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MATERNAL AND NEONATAL ANTHROPOMETRY AND GROWTH FACTOR EXPRESSION AND APOPTOSIS IN HUMAN PLACENTA: A COMPARISON BETWEEN TWO POPULATIONS

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Cover illustration: Mother and child; an ultrasound scan of a normal foetus at gestational week 20 (© Akram 2011).

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To my father:
My mentor, role model, and friend.

To my wife:
Without whose support and patience this thesis would not have been completed.

To my supervisors:
For their support and guidance, not only in academia, but in everyday life.

ABSTRACT

The insulin-like growth factors (IGFs), IGF-binding proteins (IGFBPs), and IGFBP proteases, are key endocrine regulators of somatic growth and cellular proliferation. IGFs are involved in growth both pre- and postnatally. Dysregulation of the IGF axis can lead to growth disorders such as intrauterine growth restriction (IUGR), prenatally, and small for gestational age (SGA), postnatally. This hormone axis is dependent on essential micronutrients and trace elements for proper growth and functioning of cells and organs. A deficiency in micronutrients may therefore lead to deficiencies in the axis. The IGFs have been further associated with anti-apoptotic activity with deficiencies in the axis possibly resulting in the activation of apoptosis and the subsequent inhibition of normal placental and foetal growth leading to babies being born small for their gestational age. Determining the aetiologies behind SGA may help our understanding of this vast clinical problem and may also help in suggesting possible interventions to improve pregnancy, and life, outcomes.

The aim of this study was to look in to the associations between the insulin-like growth factors and the regulation of apoptosis, looking at newborn and maternal anthropometric outcomes in the Swedish and Pakistani populations.

Human placental samples were obtained from 89 women at rural field sites in Pakistan (papers I, II, & IV). Furthermore, 33 samples were obtained from the Swedish population (papers III and IV). Maternal and neonatal anthropometric variables were noted at the time of delivery. Umbilical cord blood samples were also taken to assess levels of certain key micronutrients, namely zinc and iron. IGF mRNA expression levels were assessed using RT-PCR techniques. Oestrogen receptor (ER) and progesterone receptor (PR) expression levels were quantified using solution hybridization. Additionally, protein analysis was conducted using Western immunoblot analysis, ELISA, and radioimmunoassay studies. For the purposes of experimentation and analysis, samples were divided into small, appropriate, and large for gestational age groups (SGA, AGA, & LGA, respectively). TUNEL and immunohistochemical staining were also employed for the assessment of apoptotic proteins and factors.

In the Pakistani population, we have shown significantly lower expression levels of placental IGF-I and IGF-II in the SGA group. Furthermore, we have shown lower IGF-I protein levels and significant associations of maternal and newborn anthropometry to IGF expression and protein levels, as well as placental IGFBP-1 levels. These findings suggest the importance of the IGF-axis in birth weight outcomes. Significant correlations of maternal anthropometry and birth anthropometry may indicate the potential use of maternal anthropometry as a screening tool for low birth weight. We have also shown significant differences in cord blood haemoglobin, iron, and zinc levels in the two groups (though all values were within normal ranges), indicating the importance of an adequate nutritional status in pregnancy. In the Swedish population, we have shown similar significant differences in IGF-I expression, with lower levels in the SGA group. We have, in addition, shown significant correlations of PR and IGF-I expression, and ER and maternal anthropometry. These results further suggest the complex multi-factorial regulation of the IGF-axis and indicate the possible role of the ERs and PR in the pathogenesis of foetal growth restriction. Furthermore, placentas of Pakistani mothers have higher levels of placental apoptosis than their Swedish counterparts. Pakistani mothers were also significantly smaller than Swedish mothers in our population groups. These differences in apoptotic activity and anthropometry may thus be associated with the differences in birth weights between these populations.

In summary, this thesis adds to our overall understanding of the correlations between maternal anthropometric and newborn biometric measurements, along with placental levels of components of the IGF-axis and apoptosis. Our data supports previous data on the role of nutrient supplementation in pregnancy and offers an explanation to the possible mechanisms behind the complex problem of foetal growth restriction. Furthermore, our results appreciate the fine balance of growth promoting and growth inhibiting factors in foeto-placental growth and development.

LIST OF PAPERS

I **Akram SK,** Akram, M, Bhutta ZA, Söder O. *Human Placental IGF-I and IGF-II expression: a correlation with Maternal and Infant Anthropometric Variables and micronutrients at birth in the Pakistani population.* Acta Paediatrica. 2008. 97(10): 1443-1448.

II **Akram SK,** Skwirut-Carlsson C, Bhutta ZA, Söder O. *Placental IGF-I, IGFBP-1, zinc, and iron, and maternal and infant anthropometry at birth.* Manuscript submitted. Under review for publication. 2011.

III **Akram SK,** Sahlin L, Östlund E, Hagenäs L, Fried G, Söder O. *Placental IGF-I, oestrogen receptor, and progesterone receptor mRNA expression, and maternal anthropometry in intrauterine growth restricted pregnancies in the Swedish population.* Hormone Research in Paediatrics. 2011. 75(2):131-7.

IV **Akram SK,** Simatou E, Stukenborg JB, Hagenäs L, Bhutta ZA, Söder O. *Placental apoptosis in small for gestational age babies: a comparison between Swedish and Pakistani populations.* Manuscript to be submitted for publication.

CONTENTS

LIST OF ABBREVIATIONS

AGA	Appropriate for gestational age
AKT	(Serine/threonine protein kinase protein)
APAF-1	Apoptosis protease-activating factor 1
ATP	Adenosine triphosphate
BCL-2	B-cell lymphoma 2
BMI	Body Mass Index
cDNA	Complementary deoxyribonucleic acid
cRNA	Complementary ribonucleic acid
CTP	Cytidine triphosphate
Da	Dalton (measure of weight of a protein molecule)
dATP	Deoxyadenosine triphosphate
DISC	Death-induced signalling complex
DNA	Deoxyribonucleic acid
ER	Oestrogen receptor
FADD	Fas-associated death domain
Fe^{++}	Ferritin (Iron)
FGR	Foetal growth restriction
GDM	Gestational diabetes mellitus
GH	Growth hormone
GTP	Guanosine triphosphate
HCG	Human chorionic gonadotrophin
IAP	Inhibitors of apoptosis
IGF-I	Insulin-like growth factor - I
IGF-II	Insulin-like growth factor - II
IGF-I-R	Insulin like growth factor receptor, type 1
IGFBP	Insulin-like growth factor binding protein
IU	International Units
IUGR	Intrauterine growth restriction
kDa	Kilo-Dalton
kg	Kilogram
LBW	Low birth weight
LGA	Large for gestational age
m	Meter
mRNA	Messenger ribonucleic acid
NF-κB	Nuclear factor kappa-light-chain-enhancer of activated B cells
PCR	Polymerase chain reaction
PR	Progesterone receptor
PVDF	Polyvinyldine fluoride
RDA	Recommended daily allowance
RIA	Radioimmunoassay
RNA	Ribonucleic acid
RT-PCR	Reverse transcriptase polymerase chain reaction
SD	Standard deviation
SGA	Small for gestational age
SI	Système International
TCA	Trichloroacetic acid
TGF	Transforming growth factor
TNF	Tumour necrosis factor
UNICEF	United Nations Children's Fund
USDA	United States Department of Agriculture
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation

Foreword

The work in this thesis is the culmination of collaborative clinical and experimental research between the Aga Khan University, Karachi, Pakistan, and Karolinska Institutet, Stockholm, Sweden. It further reflects the efforts and contribution of many people. The underlying aim was to attempt to contribute to the understanding of the aetiology of, and to provide possible suggestions of treatment of the widespread clinical problem of children being born small for their gestational ages. This vast clinical problem affects millions of pregnancies globally, and results in a vicious cycle of growth related problems. The methodologies employed in this thesis included the measurement of an array of clinical and experimental parameters, which are discussed in detail. The results and accompanying discussion represent an overview of the papers, which are presented in the appendices. All work has been conducted following strict ethical protocols and guidelines. All samples were obtained following consent. Patient details have been excluded to maintain confidentiality. All previously published work has been reproduced following permission from the publishers.

1. INTRODUCTION

1.1 Normal vs. Abnormal Growth

Human growth is an extraordinary phenomenon starting from the fusion of two cells, the male sperm and the female egg. These cells then begin to divide, culminating in a living breathing neonate. The period of growth and cellular multiplication within the uterus is known as the *gestational period*. The gestational period is further divided into three time periods, or *trimesters*, based on development of the foetus. The early gestational period, also known as the first trimester, is of unprecedented importance as it influences many factors later in life. The final outcome of adult height is determined not only by genetic factors, but also by the adequacy of foetal growth. The fastest growth rate is during embryonic life. To quantify it, if the intrauterine growth rate was sustained, a person would grow by approximately *50-60 centimetres* per year. As one can imagine, this proportion of growth requires many factors, the most important being a healthy nutrient rich environment. This encompasses an adequate blood supply for tissue nutrition and oxygen and is grossly reflected by the mother's overall health. The details of the placental environment will be discussed below.

Growth is principally regulated by multiple hormones and their associated proteins. An important group of such proteins is the *insulin-like growth factor* (*IGF*) axis and the *growth hormone* (*GH*)(Frago 2005). Problems with the production of any of these growth factors, or problems with maternal nutrition, are associated with intrauterine growth restriction (*IUGR*), also known as foetal growth restriction (*FGR*). IUGR is associated with an increased risk of both short-term and long-term health problems. The immediate birth outcome is that of a baby being born small for gestational age (*SGA*), increasing the risks of neonatal morbidity and mortality. These problems perpetuate throughout life resulting in increased blood pressure, cardiovascular and cerebrovascular disease, insulin resistance, and an increased incidence in type II diabetes mellitus. SGA is defined as a birth weight below the 10^{th} percentile for the gestational age, based on population specific growth charts (Engle 2004).

Some basic definitions for birth weight are as follows (Goldenring 2004):

- *Appropriate for gestational age (AGA):* Normal birth weight (*10th – 90th percentile*)
- *Small for gestational age:* Weight below the *10th percentile* at gestational age *(-2 SD)*
- *Low birth weight:* Weight below a defined limit (*2.5kg*) at any gestational age
- *Large for gestational age:* Weight above the *90th percentile* at gestational age *(+ 2SD)*
- *Macrosomia:* Weight above a defined limit (*4.5kg*) at any gestational age

Percentiles, along with normal growth curves for the Pakistani population can be seen in *Figure 1*. *Figure 1a* shows the standard weight (*kg*), and age (*months*), growth chart issued by the Ministry of Health, Government of Pakistan (http://www.health.gov.pk/). Weight to age growth between the *10th* and the *90th* percentiles is considered within 'normal' ranges (*AGA*). *Figure 1b* shows standard growth curves for the British Pakistani population, showing height (*m*), weight (*kg*), and body mass index (*BMI*) for boys and girls (modified from: (Kelly, Shaw et al. 1997)). *Figure 1c* shows the standard growth charts for the Swedish population, comparing girls and boys between the ages *24 weeks of gestation* and *24 months* (modified from: (Niklasson and Albertsson-Wikland 2008)).

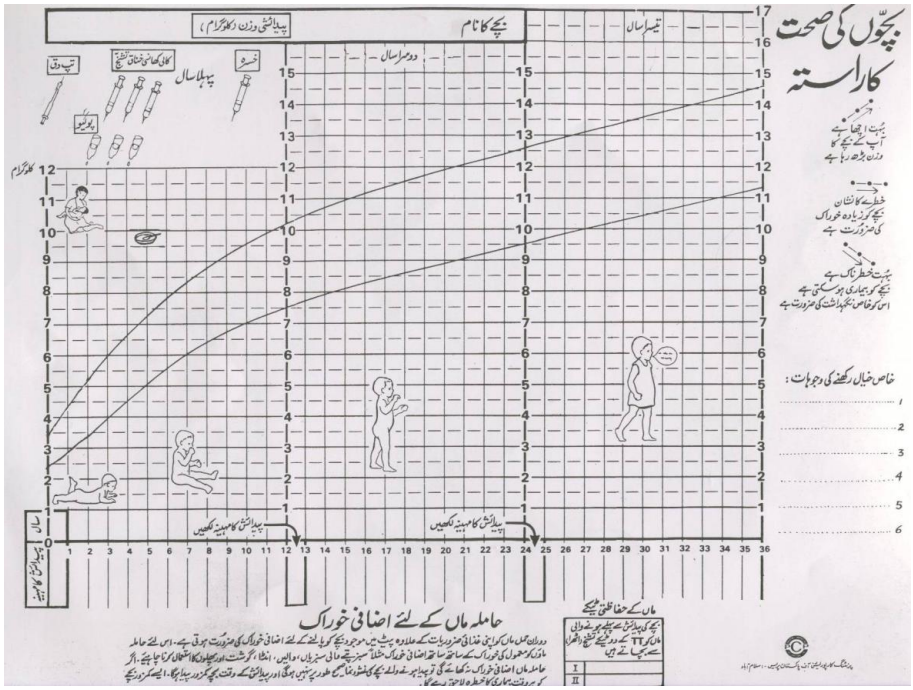
The global incidence of SGA is startling, affecting more than *30 million* newborns per annum (de Onis, Blossner et al. 1998). Furthermore, the consequences of maternal under-nutrition are estimated to contribute to a substantial *3.5million* deaths a year, globally (Black, Allen et al. 2008). The vast majority of babies succumbing to foetal growth restriction, with subsequent low birth weights, are in central and South-east Asia. These regions account for approximately *75%* of all known incidences of foetal growth restriction (WHO 2004). Table 1 emphasises some of the principal maternal and foetal factors contributing towards growth outcomes.

Table 1. Key maternal and foetal factors contributing to growth outcomes

Maternal Factors	Foetal factors
• Maternal size (*Maternal Constraint*). • Adequate nutrition throughout pregnancy. • Maternal glucose metabolism • Thyroid hormone levels - essential for normal foetal growth and development.	• Genetic and environmental factors. • Foetal and infant nutrition - important for adequate growth. • Thyroid hormone levels - essential for growth stature and neurological development.

Legend to Table 1. This table emphasises key maternal and foetal factors contributing to foetal and infant growth outcomes and development.

1A.



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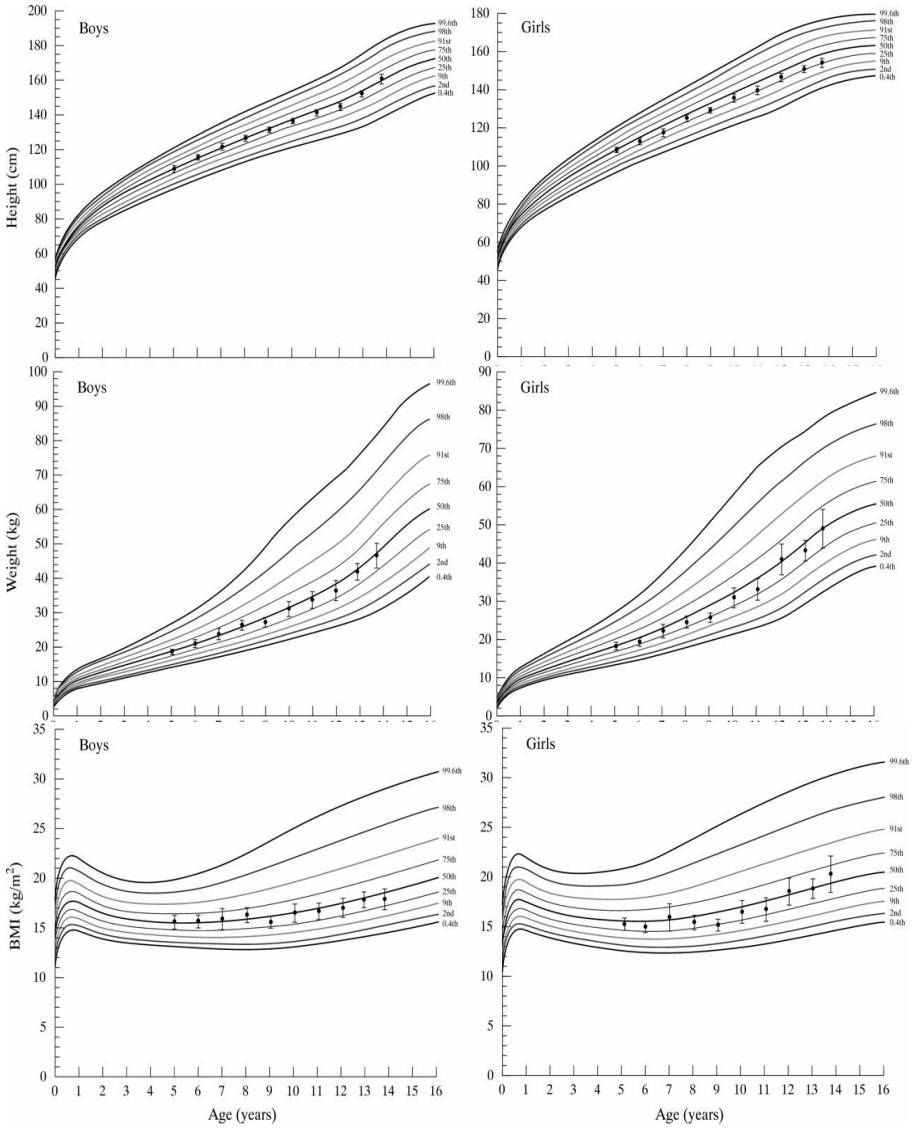

Boys
Girls
Height (cm)
Weight (kg)
BMI (kg/m²)
Age (years)
99.6th
98th
91st
75th
50th
25th
9th
2nd
0.4th

1C.

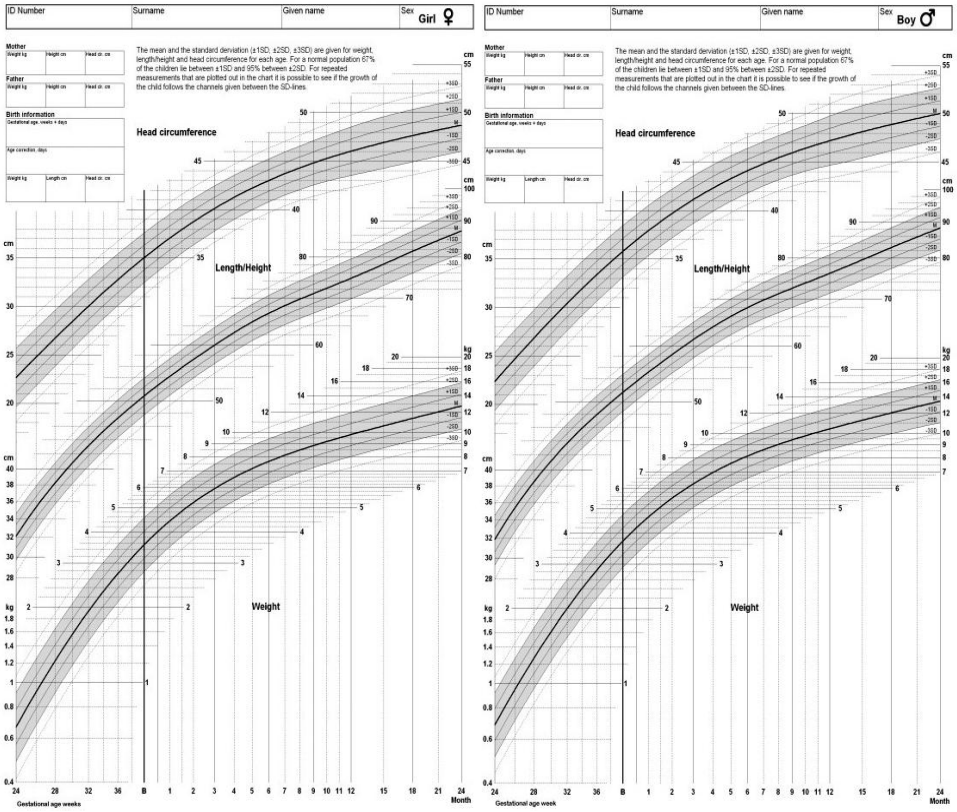


Figure 1a. A general growth chart depicting age (months) and weight (kg) in the Pakistani population showing the upper and lower limits (10th percentile and 90th percentile; Government of Pakistan; http://www.health.gov.pk/). Figure 1b. Growth charts of Pakistani children born in the UK (ages 0-18 years). The charts depict height (m), weight (kg), and body mass index (BMI) in boys and girls, and indicates that the Pakistani children are, on average, smaller than the local British population (modified; (Kelly, Shaw et al. 1997)). Figure 1c. Swedish growth charts showing head circumference (*cm*), length (*cm*), and weight (*kg*), for girls and boys between gestational week 24 and 24 months of age (modified from: (Niklasson and Albertsson-Wikland 2008)).

1.2 The Human Placenta: a brief description

The placenta is a unique organ, as it is only temporarily present in the body, during the gestational period. It consists of two parts, the *trophoblastic* placenta, which is genetically and biologically part of the foetus, and the *decidual* placenta, which is part of the mother. It is a complex organ, tapping into the maternal blood supply, providing essential oxygen and nutrients to a growing foetus. Beyond this, it also serves as a unique barrier, protecting the foetus from potentially harmful substances. The two parts of the placenta are distinctly separate from one another, isolating the foetal blood supply, and yet providing a means to transfer gases and nutrients between maternal and foetal blood (Boyd 1970). Furthermore, placental growth is similar to that of tumour growth, as it is dependent on a complex balance of growth promoting and growth inhibiting factors, which will be discussed in more detail.

As can be seen in *Figure 2*, there is no contact between the foetal and maternal blood circulation. In the intervillous space, the villi are bathed in maternal blood, decreasing the distance between the two circulatory systems. There are, however, microporous adventitial layers preventing foetal and maternal blood cells from mixing. These micropores allow only the smallest molecules to pass through, which consists principally of gases and essential nutrients. Unfortunately, alcohol, certain medications, toxins, and several types of viruses can also cross this barrier. Foetal blood cells may also escape into the maternal blood supply, particularly in cases of improper placental implantation and at the time of delivery.

The functions of the placenta go beyond simple gas and nutrient exchange. It is also a significant endocrine organ producing progesterone, placental lactogen, oestrogen, and growth hormone analogues. Progesterone serves to maintain the placental blood flow throughout pregnancy, thus maintaining gestational growth. Placental lactogen, also known as *somatomammotropin*, causes an increase in sugar and fat content within maternal blood, allowing an abundance of nutrition to be passed on to the rapidly growing foetus. A significant increase in its levels can result in gestational diabetes. The growth hormone analogues are essential in maintaining placental growth, and adequate foetal cellular and tissue growth

throughout pregnancy. Another essential hormone synthesised within the placenta is human chorionic gonadotrophin (*hCG*). The principal form of this hormone, beta-hCG (*β-hCG*) is secreted by the precursor trophoblastic cells as early as the third day of fertilization. Its major function is to ensure that the endometrial vessels engorge, priming the endometrium for embryo implantation. Essentially, normal implantation and placentation is a balance between regulatory gradients created by both the trophoblasts and endometrium (Kliman 1994).

Foetal blood circulation and chorionic villi are evident as early as the third week of gestation (*Figure 2*). The trophoblastic cells form the distinct layers separating the maternal blood supply from that of the foetus. By the fourth week of gestation, the basic structure of the placenta is complete, though the foetus is a mere 2cm in length. Chorionic villi and intervillous spaces have developed to allow for adequate growth of the placenta and foetus for the remainder of the pregnancy.

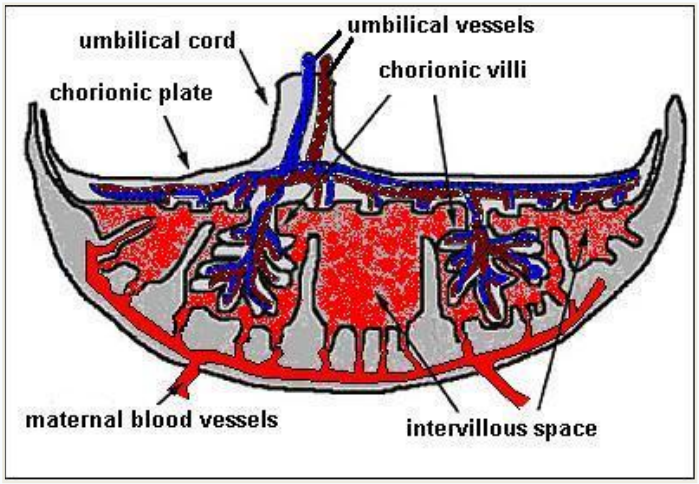


Figure 2. A cross section of the placental barrier. The umbilical vessels are within the villi and are distinctly separate from the maternal vessels. Maternal blood is 'streamed' into the intervillous spaces and allows for the transfer of key nutrients and gas exchange.

The blood supply to the placenta consists of uterine spiral arteries and the uterine vein. These arteries stream blood into the intervillous space, bathing the villi in blood. The blood is then reabsorbed into the uterine vein, providing a constant flow of gases and nutrients to the villi. The foetal vessels consist of two umbilical arteries and a single vein. The arteries deliver the de-oxygenated blood to the placenta and, once it has passed through the villous circulation, the oxygen and nutrient-rich blood is carried back to the foetus via the larger singular umbilical vein. To maintain an adequate supply of nutrients to the foetus, as much as 35% of maternal blood flows through the intervillous spaces (Moore 1993).

The complex system of the placento–foetal circulation is not without complications. Poor maternal health and nutrition, in particular, are contributing factors towards IUGR and subsequent SGA. Additionally, there may be problems with the placental circulation itself, whereby there is a reduced utero-placental blood flow. The most common manifestation of this is *preeclampsia*, a clinical syndrome, in the mother, presenting with high blood pressure, proteinuria, and oedema. This may lead to the more significant problem of *eclampsia* which differs from preeclampsia due to seizure manifestation, which poses an immediate threat to both the mother's life and to foetal viability. Though the exact aetiology of this disease is yet unknown, one of the most common findings is that of poor trophoblastic invasion (Feinberg, Kliman et al. 1991). Furthermore, placental growth and development is somewhat similar to that of a tumour, as mentioned above, with a vast expression of cellular and vascular growth promoting factors and rapid tissue growth. In the later stages of pregnancy, the natural inhibitors of growth reach a balance with the growth promoting factors, preventing further uncontrolled growth, which would otherwise be the case in a tumour. Excessive growth inhibition in the earlier stages gestation would therefore lead to suppression of growth of the foeto-placental unit, resulting in IUGR (Garcia-Lloret, Yui et al. 1996).

1.3 Growth Hormone and the IGF-axis

Key factors in promoting and regulating foetal growth are the *growth hormone (GH),* and the *insulin-like growth factors* (*IGFs*). Growth hormone is a protein,

consisting of approximately 190 amino acids, synthesised in both the mother and foetus. The principal source of this hormone is the pituitary gland. The IGFs are approximately 70 amino acids and are discussed in detail below. *Figure 3* represents the hypothalamic-pituitary-peripheral tissue IGF-axis, which is paramount in cellular and tissue growth. Problems in this cycle may have major effects on cellular growth and modulation as many tissues do not receive the appropriate stimuli they require to grow leading to poor cellular and tissue growth and development (Holt 2002).

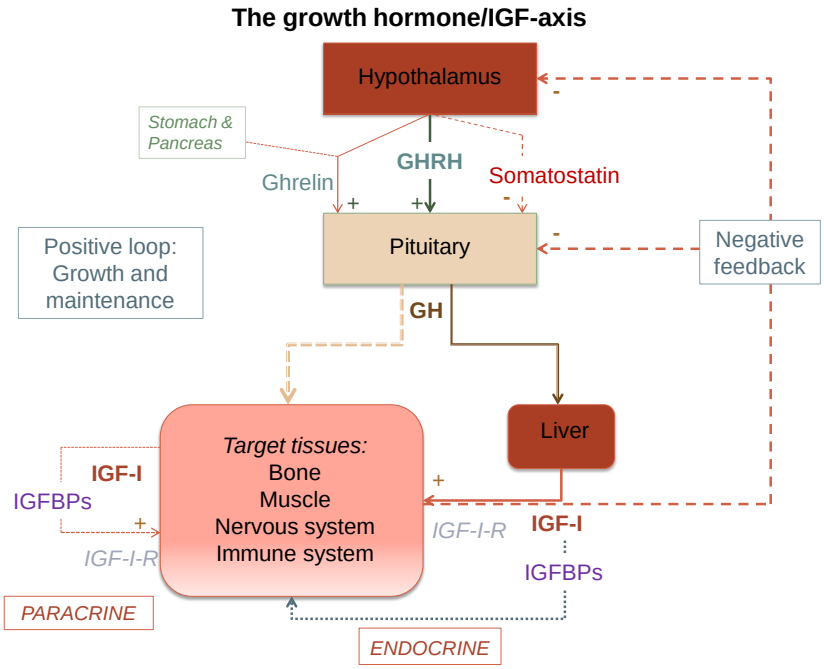


Figure 3. A schematic depicting the insulin-like growth factor/growth hormone (*IGF/GH*) axis. The *hypothalamus* releases growth promoting and growth inhibiting factors, which act downstream on the *pituitary gland*, influencing the release of growth hormone (*GH*). GH further stimulates insulin-like growth factor (*IGF-I and IGF-II*) production in the liver, exerting *endocrine actions*, and local tissues (*paracrine actions*). The IGFs are carried by their binding proteins (*IGFBPs*) and are then released to act on cell membrane receptors (*IGF-I-R)*. As can be seen in the diagram, growth is a complex process of positive and negative feedback loops. Abbreviations are: GH, growth hormone; GHRH, GH releasing hormone; IGF, insulin-like growth factor; IGFBP, IGF binding protein; IGF-I-R, IGF-I-

receptor. GH plays a role in foetal growth towards the end of the gestational period and becomes an important growth promoter after the first year of life, sustained throughout childhood and adolescence. Prior to this, foetal growth is principally controlled by other growth promoting factors, namely the insulin-like growth factors (*IGF-I and IGF-II*). As the name suggests, these polypeptides are structurally very similar to insulin, though they have very different actions. *IGF-I* is the principal growth factor in the early stage of pregnancy, ensuring tissue development and proliferation. It is synthesised in both the maternal and foetal livers, as well as the placenta. *IGF-II*, on the other hand, is a much more potent stimulant of organ proliferation, and is the principal growth factor throughout the remainder of gestation (Forbes and Westwood 2008). It is of particular importance in the development of the brain, kidneys, and liver. It is synthesised in the placenta, pancreas, muscles, and brain, ensuring that these organs develop and that the foetus grows appropriately and does not succumb to growth restriction. These growth factors are relatively small molecules and are carried through the circulatory system via *IGF binding proteins* (*IGFBPs*). In humans, six forms of IGFBPs have been identified, each with a specific role in different organs (*IGFBP 1-6*). These binding proteins, as the name suggests, serve the purpose of binding the IGFs and carrying them within the circulation (Beattie, Allan et al. 2006).

A key binding protein of the placenta is IGFBP-1, as it is synthesised within the decidual placenta (Giudice, Martina et al. 1997). It is polypeptide of approximately *25kDa* in size and is therefore small enough to cross through capillary membranes. For this reason it is thought to be able to carry IGF-I and IGF-II into the extra-cellular space, thereby greatly increasing the chances of ligand receptor interaction in foetal tissues (Lee, Conover et al. 1993). Furthermore, IGF-I and IGF-II have a much greater affinity to the highly phosphorylated isoform of IGFBP-1 (*pIGFBP-1*), than to the IGF-I-receptor (*IGF-I-R*). This means that the free IGF-I is 'mopped' up and is thus unable to interact with the receptor, restricting cellular growth. This is a normal feedback inhibitory mechanism to prevent unregulated growth. Interestingly, the non- and lesser phosphorylated isoforms of IGFBP-1 have a low affinity to IGF-I and a high affinity for IGF-II. The non-phosphorylated form of this binding protein is predominant in pregnancy, presumably through dephosphorylation by placental alkaline phosphatase, which is expressed by

syncytiotrophoblasts (Forbes and Westwood 2008). This may allow for the selective release of IGF-I, and the binding of IGF-II, in the earlier stages of pregnancy, inhibiting apoptosis and promoting placentation and growth (Forbes, Westwood et al. 2008). Furthermore, IGFBP has also been shown to have a dual role, as it has been shown to interact directly with certain tyrosine kinase receptors, thereby activating the cellular reproductive cycle directly, particularly in foetal growth restriction (Lee, Giudice et al. 1997).

Once the ligand-binding protein complex reaches the cell surface, IGF is released from the binding protein when required. The IGFs are, in this manner, in a constant state of bioavailable (*free*) and bound (*to the IGFBPS*) forms (Clemmons 1998). Release of IGF depends on the post-translational modifications of IGFBP-1, principally through *phosphorylation/dephosphorylation*, allowing for easy interaction and binding to specific cell surface receptors, which then activate the internal cellular mechanisms promoting cellular mitosis, thereby preventing apoptosis. There are two specific cell surface receptors, *IGF-I-R and IGF-II-R*. The IGF-I-R is a tyrosine kinase receptor closely related to the insulin receptor that binds IGF-I and IGF-II with high affinity and mediates IGF survival and metabolic signalling (Randhawa 2008). The IGF-II-R is a large transmembrane receptor that is unrelated to the IGF-I-R and selectively binds IGF-II. It thus acts as a clearance factor for IGF-II by internalising and degrading cell-surface IGF-II, thereby acting as a signalling antagonist (Blakesley, Scrimgeour et al. 1996). The growth stimulating effects of both ligands (*IGF-I and IGF-II*) appear to be principally regulated through the IGF-I-R. Following birth, IGF-I regains the role as the principal growth factor, primarily synthesised in the liver (though peripheral production has also been shown), promoting growth in many tissues, including muscles, cartilage, bone, liver, kidney, nerves, skin, and lungs (Giudice, de Zegher et al. 1995).

1.4 Micronutrients

Micronutrients encompass a vast number of microscopic elements and vitamins essential for cellular reproduction and maintaining cellular health. They are therefore paramount in tissue and organ development, as both deficiencies and excesses may have significant effects on growth outcomes (Fall, Yajnik et al. 2003). The most common trace elements include zinc, iron, molybdenum, chromium, calcium, copper, iodine, magnesium, phosphorus, manganese and selenium. Essential vitamins include the fat soluble vitamins, A, D, E, and K, and the water soluble vitamins, B1, B2, B3, B6, B12, and C. Each trace element and vitamin plays a key constitutive role in the genetics of cellular mitosis and growth, comprising of essential structural and functional components of these mitotic processes. The essential vitamins and minerals are listed in Table 2. In this thesis, we focus on two principal micronutrients, zinc and iron, as deficiencies of these have been implicated with adverse pregnancy outcomes (Ploysangam, Falciglia et al. 1997; Scholl 2005).

1.4.1 Zinc

As mentioned above, zinc is an essential trace element required for normal cellular function and reproduction. Zinc prosthetic groups are fundamental in the structure of thousands of proteins, the principal one being the *zinc finger,* an important structural part of DNA. It is also a key constituent in thyroxin. Deficiencies of this micronutrient have been seen in children born SGA. Additionally, excessive amounts have toxic effects on normal physiological function, as can be seen with excessive zinc levels leading to hyperthyroidism. The thyroid hormone is essential for normal cellular metabolism and function, as mentioned in *Table 1*, above. Babies born hypothyroid develop significant mental and physical handicaps. Additionally, zinc ions (Zn^{2+}) are believed to act as neurotransmitters, and are therefore essential in maintaining a healthy neuronal environment. Zinc is also known to be a key activator of carbonic anhydrase, an essential enzyme in the transportation of carbonic dioxide and in maintaining acid-base balance within vertebrates (Adamo and Oteiza 2010).

1.4.2 Iron

Iron deficiency is the most common nutrient deficiency in the world, affecting up to *300 million* people per annum. The principal form of this trace element in humans is the oxidised form, *ferritin* (Fe^{2+}). The key component of the ferritin protein is required to synthesise haemoglobin and is thus essential in blood oxygen transportation. The protein consists of 24 subunits, both light (L) and heavy (H) chain. Deficiencies in iron (*ferritin*), and thus haemoglobin, result in anaemia and a decrease in the number of haemoglobin carrying cells. Anaemia causes a wide array of symptoms ranging from tiredness to fatigue and shortness of breath, as the oxygen carrying capacity can be markedly reduced. Severe anaemia, therefore, is life-threatening. Furthermore, deficiencies have been associated with *premature labour* and increased maternal mortality (Scholl 2005). Excesses of this trace element can lead to haemochromatosis and are also seen in porphyrias. Furthermore, the ferritin protein is a remarkable protein that may become elevated in inflammatory responses, hence its use as an acute-phase reactant. It may therefore also indicate the severity or progress of a disease (Munoz, Villar et al. 2009).

1.4.3 Other micronutrients

Aside from the above mentioned micronutrients, recent reviews have shown that the majority of vitamins and minerals play a role in the regulation of pregnancy and birth outcomes. A summary of the essential minerals and vitamins, along with their recommended daily allowances (*RDAs*), and correlations to maternal and newborn anthropometry, can be seen in *Table 2*.

Table 2. Vitamins and minerals, RDA, and correlations with anthropometry.

Vitamins	RDA	Correlation with maternal and newborn anthropometry	Minerals	RDA	Correlation with maternal and newborn anthropometry
A	770 µg	Yes (Dancheck, Nussenblatt et al. 2005)	*Calcium*	1000 mg	Yes (Atallah, Hofmeyr et al. 2002)
D	5 µg	Yes (Scholl and Chen 2009)	*Copper*	1000 µg	Yes (Zadrozna, Gawlik et al. 2009)
E	15 mg	Yes (Dancheck, Nussenblatt et al. 2005)	*Chromium*	30 µg	No available data
K	90 µg	No available data	*Iodine*	220 µg	Yes (Alvarez-Pedrerol, Guxens et al. 2009)
B1	1.4 mg	Yes (Fawzi, Msamanga et al. 2007)	*Iron*	27 mg	Yes (Scholl and Hediger 1994)
B2	1.4 mg	Yes (Fawzi, Msamanga et al. 2007)	*Magnesium*	350 – 400 mg	Yes (Makrides and Crowther 2001)
B3	18 mg	Yes (Fawzi, Msamanga et al. 2007)	*Manganese*	2 mg	No available data
B5	6 mg	Yes (Baker, Thind et al. 1977)	*Molybdenum*	50 µg	No available data
B6	1.9 mg	No (Thaver, Saeed et al. 2006)	*Phosphorus*	700 mg	No available data
B12	2.6 µg	Yes (Baker, Thind et al. 1977)	*Selenium*	60 µg	Yes (Zadrozna, Gawlik et al. 2009)
Folate	400 – 600 µg	Yes (Tamura and Picciano 2006)	*Zinc*	11 mg	Yes (Akram, Akram et al. 2008)
Biotin	30 µg	No (Baker, Thind et al. 1977)			
C	85 mg	Yes (Fawzi, Msamanga et al. 2007)			

Legend to Table 2. An overview of key vitamins and minerals and their *recommended daily allowances* (*RDAs*) for women in the pregnancy age group (*15-40 years*), along with their correlations with maternal and infant anthropometry. The RDAs are based on North American recommendations (USDA 2009). All units are given in SI units.

1.5 Steroid hormones and their receptors

Steroid hormones play a key role in tissue regulation and functioning from the early stages of foetal life onwards. In pregnancy, rises in both oestrogen (o*estriol*) and progesterone have been well documented, along with increases in their receptors (Akram, Sahlin et al. 2010). The predominant source of oestrogen during pregnancy is the placenta. Oestradiol, which is the predominant form of oestrogen in non-pregnant women, is responsible for the appropriate function of the menstrual cycle, particularly ovulation. Furthermore, oestrogens play significant roles in the normal structure and functioning of growth processes. These include normal functioning of bone and mineral metabolism, muscle growth, uterine growth, and the appropriate functioning of the coagulation cascade, to name a few (Nelson and Bulun 2001). Oestrogen is further believed to act through specific growth factors, oestromedins, which act through the oestrogen receptor (*ER*). In the endometrium, IGF-I is believed to be one of the principal endometrial synthesised growth promoting oestromedins (Giudice, Dsupin et al. 1993). The oestrogen receptor is a nuclear hormone receptor and consists of 2 isoforms, *ERα and ERβ*, both of which are structurally similar, with an equal affinity to the oestrogens (Dechering, Boersma et al. 2000). The actions of oestrogens, the oestromedins, and their receptors are thus essential for the normal functioning and growth of the pregnant uterus and foeto-placental development.

A second key steroid hormone involved in the female reproductive cycle and pregnancy is progesterone. It is of particular importance in gestation and embryogenesis, whereby increases in progesterone levels are seen. Furthermore, decreases in levels of this hormone are believed to play a key role in the initiation of parturition (Graham and Clarke 1997). Progesterone acts through its receptor, which belongs to the nuclear subfamily group of receptors. The progesterone receptor (*PR*) consists of two clinically important isoforms, *PRA and PRB*, both of which are regulated by oestrogens (Mylonas, Makovitzky et al. 2009). Progesterone thus plays an important role in the normal development of the foetus and regulation of pregnancy, though is dependent on the presence of oestrogen.

The principal hormones in pregnancy and their relative concentrations

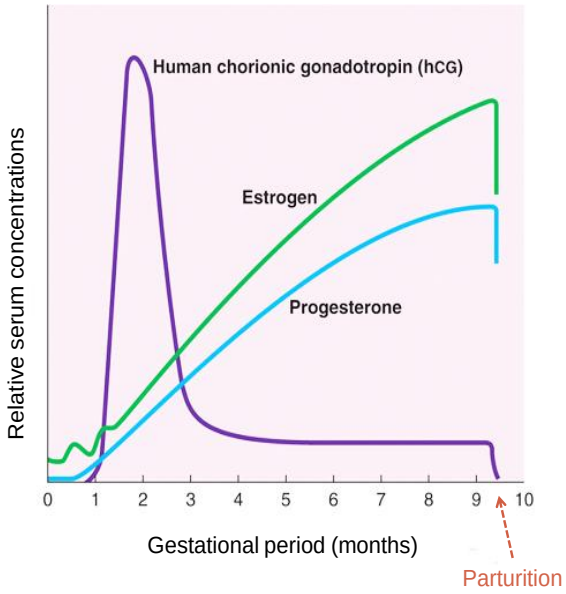


Figure 4. The relative concentrations of oestrogen and progesterone (*and human chorionic gonadotrophin, hCG*), from the last menstrual period (gestational age = 0) to partum. Oestrogen and progesterone are required for normal uterine and foeto-placental growth and decreased levels of these hormones, or antagonism of their receptors, will induce labour, as can be seen in the figure (modified: (Sherwood 2007)).

1.6 Apoptosis

Apoptosis is a controlled process of programmed cell death seen in the vast majority of cells in the body. One the process has begun, cells undergo various changes, including shrinking, nuclear and chromatin fragmentation and eventually fragmentation and engulfment by macrophages (Savill and Fadok 2000). Apoptosis is therefore essential in maintaining normal cell homeostasis and failures in these regulatory processes can result in pathological problems, such as is seen in cancer (Prindull 1995). Furthermore, apoptosis differs greatly from *necrosis*, which is associated with the uncontrolled release of cellular contents secondary to tissue injury, resulting in surrounding cellular damage and inflammation (Leist and Jaattela 2001).

Programmed cell death (*apoptosis*) is initiated by two major pathways; the intrinsic and extrinsic pathways (*Figure 5*). The latter is better known as the *death receptor pathway* as it activated by extra-cellular apoptotic ligands binding to the cell surface thus initiating the downstream cell-death cascade. Of particular importance in this pathway is the *initiator protein caspase 8*, which binds to an adaptor protein FAS-associated death domain (*FADD*) forming a death-inducing signalling complex (*DISC*). This then activates the *effecter caspases 3, 6, and 7* (Ashkenazi and Dixit 1998). In contrast to this, the intrinsic, or *mitochondrial*, pathway is triggered by intracellular signalling as a result of damaged DNA, oxidative stress, or growth factor deprivation. Mitochondrial damage results in the release of cytochrome C and activation of the *initiator caspase 9* which subsequently bind together, along with the apoptosis protease-activating factor 1 (*APAF-1*) to form an *'apoptosome'* (*intracellular DISC*) activating *caspase 3* (Shi 2002). Both pathways culminate in the packaging of cell content into apoptotic bodies with subsequent phagocytosis. These cell death processes must therefore be kept in check. At every stage of the apoptotic pathway there are various anti-apoptotic molecules and mechanisms delivering pro-survival signals. These include *NF-κB, AKT, BCL-2* and the *IAP* group of proteins (Vogelstein and Kinzler 2004). Apoptosis is thus essential for normal cell and tissue homeostasis and development and is regulated by both pro- and anti-apoptotic factors. The normal apoptotic zones in foetal growth can be seen in *Figure 6*.

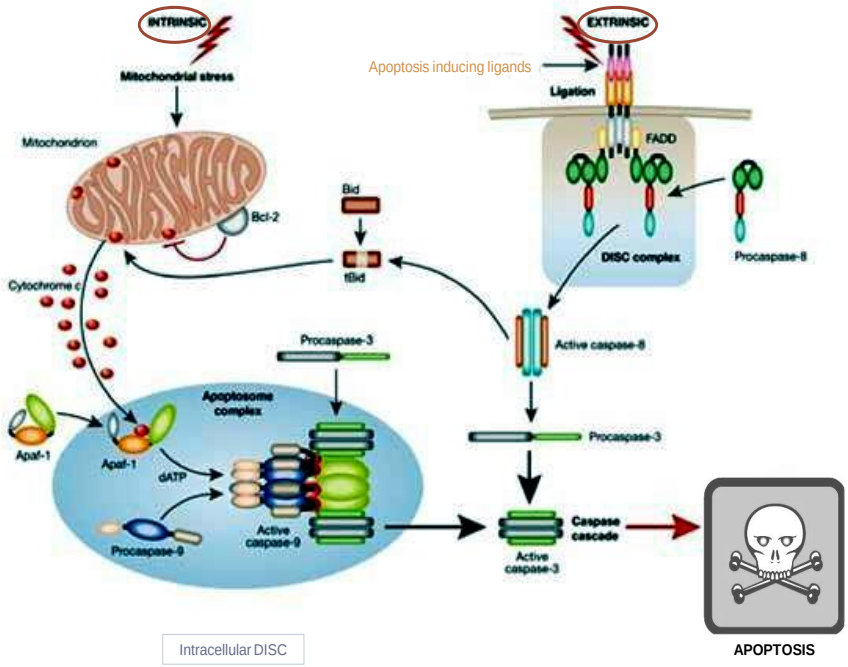


Figure 5: A diagram depicting the principal pathways of apoptosis: the intrinsic, *mitochondrial pathway*, and the extrinsic, *death receptor pathway*. (Modified: (MacFarlane and Williams 2004)). Abbreviations are: FAS-associated death domain protein, FADD; death-inducing signalling complex, DISC; B-cell lymphoma 2, BCL-2; apoptosis protease-activating factor 1, APAF-1; deoxyadenosine triphosphate, dATP.

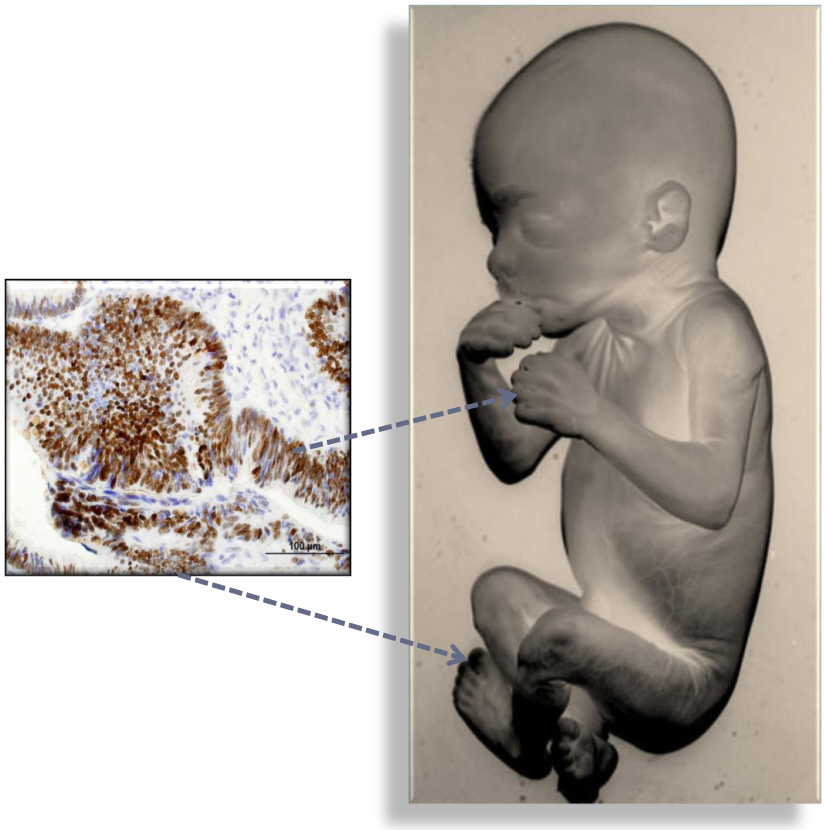


Figure 6. A picture of a foetus showing sites of natural apoptosis (*inlay, unpublished data*). The brown cells represent apoptosis and actively occur in areas of excessive growth, such as webbing between fingers and toes (Immunohistochemical staining slide from unpublished data; *40X magnification*; foetal image modified from *The Science Library*, http://www.sciencephoto.com/).

1.7 Anthropometry and Growth Restriction

Aside from the above mentioned factors affecting foetal growth, a principal non-genetic factor determining foetal size is *maternal constraint*. This phenomenon occurs as a result of maternal and utero-placental factors limiting foetal growth. This is particularly seen in cases of nutritional deprivation, whereby poor maternal nutrition results in decreased nutrition to the foeto-placental environment limiting growth. Maternal constraint can thus be divided into two major types. In the first, *maternal size* accounts for a physical constraint impeding foeto-placental growth (*supply driven constraint*). In the second, a discrepancy in the supply and demand of nutrients is seen, as is the case in twin pregnancies (*demand-driven constraint*) (Gluckman and Hanson 2004).

Maternal size may reflect nutritional state and certain maternal parameters may thus be used as indicators of a nutritionally deprived foeto-placental environment. These parameters include maternal height, weight (and weight gain during pregnancy), triceps skin-fold thickness, and placental weight, to name a few (Yajnik, Fall et al. 2003; Akram, Akram et al. 2008). These maternal anthropometrical parameters may therefore be measured as a potential screening for foeto-placental growth restriction during pregnancy. Furthermore, smaller maternal sizes may result in lower birth weights, with subsequent growth restriction of the child, perpetuating a continuous cycle of growth deficiency from generation to generation (*Figure 7*).

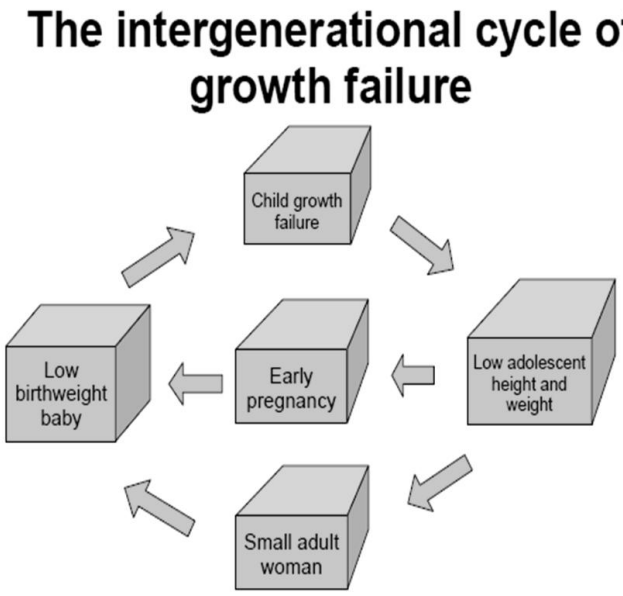


Figure 7. A schematic representing the vicious cycle of poor growth from one generation to the next.

2. AIMS

The general aim of this thesis was to study the relationships between maternal and neonatal anthropometry and biometrics, looking at maternal micronutrient deficiencies, namely zinc and iron, as well as the placental IGFs and IGFBP-1. The specific aims were as follows:

Paper I To correlate infant birth weight with maternal anthropometric and infant biometric data, including the mRNA expression of placental IGF-I and IGF-II at birth and the umbilical cord levels of the micronutrients zinc and iron, in the Pakistani population.

Paper II To correlate the placental protein levels of IGF-I and IGFBP-1 with mRNA expression of IGF-I and IGF-II (*paper I*), the micronutrients zinc and iron, and newborn and maternal anthropometry in the Pakistani population.

Paper III To correlate maternal and neonatal anthropometric variables with placental mRNA expression levels of oestrogen receptors (*ERα and ERβ*), the progesterone receptor (*PR*), and IGF-I, in the Swedish population.

Paper IV To ascertain levels of placental apoptosis, looking at p53, caspase 8, and caspase 3, in placentas of infants born small for gestational age (*SGA*) and appropriate for gestational age (*AGA*), comparing samples from two population groups.

3. MATERIALS & METHODS

The following methods were used and are briefly discussed in this section. Please refer to individual papers for more details of each method:

- *Clinical parameters*
- *RT-PCR (Reverse transcriptase-PCR)*
- *Solution hybridisation*
- *Western Immunoblotting*
- *Radioimmunoassay*
- *Ion chromatography*
- *TUNEL staining*
- *Immunohistochemical staining*
- *ELISA*

3.1 Population & samples

The sample cohort consisted of data from two populations. The first population cohort consisted of eighty-nine sampled pregnant women selected from rural field sites around Karachi, Pakistan. The second cohort was from placental samples from thirty-three women from the Swedish population at the Karolinska Hospital, Stockholm, Sweden. Ethical approval was obtained within both countries. Following informed consent, placental samples were taken and frozen, along with umbilical cord blood samples, immediately following delivery. The samples were frozen in liquid nitrogen and were stored for subsequent analysis. Samples were subsequently stored in RNA later®, at a temperature of *-70° Celsius*. Newborn and maternal anthropometry was also documented at the time of delivery. The following maternal anthropometric variables were considered: *height, weight, body mass index (BMI), mid-arm circumference, and triceps skin fold thickness*. Newborn anthropometry was documented as follows: *height, weight, BMI, occipito-frontal circumference, mid-arm circumference, mid-chest circumference, and triceps skin fold thickness*. All samples were subsequently plotted on population specific growth charts (*Figure 1*), and divided into groups based on

their birth weights and gestational ages. The *small for gestational age (SGA)* group consisted of neonates with less than or equal to the 10th percentile (≤ *10th percentile*), whilst the *large for gestational age (LGA)* group consisted of infants with birth weights greater than or equal to the 90th percentile (≥ *90th percentile; papers I and II*). All remaining infants were then assigned to the *appropriate for gestational age (AGA, or normal control)* group (> *10th percentile; < 90th percentile*). Comparisons between SGA and AGA groups were also done (*papers III and IV*). *Figure 8* depicts a simple and cost effective way of measuring anthropometric variables (*skin fold thickness*).

Figure 8. The use of skin fold callipers to measure the mid-triceps skin fold thickness as a measurement of body fat. Skin fold thickness is a key anthropometric variable.

3.2 RNA Expression

For the purpose of quantifying expression levels of the growth factors, IGF-I and IGF-II, mRNA expression was studied in the placental samples. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, Venlo, Holland), according to the manufacturer's recommended protocol. RNA quantification was done using absorbance photospectrometery at an absorbance of 260nm (HITACHI – U200 spectrophotometer, Hitachi, Hialeah, FL). Further analysis was done using *reverse-transcriptase polymerase chain reaction (RT-PCR)*, as described below.

3.2.1 RT-PCR

Reverse transcriptase-PCR was used for quantifying amounts of ribonucleic acid (*RNA*). A detailed description can be seen in *paper I*. In brief, cDNA was synthesized from total RNA using the Superscript cDNA kit (Invitrogen Inc., U.K.). Primer pairs specific for human IGF-I and IGF-II, and the internal standards, β-actin and GAPDH, were used (Genset Oligos, Paris, France; *Table 3*). PCR was performed using the Gene Amp PCR 2400 system (PerkinElmer, Palo Alto, CA) in a total volume of *50µl*. The PCR products were characterized by electrophoresis on *2%* agarose gels stained with ethidium bromide. Optical densities of the resulting bands were recorded using the EDAS 120 Kodak Gel documentation system (Eastman Kodak Co., Rochester, NY) and quantified using *Image J* (NIH, USA; http://rsb.info.nih.gov/ij/).

Table 3. Primer sequences.

Gene	Oligonucleotide sequence	Product Size (bp)
β-actin	5' primer: CAC ACT GTG CCC ATC TAC GA 3' primer: GTT TCA TGG ATG CCA CAG GA	349
GAPDH	5' primer: CAA TGA CCC CTT CAT TGA CC 3' primer: CCT GCT TCA CCA CCT TCT TG	594
IGF-I	5' primer: AAA TCA GCA GTC TTC CAA CC 3' primer: CTT CTG GGT CTT GGG CAT GT	395
IGF-II	5' primer: CTG GAG ACG TAC TGT GCT AC 3' primer: GGT GTT TAA AGC CAA TCG AT	547

Legend to Table 3. Primers used for amplification of β-actin, GAPDH, insulin-like growth factors (*IGF-I, IGF-II*), in reverse transcriptase-polymerase chain reaction (*RT-PCR*).

3.2.2 Solution Hybridisation

Solution Hybridisation was conducted as previously described (*paper III*; (Sahlin 1995)). In brief, tissues were thawed and homogenised. DNA extraction was done using phenol-chloroform and DNA content was determined by a fluorometric assay at the wavelength 458 nm with Hoechst Dye 33258 (Labarca and Paigen 1980).

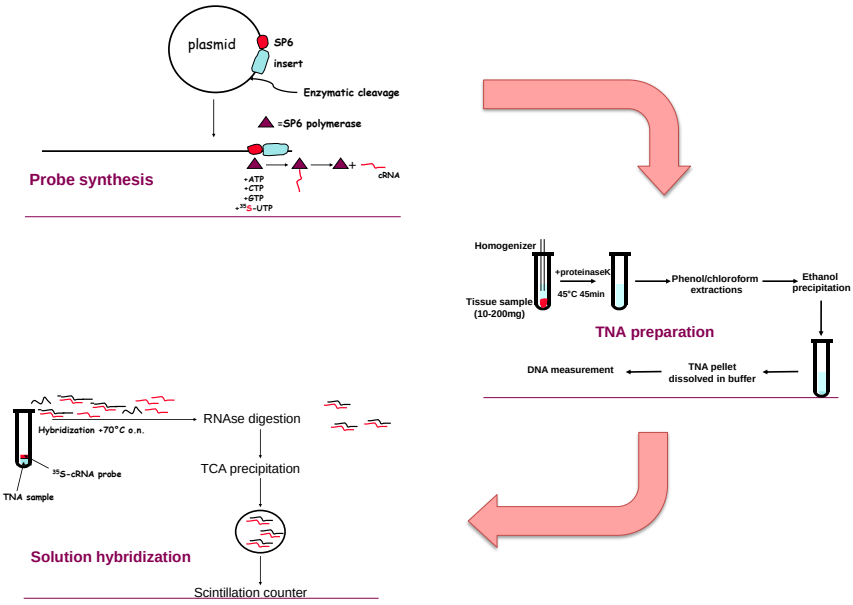


Figure 9. A brief overview of the steps of solution hybridisation analysis: *probe synthesis*, *TNA preparation*, and *solution hybridisation*. (Modified from Sahlin, L. 2010). Abbreviations are: adenosine, cytidine, guanosine triphosphate, ATP, CTP & GTP, respectively; ribonucleic acid, RNA; deoxyribonucleic acid, DNA; total nucleic acids, TNA; complementary RNA, cRNA; trichloroacetic acid, TCA.

3.3 Protein analysis

The largest of the proteins studied in this thesis was that of IGFBP-1 (*30 kDa*). To analyse levels of this binding protein in our placental samples, protein extraction was first carried out on ice, as to ensure that the cellular proteins did not denature. The concentration of purified protein from each sample was then quantified using the *Bradford assay* (Bradford 1976). Specific analytical methods are described below. *ELISA analysis* has been explained with apoptosis (below).

3.3.1 Western blot

Frozen sections of placental decidua were thawed on ice for subsequent protein extraction. In brief, protein was extracted from tissues using gentle mortaring and micro centrifugation. The supernatatant was subsequently separated by SDS-PAGE (*4 -12%* Bis-Tris Gels; Invitrogen, U.K.) for Western blot analysis. Western blot analysis was carried out by taking *20µg* of protein and transferring it onto polyvinyldine fluoride (*PVDF*) membranes (BIORAD, CA) in a Hoefer Semi-Dry Transphor unit at *200mA* (Amersham Biosciences, Sweden). Membranes were then blocked in *3%* non-fat milk in Tris-buffered saline containing *0.1%* Tween20 (*TBS-T*) and *10ng/ml* of a primary mouse anti-human antibody (IDS Ltd., U.K.; at 1:1000 dilution) overnight at *4°C*. Thereafter, membranes were washed with TBS-T and incubated with secondary antibody (donkey anti-mouse antibody at a 1:10000 dilution, IDS Ltd., U.K.) for 90 min at room temperature, followed by washing with TBS-T and water. Finally, the membranes were exposed to enhanced chemiluminescence reagents (Amersham Biosciences, Sweden) for 1 min at *20°C*. Band densities were analysed with *ImageJ* (NIH, USA; http://rsb.info.nih.gov/ij/).

3.3.2 Radioimmunoassay (RIA)

The RIA was carried out to assess differences in levels of *all* phosphorylated, and the *lesser and non*-phosphorylated isoforms of IGFBP-1. Serum samples (*50 µl*) were assayed in triplicates. Human IGFBP-1 (Gropep, Adelaide, Australia) was labelled with ^{125}I using the Chloramine-T method, purified on a PD-10 column (Amersham Biosciences, Sweden) and then diluted in phosphate buffered saline (*0.01 M, pH 7.4*) containing *0.5%* bovine serum albumin (PBS-BSA), was used as a tracer. Standards (*3- 400 ng/ml* human IGFBP-1) in PBS-BSA and placental protein samples (*50 µl*) were incubated overnight (*4°C*) with *100 µl* α-hIGFBP-1 (*20 ng/ml*) (*Mab 6303 and 6305*, Medixbiochemica, Finland) and *100 µl* ^{125}I – IGFBP-1 tracer (*10 000 CPM*). The *Mab 6303* recognizes all phosphorylated isoforms of the protein, whereas the *Mab 6305* is specific to the less and non-phosphorylated (*npIGFBP-1*) isoforms. The next day, *50 µl* of secondary antibody (α-mouse/ rat; Sac-Cel #4, IDS Ltd., U.K.) was added and samples were incubated for 30 min. Water (*1 ml*) was then added and the samples were centrifuged (*3000 x g, 4°C*) for

15 min and the supernatant was aspirated. Radioactivity in the precipitant was measured using a γ-counter (1470 Wizard, PerkinElmer, Sweden). The detection limit was *6 ng/ ml* and the intra- and inter-assay variance were *5.6 and 11.8%*, respectively (sensitivity of *1.0 ng/ ml*).

3.4 Ion Chromatography

Ion quantification and analysis was carried out for both zinc and iron (*papers I and II*). Analysis was carried out at the Aga Khan Hospital laboratory, Karachi, Pakistan, using standardised protocols (Beckman Synchron CX7 autoanalyser, Beckman Coulter, Ca, USA).

3.5 Cell Death Analysis

There are multiple methods one can utilise to analyse apoptosis. In this thesis, we focused principally on TUNEL staining, immunohistochemical staining, and cell death ELISA analysis (described as follows).

3.5.1 TUNEL

To examine the up-regulation of p53, Caspase 3, and Caspase 8, we used placental samples (as previously described). Paraffin embedded slides were stained with (antibody). The slides were counterstained with DAPI for 15 min, and the resulting fluorescent signals were detected by fluorescence microscopy. Ten separate areas in each sample were then independently counted for positive and negative cells, using *40X* microscopy.

3.5.2 Immunohistochemical staining

Immunohistochemical staining was done using the BOND MAX probe system (Leica Microsystems, Germany). The specific protocol can be seen in *paper IV*. In brief, peroxidase activity was inhibited using methanol and *1.5%* hydrogen peroxide. Following antigen retrieval (citrate-based buffer, *pH 5.9-6.1, at 94°C - 96°C*), the primary antibody was run for 30 minutes. Details of antibodies are as

follows: p53 DO-7 (Dako Sweden AB, 1:500), caspase 8 AB61755 (Abcam PLC, UK, 1:500), caspase 3 JHM62 (Novocastra, UK, 1:50). Post primary antibodies were then used as per the manufacturer's protocol (Dako Sweden AB). All slides were then developed with *3'3*-diaminobenzidine (*DAB*) and counterstained with haematoxylin, and analysed using *40X* magnification microscopy, as with TUNEL staining (above).

3.5.3 ELISA

Enzyme-linked immunosorbent assay (*ELISA*), was used to assess both IGF-I protein concentrations (*paper II*), and levels of apoptosis (*paper IV*). The methodologies employed were in accordance to the respective manufacturers' protocols (details in *papers II and IV*). In brief, protein was extracted from thawed tissue (*on ice*). All reagents and steps were according to the manufacturers' protocols. All samples were analyzed in triplicates and experiments were repeated three times. Results of the IGF-I analysis are expressed as a quantified amount based on the standard curve (*ng/ml*; Mediagnost, Reutlingen, Germany). Results of the programmed cell death analysis are expressed as levels of apoptotic activity (*enrichment factor)*, calculated as an optical absorbance ratio of the absorbance (10^{-3}) of the dead/dying cells divided by the absorbance from the corresponding negative control (based on the manufacturer's protocol (*Cell Death Detection Kit ELISAPLUS*, Roche Diagnostics Scandinavia AB).

4. RESULTS & DISCUSSION

4.1 Maternal anthropometry and infant birth parameters

To gain a better understanding of population demographics and the associations of maternal and newborn anthropometry, we correlated several maternal and infant anthropometric variables: *height, weight, BMI, triceps skin fold thickness, mid-arm circumference, mid-chest circumference, and occipito-frontal circumference.* These correlations were compared within the respective groups, *SGA, AGA, and LGA*, based on birth weights, as mentioned previously. Out of the 89 sampled cases (Pakistani population) the mean birth weight of the sample cohort was 2.79kg; the prevalence of SGA being 13.4% (≤ *10^{th} percentile*), and LGA being 23.6% (≥ *90^{th} percentile*). We have shown significant differences in maternal and newborn anthropometry comparing the growth restricted (*SGA*) and LGA groups. We have also shown significant correlations of newborn birth weight and maternal anthropometric variables, namely, age at delivery, height, weight, and mid-arm circumference ($p < 0.05$, *paper I*). As mentioned in the paper, we associate these positive correlations to the poorly defined process of *maternal constraint*. In brief, maternal constraint is a process whereby foeto-placental growth is physically limited by maternal size. This may be a natural protective mechanism, as smaller mothers would have problems delivering larger babies due to decreased pelvic size. Without these constraints, the risks of immediate neonatal morbidity and mortality would otherwise increase, as is seen in babies born post term and with large birth weights (Olesen, Westergaard et al. 2003). Furthermore, maternal constraint may aid in decreasing immediate intra-uterine complications, controlling foetal growth and nutrient distribution, as can be seen in multiple pregnancies (Gluckman and Hanson 2004). This process, however, has long term complications, as can be seen with increased infant and childhood morbidity and mortality in low birth weight infants (McCormick 1985).

These differences in size of the mothers seen in our subgroups may also, in part, be due to genetics, as smaller parents are likely to carry genes encoding height and size outcomes of their offspring, resulting in smaller children (Visscher 2008). This is further seen in obese people, as this too, is hereditary, reflecting the

importance of genetics on growth and size outcomes (Bouchard and Perusse 1988). The majority of these genetic influences, however, come in to play in the later stages of life, beyond the neonatal period. There are, however, other genetic factors that influence birth weight outcomes, such as the appropriate functioning of the IGF-axis (discussed below).

Our results suggest that newborn anthropometry may reflect maternal anthropometry, and vice versa. This is interesting, as maternal anthropometry is the simplest clinical tool one can employ. Maternal body fat composition, such as triceps skin fold thickness, amongst other anthropometrical variables, may therefore be used as simple and cost effective tools to screen for growth restricted pregnancies, particularly in impoverished countries lacking resources for more advanced radiological diagnostic techniques. Health care workers in primary care centres in rural areas may thus be trained to monitor and screen pregnancies in their areas using these simple tools. This could, in turn, allow for the timely identification and referral of mothers with 'lower' body fat composition and anthropometry (higher risk for low birth weight). It is therefore of utmost importance that local pregnancy growth charts with the various anthropometrical variables be established and distributed to all health care centres and workers. The basic principles of maternal constraint and nutrition can be seen more simply in *Figure 10*. The significant correlations of maternal anthropometry and newborn birth weight can be seen in *Figure 11*.

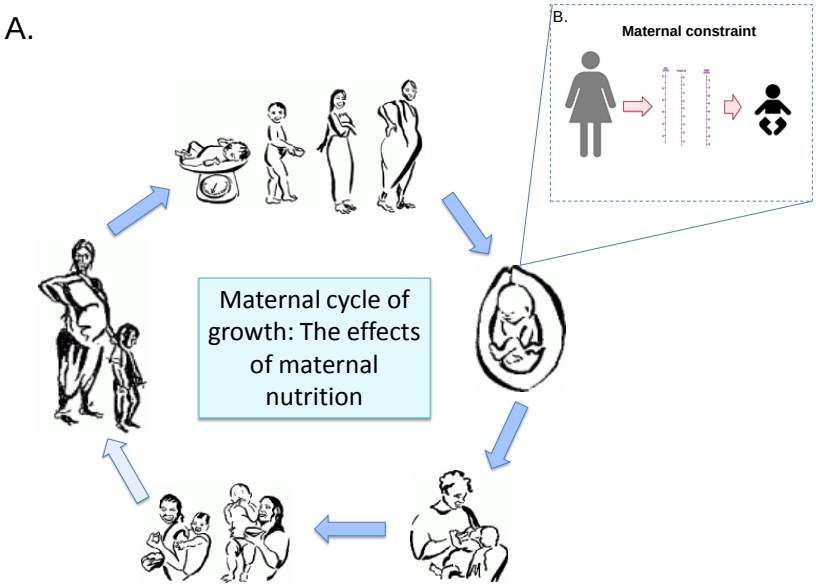


Figure 10a. A simplified schematic representing maternal size and nutrition influencing growth. Figure 10b. (*Inlay*) maternal anthropometry influencing newborn anthropometry (*maternal constraint*).

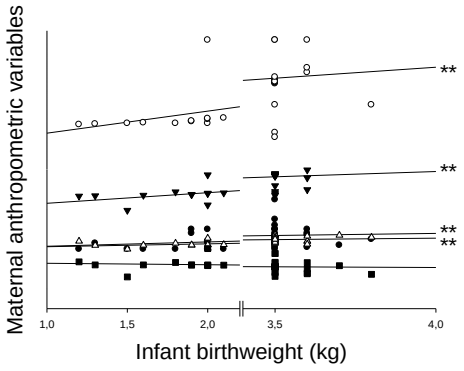


Figure 11. Infant birth weight correlated with maternal anthropometric variables. (maternal age ●; height ○; mid-arm circumference Δ; triceps skin-fold thickness ■; weight ▼). ***p<0.01. (Figure reproduced with permission from paper I, (Akram, Akram et al. 2008)).*

4.2 Anthropometry and the growth hormones, IGF-I & IGF-II

As mentioned above, there are multiple genetic, nutritional, and environmental factors influencing growth. Genetic deficiencies in insulin metabolism can lead to profoundly decreased birth weight outcomes (Weedon, Frayling et al. 2005). This can be seen more markedly with deficiencies of the IGF-I gene, which results in both prenatal and long term growth deficiencies, as well as increased morbidity (Vaessen 2002). Furthermore, decreased levels of IGF-I and IGF-II have been associated with decreased foeto-placental growth, and newborn size and weight (Fowden 2003). Our results of significantly lower protein levels of IGF-I, as well as decreased mRNA expression of IGF-I and IGF-II, in the SGA groups support these previous findings of the IGF-axis influencing birth anthropometry (*paper I*). *Figure 12* represents the multiple regression correlation of newborn birth weight and maternal height, placental IGF-I mRNA expression, and birth height. The significant regression correlation emphasises the association of IGF-I and newborn anthropometry. More specifically, a significant multiple regression correlation was noted between birth weight and maternal height ($p < 0.01$). Furthermore, our results showed a significant positive correlation of maternal body mass index (*BMI*) and infant birth weight ($r = 0.32$, $p < 0.01$).

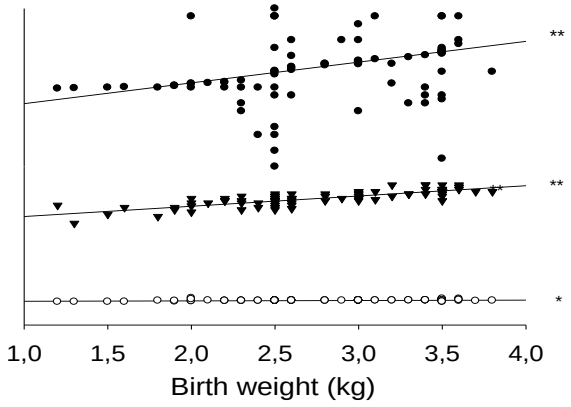


Figure 12. Multiple regression analysis of birth weight with biometric variables: maternal height (●; ***p<0.01*); IGF-I (○; **p<0.05*); infant height (▼; ***p<0.01*). *Unpublished data.*

We have also shown significant associations of maternal and newborn anthropometry with placental protein levels of IGF-I, IGFBP-1, and mRNA expression levels of IGF-I and IGF-II ($p < 0.05$, *papers I and II*). The most signficant of these maternal parameters are height and triceps skin fold thickness. These represent overall size and body fat composition. As we have discussed above, maternal size may influence birth weight, possibly through maternal constraint or genetic factors. Maternal size may thus influence foetal size and birth outcomes through the IGF-axis, as both mRNA expression, and protein levels of components of the IGF-axis appear to be significantly associated with maternal and newborn anthropometry. The mechanism of maternal regulation of the IGF-axis is, however, still unknown. A possible mechanism may be through maternal body fat regulation of the insulin axis, whereby increasing maternal size (and thus body fat) result in increasing insulin resistance and stimulation of the IGF-I axis (Evans, Hoffmann et al. 1984; Cnop, Havel et al. 2003) This mechanism is evident in the development of gestational diabetes, with the affected neonates being born large for their gestational ages (Tamas and Kerenyi 2001).

We have thus shown that maternal and newborn anthropometry appear to be associated with the IGF-axis, suggesting that maternal anthropometry may possibly regulate foetal-placental growth through this growth hormone axis. These results may explain the mechanisms behind maternal constraint and re-emphasise the importance of establishing maternal growth curves to aid in the potential diagnosis of foetal growth restriction through the regular measurement of maternal anthropometry. Furthermore, circulating levels of the placento-foetal IGF-axis (through maternal blood samples), may also support and aid in the timely diagnosis of foetal growth restriction.

The relative mRNA expression levels of IGF-I and IGF-II, in the placenta, can be seen in *Figure 13*. Signficant differences were seen whilst comparing the SGA and LGA groups ($p < 0.05$).

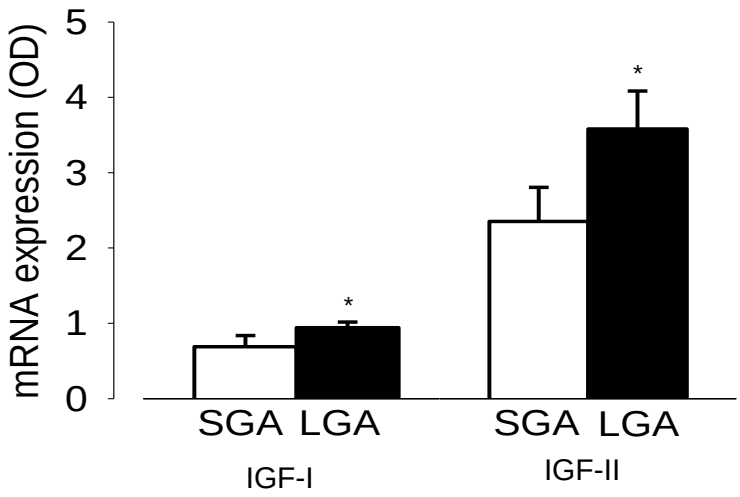


Figure 13. Placental expression of IGF-I and IGF-II mRNA. **p<0.05*. Abbreviations are: SGA, small for gestational age; LGA, large for gestational age; IGF, insulin-like growth factor; OD optical density ratio. *(Figure reproduced with permission from paper I, (Akram, Akram et al. 2008)).*

4.3 Anthropometry and IGFBP-1

To assess the differences in placental IGFBP-1 levels, comparing the SGA and LGA groups, we employed radioimmunoassay (*RIA*) techniques to quantify protein levels, along with their respective isoforms (*Figure 14a*). These results have been represented in comparison to total IGFBP-1 levels, which were quantified using Western immunoblotting techniques, (*WIB; Figure 14b*). IGFBP-1 levels (*WIB*) were significantly higher in the SGA group as compared to the LGA group ($p < 0.01$; Student's t-test). On comparing the radiometric levels of total IGFBP-1 (*Mab 6303, RIA*) and npIGFBP-1 (*Mab 6305, RIA*), within the SGA and LGA groups, no significant differences were seen between the two groups. In *paper II* we have shown that total placental IGFBP-1 levels, using *WIB*, were significantly higher in the SGA group, as compared to the LGA group, showing a negative correlation between the binding protein levels and birth weight. A similar negative correlation was seen between IGFBP-1 levels, using RIA, and birth weight, though this was not statistically significant. Though we have not shown any differences in the isoforms of IGFBP-1 comparing between the groups, it is still possible that the phosphorylation state of IGFBP-1 may play a key role in the pathogenesis of growth restriction, as has been previously suggested (Wang and Chard 1992; Giudice, de Zegher et al. 1995; Forbes and Westwood 2008).

Furthermore, our results of IGFBP-1 correlating significantly with maternal anthropometry, in particular, maternal height and triceps skin fold thickness (*paper II*), suggest that this binding protein may also be regulated by 'maternal constraining' factors. Higher levels of the binding protein were also noted in the SGA group, which, as discussed in the paper, may indicate that IGFBP-1 has a dual functional role. *Firstly*, IGFBP-1 may directly stimulate tissue growth in response to growth restriction, which has been seen in other tissues (Wheatcroft and Kearney 2009). *Secondly*, levels of the binding protein may increase in response to nutritional and growth deficits to 'mop up' free IGFs allowing for essential nutrients to be delivered to the brain and other vital organs, which, in the presence of excessive IGF, would otherwise be shunted to muscle tissue. IGFBP-1 may therefore play an important role in glucoregulation and insulin metabolism.

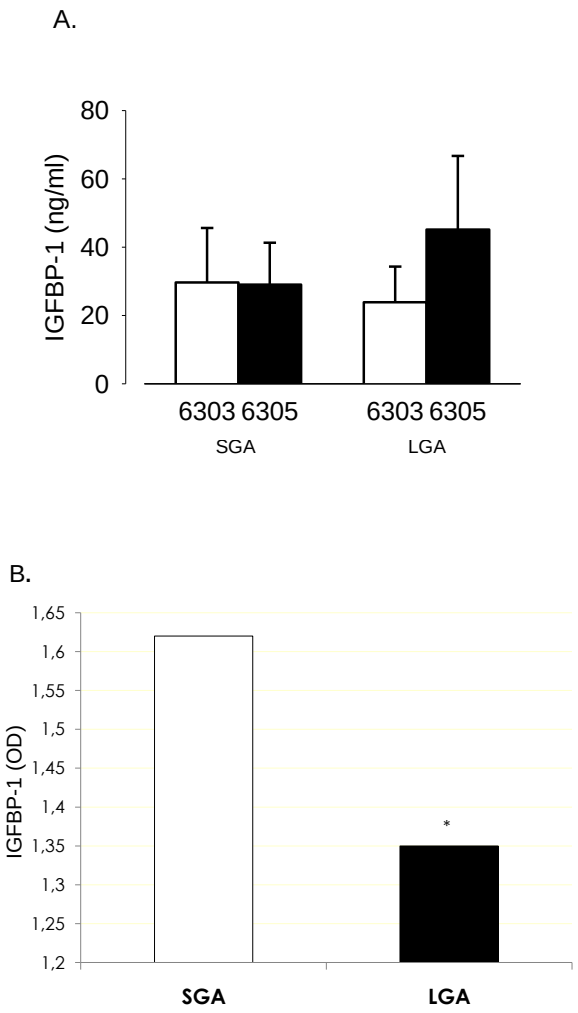


Figure 14a. IGFBP-1 RIA results comparing the phosphorylated, and lesser and non-phosphorylated isoforms (*6303 and 6305, respectively*) in the SGA and LGA groups (□ SGA; ■ LGA). Figure 14b. IGFBP-1 (WIB) comparing SGA and LGA groups; optical density (OD; **p<0.01).* Abbreviations are: SGA, small for gestational age; LGA, large for gestational age; IGFBP-1, insulin-like growth factor binding protein-1. *Unpublished data.*

4.4 Growth hormones and micronutrients

Micronutrient deficiencies have been previously associated with adverse pregnancy outcomes, as discussed previously. This has been shown specifically in the cases of iron, zinc, and vitamin A, to name a few. We have described key micronutrients and their effects on pregnancy outcomes in table 2 (above). In this thesis, we looked at two key micronutrients, iron and zinc, and their associations with the IGF-axis and anthropometry (*papers I and II*). Multiple micronutrient deficiencies and relationship to pregnancy outcomes have been published previously (Haider and Bhutta 2006). Our results showed significantly higher cord blood levels of zinc and iron in the LGA group ($p < 0.05$), with all levels being in normal ranges. These results re-emphasise the importance of these micronutrients and suggest their role in foeto-placental growth and pregnancy outcomes.

Whilst looking at only two common micronutrient deficiencies, we appreciate the short-fall this may represent, as single-micronutrient supplementation has not been shown to benefit pregnancy outcomes. Furthermore, isolated mineral and trace deficiencies have not been found either, suggesting that multiple micronutrient deficiencies are a result of widespread poor nutrition, as is the case in many developing countries (Kontic-Vucinic, Sulovic et al. 2006). Multiple micronutrient supplementation, particularly in areas of poverty and poor dietary intake, is therefore paramount in improving pregnancy and growth outcomes, in an attempt to break the viscious cycle of growth restriction seen from generation to generation (Hindle, Gitau et al. 2006; Katz, Christian et al. 2006).

4.5 Sex steroid hormone receptors and IGF-I

In an attempt to further assess the regulation of IGF-I, we looked at placental mRNA expression of IGF-I and the sex steroid receptors, oestrogen receptor (*ERα and ERβ*) and progesterone receptor (*PR*). The study population included placental samples and biometric data from *33* Swedish patients (*paper III*). The results of this paper indicate lower IGF-I mRNA expression in the growth restricted group (*GR; SGA*) as compared to the control group of health pregnancies (*appropriate for gestational age, AGA*). These results are similar to those we have demonstrated earlier, looking at placental samples in the Pakistani population, and re-emphasise the importance of IGF-I in foeto-placental growth and pregnancy outcomes. In this data series, our SGA group consisted of placenta from pregnancies suffering from mild and severe pre-eclampsia, as well as intrauterine growth restriction (*IUGR*) not related to this clinical syndromes. Growth restriction with or without pre-eclampsia appears to equally affect IGF-I levels, with significantly lower levels as compared to the control group, suggesting a common pathogenesis behind growth restriction and decreased birth weight. These differences can be seen more clearly in *Figure 15*. It thus appears that, whether the cause is genetic, enivornmental, or nutritional, the IGF-axis may be the pincipal regulator of foeto-placental growth. However, the exact mechanisms behind the regulation of this growth hormone axis are still unclear.

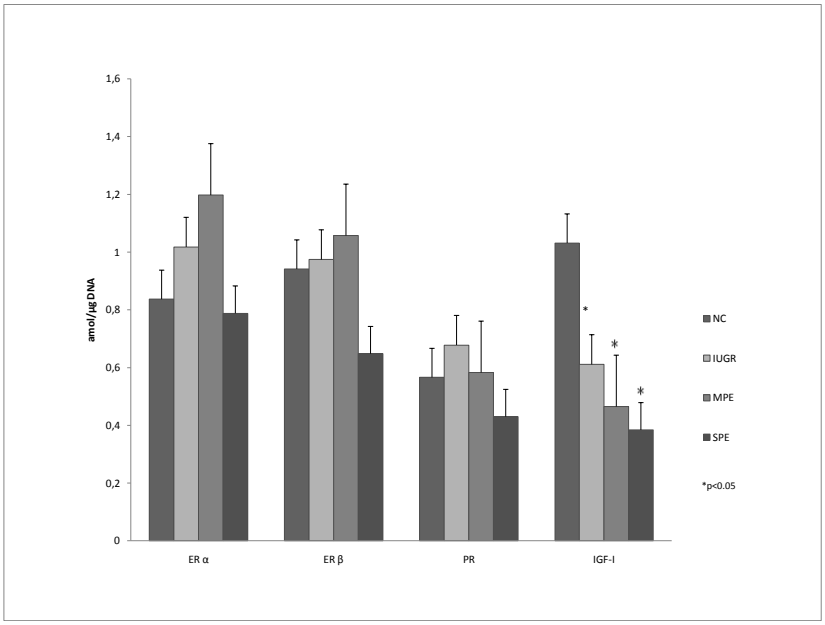


Figure 15: The relative mRNA expression of placental oestrogen receptors (*ERα and ERβ*), progesterone receptor (*PR*), and insulin-like growth factor-I (*IGF-I*). The graph compares the four groups, normal control (*NC/AGA, n=14*), intrauterine growth restriction (*IUGR, n=9*), mild preeclampsia with IUGR (*MPE, n=5*), and severe preeclampsia with IUGR (*SPE, n=5*). All bars are given with the standard error of the mean (SEM). The level of significance was set as *p<0.05* (ANOVA with post-hoc Waller-Duncan correction). *(Reproduced with permission from paper III, (Akram, Sahlin et al. 2010))*.

We have shown significant positive correlations of PR and IGF-I mRNA expression levels ($p < 0.05$). This association may suggest a common stimulatory and regulatory pathway, whereby changes in placental progesterone levels, and thus receptor expression levels, may affect IGF expression. Our results support previous findings of PR down-regulation of IGFBP-1, with subsequent upregulation of IGF-I, emphasising the growth promoting effects of this steroid hormone receptor through the regulation of the IGF-axis (Gao and Tseng 1997). Our results of PR mRNA expression correlating with infant anthropometry and gestational age, in the SGA group ($p < 0.05$), may also suggest that the progesterone receptor may be associated with birth weight outcomes.

Though we did not find any direct correlations of oestrogen receptor mRNA expression and IGF-I, we have shown that ERα positively correlated with PR expression, in the AGA group. We have also shown signficant correlations of ERα and maternal BMI at delivery (*SGA group*). The oestrogen hormone, and it's receptors, have been shown to play an important role in stimulating trophoblast vascular endothelial growth factor (*VEGF*) expression and, consequently, villous placental angiogenesis, to promote foetal growth and development in early primate pregnancy (Albrecht, Robb et al. 2004). We have not looked in to the expression of VEGF, but suggest that the ER may also regulate other placental growth factors (oestromedins), possibly through the regulation of PR expression, thereby influencing foeto-placental growth. This is further supported by our results of ERβ correlating positively with maternal delivery weight and gestational age, in the AGA group, suggesting that maternal size (and the influence of maternal constraint), may regulate placental growth through levels of oestrogen hormones and thus the levels of the hormone receptors. We further suggest that these hormone receptors (*ER and PR*) may influence their growth promoting effects through regulation of IGF-I.

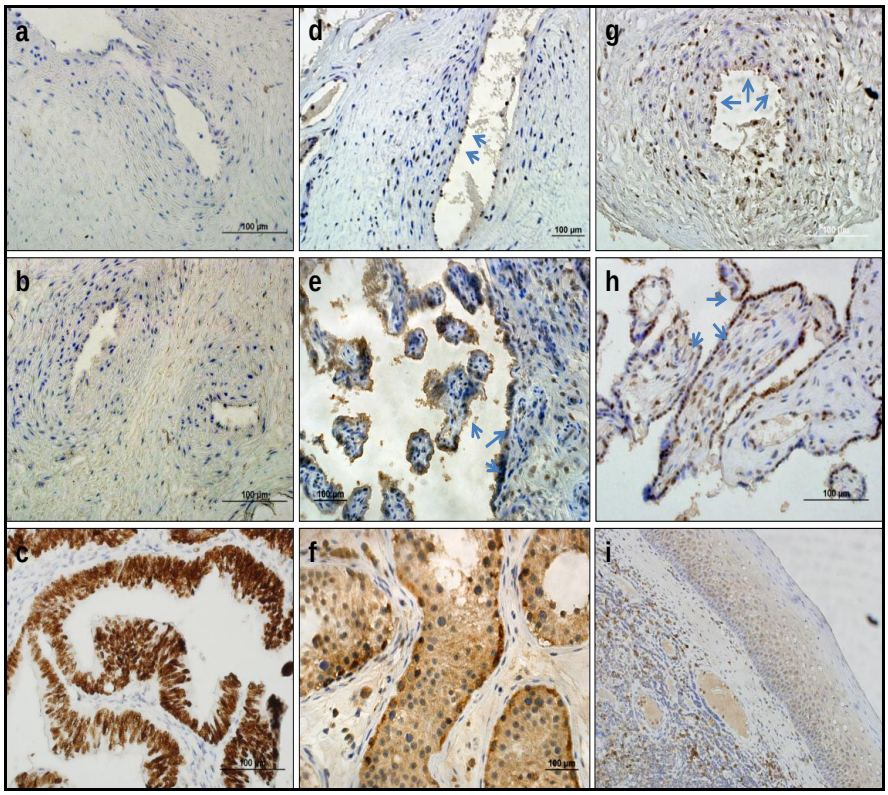
4.6 Apoptosis and IGF-I

As mentioned above, apoptosis is essential for normal growth and tissue regulation, with a fine balance in growth promoting and growth suppressing factors. In this study (*paper IV*), we looked at the levels of apoptotic activity in the placenta of SGA and AGA babies, by means of immunohistochemical staining and cell death ELISA. We have shown a higher apoptotic index, for caspase 8 and caspase 3, in the Pakistani samples, as compared to the Swedish samples *($p<0.01$)*. We have also shown a tendency to increased apoptotic activity in the placenta of SGA babies as compared to those born AGA, though these differences are not statistically significant. *Figure 16* represents the immunohistochemical staining of p53, caspase 8, and caspase 3, comparing the SGA and AGA groups in both the Pakistani and Swedish populations. These results, along with those of the cell death ELISA, can be more clearly seen in *Figures 17* and *18*. Cell death ELISA analysis showed a greater level of apoptotic activity in placenta from the Pakistani population as compared to the Swedish groups (*$p<0.05$*) along with increased apoptotic activity in the SGA groups (*$p<0.05$*). Our results support those previously seen, whereby higher levels of placental apoptosis were associated with unfavourable foetal growth and birth weight outcomes (Smith, Baker et al. 1997). We did not see any differences in preliminary TUNEL staining, and these results have therefore not been discussed here. Our results suggest that the increased apoptotic activity in the placenta of the Pakistani population may be associated with decreased foeto-placental growth, particularly in babies born SGA. Furthermore, the negative results of the p53 staining, along with the positive results of the caspase 8 staining, suggest that the key pathway of placental apoptosis is the *extrinsic pathway*, through activation of a cell-surface tyrosine kinase receptor (*death receptor*), with downstream activation of caspase 8 and activation of the apoptotic cascade (Ashkenazi and Dixit 1998).

The IGF-I axis has been shown to have growth promoting effects, not only in foeto-placental growth, but also in other tissues (Mullis 2011). IGF-I has therefore been suggested as having an *anti-apoptotic* role, whereby down regulation of this growth factor may lead to activation of the apoptotic cascade (Feng 2010). Our results of decreased IGF-I, IGF-I mRNA expression, and increase apoptosis in the

SGA groups in both populations support these findings. These results further suggest that this IGF-regulation of apoptosis may be a possible mechanism for placental and foetal growth restriction.

16A.



16B.

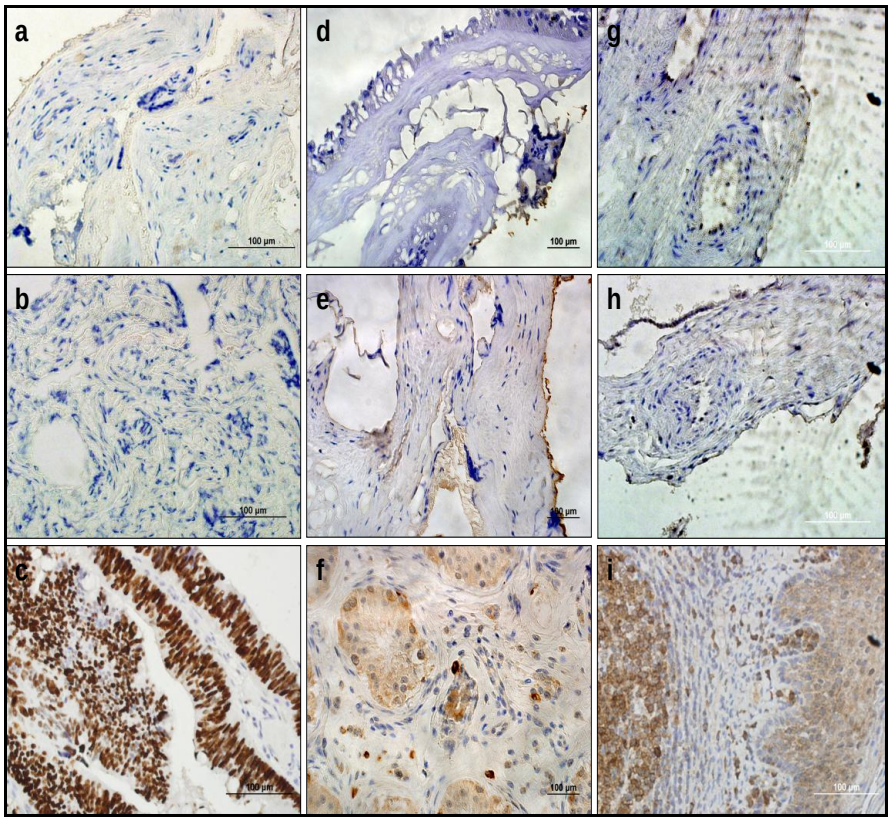


Figure 16. Immunohistochemical staining showing *positive (brown)* and *negative (blue)* cells for p53, caspase 8 and caspase 3, comparing small and appropriate for gestational age placental samples (*SGA and AGA, respectively*). Figure **16a**: *Pakistani population* samples. (*a)* p53 AGA group; (*b)* p53 SGA group; (*c)* p53 positive control; (*d)* Caspase 8 AGA group; (*e)* Caspase 8 SGA group; (*f)* Caspase 8 positive control; *(g)* Caspase 3 AGA group; *(h)* Caspase 3 SGA group; *(i)* Caspase 3 positive control. Figure **16b**: *Swedish population* samples. (*a)* p53 AGA group; (*b)* p53 SGA group; (*c)* p53 positive control; (*d)* Caspase 8 AGA group; (*e)* Caspase 8 SGA group; (*f)* Caspase 8 positive control; *(g)* Caspase 3 AGA group; *(h)* Caspase 3 SGA group; *(i)* Caspase 3 positive control. As can be seen in the figures, p53 stained negatively for both populations. Furthermore, relatively more positive cells were seen in the SGA group as compared to the AGA group for both caspase 8 and caspase 3. The Pakistani placental samples showed a higher apoptotic index as compared to the Swedish placental samples (**$p<0.01$). *Unpublished data (paper IV).*

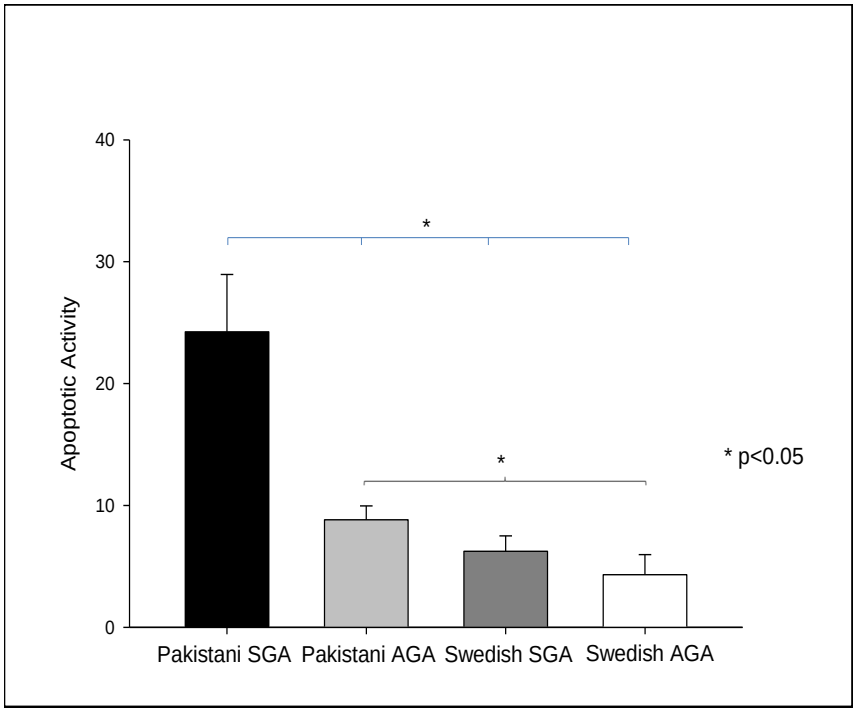


Figure 17. A comparison between apoptotic activities (enrichment factor) in the placentas of different groups, as assessed by cell death ELISA. The histogram shows apoptotic activity in the SGA (*Pakistani n = 12; Swedish n = 7*) and AGA (*Pakistani n = 12; Swedish n = 5*) groups, comparing between populations (*$p < 0.05$). All charts represent means with their standard errors (SEM). Abbreviations are: SGA, small for gestational age; LGA, large for gestational age. *Unpublished data (paper IV).*

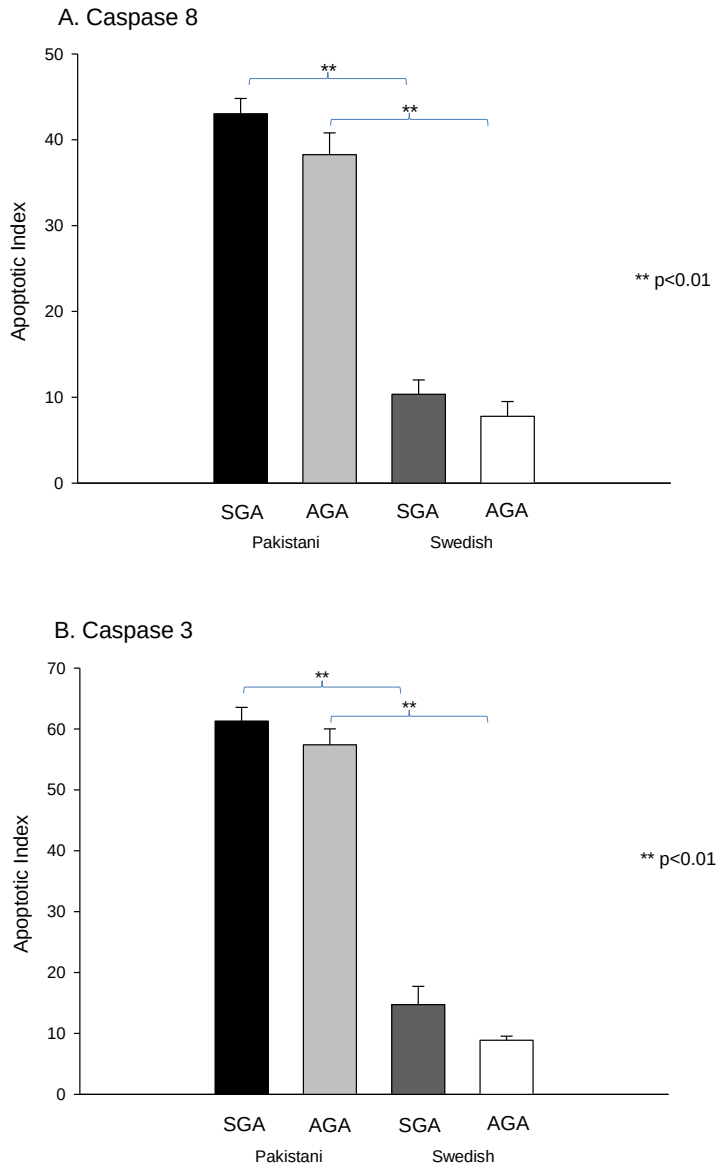


Figure 18. Histograms representing the apoptotic indices for caspase 8 and caspase 3 (*relative degrees of apoptosis*) in the SGA and AGA groups (*Pakistani and Swedish populations*). 18a. *Caspase 8.* 18b. *Caspase 3*. Significant differences were seen between the two populations (***p<0.01*). All indices are represented with their standard errors (SEM). Abbreviations are: SGA, small for gestational age; LGA, large for gestational age. *Unpublished data (paper IV).*

4.7 Population differences

Throughout the study, we have divided populations based on birth weight percentiles, based on gestational ages. These include SGA, AGA, and LGA. In *paper IV*, differences in the levels of apoptosis and anthropometry were looked at, comparing the SGA and AGA groups. We have not, however, compared the IGF results, as these were conducted on the LGA group in the Pakistani population, and the AGA group in the Swedish population, making it difficult to compare those results. The results of the comparisons of the SGA and AGA groups can be seen in *paper IV*, where Pakistani mothers are seen to be significantly shorter than their Swedish counterparts in both the SGA and AGA groups ($p < 0.01$). Furthermore, Pakistani mothers were also seen to have significantly lower weights at the time of delivery, as compared to their Swedish counterparts (*SGA group*; $p < 0.01$). Similar differences are also seen whilst comparing pre-pregnancy weights ($p < 0.05$). These differences suggest that the Pakistani mothers are smaller than their Swedish counterparts, particularly with babies born SGA. Ethnic differences in birth weight outcomes have been previously shown comparing two distinct ethnic groups in the United States of America (Lu and Halfon 2003). Furthermore, both maternal and foetal genetic factors contribute towards birth weight and growth outcomes, with an estimated *22%* of birth weight being attributed to maternal factors (Lunde, Melve et al. 2007). This has been further supported by Adam et al., who have shown the genetics of preterm labour (Adams and Eschenbach 2004). Of interest to note, in our results, is that no significant differences were seen in basic infant anthropometry comparing between the groups.

The significant difference in apoptotic activity between populations is interesting and may further indicate genetic differences. Though all samples looked appropriately intact during the experimental stages, we cannot exclude that these differences may also be partially due to different handling and storing of the samples at the time of collection. If not stored immediately, the placenta would develop increased levels of apoptosis, as well as necrosis, once the blood-hypoxic strain (after delivery and parturition of placenta) is present. We did not, however, see any direct indication of this in our samples. We therefore suggest that certain ethnic populations may possibly be at a higher risk of placental apoptosis and

foeto-placental growth restriction, which is seen across the world, as the incidence of growth restriction is significantly higher in less developed countries, such as Pakistan. Both SGA groups were also seen to have lower IGF-I (and IGF-I mRNA expression) levels, as well as higher apoptosis, as compared to the control groups. Our results comparing the SGA and AGA groups (*papers III and IV*) therefore suggest that the differences in levels of IGF-I and apoptosis may be a possible aetiology to growth restriction, irrespective of ethnicity.

5. SUMMARY

Foetal implantation, growth, and development rely on an extremely complicated and intricate balance of growth promoting factors (*IGF-axis*), growth suppressing factors (*apoptosis*), nutritional status, and steroid hormones and their receptors. The studies presented in this thesis go through several of these factors, starting with the growth factors, IGF-I and IGF-II, the micronutrients, zinc and iron, and moving on to IGFBP-1, the steroid hormone receptors, *ER and PR*, and several markers of apoptosis, namely p53 and the caspase enzymes (*caspases 3 and 8*). *Papers I and II* include experimental and biometric data from the Pakistani population, whilst *paper III* looks at data in the Swedish population. *Paper IV* consists of experimental data and anthropometric comparisons between both populations. Our overall results emphasise the fine homeostasis and balance of all these factors, and suggest their importance in pregnancy birth weight outcomes. This is represented more simply as a schematic, *Figure 19*. Discrepancies in any one, or several, of these factors leads to poor foetal growth, diseases of pregnancy, and babies being born SGA. Furthermore, maternal anthropometry, in both the Pakistani and Swedish populations, may be indicative of a strained foeto-placental environment and could therefore possibly be used as a primary care tool to identify pregnancies at risk of foetal growth restriction. This is especially important in developing countries, where the burden of FGR is high and the availability of secondary care is sparse. Timely identification and treatment of high risk pregnancies could potentially break the vicious cycle of poor growth. Our results, summarised, not only identify clinical parameters of growth restriction, but also suggest a possible aetiology behind this vast problem. One can hope that, in time, with increased research in the field, and with a better understanding of the mechanism of pathogenesis, foetal growth restriction and babies being born small for their gestational ages will no longer be global health burdens.

In summary:

- *We have shown lower levels of IGF-I, as well IGF-I and IGF-II mRNA expression, in the placenta of babies born small for their gestational ages.*
- *We have shown higher levels of IGFBP-1 in the SGA group.*
- *We have shown lower levels of the micronutrients, zinc and iron, in the SGA group.*
- *We have shown correlations of IGF-I and IGF-II and maternal and newborn anthropometry.*
- *We have shown lower levels of progesterone receptor expression, along with positive correlations to IGF-I expression and maternal anthropometry.*
- *We have shown higher levels of placental apoptotic activity in the SGA groups, as well as higher levels of apoptosis in placenta of the Pakistani population.*
- *We have shown significant differences in anthropometric size between Swedish and Pakistani mothers.*
- *Our results constitute additional support to the role of the IGF-axis in foeto-placental growth, and further suggest the regulation of this axis through multiple pathways.*
- *The results also indicate that maternal anthropometry may possibly be used as an indicator of foeto-placental growth.*

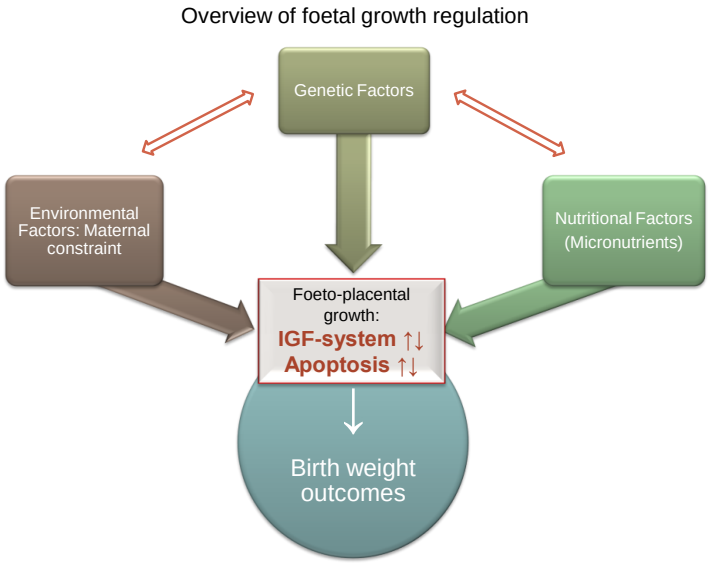


Figure 19. A simplified schematic showing key regulators of birth weight outcomes in pregnancy. The diagram emphasises the major factors that regulate foeto-placental growth through the fine balance of the IGF-system and apoptosis. Increases in growth stimulation may result in decreased apoptosis with a possible increase in foeto-placental growth and subsequent birth weight, and vice versa. Furthermore, the regulators may influence one another resulting in several stimulatory factors affecting foeto-placental growth outcomes. All pathways, regardless of cause, appear to act through the influence of the balance of the IGF-axis and apoptosis.

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(Shahzad K. Akram)

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APPENDIX

I

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REGULAR ARTICLE

Human placental IGF-I and IGF-II expression: correlating maternal and infant anthropometric variables and micronutrients at birth in the Pakistani population

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Abstract

Aim: To correlate infant birth weight with maternal and infant biometric data, including the expression of placental IGF-I and IGF-II at birth, and levels of serum zinc and ferritin.

Methods: The data consisted of observations from 89 women from Karachi, Pakistan. Placental and cord blood samples were taken immediately following delivery and were subsequently divided into two groups, small and large for gestational age (SGA and LGA).

Results: The mean birth weight was 2.79 kg; the prevalence of SGA being 13.4% (≤10th percentile); the prevalence of LGA being 23.6% (≥90th percentile). Placental IGF-I and IGF-II mRNA expression was greater in the LGA group ($p < 0.05$). Furthermore, a significant correlation was noted between infant birth weight and maternal anthropometric parameters ($p < 0.01$). Cord zinc levels were also significantly higher in the LGA group ($p < 0.05$).

Conclusion: Maternal anthropometry, along with placental IGF-I and IGF-II mRNA levels, correlated significantly with infant birth weight suggesting the importance of these growth factors for birth weight outcomes. The higher zinc levels in the LGA group also suggest the importance of this micronutrient in foetal growth. Our results suggest that growth problems have a multifactorial aetiology arising from within the infant rather than due to maternal constraint alone.

INTRODUCTION

Small for gestational age (SGA) babies constitute a major clinical and public health problem, particularly in developing countries where the prevalence has been shown to be as high as 40% (Sub-Saharan Africa and South–East Asia), compared to less than 10% in developed countries (1). The worldwide incidence based on the definitions of percentiles is estimated at approximately *24% or 30 million newborns per year*. Overall, nearly 75% of all affected newborns are born in rural Asia – mainly South–Central Asia – 20% in Africa and about 5% in Latin America (2). In Pakistan, the incidence of SGA is approximately 25%, which is significantly greater than the WHO criteria for triggering a 'public health action'. The overall incidence may in fact be more than this as it is greatly under-reported (3,4). A vast range of subsequent complications, from the time of birth to adult life, are a huge burden on the health systems of many countries (5–10). Although SGA is a preventable problem, the incidence and prevalence remains extremely high in many parts of the world and the exact mechanisms of this complex problem are still unclear.

SGA is a complex problem, with multiple aetiologies. It is defined as a birth weight of less than or equal to the 10th percentile for the gestational age, using population-specific growth charts and normal distribution curves, as there may be variations of 'normal ranges' between populations (2,11,12). Furthermore, the outcomes of growth restriction vary depending on the onset during gestation, leading to symmetrical or asymmetrical growth restriction. The causative factors, and therefore the interventions, for both outcomes vary (12–14). It is well known that foetal and infant growth is regulated by the insulin-like growth factor (IGF) family of growth factors, as well as genetic and environmental factors (15–17). The IGFs, IGF-binding proteins (IGFBPs) and IGFBP proteases are important regulators of somatic growth and cellular proliferation. IGF-I and, more specifically, IGF-II play a key role in foetal-placental growth throughout gestation (18). Both these growth factors exert their actions through the common IGF-I receptor (IGF-IR). Dysregulation of the GH axis, including abnormal expression of IGFs, structural defects in their receptors and impairment in the postreceptor signalling machinery or abnormal binding with IGFBPs may lead to abnormal foetal growth (19,20). Prenatally, this dysregulation can lead to intrauterine growth restriction or subsequently resulting in SGA babies at birth (21).

There have been many studies conducted on the possible aetiologies and pathogenesis of processes leading to SGA, including correlations of maternal and infant anthropometric parameters. Furthermore, the role of certain key micronutrients looking at infant birth weight as an outcome has been studied (22–24). In this study, we correlated mRNA

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expression levels of the principal growth factors, IGF-I and IGF-II in the placenta, with key clinical and biochemical factors to determine any associations in babies born SGA. More specifically, the mRNA expression of the IGFs from human placental tissue was investigated in an attempt to understand the complex relationship between micronutrients, the growth factors and maternal and infant anthropometric variables, looking at infant birth weight as an outcome. The study population consisted of women living in rural Karachi, Pakistan, as these women represent the general rural population across much of Asia, culturally, socioeconomically and in their habitats, where the greatest prevalence of SGA lies.

METHODS

Population characteristics

This study was a prospective, observational study, undertaken after approval by the Ethics Committee of The Aga Khan University (AKU), Karachi, Pakistan. The study population consisted of 89 women from whom placental specimens were taken, following informed consent, along with cord blood samples taken immediately following delivery. The placental samples were collected from rural maternity field sites around Karachi and were subsequently frozen and transported to AKU. These were then analysed for certain factors, as described below. All babies born were at term gestation (37–42 weeks) and were subsequently subgrouped based on birth weight. Out of the 89 samples, three groups were created, SGA, normal birth weight (NBW) and large for gestational age (LGA). The first group, SGA, was that which had a birth weight less than or equal to 2.10 kg ($\leq$10th percentile; n = 12); the second group, LGA, had a birth weight greater than or equal to 3.50 kg ($\geq$90th percentile; n = 21); the remainder, NBW, consisted of the remaining 56 subjects ($>$ 10th and $<$ 90th percentiles). For the purpose of analysis, the majority of the experimentation was conducted on the SGA and LGA groups. The growth charts used were based on the World Health Organization (WHO) growth standards for the Indian subcontinent.

Anthropometric variables

Prior to analysis of tissue samples, maternal and infant biometric data were as recorded at the time of birth. These variables included maternal and infant weight, height, upper midarm circumference and midtriceps skinfold thickness. The values of limb circumference measurement were taken from both upper and lower limbs, but for the purpose of analysis only upper limb circumferences were used as both measurements represent the same clinical parameters. Maternal age was also noted. Other infant variables recorded included occipitofrontal diameter and chest circumference.

RNA quantification

Tissue specimens were dissected from the placental decidua immediately following delivery and were stored in RNA*later*™ (Ambion Inc., Austin, TX, USA) and frozen in liquid nitrogen. RNA extraction was performed using the RNeasy Mini Kit (Qiagen, NV, Venlo, The Netherlands) according to the manufacturers recommended protocol (Qiagen 08/2003). In brief, RNA quantification was carried out using absorbance photospectrometery at an absorbance of 260 nm (HITACHI – U200 spectrophotometer, Hitachi, Hialeah, FL, USA). Subsequently, reverse transcription was carried out using a cDNA kit (First-Strand cDNA synthesis kit, Amersham Biosciences, Sweden), according to the manufacturer's instructions. Primer sequences for the housekeeping genes (β-actin and GAPDH), IGF-I and IGF-II, were as previously used (25). cDNA was synthesized from total RNA using the Superscript cDNA kit (Invitrogen Inc., UK). Primer pairs specific for human IGF-I and IGF-II and the internal standards, β-actin and GAPDH were ordered from Genset Oligos (Paris, France). PCR was performed using the Gene Amp PCR 2400 system (PerkinElmer, Palo Alto, CA, USA) using a total volume of 50 μL. The following PCR protocol was utilized: 30 cycles, 96°C for 5 min, 54°C for 31 sec, 72°C for 1 min, with a final heating of 72°C for 5 min followed by cooling to 4°C. The PCR products were characterized by electrophoresis on 2% agarose gels stained with ethidium bromide. Optical densities of the resulting bands were recorded using the EDAS 120 Kodak Gel documentation system (Eastman Kodak Co., Rochester, NY, USA) and quantified using *Image J* (NIH, USA; http://rsb.info.nih.gov/j/).

Biochemical analysis

The cord venous blood levels of specific factors were evaluated, namely, zinc and ferritin, from the samples obtained. Cord blood samples were placed in potassium EDTA and heparin bottles for subsequent ion chromatography analysis for the micronutrients zinc and ferritin and to assess haemoglobin levels as a marker of general infant health.

Statistical analysis

Statistical analysis was undertaken using both descriptive and regression tools. These included simple linear regression, stepwise regression and the more complex multiple regression model. These methods were conducted using the statistical software package SPSS (SPSS Inc., Chicago, IL, USA). The level of statistical significance was taken to be $p < 0.05$.

RESULTS

Population cohort

Out of the 89 sampled cases, the mean birth weight of the sample cohort was 2.79 kg; the prevalence of SGA being 13.4% ($\leq$10th percentile), and LGA being 23.6% ($\geq$90th percentile). There was a significant statistical difference between the groups at birth with regards to the majority of the variables. All maternal variables, except triceps skinfold thickness, were significantly higher in the LGA group. Details of the descriptive statistics can be seen in Table 1, as a comparison of various anthropometric variables between the two groups, SGA and LGA.

Table 1 Descriptive statistics of maternal and infant anthropometric variables at birth; comparing the small for gestational age (SGA) and large for gestational age (LGA) groups

	SGA Mean (2 SE)	LGA Mean (2 SE)	p-value
Maternal age (years)	25.17 (*1.02*)	26.98 (*2.50*)	**<0.05**
Maternal height (m)	1.51 (*0.01*)	1.55 (*0.01*)	**<0.01**
Maternal weight (kg)	53.15 (*1.84*)	56.67 (*2.20*)	**<0.01**
Maternal body mass index (BMI)	20.80 (*0.77*)	23.65 (*0.93*)	**<0.01**
Maternal midarm circumference (m)	0.22 (*0.06*)	0.25 (5.00×10^{-3})	**<0.01**
Maternal triceps skinfold thickness (m)	0.12 (*0.07*)	0.11 (*0.01*)	0.23
Infant weight (kg)	1.78 (*0.17*)	3.54 (*0.04*)	**<0.01**
Infant height (m)	0.46 (*0.07*)	0.49 (*0.01*)	**<0.01**
Infant occipitofrontal circumference (m)	0.33 (*0.05*)	0.36 (*0.01*)	**<0.01**
Infant midarm circumference (m)	0.97 (*0.02*)	0.11 (3.00×10^{-3})	**<0.01**
Infant chest circumference (m)	0.31 (6.10×10^{-3})	0.34 (8.00×10^{-3})	**<0.01**

All of the variables, except maternal triceps skinfold thickness, were significantly higher in the LGA group (n = 21) as compared to SGA group (n = 12). Levels of significance are given in the right-hand column (a p-value of < 0.05 is regarded as significant).

IGF-I and IGF-II expression

The placental expression of both IGF-I and IGF-II mRNA was significantly greater in the LGA group, when compared to the SGA group ($p < 0.05$). This difference can be seen more clearly in Figure 1. Bi-variate linear correlation showed a statistically significant positive correlation between several of the maternal and infant variables: IGF-I and IGF-II mRNA levels with respect to birth weight, height and infant midarm circumference ($p < 0.01$; Pearson correlation). IGF-I mRNA expression also significantly positively correlated with baby chest circumference at birth ($p < 0.01$). Maternal height also positively correlated with infant birth weight and height ($p < 0.01$). No significant correlation was seen between growth factor expression and maternal anthropometric variables.

Anthropometric variables

When looking at maternal and infant anthropometric variables, significant positive correlations were seen between baby weight and height, with maternal age, weight, height and upper midarm circumference ($p < 0.01$) (Fig. 2). Infant birth weight showed the following significant correlations: infant birth height ($r = 0.83$), occipitofrontal diameter ($r = 0.72$) and chest circumference ($r = 0.83$), maternal midarm circumference ($r = 0.82$). Infant height at birth also demonstrated a significant positive correlation with several of the infant and maternal clinical variables, including infant occipitofrontal diameter ($r = 0.72$), chest circumference ($r = 0.94$) and maternal midarm circumference ($r = 0.69$). Furthermore, in keeping with the positive maternal correlations with infant birth weight, a significant positive correlation

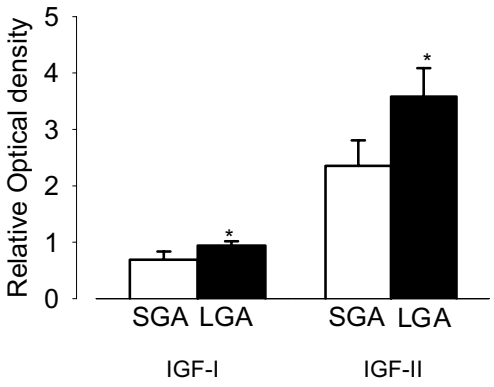

Figure 1 Placental expression of IGF-I and IGF-II mRNA in the small for gestational age (SGA) and large for gestational age (LGA) groups as investigated by RT-PCR. A simple error (2 SE) bar chart comparing SGA (n = 12) and LGA (n = 21) groups (□ SGA; ■ LGA). Both IGF-I and IGF-II expression levels were significantly higher in the LGA group, as compared to the SGA group (*$p < 0.05$).

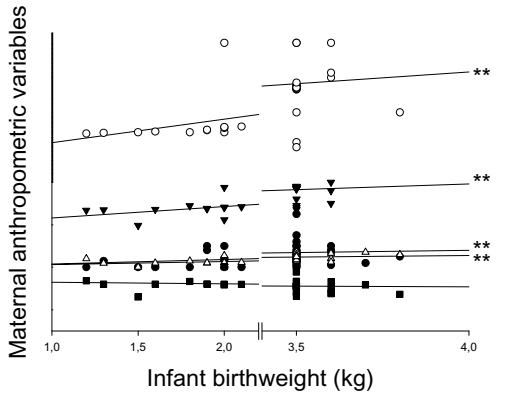

Figure 2 Infant birth weight correlated with maternal anthropometric variables, comparing the SGA and LGA groups (n = 12 and 21, respectively). A scatter plot depicting the regression lines for each correlation. All correlations were highly significant (**$p < 0.01$), except for maternal triceps skinfold thickness (maternal age ●; height ○; midarm circumference Δ; triceps skinfold thickness ■; weight ▼).

was seen with maternal body mass index (BMI; $r = 0.32$, $p < 0.01$).

Biochemical analysis

A significant difference in the zinc levels at birth was noted in the two groups ($p < 0.05$), with serum zinc levels being significantly lower in the SGA babies as compared to the LGA babies. There were no significant differences when correlating ferritin or haemoglobin levels between the two groups. Infant zinc and ferritin levels did not correlate with IGF-I and IGF-II mRNA levels, nor was there any statistical significance seen when comparing between the SGA and LGA infants.

Birth weight correlations

Stepwise regression analysis was carried out on infant birth weight, taken as the dependant variable. The entire sample population ($n = 89$) was used to ensure an adequate number of cases for both stepwise regression and multiple regression. Out of all variables, the following were found to have significant regression coefficients and were selected for the estimation of the multiple regression models: placental IGF-I mRNA levels, infant chest circumference, height, arm circumference and maternal height. The regression model showed that these variables accounted for 87% of the variation in birth weight. This means that changes in birth weight were positively and significantly related to the five variables, with an overall regression coefficient (β) of 0.87 (Fig. 3). Independent regression correlation of the growth factors expressions with all anthropometric variables in the individual groups, SGA and LGA, showed that the only significant correlation was that of IGF-I mRNA expression and infant birth weight ($p < 0.01$). The missing values, in the analysis, were replaced using trend generated values from a best fit line, for the purpose of regression correlation and multiple regression analysis.

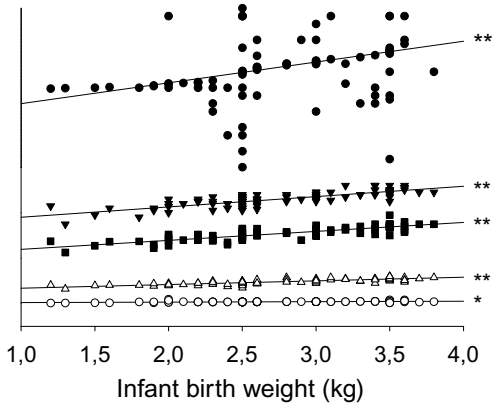

Figure 3 Multiple regression analysis of infant birth weight with biometric variables. A scatter plot depicting the corresponding correlation lines. All correlations were positively significant (*$p < 0.05$, **$p < 0.01$). The regression coefficient (β), and levels of significance (p), for each of the variables is as follows: maternal height (● $\beta = 0.09$; $p < 0.01$); IGF-I (○ $\beta = 1.22$; $p < 0.05$); infant height (▼ $\beta = 0.39$; $p < 0.01$); infant midarm circumference (Δ $\beta = 0.18$; $p < 0.01$); infant chest circumference (■ $\beta = 0.25$; $p < 0.01$). The overall regression coefficient was $\beta = 0.87$ ($p < 0.01$; $n = 89$).

DISCUSSION

The study at hand was a novel study carried out on a rural population in Pakistan, as there are little previous data on the Pakistani population. All infants born were term spontaneous vaginal deliveries. The results, as the aims, were twofold; first, the correlation of biochemical variables, zinc and ferritin, with one another and with certain maternal and infant anthropometric measurements; second, the correlation of the anthropometric and biochemical variables with placental IGF-I and IGF-II mRNA expression to assess possible relationships between these variables, comparing SGA and LGA babies. Our results showed a significant difference in the levels of IGF-I and IGF-II mRNA expression and cord zinc levels between the two groups, as well as a significant regression correlation between infant birth weight and certain infant and maternal variables.

Our data show lower levels of mRNA expression of IGF-I and IGF-II in infants born SGA, thus implying that there is decreased transcription resulting in decreased growth factor levels in this group. These findings are in line with previous data seen by Leger et al. and Ong et al. (26,27), and support current knowledge on the roles of IGF-I and IGF-II as they are known to play a significant part in regulating placental and foetal growth (15–18,21,28). These growth factors play a more important role in gestational growth than growth hormone alone, as they are regulated and synthesized within the placenta. They are therefore not impeded by the natural barriers that the placenta presents to many other hormones and substances and can circulate within the foetus (15,17). However, it is has remained unclear whether or not these differences are a cause or effect of SGA as there have been few studies done throughout the gestational period to assess levels correlating with foetal size. The significant positive correlations between the growth factors mRNA expressions and infant anthropometric variables support the above-stated findings of the role of IGF-I and IGF-II in gestational growth, as lower levels were seen in SGA babies in our study. All babies were symmetrically growth restricted as all infant anthropometric variables significantly correlated with one another. Symmetrical growth restriction, as opposed to asymmetrical, arises from early gestation, whereby all aspects of foetal growth are affected to the same level (2,17,20, 29). This implies that the foetus has been nutritionally deprived from early gestation throughout pregnancy. Although we do not have levels of maternal IGF-I and IGF-II throughout the course of pregnancy, we can suggest further trials be conducted to correlate and predict growth outcomes based on growth factor levels and to ascertain the cause/effect relationship.

Our results show that infant birth weight and height significantly positively correlate with maternal anthropometric variables. These correlations, along with the correlations

in maternal and infant anthropometric variables between the SGA and LGA group, show that infant birth weight, among other parameters, is significantly related to maternal weight and size, and represents *maternal constraint* (30–32). This is a natural process whereby smaller maternal sizes result in smaller foetal and infant sizes to allow both gestational growth and the delivery to be viable processes. These findings have many implications in developing countries where it is not always possible to obtain tests or radiological investigations. In these situations, smaller mothers, or those with a lower BMI, who are at a higher risk of foetal growth restriction may be picked up by midwives or health care workers and subsequently referred to appropriate care facilities.

Furthermore, we found that a significant proportion (87%) of the variation in infant birth weight can be explained by infant and maternal variables, as was seen in the multiple regression models. These included infant IGF-I mRNA expression levels, height, chest circumference, midarm circumference, and maternal height. In essence, these results support our findings of symmetrical growth disorders in the infants, both small and large, as the majority of regressors are infant variables, correlating with infant birth weight. Our findings were similar to those of Boyne et al. (33), whereby infant IGF-I levels were seen to have a highly significant positive correlation with infant birth weight. As maternal height appeared to influence infant weight greater than any other maternal parameter, we can suggest that population variations in maternal height, along with body mass indices, may account for variations in the infant birth weight, or be used as a predictor for birth weight. This finding supports the idea of maternal constraint affecting infant growth. Based on our results, we can suggest that maternal constraint causes lower levels of IGF-I with a resultant symmetrical decrease in foetal size, suggesting that lower levels of IGF-I are in fact an effect of a much larger and complex problem. To confirm these results, we suggest further trials on IGF binding proteins and receptors to assess the effects of maternal anthropometry on the placental growth factor axis.

Although we did not find a significant correlation between infant zinc levels and the levels of IGF-I and IGF-II mRNA expression, significantly higher zinc levels were seen in the LGA group when compared with the SGA group. Zinc is an important constituent of both cellular maintenance and cellular growth, as it is an integral element in normal transcription processes (1,15). Our findings are in line with those of Akman et al. (34), who suggested that zinc may not necessarily influence the IGF-axis directly, but may influence growth through other pathways. Krebs et al. (35), reported that a small 'exchangeable zinc pool', as a result of zinc deficiency, was seen to significantly influence growth outcomes at birth, with significantly lower levels in SGA babies. In our study, the LGA group was also seen to have higher cord ferritin and haemoglobin levels, though this difference was not statistically significantly. Scholl et al. (36) suggested that lower ferritin levels and anaemia result in an oxidative stress on the foetoplacental unit resulting in adverse foetal outcomes. Although this mechanism does not appear to affect the expression of IGF-I and IGF-II mRNA, it may affect other aspects of the growth factor axis, thus resulting in SGA babies. Though growth factor mRNA expression does not appear to be directly influenced by zinc and ferritin deficiencies, our results suggest that these micronutrients appear to influence growth outcomes through other mechanisms.

Our study carries several limitations, predominantly the limited sample size. It is therefore appropriate to suggest further studies correlating similar variables to establish a more sound predictive method for the possible diagnosis of foetal and infant growth restriction based on maternal anthropometric variables throughout the gestational period. This may in fact lead to the establishment of a relatively quick and cost-effective method of screening patients and identifying those at a higher risk of having SGA, or conversely, LGA babies. There have been studies assessing maternal and paternal anthropometric variables and their correlations with foetal growth, but these are still not applied in a clinical setting, particularly as screening parameters for SGA (30,37,38). Maternal constraint appears to play an important role in growth outcomes, though it is not the sole aetiological factor. In conclusion, we have shown that the birth weight outcome is in fact a complex multifactorial process with many key intrinsic factors in the infant and placenta contributing to final neonatal anthropometry.

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II

Full title: **Placental IGF-I, IGFBP-1, zinc, and iron, and maternal and infant anthropometry at birth.**

Short title: **IGF-I, IGFBP-1, Zinc, Iron, and anthropometry at birth**

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ABSTRACT

Aim: To correlate placental protein levels of IGF-I and IGFBP-1, with previously determined levels of IGF-I and IGF-II mRNA expression, and the micronutrients zinc and iron, along with maternal and infant anthropometry. ***Methods:*** Placental samples were obtained from women in rural field sites in Pakistan. Samples were divided into small for gestational age (SGA) and large for gestational age (LGA) groups. IGFBP-1 protein levels were assessed using Western immunoblotting, and IGF-I protein levels were quantified using ELISA techniques. IGF mRNA expression, zinc and iron were quantified as previously described and were used for comparative purposes only. ***Results:*** 33 subjects were included in the study (SGA, n=12; LGA n=21). Higher levels of IGFBP-1 were seen in the SGA group (*p<0.01*). Correlations of IGFBP-1 and anthropometry showed positive correlations with maternal and infant triceps skin-fold thickness, in the LGA and SGA groups, respectively *(p<0.05)*. Significantly lower IGF-I protein levels were seen in the SGA group. IGF-I protein levels correlated significantly with maternal and newborn anthropometry. IGFBP-1 correlated significantly with IGF-II mRNA expression ($p<0.05$). ***Conclusion:*** Placental protein levels of IGF-I and IGFBP-1 appear to have an association with maternal anthropometry. Maternal anthropometry may thus influence IGFBP-1 and IGF-I levels and may possibly be used for screening of pregnancies, with the potential for timely identification of these high-risk pregnancies.

INTRODUCTION

Newborn growth disorders comprise a devastating set of problems that continue to burden much of the world, particularly in low-income countries. One problem in particular is that of children being born with low birth weights (LBW). Foetal growth restriction and subsequent LBW encompass vast clinical and public health problems, increasing infant mortality and morbidity, as well as subsequent adult morbidity (1, 2). The global incidence of LBW, and babies born small for their gestational ages (SGA), is currently estimated to be *30 million newborns per year (23.8%)* (3). Overall, approximately 75% of all affected babies are born in Asia, mainly south-central Asia, with the prevalence estimated at 40% in certain regions (4). The pathogenesis of LBW, and SGA, is complex, resulting from nutritional, environmental, and genetic factors. Nutritional deficiencies have been shown to decrease foeto-placental growth and subsequent birth weight. This has been shown more clearly through supplementation of multiple micronutrients resulting in improved maternal nutritional status and increased birth weight outcomes in comparison to placebo (5). Nutritional (multivitamin) supplementation has thus been widely recommended in pregnancy. A micronutrient of particular importance in pregnancy is zinc. Timely supplementation has been shown to decrease the risks of preterm labour and may thereby decrease the incidence of SGA (6). A second key micronutrient in pregnancy is iron (ferritin), which is essential in the production of haemoglobin. Deficiencies result in anaemia leading to increased risks of preterm deliveries, low birth weight, and maternal mortality in pregnancy (7). Iron and zinc supplementation may therefore improve birth weight outcomes in impoverished and nutritionally deprived areas. In addition to these key micronutrients, maternal size is believed to contribute significantly towards foeto-placental size and birth weight, as smaller mothers are at an increased risk of delivering babies with low birth weight. The mechanism for this 'maternal' constraint is, however, not clearly known (8).

On a molecular level foetal and post-natal growth are controlled by complex axes of hormones, their binding proteins, receptors, and proteases. The most important hormone axis of growth in humans is the insulin-like growth factor (IGF) axis, which consists of the insulin-like growth factor binding proteins (IGFBPs), the growth factors IGF-I and IGF-II, the IGF receptor (IGF-1-R), and IGFBP proteases (9). The growth factors, IGF-I and IGF-II, are essential in normal growth processes and development (10). There are six known IGF binding proteins in humans (IGFBP-1 to -6), each with unique roles in different organs. The most significant in pregnancy is IGFBP-1, as it is secreted by the placental decidua and enters directly into the foetal circulation. IGF-I has a greater affinity to IGFBP-1 than to its receptor (IGF-I-R) and is thus sequestered by the binding protein. This binding indirectly regulates tissue growth by decreasing the amount of free IGF (11). IGFBP-1 has thus been shown to have an inverse relationship to placental and foetal growth, particularly in the second and third trimesters. Increases in the binding protein may therefore

potentially lead to growth restriction and a low birth weight (12). We hypothesise that certain key micronutrients, namely zinc and iron, are implicated in the appropriate functioning of the IGF system, thereby indirectly regulating foeto-placental growth. In an attempt to better understand the regulation of the IGF-system, and the possible roles of zinc and iron, we analysed associations between placental protein levels of IGFBP-1, IGF-I, and the two micronutrients, along with previously determined levels of placental IGF-I and IGF-II mRNA expression (13). We further correlated these factors with newborn and maternal anthropometry assessing for possible associations.

METHODS

Study design and methods

The present study was a cross-sectional observational study, undertaken after approval by the Ethical Review Committee of The Aga Khan University, Karachi, Pakistan. Following informed consent, placental samples were taken from 89 women living in rural Karachi, Pakistan. Cord blood samples were also taken for subsequent analysis. Samples were immediately frozen in liquid nitrogen and transported to the Aga Khan University for analysis. The samples were analysed for protein levels of IGFBP-1 and IGF-I. mRNA expression of the growth factors, IGF-I and IGF-II, and the micronutrients, zinc and iron, are as previously described by us and the data was used for comparative purposes only (13).

Population Characteristics

The placental samples were collected immediately following delivery. Inclusion criteria included placental samples from term pregnancies (gestational age 37-42 weeks), to healthy mothers with no history of pregnancy-related illnesses or infections. Exclusion criteria included preeclampsia, eclampsia, and pre- and post-term deliveries. Gestational diabetes was not checked for. A total of 33 samples were included in the study. The samples were subsequently divided into SGA and large for gestational age (LGA) groups, based on population specific growth charts. The first group, SGA, consisted of samples with birth weights of less than or equal to 2.50kg (≤10th percentile based on our population specific growth charts, n = 12); the second group, LGA, consisted of neonates with birth weights ≥ 90th percentile (population specific growth chart, birth weight ≥ 3.5kg, n = 21) (14). Whilst the definitions of SGA and LGA are standard, we appreciate that the birth weights (kg) included in our study are lower than those of the WHO growth charts. This may create a potential bias by including/excluding certain pregnancies, but as these were the weights defined by percentiles in our population subgroup we considered these categories appropriate for further comparison and analysis. Maternal and infant biometric data was recorded at the time of birth, and was used for correlation analysis with IGFBP-1 and IGF-I protein levels. These variables included maternal and infant weight, height, upper mid-arm circumference, and mid-triceps skin fold thickness and have been described in detail previously (13). IGF-I and IGFBP-1 protein analyses were carried out as described below.

Determination of IGFBP-1 by Western immunoblotting (WIB)

Western immunoblotting was utilized to quantify IGFBP-1 protein levels in the placental tissue. Frozen sections of placental decidua were thawed on ice for subsequent protein extraction. In brief, protein was extracted from tissues using gentle mortaring in lysis buffer, followed by micro centrifugation. The supernatatant was subsequently separated by SDS-PAGE (4 -12% Bis-Tris Gels; Invitrogen, U.K.). Protein quantification was conducted using the Bradford assay. Western immunoblot analysis was carried out using 20µg of protein on polyvinylidene fluoride (PVDF) membranes (BIORAD, CA) in a Hoefer Semi-Dry Transphor unit at 200mA (Amersham Biosciences, Sweden). Membranes were then blocked in 5% non-fat milk in Tris-buffered saline containing 0.1% Tween20 (TBS-T) and 10ng/ml of a primary mouse anti-human antibody (IDS Ltd., U.K.; at 1:1000 dilution) overnight at 4°C. Thereafter, membranes were washed with TBS-T and incubated with secondary antibody (goat anti-mouse antibody at 1:10000 dilution, IDS Ltd., U.K.) for 90 min at room temperature, followed by washing with TBS-T and water. Finally, the membranes were exposed to enhanced chemiluminescence reagents (Amersham Biosciences, Sweden) for 1 min at 20°C. Band densities were analysed using the quantification programme *ImageJ* (NIH, USA; http://rsb.info.nih.gov/j/). Results are

presented as a ratio of optical density (band intensity) measurement between sample and control.

Quantification of IGF-I and IGF-II mRNA expression by RT-PCR

RNA extraction and quantification was as previously described (13). In brief, cDNA was synthesized from total RNA using the Superscript cDNA kit (Invitrogen Inc., U.K.). This was then reverse transcribed using reverse transcription polymerase chain reaction (RT-PCR) techniques. The specific primer pairs for human IGF-I and IGF-II were as used before. PCR products were characterized by agarose gel electrophoresis, and the resulting bands were recorded for their optical density and quantified using *ImageJ*. Previous data have not been included and were used for analysis with IGFBP-1 and IGF-I protein levels only.

IGF-I ELISA analysis

For the purposes of IGF-I protein analysis, we used enzyme-linked immunosorbant assay (ELISA) techniques. Protein extraction and quantification were done as described above. Protein standards and duplicates of all samples were then analysed according to the manufacturer's protocol (IGF-I ELISA, Mediagnost, Reutlingen, Germany). IGF-I levels were quantified using absorbance photospectrometry techniques, using an ELISA plate reader at an absorbance of 450nm (room temperature; 22.5º Celsius). Levels are expressed as concentrations in *ng/ml* as calculated against the standard curve (based on the manufacturer's protocol).

Micronutrient analysis

Cord blood samples were placed in potassium EDTA and heparin bottles for subsequent ion chromatography analysis for the micronutrients zinc and ferritin (13). Ion quantification and analysis were carried out at the Aga Khan Hospital laboratory, Karachi, Pakistan, using standardised protocols (Beckman Synchron CX7 autoanalyser, Beckman Coulter, Ca, USA).

Statistical analysis

Statistical analysis was undertaken using both descriptive and regression tools, including the Student's t-test, ANOVA, Pearson's correlation, and multivariate linear regression with the Durbin-Watson statistic to calculate for autocorrelation. Results are presented as the mean and standard error (SE), along with levels of significance *($p < 0.05$)*. Regression coefficients are represented with the letter 'r'. The analysis was done using the statistical software package PASW, version 18.

RESULTS

Population characteristics

Thirty-three samples were included in this study (SGA, n = 12; LGA, n = 21). Details of IGF-I and IGF-II mRNA expression, anthropometric variables, and correlations with birth weight are as previously described by us (13). IGFBP-1 and IGF-I protein levels, along with zinc, iron, and haemoglobin, can be seen in table 1.

Table 1

	SGA *Mean (SE)*	***LGA*** *Mean (SE)*	***p-value***
Infant body mass index (BMI)	0.46 *(0.03)*	0.49 *(0.01)*	***<0.01***
Placental IGFBP-1 (band intensity)	1.62 *(0.11)*	1.36 *(0.05)*	***<0.01***
IGF-I levels (ng/mL)	6.35 *(0.29)*	14.08 *(2.61)*	***<0.05***
Zinc (µmol/L)	0.12 *(1.75 x10^{-3})*	0.14 *(0.01)*	***<0.05***
Iron (ferritin) (µmol/L)	1.62 *(0.02)*	2.37 *(0.11)*	***<0.05***
Haemaglobin (Hb) (g/L)	13.92 *(0.87)*	15.89 *(0.76)*	***<0.01***

Legend to Table 1. Descriptive statistics of infant birth body mass index (BMI) and biochemical variables, comparing the small for gestational (SGA, n= 12) and large for gestational age (LGA, n= 21) groups. Details of maternal and infant anthropometry, and mRNA expression of IGF-I and IGF-II are as previously mentioned and have therefore not been included in the table (13). All values are given as means along with their standard errors (SE) and are expressed in the international system (SI) of units. IGF-I levels were calculated against a standard curve and are therefore expressed as *ng/ml*. The level of significance was taken to be $p<0.05$.

The Micronutrients and birth weight outcomes

Zinc and iron levels were significantly lower in the SGA group as compared to the LGA group (*$p < 0.05$*, table 1). All micronutrient levels were within normal ranges in both groups.

Haemoglobin levels were markedly lower in the SGA group as compared to the LGA group, though all values were within normal ranges ($p < 0.01$; table 1). IGFBP-1 protein levels did not correlate with cord blood levels of the micronutrients zinc and iron (Pearson's correlation).

IGFBP-1 analysis

IGFBP-1 protein levels were significantly higher in the SGA group as compared to the LGA group, as can be seen in table 1 ($p < 0.01$; Student's t-test). ANOVA analysis using IGFBP-1 as the dependant variable, with the maternal anthropometric variables as the independent variables, was highly significant ($p < 0.01$). No correlation was seen between infant birth weight and IGFBP-1 ($r = -0.12, p = 0.63$). Pearson's correlation of IGFBP-1 and maternal anthropometry showed significant correlations of the binding protein and maternal triceps skin-fold thickness ($r = 0.55, p < 0.05$), and infant mid-chest circumference at birth ($r = 0.57, p < 0.05$), in the LGA group. Additionally, in the SGA group, infant triceps skin-fold thickness at birth correlated positively with IGFBP-1 levels ($r = 0.65, p < 0.05$). Multivariate regression analysis of IGFBP-1 showed significant correlations between IGFBP-1 and maternal mid-arm circumference, and triceps skin-fold thickness ($p < 0.01$).

Figures 1a and 1b. Placental IGF-I and IGF-II mRNA expression, and IGFBP-1 correlations in the SGA and LGA groups.

Figure 1a. SGA

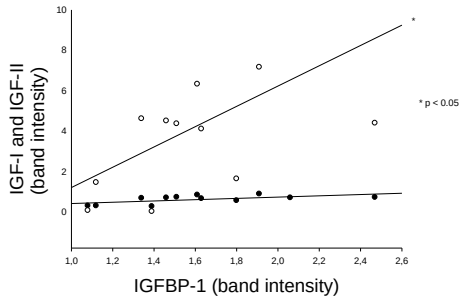

Figure 1b. LGA

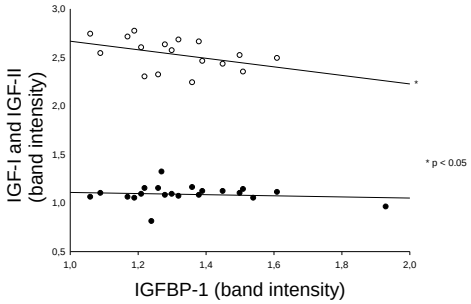

Figure 1. Multivariate linear regression correlation of placental IGFBP-1 with mRNA expression levels of placental IGF-I and IGF-II (● IGF- I; ○ IGF- II). **Figure 1a:** Regression correlation within the small for gestational age (SGA) group (n=12). A significant *positive* correlation was seen between IGFBP-1 and IGF-II mRNA expression (*p<0.05). **Figure 1b:** Regression correlation of the large for gestational age (LGA) group (n=21). A significant negative correlation was seen between IGFBP-1 and IGF-II mRNA expression (*p<0.05).

IGF analysis

IGF mRNA expression levels assessed in a previous study (13) were used for comparisons. IGF-I protein levels (ELISA) were significantly lower in the SGA group, as compared to the LGA group ($p < 0.05$, *table 1*). There were no significant correlations between IGFBP-1 and IGF-I protein or mRNA expression levels in the respective groups. IGFBP-1 levels significantly correlated with IGF-II mRNA expression in both the SGA and LGA groups ($p<0.05$ figure 1). Of interest to note are the significant positive correlations in the SGA group and the significant negative correlation in the LGA group (figures 1a and 1b). Pearson's correlation analysis of IGF-I protein levels showed positive correlations with maternal height ($r = 0.62, p = 0.03$), maternal weight ($r = 0.58; p = 0.04$), newborn height ($r = 0.63, p = 0.03$), and newborn weight ($r = 0.74, p < 0.01$) in the SGA group. In the LGA group, IGF-I protein levels also significantly correlated to newborn weight ($r = 0.64, p < 0.01$).

DISCUSSION

The objectives were to quantify and correlate protein levels of IGFBP-1 and IGF-I in human placenta with previously determined mRNA expression levels of the growth factors, IGF-I and IGF-II, and the cord blood levels of the micronutrients, zinc and iron, along with maternal and infant anthropometry at birth. We have shown that IGFBP-1 levels were significantly higher in the SGA group, as compared to the LGA group. Furthermore, IGFBP-1 levels correlated significantly with IGF-II mRNA expression levels in the both groups. We appreciate that though our population distributions may not be representative of global growth charts; they represent different clinical groups and were thus described as above for the purposes of comparison.

Lee, et al., have shown that IGFBP-1 has an inverse relationship to the amount of free IGF, suggesting that increases in levels of this binding protein may result in subsequent increases in ligand sequestration (15). This, in turn, reduces receptor – ligand interaction compromising cellular proliferation and growth (16). In the absence of this mechanism, cellular growth and development may potentially go unchecked leading to cellular and tissue hypertrophy (17). We have shown similar results in our LGA group. Furthermore, we have shown a significant positive correlation between IGF-II mRNA expression and the binding protein levels in the SGA group. These results, though contrary to those previously described, may be due to the fact that IGFBP-1 sequesters IGF-II carrying it through capillary barriers and aiding in consistent growth factor release at a cellular level, particularly in catabolic states. This increased binding may further explain the increase in IGF-II mRNA expression, possibly as a result of feedback regulation. Additionally, IGFBP-1 may directly regulate growth as it is believed to act independently of the growth factors, IGF-I and IGF-II, as has been shown in animal models (18, 19). The binding protein may thereby directly influence IGF-II transcription resulting in increased IGF-II mRNA expression in catabolic states, as is the case in growth restricted pregnancies (20). Increases in IGFBP-1 may further protect against the insulin effects of IGF-I and IGF-II by binding to them, particularly in SGA as nutrition is predominantly required in the brain and vital organs. In the absence of the binding protein, increases in IGF-I and IGF-II would cause increased glucose uptake in muscles, diverting essential nutrition from the vital organs (21).

We have found significant associations of IGFBP-1 with maternal anthropometry. These correlations may suggest the influence of maternal body composition, particularly fat and muscle composition, on insulin (and IGF) metabolism. This altered metabolism may thus influence the IGF-axis, particularly in states of growth, such as the pregnant uterus and foeto-placental growth. Placental IGFBP-1 may thus be directly regulated by circulating levels of maternal insulin and the IGFs. Furthermore, increasing levels of placental IGFBP-1 in association to increases in maternal anthropometry may represent a complex maternal growth regulation, whereby smaller mothers may have decreased placental IGFBP-1 levels in an attempt to increase free IGFs, asserting a growth promoting affect. These results, though contradictory to the higher levels of binding protein in the SGA group, are in keeping with previously published results and thus support the dual role that IGFBP-1 may play. Maternal size may thus regulate foeto-placental size, probably through the IGF-axis, thereby affecting flow of nutrients through the placenta. This may affect foeto-placental growth, contributing to decreased foetal growth in smaller mothers (22). We recommend further studies be done to ascertain the sensitivity and specificity of these associations with anthropometry.

IGF-I protein levels were significantly lower in the SGA group, and corresponded to lower IGF-I mRNA expression, as has been previously described (13). We have also shown significant correlations between IGF-I protein levels and maternal and newborn anthropometry. Inguinez, et al., has previously shown similar significant correlations of placental and serum IGF-I protein levels (and mRNA expression) in children born small for gestational age (23). These correlations reflect the importance of IGF-I regulation of growth, particularly in the final stages of pregnancy. They further indicate that IGF-I may be regulated by *maternal constraint*, with lower levels seen in smaller mothers, thus resulting in decreased placental and foetal growth and babies being born SGA. Furthermore, our results showed significant correlations of IGF-I protein levels and infant height in both groups, re-

emphasising the important role of this growth factor in growth outcomes.

The association of zinc deficiency with foetal growth restriction has been previously described (24). The lower zinc levels in the SGA population, as seen in our results, support previous findings of the role of zinc in foeto-placental growth (25). Higher zinc levels thus appear to be associated with increased birth weight. We therefore believe that timely supplementation of this micronutrient may reduce the risks of preterm labour thereby improving pregnancy outcomes. Iron is a second essential micronutrient in normal pregnancy growth and physiology. Iron deficiency anaemia is the most clinically significant micronutrient deficiency, globally, affecting more than one third of the world's populous, and more than a billion pregnancies (26). Though it has been shown that supplementing iron reduces maternal and newborn mortality rates, the association of this micronutrient with foetal growth restriction is unclear (5). Numerous studies have outlined the poor pregnancy outcomes of both iron deficiency and the consequences of excesses (27-29). Scholl et al., (30), has shown that both extremely low and high levels of serum iron result in increased levels of IGFBP-1 and subsequent growth restriction. We have shown significantly lower iron and haemoglobin levels in the SGA group, though all levels were within normal ranges. These correlations reflect the importance of adequate nutrition in pregnancy, re-emphasising the importance of iron supplementation in pregnancy. Furthermore, our results may suggest that the 'normal ranges' of zinc, iron, and haemoglobin may need to be reconsidered in pregnancy, or possibly adjusted based on population specific levels, as lower levels, whilst still within normal ranges, are associated with decreased birth weights.

In conclusion we have shown significant correlations of IGFBP-1 protein levels with IGF-II mRNA expression and maternal anthropometry. We have also shown significant correlations of IGF-I protein levels with maternal anthropometry. These results emphasise the complex regulation of the IGF-axis, through nutritional and environmental factors. IGFBP-1 and IGF-I are two key components of the pregnancy growth hormone axes and appear to have independent growth promoting actions. We further suggest that maternal anthropometry may be used as cost effective and simple tool to identify high risk pregnancies, as mothers of SGA babies are clinically significantly smaller than those of normal birth weight pregnancies and have been shown to have lower levels of placental IGF-I. We re-emphasize the importance of appropriate intervention and dietary supplementation in an attempt to break the generation-to-generation cycle of growth restriction (5, 6, 31). Finally, we suggest the need for further studies to look into the use of anthropometrical parameters as diagnostic tools for LBW and SGA, as these tools may be easily applied in impoverished areas and may allow for the timely diagnosis and treatment of millions of affected pregnancies.

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III

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Placental IGF-I, Estrogen Receptor, and Progesterone Receptor Expression, and Maternal Anthropometry in Growth-Restricted Pregnancies in the Swedish Population

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Key Words
Insulin-like growth factor-I · Estrogen receptor · Progesterone receptor · Placenta · Small for gestational age

Abstract

Background/Aims: Fetal growth restriction is a complex problem of pregnancy arising from multiple etiologies. Key regulatory elements of growth are the insulin-like growth factor (IGF) axis, and estrogen and progesterone receptors. The aims were to determine the relations of expression of IGF-I, estrogen receptors α and β (ERα and ERβ, respectively), and progesterone receptor (PR), with maternal anthropometry, focusing on birth weight outcomes. ***Methods:*** Placental samples were obtained from 33 patients following delivery. mRNA expression was determined by a solution hybridization technique. Samples were divided into normal control (NC) and growth-restricted (GR) groups. ***Results:*** IGF-I expression was lower in the GR as compared to the NC group. PR levels correlated positively with IGF-I expression, infant anthropometry, and gestational age (GR). ERα correlated positively with PR expression (NC), and maternal BMI at delivery (GR). ERβ correlated positively with maternal delivery weight and gestational age (NC). ***Conclusion:*** The differences in placental expression of IGF-I emphasize its key role in birth weight outcomes. We further suggest the importance of PR expression in the pathogenesis of intrauterine growth restriction, as there were direct correlations of PR expression with both IGF-I expression and infant anthropometric parameters, as well as gestational age.

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Introduction

Fetal growth restriction is a complex problem of pregnancy, arising from multiple etiologies, including environmental, nutritional and genetic problems [1]. The genetic factors tend to contribute towards early growth restriction, particularly in the first and second trimesters, whereas nutritional and environmental factors predominantly influence growth in the latter part of pregnancy, contributing to the phenomenon of maternal constraint, whereby normal fetal growth is affected by maternal size. A significant component of metabolic regulation is that of hormone-endocrine pathways, the most significant of these being the paracrine insulin-like growth factor (IGF) system. Deficiencies in this system contribute to fetal growth problems, with the outcome being that of babies born with a low birth weight (LBW), or, more specifically, small for their gestational age (SGA). Additionally, increases in metabolic factors may also adversely contrib-

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ute to excessive fetal growth [2]. These growth disorders may have profound effects on postnatal growth and development, increasing the risks of both immediate perinatal morbidity and mortality. SGA therefore constitutes a significant clinical and public health problem, globally, affecting more than *30 million* newborns per year [3, 4]. Furthermore, a well-established cause of intrauterine growth restriction (IUGR) is that of the clinical syndrome *preeclampsia*, which is defined as a combination of an increased diastolic blood pressure (>90 mm Hg), along with proteinurea [5]. The degrees of proteinurea further grade the syndrome into *mild* and *severe* [6, 7].

The IGF system consists of the growth factors, IGF-I and IGF-II, the binding proteins (IGFBPs 1–6), proteases, and their signaling receptor (IGF-I-R). In pregnancy, the key binding protein is that of IGFBP-1, which binds to and delivers the growth factors, IGF-I and -II, directly to fetal tissue cell surfaces, activating cellular mitotic processes, promoting cellular and tissue growth. The IGFs are therefore paramount to normal cellular growth, with the relationship between IGF-I and -II, and IGFBP-1 in the placenta of children born SGA being a well-established one [8]. In addition to the IGF system, key maternal steroid hormone receptors are believed to influence placental function and growth, namely, the estrogen and progesterone receptors. Estrogen is believed to indirectly promote growth, both in utero and in postnatal life, through the activation of *estromedins*, a group of estrogen-induced growth factors, which principally act through the actions of the estrogen receptor in the maternal endometrium [9]. The estrogen receptor comprises of two main isoforms, α and β (ERα and ERβ, respectively), which show significant overall sequence homology and function, and therefore share similar affinities in estradiol binding. Additionally, the estromedins are thought to activate paracrine growth factors, namely IGF-I, which further mediate stromal-epithelial cell interactions to facilitate the growth response, particularly in the later stages of pregnancy [9].

Increases in progesterone hormone levels are also seen in pregnancy, with a subsequent up-regulation of its receptor in the endometrium. This PR comprises of two major isoforms (PRA and PRB), both of which are dependent on estrogen for transcription and expression [10]. More specifically, PRA has been shown to down-regulate transcription of the growth-promoting PRB. In contrast, PRB levels inhibit IGFBP-1 synthesis, with a resultant increase in free IGF-I and -II, thereby asserting a growth-promoting effect [11]. PRB is more abundantly seen in the uterus throughout pregnancy, whereas PRA levels have been seen to increase after the onset of labor [12]. We therefore hypothesize that lower levels of ERα, ERβ, and PRB are contributing factors towards the development of FGR and SGA pregnancies. The principal aims of this study were, therefore, to quantify mRNA expression levels of ERα, ERβ, PR and IGF-I in the placentas of growth-restricted and normal-birth-weight babies in the Swedish population. Additionally, to correlate these expression levels with maternal and infant anthropometric variables for potential uses as markers of growth restriction.

Materials and Methods

Population Characteristics

Placental samples were obtained following informed consent from 33 patients, following delivery during 1997–1998, at the Karolinska University Hospital, Stockholm, Sweden. The samples were divided into two major groups: normal control (NC, n = 14), and growth-restricted (GR, n = 19). For descriptive purposes, the GR group was further divided on the basis of the presence of preeclampsia as follows: intrauterine growth-restricted (IUGR, n = 9), mild preeclampsia with IUGR (MPE, n = 5), and severe preeclampsia with IUGR (SPE, n = 5). The GR samples included babies with IUGR with or without preeclampsia. Tissue specimens were immediately frozen in liquid nitrogen and stored at –70°C for subsequent transport and analysis. Furthermore, maternal height, weight, and gestational age were recorded at the time of admission, prior to delivery. Additionally, mode of delivery was also documented. Archived birth records were consulted for information regarding all anthropometric variables.

Solution Hybridization Analyses of mRNAs

Total Nucleic Acid Purification

Total nucleic acids (TNA) were prepared as described by Sahlin [13]. Briefly, after thawing the tissues were homogenized and digested with proteinase K (Merck, Darmstadt, Germany) in an SDS-containing buffer, followed by subsequent extraction with phenol-chloroform as described by Durnam and Palmiter [14]. The DNA content of the TNA samples was determined by a fluorometric assay at the wavelength 458 nm with Hoechst Dye 33258 [15].

Hybridization Probes

ERα. The probe used for the ERα mRNA determinations was derived from *bcpe1*, a full-length cDNA containing the whole open reading frame of the human estrogen receptor. The cDNA was inserted in a pGEM7zf vector. Restriction of this vector with *Bgl*II allows the synthesis of a 576-bp antisense probe, complementary to the sequence encoding the C-terminal half of the steroid-binding domain (E) and all of domain F [16].

ERβ. The probe used for ERβ mRNA determinations was derived from a pBS plasmid with an insert of a 187-bp *Pvu*II/*Eco*RI fragment corresponding to nucleotides 774–979 in the human ERβ gene [17].

PR. The probe utilized to assess the PR mRNA expression was derived from a full-length cDNA containing the entire open read-

ing frame of the human PR. The cDNA was subsequently inserted into a pGEM3Z vector, which was further restricted with *Bcl*I. The synthesized probe corresponded to nucleotides 2065–2838, which encode the C-terminal part of the ligand-binding domain (E) [18]. Thus, the probe recognizes the PR isoforms that are truncated at the N-terminal.

IGF-I. IGF-I mRNA expression was determined using a probe derived from a 775-bp *Rsa*I-*Eco*RI cDNA fragment of human IGF-I. This fragment was cloned into the *Hinc*II and *Eco*RI sites of a Bluescript KS vector, with subsequent restriction with *Xho*I to synthesize the probe [19].

Hybridization Analysis of mRNA

For measurements of specific mRNA, probes were synthesized in vitro and radiolabeled with ^{35}S-UTP (Amersham, Bucks., UK). The in vitro synthesis of radioactive cRNA was performed as described by Melton et al. [20], using reagents supplied from Promega Biotech (Madison, Wisc., USA). ^{35}S-UTP-labeled cRNA was hybridized (20,000–40,000 cpm per incubation) at 70°C to TNA samples as described by Mathews et al. [21]. Incubations were performed in duplicate in microcentrifuge tubes (Treff AG, Degersheim, Switzerland) in a total volume of 40 μl containing 0.6 mol l^{-1} NaCl, 22 mmol l^{-1} Tris-HCl pH 7.5, 5 mmol l^{-1} EDTA, 0.1% SDS, 0.75 mmol l^{-1} dithiothreitol, and 25% formamide. After overnight incubation under paraffin oil, each sample was treated for 45 min at 37°C with 1 ml RNase buffer containing 0.3 mol l^{-1} NaCl, 10 mmol l^{-1} Tris pH 7.5, 2 mmol l^{-1} EDTA, 40 μg RNase A, 118 units RNase T1 (Boehringer-Mannheim, Mannheim, Germany) and 100 μg calf thymus DNA, to digest non-hybridized RNA. Labeled hybrids protected from RNase digestion were precipitated by addition of 100 μl of 6 mol l^{-1} trichloroacetic acid and collected on filters (Whatman GF/C). The radioactivity on the filters was determined in a liquid scintillation counter and the results were compared with a standard curve of known amounts of in vitro synthesized mRNA complementary to the probe used. Results are expressed as amol (10^{-18}) mRNA/μg DNA in the TNA samples.

Statistical Analysis

Statistical analysis was performed using the Student's t test, Pearson's correlation test, and one-way ANOVA. ANOVA was conducted using the NC group as the dependent factor. Post-hoc significance for ANOVA was evaluated using the Waller-Duncan correction. Values are given as mean ± SEM. A p value of <0.05 was considered as statistically significant.

Ethical Considerations

Ethical approval was obtained from the Northern Stockholm Ethical Committee, Stockholm, Sweden. Written consent was obtained from all patients.

Results

Population Characteristics

The study population was divided into four groups, as mentioned above. Comparisons of maternal weight (kg), height (m), and BMI between these groups showed no statistical significance, though maternal BMI was marginally lower in the SPE group. The placental mRNA expression for the receptors, ERα, ERβ, and PR, and the peptide hormone, IGF-I, are shown in figure 1. Significant differences in IGF-I expression were seen between all groups when compared to the NC ($p < 0.05$, ANOVA). No statistically significant differences were seen with ERα, ERβ, or PR, comparing between the groups. Of interest to note is the decreased expression of ERα, ERβ, PR and IGF-I in the SPE group (fig. 1). Significant differences in modes of delivery were also noted, with the majority of babies being born with acute cesarean sections in the GR groups, as compared to elective cesarean sections and spontaneous vaginal deliveries in the NC group ($p < 0.001$).

Descriptive statistics of maternal and infant anthropometric characteristics, receptor expression levels, and measured hormone levels along with their levels of significance are shown in table 1 (NC and GR groups). As can be seen in the table, birth weight, birth height and

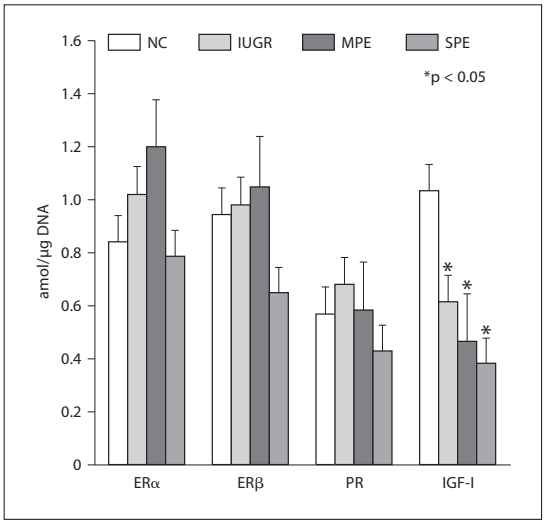

Fig. 1. Relative mRNA expression of placental estrogen receptors (ERα and ERβ), progesterone receptor (PR), and insulin-like growth factor-I (IGF-I). The graph compares the four groups: normal control (NC, n = 14), intrauterine growth restriction (IUGR, n = 9), mild preeclampsia with IUGR (MPE, n = 5), and severe preeclampsia with IUGR (SPE, n = 5). All bars are given with the SEM. The level of significance was set as $p < 0.05$ (ANOVA with post-hoc Waller-Duncan correction).

Table 1. Descriptive statistics of maternal and infant anthropometric variables, along with IGF-I and hormone receptor mRNA levels, comparing the normal controls (NC, n = 14) and the growth-restricted babies (GR, n = 19)

	NC	GR	p value
Maternal height, m	1.65 ± 0.03	1.65 ± 0.02	0.43
Maternal weight, kg	61.63 ± 4.78	60.25 ± 2.01	0.37
Maternal weight at delivery, kg	70.81 ± 3.54	67.41 ± 1.85	0.22
Maternal BMI pre-pregnancy	22.69 ± 1.40	22.27 ± 1.08	0.30
Maternal BMI at delivery	25.96 ± 1.12	24.57 ± 0.56	0.23
Gestational age at delivery, weeks	38.21 ± 0.19	35.05 ± 1.15	0.06
Infant birth weight, kg	3.23 ± 0.09	2.00 ± 0.17	<0.01
Infant birth height, m	0.49 ± 0.04	0.44 ± 0.09	<0.01
ERα, amol/μg DNA	0.84 ± 0.09	0.99 ± 0.11	0.15
ERβ, amol/μg DNA	0.94 ± 0.15	0.90 ± 0.08	0.41
PR, amol/μg DNA	0.57 ± 0.04	0.59 ± 0.06	0.40
IGF-I, amol/μg DNA	1.03 ± 0.09	0.52 ± 0.05	<0.01

For the purposes of statistical analysis, all the intrauterine growth-restricted infants were combined into one group (GR). All means are given with their SEM. All values are given in SI units. Significant differences were seen in infant birth weight, birth height, and IGF-I mRNA levels when comparing the two groups. The level of significance was defined as $p < 0.05$, evaluated by Student's t test.

IGF-I levels were significantly lower in the GR group as compared to the NC group ($p < 0.01$). Additionally, comparisons of mean gestational ages at the time of delivery (in weeks) between the two groups showed a tendency towards a lower mean gestational age in the GR group ($p = 0.06$). No significant differences were seen in the expression levels of the receptors, ERα, ERβ, and PR, comparing the GR group NC group.

Anthropometric Correlations with the ERα, ERβ, PR, and IGF-I

Further analysis using Pearson's correlation showed significant relationships between several of the anthropometric factors within both groups. These can be seen more clearly in table 2. Table 2a, representing the NC, shows significant correlations between placental ERβ expression and maternal weight at delivery ($r = -0.87$, $p < 0.05$) and gestational age (in weeks, $r = 0.50$, $p < 0.05$); placental ERα and PR mRNA levels ($r = 0.70$, $p < 0.05$); IGF-I expression and infant birth height ($r = 0.99$, $p < 0.01$). Of interest to note is that the correlation between maternal weight and placental ERβ mRNA is negative, whereas that of placental IGF-I mRNA and birth height is positive. Furthermore, a positive correlation trend is noted between IGF-I and ERα ($r = 0.52$, $p = 0.07$). Though not significant, a positive correlation was seen between IGF-I expression and the gestational age in weeks ($r = 0.44$, $p = 0.07$). Table 2b shows the correlations within the GR group. Significant positive correlations are seen between maternal BMI at delivery and placental ERα expression ($r = 0.76$, $p < 0.05$); PR expression and infant birth weight ($r = 0.89$, $p < 0.01$), and birth height ($r = 0.83$, $p < 0.01$); gestational age in weeks and PR expression ($r = 0.55$, $p < 0.05$), and IGF-I expression ($r = 0.52$, $p < 0.05$). Significant positive correlations are also seen between IGF-I expression and infant birth weight ($r = 0.75$, $p < 0.05$) and birth height ($r = 0.79$, $p < 0.05$). In addition, placental IGF-I and PR mRNA levels are also positively correlated ($r = 0.77$, $p < 0.05$). Furthermore, positive correlation trends can be seen between ERβ and maternal weight ($r = 0.56$, $p = 0.08$), IGF-I and maternal BMI ($r = 0.52$, $p = 0.08$), and IGF-I and maternal BMI at delivery ($r = 0.55$, $p = 0.06$). No significant correlations were seen between mode of delivery and IGF and hormone receptor expression.

Discussion

The aims of the study were to ascertain the placental expression levels of IGF-I, ERα, ERβ, and PR, comparing NC and GR groups in the *Swedish* population. Furthermore, we correlated these expression levels with anthropometric variables. This is the first study of its kind looking into the associations of placental expression of the

Table 2. Pearson's correlation of maternal and infant anthropometry along with the analyzed placental hormone and receptor mRNA levels within the normal control group (**a**) and the growth-restricted group (**b**)

a Normal control (NC) group

	ERα	ERβ	PR	IGF-I
ERα	–			
ERβ	0.35 (0.28)	–		
PR	**0.02 (0.70)**	0.70 (–0.13)	–	
IGF-I	0.07 (0.52)	0.19 (0.38)	0.95 (0.02)	–
Maternal height	0.17 (0.43)	0.25 (0.35)	0.79 (0.08)	0.48 (0.22)
Maternal weight	0.95 (–0.02)	0.46 (–0.25)	0.18 (–0.43)	0.88 (–0.05)
BMI	0.46 (–0.22)	0.27 (–0.33)	0.13 (–0.43)	0.84 (–0.06)
Delivery weight	0.45 (0.55)	**0.04 (–0.87)**	0.92 (–0.02)	0.92 (0.06)
Delivery BMI	0.98 (0.07)	0.28 (–0.32)	0.16 (–0.41)	0.98 (–0.01)
Gestational age	0.78 (0.08)	**0.04 (0.50)**	0.91 (–0.03)	0.07 (0.44)
Birth weight	0.63 (0.16)	0.20 (0.38)	0.33 (0.29)	0.31 (0.31)
Birth height	0.35 (–0.25)	0.41 (0.25)	0.97 (0.03)	**0.01 (0.99)**

b Growth-restricted (GR) group

	ERα	ERβ	PR	IGF-I
ERα	–			
ERβ	0.15 (0.36)	–		
PR	0.13 (0.37)	0.43 (0.20)	–	
IGF-I	0.21 (0.31)	0.71 (0.09)	0.04 (0.77)	–
Maternal height	0.11 (–0.45)	0.97 (0.02)	0.55 (–0.17)	0.35 (–0.27)
Maternal weight	0.70 (–0.11)	0.08 (0.56)	0.20 (–0.35)	0.95 (0.02)
BMI	0.65 (0.18)	0.44 (0.29)	0.81 (0.06)	0.08 (0.52)
Delivery weight	0.46 (0.23)	0.32 (0.30)	0.61 (–0.15)	0.35 (0.28)
Delivery BMI	0.04 (0.76)	0.34 (0.24)	0.79 (0.07)	0.06 (0.55)
Gestational age	0.11 (0.38)	0.73 (–0.09)	0.02 (0.55)	0.03 (0.52)
Birth weight	0.75 (0.25)	0.30 (–0.29)	0.01 (0.89)	0.03 (0.75)
Birth height	0.61 (0.21)	0.85 (0.08)	**0.01 (0.83)**	**0.04 (0.79)**

The table represents the corresponding p values and the correlation coefficients (r) in parentheses. Levels of significance are defined as $p < 0.05$. Bold values indicate significant correlations.

steroid receptors, ERα, ERβ, and PR, with maternal and infant anthropometry. We have shown significant differences in expression levels of IGF-I, along with significant correlations of receptor expression and both maternal and infant anthropometry, and gestational age at the time of delivery. Previous studies have emphasized the significance of the steroid hormones and their receptors on the regulation of growth factors in endometrial tissue, though none have looked at correlations with birth weight outcomes and anthropometry [9, 22]. Furthermore, we have previously shown similar significant differences in IGF-I levels in a different ethnic group, whereby IGF-I significantly correlated to maternal and infant anthropometry in the *Pakistani* population [23]. Our previous results also emphasize the differences in levels of placental IGF-I with respect to birth weight and height outcomes.

The growth factor, IGF-I, plays a key role in fetal and placental growth and development throughout pregnancy [24]. Our results showed significantly higher levels of IGF-I in the NC group as compared to the GR groups. These results are in keeping with previous findings, where lower IGF-I and IGF-II placental mRNA levels have been associated with babies being born SGA [23]. Ozkan et al. [25] have also shown decreased IGF-I expression in preeclampsia, where IGF-I inhibition is believed to be one of the key mechanisms behind the development

of this clinical problem, though the exact mechanism is still unclear. The differences in expression levels of IGF-I, with lower levels seen in the GR group, could be due to increased fetal hypoxic strain leading to a subsequent down-regulation of the growth factor pathway, modulating cellular and tissue growth in an already 'strained' fetal environment [26]. Allan et al. [27] have emphasized the importance of this fine balance and the physiological and genetic role of IGF-I in cellular and tissue growth and development.

The estrogen receptors play an important role in cellular and tissue growth as well as in pathological conditions, namely preeclampsia and HELLP syndrome (hemolysis, elevated liver enzymes, and low platelets) [28, 29]. These findings outline the regulatory role of the receptor on both cellular development and 'normal' functioning. We found significant correlations of ERα and ERβ expression and maternal weight and BMI in the subgroups at the time of delivery, though, interestingly, the correlation of ERβ with maternal weight at the time of delivery, in the NC group, was a negative one. This suggests that increased maternal weight negatively regulates the expression of ERβ in the human placenta. The pivotal role of these receptors in the pathogenesis of pregnancy-related diseases and outcomes is evident, and we therefore suggest that further trials be conducted to study the mechanisms underlying this relationship.

A second key steroid receptor in the regulatory pathway of normal growth and development in pregnancy is that of the PR. Gao and Tseng [11] have shown the importance of this receptor in regulating IGFBP-1 in human endometrial cells. The PR belongs to a superfamily of ligand-induced transactivators, thereby allowing progesterone to bind to specific DNA sequences, activating gene transcription [30]. This, in turn, regulates the transcription and expression of other growth-promoting factors. Our results showed no significant difference in levels of PR expression between the groups, GR and NC. This may be due to the fact that increases in PR are principally associated with the onset of labor and may thus explain the lack of difference in levels in both patient groups [12, 31]. Interestingly, placental PR mRNA expression levels were significantly correlated to IGF-I mRNA expression, gestational age, and to infant birth height and weight, in the GR group, suggesting that this nuclear receptor may indeed play a role in placental IGF-I regulation and fetoplacental growth. Lower placental expression levels of IGF-I were seen in the GR as compared to NC group in the absence of any significant differences in PR expression between the groups. This further suggests the complex multifactorial relationship of IGF-I expression, independent of steroid hormone expression, in the pathogenesis of growth restriction.

In summary, we have, for the first time, shown significant differences in placental IGF-I expression levels, and significant correlations of the placental expression of the steroid receptors, ERα, ERβ, and PR, with both IGF-I and maternal and infant anthropometry in GR infants. We have also shown significant correlations of hormone receptor expression and IGF-I with gestational age, in both groups. Our data supports previous results of the role of IGF-I in fetoplacental growth [24]. Additionally, we looked at these variables within a population subgroup with the clinical syndrome, preeclampsia. The changes in levels of these receptors and hormones in GR and preeclampsia suggest their role in the pathogenesis of these clinical problems. We have shown that the differences in placental growth factor expression are present irrespective of ethnicity and therefore suggest a common underlying mechanism of growth restriction, irrespective of differences in population genetics. Our results suggest the possible roles of ER and PR expression in the pathogenesis of both fetal growth restriction and preeclampsia, with lower levels contributing to higher likelihoods of disease outcome. Modalities to increase the expression of these receptors, with a subsequent increase in hormone receptor binding and activation of cellular growth pathways, could therefore be used as possible interventions in the prevention of fetal growth restriction.

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IV

Full title: **Placental apoptosis in small for gestational age babies: a comparison between Swedish and Pakistani populations.**

Short title: **Placental apoptosis in small for gestational age babies**

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Key Words: SGA, AGA, Apoptosis, Caspase 3, Caspase 8.

ABSTRACT

Background: Foeto-placental growth is regulated by a complex balance of growth promoting and growth inhibiting factors and hormones, namely the insulin-like growth factors and the intracellular caspase proteins. Changes in the IGF-axis appear to affect this balance, with deficiencies possibly triggering *apoptosis*. ***Aim:*** To ascertain levels of apoptosis in the placenta of infants born small for gestational age (SGA) and appropriate for gestational age (AGA), comparing samples from two population groups, Pakistani and Swedish, in an attempt to better understand the mechanism behind foeto-placental growth restriction. ***Methods:*** Placental samples were taken immediately following delivery in both Karachi and Stockholm. In total 36 samples were included for further analysis (Pakistani: SGA n=12, AGA n=12; Swedish: SGA n=7, AGA n=5). Protein extraction was conducted for cell-death ELISA, and the remaining tissue samples were then paraffin embedded for further immunohistochemical and immunoflourescent analysis, looking at the apoptotic proteins, p53, caspase 8, and caspase 3. Furthermore, we compared maternal and newborn anthropometry between populations. ***Results:*** A higher apoptotic index, for caspase 8 and caspase 3, was seen in the Pakistani samples, as compared to the Swedish samples *(p<0.01)*. Cell death ELISA analysis showed greater apoptotic activity in placenta from the Pakistani population as compared to the Swedish groups ($p<0.05$) as well as increased apoptotic activity was seen in the SGA groups as compared to the AGA groups within each population (ELISA, *p<0.05*). Pakistani mothers were shorter than their Swedish counterparts (*p<0.01*). No differences were seen in p53 levels as assessed by immunohistochemistry. ***Conclusion:*** Increased apoptotic activity in the placenta of the Pakistani population may be associated with decreased foeto-placental growth seen in this population, particularly in babies born SGA. These findings, along with previously published results of the IGF-axis, and birth weight outcomes, suggests that lower IGF levels may be involved in the extracellular triggering of apoptosis, through caspase 8, and may indicate a mechanism behind the development of foeto-placental growth restriction.

INTRODUCTION

Foetal growth restriction (FGR) is a complex problem of pregnancy with many short and long term consequences. These include perinatal morbidity and mortality, and long term health complications, including cardiovascular disease. There are multiple aetiologies underlying this vast clinical problem, though the exact molecular mechanisms are still unknown. FGR further leads to babies being born small for their gestational ages (SGA) affecting an estimated 30 million pregnancies each year (1). Placental, and foetal, growth regulation is a complex process, controlled by genetic, environmental and hormonal factors (2). The hormonal factors include growth hormone axes and their receptors. A key regulatory axis of normal growth is the insulin-like growth factor (IGF) hormone axis, comprised of the insulin-like growth factors (IGF-I and IGF-II), the IGF binding proteins (IGFBPS 1-6), IGFBP proteases, and the IGF-I receptor. Deficiencies, or excesses, of this axis thus affect foetal growth outcomes and viability (3).

Cellular and tissue growth is further regulated by a balance of growth promoting and suppressing factors. Changes in this balance may therefore tip growth in either direction, as can be seen with decreases of IGF-I and IGF-II leading to SGA in pregnancy (4). Furthermore, increases in placental apoptosis have also been seen in pregnancies affected with the clinical syndrome *pre-eclampsia*, which is widely associated with growth inhibition (5). Though the exact mechanism is unknown, placental apoptosis is believed to be a key regulatory element in foeto-placental growth (6). The two major pathways of apoptosis are the *intrinsic* and *extrinsic* pathways. The intrinsic pathway is regulated through intracellular mitochondrial stress, with subsequent activation of the B-cell lymphoma 2 and apoptotic protease activating factor 1 (BCL-2/APAF1) pathway, leading to the nuclear activation of the caspase family of enzymes with subsequent cellular destruction. The extrinsic pathway is stimulated through cell surface tyrosine-kinase receptors with direct downstream activation of the caspase enzymes. Activation of either, or both, of these pathways triggers apoptosis leading to controlled cell and tissue death (7).

A principal regulator of apoptosis is *protein 53* (p53, pro-apoptotic), which plays a role in tumour genesis. Down regulation of the gene results in decreased apoptosis and increased cell and tissue growth (8). Levels of p53 and the activator protein, Bcl-2, can therefore be used as markers to assess the *intrinsic* activation of the apoptotic cascade. In contrast,

the *extrinsic* pathway is mediated through cell surface death receptors, leading to activation of caspase 8 (9). Levels of caspase 8 may therefore be used as markers of the *extrinsic* activation of the apoptotic cascade. Aside from the regulators of apoptosis, certain key *executor proteins* may be seen in increased programmed cell death. Caspase 3 is one particular down-stream executioner protein of importance, as it is activated by both the intrinsic and extrinsic pathways and can be used to differentiate apoptosis from necrosis (10).

The aim of this study was to analyse components of the apoptotic axes, namely p53, caspase 8, and caspase 3, in the placenta of babies born small and appropriate for their gestational ages (SGA and AGA, respectively), in the Pakistani and Swedish populations in an attempt to further understand the mechanisms behind foetal growth restriction.

METHODS

Population Demographics

Following informed and written consent, thirty-six placental samples were taken from two populations immediately after delivery. The samples were then frozen in liquid nitrogen for further storage and transport. Categorization of samples for further analysis was done based on the respective newborn birth weights. Inclusion criteria in the AGA groups included: term delivery, normal birth weights, exclusion of any clinical diseases/syndromes, and normal progression of pregnancy. Inclusion criteria in the SGA groups included all pregnancies that had a birth weight of less than the 10^{th} percentile for their gestational ages (≤ 10^{th} percentile, based on population specific growth charts). Twelve Swedish samples (7 growth restricted, and 5 normal controls/appropriate for gestational age, AGA), and 24 Pakistani samples (12 growth restricted, and 12 normal controls/AGA), were included for further analysis, as described below. Population demographics are as previously described (11, 12). Infant and maternal height, weight, and body mass index (BMI) at delivery were measured.

Cell Death ELISA

Protein extraction and quantification were conducted according to the Bradford assay (Bradford protein assay kit, Bio-Rad Laboratories, CA, USA). Cellular apoptosis was assessed by the detection and quantification of cytoplasmic histone-associated DNA fragments (mono- and oligonucleosomes) by photometric enzyme immunoassay (Cell Death Detection Kit ELISA^{PLUS}, Roche Diagnostics Scandinavia AB). Tissue lysis was performed according to the manufacturer's protocol (20µl sample + 80µl lysis buffer). Following centrifugation, the supernatant was then removed for analysis of cytoplasmic histone-associated DNA fragments. Necrosis was excluded by the negative determination of mono- and oligonucleosomes in the protein extract from tissue samples. All samples were analyzed in triplicates. Results are expressed as *'apoptotic activity'*, an optical absorbance ratio of the absorbance (10^{-3}) of the dead/dying cells divided by the absorbance from the corresponding positive and negative controls (*enrichment factor ratio*, based on manufacturer's protocol).

Immunohistochemical staining

Prior to immunohistochemical analysis, samples were fixed in 4% paraformaldehyde for 24 hours following which they were transferred to 70% ethanol at 4ºC. The samples were then trimmed and routinely embedded in paraffin using an automated processor, sectioned at *5 µm* thickness. Immunohistochemical staining for p53, caspase 8, and caspase 3, was conducted using the BOND-MAX analyser (Leica Microsystems, Germany). Endogenous peroxidase activity was quenched for 10 min at room temperature in methanol containing 1.5% hydrogen peroxide. Antigen retrieval was done in the buffer ERI (citrate-based, pH 5.9-6.1) for 20 minutes at 94°C to 96ºC. The automated analyser was then programmed to run the primary antibody for 30 minutes. The antibody DO-7 (p53; Dako Sweden AB), was used at a dilution of 1:500, the antibody AB61755 (caspase 8; Abcam PLC, UK), was used at a dilution of 1:500, and the antibody JHM62 (caspase 3; Novocastra, UK) was used at a dilution of 1:50. The sections were washed and then incubated with goat anti-rabbit immunoglobulins conjugated to peroxidase-labeled dextran polymer (Dako Sweden AB) for 15 minutes at room temperature. The peroxidase reaction was then developed with 3'3-diaminobenzidine (DAB) and counterstained with haematoxylin, for 10 minutes each, respectively (figure 1).

Figure 1a

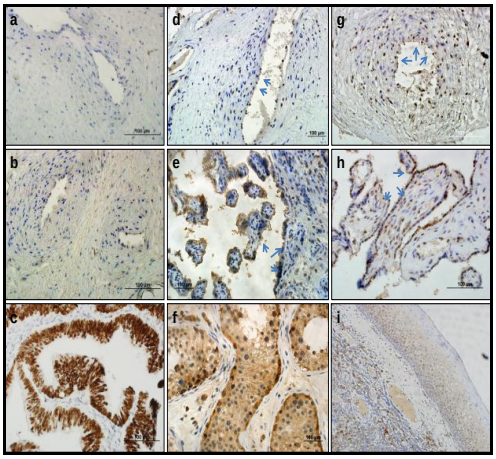

Figure 1b

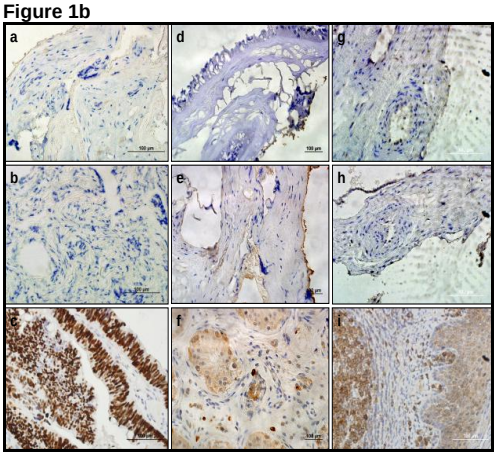

Figure 1. Immunohistochemical staining showing positive (brown) and negative (blue) cells for p53, caspase 8 and caspase 3, comparing small and appropriate for gestational age placental samples (SGA and AGA, respectively).**1a**: *Pakistani population* samples. (*a)* p53 AGA group; (*b)* p53 SGA group; (*c)* p53 positive control; (*d)* Caspase 8 AGA group; (*e)* Caspase 8 SGA group; (*f)* Caspase 8 positive control; *(g)* Caspase 3 AGA group; *(h)* Caspase 3 SGA group; *(i)* Caspase 3 positive control.**1b**: *Swedish population* samples. (*a)* p53 AGA group; (*b)* p53 SGA group; (*c)* p53 positive control; (*d)* Caspase 8 AGA group; (*e)* Caspase 8 SGA group; (*f)* Caspase 8 positive control; *(g)* Caspase 3 AGA group; *(h)* Caspase 3 SGA group; *(i)* Caspase 3 positive control. As can be seen in the figures, p53 stained negatively for both populations. Furthermore, relatively more positive cells were seen in the SGA group as compared to the AGA group for both caspase 8 and caspase 3. The Pakistani placental samples showed a higher apoptotic index as compared to the Swedish placental samples ($p < 0.01$).

Image analysis and Scoring

Ten separate fields in each of three nonadjacent sections from each sample were examined for staining employing a Leica microscope (40X magnification; Leica Microsystems GmBH, Wetzlar, Germany) equipped with a Sony digital video camera (Diagnostic Instruments, Inc., Sony, Japan) interfaced to a computer. For the purposes of accurate image analysis, immunostained cells were manually evaluated by two blinded observers (S.A & E.S) to the groups. A semi-quantitative three scale manual scoring system was used: (-) negative, (+) faint, and (++) moderate to strong positive staining. Counts from both observers were then averaged and are as described below. The results are expressed as an *apoptotic index* (relative ratio of the apoptotic cells to the total number of cells).

Ethical considerations

All placental samples were obtained following ethical approval from the Northern Stockholm Ethical Committee, Stockholm, Sweden, and the Aga Khan University Ethical Review Committee, Karachi, Pakistan. Furthermore, written consent was obtained from all mothers prior to sample acquisition.

Statistical analysis

Statistical comparisons between two groups were done using Student's t-test. Further comparisons were done using the Kruskall-Wallis one way analysis of variance (ANOVA), and the Holm-Sidak post hoc test. Statistical significance was taken as a *p-value* of less than 5% ($p < 0.05$).

RESULTS

Population demographics

The study group consisted of samples from both the Swedish and Pakistani populations, as previously described (11, 12). In total 24 Pakistani samples and 12 Swedish samples were included in the study (table 1). Swedish mothers were significantly taller than the Pakistani mothers in the appropriate for gestational age (AGA) group ($p < 0.01$; table 1a). Swedish mothers were also both significantly taller and weighed more than their Pakistani counterparts, in the SGA group ($p < 0.01$). Furthermore, differences, though not

statistically significant, were seen in maternal body mass index (BMI, $p = 0.06$) and infant weight ($p = 0.06$) comparing populations in the SGA groups (table 1b). Maternal body mass index was also significantly lower in the mothers of the SGA group, within each respective population ($p < 0.01$, *ANOVA*).

Table 1.

1a AGA		**Pakistani** (±SEM)	**Swedish** (±SEM)	p-value
Newborn	Height (m)	0.49 (0.01)	0.49 (0.04)	0.82
	Weight (kg)	3.15 (0.09)	3.23 (0.09)	0.38
	Body Mass Index	12.98 (0.19)	13.03 (1.00)	0.89
Maternal	Height (m)	1.51 (0.01)	1.65 (0.03)	***<0.01***
	Weight (kg)	55.51 (1.05)	70.81 (3.54)	0.14
	Body Mass Index	23,28 (0.41)	25.96 (1.12)	0.97

1b SGA		**Pakistani** (±SEM)	**Swedish** (±SEM)	p-value
Newborn	Height (m)	0.41 (0.01)	0.44 (0.09)	0.31
	Weight (kg)	1.78 (0.09)	2.00 (0.17)	0.06
	Body Mass Index	10.39 (0.46)	10.03 (0.68)	0.74
Maternal	Height (m)	1.51 (0.01)	1.66 (0.03)	***<0.01***
	Weight (kg)	47.41 (0.92)	60.18 (2.01)	***<0.01***
	Body Mass Index	20.80 (0.39)	22.27 (0.56)	0.06

Legend to Table 1. Population demographics showing newborn and maternal height, weight, and body mass index (BMI) at delivery. (1a) comparison of the anthropometric variables in the appropriate for gestational age (AGA) groups in the Pakistani (n=12) and Swedish (n=5) populations. (1b) comparison of anthropometric variables between small for gestational age (SGA) groups in both populations (Pakistani: n = 12; Swedish n = 7). The corresponding p-values are given (Student's t-test). A p-value of <0.05 is considered statistically significant.

Cell death ELISA

Apoptotic activity was significantly higher in the Pakistani samples, as compared to the Swedish placental samples ($p < 0.05$, figure 2). Further analysis comparing groups showed a significantly higher level of apoptosis in the Pakistani SGA placental samples as compared to the Pakistani AGA group ($p < 0.01$), the Swedish AGA group ($p < 0.05$), and the Swedish growth restricted placental samples ($p < 0.05$). Furthermore, apoptotic activity was significantly higher in the Pakistani control (AGA) group as compared to the Swedish control (AGA) group ($p < 0.05$). There was, however, no significant difference in apoptotic activity comparing the Swedish AGA and SGA groups ($p = 0.16$).

Figure 2

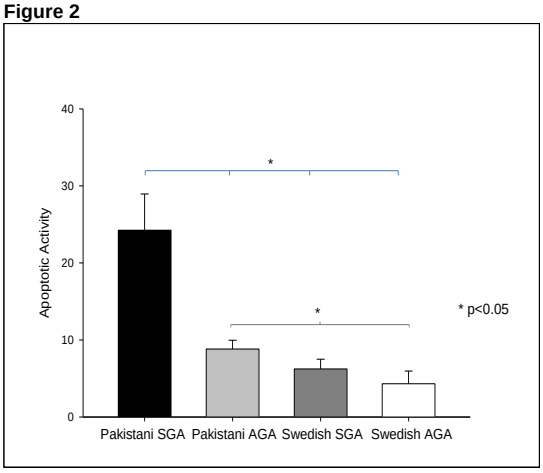

Figure 2. Comparison between apoptotic activity (enrichment factor) in placenta as assessed by cell death ELISA. The histogram shows apoptotic activity in the SGA (Pakistani n = 12; Swedish n = 7) and AGA (Pakistani n = 12; Swedish n = 5) groups, comparing between populations (*$p < 0.05$). All charts represent means with their standard errors (SEM).

Immunohistochemical analysis of apoptotic markers

The apoptotic index for p53 was zero in both populations, and comparing the SGA and AGA groups. Immunohistochemical staining for p53, caspase 8, and caspase 3 can be seen in figure 1, showing the control group (AGA), the growth restricted group (SGA), and the positive controls, for each respective marker. Caspase 8 and caspase 3 showed significantly higher apoptotic indices in the Pakistani population (SGA and AGA), as compared to the Swedish population. This can be more clearly seen in figures 3a and 3b, respectively ($p < 0.01$). The figures indicate higher levels of apoptosis in the Pakistani population, as well as higher levels in the SGA groups, as compared to the AGA groups, though these difference were not statistically significant.

Figure 3.

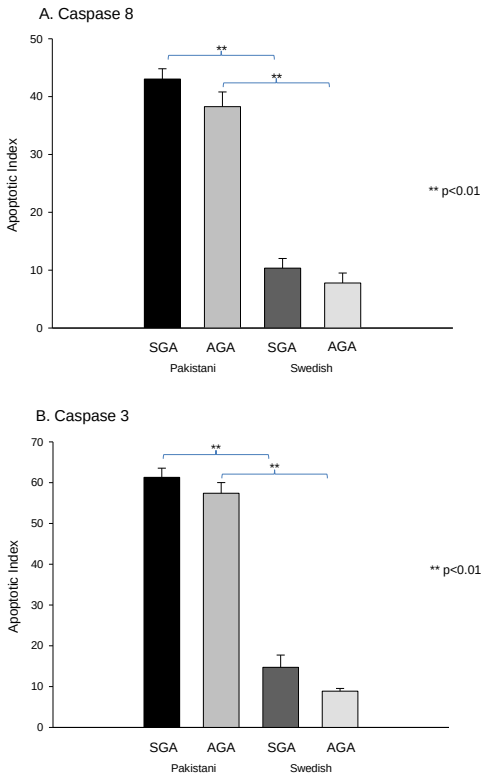

Figure 3. Histograms representing the apoptotic index for caspase 8 and caspase 3 (relative degree of apoptosis) in the SGA and AGA groups (Pakistani and Swedish populations). Figure 3a. Capsase 8. Figure 3b. Caspase 3. Significant differences were seen between the two populations (***p < 0.01*). All indices are represented with their standard errors (SEM).

DISCUSSION

This is one of the first studies of its kind comparing levels of placental apoptosis in the placenta of two ethnically different population subgroups. The aims of this study were to quantify levels of placental apoptosis comparing growth outcomes in Pakistani and Swedish population sub-groups. Furthermore, we attempted to ascertain which apoptotic pathway was triggered in the placenta by looking at two key initiators of apoptosis, p53 and caspase 8. Our results show increases in apoptotic activity in the placenta of babies born SGA, as well as higher levels of apoptotic activity in the placenta from the Pakistani population, as compared to the Swedish population. Furthermore, we have shown that the *extrinsic pathway* of apoptosis appears to be the principal pathway of apoptosis in the placenta.

The difference in apoptotic activity between populations is highly interesting and may suggest a relationship between maternal size and levels of placental apoptotic activity in an attempt to control foeto-placental growth (13). The smaller Pakistani mothers, on average, give birth to smaller babies, and this may, hypothetically, be a result of increased placental apoptosis. These results suggest the role of population genetics and environmental factors in birth weight outcomes, particularly that of *maternal constraint*. Gluckman, *et al*., describe maternal constraint as 'a set of poorly defined processes by which maternal and uteroplacental factors act to limit the growth of the foetus, presumably by limiting nutrient availability and/or the metabolic-hormonal drive to grow' (14). They further associate this clinical phenomenon to smaller maternal size, as was seen in the SGA groups of both populations. Smaller mothers amount to smaller pelvis size and structure and thus cannot accommodate larger babies, possibly controlling foeto-placental growth and size. Smaller parents are also genetically more likely to have smaller children, though our results showed no differences in anthropometric parameters of babies born in the AGA groups comparing both populations, suggesting the importance of other factors beyond population genetics influencing foeto-placental growth (15). We have also shown that placental apoptosis is principally controlled through the extrinsic pathway, through the activation of caspase 8. The significantly increased apoptotic index in the Pakistani population may indicate that these placentas have a higher baseline level of apoptosis, particularly towards the end of pregnancy. They may also indicate differences in the handling of tissues from both populations, whereby small delays in the appropriate transporting and storing of tissues influences levels of apoptosis and tissue necrosis.

Barrio, *et al.*, amongst others, has shown significantly higher levels of caspase 3, the apoptotic executioner, in placenta of SGA babies (16, 17). We did not, however, find any significant differences in our population groups.. Furthermore, despite the lack of difference in caspase 3 positive cells in our population subgroups, we have shown significant differences in levels of DNA histone complexes in our subgroups, through the use of the apoptosis ELISA. These results indicate

the final products of apoptosis and suggest higher levels of apoptosis in the SGA groups as compared to the AGA groups, and in the Pakistani subgroup in comparison to the Swedish subgroup. There is little data on the direct comparison of placental apoptosis in different populations, suggesting that the significant differences we have shown in our sub-groups may be due to ethnic factors. As mentioned previously, comparisons of the normal control groups (AGA) between populations showed no difference, indicating that genetics may not play a significant role in the pathogenesis of FGR. We appreciate the limitations our small sample size may pose and suggest further studies be conducted to compare apoptosis across populations.

Protein 53 (p53) is a well known pro-apoptotic tumour suppressor and has been shown to promote growth arrest and cellular senescence (18). Furthermore, it has been shown to also block angiogenesis, restricting tissue growth. Increases in p53 gene expression have further been associated with the development of gestational trophoblastic disease and trophoblast apoptosis in association with foetal growth restriction (19, 20). P53 may thus act as an intrinsic initiator of apoptosis in higher risk pregnancies. We did not, however, find any p53 positive cells in our subgroups, suggesting that the placental activation of apoptosis is principally regulated through the extrinsic pathway, possibly through decreased expression of growth promoting factors, as has previously been described by us (11, 21).

The IGF-axis plays a key role in foeto-plaental growth, with down-regulation resulting in increases in placental apoptosis as a possible cause for intrauterine growth restriction (22). Furthermore, IGF-I has been previously shown to have anti-apoptotic activity through the activation of an intracellular tyrosine kinase receptor, playing a key role in cell and tissue growth and modulation (23).We have previously shown similar results of the effects of decreased IGF-I and IGF-II mRNA expression, along with decreased IGF-I protein levels, correlating to low birth weight (21, 24). The increased apoptotic activity in the placenta of SGA babies, seen in our results, suggests that this increase may be secondary to the down-regulation of the IGF-axis, thereby resulting in decreased growth and subsequent growth restriction. Furthermore, this fine balance between growth promoting and suppressing factors appears to be associated with maternal anthropometry, re-emphasising the importance of establishing maternal pregnancy growth charts to aid in the timely diagnosis of IUGR. This is of particular importance in areas where ethnic and environmental factors appear to play a larger role in the pathogenesis of this devastating clinical problem. In developing countries, such as Pakistan, where advanced radiological diagnostic tools are scarcely available, early screening and identification of high-risk pregnancies through clinical screening may allow for timely intervention. This, in turn, may help break this vicious cycle of poor growth, aiding towards a healthier population. Finally, maternal blood samples screening for biomarkers of foeto-placental growth (such as placental growth factors) may be an additional useful screening tool, globally, to help improve the diagnosis of IUGR and pregnancy related disorders affecting foeto-placental growth, such as pre-eclampsia (25).

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