Placental Restriction and Endocrine Control of Postnatal Growth

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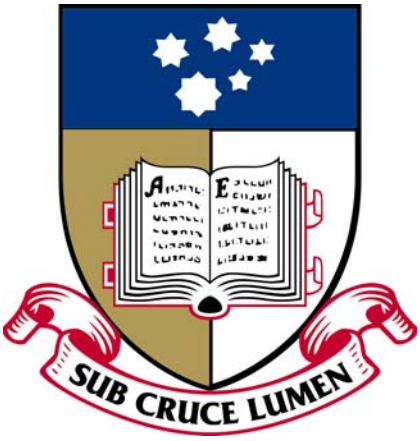

For my wife Zoe and my family and friends

TABLE OF CONTENTS

LIST OF TABLES AND FIGURES

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STATEMENT OF ORIGINALITY AND AUTHENTICITY

I declare that this thesis contains no material which has been accepted for the award of any other degree or diploma in any university and or tertiary institution and, to the best of my knowledge and belief, the thesis contains no material previously published or written by another person, except where due reference is made in the text of the thesis.

I give consent to this copy of my thesis, when deposited in the University Library, being available for loan and photocopying if accepted for the award of the degree.

Signed,

Miles J De Blasio,

Date:

TABLE OF ABBREVIATIONS AND BIOCHEMICAL NAMES

α-aN	Plasma α-amino nitrogen concentration
$\alpha\text{-aN}_{60'\text{-}120'}$	Plasma α-amino nitrogen concentration during the second hour of the hyperinsulinaemic euglycaemic clamp
AGA	Appropriate for gestational age
AGR	Absolute growth rate
ANOVA	Analysis of Variance (statistical test)
BMI	Body mass index
CFGR	Current fractional growth rate
CO_2	Carbon dioxide
CRL	Crown-rump length
CVD	Cardiovascular disease
EDTA	Ethylenediamine tetra-acetic acid
ELISA	Enzyme-linked immuno-sorbent Assay
FFA	Free fatty acid concentration
$FFA_{60'\text{-}120'}$	Free fatty acid concentration during the second hour of the hyperinsulinaemic euglycaemic clamp
GH	Growth hormone
GIR	Glucose infusion rate
$GIR_{60'\text{-}120'}$	Glucose infusion rate during the second hour of the hyperinsulinaemic euglycaemic clamp
$GIR_{70\text{-}130'}$	Glucose infusion rate during the second hour of the hyper-IGF-I euglycaemic clamp
GLUT4	Glucose transporter protein 4
HEAAC	Hyperinsulinaemic euglycaemic aminoacidaemic clamp
HEC	Hyperinsulinaemic euglycaemic clamp
HIEC	Hyper-IGF-I euglycaemic clamp
HPAA	Hypothalamo-pituitary adrenal axis
HPLC	High performance liquid chromatography
HPTA	Hypothalamo-pituitary thyroid axis

i.m.	Intramuscular
ID	Internal diameter
IGFBP	Insulin-like growth factor binding protein
IGF-I	Insulin-like growth factor-I
IGF-II	Insulin-like growth factor-II
IGF-IR	Type 1 Insulin-like growth factor receptor
IGF-IIR	Type 2 Insulin-like growth factor receptor
IgG	Immunoglobulin G
IMVS	Institute of Medical and Veterinary Science
IR	Insulin receptor
IRS	Insulin receptor substrate
IUGR	Intrauterine growth restriction (or retardation)
IVGTT	Intravenous glucose tolerance test
kda	Kilodalton
kg	Kilogram
KHz	Kilohertz
M	Molar
Man-6-P	Mannose-6-Phosphate
mCi	Milli Curie
meq	Milli Equivalent
mg	Milligram
ml	Milli Litre
mM	Millimolar
mRNA	Messenger ribonucleic acid
ms	Millisecond
mU	Milli Unit
NFGR	Neonatal fractional growth rate
NIDDM	Non-insulin dependent diabetes mellitus
nmol	Nanomole
NQS	β-Napthoquinone sulphonate
O_2	Oxygen
^oC	Degrees centigrade
OH	Hydroxyl

pg	Picogram
PI	Ponderal index
pO_2	Partial pressure of oxygen
PR	Placental restriction or placentally restricted
rhIGF-I	Human recombinant insulin-like growth factor-I
RIA	Radioimmunoassay
SD	Standard deviation
SEM	Standard error of the mean
SGA	Small for gestational age
SSGIR	Steady state glucose infusion rate
TBG	Thyroxine-binding globulin
TG	Triglyceride
TH	Thyroid hormone
TPO	Thyroid peroxidase
TRH	Thyrotropin-releasing hormone
TSH	Thyroid (thyrotropin)-stimulating hormone
TTR	Transthyrectin
μCi	Micro Curie
μg	Microgram
μl	Microlitre
%CV	Coefficient of variance
L-thyroxine (T4)	L-3,5,3',5'-tetraiodothyronine
L-triiodothyronine (T_3)	L-3,5,3'-triiodothyronine
3-(^{3}H)-Glucose	Carbon 3 tritiated labelled glucose
^3H_2O	Tritiated labelled water

PAPERS ARISING FROM THIS THESIS

De Blasio MJ, Walker MR, Gatford KL, Robinson JS, Owens JA. (2004). Placental restriction of fetal growth reduces size at birth and increases postnatal growth and adiposity in the young lamb. *'Accepted American Journal of Physiology – Regulatory, Integrative and Comparative Physiology'.*

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De Blasio MJ, Walker MR, Gatford KL, Robinson JS, Owens JA. (2004). Placental restriction alters circulating thyroid hormone in the young lamb postnatally. *'Accepted American Journal of Physiology – Regulatory, Integrative and Comparative Physiology'.*

RELATED PUBLICATIONS

Gatford, K. L., E. M. Wintour, **M. J. De Blasio,** J. A. Owens, M. Dodic, (2000). Differential timing for programming of glucose homeostasis, sensitivity to insulin and blood pressure by in utero exposure to dexamethasone in sheep. Clin Sci **98(5):** 553-560.

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Gatford, K. L., **M. J. De Blasio,** P. Thavaneswaran, S. Fielke, I. C. McMillen, J. S. Robinson, and J. A. Owens. (2004). Postnatal ontogeny of glucose homeostasis and insulin action in the sheep. Am J Physiol. Accepted December 8 2003, first published online February 3 2004 10.1152/ajpendo.00340.2003.

OTHER PUBLICATIONS

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Gatford, K. L., J. E. Ekert, K. Blackmore, **M. J. De Blasio,** J. M. Boyce, J. A. Owens, R. G. Campbell, P. C. Owens. (2003). Variable maternal nutrition and growth hormone treatment in the second quarter of pregnancy in pigs alter semitendinosus muscle in adolescent progeny. *Br J Nutr* **90:** 1-12.

ABSTRACT

Intrauterine Growth Restriction (IUGR) is evident in infants born with a reduced weight or length, and/or increased thinness for gestational age. IUGR is associated with altered postnatal growth and regulation, due to unknown mechanisms. Much clinical IUGR results from the reduced delivery of essential substrates (oxygen and nutrients) to the fetus, due to either maternal or placental limitations. Catch-up growth (accelerated rate of growth in absolute or fractional terms) occurs in the majority of IUGR infants, and returns an infant to their predetermined growth curve. IUGR is associated with increased risks of morbidity and mortality in the perinatal period, and with a reduced final adult stature and increased risk of adult onset diseases, particularly diabetes and cardiovascular disease. Catch-up growth after IUGR predicts improved health in terms of reduced hospital visits in infants and children, and an increased final adult stature but also predicts an increased risk of developing obesity, as well as diabetes and cardiovascular disease. The underlying mechanisms for catch-up growth may contribute to this range of outcomes in later life, but are poorly understood. Studies in IUGR infants have demonstrated increased absolute and/or fractional growth rates following birth, termed catch-up growth, in the presence of reduced or normal plasma concentrations of the thyroid hormones and major anabolic hormones (insulin and/or IGF-I). This suggests that increased sensitivity to, rather than increased production of insulin, IGF-I and thyroid hormone, causes catch-up growth following IUGR. We therefore hypothesised that placental restriction of fetal growth would reduce size at birth and increase postnatal growth and adiposity in association with increased metabolic sensitivity to insulin, IGFs and thyroid hormones. This study has

shown that the placentally restricted (PR) lamb has a reduced size at birth in terms of soft and skeletal tissues, has increased rates of growth postnatally, and has increased adiposity by six weeks of age. We have also shown that PR of fetal growth in the sheep did not alter gestational age at delivery, but reduced survival rate. PR lambs demonstrated catch-up growth in most parameters by 30 days of age and increased adiposity at six weeks of age compared to the control lambs. Placental restriction increased insulin and IGF sensitivity of circulating free fatty acids, which in turn, predicts increased adiposity. Neonatal catch-up growth after fetal growth restriction was substantially predicted by both abundance of, and metabolic sensitivity to insulin, suggesting increased insulin action as an underlying cause. Catch-up growth occurs in the neonate despite reduced concentrations of fasting plasma IGFs, along with increased IGF sensitivity of free fatty acid metabolism and adiposity. Plasma TH concentrations predicted growth of soft and skeletal tissue in lambs during early postnatal life, particularly in those undergoing catch-up growth following PR. Therefore neonatal catch-up growth after IUGR is associated with increased sensitivity to both insulin and IGFs, particularly of circulating free fatty acids, and appears to occur to the extent allowed by the prevailing abundance of these hormones and of thyroid hormones. If this altered endocrine state persists, increased adiposity and its subsequent amplification may contribute to the development of obesity, and related adverse metabolic and cardiovascular outcomes in adult life.

Chapter 1

INTRODUCTION

1.1 Intrauterine Growth Restriction (overview)

Intrauterine Growth Restriction (IUGR) is evident in infants by definition as reduced weight, reduced length, and/or increased thinness at birth for a given gestational age, and is associated with altered postnatal growth ([1]Chernausek, 1996), due to as yet unknown mechanisms. It is estimated that at least 30 million infants worldwide are born every year at term with a low birth weight resulting from IUGR, representing approximately 24% (ranging from 9.4% in China to 54.2% in India) of all newborns in developing countries ([2]WHO, 1995, [3]de Onis*, et al.*, 1998). Overall, nearly 75% of all affected newborns are born in Asia, with 20% in Africa, and about 5% in Latin America ([3]de Onis*, et al.*, 1998). Although some of these may be healthy infants who are small due to genetic constraints, a large proportion of newborns suffer from some degree of intrauterine growth retardation ([3]de Onis*, et al.*, 1998). Most of the infants born after IUGR have experienced a reduction in the delivery of essential substrates (eg. oxygen and nutrients from the blood) as a fetus due to either maternal or placental limitations ([4]Owens*, et al.*, 1986, [5]Owens*, et al.*, 1987). IUGR is associated with substantially increased risks of morbidity and mortality in the perinatal period, which can progress to other adult onset diseases ([6]Barker, 1991). It is also associated with a reduction in final adult stature ([7]Karlberg*, et al.*, 1995), which varies depending on the extent of accelerated growth, termed catch-up growth, which occurs in the first few months of life in the IUGR infant. There is also increasing evidence that catch-up growth itself is also an independent risk factor for adult onset diseases, separate from that associated with small size at birth due to IUGR ([8]Ong*, et al.*, 2000, [9]Eriksson*, et al.*, 1999,

[10]Forsén, *et al.*, 1999). Understanding the underlying causes of catch-up growth after IUGR may help determine why it adds to the risk of developing diseases later in life.

1.2 "Catch-up" growth after IUGR (overview)

Infants born small due to IUGR, undergo increased rates of growth after birth, termed catch-up growth ([7]Karlberg, *et al.*, 1995). Catch-up growth is commonly evident in infants or children born following a transient period of growth inhibition ([11]Prader, *et al.*, 1963). This phase of abnormally rapid growth can continue until the child has "caught up" to their normal growth curve or to the size of their normal counterparts. Catch-up growth following IUGR occurs predominantly during the first year of life. Studies have shown that catch-up growth in terms of weight occurs in the majority (86%) of IUGR infants, begins as early as 2 weeks and is largely complete by 5 months of age, with catch-up growth in terms of height evident by 6 to 12 months of age ([7]Karlberg, *et al.*, 1995, [12]Hokken-Koelega, *et al.*, 1995). Some infants (~15%) do not catch-up however and may have a reduced final adult stature, while those who do catch-up, may have increased adiposity as measured by skin fold thickness and waist circumference ([8]Ong, *et al.*, 2000, [13]Albertsson Wikland, *et al.*, 1998). Children who showed catch-up growth in terms of weight and length between zero and two years of age are fatter and have more central fat distribution at five years (body mass index, percentage of body fat and waist circumference) than those children that did not catch-up ([8]Ong, *et al.*, 2000). Catch-up growth and increased adiposity is most evident in developing countries that are increasingly adopting a high fat content Western diet ([14]Law, 2001). Changing economic

circumstances and lifestyles in developing countries may be increasing the tendency towards rapid postnatal weight gain in their population. In developed countries, the incidence of obesity and type 2 diabetes already is high and increasing, and the individuals who appear to be at the greatest risk are those who are born small after intrauterine growth restriction, have increased postnatal growth and grow up in a society which is increasingly characterised by low activity levels and diets high in fat ([14]Law, 2001). Understanding why infants that are born small undergo catch-up growth following IUGR and tend to have increased adiposity may enable the development of measures to prevent or ameliorate these adverse consequences including the development of related diseases in adult life. The mechanisms responsible for neonatal catch-up growth after IUGR are unknown, but potentially may involve alterations in nutrient intake and utilisation for growth and energy production, energy expenditure, and in the activity of the major endocrine and neuro-endocrine axes regulating these processes and neonatal growth. Currently we have limited insight into how catch-up growth occurs and the contributions if any of these potential mechanisms. Before reviewing the present understanding of this, it is pertinent to firstly review how fetal growth occurs and is controlled, and the risk factors associated with, and mechanisms leading to IUGR, before addressing postnatal growth and its control. Finally I will summarise our current understanding of the mechanisms underlying catch-up growth.

1.3 Fetal growth and IUGR

1.3.1 Brief overview of fetal growth in humans

The human embryonic phase of development occurs during the first 8 weeks of gestation and the fetal phase of development continues after this (week 9) until term ([15]Kuller*, et al.*, 1996). Embryonic development and morphogenesis require cellular interactions with the microenvironment. The growth of the individual begins as a single fertilised cell that differentiates into more than 200 different cell types. By approximately 4-5 days after fertilization, the embryo (blastocyst) has differentiated into two distinct cell types: the inner cell mass which will develop into the fetus and eventually become the newborn, and the outer cell mass or trophectoderm, which will develop into the placenta and external membranes ([15]Kuller*, et al.*, 1996). The blastocyst then implants itself to the endometrium and begins to grow. Fetal growth occurs through cellular proliferation or hyperplasia, and hypertrophy, with concomitant apoptosis and migration of cells to ensure tissue remodelling as appropriate, and accompanied by the process of differentiation ([16]Garnica*, et al.*, 1996). Fetal growth, in terms of increasing mass or size, occurs largely in the second half of gestation in mammalian species, whereas placental growth in terms of mass occurs predominantly in the first half of pregnancy. There is extensive remodelling of the placenta in the second half of gestation however, to increase its functional capacity to meet fetal demand for nutrients ([17]Robinson*, et al.*, 1996). The rate of growth of the fetus is regulated primarily by the availability of oxygen and nutrients along with the modulating effects of fetal hormones and growth factors on the utilisation of these substrates for metabolism and growth ([18]Robinson*, et al.*, 1985). The normal human fetus adds 5 grams of weight per day at 15

weeks and up to 30-35 grams of weight per day at 34 weeks, and then down to zero or even weight loss at 42 weeks of gestation ([19]Styne, 1998). The caloric requirements of a human fetus are approximately 95 kcal/kg/day of which about 40 kcal are for growth and the remainder are oxidised for energy production ([20]Milner*, et al.*, 1996). Therefore at any given gestational age, normal intrauterine growth will occur only if there are sufficient nutrients to meet the needs of oxidation and tissue growth ([21]Fowden, 1995).

1.3.2 Factors regulating fetal growth

Fetal growth rate is characterised by measurement of a range of anthropometric measures of size at birth such as birth weight, length, and head width and circumference. These variables reflect growth of various organs and systems of the body. The rate and pattern of fetal growth is determined by the fetal genome and the availability of nutrients for growth ([22]Owens*, et al.*, 1989). The mother and her environment, and the placenta, are major determinants of the fetal growth rate and the extent to which it can occur to its full potential. The placenta supplies the fetus with substrates from the mother but competes with the fetus for her oxygen and nutrients ([17]Robinson*, et al.*, 1996). Fetal growth and development is also determined by the interaction between the fetal neural and endocrine regulatory systems and the supply of oxygen and nutrients from the placenta ([22]Owens*, et al.*, 1989). The major substrates for fetal growth are oxygen, glucose, lactate and amino acids, with other essential substrates including lipids, ketone bodies and a range of micronutrients ([22]Owens*, et al.*, 1989). These are delivered to the fetus mainly by the placenta, which is a specialised organ of exchange that plays a key role in the nutrition of the fetus

by mediating the transport of nutrients and metabolic wastes across the barrier, while separating the maternal and fetal compartments ([16]Garnica*, et al.*, 1996). The efficiency of oxygen and substrate transfer by the placenta is determined by its surface area, vascularity, permeability ([4]Owens*, et al.*, 1986, [5]Owens*, et al.*, 1987), uterine and umbilical blood flow rates, the activity of various substrate transport mechanisms (active, passive, facilitated), placental consumption, and the placental production of these substrates ([23]Hay Jr*, et al.*, 1985), most of which increase throughout gestation. The placenta develops from a highly vascularized membrane known as the chorion that attaches to discrete or diffuse areas of the epithelium of the uterine mucosa. The process of placentation in mammals involves the formation of a fetal-maternal interface ([24]Lim*, et al.*, 1996). In the human, a hemochorial placenta forms in which the fetal trophoblasts from chorionic villi come into contact with the maternal circulation ([24]Lim*, et al.*, 1996). Placental growth is rapid during early gestation and then continues at a slower rate until term in humans or is completed before the end of gestation in other species, including ruminants ([20]Milner*, et al.*, 1996, [25]Hay Jr, 1996).

While the environment of the developing fetus is the major influence on its growth and development, genetics also play a part. Thirty-eight percent of the variation of birth weight in humans has been attributed to genetic factors of which about half of the remaining 62% was accounted for by maternal factors ([19]Styne, 1998). Multiple gene loci contribute to the birth weight of the normal fetus, with the maternal genotype and environment being most influential ([20]Milner*, et al.*, 1996).

The major endocrine and neuro-endocrine axes regulating fetal growth include insulin, thyroid hormones, insulin-like growth factors (IGFs), catecholamines and glucocorticoids ([26]Fowden*, et al.*, 2001). In fetal life, insulin, the IGFs and thyroid hormones have major roles in promoting tissue accretion, while the latter together with thyroid hormones and glucocorticoids, are important regulators of differentiation ([27]Fowden*, et al.*, 1995). The insulin-like growth factors regulate growth and development by acting both locally and systemically to promote growth by metabolic and non-metabolic mechanisms ([26]Fowden*, et al.*, 2001, [28]Han, 1996). Catecholamines influence and restrict fetal growth, by redistributing cardiac output away from growing tissues ([29]Hoet, 1999) and reducing the circulating concentrations of insulin and IGF-I, while increasing the levels of IGF binding proteins and hence decreasing the effects of these major anabolic hormones ([30]Hooper*, et al.*, 1994, [31]Bassett*, et al.*, 1998). Thyroid hormones have an important role in fetal growth and development by affecting both tissue accretion and differentiation in the fetus by a combination of metabolic and non-metabolic mechanisms ([32]Fowden, 1995). Cortisol appears to act directly on cells of the body to alter gene transcription or post-translational processing of the gene products, but may also initiate the transition from the fetal to the adult modes of growth regulation by switching from IGF-II to IGF-I gene expression in the fetal liver ([32]Fowden, 1995). The rates of production and hence activity of these growth regulatory hormones within the fetus are regulated by and hence mediate the influence of substrate availability on fetal metabolism and growth ([17]Robinson*, et al.*, 1996, [20]Milner*, et al.*, 1996, [33]Robinson*, et al.*, 1995, [34]Jackson, 1996).

1.3.3 Definition and consequences of IUGR in humans

The classic definition of intrauterine growth retardation or restriction (IUGR) is a birth weight below the 10th percentile (or below 2500g for a term baby in the United States) ([19]Styne, 1998). The indicator of IUGR most commonly used is low birth weight ([35]Kramer*, et al.*, 1990, [36]Buehler*, et al.*, 1987, [37]Starfield*, et al.*, 1982) followed by short stature ([38]Leger*, et al.*, 1997, [39]Leger*, et al.*, 1997, [40]Paz*, et al.*, 1993). Most human IUGR is also asymmetrical in nature, where the head is spared compared to the body. Thus in asymmetrical or disproportionate IUGR, infants are short and have a reduced head circumference or have relatively normal length and head circumference for gestational age, but are thin with low weight to length ratio ([41]Kramer, 1987). These disproportionate IUGR infants undergo increased postnatal growth and suffer from less severe cognitive deficits than those that are proportionally small ([41]Kramer, 1987).

1.3.3.1 Short term consequences of IUGR

IUGR is associated with increased morbidity and mortality in the short and long term and hence is a very costly condition in emotional, social and financial terms ([19]Styne, 1998). The immediate and short-term consequences of IUGR in infants have been recently reviewed ([42]Ashworth, 1998, [43]Greenwood*, et al.*, 2003, [44]Kanaka-Gantenbein*, et al.*, 2003). The risks of neonatal and postnatal morbidity and mortality are approximately 11% higher in low birth weight singleton infants, compared with normal birth weight singleton infants ([36]Buehler*, et al.*, 1987, [37]Starfield*, et al.*, 1982, [45]Platt*, et al.*, 1995, [46]Erickson*,*

et al., 1982). The risks of morbidity and mortality also appear to increase with increasing severity of IUGR ([35]Kramer*, et al.*, 1990). Infants born with IUGR have increased risks of stillbirth, fetal distress, in-hospital mortality, and adverse metabolic and asphyxic neonatal outcomes ([35]Kramer*, et al.*, 1990). Other short-term consequences of IUGR include decreased mental capability ([47]Lundgren*, et al.*, 2001), increased incidence of diarrhea and respiratory infections ([42]Ashworth, 1998, [48]Barros*, et al.*, 1992), and metabolic changes including altered glucose, growth hormone and insulin secretion that occurs in children between 9 weeks and 11 years of age ([13]Albertsson Wikland*, et al.*, 1998, [39]Leger*, et al.*, 1997, [49]Whincup*, et al.*, 1997, [50]Yajnik*, et al.*, 1995, [51]Arends*, et al.*, 2002, [52]Kajantie*, et al.*, 2003).

1.3.3.2 Long term consequences of IUGR

IUGR is associated with increased risks of a range of adult onset diseases. The finding that IUGR is associated with adult onset diseases led to the 'Fetal Origins of Adult Disease' hypothesis originally developed by Barker and colleagues. It was developed as a result of findings from epidemiological data obtained from various areas within Britain, where high rates of infant mortality occurred between 1921 and 1925, and were related to the subsequent prevalence of ischaemic heart disease between 1968 and 1978 ([53]Barker*, et al.*, 1986). This hypothesis postulates that exposure to a sub-optimal intrauterine environment results in fetal adaptation to promote survival, but alters fetal growth and development, increasing the long-term risks of developing adult onset diseases such as hypertension, cardiovascular disease (CVD) and non-insulin dependent diabetes mellitus (NIDDM) ([54]Barker, 1995, [55]Barker, 1998).

Barker proposed that the fetus responds to undernutrition with permanent changes in its physiology and metabolism which may lead to coronary heart disease and stroke in adult life' ([55]Barker, 1998). The process by which this occurs was termed programming, which is when an early stimulus or insult, occurring at a critical or sensitive period in fetal life, results in permanent or long-term changes in the structure or function of organs and/or processes in the body ([56]Lucas*, et al.*, 1999). The events that occur during these 'critical periods of development' may be causally linked to chronic degenerative conditions such as non-insulin dependent diabetes mellitus and cardiovascular disease, which do not appear usually until mid-life or later.

Numerous studies, from different populations around the world, indicate a relationship between low birth weight and low weight at 1 year of age and chronic adult disease ([57]Fall*, et al.*, 1995, [58]Newnham, 1998, [59]Hattersley*, et al.*, 1999, [60]Wynn, 1997, [61]Flanagan*, et al.*, 2000, [62]McCowan*, et al.*, 1999). Birth weight at term is inversely related to the risk of cardiovascular mortality and the insulin resistance syndrome (elevation of systolic and diastolic pressure, impaired glucose tolerance, and elevated triglycerides), also known as Syndrome X ([54]Barker, 1995). Another consequence of low birth weight involves altered function of the reproductive system. Low birth weight is associated with an increase in the incidence of premature pubarche, which in addition to insulin insensitivity may lead to ovarian hyperandrogenism in girls ([63]Francois*, et al.*, 1997). Boys born with IUGR may have small testes and elevated plasma gonadotropins, which are associated with reduced fertility as adults ([64]Francois, 1997). Other studies investigating the long term

consequences of IUGR show that these infants have an increased risk of developing higher systolic blood pressure ([54]Barker, 1995, [65]Barker, *et al.*, 1989), increased cardiovascular mortality ([66]Barker, *et al.*, 1989), and glucose intolerance, hyperinsulinaemia, and non-insulin dependent diabetes mellitus (NIDDM) ([67]Hales, *et al.*, 1991, [68]Valdez, *et al.*, 1994, [69]Barker, *et al.*, 1993) as adults. Recent research has shown that adults who had a low birth weight or who were thin at birth are insulin resistant and have an increased risk of developing NIDDM ([70]Cianfarani, *et al.*, 1999, [71]Barker, 1999, [72]Bavdekar, *et al.*, 1999, [73]Newsome, *et al.*, 2003). Later consequences of IUGR infants exhibiting rapid postnatal growth are adult onset diseases such as hypertension, cardiovascular disease (CVD), NIDDM, and obesity ([8]Ong, *et al.*, 2000, [9]Eriksson, *et al.*, 1999, [54]Barker, 1995, [55]Barker, 1998, [59]Hattersley, *et al.*, 1999, [74]Dennison, *et al.*, 1997, [75]Yarbrough, *et al.*, 1998, [76]McCance, *et al.*, 1994, [77]Hyppönen, *et al.*, 2001, [78]Leon, *et al.*, 1996, [79]Leon, 1998, [80]Jensen, *et al.*, 2003). To understand the mechanisms that link IUGR and these long-term consequences in later adult life, we must firstly understand the factors and mechanisms that lead to IUGR.

1.3.4 Risk factors for IUGR

There are many factors associated with human fetal growth restriction (IUGR), including intrinsic factors, which originate or act from within the fetus, and extrinsic factors, which are physiological or pathophysiological changes external to the fetus, such as impaired nutrient delivery and supply to the fetus or placental insufficiency ([81]Owens, *et al.*, 1995). Intrinsic factors associated with IUGR include intrauterine infections, genetic and chromosomal anomalies,

anaemia, and congenital malformations ([81]Owens*, et al.*, 1995). Extrinsic factors associated with IUGR include environmental factors, such as high altitude, pollution and irradiation, and maternal factors, such as undernutrition, low maternal weight gain, maternal age, smoking, alcohol and drug abuse, low socio-economic status, various medical complications, and abnormal placentation ([81]Owens*, et al.*, 1995).

1.3.5 Underlying mechanisms responsible for IUGR

The hormones involved in fetal tissue accretion and differentiation include insulin, thyroid hormones, IGFs, the catecholamines, and the glucocorticoids ([26]Fowden*, et al.*, 2001). These hormones have altered concentrations when there is a change in the nutrient availability to the fetus ([26]Fowden*, et al.*, 2001). The metabolic changes seen in the growth-retarded fetus include hypoglycaemia, elevated blood levels of triglycerides, along with decreased branched chain amino acid (BCAA) concentrations ([17]Robinson*, et al.*, 1996). Changes in the fetal hormone environment are designed to reduce oxygen and nutrients to the tissues for growth and redirect them to the essential tissues such as the brain, heart and the placenta ([26]Fowden*, et al.*, 2001). This fall in the levels of these hormones reduces the uptake of nutrients into fetal tissues, therefore reducing the growth rate and helping to increase in utero survival ([26]Fowden*, et al.*, 2001).

1.4 Catch-up growth after IUGR

Infants born small for gestational age, undergo increased rates of growth in terms of weight or length after birth, termed catch-up growth, which occurs during the first few months of life ([7]Karlberg*, et al.*, 1995, [11]Prader*, et al.*, 1963). Catch-up growth has been defined as: "a growth velocity in terms of height and/or length above the statistical limits of normality for age and/or maturity during a defined period of time following a transient period of growth inhibition ([11]Prader*, et al.*, 1963)". This rapid phase of catch-up growth can continue until the child has caught up to their normal growth curve, and allows an individual, temporarily deflected from their genetically predetermined growth curve, to literally catch up to the centiles reflecting measures of size of their normal birth weight counterparts, once the inhibitory influence has been overcome ([82]Colle*, et al.*, 1976). Catch-up growth after IUGR has been shown by most studies to commence during, and be complete by, the end of the first 2 years of life, with most growth largely occurring in the first few months of life ([7]Karlberg*, et al.*, 1995, [13]Albertsson Wikland*, et al.*, 1998, [83]Albertsson Wikland*, et al.*, 1994, [84]Albertsson Wikland*, et al.*, 1993, [85]Karlberg*, et al.*, 1997). Most IUGR infants grow at faster rates than normal infants in the first few months of life, in terms of z-scores for weight or length. A study of Hong Kong children (studied from birth to 8 years of age) born small for gestational age (SGA), found that the majority (65%) of children had reached within 1SD of their normal length by 5 months of age ([85]Karlberg*, et al.*, 1997). The majority (85%) of IUGR infants born in Sweden had caught-up completely in terms of height and weight by 3 to 9 months of age ([84]Albertsson Wikland*, et al.*, 1993). Subsequent studies have shown that catch-up growth occurs in more than 86% of IUGR infants in terms

of weight, begins as early as 2 weeks and is largely complete by 5 months of age, with catch-up growth in terms of height evident by 6 to 12 months of age ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). Another more recent study ([38]Leger*, et al.*, 1997) supports this conclusion, by demonstrating that on average, catch-up growth in terms of weight and head circumference in IUGR infants occurred during the first 3 months of life. Nevertheless, 47% of IUGR children exhibited a slight and continued catch-up in growth in terms of height between 1.5 and 2 years of age ([38]Leger*, et al.*, 1997). In the first year of life therefore, the majority of IUGR infants (57 - 84%) undergo some degree of catch-up growth in terms of weight or height, which may persist for linear growth at least ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). The remainder, who do not catch-up, may have persistent short stature in adulthood and constitute a group with a 7-fold higher risk of persistent short stature ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995).

1.4.1 Consequences of catch-up growth

Recent studies suggest that infants born after IUGR and who undergo rapid postnatal growth may have an increased risk of long-term adverse consequences such as chronic diseases in adulthood, as described earlier ([8]Ong*, et al.*, 2000, [9]Eriksson*, et al.*, 1999, [10]Forsén*, et al.*, 1999, [70]Cianfarani*, et al.*, 1999, [86]Adair*, et al.*, 2003, [87]Parker*, et al.*, 2003, [88]Dietz, 1994, [89]Crowther*, et al.*, 1998, [90]Forsén*, et al.*, 2000). Some studies have assumed that catch-up growth is desirable for low birth weight children in the short term, but the literature and data on this possibility is limited ([91]Victora*, et al.*, 2001, [92]Harding*, et al.*, 2003). A population-based cohort in southern Brazil of 3582

children who were studied at birth, 20 and 42 months of age, showed that catch-up growth from 0 to 20 months was related to a reduced risk of hospital admissions and mortality ([91]Victora*, et al.*, 2001). Thus children who were SGA, but presented substantial weight gain (0.66 z-score) up to the age of 20 months had 65% fewer hospital admissions than other SGA children ([91]Victora*, et al.*, 2001). Mortality to age 5 years was also 75% lower (3 versus 13 per 1000) for rapid-growing SGA children compared to the remaining SGA children ([91]Victora*, et al.*, 2001). The hospital admission and mortality rates of SGA children who caught up were similar to those observed for children born with an appropriate birth weight for their gestational age (AGA) ([91]Victora*, et al.*, 2001). A recent study found that delayed catch-up growth in IUGR infants was associated with prolonged nursery stay in the neonatal period ([92]Harding*, et al.*, 2003). Clinical records showed that 48% of infants in this study had feeding problems, poor growth, and delayed or late onset catch-up growth, and these appeared to be the cause of the prolonged hospital stay ([92]Harding*, et al.*, 2003). Growth promotion efforts for infants who are born small should take into account their possible long-term as well as short-term consequences ([91]Victora*, et al.*, 2001). Catch-up growth in infancy, together with size at birth, may determine the extent to which target adult height is attained following IUGR ([7]Karlberg*, et al.*, 1995), with those who do not catch-up having a higher risk of persistent short stature into adulthood ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). The extent of catch-up growth after IUGR in infants in standard deviation scores (SDS) is a strong predictor of their final adult stature ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). Overall, IUGR infants achieve a final adult height of approximately 1SD score below their mid-parental or target adult

height, but can be as much as 2 or 3SD shorter than this ([7]Karlberg*, et al.*, 1995). Short prepubertal children who were born with IUGR account for approximately 25% of short children referred to paediatric endocrinologists for growth hormone treatment. Growth hormone (GH) treatment to restore growth has had variable outcomes in terms of increased final height to date with very high doses required for modest increments in final stature. A recent study has shown that GH treatment of IUGR infants that presented with short stature due to GH deficiency at a dosage of 0.4 U/kg/week for a period of 4.6 $\pm$ 2.5 years has a limited effect on the final height of these infants ([93]Coutant*, et al.*, 1998). Understanding the basis of catch-up growth and the origins of its variability is important therefore as it may indicate alternative and more effective approaches to treatment of the failure of infants to catch-up ([1]Chernausek, 1996, [94]Karlberg*, et al.*, 1993). A recent study has indicated that infants born SGA have a higher risk of minimal neurological dysfunction later in life compared with normal sized babies ([47]Lundgren*, et al.*, 2001). Lundgren and colleagues have shown that males born below -2SDS in terms of weight, length and head circumference for gestational age had a lower intellectual performance scores ([47]Lundgren*, et al.*, 2001). They also have shown that among men born SGA, low mean intellectual performance scores were seen in those that did not undergo catch-up, compared to those that did undergo catch-up growth ([47]Lundgren*, et al.*, 2001). Catch-up growth was associated with increased mean psychological performance in those born SGA ([47]Lundgren*, et al.*, 2001). On the other hand, those IUGR newborns that present with rapid postnatal growth during infancy appear to have an even greater increased risk of chronic diseases in adulthood, independent of that associated with IUGR ([8]Ong*, et al.*, 2000, [9]Eriksson*, et al.*,

1999, [10]Forsén, *et al.*, 1999). Other studies suggest that catch-up growth after IUGR may independently increase the risk of developing adult-onset diseases such as diabetes, insulin resistance, hypertension and cardiovascular disease ([9]Eriksson, *et al.*, 1999, [70]Cianfarani, *et al.*, 1999, [86]Adair, *et al.*, 2003, [87]Parker, *et al.*, 2003, [95]Rasmussen, 2001, [96]Forsén, *et al.*, 1997). This increased disease risk may be mediated in part through altered body composition, as a recent critical review of published studies concluded that catch-up growth after IUGR predicts increased subcutaneous and visceral obesity in later life ([97]Rogers, *et al.*, 2003). Thus small babies, who have rapid rates of growth or catch-up growth, exhibit greater adiposity and a taller childhood stature than is predicted from parental heights ([8]Ong, *et al.*, 2000), and have an increased risk of obesity ([88]Dietz, 1994), cardiovascular disease and type 2 diabetes as adults ([9]Eriksson, *et al.*, 1999, [89]Crowther, *et al.*, 1998, [90]Forsén, *et al.*, 2000). A recent study investigated a large British birth cohort and showed that men aged 33 years who were light at birth, have a greater risk of developing obesity if they grew rapidly during childhood ([98]Parsons, *et al.*, 2001).

Therefore in summary, there are beneficial and adverse consequences in the short and long term for children who undergo spontaneous catch-up growth following IUGR (summarized in Figure 1.1). It is not clear to what extent all catch-up growth is associated with this full range of outcomes or if catch-up growth and its underlying causes are heterogeneous in nature. Increasingly however, it appears that those infants that undergo catch-up growth after IUGR may have reduced short term adverse consequences but may be increasing their risk of longer term more adverse outcomes. Why this occurs and whether

it is possible to promote catch-up growth and its beneficial effects while avoiding the adverse consequences requires better understanding of what drives catch-up growth and its relationships to these outcomes. An understanding of the mechanisms responsible for catch-up growth may identify alternative approaches to the treatment of catch-up growth failure after IUGR, and give insight into why it may increase the risk of later disease ([1]Chernausek, 1996, [94]Karlberg*, et al.*, 1993).

Beneficial consequences of catch-up growth	*Adverse consequences of catch-up growth*
↓ risk of hospital admissions during infancy (up to 42 months) ([91]Victora*, et al.*, 2001, [92]Harding*, et al.*, 2003)	↑ fat deposition (2 to 7 years) ([8]Ong*, et al.*, 2000, [88]Dietz, 1994, [97]Rogers*, et al.*, 2003)
↓ morbidity and mortality in childhood (42 months) ([91]Victora*, et al.*, 2001)	Altered body composition (adulthood) ([97]Rogers*, et al.*, 2003)
↑ cognitive function (up to 9 years) ([99]Frisk*, et al.*, 2002)	↑ blood pressure (16 years) ([86]Adair*, et al.*, 2003)
↑ psychological performance (behaviour) (aged 18 years) ([47]Lundgren*, et al.*, 2001)	↑ insulin resistance (50 years) ([87]Parker*, et al.*, 2003)
↑ stature (long bones) (up to 18 years) ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995)	↑ risk of diabetes (predicted at 7 years, 55 to 60 years) ([9]Eriksson*, et al.*, 1999, [89]Crowther*, et al.*, 1998, [90]Forsén*, et al.*, 2000)
↑ weight (42 months) ([91]Victora*, et al.*, 2001)	↑ risk of obesity, CVD, stroke, etc. (predicted at 7 years, 55 to 60 years) ([8]Ong*, et al.*, 2000, [9]Eriksson*, et al.*, 1999, [10]Forsén*, et al.*, 1999)

Figure 1.1. Consequences of catch-up growth in infants following IUGR.

The table lists the beneficial and adverse consequences for infants that undergo catch-up growth after being born with IUGR.

1.4.2 Postnatal growth and its regulation

To understand what might determine the extent of postnatal catch-up growth seen in IUGR infants and related short and long term outcomes, the mechanisms by which postnatal growth occurs and the factors that normally regulate growth must be considered. Normally, postnatal growth velocity in humans is highest immediately after birth during early infancy, decreases rapidly until about 4 years of age and then gradually declines further to a steady level just before the onset of the pubertal growth spurt ([100]Tanaka, 1996). At puberty there is a rapid increase in growth velocity until the late teens, followed by a decrease, and then a cessation in early adulthood ([100]Tanaka, 1996). Alterations in the endocrine regulation of growth may be an important mechanistic element in catch-up growth after IUGR. Normal postnatal growth requires the action of major anabolic hormones such as insulin, the IGFs, growth hormone (GH) and thyroid hormone, but other factors such as catabolic hormones including cortisol, and nutritional availability and intake are important influences on postnatal growth ([100]Tanaka, 1996). Understanding the effect of IUGR on the activity of these hormonal axes postnatally may therefore help to identify the determinants of catch-up growth. Growth in the infancy phase is dependent on nutrition and hormones such as growth hormone and thyroid hormone, growth during childhood primarily depends on growth hormone, while pubertal growth is primarily dependent on sex hormones ([100]Tanaka, 1996). Much of the growth rate of the neonate is determined by nutritional availability along with the actions of these anabolic and catabolic hormones ([34]Jackson, 1996). Studies of the endocrine basis of catch-up growth after IUGR in human

infants have focussed mainly on the production or abundance of these anabolic hormones, particularly insulin, IGFs, and thyroid hormones, that of the catabolic hormone, cortisol, and other factors, such as nutrition, which influence postnatal growth and may be involved in catch-up growth ([13]Albertsson Wikland*, et al.*, 1998, [101]de Waal*, et al.*, 1994, [102]Garcia*, et al.*, 1996, [103]Leger*, et al.*, 1996, [104]Cianfarani*, et al.*, 2002, [105]Cianfarani*, et al.*, 2001, [106]Ong*, et al.*, 2002, [107]Cianfarani*, et al.*, 2003, [108]Soto*, et al.*, 2003). Growth involves the net synthesis of protein and other macromolecules and includes lengthening of bones (at the extremities) as well as increases in the number and size of cells in the soft tissues throughout the body. Growth of soft tissues (muscle and organs) is accomplished by hyperplasia (increasing the number of cells) by stimulating cell division, and hypertrophy (increasing the cell size). The most important environmental factor that influences postnatal growth is nutrition, which interacts with the genetic drive to determine the final size achieved, especially in terms of height ([34]Jackson, 1996, [100]Tanaka, 1996). Undernutrition during infancy leads to growth failure due to decreased growth rate, delayed bone age, delayed puberty and short adult height ([100]Tanaka, 1996). Growth requires the proliferation of chondrocytes in the growth plate of long bones, which is controlled by growth factors and other hormones such as insulin, growth hormone, IGF-I, thyroid hormone, sex steroids and corticosteroids ([100]Tanaka, 1996).

1.4.2.1 Skeletal muscle growth

Skeletal muscles comprise the largest group of tissues in the body, accounting for approximately 40% of total adult body weight ([109]Sherwood, 1997). The

majority of skeletal muscle originates from mesodermal cells in the somites of the early developing embryo ([110]Sejersen*, et al.*, 1996). In the human, somite pairs begin to develop adjacent to the notochord and neural tube at day 20 of gestation ([110]Sejersen*, et al.*, 1996). This continues in a caudal direction to produce 37 pairs of somites by day 30 of gestation, with cells in the dorsal-lateral region forming the dermamyotome ([110]Sejersen*, et al.*, 1996). Muscle progenitor cells form the muscle mass and are located in the dermamyotome, which receive dorsal signals to enter myogenesis ([111]Buckingham, 2001). Early differentiating myogenic cells underlie the dermamyotome, which undergo myogenesis to eventually form muscle ([111]Buckingham, 2001). Migrating myogenic cells undergo proliferation and then fuse into multinucleated myotubes, and then mature into muscle fibres ([110]Sejersen*, et al.*, 1996). In the human fetus, myotubes of the gastrocnemius muscle are first seen at approximately 45 days of gestation, with the formation of myotubes occurring in three phases, called the primary, secondary and tertiary ([110]Sejersen*, et al.*, 1996). The myofibres derived from these myotubes will become indistinguishable in the adult ([110]Sejersen*, et al.*, 1996). Satellite cells are a group of proliferative cells located in skeletal muscle that contribute to postnatal growth, the maintenance of adult skeletal muscle, and the repair of damaged myofibres ([112]Hawke, 2001). After birth, these neonatal satellite cells fuse to growing myofibres to contribute to additional nuclei during postnatal skeletal muscle growth ([112]Hawke, 2001). Muscle growth requires the increase in diameter due to hypertrophy and to a small extent hyperplasia of the fast-glycolytic fibres. It is due to the increase in myosin and actin filaments, which are involved in muscle strength ([109]Sherwood, 1997). The growth of skeletal

muscle can be stimulated by anaerobic, short-duration, high intensity exercise ([109]Sherwood, 1997).

1.4.2.2 Long bone growth

Growth of long bones (growth in terms of length and height) occurs as the result of proliferation of chondrocytes, which are the cartilage cells located in the epiphysis of bones ([113]Abad*, et al.*, 1999, [114]Ohlsson*, et al.*, 1998). These chondrocytes enlarge as they are pushed towards the diaphysial plate. The combination of proliferation and hypertrophy of chondrocytes causes the epiphysis to increase in width, which then undergoes ossification ("hardening") to produce new bone ([113]Abad*, et al.*, 1999, [114]Ohlsson*, et al.*, 1998). Endochondral bone formation is a highly complex process that requires the coordination of maturation, proliferation, and differentiation of chondrocytes to produce hypertrophic cells in the epiphyseal growth plate ([113]Abad*, et al.*, 1999, [114]Ohlsson*, et al.*, 1998, [115]van der Eerden*, et al.*, 2003). The normal epiphyseal growth plate has chondrocytes organised into layers. The distal epiphyseal end has chondroblast progenitor cells occurring singly and in small clusters to form the reserve cell zone. Towards the metaphysis and adjacent to the reserve zone, are small flat proliferating cells which form columns, this is the proliferative zone ([113]Abad*, et al.*, 1999, [114]Ohlsson*, et al.*, 1998, [115]van der Eerden*, et al.*, 2003). When the proliferative cells lose their ability to proliferate they undergo the process of differentiation to produce large hypertrophic chondrocytes, which secrete a collagen rich matrix that undergoes apoptosis to leave a scaffold of cartilage that mineralises to form new bone ([114]Ohlsson*, et al.*, 1998, [115]van der Eerden*, et al.*, 2003, [116]Karsenty, 2001). The largest, most

distal chondrocytes undergo apoptosis and leave behind a cartilaginous framework ([116]Karsenty, 2001). Then metaphyseal trabecular bone forms on this framework due to invading osteoblasts derived from bone-marrow stromal cells that enter via capillaries. Postnatally, growth velocity rapidly decreases due to growth plate maturation in long bones and spine, leading to growth plate fusion and cessation of longitudinal growth ([115]van der Eerden*, et al.*, 2003).

1.4.3 Endocrine regulation of neonatal growth

The major anabolic hormones that regulate early postnatal growth are insulin, the insulin-like growth factors, thyroid hormone, and in later infancy, growth hormone, while cortisol is an influential catabolic hormone, and sex steroids are influential later in childhood ([100]Tanaka, 1996). There are developmental changes in circulating levels and tissue responsiveness to these hormones, which parallel the changes in postnatal growth rates.

1.4.3.1 Insulin axis

Insulin is a small protein, with a molecular weight of about 6000 Daltons, composed of two chains held together by two disulfide bonds, with a third bond present in the A-chain (Figure 1.2). The human insulin receptor is a 400-kDa glycoprotein consisting of four glycosylated peptide chains covalently linked by disulfide bonds (Figure 1.3). The molecules are a dimer consisting of two alpha-subunits (120 kDa) including binding sites for insulin, and two beta-subunits (80 kDa), linked by disulfide bonds. The alpha chains are entirely extra cellular and contain insulin-binding domains, while the linked beta chains penetrate through the plasma membrane. The insulin receptor is a tyrosine

kinase which functions as an enzyme that transfers phosphate groups from ATP to tyrosine residues on intracellular target proteins. Binding of insulin to the alpha subunits of the insulin receptor causes the beta subunits to phosphorylate by a process called autophosphorylation, in turn activating the receptor. Upon insulin binding to the alpha subunit, tyrosine kinase is activated and plays a major role in mediating insulin signal transduction ([117]Kishimoto*, et al.*, 1994). The activated receptor then phosphorylates a number of intracellular proteins, which in turn alters their activity, thereby generating a biological response. Several intracellular proteins have been identified as phosphorylation substrates for the insulin receptor, such as insulin receptor substrate-1 (IRS-1). When IRS-1 is activated by phosphorylation, it recruits and activates other enzymes that mediate insulin's effects such as PI3-kinase, various protein kinases, which lead to GLUT4 translocation. This is described in further detail in a subsequent section (see Introduction 1.4.4.1.2).

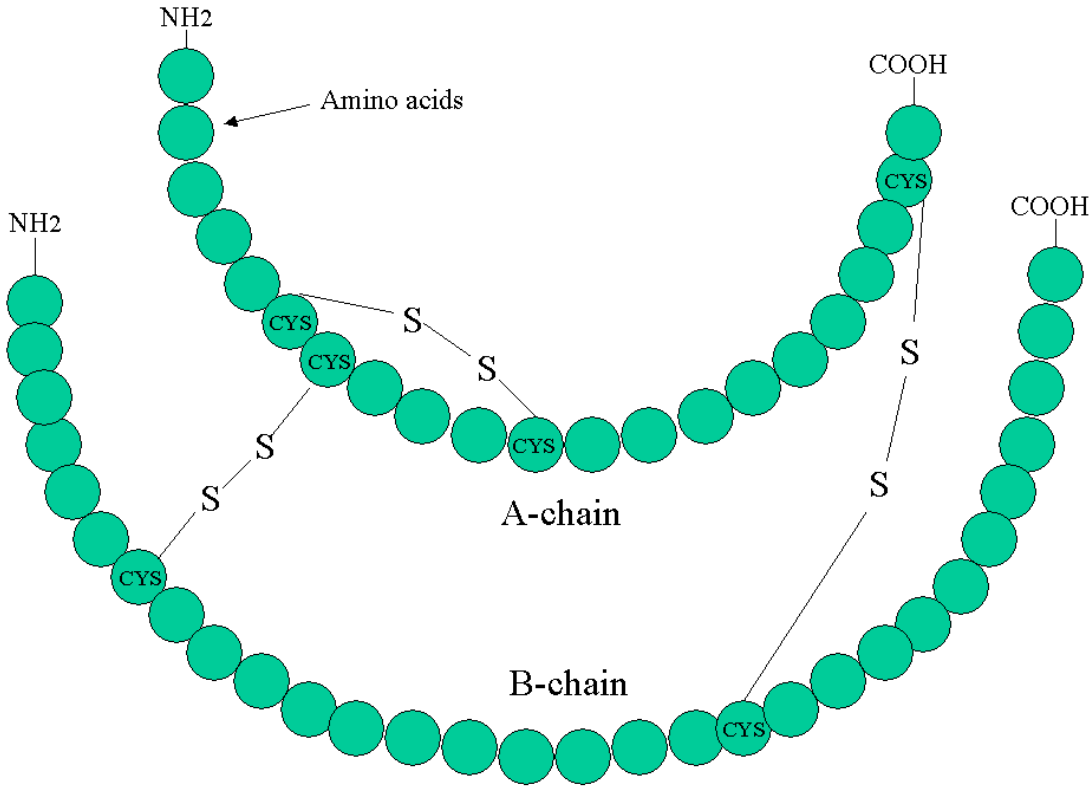

Figure 1.2. Schematic structure of human insulin indicating the N-terminal and C-terminal and the A and B chains with three disulphide bonds (S-S).

Adapted from ([109]Sherwood, 1997).

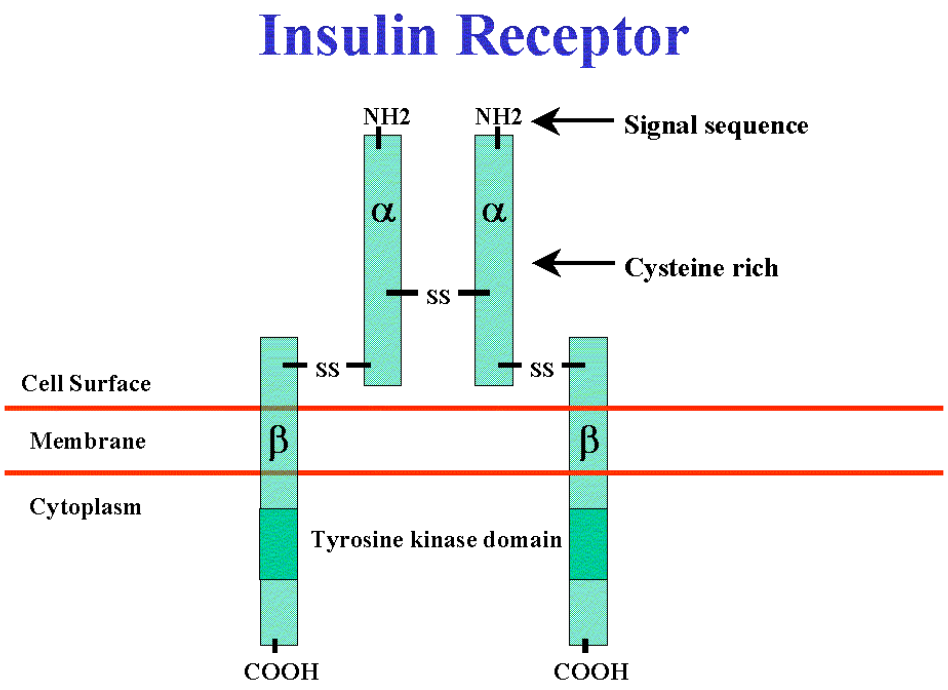

Figure 1.3. Structure of insulin receptor.

Demonstrates the four-glycosylated peptide chains covalently linked by disulfide bonds, the two extracellular alpha-subunits, which include binding sites for insulin, and two beta-subunits, which are linked by disulfide bonds and penetrate through the plasma membrane. Adapted from ([118]Le Roith, 1997).

1.4.3.1.1 Metabolic actions of insulin

Insulin is produced by the β-cells of the Islets of Langerhans in the pancreas in response to various metabolic stimuli, particularly a rise in blood glucose. Once insulin is secreted into the blood, it contributes to glucose homeostasis by stimulating the clearance of glucose from the blood into skeletal muscle while inhibiting glucose production and release by the liver and kidney. While insulin also stimulates glucose uptake and utilisation by adipose tissue, this makes little quantitative contribution to the control of blood glucose concentration in the short term.

The primary stimuli for insulin secretion are an increase in blood levels of glucose and amino acids, with glucose being the most important quantitatively. Insulin stimulates glucose uptake in muscle cells and adipocytes by increasing the amounts of glucose transporters, specifically GLUT4, that are transported to the cell surface ([20]Milner*, et al.*, 1996). Insulin also influences nutrient storage and immobilization, such that at low insulin concentrations, lipolysis is unrestrained, whereas at high insulin concentrations, lipolysis is inhibited and lipogenesis stimulated. The combination of amino acids appearing in the blood following a meal is also a very powerful stimulator for insulin secretion, whereas lipids have little role in affecting insulin secretion ([20]Milner*, et al.*, 1996). Protein synthesis in muscles is stimulated by the high plasma insulin concentrations that occur postprandially, where there is an increase in the cellular uptake of amino acids from plasma into muscle and liver. Conversely, when plasma insulin concentrations are low such as in the fasting state, skeletal muscle

releases amino acids into the blood as a result of protein breakdown that is no longer inhibited by insulin ([20]Milner*, et al.*, 1996).

1.4.3.1.2 Glucose transport

The regulation of glucose transport is a primary function of insulin and is performed by glucose-transporter proteins. In muscle and adipocytes insulin-stimulated glucose uptake is achieved by the translocation of the insulin-sensitive glucose transporter GLUT-4 from the intracellular storage vesicles to the cell surface ([119]Zorzano, 1996). Skeletal muscle is the major insulin-sensitive consumer of glucose in the body (with the brain also consuming a large percentage) and is the main tissue involved in the insulin-induced stimulation of glucose uptake ([119]Zorzano, 1996). Glucose transport into skeletal muscle occurs through the GLUT1 and GLUT4 glucose transporters. GLUT4 is the most abundant glucose transporter and is located intracellularly in both muscle and adipocytes where it can be rapidly translocated to the cell surface in response to insulin binding to its receptor, and in response to exercise or hypoxia ([120]Flier*, et al.*, 1987, [121]James*, et al.*, 1985, [122]Douen*, et al.*, 1990, [123]Handberg*, et al.*, 1992). This insulin-stimulated glucose transport into skeletal muscle fibres controls the utilisation rate of glucose in skeletal muscle, which is reduced in many insulin resistant states, such as NIDDM. When insulin binds to its receptor it activates tyrosine kinase phosphorylation at the intracellular portion of the insulin receptor (Figure 1.4) ([124]Shepherd*, et al.*, 1999). The receptor then phosphorylates and activates the insulin receptor substrate-1 molecule (IRS-1) (Figure 1.4) ([124]Shepherd*, et al.*, 1999). The IRS-1 forms a complex via SH2 molecules with docking proteins such as the p85

subunit of the phosphoinositide-3 kinase (PI-3 kinase) (Figure 1.4). The p85 subunit then binds to the catalytic subunit p110. These two subunits make up the PI-3 kinase. This PI-3 kinase then activates phosphoinositide-dependent kinases that participate in the activation of protein kinase B (or Akt kinase) and atypical forms of protein kinase C (Figure 1.4). Intracellular translocation of GLUT4 to the plasma membrane is stimulated by the expression of the active forms of protein kinase B (Akt kinase) or protein kinase C. This suggests that one or both of these kinases may be the *in vivo* mediator of the process by which GLUT4 is translocated by the stimulation of insulin ([124]Shepherd*, et al.*, 1999). There are also other areas that may affect the docking of the GLUT4 transporter to the cell membrane. These being synaptobrevin (v-SNARE), a small guanosine triphosphate-binding protein called Rab-4, or syntaxin-4 (t-SNARE) (Figure 1.4). If one or more of these three molecules is down regulated then the GLUT4 transporters will not bind and incorporate themselves into the cell membrane therefore reducing GLUT4-mediated glucose transport into the cell. When blood levels of insulin decrease and insulin receptors are no longer occupied, the glucose transporters are recycled back into the cytoplasm (Figure 1.4). Degradation of insulin occurs through reversal of the insulin signal at the cellular level by a class of enzymes termed phosphotyrosine phosphatases ([125]Kahn*, et al.*, 1993).

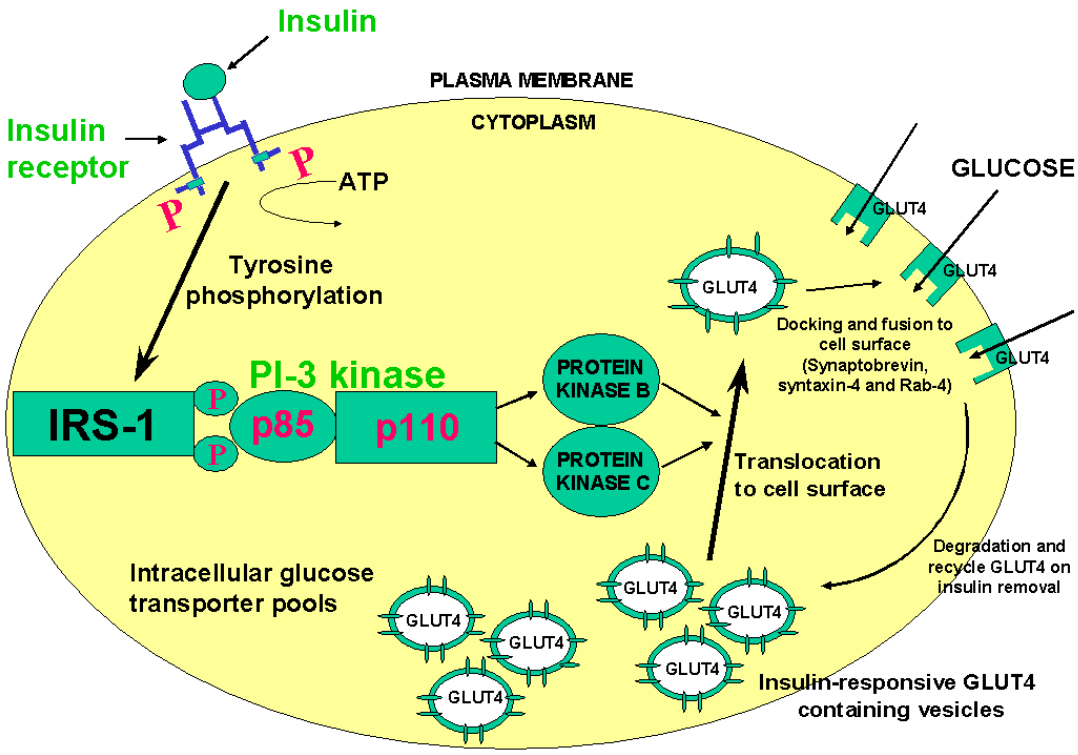

Figure 1.4. Insulin signalling pathway that regulates glucose metabolism in muscle cells and adipocytes.

Insulin binds to its receptor in the plasma membrane, phosphorylating the receptor and the insulin-receptor substrates (IRS). These form complexes with docking proteins such as PI-3 kinase and its 85kd subunit (p85), which is bound to the p110 subunit. This activates phosphoinositide-dependent kinases that participate in activation of protein kinases B and C. The vesicles translocate and form a complex with syntaxin-4, synaptobrevin and Rab-4. The vesicles fuse with the membrane allowing glucose to be transported into the cell. On insulin removal GLUT-4 is internalized from the plasma membrane, and GLUT-4 is re-sorted into vesicles. Figure adapted from ([124]Shepherd*, et al.*, 1999).

1.4.3.1.3 Effect of insulin on growth

In the neonate, insulin is a major anabolic hormone. Upon feeding, the concentration of insulin increases acutely in blood, stimulating amino acid uptake and protein synthesis, promoting storage of glucose as glycogen in skeletal muscle, increasing triglyceride synthesis and storage into adipose tissue, and promoting chondrocyte proliferation and hypertrophy in long bones ([126]Davis*, et al.*, 1998, [127]Henson*, et al.*, 1997). Insulin's effects on postnatal growth tend to be mediated by regulating growth hormone receptor number, leading to growth hormone sensitivity, and then IGF-I secretion ([100]Tanaka, 1996). Neonatal pigs utilize their dietary amino acids for protein accretion with high efficiency, and this efficiency declines during early life ([128]Wray-Cahen, 1997). Recent studies on pigs have focused on identification of the factors that mediate this response in the neonate ([129]Davis*, et al.*, 1998). The stimulation of protein synthesis in skeletal muscle, but not liver, can be reproduced by insulin infusion when essential amino acids and glucose are maintained at fasting levels ([128]Wray-Cahen, 1997, [130]Davis*, et al.*, 2001, [131]Wray Cahen*, et al.*, 1998). Tissue protein synthesis was measured using a flooding dose of L-(4-3H)phenylalanine in 7 and 26 day old pigs ([130]Davis*, et al.*, 2001, [131]Wray Cahen*, et al.*, 1998). Insulin infusion alone increased protein synthesis in various skeletal muscles (from 35 to 64%), as well as cardiac muscle (50%), skin (34%), and spleen (26%) ([132]Davis*, et al.*, 2001). Infusion of amino acids alone increased protein synthesis in skeletal muscles (from 28 to 50%), as well as liver (27%), pancreas (28%), and kidney (10%) ([132]Davis*, et al.*, 2001). Co-infusion of both insulin and amino acids did not have an additive effect on

protein synthesis ([132]Davis*, et al.*, 2001) and another recent study also suggests that insulin and amino acids independently stimulate protein synthesis in skeletal muscle of the neonate ([133]O'Connor*, et al.*, 2003). However, insulin does not appear to be involved in the stimulation of protein synthesis in visceral tissues. Thus different mechanisms may be responsible for the regulation of peripheral and visceral tissue growth in the neonate ([130]Davis*, et al.*, 2001). Therefore the rate of skeletal muscle protein deposition in the neonate is largely due to a stimulation of skeletal muscle protein synthesis by insulin and amino acids ([129]Davis*, et al.*, 1998).

1.4.3.2 The Somatotropic Axis

Human Growth Hormone (hGH) (also called somatotropin) is a single 22kD chain peptide of 191 amino acids that is produced in the pituitary gland ([134]DeZegher, 1996). GH promotes growth in children and plays an important role in adult metabolism and in regulating normal growth and development. It also promotes protein synthesis in skeletal muscle by enhancing amino acid uptake ([135]Kostyo, 1968). Growth hormone insufficiency is a condition caused by a deficiency of natural growth hormone. Growth hormone deficient adults have excess adipose tissue stored viscerally and a reduced lean body mass, and with GH replacement, adipose tissue decreases and muscle mass increases ([136]Russell-Jones*, et al.*, 1993, [137]Nilsson*, et al.*, 1996). The hypothalamus controls the secretion of growth hormone by two peptides that have opposing effects ([137]Nilsson*, et al.*, 1996). Growth hormone releasing factor (GRF), stimulates GH production and secretion, and growth hormone inhibiting hormone (GHIH), blocks spontaneous and GRF-induced GH release

([137]Nilsson, *et al.*, 1996). The interaction between these two peptides results in GH being secreted in a pulsatile fashion at about 3-4 hour intervals ([137]Nilsson, *et al.*, 1996).

According to the somatomedin hypothesis, proposed some 30 years ago, GH stimulates skeletal growth by stimulating liver production of IGF-I (originally named somatomedin), which in turn, stimulates longitudinal bone growth in an endocrine manner ([137]Nilsson, *et al.*, 1996, [138]Guler, *et al.*, 1988, [139]Daughaday, *et al.*, 1972, [140]Daughaday, *et al.*, 1989, [141]Phillips, *et al.*, 1990). Most tissues throughout the body produce IGF-I which is secreted and acts peripherally in response to GH and other hormones, as well as nutrients, to promote postnatal growth ([142]Le Roith, *et al.*, 2001).

1.4.3.2.1 Insulin-like growth factor –I (IGF-I)

Insulin-like growth factor (IGF) –I is a 70 amino acid basic peptide (7648 Da) with three intra-chain disulfide bridges, consisting of amino-terminal B (29 amino acids) and A (21 amino acids)-regions (similar to proinsulin) separated by a short connecting C-region (12 amino acids), and a D-region (8 amino acids) extension peptide at the carboxy-terminus ([140]Daughaday, *et al.*, 1989, [143]Sara, *et al.*, 1990, [144]Rinderknecht, *et al.*, 1978) (Figure 1.5). *In vivo*, IGF-I is synthesised by the liver and many other tissues in postnatal life, including skeletal muscle and bone, and is postulated to have potent mitogenic and metabolic actions at or near the sites of synthesis; this has been termed the paracrine role of IGF-I ([140]Daughaday, *et al.*, 1989). IGF-I also appears in the peripheral circulation, where it circulates primarily in a high molecular weight

tertiary complex with IGF-binding protein-3 (IGFBP-3) and acid-labile subunit (ALS) ([145]Baxter*, et al.*, 1989). A smaller proportion of IGF-I may circulate in association with other IGF-binding proteins. It has been estimated that <5% of plasma IGF-I circulates unbound ([146]Zapf*, et al.*, 1986). Plasma IGF-I levels are stabilized by the IGF-binding proteins and there is negligible diurnal variation ([147]Lee*, et al.*, 1987). *In vivo* synthesis of IGF-I is stimulated by GH, and is also dependent on and regulated by other factors, including nutrition, where fasting decreases IGF-I ([140]Daughaday*, et al.*, 1989, [147]Lee*, et al.*, 1987, [148]Blum*, et al.*, 1996). Other known regulators of IGF-I synthesis include thyroid stimulating hormone (TSH) which stimulates IGF-I production by thyroid follicular cells, luteinizing hormone (LH) which stimulates IGF-I expression in granulosa and Leydig cells, parathyroid hormone (PTH) which stimulates IGF-I synthesis in osteoblasts, and follicle stimulating hormone (FSH) which stimulates IGF-I in granulosa and Sertoli cells ([148]Blum*, et al.*, 1996).

In humans, plasma IGF-I levels are low during fetal and neonatal life, increase gradually during childhood, peak during mid-puberty, and decline gradually through adult life ([140]Daughaday*, et al.*, 1989, [147]Lee*, et al.*, 1987, [149]Rosenfeld*, et al.*, 1986, [150]Lee*, et al.*, 1990). Serum levels of IGF-I are relatively low in the human fetus, increasing from 20 ng/ml in week 30 of gestation to over 100 ng/ml at term. After birth, serum IGF-I levels increase up to 200 ng/ml and those of IGF-II rise to 700 ng/ml ([151]Bennett*, et al.*, 1983). In the first 7 days of postnatal life in term neonates, IGF-I levels decrease on day 1, remain low during the first 3 days of life, and then return to birth levels by the end of the first week ([152]Giudice*, et al.*, 1995).

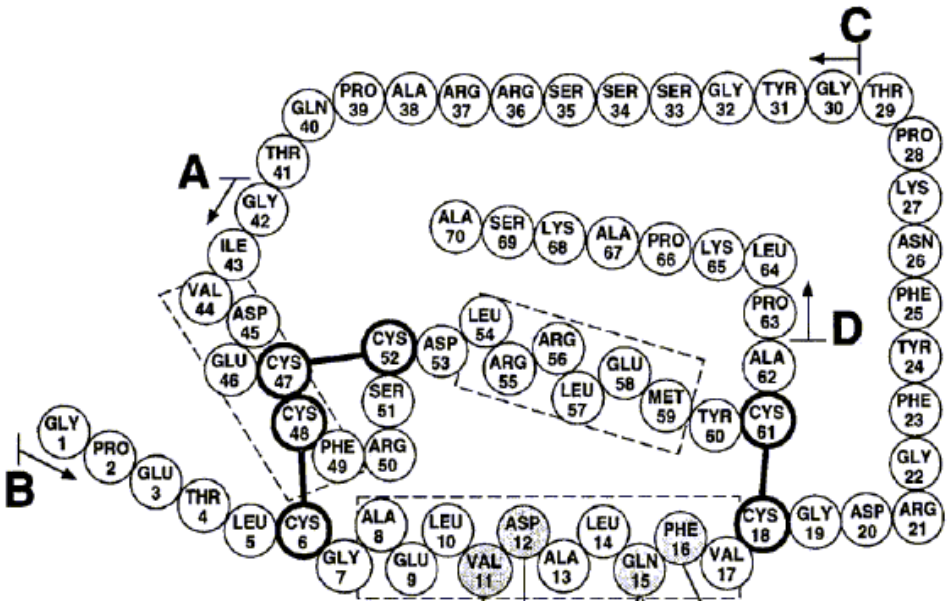

Figure 1.5. Structure of IGF-I

Schematic representation of the structure of IGF-I demonstrating the amino acid sequence and sulphide bonds. A, B, C and D domains are indicated. Figure adapted from ([153]Sara*, et al.*, 1988).

The IGF axis plays a major role in controlling the growth and differentiation of skeletal muscles in children (young and even post-puberty) to increase skeletal muscle mass, as well as other tissues such as bone for development, only before epiphyseal fusion ([154]Florini*, et al.*, 1996). The insulin-like growth factors (IGF), IGF-I and –II are known to be major growth promoting hormones of prenatal and postnatal growth ([155]Baker*, et al.*, 1993). Gene deletion studies in mice confirm that IGF-I at least is essential for pre- and postnatal growth ([156]Wang, 1999). The insulin-like growth factor-I (IGF-I) receptor (IGF-IR) is a transmembrane glycoprotein of 350 kDa generated from a precursor of 1367 amino acids (Figure 1.6). The IGF-IR is a heterotetrameric disulfide-linked protein consisting of two alpha subunits (135 kDa each), which bind IGF-I, and two beta subunits (90 kDa each). The IGF-IR has approximately 84% homology with the insulin receptor. Both IGF-I and IGF-II act via the type-1 IGF receptor to promote proliferation and inhibit apoptosis of a variety of cells in a wide range of tissues ([157]Le Roith, 2000). The IGFs also stimulate glucose and amino acid uptake and protein accretion by a variety of cell types, including myofibres ([146]Zapf*, et al.*, 1986, [158]Jacob*, et al.*, 1989, [159]Guler, 1987). In lambs, IGF-I infusion decreases whole-body catabolism and increases skeletal muscle protein synthesis ([160]Douglas*, et al.*, 1991, [161]Douglas*, et al.*, 1991). A number of studies have established that IGFs stimulate anabolic responses in myoblasts, as they do in other cell types. IGFs have the unusual property of stimulating both proliferation and differentiation of myoblasts ([154]Florini*, et al.*, 1996), and can increase chondrocyte proliferation and hypertrophy, stimulating bone elongation ([162]Wang*, et al.*, 1999). In addition, IGF-I is a potent stimulator of pre-adipocyte proliferation and early differentiation *in vivo* and *in vitro*

([163]Stewart, *et al.*, 1999, [164]Benito, *et al.*, 1996, [165]Smith, *et al.*, 1988, [166]Holzenberger, *et al.*, 2001, [167]Schmidt, *et al.*, 1990). Therefore alterations in IGF-I abundance and action could potentially mediate alterations in growth and adiposity postnatally following IUGR ([168]Sandhu, *et al.*, 2003, [169]Ben-Shlomo, *et al.*, 2003).

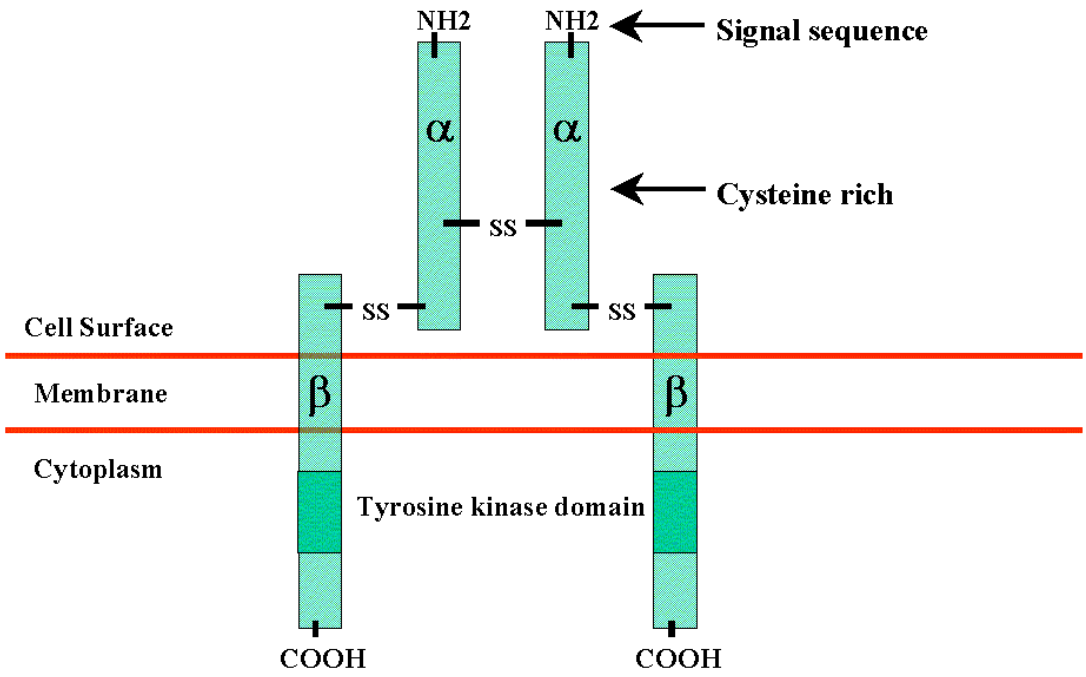

Figure 1.6. Structure of the Type I IGF receptor.

Schematic diagram demonstrates the heterotetrameric disulfide-linked protein consisting of two alpha subunits with binding sites for IGF-I and two beta subunits that penetrate the plasma membrane. Adapted from ([118]Le Roith, 1997).

1.4.3.2.1.1 Metabolic actions of IGF-I

IGF-I has acute metabolic actions as well as long-term anabolic effects. Intravenous infusion of IGF-I, which increases circulating IGF-I, decreases circulating glucose in several species. Studies in the rat, dog and human have shown that the decrease in blood glucose induced by circulating IGF-I is mainly due to increased glucose uptake by muscles as well as a decrease in hepatic glucose production, free fatty acid levels, and fat oxidation rates ([158]Jacob*, et al.*, 1989, [170]Boulware, 1994). The administration of IGF-I also decreases insulin and growth hormone secretion and increases insulin sensitivity in both rats and normal human subjects ([171]Zenobi*, et al.*, 1992, [172]Moxley III*, et al.*, 1990, [173]Froesch*, et al.*, 1994), and may be therapeutically useful in disease states such as insulin resistance, obesity, and hyperlipidemia. In type 2 diabetic patients, IGF-I administration improves glycaemic profiles, and in normal subjects, it increases energy expenditure and lipid oxidation and has a protein-sparing effect ([173]Froesch*, et al.*, 1994). Total and VLDL-triglycerides as well as LDL-cholesterol were also decreased by IGF-I administration ([173]Froesch*, et al.*, 1994). IGF-I also lowers circulating amino acids, and it does this more potently than insulin ([170]Boulware, 1994). In the very young pig, at 7 days of age, IGF-I stimulates amino acid uptake and protein synthesis by 25-60% in various skeletal muscles, as well as in cardiac muscle (+38%), skin (+24%), and spleen (+32%) ([174]Davis*, et al.*, 2002). Insulin replacement during an IGF-I infusion does not alter the response of protein synthesis to IGF-I in any tissue (liver, jejunum, pancreas, or kidney), and IGF-I infusion, with or without insulin replacement, has no effect on protein synthesis in the liver, jejunum, pancreas,

or kidney ([174]Davis*, et al.*, 2002). Therefore the magnitude and tissue specificity of the response of protein synthesis to physiological increases in plasma IGF-I are similar to those of insulin. These studies provide evidence that insulin and IGF-I each act to stimulate protein synthesis and the response is greater in skeletal muscle of the neonate ([174]Davis*, et al.*, 2002).

1.4.3.2.2 Insulin-like growth factor –II (IGF-II)

Insulin-like growth factor (IGF) –II is an acidic peptide of some 67 amino acids (7469 Da) that has a 60% homology with IGF-I, and is secreted by many tissues but is a less effective anabolic agent than IGF-I ([143]Sara*, et al.*, 1990, [144]Rinderknecht*, et al.*, 1978, [148]Blum*, et al.*, 1996, [160]Douglas*, et al.*, 1991). In the human fetus, the IGF-II levels are about 100 ng/ml at 30 weeks to over 300 ng/ml at term. In humans, IGF-II concentrations rapidly increase after birth for the first few weeks of life ([148]Blum*, et al.*, 1996). IGF-II may also play a role in the regulation of body weight and composition in men and women, since higher IGF-II levels are associated with a reduced risk of gaining weight, meaning those who gained weight and developed obesity had reduced baseline fasting serum IGF-II concentrations ([168]Sandhu*, et al.*, 2003).

The insulin-like growth factor-II (IGF-II) receptor (IGF-IIR) is a single polypeptide chain identical to the cation-independent mannose-6-phosphate (Man-6-P) receptor, and binds IGF-II with high affinity and to a lesser extent IGF-I (Figure 1.7). The receptor has a large extracellular domain and a small cytoplasmic domain, which lacks signaling capacity, but is coupled via a G-protein in the cell membrane to a calcium channel ([175]Jones*, et al.*, 1995). The main function of

the IGF-II receptor appears to be recycling of lysosomal enzymes containing Man-6-P residues, and a degradative pathway for IGF-II, with the proteolytic cleavage of the extracellular portion of the receptor producing a soluble form that may function as an IGF-II carrier protein ([175]Jones*, et al.*, 1995, [176]Blakesley*, et al.*, 1999).

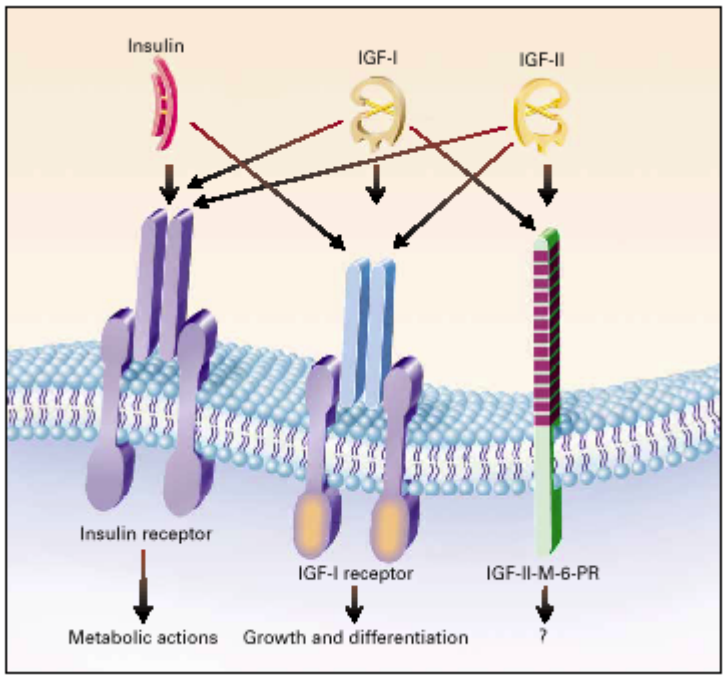

Figure 1.7. Insulin, IGF-I and IGF-II receptor binding.

Demonstrates the cross binding of insulin, IGF-I and IGF-II to exert their actions. Also demonstrates the schematic representation of the insulin receptor and IGF-I and IGF-II receptor. Figure taken from ([118]Le Roith, 1997).

1.4.3.2.2.1 Metabolic actions of IGF-II

The metabolic actions of IGF-II in fetal life and in postnatal life are unclear. Infusion of IGF-II (50 μg.kg^{-1}.hr^{-1}) concurrently with IGF-I (15 μg.kg^{-1}.hr^{-1}) was associated with a rise in plasma IGF-I concentration in male castrated lambs ([177]Koea*, et al.*, 1992). Plasma IGF-II increased over the infusion and completely blocked the anabolic effect of IGF-I, with the rate of net protein catabolism remaining unchanged throughout the combined IGF-I and IGF-II infusion ([177]Koea*, et al.*, 1992). This suggests that IGF-II may modulate the anabolic effects of IGF-I ([177]Koea*, et al.*, 1992). IGF-II infusion alone did not alter the rate of net protein loss in fasted lambs, indicating that IGF-II is a less effective anabolic agent than IGF-I ([160]Douglas*, et al.*, 1991).

1.4.3.2.3 Insulin-like Growth Factor Binding Proteins (IGFBPs)

There are six different IGF binding proteins (IGFBP1-6), which bind IGFs with high affinity. All six IGFBPs have a core protein structure with 18 cysteine residues whose alignment is preserved, indicating a similar tertiary structure. IGFBPs have four broad functions *in vivo* ([175]Jones*, et al.*, 1995). These IGF binding proteins serve as carrier (or transport) proteins for the IGFs (IGF-I and IGF-II) and modulate the actions of IGFs by competing for and interacting with the IGF receptors ([148]Blum*, et al.*, 1996). They regulate the levels of IGF-I and IGF-II in the circulation, they localise IGFs to cells and tissues, they extend the half-life of IGFs and regulate metabolic clearance of IGFs, and they directly modulate the interactions of IGFs with their receptors both positively and negatively ([175]Jones*, et al.*, 1995, [178]Le Roith, 1996).

1.4.3.3 Thyroid Hormone axis

Thyroid hormones (TH) play an important role in growth and development of the infant both before and after birth ([27]Fowden*, et al.*, 1995, [179]Zhang*, et al.*, 2000). The follicular cells (or secreting cells) of the thyroid gland produce two iodine-containing hormones derived from the amino acid tyrosine: L-3,5,3',5'-tetraiodothyronine (L-thyroxine (T_4)) and L-3,5,3'-triiodothyronine (L-triiodothyronine (T_3)). The thyroid hormones are very hydrophobic and those that exhibit biological activity are T_4, T_3, L-3,5,3'-triiodothyronine (rT3) and L-3,5-diiothyronine (3,5-T2) ([180]Hulbert, 2000). Thyroid Hormones (TH) are important regulators of overall basal metabolic rate ([27]Fowden*, et al.*, 1995, [181]Reed-Larsen*, et al.*, 1998), enhance muscle growth and are essential for the structural and biochemical development of the cardiovascular, central and peripheral nervous systems. Postnatally, the growth-promoting actions of TH are believed to be due, in part, to their effects on cell metabolism ([182]Dauncey, 1990). Thyroid hormones stimulate protein synthesis, glucose utilization and oxidative phosphorylation in the majority of adult tissues ([183]Brent*, et al.*, 1991, [184]Harper*, et al.*, 1993). This then leads to increased calorigenesis and oxygen consumption by the whole animal ([182]Dauncey, 1990). Most of the actions of the active thyroid hormone T_3 are exerted via ligand-activated nuclear T_3 receptors ([185]Kohrle, 2000). Activation of T_4, is catalyzed by two enzymes, iodothyronine-5'-deiodinases type I and type II, whereas inactivation occurs via type III iodothyronine-5-deiodinase and to some extent by type I 5'deiodinase ([185]Kohrle, 2000). Characterization of tissue-specific expression patterns

indicates that these selenium-dependent enzymes exert tight control on local and systemic availability of active T_3 ([185]Kohrle, 2000).

1.4.3.3.1 Thyroid hormone synthesis

The thyroid gland in adult humans consists of two lobes divided into lobules that have a rich blood supply. Each lobule within the gland contains 20-40 follicles that are lined by epithelial cells. These surround central deposits of a secretory substance called colloid that is secreted into the interior of the follicle. The colloid consists of large glycoproteins called thyroglobulin containing the thyroid hormone that are synthesized by the follicular epithelial cells of the thyroid gland (Figure 1.8).

Iodine (I) is essential for the synthesis of thyroid hormones. T_4 is 65% iodine by weight with 4 iodines per molecule, while T_3 is 58% iodine with 3 iodines per molecule. Iodine is not always available in sufficient quantities from dietary sources therefore between 5000 and 10,000 μg of concentrated hormonal iodine is stored in the thyroid gland for periods when there is iodine insufficiency. Thyroid Hormone synthesis and secretion is regulated by a negative feed back system involving the hypothalamus, the pituitary and the thyroid gland, known as the Hypothalamo-Pituitary-Thyroid (HPT) axis. Thyroid hormone release requires the synthesis of Thyrotropin-Releasing hormone (TRH), a tripeptide synthesized from the paraventricular nucleus of the hypothalamus. TRH is transported via axons to the median eminence and then to the anterior pituitary in turn stimulating the release of Thyroid-Stimulating Hormone (thyrotropin; TSH) (Figure 1.9). TSH binds to the TSH receptor in the

thyroid gland and stimulates the synthesis of thyroglobulin (Tg) and thyroid peroxidase (TPO), which are then bound to the iodine molecules, then secreted as T_4 and T_3 from the thyroid gland. In adult humans, thyroid hormones are almost entirely (~99%) bound to plasma proteins such as Thyroxine-Binding Globulin (TBG), Transthyretin (TTR), and Albumin. There is a very small percentage of TH, which remains free or unbound and is termed Free T_3 (FT_3) and Free T_4 (FT_4). Only 0.03% of the total serum T_4 is free (unbound) whereas 0.3% of total T_3 is free. The majority of TH is released as T_4, which is some 40-fold higher in concentration than T_3 in plasma.

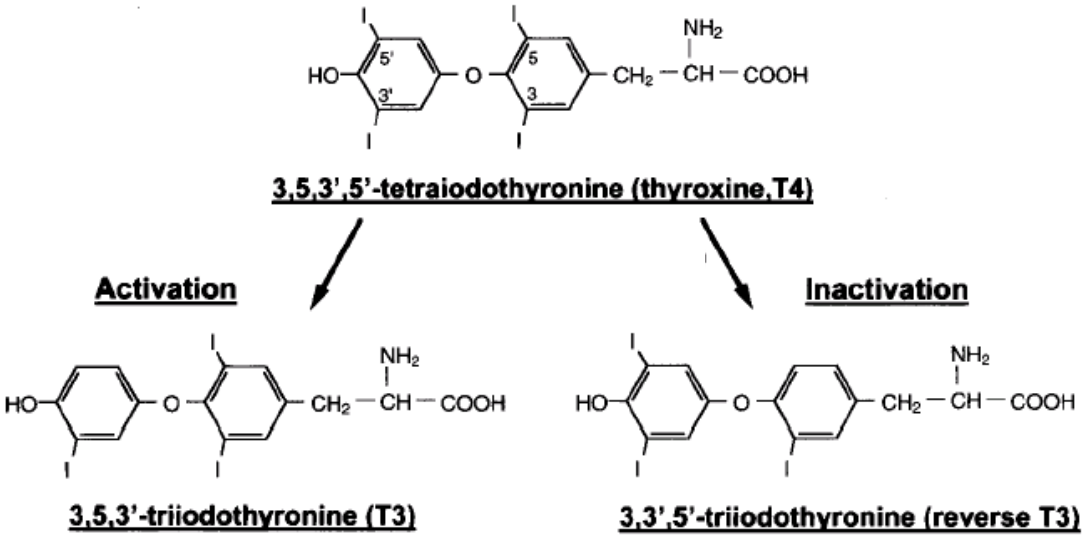

Figure 1.8. Chemical structures of thyroxine (T_4) and its activation to triiodothyronine (T_3) and inactivation to reverse T_3.

Adapted from ([186]Bianco*, et al.*, 2002).

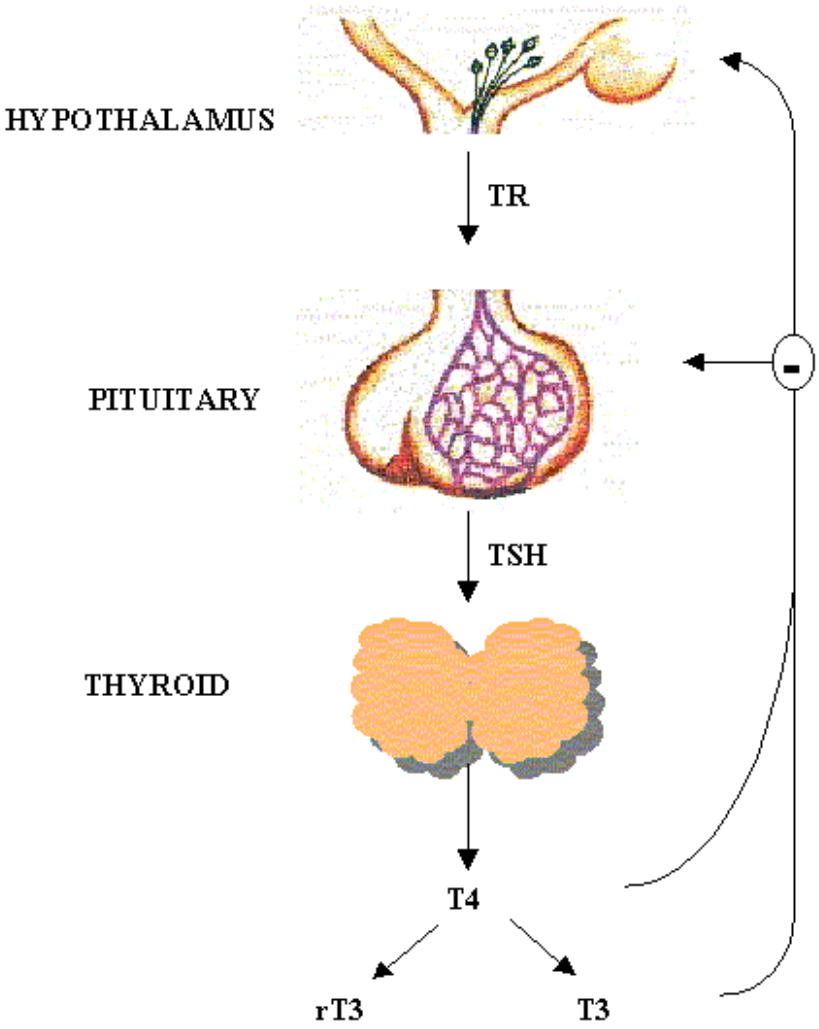

Figure 1.9. Classic feedback system for the control of thyroid hormone release from the thyroid gland.

When thyroid hormone levels are low the negative feedback effect of T_4 and T_3 on the hypothalamus and pituitary is reduced. This stimulates the release of TRH in turn stimulating TSH, which activates thyroxine synthesis in the thyroid gland. When thyroid levels are high, inhibition of TRH and TSH occurs.

Circulating TSH displays both pulsatile and circadian secretions in the human. In humans, the TSH production rates are about 40-150 mU/day. The pulsatile secretions fluctuate at 1-2 hour intervals, with secretion decreased during fasting or illness. The circadian TSH secretions display a nocturnal surge before the onset of sleep that is independent of cortisol secretion and T_3 and T_4 secretion ([181]Reed-Larsen*, et al.*, 1998). When TSH is stimulated by the action of TRH on the pituitary, the thyroid cells remove iodide from the capillaries at the base of the cell and move it to the apex of the cell where it joins with tyrosine molecules to make T_4, and to a lesser extent T_3. Both T_4 and T_3 are stored together in the colloid and are released together, or some T_4 is converted to T_3 as required. These two processes are under the control of TSH ([181]Reed-Larsen*, et al.*, 1998). Approximately 120 nmol of T_4, and 47 nmol of T_3 are secreted into the circulation in a typical 70 kg person each day. Postnatally, 70-90% of the circulating T_3 is from peripheral monodeiodination of T_4 ([187]Sack, 1996). Thyroid hormone is essential for the normal development, differentiation, and metabolic balance within the body. T_3 is an important regulator of endochondral bone formation in the epiphyseal growth plate of bones ([100]Tanaka, 1996). T_3 can inhibit clonal expansion and promote differentiation of chondrocytes in the growth plate, which suggests the thyroid hormone may recruit chondrocytes more rapidly into the differentiation pathway to enhance endochondral bone formation and linear bone growth ([188]Robson*, et al.*, 2000). This may partially explain the accelerated bone maturation in childhood thyrotoxicosis and the rapid initial period of catch-up growth in hypothyroid children treated with T_4 ([188]Robson*, et al.*, 2000).

Thyroid hormones and the late gestation cortisol surge play an important role in the change from the intra-uterine to extra-uterine environment. Umbilical cord blood TSH levels are low at birth, and after the cord is cut there is a rapid increase in TSH about 10-15 minutes after birth ([187]Sack, 1996). The dramatic increase in serum TSH is stimulated by the rapid temperature drop experienced when moving from the intra-uterine to the extra-uterine environment, and stimulates the secretion of T_3 and T_4 by the thyroid gland. Plasma TSH, T_3 and T_4 increase in gestation until birth where TSH and T_4 peak and decrease, while T_3 continues to increase, after birth (Figure 1.10) ([187]Sack, 1996, [189]Fisher*, et al.*, 1994). TSH concentrations remain constant from about 1 month of age throughout childhood ([187]Sack, 1996). Fetal plasma reverse T_3 peaks during gestation and gradually declines thereafter to adult levels (Figure 1.10) ([187]Sack, 1996, [189]Fisher*, et al.*, 1994). If the peripheral conversion of T_4 concentration to the more biologically active T_3 occurs in early life after IUGR, it may be a driver of growth and hence may be a mechanism that might partially explain catch-up growth seen in hypothyroid infants treated with thyroid hormone, and possibly the catch-up growth seen in infants born after IUGR.

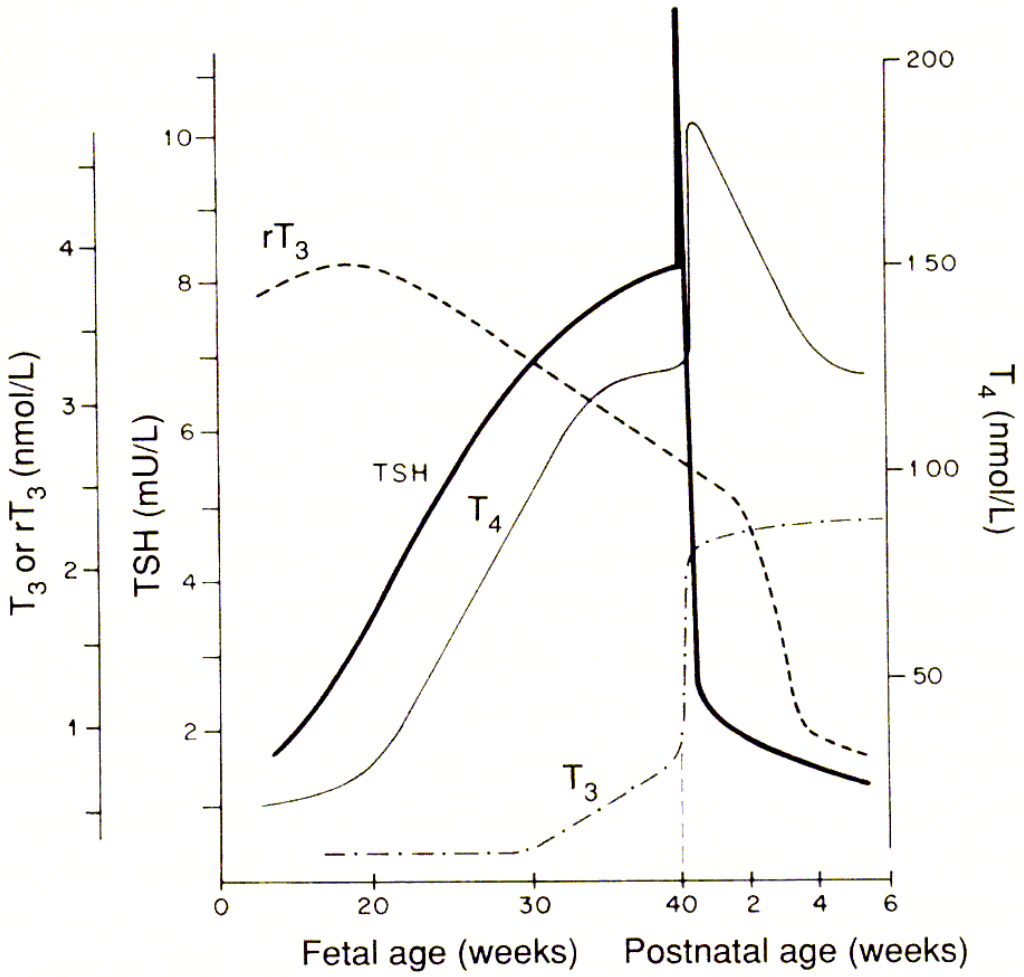

Figure 1.10. Thyroid Hormone concentrations during gestation and early postnatal life in the human fetus and infant.

TSH, T_3 and T_4 increase in gestation until birth where TSH and T_4 peak and decrease while T_3 continues to increase after birth. Reverse T_3 peaks during gestation and gradually declines thereafter. (Figure taken from ([189]Fisher*, et al.*, 1994))

1.4.3.4 HPA axis and Cortisol

Cortisol is a catabolic hormone produced by the adrenal cortex that plays an important role in carbohydrate, protein and fat metabolism ([190]Hughes*, et al.*, 1996). Cortisol increases the concentration of blood glucose by stimulating gluconeogenesis (the conversion of amino acids to carbohydrates within the liver) and inhibits glucose uptake, stimulates protein degradation in muscle especially, and also facilitates lipolysis of fat stores ([190]Hughes*, et al.*, 1996). There is a normal prepartum rise in plasma cortisol in the fetus that induces maturation of the somatotropic axis in the liver via increases in GH receptor number and GH dependent IGF-I gene expression in the liver ([191]Li*, et al.*, 1998). Cortisol also up-regulates the insulin-sensitive glucose transporter GLUT4 in skeletal muscle in the late gestation fetus ([191]Li*, et al.*, 1998). At present, little is known of the actions of cortisol on growth in the neonate of species that are mature at birth.

The overproduction of cortisol in children, such as in Cushing Syndrome, causes a deceleration in linear growth by inhibiting the action of growth factors at the growth plate of bones ([100]Tanaka, 1996), and catch-up and favourable long-term growth is seen after treatment with human growth hormone ([192]Lebrethon*, et al.*, 2000). In rabbits, glucocorticoids suppress the proliferation of growth plate chondrocytes ([193]Baron*, et al.*, 1994). After stopping local glucocorticoid administration, the cumulative number of stem cell divisions in the affected growth plate is reduced compared with the contralateral growth plate ([193]Baron*, et al.*, 1994). The cells in the affected growth plate begin to

proliferate faster than the non-exposed cells, leading to local catch-up growth, occurring due to the delay in normal growth plate senescence ([194]Boersma*, et al.*, 1997). Baron and colleagues proposed that glucocorticoid administration delays senescence by suppressing stem cell proliferation, resulting in catch-up growth after glucocorticoid administration is removed ([193]Baron*, et al.*, 1994). Therefore excess glucocorticoids may limit the extent that an infant can undergo catch-up growth and hence reduce final adult stature.

1.4.3.5 Sex steroids

Steroid hormones are powerful mediators of fetal programming and can be altered by physiological stimuli during the course of development ([195]Matthews*, et al.*, 2001). Oestrogen and androgens regulate proliferation, morphogenesis, differentiation and apoptosis ([196]Lorenzo, 2003). The mechanisms of action of sex steroids on growth are not clear but a previous study has hypothesised that sex steroid hormones may prevent bone loss by inhibiting apoptosis in osteoblasts and stimulating apoptosis in osteoclasts ([197]Kousteni*, et al.*, 2001). Testosterone exerts several effects on the body not related to reproduction including a protein anabolic effect, increasing muscle mass, promoting bone growth, but also sealing the epiphyseal growth plate of bones ([100]Tanaka, 1996). Oestrogen also closes the epiphyseal growth plates of bones, but promotes fat accretion, and at higher concentrations reduces growth rate ([100]Tanaka, 1996).

Under conditions of good nutrition, male (uncastrated) lambs will grow at a faster rate than female lambs, and as a result have a carcass weight that is

heavier than females at the same age ([198]Arnold*, et al.*, 1988, [199]Arnold*, et al.*, 1988). Administration of either androgens or oestrogens in growing lambs increases their growth rate ([200]Hutcheson*, et al.*, 1992) resulting in the production of a carcass with more muscle and less fat ([200]Hutcheson*, et al.*, 1992). Oestrogen treatment in lambs decreases fatty acid esterification, resulting in increased plasma non-esterified fatty acid concentrations, and decreases net adipose tissue deposition by either increasing lipolysis or decreasing lipogenesis ([201]DeHaan*, et al.*, 1990). Therefore alterations in sex steroids after IUGR may have an influence on the postnatal growth rate and body composition in early life.

1.4.4 Potential mechanisms of catch-up growth

Catch-up growth following growth inhibition in childhood (or its equivalent) has been described quite extensively in both human and animal studies, but the mechanisms involved are still not fully understood. Tissues are affected by growth restriction dependent on their metabolic activity ([202]Drouillard*, et al.*, 1991). At the initial stages of catch-up growth, tissue deposition is mostly of muscle and protein and then fat deposition takes over, while the final outcome for body composition depends on refeeding duration ([203]Wright*, et al.*, 1991). In a recent study, catch-up fat in terms of deposition, has been shown to occur during the early catch-up growth period in the rat due to reduced energy expenditure, possibly as a result of suppressed thermogenesis, and not hyperphagia ([204]Crescenzo*, et al.*, 2003). Many studies have also investigated catch-up growth of long bones, but the mechanisms are also unclear.

1.4.4.1 Skeletal muscle catch-up growth

Accelerated growth of skeletal muscle is observed in infants following IUGR ([205]Georgieff*, et al.*, 1989). Catch-up growth of muscle at 4 and 12 months of age was studied indirectly in three groups of infants that included 30 very low birth weight (less than 1500 grams), 30 low birth weight (1500 to 2499 grams) and 30 normal birth weight (greater than or equal to 2500 grams) infants ([205]Georgieff*, et al.*, 1989). All three groups had significant increases in mean upper mid-arm circumferences, mid-arm muscle circumferences, and arm muscle areas between 4 and 12 months of age ([205]Georgieff*, et al.*, 1989). The mechanisms are not fully understood but it is likely that altered abundance of growth regulatory hormones and growth factors, or the abundance of receptors in target tissues are involved ([206]Prader, 1978). It has been proposed that tissues depleted of insulin and IGFs during fetal life and then exposed to increased concentrations of these hormones after birth will show catch-up growth ([70]Cianfarani*, et al.*, 1999). In the mouse C2C12 skeletal muscle cell line, insulin has been shown to rapidly stimulate beta-chain insulin receptors, activate the PI3-kinase/Akt/p70S6-kinase signaling pathway, as well as phosphorylate both mitogen-activated protein kinases, resulting in the formation of multinucleated myotubes and the induction of the creatine kinase activity ([207]Conejo*, et al.*, 2001). This suggests that insulin may induce myogenesis through the PI 3-kinase/p70S6-kinase and p38-MAPK pathways, with signaling through the p44/p42-MAPK pathways being inhibited ([207]Conejo*, et al.*, 2001).

1.4.4.2 Long bone catch-up growth

Catch-up growth in terms of height has been observed following IUGR due to placental restriction in humans ([13]Albertsson Wikland*, et al.*, 1998, [84]Albertsson Wikland*, et al.*, 1993, [85]Karlberg*, et al.*, 1997, [208]Fitzhardinge*, et al.*, 1989). It has been proposed by recent studies in rabbits that catch-up of long bones may be a result of delayed senescence at the growth plate ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). Senescence (or epiphyseal fusion) is a process where the proliferative capacity of chondrocytes declines with each generation of cells and may be dependent on the cumulative number of cell divisions that the chondrocytes of long bones undergo ([209]Gafni*, et al.*, 2001). This delayed senescence has been suggested to be the underlying cause of catch-up growth following restriction due to dexamethasone administration to growing rabbits in order to suppress their linear growth ([209]Gafni*, et al.*, 2001). Catch-up growth occurring after cessation of dexamethasone-induced growth restriction, was characterized by a delay in the age related senescent decline in the heights of the proliferative zone, hypertrophic zone, and total growth plate, in the distal femoral growth plates ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). These findings suggest that linear catch-up growth after placental restriction and IUGR may be due in part, to a delay in growth plate senescence ([209]Gafni*, et al.*, 2001). Glucocorticoids reduce bone formation and increase resorption resulting in decreased bone density, and retarded linear growth of bone ([210]Reid, 1996). A previous study measured cord blood concentrations of CRH, ACTH and cortisol in growth retarded human newborns and compared them with normally grown fetuses, which were matched for gestational age, presence or absence of labour, and mode of delivery ([211]Goland*, et al.*, 1993). The mean umbilical cord

plasma CRH and ACTH level was significantly higher in the growth-retarded newborns, while the mean cortisol concentration in the growth-retarded fetuses tended to be higher ([211]Goland*, et al.*, 1993). This increased circulating level of cortisol suggests that growth-retarded newborns may have reduced bone density due to increased resorption of bone.

1.5 Endocrine state of the IUGR infant

IUGR alters other postnatal characteristics that independently affect human infant growth, such as appetite and the activity of major neuroendocrine and endocrine axes ([50]Yajnik*, et al.*, 1995, [57]Fall*, et al.*, 1995, [82]Colle*, et al.*, 1976, [108]Soto*, et al.*, 2003, [152]Giudice*, et al.*, 1995). Endocrine axes which are known to regulate growth and which are altered in the IUGR infant, include the insulin, IGF and thyroid hormone axes.

1.5.1 Insulin axis

In the IUGR infant at birth, plasma insulin concentrations are low and remain so for the first 6 months compared with infants of average size at birth ([82]Colle*, et al.*, 1976). However, the IUGR infants that undergo catch up growth have an increased rate of insulin secretion in response to a glucose challenge at 6 months of age, compared to those who do not undergo catch-up growth, suggesting that this growth is influenced and perhaps limited by insulin abundance ([82]Colle*, et al.*, 1976). Thus IUGR infants with the poorest postnatal growth were those who had the lowest insulin response to glucose in one study at least ([82]Colle*, et al.*, 1976). Alternatively, the increased insulin secretory

response could reflect insulin resistance, which is unlikely to contribute to catch-up growth. Catch-up growth after IUGR is not consistently characterised by increased serum insulin levels ([82]Colle*, et al.*, 1976, [152]Giudice*, et al.*, 1995, [212]Deiber*, et al.*, 1989, [213]Adcock*, et al.*, 1997, [214]Ogilvy-Stuart*, et al.*, 1998), suggesting that increased insulin sensitivity may be a mechanism that accelerates neonatal growth after IUGR.

1.5.1.1 Insulin sensitivity

Since catch-up growth occurs with reduced or normal serum insulin levels, increased sensitivity to insulin, rather than altered production, may be a mechanism that underlies catch-up growth. This has been supported by numerous animal studies, whereby increased sensitivity to insulin is observed following IUGR, albeit in adults. This may be due to an increased abundance of insulin receptors seen in skeletal muscle ([215]Ozanne*, et al.*, 1996) and adipose tissue ([216]Ozanne*, et al.*, 1997) in adult animals born after IUGR. Increased sensitivity to insulin has also been reported in human studies of IUGR infants. A recent study investigated insulin sensitivity at 1 year of age in infants born small for gestational age ([108]Soto*, et al.*, 2003). Fasting plasma insulin was significantly higher in those SGA infants who had caught up in terms of weight, compared with those who did not catch up, and insulin sensitivity was associated with catch-up in terms of weight and current body mass index during this first year of postnatal life ([108]Soto*, et al.*, 2003). Another study of SGA infants investigated insulin sensitivity of glucose metabolism in the first 48 hours after birth ([217]Bazaes*, et al.*, 2003). SGA infants displayed an increased insulin sensitivity of glucose metabolism at 48 hours compared with AGA infants

([217]Bazaes*, et al.*, 2003), but whether this persists during the first few months of life and promotes catch-up growth is not known.

1.5.2 GH/IGF axis

There is evidence that GH responsiveness and secretion are enhanced in human IUGR infants ([212]Deiber*, et al.*, 1989, [218]DeZegher, 1990). Furthermore, in children born with IUGR who do not catch up, there is evidence of GH resistance ([219]Balsamo, 1995, [220]Chatelain*, et al.*, 1998, [221]Czernichow, 1997). In lambs, hepatic GH receptors are not fully functional up to day 63 of life ([222]Min*, et al.*, 1999). In the IUGR infant, plasma IGF-I concentrations at 5 days of age postnatally were significantly lower compared to controls, but increased to reach those of controls by 9 months of age ([223]Thieriot-Prevost*, et al.*, 1988). Furthermore, catch-up growth in the human IUGR infant has been associated with an increase in plasma IGF-I concentrations into the normal range compared to persistent low IGFs in those infants who did not catch-up ([82]Colle*, et al.*, 1976). Plasma IGF-II levels were significantly reduced in children with short stature born after IUGR, compared to controls ([101]de Waal*, et al.*, 1994). In human IUGR infants, catch-up growth seems to occur with reduced or normal serum IGF-I levels ([152]Giudice*, et al.*, 1995, [214]Ogilvy-Stuart*, et al.*, 1998). The role of the IGF-I receptor (IGF-IR) in regulating human growth was examined in African Efe Pygmies, which are a population with the smallest adult height in the world. These pygmy children are small at birth and growth retarded at 6 months ([224]Hattori*, et al.*, 1996). They have a reduction in IGF-IR on the cell surface of immortalized T lymphocytes, decreased IGF-IR gene transcription and reduced receptor signaling, but normal affinity for IGF ([224]Hattori*, et al.*, 1996). This

suggests that human stature is partly genetically controlled by the expression of IGF-IR ([224]Hattori*, et al.*, 1996). Another study has shown that mutations in the gene for the IGF-IR might underlie some cases of prenatal and postnatal growth failure ([225]Abuzzahab*, et al.*, 2003). One boy and one girl who had mutations in the IGF-IR gene were identified. Fibroblasts from both children had decreased IGF-IR function, as compared with that in control fibroblasts. Both of these children had IUGR and poor postnatal growth, which may be due to mutations in the IGF-IR, hence retarding intrauterine and postnatal growth in humans ([225]Abuzzahab*, et al.*, 2003). Similarly, IGF-IR deficiency in mice resulted in disproportionate postnatal organ growth, and a major deficit in adipose tissue ([166]Holzenberger*, et al.*, 2001).

IUGR children require both a greater basal and GH-induced plasma IGF-I concentration in order to achieve a growth velocity of similar magnitude to that of growth hormone deficient children ([220]Chatelain*, et al.*, 1998). These data suggest a different sensitivity to GH in IUGR children, and imply that these children may be partially IGF-I resistant ([220]Chatelain*, et al.*, 1998). A recent study investigating IGF-I concentrations in pigs born following IUGR showed that circulating IGF-I concentrations were significantly lower in IUGR than in controls ([226]Schoknecht*, et al.*, 1997). When these IUGR pigs were infused with IGF-I, there was a significant increase in circulating IGF-I, along with an increase in growth rate, protein and fat accretion to the control levels ([226]Schoknecht*, et al.*, 1997).

Altered activity of the IGFBPs that modulate IGF bioactivity may also occur in catch-up growth after IUGR. A recent study has shown that at 5 days of age plasma IGFBP-3 levels were significantly lower in SGA neonates than in AGA neonates ([227]Cance-Rouzaud*, et al.*, 1998). A recent study in infants showed however, no difference in IGFBP-3 in those infants that underwent catch-up growth (measured by z-score), compared to those who did not undergo catch-up growth, measured at both 3 and 6 months of age ([102]Garcia*, et al.*, 1996). Serum IGFBP-3 was not a predictor for later postnatal growth parameters ([103]Leger*, et al.*, 1996).

In summary, catch-up growth in IUGR infants has been described as occurring in the presence of reduced or at best, normal serum IGF-I concentrations in the first few months of life and up to one year of age ([152]Giudice*, et al.*, 1995, [214]Ogilvy-Stuart*, et al.*, 1998, [228]Ozkan*, et al.*, 1999). Therefore if IGF-I has a role in catch-up growth, this suggests that it is via increased sensitivity rather than increased production of IGF-I. Possible pathways that this could result from are the reduced abundance of inhibitory binding proteins, the increased abundance of facilitatory binding proteins or IGF-IR, or an enhanced post-receptor signalling.

1.5.3 Thyroid Hormone axis

The effect of IUGR on circulating thyroid hormone abundance has been studied in the human infant at birth, but less so in the first few months of life during catch-up growth ([229]Bongers-Schokking*, et al.*, 1984, [230]Jacobsen*, et al.*, 1979). The thyroid hormone axis ([231]Berthon*, et al.*, 1993, [232]Pracyk*, et al.*, 1992) acts

via T_3 and possibly other forms of TH to regulate growth and differentiation of major tissues, as well as fuel metabolism and metabolic efficiency ([233]Williams*, et al.*, 1998, [234]Silva, 2003, [235]Casas*, et al.*, 2003). An important element of the TH axis and determinant of its activity are circulating levels of TH and the activation of T_4 to T_3, the more biologically active form ([180]Hulbert, 2000). Plasma T_3 and T_4 concentrations are reduced at birth in the IUGR infant ([229]Bongers-Schokking*, et al.*, 1984, [230]Jacobsen*, et al.*, 1979), reflecting the hypoxic and hypoglycaemic intrauterine environment. Following birth, plasma T_3 concentrations are similar in IUGR and normal infants from 1 week to 8 months of age, while plasma T_4 is reduced in IUGR infants up to 50 days of age after which they normalise ([230]Jacobsen*, et al.*, 1979). This suggests that in early postnatal life following IUGR, there may be normal TH production, but enhanced conversion of T_4 to T_3, which may help to maintain TH bioavailability and action ([230]Jacobsen*, et al.*, 1979). While the IUGR infants in this study gained as much weight as normal infants over the period of study, suggestive of catch-up growth, whether this was related to circulating T_3 or the degree to which it may have been limited by T_4, was not examined ([230]Jacobsen*, et al.*, 1979). It is therefore possible that increased conversion of T_4 to T_3 and hence increased plasma T_3 levels occur and are highest in those IUGR infants that undergo catch up growth ([230]Jacobsen*, et al.*, 1979).

1.5.4 Summary of catch up growth and endocrine state of IUGR

In summary, catch-up growth in IUGR infants occurs during the first year of life following IUGR, despite reduced or at best normal abundance of insulin, insulin-like growth factors and thyroid hormones in the blood. Therefore catch-up

growth of IUGR infants occurs in the first year of life despite evidence of a reduced or normal abundance of the major anabolic hormones of infancy, which partly mediate the actions of several major factors of postnatal growth, including nutrition, the somatotropic axis, and steroids. It is therefore proposed that restricted growth *in utero* may permanently alter or program key tissues involved in growth and growth regulation of the neonate, specifically those of the growth regulatory endocrine axes and their major tissue targets. Because of the invasive nature of studies required to investigate this hypothesis, the effect of restriction of fetal growth on birth phenotype, postnatal growth velocity, and sensitivity to somatotropic hormones in a suitable experimental animal model of IUGR and neonatal catch-up growth needs to be investigated.

1.6 Experimental IUGR (placental restriction), postnatal growth and endocrine state in animals

To directly investigate the consequences of IUGR for growth and its endocrine regulation postnatally, animal studies have to be employed since it is not ethical or feasible to perform highly invasive studies on human infants. Many studies of the patterns of postnatal growth in IUGR infants have been performed but few of these have examined very early postnatal growth and sensitivity to, as well as production of the major anabolic hormones of this age, and virtually none have examined these in the first month of life. Therefore an animal species that shows similar metabolic and endocrine responses to human IUGR before and after birth would be useful to investigate the mechanisms of fetal growth restriction and subsequent postnatal catch-up growth in early life.

1.6.1 Sheep model of placental restriction

Placental insufficiency is a major cause of IUGR in most species, due to either undernutrition or reduced supply of oxygen and nutrients. The placenta found in ruminants is the cotyledonary type (Figure 1.11). In the cotyledonary placenta, there are multiple, discrete areas of attachment called cotyledons which are formed by the interaction of areas of the allantochorion with the endometrium. The fetal portions of this type of placenta, or the cotyledons, along with the maternal contact sites termed caruncles, form the complex termed a placentome (Figure 1.11). The sheep placenta consists of 40-90 placentomes (cotyledons), which form specialized areas of the non-pregnant uterus known as caruncles. Excision of the majority of these caruncles before pregnancy restricts total placental size by limiting the number of placentomes in the subsequent pregnancy ([5]Owens*, et al.*, 1987, [18]Robinson*, et al.*, 1985, [236]Robinson*, et al.*, 1979). The placentomes of the sheep placenta reach their maximum diameter in early pregnancy, and grow to reach their maximum size by 70 days of pregnancy (term 145-150 days in the sheep). Removal of the caruncles prior to pregnancy in the sheep enables us to implement an experimental restriction of placental and fetal growth (placental restriction) ([5]Owens*, et al.*, 1987, [18]Robinson*, et al.*, 1985, [236]Robinson*, et al.*, 1979). This leads to a reduction in oxygen and substrate supply to the fetus, reducing fetal growth and hence size at birth, leading to intra-uterine growth retardation (IUGR) ([22]Owens*, et al.*, 1989). Placental delivery of oxygen and nutrients (in particular, glucose) to the fetus is reduced, and restricts fetal growth from about mid-gestation at least ([5]Owens*, et al.*, 1987). Experimental restriction of

placental and hence fetal growth in the sheep has been shown to have similar growth, metabolic and endocrine consequences for the fetus as that for the human IUGR fetus ([237]Owens*, et al.*, 1994). Specifically, fetal growth retardation due to restriction of placental growth after removal of endometrial caruncles in the sheep, results in the fetus being chronically hypoxaemic, polycythaemic and hypoglycaemic in late gestation ([5]Owens*, et al.*, 1987, [18]Robinson*, et al.*, 1985, [236]Robinson*, et al.*, 1979).

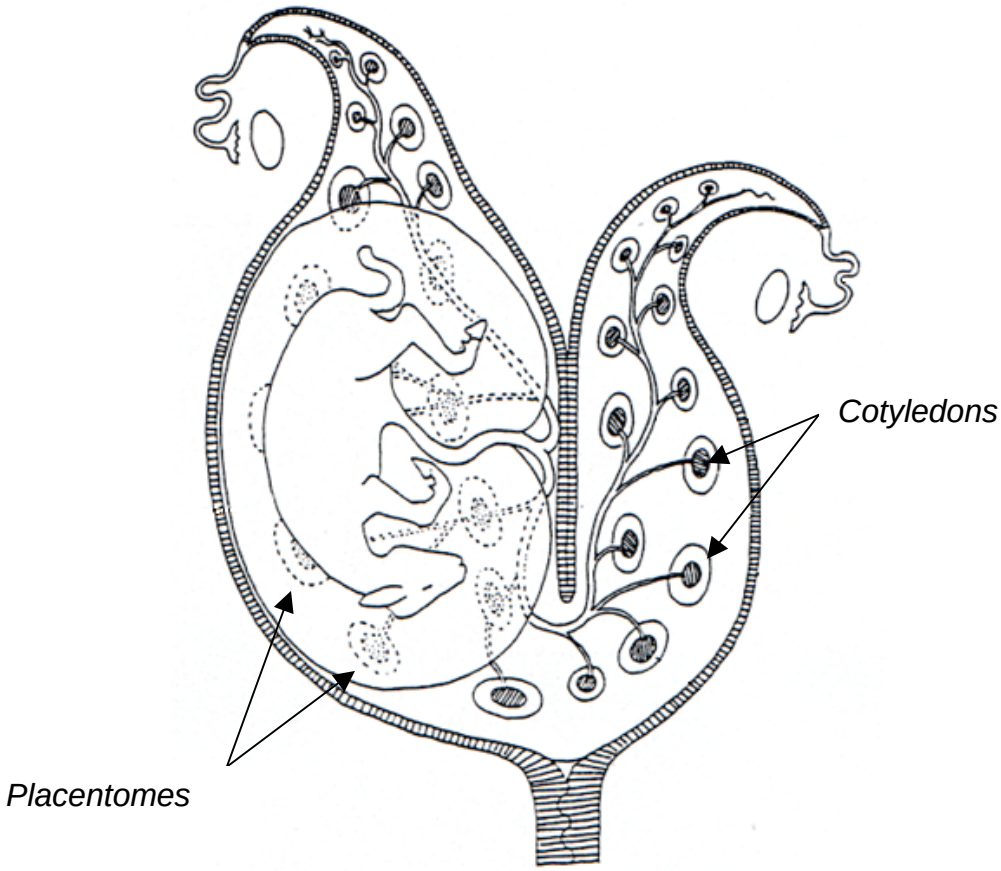

Figure 1.11. Schematic diagram of the bicornuate uterus of the sheep. Demonstrates fetus surrounded by placenta, and caruncles, which form placentomes with cotyledons, and supply oxygen and nutrients to the fetus. Adapted from ([238]Steven*, et al.*, 1975).

The restricted sheep placenta has reduced spiral arterioles, which have failed to undergo the normal physiological changes that increase vessel diameter and reduce vascular resistance to flow ([17]Robinson*, et al.*, 1996). Placental restriction in the sheep is characterised with a reduced supply of oxygen to both the pregnant uterus and fetus and a redistribution of oxygen to the fetus ([5]Owens*, et al.*, 1987). Glucose delivery and consumption by the fetus is similar to that of oxygen, with hypoglycaemia evident by 110 days gestation, but the consumption of glucose being matched to growth of the fetus. If there is a reduction in available nutrients to the fetus as in growth retarded infants, then growth of the fetus is compromised. Other hormones that promote or are essential for fetal growth, such as insulin, insulin-like growth factors (IGF-I and -II), thyroxine (T_4) and tri-iodothyronine (T_3), are also reduced in plasma of the growth-restricted fetus, while hormones that are increased include cortisol, and noradrenalin ([13]Albertsson Wikland*, et al.*, 1998, [30]Hooper*, et al.*, 1994, [32]Fowden, 1995, [103]Leger*, et al.*, 1996, [236]Robinson*, et al.*, 1979, [239]Harding*, et al.*, 1985, [240]Cetin*, et al.*, 2001, [241]Mellor*, et al.*, 1977, [242]Leger*, et al.*, 1996, [243]Bauer*, et al.*, 1995, [244]Cetin, 1990, [245]Rochiccioli, 1989, [246]Houang*, et al.*, 1999, [247]Phillips*, et al.*, 1996) (Figure 1.12).

1.6.1.1 Postnatal consequences of placental restriction in the sheep

The consequences of placental restriction in the postnatal period are relatively unknown. Many studies have focussed on the effect of placental restriction in fetal life but the metabolic and endocrine effects are still unclear. The effect of placental restriction on postnatal growth rate has been studied to a limited extent in other species also. Placental restriction of growth of the sheep fetus is

similar to the IUGR human fetus in that there is asymmetrical growth retardation ([17]Robinson*, et al.*, 1996). Organs such as the spleen, thymus, liver and gut are restricted the most, whereas organs such as the heart and kidney grow in proportion to body weight. The brain is also spared in the sheep and is larger relative to body weight being similar to the human IUGR infant. Immediately after birth, placentally restricted lambs take longer to stand and longer until the onset of suckling, and have a higher rectal temperature during the first hour of life ([241]Mellor*, et al.*, 1977). Plasma insulin, corticosteroids and lactate are not different during the first 24 hours after birth, but plasma T_4 and fructose are reduced in placentally restricted compared with control lambs at this age ([241]Mellor*, et al.*, 1977). A recent study of low and high birth weight lambs that were fed a liquid diet to grow fast or slow has shown that at birth the low birth weight lambs are less mature with respect to some aspects of endocrine and metabolic development ([248]Greenwood*, et al.*, 2002). Fast-grown low birth weight lambs had higher concentrations of glucose, insulin, urea nitrogen, growth hormone, and IGF-I, compared with slow-grown low birth weight lambs ([248]Greenwood*, et al.*, 2002). An adverse effect of neonatal catch-up growth on subsequent adiposity is supported by the latter study in the sheep, where those of low birth weight due to multiple pregnancy were allowed either *ad libitum* milk intake or were restricted in their feeding ([249]Greenwood*, et al.*, 1998). Regardless of feed intake, low birth weight lambs grew more quickly for their size and had a higher percentage of body fat at 20 kg live weight, than high birth weight lambs ([249]Greenwood*, et al.*, 1998). The increased adiposity of low birth weight lambs was also exacerbated when lambs were allowed to feed *ad libitum* compared to restricted feeding ([249]Greenwood*, et al.*, 1998).

Figure 1.12. Fetal blood gas, metabolic, and endocrine changes associated with intrauterine growth restriction in the sheep and the human infant.

Decreased with IUGR	Unchanged with IUGR	Increased with IUGR
PO_2 [237, 248]	Adrenocorticotropic hormone (ACTH) [248]	O_2 carrying capacity (Hb concentration) [237]
O_2 content [237, 241, 248]	Placental lactogen [244]	Cortisol [30, 32, 240, 247, 248]
Glucose [237]		Leptin [241]
T_4 and T_3 [240]		
IGF-I, IGF-II [13, 109, 243, 244]		
Growth Hormone [13, 246]		
Lactate [237]		
Prolactin [240]		
Amino acids [241, 245]		

References for table: ([13]Albertsson Wikland*, et al.*, 1998, [30]Hooper*, et al.*, 1994, [32]Fowden, 1995, [103]Leger*, et al.*, 1996, [236]Robinson*, et al.*, 1979, [239]Harding*, et al.*, 1985, [240]Cetin*, et al.*, 2001, [241]Mellor*, et al.*, 1977, [242]Leger*, et al.*, 1996, [243]Bauer*, et al.*, 1995, [244]Cetin, 1990, [245]Rochiccioli, 1989, [246]Houang*, et al.*, 1999, [247]Phillips*, et al.*, 1996).

1.6.2 Other animal models of placental restriction

Many models of placental restriction have been studied to date, such as maternal protein and feed restriction, placental embolisation, caruncle ctomy, uterine artery ligation, and multiple litter (natural) restriction, and these models of restriction have been studied in many different animal models, such as the rat, sheep, pig and guinea pig ([18]Robinson*, et al.*, 1985, [226]Schoknecht*, et al.*, 1997, [243]Bauer*, et al.*, 1995, [247]Phillips*, et al.*, 1996, [250]Woodall*, et al.*, 1996, [251]Muaku*, et al.*, 1996, [252]Shepherd*, et al.*, 1997, [253]Ritacco*, et al.*, 1997, [254]Gatford*, et al.*, 2002, [255]Kind*, et al.*, 1995, [256]Muaku*, et al.*, 1995, [257]Owens*, et al.*, 1987, [258]Combes, 1997, [259]Huizinga*, et al.*, 2000).

Recent studies ([250]Woodall*, et al.*, 1996, [251]Muaku*, et al.*, 1996, [252]Shepherd*, et al.*, 1997) have examined the consequences of severe chronic maternal undernutrition (30% of *ad libitum* feed) on postnatal growth in the rat. This intervention reduced size at birth in pups, which then showed persistent growth failure and remained markedly smaller than control offspring up to adulthood ([260]Woodall*, et al.*, 1996). Body weights of offspring of restricted dams were reduced in late gestation, at birth and up to adulthood at 90 days of age, compared to that of control offspring ([260]Woodall*, et al.*, 1996). Nose-rump length was also reduced in the offspring of restricted dams from day 22 of gestation until weaning ([260]Woodall*, et al.*, 1996). Plasma IGF-I levels were significantly reduced in the pups of restricted fed dams from day 22 of gestation until postnatal day 9, but were not significantly different at later ages ([260]Woodall*, et al.*, 1996). Plasma insulin levels were also significantly reduced

in the pups of restricted fed dams at birth, but not subsequently ([260]Woodall*, et al.*, 1996). Even though IGF-I levels returned to normal levels by day 9 postnatally, the rat pups did not demonstrate catch-up growth, since they showed persistent growth failure and remained markedly smaller than control offspring up to adulthood ([260]Woodall*, et al.*, 1996). So the rat is probably not a good model to determine catch-up growth in during early postnatal life after placental restriction due to maternal feed restriction. While food was available *ad libitum* after birth, restricted dams lost a significant amount of body weight throughout gestation due to undernutrition. The rat pups only caught up to the control group by day 10 postnatally ([260]Woodall*, et al.*, 1996). Therefore the offspring of restricted mothers may have been restricted postnatally as well.

In the pig, natural runting can occur, which are pigs that are born small and undergo compensatory growth in terms of fractional but not absolute growth rates for weight for the first two weeks of life. This catch-up growth in the spontaneous IUGR pig is not due to increased abundance of anabolic hormones, since they have reduced plasma and hepatic levels of IGF-I mRNA expression ([226]Schoknecht*, et al.*, 1997, [253]Ritacco*, et al.*, 1997). Therefore catch-up growth in fractional terms for weight occurs in IUGR neonatal pigs despite low plasma IGF-I. This suggests that the IUGR piglets may be more sensitive to IGF-I than control piglets and that IGF-I may play a role in the regulation of whole body metabolism. Consistent with this, exogenous IGF-I infusion significantly increased protein and fat accretion (+15-20%) in the IUGR piglets, but not in the control pigs ([226]Schoknecht*, et al.*, 1997). Exogenous IGF-I (4 μg/hour) did not alter concentrations of insulin, glucose, IGF-II, or the

thyroid hormones in plasma up until day 7 postnatally, in control or IUGR pigs ([226]Schoknecht, *et al.*, 1997). At birth however, plasma triiodothyronine (T_3) concentration was greater than controls, and at day 7 the infusion of IGF-I increased circulating levels of insulin and glucose to a greater extent in the IUGR piglets compared to the controls ([226]Schoknecht, *et al.*, 1997). In addition, the increased growth in terms of body weight, protein and fat accretion, measured by using proximate analysis of ground carcasses, may be the result of IUGR piglets being more sensitive to IGF-I than controls, since the IUGR piglets had the same if not lower concentrations of IGF-I compared to controls. In this study however, the precise mechanism of the observed increased growth in response to IGF-I administration could not be determined since they did not measure feed intake ([226]Schoknecht, *et al.*, 1997). They did suggest that the growth could be due to the IGF increasing their feed intake, thus providing the IUGR pigs with increased nutrients ([226]Schoknecht, *et al.*, 1997).

In animals such as the guinea pig, the rate of blood flow is a major determinant of fetal growth ([261]Saintonge, *et al.*, 1981). Spontaneous fetal growth retardation in the guinea pig due a large litter size, is associated with a smaller placenta, a reduced placental blood flow, and a reduced transfer of amino acids to the fetus ([261]Saintonge, *et al.*, 1981). A recent study has demonstrated that maternal feed restriction in the guinea pig, restricts fetal growth, and causes hyperinsulinaemia in the young adult male offspring, suggestive of insulin resistance ([262]Kind, *et al.*, 2003). Also guinea pigs that were small at birth did undergo catch-up growth in terms of weight ([262]Kind, *et al.*, 2003).

We therefore hypothesised that placental restriction of fetal growth in the sheep, specifically increases the activities of the insulin, IGF, growth hormone, and thyroid hormone axes and so promotes growth. The effect of placental restriction on size at birth, neonatal growth rates, *in vivo* metabolic sensitivity to insulin and IGF-I, plasma concentrations of these hormones and T_3 and T_4 will therefore be determined.

1.7 General hypothesis

Placental restriction of fetal growth will reduce size at birth and increase neonatal growth rates of soft and skeletal tissues due to either the increased abundance of and/or increased sensitivity to insulin, IGF-I, and TH.

1.8 Specific hypotheses

Placental restriction of fetal growth will reduce size at birth in sheep.

Placental restriction of fetal growth will increase the postnatal growth rates of both soft and skeletal tissues in sheep.

Placental restriction of fetal growth will increase the postnatal sensitivity of soft and skeletal tissues and hence increase postnatal growth of these tissues.

Placental restriction of fetal growth will increase postnatal adiposity in sheep.

1.9 Significance of project

This project will determine if placental restriction of fetal growth, a major identifiable cause of IUGR, alters neonatal growth by altering the functional development of major endocrine regulators of postnatal growth, the insulin, IGF, and TH axes. This will provide the scientific basis for the subsequent design and testing of alternative therapies to overcome the adverse consequences of IUGR for adult stature.

1.10 Proposed studies

1.10.1 Aims

To determine the effects of placental restriction of fetal growth in sheep on size at birth, postnatal growth, and the metabolic and endocrine state of the neonate during the catch-up growth period (0-45 days).

Specifically,

1.10.2 Size at birth

To determine the weight, crown-rump length, shoulder height, metatarsal and tibia lengths, radius/ulna and metacarpal lengths, skull length and width, abdominal and lower thoracic circumferences, and hind limb and radius/ulna circumferences (at birth and every 5 days up to 45 days of age) of male and female control lambs and male and female lambs born after placental restriction of fetal growth.

1.10.3 Neonatal growth

To determine the absolute and fractional rates of growth in early postnatal life (0-45 days), particularly of weight, crown-rump length, shoulder height, metatarsal and tibia length, radius/ulna and metacarpal length, skull length and width, abdominal and lower thoracic circumference, and hind limb and radius/ulna circumference, of male and female control and placentally restricted lambs.

1.10.4 Body composition

To determine and compare the lean and fat masses and body compositions (at 45 days of age) of control and placentally restricted male and female lambs. Lean mass will be calculated by the summation of weights or various muscles of varying muscle fibre types removed at post mortem (biceps, M flexor Carpi Radialis, Semitendinosus, Tibialis, Gastrocnemius, Soleus, EDL, Biceps femoris, and Vastus lateralis). Fat mass (visceral fat mass) will be calculated by the summation of fat depots that can be completely removed at post mortem (omental, perirenal, retroperitoneal).

1.10.5 Hormone production and sensitivity

To determine in male and female control and placentally restricted lambs, the basal plasma insulin concentrations, and insulin secretion in response to IVGTT, the basal plasma IGF-I and IGF-II concentrations, the basal plasma thyroid hormone concentrations - free and total T_3 and T_4, basal insulin and IGF stimulated uptake of α-amino nitrogen concentrations and free fatty acid concentrations. Also, the whole body insulin and IGF-I sensitivities of glucose, amino acid and free fatty acid metabolism in male and female lambs undergoing catch-up growth after placental restriction.

Chapter 2

MATERIALS AND METHODS

2.1 Treatment of Animals

All surgical and experimental procedures performed in this project were approved by the Adelaide University Animal Ethics Committee (Animal Ethics Approval Number: M/1/97A and M/10/00).

2.1.1 Restriction of Placental Growth

Restriction of placental implantation (caruncleclectomy): The non-pregnant sheep uterus is a bicornuate, pear-shaped tubular organ, which is covered by a columnar epithelium with up to 100-160 cup-like elevations of endometrium, termed caruncles. Implantation of the ovine blastocyst to form the placenta occurs at these sites. These endometrial caruncles can be removed surgically to restrict placental implantation and development in a subsequent fetus ([4]Owens*, et al.*, 1986, [236]Robinson*, et al.*, 1979). Restriction of placental growth and development restrains fetal growth and produces fetal growth restriction. Anaesthesia in ewes was induced with sodium thiopentone (1g in 20 ml H_2O; Pentothal, Abbot, Sydney, Australia) injected intravenously via the jugular vein. The ewe was intubated with size 6.0 endotracheal tube (Critical Assist Group, Thailand) and anaesthesia maintained with halothane in oxygen (Fluothane, ICI, Villawood, Australia), with the sheep breathing spontaneously via the endotracheal tube in a closed circuit system with soda lime as the carbon dioxide absorber. The abdominal area was shaved, scrubbed with Betadine Surgical Scrub and Betadine Antiseptic Solution (Faulding Pharmaceuticals,

Australia), and then swabbed with a 1:10 solution of alcoholic hibitane (Chlorhexidine gluconate 5% w/v) (Johnson and Johnson, Australia). A 10 cm mid-line incision was made along the abdomen and the uterus exteriorised. Each uterine horn was opened along the length from the cervix to near the utero-tubular junction. The majority of visible endometrial caruncles (between 65 and 148) were then removed from the non-pregnant uterus, leaving 6-8 caruncles (creating moderate restriction) in each horn of the bicornuate uterus ([236]Robinson*, et al.*, 1979). Each horn was closed in a single layer through the myometrium and the visceral peritoneum, using continuous sutures of 3/0 chromic catgut. The abdominal cavity was closed with sutures (size 0) in two layers; the first consisted of continuous sutures through the peritoneum and rectus sheath, and the second through the skin and subcutaneous tissues.

2.1.2 Mating and housing of ewes

After a 10-week recovery period from the carunclectomy operation, the ewes were entered into a mating program, and pregnancies were confirmed by ultrasound. Ewes were housed in individual pens in animal holding rooms from 110 days of gestation, with a 12:12 light/dark lighting cycle, and fed lucerne chaff twice daily *ad libitum*, with water *ad libitum*. These ewes delivered 97 lambs, of which 48 lambs (controls) were from control ewes, and 49 lambs (placentally restricted, PR) were from the placentally restricted ewes. Of these, twins and non-surviving lambs were removed so that 37 control lambs and 34 PR lambs were used in these studies. Lambs were housed in the pens with their mothers throughout the study. During lactation, ewes were fed lucerne chaff twice daily *ad libitum*, with water *ad libitum*.

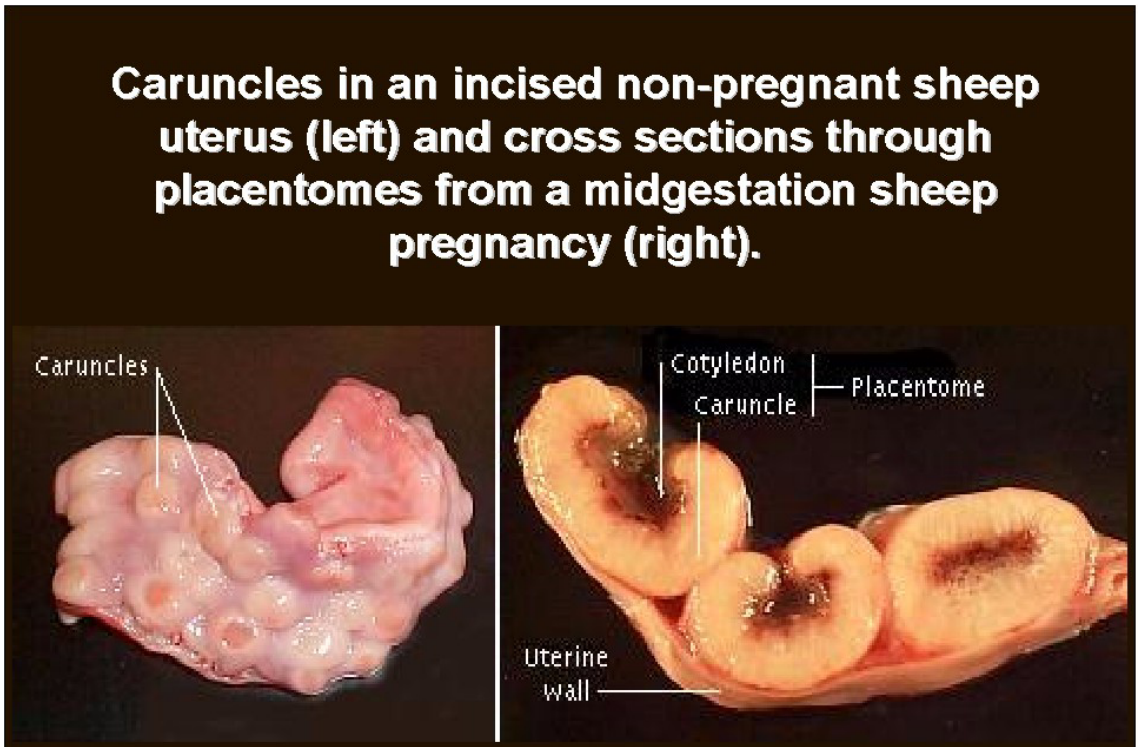

Figure 2.1. Caruncles of a sheep uterus.

Demonstrates caruncles lining the non-pregnant uterus of a sheep (left) and a magnified picture demonstrating a cross-sectional view of the placentomes lining the uterine wall (right). The caruncles were removed prior to pregnancy to restrict fetal growth. Figure from website ([263]Bowen, 1987).

2.1.3 Insertion of vascular catheters

At approximately 5 days of age, catheters (1.52 mm OD x 0.86 mm ID, Critchley Electrical Products Pty Ltd Silverwater NSW, Australia) were inserted into the left femoral artery and vein of the lamb under general anaesthesia induced and maintained by Halothane inhalation anaesthetic (Fluothane, ICI, Villawood, Australia). All lambs received an intramuscular injection (0.5 mL) of antibiotics (Norocillin LA injection, Norbrook Laboratories, UK) before surgery and then for 3 days post surgery, and an intramuscular injection (100 μl) of Xylazine diluted 1:10 (Troy Laboratories Pty Ltd, Smithfield NSW, Australia) was administered when lambs were fully conscious. Patency of the catheters in the lambs was maintained by flushing the catheters with heparinized saline (500 U/mL) daily for 3 days after surgery and then every second day.

2.1.4 Measurement of size at birth and postnatal growth rate

At birth and at 5-day intervals to 45 days of age, birth phenotype (day 0) and postnatal growth parameters were measured (Figures 2.2 and 2.3). Placental weight, body weight, crown-rump length (CRL), tibia and metatarsal lengths, radius/ulna and metacarpal lengths, shoulder height, lower thoracic and abdominal circumferences, skull length and width, hind limb circumference (knee joint) (around the lateral malleolus of tibia), tibia circumference (equidistant between the proximal end of fibula and lateral malleolus of tibia), and radius/ulna circumference were measured (Figures 2.2 and 2.3). Body mass index (BMI) was calculated as weight/CRL^2 (kg/cm^2), while ponderal index

(PI) was calculated as weight/CRL^3 (kg/cm^3). Each parameter of size was measured in duplicate for each age, and then averaged to get a value for the parameter, with repeatability ranging from 4% for weight to 8% for other parameters. The absolute growth rate (AGR) for all parameters was linear for 45 $\pm$ 3 days following birth and was determined by linear regression analysis for each parameter within individual sheep. Neonatal fractional growth rate (NFGR) was calculated as the AGR for a parameter relative to size of that parameter at birth, while current fractional growth rate (CFGR) was calculated as the AGR for a parameter relative to the current size of that parameter.

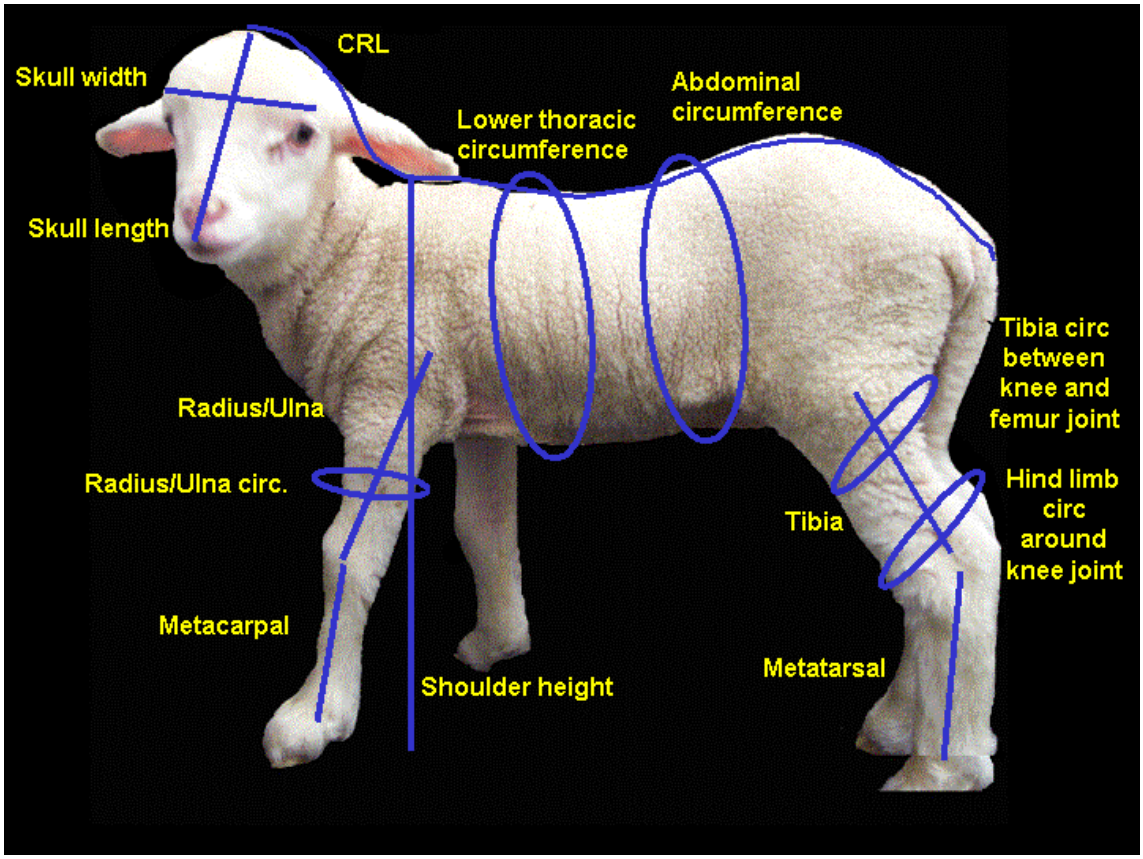

Figure 2.2. Growth measurements of the neonatal lamb.

Photograph of a lamb demonstrating the areas where growth measures were taken (crown-rump length (CRL), tibia and metatarsal lengths, radius/ulna and metacarpal lengths, shoulder height, lower thoracic and abdominal circumferences, skull width and length, hind limb circumference (knee joint) (around the lateral malleolus of tibia), tibia circumference (equidistant between the proximal end of fibula and lateral malleolus of tibia), and radius/ulna circumference.

Table 2.1. Measurements of lambs

Measurement	**Measurement description**
Weight:	Lambs were weighed using hanging scales (Salter Scales Model 235 6S Australia) in which the lambs were suspended by a hessian bag.
Crown-rump length:	Measured from between the cornual processes (horns) along the neck, down the back and to the second coccygeal vertebrae (second joint in tail) using a tailor's tape measure.
Shoulder height:	Measured from dorsal point of the scapula to the ground (with lamb standing) using a tailor's tape measure.
Lower thoracic circumference:	Measured around the trunk of the lamb in line with the caudal extremity of the sternum (end of breastbone closest to rear of lamb) using a tailor's tape measure.
Abdominal circumference:	Measured around the trunk of the lamb in line with the umbilicus using a tailor's tape measure.
Skull width:	Measured midway between the cornual processes (horns) and the supraorbital process of the frontal bone (eye sockets) using Vernier callipers.
Skull length:	Measured from the dorsal point of the ethmoid bone (nose) to the caudal point of the parietal bone (back of head) using Vernier callipers.

Hind limb circumference (around knee joint):	Measured around the lateral malleolus of tibia above the extensor ligament (centre of bone) using a tailor's tape measure.
Tibia circumference (between knee and tibia/femur joint):	Measured equidistant between the proximal end of the fibula and lateral malleolus of the tibia using a tailor's tape measure.
Radius/Ulna circumference:	Measured around the radius/ulna equidistant between the styloid process of the ulna and the olecranon using a tailor's tape measure.
Radius/Ulna length:	Measured from the styloid process of the ulna to the olecranon using a tailor's tape measure.
Metacarpus length:	Measured from the proximal extremity (carpal bones) to the distal extremity (two condyles which meet the 1st phalanx) using a tailor's tape measure.
Tibia length:	Measured from proximal extremity (tibial tuberosity) to the distal extremity (lateral malleolus) using a tailor's tape measure.
Metatarsus length:	Measured from the proximal extremity (tarsal bones) to the distal extremity (1st phalanx) using a tailor's tape measure.

2.1.5 Post-mortem

At 45 ± 3 days of age, a subset of lambs (9 controls and 8 PR lambs) were killed by intravenous administration of an overdose of barbiturates (Pentobarbitone sodium 325 mg/mL Lethabarb, Virbac Australia Pty Ltd, Peakhurst NSW, Australia). Organs were removed, weighed and measured. Fat depots and muscles that could be completely removed as a whole entity were weighed. A sample of each organ was fixed in 4% paraformaldehyde and another sample frozen in liquid nitrogen, and fat depots were placed in OCT compound and frozen with iso-Pentane (BDH Laboratory Supplies, UK) in liquid nitrogen, and then stored at –80^{o}C for later analysis.

2.2 Assessment of Endocrine Axes and function

2.2.1 Insulin Axis

2.2.1.1 Hyperinsulinaemic euglycaemic clamp and insulin sensitivity of glucose metabolism

At 30 ± 2 days of age, a hyperinsulinaemic euglycaemic clamp (HEC) was performed. Lambs were fasted for 1 hour before the experiment, but were allowed water *ad libitum*. Using the hyperinsulinaemic euglycaemic clamp the insulin sensitivity of whole body glucose metabolism can be determined. After an initial 1-hour fast arterial blood was sampled (2 mL) at -10, -5 and 0 minutes from the femoral artery. Human insulin (Actrapid, Novo Nordisk A/S, Denmark) was then continuously infused intravenously (2 mU/kg/min) and blood sampled

(0.2 mL) every 5 minutes for 120 minutes ([264]DeFronzo*, et al.*, 1979). Blood glucose at each time point was measured using a HemoCue glucometer (HemoCue AB, Sweden). At 15 minutes after the start of the insulin infusion, glucose (25%) was infused intravenously, initially at 2 mg/kg/min. The glucose infusion rate (GIR) was then adjusted each 5 minutes after the measurement of blood glucose, to maintain euglycaemia at the fasting blood glucose concentration, using a modification of a previously published algorithm ([264]DeFronzo*, et al.*, 1979). All syringe infusions were carried out using a fully programmable infusion pump (World Precision Instruments SP220i). The mean coefficient of variation in blood glucose during the second hour of the clamp was 2.6%. Plasma insulin measured prior to the start of (-10, -5, 0 minutes) and during the second hour of the hyperinsulinaemic euglycaemic clamp (60, 75, 90, 105, 120 minutes), was used to determine the fasting plasma insulin concentration and plateau plasma insulin concentration achieved respectively. The insulin sensitivity of whole body glucose metabolism was determined as the glucose infusion rate from 60 to 120 minutes ($GIR_{60\text{-}120}$) during the hyperinsulinaemic euglycaemic clamp, corrected for the mean plasma insulin in the second hour of infusion measured every 15 minutes. The mean coefficient of variation for $GIR_{60\text{-}120}$ in each lamb during the hyperinsulinaemic euglycaemic clamp was less than 5.8%.

2.2.1.2 Plasma insulin concentration

Plasma insulin concentrations were measured prior to (-10, -5, 0) and during the second hour of the hyperinsulinaemic euglycaemic clamp (60, 75, 90, 105, 120 minutes). Plasma from these time points was measured for insulin using a

commercially available radioimmunoassay kit (Pharmacia, Uppsala, Sweden). Standard (100 μl) and unknown (100 μl) plasma samples were pipetted into tubes, ^{125}I-insulin (100 μl) and then 100 μl of antibody were added and allowed to incubate on a shaking rack for 2 hours at room temperature. Double antibody suspension (2 ml) was then added and allowed to incubate for a further ½ hour at room temperature. Samples were centrifuged for 10 minutes at 1500g. Tubes were then decanted and left upside down on absorbent paper for approximately 10 minutes, being careful not to dislodge the pellet at the bottom of the tube. The radioactivity of the pellet was determined using a gamma counter. The mean coefficient of variation was less than 8.3%.

2.2.1.3 Insulin sensitivity of circulating amino acids

Plasma α-amino nitrogen concentrations were measured prior to and during the second hour of the hyperinsulinaemic euglycaemic clamp (-10, -5, 0, 60, 75, 90, 105, 120 minutes). The insulin sensitivity of circulating amino acids was calculated as the percentage change from fasting plasma α-amino nitrogen concentration to those of the second hour of the HEC, corrected for the plateau plasma insulin concentration (α-a$N_{60\text{-}120}$).

2.2.1.4 Insulin sensitivity of circulating free fatty acids

Plasma free fatty acid (FFA) concentrations were measured prior to and during the second hour of the hyperinsulinaemic euglycaemic clamp (-10, -5, 0, 60, 75, 90, 105, 120 minutes). The insulin sensitivity of circulating FFA was calculated as the percentage change from fasting FFA concentrations to those of the second hour of the HEC, corrected for the plateau plasma insulin ($FFA_{60\text{-}120}$).

2.2.1.5 Insulin clearance and post-hepatic insulin secretory rate

Insulin clearance was calculated as the rate of insulin infusion (2 mU/kg/min) divided by the plateau plasma insulin concentration in the second hour of the clamp. Post-hepatic insulin delivery rate was calculated as the fasting plasma insulin concentration multiplied by the insulin clearance rate. In addition, the post-hepatic insulin secretory rate in the stimulated state was calculated as the maximum plasma insulin concentration during the glucose tolerance test multiplied by the insulin clearance rate.

2.2.1.6 Disposition Index

The disposition index in the basal state, an index of insulin action reflecting both insulin abundance and sensitivity, was calculated for glucose, amino acid, and free fatty acid metabolism as the post-hepatic insulin delivery rate in the fasting state multiplied by the insulin sensitivity of glucose, amino acids, or free fatty acid metabolism. In addition, the stimulated disposition indices for glucose, amino acid and free fatty acid metabolism were calculated as the post-hepatic insulin secretory rate in the stimulated state, multiplied by the insulin sensitivity of glucose, amino acid and free fatty acid metabolism.

2.2.1.7 Insulin secretion in response to an intravenous glucose tolerance test

At 32 $\pm$ 3 days of age an intravenous glucose tolerance test (IVGTT) was performed. Lambs were fasted for 3 hours before the experiment, but were allowed water *ad libitum*. Arterial blood samples (2 mL) were taken at -5, -3,

and 0 minutes. At 0 minutes, 0.25 g glucose (25% dextrose) per kg live weight was rapidly infused intravenously. Blood (2 mL) was sampled at 5 minute intervals for the first 30 minutes, at 10 minute intervals for the next 70 minutes, then at 20-30 minute intervals until at least 240 minutes after the infusion of glucose or until at least 2 consecutive blood glucose concentrations were below the mean fasting blood glucose concentration. Blood glucose concentration at each time point was measured using a HemoCue glucometer (HemoCue AB, Sweden). Remaining blood was centrifuged, and plasma collected for subsequent measurement of insulin at each time point, using a commercially available radioimmunoassay kit (Phadeseph, Pharmacia-Upjohn Diagnostics AB, Sweden). The inter-assay coefficient of variation for the insulin RIA is 3.7%. Insulin secretion during the IVGTT was calculated as the area under the insulin concentration curve versus time, and corrected for the glucose area under the curve. Areas under the curve were determined using the Sigma Scan Pro v4 software package (Jandel Scientific Software).

2.2.2 Insulin-like Growth Factor Axis

2.2.2.1 Hyper-IGF-I euglycaemic clamp and IGF-I sensitivity of glucose metabolism

At 35 $\pm$ 3 days of age, a hyper-IGF-I euglycaemic clamp was performed. Lambs were fasted for 1 hour before the experiment, but were allowed water *ad libitum*. After the 1-hour fast arterial blood was sampled (2 ml) from the femoral artery at -10, -5 and 0 minutes prior to the commencement of an infusion of human recombinant IGF-I (rhIGF-I; Gropep Australia). The IGF-I infusate was prepared from a stock solution of 4mg/mL and was diluted to 10 mL with sterile

saline to provide a concentration that would deliver 3 μg/kg/min (calculated from unpublished observations), calculated according to the weight of the lamb. The IGF-I was infused from t=0 minutes across a 130 minute infusion period into the femoral vein using a fully programmable infusion pump (World Precision Instruments SP220i). At t=25 minutes, a fixed concentration of glucose (25% dextrose) was also infused into the femoral vein at 2 mg/kg/min using an infusion pump (World Precision Instruments SP220i). The glucose infusion rate (GIR) was then adjusted every 5 minutes after the measurement of blood glucose, to maintain euglycaemia at the fasting blood glucose concentration, using a modification of a previously published algorithm ([264]DeFronzo*, et al.*, 1979). The IGF-I sensitivity of glucose metabolism was determined as the glucose infusion rate from 70 to 130 minutes (GIR) for the hyper-IGF-I euglycaemic clamp. All syringe infusions were carried out using a fully programmable infusion pump (World Precision Instruments SP220i). The coefficient of variation for the GIR in each lamb during the hyper-IGF-I euglycaemic clamp was less than 3.6%.

2.2.2.2 IGF-I sensitivity of circulating amino acids

Alpha-amino nitrogen (α-aN) concentrations in plasma were measured in deproteinised plasma samples taken during the second hour of the hyper-IGF-I euglycaemic clamp (t = -10, -5, 0, 70, 85, 100, 115, 130 minutes). IGF-I sensitivity of amino acid metabolism was calculated as the percentage change from baseline to the average of α-aN for 70 to 130 minutes.

2.2.2.3 IGF-I sensitivity of circulating free fatty acids

Plasma free fatty acid (FFA) concentrations were measured prior to and during the second hour (t = -10, -5, 0, 70, 85, 100, 115, 130 minutes) of the hyper-IGF-I euglycaemic clamp. The IGF-I sensitivity of circulating FFA was calculated as the percentage change from fasting FFA concentrations to those during 70-130 minutes of the HIEC.

2.2.2.4 Plasma Insulin-like Growth Factors (IGF)-I and -II (IGF-I and IGF-II) concentrations

2.2.2.4.1 Extraction of IGFs from plasma using size exclusion chromatography under acidic conditions

Plasma from lambs was acidified by dilution to a final concentration of between 15 and 30% (v/v) in a solution to achieve the same final concentration as acidic chromatography mobile phase (200 mM acetic acid, 50 mM trimethylamine, 5ml/L Tween-20, pH 2.8) ([237]Owens*, et al.*, 1994). The diluted plasma samples were mixed with an equal volume of Freon (1,1,2-trichloro-1,2,2-trifluoroethane) and centrifuged at 10,000g for 10 minutes and the upper aqueous phase recovered ([237]Owens*, et al.*, 1994). Defatted plasma was clarified by centrifugation through a microfilter containing a 0.45 μm cellulose acetate filter membrane (Alltech Associates Inc.). Between 200 and 350 μl of the acidified, defatted, filtered plasma (containing 30-40 μl of plasma) was injected onto a Protein-Pak 125 HPLC column (Waters/Millipore) using an automatic injector (ICI AS 2000, ICI instruments) ([237]Owens*, et al.*, 1994). Samples were eluted at 1ml/min in acidic chromatography mobile phase (pH 2.8) ([237]Owens*, et al.*, 1994). Fractions of 0.2 ml were initially collected between 6 and 12 minutes

after injection of plasma samples. IGF-I and IGF-II content was measured in all fractions after neutralisation with 0.6 volumes of 0.4M Tris-base. Two peaks of activity were detected, the first being eluted between 6 and 8.5 minutes after injection, containing the IGFBPs, the second peak being eluted between 9 and 11 minutes after injection, containing IGF-I and IGF-II devoid of binding proteins ([237]Owens*, et al.*, 1994).

2.2.2.4.2 Measurement of IGF-I

Recombinant human IGF-I (Gropep Pty. Ltd, Adelaide) was used to prepare the standard and radiolabelled ligand for RIA. IGF-I was iodinated using chloramine-T and $Na^{125}I$ ([265]Gargosky*, et al.*, 1990). Specific radioactivity of (^{125}I)-iodo-IGF-I was 80 Ci/g. Rabbit anti-human IGF-I antibody (MAC Ab 89/1) was used at a final dilution of 1:60 000. All measurements were performed in triplicate. 100 µl of the HPLC fraction was added to a polystyrene tube, followed by 200 µl of RIA buffer (30mM Na_2HPO_4; 0.02% (w/v) protamine sulphate; 10 mM disodium EDTA; 0.05% (v/v) Tween-20; 0.02% (w/v) NaN_3; pH 7.5) and 60 µl of 0.4 M Tris base to bring the pH to 7.4. A stock solution of 10 ng/ml IGF-I standard in RIA buffer stored at –20^{o}C was serially diluted in IGF RIA buffer to prepare a standard of <5 to 500 pg/tube, which was added in 200 µl. Mobile phase (100 µl) and 60 µl of 0.4 M Tris base was added to each standard tube as neutralised eluate blank. Anti-human IGF-I (50 µl) and 50 µl of radio-iodinated h-IGF-I (20 000 cpm) were added per tube. Tubes containing 50 µl radio labelled IGF-I only, provided a measure of total radioactivity added and tubes with RIA buffer and radio labelled IGF-I only provided a blank (no IGF-I antiserum). The tubes were mixed and incubated for 22 hours at 4^{o}C. 10

μl of a 1:20 dilution in RIA buffer of rabbit IgG (cat no. P0448, DAKO, Australia Pty. Ltd) and 50 μl of a 1:20 dilution in RIA buffer of sheep anti-rabbit IgG (Silenus, Victoria, Australia) were added. The tubes were mixed and incubated for 30 minutes at 4^{o}C. One ml of ice-cold polyethylene glycol 6000 (6% w/v) in 150 mM of aqueous NaCl was added and the tubes were centrifuged at 4000 rpm for 25 minutes at 4^{o}C (J-6B Beckman Instruments, USA). The supernatant was aspirated and radioactivity in the pellet was measured in a gamma scintillation spectrometer (1261 Multigamma, LKB Pharmacia and Wallace Oy). The RiaCalc II data management program (Pharmacia and Wallace Oy) calculated IGF-I concentrations and the minimal detectable concentration, which was 6.5 pg/tube on average.

2.2.2.4.3 Measurement of IGF-II

Recombinant human IGF-II receptor grade (Gropep Pty. Ltd, Adelaide) was used to prepare the standard and radio labelled ligand. IGF-II was iodinated using chloramine-T and Na^{125}I ([265]Gargosky*, et al.*, 1990). Specific radioactivity of radio-ligand was 70 Ci/g. A mouse anti-rat IGF-II monoclonal antibody (Dr K Nishikawa, Kanazawa, Medical University, Ishikawa, Japan) was used at a final dilution of 1:500 in RIA buffer. All measurements were performed in triplicate. 50 μl of the appropriate HPLC fraction was added to a polystyrene tube, followed by 200 μl of RIA buffer and 30 μl of 0.4 M Tris base to bring the pH to 7.4. A stock solution of 10 ng/ml IGF-I standard in RIA buffer stored at –20^{o}C was serially diluted in IGF RIA buffer to prepare a standard of <5 to 500pg/tube, which was added in 200 μl. Mobile phase (50 μl) and 30 μl of 0.4 M Tris base was added to each standard tube as neutralised eluate blank. Anti-rat IGF-II

(50 μl) and 50 μl of radio-iodinated h-IGF-II (20 000 cpm) were added per tube. Tubes containing radio labelled IGF-II, provided a measure of total radioactivity added and tubes with RIA buffer and radio-labelled IGF-II only provided a blank (no IGF-II antiserum). The tubes were mixed and incubated for 22 hours at 4^{o}C. 10 μl of a 1:20 dilution in RIA buffer of mouse serum (IMVS, Gilles Plains, South Australia) and 50 μl of a 1:20 dilution in RIA buffer of sheep anti-mouse IgG (Silenus, Victoria, Australia) were added. The tubes were mixed and incubated for 30 minutes at 4^{o}C. One ml of ice-cold polyethylene glycol 6000 (6% w/v) in 150 mM of aqueous NaCl was added and the tubes were centrifuged at 4000 rpm for 25 minutes at 4^{o}C (J-6B Beckman Instruments, USA). The supernatant was aspirated and radioactivity in the pellet was measured in a gamma scintillation spectrometer (1261 Multigamma, LKB Pharmacia and Wallace Oy). The RiaCalc II data management program (Pharmacia and Wallace Oy) calculated IGF-I concentrations and the minimal detectable concentration, which was 19 pg/tube on average.

2.2.3 Assay of blood glucose

The arterial blood samples taken from both the Hyperinsulinaemic and Hyper-IGF-I euglycaemic clamps, and the IVGTT were measured for blood glucose using a glucometer (HemoCue AB, Sweden) which is used to quantify the formation of a coloured formazan using two wavelengths, 660 and 840nm. The mean coefficient of variation was less than 5%.

2.2.4 Assay of plasma glucose

The quantitative determination of plasma glucose was performed with a COBAS MIRA automated sample system using the Glucose HK assay kit, with the C.f.a.s. Calibrator, and quality controls: Precinorm U and Precipath U (Roche Diagnostics, NSW, Australia). The mean coefficient of variation was less than 3.3%.

2.2.5 Assay of plasma Free Fatty Acid

The quantitative determination of plasma free fatty acids was performed with a COBAS MIRA automated sample system using the NEFA-C Free Fatty Acid assay kit (NovoChem) and quality controls: QCS 1 and 2 (Bio-Rad, Australia). The mean coefficient of variation was less than 4.6%.

2.2.6 Assay of plasma α-amino Nitrogen (α-aN) concentration

Plasma samples (100 μl) were deproteinised by adding 800 μl of 0.04 M H_2SO_4 and 100 μl of Sodium Tungstate and centrifuged for 15 minutes at 3000g (4^{o}C). The supernatant was removed and pipetted in triplicate into 96 well ELISA plates. Sodium tetraborate (24 μl) and NQS (β-napthoquinone sulphonate) (20 μl) were added to each well, and then the plates were placed in a dry oven for 20 minutes at 80^{o}C followed by 5 minutes in a -20^{o}C freezer. Water (120 μl), acid formaldehyde (36 μl) and sodium thiosulphate (20 μl) were added to each well and the plates were left to stand at room temperature for 30 minutes. The plates were read with a dual wavelength ELISA reader at 490 nm and 660 nm

([266]Evans, *et al.*, 1993). The intra-assay coefficient of variation for the α-amino nitrogen assay is 3.9%.

2.3 Thyroid Hormone Axis

2.3.1 Assay of Thyroid hormone (Total T_3, Free T_3, Total T_4, Free T_4) concentrations

Fasting basal blood samples were taken at postnatal day 30 for determination of thyroid hormones. Free and Total T_3 and T_4 in plasma were determined by commercially available RIA kits (CIS bio-international, France). The intra-assay coefficients of variation for the FT_3, TT_3, FT_4, and TT_4 assays were 1.9%, 1.6%, 5.4%, and 6.2% respectively.

2.3.1.1 Total T_3

The radioimmunoassay kit RIA-gnost T_3 (coated tube) measures total triiodothyronine (T_3) in serum by competitive protein binding analysis. With the displacement reagent 8-anilion-1-sulphonic acid (ANSA) the T_3 to be measured is displaced from the binding proteins and competes with ^{125}I-T_3 for binding sites of a specific T_3 antibody that are available in limited numbers. The quantity of bound T_3 tracer is consequently inversely proportional to the T_3 concentration in the sample or standard. Standards and unknown plasma samples (50 μl) were pipetted into the bottom of test tubes coated with rabbit anti-T_3 antibodies. One ml of ^{125}I-T_3 tracer was added to each tube and the tubes were incubated on a shaker (150-400 rpm) for 2 hours at room temperature. The tubes were then

decanted and left on absorbent paper for 10 minutes. Tubes were then counted with a gamma counter.

2.3.1.2 Free T_3

The radioimmunoassay kit RIA-gnost FT_3 (coated tube) measures free triiodothyronine (T_3) in serum by a polyclonal solid-phase antibody. Standards and unknown plasma samples (50 µl) were pipetted into the bottom of test tubes coated with rabbit anti-T_3 antibodies. One ml of ^{125}I-FT_3 tracer was added to each tube and the tubes were incubated on a shaker (150-400 rpm) for 2 hours at room temperature. The tubes were then decanted and left on absorbent paper for 10 minutes. Tubes were then counted with a gamma counter.

2.3.1.3 Total T_4

The radioimmunoassay kit RIA-gnost T_4 (coated tube) measures total thyroxine (T_4) in serum by competitive protein binding analysis. With the displacement reagent 8-anilion-1-sulphonic acid (ANSA) the T_4 to be measured is displaced from the binding proteins and competes with ^{125}I-T_4 for binding sites of a specific T_4 antibody that are available in limited numbers. The quantity of bound T_4 tracer is consequently inversely proportional to the T_4 concentration in the sample or standard. Standards and unknown plasma samples (20 µl) are pipetted into the bottom of test tubes coated with sheep anti-T_4 antibodies. One ml of ^{125}I-T_4 tracer was added to each tube and the tubes were incubated on a shaker (150-400 rpm) for 2 hours at room temperature. The tubes were then

decanted and left on absorbent paper for 10 minutes. Tubes were then counted with a gamma counter.

2.3.1.4 Free T_4

The radioimmunoassay kit RIA-gnost FT_4 (coated tube) measures free thyroxine (FT_4) in serum by a polyclonal solid-phase antibody. Standards and unknown plasma samples (100 μl) were pipetted into the bottom of test tubes coated with sheep anti-T_4 antibodies. One ml of ^{125}I-FT_4 tracer was added to each tube and the tubes were incubated on a shaker (150-400 rpm) for 2 hours at room temperature. The tubes were then decanted and left on absorbent paper for 10 minutes. Tubes were then counted with a gamma counter.

2.4 Summary of animals used and measurements in lambs for each experimental chapter in this thesis.

The following table summarises the number of animals used for this thesis and the break-up of animals used in each chapter (Table 2.2). The table shows the parameters such as size at birth, growth rates, as well as plasma metabolites, plasma hormones, and insulin and IGF sensitivities (Table 2.2).

All lambs born in this study had size at birth measurements recorded, of these most had absolute and fractional growth measures at most ages, except where there were insufficient resources or lambs died or were put down due to illness before 45 days of age. A subset of animals was used to measure body composition, with the remainder continuing on to older ages for other studies.

Insulin sensitivity was measured in most animals except for when catheters did not remain patent throughout the 45 days of age. The hyper IGF-I euglycaemic clamp was a new technique developed midway through the cohort of lambs hence only the last group of lambs had this experiment performed on them. Thyroid hormone concentrations at day 30 were measured in fed and fasted samples in most of the cohort, whereas at day 8 thyroid hormones were measured in fed and fasted samples, only in the last group of lambs in the cohort.

Table 2.2. Table summarising the measurements and number of lambs used in each chapter of this thesis.

Total number of lambs	97			
Surviving lambs	71			
Control/Placentally restricted	37 Control		34 Placentally restricted	
Male/female	20 Male	17 Female	14 Male	20 Female
Chapter 3				
Size at birth	20	17	14	20
Absolute Growth rates	18	14	13	17
Neonatal fractional growth rates	18	14	13	17
Body composition	5	4	4	4
Chapter 4				
Size at birth	16	11	9	16
Absolute Growth rates	14	9	9	15
Neonatal fractional growth rates	14	9	9	15
Plasma glucose/amino acids/free fatty acids	8	6	5	9
Plasma insulin	8	6	5	9
Insulin sensitivity (glucose)	8	5	6	10
Insulin sensitivity (amino acids)	8	7	6	8
Insulin sensitivity (free fatty acids)	7	4	5	8
Chapter 5				
Size at birth	12	10	10	14
Absolute Growth rates	12	10	10	14
Neonatal fractional growth rates	12	10	10	14
Plasma IGF-I and -II	5	5	5	9
IGF sensitivity (glucose)	10	8	5	5
IGF sensitivity (amino acids)	10	7	4	4
IGF sensitivity (free fatty acids)	6	7	4	3
Chapter 6				
Size at birth	12	10	10	12
Plasma thyroid hormones (day 30)	12	10	10	12
Plasma thyroid hormones (day 8)	5	4	4	4

2.5 Statistical Analysis

Data is expressed as mean ± standard error of the mean (SEM), unless otherwise stated. The effects of placental restriction (moderate, severe, overall) and gender were assessed by analysis of variance (ANOVA) (SPSS 11.5 software package for Windows). Associations between parameters were assessed by Pearson's correlation or multiple linear regression analysis, with Bonferronis correction for multiple comparisons (SPSS 11.5 software package for Windows). In addition, lambs were classified according to birth weight, CRL, and shoulder height as low, medium or high according to size at birth: low, more than 1SD below the mean; medium, within 1SD of the mean; high, more than 1SD above the mean. Within each birth weight class, lambs were also classed as low and high neonatal FGR, according to whether their FGR fell below or above the line of best fit for FGR versus size at birth for that parameter. The effects of size at birth and FGR category were also assessed by ANOVA (SPSS 11.5 software package for Windows). The coefficient of variation (% CV) was calculated as the standard deviation divided by the mean multiplied by 100. Statistical significance was assumed at *$p<0.05$ and **$p<0.001$.

Chapter 3

PLACENTAL RESTRICTION OF FETAL GROWTH REDUCES SIZE AT BIRTH AND INCREASES POSTNATAL GROWTH AND ADIPOSITY IN THE YOUNG LAMB

3.1 INTRODUCTION

Intrauterine growth restriction (IUGR) is evident as reduced weight, length, and/or increased thinness for gestational age and is associated with altered postnatal growth in the first few months of life ([1]Chernausek, 1996, [7]Karlberg*, et al.*, 1995, [11]Prader*, et al.*, 1963). Many IUGR infants undergo accelerated growth for their size in early life, termed catch-up growth ([7]Karlberg*, et al.*, 1995, [11]Prader*, et al.*, 1963), which together with size at birth, is a major predictor of later functional and health outcomes, including obesity ([87]Parker*, et al.*, 2003). The latter most probably results from the underlying drivers of catch-up growth, as well as the accelerated growth itself ([267]Cameron*, et al.*, 2002), which varies in its extent, timing and pattern for unknown reasons. These could include differences in the factors perturbing the intrauterine environment to cause IUGR and altered growth and function postnatally ([268]Ong*, et al.*, 2002). One major cause of IUGR is placental insufficiency, but whether it induces catch-up growth in early postnatal life and alters body composition or when, has not been directly determined.

The incidence of clinical IUGR is approximately 10% of live births in many developed countries and up to 40% in some developing countries ([269]Roder*, et al.*, 1997). Increasingly, IUGR is followed by accelerated or catch-up growth in terms of weight and length, which occurs in the majority of infants (57% - 88%) and in the first two to five months of age ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [38]Leger*, et al.*, 1997, [62]McCowan*, et al.*, 1999,

[84]Albertsson Wikland*, et al.*, 1993, [85]Karlberg*, et al.*, 1997). Consequently, most reach within 1SD of normal weight or length by 3-9 months of age ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [38]Leger*, et al.*, 1997, [62]McCowan*, et al.*, 1999, [84]Albertsson Wikland*, et al.*, 1993, [85]Karlberg*, et al.*, 1997). A population-based study of healthy Swedish boys and girls showed that 3.1% were of low birth weight (below -2 SDS) and 3.5% were of low birth length (below -2 SDS) and that most (88%) experienced catch-up growth in weight and length during the first 2 years of life, and largely in the first 2 months of age ([7]Karlberg*, et al.*, 1995). Similarly, in Hong Kong, 79% of infants who were short at birth began to catch-up within 2 weeks of birth and reached a height greater than -2 SDS by 5 months of age, and a third of these children reached the normal height range (to within –2SD) by 5 months of age ([85]Karlberg*, et al.*, 1997). A more recent study of IUGR infants showed that most had caught up by 6 months of age, although 20% were still short, 16% had reduced weight, and 18% still had a low head circumference ([62]McCowan*, et al.*, 1999). Thus most IUGR infants catch-up in terms of weight by five months of life and in height by 12 months of age ([7]Karlberg*, et al.*, 1995, [13]Albertsson Wikland*, et al.*, 1998, [84]Albertsson Wikland*, et al.*, 1993).

Catch-up growth in infancy, together with size at birth, determines the extent to which target adult height is attained following IUGR ([7]Karlberg*, et al.*, 1995), with those who do not “catch-up” having a 7-fold higher risk of persistent short stature into adulthood ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). Other studies suggest, however, that catch-up growth may increase the risk of developing adult-onset diseases such as diabetes, hypertension and

cardiovascular disease ([9]Eriksson*, et al.*, 1999, [70]Cianfarani*, et al.*, 1999, [95]Rasmussen, 2001, [96]Forsén*, et al.*, 1997). This increased disease risk may be mediated in part through altered body composition, as a recent critical review of published studies concluded that catch-up growth after IUGR predicts increased subcutaneous and visceral obesity in later life ([97]Rogers*, et al.*, 2003). Thus small babies who have rapid rates of growth or catch-up growth, exhibit greater adiposity and a taller childhood stature than is predicted from parental heights ([8]Ong*, et al.*, 2000), and have an increased risk of obesity ([88]Dietz, 1994), cardiovascular disease and type 2 diabetes as adults ([9]Eriksson*, et al.*, 1999, [89]Crowther*, et al.*, 1998, [90]Forsén*, et al.*, 2000). A recent study investigated a large British birth cohort and showed that men aged 33 years who were light at birth, have a greater risk of developing obesity if they grew rapidly during childhood ([98]Parsons*, et al.*, 2001). Whether this increased adiposity associated with catch-up growth following IUGR is established early in infant life at the time of greatest catch-up, or emerges later, is unclear however.

An adverse effect of neonatal catch-up growth on subsequent adiposity is supported by a study in sheep, where those of low birth weight due to multiple pregnancy were allowed either *ad libitum* milk intake or were restricted in their feeding ([249]Greenwood*, et al.*, 1998, [270]Bell, 1992). Regardless of feed intake, low birth weight lambs grew more quickly for their size and had a greater percentage of body fat at 20 kg live weight, than did high birth weight lambs ([249]Greenwood*, et al.*, 1998). The increased adiposity of low birth weight lambs was also exacerbated when lambs were allowed to feed *ad libitum* compared to restricted feeding ([249]Greenwood*, et al.*, 1998). In this study, sheep were

compared at the same live weight rather than age, with the food restricted and *ad libitum* fed lambs of low birth weight killed at approximately 50 and 100 days respectively, compared to approximately 40 and 110 days for the high birth weight lambs ([249]Greenwood*, et al.*, 1998). It is therefore unclear whether catch-up growth after IUGR increases adiposity or simply accelerates the acquisition of fat in early postnatal life.

An additional question is the nature of the prenatal factors causing IUGR, which is characterised by catch-up growth and increased adiposity postnatally, and whether they are environmental or genetic in nature. Because placental size accounts for much of the variation in size at birth in mammalian species ([271]Pardi*, et al.*, 1997, [272]Prada*, et al.*, 1998), and most IUGR infants catch-up, it is likely that placental restriction, which restricts fetal growth, can induce catch-up growth in early postnatal life. Previous studies have demonstrated that restriction of implantation and placental growth in the sheep limits delivery of oxygen and nutrients to the fetus, which alters metabolism, endocrine state and growth of the fetus ([4]Owens*, et al.*, 1986, [5]Owens*, et al.*, 1987, [257]Owens*, et al.*, 1987) similarly to that observed in human IUGR ([273]Barker, 1997, [274]Harding*, et al.*, 1992). We therefore hypothesised that restriction of placental and hence fetal growth would reduce size at birth and increase postnatal growth rate and fat mass in the young lamb.

3.2 MATERIALS AND METHODS

3.2.1 Animals and Surgery

All procedures performed in this project were approved by the Adelaide University Animal Ethics Committee (Animal Ethics Approval Number: M/1/97A). Placental growth was restricted in 45 Merino ewes by removal of the majority of visible endometrial caruncles (65-148) from the non-pregnant uterus, leaving either 3 to 8 caruncles in each horn of the bicornuate uterus ([236]Robinson*, et al.*, 1979) (See Materials and Methods; 2.1.1). Ewes were housed in individual pens in animal holding rooms from approximately a week before giving birth (See Materials and Methods; 2.1.2). Control ewes delivered 37 lambs and the placentally restricted delivered 34 lambs. The lambs were housed in the pens with their mothers throughout the study.

3.2.2 Growth Measures

At birth and at 5-day intervals up to 45 days of age, size at birth (day 0), and subsequently size in terms of body weight, crown-rump length (CRL), tibia and metatarsal lengths, shoulder height, and abdominal, hind limb, tibia, and radius/ulna circumferences were measured (See Materials and Methods; 2.1.4). The postnatal growth of lambs was calculated from birth to 45 days of age for each parameter, relative to that parameter at birth (See Materials and Methods; 2.1.4).

3.2.3 Post mortem

At 43 ± 2 days of age, a subset of the lambs (9 controls and 8 PR) was killed by intravenous administration of an overdose of barbiturate (Pentobarbitone, Lethabarb) (See Materials and Methods; 2.1.5). Organs were weighed and measured (See Materials and Methods; 2.1.5). Individual muscles were removed and weighed (biceps, M flexor carpi radialis, tibialis, semitendinosus, gastrocnemius, soleus, M extensor digitorum longus, biceps femoris, and vastus lateralis). The summed mass of the individual muscles is termed the summed muscle mass. The fat depots that could be completely dissected were weighed (retroperitoneal, perirenal and omental fat depots). Visceral fat was calculated as the sum of these three fat depots.

3.2.4 Statistical Analysis

Data is expressed as mean ± standard error of the mean (SEM), unless otherwise stated. Associations between parameters were assessed by Pearson correlation or multiple linear regression analysis (SPSS 11.0 software package for Windows). The effects of placental restriction and sex, as between factors, on parameters were assessed by analysis of variance (ANOVA), as were their effects, together with age, as a repeated measures factor (9 levels) on CFGR (SPSS 11.0 for Windows). The effects of placental restriction and sex as between factors on individual muscle weights and adipose depot weights (in absolute or relative to body weight) were determined using repeated measures factor ANOVA (muscle: 9 levels; fat: 3 levels). The coefficient of variation (% CV) was calculated as the standard deviation divided by the mean. Statistical significance was assumed at $p<0.05$.

3.3 RESULTS

3.3.1 Effect of placental restriction on perinatal survival, size at birth and postnatal growth

Placental restriction reduced survival rate (still born and neonatal deaths) overall (control 95%, PR 82%, $p<0.001$), which was also reduced to a greater extent in twins (control 91%, PR 61%, $p<0.05$) than for singletons (control 95%, PR 75%, $p<0.05$) (twins were excluded from all experimental chapters). Placental restriction reduced placental weight (-37%) ($p<0.05$) and reduced size at birth, in terms of weight (-25%), hind limb (-14%) and abdominal circumference (-10%), crown-rump length (CRL) (-9%), tibia and metatarsal lengths (-6%) (Table 3.1). Body mass index (-11%) was reduced, while skull width was relatively spared (-5%) in placentally restricted lambs (Table 3.1). Weight, CRL, skull width, shoulder height, tibia and metatarsal length, and abdominal circumference at birth were greater in males than females ($p<0.05$ for all) (Table 3.1). Placental restriction reduced body mass index ($p<0.05$) at birth and differently with sex, such that this decrease was greater in females ($p=0.032$) than males (Table 3.1). Placental restriction tended to reduce ponderal index ($p<0.1$) at birth and differently with sex, such that this ponderal index decreased in females ($p=0.007$) but not males (Table 3.1).

Placental restriction reduced the absolute growth rate of skull width ($p<0.05$) (Table 3.2), and tended to increase absolute growth rate for skull length, tibia length, and hind limb (knee joint) and radius/ulna circumference ($p<0.1$) (Table

3.2). Males had greater absolute growth rates for weight and both measures of hind limb circumferences than females ($p=0.001$, $p=0.03$, $p=0.03$, respectively) (Table 3.2).

Placental restriction increased neonatal fractional growth rates for weight, skull length, tibia length, hind limb circumference (knee joint), radius/ulna and abdominal circumference, decreased neonatal fractional growth rate for skull width ($p<0.05$ for all), and tended to increase neonatal fractional growth rate for CRL and thoracic circumference ($p<0.1$) and similarly in males and females (Table 3.3).

Table 3.1. The effect of experimental placental restriction and sex on phenotype at birth in sheep.

Birth phenotype	Male		Female		ANOVA (p value)		
	Controls	PR	Control	PR	PR	S	PRxS
Placental weight (kg)	0.54 ± 0.08 (6)	0.32 ± 0.07 (7)	0.54 ± 0.09 (4)	0.36 ± 0.14 (2)	0.05	ns	ns
Weight (kg)	5.53 ± 0.23 (20)	4.15 ± 0.28 (14)	5.06 ± 0.25 (17)	3.58 ± 0.23 (20)	0.000	0.02	ns
Crown rump length (cm)	56.3 ± 0.92 (20)	50.1 ± 1.11 (14)	53.3 ± 1.03 (16)	49.3 ± 0.93 (20)	0.000	0.03	ns
Shoulder height (cm)	40.4 ± 0.68 (20)	37.9 ± 0.82 (14)	39.6 ± 0.76 (16)	36.3 ± 0.68 (20)	0.000	0.05	ns
Tibia length (cm)	13.6 ± 0.26 (20)	12.8 ± 0.31 (14)	13.3 ± 0.29 (16)	12.2 ± 0.26 (20)	0.001	0.05	ns
Metatarsal length (cm)	12.3 ± 0.21 (20)	11.9 ± 0.25 (14)	12.1 ± 0.24 (16)	11.3 ± 0.21 (20)	0.004	0.05	ns
Metacarpal length (cm)	11.06 ± 0.51 (5)	10.98 ± 0.57 (4)	10.65 ± 0.46 (6)	9.90 ± 0.57 (4)	ns	p<0.1	ns
Radius/ulna length (cm)	13.51 ± 0.66 (5)	13.18 ± 0.74 (4)	13.56 ± 0.61 (6)	11.64 ± 0.74 (4)	p<0.1	ns	ns
Skull width (cm)	6.64 ± 0.08 (20)	6.25 ± 0.09 (14)	6.48 ± 0.08 (16)	6.07 ± 0.08 (20)	0.000	0.02	ns
Skull length (cm)	14.1 ± 0.20 (20)	13.3 ± 0.24 (14)	13.9 ± 0.23 (16)	13.1 ± 0.20 (20	0.001	ns	ns
Abdominal circumference (cm)	39.3 ± 0.89 (20)	35.9 ± 1.06 (14)	37.5 ± 0.99 (16)	32.9 ± 0.89 (20)	0.000	0.008	ns
Thoracic circumference (cm)	39.3 ± 0.76 (20)	34.9 ± 0.91 (14)	37.8 ± 0.85 (16)	33.9 ± 0.76 (20)	0.000	p<0.1	ns
Hind limb circumference (knee joint) (cm)	10.4 ± 0.30 (20)	9.01 ± 0.36 (14)	10.28 ± 0.34 (16)	8.39 ± 0.30 (20)	0.000	ns	ns
Tibia circumference (cm)	10.84 ± 0.87 (5)	10.25 ± 0.98 (4)	10.80 ± 0.80 (6)	8.69 ± 0.98 (4)	p<0.1	ns	ns
Radius/ulna circumference (cm)	9.36 ± 0.63 (5)	8.09 ± 0.71 (4)	9.63 ± 0.58 (6)	7.83 ± 0.71 (4)	0.017	ns	ns
Body Mass Index ($kg.m^{-2}$)	17.5 ± 0.55 (20)	16.3 ± 0.66 (14)	17.7 ± 0.62 (16)	14.3 ± 0.55 (20)	0.000	p<0.1	0.032
Ponderal Index ($kg.m^{-3}$)	31.2 ± 1.07 (20)	32.5 ± 1.27 (14)	33.5 ± 1.19 (16)	29.03 ± 1.07 (20)	p<0.1	ns	0.007

All values are expressed as mean ± SEM; number of animals are in parentheses. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex.

Table 3.2. The effect of experimental placental restriction and sex on absolute growth rate from birth to 45 days of age in sheep.

Absolute Growth Rate	Male		Female		ANOVA (p value)		
	Controls	PR	Control	PR	PR	S	PRxS
Weight (kg/day)	0.30 ± 0.01 (18)	0.30 ± 0.02 (13)	0.26± 0.01 (14)	0.25 ± 0.01 (17)	ns	0.001	ns
Crown rump length (cm/day)	0.75 ± 0.04 (18)	0.76 ± 0.01 (13)	0.73 ± 0.05 (14)	0.77 ± 0.04 (17)	ns	ns	ns
Skull width (cm/day)	0.04 ± 0.01 (18)	0.04 ± 0.01 (13)	0.04 ± 0.01 (14)	0.03 ± 0.01 (17)	0.03	ns	ns
Skull length (cm/day)	0.09 ± 0.01 (18)	0.13 ± 0.01 (13)	0.11 ± 0.01 (14)	0.13 ± 0.01 (17)	p<0.1	ns	ns
Shoulder height (cm/day)	0.31 ± 0.02 (18)	0.32 ± 0.02 (13)	0.29 ± 0.02 (14)	0.29 ± 0.02 (17)	ns	p<0.1	ns
Tibia length (cm/day)	0.12 ± 0.01 (18)	0.14 ± 0.01 (13)	0.12 ± 0.01 (14)	0.13 ± 0.01 (17)	P<0.1	ns	ns
Metatarsal length (cm/day)	0.06 ± 0.01 (18)	0.08 ± 0.01 (13)	0.06 ± 0.01 (14)	0.06 ± 0.01 (17)	ns	ns	ns
Metacarpal length (cm/day)	0.10 ± 0.01 (5)	0.08 ± 0.02 (3)	0.09 ± 0.01 (4)	0.07 ± 0.02 (2)	ns	ns	ns
Radius/ulna length (cm/day)	0.12 ± 0.01 (5)	0.12 ± 0.01 (3)	0.09 ± 0.01 (4)	0.10 ± 0.02 (2)	ns	p<0.1	ns
Hind limb circ. (knee joint) (cm/day)	0.09 ± 0.01 (18)	0.10 ± 0.01 (13)	0.07 ± 0.01 (14)	0.09 ± 0.01 (17)	p<0.1	0.03	ns
Tibia circumference (cm/day)	0.23 ± 0.03 (5)	0.23 ± 0.03 (3)	0.15 ± 0.03 (4)	0.18 ± 0.04 (2)	ns	0.03	ns
Radius/ulna circumference (cm/day)	0.11 ± 0.02 (5)	0.14 ± 0.02 (3)	0.12 ± 0.02 (4)	0.16 ± 0.03 (2)	p<0.1	ns	ns
Abdominal circumference (cm/day)	0.66 ± 0.04 (18)	0.67 ± 0.04 (13)	0.63 ± 0.04 (14)	0.65 ± 0.04 (17)	ns	ns	ns
Thoracic circumference (cm/day)	0.68 ± 0.05 (18)	0.70 ± 0.06 (13)	0.69 ± 0.05 (14)	0.63 ± 0.05 (17)	ns	ns	ns

All values are expressed as mean ± SEM; number of animals are in parentheses. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex.

Table 3.3. The effect of placental restriction and sex on neonatal fractional growth rate from birth to 45 days of age in sheep.

Neonatal Fractional Growth Rate	Male		Female		ANOVA (p value)		
	Controls	PR	Control	PR	PR	S	PRxS
Weight (%/day)	5.55 ± 0.40 (18)	7.04 ± 0.40 (13)	5.34 ± 0.40 (14)	7.24 ± 0.40 (17)	0.000	ns	ns
Crown rump length (%/day)	1.37 ± 0.10 (18)	1.51 ± 0.10 (13)	1.44 ± 0.10 (14)	1.57 ± 0.10 (17)	p<0.1	ns	ns
Skull width (%/day)	0.62 ± 0.10 (18)	0.57 ± 0.10 (13)	0.57 ± 0.10 (14)	0.50 ± 0.10 (17)	0.05	ns	ns
Skull length (%/day)	0.64 ± 0.10 (18)	0.95 ± 0.10 (13)	0.78 ± 0.10 (14)	0.95 ± 0.10 (17)	0.003	ns	ns
Tibia length (%/day)	0.89 ± 0.10 (18)	1.09 ± 0.10 (13)	0.91 ± 0.10 (14)	1.06 ± 0.10 (17)	0.03	ns	ns
Hind limb circumference (knee joint) (%/day)	0.92 ± 0.10 (18)	1.14 ± 0.10 (13)	0.71 ± 0.10 (14)	1.10 ± 0.10 (17)	0.01	ns	ns
Radius/ulna circumference (%/day)	1.21 ± 0.20 (5)	1.75 ± 0.30 (3)	1.26 ± 0.30 (4)	1.99 ± 0.4 (2)	0.03	ns	ns
Abdominal circumference (%/day)	1.71 ± 0.10 (18)	1.84 ± 0.10 (13)	1.70 ± 0.10 (14)	1.99 ± 0.10 (17)	0.05	ns	ns
Thoracic circumference (%/day)	1.74 ± 0.10 (18)	1.97 ± 0.20 (13)	1.97 ± 0.20 (14)	1.85 ± 0.10 (17)	p<0.1	ns	ns

All values are expressed as mean ± SEM; number of animals are in parentheses. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex.

In placentally restricted lambs, weight, CRL, and abdominal circumference were reduced until day 30, indicating that they had 'caught-up' to control lambs in terms of these parameters by day 35 postnatally (Figure 3.1). Tibia length of placentally restricted lambs was similar to that of controls by day 20 (Figure 3.1). In contrast, skull width was reduced in placentally restricted lambs compared to controls up to and including day 45 (Figure 3.1). Females had reduced weight, shoulder height, tibia length, and abdominal circumference compared to males from birth to 45 days of age, but CRL was similar to males by day 10 and skull width by day 35 (Figure 3.2).

Placental restriction increased weekly current fractional growth rate for weight throughout the first 45 days of life (repeated measures ANOVA, $p=0.05$) (Figure 3.3). Placental restriction did not alter weekly current fractional growth rates for CRL, shoulder height and skull width throughout the first 45 days of life, but increased those of CRL and decreased those of skull width at day 25 (repeated measures ANOVA, $p<0.05$) (Figure 3.3).

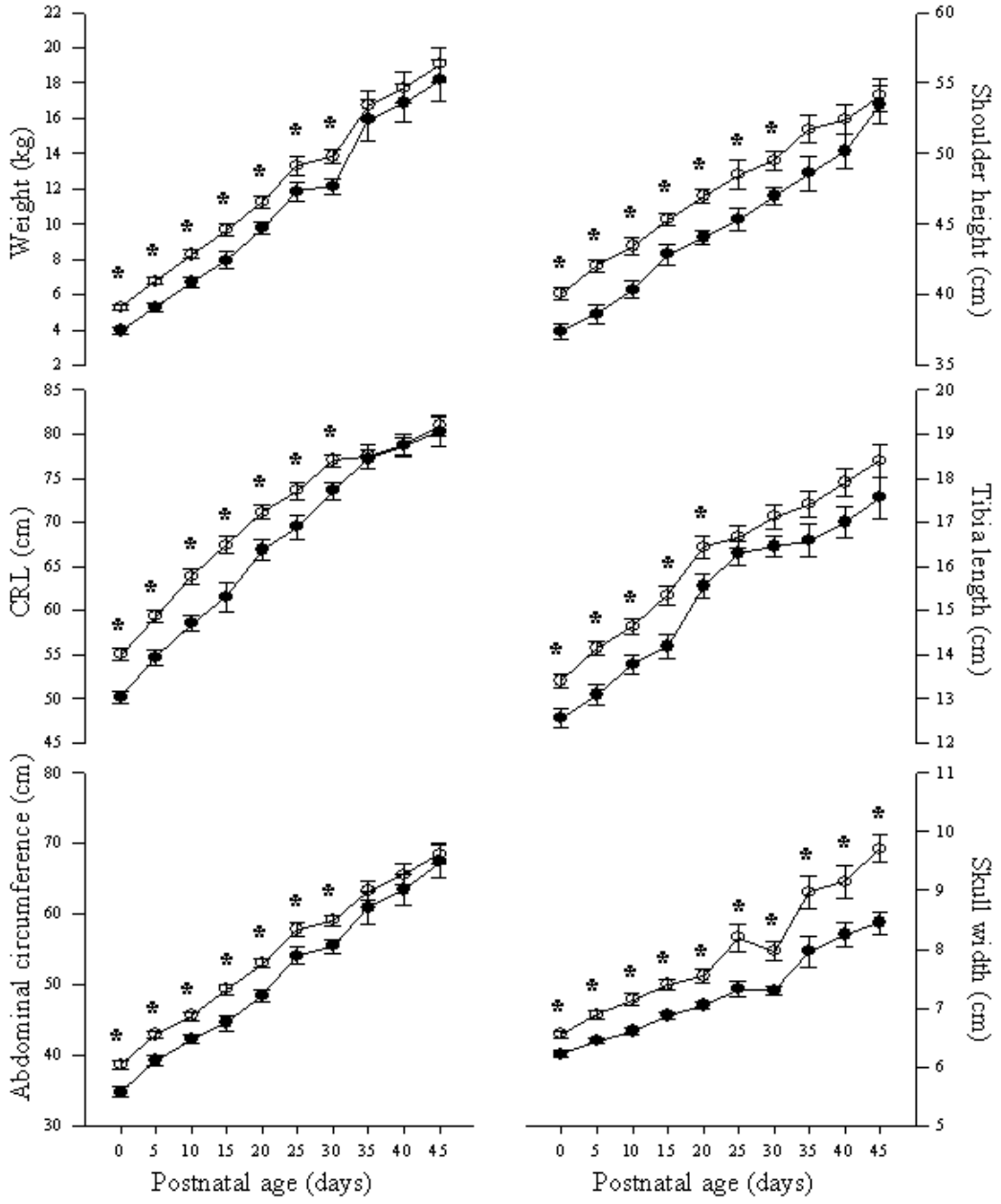

Figure 3.1. Effect of placental restriction on postnatal growth in lambs. Controls are represented by open, and placentally restricted by closed circles. Males and females are combined. Data are mean ± SEM, *p<0.05, determined by addition of age as a repeated measures factor (9 levels) by ANOVA.
Number of animals at each time point: day 0 (37 control, 34 PR), day 5 (32 control, 31 PR), day 10 (27 control, 31 PR) day 15 (27 control, 18 PR), day 20 (31 control, 30 PR), day 25 (15 control, 15 PR), day 30 (31 control, 32 PR), day 35 (11 control, 7 PR), day 40 (12 control, 10 PR), day 45 (9 control, 8 PR).

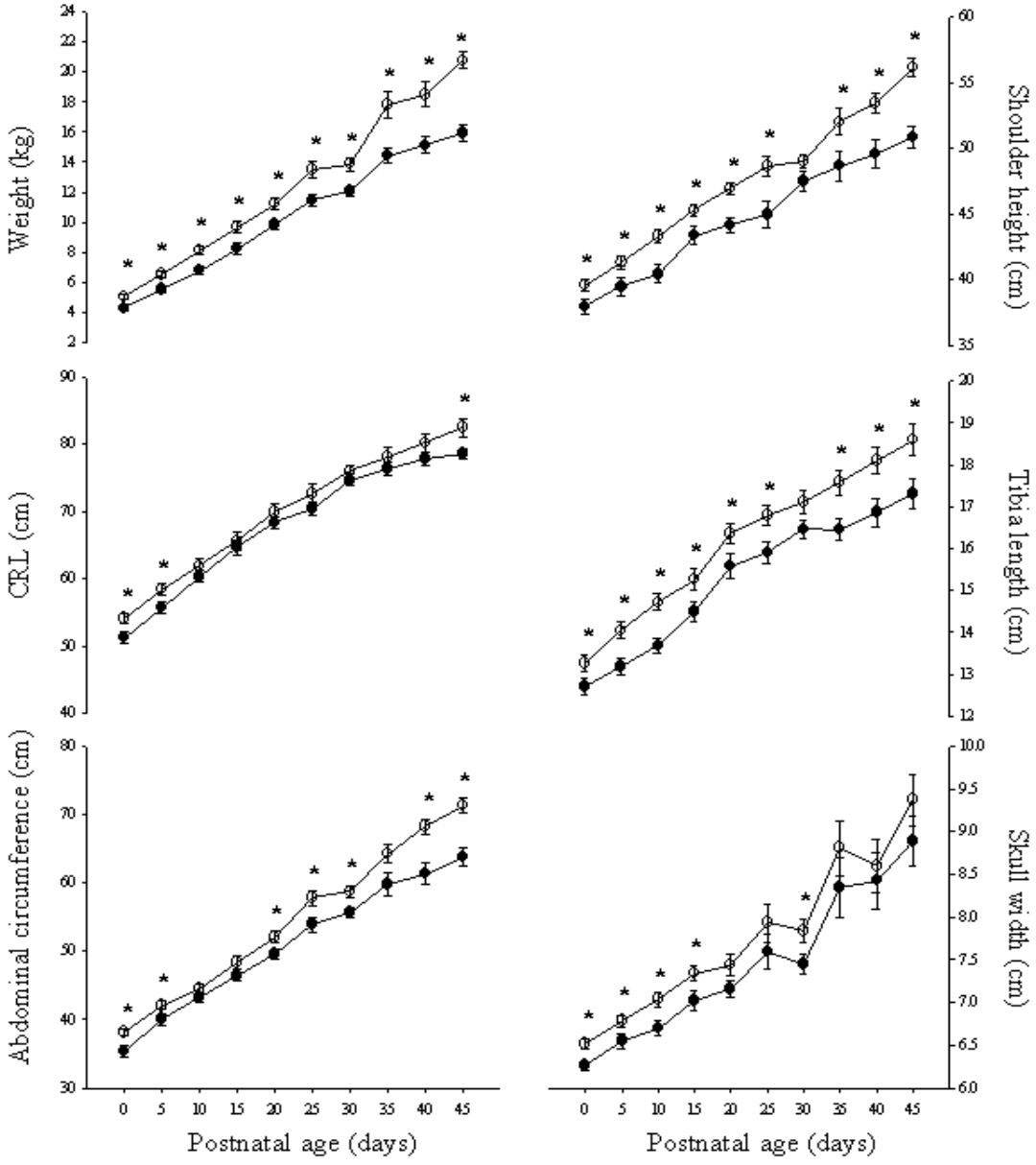

Figure 3.2. Effect of sex on postnatal growth in lambs.

Males are represented by open, and females by closed circles. Data are mean ± SEM, *p<0.05, determined by addition of age as a repeated measures factor (9 levels) by ANOVA.

Number of animals at each time point: day 0 (34 male, 37 female), day 5 (33 male, 30 female), day 10 (28 male, 30 female) day 15 (24 male, 21 female), day 20 (31 male, 30 female), day 25 (16 male, 14 female), day 30 (32 male, 31 female), day 35 (10 male, 8 female), day 40 (11 male, 11 female), day 45 (9 male, 8 female).

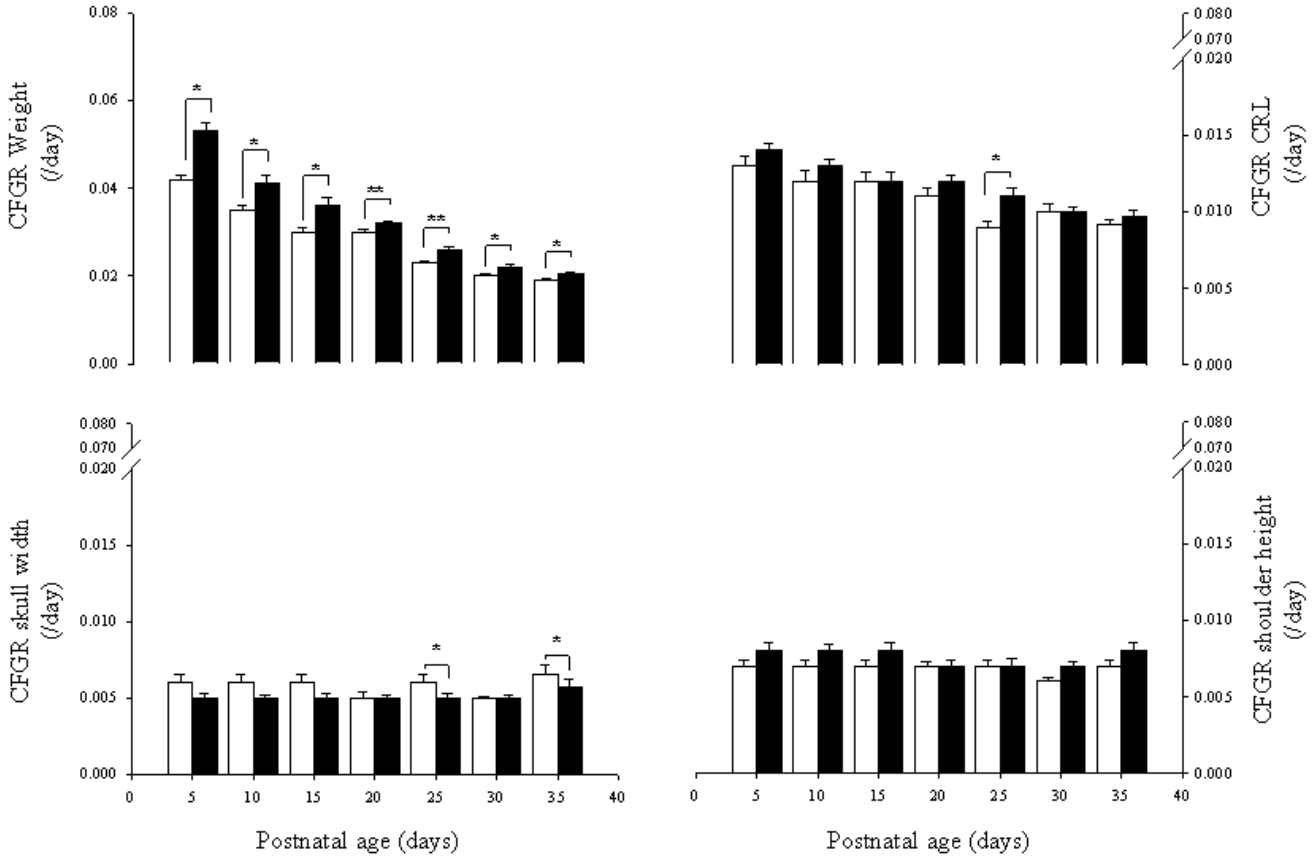

Figure 3.3. The effect of placental restriction on postnatal growth rate in the young lamb.

Current fractional growth rate (CFGR) for weight, CRL, skull width and shoulder height for 5-day periods are shown. Controls are represented by white, and placental restriction by black bars. Males and females are combined. Data are mean ± SEM, *p<0.05.

3.3.2 Effect of placental restriction and gender on organ weights at 45 days of age

At 45 days of age organ weights of placentally restricted lambs were not different from controls (Table 3.4). When organ weights were analysed according to gender, total brain, liver and gut weights were reduced in females compared to males ($p<0.05$), whereas when corrected for live weight, the brain, adrenals and heart weights were increased in females compared to males ($p<0.05$) (Table 3.4).

3.3.3 Effect of placental restriction on skeletal muscle mass and adiposity at 45 days of age

Placental restriction tended to reduce the summed weight of dissected muscles ($p<0.1$) (Table 3.5). When individual skeletal muscle weights were considered as repeated measures, placental restriction tended to reduce skeletal muscle mass overall ($p=0.078$), in males in particular ($p<0.05$), and in semitendinosus and biceps femoris specifically ($p<0.05$) (Table 3.5). Placental restriction tended to alter individual muscle mass in terms of M flexor Carpi Radialis and tibialis ($p<0.1$ for both) differently with sex, such that the decrease was greater in males than females (Table 3.5). When individual skeletal muscle weights as a percentage of body weight were considered as repeated measures, placental restriction reduced that of M flexor Carpi Radialis, tibialis, and semitendinosus in males specifically ($p<0.05$) (Table 3.5). Placental restriction altered muscle mass of gastrocnemius (relative to live weight) differently with sex ($p<0.005$),

and tended to alter muscle mass of gastrocnemius ($p<0.1$) differently with sex, such that both decreased in females, but not males (Table 3.5). Males had heavier muscles (biceps, gastrocnemius, and soleus) and a greater combined muscle mass compared with females ($p<0.05$ for all) (Table 3.5). Biceps femoris (corrected for live weight) correlated positively (control: $r=0.87$, $n=7$, $p<0.0025$; placentally restricted: $r=0.86$, $n=4$, $p<0.05$) with placental weight in lambs. In control lambs, weights of biceps, gastrocnemius, soleus, and biceps femoris correlated positively with size at birth in terms of weight, CRL, and shoulder height ($p<0.05$). In placentally restricted lambs, combined muscle mass, gastrocnemius, soleus, and biceps femoris were positively correlated with size at birth in terms of birth weight, CRL, shoulder height, and abdominal circumference ($p<0.05$ for all) (Table 3.7).

Placental restriction increased the mass of individual fat depots (perirenal) and combined visceral fat mass at 45 days of age ($p<0.05$) (Table 3.5, Figure 3.4). When individual adipose tissues were considered as repeated measures, placental restriction increased adipose depot mass overall ($p<0.05$), in both males ($p<0.05$) and females ($p<0.05$), and increased omental fat mass in males ($p<0.05$) and total perirenal fat and omental fat masses in females specifically ($p<0.05$) (Table 3.5). Placental restriction also increased visceral fat mass relative to live weight ($p<0.05$), and increased total perirenal fat mass relative to live weight ($p<0.05$) (Table 3.5, Figure 3.4). When individual adipose tissues relative to live weight were considered as repeated measures, placental restriction increased adipose depot mass relative to live weight overall ($p=0.05$), in males in particular ($p<0.05$), and that of total perirenal fat and omental fat in

males ($p<0.05$) and of total retroperitoneal fat in females ($p<0.05$) specifically (Table 3.5). Retroperitoneal fat (corrected for live weight) correlated negatively (control: $r=-0.67$, $n=7$, $p<0.05$; placentally restricted: $r=-0.89$, $n=4$, $p<0.025$) with placental weight in lambs. In controls, omental fat and retroperitoneal fat mass tended to be correlated negatively with placental weight ($p<0.1$ for both) (Table 3.6). Following placental restriction, total omental fat mass correlated positively with placental weight ($p<0.05$) (Table 3.7). Males were also fatter in terms of total omental and visceral fat mass compared to females at 45 days of age ($p<0.05$) (Table 3.5).

3.3.4 Associations of skeletal muscle mass and adiposity at 45 days of age with size at birth and postnatal growth

In controls, summed muscle mass and individual weights of gastrocnemius, soleus, and biceps femoris correlated positively with AGR in terms of weight ($p<0.05$ for all) (Table 3.6). Following placental restriction, the combined muscle mass, weights of gastrocnemius, biceps femoris, and vastus lateralis positively correlated with AGR in terms of weight and negatively with FGR in terms of weight ($p<0.05$ for all) (Table 3.7).

In the placentally restricted lambs, visceral fat mass correlated positively with size at birth in terms of shoulder height and abdominal circumference ($p<0.05$ for both) (Table 3.7), while omental fat mass correlated positively with weight, shoulder height, and abdominal circumference at birth ($p<0.05$ for all) (Table 3.7).

In the placentally restricted lambs, visceral fat mass tended to correlate positively with absolute growth in terms of weight ($p<0.1$) (Table 3.7). In the placentally restricted lambs, omental fat mass correlated positively with absolute growth in terms of weight ($p<0.05$ for all), while tending to correlate negatively with neonatal fractional growth in terms of weight ($p<0.1$) (Table 3.7).

Table 3.4. The effect of placental restriction and sex on size of organs at 45 days of age in the young sheep.

Weight of organs	Male		Female		ANOVA (p value)		
(grams)	Control (5)	PR (4)	Control (4)	PR (4)	PR	S	PRxS
Brain (g)	88.7 ± 3.0	91.5 ± 3.3	81.1 ± 3.9	79.2 ± 3.9	ns	0.02	ns
(%LW)	0.42 ± 0.02	0.45 ± 0.02	0.50 ± 0.02	0.51 ± 0.02	ns	0.004	ns
Thyroid (total) (g)	2.89 ± 0.29	2.92 ± 0.33	2.85 ± 0.46	2.18 ± 0.38	ns	ns	ns
(%LW)	0.01 ± 0.002	0.01 ± 0.002	0.02 ± 0.002	0.01 ± 0.002	ns	ns	ns
Adrenals (total) (g)	1.74 ± 0.22	1.42 ± 0.25	1.23 ± 0.25	1.50 ± 0.29	ns	ns	ns
(%LW)	0.008 ± 0.001	0.007 ± 0.001	0.01 ± 0.001	0.01 ± 0.001	ns	0.02	ns
Kidneys (total) (g)	113.5 ± 15.5	123.3 ± 17.3	84.3 ± 17.3	94.8 ± 19.9	ns	ns	ns
(%LW)	0.53 ± 0.07	0.61 ± 0.08	0.69 ± 0.09	0.64 ± 0.09	ns	ns	ns
Pancreas (g)	27.4 ± 3.8	19.3 ± 4.3	16.6 ± 4.3	11.9 ± 4.9	ns	ns	ns
(%LW)	0.13 ± 0.02	0.09 ± 0.02	0.10 ± 0.02	0.08 ± 0.03	ns	ns	ns
Spleen (g)	110.8 ± 7.5	130.0 ± 8.3	96.2 ± 9.6	110.7 ± 9.6	ns	ns	ns
(%LW)	0.52 ± 0.05	0.65 ± 0.05	0.59 ± 0.06	0.72 ± 0.06	ns	ns	ns
Liver (total) (g)	466.0 ± 16.9	461.4 ± 18.9	368.2 ± 21.8	404.8 ± 21.8	ns	0.003	ns
(%LW)	2.18 ± 0.14	2.31 ± 0.16	2.25 ± 0.18	2.66 ± 0.18	ns	ns	ns
Heart (g)	113.2 ± 7.8	114.1 ± 8.8	122.3 ± 10.1	100.2 ± 10.1	ns	ns	ns
(%LW)	0.53 ± 0.03	0.56 ± 0.04	0.75 ± 0.04	0.65 ± 0.04	ns	0.002	ns
Gut (total) (g)	2933 ± 274	2785 ± 307	2062 ± 354	1703 ± 354	ns	0.01	ns
(%LW)	13.5 ± 1.1	13.8 ± 1.2	12.5 ± 1.4	11.3 ± 1.4	ns	ns	ns

All values are expressed as mean ± SEM. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex.

Table 3.5. The effect of placental restriction and sex on size of individual skeletal muscles and fat depots at 45 days of age in the young sheep.

	Male		Female		ANOVA (p value)		
	Control (5)	PR (4)	Control (4)	PR (4)	PR	S	PRxS
Biceps (g)	17.0 ± 1.1	16.2 ± 1.2	13.7 ± 1.4	13.8 ± 1.4	ns	0.05	ns
(%LW)	0.08 ± 0.005	0.08 ± 0.006	0.08 ± 0.005	0.09 ± 0.006	ns	ns	ns
M Flexor Carpi Radialis (g)	8.1 ± 1.3	4.1 ± 1.7	3.5 ± 1.7	4.8 ± 1.7	ns	P<0.1	P<0.1
(%LW)	0.04 ± 0.007	0.02 ± 0.009	0.02 ± 0.009	0.03 ± 0.009	ns	ns	P<0.1
Tibialis (g)	24.2 ± 2.8	16.4 ± 3.2	14.5 ± 3.7	16.7 ± 3.7	ns	P<0.1	P<0.1
(%LW)	0.11 ± 0.01	0.08 ± 0.02	0.09 ± 0.02	0.11 ± 0.02	ns	ns	ns
Semitendinosus (g)	55.4 ± 5.2	45.3 ± 5.7	41.3 ± 6.7	39.1 ± 6.7	ns	P<0.1	ns
(%LW)	0.26 ± 0.02	0.22 ± 0.03	0.25 ± 0.03	0.25 ± 0.03	ns	ns	ns
Gastrocnemius (g)	68.2 ± 3.5	70.4 ± 3.9	57.8 ± 4.5	47.2 ± 4.5	ns	0.002	P<0.1
(%LW)	0.32 ± 0.009	0.35 ± 0.01	0.35 ± 0.01	0.31 ± 0.01	ns	ns	0.005
Soleus (g)	21.6 ± 1.2	22.0 ± 1.4	17.0 ± 1.6	14.4 ± 1.6	ns	0.001	ns
(%LW)	0.10 ± 0.004	0.11 ± 0.005	0.10 ± 0.006	0.09 ± 0.006	ns	ns	ns
EDL (g)	5.9 ± 1.8	9.1 ± 2.0	5.5 ± 2.3	6.2 ± 2.3	ns	ns	ns
(%LW)	0.03 ± 0.008	0.04 ± 0.009	0.03 ± 0.01	0.04 ± 0.01	ns	ns	ns
Biceps femoris (g)	167 ± 12	163 ± 13	130 ± 15	120 ± 15	ns	0.01	ns
(%LW)	0.78 ± 0.04	0.80 ± 0.04	0.79 ± 0.05	0.78 ± 0.05	ns	ns	ns
Vastus lateralis (g)	108.6 ± 15.1	94.1 ± 16.9	83.8 ± 19.5	58.7 ± 19.5	ns	P<0.1	ns
(%LW)	0.51 ± 0.07	0.46 ± 0.08	0.51 ± 0.09	0.36 ± 0.09	ns	ns	ns
Summed muscle mass (g)	476 ± 29	439 ± 33	368 ± 38	321 ± 38	P<0.1	0.03	ns
(%LW)	22.3 ± 1.0	21.7 ± 1.2	22.3 ± 1.4	20.7 ± 1.4	ns	ns	ns
Total perirenal fat (g)	112 ± 17	146 ± 17	90 ± 19	126 ± 19	0.03	ns	ns
(%LW)	0.52 ± 0.07	0.73 ± 0.08	0.55 ± 0.09	0.82 ± 0.09	0.02	ns	ns
Omental fat (g)	179 ± 15	220 ± 15	122 ± 15	136 ± 21	0.05	0.002	ns
(%LW)	0.84 ± 0.09	1.09 ± 0.09	0.76 ± 0.09	0.9 ± 0.1	P<0.1	ns	ns
Total retroperitoneal fat (g)	94.2 ± 19.5	128.5 ± 21.9	56.1 ± 21.9	109 ± 25	P<0.1	ns	ns
(%LW)	0.4 ± 0.1	0.7 ± 0.1	0.5 ± 0.2	0.7 ± 0.2	ns	ns	ns
Visceral fat (g)	385 ± 43	494 ± 48	268 ± 48	371 ± 55	0.03	0.02	ns
(%LW)	1.6 ± 0.3	2.5 ± 0.3	1.5 ± 0.3	2.2 ± 0.4	0.03	ns	ns

All values are expressed as mean ± SEM. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex.

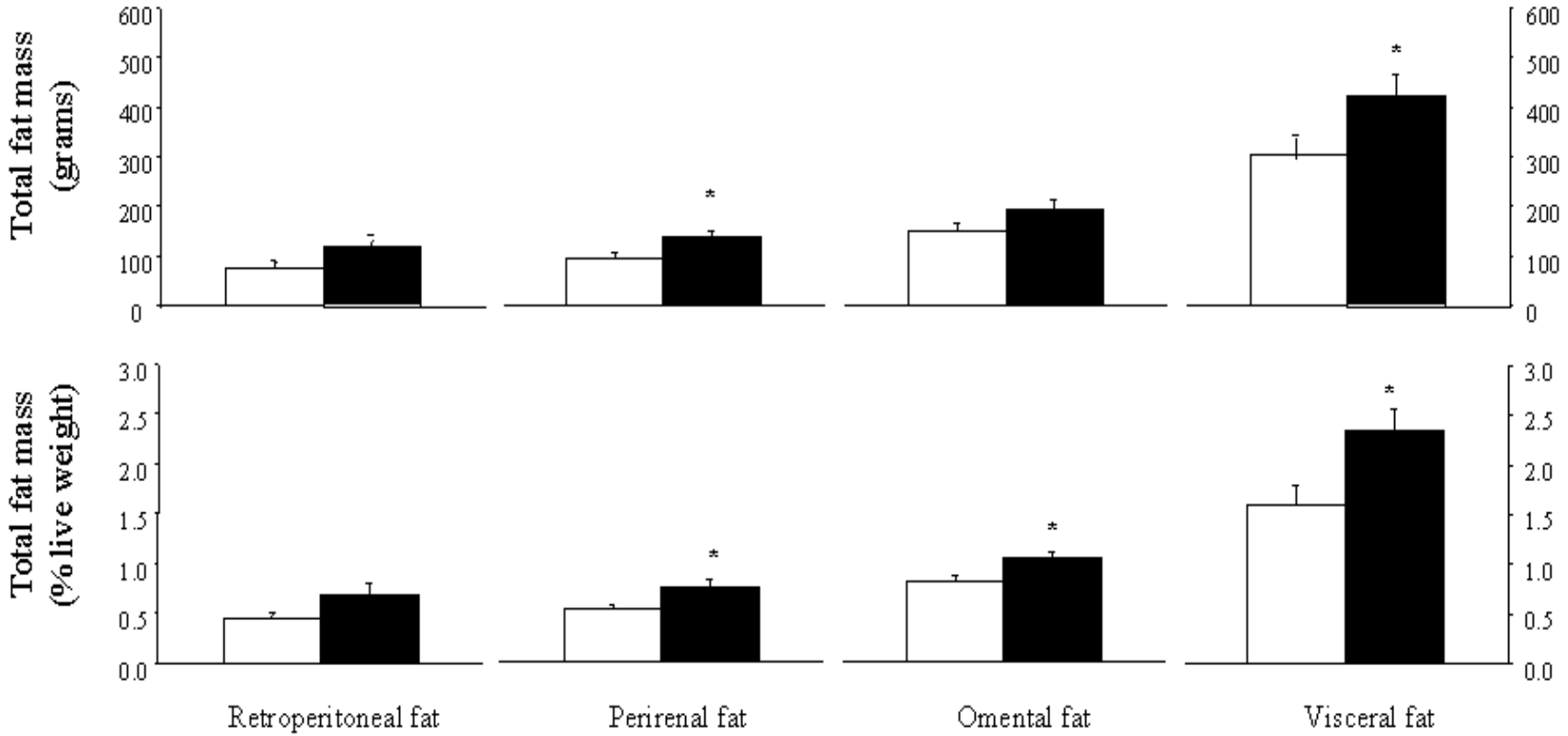

Figure 3.4. Effect of placental restriction on adiposity in lambs at 43 days of age.

Visceral fat mass was calculated as the sum of retroperitoneal, perirenal and omental fat weights. Controls (n=9) are represented by white, and placentally restricted (n=8) by black bars. Males and females are combined. Data are mean $\pm$ SEM, *$p<0.05$.

Table 3.6. Correlations of muscle and adipose depots weights with size at birth and postnatal growth rate in control lambs.

Control (n=9)	Size at birth					Growth rate	
	Placental weight	Weight	Crown-rump length	Shoulder height	Abdominal circumference	AGR weight	NFGR weight
	r	r	r	r	r	r	r
Summed muscle mass (g)	-0.20	0.43	0.57+	0.52+	0.41	0.81**	0.06
(%LW)	0.26	0.05	0.06	-0.03	0.08	0.11	0.17
Biceps (g)	0.08	0.64*	0.60*	0.61*	0.62*	0.53+	-0.31
M flex Carpi Radialis (g)	0.06	0.36	0.38	0.42	0.39	0.57	-0.10
Tibialis (g)	-0.35	0.17	0.16	0.23	0.16	0.57	0.15
Semitendinosus (g)	0.03	0.24	0.43	0.31	0.09	0.78	0.21
Gastrocnemius (g)	0.09	0.62*	0.78**	0.60*	0.48	0.83#	-0.08
Soleus (g)	-0.07	0.54+	0.67*	0.63*	0.46	0.83#	-0.12
EDL (g)	-0.16	0.16	0.11	0.35	0.26	-0.13	-0.20
Biceps femoris (g)	0.15	0.69*	0.84#	0.79**	0.62*	0.76*	-0.23
Vastus lateralis (g)	-0.35	0.02	0.10	0.08	0.09	0.43	0.26
Visceral fat (g)	-0.29	-0.06	0.09	0.14	0.04	0.41	0.19
(%LW)	-0.22	-0.30	-0.23	-0.17	-0.17	-0.16	0.22
Omental fat (g)	-0.62+	-0.23	-0.05	0.05	-0.18	0.74*	0.47
Total perirenal fat (g)	-0.12	0.32	0.45	0.46	0.34	0.46	-0.14
Total retroperitoneal fat (g)	-0.51+	0.07	0.14	0.15	0.14	0.46	0.08

Values are correlation coefficients (demonstrating positive or negative correlations) of size at birth, absolute (AGR) and neonatal fractional (NFGR) growth rate in terms of weight, with the weight of individual skeletal muscles and fat depots at 45 days of age postnatally. *p<0.05, **p<0.01, #p<0.005, +p<0.1.

Table 3.7. Correlations of muscle and adipose depots weights with size at birth and growth rate in placentally restricted lamb.

Placentally restricted (n=8)	Size at birth					Growth rate	
	Placental weight	Weight	Crown-rump length	Shoulder height	Abdominal circumference	AGR weight	NFGR weight
	r	r	r	r	r	r	r
Summed muscle mass (g)	0.88*	0.95#	0.81*	0.75*	0.76*	0.89#	-0.82**
(%LW)	0.77	0.68*	0.50	0.57+	0.53+	0.47	-0.70*
Biceps (g)	0.34	0.51	0.69*	0.15	0.27	0.72*	-0.29
M flex Carpi Radialis (g)	0.99*	0.10	-0.08	-0.02	-0.34	-0.07	-0.44
Tibialis (g)	0.98*	0.42	0.20	0.43	0.04	0.15	-0.65+
Semitendinosus (g)	0.27	0.64+	0.81*	0.02	0.69	0.62+	-0.49
Gastrocnemius (g)	0.78	0.92#	0.82**	0.76*	0.78*	0.96#	-0.69*
Soleus (g)	0.61	0.86**	0.80*	0.65+	0.81*	0.92#	-0.59+
EDL (g)	0.80+	0.75*	0.61+	0.55	0.67*	0.53	-0.77*
Biceps femoris (g)	0.75	0.90#	0.81*	0.65+	0.82**	0.84**	-0.74*
Vastus lateralis (g)	0.93*	0.73*	0.45	0.88#	0.40	0.66*	-0.70*
Visceral fat (g)	0.52	0.57	0.37	0.77*	0.74*	0.65+	-0.28
(%LW)	-0.77	-0.09	-0.26	0.34	0.31	-0.04	0.26
Omental fat (g)	0.97*	0.84*	0.65+	0.88**	0.78*	0.81*	-0.66+
Total perirenal fat (g)	0.60	0.25	0.26	0.33	0.16	0.27	-0.10
Total retroperitoneal fat (g)	-0.62	-0.14	-0.13	0.13	0.19	-0.01	0.39

Values are correlation coefficients (demonstrating positive or negative correlations) of size at birth, absolute (AGR) and neonatal fractional (NFGR) growth rate in terms of weight, with the weight of individual skeletal muscles and fat depots at 45 days of age postnatally. *p<0.05, **p<0.01, #p<0.005, +p<0.1.

3.4 DISCUSSION

This study has shown that experimental restriction of placental implantation and growth in the sheep reduces placental weight and size of offspring at birth and induces catch-up growth in terms of weight and length in the first month of life. Furthermore, placental restriction reduces skeletal muscle mass and increases visceral adiposity in absolute and relative terms in lambs at one month of age. Therefore placental insufficiency may be a significant cause of the catch-up growth observed following IUGR in human populations and of early onset increased adiposity ([8]Ong*, et al.*, 2000, [267]Cameron*, et al.*, 2002).

Reduced weight, length and increased thinness at birth are common features of the human IUGR infant ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [83]Albertsson Wikland*, et al.*, 1994) and of the newborn in other species following experimental restriction of fetal growth ([241]Mellor*, et al.*, 1977, [253]Ritacco*, et al.*, 1997, [275]Muaku*, et al.*, 1997). We have previously found that placental restriction in the sheep disproportionately reduced growth of soft tissue relative to skeletal tissues, and the current study demonstrates that this pattern persists until birth ([236]Robinson*, et al.*, 1979). The current study is the first to demonstrate that restricted implantation and growth of the placenta induces postnatal catch-up growth in terms of weight and other parameters. While measures were available only in some animals after day 30, catch-up growth had clearly occurred in these animals. In addition, catch-up growth had occurred in a former cohort of lambs by 60 days of age (unpublished observations). Growth overall slows between 25 to 30 days of age, which is prior to the insulin clamp, then resumes subsequently and continues despite the

IGF-I clamp at 35 days of age. Therefore the clamps are unlikely to have caused this slowing of growth so there may be other factors involved such as increased peripheral sensitivity to major growth promoting hormones such as insulin, IGFs, and thyroid hormones. This catch-up growth normalised size in terms of most parameters by 45 days of age, with the exception of skull width. An increase in neonatal growth rate in terms of weight, height and head size is also observed in most human infants following IUGR ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [83]Albertsson Wikland*, et al.*, 1994), but similar to our observations here in the sheep, catch-up growth in head size in humans is incomplete at 6 months of age ([62]McCowan*, et al.*, 1999). Another study of the effects of spontaneous variations in birth weight on postnatal growth in pigs, found that while absolute growth rate was reduced, fractional growth rate in the first month of life was increased in low birth weight pigs compared to those of high birth weight ([276]Poore, 2002). In the latter study, the consequences of spontaneous IUGR in the pig for growth of long bones and other parameters of size were not determined ([276]Poore, 2002). The consequences of being fed *ad libitum* or given a restricted feed intake for growth of low and high birth weight male lambs during the neonatal period have been examined ([249]Greenwood*, et al.*, 1998). Low birth weight lambs grew at absolute rates equivalent to that of high birth weight lambs if fed milk *ad libitum* in the first few months of life ([249]Greenwood*, et al.*, 1998). In the current study, placentally restricted lambs were not able to do so, which may in part reflect reduced mammary size which has previously been reported ([277]Mellor*, et al.*, 1987), and may have reduced milk quantity and quality, and reduced nutrient availability to the lambs ([277]Mellor*, et al.*, 1987). The placentally restricted lambs therefore may have

the potential to grow at even faster rates in terms of weight than observed in the present study, if they were to have access to additional nutrients.

In the current study, catch-up growth in terms of length of long bones was observed to some extent, although the consequences for bone density and composition were not determined. In the study of Greenwood and colleagues of growth of low and high birth weight lambs, bone growth was not measured directly ([249]Greenwood*, et al.*, 1998), however reduced ash content was observed in the carcass in low birth weight lambs. Together with the current study, this suggests that bone is more limited than soft tissues in its capacity to catch-up following birth ([249]Greenwood*, et al.*, 1998), and that nutrient availability, and possibly calcium supply, limits bone matrix accumulation and particularly bone density ([249]Greenwood*, et al.*, 1998).

In the current study, we have shown that by 45 days of age there were no differences in weight of bodily organs. We have also shown that placental restriction increased visceral fat mass in absolute and relative terms in the young lamb at one month of age. Thus increased absolute and relative adiposity is of early onset following IUGR due to placental restriction, while catch-up growth in terms of weight at least is still occurring. This increased adiposity (omental and visceral) was predicted by increased rates of absolute growth in terms of weight in both control and placentally restricted lambs. This is consistent with the findings from the British birth cohort, where men who were light at birth had a greater risk of developing obesity if they grew rapidly during childhood ([98]Parsons*, et al.*, 2001) and other studies investigating the effects of

catch-up growth on stature and adiposity ([8]Ong*, et al.*, 2000, [13]Albertsson Wikland*, et al.*, 1998). If this increased storage of fat continues throughout life following fetal growth restriction as indeed occurs in humans, there may be implications for the risk of developing obesity related disorders, including cardiovascular disease later in life ([273]Barker, 1997). We have also shown here that placental restriction tended to reduce skeletal muscle mass in absolute terms and that of some individual muscles in absolute or relative terms depending on the sex of the lambs. Skeletal muscle mass was also reduced with size at birth and these observations suggest that increased adiposity following IUGR is in part due to a reduction in lean tissue mass from early in life.

The mechanistic basis of catch-up growth after IUGR is poorly understood, but programming of altered activity of neuroendocrine and endocrine axes, which influence growth, appetite and satiety and metabolic efficiency are implicated in this phenomenon ([50]Yajnik*, et al.*, 1995, [57]Fall*, et al.*, 1995, [82]Colle*, et al.*, 1976, [108]Soto*, et al.*, 2003, [152]Giudice*, et al.*, 1995, [267]Cameron*, et al.*, 2002). Hyperphagia occurs following spontaneous or experimental restriction in the adult rat ([278]Vickers*, et al.*, 2000), guinea pig ([262]Kind*, et al.*, 2003), and young lamb of low birth weight due to multiple pregnancy ([249]Greenwood*, et al.*, 1998, [270]Bell, 1992). This has led to the suggestion that adverse prenatal environments, which restrict fetal growth, also permanently alter the set point and activity of neuroendocrine mechanisms regulating satiety and appetite ([268]Ong*, et al.*, 2002). The increased relative growth rate and increased adiposity of low birth weight lambs fed *ad libitum* were attributed to hyperphagia

and reduced energy requirements for maintenance ([249]Greenwood*, et al.*, 1998). This increased nutrient intake due to prenatally induced hyperphagia could further contribute to both increased growth and adiposity if the capacity for growth of lean tissues was exceeded. Reduced thermogenesis and increased metabolic efficiency, is observed during refeeding after semi-starvation in young rats ([204]Crescenzo*, et al.*, 2003). Consistent with this contributing to increased growth and adiposity following IUGR, low birth weight lambs fed either *ad libitum* or restricted high-quality liquid diets from birth, had energy requirements for maintenance that were 30% lower than those of high birth weight lambs in early postnatal life ([249]Greenwood*, et al.*, 1998).

An important additional element resulting in increased adiposity following IUGR and catch-up growth may be a limited capacity of skeletal muscle to utilise nutrients for growth ([279]Greenwood*, et al.*, 2000). This appears consistent with a recent review, which concluded that small size at birth predicts reduced lean tissue in later life ([97]Rogers*, et al.*, 2003). In the current study, reduced size at birth in terms of a range of parameters also predicted reduced individual and summed skeletal muscle mass in the young lamb. In the current study, reduced skeletal muscle mass occurred following placental restriction despite individual skeletal muscle size being predicted by postnatal growth, as for adipose tissue, suggesting that lean tissue growth is restrained or limited following IUGR and especially relative to that of adipose tissue. Consequently, although postnatal growth rate also predicted increased skeletal muscle mass, the extent of this was apparently not sufficient to normalise lean tissue size. Greenwood and colleagues found that low birth weight lambs have reduced

DNA content and a higher protein to DNA ratio in muscle than high birth weight lambs, suggesting reduced myonuclei number as a possible explanation for a limited capacity for postnatal growth of skeletal muscle following IUGR ([279]Greenwood*, et al.*, 2000). Thus although catch-up growth predicted increased skeletal muscle size as well as adipose tissue in the current study, the former clearly occurred to a much lesser extent and would further increase the relative adiposity of the IUGR lambs undergoing catch-up growth.

Accelerated growth of long bones was also observed following placental restriction as in IUGR more generally in humans ([13]Albertsson Wikland*, et al.*, 1998, [84]Albertsson Wikland*, et al.*, 1993, [85]Karlberg*, et al.*, 1997, [208]Fitzhardinge*, et al.*, 1989). This may be due to fetal growth restriction delaying the senescence of the growth plate, which then subsequently resumes after birth ([209]Gafni*, et al.*, 2001). Senescence (or epiphyseal fusion) is a process where the proliferative capacity of chondrocytes declines with each generation of cells and may be dependent on the cumulative number of cell divisions that the chondrocytes of long bones undergo ([209]Gafni*, et al.*, 2001). This delayed senescence has been suggested to be the underlying cause of catch-up growth following restriction due to dexamethasone administration to growing rabbits in order to suppress their linear growth ([209]Gafni*, et al.*, 2001). Catch-up growth occurring after cessation of dexamethasone-induced growth restriction, was characterized by a delay in the age related senescent decline in the heights of the proliferative zone, hypertrophic zone, and total growth plate in the distal femoral growth plates ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). These findings support linear catch-up growth after placental restriction and

IUGR being caused in part, by a delay in growth plate senescence ([209]Gafni*, et al.*, 2001).

Other mechanisms may help drive catch-up growth after IUGR, including increased activity of the major endocrine axes which influence infant/early postnatal growth such as the insulin, insulin-like growth factor (IGF) and growth hormone axes. In human infants undergoing catch-up growth after IUGR however, circulating insulin and IGF levels are at best normal, although stimulated levels of insulin do predict increased growth ([152]Giudice*, et al.*, 1995, [212]Deiber*, et al.*, 1989, [213]Adcock*, et al.*, 1997, [214]Ogilvy-Stuart*, et al.*, 1998, [280]Dotsch*, et al.*, 1998). There is also limited evidence for increased metabolic sensitivity to insulin in SGA infants in the first few days of life ([108]Soto*, et al.*, 2003, [217]Bazaes*, et al.*, 2003). Therefore increased insulin action, especially when nutrient availability is abundant and hyperphagia occurs, may also contribute significantly to catch-up growth in terms of weight and increased adiposity ([108]Soto*, et al.*, 2003). In contrast, the elevated plasma growth hormone levels in infants and lambs of low birth weight in the first two weeks of life ([227]Cance-Rouzaud*, et al.*, 1998, [281]Varvarigou*, et al.*, 1994) suggest that growth hormone resistance is present and is unlikely to promote catch-up growth of lean or skeletal tissues, although may contribute to increased adiposity. Reduced plasma TH levels have also been described in placentally restricted lambs in the first 24 hours of life ([241]Mellor*, et al.*, 1977) and in preterm and SGA infants in early postnatal life ([229]Bongers-Schokking*, et al.*, 1984, [230]Jacobsen*, et al.*, 1979), suggesting altered TH activity is also not contributing to catch-up growth. If they persist however, the decreased TH

concentrations might reduce energy expenditure and thermogenesis however, and increase energetic efficiency.

In summary, placental restriction reduces size at birth and induces neonatal catch-up growth in terms of weight and to a lesser extent of skeletal tissues in the young lamb. While catch-up growth normalises size in terms of most parameters after one month of age, skeletal muscle mass is reduced and visceral adipose tissue increased, in the young lamb following placental restriction. If these alterations in body composition persist, they may contribute to adverse metabolic and cardiovascular outcomes in later life.

Chapter 4

PLACENTAL RESTRICTION OF FETAL GROWTH INCREASES GROWTH RATE AND INSULIN ACTION IN THE NEONATAL LAMB

4.1 INTRODUCTION

Intrauterine Growth Restriction (IUGR), evident as reduced weight, reduced length, and/or increased thinness at birth for a given gestational age, is associated with altered postnatal growth ([1]Chernausek, 1996), due to as yet unknown mechanisms. The incidence of clinical IUGR is approximately 10% of live births in many developed countries and up to 40% in some developing countries ([269]Roder*, et al.*, 1997). Much IUGR results from the reduced delivery of essential substrates (eg. oxygen and nutrients from the blood) to the fetus due to either maternal or placental limitations ([5]Owens*, et al.*, 1987) and is characterised by accelerated growth in infancy, termed catch-up growth ([7]Karlberg*, et al.*, 1995). Catch-up growth in terms of weight and length occurs in the majority of IUGR infants (57 - 84%), begins as early as 2 weeks and is largely complete by 3 to 5 months of age, when many reach within 1SD of the normal weight or length ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [38]Leger*, et al.*, 1997, [62]McCowan*, et al.*, 1999, [84]Albertsson Wikland*, et al.*, 1993, [85]Karlberg*, et al.*, 1997). Together with size at birth, catch-up growth in infancy substantially predicts the extent to which target adult height is attained following IUGR ([7]Karlberg*, et al.*, 1995), and those who do not catch-up constitute a category with a 7-fold higher risk of persistent short stature into adulthood ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). Those infants who do undergo catch-up growth may have increased adiposity ([8]Ong*, et al.*, 2000, [13]Albertsson Wikland*, et al.*, 1998) with a greater risk of developing obesity ([88]Dietz, 1994), cardiovascular disease and type 2 diabetes as adults ([9]Eriksson*, et al.*, 1999, [89]Crowther*, et al.*, 1998, [90]Forsén*, et al.*, 2000). Greater

understanding of the underlying drivers of catch-up growth may identify alternative approaches to the treatment of catch-up failure after IUGR, and give insight into why it may increase the risk of later disease ([1]Chernausek, 1996, [94]Karlberg*, et al.*, 1993). Studies of the possible endocrine basis of catch-up growth after IUGR in human infants have focussed mainly on the production and/or abundance of anabolic hormones, particularly by insulin, which is essential for neonatal growth ([126]Davis*, et al.*, 1998) and is therefore implicated in this phenomenon. In infancy, insulin deficiency is characterised by profound growth restriction and acute post-prandial rises in insulin can account for much of the associated increased amino acid uptake and protein synthesis which underlies growth of soft tissues in the neonate ([126]Davis*, et al.*, 1998). Insulin also promotes triglyceride synthesis and storage in adipose tissue and chondrocyte proliferation and hypertrophy in long bones ([126]Davis*, et al.*, 1998, [127]Henson*, et al.*, 1997). In the IUGR infant at birth, plasma insulin concentrations are usually low and often remain so for the first 6 months compared with those of infants of average size at birth ([82]Colle*, et al.*, 1976). The IUGR infant that catches up has increased insulin secretion to glucose at 6 months of age, compared to those who do not catch-up, suggesting that catch-up growth is influenced and perhaps limited by insulin abundance ([82]Colle*, et al.*, 1976). Alternatively, this increased insulin secretory response could reflect insulin resistance, unlikely to contribute to catch-up growth. Because catch-up growth after IUGR is not consistently characterised by increased serum insulin levels ([82]Colle*, et al.*, 1976, [152]Giudice*, et al.*, 1995, [212]Deiber*, et al.*, 1989, [213]Adcock*, et al.*, 1997, [214]Ogilvy-Stuart*, et al.*, 1998), we suggest that increased sensitivity to, rather than increased production of insulin, accelerates neonatal

growth after IUGR. Consistent with this proposal, a recent study showed that at 48 hours of age, IUGR infants display evidence of increased insulin sensitivity of glucose metabolism, while glucose and insulin concentrations were lower than normally grown infants ([217]Bazaes*, et al.*, 2003). It is not known if this persists, and if it extends to utilisation of other nutrients for growth and whether insulin action overall is increased, during the catch-up growth following IUGR. To determine this requires assessment of both insulin secretion and sensitivity, which is more readily undertaken in an experimental paradigm rather than the human infant. Placental insufficiency is a major cause of IUGR ([5]Owens*, et al.*, 1987) in humans and other mammalian species. Experimental restriction of placental and fetal growth in sheep has been shown to have similar metabolic, endocrine and growth consequences for the fetus to those commonly observed in human IUGR ([237]Owens*, et al.*, 1994). Furthermore, catch-up growth in terms of weight and length occurs following placental restriction in the sheep. We therefore hypothesised that placental restriction of fetal growth in sheep would reduce size at birth, increase growth rates of soft and skeletal tissues and increase metabolic sensitivity to insulin, while not altering production and circulating levels of insulin in the neonate.

4.2 MATERIALS AND METHODS

4.2.1 Animals and Surgery

All procedures performed in this project were approved by the Adelaide University Animal Ethics Committee (Animal Ethics Approval Number: M/1/97A). Placental growth was restricted in 45 Merino ewes by removal of the majority of visible endometrial caruncles (65-148) from the non-pregnant uterus, leaving either 3 to 8 caruncles in each horn of the bicornuate uterus ([236]Robinson*, et al.*, 1979) (See Materials and Methods; 2.1.1). Ewes were housed in individual pens in animal holding rooms from approximately a week before giving birth (See Materials and Methods; 2.1.2). Control ewes delivered 27 lambs (16 males and 11 females) and the placentally restricted delivered 25 lambs (9 males and 16 females). At approximately 5 days of age, catheters were inserted into the lambs' femoral artery and vein under general anaesthesia (See Materials and Methods; 2.1.3).

4.2.2 Measurement of Growth

At birth and at 5-day intervals up to 45 days of age, size at birth (day 0), and subsequently size in terms of body weight, crown-rump length (CRL), tibia and metatarsal lengths, shoulder height, and abdominal, tibia, radius/ulna and hind limb circumferences were measured (See Materials and Methods; 2.1.4). The postnatal growth of lambs was calculated from birth to 45 days of age for each parameter relative to that parameter at birth (See Materials and Methods; 2.1.4).

4.2.3 Insulin secretion in response to an intravenous glucose tolerance test

At 32 ± 3 days of age, an intravenous glucose tolerance test (IVGTT) was performed (See Materials and Methods; 2.2.1.7). Arterial blood samples (2 mL) were taken before and after a glucose bolus (0.25 g glucose per kg live weight). Blood glucose concentration was measured (See Materials and Methods; 2.2.3) and the remaining blood was centrifuged, and plasma collected for subsequent measurement of plasma glucose and insulin concentrations (See Materials and Methods; 2.2.4 and 2.2.1.2, respectively).

4.2.4 Hyperinsulinaemic euglycaemic clamp and insulin sensitivity of glucose metabolism

At 30 ± 2 days of age, a hyperinsulinaemic euglycaemic clamp (HEC) was performed (See Materials and Methods; 2.2.1.1). Arterial blood was sampled and measured (2 mL) at -10, -5 and 0 minutes, then human insulin was continuously infused (2 mU/kg/min) and blood sampled (0.2 mL) every 5 minutes for 120 minutes. At 15 minutes after the start of the insulin infusion, glucose (25%) was infused intravenously and was adjusted each 5 minutes to maintain euglycaemia. The insulin sensitivity of whole body glucose metabolism was determined as the mean glucose infusion rate from 60 to 120 minutes during the hyperinsulinaemic euglycaemic clamp, divided by the mean plasma insulin concentration in the second hour of infusion measured every 15 minutes.

4.2.5 Insulin sensitivity of circulating amino acids

Plasma α-amino nitrogen concentration was measured prior to and during the second hour of the hyperinsulinaemic euglycaemic clamp (-10, -5, 0, 60, 75, 90, 105, 120 minutes) by colorimetric assay ([266]Evans*, et al.*, 1993) (See Materials and Methods; 2.2.1.3). The insulin sensitivity of circulating amino acids was calculated as the percentage change from fasting plasma α-amino nitrogen concentrations to those during the second hour of the HEC, corrected for the plateau plasma insulin concentration.

4.2.6 Insulin sensitivity of circulating free fatty acids

Plasma free fatty acid (FFA) concentration was measured prior to and during the second hour of the hyperinsulinaemic euglycaemic clamp (-10, -5, 0, 60, 75, 90, 105, 120 minutes) by enzymatic colorimetric analysis (See Materials and Methods; 2.2.1.4). The insulin sensitivity of circulating FFA was calculated as the percentage change from fasting FFA concentrations to those during the second hour of the HEC, corrected for the plateau plasma insulin concentration (FFA 60-120 minutes).

4.2.7 Insulin clearance and post-hepatic insulin secretory rate

Insulin clearance and post-hepatic insulin delivery rate was calculated from data collected during the hyperinsulinaemic euglycaemic clamp (See Materials and Methods; 2.2.1.5).

4.2.8 Disposition Index

The disposition index in the basal and insulin stimulated state were calculated from data collected during the hyperinsulinaemic euglycaemic clamp and the post-hepatic insulin delivery rate (See Materials and Methods; 2.2.1.6).

4.2.9 Statistical Analysis

Data are expressed as mean ± standard error of the mean (SEM), unless otherwise stated. The effect of placental restriction and gender were assessed by using two between factor analysis of variance (ANOVA) (SPSS 11.5 software package for Windows). Associations between parameters were assessed by Pearson correlation or multiple linear regression analysis (SPSS 11.5 software package for Windows). The effects of size at birth and FGR category were assessed by two between factors ANOVA (SPSS 11.5 software package for Windows). Hyperbolic decay (2 parameter) curves and overall mean with bi-directional error bars were fitted to the insulin secretion and sensitivity data (Sigmaplot 2001 for Windows version 7.101). Equation of the hyperbolic curve is in the form $y=ab/(b+x)$ (Sigmaplot 2001 for Windows version 7.101). Statistical significance was assumed at $*p<0.05$, and $**p<0.001$.

4.3 RESULTS

4.3.1 Effect of placental restriction on perinatal survival, size at birth and postnatal growth

Placental restriction reduced weight (-25%, $p<0.001$), abdominal circumference (-11%, $p<0.001$), hind limb circumference (-17%, $p<0.001$), crown-rump length (-10%, $p<0.001$), and body mass index (-10%, $p<0.05$), at birth (Table 4.1). PR also reduced length of long bones at birth (-7% for all) (Table 4.1) as well as skull width and length ($p<0.05$ for both). At 30 days of age, the placentally restricted lambs were not different from controls in terms of all parameters with the exception of skull width ($p<0.05$) (Table 4.1). Females were smaller than males in terms of weight, abdominal circumference, and crown-rump length and skull width at birth, and in terms of weight, abdominal and hind limb circumferences, and metatarsal length at 30 days of age ($p<0.05$ for all) (Table 4.1). PR increased AGR for weight, hind limb circumference tibia length and skull length ($p<0.05$ for all) (Table 4.2). PR increased neonatal FGR for weight, abdominal and hind limb circumferences, crown-rump length, shoulder height, tibia and metatarsal lengths, and skull length ($p<0.05$ for all) (Table 4.2).

Table 4.1. The effect of experimentally induced placental restriction on size at birth and at 30 days of age in sheep.

Parameter	Controls (n = 27)				Placentally Restricted (n=25)				Size at birth			Size at 30 days		
	Males (n=16)		*Females (n=11)*		*Males (n=9)*		*Females (n=16)*		*PR*	*S*	*PRxS*	*PR*	*S*	*PRxS*
	Birth	*30 days*	*Birth*	*30 days*	*Birth*	*30 days*	*Birth*	*30 days*						
Weight (kg)	5.53 ± 0.22	14.5 ± 0.5	5.00 ± 0.26	12.9 ± 0.6	4.33 ± 0.27	13.1 ± 0.6	3.71 ± 0.23	11.4 ± 0.5	0.000	0.02	ns	ns	0.006	ns
Abdominal circ. (cm)	39.3 ± 0.8	60.2 ± 1.1	37.8 ± 0.9	57.5 ± 1.3	36.5 ± 0.9	56.8 ± 1.3	33.5 ± 0.8	54.3 ± 1.1	0.000	0.014	ns	ns	0.04	ns
Hind limb circ. (cm)	10.4 ± 0.3	13.5 ± 0.3	10.4 ± 0.3	12.8 ± 0.3	9.1 ± 0.3	12.1 ± 0.3	8.5 ± 0.3	11.3 ± 0.3	0.000	ns	ns	ns	0.007	ns
CRL (cm)	56.3 ± 0.9	77.9 ± 1.1	53.3 ± 1.0	75.8 ± 1.3	50.9 ± 1.0	73.4 ± 1.3	49.7 ± 0.9	73.7 ± 1.1	0.000	0.03	ns	ns	ns	ns
Shoulder ht (cm)	40.4 ± 0.6	50.3 ± 0.8	39.5 ± 0.7	48.4 ± 0.9	38.3 ± 0.7	47.2 ± 0.9	36.8 ± 0.6	46.8 ± 0.8	0.001	P<0.1	ns	ns	ns	ns
Tibia length (cm)	13.6 ± 0.2	17.3 ± 0.4	13.2 ± 0.3	17.0 ± 0.4	12.9 ± 0.3	17.0 ± 0.4	12.3 ± 0.2	16.1 ± 0.3	0.004	ns	ns	ns	ns	ns
Metatarsal length (cm)	12.3 ± 0.2	14.4 ± 0.2	12.1 ± 0.2	13.9 ± 0.3	11.9 ± 0.2	14.1 ± 0.3	11.3 ± 0.2	13.3 ± 0.2	0.02	P<0.1	ns	ns	0.008	ns
Skull width (cm)	6.6 ± 0.07	8.1 ± 0.2	6.43 ± 0.08	7.8 ± 0.2	6.3 ± 0.08	7.5 ± 0.2	6.1 ± 0.07	7.2 ± 0.2	0.000	0.008	ns	0.05	P<0.1	ns
Skull length (cm)	14.1 ± 0.2	17.2 ± 0.4	13.8 ± 0.2	17.2 ± 0.4	13.4 ± 0.2	17.1 ± 0.4	13.2 ± 0.2	17.2 ± 0.4	0.002	ns	ns	ns	ns	ns
BMI (kg/cm^2)	17.4 ± 0.5	21.6 ± 0.4	17.5 ± 0.6	22.2 ± 0.3	16.5 ± 0.6	20.7 ± 0.5	14.7 ± 0.6	20.1 ± 0.3	0.002	ns	ns	ns	ns	ns
PI (kg/cm^3)	31.2 ± 1.0	27.8 ± 0.7	33.2 ± 1.2	25.1 ± 0.3	32.4 ± 1.3	28.1 ± 0.6	29.6 ± 1.1	27.8 ± 0.4	ns	ns	ns	ns	ns	ns

All values are expressed as mean ± SEM, the numbers of animals are in parentheses. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex, ns represents non-significance. Statistical significance was assumed at $p<0.05$.

Table 4.2. Effect of placental restriction and gender on postnatal growth of lambs.

Parameter	Absolute Growth Rate (AGR) (kg/day) or (cm/day)				Fractional Growth Rate (NFGR) (0-45 days) (AGR/birth parameter) (%/day)				AGR			NFGR		
	Controls		Placentally Restricted		Controls		Placentally Restricted		PR	S	PRxS	PR	S	PRxS
	Males (n=18)	Females (n=14)	Males (n=13)	Females (n=17)	Males (n=18)	Females (n=14)	Males (n=13)	Females (n=17)						
Weight (kg)	0.30 ± 0.01	0.26 ± 0.01	0.30 ± 0.02	0.25 ± 0.01	5.54 ± 0.4	5.34 ± 0.4	7.04 ± 0.4	7.24 ± 0.4	0.05	ns	ns	0.05	ns	ns
Abdominal circ. (cm)	0.66 ± 0.04	0.63 ± 0.04	0.67 ± 0.04	0.65 ± 0.04	1.71 ± 0.01	1.70 ± 0.01	1.84 ± 0.01	2.00 ± 0.01	ns	ns	ns	0.05	ns	ns
Hind limb circ. (cm)	0.09 ± 0.008	0.07 ± 0.009	0.10 ± 0.01	0.09 ± 0.009	0.09 ± 0.001	0.07 ± 0.001	1.14 ± 0.01	1.11 ± 0.01	0.05	ns	ns	0.05	ns	ns
Crown-rump length(cm)	0.76 ± 0.04	0.73 ± 0.05	0.76 ± 0.05	0.77 ± 0.04	1.36 ± 0.1	1.43 ± 0.1	1.50 ± 0.1	1.57 ± 0.1	ns	ns	ns	0.05	ns	ns
Shoulder height (cm)	0.31 ± 0.02	0.30 ± 0.02	0.32 ± 0.02	0.29 ± 0.02	0.78 ± 0.1	0.74 ± 0.1	0.86 ± 0.1	0.81 ± 0.1	ns	ns	ns	0.05	ns	ns
Tibia length (cm)	0.12 ± 0.01	0.12 ± 0.01	0.14 ± 0.01	0.13 ± 0.01	0.90 ± 0.1	0.91 ± 0.1	1.1 ± 0.1	1.1 ± 0.1	0.05	ns	ns	0.05	ns	ns
Metatarsal length (cm)	0.06 ± 0.007	0.06 ± 0.008	0.07 ± 0.008	0.06 ± 0.007	0.52 ± 0.1	0.52 ± 0.1	0.65 ± 0.1	0.56 ± 0.1	ns	ns	ns	0.05	ns	ns
Skull width (cm)	0.04 ± 0.004	0.04 ± 0.004	0.04 ± 0.004	0.03 ± 0.004	0.62 ± 0.1	0.64 ± 0.1	0.57 ± 0.1	0.50 ± 0.1	ns	ns	ns	ns	ns	ns
Skull length (cm)	0.09 ± 0.01	0.11 ± 0.01	0.13 ± 0.01	0.13 ± 0.01	0.64 ± 0.1	0.77 ± 0.1	0.95 ± 0.1	0.95 ± 0.1	0.05	ns	ns	0.05	ns	ns

The absolute and fractional growth rates (AGR and NFGR) of the control (n = 27) and placentally restricted lambs (n = 25) in terms of phenotype at birth. Measurements were calculated from birth to 45 days. Data is expressed as the mean ± SEM. Statistical significance was assumed at $p<0.05$*, compared to controls.

4.3.2 Effect of placental restriction on fasting plasma metabolites, insulin abundance, sensitivity and disposition indices

PR increased fasting plasma α-amino nitrogen concentrations ($p<0.05$), but did not alter fasting plasma glucose or FFA concentrations in lambs at 30 days of age (Table 4.3). PR did not alter fasting plasma insulin concentrations, but reduced insulin secretion during the IVGTT, with and without correction for glucose area under the curve ($p<0.05$ for both) and did not alter basal or stimulated post-hepatic insulin secretory rates at this age (Table 4.3). PR did not alter the insulin sensitivities of glucose metabolism and of circulating amino acids, but increased that of circulating FFA (+83%)($p<0.05$) (Table 4.3). PR did not alter basal disposition indices for glucose and amino acid metabolism, but increased basal disposition indices for free fatty acid metabolism ($p<0.05$) (Table 4.3). The basal disposition index for glucose metabolism correlated negatively with skull length ($r=-0.31$, $p=0.05$), abdominal circumference ($r=-0.35$, $p=0.04$), ponderal index ($r=-0.40$, $p=0.02$), and body mass index ($r=-0.45$, $p=0.008$) at birth. The basal disposition index for amino acid metabolism correlated negatively with tibia length ($r=-0.30$, $p=0.05$) at birth. The basal disposition index for free fatty acid metabolism correlated negatively with shoulder height ($r=-0.35$, $p=0.04$), skull width ($r=-0.47$, $p=0.008$), abdominal circumference ($r=-0.35$, $p=0.04$) and ponderal index ($r=-0.36$, $p=0.04$) at birth. PR did not alter insulin stimulated disposition indices for free fatty acid metabolism, but tended to decreased insulin stimulated disposition indices for glucose and amino acid metabolism ($p<0.1$ for both) (Table 4.3). There were no gender or gender-treatment interactions for the metabolites, insulin

sensitivities or disposition indices (Table 4.3). Post-hepatic insulin delivery rate was negatively correlated with insulin sensitivity of glucose metabolism in a hyperbolic fashion, particularly in placentally restricted lambs (r=0.62, p=0.006) (Figure 4.3). Post-hepatic insulin delivery rate was also negatively correlated with insulin sensitivity of free fatty acids in placentally restricted lambs (r=0.59, p=0.019) (Figure 4.3).

Table 4.3. The effect of placental restriction on plasma insulin, metabolite concentrations and insulin sensitivity in the lamb.

	Controls		Placentally restricted		PR	S	PRxS
	Male (n)	Female (n)	Male (n)	Female (n)			
Plasma glucose (mmol/l)	4.87 ± 0.24 (8)	5.03 ± 0.26 (6)	5.00 ± 0.26 (5)	4.51 ± 0.21 (9)	ns	ns	ns
Plasma free fatty acids (meq/l)	1.09 ± 0.07 (8)	1.13 ± 0.08 (6)	1.03 ± 0.08 (5)	1.06 ± 0.04 (9)	ns	ns	ns
Plasma α-amino nitrogen (mM)	3.23 ± 0.22 (8)	3.64 ± 0.26 (6)	4.05 ± 0.23 (5)	4.51 ± 0.23 (9)	0.05	ns	ns
Insulin abundance							
Plasma insulin (μU.mL^{-1})	10.0 ± 2.5 (8)	9.5 ± 2.7 (6)	12.4 ± 2.0 (5)	8.6 ± 2.3 (9)	ns	ns	ns
Plateau plasma Insulin (μU.mL^{-1})	69.1 ± 8.6 (8)	62.4 ± 9.2 (6)	64.8 ± 8.8 (5)	59.1 ± 4.9 (9)	ns	ns	ns
Insulin secretion during IVGTT (μU.mL^{-1}.min)	36.4 ± 9.3 (8)	23.6 ± 9.4 (6)	17.3 ± 8.8 (5)	12.3 ± 7.1 (9)	0.05	ns	ns
Insulin secretion during IVGTT relative to glucose (μU.mmol^{-1}.L)	3562 ± 968 (8)	2983 ± 972 (6)	1584 ± 483 (5)	1239 ± 398 (9)	0.05	ns	ns
Insulin clearance (ml.kg^{-1}.min^{-1})	30.6 ± 3.1 (8)	32.8 ± 3.4 (6)	34.0 ± 3.1 (5)	35.7 ± 2.7 (9)	ns	ns	ns
Post-hepatic insulin delivery rate (μU.kg^{-1}.min^{-1})	278 ± 54 (8)	293 ± 58 (6)	421 ± 54 (5)	280 ± 48 (9)	ns	ns	ns
Insulin sensitivity:							
Glucose (mg.mL.μU^{-1}.min^{-1}.kg^{-1})	0.09 ± 0.02 (8)	0.11 ± 0.02 (5)	0.07 ± 0.02 (6)	0.12 ± 0.02 (10)	ns	ns	ns
Amino acids (%ΔmM.μU^{-1}.mL^{-1})	0.37 ± 0.07 (8)	0.42 ± 0.07 (7)	0.22 ± 0.07 (6)	0.35 ± 0.06 (8)	ns	ns	ns
Free fatty acids (%Δmeq.μU^{-1}.mL^{-1})	0.28 ± 0.07 (7)	0.32 ± 0.09 (4)	0.43 ± 0.08 (5)	0.42 ± 0.06 (8)	0.05	ns	ns
Basal Insulin Disposition Index:							
Glucose (mg.ml.kg^{-2}.min^{-2})	23.9 ± 4.4 (7)	27.6 ± 3.0 (6)	28.0 ± 3.5 (7)	27.6 ± 3.0 (9)	ns	ns	ns
Amino acid (mg.mU.kg^{-1})	99 ± 24 (7)	121 ± 26 (6)	79 ± 26 (7)	103 ± 21 (9)	ns	ns	ns
Free fatty acids (meq.ml^{-2}.kg^{-1}.min^{-1})	94 ± 20 (7)	130 ± 22 (6)	171 ± 27 (7)	148 ± 26 (9)	0.05	ns	ns
Insulin Stimulated Disposition Index							
Glucose (mg.ml.kg^{-2}.min^{-2})	343 ± 69 (7)	374 ± 74 (6)	217 ± 91 (7)	224 ± 64 (9)	p<0.1	ns	ns
Amino acid (mM.mU^{-1}.kg^{-1})	1664 ± 411 (7)	1245 ± 444 (6)	596 ± 498 (7)	669 ± 384 (9)	p<0.1	ns	ns
FFA (meq.ml^{-2}.kg^{-1}.min^{-1})	1242 ± 407 (7)	1985 ± 447 (6)	1834 ± 577 (7)	1139 ± 353 (9)	ns	ns	ns

Results are from measurements made during the hyperinsulinaemic euglycaemic clamp. All values are expressed as mean ± SEM; number of animals are in parentheses. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex, ns represents non-significance.

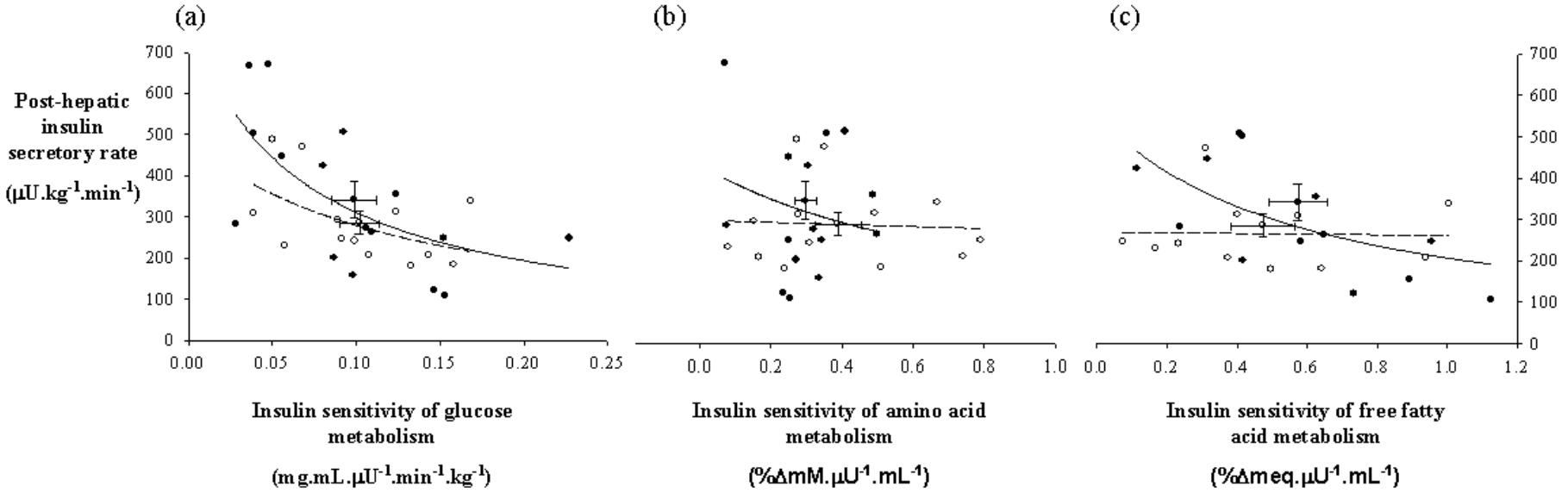

Figure 4.1. Post-hepatic insulin secretory rate and insulin sensitivities of (a) glucose, (b) amino acid, (c) and free fatty acids. Mean ± SEM shown for controls (open circles and dashed line) and placentally restricted (closed circles and solid line).

The following predictive models were obtained by regression analysis:

(a) Controls: Post-hepatic insulin delivery rate x (0.13+Insulin sensitivity of glucose metabolism)=64.8, r=0.44, p=0.076.

PR: Post-hepatic insulin delivery rate x (0.066+Insulin sensitivity of glucose metabolism)=51.4, r=0.62, p=0.006.

(b) Controls: Post-hepatic insulin delivery rate x (11.0+Insulin sensitivity of amino acid metabolism)=3257, r=0.000, N.S.

PR: Post-hepatic insulin delivery rate x (0.84+Insulin sensitivity of amino acid metabolism)=359, r=0.000, N.S.

(c) Controls: Post-hepatic insulin delivery rate x (31.8+Insulin sensitivity of free fatty acid metabolism)=8585, r=0.000, N.S.

PR: Post-hepatic insulin delivery rate x (0.60+Insulin sensitivity of free fatty acid metabolism)=331, r=0.59, p=0.019.

4.3.3 Effect of birth category on size at birth and postnatal growth rate in the lamb

When sheep were classed according to birth weight size category, all measures of size at birth were reduced with decreasing size category ($p<0.001$) (Table 4.4). Decreasing birth weight category reduced AGR for weight and metatarsal length ($p<0.05$) (Table 4.5). Decreasing birth weight category increased neonatal FGR for weight, metatarsal length and FGR at 30 days of age for shoulder height ($p<0.05$) (Table 4.5). When lambs were classed into size at birth categories according to CRL or shoulder height at birth, most measures of size at birth were reduced in those of low size category compared to those of medium and/or high size category (data not shown). In addition, decreasing size at birth category for CRL or shoulder height at birth increased AGRs for shoulder height and long bones and neonatal FGRs for most parameters of size (data not shown).

Table 4.4. Effect of birth weight category on size at birth in lambs.

	Birth weight category			
	Low	Medium	High	ANOVA (Overall model)
Weight (kg)	3.00 ± 0.13 (13)	4.70 ± 0.09 (25)	5.9 ± 0.1 (14)	
Crown rump length (cm)	48.0 ± 0.9 (13)	53.2 ± 0.6 (24)*	57.5 ± 0.9 (14)+#	p<0.001
Shoulder height (cm)	34.5 ± 0.6 (13)	39.3 ± 0.4 (24)*	41.2 ± 0.5 (14)+#	p<0.001
Ponderal index ($kg.m^{-3}$)	27.2 ± 1.2 (13)	31.3 ± 0.8 (24)*	31.0 ± 1.2 (14)+	p=0.07
Body mass index ($kg.m^{-2}$)	13.0 ± 0.5 (13)	16.5 ± 0.3 (24)*	17.8 ± 0.4 (14)+#	p<0.001
Metatarsal length (cm)	10.7 ± 0.2 (13)	12.2 ± 0.1 (24)*	12.5 ± 0.2 (14)+	p<0.001
Tibia length (cm)	11.1 ± 0.3 (13)	13.3 ± 0.2 (24)*	13.7 ± 0.2 (14)+#	p<0.001
Abdominal circumference (cm)	32.0 ± 0.7 (13)	37.7 ± 0.5 (24)*	40.2 ± 0.7 (14)+#	p<0.001
Hind limb circ. (knee joint) (cm)	8.3 ± 0.4 (13)	9.9 ± 0.2 (24)*	10.7 ± 0.3 (14)+	p<0.001
Skull length (cm)	12.9 ± 0.2 (13)	13.9 ± 0.1 (24)*	14.5 ± 0.2 (14)+#	p<0.001
Skull width (cm)	6.0 ± 0.06 (13)	6.4 ± 0.04 (24)*	6.4 ± 0.06 (14)+	p<0.001

Lambs were classed into categories according to their birth weight. Data is expressed as mean ± SEM, with n in brackets and significance symbol after the brackets (*low and medium, +low and high, #medium and high, p<0.05 for all).

Table 4.5. Effect of birth weight category on postnatal growth rates in lambs.

	Birth weight category			ANOVA
	Low	Medium	High	(Overall)
AGR:				
Weight (kg/day)	0.22 ± 0.013 (13)	0.28 ± 0.01 (22)*	0.27 ± 0.01 (13)+	p<0.01
Crown rump length (cm/day)	0.70 ± 0.06 (13)	0.82 ± 0.04 (22)	0.73 ± 0.06 (13)	p=0.61
Shoulder height (cm/day)	0.34 ± 0.03 (13)	0.30 ± 0.02 (22)	0.28 ± 0.02 (13)	p=0.24
Metatarsal length (cm/day)	0.07 ± 0.007 (13)	0.06 ± 0.005 (22)	0.05 ± 0.009 (13)+	p<0.05
Tibia length (cm/day)	0.14 ± 0.01 (13)	0.13 ± 0.009 (22)	0.13 ± 0.01 (13)	p=0.98
Abdominal circ. (cm/day)	0.56 ± 0.04 (13)	0.64 ± 0.03 (22)	0.58 ± 0.04 (13)	p=0.33
Hind limb circ. (knee joint) (cm/day)	0.09 ± 0.01 (13)	0.09 ± 0.008 (22)	0.07 ± 0.01 (13)	p=0.27
Skull length (cm/day)	0.11 ± 0.01 (13)	0.13 ± 0.01 (22)	0.11 ± 0.01 (13)	p=0.35
Skull width (cm/day)	0.03 ± 0.002 (13)	0.03 ± 0.001 (22)	0.03 ± 0.002 (13)	p=0.25
NFGR: (x100)				
Weight	7.36 ± 0.40 (13)	6.07 ± 0.30 (22)*	4.61 ± 0.40 (13)+#	p<0.001
Crown rump length	1.47 ± 0.10 (13)	1.60 ± 0.10 (21)	1.27 ± 0.10 (13)	p=0.26
Shoulder height	0.98 ± 0.10 (13)	0.74 ± 0.10 (21)	0.69 ± 0.10 (13)+	p<0.05
Metatarsal length	0.71 ± 0.10 (13)	0.45 ± 0.10 (21)*	0.36 ± 0.10 (13)+	p<0.01
Tibia length	1.26 ± 0.10 (13)	1.01 ± 0.10 (21)	0.96 ± 0.10 (13)	p=0.36
Abdominal circ.	1.81 ± 0.10 (13)	1.72 ± 0.10 (21)	1.45 ± 0.10 (13)+	p<0.05
Hind limb circ. (knee joint)	1.17 ± 0.20 (13)	0.96 ± 0.10 (21)	0.65 ± 0.20 (13)+	p<0.05
Skull length	0.87 ± 0.10 (13)	0.94 ± 0.10 (21)	0.77 ± 0.10 (13)	p=0.29
Skull width	0.53 ± 0.10 (13)	0.50 ± 0.10 (21)	0.51 ± 0.10 (13)	p=0.08
CFGR: (x100)				
Weight	2.22 ± 0.07 (16)	2.18 ± 0.04 (15)	1.97 ± 0.04 (15)+#	p<0.001
Crown rump length	1.05 ± 0.05 (16)	1.08 ± 0.06 (15)	0.93 ± 0.07 (15)	p=0.19
Shoulder height	0.71 ± 0.05 (16)	0.56 ± 0.03 (15)*	0.59 ± 0.04 (15)	p<0.05
Metatarsal length	0.52 ± 0.04 (16)	0.39 ± 0.04 (15)	0.37 ± 0.04 (15)+	p<0.05
Tibia length	0.80 ± 0.06 (16)	0.82 ± 0.06 (15)	0.76 ± 0.05 (15)	p=0.74
Abdominal circ.	1.19 ± 0.07 (16)	1.15 ± 0.03 (15)	0.92 ± 0.04 (15)+#	p<0.001
Hind limb circ. (knee joint))	0.66 ± 0.09 (16)	0.76 ± 0.08 (15)	0.64 ± 0.06 (15)	p=0.54
Skull length	0.50 ± 0.03 (16)	0.47 ± 0.03 (15)	0.49 ± 0.04 (15)	p=0.08
Skull width	0.67 ± 0.04 (16)	0.74 ± 0.06 (15)	0.52 ± 0.04 (15)	p<0.05

Lambs were divided into 3 groups according to their birth weight. Data is mean ± SEM, n in parentheses and significance symbol after parentheses (*low and medium, +low and high, #medium and high, p<0.05 for all).

4.3.4 Effect of birth weight category on fasting plasma metabolites, insulin abundance, secretion, clearance, and sensitivity in the lamb

Birth weight category did not alter fasting plasma glucose, fasting plasma FFA, or α-amino nitrogen concentrations, insulin secretion during the IVGTT or basal post-hepatic insulin secretory rate (Table 4.6). Fasting and plateau plasma insulin concentrations achieved during the HEC were reduced, and insulin clearance rate, insulin sensitivity of glucose, amino acid, and FFA metabolism were increased in low compared to medium and high birth weight category ($p<0.05$ for all) (Table 4.6). Birth weight category did not alter the basal or stimulated glucose and amino acid disposition indices, but the free fatty acid disposition index tended to be increased in the low birth weight category ($p<0.1$) (Table 4.6).

4.3.5 Effect of birth weight category and growth on fasting plasma metabolites, insulin abundance and sensitivity in the young lamb

Lambs in the high neonatal growth rate category had increased fasted plasma insulin concentrations overall than those in the low neonatal growth category ($p<0.05$) (Table 4.7). Within the low birth weight category, lambs with a high neonatal growth rate had increased fasted plasma insulin concentrations, and lower insulin sensitivities of glucose and free fatty acid metabolism than those of low growth rate ($p<0.05$ for all) (Table 4.7). Within the medium birth weight category, lambs with a high neonatal growth rate had higher fasted plasma insulin concentrations, and lower insulin sensitivities of glucose, amino acid and

free fatty acid metabolism than lambs with a low neonatal growth rate ($p<0.05$ for all) (Table 4.7). Within the high birth weight category those lambs with a high neonatal growth rate had higher insulin sensitivity of amino acid and reduced free fatty acid metabolism compared to those lambs with a low neonatal growth rate ($p<0.05$) (Table 4.7).

4.3.6 Postnatal growth, insulin abundance, sensitivity and disposition indices

Neonatal FGR for weight and skull length were independently and positively correlated with fasting plasma insulin concentration and insulin sensitivity of amino acid metabolism ($p<0.01$, respectively) ($NFGR_{weight}$=0.043+0.0006 x Basal insulin+0.012 x Insulin sensitivity of amino acid metabolism) (overall model: $r = 0.56$, $p<0.05$), ($NFGR_{skull\ length}$=0.002+0.0003 x Basal insulin+0.004 x Insulin sensitivity of amino acid metabolism) (overall model: $r = 0.74$, $p<0.0001$) (data not shown). Current FGR for weight at one month of age was independently and positively correlated with fasting plasma insulin concentration ($p<0.001$) and the insulin sensitivity of amino acid metabolism ($p<0.005$) ($CFGR_{weight}$=0.02+0.0002 x Basal insulin+0.002 x Insulin sensitivity of amino acid metabolism) (overall model: $r = 0.73$, $p<0.001$) (data not shown). The basal disposition index for amino acid metabolism correlated positively with current FGR for weight ($r = 0.33$, $n = 28$) and skull length ($r = 0.46$, $n = 27$) ($p<0.05$ for both) (data not shown). The basal disposition index for free fatty acid metabolism correlated positively with current FGR for weight ($r = 0.33$, $n = 24$) ($p<0.05$).

Table 4.6. Effect of birth weight category on metabolic and endocrine state of neonatal lambs.

Hormone and metabolite abundance and sensitivity	Low birth weight category	Medium birth weight category	High birth weight category	ANOVA (Overall model)
Fasting blood glucose (mmol/l)	4.52 ± 0.26 (8)	4.97 ± 0.17 (15)	4.87 ± 0.28 (5)	p=0.53
Fasting plasma FFA (meq/l)	0.99 ± 0.16 (8)	1.09 ± 0.10 (15)	1.13 ± 0.18 (5)	p=0.47
Plasma FFA 60-120'(meq/l)	0.62 ± 0.13 (8)	0.89 ± 0.08 (15)	0.81 ± 0.15 (5)	p=0.46
Basal plasma insulin ($\mu U.ml^{-1}$)	4.75 ± 2.70 (8)	14.43 ± 1.71 (15)*	11.07 ± 3.02 (5)+	p<0.05
Insulin 60-120' ($\mu U.ml^{-1}$)	49.1 ± 9.1 (8)	77.3 ± 5.8 (15)*	80.8 ± 10.2 (5)+	p<0.05
Insulin secretion during IVGTT ($\mu U.mL^{-1}.min$)	746 ± 150 (9)	4800 ± 2100 (8)	2640 ± 626 (9)	p=0.07
Insulin secretion during IVGTT (corrected for glucose) ($\mu U.mmol^{-1}.L^{-1}$)	8.4 ± 2.2 (9)	53.9 ± 21.3 (8)*	31.6 ± 8.0 (9)	p<0.05
Insulin Clearance ($ml.kg^{-1}.min^{-1}$)	41.3 ± 2.7 (8)	28.33 ± 1.70 (15)*	25.6 ± 3.0 (5)+	p<0.01
Post-hepatic insulin delivery rate ($\mu U.kg^{-1}.min^{-1}$)	270 ± 46 (10)	353 ± 48 (10)	289 ± 32 (8)	p=0.54
Insulin sensitivity				
Glucose ($mg.mL.\mu U^{-1}.min^{-1}.kg^{-1}$)	0.15 ± 0.02 (8)	0.09 ± 0.01 (15)*	0.07 ± 0.02 (5)+	p<0.01
Amino acids ($\%\Delta mM.\mu U^{-1}.mL^{-1}$)	0.017 ± 0.001(8)	0.008 ± 0.002(15)*	0.011 ± 0.001(5)+#	p<0.05
Free fatty acids ($\%\Delta meq.\mu U^{-1}.mL^{-1}$)	0.81 ± 0.14 (8)	0.24 ± 0.09 (15)*	0.36 ± 0.15 (5)	p<0.05
Basal Disposition Index				
Glucose ($mg.ml.kg^{-2}.min^{-2}$)	30.5 ± 4.2 (11)	26.9 ± 2.6 (10)	25.3 ± 5.1 (8)	p=0.64
Amino acid ($mM.mU^{-1}.kg^{-1}$)	104 ± 21 (10)	96 ± 15 (10)	103 ± 28 (8)	p=0.95
Free fatty acids ($meq.ml^{-2}kg^{-1}.min^{-1}$)	166 ± 16 (10)	103 ± 20 (7)	126 ± 42 (7)	p=0.23
Insulin Stimulated Disposition Index				
Glucose ($mg.ml.kg^{-2}.min^{-2}$)	246 ± 42 (10)	331 ± 101 (7)	317 ± 58 (8)	p=0.60
Amino acid ($mM.mU^{-1}.kg^{-1}$)	715 ± 119 (10)	1091 ± 306 (7)	1510 ± 623 (8)	p=0.34
FFA ($meq.ml^{-2}.kg^{-1}.min^{-1}$)	1399 ± 309(10)	1736 ± 617 (5)	1331 ± 314 (7)	p=0.78

Lambs were divided into 3 groups according to their birth weight. Data is mean ± SEM, n in parentheses and significance symbol after parentheses (*low and medium, +low and high, #medium and high, p<0.05 for all).

Table 4.7. The interaction of birth weight and growth rate on the metabolic and endocrine state of young lambs.

	Growth rate category (Weight)	Birth weight category			Birth weight	Growth rate	Birth weight x Growth rate	ANOVA (overall model)
		Low	Medium	High				
Plasma insulin concentration ($\mu U.ml^{-1}$)	Low	3.47 ± 0.55 (4)	7.83 ± 3.01 (4)	10.71 ± 2.28 (4)	0.081	0.031	ns	0.007
	High	11.55 ± 2.46 (6) *	20.19 ± 2.46 (6) *	9.75 ± 6.03 (6) #				
Insulin sensitivity of glucose metabolism ($mg.mL.\mu U^{-1}.min^{-1}.kg^{-1}$)	Low	0.156 ± 0.021 (4)	0.114 ± 0.021 (4)	0.088 ± 0.016 (4)	0.067	ns	ns	0.067
	High	0.112 ± 0.017 (6) *	0.069 ± 0.017 (6) *	0.089 ± 0.042 (6)				
Insulin sensitivity of amino acid metabolism ($\%\Delta mM.\mu U^{-1}.mL^{-1}$)	Low	0.020 ± 0.001 (4)	0.014 ± 0.001 (4)	0.010 ± 0.001 (4)	0.001	0.01	0.012	0.000
	High	0.012 ± 0.001 (6) *	0.008 ± 0.001 (6) *	0.014 ± 0.003 (6) *#				
Insulin sensitivity of free fatty acid metabolism ($\%\Delta meq.\mu U^{-1}.mL^{-1}$)	Low	0.075 ± 0.001 (4)	0.052 ± 0.001 (4)	0.049 ± 0.001 (4)	ns	ns	0.022	0.043
	High	0.075 ± 0.001 (6)	0.012 ± 0.001 (6) *	0.036 ± 0.003 (6) *#				

*$p<0.05$ high FGR_{weight} is different from low FGR_{weight} within that birth weight category only, # $p<0.05$ high FGR_{weight} in all categories is different from low FGR_{weight} categories.

4.4 DISCUSSION

This study has demonstrated that experimentally restricting placental growth reduces size at birth in terms of weight, length and thinness, and increases postnatal growth rate and insulin sensitivity and action in the first month of life. Insulin action in terms of glucose metabolism and circulating free fatty acids was enhanced in lambs that were small at birth, due to increased insulin sensitivity and not altered insulin production. Although placental restriction did not affect some parameters of insulin action, variations in this across the whole birth weight range may exist and relate to growth. Nevertheless, catch-up growth in terms of soft tissues was substantially predicted by both insulin abundance as well as insulin sensitivity. Notably it was insulin sensitivity and disposition index of circulating free fatty acids that was most enhanced in the placentally restricted lambs, which may partly cause the substantial increase in their visceral adiposity, as previously described ([8]Ong*, et al.*, 2000, [13]Albertsson Wikland*, et al.*, 1998, [98]Parsons*, et al.*, 2001).

Placental restriction disproportionately reduced size at birth as seen previously in the fetal sheep in terms of soft compared to skeletal tissues, and with relative head sparing ([236]Robinson*, et al.*, 1979). This pattern is characteristic of most human IUGR ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [83]Albertsson Wikland*, et al.*, 1994), and in a variety of experimental paradigms in other various species ([241]Mellor*, et al.*, 1977, [253]Ritacco*, et al.*, 1997, [275]Muaku*, et al.*, 1997). IUGR due to PR in sheep also resulted in catch-up growth in terms of both weight and length during the first month of life as has

been observed in infants following IUGR ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [83]Albertsson Wikland*, et al.*, 1994). "Catch-up" growth in terms of weight in the first few weeks of life following spontaneous fetal growth restriction has also been observed, in the IUGR pig ([253]Ritacco*, et al.*, 1997, [282]Dauncey*, et al.*, 1994) although not consistently. Lambs born small due to multiple pregnancy also grow at an accelerated rate following birth, especially when allowed unlimited access to milk replacement ([249]Greenwood*, et al.*, 1998, [270]Bell, 1992). As placental restriction limits mammary gland growth ([241]Mellor*, et al.*, 1977, [283]Mellor, 1987), it is likely that even greater catch-up growth may have occurred in the current study, if lambs had been cross fostered or fed artificially. There was no evidence of substantially inadequate nutritional state in the placentally restricted lambs however, as circulating levels of glucose and free fatty acids were unchanged and those of alpha-amino nitrogen actually increased in the fasting state, which has also been observed in lambs born small due to multiple pregnancy ([249]Greenwood*, et al.*, 1998, [284]Ravelli*, et al.*, 1976, [285]Law*, et al.*, 1992).

Placental restriction did not alter fasting plasma insulin or post-hepatic insulin secretory rate, suggesting that increased insulin abundance was not contributing to the observed catch-up growth following placental restriction in the postnatal lamb. Furthermore, insulin secretion in response to a glucose load was reduced, consistent with observations in human IUGR infants ([82]Colle*, et al.*, 1976, [217]Bazaes*, et al.*, 2003). The reduction in stimulated disposition indexes for glucose metabolism and circulating amino acids following placental

restriction, despite enhanced insulin sensitivity, is consistent with an early onset defect in insulin secretion postnatally, as has been described in young adults with IUGR ([286]Jaquet*, et al.*, 2000). This further suggests that any increased growth in the placentally restricted lamb due to increased insulin action may occur substantially in the post-prandial state. Importantly, catch-up growth of soft tissues was predicted by circulating insulin levels or post-hepatic insulin secretory rate, and insulin sensitivity of amino acids. This enhanced insulin sensitivity of amino acid metabolism may be a major driver of neonatal growth, but insulin abundance is also an important determinant of the extent to which "catch-up" growth can occur.

Placental restriction substantially increased insulin sensitivity of free fatty acids, reflecting sensitivity of suppression of lipolysis and/or triglyceride synthesis in the young lamb. This would promote accretion of lipid and adipose tissue and may contribute to the increased adiposity of these placentally restricted lambs at one month of age and of lambs small-at-birth due to multiple pregnancy and of human infants following catch-up growth ([249]Greenwood*, et al.*, 1998, [284]Ravelli*, et al.*, 1976, [285]Law*, et al.*, 1992). It is unclear if this increased storage of fat continues throughout life, however, triplet lambs are fatter than singletons by the end of the first few months of life ([249]Greenwood*, et al.*, 1998). If this excess storage of fat persists, the increased adiposity may contribute to the adverse metabolic and cardiovascular outcomes for later adult life that have been associated with IUGR in humans ([9]Eriksson*, et al.*, 1999, [70]Cianfarani*, et al.*, 1999, [95]Rasmussen, 2001, [96]Forsén*, et al.*, 1997).

The IUGR lamb is more sensitive to insulin in general in the first month of life as insulin sensitivity of glucose metabolism in terms of whole body glucose uptake, circulating amino acids, as well as of free fatty acids, all increased with decreasing size at birth. The tissue site(s) of this enhanced metabolic sensitivity to insulin were not determined in the current study, but are consistent with the increased growth rate of soft tissues being supported by increased glucose utilisation for energy production and storage, and increased amino acid accretion in growing tissues. A preliminary study, using radio-labelled glucose, demonstrated increased glucose utilisation by skeletal muscle accounts for the majority of the increase in whole body glucose uptake in response to insulin (unpublished observations). Increased insulin sensitivity of glucose metabolism, assessed indirectly, has now been described in the human IUGR infant in the first two days of life ([217]Bazaes*, et al.*, 2003), which if it persists, suggests both the neonatal human and lamb are similar in the consequences of IUGR for the insulin axis in the first month of life. The enhanced insulin sensitivity of circulating amino acids may also substantially reflect that of skeletal muscle. Previous studies in young sheep have shown that insulin stimulates predominantly amino acid uptake in non-visceral tissues ([287]Wilkening*, et al.*, 1994). In the neonatal pig, insulin infusion stimulates protein synthesis in skeletal muscle ([130]Davis*, et al.*, 2001) and in cardiac muscle and skin, but not in liver, intestine, spleen, pancreas, or kidney ([130]Davis*, et al.*, 2001) at least when circulating amino acids and glucose are maintained. This suggests that the enhanced insulin sensitivity of circulating

amino acids observed in the current study may be a consequence of increased sensitivity of skeletal muscle, and this may be driving the accelerated growth of skeletal muscle ([130]Davis*, et al.*, 2001).

Recently it has been shown that IUGR alters the development and function of skeletal muscles in pigs at birth ([288]Wank*, et al.*, 2000), increasing the proportion of type 1 muscle fibres, which are highly insulin sensitive. If this pattern persists, it may partly underlie the increased sensitivity of glucose and amino acids to insulin observed here in the young IUGR lamb. The factors responsible for this and for enhanced insulin action in the young lamb following placental restriction are unknown but some aspect of the altered metabolic and endocrine environment before birth is presumably responsible. The placentally restricted fetal sheep is hypoxaemic and hypoglycaemic with elevated circulating cortisol and reduced plasma concentrations of insulin, insulin-like growth factors, thyroid hormones, and prolactin ([18]Robinson*, et al.*, 1985, [255]Kind*, et al.*, 1995, [289]Phillips*, et al.*, 2001). Both cortisol and thyroid hormones have well established roles in modulating the development of numerous tissues, including skeletal muscle and liver before and after birth ([32]Fowden, 1995, [290]Fowden*, et al.*, 1993, [291]Forhead*, et al.*, 2003). Glucocorticoids have also been shown to affect GLUT1 and GLUT4 in the liver and skeletal muscle before birth ([292]Sakoda*, et al.*, 2000, [293]Hahn*, et al.*, 1999, [294]Weinstein*, et al.*, 1998), but have relatively little effect on basal or insulin stimulated insulin secretion ([290]Fowden*, et al.*, 1993, [295]Fowden, 1993, [296]Fowden*, et al.*, 1996). The infusion of cortisol into the preterm fetal sheep

increased fetal plasma cortisol to term levels but did not alter fetal arterial blood concentrations of plasma glucose or insulin ([296]Fowden*, et al.*, 1996). GLUT-4 is up-regulated in rat skeletal muscle during the perinatal period ([297]Santalucia*, et al.*, 1992). Cortisol infusion into control and adrenalectomized sheep fetuses at 127-130 days gestation, increased plasma cortisol and muscle GLUT-4 mRNA expression ([191]Li*, et al.*, 1998). If this persists, elevated cortisol prenatally as in IUGR may enhance insulin sensitivity of glucose postnatally.

The mechanism underlying the very substantial "catch-up" of long bones is unclear. Although long bone growth was predicted by insulin sensitivity of glucose and amino acid metabolism, no specific marker of growth plate sensitivity to insulin was measured in the current study. Insulin has mitogenic actions on chondrocytes isolated from the proximal tibial growth plate of fetal lambs ([298]Hill*, et al.*, 1990), acting in part by endogenously produced IGF-II, and regulated by glucose availability. Persistence of this insulin dependence in neonatal life and similar programming of enhanced insulin sensitivity in the growth plates of long bones, as occurs in other tissues in the young lamb following placental restriction, would help explain the "catch-up" growth in skeletal tissues. The "catch-up" growth of long bones may also be due to delayed senescence of the growth plate. Senescent growth plates are replaced with bone, termed epiphyseal fusion with the timing of this dependent not on age, but on the cumulative number of cell divisions that the chondrocytes of long bones undergo ([209]Gafni*, et al.*, 2001). This has been suggested to be the underlying cause of catch-up growth following restriction due to dexamethasone

administration to growing rabbits in order to suppress their linear growth ([209]Gafni*, et al.*, 2001). Catch-up growth occurring after cessation of dexamethasone induced growth restriction, was characterized by a delay in the age related senescent decline in the heights of the proliferative zone, hypertrophic zone, and total growth plate in the distal femoral growth plates ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). This was due to locally acting mechanisms, as transplantation of the growth plates into untreated animals did not alter this response ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). These findings support linear catch-up growth being caused in part, by a delay in growth plate senescence ([209]Gafni*, et al.*, 2001), which may be accompanied by increased sensitivity to anabolic hormones such as insulin, since this is typically higher earlier in development and declines with age.

In conclusion, this study has shown that, experimental placental restriction reduces size at birth and increases neonatal growth rate for soft and skeletal tissues and insulin action on adipose tissue in the young lamb. Neonatal 'catch-up' growth after fetal growth restriction as indicated by low birth weight is substantially predicted and may be limited by both abundance of and sensitivity to insulin, and increased insulin action.

Chapter 5

PLACENTAL RESTRICTION ALTERS CIRCULATING INSULIN-LIKE GROWTH FACTORS AND METABOLIC SENSITIVITY TO IGF-I AND THEIR RELATIONSHIP TO GROWTH AND ADIPOSITY IN THE YOUNG LAMB

5.1 INTRODUCTION

Intrauterine Growth Restriction (IUGR), evident as reduced weight, reduced length, or increased thinness at birth for a given gestational age, is characterized by accelerated growth in the first few months of life, due to poorly understood mechanisms ([1]Chernausek, 1996, [7]Karlberg*, et al.*, 1995, [11]Prader*, et al.*, 1963). Both IUGR and this subsequent catch-up growth are independent predictors of increased morbidity and mortality in adult life ([87]Parker*, et al.*, 2003, [267]Cameron*, et al.*, 2002, [268]Ong*, et al.*, 2002). Thus IUGR infants who catch-up develop increased adiposity in childhood ([8]Ong*, et al.*, 2000, [13]Albertsson Wikland*, et al.*, 1998) and have an increased risk of developing type 2 diabetes, obesity and cardiovascular disease as adults ([8]Ong*, et al.*, 2000, [13]Albertsson Wikland*, et al.*, 1998, [62]McCowan*, et al.*, 1999, [94]Karlberg*, et al.*, 1993). The underlying causes of catch-up growth after IUGR are currently unknown, but may contribute to the subsequent adverse metabolic and cardiovascular sequelae. Evidence from human infants and lambs with IUGR suggests that possible mechanisms may be increased appetite and nutrient ingestion, increased metabolic efficiency and increased activity of anabolic hormones ([108]Soto*, et al.*, 2003, [152]Giudice*, et al.*, 1995, [204]Crescenzo*, et al.*, 2003, [212]Deiber*, et al.*, 1989, [213]Adcock*, et al.*, 1997, [214]Ogilvy-Stuart*, et al.*, 1998, [217]Bazaes*, et al.*, 2003, [249]Greenwood*, et al.*, 1998, [268]Ong*, et al.*, 2002, [280]Dotsch*, et al.*, 1998).

The major anabolic hormones in infancy include the insulin-like growth factors (IGF), IGF-I and -II. *In vivo*, IGF-I is secreted by the liver and other tissues in postnatal life, including skeletal muscle and bone and is postulated to have mitogenic and metabolic actions at or near its sites of synthesis ([140]Daughaday*, et al.*, 1989). The highest concentrations of the IGFs are found in blood, where they are present mostly in the bound form, associated with up to 6 IGF binding proteins (IGFBPs). Both IGF-I and IGF-II act via the type-1 IGF receptor to promote proliferation and inhibit apoptosis of a variety of cells in a wide range of tissues ([157]Le Roith, 2000) and stimulate glucose and amino acid uptake and protein accretion into muscle and other tissues ([146]Zapf*, et al.*, 1986, [158]Jacob*, et al.*, 1989, [159]Guler, 1987). The IGFs also stimulate bone elongation, increasing chondrocyte proliferation and hypertrophy ([162]Wang*, et al.*, 1999). In addition, IGF-I is a potent stimulator of pre-adipocyte proliferation and early differentiation *in vivo* and *in vitro* ([163]Stewart*, et al.*, 1999, [164]Benito*, et al.*, 1996, [165]Smith*, et al.*, 1988, [166]Holzenberger*, et al.*, 2001, [167]Schmidt*, et al.*, 1990). IGF-II may also play a role in body weight regulation and development of obesity in men and women, since higher IGF-II levels are associated with a reduced risk of gaining weight ([168]Sandhu*, et al.*, 2003), and IGF-II over expression in mice reduce adiposity ([299]Devedjian*, et al.*, 2000). Therefore programmed alterations in the abundance and action of each IGF could potentially mediate the altered growth and adiposity observed postnatally following restriction of fetal growth ([168]Sandhu*, et al.*, 2003, [169]Ben-Shlomo*, et al.*, 2003).

These possibilities have been investigated to a limited extent in humans and other species. In humans, catch-up growth in IUGR infants has been described as occurring in the presence of reduced or at best, normal serum IGF-I concentrations in the first few months of life and up to one year of age ([152]Giudice*, et al.*, 1995, [214]Ogilvy-Stuart*, et al.*, 1998, [228]Ozkan*, et al.*, 1999). Therefore if IGF-I has a role in catch-up growth after IUGR, this suggests that it is via increased sensitivity to IGFs rather than by increased production of IGF-I. Increased sensitivity to IGFs could result from reduced abundance of inhibitory binding proteins, increased abundance of facilitatory binding proteins or increases in the expression of the type 1 IGF receptor or enhanced post-receptor signalling. Serum IGF-II levels were higher in IUGR infants undergoing catch-up growth (defined as an increase in length z score greater than 1 SD between birth and 6 months of age) compared to those infants not undergoing catch-up growth, at 3 months of age but were not compared to those of normal birth weight infants ([102]Garcia*, et al.*, 1996). However, plasma IGF-II levels were reduced in 7.5-year-old children born after IUGR who had not undergone catch-up growth, compared with normal children ([101]de Waal*, et al.*, 1994). Plasma IGF-II concentrations were not different between normal and IUGR children by 9 years of age ([104]Cianfarani*, et al.*, 2002). It is therefore unclear if the abundance of IGF-II is altered during catch-up growth after IUGR. The persistent deficit in IGF-II abundance evident in children with IUGR may contribute to increased adiposity however as reported for adults and genetically modified mice ([106]Ong*, et al.*, 2002, [299]Devedjian*, et al.*, 2000).

Because the investigation of the impact of IUGR on hormone abundance and sensitivity in early postnatal life is difficult in the human infant, we have established and characterized an experimental paradigm in which fetal growth is restricted by restriction of placental implantation and subsequent growth in the sheep ([236]Robinson*, et al.*, 1979). This restricts placental functional development, which in turn, increasingly restrains fetal growth increasingly in the second half of gestation ([4]Owens*, et al.*, 1986, [5]Owens*, et al.*, 1987, [18]Robinson*, et al.*, 1985, [257]Owens*, et al.*, 1987). Placental insufficiency is a common cause of IUGR in humans and other species and hence is implicated in the initiation of changes leading to subsequent postnatal catch-up growth and other sequelae. In addition, in humans, late gestational onset IUGR is associated with catch-up growth by 6 months of age ([92]Harding*, et al.*, 2003). Experimental restriction of placental in sheep has similar growth, metabolic and endocrine consequences for the fetus as observed in human IUGR ([237]Owens*, et al.*, 1994). We have recently shown that placental restriction in the sheep reduces size at birth and causes neonatal catch-up growth in weight and length, as well as increased adiposity in the young lamb (See Chapter 3). We therefore hypothesized that restriction of placental growth, which is a major cause of IUGR, would reduce size at birth, induce catch-up growth, with unaltered circulating IGF-I and IGF-II, but increased metabolic sensitivity to IGF-I, in the young lamb postnatally.

5.2 MATERIALS AND METHODS

5.2.1 Animals and Surgery

All procedures performed in this project were approved by the Adelaide University Animal Ethics Committee (Animal Ethics Approval Number: M/1/97A). Placental growth was restricted in 45 Merino ewes by removal of the majority of visible endometrial caruncles (65-148) from the non-pregnant uterus, leaving either 3 to 8 caruncles in each horn of the bicornuate uterus ([236]Robinson*, et al.*, 1979) (See Materials and Methods; 2.1.1). Ewes were housed in individual pens in animal holding rooms from approximately a week before giving birth (See Materials and Methods; 2.1.2). Control ewes delivered 22 lambs and the placentally restricted delivered 24 lambs. The lambs were housed in the pens with their mothers throughout the study.

5.2.2 Measurement of growth

At birth and at 5-day intervals up to 45 days of age, size at birth (day 0), and subsequently size in terms of body weight, crown-rump length (CRL), tibia and metatarsal lengths, shoulder height, and abdominal, tibia, radius/ulna and hind limb circumferences were measured (See Materials and Methods; 2.1.4). The postnatal growth of lambs was calculated from birth to 45 days of age for each parameter relative to that parameter at birth (See Materials and Methods; 2.1.4).

5.2.3 Hyper-IGF-I euglycaemic clamp and IGF-I sensitivity

At 35 ± 3 days of age, a hyper-IGF-I euglycaemic clamp was performed (See Materials and Methods 2.2.2.1). Arterial blood samples were measured for blood glucose using a glucometer (HemoCue AB, Sweden). Human recombinant IGF-I (rhIGF-I; Gropep Australia) was continuously infused (t=0 minutes) to deliver 3 μg/kg/min across a 130-minute infusion. At 25 minutes after the start of the IGF-I infusion, glucose (25%) was variably infused into the femoral artery with adjustment of the rate every 5 minutes to maintain the fasting blood glucose concentration (See Materials and Methods 2.2.2.1). The IGF-I sensitivity of glucose metabolism was determined as the mean glucose infusion rate from 70 to 130 minutes (GIR) for the hyper-IGF-I euglycaemic clamp (See Materials and Methods 2.2.2.1).

5.2.4 IGF-I sensitivity of circulating amino acids

Alpha-amino nitrogen (αaN) concentrations, reflecting total amino acid concentrations, were measured in plasma taken at 15-minute intervals from 70 to 130 minutes of the hyper-IGF-I euglycaemic clamp ([266]Evans*, et al.*, 1993) (See Materials and Methods 2.2.2.2). The sensitivity of circulating amino acids to IGF-I was calculated as the percentage change from the mean fasting plasma αaN to the mean of the last 60 minutes (70 to 130 minutes) (See Materials and Methods 2.2.2.2).

5.2.5 IGF-I sensitivity of circulating free fatty acids

Plasma free fatty acid (FFA) concentrations were measured prior to and during the second hour of the hyper-IGF-I euglycaemic clamp (See Materials and Methods 2.2.2.3). The IGF-I sensitivity of circulating FFA was calculated as the percentage change from fasting FFA concentrations to those during final 60 minutes (70-130 minutes) of the HIEC (See Materials and Methods 2.2.2.3).

5.2.6 Plasma IGF-I and IGF-II concentrations

Plasma IGF-I and IGF-II were measured at 30 days of age following acidification and size exclusion high performance liquid chromatography (HPLC) of plasma at pH 2.5 to dissociate and separate the IGFs from binding proteins (See Materials and Methods 2.2.2.4). The chromatography fractions containing free IGFs were neutralized, and then assayed by specific radioimmunoassay (See Materials and Methods 2.2.2.4.1 and 2.2.2.4.2).

5.2.7 Statistical Analysis

Data is expressed as mean $\pm$ standard error of the mean (SEM), unless otherwise stated. The effects of placental restriction and sex were assessed by analysis of variance (ANOVA) (SPSS 11.5 software package for Windows). Pearson correlation analysis and multiple linear regression analysis were performed between variables of growth, IGF abundance and sensitivity, and adiposity (SPSS 11.5 software package for Windows). The coefficient of variation (% CV) was calculated as the standard deviation divided by the mean multiplied by 100. Statistical significance was assumed at $p<0.05$.

5.3 RESULTS

5.3.1 Effect of placental restriction on size at birth

Restriction of implantation sites reduced placental weight (-39%) ($p<0.05$) and reduced size at birth, in terms of weight, abdominal and hind limb circumference (knee joint), thoracic circumference (-11% to -28%) ($p<0.05$), CRL, shoulder height, tibia length, skull width, skull length, and body mass index (-6% to -11%)($p<0.05$) (Table 5.1). Weight, CRL, skull width, and abdominal circumference at birth were greater in males than females ($p<0.05$ for all) (Table 5.1).

5.3.2 Effect of placental restriction on postnatal growth

Placental restriction reduced absolute growth rates (AGR) for skull width ($p<0.05$) (Table 5.2), but increased current fractional growth rates (FGR) at day 30 for weight, skull length ($p<0.05$ for both) (Table 5.3). Males had greater absolute growth rates for weight and hind limb circumference (knee joint) than females ($p=0.001$, $p=0.01$, respectively) (Table 5.2). Males had greater current fractional growth rate at day 30 for hind limb circumference (knee joint) ($p=0.03$) than females (Table 5.3).

5.3.3 Effect of placental restriction on fasting metabolites and insulin-like growth factors

Placental restriction did not alter fasting plasma free fatty acids or α-amino nitrogen concentrations at 35 days of age, but tended to increase fasting plasma glucose concentrations (Table 5.4). Placental restriction reduced plasma IGF-II concentrations ($p<0.05$) at 35 days of age, but did not alter plasma IGF-I concentrations (Table 5.4). Plasma IGF-I and IGF-II concentrations were reduced in female lambs compared to males at 30 days of age ($p<0.05$ for both) (Table 5.4).

Table 5.1. The effect of experimental restriction of placental growth on birth phenotype in sheep.

Birth phenotype	Male		Female		Anova (p value)		
	Controls	PR	Control	PR	PR	S	PRxS
Weight (kg)	5.49 ± 0.30 (12)	4.29 ± 0.33 (10)	4.95 ± 0.33 (10)	3.62 ± 0.28 (14)	0.000	0.05	ns
Crown rump length (cm)	55.8 ± 1.2 (12)	51.8 ± 1.3 (9)	52.6 ± 1.3 (10)	49.5 ± 1.1 (14)	0.002	0.05	ns
Shoulder height (cm)	40.3 ± 0.8 (12)	38.0 ± 0.9 (9)	38.8 ± 0.9 (10)	36.3 ± 0.8 (14)	0.014	p<0.1	ns
Tibia length (cm)	13.7 ± 0.3 (12)	12.7 ± 0.4 (9)	13.2 ± 0.4 (10)	12.3 ± 0.3 (14)	0.008	ns	ns
Metatarsal length (cm)	12.1 ± 0.3 (12)	11.8 ± 0.3 (9)	11.9 ± 0.3 (10)	11.2 ± 0.3 (14)	p<0.1	ns	ns
Skull width (cm)	6.68 ± 0.10 (12)	6.34 ± 0.11 (9)	6.47 ± 0.12 (10)	6.13 ± 0.09 (14)	0.003	0.04	ns
Skull length (cm)	14.0± 0.3 (12)	13.3 ± 0.3 (9)	13.7 ± 0.3 (10)	13.1 ± 0.2 (14)	0.03	ns	ns
Abdominal circumference (cm)	38.5 ± 1.2 (12)	36.7 ± 1.3 (9)	37.6 ± 1.3 (10)	32.8 ± 1.1 (14)	0.009	0.05	ns
Hind limb circumference (knee joint) (cm)	9.64 ± 0.35 (12)	8.90 ± 0.38 (9)	10.3 ± 0.40 (10)	8.77 ± 0.32 (14)	0.003	ns	ns
Thoracic circumference (cm)	39.0 ± 0.9 (12)	36.1 ± 1.0 (9)	37.7 ± 1.0 (10)	34.0 ± 0.8 (14)	0.001	p<0.1	ns
Body Mass Index ($kg.m^{-2}$)	17.7 ± 0.8 (12)	16.2 ± 0.8 (9)	17.6 ± 0.9 (10)	14.6 ± 0.7 (14)	0.006	ns	ns
Ponderal Index ($kg.m^{-3}$)	31.8 ± 1.5 (12)	31.6 ± 1.7 (9)	33.7 ± 1.8 (10)	29.6 ± 1.4 (14)	ns	ns	ns

Birth phenotypes of control (n = 22) and placentally restricted (n = 24) lambs. All values are expressed as mean ± SEM. Statistical significance was assumed at p<0.05 compared to controls.

Table 5.2. The effect of experimental restriction of placental growth on postnatal growth of lambs.

Absolute Growth Rate	Male		Female		Anova (p value)		
	Controls	PR	Controls	PR	PR	S	PRxS
Weight (kg/day)	0.311 ± 0.016 (12)	0.323 ± 0.018 (10)	0.263 ± 0.018 (10)	0.250 ± 0.015 (14)	ns	0.001	ns
Crown rump length (cm/day)	0.697 ± 0.046 (12)	0.760 ± 0.050 (10)	0.734 ± 0.050 (10)	0.714 ± 0.050 (14)	ns	ns	ns
Shoulder height (cm/day)	0.334 ± 0.024 (12)	0.350 ± 0.026 (10)	0.315 ± 0.026 (10)	0.288 ± 0.022 (14)	ns	p<0.1	ns
Tibia length (cm/day)	0.135 ± 0.012 (12)	0.128 ± 0.013 (10)	0.132 ± 0.013 (10)	0.117 ± 0.011 (14)	ns	ns	ns
Metatarsal length (cm/day)	0.073 ± 0.009 (12)	0.086 ± 0.010 (10)	0.072 ± 0.010 (10)	0.069 ± 0.008 (14)	ns	ns	ns
Skull width (cm/day)	0.049 ± 0.005 (12)	0.038 ± 0.005 (10)	0.043 ± 0.005 (10)	0.053 ± 0.005 (14)	0.05	ns	ns
Skull length (cm/day)	0.087 ± 0.012 (12)	0.118 ± 0.013 (10)	0.092 ± 0.013 (10)	0.106 ± 0.011 (14)	p<0.1	ns	ns
Hind limb circ. (knee joint) (cm/day)	0.096 ± 0.010 (12)	0.114 ± 0.011 (10)	0.075 ± 0.011 (10)	0.078 ± 0.010 (14)	ns	0.01	ns
Abdominal circumference (cm/day)	0.690 ± 0.045 (12)	0.708 ± 0.049 (10)	0.637 ± 0.049 (10)	0.652 ± 0.041 (14)	ns	ns	ns
Thoracic circumference (cm/day)	0.658 ± 0.058 (12)	0.647 ± 0.070 (10)	0.687 ± 0.063 (10)	0.568 ± 0.055 (14)	ns	ns	ns

Absolute growth rates (AGR) of control (n = 22) and placentally restricted lambs (n = 24). Data is expressed as the mean ± SEM. Statistical significance was assumed at $p<0.05$ compared to controls.

Table 5.3. The effect of experimental restriction of placental growth on postnatal growth of lambs.

Current Fractional Growth Rate (day 30)	Male		Female		Anova (p value)		
	Controls	PR	Controls	PR	PR	S	PRxS
Weight (%/day)	5.5 ± 0.4 (12)	7.0 ± 0.4 (10)	5.3 ± 0.4 (10)	7.2 ± 0.4 (14)	0.000	ns	ns
Crown rump length (%/day)	1.4 ± 0.1 (12)	1.5 ± 0.1 (10)	1.4 ± 0.1 (10)	1.6 ± 0.1 (14)	ns	ns	ns
Shoulder height (%/day)	0.8 ± 0.1 (12)	0.9 ± 0.1 (10)	0.7 ± 0.1 (10)	0.8 ± 0.1 (14)	ns	ns	ns
Tibia length (%/day)	0.9 ± 0.1 (12)	1.1 ± 0.1 (10)	0.9 ± 0.1 (10)	1.1 ± 0.1 (14)	ns	ns	ns
Metatarsal length (%/day)	0.5 ± 0.1 (12)	0.7 ± 0.1 (10)	0.5 ± 0.1 (10)	0.6 ± 0.1 (14)	ns	ns	ns
Skull width (%/day)	0.6 ± 0.1 (12)	0.6 ± 0.1 (10)	0.6 ± 0.1 (10)	0.5 ± 0.1 (14)	p<0.1	ns	ns
Skull length (%/day)	0.6 ± 0.1 (12)	0.9 ± 0.1 (10)	0.8 ± 0.1 (10)	0.9 ± 0.1 (14)	0.03	ns	ns
Hind limb circ (knee joint) (%/day)	0.9 ± 0.1 (12)	1.1 ± 0.1 (10)	0.7 ± 0.1 (10)	1.1 ± 0.1 (14)	ns	0.03	ns
Abdominal circumference (%/day)	1.7 ± 0.1 (12)	1.8 ± 0.1 (10)	1.7 ± 0.1 (10)	2.0 ± 0.1 (14)	ns	ns	ns
Thoracic circumference (%/day)	1.7 ± 0.1 (12)	2.0 ± 0.2 (10)	1.7 ± 0.2 (10)	1.9 ± 0.1 (14)	ns	ns	ns

The current fractional growth rates (x100%) at day 30 for control (n = 22) and placentally restricted lambs (n = 24). Data is expressed as the mean ± SEM. Statistical significance was assumed at $p<0.05$ compared to controls.

Table 5.4. Effect of experimental restriction of placental growth on circulating insulin-like growth factors and metabolic sensitivity to IGF-I in the young lamb.

	Males		Females				
	Controls	PR	Controls	PR	PR	Sex	PRxS
Plasma glucose ($mmol.L^{-1}$)	5.55 ± 0.27 (12)	5.93 ± 0.29 (10)	5.21 ± 0.29 (10)	5.77 ± 0.25 (14)	p<0.1	ns	ns
Plasma α-amino Nitrogen (mM)	3.28 ± 0.39 (12)	3.72 ± 0.47 (8)	3.61 ± 0.45 (9)	3.80 ± 0.37 (13)	ns	ns	ns
Plasma free fatty acids ($meq.L^{-1}$)	1.49 ± 0.18 (12)	1.38 ± 0.21 (9)	1.32 ± 0.21 (9)	1.41 ± 0.17 (14)	ns	ns	ns
Plasma IGF-I ($ng.ml^{-1}$)	378 ± 55 (5)	396 ± 55 (5)	256 ± 55 (5)	222 ± 41 (9)	ns	0.05	ns
Plasma IGF-II ($ng.ml^{-1}$)	401 ± 28 (5)	356 ± 28 (5)	351 ± 28 (5)	247 ± 21 (9)	0.04	0.05	ns
IGF-I sensitivity:							
Glucose ($mg.kg^{-1}.min^{-1}$)	5.08 ± 0.75 (10)	3.25 ± 1.06 (5)	6.39 ± 0.84 (8)	3.91 ± 1.06 (5)	0.04	p<0.1	ns
Amino acids ($\%\Delta mM.ml^{-1}.min^{-1}$)	32.6 ± 3.0 (10)	22.2 ± 4.7 (4)	34.4 ± 3.6 (7)	29.4 ± 4.7 (4)	p<0.1	ns	ns
Free fatty acids ($\%\Delta meq.L^{-1}.min^{-1}$)	24.6 ± 8.0 (6)	42.9 ± 9.8 (4)	36.0 ± 7.4 (7)	30.1 ± 11.4 (3)	ns	ns	ns

Data is expressed as the mean ± SEM. Statistical significance was assumed at p<0.05 compared to controls.

5.3.4 Plasma IGFs and size at birth

Plasma IGF-I concentrations at 30 days of age correlated positively with size at birth, in terms of weight ($r=0.85$, $p<0.001$), tibia length ($r=0.60$, $p<0.05$), abdominal circumference ($r=0.72$, $p=0.01$) and body mass index ($r=0.84$, $p=0.002$) in control lambs, and with size at birth in terms of tibia length ($r=0.47$, $p<0.05$) and body mass index ($r=0.46$, $p<0.05$) in placentally restricted lambs (Figure 5.1a-c; Figure 5.2a). Plasma IGF-II concentrations 30 days of age correlated positively with size at birth in terms of abdominal circumference in control ($r=0.52$, $p<0.05$) and in placentally restricted lambs ($r=0.48$, $p<0.05$) (Figure 5.2b), and in males when analysed separately (see Figure 5.2b).

5.3.5 IGF-I sensitivity and size at birth

Placental restriction reduced IGF-I sensitivity of glucose metabolism ($p=0.04$), which also tended to be lower in males compared with females ($p<0.1$) (Table 5.4). Placental restriction also tended to reduce IGF-I sensitivity of amino acid metabolism ($p<0.1$), but did not alter that of IGF-I sensitivity of free fatty acid metabolism (Table 5.4). IGF-I sensitivity of glucose metabolism correlated negatively with ponderal index at birth in controls only ($r=0.49$, $p<0.025$) (Figure 5.3). When control and placentally restricted lambs were combined, IGF-I sensitivity of glucose metabolism correlated negatively with ponderal index at birth ($r=-0.32$, $n=27$, $p=0.05$), while IGF-I sensitivity of free fatty acid metabolism correlated negatively with weight ($r=-0.57$, $n=12$, $p=0.03$), CRL ($r=-0.70$, $n=11$, $p=0.008$), tibia ($r=-0.52$, $n=11$, $p=0.05$), metatarsal length ($r=-0.74$, $n=11$,

p=0.004), and skull width (r=-0.60, n=11, p=0.03) and length (r=-0.51, n=11, p=0.05) at birth. In control lambs, IGF sensitivity of glucose metabolism tended to correlate negatively with body mass index at birth (r=-0.38, n=18, p<0.1), correlated negatively with ponderal index (r=-0.49, n=18, p=0.02), and tended to correlate positively with CRL at birth (r=0.35, n=18, p<0.1). In control lambs, IGF-I sensitivity of free fatty acid metabolism tended to correlate negatively with ponderal index at birth (r=-0.34, n=13, p<0.05), and correlated positively with metatarsal length (r=0.55, n=13, p=0.03) at birth. IGF-I sensitivity of amino acid metabolism was not related to any measure of size at birth.

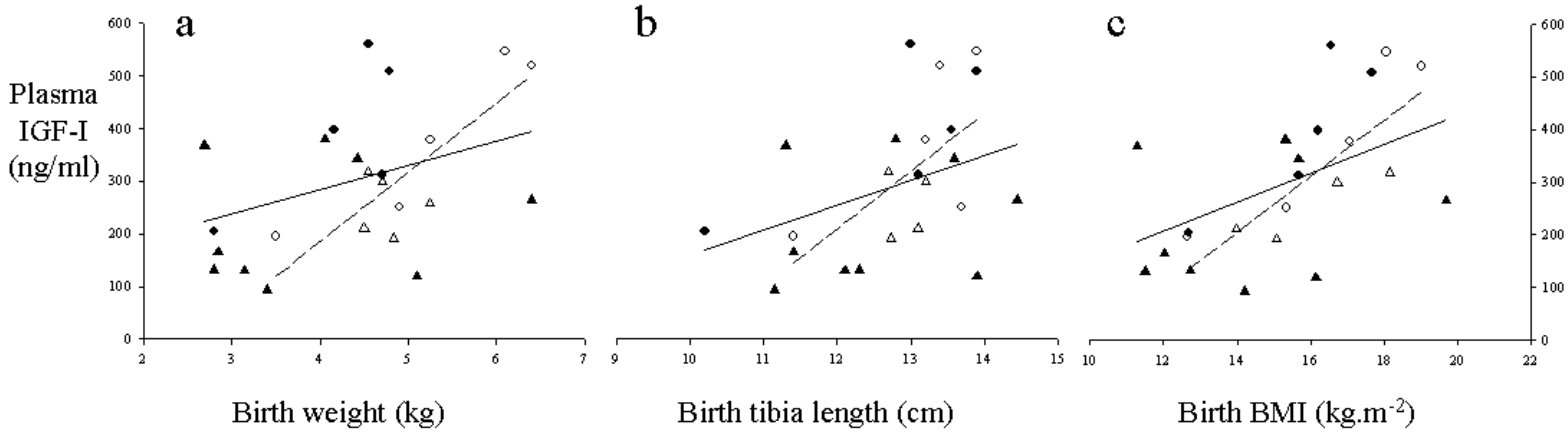

Figure 5.1. Plasma IGF-I concentration at 30 days of age and size at birth for control and placentally restricted lambs.

Controls are represented by the dashed regression line and open symbols and placentally restricted are represented by the solid regression line and closed symbols. Males are represented by circles and females by triangles. Plasma IGF-I correlated positively with (a) birth weight (controls ($r=0.85$, $p<0.001$) and placentally restricted ($r=0.33$, ns)), (b) birth tibia length (controls ($r=0.60$, $p<0.05$) and placentally restricted ($r=0.40$, ns)), and (c) BMI at birth (controls ($r=0.84$, $p<0.001$) and placentally restricted ($r=0.46$, $p<0.05$)). Birth weight was positively correlated with plasma IGF-I for control ($r=0.94$, $p=0.002$) and placentally restricted ($r=0.74$, $p<0.05$) males only. Tibia length was positively correlated with plasma IGF-I for placentally restricted ($r=0.74$, $p<0.05$) males only. Body mass index was positively correlated with plasma IGF-I for control ($r=0.93$, $p=0.001$) and placentally restricted ($r=0.88$, $p=0.025$) males only.

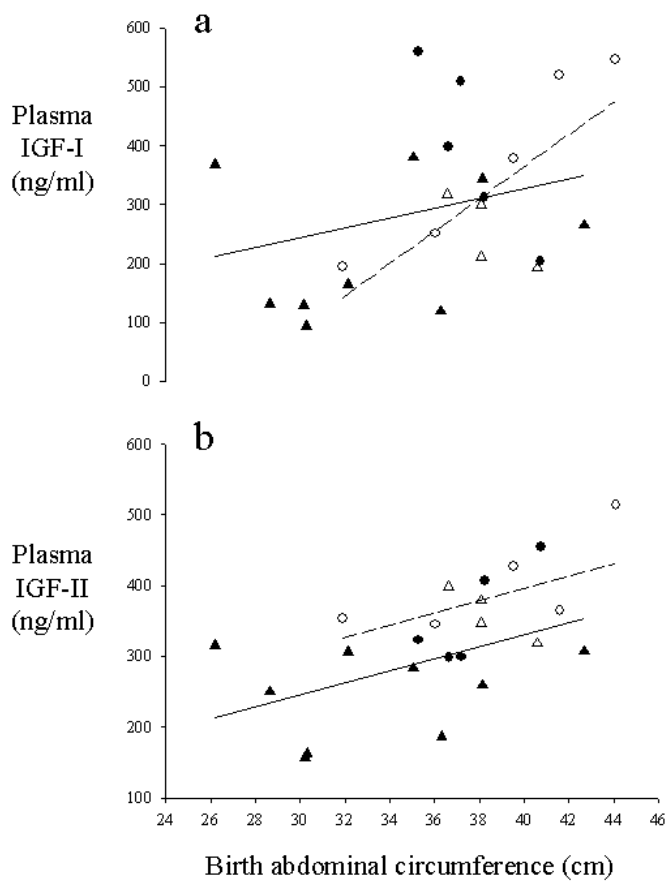

Figure 5.2. Plasma IGF-I and IGF-II concentrations at 30 days of age and abdominal circumference at birth for control and placentally restricted lambs. Controls are represented by the dashed regression line and open symbols and placentally restricted are represented by the solid regression line and closed symbols. Males are represented by circles and females by triangles. Abdominal circumference correlated positively with (a) plasma IGF-I (controls ($r=0.72$, $p=0.01$) and placentally restricted ($r=0.26$, ns)), and (b) plasma IGF-II (controls ($r=0.52$, $p<0.05$) and placentally restricted ($r=0.48$, $p<0.05$)). Abdominal circumference was positively correlated with plasma IGF-I for control ($r=0.96$, $p=0.001$) males only. Abdominal circumference was positively correlated with plasma IGF-II for control ($r=0.77$, $p=0.01$) and placentally restricted ($r=0.86$, $p=0.03$) males only.

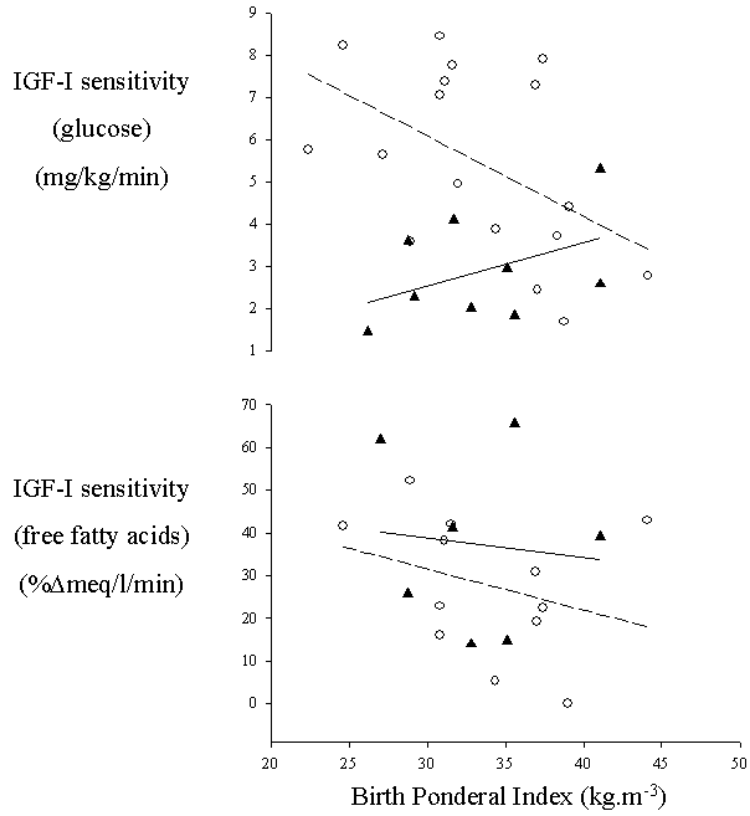

Figure 5.3. Size at birth and IGF-I sensitivity at 35 days of age in the lamb. Controls are represented by the dashed regression line and open circles and placentally restricted are represented by the solid regression line and solid triangles. IGF sensitivity of glucose metabolism (control: r=0.49, p<0.025; placentally restricted: r=0.42, NS) and free fatty acid metabolism (control: r=0.31, NS; placentally restricted: r=0.10, NS) with ponderal index at birth in lambs is shown.

5.3.6 Plasma IGFs, IGF-I sensitivity and postnatal growth

In all lambs combined, AGR in terms of weight (r=0.50, n=28, p=0.003), shoulder height (r=0.61, n=28, p=0.001), tibia length (r=0.51, n=28, p=0.003), and abdominal circumference (r=0.69, n=28, p=0.001), and current FGR in terms of weight (r=0.50, n=28, p=0.004) and tibia length (r=0.34, n=28, p=0.04) correlated positively with IGF-I sensitivity of glucose metabolism. AGR in terms of abdominal circumference (r=0.41, n=25 p=0.05) and in terms of weight (r=0.28, n=25, p<0.1) correlated positively with IGF-I sensitivity of amino acid metabolism. AGR in terms of weight (r=0.35, n=20, p=0.05), shoulder height (r=0.41, n=20, p=0.03), and abdominal circumference (r=0.35, n=20, p=0.05), and current FGR in terms of weight (r=0.39, n=20, p=0.04) correlated positively with IGF-I sensitivity of free fatty acid metabolism.

In control lambs, AGR in terms of tibia length (r=0.48), and current FGR in terms of tibia length (r=0.39, n=18, p=0.05) correlated positively with IGF-I sensitivity of glucose metabolism. AGR in terms of weight (r=-0.57, n=18, p=0.007) (Figure 5.4), and current FGR in terms of weight (r=-0.45, n=18, p=0.05) (Figure 5.4), shoulder height (r=-0.48, n=18, p=0.02) and metatarsal length (r=-0.53, n=18, p=0.01) correlated negatively with IGF-I sensitivity of glucose metabolism. AGR in terms of abdominal circumference tended to correlate positively with IGF-I sensitivity of amino acid metabolism (r=0.36, n=17, p<0.1), while AGR in terms of tibia length (r=-0.41, n=17, p=0.05), and shoulder height (r=-0.38, n=17, p<0.1) tended to correlate negatively with IGF-I sensitivity of amino acid metabolism. In control lambs, AGR in terms of weight (r=-0.60, n=13, p=0.02) correlated negatively with IGF-I sensitivity of free fatty

acid metabolism (Figure 5.4). AGR in terms of shoulder height (r=0.63, n=13, p=0.01), and tibia length (r=0.55, n=13, p=0.03) and current FGR in terms of tibia length (r=0.61, n=13, p=0.02) correlated positively with IGF-I sensitivity of free fatty acid metabolism. AGR in terms of metatarsal length (r=-0.49, n=13, p=0.05) and abdominal circumference (r=-0.49, n=13, p=0.05), and current FGR in terms of weight (r=-0.49, n=13, p=0.02), and abdominal circumference (r=-0.53, n=13, p=0.04) correlated negatively with IGF-I sensitivity of free fatty acid metabolism.

In placentally restricted lambs, current FGR in terms of weight correlated positively with IGF-I sensitivity of glucose metabolism (r=0.56, n=10, p=0.04) (Figure 5.4). AGR in terms of metatarsal length (r=0.82, n=8, p=0.006) and tibia length (r=0.67, n=8, p=0.04), and current FGR in terms of metatarsal length (r=0.62, n=8, p=0.05) and abdominal circumference (r=0.74, n=8, p=0.02) correlated positively with IGF-I sensitivity of amino acid metabolism. AGR in terms of abdominal circumference (r=0.58, n=8, p<0.1), and current FGR in terms of shoulder height (r=0.59, n=8, p<0.1) and tibia length (r=0.58, n=8, p<0.1) tended to correlate positively with IGF-I sensitivity of amino acid metabolism. AGR in terms of metatarsal length (r=-0.78, n=7, p=0.02) and current FGR in terms of weight (r=-0.62, n=7, p=0.03) (Figure 5.4) and tibia length (r=-0.68, n=7, p=0.02) correlated negatively with IGF-I sensitivity of free fatty acid metabolism, while AGR in terms of weight (r=0.58, n=7, p<0.1) tended to correlate positively with IGF-I sensitivity of free fatty acid metabolism (Figure 5.4).

AGR in terms of weight (r=0.65, n=24, p=0.001) correlated positively with plasma IGF-I concentrations at 30 days of age in all lambs. AGR in terms of shoulder height (r=0.35, n=24, p=0.05), tibia length (r=0.49, n=24, p=0.008), and metatarsal length (r=0.34, n=24, p=0.05), and current FGR in terms of tibia length (r=0.40, n=24, p=0.03) correlated positively with plasma IGF-II concentrations at 30 days of age in all lambs.

AGR in terms of weight (r=0.45, n=10, p<0.1) tended to correlate positively with plasma IGF-I concentrations at 30 days of age, current FGR in terms of weight (r=-0.60, n=10, p=0.04) correlated negatively with plasma IGF-I concentrations at 30 days of age in control lambs (Figure 5.5). AGR (r=0.53) and CFGR (r=0.48) in terms of tibia length correlated positively (Figure 5.6), while current FGR in terms of abdominal circumference (r=-0.56, n=10, p=0.05) correlated negatively with plasma IGF-II at 30 days of age in control lambs. AGR in terms of weight (r=0.77, p<0.001) (Figure 5.5) and abdominal circumference (r=0.48, n=10, p=0.05) in placentally restricted lambs correlated positively with plasma IGF-I concentrations at 30 days of age. AGR in terms of shoulder height (r=0.48, n=14, p=0.04) and metatarsal length (r=0.54, n=14, p=0.02), and current FGR in terms of weight (r=0.44, p<0.05) (Figure 5.5), shoulder height (r=0.48, n=14, p=0.04), tibia length (r=0.42, n=14, p=0.05), and metatarsal length (r=0.53, n=14, p=0.03) correlated positively with plasma IGF-II concentrations at 30 days of age. AGR in terms of weight (r=0.41, n=14, p<0.1) tended to correlate positively with plasma IGF-II concentrations at 30 days of age in placentally restricted lambs (Figure 5.5).

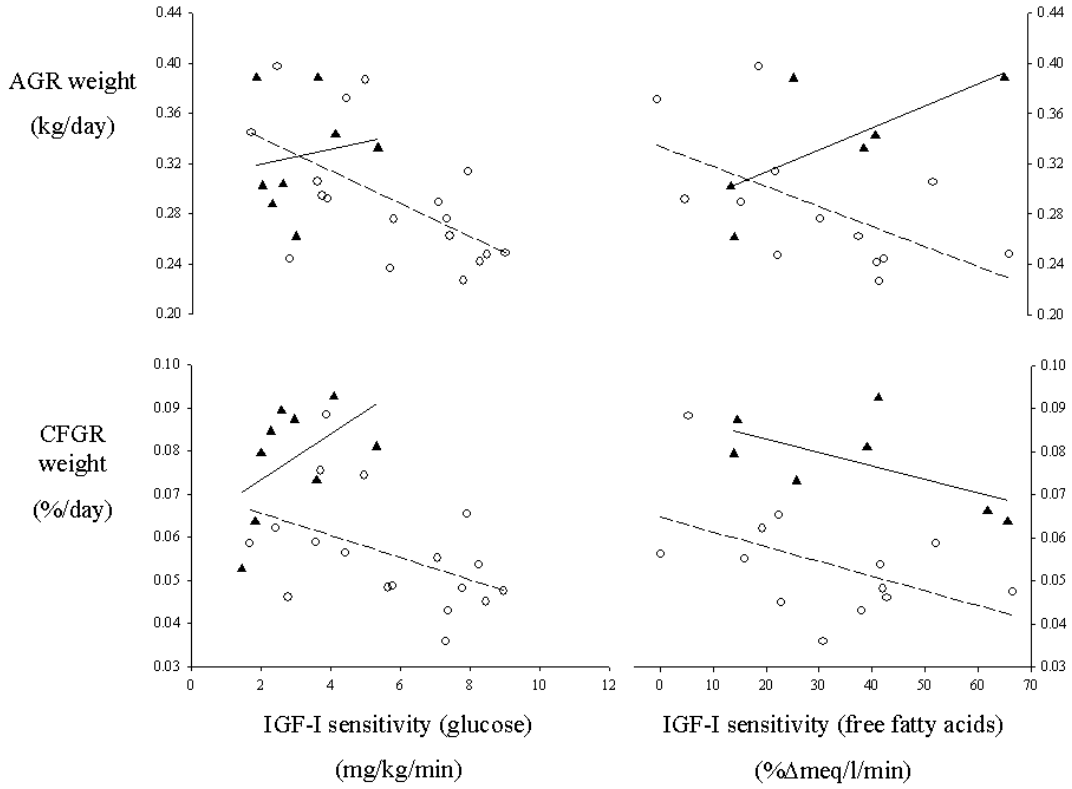

Figure 5.4. Postnatal growth and IGF-I sensitivity in lambs.

Controls are represented by the dashed regression line and open circles and placentally restricted are represented by the solid regression line and solid triangles. IGF sensitivity of glucose (control: r=-0.57, p<0.01; placentally restricted: r=0.14, NS) and free fatty acid metabolism (control: r=-0.60, p<0.05; placentally restricted: r=0.58, p<0.1) with absolute growth rate in terms of weight in lambs is shown. IGF sensitivity of glucose (control: r=-0.45, p<0.05; placentally restricted: r=0.56, p<0.05) and free fatty acid metabolism (control: r=-0.49, p<0.05; placentally restricted: r=-0.62, p<0.05) with current fractional growth rate in terms of weight in lambs is shown.

5.3.7 IGF-I sensitivity of free fatty acid metabolism and adiposity

IGF-I sensitivity of free fatty acid metabolism correlated positively with total retroperitoneal fat mass ($r=0.31$, $p<0.05$), and tended to correlate positively with total visceral fat mass ($r=0.27$, $p<0.1$) in all lambs. IGF-I sensitivity of free fatty acid metabolism correlated positively with total perirenal fat mass ($r=0.94$, $n=6$, $p=0.003$), total omental fat mass ($r=0.82$, $n=6$, $p=0.02$), and total visceral fat mass ($r=0.90$, $n=5$, $p<0.01$) (Figure 5.7), in placentally restricted lambs.

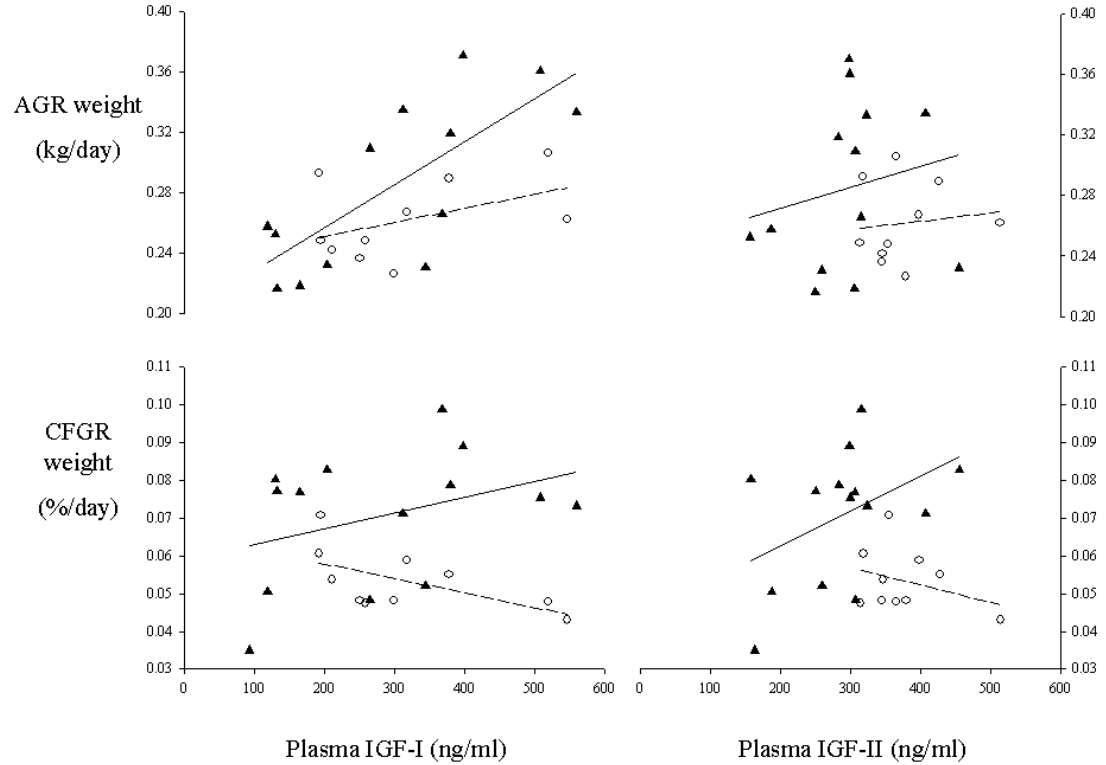

Figure 5.5. Postnatal growth and plasma IGF concentrations in lambs.

Controls are represented by the dashed regression line and open circles and placentally restricted are represented by the solid regression line and solid triangles. Plasma IGF-I (control: $r=0.45$, $p<0.1$; placentally restricted: $r=0.77$, $p<0.001$) and IGF-II concentration (control: $r=0.12$, NS; placentally restricted: $r=0.41$, $p<0.1$) with absolute growth rate in terms of weight in lambs is shown. Plasma IGF-I (control: $r=-0.60$, $p<0.05$; placentally restricted: $r=0.36$, NS) and IGF-II concentration (control: $r=-0.32$, NS; placentally restricted: $r=0.44$, $p<0.05$) with current fractional growth rate in terms of weight in lambs is shown.

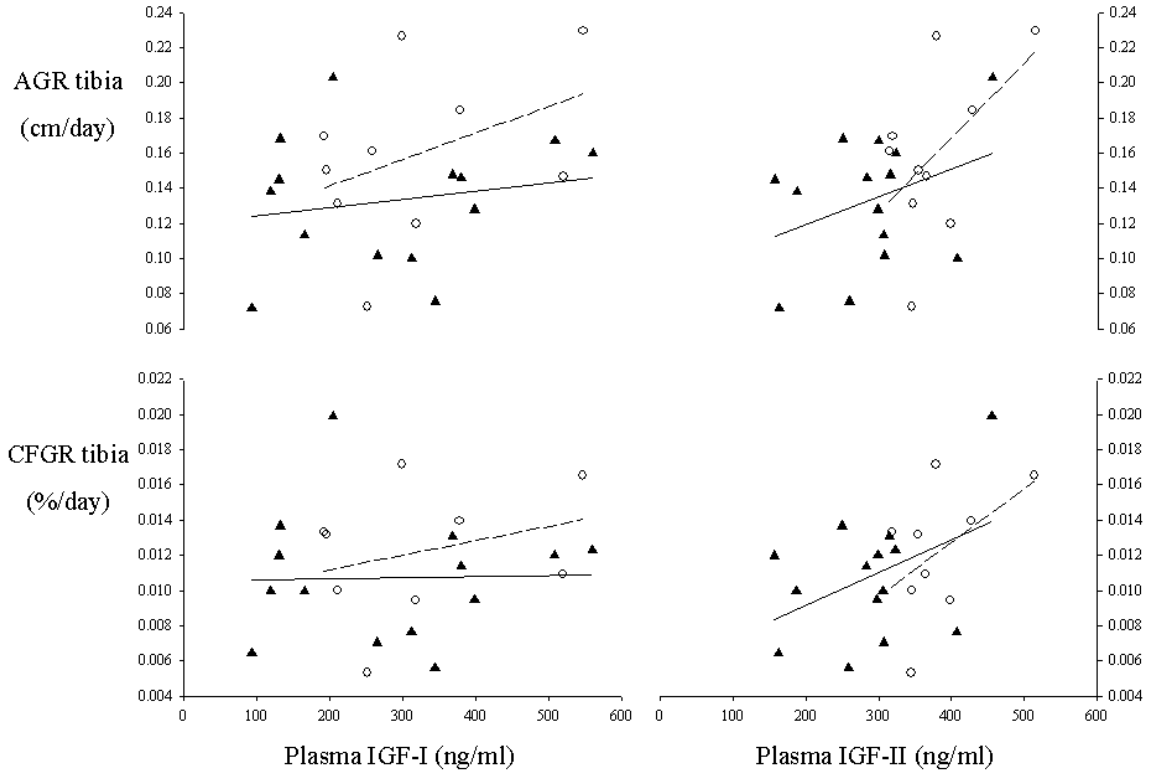

Figure 5.6. Postnatal growth and plasma IGF concentrations in lambs.

Plasma IGF-I (control: r=0.41, NS; placentally restricted: r=0.19, NS) and IGF-II concentration (control: r=0.53, $p<0.05$; placentally restricted: r=0.36, NS) with absolute growth rate in terms of tibia length in lambs is shown. Plasma IGF-I (control: r=0.30, NS; placentally restricted: r=0.03, NS) and IGF-II concentration (control: r=0.48, $p<0.05$; placentally restricted: r=0.42, $p<0.05$) with current fractional growth rate in terms of tibia length in lambs is shown.

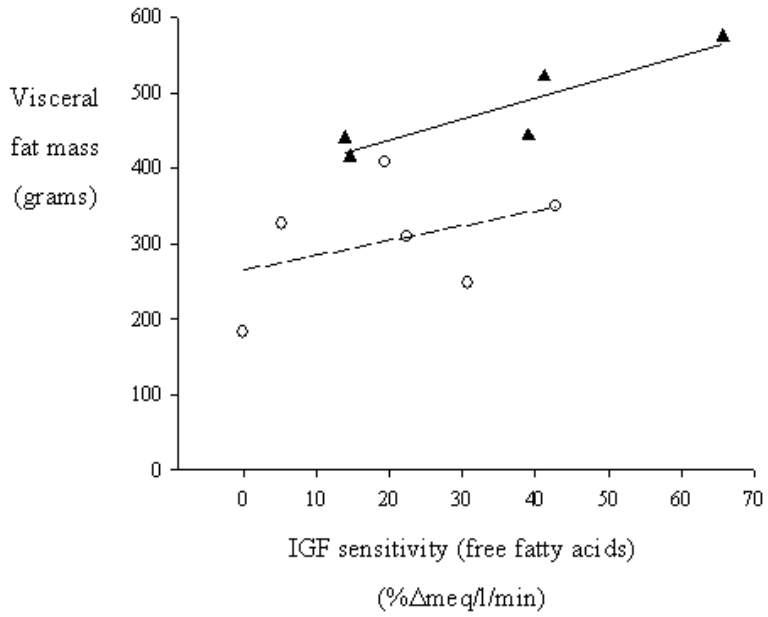

Figure 5.7. IGF-I sensitivity of free fatty acid metabolism and visceral adiposity in lambs.

Controls are represented by the dashed regression line and open circles and placentally restricted are represented by the solid regression line and solid triangles. IGF-I sensitivity of free fatty acid metabolism (control: r=0.40, NS; placentally restricted: r=0.90, $p<0.01$) and visceral adiposity (from post mortem; 45 days of age) in lambs is shown.

5.4 DISCUSSION

This study has demonstrated that experimentally restricting placental growth in the sheep, which reduces size at birth and induces neonatal catch-up growth, alters circulating insulin-like growth factors and metabolic sensitivity to IGF-I and their relationship to growth and adiposity in the young lamb. While plasma IGFs, size at birth and growth are similarly related in both control and placentally restricted lambs to some extent, the metabolic sensitivities to IGF-I are not. The apparent differences may be due to regression analyses on limited numbers of animals and lack of overlap of treatment groups. Placental restriction reduced circulating IGF-II levels and IGF sensitivity of glucose metabolism in the young lamb, suggesting that the longer-term catch-up growth after IUGR still occurring at one month of age is being driven primarily by other factors. However, thinness at birth was associated with increased IGF sensitivity of glucose and free fatty acid metabolism after one month of age. This suggests that disproportionate restriction of soft tissue growth before birth and subsequent catch-up growth is driven by increased IGF action. Furthermore, absolute and fractional growth of soft and skeletal tissues was predicted by circulating IGF abundance and metabolic sensitivity to IGFs in placentally restricted lambs and to some extent in control lambs at this age. This is consistent with catch-up growth occurring to the extent allowed by the prevailing abundance of and sensitivity to the IGFs. Placental restriction reduced plasma IGF-II levels at one month of age, while both plasma IGF-I and IGF-II decreased with decreasing size at birth in the young lamb. This suggests that reduced tissue expression and secretion of the IGFs may persist

postnatally following placental restriction. Whether this altered abundance and action of factors known to stimulate or inhibit synthesis of each IGF is not known. It is also possible that reduced systemic levels of the IGFs reflect increased clearance rather than reduced production. This could arise from increased binding to and uptake by receptors or to reduced blood levels of the IGF binding proteins or indeed both. Determining the mechanistic basis of reduced IGF abundance in the young lamb following placental restriction will be necessary to definitely assess whether IGF action, reflecting both production and sensitivity, is actually altered and how.

The effect of experimental IUGR on birth phenotype, postnatal growth velocity and abundance of, or increased sensitivity to, somatotropic hormones has been addressed in several recent studies. Chronic and severe maternal undernutrition in the rat ([250]Woodall*, et al.*, 1996, [251]Muaku*, et al.*, 1996, [252]Shepherd*, et al.*, 1997) reduces birth weight, but results in persistent growth failure, such that the offspring of restricted mothers remained very small when compared with controls as adults. Thus, in contrast to catch-up growth in humans occurring during infancy, catch-up growth in offspring of severely feed restricted pregnant rats does not occur until adulthood when it may represent predominantly adipose tissue (13 and 30 weeks of age). In IUGR piglets, increased fractional growth rates occurred in terms of weight but not length in the first two weeks of postnatal life ([253]Ritacco*, et al.*, 1997). These increased fractional growth rates were not due to increased abundance of plasma IGF-I ([253]Ritacco*, et al.*, 1997), but it was speculated that it may be increased sensitivity to IGF-I ([253]Ritacco*, et al.*, 1997).

In the current study, placentally restricted lambs or lambs that are small at birth had reduced circulating concentrations of the IGFs, at least towards the end of the catch-up growth period. Nevertheless, they still demonstrate catch-up growth in terms of soft and skeletal tissues, possibly due to these tissues being possible sites of increased IGF-I sensitivity. The precise sites responsible for this increased metabolic sensitivity to IGF-I in terms of glucose and free fatty acid metabolism in particular, and whether they consist of those tissues undergoing catch-up growth cannot be deduced from the current study. In fasted lambs, infusion of IGF-I at 6.7 $nmol.kg^{-1}.h^{-1}$ induced hypoglycaemia to the same extent as insulin at 0.17 $nmol.kg^{-1}.h^{-1}$, whereas 2 $nmol.kg^{-1}.h^{-1}$ did not ([160]Douglas*, et al.*, 1991). IGF-I at the higher dose lowered blood glucose by increasing the rate of glucose clearance to a greater extent than insulin, while only insulin increased clearance and reduced glucose production, to induce hypoglycaemia. In the current study, IGF-I was infused into fasted lambs at approximately 24 $nmol.kg^{-1}.h^{-1}$, suggesting that glucose uptake by tissues, such as skeletal muscle would certainly have been increased, while the extent to which glucose release by the liver was inhibited is unclear. Infusion of IGF-I at a similar dose (5 $\mu g.kg^{-1}.min^{-1}$, or approximately 39 $nmol.kg^{-1}.h^{-1}$) to chronically catheterised fasted rats reduced plasma glucose by 30-40 mg/dl due to an increase in glucose uptake, while hepatic glucose production was unchanged ([158]Jacob*, et al.*, 1989). Furthermore, IGF-I infusion with the maintenance of euglycaemia increased glucose uptake and stimulated (3-^{3}H)glucose incorporation into tissue glycogen, but still failed to suppress glucose production and FFA levels ([158]Jacob*, et al.*, 1989). In another study, infusion of IGF-I at a

comparable rate to the current study of 0.57 nmol.kg^{-1}.min^{-1} (or approximately 34 nmol.kg^{-1}.h^{-1}) stimulated whole body glucose uptake similar to that produced by an infusion of 0.01 nmol.kg^{-1}.min^{-1} (or approximately 0.6 nmol.kg^{-1}.h^{-1}) of insulin ([172]Moxley III*, et al.*, 1990). Furthermore, the glucose metabolic rate, as measured by 2-deoxy-D-glucose uptake, was comparable in quadriceps, soleus, and diaphragm muscles during the infusion of IGF-I and insulin at these doses ([172]Moxley III*, et al.*, 1990). However, IGF-I at this dose had a lesser effect on liver causing a 38% inhibition of hepatic glucose output compared with 66% inhibition by insulin. This study confirms that skeletal muscle is more responsive than the liver to IGF-I, which agrees with the distribution of the type 1 IGF-I receptors in the two tissues in this species ([172]Moxley III*, et al.*, 1990). This suggests that in the current study, IGF-I was probably targeting predominantly skeletal muscle at the dose used in stimulating glucose utilisation, although use of tracer methodology will be required to confirm this. Furthermore, placental restriction may alter relative sensitivity to IGF-I of these tissues. Net protein loss was reduced after infusion of low and high dose IGF-I (6.7 nmol.kg^{-1}.h^{-1}, which induced hypoglycemia, and 2.0 nmol.kg^{-1}.h^{-1}, which did not) and insulin by 11%, 15%, and 12%, respectively, in awake, fasted lambs ([160]Douglas*, et al.*, 1991). In contrast to the insulin infusion, the high dose rhIGF-I infusion increased the rate of protein synthesis in skeletal and cardiac muscle ([160]Douglas*, et al.*, 1991). Protein metabolism is more sensitive than glucose metabolism to IGF-I infusion, as protein loss was reduced by an IGF-I infusion and protein synthesis was increased by IGF-I infusion but not by insulin infusion ([160]Douglas*, et al.*, 1991).

A study to determine the response of protein synthesis to insulin-like growth factor I (IGF-I) in skeletal muscle of neonatal pigs was performed ([174]Davis*, et al.*, 2002). Overnight fasted 7 and 26-day-old pigs were infused with either a low or high dose of IGF-I (20, or 50 $\mu g.kg^{-1}.h^{-1}$) (or approximately 2.6, or 6.5 $nmol.kg^{-1}.h^{-1}$), with amino acids and glucose being clamped at fasting levels ([174]Davis*, et al.*, 2002). An additional group of the high-dose IGF-I-infused pigs was also provided replacement insulin since IGF-I infusion is known to lower circulating insulin ([174]Davis*, et al.*, 2002). It was shown that the low dose of IGF-I increased protein synthesis by 25-60% in skeletal muscle, cardiac muscle (+38%), skin (+24%), and spleen, (+32%) in 7-day-old pigs, but had no effect in liver, jejunum, pancreas, and kidney ([174]Davis*, et al.*, 2002). The higher dose of IGF-I did not further increase protein synthesis in skeletal muscle above that of the low dose, and insulin replacement did not alter the response to IGF-I ([174]Davis*, et al.*, 2002). This suggests that at the dose used in the current study ($3\mu g.kg^{-1}.min^{-1}$ or 24 $nmol.kg^{-1}.h^{-1}$), the IGF-I effect on amino acids is reflecting predominantly skeletal muscle and not liver. Therefore IGF-I sensitivity of glucose and amino acid metabolism in the current study predominantly reflects skeletal muscle and not visceral tissue sensitivity.

Maternal protein restriction (0.5% versus 13% protein) reduced body weight (-10%) and circulating IGF-I (-30%) in the pig ([226]Schoknecht*, et al.*, 1997). IGF-I infusion ($4\mu g.hr^{-1}$, or approximately 0.52 $nmol.h^{-1}$) to IUGR neonatal pigs from day 3 to 10 increased circulating IGF-I levels, growth rate, and protein and fat accretion to those of controls ([226]Schoknecht*, et al.*, 1997). The IGF-I infusion significantly increased protein and fat accretion in the IUGR pigs, but not in

controls ([226]Schoknecht*, et al.*, 1997) possibly indicating that after IUGR, neonatal pigs have increased IGF sensitivity for the first week of life.

A major question that remains unsolved is how these tissues may become more sensitive to these anabolic hormones and the manner in which or how the increased rates of growth that constitute catch-up cease. There may be some signal from the tissues to the systems promoting growth, which halts growth when the organism has "caught-up". Myostatin produced by muscle and released into blood has been proposed to be a bodyweight/skeletal muscle mass signaller and to act as a negative regulator of skeletal muscle mass ([300]Lee*, et al.*, 2001). It can act back on skeletal muscle to inhibit anabolic actions of insulin/IGFs, and when blood levels are high enough, signals that the normal body muscle mass has been restored. The release of myostatin inhibition of skeletal muscle growth before birth in the placentally restricted lambs following birth could contribute to a drive to grow more rapidly. A previous study has shown that expression of myostatin at birth was 65% higher in the longissimus muscle of piglets of low birth weight compared to normal size littermates ([301]Ji*, et al.*, 1998). This suggests that the increased concentrations of myostatin in these low birth weight animals may help contribute to and help explain their reduced muscle mass seen at birth. In chickens, when myostatin concentrations are low and IGF-I levels are high there is a large degree of muscle growth up to 6 weeks post hatch ([302]Guernec*, et al.*, 2003). If an increased IGF-I/myostatin ratio occurs in early postnatal life following placental restriction, it may help to explain the mechanism of increased muscle mass seen during the catch-up growth period after IUGR.

Normally, growth of long bones occurs as a result of proliferation of chondrocytes located in the epiphysis of bones ([113]Abad*, et al.*, 1999, [114]Ohlsson*, et al.*, 1998). IGFs can increase chondrocyte proliferation and hypertrophy, stimulating bone elongation ([162]Wang*, et al.*, 1999). The proximal growth plates of the tibiae of liver IGF-I-deficient mice are smaller in total height, as well as in the height of the proliferative and hypertrophic zones of chondrocytes ([303]Yakar*, et al.*, 2002). There was a 10% decrease in bone mineral density and a greater than 35% decrease in periosteal circumference and cortical thickness. IGF-I treatment for 4 weeks restored the total height of the proximal growth plate of the tibia demonstrates that circulating IGF-I is necessary for normal bone growth ([303]Yakar*, et al.*, 2002). Accelerated growth of long bones was also observed in the young lamb following placental restriction. It has been proposed by recent studies in rabbits that catch-up of long bones may be a result of delayed senescence at the growth plate ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). Senescence (or epiphyseal fusion) is a process where the proliferative capacity of chondrocytes declines with each generation of cells and may be dependent on the cumulative number of cell divisions that the chondrocytes of long bones undergo ([209]Gafni*, et al.*, 2001). This may be due to delayed senescence of the growth plate (senescent growth plates are replaced with bone, termed epiphyseal fusion), which may not be dependent on age, but rather the cumulative number of cell divisions that the chondrocytes of long bones undergo ([209]Gafni*, et al.*, 2001). Catch-up growth occurring after cessation of dexamethasone-induced growth restriction, was characterized by a delay in the age related senescent decline in the heights of

the proliferative zone, hypertrophic zone, and total growth plate, in the distal femoral growth plates ([113]Abad*, et al.*, 1999, [209]Gafni*, et al.*, 2001). These findings suggest that linear catch-up growth after placental restriction and IUGR may be due in part, to a delay in growth plate senescence ([209]Gafni*, et al.*, 2001). Therefore in the current study, a reduced abundance of IGF-I in the blood, and increased rates of growth of long bones, suggests that either the local abundance and/or increased sensitivity or other factors may be responsible for catch-up growth.

Placentally restricted lambs were also fatter than controls in terms of visceral fat (combined fat mass of retroperitoneal, perirenal, and omental fat depots) at 45 days of age (See Chapter 3). Increased adiposity in both control and placentally restricted lambs was predicted by increased IGF-I sensitivity of circulating FFA at one month of age. Notably, a consistently greater extent of adiposity seen for the IGF sensitivity of circulating free fatty acids in the placentally restricted compared to the control lambs. This suggests that increased local action of IGFs in adipose tissue may help expand these visceral depots, and that it occurs to a greater extent following placental restriction. The reduced concentrations of IGF-II may also be contributing to the increased adiposity in the placentally restricted lambs as in human adults and genetically modified mice deficient in IGF-II ([168]Sandhu*, et al.*, 2003, [169]Ben-Shlomo*, et al.*, 2003, [299]Devedjian*, et al.*, 2000). The mechanisms by which these could occur however are at present unknown. Recent studies have suggested that IGF-I stimulates proliferation and differentiation of 3T3-L1 preadipocytes (adipocyte precursor cells), through the activation of MAPK (mitogen-activated protein

kinase), and MAPK activation by IGF-I is mediated through the Src family of non-receptor tyrosine kinases ([304]Boney*, et al.*, 2000, [305]Sekimoto*, et al.*, 2003). It has also been demonstrated that IGF-I is equipotent with insulin in stimulating glucose uptake, and IGF-I more potently than IGF-II stimulated clonal expansion in 3T3-L1 pre-adipocytes ([306]Siddals*, et al.*, 2002). These placentally restricted lambs may have increased proliferation and differentiation of adipocytes and along with increased IGF-I and insulin sensitivity of lipolysis and hence adipocytes, may partially explain the increased adiposity seen in lambs at one month of age.

In conclusion, the longer-term catch-up growth in terms of soft and skeletal tissues of the placentally restricted lamb may be partially explained by the relationships between growth and IGF abundance and sensitivity. The increased adiposity after placental restriction may be driven by increased IGF as well as insulin action. Disproportionate fetal growth restriction in sheep is followed by catch-up growth of soft and skeletal tissues in the neonate despite reduced or normal concentrations of fasting plasma IGF-I and –II, and may be due to an increase in local abundance or sensitivity. The extent to which earlier catch-up growth is driven by increased IGF action and whether IGF production is altered and how following placental restriction throughout catch-up growth remain to be determined.

Chapter 6

PLACENTAL RESTRICTION ALTERS CIRCULATING THYROID HORMONE IN THE YOUNG LAMB POSTNATALLY

6.1 INTRODUCTION

Children who are born short or light at birth due to intrauterine growth restriction (IUGR) grow at an accelerated rate during infancy (termed 'catch-up' growth) ([7]Karlberg*, et al.*, 1995), but the physiological basis for this is unknown. Both clinical IUGR and catch-up growth are common, with IUGR occurring in approximately 10% of live births in Australia and up to 40% in some developing countries ([269]Roder*, et al.*, 1997). The majority of IUGR infants (57-84%) undergo some 'catch-up' growth which begins as early as 2 weeks and is largely complete by 5 months of age for weight and head circumference, and by 6 to 12 months for height ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [38]Leger*, et al.*, 1997, [62]McCowan*, et al.*, 1999, [84]Albertsson Wikland*, et al.*, 1993). Catch-up growth is associated with a reduced risk of morbidity and mortality in childhood ([91]Victora*, et al.*, 2001), and increases attainment of target adult height ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995). However, it also predicts increased childhood and adult obesity ([97]Rogers*, et al.*, 2003) and may independently add to the risk of developing adult-onset diseases such as diabetes, hypertension and cardiovascular disease ([9]Eriksson*, et al.*, 1999, [70]Cianfarani*, et al.*, 1999, [95]Rasmussen, 2001, [96]Forsén*, et al.*, 1997). Understanding the mechanistic basis of catch-up growth following IUGR may clarify why it gives rise to these different later outcomes.

The association of catch-up growth with IUGR suggests that the underlying causes originate in an adverse intrauterine environment, which restricts growth and alters functional development ([104]Cianfarani*, et al.*, 2002, [208]Fitzhardinge*, et*

al., 1989, [307]Lapillonne*, et al.*, 1997). A variety of prenatal perturbations restrict fetal growth and change postnatal characteristics that affect infant growth, such as appetite and the activity of major neuroendocrine and endocrine axes ([50]Yajnik*, et al.*, 1995, [57]Fall*, et al.*, 1995, [82]Colle*, et al.*, 1976, [108]Soto*, et al.*, 2003, [152]Giudice*, et al.*, 1995). One such axis is the thyroid hormone (TH) axis ([231]Berthon*, et al.*, 1993, [232]Pracyk*, et al.*, 1992), which acts via T_3 and possibly other forms of TH to regulate growth and differentiation of major tissues, as well as fuel metabolism and metabolic efficiency ([233]Williams*, et al.*, 1998, [234]Silva, 2003, [235]Casas*, et al.*, 2003). An important element of the TH axis and determinant of its activity are circulating levels of TH and the activation of T_4 to T_3, the more biologically active form ([180]Hulbert, 2000).

The effect of IUGR on circulating TH abundance has been studied in the human infant at birth but less so in the first few months of life ([229]Bongers-Schokking*, et al.*, 1984, [230]Jacobsen*, et al.*, 1979). Plasma T_3 and T_4 are reduced at birth in the IUGR infant ([229]Bongers-Schokking*, et al.*, 1984, [230]Jacobsen*, et al.*, 1979), reflecting the hypoxic and hypoglycaemic intrauterine environment. Following birth, plasma T_3 concentrations were similar in IUGR and normal infants from 1 week to 8 months of age, while plasma T_4 was reduced in IUGR infants up to 50 days of age after which they normalise ([230]Jacobsen*, et al.*, 1979). Plasma TSH, T_3 and T_4 increase in gestation until birth where TSH and T_4 peak and decrease while T_3 continues to increase after birth ([189]Fisher*, et al.*, 1994). Plasma reverse T_3 peaks during gestation and gradually declines thereafter to adult levels ([189]Fisher*, et al.*, 1994). This suggests that following IUGR there may be normal TH production but enhanced conversion of T_4 to T_3, which may

help maintain TH bioavailability and action ([230]Jacobsen*, et al.*, 1979). While the IUGR infants in this study gained as much weight as normal infants over the period of study, suggestive of catch-up growth, whether this was related to circulating T_3 or the effect to which it may have been limited by T_4, was not examined ([230]Jacobsen*, et al.*, 1979). It is therefore possible that increased conversion of T_4 to T_3 and hence increased plasma T_3 levels occur and are highest in those IUGR infants that catch up ([230]Jacobsen*, et al.*, 1979).

Limited data from other species also suggests a role for TH action in regulating catch-up growth. In a study of Romanov lambs with spontaneous IUGR, born between 135 and 145 days of gestation and fed artificially, plasma T_3 and T_4 were measured from birth to 30 days of age. Plasma T_3 at 2, 4, 8 hours after birth and at 11 days, and plasma T_4 at 4 hours after birth were positively correlated with birth weight ([308]Cabello*, et al.*, 1981). Plasma T_3 at 2, 4 and 8 hours after birth also correlated positively with weight gain during the first month of life ([308]Cabello*, et al.*, 1981). The relationship between size at birth, postnatal growth and plasma TH at later ages was not described however ([308]Cabello*, et al.*, 1981). The longer-term consequences of IUGR for peripheral TH abundance in early postnatal life and their possible contribution to catch-up growth are unknown. Therefore, before we address this issue, the common cause of IUGR in humans and other species need to be considered.

While there are many conditions that can restrict fetal growth, most act to reduce delivery of essential substrates to the fetus, because of either maternal or placental limitations ([257]Owens*, et al.*, 1987). Consequently, placental

insufficiency is a major cause of IUGR, which reduces delivery of oxygen and nutrients to the fetus ([257]Owens*, et al.*, 1987), and placental weight in late gestation accounts for much of the overall variation in size at birth. Experimental restriction of placental and hence fetal growth in sheep and other species has similar growth, metabolic and endocrine consequences for the fetus, as observed in human IUGR in late gestation ([237]Owens*, et al.*, 1994). We have previously shown that placental restriction reduces size at birth and increases postnatal growth and adiposity in the first month of life in sheep (See Chapter 3). Placental restriction in sheep decreases plasma total T_4 levels in the lamb in the first 24 hours following birth, but whether plasma T_3 is also altered and whether such changes persist is unknown ([241]Mellor*, et al.*, 1977). We therefore hypothesised that placental restriction (PR) of fetal growth in sheep, which reduces size at birth and increases postnatal growth, would increase circulating TH concentrations, and that these would relate to growth and body composition in the young lamb in the first month of life. We further hypothesised that short-term fasting, which inhibits activation of T_4 to T_3 and reduces plasma total or free T_3 in rats and adult men, in adaptation to fuel shortage ([309]Hugues*, et al.*, 1984, [310]Kmiec*, et al.*, 1996), would reduce THs to a lesser extent in placentally restricted lambs compared to control lambs.

6.2 MATERIALS AND METHODS

All procedures performed in this project were approved by the Adelaide University Animal Ethics Committee (Animal Ethics Approval Number: M/1/97A). Placental growth was restricted in 45 Merino ewes by removal of the majority of visible endometrial caruncles (65-148) from the non-pregnant uterus, leaving either 3 to 8 caruncles in each horn of the bicornuate uterus ([236]Robinson*, et al.*, 1979) (See Materials and Methods; 2.1.1). Ewes were housed in individual pens in animal holding rooms from approximately a week before giving birth (See Materials and Methods; 2.1.2). Control ewes delivered 22 lambs (12 males and 10 females) and the placentally restricted delivered 22 lambs (10 males and 12 females). The lambs were housed in the pens with their mothers throughout the study.

6.2.1 Growth Measures

At birth and at 5-day intervals up to 45 days of age, size at birth (day 0), and subsequently size in terms of body weight, crown-rump length (CRL), tibia and metatarsal lengths, shoulder height, and abdominal, tibia, radius/ulna and hind limb circumferences were measured (See Materials and Methods; 2.1.4). The postnatal growth of lambs was calculated from birth to 45 days of age for each parameter relative to that parameter at birth (See Materials and Methods; 2.1.4).

6.2.2 Plasma Thyroid hormones (Free and total T_3 and T_4).

Arterial blood samples were taken (22 control, 22 PR) and plasma removed for determination of thyroid hormone concentrations (See Materials and Methods; 2.3.1). Free T_3 and T_4 (FT_3, FT_4), and total T_3 and T_4 (TT_3, TT_4) were determined in plasma by specific radioimmunoassay (RIA-gnost FT_3, FT_4, TT_3, TT_4 CIS bio International, France) (See Materials and Methods 2.3.1.1, 2.3.1.2, 2.3.1.3, 2.3.1.4). In a subset of these animals (9 control, 9 PR), fed (5, 15, 20, 35, 40, 45 days) and fasted (1 hour) (8 and 30 days) arterial blood samples were taken and plasma removed for determination of thyroid hormone concentrations. Total T_3 and T_4 in this subset of animals were measured throughout the period of catch-up growth to assess the possible role of thyroid binding globulins and altered size at birth and catch-up growth.

6.2.3 Statistical Analysis.

Unless otherwise stated, data is expressed as mean $\pm$ standard error of the mean (SEM). The effect of placental restriction on parameters was assessed by analysis of variance (ANOVA) (SPSS 11.0 software package for Windows). The effect of gender and its interaction with placental restriction were examined by ANOVA. In addition, the effect of placental restriction on thyroid hormone concentration over 40 days was analysed by ANOVA (6 levels). Data was also divided into tertiles based on birth weight. Where appropriate, current weight was adjusted for by its addition as a covariate. Statistical significance was assumed at $p<0.05$.

6.3 RESULTS

6.3.1 Effect of placental restriction on size at birth

Placental restriction reduced placental weight and size at birth, with greater reductions in soft tissues: weight, hind limb, thoracic and abdominal circumferences, crown-rump length (CRL), tibia and metatarsal lengths, reduced body mass index ($p<0.05$ for all), with relative sparing of skull width and length (Table 6.1). Females had reduced size at birth in terms of weight, CRL, skull width and abdominal circumference ($p<0.05$) (Table 6.1). Placental restriction did not alter absolute growth rates (AGR), but increased neonatal fractional growth rates (AGR relative to size at birth) for weight (+24%), tibia (+15%) and metatarsal (+18%) lengths, hind limb (knee joint) (+23%), and abdominal circumferences (+19%) between birth and 45 days ($p<0.05$ for all) (data not shown here). Placental restriction increased weekly current fractional growth rate for weight throughout the first five weeks of life, ($p<0.05$) particularly in the first two weeks of life (data not shown here).

6.3.2 Effect of placental restriction on plasma thyroid hormone concentrations

In the fed state, plasma total T_4 did not change with age, and was not altered by placental restriction (Figure 6.1). Plasma total T_3 decreased with age ($p<0.0001$) and was reduced at days 35 and 40 compared to that at day 5 in the fed state ($p<0.05$ for both) (Figure 6.1). In the fed state, placental restriction did

not alter plasma total T_3 overall, but tended to increase plasma total T_3 at days 35 and 40 ($p<0.1$ for both) (Figure 6.1). Plasma total T_3/T_4 ratio declined with age ($p<0.0001$) and was reduced at days 20, 25, 35, 40 compared with that at day 5 in the fed state ($p<0.05$ for all) (Figure 6.1). Placental restriction did not alter the plasma total T_3/T_4 ratio overall, but increased plasma total T_3/T_4 ratio at day 20 ($p<0.05$) and tended to increase that at day 40 ($p<0.1$) in the fed state (Figure 6.1).

In the fasted state, placental restriction did not change plasma total or free T_4 or T_3 concentrations at day 8 or day 30, but tended to increase the plasma total T_3/T_4 ratio at day 8 ($p<0.1$) (Table 6.2). PR did not alter the plasma ratios of total T_3/total T_4, free T_3/total T_3, free T_4/total T_4, or free T_3/free T_4 in the fasted state (Table 6.2). There were no differences between males and females in plasma free and total T_3 and T_4 concentrations or total T_3/T_4 ratios in the fed and fasted states nor did any interactions between PR, age and sex, influence these parameters (data not shown).

Table 6.1 The effect of placental restriction on size at birth in sheep.

Parameter	Control		Placentally restricted		PR	S	PRxS
	Males (n=12)	Females (n=10)	Males (n=10)	Females (n=12)			
Placental weight (kg)	0.50 ± 0.07	0.54 ± 0.1	0.33 ± 0.08	0.33 ± 0.1	0.05	ns	ns
Weight (kg)	5.45 ± 0.22	5.06 ± 0.25	4.27 ± 0.26	3.62 ± 0.21	0.001	0.03	ns
Crown-rump length (cm)	54.9 ± 0.9	53.3 ± 1.0	51.2 ± 1.1	49.1 ± 0.9	0.001	0.02	ns
Shoulder height (cm)	40.1 ± 0.7	39.6 ± 0.8	38.1 ± 0.8	36.2 ± 0.6	0.001	ns	ns
Tibia length (cm)	13.5 ± 0.3	13.3 ± 0.3	12.8 ± 0.3	12.2 ± 0.2	0.001	ns	ns
Metatarsal length (cm)	12.5 ± 0.2	12.1 ± 0.2	12.0 ± 0.2	11.3 ± 0.2	0.01	ns	ns
Skull width (cm)	6.6 ± 0.08	6.5 ± 0.09	6.3 ± 0.09	6.4 ± 0.07	ns	ns	ns
Skull length (cm)	14.0 ± 0.2	13.8 ± 0.2	13.3 ± 0.2	13.1 ± 0.2	ns	ns	ns
Abdominal circumference (cm)	38.8 ± 0.9	37.5 ± 1.0	36.4 ± 1.0	33.0 ± 0.8	0.001	0.02	ns
Hind limb circ. (knee joint)(cm)	10.3 ± 0.3	10.3 ± 0.3	9.1 ± 0.3	8.4 ± 0.3	0.001	ns	ns
Thoracic circumference (cm)	39.0 ± 0.7	37.8 ± 0.8	35.4 ± 0.9	33.9 ± 0.7	0.001	ns	ns
Ponderal Index (kg/cm^3)	31.2 ± 1.1	33.5 ± 1.2	31.7 ± 1.3	29.8 ± 1.1	ns	ns	ns
Body Mass Index (kg/cm^2)	17.4 ± 0.5	17.7 ± 0.6	16.2 ± 0.6	14.6 ± 0.5	0.001	ns	ns

All values are expressed as mean ± SEM; number of animals are in parentheses. PR: represents placentally restricted, S: represents sex, PRxS: represents the interaction of PR and sex, ns represents non-significance.

Table 6.2. The effect of placental restriction on plasma thyroid hormone concentrations in fasted lambs.

Day 8	*Control (n=9)*	*Placentally Restricted (n=8)*
Plasma Total T_4 (nmol/l)	112.7 ± 7.9	93.8 ± 14.4
Plasma Total T_3 (nmol/l)	2.71 ± 0.06	2.64 ± 0.13
Total T_3/Total T_4 ratio	0.025 ± 0.001	0.032 ± 0.002 #
Day 30	*Control (n=22)*	*Placentally Restricted (n=22)*
Plasma Total T_4 (nmol/l)	87.6 ± 4.4	97.3 ± 5.2
Plasma Free T_4 (pmol/l)	27.1 ± 1.9	27.3 ± 2.4
Plasma Total T_3 (nmol/l)	1.52 ± 0.07	1.55 ± 0.08
Plasma Free T_3 (pmol/l)	9.61 ± 0.31	10.15 ± 0.42
Total T_3/Total T_4 ratio	0.019 ± 0.002	0.020 ± 0.001
FreeT_3/Total T_3 ratio	6.54 ± 0.35	6.75 ± 0.30
Free T_4/Total T_4 ratio	0.31 ± 0.02	0.29 ± 0.02
Free T_3/Free T_4 ratio	0.35 ± 0.16	0.36 ± 0.18

Values are expressed as mean ± SEM; #p<0.1. No differences between males and females in plasma free and total T_3 and T_4 concentrations or total T_3/T_4 ratios in the fed and fasted states nor any interactions between PR, age and sex, influence these parameters (data not shown).

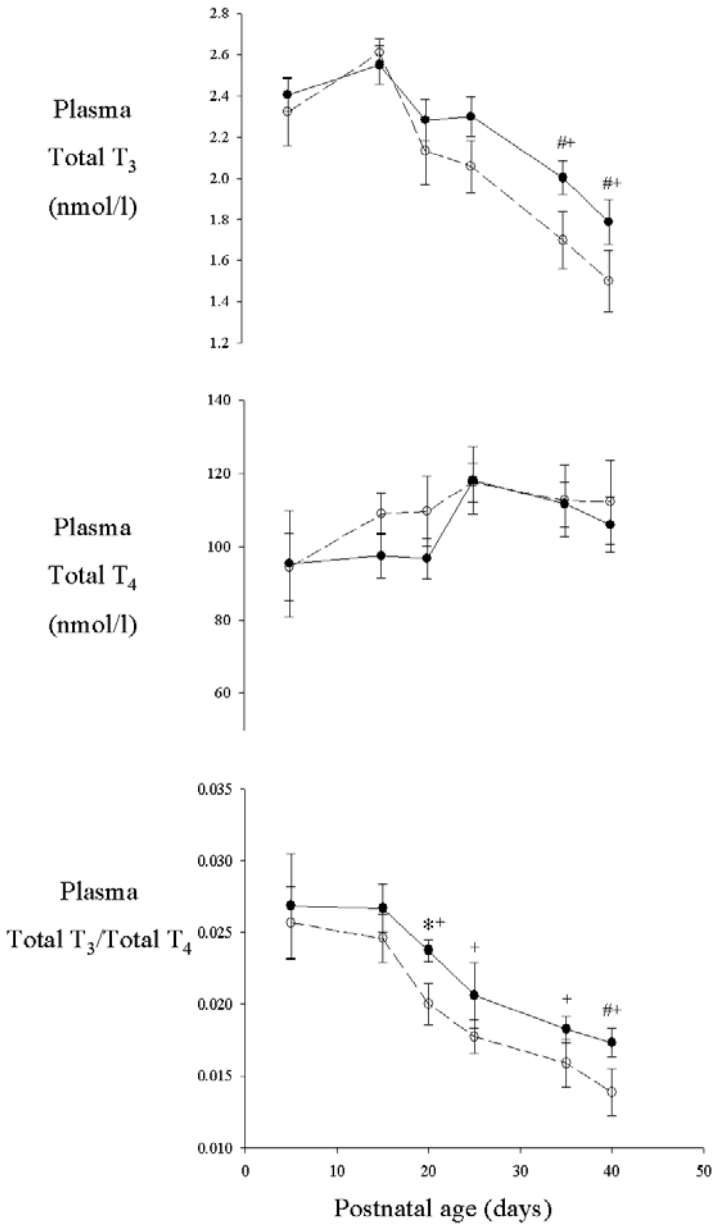

Figure 6.1. Plasma thyroid hormone concentrations during the first 6 weeks of life in the fed lambs.

Controls are represented by open circles and dashed line and placentally restricted by closed circles and solid line. Statistical significance: *$p<0.05$ and $^{\#}p<0.1$. Number of animals at each time point: day 5 (8 control, 4 PR), day 15 (9 control, 7 PR), day 20 (8 control, 7 PR), day 25 (9 control, 8 PR), day 35 (9 control, 9 PR), day 40 (7 control, 6 PR). Plasma total T_3 and total T_3/T_4 ratio decreased with age, $^{+}p<0.05$ compared with total T_3 and T_3/T_4 ratio at day 5 (overall ANOVA; $p<0.05$ and $p<0.0001$, respectively).

6.3.3 Size at birth and plasma thyroid hormone concentrations

In control lambs, plasma total T_4 concentrations in the fed state did not correlate with size at birth (Table 6.3, Figure 6.2), whereas those in the fasted state at days 8 and 30 correlated positively with size at birth in terms of weight, CRL, shoulder height and tibia and metatarsal lengths (Table 6.3, Figure 6.2). Similarly in placentally restricted lambs, plasma total T_4 concentrations in the fed state generally did not correlate with size at birth (Table 6.3, Figure 6.2), whereas those in the fasted state at day 8 correlated positively with size at birth in terms shoulder height (Table 6.3, Figure 6.2).

In control lambs, plasma total T_3 concentrations in the fed state during the first month of life (days 5, 15, 25) generally correlated negatively with size at birth, in terms of weight, CRL, shoulder height and metatarsal length (Table 6.4, Figure 6.2). In placentally restricted lambs, plasma total T_3 in the fed state during the first month of life also correlated negatively with size at birth, in terms of weight, CRL, shoulder height, metatarsal and tibia length, but predominantly at days 15, 20 and 25 (Table 6.4, Figure 6.2). In the fasted state, plasma T_3 concentrations during the first month of life did not correlate with size at birth in either control or placentally restricted lambs (Table 6.4, Figure 6.2).

Table 6.3. Correlations of plasma total T_4 concentrations and size at birth in control and placentally restricted lambs.

Postnatal age (days)	Size at birth																			
	Weight				CRL				Shoulder height				Tibia length				Metatarsal length			
	Control		PR		Control		PR		Control		PR		Control		PR		Control		PR	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
5	0.22	ns	0.31	ns	0.14	ns	-0.16	ns	0.05	ns	0.70	ns	0.59	P<0.1	0.53	ns	0.57	P<0.1	0.16	ns
8 (fasted)	0.77	0.008	0.43	ns	0.78	0.007	0.008	ns	0.66	0.03	0.81	0.008	0.78	0.007	0.54	P<0.1	0.57	0.05	0.15	ns
15	0.19	ns	0.32	ns	0.12	ns	0.04	ns	-0.02	ns	0.64	P<0.1	0.63	0.04	0.24	ns	0.37	ns	0.05	ns
20	-0.13	ns	-0.49	ns	-0.17	ns	-0.53	ns	-0.33	ns	-0.31	ns	0.14	ns	-0.33	ns	0.06	ns	-0.86	0.006
25	0.07	ns	-0.31	ns	-0.07	ns	-0.45	ns	-0.22	ns	0.07	ns	-0.13	ns	-0.23	ns	-0.21	ns	-0.53	P<0.1
30 (fasted)	0.72	0.01	0.05	ns	0.70	0.02	0.18	ns	0.42	ns	-0.37	ns	0.54	P<0.1	-0.29	ns	0.28	ns	-0.06	ns
35	0.06	ns	0.24	ns	0.08	ns	-0.07	ns	-0.09	ns	0.23	ns	0.39	ns	0.07	ns	-0.22	ns	-0.32	ns
40	0.33	ns	0.22	ns	0.33	ns	0.16	ns	-0.06	ns	-0.05	ns	0.28	ns	-0.24	ns	-0.23	ns	0.09	ns

R values and P values are shown, negative sign indicates negative correlation.

Table 6.4. Correlations of plasma total T_3 concentrations and size at birth in control and placentally restricted lambs.

Postnatal age (days)	Size at birth																			
	Weight				CRL				Shoulder height				Tibia length				Metatarsal length			
	Control		PR		Control		PR		Control		PR		Control		PR		Control		PR	
	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p	r	p
5	-0.58	P<0.1	-0.26	ns	-0.55	P<0.1	-0.51	ns	-0.60	0.05	-0.05	ns	-0.22	ns	-0.19	ns	0.05	ns	-0.54	ns
8 (fasted)	0.21	ns	0.25	ns	0.27	ns	-0.15	ns	0.23	ns	0.71	0.02	0.11	ns	0.37	ns	0.30	ns	0.05	ns
15	-0.83	0.003	-0.72	0.03	-0.72	0.02	-0.73	0.03	-0.77	0.008	-0.49	ns	-0.54	P<0.1	-0.67	0.05	-0.73	0.01	-0.50	ns
20	0.15	ns	-0.62	P<0.1	0.21	ns	-0.79	0.02	0.09	ns	-0.34	ns	0.11	ns	-0.51	ns	0.17	ns	-0.96	0.001
25	-0.65	0.03	-0.61	0.05	-0.57	ns	-0.28	ns	-0.74	0.01	-0.87	0.003	-0.35	ns	-0.67	0.03	-0.64	0.03	-0.46	ns
30 (fasted)	-0.06	ns	0.30	ns	0.03	ns	0.10	ns	-0.17	ns	0.33	ns	-0.11	ns	0.12	ns	-0.27	ns	0.07	ns
35	-0.15	ns	-0.14	ns	0.07	ns	-0.12	ns	-0.07	ns	-0.47	ns	-0.01	ns	-0.57	0.05	-0.30	ns	-0.48	P<0.1
40	-0.12	ns	0.46	ns	0.13	ns	0.42	ns	-0.09	ns	-0.42	ns	-0.02	ns	-0.04	ns	-0.77	0.02	0.34	ns

R values and P values are shown, negative sign indicates negative correlation.

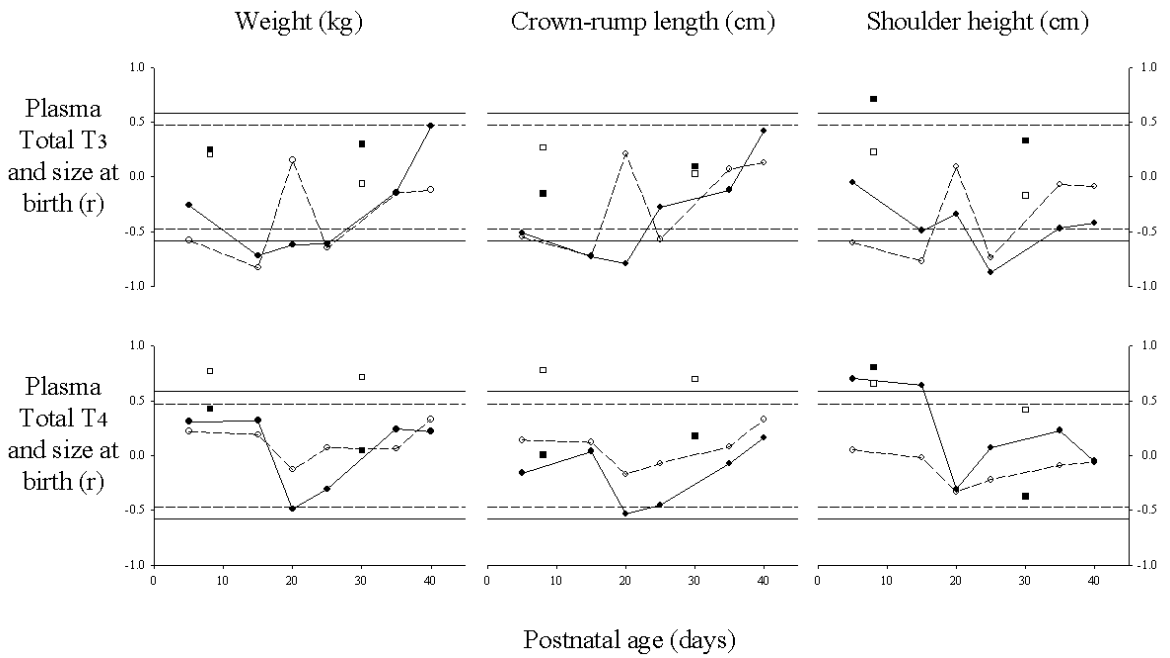

Figure 6.2. Correlation coefficients of the association between plasma total T_3 and T_4 and size at birth in control and placentally restricted lambs during the first 6 weeks of life.

Controls (n=9) are represented by open circles and dashed line and placentally restricted (n=8) by closed circles and solid line. Open boxes represent fasted control and solid boxes represent fasted placentally restricted samples (day 8, 30). Correlation coefficient range from –1 to +1, for size at birth in terms of weight, crown-rump length, and shoulder height, and total T_3 and T_4 over 40 days of life. (——) p=0.05, (-----) p=0.1.

In control lambs, the plasma total T_3/T_4 ratio in the fed state during the first month of life did not correlate with size at birth (data not shown). In placentally restricted lambs, the plasma total T_3/T_4 ratio in the fed state during the first month of life (days 5 and 25) correlated negatively with size at birth in terms of weight ($r=-0.91$, $n=4$, $p<0.05$; $r=-0.69$, $n=8$, $p<0.05$), CRL ($r=-0.92$, $n=4$, $p<0.05$; $r=-0.74$, $n=8$, $p<0.05$), metatarsal ($r=-0.91$, $n=4$, $p<0.05$; $r=-0.64$, $n=8$, $p<0.05$) and radius/ulna length ($r=-0.97$, $n=4$, $p<0.05$; $r=-0.73$, $n=8$, $p<0.05$), skull length ($r=-0.88$, $n=4$, $p<0.05$; $r=-0.71$, $n=8$, $p<0.05$), abdominal ($r=-0.91$, $n=4$, $p<0.05$; $r=-0.66$, $n=8$, $p<0.05$) and thoracic circumference ($r=-0.92$, $n=4$, $p<0.05$; $r=-0.82$, $n=8$, $p<0.05$). In control or placentally restricted lambs, plasma total T_3/T_4 ratios in the fasted state at days 8 and 30 did not correlate with any measure of size at birth (data not shown). In all lambs, plasma free T_3 to free T_4 ratio correlated positively with size at birth for abdominal circumference ($r=0.29$, $n=44$, $p=0.03$), and negatively with placental weight ($r=-0.49$, $n=44$, $p=0.02$), while tending to correlate positively with birth weight ($r=0.21$, $n=44$, $p=0.08$). In control lambs, plasma free T_3 to free T_4 ratio correlated positively with size at birth for weight ($r=0.36$, $n=22$, $p=0.05$), abdominal ($r=0.45$, $n=22$, $p=0.02$) and thoracic ($r=0.44$, $n=22$, $p=0.02$) circumference, while tending to correlate positively with size at birth for CRL ($r=0.33$, $n=22$, $p=0.07$), shoulder height ($r=0.32$, $n=22$, $p=0.08$), and hind limb circumference (knee joint) ($r=0.35$, $n=22$, $p=0.06$). Plasma free T_3 to free T_4 ratio did not correlate with size at birth in placentally restricted lambs.

6.3.4 Postnatal growth and plasma thyroid hormone concentrations in the young lamb

Absolute growth rate for all lambs in terms of weight correlated positively with plasma total T_3 concentrations at day 35 (r=0.39), and plasma total T_4 concentrations at days 30 (r=0.37), 35 (r=0.48), and 40 (r=0.45) ($p<0.05$ for all). Absolute growth rate for all lambs in terms of CRL correlated positively with plasma total T_3 concentrations at days 15 (r=0.48), 20 (r=0.51), and 35 (r=0.42), and plasma total T_4 concentrations at day 20 (r=0.64) ($p<0.05$ for all).

In placentally restricted lambs, absolute growth rate in terms of weight tended to correlate positively with plasma total T_3 in the fasted state at day 30 (r=0.54, n=8, p=0.07) and that of tibia length, with plasma total T_3 concentrations at day 35 (r=0.75, n=8, $p<0.05$). In placentally restricted lambs, absolute growth rate in terms of abdominal circumference correlated positively with plasma total T_3 and T_4 concentrations in the fasted state at day 8 (r=0.76, n=8, $p<0.05$, for both) and with that of T_3 at day 30 (r=0.60, n=8, $p<0.05$).

Neonatal fractional growth rates for any size parameters did not correlate with plasma total T_4 concentrations during the first month of life in either control or placentally restricted lambs in the fed or fasted states (data not shown). In control and placentally restricted lambs, neonatal fractional growth rate in terms of weight correlated positively with plasma free T_4 concentrations (day 30) (controls r=0.49, $p<0.025$, PR r=0.44, $p<0.025$) (Figure 6.3). In placentally restricted lambs, neonatal fractional growth rate in terms of metatarsal length

correlated positively with plasma free T_4 (day 30) (controls r=0.20, NS, PR r=0.48, p<0.025) (Figure 6.4).

In control and placentally restricted lambs, neonatal fractional growth rates for weight (control: r=0.92, p=0.0005; PR: r=0.80, p=0.025), CRL (control: r=0.88, p=0.0005; PR: r=0.67, p=0.05), and shoulder height (control: r=0.78, p=0.005; PR: r=0.14, NS) generally correlated positively with plasma total T_3 at day 15 (Figure 6.5). In placentally restricted but not control lambs, neonatal fractional growth rate in terms of weight correlated positively with plasma total and free T_3 concentrations (day 30) (controls r=0.30, NS, PR r=0.54, p<0.005; controls r=0.09, NS, PR r=0.54, p<0.005 respectively) (Figure 6.3). In control and placentally restricted lambs, neonatal fractional growth rate in terms of metatarsal length correlated positively with plasma free T_3 (day 30) (controls r=0.35, p<0.05, PR r=0.56, p<0.005), but with plasma total T_3 (day 30) in controls only (controls r=0.41, p<0.05, PR r=0.30, NS) (Figure 6.4).

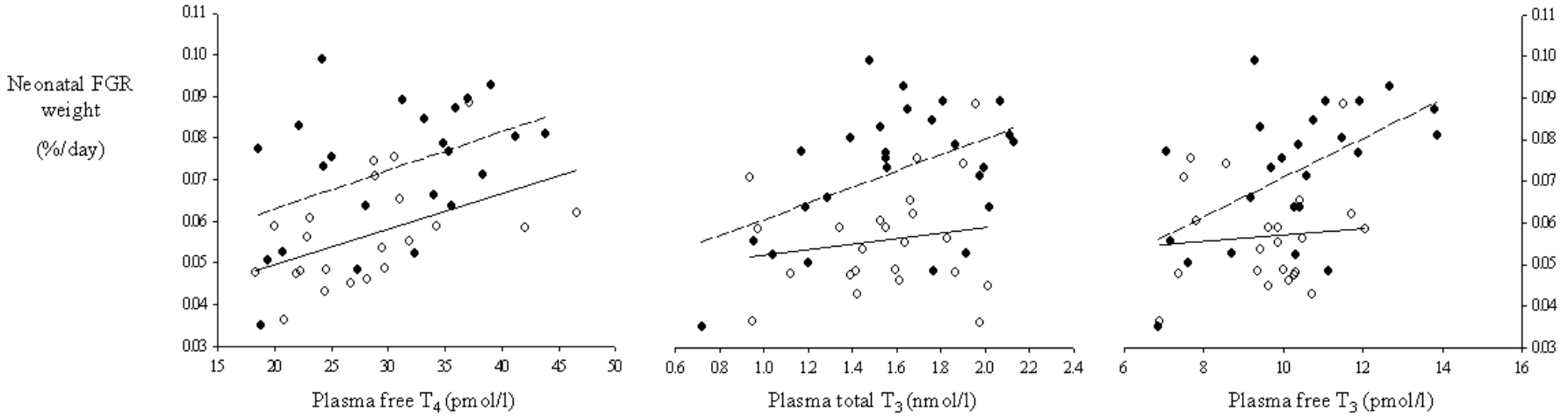

Figure 6.3. The relationship between neonatal fractional growth rate in terms of weight and plasma free T_4, total T_3, and free T_3 concentrations at day 30 (fasted).

Controls are represented by open circles and dashed regression line, and placentally restricted by closed circles and solid regression line. Free T_4: controls r=0.49, p<0.025, PR r=0.44, p<0.025, total T_3: controls r=0.30, NS, PR r=0.54, p<0.005, free T_3: controls r=0.09, NS, PR r=0.54, p<0.005.

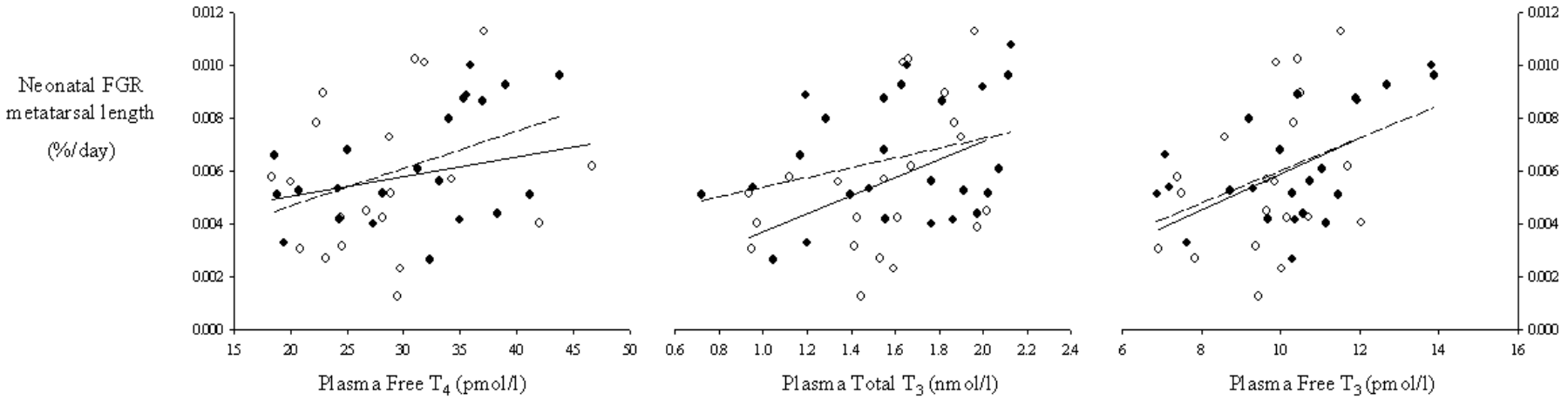

Figure 6.4. The relationship between neonatal fractional growth rate in terms of metatarsal length and plasma free T_4, total T_3, and free T_3 concentrations at day 30 (fasted).

Controls are represented by open circles and dashed regression line, and placentally restricted by closed circles and solid regression line. Free T_4: controls r=0.20, NS, PR r=0.48, p<0.025, total T_3: controls r=0.41, p<0.05, PR r=0.30, NS, free T_3: controls r=0.35, p<0.05, PR r=0.56, p<0.005.

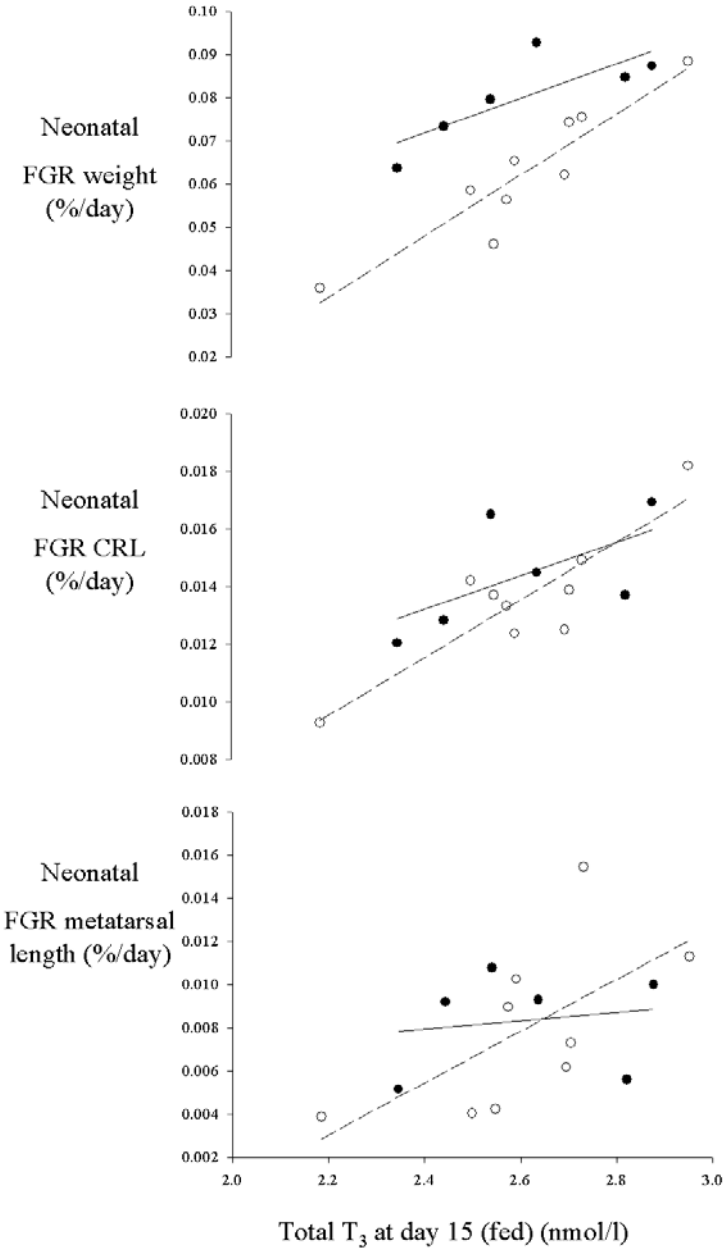

Figure 6.5. Neonatal catch-up growth and plasma total T_3 in fed lambs.

Controls are represented by open circles and dashed regression line, and placentally restricted by closed circles and solid regression line. Neonatal fractional growth for weight (control: r=0.92, p=0.0005; PR: r=0.80, p=0.025), CRL (control: r=0.88, p=0.0005; PR: r=0.67, p=0.05), and metatarsal length (control: r=0.63, p=0.05; PR: r=0.17, NS) and total T_3 at day 15.

Current fractional growth rates for any parameters of size generally did not correlate with plasma total T_4 in either control or placentally restricted lambs in the fed or fasted states throughout the first month of life (data not shown). However, in placentally restricted lambs at day 20, plasma total T_4 correlated with current fractional growth rate for CRL ($r=0.92$, $n=7$, $p<0.005$) and tibia length ($r=0.66$, $n=7$, $p<0.05$) (Figure 6.6).

In control lambs, current fractional growth rate for weight correlated positively with plasma total T_3 concentrations at days 15 ($r=0.61$, $n=9$, $p<0.05$), 25 ($r=0.63$, $n=9$, $p<0.05$), and 35 ($r=0.69$, $n=9$, $p<0.05$) and 40 ($r=0.81$, $n=9$, $p<0.05$) (Figure 6.6, 6.7). Similarly, in control lambs, current fractional growth rate for CRL correlated positively with plasma total T_3 concentrations at day 5 ($r=0.69$, $n=8$, $p<0.05$), 15 ($r=0.78$, $n=9$, $p<0.05$) and 25 ($r=0.75$, $n=9$, $p<0.05$) (Figure 6.6, 6.7). In control lambs, current fractional growth rates for metatarsal length (control: $r=0.66$, $p=0.05$; PR: $r=0.15$, NS) correlated positively with plasma total T_3 concentrations at day 15 (Figure 6.6, 6.7).

In placentally restricted lambs at day 20, current fractional growth rates for CRL correlated positively with plasma total T_3 concentrations ($r=0.69$, $n=7$, $p<0.05$) (Figure 6.6). Similarly, in placentally restricted lambs, current fractional growth rates for metatarsal length correlated positively with plasma total T_3 concentrations at day 15 ($r=0.42$) and 25 ($r=0.44$) ($p<0.05$ for all). In placentally restricted lambs at day 35, current fractional growth rates for tibia length correlated positively with plasma total T_3 ($r=0.74$, $n=7$, $p<0.05$) (Figure 6.6).

6.3.5 Whole body insulin sensitivity and plasma thyroid hormone concentrations

Plasma free T_3 correlated negatively with insulin sensitivity of glucose metabolism in controls only ($r=-0.73$, $p<0.025$) at 30 days of age. Plasma total T_4 and free T_4 correlated negatively with insulin sensitivity of free fatty acid metabolism in placentally restricted lambs only ($r=-0.66$, $p<0.025$, $r=-0.96$, $p<0.025$, respectively) at 30 days of age (Figure 6.8). Plasma free T_3 correlated negatively with insulin sensitivity of free fatty acid metabolism in control ($r=-0.63$, $p<0.05$) and placentally restricted lambs ($r=-0.93$, $p<0.05$) at 30 days of age (Figure 6.8).

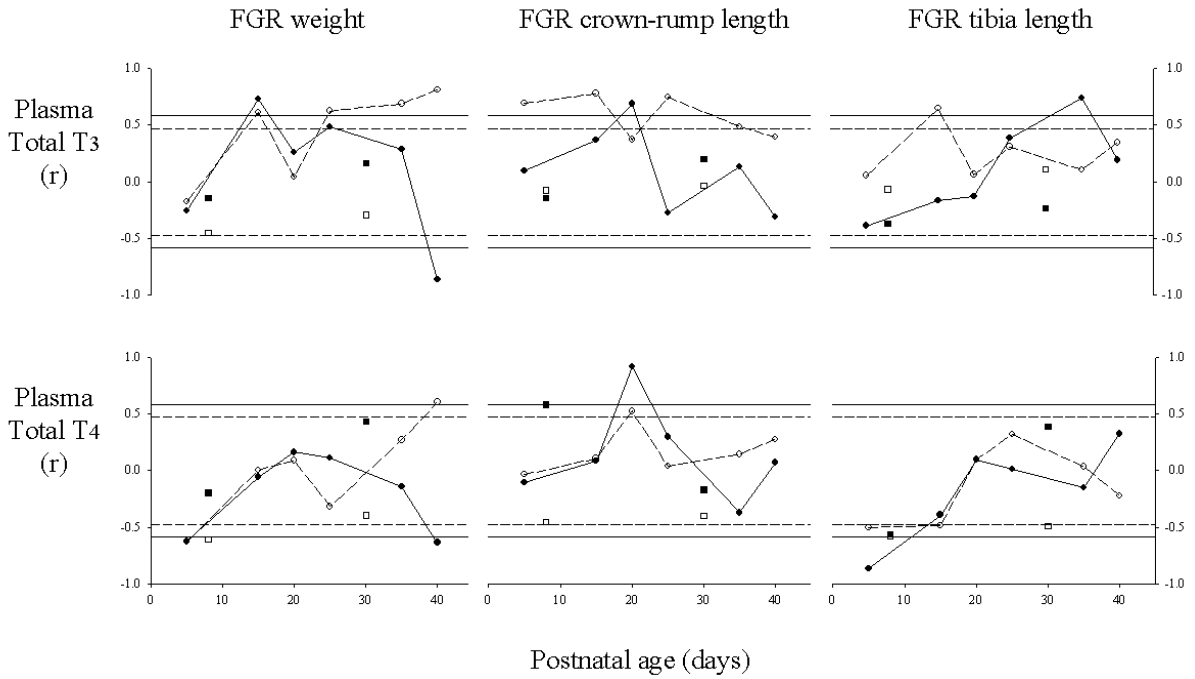

Figure 6.6. Correlation coefficients of the association between plasma total T_3 and T_4 and current fractional growth rates in control and placentally restricted lambs during the first month of life.

Controls (n=9) are represented by open circles and dashed line and placentally restricted (n=8) by closed circles and solid line. Open boxes represent fasted control and solid boxes represent fasted placentally restricted samples (day 8, 30). Correlation coefficients range from –1 to +1, for current fractional growth rate in terms of weight, crown-rump length, and tibia length, and total T_3 and T_4 over 40 days of life. (——) p=0.05, (-----) p=0.1.

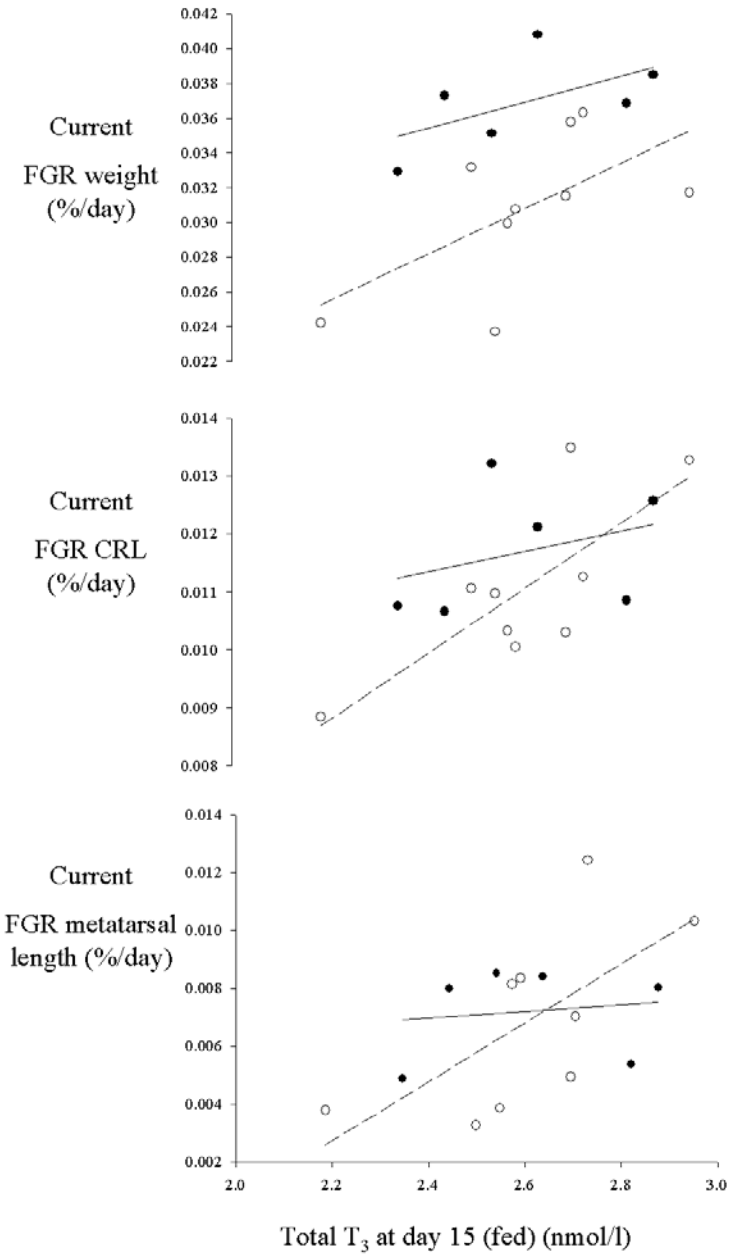

Figure 6.7. Current fractional growth and total T_3 in fed lambs (day 15).

Controls are represented by open circles and dashed regression line, and placentally restricted by closed circles and solid regression line. Current fractional growth for weight (control: r=0.61, p=0.05; PR: r=0.57, NS), CRL (control: r=0.78, p=0.005; PR: r=0.38, NS), and metatarsal length (control: r=0.66, p=0.05; PR: r=0.15, NS) and total T_3 at day 15.

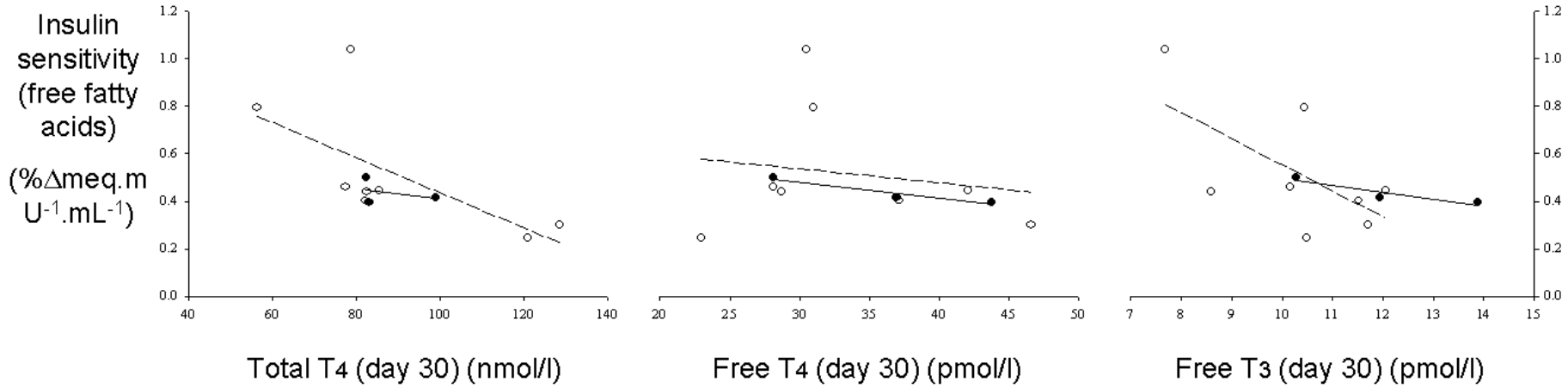

Figure 6.8. Insulin sensitivity of free fatty acid metabolism and plasma thyroid hormone concentration at day 30.

Controls are represented by open circles and dashed regression line, and placentally restricted by closed circles and solid regression line. Total T_4 (control: r=-0.37, NS; PR: r=-0.66, p<0.025), free T_4 (control: r=-0.17, NS; PR: r=-0.96, p<0.025), and free T_3 (control: r=-0.63, p<0.05; PR: r=-0.93, p<0.05) and insulin sensitivity of free fatty acids at day 30.

6.4 DISCUSSION

This study has shown that placental restriction and small size at birth alter thyroid hormone abundance in the first month of life in sheep, increasing plasma total T_3 and the ratio of plasma T_3 to T_4, after two to three weeks of age. Previously, plasma T_3 and T_4 have been found to be reduced in the first day of life in IUGR in sheep and other mammals ([230]Jacobsen*, et al.*, 1979, [308]Cabello*, et al.*, 1981), which the current study shows does not persist. Rather, there is normal plasma T_4 and increased plasma T_3 peripherally in early postnatal life following fetal growth restriction, possibly due to increased TH activation. Because this increase in peripheral T_3 is late onset and occurs mainly after the period of maximal catch-up growth in fractional terms during the first two to three weeks (See Chapter 3), it may not have a primary role in the early onset catch-up growth. However, subsequent growth throughout the first month of life is related to and may partly depend on circulating total or free T_3 in control and in placentally restricted lambs.

Exogenous thyroxine (T_4) administration in conjunction with two feeding regimes on adipose tissue (perirenal) and liver growth in postnatal lambs was examined in a recent study ([311]Gate*, et al.*, 2000). Pairs of lambs were fed either 100 grams (low fed) or 200 grams (high fed) of milk powder per litre of reconstituted milk replacer over the first month of life, with half of the pairs fed a daily bolus dose of T_4 (15 mg/kg body weight) until 8 days of age ([311]Gate*, et al.*, 2000). The high fed lambs grew faster, were fatter and had larger livers than low fed lambs at 8 and 35 days of age. The T_4 administration resulted in a lower thermogenic activity (GDP binding) in adipose tissue at 8 days of age in the low,

but not high fed lambs. Between 17 and 35 days of age high fed lambs previously treated with T_4 had lower daily milk consumption than untreated siblings, but still had the same growth rate. Following withdrawal of T_4 treatment, the high fed T_4-treated lambs were able to maintain the same growth rate as untreated lambs with a lower food intake ([311]Gate*, et al.*, 2000). Therefore the high fed lambs with T4 treatment have a reduced appetite without any detrimental effects on growth, suggesting there may be an improved efficiency of nutrient utilisation ([311]Gate*, et al.*, 2000).

Normally, plasma T_3 and the ratio of plasma total T_3 to T_4 increase in the first 8 hours of life in the sheep and other species ([230]Jacobsen*, et al.*, 1979, [231]Berthon*, et al.*, 1993, [308]Cabello*, et al.*, 1981), reflecting in part an increase in TSH which stimulates T_4 and T_3 secretion from the thyroid and the loss of the placenta with its high D3 activity which inactivates T_3 and T_4 ([186]Bianco*, et al.*, 2002). Subsequently, plasma T_3 and the ratio of plasma T_3 to T_4 reportedly increase to a maximum at one to two weeks of age then decline, in the sheep ([308]Cabello*, et al.*, 1981). The findings of the current study were consistent with this, as plasma T_4 did not change, while plasma T_3 and the ratio of T_3 to T_4 were maximal in the first one to two weeks, then decreased with increasing age in the young lamb. A broadly similar pattern occurs in the human infant where after the initial surge of serum T_3 in the neonate, free and total serum T_3 subsequently increase again to maximal levels at 50 to 80 days of age ([230]Jacobsen*, et al.*, 1979). These changes in peripheral TH levels and the relative amounts of T_3 and T_4 presumably reflect the secretion of T_4 and T_3 by the thyroid under TSH stimulation, and the production of T_3 in extrathyroidal tissues via 5' deiodination

catalysed by D1 and D2, with the former in particular contributing to circulating T_3. Together with D3, these selenodeiodinases also inactivate T_3 and T_4 and help determine their levels in blood ([186]Bianco*, et al.*, 2002). Little is known of the developmental changes in these various determinants of circulating T_3 and T_4 in sheep or humans or of the effect of an adverse prenatal environment, which restricts growth.

Although the findings of the current study suggests that the reduced plasma T_4 evident in the first 24 hours of life following fetal growth restriction does not persist, plasma T_4 at days 8 and 30 in the fasted state was reduced in association with reduced size at birth. In another study of lambs exhibiting spontaneous fetal growth restriction, plasma total T_4 and also T_3 were reduced in the first few hours of life and at 11 days of age, correlated positively with birth weight, but were apparently not altered at later ages ([308]Cabello*, et al.*, 1981). Therefore a mild deficit in peripheral T_4 may persist for several weeks following fetal growth restriction, due to reduced production or increased metabolism of TH. In support of the latter possibility, in the current study, placental restriction tended to increase plasma T_3 at one month of age and plasma T_3 was generally higher with reduced size at birth, from day 5 onwards in control and from day 15 onwards in placentally restricted lambs. This contrasts with previous findings in spontaneously growth restricted lambs ([308]Cabello*, et al.*, 1981) and suggests that placental restriction may have increased activation of T_4 to T_3, increasing peripheral T_3. Consistent with this is the increased plasma T_3 to T_4 ratio with placental restriction and with decreased size at birth in placentally restricted lambs at some ages in the first month of life. This also suggests that there may

be increased production of T_4 since normal levels were mostly maintained, despite increased levels of T_3. In the human IUGR infant, serum T_4 is reduced and serum T_3 is unchanged suggesting an increased T_3 to T_4 ratio in the first two months of life, which resembles the pattern observed here and in previous studies of experimental and spontaneous IUGR in the sheep ([230]Jacobsen*, et al.*, 1979). It is therefore possible that in the sheep and human infant, restriction of fetal growth increases D1 activity in tissues such as liver and kidney and that of D2 in the thyroid, skeletal muscle and brown adipose tissue, contributing to the observed increases in plasma T_3 and plasma T_3 to T_4.

The mechanisms by which placental restriction could alter production and activation of thyroid hormone postnatally are unknown. In the rat, a critical period exists during perinatal development, where the regulation of pituitary-thyroid axis function in later life can be permanently altered or programmed by perturbation ([232]Pracyk*, et al.*, 1992). Administration of propylthiouracil perinatally to rat dams and their pups from gestational day 17 through to postnatal day 5 produced hypothyroidism in offspring ([232]Pracyk*, et al.*, 1992). Plasma concentrations of T_3 and T_4 in offspring were suppressed from the beginning of propylthiouracil administration through to postnatal day 10, then rose over the next two weeks compared to controls but showed a deficit into adulthood ([232]Pracyk*, et al.*, 1992). Thus, perinatal hypothyroidism in the rat results in reduced plasma T_3 and T_4 levels into adulthood, despite normal levels of plasma TSH ([232]Pracyk*, et al.*, 1992). In contrast, in the hypothyroid fetal pig, induced by feeding the sow a high glucosinolate rapeseed diet in late gestation, after birth there is a surge in the circulating levels of total and free T_3 in offspring

during the first 6 hours of life ([231]Berthon, *et al.*, 1993). This surge was greater for plasma total T_3 than total T_4 and was followed by a transient decrease in both T_3 and T_4 at 12 hours and a second rise in T_3 by 24 hours after birth ([231]Berthon, *et al.*, 1993), but whether this persists and was related to growth rate is not known. Placental restriction in the sheep reduces the rates of delivery of oxygen and glucose to the fetus in late gestation, which responds with a range of adaptations including hypothyroidism ([5]Owens, *et al.*, 1987, [239]Harding, *et al.*, 1985, [257]Owens, *et al.*, 1987). Therefore the hypothyroidism of the growth restricted fetal sheep may be at least partly responsible for the alterations in peripheral determinants of plasma T_3 in early postnatal life, acting via similar albeit unknown mechanisms to those observed in the pig perinatally ([231]Berthon, *et al.*, 1993). The mechanisms by which placental restriction and fetal hypothyroidism increase plasma T_3 and T_3 to T_4 ratio may be direct, targeting and altering the functional capacity of tissues and cells which determine T_3 abundance or indirect, or as a result of programmed changes in the activity of regulatory factors known to regulate these or target responsiveness to such factors ([186]Bianco, *et al.*, 2002). Factors known to promote D1 activity and activation of T_4 to T_3 include T_3 itself and GH, while glucocorticoids have more variable effects ([312]Darras, *et al.*, 1992, [313]Van der Geyten, *et al.*, 1999). Glucocorticoids acutely decrease the ratio of T_3 to T_4 in plasma, which had been ascribed to inhibition of D1, but more recently appears due largely to induction of D3 and increased clearance of T_3 due to 5' deiodination, in humans at least ([314]Duick, *et al.*, 1974, [315]Chopra, *et al.*, 1975, [316]LoPresti, *et al.*, 1989). While studies have shown that fetal growth restriction is associated with increased peripheral glucocorticoids ([308]Cabello, *et al.*, 1981) and some evidence of GH

resistance in children and adults ([103]Leger*, et al.*, 1996, [227]Cance-Rouzaud*, et al.*, 1998), its effects and that of placental restriction on these axes in early postnatal life in sheep or other species are mostly unknown. Spontaneous fetal growth restriction in sheep was characterised by increased circulating cortisol in the first 48 hours of life but not beyond ([308]Cabello*, et al.*, 1981). High circulating GH levels have been reported as persisting for several days following birth in the human infant with IUGR but did not predict either of later growth or of short stature at 2 years of age ([103]Leger*, et al.*, 1996, [227]Cance-Rouzaud*, et al.*, 1998).

In the current study, postnatal growth was related to circulating TH abundance, particularly after the first 2 to 3 weeks of age in the young lamb. Thus overall, absolute growth rate for vertebral column length in all lambs was related to both plasma T_3 and T_4 from 2 to 3 weeks onwards, while that for weight was related to that in weeks 5 to 6 consistent with the essential role of TH in neonatal growth ([233]Williams*, et al.*, 1998, [317]Cabello*, et al.*, 1989, [318]Dauncey*, et al.*, 1993, [319]Yen, 2001). In contrast, neonatal and current fractional growth rates for both soft and skeletal tissues were predicted by plasma free T_4 and free and total T_3, but not plasma total T_4, which possibly reflects the greater biological activity of the unbound form of TH in plasma and of the more active metabolite T_3 in comparison to T_4 ([180]Hulbert, 2000). It should also be noted that placental restriction did not alter the percentage of total T_3 and T_4 present in plasma as free, suggesting that normal transport of TH occurs in lambs following placental restriction. It remains possible however, that placental restriction alters the binding capacity postnatally and that this contributes to the changes in total TH ([230]Jacobsen*, et al.*, 1979). For example, a reduction in TH binding in plasma

would reduce total T_3 and T_4 and obscure an increase in T_4 production and activation to T_3 due to placental restriction.

Interestingly, for catch-up growth in terms of weight, a higher rate of neonatal or current fractional growth was seen at a given plasma concentration of TH in the placentally restricted lambs compared to the control lambs (Figure 6.1). This raises the possibility that the placentally restricted lambs are more 'sensitive' to TH than the control lambs. This could be due to increased expression of thyroid hormone receptors (THRs) in tissues responsible for much of the increase in weight or of D2, the deiodinase responsible for increasing nuclear T_3 levels in tissues such as skeletal muscle and brown adipose tissue, at least in humans ([186]Bianco*, et al.*, 2002, [234]Silva, 2003), following placental restriction. Whether this occurs is yet to be investigated, but increased expression of THRs in skeletal muscle has been observed in the neonatal pig, following experimentally induced hypothyroidism ([320]Harrison*, et al.*, 1996). Piglets were made hypothyroid by providing methimazole and iopanoic acid in their feed between 4 and 14 days of age, which resulted in a reduction in plasma concentrations of T_3, T_4, and free T_3, and an increase in the proportion of type I slow-twitch oxidative fibres in longissimus dorsi and soleus which increased the nuclear T_3 receptor by 46%, possibly making the skeletal muscle more sensitive to thyroid hormone binding ([320]Harrison*, et al.*, 1996). This period of hypothyroidism may induce changes in myofibre differentiation during early postnatal life and may be a mechanism making skeletal muscle more sensitive to circulating thyroid hormones in future.

Placental restriction may also increase the energetic efficiency of soft tissues postnatally, increasing that of soft tissue accretion and growth, due to reduced thermogenesis and uncoupling of ATP phosphorylation, as a result of altered mitochondrial energetics and metabolism ([234]Silva, 2003). Alternatively, the proportions of various tissues undergoing growth in the placentally restricted lambs may differ from that in controls, with a greater representation of more TH sensitive tissues in the increase in weight. There is little information on the impact of IUGR on subsequent body composition and growth in the neonatal sheep or other species however. We have observed increased visceral adiposity in the placental restricted lamb at one month of age and in humans, low birth weight predicts reduced skeletal tissue or lean tissue mass ([97]Rogers*, et al.*, 2003) and increased relative central adiposity in later life ([8]Ong*, et al.*, 2000). Consistent with this, when nutrition during the first month of life is unlimited, the twin or triplet neonatal lamb that has experienced fetal growth restriction is relatively fat compared to normal singleton lambs at any given body weight ([249]Greenwood*, et al.*, 1998, [270]Bell, 1992). This suggests a greater proportion of tissue accretion in adipose tissue following IUGR. While there were consistently higher rates of neonatal and current fractional growth rates for weight in placentally restricted lambs, whatever the prevailing plasma THs concentrations, this was not the case for growth of skeletal tissues. This suggests that the primary determinant of increased growth of long bones or the vertebral column in the young lamb following placental restriction, is the increased abundance of free T_3 and T_4 and of total T_3 in the periphery rather than intrinsic changes in these and other determinants of local TH action in the growth plate and elsewhere within skeletal tissues ([188]Robson*, et al.*, 2000).

Alternatively, the latter may be differentially altered by placental restriction in comparison with soft tissues.

Increased abundance and action of thyroid hormones may also influence the growth and development of skeletal tissues and other tissues via changes in the activity of the somatotropic axis. Studies have shown that TH stimulates postnatal growth and affects GH and IGF production and their tissue activity ([317]Cabello*, et al.*, 1989). It is well established from *in vitro* studies that T_3 influences the synthesis and secretion of insulin-like growth factor ([321]Binoux*, et al.*, 1985). It has been demonstrated that growth can be stimulated by T_3 and or growth hormone administration in the dwarf mouse ([322]Fouchereau-Peron, 1981) and hypophysectomized chickens ([323]Scanes*, et al.*, 1986), however the effects of growth hormone were significantly lower than that of the thyroid hormone. This means that TH could promote growth of tissues indirectly by influencing the synthesis and secretion of growth factors *in vivo*. In addition, GH acting via IGF-I and T_3 both inhibit the activity of 11βHSD-1 in tissues such as liver and kidney, reducing local production of cortisol from cortisone and hence its inhibitory actions on growth ([324]Moore*, et al.*, 1999, [325]Stewart*, et al.*, 2001).

Therefore in conclusion, placental restriction in the sheep reduced size at birth and increased postnatal growth rates in terms of weight and long bones in lambs. Plasma TH was associated with absolute and fractional growth of soft and skeletal tissue in lambs during early postnatal life, particularly in those undergoing catch-up growth. Thyroid hormone has long been known to be essential for infant growth and this study has now shown that the increased rates

of growth seen following IUGR may be partly due to the increased abundance, and/or sensitivity of tissues to TH.

Chapter 7

GENERAL DISCUSSION

General Discussion

Determining the consequences of experimental restriction of placental growth for size at birth and the metabolic and endocrine state of the neonate and its relationship to growth will help to delineate the contribution of endocrine axes to catch-up growth and the mechanisms by which they act. This will provide the scientific basis for the subsequent design and testing of alternative therapies to overcome the deleterious consequences of IUGR; reduced adult stature and endocrine and metabolic problems in later adult life.

The experimental paradigm used in this series of studies, the placentally restricted sheep, results in a growth restricted lamb which has qualities similar to the human IUGR infant, i.e. small size at birth due to poor nutrient supply before birth, an increased rate of postnatal growth (catch-up growth), and endocrine and metabolic changes which lead to the development of adult onset diseases such as obesity, cardiovascular disease, and NIDDM.

The results of these studies has demonstrated that experimentally inducing placental restriction by the removal of most of the endometrial caruncles from the uterus of the sheep restricts fetal growth and disproportionately reduces size at birth. Reduced body size and weight at birth are common features for most studies with human IUGR infants ([7]Karlberg*, et al.*, 1995, [12]Hokken-Koelega*, et al.*, 1995, [83]Albertsson Wikland*, et al.*, 1994) and experimentally growth-restricted animals ([241]Mellor*, et al.*, 1977, [253]Ritacco*, et al.*, 1997, [275]Muaku*, et al.*, 1997). Placental restriction makes sheep proportionately

smaller in terms of body mass index and ponderal index, but also makes females thinner. By six weeks of age there was no difference in terms of size, ponderal index or body mass index between IUGR and control lambs. Thus after placental restriction, lambs undergo catch-up growth and have caught up to controls in terms of weight and length for soft and skeletal tissues by this age. Placentally restricted lambs had higher rates of growth during postnatal life and males had increased adiposity at six weeks, supporting previous studies in humans where men who were light at birth have the greater risk of developing obesity if they grew rapidly during childhood ([98]Parsons*, et al.*, 2001). This increased adiposity in the young lamb may be an indication of things to come in adult life.

The elevated insulin-sensitivity of FFA metabolism in the placentally restricted and small lambs may partly explain the observations of increased adiposity following catch-up growth described in the first experimental chapter of this thesis (Chapter 3) and also in humans ([249]Greenwood*, et al.*, 1998, [284]Ravelli*, et al.*, 1976, [285]Law*, et al.*, 1992). The circulating levels of FFA were decreased to a greater extent by insulin in the placentally restricted lambs compared with the control lambs, indicating a greater sensitivity of FFA metabolism, and probably reflecting an increased insulin-suppression of lipolysis in the placentally restricted lambs of this age, if the site of increased insulin sensitivity is adipocytes. The storage of fat occurs in the presence of normal plasma insulin levels and normal post-hepatic insulin secretion suggesting that insulin action will be increased. Restriction of fetal growth did not alter fasting plasma glucose, suggesting that increased nutrient availability was not a contributor to

their increased growth and adiposity. However, if appetite and nutrient intake is increased in the placentally restricted lamb, as in IUGR rats ([278]Vickers*, et al.*, 2000), post-prandial blood glucose may be higher and add to expansion of adipose tissue.

Placental restriction did not alter insulin sensitivity of glucose or amino acid metabolism, but low birth weight did, increasing these at one month of age. The increased circulating concentrations of amino acids, along with increased insulin sensitivity of amino acid metabolism, may be promoting growth of their skeletal muscle, causing catch-up growth. Therefore the results of this study, suggest that it is possible that increased growth of soft tissue, especially skeletal muscle may be closely related to insulin sensitivity of glucose and amino acid metabolism.

Catch-up growth in terms of soft and skeletal tissues of the placentally restricted lamb may also be partially explained by the relationships between growth and IGF abundance and sensitivity. The increased adiposity after placental restriction may be driven by increased IGF as well as insulin action. The current study has shown that catch-up growth of soft and skeletal tissues in the neonate occurs despite reduced or normal concentrations of fasting plasma IGF-I and –II, which may be due to an increase in local abundance or sensitivity. The extent to which earlier catch-up growth is driven by increased IGF action and whether IGF production is altered is still unknown.

Placental restriction of fetal growth increased circulating TH concentrations in young lambs only after approximately 3 weeks of age and maximal catch-up growth. The increased circulating TH concentrations were associated with increased neonatal and current growth rates in terms of weight and long bones, suggesting the TH axis may play a role in catch-up growth. This association with accelerated growth in terms of weight supports a previous study in which plasma total T_3 levels were related to weight gain during the 1st month of life ([308]Cabello*, et al.*, 1981). If these elevated levels of TH persist throughout the first month of life they may also contribute to catch-up following restriction of fetal growth. Catch-up in terms of long bone length was also predicted by the total T_3 levels in the placentally restricted lambs, suggesting a role for TH in the control of accelerated bone growth after IUGR. Increased neonatal growth rate for soft and skeletal tissues was predicted by free T_4 and free and total T_3, but not total T_4 which possibly reflects the greater biological activity of the unbound form of TH in plasma and of the more active metabolite T_3 in comparison to T_4 ([180]Hulbert, 2000). Another finding of the present study was that the total T_3/T_4 ratio, which demonstrates the conversion of T_4 into the active form T_3 by tissues, was found to be higher in the placentally restricted lambs compared to controls, suggesting that the placentally restricted lambs may have a greater ability to convert T_4 into T_3 in the tissues. This along with increased sensitivity to TH may allow them to grow at a faster rate compared with controls, as demonstrated in Chapter 6. Another possibility is that the placentally restricted lambs may be losing less energy in the form of heat (thermogenesis) and using this energy to promote growth. When brown adipose tissue thermogenesis is required, the enzyme type II 5' iodothyronine is stimulated. This enzyme increases the

concentrations of intracellular T_3 to very high levels to increase ATP consumption and therefore produce heat ([234]Silva, 2003). It is possible that placentally restricted lambs may have reduced fat stores at birth, which may trigger an increase in T_3, thereby increasing thermogenesis to counteract cold, but concomitantly increasing the rate of growth in the placentally restricted lambs. This may persist over a period of a few weeks until the lambs have put on weight and increased their fat stores, and may partially explain why catch-up growth period of muscle, bone and fat occurs mostly in the first month of life. Interestingly, for catch-up in terms of weight, a higher rate of growth was observed for a given plasma concentration of TH in the PR lambs. The higher rate of growth for a given concentration of TH raises the possibility that PR lambs are more 'sensitive' to TH than controls, but this could not be examined directly. Alternatively, the proportions of various tissues undergoing growth in the PR lambs may differ from that of controls, with a greater representation of more TH sensitive tissues. Therefore, thyroid hormone may be a hormone that promotes growth during early postnatal life and catch-up growth following IUGR may partly be due to the increased abundance, and/or sensitivity of TH to growth promoting tissues. It is also possible that the TH axis may be an important contributor to catch-up growth and later adult onset diseases.

Therefore the four studies described in this thesis examining the endocrine consequences of fetal growth restriction demonstrate that placental restriction and/or small size at birth is associated with increased postnatal growth rate (catch-up growth), which leads to increased adiposity by 45 days of age in the lamb. We have shown for the first time that this increased growth and adiposity

may be due to these lambs being more sensitive to insulin, IGF-I, and possibly thyroid hormone. There is no doubt that if these lambs are more sensitive to these hormones, the excess nutrients being taken into the tissues will promote growth of these tissues, and any excess nutrients would be stored as fat in adipocyte stores. Furthermore, this fat seems to be directed towards storage in the visceral fat store. Increased visceral adiposity has been shown to have strong correlations with more deleterious effects on the body and on cardiovascular and metabolic health than other fat storage sites ([326]Kelley*, et al.*, 2000, [327]Banerji*, et al.*, 1999, [328]Ross*, et al.*, 2002). Therefore the increased sensitivity to anabolic hormones in these small (or placentally restricted) lambs that is promoting catch-up growth, maybe detrimental in terms of later health outcomes.

Another area of particular interest for investigation would be the feed intake of these restricted lambs during the first 45 days of life. Do the placentally restricted lambs eat more, and/or do they have an increased appetite? Is the quality of the milk from the ewe better, providing more nutrients to the lamb? As a pilot study (data presented here only) a subset of our lambs had a basic feed intake experiment performed, which recorded the amount of suckling events and the total time a lamb suckled during a 2-hour period at 15 days of age following a 60 minute fast. We demonstrated that PR lambs had a greater number of suckling events overall (PR=7.7 ± 1.5 events, CON=6.3 ± 2.2 events. P=0.045) and had a tendency towards greater suckling time (PR = 190.0 ± 11.0 secs, CON = 154.0 ± 8.0 secs, P=0.08) during the first 30 minutes after being returned to the ewe. This may be an indication of an increased appetite in

these lambs and suggests that neuroendocrine regulation of appetite may be altered in these lambs. This may involve alterations in leptin and neuropeptide Y (NPY) concentrations. Leptin is a hormone secreted by adipocytes in direct proportion to the amount of adipose tissue in the body, whereas NPY is a hormone that is secreted by the hypothalamus and is an extremely potent stimulator of feeding behaviour. These hormones may give a better indication of the feeding stimulus and nutrition level in these placentally restricted lambs. If this were the case, these animals would have increased nutrient availability for the tissues for growth, including adipose tissue, but this may add to the deleterious outcomes for later health. If these prenatally restricted lambs continue to experience restricted nutrient availability in early postnatal life, as during fetal life, and hence did not undergo catch-up growth in early postnatal life, more positive longer-term health outcomes may result. This scenario of continual nutrient restriction in postnatal life may be better for the growth restricted offspring than having increased or unlimited nutrient supply postnatally which may lead to obesity and increased rates of growth. On the other hand, it is widely assumed that catch-up growth is desirable for low birth weight children, but the literature on this subject is limited. But more recently, studies have suggested that human populations having undergone catch-up growth exhibit adverse long-term outcomes in terms of blood pressure ([78]Leon*, et al.*, 1996), death from cardiovascular disease ([9]Eriksson*, et al.*, 1999) and the occurrence of type 2 diabetes ([8]Ong*, et al.*, 2000, [90]Forsén*, et al.*, 2000). This supports the British birth cohort where men who were light at birth have the greater risk of developing obesity if they grew rapidly during childhood ([98]Parsons*, et al.*, 2001), and other studies investigating the effects of catch-up

growth on stature and adiposity ([8]Ong*, et al.*, 2000, [13]Albertsson Wikland*, et al.*, 1998). The highest death rates from coronary heart disease occurred in boys who were thin at birth but whose weight caught up so that they had an average or above average body mass from the age of 7 years ([9]Eriksson*, et al.*, 1999). Death from coronary heart disease may therefore be a consequence of poor prenatal nutrition followed by improved postnatal nutrition ([9]Eriksson*, et al.*, 1999).

The major questions that remain unresolved from this study are how tissues may become more sensitive to these anabolic hormones, in early postnatal life and the manner in which the body knows when to stop the increased rates of growth seen with catch-up growth. Furthermore, another tissue characteristic that may be modified ('programmed') with placental restriction of fetal growth is receptor number. Expression of insulin, IGF-I/II, and/or thyroid hormone receptors or their post-receptor signalling may be transiently up regulated to allow accelerated growth to occur through increased hormone sensitivity. Thus there may be possible post-receptor events that could be amplified in these small lambs so that when insulin, IGFs and/or TH binds with its receptor the post-receptor signal is enhanced. The abundance and structure of these receptors in tissues (especially muscle and fat) from growth-restricted lambs should also be examined in future.

Myostatin may be the bodyweight/skeletal muscle mass signaller which may act as a negative regulator of skeletal muscle mass ([300]Lee*, et al.*, 2001). It inhibits the anabolic actions of insulin/IGFs in muscle, and may signal that the normal

body muscle mass has been restored. Therefore increased concentrations of myostatin in these low birth weight animals may help contribute to and help explain their reduced muscle mass seen at birth, whereas if low concentrations of myostatin or an increased IGF-I/myostatin ratio occurs in early postnatal life following placental restriction, it may help to explain the mechanism of increased muscle mass seen during the catch-up growth period after IUGR. The structure of long bones may also be altered with placental restriction. Delayed senescence of long bones has been suggested to be the underlying cause of catch-up growth following restriction due to dexamethasone administration to growing rabbits in order to suppress their linear growth ([209]Gafni*, et al.*, 2001). These findings suggest that linear catch-up growth after placental restriction and IUGR is being caused in part by a delay in growth plate senescence ([209]Gafni*, et al.*, 2001).

In particular, insulin and the type 1 IGF-I receptor numbers in adipose tissue should be examined to determine whether this contributes to enhanced insulin and IGF-I sensitivity of adipose tissue. The structure of skeletal muscle may also be altered by placental restriction. Recently it was shown that IUGR alters the development and function of skeletal muscles in pigs ([288]Wank*, et al.*, 2000). Muscle fibre type estimation revealed an increased proportion of type I fibres in flexor digitalis superficialis and gastrocnemius medialis in IUGR piglets at one day of age ([288]Wank*, et al.*, 2000). This increase in the insulin sensitive muscle fibre type (more type I fibres) suggests that IUGR piglets have increased sensitivity of glucose and amino acids to insulin in early neonatal life.

In conclusion, we have shown that the placentally restricted lamb is born small at birth, as indicated by the reduction in size of the majority of body parameters. The restricted lambs grow faster in postnatal life up to 45 days of age, their whole body metabolic sensitivity of free fatty acid metabolism to insulin and IGF-I is increased and they have increased circulating concentrations of thyroid hormones. In addition, they have increased adiposity, particularly visceral fat, by 45 days of age (Figure 7.1). The mechanism that promotes this increased postnatal growth and adiposity in both IUGR infants and experimentally restricted animals requires further investigation. Ideally, better growth *in utero* may alleviate some of the problems that IUGR infants undergo postnatally and later in life. Interventions that could overcome the problem of the adverse environment that restricts growth and reduces the incidence of small size at birth and catch-up growth, could normalise circulating levels of IGFs and TH, sensitivity to these hormones, and hence reduce the risk of developing adult onset obesity and related adult diseases, would be beneficial (Figure 7.1). Interventions postnatally to ameliorate the adverse long-term consequences of intrauterine growth restriction may be more feasible however. Design and development of effective postnatal interventions would require further investigation to understand more fully the mechanisms responsible for catch-up growth and its associated increased adiposity.

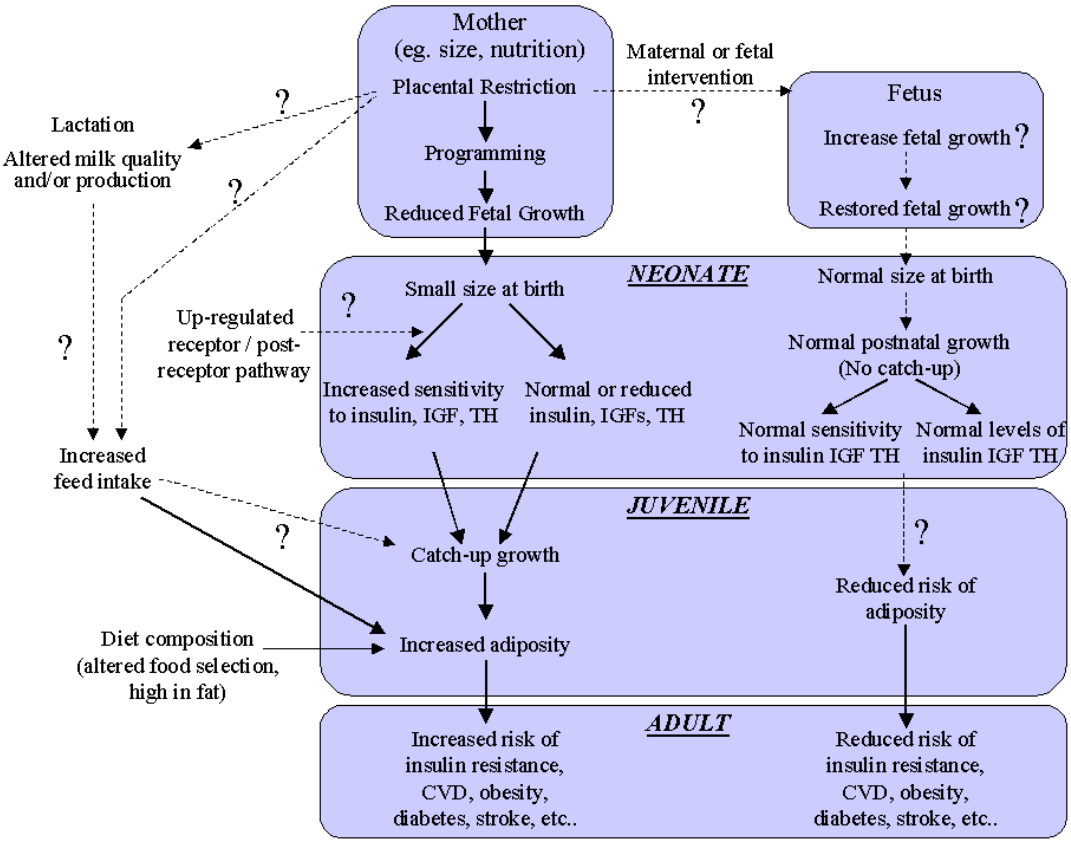

Figure 7.1. Flow diagram describing the findings of this study and what is currently unknown.

Solid arrows represent known information, and dashed arrows indicate what is currently unknown. Demonstrates the proposed effects of fetal growth restriction at neonatal, juvenile and adult stages of life.

Chapter 8

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Chapter 9

APPENDICES

9.1 Additional Tables and Figures

9.1.1 Summary of experiments timetable, protocols in the lamb

Experiment, protocol (LAMB)	Control and Placentally Restricted lambs (postnatal age (days))
Vascular catheterisation	4-5d
Body weight, Dimensions	0, 5, 10, 15, 20, 25, 30, 35, 40, 45d
GH Profile	10, 38d
Insulin sensitivity *in vivo* (Hyperinsulinaemic euglycaemic Clamp + 3-^{3}H-glucose)	8, 30d
Intravenous Glucose Tolerance Test	32d
IGF-I Sensitivity *in vivo* (Hyper-IGF-I euglycaemic Clamp + 3-^{3}H-glucose)	35d
Post-mortem	45d

9.1.2 Summary of experiments and protocols in the pregnant ewe (data not shown in this thesis)

Experiment, protocol (MATERNAL)	Placental Restriction Age (days)
Body weight, condition score	90, 110, 119, 130, 140d gestation birth, 5, 10, 30, 40d postnatal
Measure frame size	110d gestation
Measure mammary gland size	110, 120, 130, 140d gestation birth, 5, 10, 30, 40d postnatal
Measure of feed intake	Daily from 110d gestation until delivery
Ultrasound-vascular resistance	119, 130, 140d gestation
Ultrasound-fetal and placentome size	120, 130, 140d gestation
Observe length of labour	~145-150d gestation

9.2 Drug Summary

Trade name	**Active ingredients & form**	**Manufacturer**
Actrapid human insulin	Human insulin BP 10 mL vial of 100 U/mL	Novo Nordisk AS, Bagsvaerd, Denmark
Human IGF-I	Recombinant human (rh) IGF-I, Animal media grade, lyophilised powder	GroPep Pty. Ltd., Adelaide, Australia
Saline for injection	0.9% Sodium Chloride intravenous infusion	Baxter Healthcare, NSW, Australia
Glucose for injection	25% Glucose intravenous infusion	Baxter Healthcare, NSW, Australia
Heparin injection BP	Heparin sodium (porcine mucous) 50 ampoules of 25,000 IU in 5mL	CSL Limited VIC Australia
Pentothal	Thiopentone sodium BP Sterile powder 5g (made up in 50 mL sterile water for use)	Rhone Merieux, Pinkenba QLD Australia
Halothane inhalation anaesthetic	Fluothane	Zeneca Ltd, Cheshire UK
Ilium xylazil (analgesic, sedative)	Xylazine hydrochloride 20 mg/mL, solution	Troy Laboratories Pty Ltd, NSW, Australia
Ilium penstrep	Suspension	Troy Laboratories Pty

(antibiotic)	250 mg/mL Procaine penicillin 250 mg/mL Dihydrostreptomycin sulphate 20 mg/mL Procaine hydrochloride	Ltd, Smithfield NSW, Australia
Tricin triple antibiotic powder	250 IU/g bacitracin zinc 5 mg/g neomycin sulphate 5000 IU/g polymyxin B sulphate	Jurox Pty Ltd Silverwater NSW Australia
Norocillin (antibiotic)	LA (long acting) injection, broad spectrum antibiotic, Procaine Penicillin G 150mg/ml Benzathine Penicillin G 112.5mg/ml	Norbrook Laboratories Ltd. United Kingdom
Ampicillin 500mg	Ampicillin Sodium for injection, wide range bactericidal, 5 x 5ml vials	CSL Limited VIC Australia
Alcoholic Hibitane	Chlorhexidine gluconate, 5% w/v, and ethanol	Johnson and Johnson Australia
Betadine Surgical Scrub	7.5% w/v Povidone Iodine	Faulding Pharmaceuticals, SA, Australia

Betadine Antiseptic Solution	10% w/v Povidone Iodine	Faulding Pharmaceuticals SA, Australia
Lethabarb euthanasia injection	Pentobarbitone sodium 325 mg/mL	Virbac (Australia) Pty Ltd, NSW Australia
Iso-Pentane	C_5H_{12}	BDH Laboratory Supplies Australia

9.3 Instructions for use of CD

The compact disk presented here contains a copy of this thesis as a pdf (Portable Document Format) file.

In order to view this file you will require the Adobe Acrobat Reader program (available from the Adobe website: www.adobe.com).