - Stressed - Angry	49.5
How much sleep on average do you obtain each night?	**%**
• 10 or more hours per night	30.1
• 9-10 hours per night	25.2
• 7-8 hours per night	27.2
• 5-6 hours per night	14.6
• Less than 5	1.9
• Not sure- do not sleep well throughout night	1.0

Table 3.5: Details of the stress profile of the participants in our study.

3.2.6. Smoking Profile of the participants of the study

Another important factor that was investigated in our study was the smoking habits of the women before, as well as, during pregnancy (Table.3.6). The majority of women reported that they did not smoke immediately before their pregnancy and this was maintained during pregnancy. Also the majority of the prospective mothers reported that someone else in their household was smoking both before and during their pregnancy, therefore these women were exposed to passive smoking.

SMOKING PROFILE		
Immediately before pregnancy did you smoke?	**B.P. %**	**D.P. %**
• Yes	21.4	N/A
• No	78.6	N/A
Since you have been pregnant, have you smoked cigarettes?	**%**	**%**
• Yes	N/A	13.9
• No	N/A	86.1
Does anyone in your household smoke?	**%**	**%**
• Yes	59.2	58.8
• No	40.8	41.2

Table 3.6: Details of the smoking profile of the participants in our study**.**

The following pie charts depict the overall percentages of women that smoked before (BP) and during (DP) pregnancy. We would like to emphasize on the importance that only a 7% of the women reported to have stopped smoking while pregnant where the rest of the participants reported to have continued this unhealthy habit. To the question if anyone in the

household was smoking before and if they had continued to smoke during pregnancy, a high percentage reported that they were exposed to passive smoking with percentage remaining the same irrespective of wife/partner being pregnant (Fig.3.7).

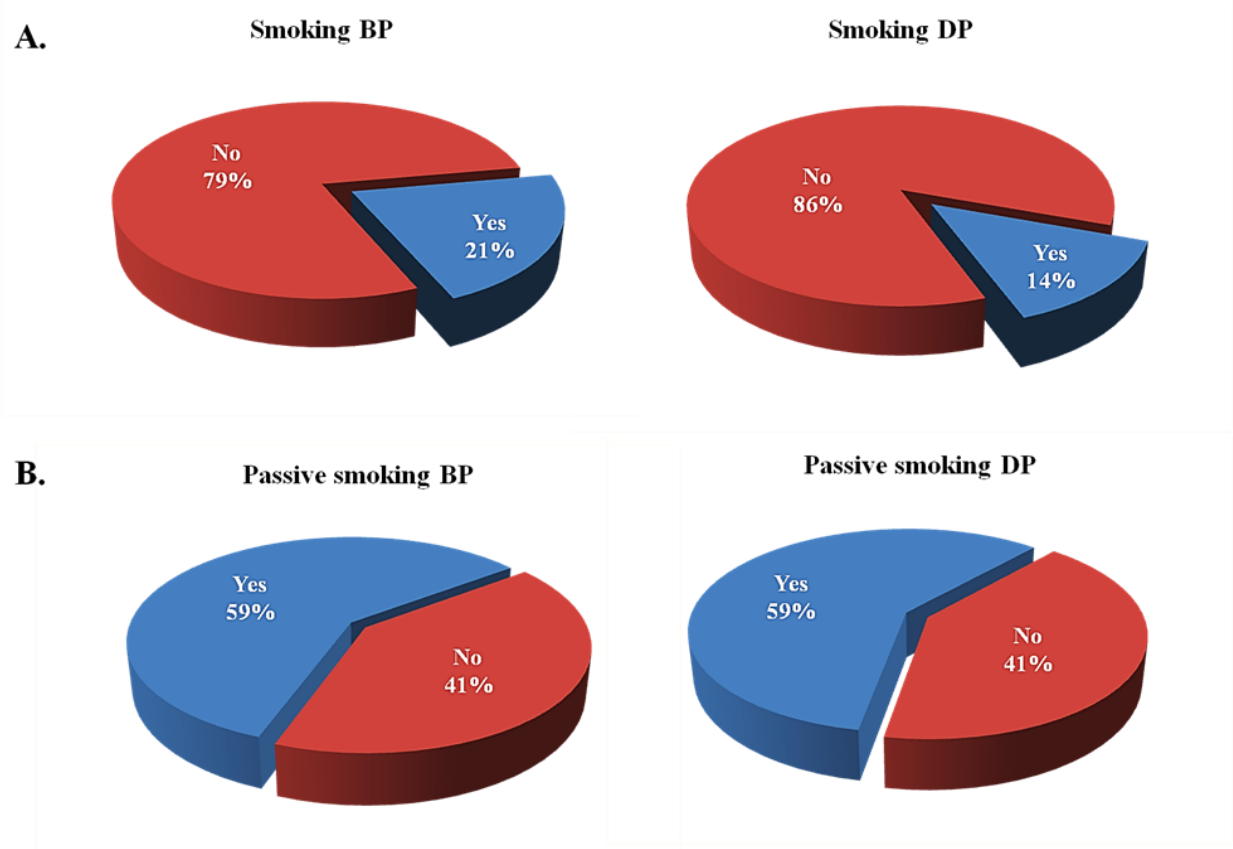


Figure 3.1: Pie charts representing the smoking habits of the participants. Panel A: Indicates the smoking habits of the women before (BP) and during (DP) pregnancy. Panel B: indicates the smoking habits in the household of the women before (BP) and during (DP) pregnancy.

3.2.7. Statistical Analysis of questionnaire data

As the questions were the same for the two states, before and during pregnancy, a student's t-test was used to evaluate the significance of the answers obtained in the different profiles of our questionnaires. The statistically significant data is presented beneath in graph formats. Pie charts were also used for each question that was significant with broke down to related sub categories to emphasize any variations. For comparison purposes the answers were grouped as before and during pregnancy and the mean values of the responses are shown in the representative graphs below.

The subjects response to the question of how many portions per week would they eat pies, pastries or fried food, before and during pregnancy, shows from the pie chart below that there is a wide variation in their responses; with the prospective mothers modifying their eating habits during pregnancy compared to before and adopting healthier eating patterns.

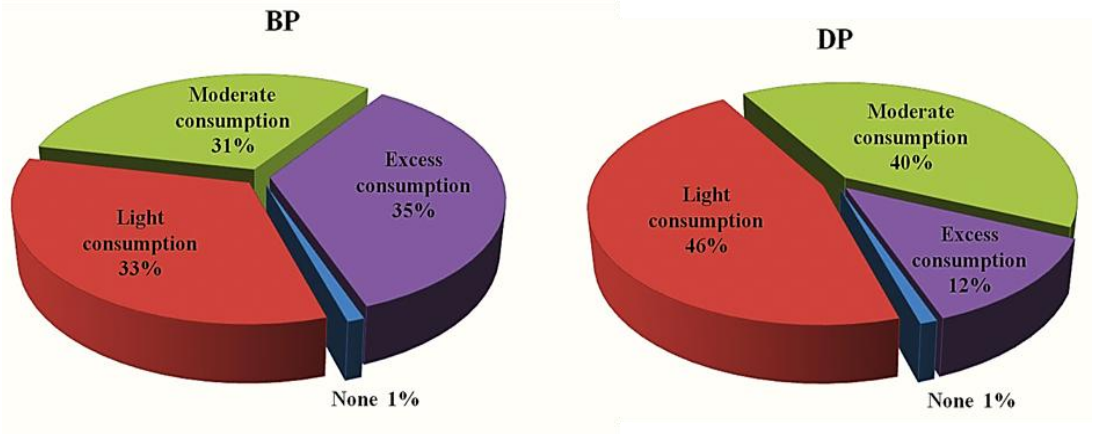


Figure 3.2: Pie charts of the answers to the question: How many meals per week would include any of following: pies, pastries, fried food? (DP: during pregnancy, BP: before pregnancy). Light consumption (1-2 portions), moderate consumption (3-4 portions) and excess consumption (5 or more portions).

When grouped according to the pregnancy state the answers indicated that the average number of portions that women were consuming during pregnancy (moderate consumption) were higher to the average portion number consumed before pregnancy (Fig. 3.3).

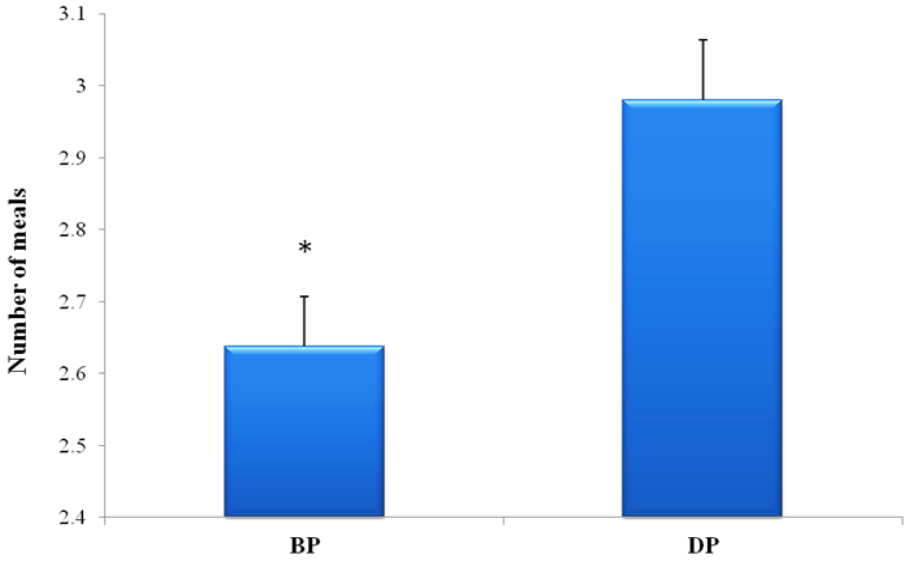


Figure 3.3: How many meals per week would include any of following: pies, pastries, fried food? (DP: during pregnancy, BP: before pregnancy), *P = 0.002*.

Pregnant women also increased their daily intake in iron rich foods such as lean red meat, chicken, etc., and this is depicted in the pie chart (Fig. 3.4) which is in agreement with the analysis performed in the grouped categories showing that the average number of iron rich food consumed on a daily basis was significanlty higher during pregnancy than before (Fig. 3.5).

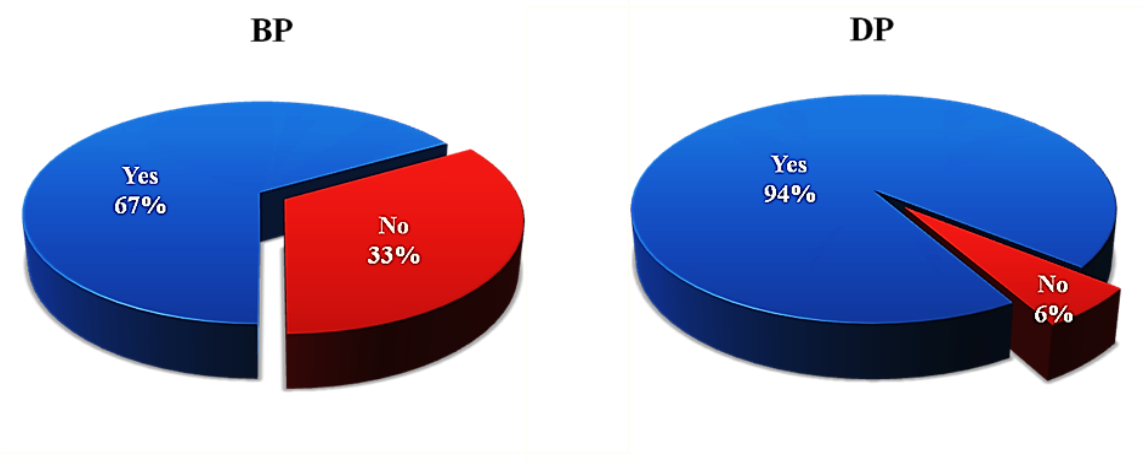


Figure 3.4: Pie charts of the answers to the question: Do you eat iron rich foods every day? (E.g. lean red meat, chicken, etc.) (DP: during pregnancy, BP: before pregnancy).

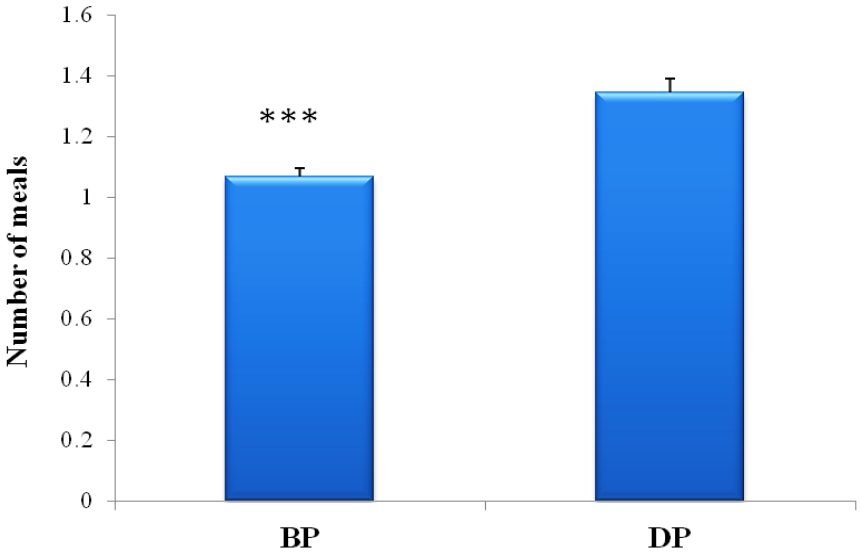


Figure 3.5: Do you eat iron rich foods every day? (E.g. lean red meat, chicken, etc.) (DP: during pregnancy, BP: before pregnancy), $P = 6.0 \times 10^{-7}$

The participants reported to have increased their daily consumption of dairy products such as: cheese, milk, yoghurt or calcium enriched milk during pregnancy. The pie charts below (Fig. 3.6) are indicative of this increase.

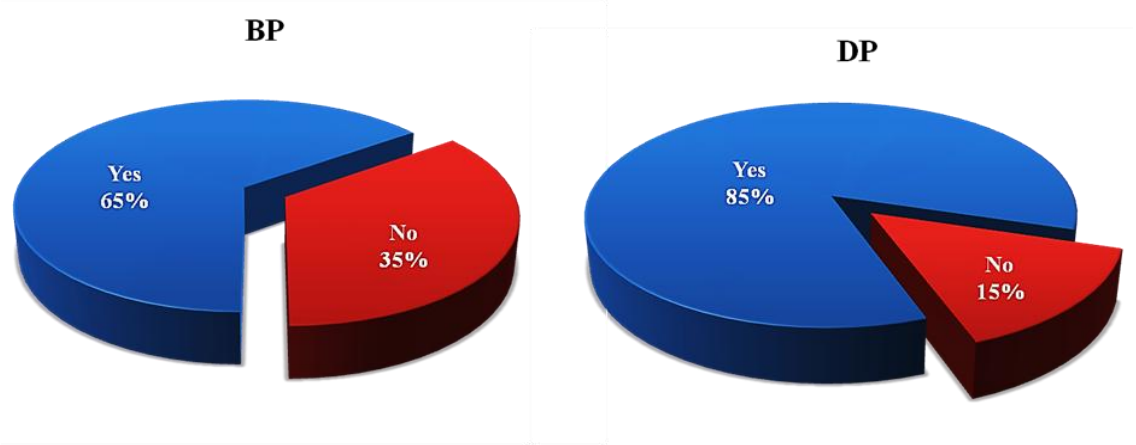


Figure 3.6: Pie charts of the answers to the question: Do you eat 2 or more serving of cheese, milk, yoghurt or calcium enriched milk/day? (DP: during pregnancy, BP: before pregnancy).

Also when the answers were examined in the grouped categories it was seen that the women increased significanlty their average servings of cheese, milk, yoghurt or calcium enriched milk/day to 2 or more during their pregnancy (Fig. 3.7).

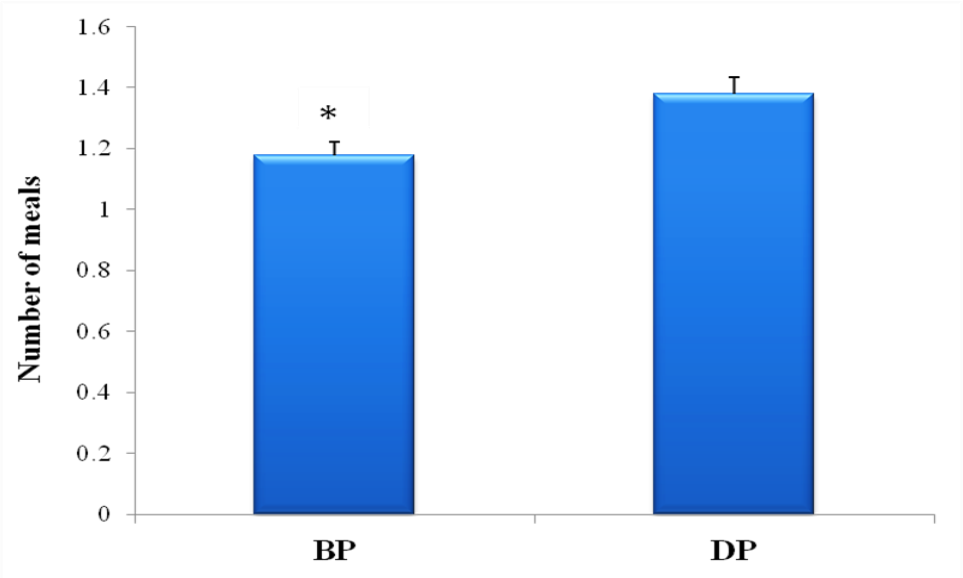


Figure 3.7: Do you eat 2 or more serving of cheese, milk, yoghurt or calcium enriched milk/day? (DP: during pregnancy, BP: before pregnancy), *P = 0.004.*

Furthermore, the daily consumption of caffeine in the form of coffee, black tea or other beverages containing caffeine showed to be modified during pregnancy compared to the

drinking habits of the women before pregnancy as seen in the pie charts presented below (Fig.3.8).

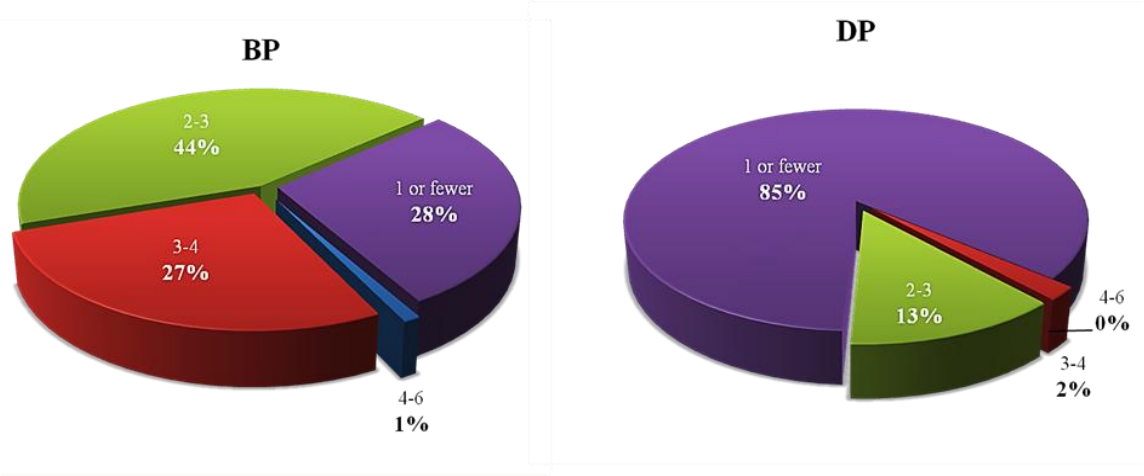


Figure 3.8: Pie charts of the answers to the question: How many cups of coffee, black tea or caffeine containing beverages do you drink/day? (DP: during pregnancy, BP: before pregnancy). Numbers indicate the number of cups.

Also the average of the responses obtained in the two categories is in agreement with the observation that the prospective mothers had significantly reduced (85% reported to drink 1 or fewer cups per day) their intake of caffeine on a daily basis (Fig. 3.9).

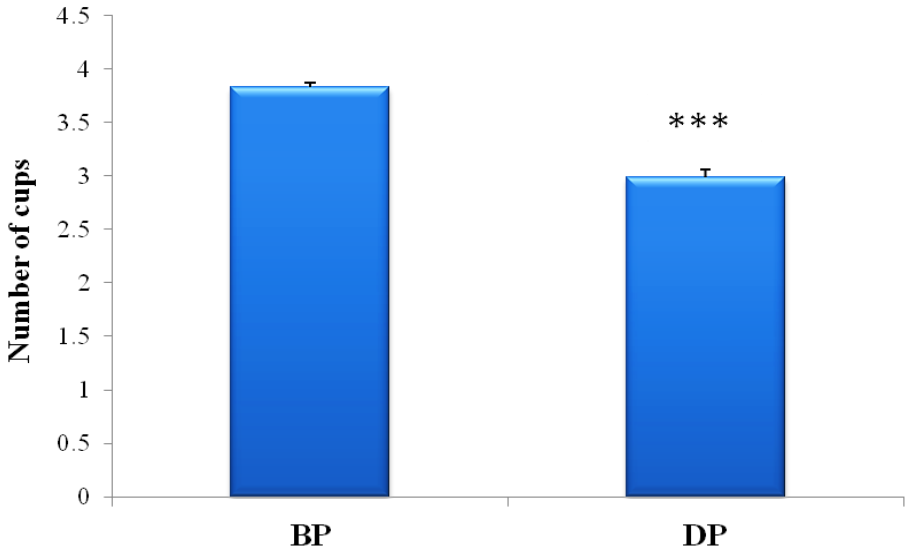


Figure 3.9: How many cups of coffee, black tea or caffeine containing beverages do you drink/day? (DP: during pregnancy, BP: before pregnancy), $P = 2.0 \times 20^{-18}$

To the question of how much soda, sugar-containing drinks do you normally have each day again it was evident that the women had modified their habits to meet the "guidelines" of a healthier approach during pregnancy as the number of responses stating to drink 1 or fewer during pregnancy was increased compared to before (Fig. 3.10).

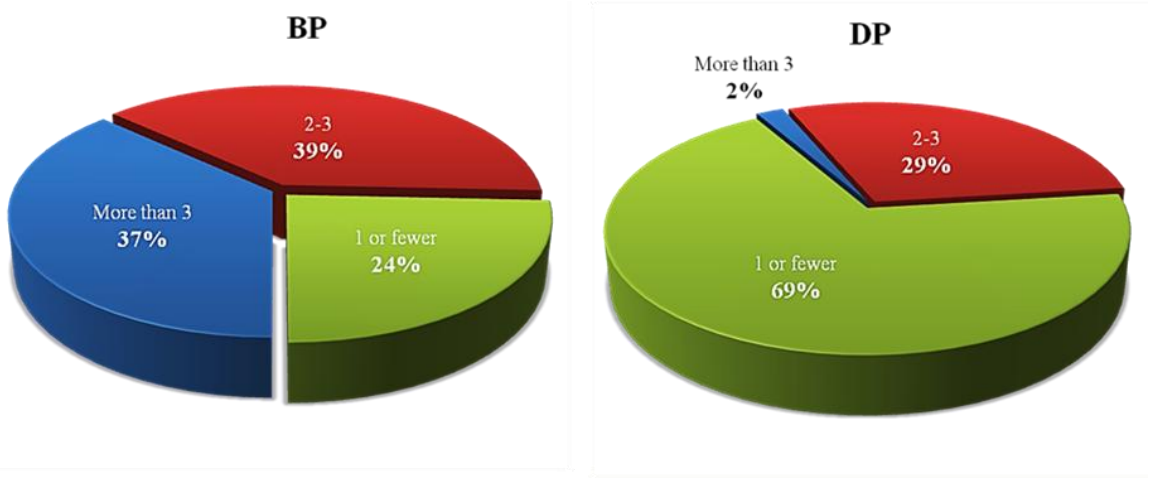


Figure 3.10: Pie charts of the answers to the question: How much soda, sugar drinks do you normally have each day? (DP: during pregnancy, BP: before pregnancy). Numbers indicate the number of drinks.

This again agrees with the two category analysis performed were a significant reduction in the average number of drinks consumed containing sugar that was reported during pregnancy in comparison with the intake before pregnancy (Fig. 3.11).

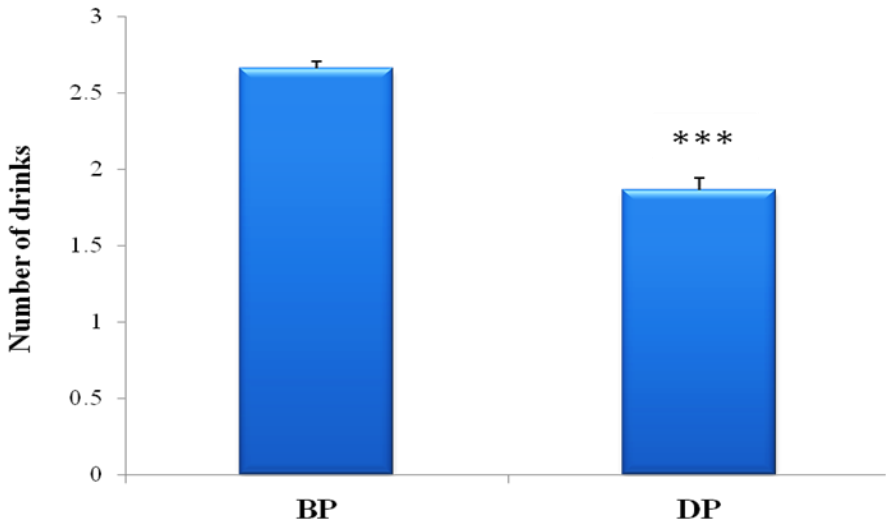


Figure 3.11: How much soda, sugar drinks do you normally have each day? (DP: during pregnancy, BP: before pregnancy), $P = 1.4 \times 10^{-15}$

In a similar trend, consumption of alcoholic beverages on a weekly basis showed a noticeable change between the two states of pregnancy and according to the numbers of beverages consumed (Fig. 3.12).

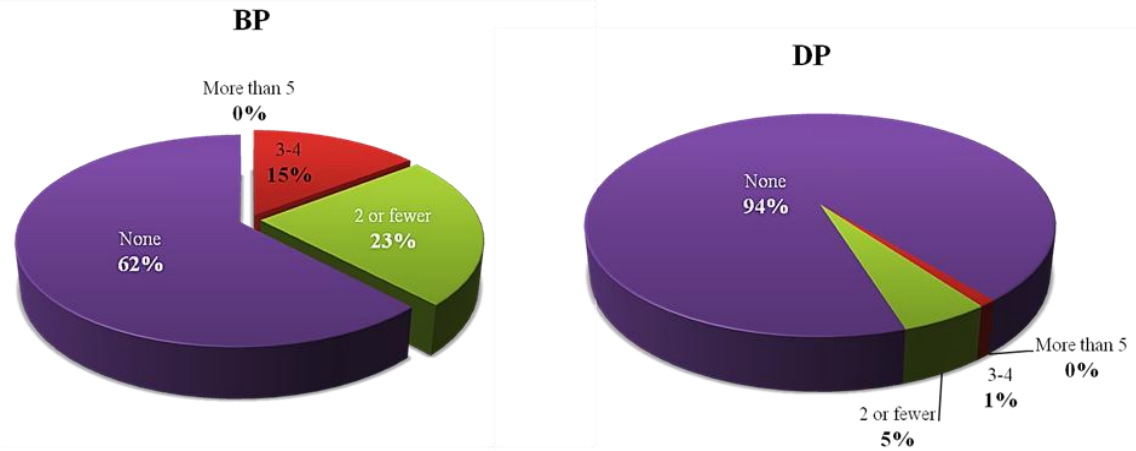


Figure 3.12: Pie charts of the answers to the question: How many alcoholic beverages do you consume on a weekly basis? (DP: during pregnancy, BP: before pregnancy). Numbers indicate the number of drinks.

Significanlty higher was the average consumption reported before pregancy of alcoholic beverages compared to during pregancy (Fig. 3.13).

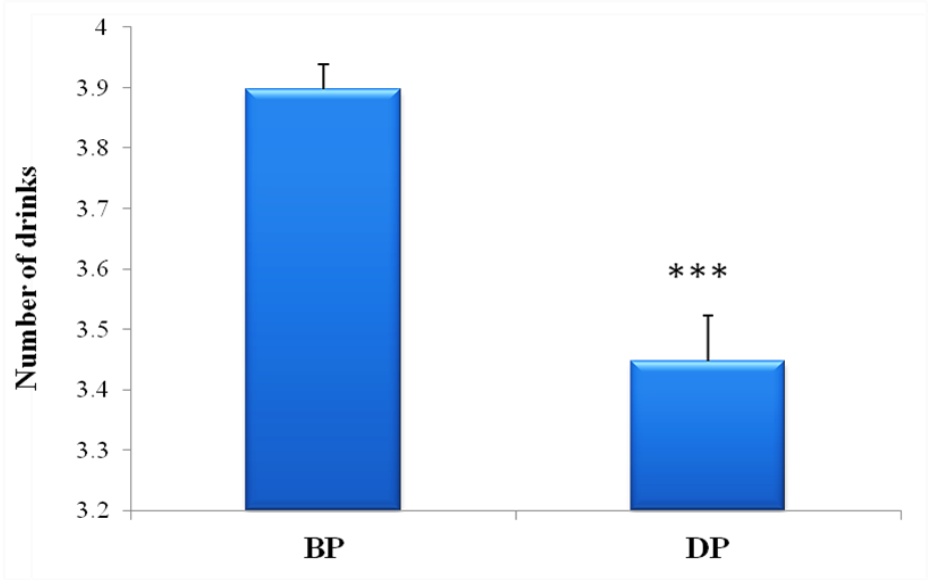


Figure 3.13: How many alcoholic beverages do you consume on a weekly basis? (DP: during pregnancy, BP: before pregnancy*), P = 9.6 x* 10^{-7}

Modifiction of the consumption of fast food was also observed when sub category analysis was performed as seen in the pie charts below (Fig. 3.14) with the percentage of women reporting not to comsume any fast food at all during their pregnancies rising when compared to consumption before pregnancy.

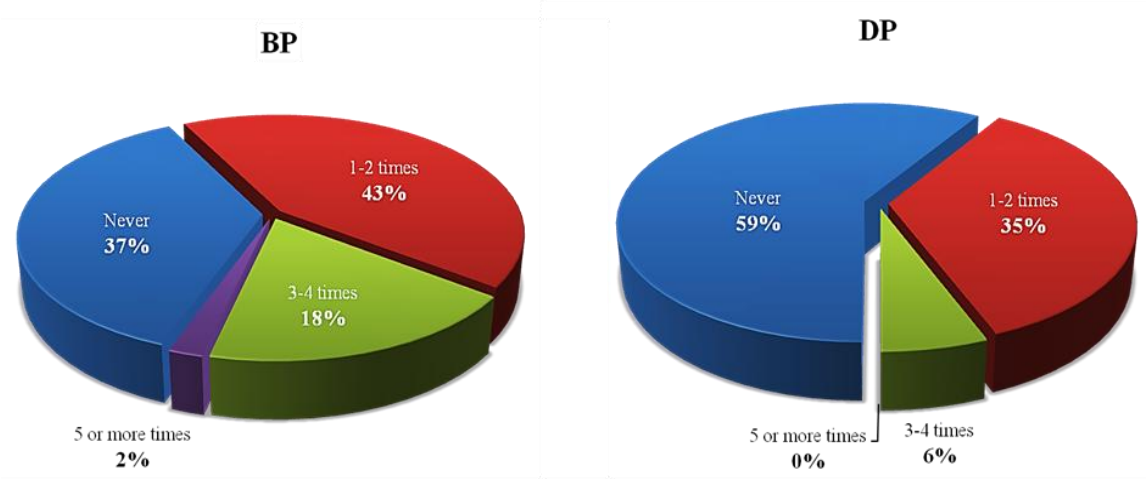


Figure 3.14: Pie charts of the answers to the question: How many times a week do you eat fast food? (DP: during pregnancy, BP: before pregnancy). Numbers indicate the frequency in a week's period.

When analysis was performed in the pregnancy states, as seen below the weekly preferance to fast food had significanlty decreased during their pregnancy compared to before pregnancy. These results are depicted in Fig. 3.15.

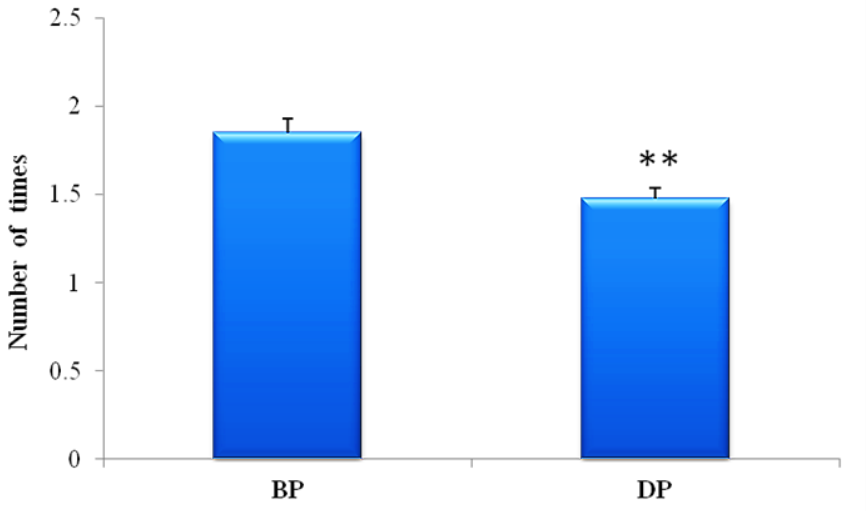


Figure 3.15: How many times a week do you eat fast food? (DP: during pregnancy, BP: before pregnancy), *P = 0.0002*

3.3. Discussion

In this study we have used an extensive range of questions to obtain as much information as possible about the cohort of the patients we dealt with. In this chapter main emphasis was on exercise and nutrition of these pregnant women before and during pregnancy. It should be noted that maternal attitudes and foetal outcome is discussed in length in Chapter 6 of the thesis. A key finding of our study is that there was a significant reduction in the intake of alcohol, caffeine-containing and sugary drinks as well as sugary refreshments during pregnancy. Moreover, in our cohort 14% of women smoked during pregnancy. This is comparable to a recent study of Australian women that showed 14.8% of nonindegenous women smoking during pregnancy (Mendelsohn, 2010). Maternal smoking during pregnancy is a well established risk factor for perinatal mortality, miscarriage, and premature births (Hackshaw *et al*., 2011). Nicotine and carbon monoxide can cross the foeto-placental barrier and reach high concentrations in the foetus, reducing placental blood flow leading to foetal hypoxia and subsequently growth restriction (Einarson, 2009). Nicotine is a neurotoxin to the developing brain and causes cognitive, emotional and behavioural problems. Children born to mothers who smoke in pregnancy also have reduced lung function and an increased risk of respiratory illness. Exposure to heavy smoking in utero also increases the risk of nicotine dependence in adulthood (Mendelsohn, 2010). In this study another interesting finding was unearthed, since parental/passive smoking was not reduced in the household during pregnancy, staying at a high rate of 59%. This could be detrimental, as all types of passive smoking have been associated with a significant increase in the risk of infants developing lower respiratory infections in the first two years of life (Jones *et al*., 2011).

In this questionnaire also incorporated seven questions relating to the immune profile of this cohort. We have done so, as acute infections in pregnant women are often associated with adverse effects such as miscarriage, pre-term labour or even stillbirth (Goldenberg *et al*.,

2010; 2000). There is also evidence from epidemiological data that infections during pregnancy might precipitate other pathologies like preeclampsia (Conde-Agudelo *et al*., 2008). Data from this self-reported questionnaire indicated that the vast majority of participants do not suffer from allergies, do not have an inflammatory disease or are prone to cystitis. The most common infection reported was a cold and 36.5% of pregnant women found it hard to shift the upper respiratory infection. Interestingly, a significant inverse correlation has been noted between difficulty to shed an infection and number of colds and number of infections during pregnancy. Similar data has been obtained by a very recent study of Australian women, where cold was the commonest infection reported using a similar self-reported method (Lain *et al*., 2011). Our study has a number of limitations, with reliance being one. In the study by Lain *et al*., only 21% of the women that reported an infection sought medical attention. We do not have such records for our cohort. Moreover, given that we have asked the patients to self-report at the end of the third trimester there might be issues with recall bias. We would like to acknowledge powerful conclusion from an earlier study using an analogous type of reporting. The authors concluded that "future studies should emphasise the importance of interviewing women as early as possible, as mothers tend to under-report infection" (Collier *et al*., 2009). However our data related to the immune profile also has certain strengths as it includes the investigation of numerous rather a single infection and incorporated both chronic and acute infections. These data can be of clinical importance as gynaecologists can use a self-reported method for infections to classify patients in a high or low group for predisposition towards pregnancy complications such as preeclampsia. With regards to caffeine effects during pregnancy, there is still some controversy in the field. Caffeine is readily absorbed from the mucosa of the gastrointestinal tract. It crosses the human placenta rapidly reaching concentration in the foetus similar to maternal plasma levels. Caffeine has been implicated as a cause of spontaneous abortion, intrauterine growth

restriction (IUGR), low birth weight (LBW) and pre-term delivery (Kuczkowski, 2009). However, other investigators failed to find any association between caffeine intake and poor pregnancy outcomes (Wen *et al*., 2001), whereas some studies have shown positive outcomes. Adeney *et al*., have shown that moderate caffeine consumption during pregnancy exerts a protective effect towards gestational diabetes mellitus (GDM) (Adeney *et al*., 2007). These mixed results may arise due to the problem of accurately assessing the caffeine intake. Moreover the amount of caffeine varies tremendously in different coffee chains. In a very recent study, analysis of 20 commercial espresso coffees using high performance liquid chromatography (HPLC) technology revealed differences in caffeine levels up to 6-fold (Crozier *et al*., 2011). The authors of this study concluded that single serving of high caffeine espresso could well place at risk individuals who are more susceptible to the effects of caffeine toxicity, including women who are pregnant. In our cohort, a significant reduction in caffeine intake has been noted however we were not able to quantify the precise amount ingested. Nawrot and colleagues have suggested that women of reproductive-aged should consume less than 300 mg of caffeine/ day (equivalent to 4.6 mg/kg body weight per day for a 65-kg person; Nawrot *et al*., 2003). Another key finding was the significant decrease of sugar-containing drinks during pregnancy. In USA for example, sugar-sweetened soft drinks are the principal energy contributor in the diet (Block, 2004) and they appear to play a role in the obesity epidemic due to their high content of readily absorbed sugars (Schulze *et al*., 2004). In a recent study involving 59.334 Danish pregnant women it has been shown that daily intake of artificially sweetened soft drinks may increase the risk of pre-term delivery (Halldorson *et al*., 2010). It appears therefore that the decreased noted in this study might indeed protect from preterm labour. Clearly further epidemiological studies are needed to confirm these effects.

Of interest, a wide range of responses related to the consumption of fried/fast-food during pregnancy were given. As mentioned previously, poor nutrition can lead to a range of health problems for mothers, including metabolic syndrome and cancer. Pregnancy results in a state of increased energy demands of ~300 kcals/day. Moreover maternal energy metabolism is altered during pregnancy and varies greatly among women. The same women that had increased consumption of fast food had also increased intake of iron-rich and dairy products. However, there is no evidence to suggest that these beneficial intakes of calcium and iron counteract poor eating habits. Our findings are comparable to an Australian study of 409 women where a substantial proportion of pregnant women consumed 2 meals of snacks (fast food/take away) per week (Wen *et al*., 2010). This finding might also reflect that young generations seem to deviate from the traditional Mediterranean dietary pattern, adopting new dietary trends (Baldini *et al.*, 2009). Moreover, dietary patterns can be influenced by various socio-demographic characteristics. Taking these into consideration it is imperative to develop dietary interventions to prevent undesirable health consequences during pregnancy. Another factor that can affect pregnancy is exercise. Regular physical activity is associated with improved physiological, metabolic and psychological parameters, and with reduced risk of morbidity and mortality (Melzer *et al*., 2010). In our study (based on the Paffenbarger *et al*. 1978) there was a clear shift towards a sedentary lifestyle during pregnancy. For example, there was an increase in overall inactivity of approximately 15% and an equal decrease of moderate exercise. Regular physical activity during pregnancy has been proven to be beneficial for the mother as well as the foetus. Maternal benefits include: better cardiovascular function, small weight gain during pregnancy, decreased musculoskeletal discomfort, and mood stability, reduction of GDM and gestational hypertension that can lead to preeclampsia (PE). Benefits for the foetus include: reduction of fat mass, coping better with stress, and advanced neurobehavioural maturation (Melzer *et al*., 2010). The adoption or

continuation of a sedentary lifestyle during pregnancy may contribute to the development of certain disorders such as hypertension, maternal and childhood obesity, GDM, dyspnoea, and PE (Melzer *et al*., 2010). In view of the global epidemic of sedentary life-style and obesity, we propose that pregnant women should increase their physical activity as prevention of adverse pathologies for the mother as well as the foetus. Further studies with larger sample sizes are needed to provide solid evidence of associations between increased physical activity and positive outcomes of labour and delivery.

CHAPTER 4

EXPRESSION AND CELLULAR LOCALISATION OF mTOR COMPONENTS IN HUMAN PLACENTA AND PLACENTAL MODELS: EFFECTS OF CORTISOL ON GENE EXPRESSION

4.1. Introduction

As discussed previously, the cross talk between the mother and the foetus is complex, as well as bidirectional. Apart from a plethora of hormonal and immunological events that take place during pregnancy, maternal stress is a powerful contributor to birth outcomes. Pregnancy-related anxiety, depression or distress can be causes for maternal psychological anxiety (Zhu *et al*., 2010). Moreover, maternal stress is linked to poor foetal development *in utero* (Dole *et al*., 2003; Edwards *et al.*, 1994). Since foetal growth is critically dependent on placental nutrient transport, placental mTOR signalling plays an important role in the regulation of foetal growth (Roos *et al*., 2009). In a hyperthermia-induced stress model, a range of changes were apparent regarding the mTOR pathway thus indicating that stress can affect the placental mTOR signalling pathway (Arroyo *et al*., 2009). Given that mTOR can function as a human placental sensor, we hypothesised that maternal stress can affect mTOR signalling at term and as a result influence placental growth. In this chapter we sought to investigate the expression of the main mTOR signalling components such as: mTOR, Deptor, Rictor, Raptor in human placental samples. We also tested the effect of stress on mTOR signalling using two *in vitro* placental models and correlated maternal and foetal cortisol levels to maternal stress profiles.

4.2. Results

4.2.1. Expression of mTOR, Deptor, Rictor and Raptor in human placentas

Quantitative RT-PCR revealed that mTOR, Deptor, Rictor and Raptor are expressed in the human placenta (n = 23). The expression of these genes is shown in Figure 4.1 below, where as seen Deptor is the primary transcript in the total cohort of patients. The ΔCt values of the gene expression of mTOR, Deptor, Rictor and Raptor are shown in Table 4.1 below.

Significantly lower levels of mTOR, Rictor and Raptor are shown upon comparison to the expression of Deptor.

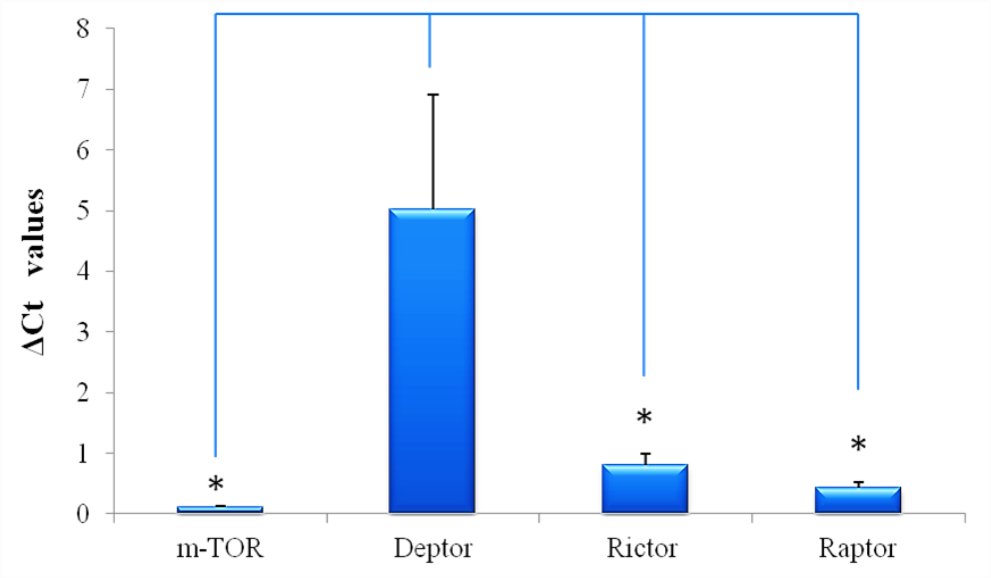


Figure 4.1: Quantitative RT-PCR analysis of mTOR, Deptor, Rictor and Raptor in human placental samples ($P < 0.05$ *) (Mparmpakas *et al*., 2011).

	mTOR	**Deptor**	**Rictor**	**Raptor**
Placentas Samples (*n=23*)	*0.10*	*5.02*	*0.79*	*0.41*
Term (*n=13*)	*0.09*	*3.93*	*0.78*	*0.33*
Preterm (*n=10*)	*0.12*	*6.42*	*0.81*	*0.53*
Labour (*n=18*)	*0.098*	*5.24*	*0.71*	*0.42*
Non-labour (*n=5*)	*0.12*	*4.22*	*1.11*	*0.40*

Table 4.1: Δ*Ct* values of the gene expression of mTOR, Deptor, Rictor, Raptor in placental tissue samples according to time and mode of delivery.

Our placental tissue bank was split according to the time of delivery into two sub-groups (term < 37 weeks of gestation and pre-term > 37 weeks of gestation) and according to the mode of delivery (labour and non-labour). Table 4.1 above summarises the Δ*Ct* of the expression of mTOR, Deptor, Rictor and Raptor in all four subgroups. When the Δ*Ct* values were analysed in the term and pre-term groups, Deptor was found to be the predominant transcript (Fig. 4.2). There was a significant correlation between Log-transformed Deptor and Raptor ($r = 0.729$, $P < 0.005$), and Rictor with Raptor ($r = 0.661$, $P = 0.014$) only in the pre-term placentas examined when compared to term.

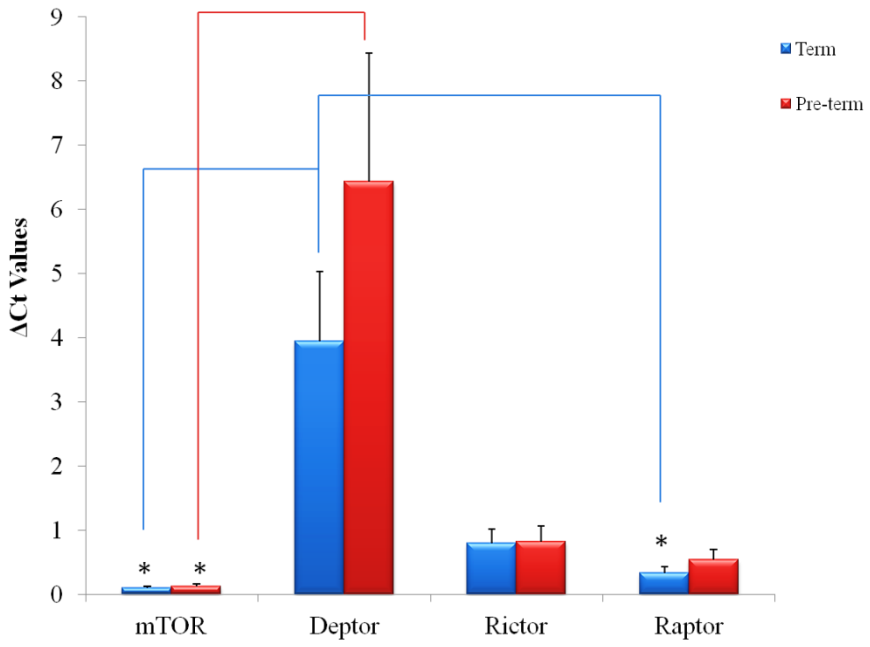


Figure 4.2: Expression of mTOR, Deptor, Rictor, Raptor in human term and pre-term placental samples. *$P < 0.05$

Also the Δ*Ct* levels (table 4.1) were analysed in the second category the labour and non-labour. Again Deptor was shown to be the predominant transcript in both categories, the labour and the non-labour (Fig. 4.3).

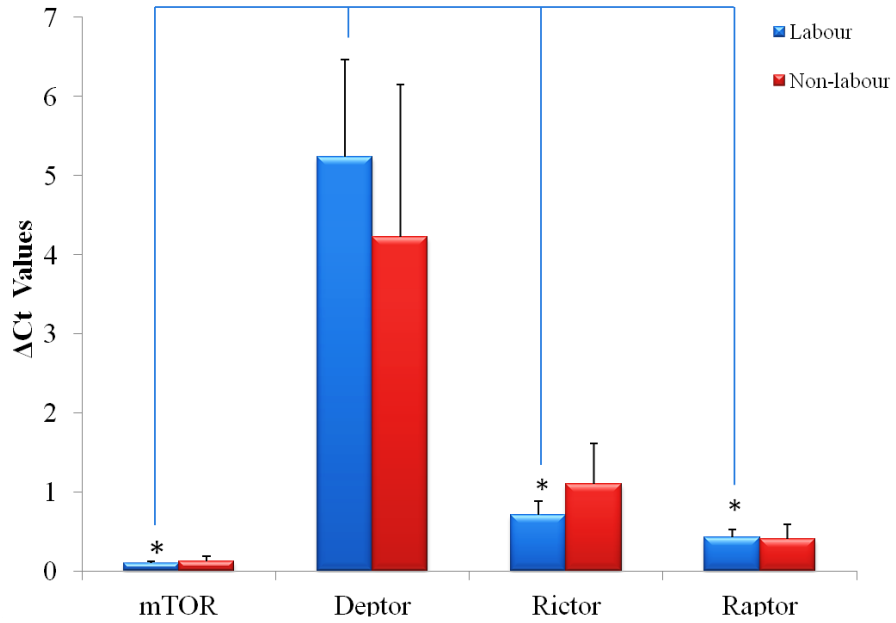


Figure 4.3: Expression of mTOR, Deptor, Rictor, Raptor in human labour and non-labour placental samples. *$P < 0.05$

In this cohort (i.e. in 23 patients), 52% reported high and very high levels of stress during pregnancy, and 48% medium and low levels. There was no correlation between maternal stress levels and body mass index (BMI). Interestingly, there was a significantly lower LogDeptor gene expression in the high stress group (-1.34) than in the low stress group (0.07; t (20) = 2.41, P = 0.026).

We then assessed whether time of delivery has an effect. In term tissues (n = 13) high stress inversely correlated with LogDeptor levels (r = -0.772, P = 0.002), placental weight (r = -0.591, P = 0.03) and foetal weight (r = -0.732, P = 0.004). In the same cohort, LogDeptor levels positively correlated with placenta weight (r = 0.497, P = 0.084) and foetal weight (r = 0.616, P = 0.025). In pre-term placentas (n = 10) maternal high stress levels inversely correlated only with placental weight (r = -0.621, P = 0.07) and foetal weight (r = -0.695, P = 0.038). Interestingly, in this group, LogDeptor levels inversely correlated with placental weight (r = -0.622, P = 0.055) and foetal weight (r = -0.575, P = 0.082).

Given the effects of timing of delivery on stress, we retested the main hypothesis. An ANCOVA (Analysis of Co-Variance) was used to test whether low/high stress correlates with Deptor, controlling for contractile status (labour/non-labour) and timing of labour (term/pre-term) entered as covariates. Taking these confounders into consideration, we still found a trend towards a significant relationship between high stress and Deptor: F (1,18) = 4.12, P = 0.055.

4.2.2. Cellular localisation of mTOR, Deptor, Rictor and Raptor in human placenta

Immunofluorescent analysis of the mTOR signalling components in human placental tissue sections revealed the cellular localization and distribution of each of the components individually. Immunofluoresence staining for mTOR revealed a cytoplasmic staining in the syncytium layer but also in the cytoplasm of the cytotrophoblast cells (Fig. 4.4). Deptor staining was almost exclusively localised in the syncytium layer exhibiting a strong cytoplasmic localization. Staining was also visible in the cytoplasm of the cytotrophoblast cells beneath the syncytium layer (Fig.4.4). Rictor appeared to have a similar expression pattern to mTOR, showing expression in the cytoplasm of both the syncytium layer and the cytotrophoblast cells. The expression of Raptor was observed mainly in the syncytium layer, with staining observed in the cytoplasm (Fig. 4.5).

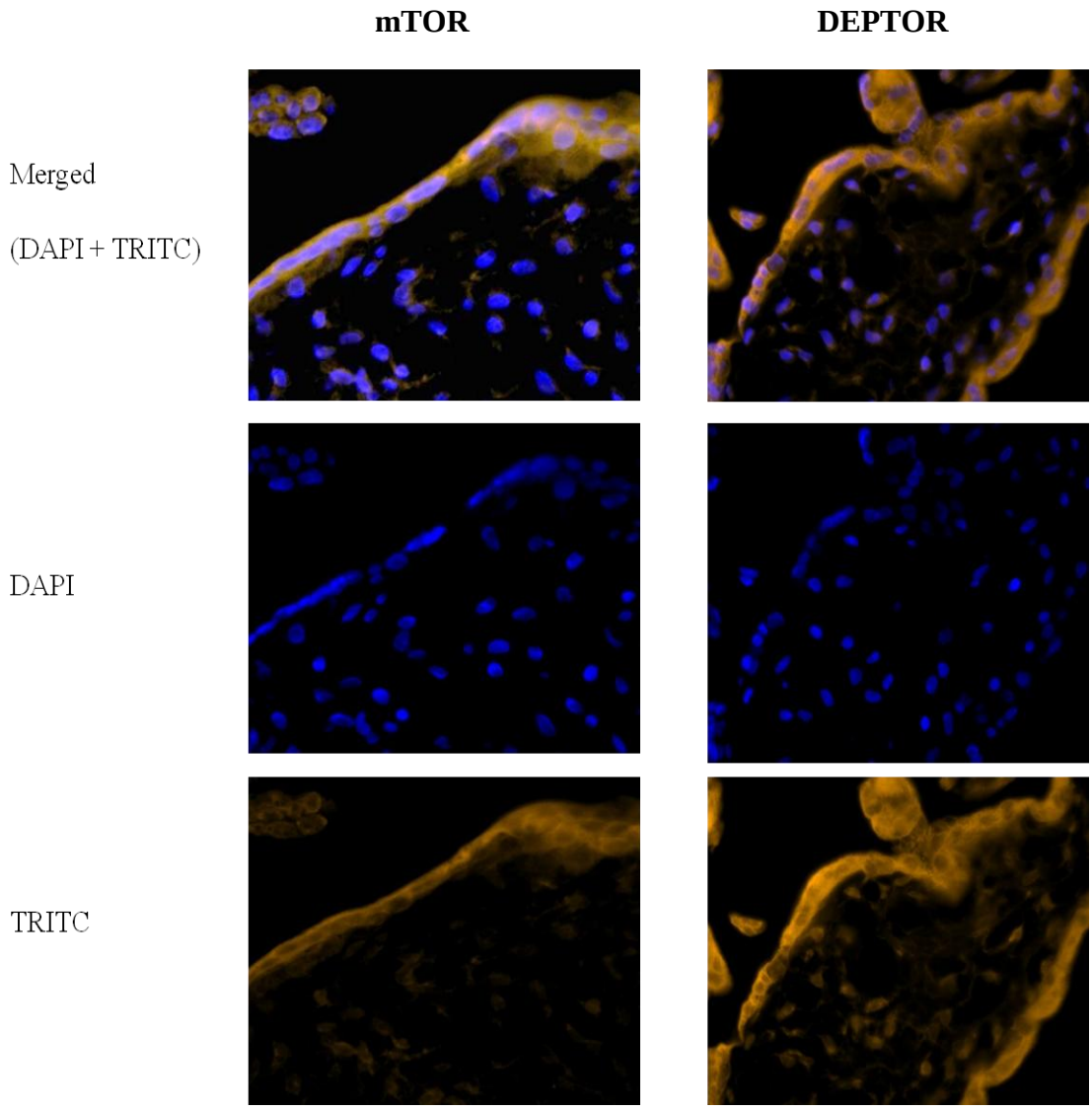


Figure 4.4: Immunofluorescence analysis in human placental tissue sections with mTOR and Deptor. Panels A and B are merged images of DAPI and Tetramethyl Rhodamine Isothiocyanate (TRITC) for mTOR and Deptor respectively. Panels C and D indicates the nuclear staining with DAPI for mTOR and Deptor respectively. Panels E and F indicates the specific staining of the antibodies (Mparmpakas *et al*., 2011).

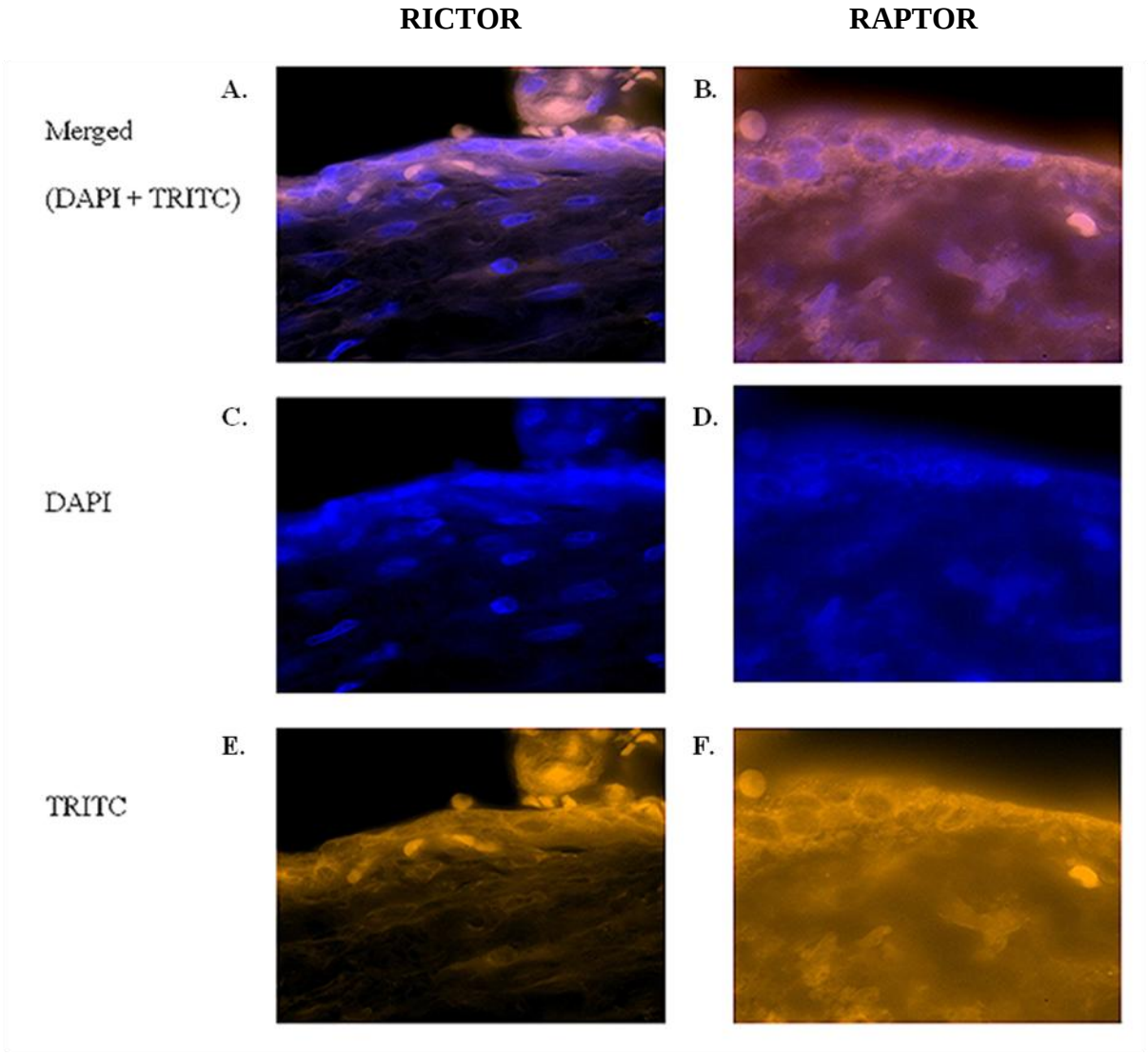


Figure 4.5: Immunofluorescence analysis in human placental tissue sections with Rictor and Raptor. Panels A and B indicative of merged images of placental sections with DAPI and TRITC for Rictor and Raptor respectively. Panels C and D indicate the nuclear staining with DAPI for Rictor and Raptor respectively. Panels E and F indicate the specific staining of the antibodies of Rictor and Raptor respectively (Mparmpakas *et al*., 2011).

Negative controls were included in the study where the primary antibody was omitted. The results are shown below in Fig. 4.6.

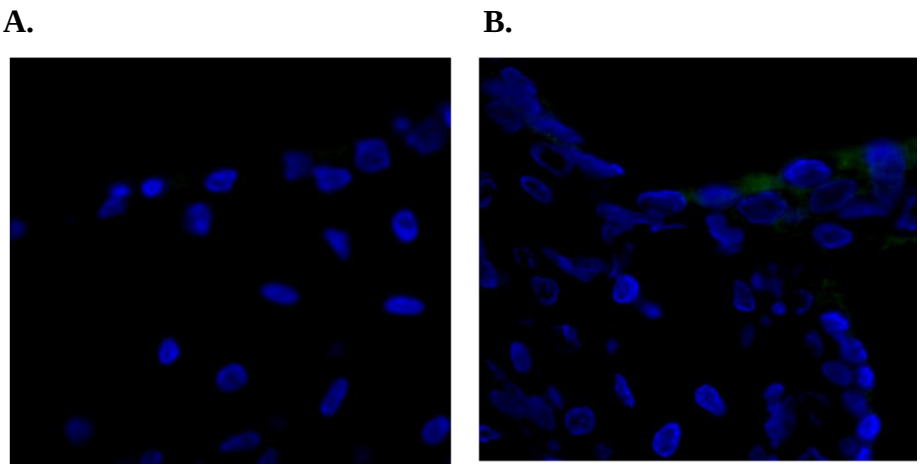


Figure 4.6: Negative controls where primary antibody was omitted, thus demonstrating staining specificity for mTOR (Panel A) and Deptor (Panel B) respectively.

4.2.3. Measurement of Cortisol levels in maternal and foetal samples.

ELISA assays were used to measure the plasma cortisol levels. Mothers with high stress had significantly (P = 0.035) elevated levels of cortisol 8.555 pg/ml ± 925 SEM compared to those with low stress 4.900 pg/ml ± 1700 SEM (Fig.4.7). The average pregnancy days for the high stress group were 223.2 ± 60 whereas the average gestational period for the low stress group was 253.7 ± 23 days. The foetal cortisol from mothers with high stress was measured at 7.440 pg/ml ± 1349 SEM, whereas foetal cortisol from mothers with low stress was measured at 7.525 pg/ml ± 1173 SEM. There was no significance amongst the two groups (P > 0.4). Interestingly, a significant positive correlation between maternal and foetal cortisol was detected in the high but not the low stress group (r = 0.678, P = 0.022). In the high stress group, there was also a significant inverse correlation between foetal cortisol and placental weight (r = -0.638, P = 0.024) as well as foetal weight (r = -0.642, P = 0.023). In

the low stress group there was a significant correlation between foetal cortisol and placental weight (r = 0.804, P = 0.029 as well as foetal weight (r = 0.784, P = 0.037).

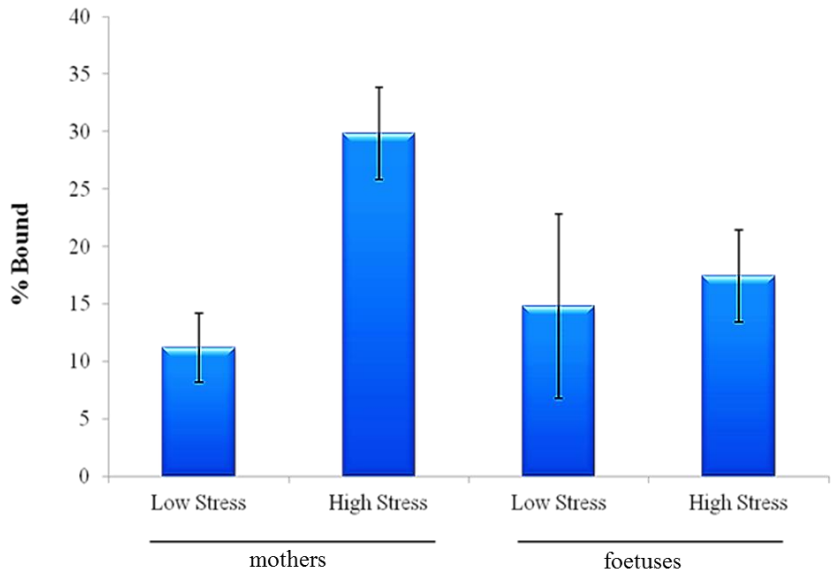


Figure 4.7: Percentage of bound cortisol in low and high stress groups of mothers and foetuses.

Also there was shown to be a significant correlation between placental and foetal weight both in the high (r = 0.983, P < 0.0001) and low maternal stress groups (r = 0.870, P < 0.011). Finally, umbilical cord (foetal) cortisol samples did not vary significantly between the term (8.254 pg/ml ±949 SEM) and pre-term groups (6.341 pg/ml ± 1822 SEM, P > 0.1).

Finally, taking into consideration the smoking status of the patients before and during pregnancy it was shown that of the 23 women, 17 did not smoke (74%) and 6 did (26%) before pregnancy. During pregnancy, 19 did not smoke (83%) whereas only 4 kept smoking (17%). Placental Deptor levels were not statistically different in the two groups during pregnancy.

4.2.4. Expression of mTOR, Deptor, Rictor, Raptor in BeWo CT and JEG-3 cells

The expression of mTOR, Deptor, Rictor and Raptor genes was examined in BeWo and JEG-3 cells. The Relative Quantity (RQ) values of BeWo cells are set at 1 as this was the calibrator cell line (Fig. 4.8). Significantly lower levels of Deptor, Rictor and Raptor are observed in JEG-3 cells when compared to BeWo cells.

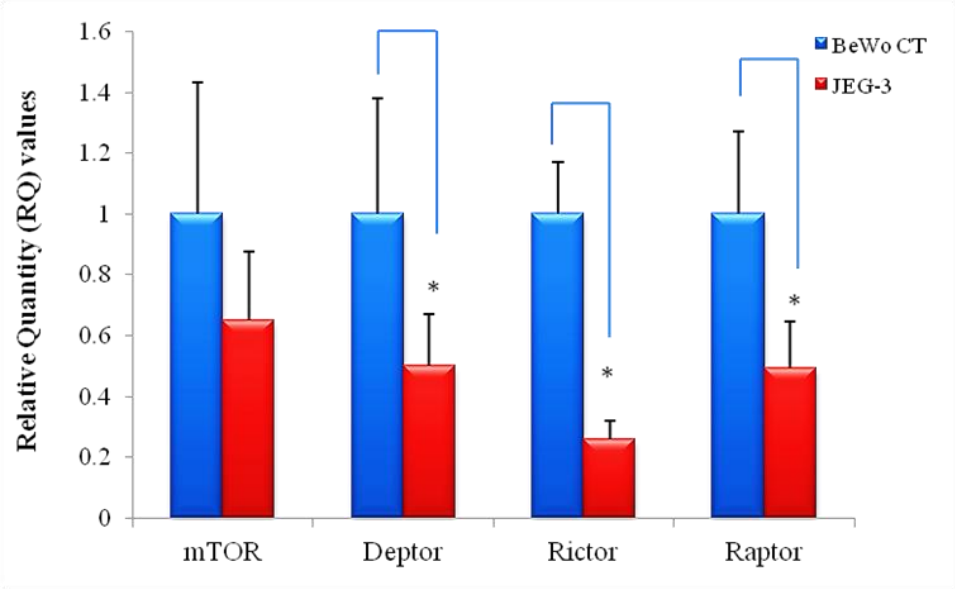


Figure 4.8: Expression of mTOR, Deptor, Rictor, Raptor in BeWo CT and JEG-3 cells. Each histogram represents the mean ± [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments ($P < 0.05$ *).

4.2.5. Cellular localisation of mTOR and Deptor in BeWo CT and JEG-3 cells.

Immunofluorescent analysis using a specific antibody for mTOR revealed cytoplasmic localisation of mTOR in both cell lines (Fig. 4.9 A & B). The staining of mTOR appeared granular and dispersed in nature. The cellular distribution of Deptor in the two cell lines was cytoplasmic. In BeWo cells Deptor localised primarily around the nucleus (Fig. 4.9 C) whereas in JEG-3 cells a more dispersed cytoplasmic staining was evident (Fig. 4.9 D).

Negative controls were included in the study where the primary antibody was omitted. The results are shown below in Fig. 4.9, panels E & F.

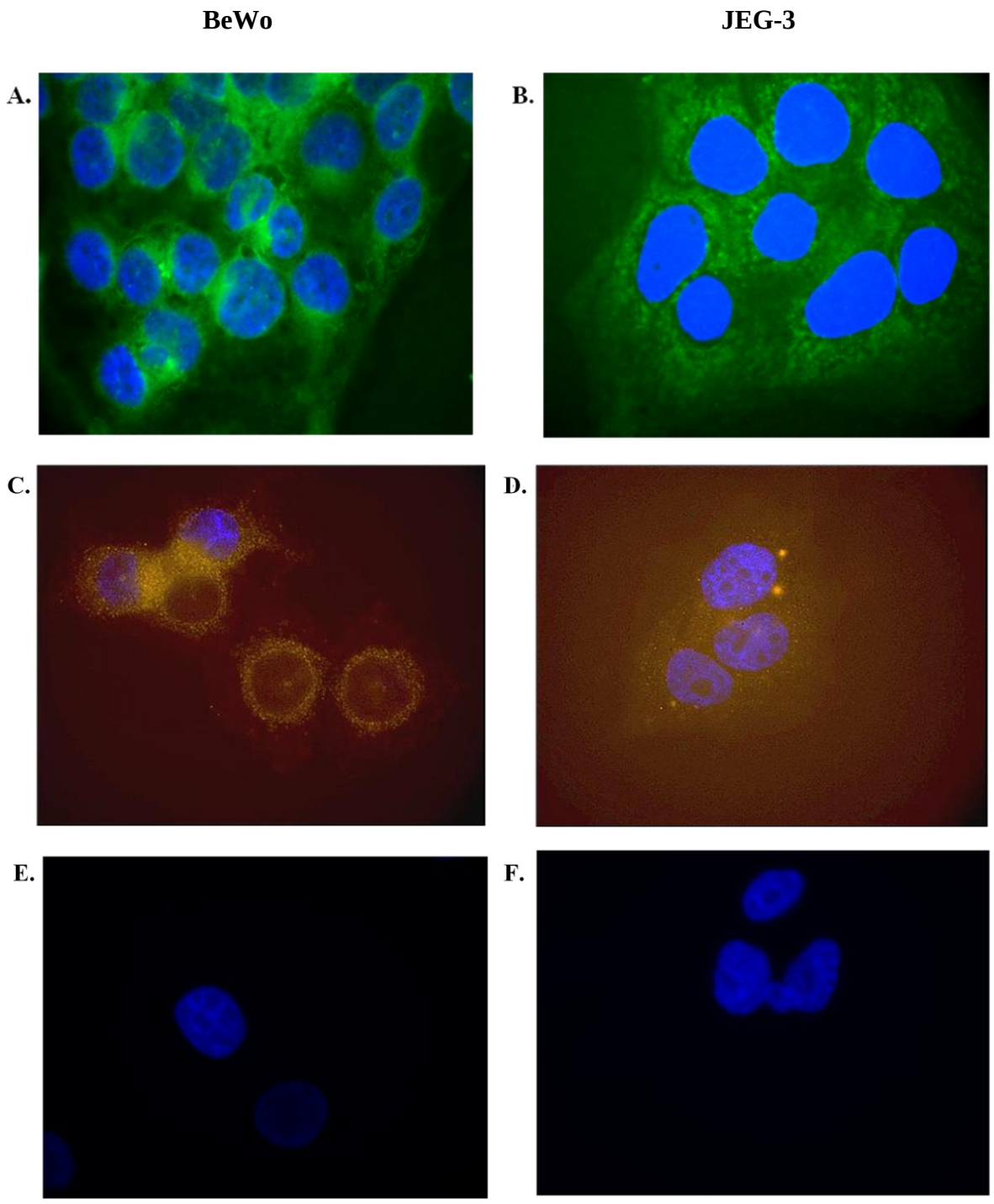


Figure 4.9: Panel A and B: Immunofluorescence analysis of mTOR in BeWo cells (A) and JEG-3 cells (B). Panel C and D: Immunofluorescence analysis of Deptor in BeWo cells (C) and JEG-3 cells (D). Panel E and F: Negative controls of BeWo and JEG-3 respectively (Mparmpakas *et al*., 2010; Mparmpakas *et al*., 2011).

4.2.6. Effects of cortisol on the expression of Deptor in BeWo CT and JEG-3 cells

Given the inverse relationship of stress and Deptor in our clinical samples, we tested the hypothesis that cortisol might affect Deptor expression directly. BeWo and JEG-3 cells were treated overnight with cortisol, in an attempt to resemble a moderate and high stress environment *in vitro*. When BeWo cells were treated with 10nM, 100nM or 1000nM cortisol, the expression of Deptor was significantly downregulated by 50%, 41% and 39% (all $P < 0.05$) respectively when compared with basal levels (Fig. 4.10).

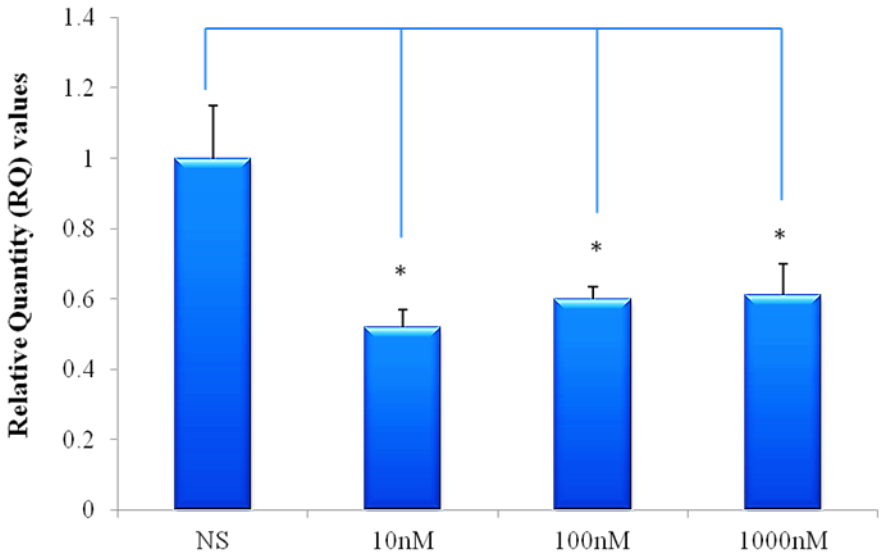


Figure 4.10: Quantitative analysis of Deptor levels in BeWo CT cells following 24 hours of cortisol treatment, significance ($P < 0.05$ *) (NS=no supplement) (Mparmpakas *et al*., 2011).

Treatment of JEG-3 cells with cortisol, lead to a significant decrease of Deptor expression at 100nM (39%, $P < 0.05$) and at 1000nM (73%, $P < 0.01$) when compared with basal levels

(Fig. 4.11). However, cortisol treatments did not exert any significant changes in the gene expression of mTOR, Rictor or Raptor in both cell lines (data not shown).

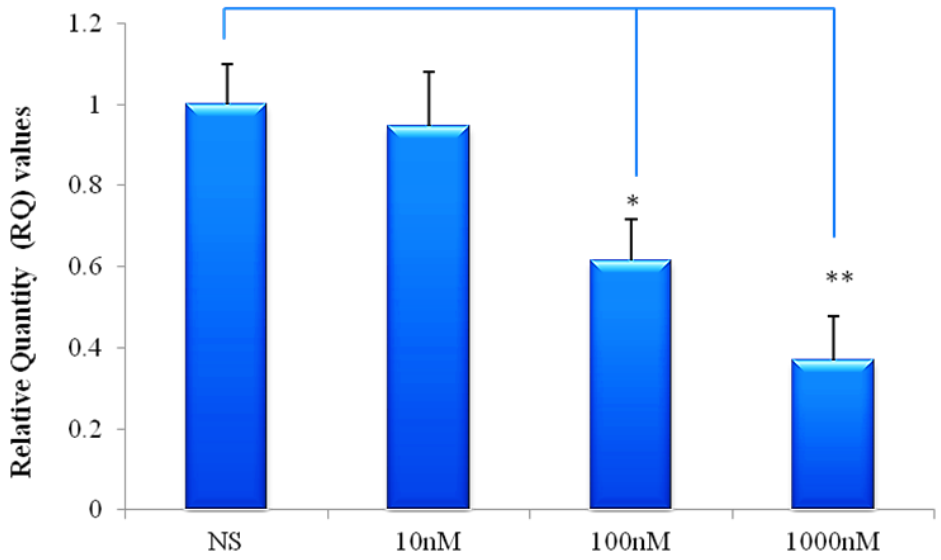


Figure 4.11: Quantitative analysis of Deptor levels in JEG-3 cells following 24 hours of cortisol treatment significance ($P < 0.05$ *) and ($P < 0.01$ **), (NS = no supplement), (Mparmpakas *et al*., 2011).

4.3. Discussion

In this chapter of our study, 23 pregnant women self-reported their stress status ranging from low and medium (Low stress response) to high and very high (High stress response). The source of the stress was not reported. Generally, maternal stress could be due to lack of social support, anxiety about the pregnancy outcome, or environment-related stressful conditions (Douglas, 2010). Nepomnaschy and colleagues have suggested that human placentation is an important period for examining the relationship between maternal stress and pregnancy outcome (Nepomnaschy *et al*., 2006). This was based on the findings of their study showing

that women with increased cortisol during the first 3 weeks after conception were more likely to result in spontaneous abortion. Indeed a major finding in our study was the significant inverse correlation between mRNA levels of placental Deptor and self-reported stress levels. None of the other mTOR components displayed any correlation with stress. It should be noted that the levels of self-reported stress were evaluated only at the third trimester. However, prenatal severe life events in the first and second but not third trimester were associated with an increased risk of pre-term birth (Zhu *et al*., 2010). Though most studies suggest that prenatal maternal stress predicts adverse infant outcomes (Dole *et al.*, 2003; Rini *et al*., 1999; Edwards *et al*., 1994), a large scale Danish study found maternal stress to predict greater infant birth weight (Tegethoff *et al*., 2010). The exact role of Deptor is relatively unknown, but emerging data suggest it plays a role in certain cells' survival (Peterson *et al*., 2009). Thus, it is possible that via reduced Deptor, stress could lead to alterations in placental growth that can impact on foetal development. In our study we provide evidence for a dual role of Deptor in term and pre-term placentas. Pre-term birth is the most important problem in maternal-child health. Numerous epidemiological studies suggested that maternal stress is associated significantly with onset of spontaneous pre-term birth (Wadhwa *et al.*, 1993). Despite extensive research, the mechanisms that drive this are still poorly understood. As a result, the prevalence rate of pre-term birth still remains high. Several studies have documented an increase of the endogenous levels of cortisol with pregnancy (Rothenberger *et al*., 2011; Ruiz *et al*., 2005). However, due to different methodological approaches, the cortisol values tend to vary. For example, one group has shown that cortisol values varied between 13 and 46 nmol/l when sampled at 37 and 38 weeks of pregnancy. The mean cortisol levels were 25.3 nmol/l (de Weerth *et al*., 2003). However, in another study, a greater variation of cortisol levels in late pregnancy was noted, ranging from 3.7 to 55 nmol/l with a median of 11.80 nmol/l (Obel *et al*., 2005). Given the importance of cortisol as a stress

marker, we hypothesised that it might affect Deptor expression directly. We tested this *in vitro,* employing two well established choriocarcinoma cell lines (BeWo and JEG-3) that have been used widely to decipher placental signalling. Both cell lines were treated over 24 hours with 10, 100 and 1000 nM of cortisol in an attempt to resemble moderate, high and very high stress levels. Treatment of JEG-3 cells with cortisol resulted in a dose-dependent downregulation of Deptor, reaching significance at 100nM and 1000nM. However, when the same treatments were repeated in BeWo cells, dose-dependence was not observed. This could be due to inherent differences that these two cell lines appear to possess. Microarray analyses revealed that up to 2700 genes are differentially expressed between the two cell lines (Burleigh *et al*., 2007). Interestingly, principal differences observed in various biological processes, including response to stress and signal transduction, were noted (Roos e*t al*., 2007). In our study, we demonstrate that Deptor is expressed in higher amounts in BeWo cells than in JEG-3 cells. It is possible that this overexpression is responsible for the partial resistance of downregulation of Deptor by cortisol in this cell line.

CHAPTER 5

EXPRESSION AND CELLULAR LOCALISATION OF GLUCOCORTICOID RECEPTOR SPLICE VARIANTS AND GROWTH ARREST-SPECIFIC 5 IN HUMAN PLACENTA AND PLACENTAL MODELS

5.1. Introduction

Cortisol is synthesised by the human adrenal cortex in response to CRH/ACTH and influences a number of responses including metabolism, immunity and is also involved in foetal development. In terms of pregnancy, cortisol plays a key role in foetal organ maturation (e.g. lung, liver and brain) and also controls synthesis of placental hormones that can affect the onset of labour (Johnson *et al*., 2008). In this study we determined the distribution of 4 mature GR mRNA spliced isoforms (GRα, GRβ, GRγ and GR-P) in order to assess labour and pregnancy-planning associated changes. We have also decided to include in our study the expression of Growth-Arrest-Specific Transcript-5 (GAS5) as it can act as a GR DNA binding decoy and compromise glucocorticoid activity.

5.2. Results

5.2.1. Glucocorticoid receptors (GRs) and the human placenta

A total of 23 human placental tissue samples were obtained from the Department of Obstetrics and Gynaecology, University Hospital of University of Crete during this study. These samples where used to investigate the expression of GRs in the human placenta. A dot plot graph was constructed in order to indicate not only the average expression of these genes in the human placental cohort, but also to determine an expression pattern per patient. Fig. 5.1 illustrates the relative expression of GRα, GRβ, GRγ and GR-P. An inter-patient variation is mainly seen in GRα whereas the expression of the other GR splice variants is more homogeneously expressed in our human placental samples.

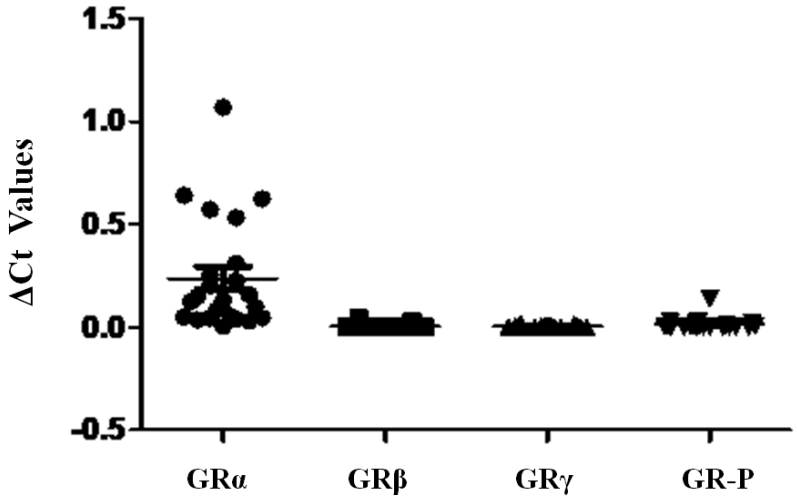


Figure 5.1: Summary graph of the ΔCt values showing the expression of GR genes in the human placentas.

Individual graphs of each gene were plotted illustrating each placental sample and the expression of the representative GR genes. As shown in the panels in Fig. 5.2, the expression of GRα is the higher observed in the human placenta in comparison to the Δ*Ct* values obtained for the other three GR genes. The y-axis represents the Δ*Ct* values of the gene expression as it was calculated from the Q-PCR analysis.

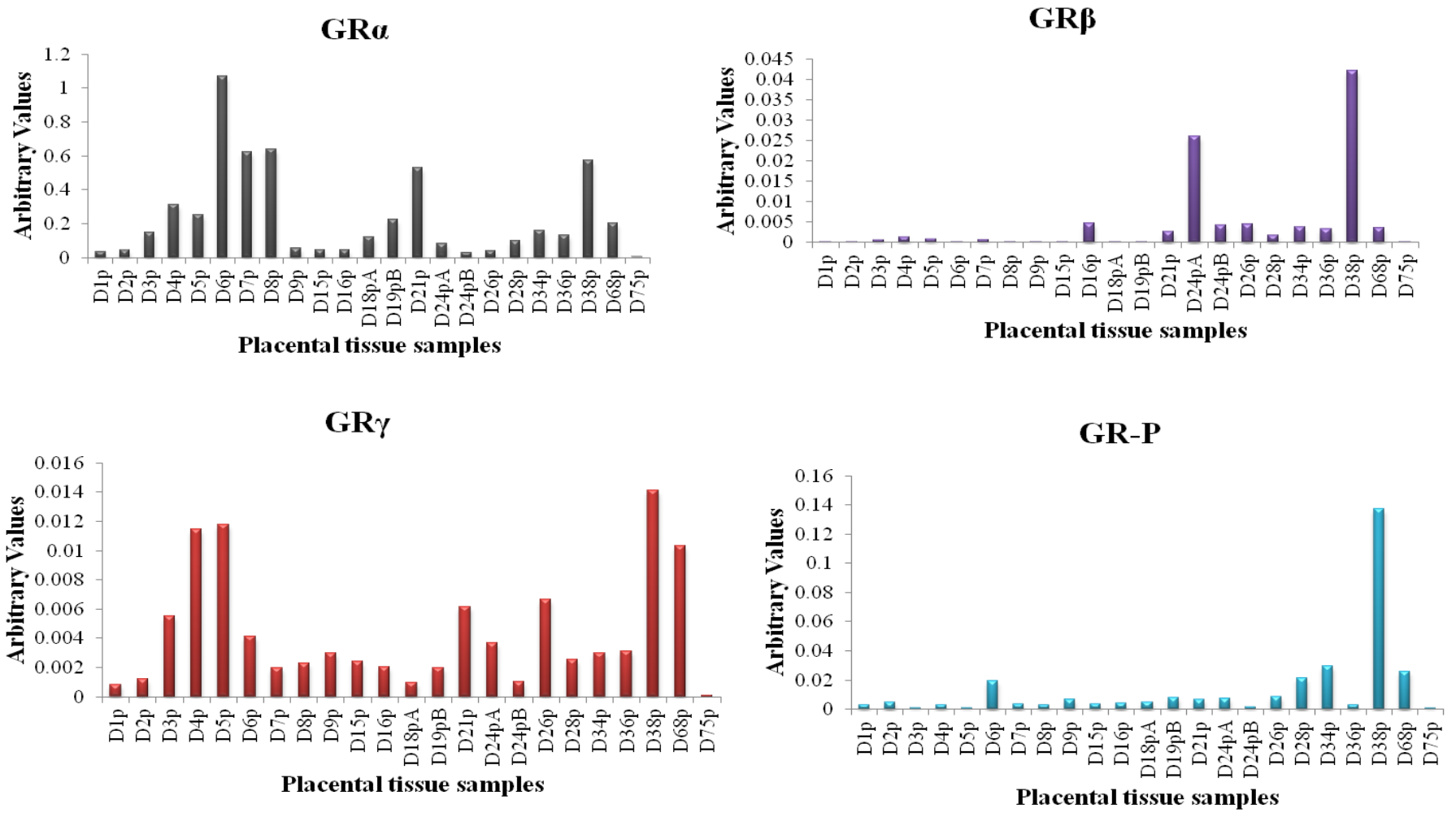


Figure 5.2: Expression of GR genes in each placental sample.

5.2.1 GAS5 and the human placenta

Placental expression of GAS5 showed an inter patient variation and this is depicted in Fig 5.3.

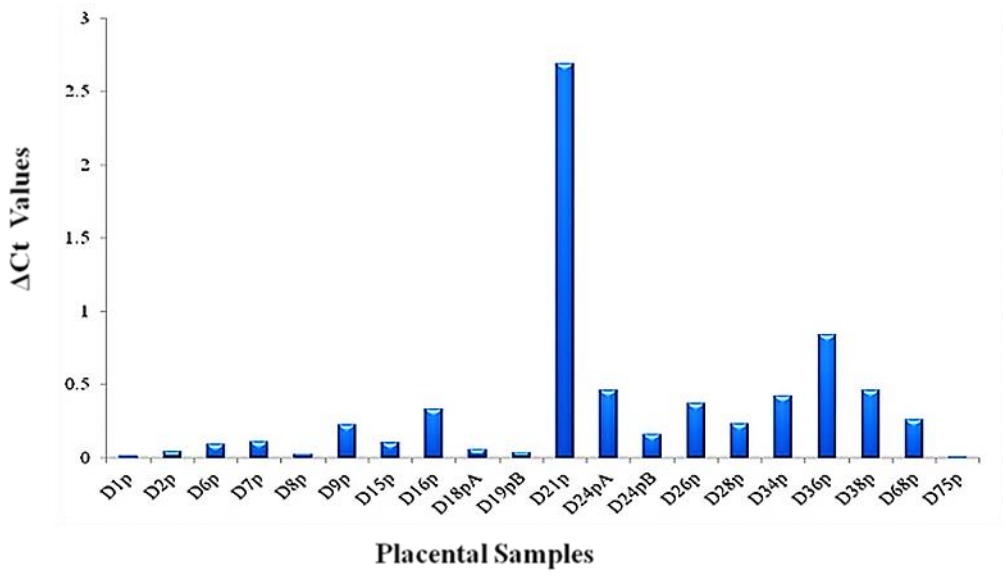


Figure 5.3: Expression of GAS-5 mRNA transcript in each placental sample.

The expression of the GR splice variants as well as the expression of GAS5, values were analysed according to the time of delivery (i.e. being at term or pre-term). Fig. 5.4 shows the results of this analysis.

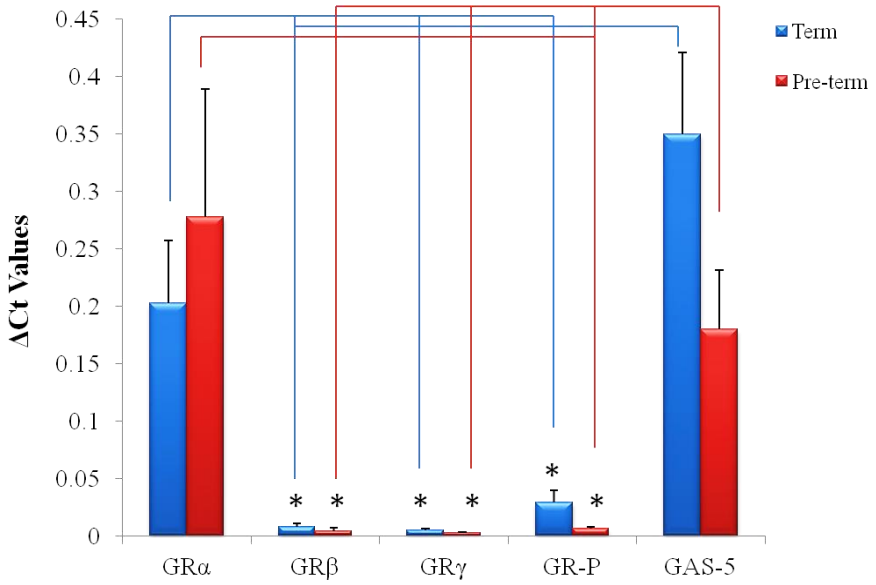


Figure 5.4: Expression of GR genes and GAS5 in the term and pre-term category of our placental samples.

The expression of these genes was also categorised according to the contractile status as performed earlier in the mTOR section.

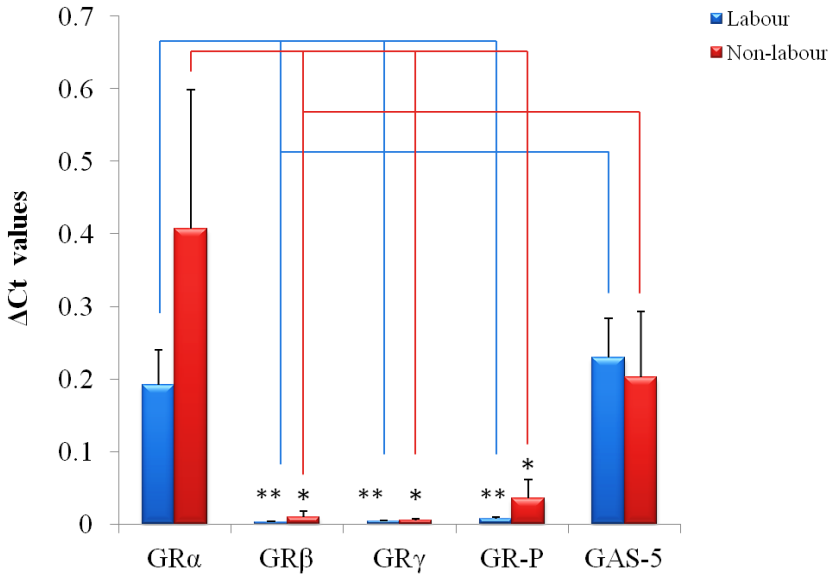


Figure 5.5: Expression of GR genes and GAS5 in the labour and non-labour category of our placental samples.

Also the expression of the GR genes, as well as the expression of GAS5 was analysed according to whether the pregnancy was planned or non-planned. Fig.5.6 shows the results of this analysis.

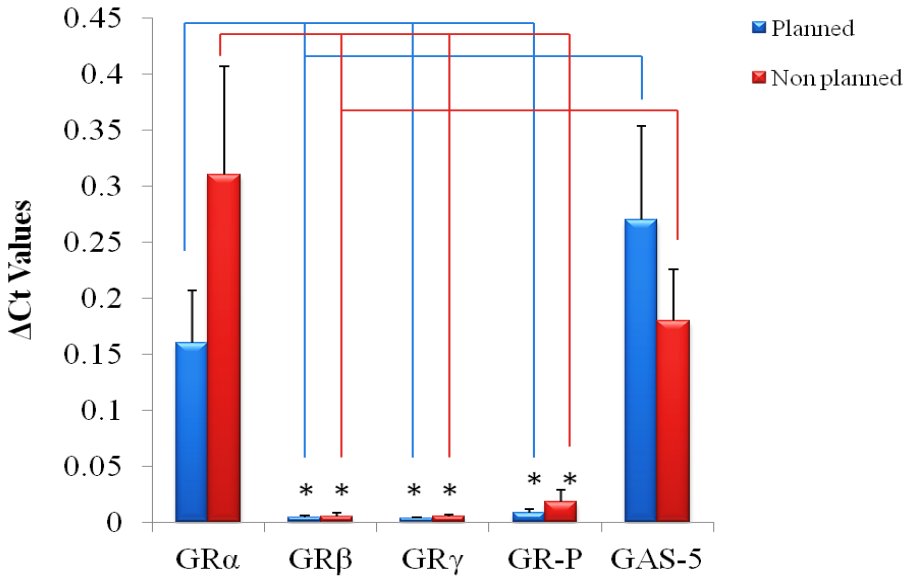


Figure 5.6: Expression of GR genes and GAS-5 in the planned and non-planned category of our placental samples.

5.2.2. Cellular distribution of GRα and GAS5 in human placentas

Immulofluorescence analysis of the GRα protein was performed in human placenta tissue sections. It is evident from Fig. 5.7 that strong homogeneous staining mainly in the cytoplasm is detected in the syncytiotrophoblast cells on the outermost layer of the placental villi. The GR protein in the cytotrophoblast cells appears to be mainly dotted in staining pattern and to be present in the cytoplasm.

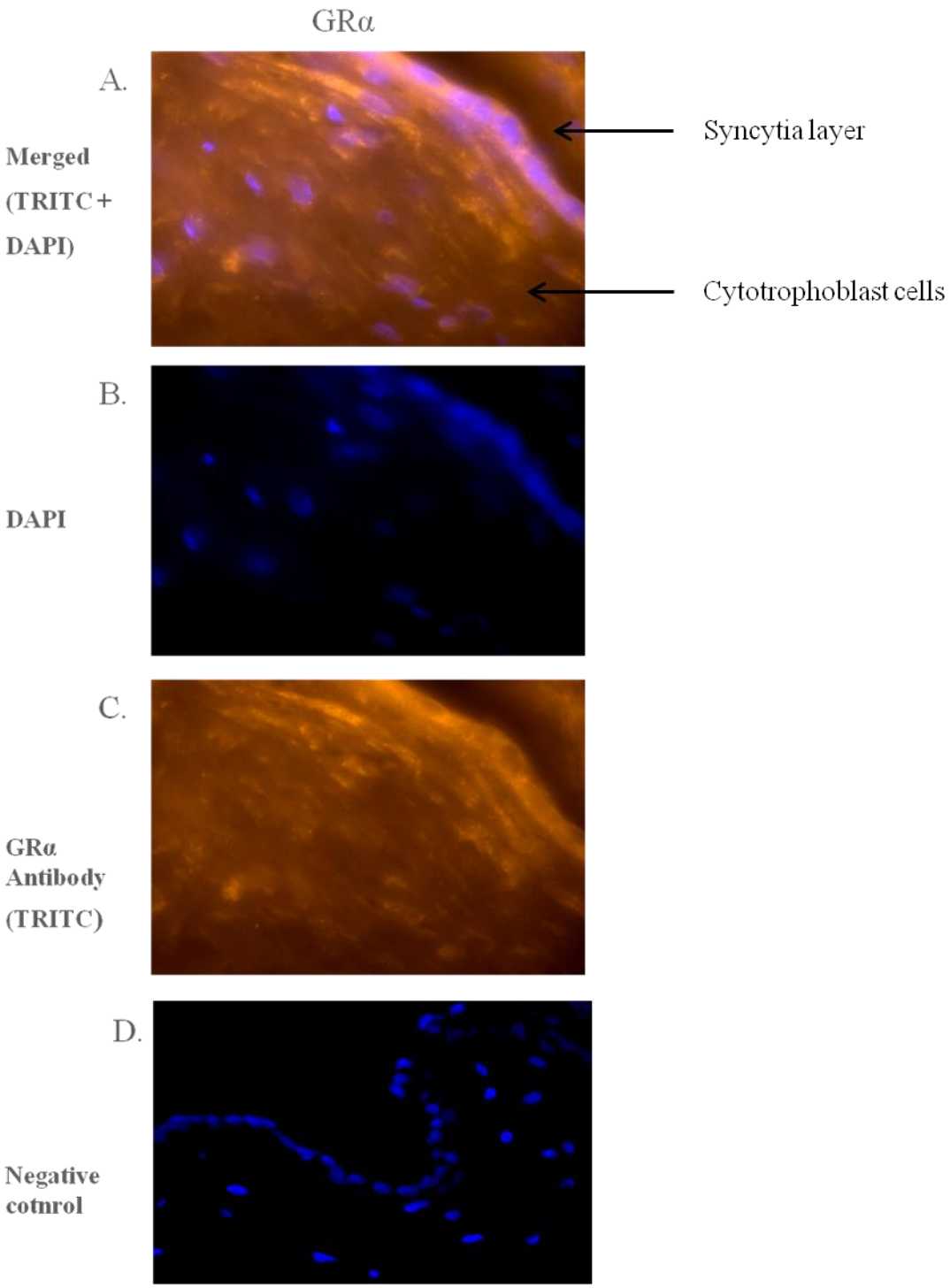


Figure 5.7: Immunostaining using GRα antibody in human placenta tissue sections. Panel A: Merged image of specific staining and nucleus staining with DAPI. Panel B: Nucleus staining. Panel C: Specific staining of the GRα protein. Panel D: Negative control.

After confirming the expression of GAS5 at gene level using Q-PCR, the RNA-FISH technique was used to localise the mRNA in human placenta tissue sections.

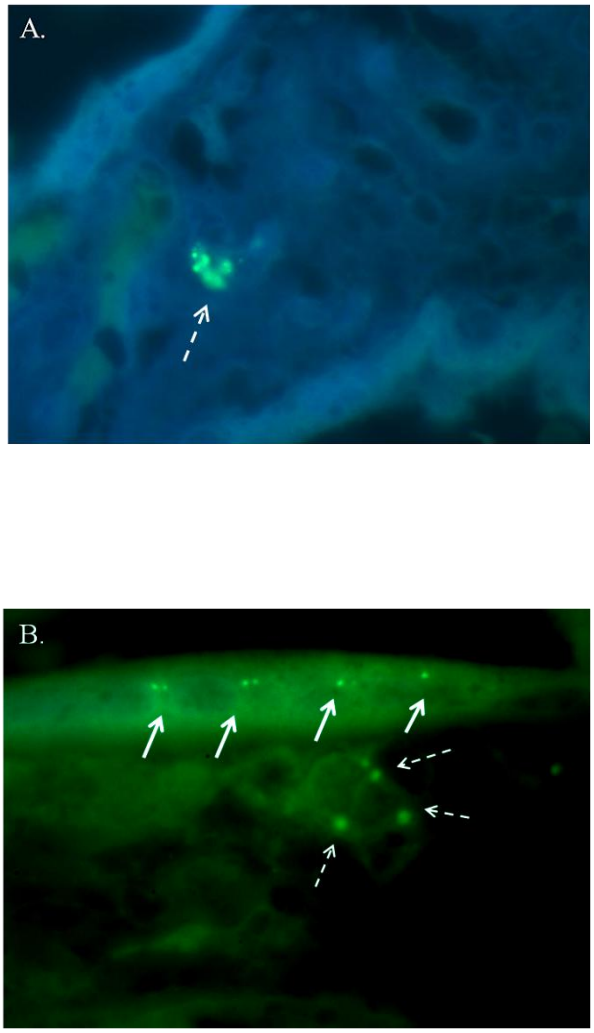


Figure 5.8: RNA FISH confirmed expression of GAS5 in cytotrophoblasts cells (Panel A & B; dotted arrows) and syncytiotrophoblasts cells (Panel B; white arrows).

5.2.3. Correlation of the placental GR expression with stress, cortisol levels, pregnancy days, placental and fetal weight, and GAS5 expression at term.

The data obtained from the questionnaire regarding the maternal stress levels, the cortisol levels, the pregnancy days, the placental and the fetal weight, were used to further analyse and to correlate the expression of all GR splice variants and GAS5 transcripts with the above variables. The correlation values (*r*) along with the significance (*P*- values) are shown in tables 5.1-5.4. We have decided to analyse our clinical data in terms of duration of pregnancy (i.e. term or pre-term deliveries) and in terms of pregnancy planning (i.e. planned or non-planned) according to the answers obtained from the queationnaire data.

		Correlations TERM									
		GRα	**GRβ**	**GRγ**	**GR-P**	**Stress**	**Cortisol**	**Pregnancy Days**	**Fetal Weight**	**Placenta Weight**	**GAS-5**
GRα	Pearson Correlation	1	0.553	0.512	0.512	0.018	0.289	-0.318	0.134	0.095	0.062
	Sig. (2-tailed)		0.050	0.073	0.074	0.955	0.389	0.371	0.663	0.757	0.865
	N	13	13	13	13	13	13	13	13	13	13
GRβ	Pearson Correlation	0.553	1	0.580*	0.980**	-0.415	0.574	0.193	0.454	0.535	0.360
	Sig. (2-tailed)	0.050		0.038	0.000	0.158	0.065	0.594	0.119	0.060	0.307
	N	13	13	13	13	13	13	13	13	13	13
GRγ	Pearson Correlation	0.512	0.580*	1	0.540	0.065	0.106	0.058	0.132	0.125	0.346
	Sig. (2-tailed)	0.073	0.038		0.057	0.834	0.757	0.873	0.666	0.685	0.328
	N	13	13	13	13	13	13	13	13	13	13
GR-P	Pearson Correlation	0.512	0.980**	0.540	1	-0.464	0.638*	0.238	0.475	0.563*	0.311
	Sig. (2-tailed)	0.074	0.000	0.057		0.110	0.035	0.507	0.101	0.045	0.381
	N	13	13	13	13	13	13	10	13	13	13
Stress	Pearson Correlation	0.018	-0.415	0.065	-0.464	1	-0.489	-0.486	-0.591*	-0.732**	-0.711*
	Sig. (2-tailed)	0.955	0.158	0.834	0.110		0.126	0.154	0.034	0.004	0.021
	N	13	13	13	13	13	13	13	13	13	13
Cortisol	Pearson Correlation	0.289	0.574	0.106	0.638*	-0.489	1	0.747*	0.309	0.393	0.290
	Sig. (2-tailed)	0.389	0.065	0.757	0.035	0.126		0.033	0.355	0.232	0.486
	N	13	13	13	13	13	13	13	13	13	13
Pregna. Days	Pearson Correlation	-0.318	0.193	0.058	0.238	-0.486	0.747*	1	0.279	0.410	0.017
	Sig. (2-tailed)	0.371	0.594	0.873	0.507	0.154	0.033		0.434	0.239	0.969
	N	13	13	13	13	13	13	13	13	13	13
Foetal Weight	Pearson Correlation	0.134	0.454	0.132	0.475	-0.591*	0.309	0.279	1	0.962**	0.444
	Sig. (2-tailed)	0.663	0.119	0.666	0.101	0.034	0.355	0.434		0.000	0.198
	N	13	13	13	13	13	13	13	13	13	13
Placenta Weight	Pearson Correlation	0.095	0.535	0.125	0.563*	-0.732**	0.393	0.410	0.962**	1	0.517
	Sig. (2-tailed)	0.757	0.060	0.685	0.045	0.004	0.232	0.239	0.000		0.126
	N	13	13	13	13	13	13	13	13	13	13
GAS-5	Pearson Correlation	0.062	0.360	0.346	0.311	-0.711*	0.290	0.017	0.444	0.517	1
	Sig. (2-tailed)	0.865	0.307	0.328	0.381	0.021	0.486	0.969	0.198	0.126	
	N	13	13	13	13	13	13	13	13	13	13

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5.1: Summary of correlation values (*r*) and significance (*P*-values, $P < 0.05$, $n = 13$) of the GR variants with other variables at term

The highest correlation observed in this cohort (i.e. term placentas) was that of GRβ with GR-P (r = 0.980). This correlation is depicted in Fig. 5.9 below.

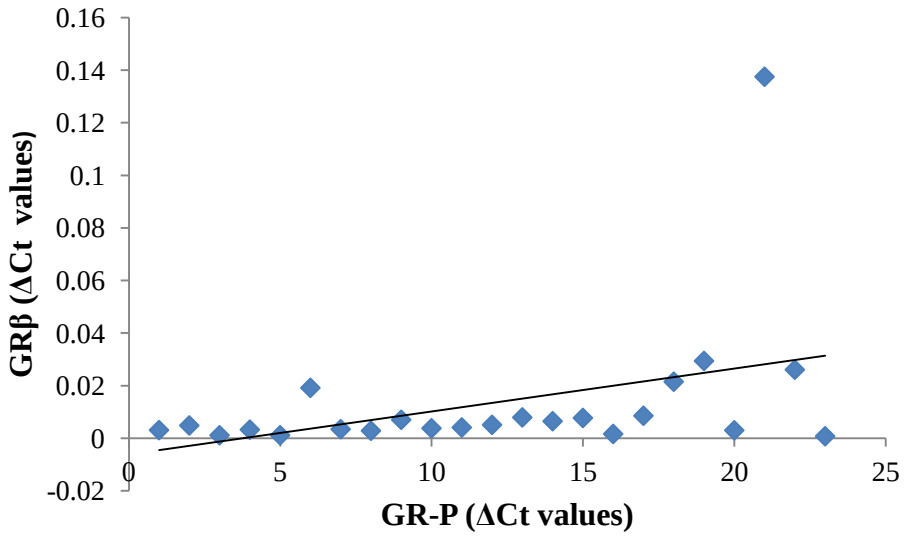


Figure 5.9: Correlation graph between GRβ and GR-P levels in term samples (r = 0.980, *P* < 0.0001, n = 13).

Another correlation observed in the term category was between the foetal and the placental weight *(r* = 0.962, *P* = 0.000, *n* =13), this is shown in Fig. 5.10 below.

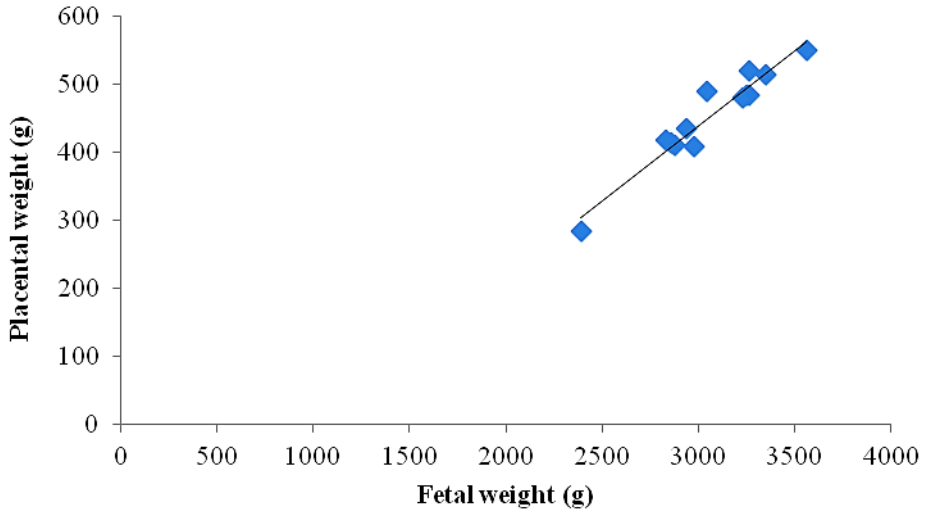


Figure 5.10: Correlation graph between the placental and fetal weight in the term samples *(r = 0.962, P = 0.000, n=13).*

5.2.4. Correlation of the placental GR expression with stress, cortisol levels, pregnancy days, placental and foetal weight, and GAS5 expression at pre-term.

Again the information received from the participants of this study was used in the pre-term category (n =10) to pursue any possible correlations between the expression of GR and GAS5 genes with variables of the questionnaire. Table 5.2. below indicates the result of the correlation in this group.

Correlations PRE-TERM											
		GRα	**GRβ**	**GRγ**	**GR-P**	**Stress**	**Cortisol**	**Pregnancy Days**	**Fetal Weight**	**Placenta Weight**	**GAS-5**
GRα	Pearson Correlation	1	-0.291	0.314	0.710*	-0.140	-0.432	0.312	0.296	0.358	-0.310
	Sig. (2-tailed)		0.415	0.376	0.021	0.719	0.568	0.381	0.407	0.310	0.417
	N	10	10	10	10	10	10	10	10	10	10
GRβ	Pearson Correlation	-0.291	1	0.215	0.024	-0.206	-0.079	-0.106	-0.132	-0.080	0.792*
	Sig. (2-tailed)	0.415		0.551	0.947	0.595	0.921	0.771	0.717	0.827	0.011
	N	10	10	10	10	10	10	10	10	10	10
GRγ	Pearson Correlation	0.314	0.215	1	0.538	0.415	-0.456	0.113	0.131	0.124	0.622
	Sig. (2-tailed)	0.376	0.551		0.108	0.267	0.544	0.756	0.718	0.734	0.074
	N	10	10	10	10	10	10	10	10	10	10
GR-P	Pearson Correlation	0.710*	0.024	0.538	1	-0.035	0.199	0.489	0.500	0.561	0.077
	Sig. (2-tailed)	0.021	0.947	0.108		0.929	0.801	0.151	0.141	0.092	0.844
	N	10	10	10	10	10	10	10	10	10	10
Stress	Pearson Correlation	-0.140	-0.206	0.415	-0.035	1	-0.830	-0.685*	-0.621	-0.695*	0.212
	Sig. (2-tailed)	0.719	0.595	0.267	0.929		0.170	0.042	0.074	0.038	0.614
	N	10	10	10	10	10	10	10	10	10	10
Cortisol	Pearson Correlation	-0.432	-0.079	-0.456	0.199	-0.830	1	0.764	0.746	0.746	0.203
	Sig. (2-tailed)	0.568	0.921	0.544	0.801	0.170		0.236	0.254	0.254	0.870
	N	10	10	10	10	10	10	10	10	10	10
Pregna. Days	Pearson Correlation	0.312	-0.106	0.113	0.489	-0.685*	0.764	1	0.986**	0.989**	-0.174
	Sig. (2-tailed)	0.381	0.771	0.756	0.151	0.042	0.236		0.000	0.000	0.654
	N	10	10	10	10	10	10	10	10	10	10
Foetal Weight	Pearson Correlation	0.296	-0.132	0.131	0.500	-0.621	0.746	0.986**	1	0.990**	-0.200
	Sig. (2-tailed)	0.407	0.717	0.718	0.141	0.074	0.254	0.000		0.000	0.606
	N	10	10	10	10	10	10	10	10	10	10
Placenta Weight	Pearson Correlation	0.358	-0.080	0.124	0.561	-0.695*	0.746	0.989**	0.990**	1	-0.186
	Sig. (2-tailed)	0.310	0.827	0.734	0.092	0.038	0.254	0.000	0.000		0.632
	N	10	10	10	10	10	10	10	10	10	10
GAS-5	Pearson Correlation	-0.310	0.792*	0.622	0.077	0.212	0.203	-0.174	-0.200	-0.186	1
	Sig. (2-tailed)	0.417	0.011	0.074	0.844	0.614	0.870	0.654	0.606	0.632	
	N	10	10	10	10	10	10	10	10	10	10

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5.2: Summary of correlation values (*r*) and significance (*P -values, P < 0.05, n = 13*) of the GR variants with other variables at pre-term.

Graphical representations of the significant correlations are presented below. From the results obtained it is shown that the expression of GRα is positively correlated to the expression of GR-P (r = 0.710, P = 0.021, n =10). Fig. 5.11 below represents this correlation.

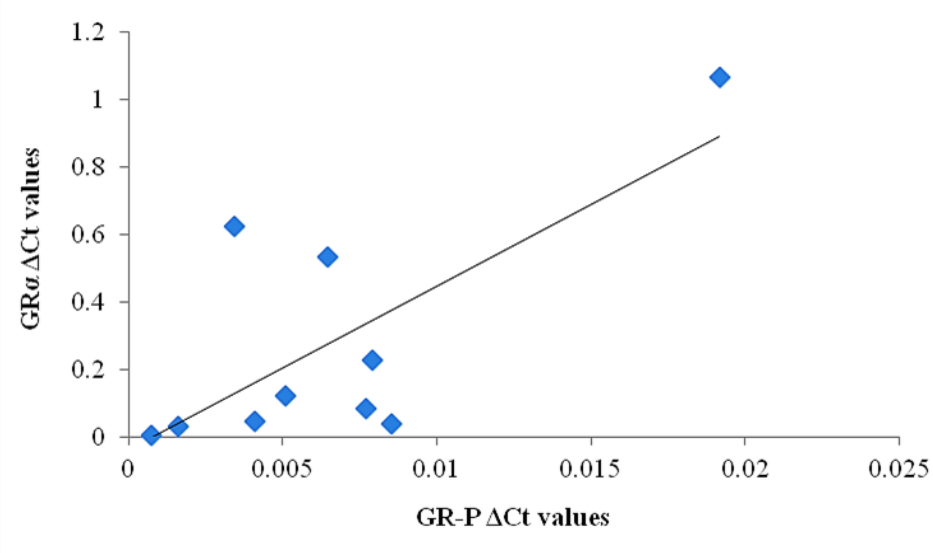


Figure 5.11: Correlation graph between GRα and GR-P in pre-term samples *(r = 0.710, P = 0.021, n=10).*

The second positive correlation in the pre-term category is the expression of GRβ with GAS5 *(r* = 0.792, *P* = 0.011, *n = 10)* and this is illustrated in Fig. 5.12 below.

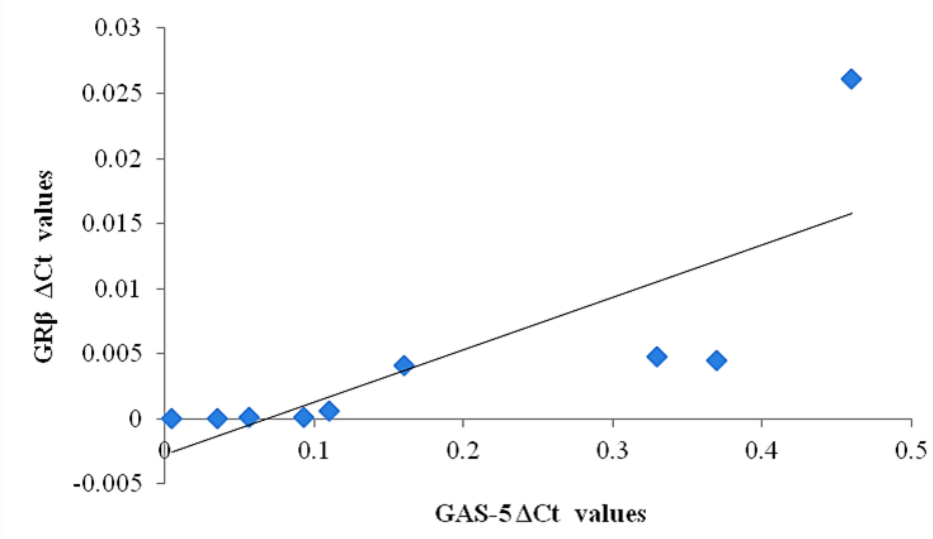


Figure 5.12: Correlation graph between GRβ and GAS5 in pre-term samples *(r = 0.792, P = 0.011, n=10).*

The duration of pregnancy measured in days is positively correlated in the pre-term category with both foetal (r = 0.986, P = 0.000, n = 10) and placental weight (r = 0.989, P = 0.000, n = 10). These correlations are depicted in Figs. 5.13 & 5.14 below.

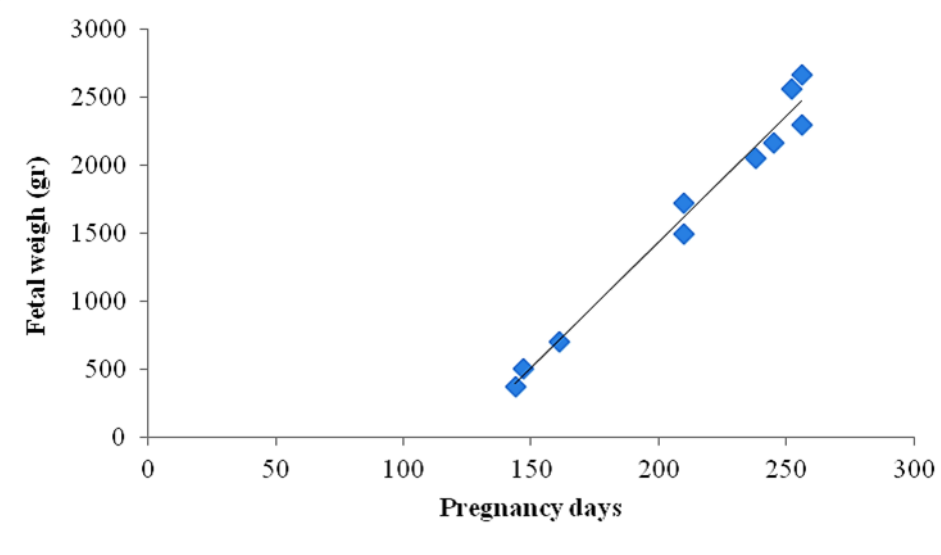


Figure 5.13: Correlation graph between foetal weight and pregnancy days in pre-term samples *(r = 0.986, P = 0.000, n = 10).*

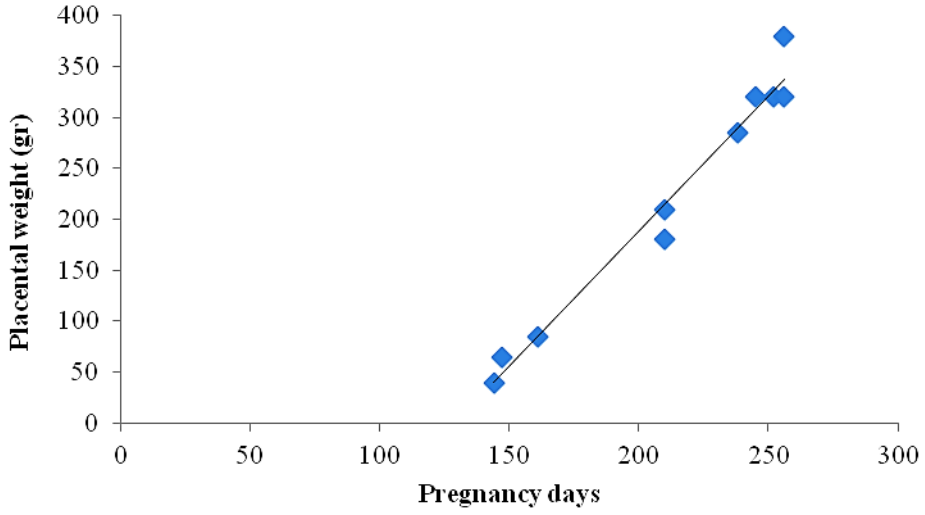


Figure 5.14: Correlation graph between placental weight and pregnancy days in pre-term samples *(r = 0.989, P = 0.000, n = 10).*

Lastly, a positive correlation was also shown between the placental and the fetal weight (r = 0.990, P = 0.000, n = 10) in the pre-term category. Fig. 5.15 below represents this correlation.

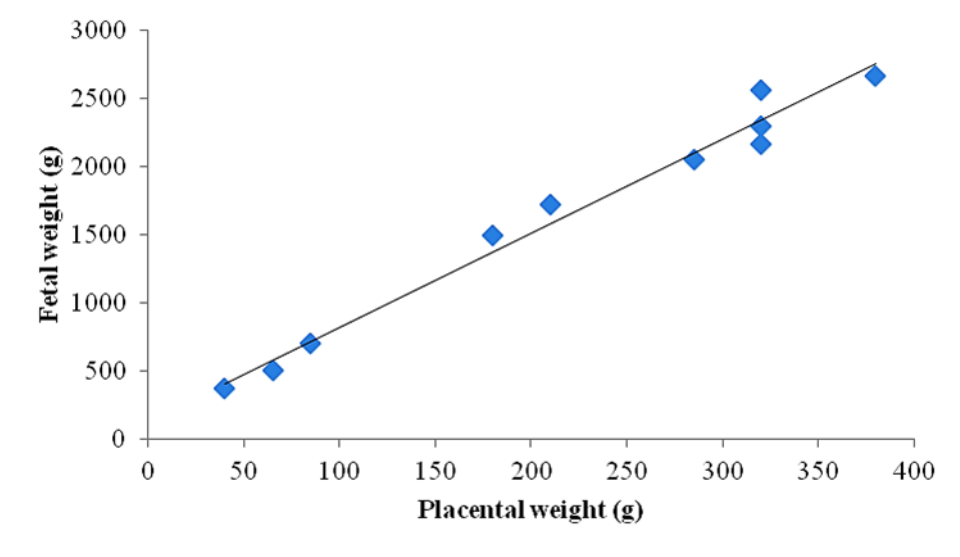


Figure 5.15: Correlation graph between foetal weight and pregnancy days in pre-term samples *(r = 0.990, P = 0.000, n = 10).*

5.2.5. Correlation of the placental GR expression with stress, cortisol levels, pregnancy days, placental and foetal weight, and GAS5 expression in planned pregnancies.

Following from the previous analysis in the term and pre-term categories, the clinical cohort was grouped and analysed based on the information in the questionnaire regarding whether the pregnancy was planned or non-planned. Possible correlations between the expression of GR and GAS-5 genes with cortisol levels, pregnancy days, foetal and placenta weight were analysed and the results are depicted in table 5.3 (*n=11*).

Correlations PLANNED											
		GRα	**GRβ**	**GRγ**	**GR-P**	**Stress**	**Cortisol**	**Pregnancy Days**	**Fetal Weight**	**Placenta Weight**	**GAS-5**
GRα	Pearson Correlation	1	-0.134	0.664*	0.015	0.521	-0.353	-0.158	-0.141	-0.152	0.881**
	Sig. (2-tailed)		0.695	0.026	0.965	0.100	0.438	0.643	0.679	0.655	0.001
	N	11	11	11	11	11	11	11	11	11	11
GRβ	Pearson Correlation	-0.134	1	0.053	0.030	-0.221	-0.433	-0.183	-0.221	-.0172	0.057
	Sig. (2-tailed)	0.695		0.877	0.931	0.514	0.332	0.590	0.513	0.614	0.876
	N	11	11	11	11	11	11	11	11	11	11
GRγ	Pearson Correlation	0.664*	0.053	1	-0.054	0.523	-0.427	0.160	0.151	0.166	0.860**
	Sig. (2-tailed)	0.026	0.877		0.876	0.099	0.339	0.638	0.657	0.627	0.001
	N	11	11	11	11	11	11	11	11	11	11
GR-P	Pearson Correlation	0.015	0.030	-0.054	1	-0.363	-0.046	0.408	0.543	0.510	-0.048
	Sig. (2-tailed)	0.965	0.931	0.876		0.272	0.922	0.213	0.084	0.109	0.896
	N	11	11	11	11	11	11	11	11	11	11
Stress	Pearson Correlation	0.521	-0.221	0.523	-0.363	1	-0.583	-0.607*	-0.544	-0.578	0.547
	Sig. (2-tailed)	0.100	0.514	0.099	0.272		0.169	0.048	0.084	0.063	0.102
	N	11	11	11	11	11	11	11	11	11	10
Cortisol	Pearson Correlation	-0.353	-0.433	-0.427	-0.046	-0.583	1	0.404	0.200	0.230	-0.455
	Sig. (2-tailed)	0.438	0.332	0.339	0.922	0.169		0.368	0.667	0.619	0.365
	N	11	11	11	11	11	11	11	11	11	11
Pregnancy Days	Pearson Correlation	-0.158	-0.183	0.160	0.408	-0.607*	0.404	1	0.952**	0.971**	-0.412
	Sig. (2-tailed)	0.643	0.590	0.638	0.213	0.048	0.368		0.000	0.000	0.237
	N	11	11	11	11	11	11	11	11	11	11
Fetal Weight	Pearson Correlation	-0.141	-0.221	0.151	0.543	-0.544	0.200	0.952**	1	0.996**	-0.329
	Sig. (2-tailed)	0.679	0.513	0.657	0.084	0.084	0.667	0.000		0.000	0.353
	N	11	11	11	11	11	11	11	11	11	11
Placenta Weight	Pearson Correlation	-0.152	-0.172	0.166	0.510	-0.578	0.230	0.971**	0.996**	1	-0.351
	Sig. (2-tailed)	0.655	0.614	0.627	0.109	0.063	0.619	0.000	0.000		0.320
	N	11	11	11	11	11	11	11	11	11	11
GAS-5	Pearson Correlation	0.881**	0.057	0.860**	-0.048	0.547	-0.455	-0.412	-0.329	-0.351	1
	Sig. (2-tailed)	0.001	0.876	0.001	0.896	0.102	0.365	0.237	0.353	0.320	
	N	11	11	11	11	11	11	11	11	11	11

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5.3: Summary of correlation values (*r*) and significance (*P -values, P < 0.05, n =13*) of the GR variants with other variables in planned pregnancies

From the results obtained the expression of GRα is positively correlated to the expression of GR-γ (r = 0.664, P = 0.026, n =11). Fig. 5.16 below represents this correlation.

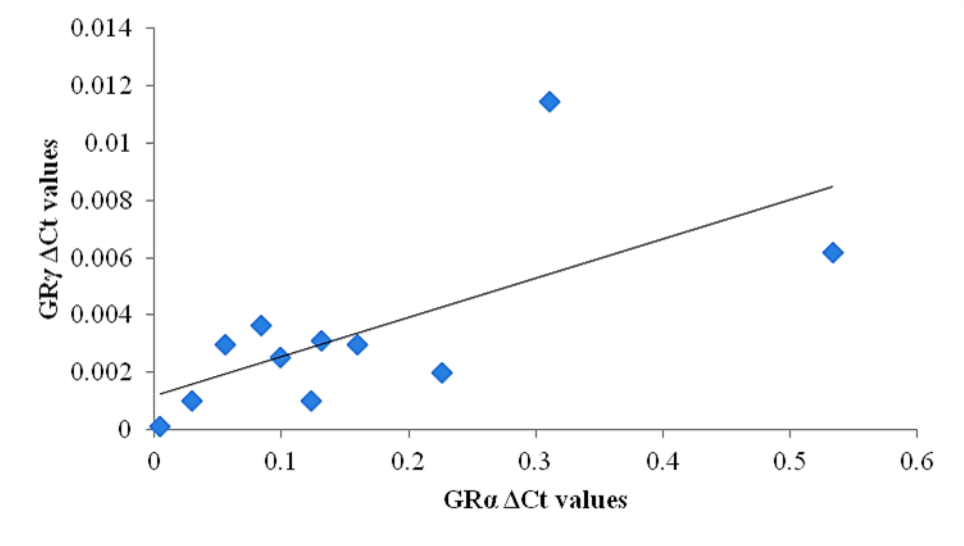


Figure 5.16: Correlation graph between GRα and GRγ in planned pregnancy samples *(r = 0.664, P = 0.026, n = 11).*

The duration of pregnancy showed positive correlations with both foetal (r = 0.952, P = 0.000, n = 11) and placental weight (r = 0.996, P = 0.000, n = 11). These correlations are illustrated in Figs 5.17 & 5.18 below.

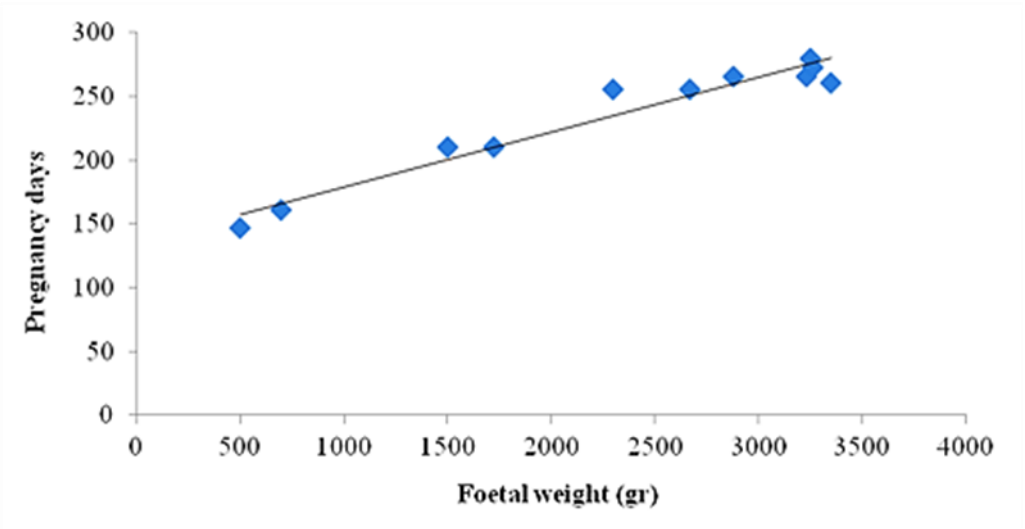


Figure 5.17: Correlation graph between pregnancy days and foetal weight in planned pregnancy samples *(r = 0.952, P = 0.000, n = 11).*

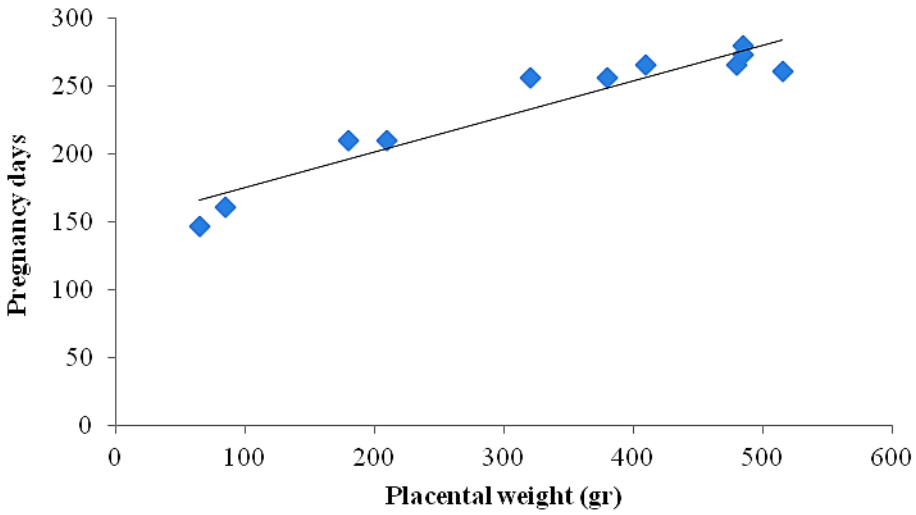


Figure 5.18: Correlation graph between pregnancy days and placental weight in planned pregnancy samples *(r = 0.996, P = 0.000, n = 11).*

The last correlation of the planned category is between the foetal and the placental weight *(r* = 0.996, *P* = 0.000, *n* = 11) .Fig. 5.19. below represents this correlation.

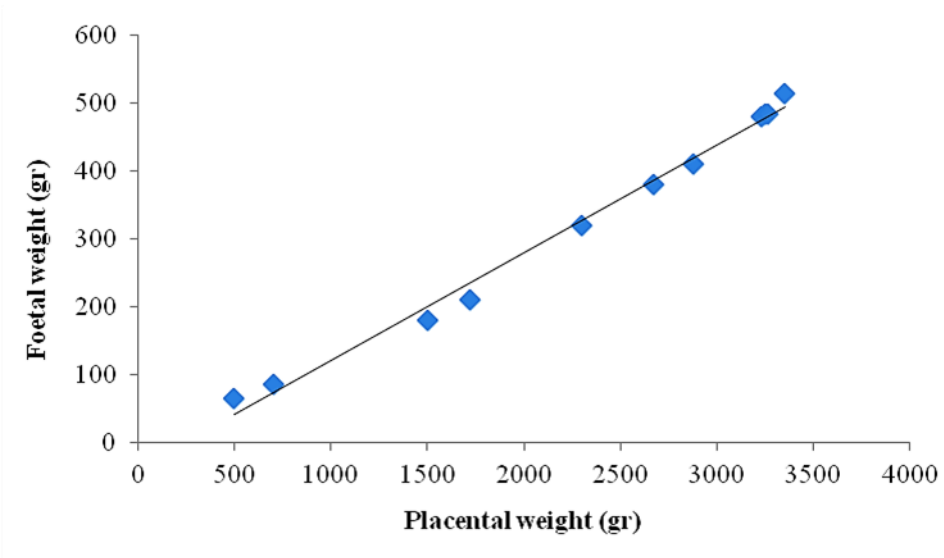


Figure 5.19: Correlation graph between pregnancy days and placental weight in planned pregnancy samples *(r = 0.996, P = 0.000, n = 11).*

5.2.6. Correlation of the placental GR expression with stress, cortisol levels, pregnancy days, placental and fetal weight, and GAS5 expression in non planned pregnancies.

The last category in which the information obtained from the questionnaire were used to determine possible correlations, was the unplanned pregnancy group (*n=12*). Table 5.4 below illustrates the results obtained.

Correlations NO PLANNED											
		GRα	**GRβ**	**GRγ**	**GR-P**	**Stress**	**Cortisol**	**Pregnancy Days**	**Fetal Weight**	**Placenta Weight**	**GAS-5**
GRα	Pearson Correlation	1	0.188	0.119	0.302	-0.707*	0.232	0.259	0.082	0.158	-0.114
	Sig. (2-tailed)		0.559	0.714	0.340	0.015	0.580	0.501	0.800	0.623	0.754
	N	12	12	12	12	12	12	12	12	12	12
GRβ	Pearson Correlation	0.188	1	0.648*	0.978**	-0.646*	0.517	0.158	0.325	0.280	0.720*
	Sig. (2-tailed)	0.559		0.023	0.000	0.032	0.190	0.684	0.302	0.378	0.019
	N	12	12	12	12	12	12	12	12	12	12
GRγ	Pearson Correlation	0.119	0.648*	1	0.663*	-0.278	0.055	0.390	0.441	0.408	0.775**
	Sig. (2-tailed)	0.714	0.023		0.019	0.409	0.896	0.300	0.152	0.188	0.008
	N	12	12	12	12	12	12	12	12	12	12
GR-P	Pearson Correlation	0.302	0.978**	0.663*	1	-0.645*	0.459	0.280	0.398	0.382	0.645*
	Sig. (2-tailed)	0.340	0.000	0.019		0.032	0.253	0.466	0.200	0.221	0.044
	N	12	12	12	12	12	12	12	12	12	12
Stress	Pearson Correlation	-0.707*	-0.646*	-0.278	-0.645*	1	-0.423	-0.146	-0.134	-0.125	-0.322
	Sig. (2-tailed)	0.015	0.032	0.409	0.032		0.296	0.730	0.694	0.713	0.398
	N	12	12	12	12	12	12	12	12	12	12
Cortisol	Pearson Correlation	0.232	0.517	0.055	0.459	-0.423	1	-0.737	-0.511	-0.582	0.802
	Sig. (2-tailed)	0.580	0.190	0.896	0.253	0.296		0.155	0.195	0.130	0.055
	N	12	12	12	12	12	12	12	12	12	12
Pregnancy Days	Pearson Correlation	0.259	0.158	0.390	0.280	-0.146	-0.737	1	0.927**	0.962**	-0.237
	Sig. (2-tailed)	0.501	0.684	0.300	0.466	0.730	0.155		0.000	0.000	0.572
	N	12	12	12	12	12	12	12	12	12	12
Fetal Weight	Pearson Correlation	0.082	0.325	0.441	0.398	-0.134	-0.511	0.927**	1	0.973**	-0.082
	Sig. (2-tailed)	0.800	0.302	0.152	0.200	0.694	0.195	0.000		0.000	0.822
	N	12	12	12	12	12	12	12	12	12	12
Placenta Weight	Pearson Correlation	0.158	0.280	0.408	0.382	-0.125	-0.582	0.962**	0.973**	1	-0.176
	Sig. (2-tailed)	0.623	0.378	0.188	0.221	0.713	0.130	0.000	0.000		0.626
	N	12	12	12	12	12	12	12	12	12	12
GAS-5	Pearson Correlation	-0.114	0.720*	0.775**	0.645*	-0.322	0.802	-0.237	-0.082	-0.176	1
	Sig. (2-tailed)	0.754	0.019	0.008	0.044	0.398	0.055	0.572	0.822	0.626	
	N	12	12	12	12	12	12	12	12	12	12

*. Correlation is significant at the 0.05 level (2-tailed).

**. Correlation is significant at the 0.01 level (2-tailed).

Table 5.4: Summary of correlation values (r) and significance (*P*-values, $P < 0.05$, n=13) of the GR variants with other variables in non planned pregnancies.

The expression of GRβ revealed positive correlations with the expression of GRγ (r = 0.648, P = 0.023, n = 12). A graph illustrating this correlation is depicted below (Fig. 5.20).

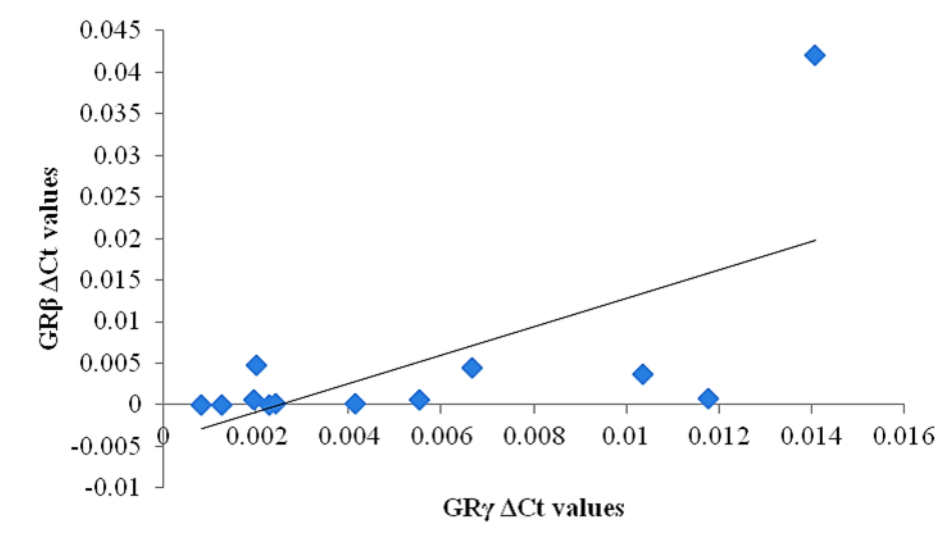


Figure 5.20: Correlation graph between GRβ and GRγ in non planned pregnancy samples *(r = 0.648, P = 0.023, n=12).*

The expression of GRγ in the non planned category shows to be positively correlated with the of GAS-5 (r = 0.775, P = 0.008, n = 12). Fig. 5.21 below depicts this correlation.

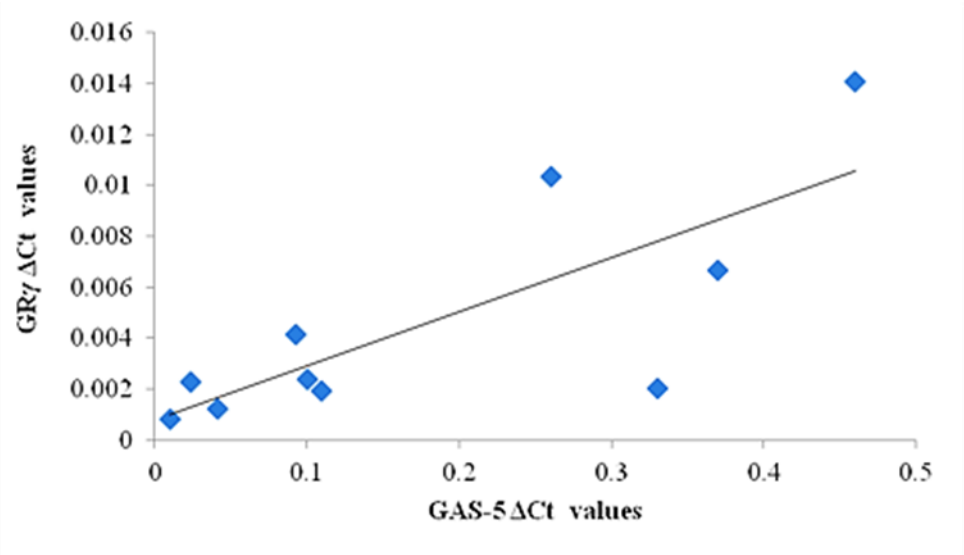


Figure 5.21: Correlation graph between GRγ and GAS5 in non planned pregnancy samples *(r = 0.775, P = 0.008, n = 12).*

The expression of GR-P is positively correlated with the expression of GAS5 in this category (r = 0.645, P = 0.044, n = 12) and this correlation is depicted in Fig. 5.22 below.

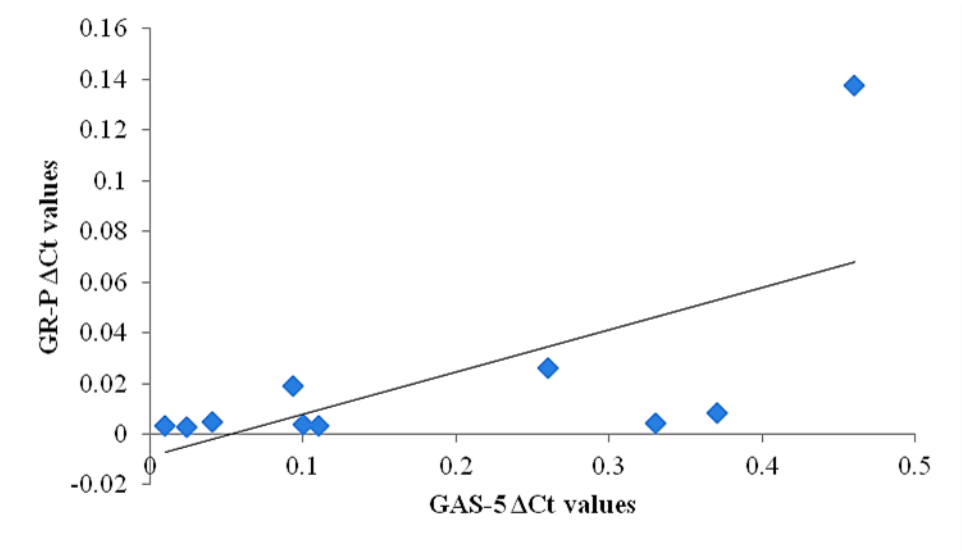


Figure 5.22: Correlation graph between GR-P and GAS5 in non planned pregnancy samples *(r = 0.645, P = 0.044, n = 12).*

The duration of pregnancy (days) shows a positive correlation with both foetal (r = 0.927, P = 0.000, n = 12) and placental weight (r = 0.962, P = 0.000, n = 12). Fig. 5.23 and 5.24 below depict these correlations.

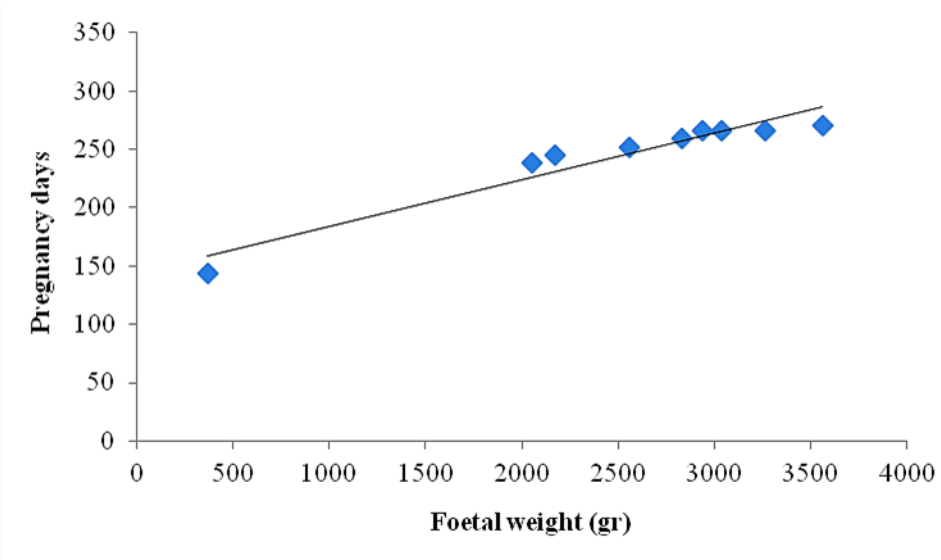


Figure 5.23: Correlation graph between pregnancy days and foetal weight in non planned pregnancy samples *(r = 0.927,P = 0.000, n = 12).*

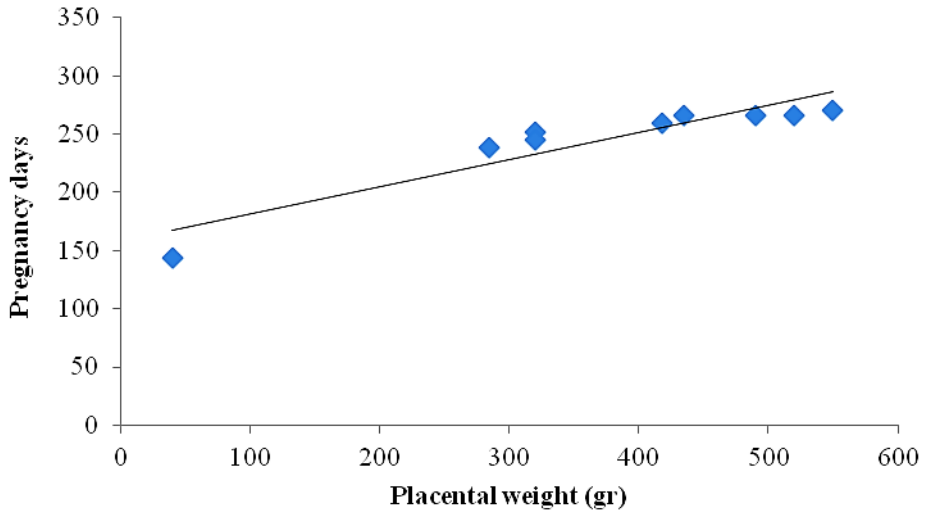


Figure 5.24: Correlation graph between pregnancy days and placental weight in non planned pregnancy samples *(r = 0.962, P = 0.000, n=12).*

Finally, the last positive correlation obtained in this category is between foetal and placental weight (r = 0.973, P = 0.000, n = 12). Fig. 5.25 below depicts this correlation.

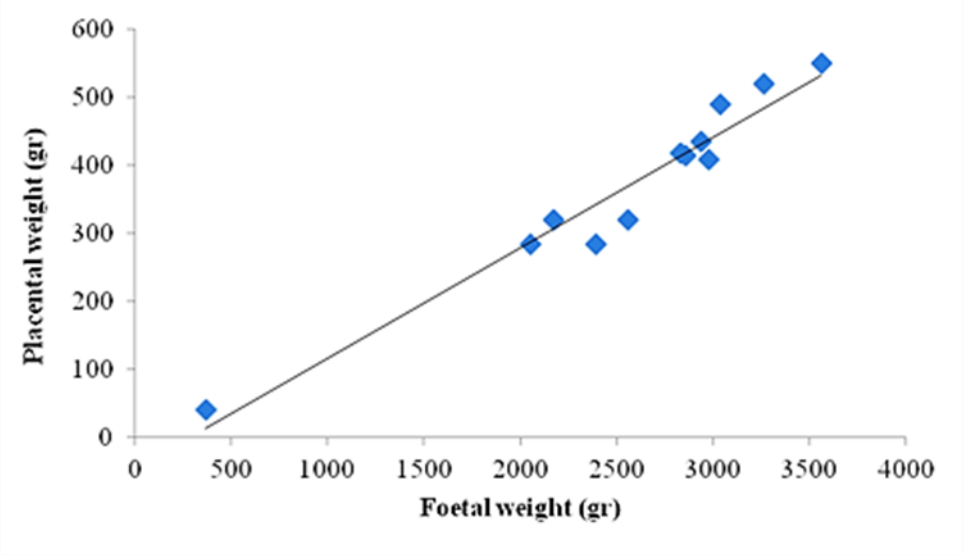


Figure 5.25: Correlation graph between placental and foetal weight in non planned pregnancy samples *(r = 0.973, P = 0.000, n = 12).*

In the term and pre-term groups the GR splice variants were examined as a ratio to the "wild-type" GRα and key changes were noted.

	Term (*n=13*)	**Pre-term (*n=10*)**
GRα / GRα	1	1
GRα / GRβ	47	65
GRα / GRγ	38	97
GRα / GR-P	11	43
GRα / GAS-5	0.8	1.5

Table 5.5: Ratio of gene expression relative to GRα in term and pre-term human placental samples.

As it is evident there is a clear shift towards a predominance of GRα in terms of GR splicing in the pre-term cohort when it was compared to term placentas, including a similar change in the GRα/GAS-5 ratio.

5.2.7. Expression of GRs and GAS-5 in BeWo cytotrophoblast (CT) and JEG-3 cells.

The expression of GAS-5 was examined in BeWo CT and JEG-3 cells. The RQ values are presented in Fig.5.26 below. As it can be seen, significant lower levels of GAS5 were detected in JEG-3 cells when compared to BeWo CT under basal (unstimulated) conditions.

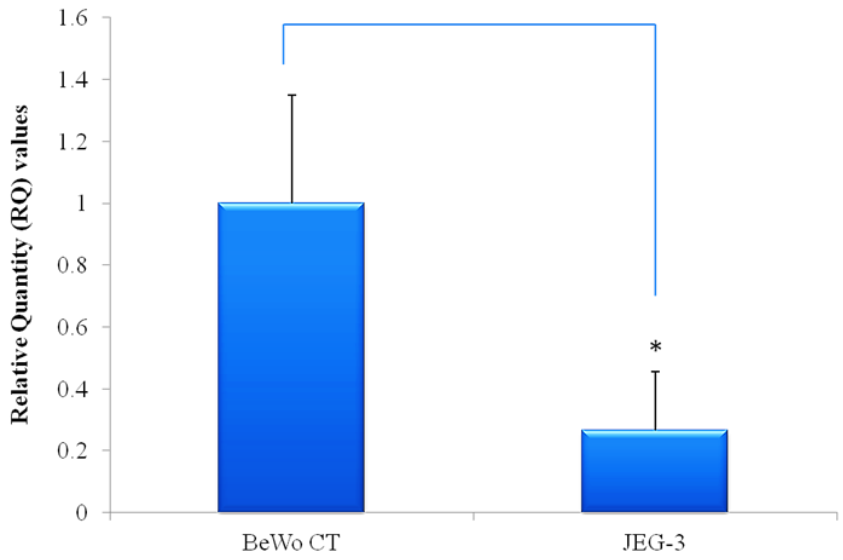


Figure 5.26: Expression of GAS-5 in BeWo CT and JEG-3 cells ($P < 0.05$*).

Also in these cDNA preparations of BeWo CT and JEG-3 cells the expression of GRα, GRβ, GRγ and GR-P was examined. Significantly higher levels are seen in the expression of GRγ and GR-P in the JEG-3 cells compared to the BeWo CT cells.

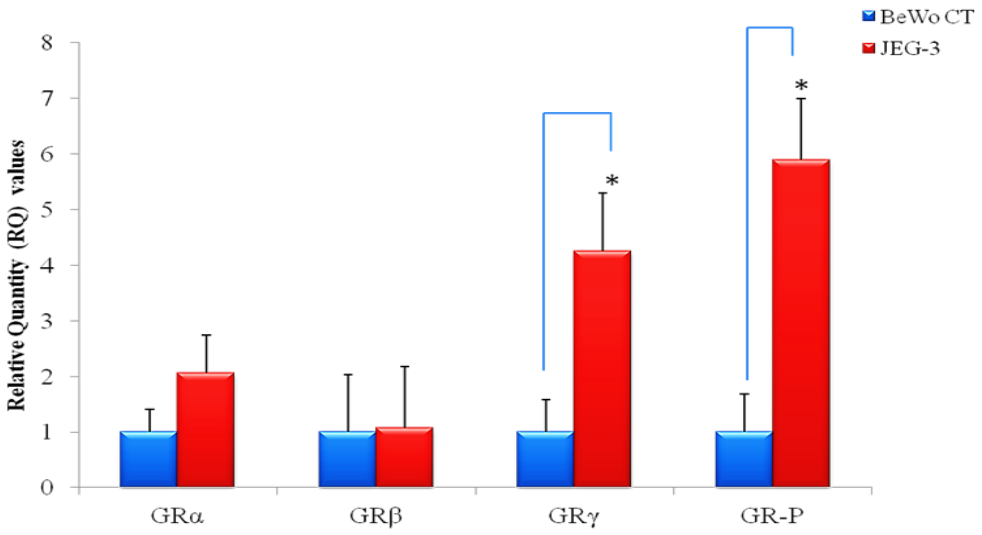


Figure 5.27: Expression of GRα, GRβ, GRγ and GR-P in BeWo CT and JEG-3 cells ($P < 0.05$ *).

5.2.8. Effect of cortisol on GRs and GAS5 gene expression in BeWo CT and JEG-3 cells.

The two well established choriocarcinoma cell lines (BeWo and JEG-3) that have been used widely to decipher placental signalling were further employed in this study. The cells were treated with cortisol in a dose-dependent manner (10nM, 100nM and 1000nM) for 24 hours in an attempt to represent a stressful environment *in vitro* ranging from a moderate to a very high stress level. The graph in Fig.5.28 depicts the results that were obtained. The expression of GAS-5 following the treatments with cortisol was significanlty higher compared to the non treated BeWo CT and JEG-3. Specifically, after treating the BeWo CT cells with 10nM, 100nM and 1000nM, a 21.7 fold, 11.2 fold and 24.9 fold increase respectively was obtained. The JEG-3 cells after the same course of treatment resulted in a 28.3 fold increase, a 7.3 and a 13.1 increase above basal levels.

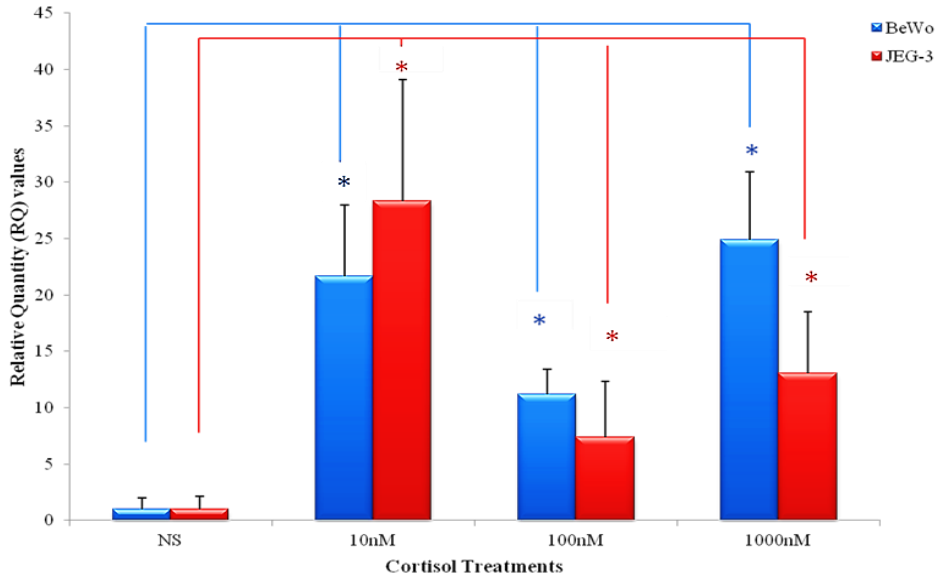


Figure 5.28: RQ values of the expression of GAS5 in BeWo CT and JEG-3 cells after 24 hour cortisol treatment with 10, 100 and 1000nM ($P < 0.05$ *). Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments, NS = non supplement.

The levels of expression of the GR genes were analysed as previously described using RQ values (Y axis). The expression of GR splice variants in both cell lines following the 24 hour treatments were obtained and the RQ values are depicted in Fig.5.29. Even though some differences are seen post cortisol treatment in the expression levels of the genes, the results do not appear to reach significance.

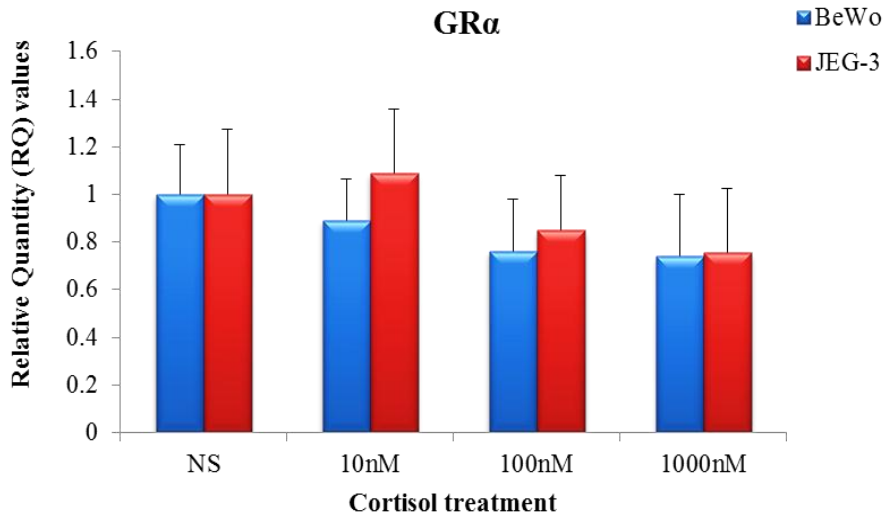


Figure 5.29: RQ values of the expression of GRα in BeWo CT and JEG-3 cells after treating with cortisol. Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments.

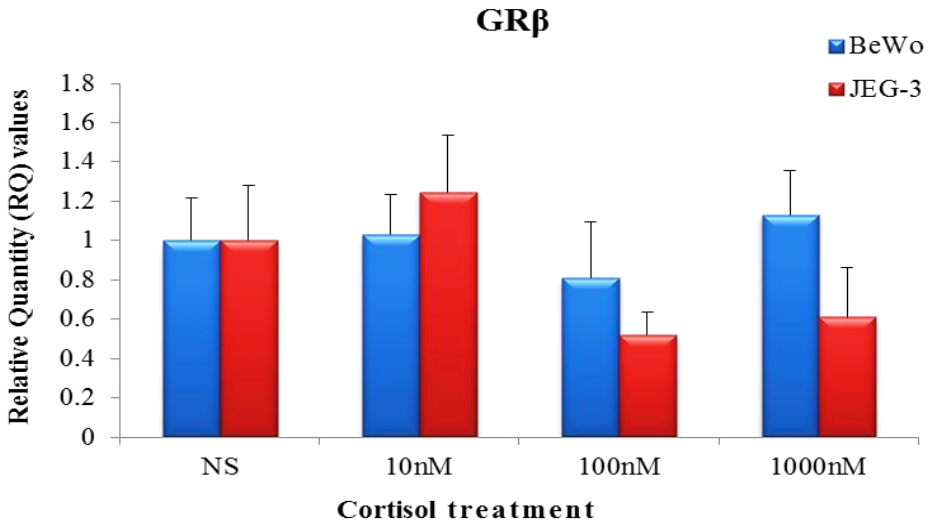


Figure 5.30: RQ values of the expression of GRβ in BeWo CT and JEG-3 cells after treating with cortisol. Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments.

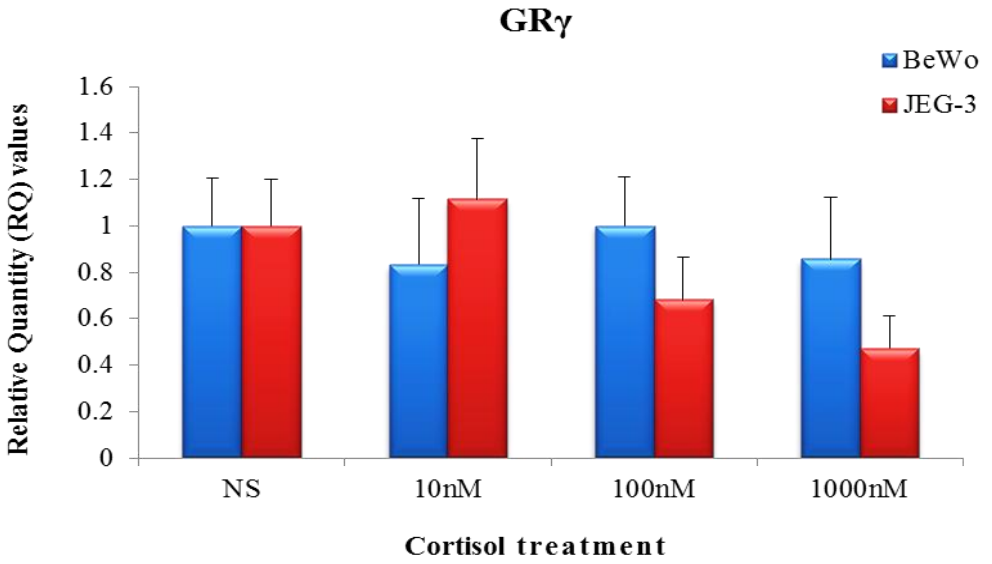


Figure 5.31: RQ values of the expression of GRγ in BeWo CT and JEG-3 cells after treating with cortisol. Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments.

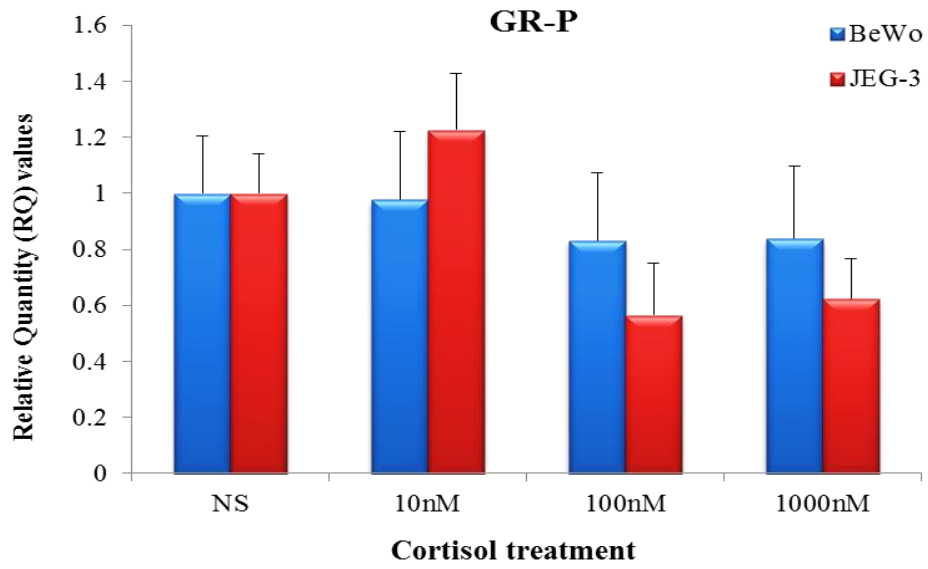


Figure 5.32: RQ values of the expression of GR-P in BeWo CT and JEG-3 cells after treating with cortisol. Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments.

5.2.9. Cellular localisation and distribution of GR in the BeWo CT and JEG-3 cells.

Immulofluorescence analysis of the GRα protein in BeWo CT and JEG-3 cells was performed. In BeWo cells this protein was localised mainly in the cytoplasm where the staining had a dotted appearance primarily around the nucleus (Fig. 5.33 A). In JEG-3 cells strong peri-nuclear staining was evident (Fig. 5.33 B).

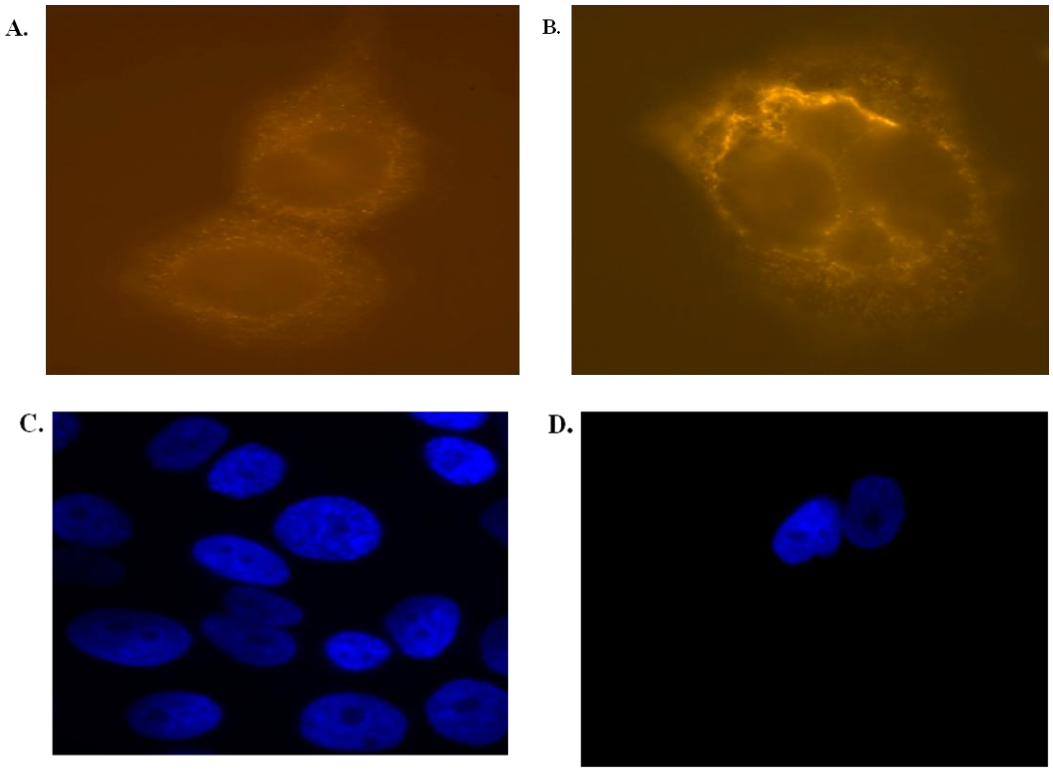


Figure 5.33: Panel A and B: Immunofluorescence analysis for GRα in BeWo CT cells (A) and JEG-3 cells (B). Panel C and D: Negative controls of BeWo and JEG-3 respectively.

5.3. Discussion

The human placenta is exposed to increased levels of cortisol as pregnancy progresses (Johnson *et al*., 2008) and its effects are mediated via binding and activating GR. However, controversy surrounds the exact mechanisms by which these responses are regulated. GR alternative splicing might also influence the subsequent activation of signalling pathways by glucocorticoids (Johnson *et al*., 2008). In our study, we have shown that all known transcripts of GR splice variants are expressed in the human placenta with GRα being the predominant transcript in all categories studied. These data corroborate a previous preliminary study of placental GRs (Johnson *et al*., 2008). The qPCR data have also been grouped under plan or non planned pregnancies but there was no apparent difference in expression levels between these groups (Appendix VI).

In addition, we demonstrate for the first time the expression of Growth-Arrest-Specific Transcript-5 (GAS5) in human placentas. We decided to incorporate GAS5 in the current study as emerging data suggests that it can act as a GR DNA binding decoy and as a result compromise its activity (Kino *et al*., 2010). Interestingly, there was equally strong expression of GAS5 when compared to GRα in both term and pre-term placentas. Pre-term labour is associated with high mortality and morbidity (Beck *et al*., 2010) and a recent study pointed towards an association between maternal stress with complications of pregnancy and especially with pre-term birth (Roy-Matton *et al*., 2011). GRβ differs from GRα on the C-terminus of the receptor protein. There is still controversy surrounding the exact function of GRβ, but it appears to exert a dominat-negative effect on GRα-induced transcriptional activity (Kino *et al*., 2010). Moreover it fails to bind hormone and subsequently activate transcriptional events (Sánchez-Vega *et al*., 2006). GRγ is a ligand-dependent transcription factor with reduced transactivating activity and its function is still under investigation. In our clinical samples, GRγ is present at much lower levels than GRα isoform in placentas studied.

Similarly, controversy surrounds the role of GR-P a truncated isoform that lacks a large part of the ligand-binding domain, including the domains for silencing of GR in the absence of hormone and transcriptional activation (Sánchez-Vega *et al*., 2006). Johnson *et al*., have shown that only placental GR-P mRNA levels were reduced significantly after spontaneous labour (Johnson *et al*., 2008).

In our study, an approximate 4-fold change in the GRα / GR-P with pre-term labour was detected. Studies in multiple myeloma cells have shown similar changes in GRα / GR-P ratio in myeloma glucocorticoid resistant and parental cell lines (Sánchez-Vega *et al*., 2006). Finally, a two-fold increase in the GRα / GAS5 ratio was also noted. This is the first study that expression and cellular distribution of GAS5 is elucidated in the human placenta. It is attractive therefore to hypothesise that the change in the ratio of the splicing isoforms alters the responsiveness of placental GRs to cortisol. We would like to propose a model where, during pre-term birth GRα is the predominant receptor since there is a decrease in negative regulators such as GRβ, GRγ as well as the "pseudo-GRE" GAS5. This ratio changes will ultimately lead to an augmented response towards glucocorticoids with potential detrimental effects for the mother and the foetus.

CHAPTER 6

POLYMORPHIC STUDIES OF THE GR GENE IN MATERNAL AND FOETAL SAMPLES: EVIDENCE FOR NATURE-NURTURE INTERACTIONS

6.1. Introduction

Normal foetal development depends partly on the ability of the mother to adapt to changes taking place on physiological as well as psychosocial levels (Lobel *et al*., 2008). Stress related to the pregnancy may be a more powerful contributor to birth outcomes such as preterm labour and low infant birth weight that general stress itself (DiPietro *et al*., 2002, 2004; Huizink *et al*., 2004; Roesch *et al*., 2004). Changes in the epigenetic regulation of the foetal GR promoter have been associated with the exposure to prenatal maternal stress (Lupien *et al*., 2009), reflecting a possible effect of maternal stress on the expression and function on the GR in the foetus. For example, the *Bcl* I polymorphism of the GR has been associated with increased glucocorticoid sensitivity and was over represented in pregnant women with pathological foeto-maternal immune adaptation (Bertalan *et al*., 2009). In this study we have examined the relationship between mothers' stress levels and the status of four different GR polymorphisms, with their infants' birth weights.

6.2. Results

6.2.1. Maternal stress, maternal attitudes towards the pregnancy and foetal birth weight

Following analysis of the questionnaire responses, the maternal attitudes were classified as following: 35.7% positive, 16.3% neutral and 48% negative. Maternal attitudes were related to self-reported stress status ranging from low and medium (Low stress response) to high and very high (High stress response). The source of the stress was not reported. Women with negative attitudes towards their pregnancy showed significantly higher levels of stress during pregnancy (2.8) compared to women with neutral (1.6) or positive attitudes towards their pregnancy (1.4; $t(95) = 9.8$, $P < 0.001$). Since women with neutral and positive attitudes

towards the pregnancy did not differ on stress levels (t (95) = 1.0, $P > 0.05$), and due to the small number of women with neutral attitudes, both groups were merged. Women forming the first group with the positive and neutral attitudes were contrasted with those with negative attitudes. Women with negative attitudes during pregnancy gave birth to infants with significantly lower birth weights (2.5Kg) than those women showing a positive or neutral attitudes towards their pregnancy (2.9Kg; F(1,71) = 4.2, $P < 0.05$; Fig. 6.1), independent of their age and their body mass index (BMI). However, from the analysis it was shown that the levels of stress were unrelated to infant's birth weights (r = -0.12, $P > 0.05$).

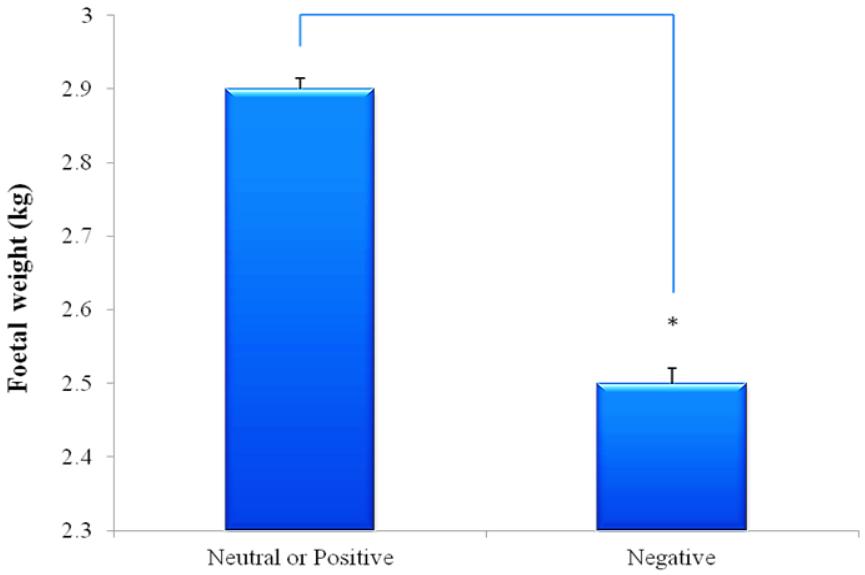


Figure 6.1: Prenatal maternal attitude towards the pregnancy and foetal weight. Neutral or positive and negative attitudes.

6.2.2. Maternal background variables, pregnancy planning and foetal birth weight

We then tested whether maternal background variables predicted infant birth weight. Age, BMI, physical activity, alcohol and smoking during pregnancy were unrelated to infant birth weight (all $P > 0.05$). Furthermore, most immune symptoms of women (i.e., difficulty

overcoming an infection, number of colds or infections during pregnancy, inflammatory diseases, proneness to cystitis/thrush, allergies, use of antibiotics) were also unrelated to infant birth weight (all $P > 0.05$).

6.2.3. Effects of maternal attitude to pregnany & pregnancy planning on infant birth weight

In planned pregnancies, stress predicted infant weight, independent of age and BMI (r = -0.44, P = 0.01), whereas in unplanned pregnancies stress did not predict infant weight (r = -0.19, P = 0.23). In mothers with neutral or positive attitudes towards the pregnancy, planning the pregnancy had no effect on infant birth weight (F(1,30) = 0.091, Not Significant). However, in mothers with negative attitudes towards the pregnancy, those with an unplanned pregnancy gave birth to infants with significantly higher weights (2681.9 gr) than those with planned pregnancies (1917.8 gr; F(1,36) = 7.074, P = 0.012; Fig. 6.2).

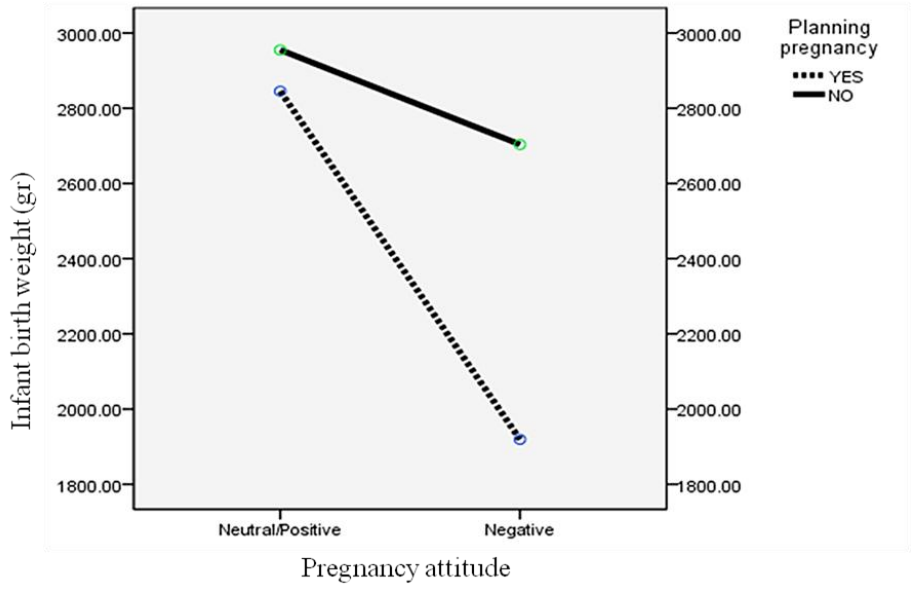


Figure 6.2: Effects of maternal attitude to pregnancy & pregnancy planning on infant birth weight.

6.2.4. Maternal and infant GR polymorphisms and foetal birth weight

Using PCR and restriction digests, we investigated four different GR polymorphisms: *Bcl*I, *N363S*, T*thIII*-I, and *ER22/23EK* and the results of this study are illustrated in Table 6.1 below. Since the maternal attitude towards the pregnancy was a significant predictor of foetal birth weight, we re-examined this association as a function of the GR gene polymorphisms, resembling a "nature-nurture" interaction, and statistically controlling for mothers' age, BMI, pregnancy planning and fast food consumption during pregnancy.

Restriction digest site	**Nucleotide change**	**Genotype**	**Mothers** n (%)	**Foetuses** n (%)
***Bcl* I**	C → G		81	87 (+ 6 twins)
		GG	5 (6.2)	7 (8.1)
		CC	19 (23.5)	23 (26.4)
		GC	57 (70.4)	57 (65.5)
N363S	Asp → Lys		81	87 (+ 6 twins)
		Wt [a]	79 (97.5)	85 (97.7)
		Hmz [b]	2 (2.5)	2 (2.5)
***ThtIII* 1**	C → T		81	87 (+ 6 twins)
		CC	35 (43.2)	33 (37.9)
		TT	2 (2.5)	8 (9.2)
		CT	44 (54.3)	46 (52.8)
ER22/23EK	Arg →Lys		81	87 (+ 6 twins)
		Wt	78 (96.3)	84 (96.5)
		Htz [c]	3 (3.7)	3 (3.4)

Table 6.1: GR gene polymorphic study results of *Bcl I, N363S, ThtIII 1, ER/22/23EK* showing the distribution of the genotyping in each of the four genetic polymorphism. When ([a]) wild type is inticated, ([b]) homozygous carriers and ([c]) heterozygous carriers.

6.2.5. Examining the variables of maternal background and the foetal birth weight.

Some of the maternal background variables collected from the questionnaire were tested as possible predictors of foetal birth weight. Variables including age, BMI, physical activity, alcohol and smoking during pregnancy were all found to be unrelated to foetal birth weight (all $P > 0.05$). Further to this, most immune symptoms that women reported (i.e., difficulty of overcoming an infection, number of colds or infections during pregnancy, inflammatory diseases, proneness to cystitis/thrush, allergies, use of antibiotics) were also found to be unrelated to foetal birth weight (all $P > 0.05$).

6.2.6. *Bcl I* Polymorphism

RT-PCR analysis followed by restriction enzyme digestion revealed the distribution of *Bcl* I polymorphisms with regards to maternal samples as well as foetal samples. The distribution of the polymorphism in our participants can be seen in Table 6.1 In our cohort, 5 women were non-carriers (GG; 6.0%), 19 were homozygous carriers (CC; 22.6%) and 57 were heterozygous carriers (GC; 71.4%). The foetal distribution of *Bcl* I polymorphism was: 7 non-carriers (6.0%), 23 homozygous carriers (28.6%) and 57 heterozygous carriers (65.4%).

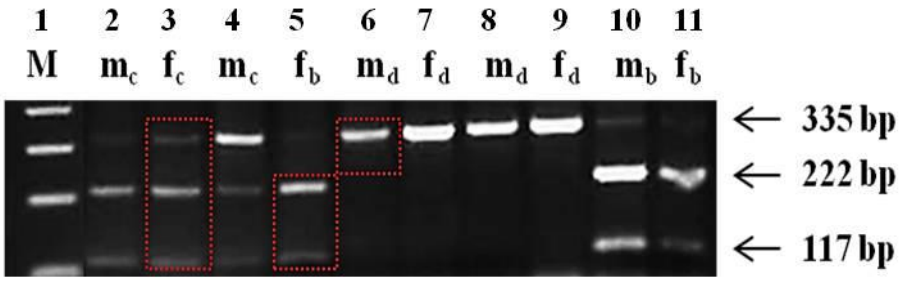


Figure 6.3: Results of electrophoresis following BclI restriction enzyme digestion of a 335bp PCR product in maternal (m) and foetal (f) samples. Lane 1 (M) shows the 1Kb DNA ladder. Lanes 2 (m_c), 3 (f_c), 4 (m_c) shows the CG heterozygote as indicated by the three bands (117, 222 and 335 bp), lanes 5 (f_b), 10 (m_b), 11 (f_b) shows the CC homozygote as indicated by the two bands (117 and 222 bp), lanes 6 (m_d),7 (f_d), 8 (m_d), 9 (f_d) shows the undigested GG homozygote with one band (335 bp). When (^{b}) homozygous carriers, (^{c}) heterozygous carriers and (^{d}) undigested GG homozygote carriers.

6.2.6.1. Examining the maternal attitude towards pregnancy, *Bcl* I polymorphism and foetal birth weight

As the maternal attitude towards the pregnancy showed to be a significant predictor of foetal birth weight, this association was re-examined as a function of the GR gene *Bcl* I polymorphism. This was performed in an attempt to resemble a "nature-nurture" interaction and statistically were controlling for the mothers' age and BMI planning and fast food.

6.2.6.2. Maternal *Bcl* I polymorphism

There is a partial inverse correlation between maternal attitude ("Happy") and foetal weight, in *Bcl* I (CC) where r = -0.51, P = 0.065, while the CG group exhibited a significant yet moderate inverse correlation; r = - 0.27, P = 0.047. For both (CC and GC) analyses we controlled for confounders mentioned previously. The GG group was not analysed as only 5 women were genotyped with this polymorphism.

6.2.6.3. Foetal *Bcl* I polymorphism

The partial correlation between maternal attitude and foetal weight, *Bcl* I (CC) is r = -0.41, P = 0.10; while in *Bcl* I (GC) it is r = -0.40, P =0 .005, controlling for the same confounders.

6.2.7. Tht*III* 1 Polymorphism

The next polymorphism that was analysed in our patient cohort was the Tht*III* 1. RT-PCR analysis followed by restriction enzyme digestion revealed the distribution of this polymorphism as can be seen by a representative agarose gel electrophoresis result shown in Fig. 6.4. The distribution of this polymorphism in our subjects is shown in Table 6.1 where it is evident that our subjects carried all three types of the Tht*III* 1 genetic polymorphism

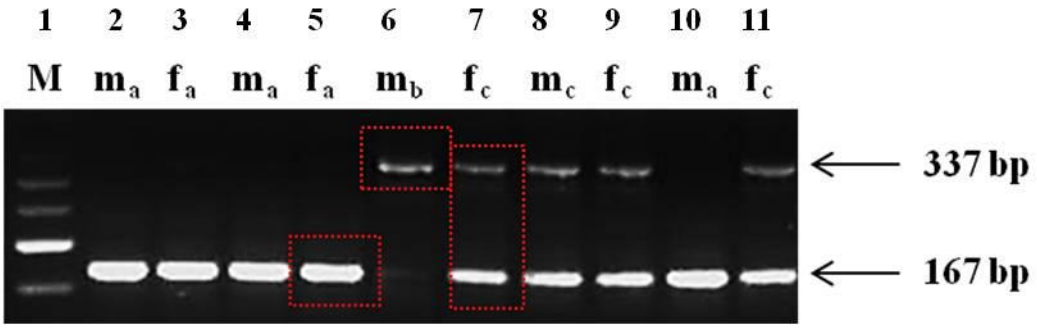


Figure 6.4: Results of electrophoresis following Tht*III*1 polymorphism genotyping in maternal (m) and foetal (f) samples. Lane 1 (M) shows the 1Kb DNA ladder. Lanes 2 (m_a), 3 (f_a), 4 (m_a), 5 (f_a), 10 (m_a) shows the CC genotype as indicated by the one band (167 bp), lane 6 (m_b) shows the TT genotype as indicated by the one band (337 bp), lanes 7 (f_c), 8 (m_c), 9 (f_c), 11 (f_c) shows the CT genotype as indicated by the two bands (167 and 337 bp). When (^{a}) wild type is inticated, (^{b}) homozygous carriers and (^{c}) heterozygous carriers.

6.2.7.1. Maternal Tht*III* 1 Polymorphism

Statistical analysis of the maternal Tht*III* 1 polymorphism has shown an inverse correlation between maternal attitudes and in Tht*III* 1 CC (r=-0.41) *P* = 0.030. In Tht*III* 1 CT, no significant correlations were noted; r = -0.16, *P* = 0.31 (NS), controlling for age, BMI, pregnancy planning, and fast food DP.

These data are also suggestive of a nature - nurture interaction since only in CC, maternal stress attitude predictes foetal weight-reduction, but not in CT, independent of confounders. Hence, the effects of maternal stress on foetal weight depend on the mother's polymorphism of GR gene.

6.2.7.2. Foetal Tht*III*1 Polymorphism

Correlation between maternal attitude and foetal weight in each group of foetal CC r = -0.43, *P* = 0.030, in CC; r = -0.29, *P* = 0.08 in CT, controlling for age, BMI, planning, and fast food during pregnancy.

6.2.8. *ER22/23EK* Polymorphism

As seen in the table 6.1 where the distributions are shown, our subjects carried both types of this genetic polymorphism with the wild type being the predominant type in both mothers and foetus. Below in Fig. 6.5 a representative image is shown after restriction enzyme digestion indicating the differences at molecular level. As only six *ER22/23EK* heterozygote subjects were identified in total, these subjects were not statistically analyzed due to the small subject number.

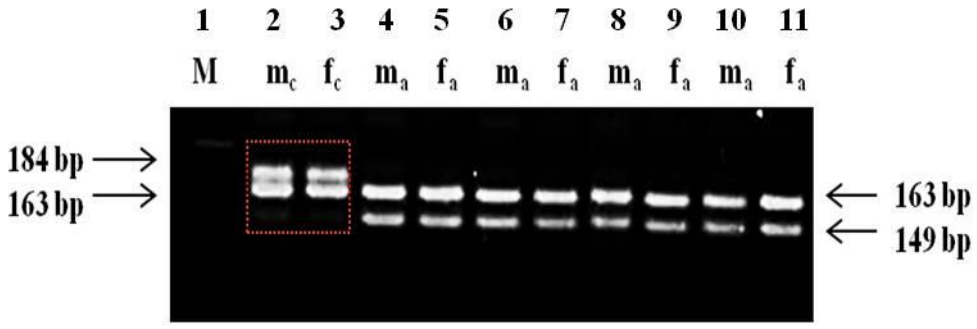


Figure 6.5: Results of electrophoresis following *ER22/23EK* polymorphism genotyping in maternal (m) and foetal (f) samples. Lane 1 (M) shows the 1Kb DNA ladder. Lanes 2 (m_c), 3 (f_c), shows the heterozygous samples as indicated by the two bands (163 and 184 bp), lanes 3-11 show the wild-type samples as indicated by the two bands (149 and 163 bp). When (^{a}) wild type is inticated, and (^{c}) heterozygous carriers.

6.2.9. *N363S* Polymorphism

The next polymorphism that was analysed in our patient cohort was the *N363S*. Again the distribution is shown in Table.6.1. Both types of this genetic polymorphism were shown to be carried by our subjects but as only four *N363S* homozygotes were identified in total again these subjects were not statistically analyzed due to the small subject number.

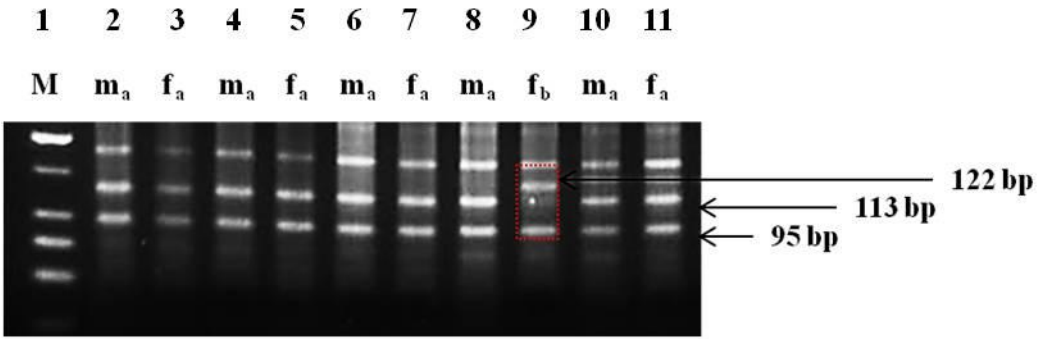


Figure 6.6: Results of electrophoresis following *N363S* polymorphism genotyping in maternal (m) and foetal (f) samples. Lane 1 (M) shows the Low Molecular Weight DNA ladder. Lanes 2-8 and 10-11 show the heterozygous samples as indicated by the two bands (113 and 95 bp), lane 9 shows the homozygous sample as indicated by the two bands (122 and 95 bp). When (^{a}) wild type is inticated and (^{b}) homozygous carriers.

6.3. Discussion

The present study extends previous findings and provides evidence for the first time how environment (i.e. stress/pregnancy planning) and genetics (i.e. polymorphisms of the GR gene) can possibly affect foetal outcome. Maternal stress during gestation period affects placental as well as embryonic/foetal development and can lead to critical complications (Gheorghe *et al*., 2010). Prenatal maternal stress has been shown to have long-term effects on the psychological as well as behavioural development of the offspring (Mulder *et al*., 2002).

One of the key findings in our study is that pregnancy planning and maternal attitudes towards pregnancy appear to affect foetal weight. Worldwide approximately 87 million unplanned pregnancies occur every year (Adetunji, 1998). Unplanned pregnancy has been reported to affect both mothers and children. Emerging studies link negative experiences of pregnant women of unplanned pregnancies before and after labour. For example two studies have linked unplanned pregnancies with poor relationships with their spouses, experienced financial and educational difficulties and problems with their professional careers (Coleman *et al*., 2005; Robbins *et al*., 2005). Recently a study of 314 women from Turkey revealed that unplanned pregnancy discouraged women to develop positive attitudes during their pregnancies as well as compromised performance of prenatal care (Karacam *et al*., 2009). Interestingly, babies born to the women with unplanned pregnancies were shorter in stature (Karacam *et al*., 2010). Data from our study suggest that there is no mother-foetal coherence in the group of unplanned pregnancies. An interesting finding are the effects of maternal attitude to pregnancy and pregnancy planning on infant birth weight. A potential interpretation would be that holding a positive or neutral (accepting) attitude towards the pregnancy buffers or protects against any potential negative effects of planning/not planning the pregnancy on fetal weight. However, in women with negative attitudes towards the pregnancy, those who planned the baby may be in conflict and hence, chronic stress and

possibly adversely affects the foetal development and weight more than those with negative attitude and an unplanned pregnancy.

In terms of the polymorphisms analyses, only the maternal Tht*III* 1 polymorphism was suggestive of a gene-environment interaction since only in Tht*III* 1 (CC), maternal stress attitude predicts foetal weight-reduction, but not in Tht*III* 1 (GC) independent of confounders. These effects were independent of women's age and BMI, and were not explained by gestational age. Additionally, multiple other immune symptoms and demographic variables tested were also not predictive of foetal weight. Hence, the effects of maternal stress on foetal weight depend on the mother's polymorphism of the GR gene. Similar gene-environment interactions were found in a study related to *ER22/23EK* polymorphism and risk for depression, which occurred only if people were additionally exposed to childhood stress (Bet *et al*., 2009).

It should be noted that apart from the well established polymorphisms that can be detected by the use of restriction digest enzymes, novel GR polymorphisms have also been reported in the literature. For example, in children with sporadic nephrotic syndrome except the known GR (*NR3C1)* polymorphisms, six novel polymorphisms have been documented (1206C > T, 1374A > G, 2382C > T, 2193T > G, IVS7-68_-63delAAAAAA, and IVS8-9C > G). Moreover, they have also identified one known *NR3C1* haplotype, [198G > A; 200G > A], and two novel *NR3C1* haplotypes (H1, [1374A > G; IVS7-68_-63delAAAAAA; IVS8-9C > G; 2382C > T] and H2, [1896C > T; 2166C > T; 2430T > C]) (Ye *et al.,* 2006). This was done by denaturing and renaturing PCR products to form heteroduplexes, followed by processing on a DHPLC apparatus. Another study using DNA sequencing showed a T to C substitution at codon 766 (position 2430) of the GR gene that is associated with systemic lupus erythematosus (Lee *et al*., 2004)".

This study posed some limitations. Firstly, our sample included small numbers in certain categories of polymorphic groups. Nevertheless, the statistically significant effect and the size of differences observed between mothers with negative versus positive and neutral attitudes in the *ThtIII* 1 polymorphism group suggest that this effect may be robust. Secondly, the distribution of *Bcl* I polymorphism seen in this sample may be unique, since there are important geographical/ethnic differences in the prevalence of these polymorphisms. It should be emphasized that in the present study, we included a homogeneous cohort of Mediterranean patients from Crete. Despite these limitations, this is the first study to demonstrate a gene-maternal environment synergism in relation to infant birth weight, using a very brief assessment of maternal attitudes to pregnancy. Should these finding be replicated in a much wider cohort, given the simplicity in assessing such attitudes and the feasibility to identify the homozygous group of women early on in pregnancy, these findings may have significant implications for public health and prevention.

CHAPTER 7

STUDIES ON SYNCYTIALISATION MODEL

7.1. Introduction

Here we have used forskolin (a syncytialisation agent) in order to fuse and differentiate BeWo cells into syncytiotrophoblasts. Previous in house studies have verified the differentiation process. We have used a cell membrane marker, pan-Cadherin to examine the levels of the cadherin protein in forskolin-treated and untreated cells to determine whether a decrease in total plasma membrane was achieved following treatment using immunofluorescence and western blotting. The cell viability/death was assessed using an MTT as well as a trypan blue assay. Finally, the amount of syncytin-2 (a syncytialisation marker) was assessed in the same cells using qPCR. This is in contrast with the undifferentiated state, as well, as the JEG-3 cells that resemble a cellular milieu of a first trimester placenta. Therefore the effect of forskolin, the inducer of syncytialisation on the expression of the mTOR main components as well as the GRs and GAS5 was assessed using quantitative PCR. Moreover, the cellular distribution of mTOR, Deptor, Rictor and Raptor was elucidated in syncytialised cells.

7.2. Results

7.2.1. BeWo and JEG-3 cell line as placental models: effect of syncytialisation on gene expression.

In this experimental model, syncytialisation was achieved using forskolin. The syncytialisation model on BeWo cells has been evaluated in detail in our laboratory, prior to elucidating the expression of mTOR signalling components in the differentiated state (syncytiotrophoblasts) of BeWo cells. The expression of PCNA was investigated using RT-PCR (Fig. 7.1). BeWo cells treated with forskolin (50 and 100 μM) for 48 hrs revealed down-

regulation of expression of PCNA at the mRNA level (Fig. 7.2). These data suggest that BeWo cells cease to proliferate, indicative of differentiation events taking place.

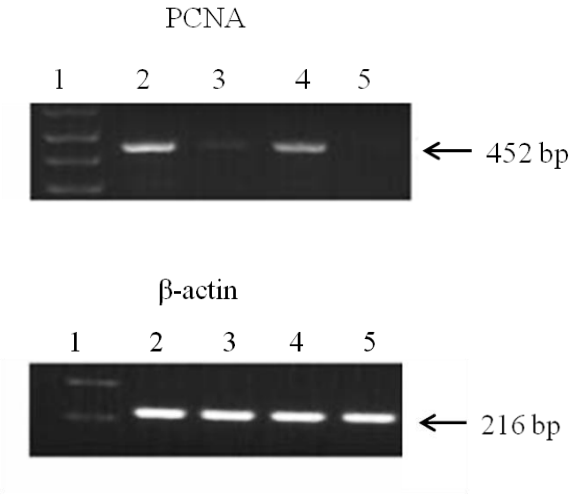


Figure 7.1: RT-PCR gel demonstrating the expression of PCNA and β-actin housekeeping gene in treated and untreated BeWo cells. Lane 1: DNA ladder; lane 2: cDNA from untreated BeWo cells (48 hours); lane 3: cDNA from stimulated BeWo cells (50 µM forskolin, 48 hours); lane 4: cDNA from untreated BeWo cells (48 hours); lane 5: cDNA from stimulated BeWo cells (100 µM forskolin, 48 hours) (Mparmpakas *et al*., 2010).

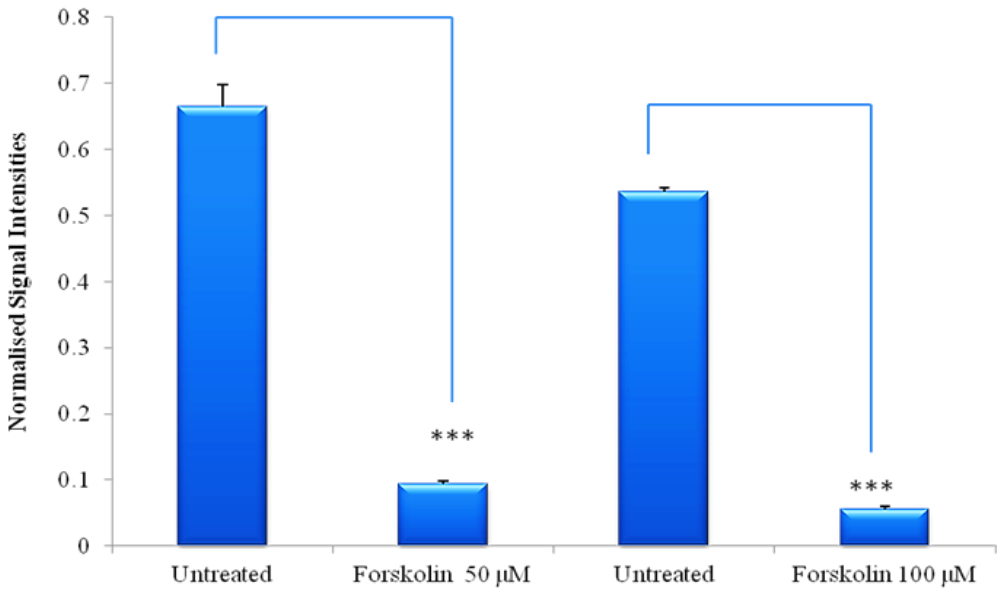


Figure 7.2: Densitometric analysis of PCNA in forskolin treated and untreated BeWo cells. Expression of PCNA was normalised against the expression of β-actin housekeeping gene. Each histogram represents the mean ± SEM of three individual experiments. Blue lines indicate significance, $P < 0.0001$***.

7.2.2. Effect of BeWo cell syncytialisation on mTOR signalling components.

Q-PCR analysis revealed that in syncytialised BeWo cells (ST) all transcripts were expressed (Fig.7.1). No significance changes were seen in the expression of mTOR, Rictor and Raptor when their expression was compared to that observed in BeWo cytotrophoblast cells (CT). However, significant down regulation in the expression of Deptor was evident in BeWo ST cells when compared to BeWo CT ($P < 0.05$*).

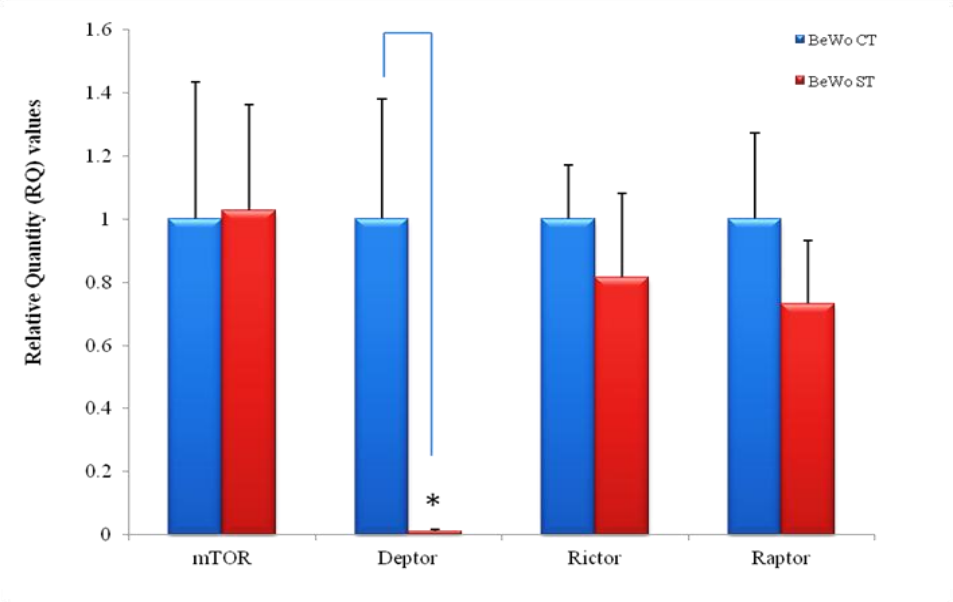


Figure 7.3: RQ values of the expression of mTOR, Deptor, Rictor, Raptor in BeWo CT and BeWo ST. Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments (P < 0.05 *).

7.2.3. Cellular distribution of mTOR, Deptor, Rictor and Raptor in BeWo ST cells

The protein expression of mTOR and each component was assessed in syncytialised BeWo cells, using immunofluorescent analysis. Similar intense cytoplasmic staining was detected in unstimulated BeWo cells as previously described (Fig. 7.4).

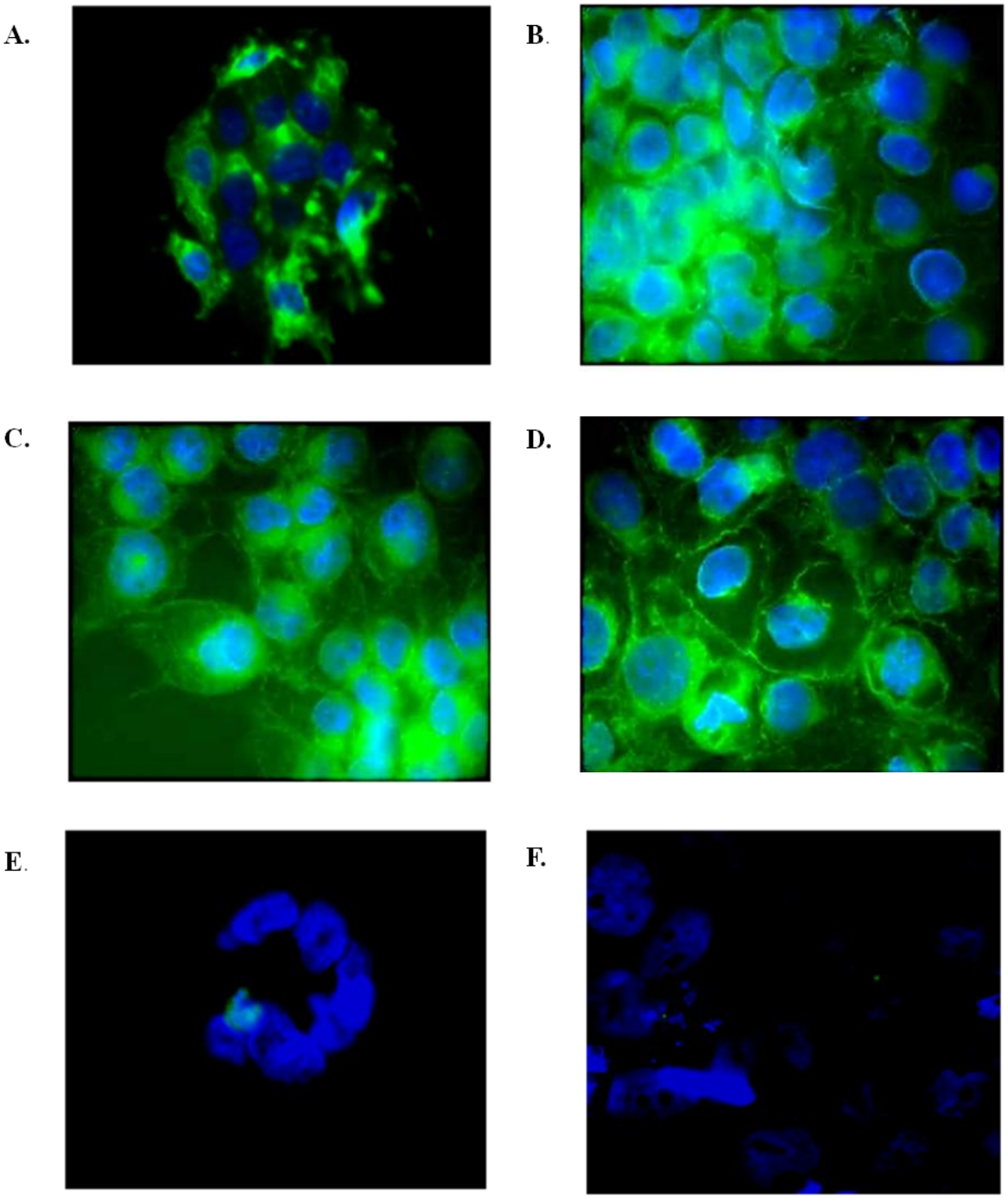


Figure 7.4: Cellular distribution of mTOR (A), Deptor (B), Rictor (C) and Raptor (D) proteins in forskolin (50μM) treated BeWo cells for 48 hrs. Also representative images are shown of the negative controls included in the study (E & F) (Mparmpakas *et al.*, 2010; Panel A & E).

7.2.4. Glucocorticoid Receptor expression in syncytialised BeWo cells

Further, the expression of the GR splice variants was examined in syncytialised BeWo cells. The graph in Fig.7.4 depicts the results of the GR expression in BeWo CT and BeWo ST. The expression of GRβ, GRγ and GR-P splice variants as seen below are significantly higher in BeWo ST when compared to BeWo CT. The individual RQ values of all the splice variants for both cell types are shown in brackets in the figure legend 7.5.

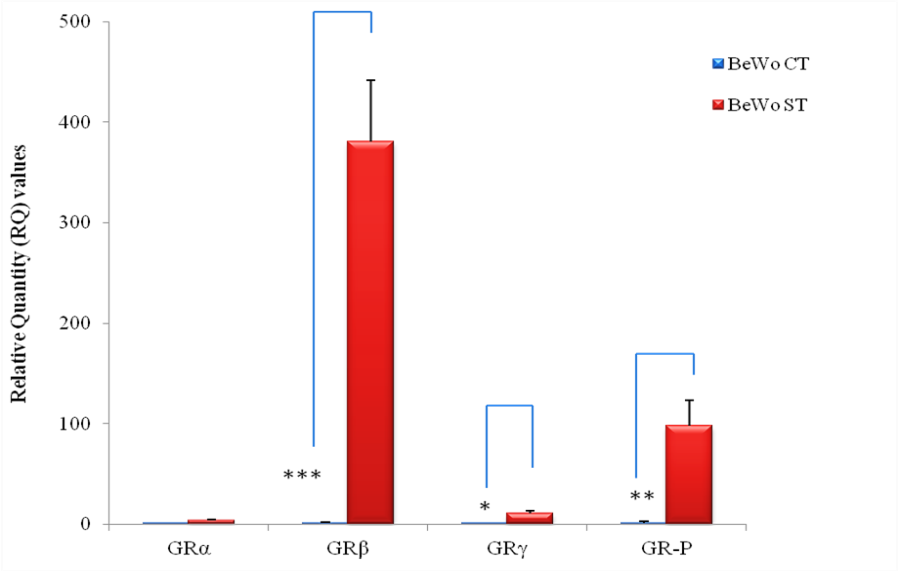


Figure 7.5: RQ values of the expression of GRα (3.43), GRβ (380.52), GRγ (10.92), and GR-P (98.25) in BeWo ST and BeWo CT (all RQ values were standardized at 1). Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments, $P < 0.05$*, $P < 0.001$**, $P < 0.0001$***).

7.2.5. Expression of GAS5 in BeWo ST and BeWo CT

The expression of GAS5 has been examined by Q-PCR in three different states: undifferentiated BeWo CT, differentiated BeWo ST as well as in the JEG-3 cell line. The results are depicted in Fig.7.6 where significantly lower levels (5.5 fold) of GAS5 were seen in BeWo CT cells when compared to the untreated BeWo ST cells. Upon comparison of BeWo CT with JEG-3 cells the expression of GAS5 in JEG-3 cells was significantly lower (3.7 fold) than that shown in BeWo CT.

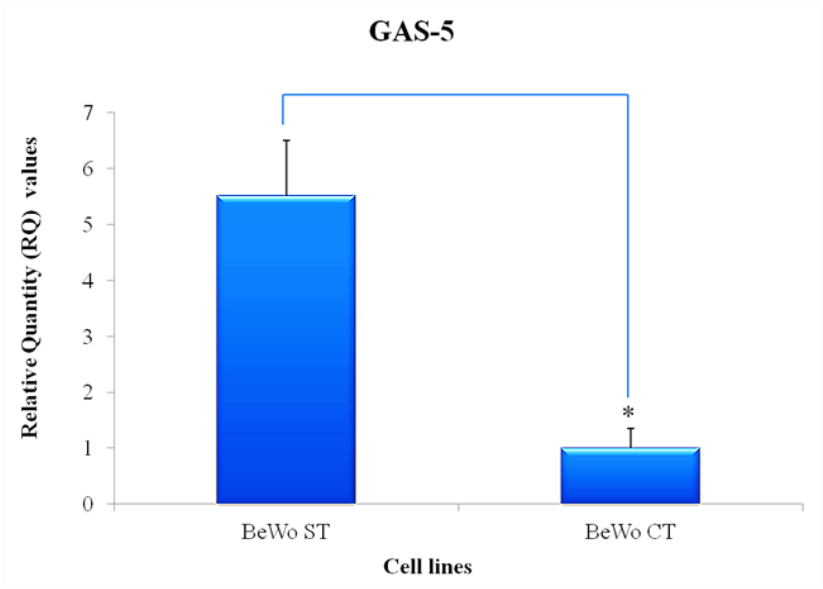


Figure 7.6: RQ values of the expression of GAS5 in BeWo ST and BeWo CT. Each histogram represents the mean + [$2^{-\Delta\Delta SD}$ (+) - $2^{-\Delta\Delta SD}$ (-)] of three individual experiments, ($P < 0.05$*).

7.3. Discussion

The two cell lines studied are widely used as *in vitro* models to investigate placental physiology and function. These cell lines have distinct fusigenic capacities. The JEG-3 cell line, despite being of a choriocarcinoma origin, is unable to morphologically differentiate and therefore it resembles the undifferentiated and hormonally inactive cytotrophoblast cells, making it an appropriate *in vitro* model to investigate first trimester placental events (Ntrivalas *et al*., 2006). BeWo cells are fusigenic and when they are fully differentiated (i.e. syncytialised) they resemble a third trimester placental model. At this stage the predominant feature of the human placenta is the hormonally active syncytiotrophoblast layer. The multinucleated syncytiotrophoblasts cover the external surface of the placental villous and are in direct interface with maternal blood (Das *et al*., 2004). Undifferentiated BeWo cells are similar in morphology to primary trophoblast cultures, with a monolayer and microvillar projections on the apical side. The capability of BeWo cells to differentiate has established these cells as an *in vitro* model to study immune, endocrine and developmental aspects (Heaton *et al*., 2008; Neelima & Rao, 2008; Ellinger *et al*., 1999), as well as placental transport mechanisms (Manley *et al*., 2005). More specifically, BeWo and JEG-3 cells were used extensively as placental models to study a variety of placental-based pathologies and cellular functions. These include: preeclampsia (Jain *et al*., 2012), hypoxia (Liao *et al*., 2012), specific maternal serum markers (Orendi *et al*., 2010), preterm labour (Lim *et al*., 2012), chorioamnionitis (Ikoma et al., 2003), trophoblast invasion (Arimoto-Ishidat *et al*., 2009), implantation events (Benaitreau *et al*., 2009) and gestational diabetes (Ma *et al*., 2010).

In this chapter we provide evidence of how the process of syncytialisation affects the expression of mTOR, Deptor, Rictor, Raptor, GR splice variants and GAS5 in BeWo cells. In our study, nuclear expression of mTOR and Deptor under basal conditions in undifferentiated BeWo was not observed. Even after forskolin induced syncytialisation, no apparent

translocation of mTOR was noted in BeWo cells at either 48 or 72 hrs. Similarly, Deptor, Rictor, Raptor also demonstrated an intense cytoplasmic staining, with Rictor demonstrating some plasma membrane localisation in a few cells. This could be due to changes in the total membrane content during the process of syncytialisation rather a true representation of where Rictor is localised.

In our experimental model, treatment with forskolin (48 h) induced a significant decrease in the gene expression of Deptor and GAS5 in BeWo CT cells, whereas a significant upregulation of GRβ was noted when compared to BeWo ST. Due to lack of commercially available antibodies of GR splice variants, we have only determined the expression of GRα in BeWo ST cells. Intense perinuclear staining was evident, with some nuclear translocation-in agreement with its role as a nuclear steroid receptor. These results support a recent study showing that forskolin altered the expression of several genes during differentiation of BeWo cells. For example, mRNA expression of SLPI, ELF-1·-1, prolyl 4-hydroxylase-ß and gastric associated differentially expressed protein was up-regulated after forskolin induced differentiation. In contrast, LMO-4 and Rab-5 interacting protein transcripts were down-regulated during cytotrophoblast differentiation (Neelima & Rao, 2008).

CHAPTER 8

GENERAL DISCUSSION

8.1. General Discussion and Concluding Remarks

8.1.1. Why is this study so important?

The key hypothesis of our study was that for a successful pregnancy outcome, there are tightly linked events at psychosocial, biochemical, genetic and molecular levels. Despite a plethora of studies identifying a number of potential risk factors for the mother, or the foetus, there has not been a comprehensive analysis of these factors from matched mothers and foetuses. Here we have studied the relative contribution of each factor for a successful pregnancy and allowed a model of their inter-relationships to be developed. As a result we have adopted a more holistic approach, investigating how stress, exercise, nutrition, immunity, placental genes, glucocorticoid receptor polymorphisms and serum levels of cortisol will influence pregnancy outcome.

8.1.2. What can we conclude from the questionnaire data?

We have used a wide repertoire of questions in order to acquire as many information as possible about the cohort of the patients we dealt with. With regards to the nutritional profile, a major finding was the significant reduction in the intake of alcohol, caffeine-containing and sugar-containing refreshments. The importance of nutrition during pregnancy with regard to pregnancy outcome has long been acknowledged. This importance has only been further emphasized by the recent changes in food quality and availability, lifestyle changes, and a new understanding of foetal programming on adult outcomes (Shapira, 2009). The Mediterranean dietary pattern (MDP) has been shown to exert certain beneficial effects, since it has appeared to be associated with the reduction in the risk of the offspring's affected by spina bifida (Vujkovic *et al*., 2009), and with children been less wheezy or asthmatic (de Batlle *et al*., 2008). A recent study proposed a diet quality index for pregnancy based on MDP evaluating the diet of a group of pregnant women by applying the Mediterranean Diet

Score (MDS) and evaluating their intake of micronutrients required in optimal amounts during pregnancy (Mariscal-Arcas *et al*., 2009). Of interest, the nature of fat in north Europe is primarily from dairy fat and lard whereas in Crete the main source was pure olive oil (Willet, 2006). It should be noted therefore, that different populations can have distinctive nutritional needs according to their age, lifestyle or specific physiologic situations or medical history. As a result analysis of our data cannot provide universally applicable results.

Another major finding of this part of the study was the effects of maternal stress on foetal weight and how pregnancy planning was implicated in this complex relation. Pregnancy is associated with major physiological and future psychosocial changes and maternal adaptation to these changes is crucial for normal foetal development. Psychological stress in pregnancy predicts earlier birth and lower birth weight. Perceived life-event stress, as well as depression and anxiety, predicted lower birth weight, decreased Apgar scores and small for gestational age babies as a recent study has shown (Marcus, 2009). It appears therefore that pregnancy-specific stress may be a more powerful contributor to birth outcomes than general stress (Lobel *et al*., 2008). In our cohort, women with negative attitudes during pregnancy gave birth to infants with significantly lower birth weights (2.5Kg) than those women showing positive or neutral attitudes towards their pregnancy (2.9Kg). However, in mothers with negative attitudes towards the pregnancy, those with an unplanned pregnancy gave birth to infants with significantly higher weights (2.7 Kg) than those with planned pregnancies. It has also been reported that women that had unplanned pregnancies had more psychological problems throughout their pregnancies when compared with those that planned their pregnancy (Karacam &Ancel, 2009). These novel findings suggest an important role of pregnancy planning with relation to foetal outcomes. This is of increasing importance since it is estimated that around 87 million pregnancies occur every year worldwide, out of which 41 million result in labour (Adetunji, 1998). So what can be done? Collectively, data from our

study suggest that there is no mother-foetal coherence in the group of unplanned pregnancies. Therefore, raising awareness of the impact of unplanned/unintended pregnancy is of key importance. This can be done by educating the public about social and health issues related to unintended pregnancy. Unintended pregnancy affects not only the individuals, but their families and communities as well. By communicating this problem to the public, increasing the understanding of the community and the individuals about prevention and improving access to necessary services, the society can ensure more positive outcomes for both mother and foetus.

8.1.3. Why is so important the pre-term labour sub-classification of clinical samples?

To this date, pre-term labour is still the leading cause of perinatal morbidity and mortality in the developed world. Despite the advances in diagnosis and molecular basis of disease, efforts to prevent pre-term birth have been compromised by a poor understanding of the underlying pathophysiology and not fully effective therapeutic interventions. The aetiopathology varies: certain clinical studies indicate that genito-urinary tract infections play a critical role in the pathogenesis of pre-term birth, or gene-environment interactions may play a key role in the onset of pre-term birth (Holst & Garnier, 2008). This is of increasing importance in our study since previous studies have pointed towards a link between maternal stress (i.e. pregnancy-related anxiety, distress or depression) and higher risk of pre-term birth or low birth weight in general (Orr *et al*., 2007; Copper *et al*., 1996). However, a number of older studies have questioned this link. For example, in a study of 1860 white women it was concluded that "in the general population of pregnant women, anxiety and depression during pregnancy, while undesirable in themselves, are of little importance in the evolution of obstetric complications" (Perkin *et al*., 1993). Similarly, Peacock and her colleagues have shown that no evidence of any association between psychosocial factors and pre-term

delivery exist (Peacock *et al*., 1995). Interestingly, in our cohort we did not observe any significant correlation between stress and gestational period. This might have to do with the fact that we have measured maternal stress only in the third trimester and might have missed the gestational time window when exposure to high stress levels can act as a "hard-wiring" process and affect the timing of birth. For example, in a recent study of 1800 Chinese women concluded that "prenatal severe life events, especially in the first trimester, may play an important role in increasing the risk of preterm birth and low birth weight" (Zhu *et al*., 2010). There is also evidence that diet might also influence the timing of birth. In a large cohort of 35.500 Danish women, it has been suggested that a shift towards a Mediterranean-type diet during pregnancy may reduce the risk of pre-term birth (Mikkelsen *et al*., 2008). We would like to propose that combining better nutritional habits and managing psychological stress through coping will lead to better management of pregnancy and reduce the chances of delivering prematurely.

8.1.4. What is the potential role of GAS5 in the human placenta?

In our study we have demonstrated for the first time the expression and cellular distribution of GAS5 (growth arrest-specific transcript 5) in the human placenta and 2 placental cell lines. As already mentioned, GAS5 encodes a single strand noncoding RNA (ncRNA) and hence its name as it can accumulate in growth-arrested cells (Schneider & King, 1988). In a recent study by Kino and colleagues it was demonstrated that GAS5 ncRNA may be a repressor for the GR by acting as a decoy "glucocorticoid response element (GRE)", thus, competing with DNA GREs for binding to the GR. The net effect is the inhibition of the association of GRs with their DNA recognition sequence (Kino *et al*., 2010).

In this study we provide further information about the regulation of this transcript by cortisol using two *in vitro* models. We demonstrate using Q-PCR that the GAS5 transcript is

upregulated by cortisol dose independently. This finding provides further evidence of regulation of GAS5 by stress and corroborates initial *in vivo* data in mice (Mayer *et al*., 2010). In a study using, C57BL/6 male mice, stress induced GAS5 RNA levels in the hippocampus and this increase was accompanied by a rise of corticosterone levels (Mayer *et al*., 2010). These two *in vitro* and *in vivo* observations are highly suggestive of a functional link between stress and this ncRNA.

We have also shown that GAS5 is expressed at levels as high as GRα in the human placenta, suggesting a higher order of complexity in the regulation of glucocorticoid signalling during pregnancy. Moreover, when we performed RNA FISH the GAS5 transcript was localised almost exclusively in the syncytiotrophoblast layer of the human placenta both at cytoplasmic and nuclear level. Interestingly, we have also demonstrated that GRα is also localised in the cytoplasm of syncytiotrophoblasts. In view of previous data in Hela cells where GAS5 translocates from the cytoplasm into the nucleus with GR in response to dexamethasone (Kino *et al*., 2010), this co-localisation is highly suggestive of a potential cross-talk at placental level. Indeed, we have shown that there is an increase in the ratio of GRα / GAS5 in pre-term labour when compared with term placental samples. This shift might affect how GRα responds to cortisol, and subsequently impact on GR-mediated signalling at placental level.

8.1.5. Is there a gene-environment interaction? Evidence from GR polymorphism studies.

As stated earlier the GR is crucial for the effects of glucocorticoids (GCs). Indeed there are several clinical features associated with GR polymorphisms. Our study provides a novel insight into the involvement of GR polymorphisms in pregnancy outcome. We have demonstrated that only the maternal Tht*III* 1 polymorphism was suggestive of a nature-nurture interaction since only in Tht*III* 1 (CC), maternal stress attitude predicts foetal weight-

reduction, but not in Tht*III* 1 (GC) independent of confounders such as BMI, pregnancy planning or fast food eating during pregnancy. Moreover, a partial correlation between maternal attitude ("Happy") and foetal weight, in *Bcl* I (CC) polymorphism was also noted. This is the first time that a gene-environment interaction between a common GR polymorphism and foetal weight was noted. In a study conducted a similar nature-nurture interaction was noted between the *ER22/23EK* polymorphism and the effect of childhood adversity on depression. In addition, patients carrying the *ER22/23EK* had decreased in Free Cortisol Index, whereas heterozygotes for the *Bcl* I polymorphism, were less vulnerable for depression in conjunction with childhood adversity, than *Bcl* I homozygotes and wild-type (Bet *et al*., 2008). In our study, no correlation between circulating plasma cortisol levels and any of the polymorphisms was detected (data not shown).

To this date, there is still controversy surrounding the potential role of these polymorphisms in terms of mediating maternal stress responses during pregnancy. It was recently shown that GR gene polymorphisms were not associated with birth weight or early postnatal weight and no associations were noted with length and head circumference. Moreover, neither were these polymorphisms associated with the risks of low birth weight or growth acceleration from birth to 24 months of age (Geelhoed *et al*., 2010). However, Bertalan and his colleagues have shown that the *Bcl* I polymorphism of the foetal GR gene is significantly associated with higher gestational age-adjusted birth weight in preterm neonates (Bertalan *et al*., 2008). The same group has later demonstrated that there was an overrepresentation of *Bcl* I polymorphism of the GR gene in pregnant women with HELLP (hemolysis, elevated liver enzymes and low platelet counts) syndrome compared to healthy pregnant women. Moreover, there were no significant differences in carrier and allelic frequencies of the *N363S* and *ER22/23EK* polymorphisms between healthy pregnant women and those with severe preeclampsia (Bertalan *et al*., 2009). As mentioned previously, *Bcl* I polymorphism

denominates a SNP in intron 2, located 647 bp inside the intron at the 3' side of exon 2. The exact mechanism via which *Bcl* I polymorphism can alter signalling is not known given that the mutation resides within an intron and therefore its location does not involve a coding, regulatory or splicing part of the GR gene. It has been suggested that this polymorphism might be in linkage with other variations, e.g., in the promoter region, or linked to other functionally important polymorphisms (Manenschijn *et al.,* 2009). It is also possible that this SNP results in an intron-mediated RNA interference (Ying & Lin, 2009). Of interest, the intron 2 of the GR gene is quite large in size and a mutation on its 5'-end might affect the splicing machinery.

In our cohort, *Bcl* I polymorphism deviated from the Hardy Weinberg equilibrium (that states that both allele and genotype frequencies in a population remain constant). However this deviation is not uncommon in genetic studies. Violations of the Hardy Weinberg equilibrium can be due to numerous causes such as: inbreeding (leads to an increase in homozygosity for all genes), mutation (can exert a subtle effect on allele frequencies), or small population size (can cause a random change in allele frequencies; genetic drift). We feel that is important to incorporate these data as they do provide a novel insight into the role of GR polymorphisms. Indeed this appears to be the general consensus. For example, in a study of 42 gene-disease associations assessed in meta-analyses of 591 studies revealed that exclusion of studies violating the equilibrium resulted in loss of statistical significance of the overall meta-analysis in three studies and changed the formal significance of the estimated between-study heterogeneity in three instances (Trikalinos *et al*., 2006). Should our findings be replicated in larger cohort, given the simplicity in assessing such attitudes and the feasibility to identify the polymorphic group of women early on in pregnancy, these findings will have significant implications for public health and prevention.

8.1.6. How is mTOR and GR signalling implicated in pre-term labour and maternal stress responses?

One of the most important findings of our study came from the preclinical studies using placental tissues. Quantitative PCR revealed that the major transcripts in the human placenta are GRα, GAS5 and Deptor. The key finding is that there are marked differences in the relative mRNA abundance of these components between term and pre-term labour. For example, when we assessed these levels as a function of a ratio of GRα over the remaining GRs there is a clear shift towards GRα, rendering it the main receptor during preterm labour. This coincides with a two-fold increase in the GRα/GAS5 ratio. This is of increasing importance since GR activity is known to be repressed by GAS5 that folds into a soluble glucocorticoid response element-like sequence and serves as a decoy for GR DNA binding. In this cohort of patients, maternal high stress levels inversely correlated with placental and fetal weights, and there is a strong correlation between GRα and GR-P as well as GRβ and GAS5, indicating possible tissue/pathology-specific splicing phenomena. Interestingly, in this preterm cohort, Deptor levels inversely correlated with placenta weight. Moreover there is an 1.6 fold increase in the expression of Deptor in the preterm samples and a notable 83% decrease of the GRα / Deptor ratio when compared to term placentas. Collectively these data point towards a convergence of mTOR and GR signalling. This is not surprising as emerging studies points towards a cross talk between these two components. In skeletal muscle "mTOR activation inhibits GR transcription function and efficiently counteracts the catabolic processes provoked by glucocorticoids" as shown by a recent study (Shimizu *et al*., 2011). Glucocorticoid resistance is caused by increased activity of mTOR (Sionov, 2008). This is of increasing importance in terms of signalling as glucocorticoid resistance is associated with weak increase in GR transcriptional activity, and no increase in GR protein levels (Miller *et al*., 2007), followed by secondary resistance driving downregulation of GRs due to prolonged exposure to glucocorticoids (Sionov, 2008). As expected, treatment with rapamycin (an

mTOR inhibitor) can resensitise the lymphoblastic leukaemia cells to glucocorticoids (Gu *et al*., 2010). An unexpected twist in this story was that inhibition of human T-cell proliferation by mTOR antagonists (e.g. rapamycin) requires GAS5 (Mourtada-Maarabouni *et al*., 2010). In view of these exciting data we would like to propose the following model for cross-talk in pre-term placenta: GRα is the predominant receptor due to a change in the ratio with the other splice variants GRβ, GRγ that act as repressors as well as the decoy GRE GAS5. This coincides with a rise in the expression of Deptor (an mTOR inhibitor). The end effect will be a sensitisation of glucocorticoid signalling and subsequent increase of GR transcriptional activity with a potential negative impact in stress conditions during pregnancy.

8.1.7. What do *in silico* analyses tell us about GR function?

In order to dissect complex cellular/tissue phenotypes, it is imperative to elucidate functional partnership amongst the proteins of interest and subsequently the potential networks formed by these interacting proteins. As we have already discussed, the exact role of GR splice variant is not fully elucidated. For this reason, we have used an *in silico* approach employing the database and web-tool STRING (Search Tool for the Retrieval of Interacting Genes-Proteins) in order to identify any potential novel interactions at protein level. STRING is a metaresource that collects the available data on protein–protein associations and predicts interactions. Over the past 11 years, STRING has grown into the most comprehensive resource of its type (Jensen *et al*., 2009). Analysis using the latest version STRING 8 has produced the following interactions (Appendix III):

GRα potentially interacts with: **HSP90AA1** (heat shock protein 90kDa alpha (cytosolic), class A member 1), **NCOA1 (**nuclear receptor coactivator 1), **SMARCA4** (matrix associated, actin dependent regulator of chromatin, subfamily a), **NFKB1** (nuclear factor of kappa light polypeptide gene enhancer in B-cells 1), **RELA** (v-rel reticuloendotheliosis viral oncogene

homolog A), **BAG1** (BCL2-associated athanogene), **TAT** (tyrosine aminotransferase), **JUN** (jun oncogene), **NRIP1** (nuclear receptor interacting protein 1), **GRIP1** (glutamate receptor interacting protein 1).

GRβ: Similar associations with GRα were noted. However, another 10 predicted interactions were provided with **FKBP4 (**FK506 binding protein 4), STAT5A (signal transducer and activator of transcription 5A), **NCOA2** (nuclear receptor coactivator 2), **CEBPB** (CCAAT/enhancer binding protein (C/EBP)), **POU2F1** (POU class 2 homeobox 1), **CREB1** (cAMP responsive element binding protein 1), **NCOA3** (nuclear receptor coactivator 3), **CALR (**calreticulin), **POMC (**proopiomelanocortin), **DAP3 (**death associated protein 3).

GRγ: Very similar interactions with GRβ were predicted. However, STRING 8 has proposed that certain associations of this gene result in an inhibitory phenotype, something that was not predicted for GRβ. For example, due ot its interactions with TAT it appears to inhibit CREB1. Similarly, NCOA3 exerts an inhibitory effect on JUN, as GRγ does in a directly. Finally, there is an inhibitory interplay between CEBP and RELA.

GR-P: GR-P appears to also interact with very similar proteins as GRα.

These findings provide potential scaffolds for modelling interactions of the GR splice variants. However, caution should be exercised as these are predicted interactions based on automatic data-mining searches, and computerised "scoring". Future studies using co-immunoprecipitation or other complex techniques such as FRET would allow us to gain a better insight of how "real" these interactions are, and how they can modify glucocorticoid pharmacology.

8.1.8. What are the limitations of this study?

We would also like to acknowledge that our study has a number of limitations. The placental samples, blood and plasma come from a fairly homogeneous population from the island of Crete in Greece. However, the uniformity of our cohort could also have certain advantages - we do not need to control for the effects of other variables such as lifestyle and ethnicity, as these have been quite similar among the participants of this study (all samples obtained from the same University Hospital, in the capital city Heraklion). In terms of the questionnaire, the source of the stress was not reported. It is accepted that individual differences may contribute to how the emotion is perceived and subsequently experienced. People with more negative personality characteristics report higher levels of emotional distress and negative emotions when faced with stressful events (Winter & Kuiper, 1997).

With regards to the polymorphic study, this could have been benefited by a larger number of participants from other European countries. Reproducibility of phenotyping for GRs has been shown to be affected by the number of individuals studied and racial heterogeneity (van Rossum *et al*., 2005). In a very recent study, generalized GC resistance accompanied with an adrenocortical adenoma was caused by a novel point mutation of the GR gene. A C to T substitution at nucleotide position of 1667 (exon 5) in GRα gene was detected in this patient by sequencing analysis (Zhu *et al*., 2011). Sequencing of the full length gene will provide a better insight into novel mutations and how might be associated with certain reproductive pathologies.

Another restriction we were faced with was the lack of access to clinical samples of the first and second trimester, due to ethical restrictions. A larger cohort of placental samples from the first, second and third trimester might have alleviated inter-patient variation as well as provided a more detailed map of these key components throughout pregnancy. When we

have used a placental synsytialisation model to represent the third trimester, certain changes in the expression of mTOR and GRs were noted.

Our *in vitro* studies provided an interesting perspective of the regulation of Deptor by cortisol. However, no direct conclusions can be drawn unless these studies are repeated in primary placental trophoblasts or using organotypic placental cultures and the effects of cortisol on mTOR and GR signalling components warrant further evaluation. Finally, the changes detected in the placental samples using QPCR should be confirmed at protein level using semi-quantitative western blotting. A drawback for this analysis is the lack of commercially available antibodies for GR splice variants and the fact that GAS5 does not translate into a protein.

8.1.9. What are the future directions?

8.1.9.1. Study further modifications of GRs.

It should be emphasised that GR function can also be affected epigenetically (i.e. DNA methylation) which can affect directly GR function (Claes, 2010). The GR gene has a number of alternative promoters that can be methylated. Therefore, methylation status of the GR promoters can be examined by bisulfite sequencing. It will also be of interest to determine using QPCR the use of different promoters of GR in relation to term or pre-term delivery as well as maternal stress levels.

In addition, it is documented that mutations in the human GR gene can impair the molecular mechanisms of GRα action and subsequently modifies tissue sensitivity to glucocorticoids (Charmandari *et al*., 2007). Therefore, apart from the polymorphism analyses, it will be of great interest to sequence the whole GR gene using sets of primers designed at the

exon/intron junction sequences as previously described (Charmandari *et al*., 2007). The identification of natural-occuring mutations in mothers' GR gene will improve our understanding of the molecular mechanisms underlying glucocorticoid action in the human placenta, with main emphasis to certain pathologies.

8.1.9.2. Elucidate further the activity of mTORC-1/mTORC-2 complexes

The activity of mTORC complexes can also be modulated post-translationally. It is important therefore to assess the phosphorylation status of the following key components: mTOR (Ser2481, Ser2448), Akt (Ser473, Thr308), S6K (Thr389, Ser371), 4E-BP1 (Thr37/46), PRAS40 (Thr246), Raptor (Ser792) and Rictor (Thr1135). Currently there are no commercially available antibodies for Deptor. The phosphorylation changes should be measured not only in placental samples but also in the cells lines treated with cortisol.

8.1.9.3. Define where in the placental cells mTOR complexes are located.

We have provided evidence of localisation of mTOR, Deptor, Rictor and Raptor in the cytoplasm of BeWo and JEG-3 cells as well as in the syncytiotrophoblasts of the human placentas. Further work is clearly required to determine where exactly mTORC-1 and mTORC-2 complexes reside, since there is evidence for localisation of mTORC-1 in mitochondria, endoplasmic reticulum, Golgi, and nucleus. The trafficking of mTOR signalling should also be investigated upon treatment of the two cell models with cortisol. It will also be of interest to determine whether placental mTOR signalling components change their trafficking/distribution with relation to the length of gestation or in conditions involving nutrient restriction such as intrauterine growth restriction.

8.1.9.4. Assess the stoichiometry of Deptor with mTORC-1/mTORC-2 at placental level.

As mentioned previously, little is known about how Deptor modulates mTOR activity. It is therefore crucial to understand the molecular proximity of Deptor with mTORC-1 and mTORC-2 complexes. The stoichiometry of mTOR complexes with Deptor in placental tissues can be measured using: a) co-immunoprecipitation studies and b) transmission electron microscopy (TEM). Moreover, we can also assess the impact of Deptor depletion and overexpression in mTORC-1 and mTORC-2 stoichiometry *in vitro*. The two placental cell lines can be used to study the hetero-dimerisation of Deptor with components of mTORC1 and mTORC2 complexes using Fluorescence Resonance Energy Transfer (FRET).

8.1.9.5. Investigate the involvement of mTOR/GR/GAS5 signalling in pathologies.

Given the involvement of these pathways in mediating stress; it will be of interest to provide a detailed map of these components in placentas from cohorts of patients diagnosed with pre-eclampsia, intrauterine growth restriction and gestational diabetes mellitus. Apart from QPCR analyses, semi-quantitative western blotting should be employed to dissect any changes in the expression at protein level at least for mTOR, Deptor, Rictor, Raptor, and GRα using a larger cohort of clinical tissues.

REFERENCES

Abraham, R.T. (2002) Identification of TOR signaling complexes: more TORC for the cell growth engine. Cell. 111: 9-12.

ACOG Committee Obstetric Practice. (2002) ACOG Committee Opinion, No. 267, January 2002: exercise during pregnancy and the postpartum period. Obstet Gynecol. 99: 171–173.

Adeney, K. L., Williams, M. A., Melissa A. Schiff, M. A., Qiu, C., Sorensen, T. K (2007) Coffee consumption and the risk of gestational diabetes mellitus Acta Obstetricia et Gynecologica Scandinavica. 86(2): 161-166

Adetunji, J.A. (1998) Unintended childbearing in developing countries: levels, trends and determinants. Calverton, MD, Macro International (Demographic and Health Surveys Analytical Report, No. 8)

Alsop, D., Ings, J.S., Vijayan, M.M. (2009) Adrenocorticotropic hormone suppresses gonadotropin-stimulated estradiol release from zebrafish ovarian follicles. PLoS ONE 4(7): e6463.

Amaldi, F., Pierandrei-Amaldi, P. (1997) TOP genes: a translationaly controlled class of genes including those coding for ribosomal proteins. In: Jeanteur, P., editor. Progress in molecular and subcellular biology. Vol. 18. Springer-Verlag; Berlin, Germany: p. 1-17.

Aranda, A. & Pascual, A. (2001). Nuclear Hormone Receptors and Gene Expression. Physiological Reviews. 81(3):1269-1304.

Arimoto-Ishida E, Sakata M, Sawada K, Nakayama M, Nishimoto F, Mabuchi S, Takeda T, Yamamoto T, Isobe A, Okamoto Y, Lengyel E, Suehara N, Morishige K,Kimura T. (2009) Up-regulation of alpha5-integrin by E-cadherin loss in hypoxia and its key role in the migration of extravillous trophoblast cells during early implantation. Endocrinology;150 (9): 4306-4315.

Arroyo, J.A., Brown, L.D., Galan, H.L. (2009) Placental mammalian target of rapamycin and related signaling pathways in an ovine model of intrauterine growth restriction. Am J Obstet Gynecol. 201, 616.e1-7.

Bain, D.L., Heneghan, A.F., Connaghan-Jones, K.D., Miura, M.T. (2007) Nuclear receptor structure: implications for function. Annu Rev Physiol. 69:201-20.

Baldini, M., Pasqui, F., Bordoni, A., Maranesi, M. (2009) Is the Mediterranean lifestyle still a reality? Evaluation of food consumption and energy expenditure in Italian and Spanish university students. Public Health Nutr. 12(2): 148-155

Ballon, D.R., Flanary,P.L., Gladue, D.P., Konopka, J.B., Dohlman H.G. & Thorner, J. (2006). DEP-domain-mediated regulation of GPCR signaling responses. Cell. 126: 1079-1093.

Bamberger, C.M., Bamberger, A.M., De Castro, M. & Chrousos, G.P. (1995). Glucocorticoid receptor b, a potential endogenous inhibitor of glucocorticoid action in humans. Journal Clinical Investigation. 95(6): 2435-2441.

Barker, D.J. (1997) The long-term outcome of retarded fetal growth. Clin Obstet Gynecol 40, 853–863.

Barker, D.J.P. (1997). Maternal nutrition, fetal nutrition, and disease in later life. Nutrition. 13(9): 807-813.

Barot, N., Luu, J., Nirmal, N. (2005) The Endocrine System .Chapter 45 - Unit 7 (Animal Form and Function (kleczekbiology.wikispaces.com/Endocrine+System).

Barrandon, C., Spiluttini, B., Bensaude, O. (2008) Non-coding RNAs regulating the transcriptional machinery. Biol Cell. 100:83–95.

Bassett, N.S., Tong, P.C., Currie, M.J., Woodall, S.M., Breier, B.H., Gluckman, P.D. (1996) Altered placental glucose transporter gene expression in maternal undernutrition and hypertension. Proc 10th Int Congr Endocrinol San Francisco, 1996, pp.3–416.

Bauer, M.K., Harding, J.E., Bassett, N.S., Breier, B.H., Oliver, M.H., Gallaher, B.H., Evans, P.C., Woodall, S.M., Gluckman, P.D. (1998) Fetal growth and placental function. MolCell Endocrinol. 140: 115-120.

Beato, M., Chalepakis, G., Schauer, M., Slater, E.P. (1989) DNA regulatory elements for steroid hormones. J Steroid Biochem. 32:737–747.

Beck, S., Wojdyla, D., Say, L., Betran, A.P., Merialdi, M., Requejo, J.H., Rubens, C., Menon, R., Van Look, P.F. The worldwide incidence of preterm birth: a systematic review of maternal mortality and morbidity. Bull World Health Organ. 88(1): 31-38.

Benaitreau, D., Dieudonné, M.N., Dos Santos, E., Leneveu, M.C., Mazancourt, P., Pecquery, R.(2009) Antiproliferative effects of adiponectin on human trophoblastic cell lines JEG-3 and BeWo. Biol Reprod.; 80(6): 1107-14.

Benirschke, K. (1998) Remarkable placenta. Clinical Anatomy.11 (3) : 194–205.

Bertalan, R., Patocs, A., Nagy, B., Derzsy, Z., Gullai, N., Szappanos, A., Rigo, J., Rac, K. (2009) Overrepresentation of BclI polymorphism of the glucocorticoid receptor gene in pregnant women with HELLP syndrome. Clinica Chimica Acta. 405 (1-2): 148-152.

Bertalan, R., Patocs, A., Vasarhelyi, B., Treszl, A., Varga, I., Szabo, E., Tamas, J., Toke, J., Boyle, B., Nobilis, A., Rigo, J. Jr., Racz, K.(2008) Association between birth weight in preterm neonates and the BclI polymorphism of the glucocorticoid receptor gene. Steroid Biochem Mol Biol. 111(1-2): 91-94.

Bet, P. M., Penninx, B.W., Bochdanovits, Z., Uitterlinden, A. G., Beekman, A.T., van Schoor. N.M., Deeg D. J., Hoogendijk W.J.(2009). Glucocorticoid receptor gene polymorphisms and childhood adversity are associated with depression: New evidence for a gene-environment interaction.(2009) Am J Med Genet B Neuropsychiatr Genet.; 150B (5): 660-669.

Beugnet, A., Wang, X. & Proud, C.G. (2003). Target of rapamycin (TOR)-signaling and RAIP motifs play distinct roles in the mammalian TOR-dependent phosphorylation of initiation factor 4E-binding protein 1. Journal of Biological Chemistry. 278: 40717-40722.

Bjornsti, M.A. & Houghton, P.J. (2004). The TOR pathway: a target for cancer therapy. Nature Reviews Cancer. 4(5): 335-348.

Block, G. (2004) Foods contributing to energy intake in the US: data from NHANES III and NHANES 1999–2000. J Food Compost Anal. 17:439–447.

Bloomfield, F.H. & Harding, J.E. (1998) Experimental aspects of nutrition and fetal growth. Fetal and Maternal Medicine Review.10: 91–107.

Bolten, M.I., Wurmser, H., Buske-Kirschbaum, A., Papoušek, M., Pirke, K.M., Hellhammer, D. (2010) Cortisol levels in pregnancy as a psychobiological predictor for birth weight. Arch Womens Ment Health.14(1): 33-41.

Bosotti, R., Isacchi, A. & Sonnhammer, E.L. (2000). FAT: a novel domain in PIK-related kinases. Trends in Biochemical Sciences. 25(5): 225-227.

Brown, E.J., Albers, M.W., Shin, T.B., Ichikawa, K., Keith, C.T., Lane, W.S., Schreiber, S.L. (1994) A mammalian protein targeted by G1-arresting rapamycin-receptor complex. Nature. 369, 756-758.

Brunn, G.J., Hudson, C.C., Sekulic, A., Williams, J.M., Hosoi, H., Houghton, P.J., Lawrence, J.C. & Abraham, R.T. (1997). Phosphorylation of the translational repressor PHAS-I by the mammalian target of rapamycin. Science. 277 : 99-101.

Buckingham, J.C. (2006) Glucocorticoids: exemplars of multi-tasking. *Br J Pharmacol*. **147** (Supplement 1): S258–68

Buemann, B., Vohl, M.C., Chagnon, M., Chagnon, Y.C., Gagnon, J., Perusse, L., Dionne, F., Despres, J.P., Tremblay, A., Nadeau, A., Bouchard, C. (1997) Abdominal visceral fat is associated with a BclI restriction fragment length polymorphism at the glucocorticoid receptor gene locus. Obes Res. 5: 186 –192

Burleigh, D. W., Kendziorski, C. M., Choi, Y. J., Grindle, K. M., Grendell, R. L., Magness, R. R., Golos, T. G. (2007) Microarray analysis of BeWo and JEG3 trophoblast cell lines: identification of differentially expressed transcripts. Placenta 28: 383–389.

Ceulemans, S., De Zutter, S., Heyrman, L., Norrback, K.F., Nordin, A., Nilsson, L.G., Adolfsson, R., Del-Favero, J., Claes, S. (2011) Evidence for the involvement of the glucocorticoid receptor gene in bipolar disorder in an isolated northern Swedish population. Bipolar Disorders.13 (7-8): 614–623.

Chambon, P. (2005). The Nuclear Receptor Superfamily: a personal retrospect on the first two decades. Molecular Endocrinology. 19(6): 1418-1428.

Charmandari, E., Kino, T., Ichijo, T., Jubiz, W., Mejia, L., Zachman, K., Chrousos, G.P. (2007) A novel point mutation in helix 11 of the ligand-binding domain of the human glucocorticoid receptor gene causing generalized glucocorticoid resistance. J Clin Endocrinol Metab. 92: 3986–3990.

Choi, K.M., McMahon, L.P. & Lawrence, J.J. (2003). Two motifs in the translational repressor PHAS-I required for efficient phosphorylation by mammalian target of rapamycin and for recognition by raptor. Journal of Biochemical Chemistry. 278 (22): 19667-19673.

Christainsen, J.J., Djurhuus, C.B., Gravholt, C.H., Iversen, P., Christiansen, J.S., Schmitz, O., Weeke, J., Jorgensen, J.O. & Moller, N. (2007). Effects of cortisol on carbohydrate, lipid, and

protein metabolism: studies of acute cortisol withdrawal in adrenocortical failure. Clinical endocrinology and metabolism. 92(9): 3553-3559.

Chrousos, G.P. & Kino, T. (2005) Intracellular glucocorticoid signaling: a formerly simple system turns stochastic. Sci STKE. pe48.

Chrousos, G.P. (2001) Glucocorticoid therapy. In: Felig, P.; Frohman, LA., editors. Endocrinology & Metablism. McGraw-Hill; New York: 2001. p. 609-632.

Clark, P.M. (1998) Programming of the hypothalamopituitary- adrenal axis and the fetal origins of adult disease hypothesis. Eur. J. Pediatr. 157(Suppl 1): S7–S10.

Coccia, E.M., Cicala, C., Charlesworth, A., Ciccarelli, C., Rossi, G.B., Philipson, L., Sorrentino, V. (1992) Regulation and expression of a growth arrest-specific gene (gas5) during growth, differentiation, and development. Mol Cell Biol. 12(8): 3514-3521.

Coleman, P.K., Reardon, D.C., Cougle, J.R., (2005). Substance use among pregnant women in the context of previous, reproductive loss and desire for current pregnancy. British Journal of Health Psychology 10(2): 255–268.

Collier, S.A., Rasmussen, S.A., Feldkamp, M.L, Honein, M.A. (2009) Prevalence of self-reported infection during pregnancy among control mothers in the National Birth Defects Prevention Study. Birth Defects Res A Clin Mol Teratol. 85(3): 193-201.

Conde-Agudelo, A., Villar, J. & Lindheimer, M. (2008) Maternal infection and risk of preeclampsia: systematic review and metaanalysis. Am J Obstet Gynecol. 198(1): 7

Constancia,M., E.Angiolini, I. Sandovici, Smith, P., Smith, R., Kelsey, G., Dean, W., Ferguson-Smith, A., Sibley, C.P., Reik, W., Fowden, A. (2005) Adaptation of nutrient supply to fetal demand in the mouse involves interaction between the Igf2 gene and placental transporter systems.Proc. Natl.Acad. Sci. USA102: 19 219– 19 224.

Copper, R. L., Goldenberg, R. L., Das, A., Elder, N., Swain, M., Norman, G., Ramsey, R., Cotroneo, P., Collins, B.A., Johnson, F., Jones, P., Meier, A.M. (1996) The preterm prediction study: maternal stress is associated with spontaneous preterm birth at less than thirty-five weeks' gestation. National Institute of Child Health and Human Development Maternal-Fetal Medicine Units Network. American Journal of Obstetrics and Gynecology, 175(5): 1286-1292.

Crozier, T.W., Stalmach, A., Lean, M.E., Crozier, A. (2011) Espresso coffees, caffeine and chlorogenic acid intake: potential health implications. Food Funct. 3(1): 30-33.

Cunningham, G.G., MacDonald, P.C., Gant, N.F. (Eds) (1993) Section II. Physiology of pregnancy. In Williams Obstetrics. Appleton and Lange, Stamford, USA. pp. 81–246.

Czeisler, C.A., Ede, M.C., Regestein, Q.R., Kisch, E.S., Fang, V.S., Ehrlich, E.N. (1976) Episodic 24-hour cortisol secretory patterns in patients awaiting elective cardiac surgery. J Clin Endocrinol Metab. 42:273–283.

Dahia,P.L.M., Honegger, J., Reincke, M., Jacobs, R.A., Mirtella, A., Fahlbusch, R., Besser, G.M., Chew, S.l. & Grossman, A.B. (1997). Expression of Glucocorticoid Receptor Gene Isoforms

in Corticotropin-Secreting Tumors. Clinical Endocrinology & Metabolism. 82(4) : 1088-1093.

Das, M., Xu, B., Lin, L., Chakrabarti, S., Shivaswamy, V. & Rote, N.S. (2004). Phosphatidylserine efflux and intercellular fusion in a BeWo model of human villous cytotrophoblast. Placenta 25(5): 396-407.

Davies,vT.H., Ning, Y.M. & Sanchez, E.R. (2002). A new first step in activation of steroid receptors: hormone-induced switching of FKBP51 and FKBP52 immunophilins. Journal of Biological Chemistry. 277 (7) : 4597-600.

de Batlle, J., Garcia-Aymerich, J., Barraza-Villarreal, A., Antó, J.M,, Romieu, I. (2008). Mediterranean diet is associated with reduced asthma and rhinitis in Mexican children. Allergy. 63(10): 1310-1316.

de Bosscher, K., Vanden Berghe, W. & Haegeman, G. (2003). The interplay between the glucocorticoid receptor and nuclear factor-kB or activator protein-1: molecular mechanisms for gene repression. Endocrine reviews. 24(4): 488-522.

de Castro, M., Elliot, S., Kino, T., Bamberger, C., Karl, M., Webster, E. and Chrousos, G. P. (1996) The non-ligand binding β-isoform of the human glucocorticoid receptor (hGR β): tissue levels, mechanism of action, and potential physiologic role. Mol Med 2: 597-607.

de Lange, P., Segeren, C.M., Koper, J.W., Wiemer, E., Sonneveld, P., Brinkmann, A.O.,White,A., Brogan, I.J., de Jong,F.H.,Lamberts, S.W.J. (2010) Expression in Hematological Malignancies of a Glucocorticoid Receptor Splice Variant That Augments Glucocorticoid Receptor-mediated Effects in Transfected Cells. *Cancer Res.*61; 3937.

de Weerth, C., van Hees, Y. and Buitelaar, J. K. (2003) Prenatal maternal cortisol levels and infant behavior during the first 5 months. Early Hum. Dev. 74: 139–151.

Denari, D., Ceballos, N.R. (2006) Cytosolic glucocorticoid receptor in the testis of Bufo arenarum: seasonal changes in its binding parameters. Gen Comp Endocrinol.147(3): 247-54.

Dennis, P.B., Jaeschke, A., Saitoh, M., Fowler, B., Kozma, S.C. & Thomas, G. (2001). MammalianTOR: a homeostatic ATP sensor. Science. 294: 1102-1105.

DiPietro, J. A., Hilton, S. C., Hawkins, M., Costigan, K. A., & Pressman, E. K. (2002) Maternal stress and affect influence fetal neurobehavioral development. Developmental Psychology, 38: 659–668.

Dittmar, K.D., Demady, D.R., Stancato, L.F., Krishna, P., Pratt, W.B. (1997) Folding of the glucocorticoidreceptor by the heat shock protein (hsp) 90-based chaperone machinery. The role of p23 is to stabilize receptor.hsp90 heterocomplexes formed by hsp90.p60.hsp70. J.Biol.Chem. 272: 21213 - 21220.

Dole, N., Savitz, D.A.,Hertz-Picciotto, I., Siega-Riz, A.M.,McMahon, M.J., Buekens, P. (2003) Maternal Stress and Preterm Birth. Am. J. Epidemiol. 157 (1): 14-24.

Dorland, W.A.N. (2007) Dorland's illustrated medical dictionary. 31st ed. Philadelphia: Saunders.

Douglas, A. J. (2010) Mother-offspring dialogue in early pregnancy: Impact of adverse environment on pregnancy maintenance and neurobiology. Prog Neuropsycho-pharmacol Biol Psychiatry. 35, 1167-1177

Edwards, C.H., Cole, O.J., Oyemade, U.J., Knight, E.M., Johnson, A.A., Westney, O.E., Laryea, H., West, W., Jones, S., Westney, L.S. (1994) Maternal stress and pregnancy outcomes in a prenatal clinic population. J Nutr.124(6 Suppl):1006S-1021S.

Einarson, A., Riordan S. (2009) Smoking in pregnancy and lactation: a review of risks and cessation strategies. Eur J Clin Pharmacol. 65(4): 325-330

Ellinger, I., Schwab, M., Stefanescu, A., Hunziker, W., Fuchs, R. (1999) IgG transport across trophoblast-derived BeWo cells: a model system to study IgG transport in the placenta. Eur J Immunol 29: 733-744.

Encio, I.J. & Detera-Wadleigh, S.D. (1991). The genomic structure of the human glucocorticoid receptor. Journal of Biological Chemistry. 266(11): 7182-7188.

Eriksson, J.G., Forsén, T., Tuomilehto, J., Winter, P.D., Osmond, C., Barker, D.J. (1999) Catch up growth in childhood and death from coronary heart disease: longitudinal study. BMJ 318: 427–431.

Escriva, H., Delaunay, F., Laudet, V. (2000) Ligand binding and nuclear receptor evolution. Bioessays. 22: 717-27.

Escriva, H., Safi, R., Hanni, C., Langlois, M.C., Saumitou-Laprade, P., Stehelin, D., Capron, A., Pierce, R., Laudet, V. (1997) Ligand binding was acquired during evolution of nuclear receptors. Proc Natl Acad Sci USA. 94: 6803-6808.

Fingar, D.C., Richardson, C.J., Tee, A.R., Cheatham, L., Tsou, C., and Blenis, J. (2004) mTOR controls cell cycle progression through its cell growth effectors S6K1 and 4E-BP1/ eukaryotic translation initiation factor 4E. Mol Cell Biol. 24: 200–216.

Flanagan-Cato, L.M. & Fluharty, S.J. (1997). Emerging mechanisms of the behavioral effects of steroids. Current Opinion in Neurobiology. 7(6): 844-848.

Ford, E.S., Giles, W.H., Dietz, W. (2002) The prevalence of metabolic syndrome in the US population. JAMA. 297(3): 356-359.

Forsen, T., Eriksson, J.G., Tuomilehto, J., Osmond, C., Barker, D. J. P. (1999) Growth in utero and during childhood among women who develop coronary heart disease: longitudinal study. BMJ 319: 1403– 1407.

Frankfurt, O. & Rosen, S.T. (2004) Mechanisms of glucocorticoid-induced apoptosis in hematologic malignancies: updates. Curr Opin Oncol. 16: 553–563.

Frias, M.A., Thoreen, C.C., Jaffe, J.D., Schroder, W., Sculley, T., Carr, S.A., Sabatini, D.M. (2006) mSin1 is necessary for Akt/PKB phosphorylation, and its isoforms define three distinct mTORC2s. Curr Biol. 16(18): 1865-70.

Frim,D.M., Emanuel, S.R.L., Robinson, B.G., Smas,C.M., Adler,G.K., Majzoub, J.A. (1988) Characterization and gestational regulation of corticotropin-releasing hormone messenger RNA in human placenta. J. Clin. Invest.2: 287-292.

Fujiwara, T., Cherrington, A.D., Neal, D.N. & Mcguinness, O.P. (1996). Role of cortisol in the metabolic response to stress hormone infusion in the conscious dog. Metabolism: clinical and experimental. 45(5): 571-578.

Fung, T.T., Rexrode, K.M., Mantzoros, C.S., Manson, J.E., Willett, W.C., Hu, F.B. (2009) Mediterranean diet and incidence of and mortality from coronary heart disease and stroke in women. Circulation.119(8): 1093-1100.

Gale,C.R., Javaid,M.K., Robinson,S.M., Law,C.M., Godfrey,K.M. & Cooper,C. (2007). Maternal Size in Pregnancy and Body Composition in Children. Journal of Clinical Endocrinology & Metabolism. 92(10): 3904-3911.

Gangloff, Y.G., Mueller, M., Dann, S.G., Svoboda, P., Sticker, M., Spetz, J.F., Um, S.H., Brown, E.J., Cereghini, S., Thomas, G., Kozma, S.C. (2004). Disruption of the mouse mTOR gene leads to early postimplantation lethality and prohibits embryonic stem cell development. Mol Cell Biol. 24(21): 9508-9516

Gao, X., Zhang, Y., Arrazola, P., Hino, O., Kobayashi, T., Yeung, R.S., Ru, B. & Pan, D. (2002). TSC tumour suppressor proteins antagonize amino acid - TOR signaling. Nature Cell Biology. 4: 699-704.

Geelhoed, M.J., Steegers, E.A., Koper, J.W., van Rossum, E.F., Moll, H.A., Raat, H., Tiemeier, H., Hofman, A., Jaddoe, V.W. (2010) Glucocorticoid receptor gene polymorphisms do not affect growth in fetal and early postnatal life. The Generation R Study. BMC Med Genet. 11: 39.

Germain, P., Staels, B., Dacquet, C., Spedding, M., Laudet, V. (2006) Overview of nomenclature of nuclear receptors. Pharmacol Rev. 58: 685-704.

Gheorghe, C.P., Goyal, R., Mittal, A., Longo, L.D. (2010) Gene expression in the placenta maternal stress and epigenetic responses. Int J Dev Biol. 54(2-3): 507–523.

Gidron, Y. & Ronson, A. (2008). Psychosocial factors, biological mediators, and cancer prognosis: a new look at an old story. Current Opinion in Oncology. 20(4): 386-392.

Godfrey, K.M. (2002). The Role of the Placenta in Fetal Programming- A Review. Placenta. 23: S20-S27.

Godfrey, K.M., & Barker, D.J. (2001) Fetal programming and adult health. Public Health Nutr. 4(2B): 611-624.

Godfrey,K., Robinson,S., Barker,D., Osmond,C. & Cox,V. (1996). Maternal nutrition in early and late pregnancy in relation to placental and fetal growth. British Medical Journal. 312(7028): 410-414.

Goldenberg, R.L., Hauth, J.C., Andrews, W.W. 2000. Intrauterine infection and preterm delivery. N Engl J Med; 342(20): 1500-1507

Goldenberg, R.L., McClure, E.M., Saleem, S., Reddy, U.M. (2010) Infection-related stillbirths. Lancet.; 375(9724): 1482-1490

Govindan, M.V., Devic, M., Green, S., Gronemeyer, H. & Chambon, P. (1985).Cloning of the human glucocorticoid receptor cDNA. Nucleic Acids Research. 13(23): 8293-8304.

Gu, L., Zhou, C., Liu, H., Gao, J., Li, Q., Mu, D., Ma, Z.(2010) Rapamycin sensitizes T-ALL cells to dexamethasone-induced apoptosis. J Exp Clin Cancer Res.29: 150.

Gu, W., Jones, C.T., Harding, J.E. (1987) Metabolism of glucose by fetus and placenta of sheep. The effects of normal fluctuations in uterine blood flow. J Dev Physiol; 9: 369–389.

Guertin, D.A., Sabatini, D.M. (2007) Defining the role of mTOR in cancer. Cancer Cell. 12: 9-22.

Guertin, D.A., Stevens, D.M., Thoreen, C.C., Burds, A.A., Kalaany, N.Y., Moffat, J., Brown, M., Fitzgerald, K.J., Sabatini, D.M. (2006) Ablation in mice of the mTORC components raptor, rictor, or mLST8 reveals that mTORC2 is required for signaling to Akt-FOXO and PKC alpha, but not S6K1. Dev Cell. 11: 859-871.

Guilliams, T.G., Edwards, L. (2010) Chronic Stress and the HPA Axis: Clinical Assessment and Therapeutic Considerations. Natural & Neutraceutical therapies for clinical practice. 9 (2):

Hackshaw, A., Rodeck, C., Boniface, S. (2011) Maternal smoking in pregnancy and birth defects: a systematic review based on 173 687 malformed cases and 11.7 million controls. Hum Reprod Update.17(5): 589-604.

Hales, C.N., Barker, D.J., Clark, P.M., Cox, L.J., Fall, C., Osmond, C., Winter, P.D. (1991) Fetal and infant growth and impaired glucose tolerance at age 64. BMJ 303: 1019–1022.

Halldorsson, T.I., Strøm, M., Petersen, S.B., Olsen, S.F. (2010) Intake of artificially sweetened soft drinks and risk of preterm delivery: a prospective cohort study in 59,334 Danish pregnant women. Am J Clin Nutr.92(3): 626-233.

Hara, K., Maruki, Y., Long, X., Yoshino, K., Oshiro, N., Hidayat, S., Tokunaga, C., Avruch, J., Yonezawa, K. (2002) Raptor, a binding partner of target of rapamycin (TOR), mediates TOR action. Cell. 110: 177-189.

Harding, J.E. (2001) The nutritional basis of the fetal origins of adult disease. Int J Epidemiol.30(1):15-23.

Heaton, S.J., Eady, J.J., Parker, M.L., Gotts, K.L., Dainty, J.R., Fairweather-Tait, S.J., McArdle, H.J., Srai, K.S., Elliott, R.M. (2008) The use of BeWo cells as an in vitro model for placental iron transport. Am J Physiol Cell Physiol 295: 1445-1453.

Hirata, M., Sato, T., Tsumagari, M., Shimada, A., Nakano, H., Hashizume, K. & Ito, A. (2003). Differential regulation of the expression of matrix metalloproteinases and tissue inhibitors of metalloproteinases by cytokines and growth factors in bovine endometrial stromal cells and trophoblast cell line BT-1 in vitro. Biology of Reproduction. 68(4): 1276-81.

Hollenberg, S.M., Weinberger, C., Ong, E.S, Cerelli, G., Oro A., Lebo R., Thompson EB., RoSenfeld MG. & Evans RM. (1985) Primary structure and expression of functional human glucocorticoid receptor cDNA. Nature. 318(6047): 635-641.

Holst D., Garnier Y. (2008) Preterm birth and inflammation-The role of genetic polymorphisms. Eur J Obstet Gynecol Reprod Biol.141(1): 3-9.

Honda, M., Orii, F., Ayabe, T., Imai, S., Ashida, T., Obara, T. and Kohgo, Y. (2000) Expression of glucocorticoid receptor β in lymphocytes of patients with glucocorticoid-resistant ulcerative colitis. Gastroenterology. 118: 859-66.

Huizink, A. C., Mulder, E. J., Robles de Medina, P. G., Visser, G. H., Buitelaar, J. K. (2004) Is pregnancy anxiety a distinctive syndrome? Early Human Development, 79: 81–91.

Hunt, J.S. (1989) Cytokine networks in the uteroplacental unit: macrophages as pivotal regulatory cells. J Reprod Immunol.16(1): 1-17.

Ikoma, Y., Nomura, S., Ito, T., Katsumata, Y., Nakata, M., Iwanaga, K., Okada, M., Kikkawa, F., Tamakoshi, K., Nagasaka, T., Tsujimoto, M., Mizutani, S. (2003) Interleukin-1 beta stimulates placental leucine aminopeptidase/oxytocinase expression in BeWo choriocarcinoma cells. Mol Hum Reprod. 9(2): 103-110.

Jacinto, E., Facchinetti, V., Liu, D., Soto, N., Wei, S., Jung, S.Y., Huang, Q., Qin, J., Su, B. (2006) SIN1/MIP1 maintains rictor- mTOR complex integrity and regulates Akt phosphorylation and substrate specificity. Cell. 127: 125-137.

Jacinto, E., Loewith, R., Schmidt, A., Lin, S., Rüegg, M.A., Hall, A., Hall, M.N. (2004) Mammalian TOR complex 2 controls the actin cytoskeleton and is rapamycin insensitive. Nat Cell Biol. 6: 1122-1128.

Jacinto, E., Lorberg, A. (2008) TOR regulation of AGC kinases in yeast and mammals. Biochem. J. 410 (19–37).

Jain, A., Olovsson, M., Burton, G.J., Yung, H.W. (2012) Endothelin-1 Induces Endoplasmic Reticulum Stress by Activating the PLC-IP(3) Pathway: Implications for Placental Pathophysiology in Preeclampsia. Am J Pathol. 180(6): 2309-2320.

Jansson, T. & Powell, T.L. (2007). Role of the placenta in fetal programming: underlying mechanisms and potential interventional approaches. Clinical Science. 113(1): 1-13.

Jensen, L.J., Kuhn, M., Stark, M., Chaffron, S., Creevey, C., Muller, J., Doerks, T., Julien, P., Roth, A., Simonovic, M., Bork, P., von Mering, C. (2009) STRING 8--a global view on proteins and their functional interactions in 630 organisms. Nucleic Acids Res.37: 412-416.

Johnson, R.F., Rennie, N., Murphy, V., Zakar, T., Clifton, V., Smith, R. (2008) Expression of glucocorticoid receptor messenger ribonucleic acid transcripts in the human placenta at term. J Clin Endocrinol Metab 93: 4887–4893.

Jones, L.L., Hashim, A., McKeever, T., Cook, D.G., Britton, J., Leonardi-Bee, J. (2011) Parental and household smoking and the increased risk of bronchitis, bronchiolitis and other lower respiratory infections in infancy: systematic review and meta-analysis. Respiratory Research 10: 12:5.

Kallen, J., Schlaeppi, J.M., Bitsch, F., Filipuzzi, I., Schilb, A., Riou, V., Graham, A., Strauss, A., Geiser, M., Fournier, B. (2004) Evidence for ligand-independent transcriptional activation of the human estrogen-related receptor alpha (ERRalpha): crystal structure of ERRalpha

ligand binding domain in complex with peroxisome proliferator-activated receptor coactivator-1alpha. J. Biol. Chem. 279 (47): 49330–7.

Karaçam Z., Ançel G. (2009) Depression, anxiety and influencing factors in pregnancy: a study in a Turkish population. Midwifery. 25(4): 344-356.

Karacam, Z., Onel, K., Gercek, E. (2009) Effects of unplanned pregnancy on maternal health in Turkey. Midwifery 27: 288-293

Karacam, Z., Sen, E., Amanak, K (2010) Effects of unplanned pregnancy on neonatal health in Turkey: A case–control study. International Journal of Nursing Practice;16: 555–563

Kato, Y., Braunstein, G. D. (1989). Discordant secretion of placental protein hormones in differentiating trophoblasts in vitro. J. clin. Endocrin. Metab 68: 814-820.

Keys, A. (1980) Seven Countries: A multivariate analysis of death and coronary heart disease. Harvard University Press. Cambridge, Massachusetts.

Kim, D.H., Sarbassov, D.D., Ali, S.M., King, J.E., Latek, R.R., Erdjument-Bromage, H., Tempst, P. & Sabatini, D.M. (2002). mTOR interacts with raptor to form a nutrient-sensitive complex that signals to the cell growth machinery. Cell. 110: 163-175.

Kino, T., Hurt, D.E., Ichijo, T., Nader, N. & Chrousos, G.P (2010) Noncoding RNA Gas5 is a growth arrest and starvation- associated repressor of the glucocorticoid receptor. Sci Signal. 3(107).

Knudsen, V.K., Orozova-Bekkevold, I.M., Mikkelsen, T.B., Wolff, S., Olsen, S.F. (2008) Major dietary patterns in pregnancy and fetal growth. Eur J Clin Nutr 62: 463–470.

Kramer, M.S. (2004) Aerobic exercise for women during pregnancy. Cochrane Database Syst Rev. (1): CD000180.

Kristof, A.S. (2010) mTOR signaling in lymphangioleiomyomatosis. Lymphat Res Biol. 8(1): 33-42

Kuczkowski K.M. (2009) Caffeine in pregnancy. Arch Gynecol Obstet. 280(5): 695-698.

Kushi, L.H., Lenart, E.B., Willett, W.C. (1995A) Health implications of Mediterranean diets in light of contemporary knowledge. 1. Plant foods and dairy products. Am J Clin Nutr. 61(6 Suppl): 1407S-1415S.

Kushi, L.H., Lenart, E.B., Willett, W.C. (1995B) Health implications of Mediterranean diets in light of contemporary knowledge. 2. Meat, wine, fats, and oils. Am J Clin Nutr. 61(6 Suppl): 1416S-1427S.

Lain, S.J., Roberts, C.L., Warning, J., Vivian-Taylor, J., Ford, J.B.(2011) A survey of acute self-reported infections in pregnancy. BMJ Open. 1(1): 083.

Laitinen, J., Pietiläinen, K., Wadsworth, M., Sovio, U., Järvelin, M.R. (2004) Predictors of abdominal obesity among 31-yr-old men and women born in Northern Finland in 1966. Eur. J. Clin. Nutr. 58: 180–190.

Lam, M & Lam, D. (2010) Adrenal Fatigue and Hormone Therapy

Laplante, M., Sabatini, D.M. (2009) mTOR signaling at a glance. J Cell Sci. 122: 3589-3594.

Laraia, B.A., Siega-Riz, A.M., Kaufman, J.S., Jones, S.J. (2004) Proximity of supermarkets is positively associated with diet quality index for pregnancy. Prev Med 39: 869–875.

Laudat, M.H., Cerdas, S., Fournier, C., Guiban, D., Guilhaume, B. & Luton, J.P. (1988). Salivary cortisol measurement: a practical approach to assess pituitary-adrenal function. Clinical endocrinology and metabolism. 66(2): 343-348.

Lazinski, M.J., Shea, A.K., Steiner, M. A. (2008). Effects of maternal prenatal stress on offspring development: a commentary. Archives of Women's Mental Health. 11(5-6): 363-375.

Lee, Y.M., Fujiwara, J., Munakata, Y., Ishii,T., Sugawara, A., Kaku, M., Kokubun, S., Sasaki, T., Funato, T.(2004) A mutation of the glucocorticoid receptor gene in patients with systemic lupus erythematosus. Tohoku J Exp Med; 203(2): 69-76.

Liao, W.X., Laurent, L.C., Agent, S., Hodges, J., Chen, D.B. (2012) Human placental expression of SLIT/ROBO signalling cues: effects of preeclampsia and hypoxia. Biol Reprod. 12; 86(4):111.

Lim, R., Riley, C., Barker, G., Rice, G.E., Lappas, M.(2012) Human labour is associated with decreased cytoplasmic FoxO4. Placenta;33(1): 52-59.

Lesage, J., Del-Favero, F., Leonhardt, M., Louvart, H., Maccari, S., Vieau, D., Darnaudery, M. (2004) Prenatal stress induces intrauterine growth restriction and programmes glucose intolerance and feeding behaviour disturbances in the aged rat. J. Endocrinol. 181: 291–296.

Lobel, M., Cannella, D.L, Graham, J.E., DeVincent, C., Schneider, J. &Meyer, B.A. (2008) Pregnancy-specific stress, prenatal health behaviors, and birth outcomes. Health Psychology. 27(5): 604-615.

Lopez-Maury, L., Marguerat, S., Bahler, J. (2008) Tuning gene expression to changing environments: from rapid responses to evolutionary adaptation. Nat Rev Genet. 9: 583–593.

Lu, N.Z., Wardell, S.E., Burnstein, K.L., Defranco, D., Fuller, P.J., Giguere, V., Hochberg, R.B., McKay, L., Renoir, J.M., Weigel, N.L., Wilson, E.M., McDonnell, D.P., Cidlowski, J.A. (2006) International Union of Pharmacology. LXV. The pharmacology and classification of the nuclear receptor superfamily: glucocorticoid, mineralocorticoid, progesterone, and androgen receptors. Pharmacol Revl **58** (4): 782–97.

Lupien, S.J., McEwen, B.S., Gunnar, M.R., Heim. C. (2009) Effects of stress throughout the lifespan on the brain, behaviour and cognition. Nature Reviews Neuroscience. 10: 434-445.

Ma, Y., Cheng, Y., Wang, J., Cheng, H., Zhou, S., Li, X. (2010) The changes of visfatin in serum and its expression in fat and placental tissue in pregnant women with gestational diabetes. Diabetes Res Clin Pract. 90(1): 60-65.

Magann, E.F., Evans, S.F., Weitz, B., Newnham, J. (2002) Antepartum, intrapartum, and neonatal significance of exercise on healthy low-risk pregnant working women. Obstet Gynecol. 99(3):466-472.

Manenschijn, L., van den Akker, E.L.T., Lamberts, S.W.J. & van Rossum, E.F.C. (2009). Glucocorticoids and mood. `Clinical features associated with glucocorticoid receptor polymorphisms. New York Academy of Sciences. 1179 : 179-198.

Manley, S.W., Li, H., Mortimer, R.H. (2005) The BeWo choriocarcinoma cell line as a model of iodide transport by placenta.Placenta 26(5): 380-386.

Marcus, S.M. & Heringhausen, J.E. (2009). Depression in childbearing women: when depression complicates pregnancy. Primary Care. 36(1): 151-165.

Marcus, S.M., (2009) Depression during pregnancy: rates, risks and consequences. Can J Clin Pharmacol. 16 (1): 15-22.

Mariscal-Arcas, M., Rivas, A., Monteagudo, C., Granada, A., Cerrillo, I., Olea-Serrano, F. (2009) Proposal of a Mediterranean diet index for pregnant women. Br J Nutr. 102(5): 744-749.

Martínez-González, M.A., de la Fuente-Arrillaga, C., Nunez-Cordoba, J.M., Basterra-Gortari, F.J., Beunza, J.J., Vazquez, Z., Benito, S., Tortosa, A., Bes-Rastrollo, M. (2008) Adherence to Mediterranean diet and risk of developing diabetes: prospective cohort study .BMJ. 336(7657): 1348-51.

Mattick, J.S. (2005) The functional genomics of noncoding RNA. Science.309: 1527–1528.

McKay, L.I. & Cidlowski, J.A. (1999). Molecular control of immune/inflammatory responses: interactions between nuclear factor- B and steroid receptor-signaling pathways. Endocrine Reviews. 20(4): 435-459.

Melzer, K., Schutz, Y., Boulvain, M., Kayser, B. (2010) Physical activity and pregnancy: cardiovascular adaptations, recommendations and pregnancy outcomes. Sports Med.; 40(6):493-507.

Mendelsohn C. (2010) Women who smoke-A review of the evidence. Aust Fam Physician; 40(6): 403-407.

Meyuhas, O. (2000) Synthesis of the translational apparatus is regulated at the translational level. Eur J Biochem. 267:6321–6330.

Mikkelsen, B.T., Østerdal L.M., Knudsen, K.V., Haugen, M., Meltzer, M, H., Bakketeig, L., & Olsen, F.S. (2008). Association between a Mediterranean-type diet and risk of preterm birth among Danish women: a prospective cohort study. Acta Obstetricia et Gynecoliga Scandinavica. 87(3): 325-330.

Miller, G. E., Chen, E., Zhou, E. S. (2007) If it goes up, must it come down? Chronic stress and the hypothalamic-pituitary-adrenocortical axis in humans. Psychological Bulletin. 133 (1): 25.

Moalli, P.A., Pillay, S., Krett, N.L., Rosen, S.T. (1993) Alternatively Spliced Glucocorticoid Receptor Messenger RNAs in Glucocorticoid-resistant Human Multiple Myeloma Cells. Cancer Res. 53; 3877.

Mouratidou, T., Ford, F., Fraser, R.B. (2006) Validation of a food-frequency questionnaire for use in pregnancy. Public Health Nutr. 9(4): 515-22.

Mourtada-Maarabouni, M., Hasan, A.M., Farzaneh, F., Williams, G.T. (2010) Inhibition of human T-cell proliferation by mammalian target of rapamycin (mTOR) antagonists requires noncoding RNA growth-arrest-specific transcript 5 (GAS5). Mol Pharmacol.;78(1): 19-28.

Mulder, E.J.H., Robles de Medina, P.G., Huizink, A.C., Van den Bergh, B.R.H., Buitelaar, J.K.,Visser, G.H.A. (2002) Prenatal maternal stress: effects on pregnancy and the (unborn) child. Early Human Development. 70: 3 –14.

Muller, A.J., Chatterjee, S., Teresky, A., Levine, A.J. (1998). The gas5 gene is disrupted by a frameshift mutation within its longest open reading frame in several inbred mouse strains and maps to murine chromosome 1. Mamm Genome. 9: 773–774.

Murakami, M., Ichisaka, T., Maeda, M., Oshiro, N., Hara, K., Edenhofer, F., Kiyama, H., Yonezawa, K., Yamanaka, S. (2004). mTOR is essential for growth and proliferation in early mouse embryos and embryonic stem cells. Mol Cell Biol, 24: 6710–6718.

Nagpal, S., Friant, S., Nakshatri, H., Chambon, P. (1993) RARs and RXRs: evidence for two autonomous transactivation functions (AF-1 and AF-2) and heterodimerization in vivo. EMBO J. 12:2349–60.

Nawrot, P., Jordan, S., Eastwood, J., Rotstein, J., Hugenholtz, A., Feeley, M. (2003) Effects of caffeine on human health. Food Additives and Contaminants. 20 (1): 1–30.

Neelima, P.S., Rao, A.J. (2008) Gene expression profiling during Forskolin induced differentiation of BeWo cells by diferential display RT-PCR. Mol Cell Endocrinol 281: 37-46.

Nepomnaschy, P. A., Welch, K. B., McConnell, D. S., Low, B. S., Strassmann, B. I., England, B. G. (2006) Cortisol levels and very early pregnancy loss in humans. Proc Natl Acad Sci USA. 103, 3938-3942

Newton, R. (2000). Molecular mechanisms of glucocorticoid action: what is important? Thorax. 55(7): 603-13.

Nojima,H., Tokunaga,C., Eguchi, S., Oshiro, N., Hidayat, S., Yoshino,K., Hara, K., Tanaka, N., Avruch, J. & Yonezawa, K. (2003). The mammalian target of rapamycin (mTOR) partner, raptor, binds the mTOR substrates p70 S6 kinase and 4E-BP1 through their TOR signaling (TOS) motif. Journal of Biochemical Chemistry. 278(18): 15461- 15464.

Ntrivalas, E., Kwak-Kim, J., Beaman, K., Mantouvalos, H., Gilman-Sachs, A. (2006) An in vitro coculture model to study cytokine profiles of natural killer cells during maternal immune celltrophoblast interactions. J Soc Gynecol Investig. 13: 196-202.

Nuclear Receptors Nomenclature Committee. (1999) A unified nomenclature system for the nuclear receptor superfamily. Cell. 97(2): 161--163.

Nussey, S.S., & Whitehead, S.A. (2001). Endocrinology, An Integrated Approach. pp.115-134. London: Bios

Oakley, R. H., Jewell, C. M., Yudt, M. R., Bofetiado, D. M. and Cidlowski, J. A. (1999) The dominant negative activity of the human glucocorticoid receptor β isoform. Specificity and mechanisms of action J Biol Chem. 274, 27857-66.

Oakley, R.H., Webster, J.C., Jewell, C.M., Sar, M., Cidlowski, J.A. (1999) Immunocytochemical analysis of the glucocorticoid receptor alpha isoform (GRα) using GRα-specific antibody. Steroids 64: 742–751.

Obel, C., Hedegaard, M., Henriksen, T. B., Secher, N. J., Olsen, J. and Levine, S. (2005) Stress and salivary cortisol during pregnancy. Psychoneuroendocrinology. 30, 647–656.

Ogden,J. (2007). Health Psychology, A Textbook. 4th ed. pp. 221-229 London: Open University Press

Olstad, R., Sexton, H. & Sogaard, A.J. (2001). The Finnmark Study. A prospective population study of the social support buffer hypothesis, specific stressors and mental distress. Social Psychiatry & Psychiatric Epidemiology. 36(12): 582-589.

Orendi, K., Gauster, M., Moser, G., Meiri, H., Huppertz, B. (2010) Effects of vitamins C and E, acetylsalicylic acid and heparin on fusion, beta-hCG and PP13 expression in BeWo cells. Placenta. 31(5): 431-438.

Orr, S.T., Reiter, J.P., Blazer, D.G., James, S.A.(2007) Maternal prenatal pregnancy-related anxiety and spontaneous preterm birth in Baltimore, Maryland. Psychosom Med.;69 (6): 566-570.

Oshiro, N., Yoshino, K., Hidayat, S., Tokunaga, C., Hara, K., Eguchi, S., Avruch, J. & Yonezawa, K. (2004). Dissociation of raptor from mTOR is a mechanism of rapamycin-induced inhibition of mTOR function. Genes Cells. 9(4): 359-366.

Owen, G.I., Zelent, A. (2000) Origins and evolutionary diversification of the nuclear receptor superfamily. Cellular and Molecular Life Sciences. 57: 809–827.

Owens, J.A., Owens, P.C., Robinson, J.S. (1989) Experimental fetal growth retardation: metabolic and endocrine aspects. In: Gluckman PD, Johnston BM, Nathanielsz PW (eds). Research in Perinatal Medicine (VIII) Advances in Fetal Physiology: Reviews in Honor of GC Liggins. Ithaca, NY: Perinatology Press, pp.263–86.

Paffenbarger, R.S.Jr., Wing, A.L., Hyde, R.T. (1978) Physical Activity as an index of Heart Attack risk in college Alumni. Am J Epidemiol. 108(3): 161-75.

Peacock, J.L., Bland, J.M., Anderson, H.R. (1995) Preterm delivery: effects of socioeconomic factors, psychological stress, smoking, alcohol, and caffeine. BMJ.; 311: 531-535.

Perkin, M.R., Bland, J.M., Peacock, J.L., Anderson, H.R. (1993) The effect of anxiety and depression during pregnancy on obstetric complications. Br J Obstet Gynaecol.100(7): 629-634.

Peterson, R.T., Beal, P.A., Comb, M.J. & Schreiber, S.L. (2000). FKBP12-rapamycin-associated protein (FRAP) autophosphorylates at serine 2481 under translationally repressive conditions. Journal of Biological Chemistry. 275: 7416-7423.

Peterson, T.R., Laplante, M., Thoreen, C.C., Sancak, Y., Kang, S.A., Kuehl, W.M., Gray, N.S., Sabatini. D.M. (2009). DEPTOR is an mTOR inhibitor frequently overexpressed in multiple myeloma cells and required for their survival. Cell. 137(5) : 873-886.

Petraglia, F., Florio, P., Gallo, R., Simoncini, T., Saviozzi, M., Di Blasio, A.M., Vaughan, J., Vale, W. (1996) Human placenta and fetal membranes express human Urocortin mRNA and peptide. Journal of Clinical Endocrinology and Metabolism. 81(10): 3807-3810.

Petroulakis, E., Mamane, Y., Le Bacquer, O., Shahbazian, D. & Sonenberg, N. (2006). mTOR signaling: implications for cancer and anticancer therapy. British Journal of Cancer. 94:195-199.

Phillips, D.I. (1998) Birth weight and the future development of diabetes. A review of the evidence. Diabetes Care 21(Suppl 2): B150–B155.

Pick, M.E., Edwards, M., Moreau, D., Ryan, E.A. (2005) Assessment of diet quality in pregnant women using the Healthy Eating Index. J Am Diet Assoc 105, 240–246.

Porterfield, S. (2001) Endrocrine Physiology, 2nd edn. St. Louis, MO: Mosby.

Pratt, W.B., Morishima, Y., Murphy, M., Harrell, M. (2006). Chaperoning of glucocorticoid receptors. *Handb Exp Pharmacol.* (172): 111–38.

Prentice, A. M. (2005). Early influences on human energy regulation: Thrifty genotypes and thrifty phenotypes. Physiology & Behavior. 86 (5) : 640-645.

Proud, C.G. (2009). Dynamic balancing: DEPTOR tips the scales. Journal of Molecular Cell Biology. 1(2): 61-63.

Pujols, L., Mullol, J., Roca-Ferrer, J., Torrego, A., Xaubet, A., Cidlowski, J.A., Picado, C. (2002) Expression of glucocorticoid receptor alpha- and beta-isoforms in human cells and tissues. Am J Physiol Cell Physiol. 283(4): C1324-31.

Rae, M.T., Hillier, S.G. (2005) Steroid signaling in the ovarian surface epithelium. Trends Endocrinol Metab 16: 327–33.

Raho, G., Barone, V., Rossi, D., Philipson, L., Sorrentino, V. (2000). The gas 5 gene shows four alternative splicing patterns without coding for a protein. Gene. 256: 13–17.

Ramsey, E.M. (1982) The placenta: human and animal. New York

Ray, D.W., Davis, J.R., White, A. & Clark, A.J. (1996). Glucocorticoid receptor structure and function in glucocorticoid - resistant small cell lung carcinoma cells. Cancer Research. 56(14): 3276-3280.

Reaven, G.M. (1998) Banting Lecture: Role of insulin resistance in human diabetes. Diabetes. 37:1595.

Reichardt, H.M. & Schutz, G. (1998). Glucocorticoid signalling-multiple variations of a common theme. Molecular & Cellular Endocrinology. 146(1-2): 1-6.

Reinisch, J.M., Simon, N.G., Karow, W.G. & Gandelman, R. (1978) Prenatal exposure to prednisone in humans and animals retards intrauterine growth. Science 202: 436–438.

Rhen, T., Cidlowski, J.A. (2005). Antiinflammatory action of glucocorticoids--new mechanisms for old drugs. N. Engl. J. Med. 353 (16): 1711–23.

Rini, C.K., Dunkel-Schetter, C., Wadhwa, P.D., Sandman, C.A. (1999) Psychological adaptation and birth outcomes: the role of personal resources, stress, and sociocultural context in pregnancy. Health Psychol. 18(4): 333-45.

Rivers, C., Levy, A., Hankock, J., Lightman, S., & Norman, M. (1999). Insertion of an amino acid in the DNA_binding domain of the glucocorticoid receptor as a result of alternative Splicing. Clinical Endocrinology & Metabolism. 84 (11): 4283 – 4286.

Robbins, A.S., Chao, S.Y., Frost, L.Z., Fonseca, V.P. (2005) Unplanned pregnancy among active duty service women, US Air Force, 2001. Military Medicine 170(1): 38–43.

Rodriguez-Bernal, C.L., Rebagliato, M., Iniguez, C., Vioque, J., Navarrete-Muñoz, E.M., Murcia, M., Bolumar, F., Marco, A., Ballester, F. (2010) Diet quality in early pregnancy and its effects on fetal growth outcomes: the Infancia y Medio Ambiente (Childhood and Environment) Mother and Child Cohort Study in Spain. Am J Clin Nutr 91, 1659–1666.

Roesch, S. C., Dunkel-Schetter, C., Woo, G., & Hobel, C. J. (2004) Modeling the types and timing of stress in pregnancy. Anxiety, Stress & Coping: An International Journal, 17, 87–102.

Rooij, W.H.,Yonker, J.E., Painter, R.C., Roseboom, T.J. (2010) Prenatal undernutrition and cognitive function in late adulthood. Proc Natl Acad Sci USA 107:16881–16886.

Rooney, B.L., Schauberger, C.W. (2002) Excess pregnancy weight gain and long term obesity: one decade later. Obstet Gynecol. 100:245–52.

Roos, S., Jansson, N., Palmberg, I., Säljö, K., Powell, T.L. & Jansson, T. (2007). Mammalian target of rapamycin in the human placenta regulates leucine transport and is down-regulated in restricted fetal growth. Journal of Physiology. 582(1): 449-459.

Roos, S., Powell, T.L., Jansson, T. (2009) Placental mTOR links maternal nutrient availability to fetal growth. Biochem Soc Trans. 37, 295-298.

Rosenwald, I.B., Lazaris-Karatzas, A., Sonenberg, N., Schmidt, E.V. (1993) Elevated levels of cyclin D1 protein in response to increased expression of eukaryotic initiation factor 4E. Mol Cell Biol 13:7358–7363.

Rosmond, R., Chagnon, Y.C., Holm, G., Chagnon, M., Perusse, L., Lindell, K., Carlsson, B., Bouchard,C., Bjorntorp, P. (2000) A glucocorticoid receptor gene marker is associated with abdominalobesity, leptin, and dysregulation of the hypothalamic-pituitary-adrenal axis. Obes Res. 8:211–218.

Rothenberger, S. E., Resch, F., Doszpod, N. And Moehler, E. (2011) Prenatal stress and infant affective reactivity at five months of age. Early Hum. Dev. 87: 129–136.

Roy-Matton, N., Moutquin, J.M., Brown, C., Carrier, N., Bell, L. (2011) The impact of perceived maternal stress and other psychosocial risk factors on pregnancy complications. J Obstet Gynaecol Can. 33(4): 344-352.

Ruiz, R.J., Fullerton, J., Brown, C.E., Schoolfield, J. (2001) Relationships of cortisol, perceived stress, genitourinary infections, and fetal fibronectin to gestational age at birth. Biol Res Nurs. 3(1): 39-48.

Sabatini, D.M., Erdjument-Bromage, H., Lui, M., Tempst, P.,and Snyder, S.H. (1994) RAFT1: A mammalian protein that binds to FKBP12 in a rapamycin-dependent fashion and is homologous to yeast TORs. Cell. 78: 35–43.

Salas-Salvadó, J., Fernández-Ballart, J., Ros, E., Martínez-González, M.A., Fitó, M., Estruch, R., Corella, D., Fiol, M., Gómez-Gracia, E., Arós, F., Flores, G., Lapetra, J., Lamuela-Raventós, R., Ruiz-Gutiérrez, V., Bulló, M., Basora, J., Covas, M.I. (2008) Effect of a Mediterranean diet supplemented with nuts on metabolic syndrome status: one-year results of the PREDIMED randomized trial. Arch Intern Med. 168(22):2449-58.

Saldana, T.M., Siega-Riz, A.M. & Adair, L.S. (2004) Effects of macronutrient intake on the development of glucose intolerance during pregnancy. Am J Clin Nutr 70, 479–486.

Sancak, Y., Thoreen, C.C., Peterson, T.R., Lindquist, R.A., Kang, S.A., Spooner, E., Carr, S.A., Sabatini, D.M. (2007) PRAS40 is an insulin- regulated inhibitor of the mTORC1 protein kinase. Mol Cell. 25:903-915.

Sánchez-Vega, B., Krett, N., Rosen, S.T., Gandhi, V. (2006) Glucocorticoid receptor transcriptional isoforms and resistance in multiple myeloma cells. Mol Cancer Ther. 5; 3062.

Sapolsky, R.M., Romero, L.M. & Munck, A.U. (2000). How do glucocorticoids influence stress responses? Integrating permissive, suppressive, stimulatory, and preparative actions. Endocrine reviews. 21(1) : 55-89.

Sarbassov, D.D., Ali, S.M., Kim, D.H., Guertin, D.A., Latek, R.R., Erdjument-Bromage, H., Tempst, P., Sabatini, D.M. (2004) Rictor, a novel binding partner of mTOR, defines a rapamycin-insensitive and raptori ndependent pathway that regulates the cytoskeleton. Curr Biol. 14:1296-1302.

Sarbassov, D.D., Ali, S.M., Sengupta, S., Sheen, J.H., Hsu, P.P., Bagley, A.F., Markhard, A.L. & Sabatini, D.M. (2006). Prolonged rapamycin treatment inhibits mTORC-2 assembly and Akt/PKB. Molecular Cell. 22 : 159-168.

Schalm, S.S. & Blenis. J. (2002). Identification of a conserved motif required for mTOR signaling. Current Biology. 12(8): 632-639.

Schalm, S.S., Fingar, D.C., Sabatini, D.M. & Blenis, J. (2003). TOS motif mediated raptor binding regulates 4E-BP1 multisite phosphorylation and function. Current Biology. 13(10): 797-806.

Schneider, C., King, R.M., Philipson, L. (1988) Genes specifically expressed at growth arrest of mammalian cells. Cell. 54(6):787-793.

Scholl, T.O. (2008) Maternal nutrition before and during pregnancy. Nestle Nutr Workshop Ser Pediatr Program 61, 79–89.

Scholl, T.O., Chen, X., Khoo, C.S., Lenders, C. (2004) The dietary glycemic index during pregnancy: influence on infant birth weight, fetal growth, and biomarkers of carbohydrate metabolism. Am J Epidemiol Mar 159, 467–474.

Schulz, L.C. (2010) The Dutch Hunger Winter and the developmental origins of health and disease. *PNAS* September 28, 2010 vol. 107 no. 39 16757-16758.

Schulze, M.B., Liu, S., Rimm, E.B., Manson, J.E., Willett, W.C., Hu, F.B. (2004) Glycemic index, glycemic load, and dietary fiber intake and incidence of type 2 diabetes in younger and middle-aged women. Am J Clin Nutr; 80: 348-356.

Scott, E. (2008) Cortisol and Stress: How Cortisol Affects Your Body, and How To Stay Healthy in the Face of Stress. Stress.about.com.

Scott, E. (2008) Cortisol and Stress: How to stay healthy (cortisol and your body). About.com Guide.

Sellick, C.A. & Reece, R.J. (2005) Eukaryotic transcription factors as direct nutrient sensors. Trends Biochem Sci.30:405–412.

Shapira, N. (2008) Prenatal nutrition: a critical window of opportunity for mother and child. Womens Health (Lond Engl). 4(6):639-56.

Shaw, G.M., Schaffer, D., Velie, E.M., Morland, K., Harris, J.A. (1995) Periconceptional vitamin use, dietary folate, and the occurrence of neural tube defects. Epidemiology 6, 219–226.

Shiell, A. W., Campbell-Brown, M., Haselden, S., Robinson, S., Godfrey, K. M. & Barker, D.J.P. (2001). High-Meat, Low-Carbohydrate Diet in Pregnancy: Relation to Adult Blood Pressure in the Offspring. Hypertension. 38(6): 1282-1288.

Shimizu, N., Yoshikawa, N., Ito, N., Maruyama, T., Suzuki, Y., Takeda, S., Nakae, J., Tagata, Y., Nishitani, S., Takehana, K., Sano, M., Fukuda, K., Suematsu, M., Morimoto, C., Tanaka, H. (2011) Crosstalk between glucocorticoid receptor and nutritional sensor mTOR in skeletal muscle. Cell Metab.13(2): 170-182.

Shiota, C.,Woo, J.T., Lindner, J., Shelton, K.D., Magnuson, M.A. (2006) Multiallelic disruption of the rictor gene in mice reveals that mTOR complex 2 is essential for fetal growth and viability. Dev. Cell. 11:583–589.

Siega-Riz, A.M., Herrmann, T.S., Savitz, D.A., Thorp, J.M. (2001) The frequency of eating during pregnancy and its effect on preterm delivery. Am J Epidemiol 153: 647–652.

Sionov R.V. (2008) The kinome and glucocorticoid-induced apoptosis (2008) Ai Zheng.27(11): 1121-1129.

Smith, C.M., Steitz, J.A. (1998) Classification of gas5 as a multi-small-nucleolar-RNA (snoRNA) host gene and a member of the 5′-terminal oligopyrimidine gene family reveals common features of snoRNA host genes. Mol Cell Biol.18: 6897–6909.

Smith, S.M. & Vale, W.W. (2006). The role of the hypothalamic-pituitary-adrenal axis in neuroendocrine responses to stress. Dialogues in Clinical Neuroscience. 8(4) : 383-395.

Snyder, S., Pendergraph, B. (2004) Exercise During Pregnancy: What Do We Really Know? Am Fam Physician. 69(5): 1053-1056

Stanner, S.A., Yudkin, J.S. (2001) Fetal programming and the Leningrad Siege study. Twin Res. 4(5):287-92. Steiger H, Gauvin L, Joober R, Israel M, Badawi G, Groleau P, Bruce KR, Yin Kin NM, Sycz L, Ouelette AS. (2012) Interaction of the BcII glucocorticoid receptor polymorphism and childhood abuse in Bulimia Nervosa (BN): relationship to BN and to associated trait manifestations. J Psychiatr Res. 46(2): 152-158.

Stein, B. & Yang, M.X. (1995) Repression of the interleukin-6 promoter by estrogen receptor is mediated by NF-kappa B and C/EBP beta. Molecular & Cellular Biology. 15(9): 4971-9.

Takahashi, T., Hara, K., Inoue, H., Kawa, Y., Tokunaga, C., Hidayat, S., Yoshino, K., Kuroda, Y. & Yonezawa, K. (2000). Carboxyl-terminal region conserved among phosphoinositide-kinase-related kinases is indispensable for mTOR function in vivo and in vitro. Genes Cells. 5 : 765-775.

Tee, A.R. & Blenis, J. (2005) mTOR, translational control and human disease. Sem Cell Dev Biol. 16 (1): 29–37.

Tegethoff, M., Greene, N., Olsen, J., Meyer, A.H., Meinlschmidt, G. (2010) Maternal Psychosocial Stress during Pregnancy and Placenta Weight: Evidence from a National Cohort Study. PLoS ONE 5(12): e14478.

Thaker, P.H., Sood, A.K. (2008) Neuroendocrine Influences on Cancer Biology. Semin Cancer Biol. 18(3): 164-70.

Thedieck, K., Polak, P., Kim, M.L., Molle, K.D., Cohen, A., Jeno, P., Arrieumerlou, C., and Hall, M.N. (2007) PRAS40 and PRR5-like protein are new mTOR interactors that regulate apoptosis. PLoS ONE. 2, e1217.

Thornton, J.W., DeSalle, R. (2000) A new method to localize and test the significance of incongruence: detecting domain shuffling in the nuclear receptor superfamily. Syst Biol.49:183-201.

Tissing, W.J.E., Meijerink, J.P.P., den Boer, M.L. & Pieters, R. (2003) Molecular determinants of glucocorticoid sensitivity and resistance in acute lymphoblastic leukemia. Leukemia. 17: 17−25.

Trichopoulou, A. (2004) Traditional Mediterranean diet and longevity in the elderly: a review. Public Health Nutr. 7(7):943-7.

Trichopoulou, A., Bamia, C., Trichopoulos, D.(2009). Anatomy of health effects of Mediterranean diet: Greek EPIC prospective cohort study. British medical journal; 23: 338: b2337.

Trichopoulou, A., Orfanos, P., Norat, T., Bueno-de-Mesquita, B., Ocké, M., Peeters, P. H., van der Schouw, Y.T., Boeing, H., Hoffmann, K., Boffetta, P., Nagel, G., Masala, G., Krogh, V., Panico, S., Tumino, R., Vineis, P., Bamia, C., Naska, A., Benetou, V., Ferrari, P., Slimani, N., Pera, G., Martinez-Garcia, Carmen Navarro, C., Rodriguez-Barranco, M., Dorronsoro, M., Spencer, E., Key, T., Bingham, S., Khaw, K., Kesse, E., Clavel-Chapelon, F., Boutron-Ruault, M., Berglund, G., Wirfalt, E., Hallmans, G., Johansson, I.,Tjonneland, A., Olsen, A., Overvad, K., Hundborg, H., Riboli, E., Trichopoulos D. (2005) Modified Mediterranean diet and survival: EPIC-elderly prospective cohort study. BMJ. 330(7498): 991.

Trikalinos, T.A., Salanti, G., Khoury, M.J., Ioannidis, J.P. (2006) Impact of violations and deviations in Hardy-Weinberg equilibrium on postulated gene-disease associations. Am J Epidemiol.; 163(4): 300-309.

Tronche, F., Kellendonk, C., Kretz, O., Gass, P., Anlag, K., Orban, P.C., Bock, R., Klein, R. & Schutz, G.(1999). Disruption of the glucocorticoid receptor gene in the nervous system results in reduced anxiety. Nature Genetics. 23(1) : 99-103.

U.S. Department of Health and Human Services. Public Health Service, Office of the Surgeon General. (2001) The surgeon general's call to action to prevent and decrease overweight and obesity. Rockville, Md. Accessed online September 7, 2011, at: www.surgeongeneral.gov/topics/obesity/calltoaction/CalltoAction.pdf.

Ukkola, O., Perusse, L., Chagnon, Y.C., Despres, J.P., Bouchard, C. (2001) Interactions among the glucocorticoid receptor, lipoprotein lipase and adrenergic receptor genes and abdominal fat in the Quebec Family Study. Intl J Obes Relat Metab Disord. 25 : 1332 –1339.

Vallee, M., W. Mayo, S. Maccari, Le Moal, M., Simon, H. (1996) Long-term effects of prenatal stress and handling on metabolic parameters: relationship to corticosterone secretion response. Brain Res. 712: 287–292.

van Rossum EF, Russcher H, Lamberts SW. (2005) Genetic polymorphisms and multifactorial diseases: facts and fallacies revealed by the glucocorticoid receptor gene. Trends Endocrinol Metab.;16(10): 445-450.

van Rossum, E.F., Koper, J.W., van den Beld, A.W., Uitterlinden, A.G., Arp, P., Ester, W., Janssen, J.A., Brinkmann, A.O., de Jong, F.H., Grobbee, D.E., Pols, H.A. & Lamberts, S.W. (2003). Identification of the BclI polymorphism in the glucocorticoid receptor gene: association with sensitivity to glucocorticoids in vivo and body mass index. Clinical Endocrinology. 59(5): 585-592.

van Rossum, E.F., Roks, P.H., de Jong, F.H., Brinkmann, A.O., Pols, H.A., Koper, J.W., & Lamberts, S.W. (2004). Characterization of a promoter polymorphism in the glucocorticoid receptor gene and its relationship to three other polymorphisms. Clinical Endocrinology. 61(5): 573-581.

van Rossum, E.F.C., & Lamberts, S.W.J. (2004). Polymorphisms in the glucocorticoid receptor gene and their association with metabolic parameters and body composition. Recent Progress in Hormone Research. 59: 333-357.

Vander Haar, E., Lee, S. I., Bandhakavi, S., Griffin, T. J. and Kim, D. H. (2007) Insulin signalling to mTOR mediated by the Akt/PKB substrate PRAS40. Nat. Cell Biol. 9, 316-323.

Vujkovic, M., Steegers, E.A., Looman, C.W., Ocké, M.C., van der Spek, P.J. & Steegers-Theunissen, R.P. (2009) The maternal Mediterranean dietary pattern is associated with a reduced risk of spina bifida in the offspring. BJOG 116 (3): 408-415

Wadhwa, P.D., Sandman, C.A., Porto, M., Dunkel-Schetter, C., Garite, T.J. (1993). The association between prenatal stress and infant birth weight and gestational age at birth: a prospective investigation. Am. J. Obstet. Gynecol., 169, 858–865.

Wang, L., Harris, T.E., Roth, R.A., Lawrence, J.C. (2007) PRAS40 regulates mTORC1 kinase activity by functioning as a direct inhibitor of substrate binding. J Biol Chem. 282:20036-20044.

Wang, L., Wang, X., Wang, W., Chen, C., Ronnennberg, A. G., Guang, W., Huang, A., Fang, Z., Zang, T., Wang, L., Xu, X. (2004) Stress and dysmenorrhoea: a population based prospective study. Occup Environ Med. 61(12): 1021-1026

Weber, M.A., Groos, S., Höpfl, U., Spielmann, M., Aumüller, G., Konrad, L. (2000) Glucocorticoid receptor distribution in rat testis during postnatal development and effects of dexamethasone on immature peritubular cells *in* vitro. Andrologia. 32 (1): 23–30.

Weerth, C., van Hees, Y., Buitelaar, J.K. (2003) Prenatal maternal cortisol levels and infant behaviour during the first 5 months. Early Human Development. 74: 139–151.

Weinman, J. & Kaptein, A. (2004). Health psychology, An introduction. London: BSB Blackwell. pp. 114-134.

Wen, H.Y, Abbasi, S., Kellems, R.E., Xia, Y. (2005). mTOR: A placental growth signaling sensor. Placenta. 26 : S63-S69.

Wen, L.M., Flood, V.M., Simpson, J.M., Rissel, C., Baur, L.A. (2010) Dietary behaviours during pregnancy: findings from first-time mothers in southwest Sydney, Australia. Int J Behav Nutr Phys Act. 3; 7:13.

Wen, L.P., Madani, K., Fahrni, J.A., Duncan, S.R., Rosen, G.D. (1997) Dexamethasone inhibits lung epithelial cell apoptosis induced by IFN-γ and Fas. Am J Physiol. 273:L921–929.

Wen, W., Shu, X.O., Jacobs, D. R, Jr., and Brown, J. E. (2001) The associations of maternal caffeine consumption and nausea with spontaneous abortion. Epidemiology, 12(1): 38–42.

Willett, W.C. (2006) The Mediterranean diet: science and practice. Public Health Nutr. (1A): 105-10.

Winter, K.A., Kuiper, N.A. (1997) Individual differences in the experience of emotions. Clin Psychol Rev.;17(7): 791-821.

Woo, S.Y., Kim, D.H., Jun, C.B., Kim, Y.M., Haar, E.V., Lee, S.I., Hegg, J.W., Bandhakavi, S., Griffin, T.J., Kim, D.H. (2007) PRR5, a novel component of mTOR complex 2, regulates platelet-derived growth factor receptor beta expression and signaling. J. Biol. Chem. 282:25604–25612

Wood, G.W. (1980) Mononuclear phagocytes in the human placenta. Placenta 1(2): 113-123.

Yang, Q., Inoki, K., Ikenoue, T., Guan, K.L. (2006) Identification of Sin1 as an essential TORC2 component required for complex formation and kinase activity. Genes Dev. 20:2820-2832.

Ye, J., Yu, Z., Ding, J., Chen, Y., Huang, J., Yao, Y., Xiao, H., Yang, J., Shen, Y., Meng, Q. (2006) Genetic variations of the NR3C1 gene in children with sporadic nephrotic syndrome. Biochem Biophys Res Commun. 348(2): 507-513.

Ying, S.Y., Lin, S.L. (2009) Intron-mediated RNA interference and microRNA biogenesis. Methods Mol Biol.;487: 387-413.

Yu, A., Rual, J.F., Tamai, K., Harada, Y., Vidal, M., He, X. & Kirchhausen, T. (2007). Association of Dishevelled with the clathrin AP-2 adaptor is required for Frizzled endocytosis and planar cell polarity signaling. Developmental Cell. 12(1): 129-141.

Zhu, H., Dai,Y., Wang, O., Li, M., Lu, L., Zhao, W., Xing X., Pan, H., Li, N.,Gong, F., (2011) Generalized glucocorticoid resistance accompanied with an adrenocortical adenoma and caused by a novel point mutation of human glucocorticoid receptor gene Chinese Medical Journal 124(4): 551-555

Zhu, P., Tao, F., Hao, J., Sun, Y., Jiang, X. (2010) Prenatal life events stress: implications for preterm birth and infant birthweight. American Journal of Obstetrics & Gynecology. 203 (1): 34.e1-34.e8.

Zong, J., Ashraf, J. &Thompson, E.B. (1990). The promoter and first, untranslated exon of the human glucocorticoid receptor gene are GC rich but Lack consensus glucocorticoid receptor element sites. Molecular and Cellular Biology. 10(10): 5580-5585.

APPENDIX I

MODELS OF HUMAN PLACENTAL

BeWo CELL LINE

Obtained	Health Protection Agency Culture Collections (www.hpacultures.co.uk)
Catalogue No.	86082803
Description	The first human, trophoblastic endocrine cell type to be maintained in continuous culture. It was initiated from a malignant gestational choriocarcinoma of the foetal placenta.
Species	Human (Choriocarcinoma)
Tissue	Endocrine
Morphology	Epithelial
Growth Mode	Adherent
Subculture Routine	Split cultures at 70-80% confluence 1:3 i.e. seeding at 4x10.000 cells/cm^2 using 0.25% trypsin/EDTA, 5% CO_2; 37°C. Vacuolisation will occur at confluence.
Culture Medium	HAM's F-12K + 2mM Glutamine + 10% Foetal Bovine Serum (FBS)
Karyotype	$2^n = 46$
Products	Gonadotrophin, lactogen and steroid hormones
Country	USA

JEG-3 CELL LINE

Obtained	Health Protection Agency Culture Collections (www.hpacultures.co.uk)
Catalogue No.	92120308
Description	One of 6 clones derived from cells implanted into a hampster cheek pouch, and then propagated on irradiated feeder layers of human fibroblasts.
Species	Human (Choriocarcinoma)
Tissue	Unknown
Morphology	Epithelial
Growth Mode	Adherent
Subculture Routine	Split cultures at 70-80% confluence 1:3 to 1:6 i.e. seeding at 1-3x10,000 cells/cm^2 using 0.25% trypsin, 5% CO_2; 37°C. Vacuolisation will occur at confluence.
Culture Medium	EMEM (EBSS) + 2mM Glutamine + 1% Non-Essential Amino Acids (NEAA) + 1mM Sodium Pyruvate (NaP) + 10% Foetal Bovine Serum (FBS)
Karyotype	$2^n = 46$
Products	HcCJ and somatomammotrophin
Country	UK

APPENDIX II

QUESTIONNAIRE

1. Birth Date……. 2. Height…… 3. Weight as a newborn……

4. Were you borne prematurely? Yes No

5. Weight before conception…… 6. Current weight……

7. Which body shape (before pregnancy) mostly matches yours?

 ☐

Apple shape
Tends to store fat around the belly and chest.

 ☐

Pear shape
Holds the majority of fat in the lower body: hips, buttocks.

 ☐

Proportionate shape
Has equal fat distribution: gains and loses weight evenly.

Immune Profile

8. How many colds did you get during pregnancy? ……..

9. How many infections did you get during pregnancy? ……..

10. Do you find it hard to shift an infection (e.g cold)? Yes No

11. Are you prone to thrush or cystitis? Yes No

12. How often did you take antibiotics in the last month?
None Once Twice More than 3 times

13. Do you have an inflammatory disease (e.g. arthritis)? Yes No

14. Do you suffer from hay fever, allergies? Yes No
If Yes, please describe……

Exercise Profile

15. How many flight of stairs do you climb every day (10 steps one flight)?

Before pregnancy…….. During Pregnancy ……

16. How many city blocks do you walk each day (1 block=130 metres)?

Before pregnancy…….. During Pregnancy ……

17. How many hours/week do you participate in any light sports (e.g. dancing, gardening, walking)?

Before pregnancy…….. During Pregnancy ……

18. How many hours/week do you participate in any strenuous sports (e.g. running, cycling, swimming, tennis)?

Before pregnancy…….. During Pregnancy ……

Nutrition Profile Before Pregnancy

19. Are you vegetarian? Yes No

20. How often do you buy full-fat dairy products?

……

21. How many meals per week would include any of the following: pies, pastries, fried foods?
0 1-2 3-4 5 or more

22. How many servings of vegetables/legumes* do you have each day?
* 1 serve of vegetables equals ½ cup cooked vegetables (e.g. broccoli, carrot) or 1 cup of salad.
0 1-2 3-4 5 or more

23. How many servings of fruit do you have each day?**
** 1 serve of fruit = 1 medium-sized apple, banana, orange or pear; 2 small apricots, kiwi fruit; 1 cup of diced pieces of canned fruit; ½ cup of juice; 4 dried apricot halves.
0 1-2 3-4 5 or more

24. How many servings of cereals* do you have each day?**
*** 1 serve cereal equals ½ cup breakfast cereal; 1 cup cooked rice, or pasta; or 2 slices of bread.
0 1-2 3-4 5 or more

25. Do you eat iron rich foods (e.g. lean red meat, chicken, green leafy vegetables) every day? Yes No
If Yes, please specify:……

26. Do you eat 2 or more servings of cheese, milk, yoghurt or calcium enriched milk every day? (a serving is 40gm cheese, 200g tub yoghurt or 1 cup milk) Yes No

27. How much water/sugar-free drinks do you drink each day?
Less than ½ litre ½-1 litre More than 1 litre

28. How many cups of coffee, black tea or caffeine containing beverages do you drink each day?
4-6 3 -4 2-3 1 or fewer

29. How much soda, sugary drinks do you normally have each day?
More than 3 2-3 1 or fewer

30. How many alcoholic beverages do you consume on a weekly basis?
More than 5 3-4 2 or fewer None

31. Immediately before pregnancy did you smoke? Yes No

If Yes, how much and for how long?.....

32. Does anyone in your household smoke? Yes No

33. How many times a week do you eat fast food?
Never 1-2 times 3-4 times 5 or more times

Nutrition Profile During Pregnancy

34. Are you vegetarian? Yes No

35. How often do you buy full-fat dairy products?

……

36. How many meals per week would include any of the following: pies, pastries, fried foods?
0 1-2 3-4 5 or more

37. How many servings of vegetables/legumes* do you have each day?
* 1 serve of vegetables equals ½ cup cooked vegetables (e.g. broccoli, carrot) or 1 cup of salad.
0 1-2 3-4 5 or more

38. How many servings of fruit do you have each day?**
** 1 serve of fruit = 1 medium-sized apple, banana, orange or pear; 2 small apricots, kiwi fruit; 1 cup of diced pieces of canned fruit; ½ cup of juice; 4 dried apricot halves.
0 1-2 3-4 5 or more

39. How many servings of cereals* do you have each day?**
*** 1 serve cereal equals ½ cup breakfast cereal; 1 cup cooked rice, or pasta; or 2 slices of bread.
0 1-2 3-4 5 or more

40. Do you eat iron rich foods (e.g. lean red meat, chicken, green leafy vegetables) every day? Yes No
If Yes, please specify:……

41. Do you eat 2 or more servings of cheese, milk, yoghurt or calcium enriched milk every day? (a serving is 40gm cheese, 200g tub yoghurt or 1 cup milk) Yes No

42. How much water/sugar-free drinks do you drink each day?
Less than ½ litre ½-1 litre More than 1 litre

43. How many cups of coffee, black tea or caffeine containing beverages do you drink each day?
4-6 3 -4 2-3 1 or fewer

44. How much soda, sugary drinks do you normally have each day?
More than 3 2-3 1 or fewer

45. How many alcoholic beverages do you consume on a weekly basis?
More than 5 3-4 2 or fewer None

46. Since you have been pregnant, have you smoked cigarettes? Yes No
If Yes, indicate the average number of cigarettes smoked per day……

47. Does anyone in your household smoke? Yes No

48. How many times a week do you eat fast food?
Never 1-2 times 3-4 times 5 or more times

Stress Profile

49. Is this a planned pregnancy? Yes No

50. How would you rate your current stress level?

Low Medium High Very high

51. What word(s) describe how you feel about being pregnant?

Happy OK Tired Satisfied
Sad Stressed Angry Other……

52. How much sleep on average do you obtain each night?

1. 10 or more hours per night
2. 9-10 hours per night
3. 7-8 hours per night
4. 5-6 hours per night
5. Less than 5
6. Not sure- do not sleep well throughout night

Medical Profile

53. Do you have a family history of any of the following health conditions?

Cancer Obesity Osteoporosis
Arthritis Heart Disease High Blood Pressure
High Cholesterol Diabetes Hypoglycemia

54. What concerns does your doctor have about your pregnancy?

What You Eat High Blood Sugar High Blood Pressure

Low Iron in Blood None Other……

55. Please list your current medication as prescribed by your doctor:

……
……

56. Which of these did you take before pregnancy?

Folic Acid Iron Pills Vitamins / Minerals
Laxatives Probiotics Home/herbal remedies (list)……..

57. Which of these do you take during pregnancy?

Folic Acid Iron Pills Vitamins / Minerals
Laxatives Probiotics Home/herbal remedies (list)……..

58. What Blood Type are you? A B A/B O Not Sure

59. Do you have anaemia caused by iron deficiency? Yes No

APPENDIX III

SOURCES OF QUESTIONNAIRE

DEPARTMENT OF HEALTH SERVICES
Division of Health Care Access and Accountability
F-1105 (02/09)

STATE OF WISCONSIN

FORWARDHEALTH
PRENATAL CARE COORDINATION PREGNANCY QUESTIONNAIRE

Instructions: Type or print clearly. Before completing this form, read the Prenatal Care Coordination Program Pregnancy Questionnaire Completion Instructions, F-1105A.

SECTION I — GENERAL INFORMATION

To Be Completed by Health Professional

1. Name — Member (Last, First, Middle Initial)
2. Date of Birth — Member
3. Age — Member

4. Ethnicity ❑ Hispanic ❑ Non-Hispanic
5. Race ❑ American Indian ❑ Asian ❑ Black ❑ Hawaiian / Pacific Islander ❑ White ❑ Other

Lim Eng
A- <20
A- >39
E- H
R- AI,A, B,HPI,O

6. Education (Indicate highest grade completed.)
❑ Primary / Secondary (1-12) _____ ❑ College (1-4 or 5+) _____
7. Marital Status
❑ Single ❑ Married

Edu<12
MS- S

8. Address — Member (Street, City, State, ZIP Code)
9. County
10. Telephone Number — Member
11. Other Telephone Number — Member
12. What is the best way to contact you? When is the best time to contact you?
13. Name and Telephone Number — Emergency Contact Person
14. Name — Medical Provider or Clinic (Doctor, Nurse Practitioner, Midwife)
❑ I do not have a medical provider.
15. Member Identification Number
16. How many times have you been to a dentist or dental clinic in the last two years?

SECTION II — CURRENT PREGNANCY

1. When is your baby due?
2. What was the date of your last menstrual period?

Tim- L,NAA
PNC- 2,3,N

3. If you could change the timing of this pregnancy, when would you want it?
❑ Earlier ❑ No change
❑ Later ❑ Not at all
4. When was your first medical appointment for prenatal care?
_____ (month / year)
❑ I have not seen anyone yet.
❑ I have an appointment set for _____. (MM/DD/YY)
5. Your Weight Before Pregnancy _____
Your Current Weight _____
Your Height _____
6. Are you pregnant with more than one baby (Twins, Triplets)?
❑ Yes ❑ No

BMI- <19.8
BMI- ≥26.1

7. Are you thinking about breastfeeding your baby?
❑ Yes ❑ No ❑ Undecided
8. Have you had a Human Immunodeficiency Virus (HIV) test during this pregnancy?
❑ Yes ❑ No
9. Have you had any bleeding or cramping?
❑ Yes ❑ No
10. Are you receiving nutrition services from the Special Supplemental Nutrition Program for Women, Infants, and Children (WIC)?
❑ Yes ❑ No

WIC- Y

Continued

PRENATAL CARE COORDINATION PROGRAM PREGNANCY QUESTIONNAIRE
F-1105 (02/09)

SECTION III — PREGNANCY HISTORY (If this is a first pregnancy, skip to Section IV.)

1. How many times have you been pregnant before?	2. Number of Full-Term Babies	3. Number of Babies Born More Than Three Weeks Early
4. Number of Miscarriages or Other Pregnancy Losses at 20 or More Weeks	5. Number of Miscarriages or Other Pregnancy Losses Before 20 Weeks	6. Number of Living Children
7. Number of Babies Weighing Less Than 5½ Pounds at Birth	8. Number of Babies Weighing More Than Nine Pounds at Birth	9. Date Last Pregnancy Ended

PreT
Loss 20+
LBW
Int<12m

10. Outcome of Last Pregnancy ❑ Live Birth ❑ Miscarriage / Other Loss

SECTION IV — CONCERNS

1. Do you have, or have you ever had, any of the following conditions? ❑ Yes ❑ No
   Check all that apply.
   - ❑ Asthma.
   - ❑ Chlamydia, gonorrhea, syphilis, or genital herpes.
   - ❑ Diabetes.
   - ❑ High blood pressure.
   - ❑ Seizures / epilepsy.
   - ❑ Urinary tract infection.
   - ❑ Other illness, infection, or condition requiring medical care.

1 — Y
2 — Y
4 — Y
7 — Y
8 — Y
9 — Y
10 — Y
11 — Y
12 — Y
13 — Y
14 — Y
15 — Y
16 — H
17 — 0

2. Do you have dental pain or bleeding gums when you eat or brush your teeth? ❑ Yes ❑ No
3. Before pregnancy, did you smoke cigarettes? ❑ Yes ❑ No
   If Yes, indicate the average number of cigarettes smoked per day. _____
4. Since you have been pregnant, have you smoked cigarettes? ❑ Yes ❑ No
   If Yes, indicate the average number of cigarettes smoked per day. _____
5. Does anyone in your household smoke? ❑ Yes ❑ No
6. In the three months before your current pregnancy, did you use any form of alcohol? ❑ Yes ❑ No
   If Yes, indicate the average number of drinks consumed per week. _____
7. Since you have been pregnant, have you used alcohol? ❑ Yes ❑ No
   If Yes, indicate the average number of drinks consumed per week. _____
8. In the past year, have you used street drugs? ❑ Yes ❑ No
9. Have you ever been physically, sexually, emotionally, or verbally abused by your partner or someone close to you? ❑ Yes ❑ No
10. Do you feel unsafe where you live? ❑ Yes ❑ No
11. During the past month, did you miss any meals, not eat when you were hungry, or use a food pantry because there was not enough food or money to buy food? ❑ Yes ❑ No
12. Have you had any housing problems in the past three months? ❑ Yes ❑ No
13. Do you have transportation, child care, or other problems that prevent you from keeping your health care or social services appointments? ❑ Yes ❑ No
14. Have you had problems with depression or received counseling or medications for mental health concerns? ❑ Yes ❑ No
15. During the past month, have you had little interest in doing things, or have you been bothered by feeling down, depressed, or hopeless? ❑ Yes ❑ No
16. How do you rate your current stress level? ❑ High ❑ Medium ❑ Low
17. How many people can you count on when you need help? ❑ 0 ❑ 1-2 ❑ 3+

Continued

PRENATAL CARE COORDINATION PROGRAM PREGNANCY QUESTIONNAIRE
F-1105 (02/09)

SECTION IV — CONCERNS (Continued)

18. Which of these things worry you a lot? Check all that apply.
    - ❑ Money problems.
    - ❑ My job.
    - ❑ My partner's job or unemployment.
    - ❑ My partner's drinking or drug use.
    - ❑ My own drinking or drug use.
    - ❑ My partner is in jail.
    - ❑ My relationship with my partner.
    - ❑ My partner did not want this pregnancy.
    - ❑ Labor and delivery.
    - ❑ Caring for this baby.
    - ❑ Caring for my other children.
    - ❑ Other _____.

19. What worries you the most?

20. What do you do to deal with your problems?

21. Who can you count on for help with everyday activities, such as child care, meals, laundry, or transportation?

22. What topics would you like to learn more about? Check all that apply.
    - ❑ Baby's growth and development.
    - ❑ Breastfeeding.
    - ❑ Caring for your newborn.
    - ❑ Family planning / birth control.
    - ❑ Getting health care for you and your baby.
    - ❑ How to stop smoking.
    - ❑ Effects of alcohol on mother and baby's health.
    - ❑ Labor and delivery.
    - ❑ Managing the discomforts of pregnancy.
    - ❑ Nutrition during pregnancy.
    - ❑ Managing stress.
    - ❑ Other _____.

23. Additional Information

SECTION V — TO BE COMPLETED BY HEALTH PROFESSIONAL

Is the member eligible for Prenatal Care Coordination (PNCC) services?

- ❑ Yes, based on a number of factors _____ or age_____.
- ❑ No.

SIGNATURE — Staff Completing Assessment	Date Signed
SIGNATURE — Qualified Health Professional (If Different from Above)	Date Signed

Pregnant Woman's Health and Diet Questions

*Your name*_____ __/__/__ *Today's Date* __/__/__ *Your Date of Birth*

Question 1 a-c is optional. Your answer will be used for reporting purposes. If you do not answer, a selection will be made for you by the staff. This does not affect you receiving WIC benefits.

Staff Use Only

1. a. Are you Hispanic or Latino? ___Yes ___ No
   b. Are you Arabic? ___Yes ___ No
   c. Check (√) all races that apply to you:
   - __ American Indian or Alaska Native
   - __ Asian
   - __ Black or African American
   - __ Native Hawaiian or other Pacific Islander
   - __ White

race

Please answer the following questions. These questions are asked to see if you may be eligible for the WIC Program. Please check (√) your answer or fill in the blank. All answers are confidential.

2. What was your weight just before you became pregnant with this baby?_____ pounds CDC

pregravid weight

3. How many weeks pregnant are you? _____ weeks

weeks gestation

4. When is your baby due? _____ month day year

5. Including this pregnancy, how many times have you been pregnant? _____
   (Count any abortions, miscarriages or stillbirths)

gravidity

6. How many previous pregnancies lasted more than 4 months? _____
   (Do not count this pregnancy)

parity

7. How many live babies have you had? _____

live births

8. If you have been pregnant before, when did your **last** pregnancy end? (CDC)
   (date of last delivery, abortion, miscarriage or stillbirth)_____ month day year

prior delivery

9. Where are you going for most of your prenatal care during this pregnancy?

prenatal place

   - __ 1. Hospital clinic
   - __ 2. Health department clinic
   - __ 3. Private Doctor's office/HMO
   - __ 4. I am not going yet
   - __ 5. Other _____

   Health Plan Name:_____ Dr. Name: _____

10. For this pregnancy, how are you paying for most of your medical care?

prenatal source

   - __ 1. Private health insurance
   - __ 2. HMO
   - __ 3. Medicaid/Healthy Kids
   - __ 4. Self or family
   - __ 5. I have no way to pay
   - __ 6. Other_____

11. When did your prenatal care begin?
    (For example, 2 months pregnant; 4 months pregnant) _____ (months pregnant)

12. Are you currently: __ 1. Not married __ 2. Married

marital status

13. How many grades of school have you completed? _____ grades completed

mother's education

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DCH-0181 Rev. 10/07 Michigan Department of Community Health Authority: Act 368 PA 1978 Page 1

14. Since you have become pregnant, have you taken any medicines (prescription or non-prescription) or street drugs?
__ No __ Yes
357+/372 - MIHP

a. If yes, for what problem _____
b. If yes, what medications/drugs _____
c. If yes, list side effects, if any_____

15. Please check (√) what is true about **this pregnancy**:

- ☐ Some weight loss during pregnancy 132+
- ☐ Severe nausea and vomiting 301+
- ☐ Gestational Diabetes 302+
- ☐ Less than 18 years of age when I became pregnant 331-MIHP
- ☐ Less than 16 months between the end of last pregnancy and beginning of this one 332
- ☐ Expecting to deliver twins or more 335 – MIHP
- ☐ Less than 20 years of age when I became pregnant and have had 3 or more previous pregnancies lasting 5 months or more 333
- ☐ Fetal Growth Restriction (Intrauterine Growth Retardation) 336+
- ☐ Breastfeeding another baby during this pregnancy 338
- ☐ High blood pressure because of this pregnancy 345+

16. What was the date of your first prenatal visit?_____
334 (after 13 weeks) - MIHP

How many times have you seen your health provider for this pregnancy?_____
(14-21 weeks: 0, 22-29 weeks: ≤1, 30-31 weeks: ≤2, 32-33 weeks: ≤3, 34 or more weeks: ≤4) 334 - MIHP

17. Please check (√) which is true about any previous **deliveries before this pregnancy:**

- ☐ I have never been pregnant before
- ☐ History of Gestational Diabetes 303
- ☐ Premature delivery (36 weeks or less gestation) 310+ - MIHP
- ☐ Delivered an infant that weighed 5 pounds, 8 ounces or less 312+ - MIHP
- ☐ Infant died after 5 months of pregnancy, infant death before 1 month, miscarriage 321 - MIHP
- ☐ Infant born with congenital or other birth defects 339
- ☐ Infant weighed 9 pounds or more 337

18. Do you have dental problems that make it difficult to eat? __ No __ Yes
381

19. Have you taken any vitamins or minerals in the past month? ___ No ___ Yes
If yes, what are you taking? _____ (Note for CPA re: 30 mg iron as a supplement daily) (CDC)

20. In the month before you got pregnant with this baby, how many times a week did you take a multi-vitamin (a pill that contains many different vitamins and minerals)? _____ times each week (CDC)

21. Do you have problems with transportation to your prenatal or WIC visits that make it hard for you to come?
__ No __Yes - MIHP

BREASTFEEDING QUESTIONS

22. WIC encourages breastfeeding because of the many benefits for mother and baby. Have you received any information about breastfeeding? __ No __ Yes

23. Where have you heard or who provided information to you about breastfeeding? Check (√) all that apply.

- __ Breastfeeding mom
- __ Magazine
- __ Health Care Provider
- __ WIC
- __ Friends and relatives
- __ TV
- __ Breastfeeding Peer Counselor
- __ Other_____

Page 2

24. My breastfeeding experience with my last baby was:

__ Wonderful __ Good __ OK __ Difficult __ I did not breastfeed

25. How long did you breastfeed your previous children? _____

ALCOHOL USE

Staff Use Only

26. Have you had any alcoholic beverages (beer, wine, liquor, wine coolers) during this pregnancy, even before you found out you were pregnant? __ No __ Yes
372 - MIHP

27. During the **three months before you were pregnant**, how many days each week or each month did you drink any alcoholic beverages on the average? CDC

Number of days each week _____ OR Number of days each month _____

Days prepregnancy

28. During the **three months before you were pregnant**, on the days when you drank, about how many drinks did you drink on the average? CDC

A drink is: 1 shot of liquor, 1 can or bottle of wine cooler, 1 can or bottle of beer, 1 glass of wine, or 1 cocktail

Average number of drinks _____ OR No drinks_____

Drinks prepregnancy

29. During the **past three months**, how many days each week or each month did you drink any alcoholic beverages on the average? CDC

Number of days each week _____ OR Number of days each month _____

Alcohol now days/week

30. During the **past three months**, on the days when you drank, about how many drinks did you drink on the average? CDC

Average number of drinks _____ OR No drinks _____

Alcohol now drinks/day

TOBACCO USE

31. Have you smoked cigarettes, pipes or cigars during this pregnancy, even before you found out you were pregnant? __ No __ Yes
371 (B-only tobacco) - MIHP

32. During the **three months before you were pregnant**, how many cigarettes, pipes, or cigars a day did you smoke? (20 cigarettes = 1 pack) CDC

Number per day _____ OR I did not smoke _____

Cigarettes prepregnancy

33. On the average, about how many cigarettes, pipes or cigars do you smoke a day **now?** CDC

Number per day _____ OR I do not smoke _____

Cigarettes now

34. How have you changed your smoking habits **during this pregnancy?**

__ I did not smoke (8)
__ Stopped completely (2)
__ Cut down (1)
__ Started smoking (4)
__ No change, tried to cut down but didn't (3)
__ No change, smoking the same (7)
__ I don't know (9)

Cigarettes change

DIET QUESTIONS

Your health:

What are your snacks and meals like:
(When, where, with who?)

What foods do you think you don't get enough of:

How do you feel about your weight/weight gain:

What activities do you like to do:

How many meals do you eat most days? _____ How many snacks do you eat most days? ____

How many times do you drink milk in a day? _____

Is your appetite usually: Good _____ Fair _____ Poor _____

Are you on a special diet (prescribed by a doctor)? _____ 403+

How many times in a week do you eat Fast Food? _____

Do you eat fish from Michigan rivers and lakes? ____

Do you eat or drink any of the following everyday or most days? (Check all that apply)

1. ____ Milk What kind _____
2. ____ Pop or other sweetened beverages
3. ____ Sweets or salty snacks
4. ____ Whole grains
5. ____ Fruits and vegetables

Do you eat or drink any of the following? (Check all that apply) 405

6. ____ Unpasteurized (raw) juice or milk
7. ____ Soft cheese (like feta, brie, camembert, blue or Mexican style cheese such as queso blanco, queso fresco or Panela unless labeled as made with pasteurized milk)
8. ____ Raw or undercooked (rare) meat, fish, poultry or eggs
9. ____ Raw sprouts or raw or undercooked tofu
10. ____ Refrigerated pate or meat spreads or refrigerated smoked seafood?
11. ____ Hot dogs, lunchmeats, and other deli meats <u>not reheated to steaming hot</u>

Do you? (Check all that apply)

12. ___ Eat a strict vegetarian diet 402+ or 403+
13. ___ Eat a low calorie/weight loss diet 403+
14. ___ Eat a low-carbohydrate, high protein diet (like Atkins, etc.) 403+
15. ___ Eat little food because of stomach surgery to lose weight 403+
16. ___ Regularly eat non-food items (ashes, carpet fibers, cigarettes or cigarettes butts, clay, dust, foam rubber, paint chips, soil, laundry starch or corn starch) 421+
17. ___ Take a vitamin or mineral supplement daily 424 (no) What kind _____
18. ___ Use herbal supplement remedies or teas 423 What kind _____

Thank you for completing this form. Please let the staff know you are finished.

WIC HEALTH QUESTIONNAIRE
Pregnant Woman

Date:_____

Woman's Name _____ Birth Date _____ Due Date _____

		Circle Yes or No		NRFs
1.	**Have you been pregnant before? (If no, skip to question 2; if yes, answer the following):** ..... How many times have you been pregnant (including this time)? _____ Date of last delivery, miscarriage or abortion: _____ How many children do you have? _____	No	Yes	System Assigned NRFs
2.	**Before you became pregnant (this pregnancy) how much did you weigh?**_____			
3.	**Do you now receive prenatal care from a doctor or clinic?** ..... In which month of pregnancy did care begin? _____ Name of doctor/clinic:_____	Yes	No	Refer
4.	**In the 3 months before pregnancy, how many cigarettes did you smoke each day? _____cigarettes or _____packs** How many cigarettes do you now smoke each day?_____cigarettes or _____packs Have you quit smoking since you became pregnant? ..... Does anyone else living in your household smoke inside the home?	Yes No	No Yes	ES
5.	**Have any of your friends or family had drinking or drug problems?**	No	Yes	
6.	**In the 3 months before pregnancy, how often did you drink beer, wine, hard liquor, or mixed drinks?** _____**days each week** ☐ **1-2 times each month** ☐ **Never** When you did, how many drinks did you have? (circle one) 1 2 3 4 5 6 7 8 or more **How often do you now drink beer, wine, hard liquor, or mixed drinks?** _____**days each week** ☐ **1-2 times each month** ☐ **Never** When you do, how many drinks do you have? (circle one) 1 2 3 4 5 6 7 8 or more			System Assigned NRFs/ Counsel/ Refer
7.	**In the three months before pregnancy did you use any of the following?** ..... Medicine (doctor prescribed) ☐ Over-the-counter drugs (aspirin, cold tablets, diet pills)☐ Vitamin/mineral pills (other than prenatal ones) ☐ Alcohol ☐ Marijuana ☐ Methamphetamine ☐ Cocaine ☐ Speed ☐ Crack ☐ Heroin ☐ LSD ☐ Valium ☐ PCP ☐ Other:_____ If yes, how often (once, daily, weekly, monthly)?_____ **Do you now use any of those drugs?** ..... If yes, how often?_____ Which ones?_____	No No	Yes Yes	
8.	**Have you had a blood lead test done in the last 12 months?** ..... Lead level or test results (if known):_____	No	Yes	
9.	**Have you thought about breastfeeding this baby?** ..... If no, why?_____	Yes	No	Counsel/ Refer
10.	**Have you ever breastfed a baby?** ..... If yes, for how long?_____ Did you have any problems?..... Why did you stop?_____	Yes No	No Yes	
11.	**Have you ever been checked for flat or inverted nipples?**	Yes	No	
12.	**With any past pregnancy, did you have any of the following:** ..... Pregnancy-related diabetes (problems with blood sugar) ☐ Infant born more than 3 weeks early ☐ Infant birth weight less than 5 lb. 8 oz. ☐ Death of fetus or infant less than one month old ☐ Infant born with neural tube defect or cleft lip or palate ☐ Preeclampsia ☐	No	Yes	Previous Pregnancy

13. With this pregnancy, has your doctor diagnosed any of the following: ..... Severe nausea or vomiting (Hyperemesis Gravidarum) ☐ Pre-diabetes ☐ Pregnancy-related diabetes (problems with blood sugar) ☐ A twin or triplet pregnancy ☐ Hypertension or Prehypertension ☐	No	Yes	79 80 83
14. Do you currently have a medical problem diagnosed by a doctor (such as diabetes, high blood pressure, hepatitis, HIV/AIDS, eating disorder or food allergy)? If yes, describe:_____	No	Yes	Medical Conditions
15. Do you have a chronic dental problem such as severe decay, tooth loss, periodontal disease or gingivitis that affects your food intake? ..... If yes, describe:_____	No	Yes	
16. Have you had major surgery in the last two months?.....	No	Yes	
17. Are you currently breastfeeding an infant or child?.....	No	Yes	66
18. Are you now in foster care? ..... If yes, when did you move to this foster care home?_____	No	Yes	94
19. How much weight do you expect to gain while pregnant?_____			
20. Are you having any of the following?..... Gas ☐ Heartburn ☐ Constipation ☐ Diarrhea ☐ Vomiting ☐	No	Yes	
21. Do you have any questions or concerns about: ..... How to breastfeed ☐ Fitting breastfeeding into your life ☐ Preparing for breastfeeding ☐ Having enough milk ☐ Having good milk ☐ Working/going to school & breastfeeding ☐ Pumping your breasts ☐ Weight gain ☐ Sore nipples ☐ Finding a doctor for your baby ☐ Breastfeeding in public ☐ Child support payments ☐ Drinking while pregnant ☐ Family planning/birth control ☐ Smoking while pregnant ☐ Using drugs while pregnant ☐ Other:_____ **Would you like information about:** Medicaid ☐ TANF ☐ Food Stamps ☐	No No	Yes Yes	Counsel/ Refer
22. Do you now receive regular dental care?	Yes	No	
23. How often do you exercise, such as walking for 20-30 minutes without stopping? _____ What kind of exercise?_____			

*Questionnaire assessed by:*_____*(staff)*

Notes:

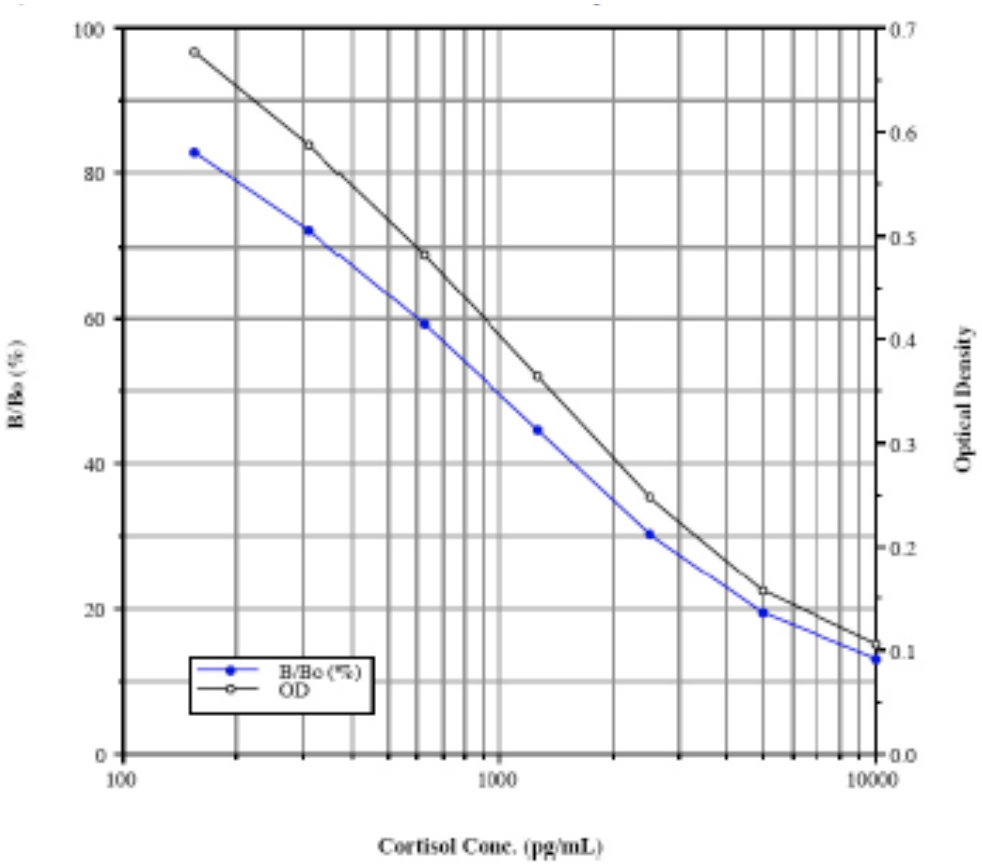
Colorado WIC Growing Healthy Families

APPENDIX IV

A. STANDARD CURVE FOR CORTISOL ASSAY

B. MAP OF RESTRICTION DIGEST ENZYMES

a) Typical standard curve used for cortisol measurements



b) Summary of restriction digest enzymes used for the polymorphic studies

Polymorphism	**Restriction Enzyme**	**Restriction Site**
Bcl I	***Bcl* I**	5′...T▼GATCA...3′ 3′...ACTAG▲T...5′
N363S	**Tsp509I**	5′...▼AATT...3′ 3′...TTAA▲...5′
Tht*III 1*	***TthIII 1***	5′...GACN▼NNGTC...3′ 3′...CTGNN▲NCAG...5′
ER22/23EK	***MnI I***	5′...CCTC(N)$_7$▼...3′ 3′...GGAG(N)$_6$▲...5′

APPENDIX V

SUMMARY OF SIGNIFICANT CORRELATION FROM:

TERM / PRE-TERM & PLAN / NO PLAN CLINICAL DATA

Summary of correlation values and significance of the GR with other variables.

Correlations TERM (n = 13)		
	(*r*)	*P* values
GRβ / GRγ	0.580*	0.038
GRβ / GR-P	0.980**	0.000
GR-P / Cortisol	0.638*	0.035
Stress / Fetal weight	- 0.591*	0.034
Stress / Placenta weight	- 0.732**	0.004
Stress / GAS5	- 0.711*	0.021
Cortisol / Pregn. days	0.747*	0.033
Fetal weight / Placenta weight	0,962**	0.000

Correlations PRE-TERM (n = 10)		
	(*r*)	*P* values
GRα / GR-P	0.710*	0.021
GRβ / GAS5	0.792*	0.011
Stress / Pregnancy days	- 0.685*	0.042
Stress / Placenta weight	- 0.695*	0.038
Pregn. days / Fetal weight	0.986**	0.000
Pregn. days / Placenta weight	0.989**	0.000
Fetal weight / Placenta weight	0.990**	0.000

*. Correlation is significant at the 0.05 level (2-tailed)

**. Correlation is significant at the 0.01 level (2-tailed)

Correlations PLAN (n = 11)		
	(*r*)	*P* values
GRα / GRγ	0.664*	0.026
GRα / GAS5	0.881*	0.001
GRγ / GAS5	0.860**	0.001
Stress / Pregn. days	- 0.607*	0.048
Pregn. days / Fetal weight	0.952**	0.000
Pregn. days / Placenta weight	0.971**	0.000
Fetal weight / Placenta weight	0.996**	0.000

Correlations NO PLAN (n = 12)		
	(*r*)	*P* values
GRα / Stress	0.707*	0.015
GRβ / GRγ	0.648*	0.023
GRβ / GR-P	0.978**	0.000
GRβ / Stress	-0.646*	0.032
GRβ / GAS5	0.720*	0.019
GRγ / GR-P	0.663*	0.019
GRγ / GAS5	0.775**	0.008
GR-P / Stress	- 0.645*	0.0032
GR-P / GAS5	- 0.645	0.044
Pregn. days / Fetal weight	0.927**	0.000
Pregn. days / Placenta weight	0.962**	0.000
Fetal weight / Placenta weight	0.973**	0.000

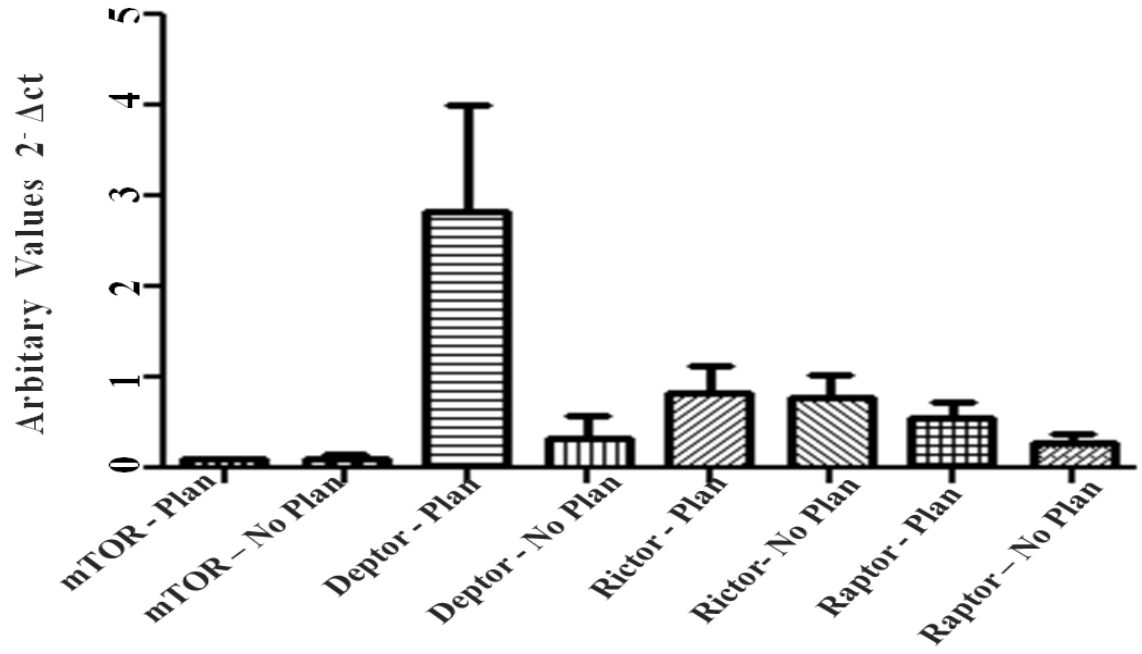
*. Correlation is significant at the 0.05 level (2-tailed)

**. Correlation is significant at the 0.01 level (2-tailed)

APPENDIX VI

EXPRESSION OF mTOR SIGNALLING COMPONENTS IN *PLAN / NO PLAN* PREGANCY

Expression of mTOR signalling components

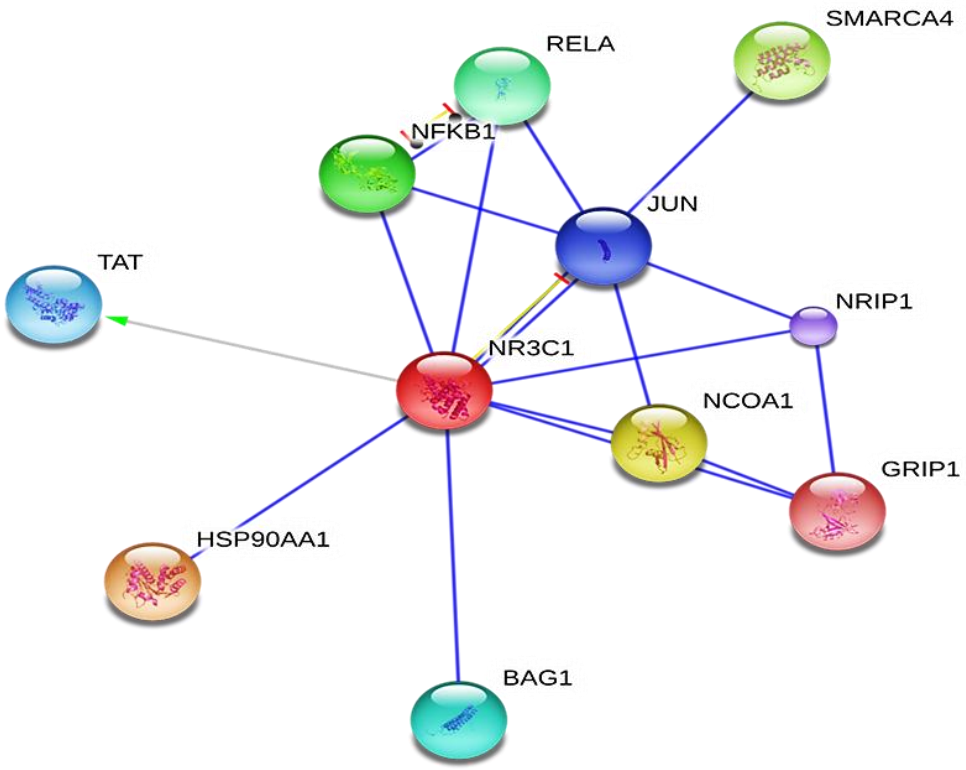


	mTOR-Plan	mTOR-No Plan	Deptor-Plan	Deptor-No Plan	Rictor-Plan	Rictor-No Plan	Raptor-Plan	Raptor-No Plan
Minimum	0.0344	0.0228	0.0033	0.0017	0.1966	0.0743	0.0339	0.0145
25% Percentile	0.0575	0.03268	0.0122	0.0047	0.2279	0.1950	0.2178	0.0615
Median	0.0857	0.07890	0.9235	0.01945	0.3784	0.4521	0.4406	0.1285
75% Percentile	0.1535	0.1468	6.793	0.2028	0.9459	1.283	0.7361	0.4043
Maximum	0.1844	0.4142	8.339	2.480	2.933	2.724	1.959	1.017
Mean	0.09801	0.1096	2.821	0.3277	0.8222	0.7784	0.5596	0.2814
Std. Deviation	0.04883	0.1129	3.523	0.7828	0.9901	0.8264	0.5339	0.3403
Std. Error	0.01472	0.03259	1.174	0.2475	0.2985	0.2385	0.1610	0.09824
Lower 95% CI of mean	0.06521	0.03785	0.1131	-0.2323	0.1570	0.2534	0.2009	0.06512
Upper 95% CI of mean	0.1308	0.1813	5.529	0.8877	1.487	1.303	0.9182	0.4976

APPENDIX VII

STRINGS FOR GR GENE

String for **GRα**



HSP90AA1: Heat shock protein 90kDa alpha (cytosolic), class A member 1;

NCOA1: Nuclear receptor coactivator 1

SMARCA4: SWI/SNF related matrix associated, actin dependent regulator of chromatin, subf. A

NFKB1: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

RELA: v-rel reticuloendotheliosis viral ongogene homolog A

BAG1: BCL2-associated athanogene;

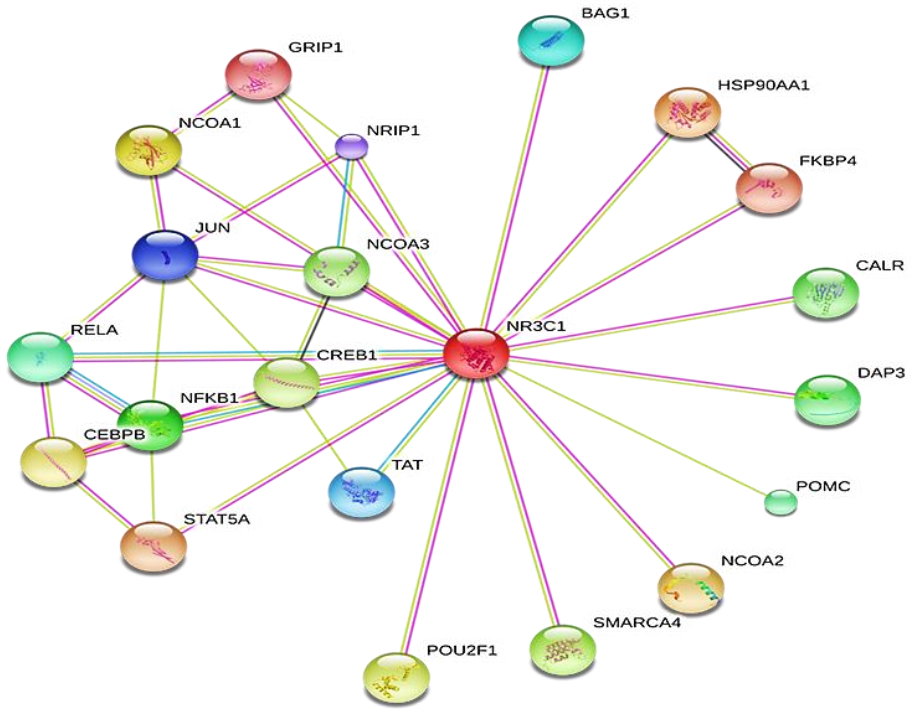
TAT: Tyrosine aminotransferase

JUN: Jun ongogene;

NRIP1: Nuclear receptor interacting protein 1;

GRIP1: Glutamate receptor interacting protein1;

String for **GRβ**



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TAT: Tyrosine aminotransferase

JUN: Jun ongogene;

NRIP1: Nuclear receptor interacting protein 1;

GRIP1: Glutamate receptor interacting protein1;

FKBP4: FK506 binding protein 4;

STAT5A: Singnal transducer and activator of transcription5A

NCOA2: Nuclear receptor coactivator 2.

CEBPB: CCAAT/enhancer binding protein(C/EBP) beta

POU2F1: POU class 2 homeobox 1.

CREB1: cAMP responsive element binding protein 1.

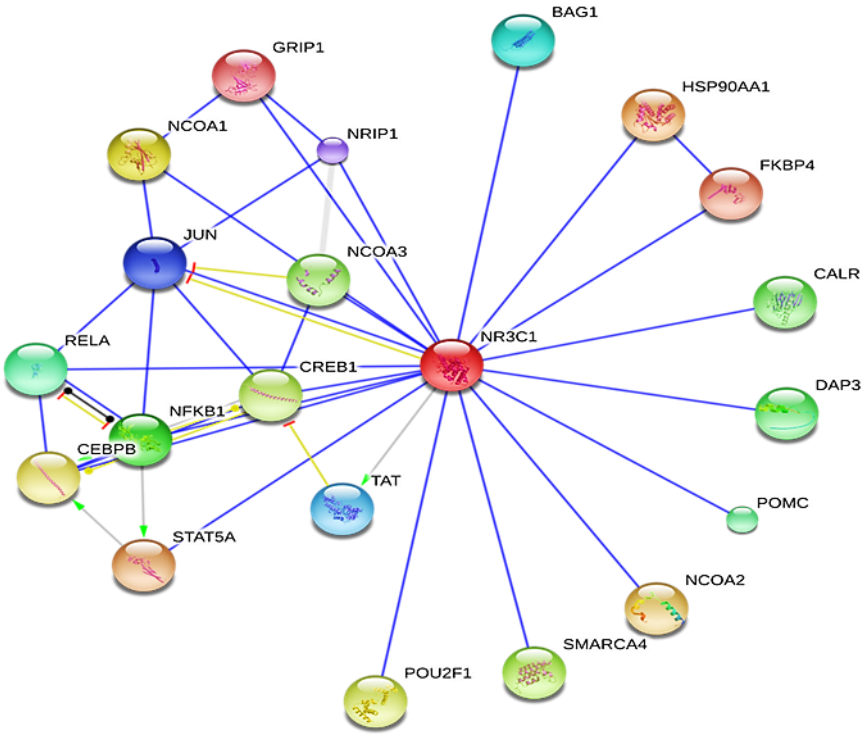
NCOA3: Nuclear receptor coactivator 3.

CALR: Calreticulin.

DAP3: Death associated protein 3.

POMC: Proopiomelanocortin.

String for **GRγ**



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NCOA1: Nuclear receptor coactivator 1

SMARCA4: SWI/SNF related matrix associated, actin dependent regulator of chromatin, subf. A

NFKB1: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

RELA: v-rel reticuloendotheliosis viral ongogene homolog A

BAG1: BCL2-associated athanogene;

TAT: Tyrosine aminotransferase

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NRIP1: Nuclear receptor interacting protein 1;

GRIP1: Glutamate receptor interacting protein1;

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CEBPB: CCAAT/enhancer binding protein(C/EBP) beta

POU2F1: POU class 2 homeobox 1.

CREB1: cAMP responsive element binding protein 1.

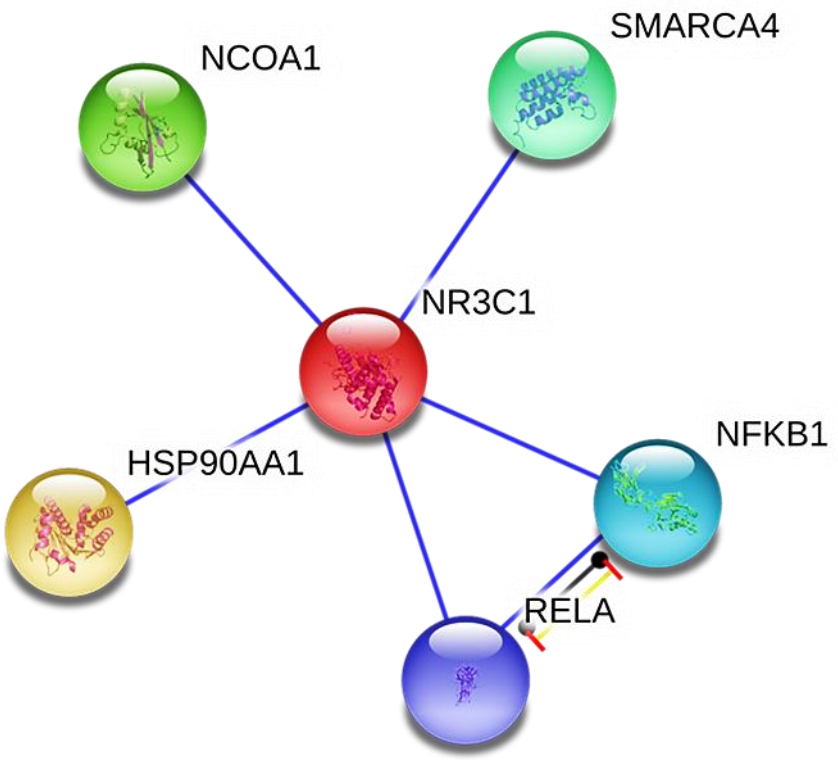
NCOA3: Nuclear receptor coactivator 3.

CALR: Calreticulin.

DAP3: Death associated protein 3.

POMC: Proopiomelanocortin.

String for **GR-P**



HSP90AA1: Heat shock protein 90kDa alpha (cytosolic), class A member 1;

NCOA1: Nuclear receptor coactivator 1

SMARCA4: SWI/SNF related matrix associated, actin dependent regulator of chromatin, subf. A

NFKB1: Nuclear factor of kappa light polypeptide gene enhancer in B-cells 1

RELA: v-rel reticuloendotheliosis viral ongogene homolog A