# REGIONAL DIFFERENCES IN ADIPOSE TISSUE DEVELOPMENT: EFFECTS OF NUTRITIONAL CHALLENGES ON GENES INVOLVED IN INSULIN, INSULIN LIKE GROWTH FACTOR AND GLUCOCORTICOID SIGNALLING

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A thesis submitted for the degree of Doctor of Philosophy

to

The University of Nottingham

in collaboration with

The University of South Australia

**September 2009**

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## Declaration

This work contains no material which has been accepted for the award of any other degree or diploma in any university or other tertiary institution and, to the best of my knowledge and belief, contains no material previously published or written by another person, except where due reference has been made in the test.

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

**Signed:**  ……

**Date:**…28/09/09……

## Acknowledgements

So many people have contributed to this work, either directly or indirectly, I would like to thank them all for their endless support and help.

Special thanks to the members of the Division of Child Health in the University of Nottingham. During the first 18 months of my research project I have received so much support and help from all of you. Thank you to Hiten Mistry, Alison Mostyn, Paula Williams, Lesia Kurlak and Vicky Wilson for always being there with advice or just to offer a listening ear. Thank you also for all the enjoyable distractions from work. Thank you to Lesia Kurlak and Sylvain Sebert for helping me to analyse and troubleshoot every experiment that failed and there were many! Thank you to Helen Budge and Michael Symonds for their critical opinion and for the numerous discussions we had. Thanks also to Don Sharkey, Julia Pyne and Pablo Fainberg for their contributions.

Thanks also to the members of Sue Ozanne's research group at the University of Cambridge. Especially to Sue Ozanne herself who was so friendly in welcoming me in her laboratory. Further thanks go to Sophia Ekizoglou for her excellent way of teaching me how to do Western Blots. Thanks to Denise Fernandez-Twinn for helping me finish the Western Blot analyses in Cambridge.

Thanks to all the members of the Early Origins of Adult Health Research Group at the University of South Australia. Special thanks to Leewen Rattanatray who has spent so much of her energy and time on the animal work that was involved in this research project. Thanks to Beverly Muhlhausler and Song Zhang for all their advice and help. Thanks to Darran Tosh for helping with every single information technology problem that I faced. Thanks to Bernard Chuang, Andrew Snell, Severence MacLaughlin and Janna Morrison for their contributions.

Special thanks to all my friends and family that have been so supporting all along. Most of all, thanks to parents for their endless moral and financial support.

Finally thanks to my supervisors; professor Caroline McMillen and associate professor David Gardner. Thank you, Caroline for welcoming me in your laboratory in Adelaide. Thank you for always helping me to keep an overview of the big picture and making me believe that this thesis could and would be finished at some point. Thank you, David for always being there with advice even from the other side of the world. Thank you for your endless patience when I seemed to have forgotten about this PhD and thank you for responding to my emails so quickly again when I decided to get started again.

Thank you all.

## Related Publications

The effects of formula feeding on differential expression of key genes involved in adipose tissue growth. Bos PM, McMillen IC, Symonds ME and Gardner DS. To be submitted.

The effect of postnatal nutrition and juvenile-onset obesity on regional adiposity in sheep: depot-specific adipose tissue sensitivity to glucocorticoid and insulin signalling. Bos PM, Ekizoglou S, Ozanne SE, McMillen IC, Symonds ME and Gardner DS. To be submitted.

**Presentations at scientific conferences related to this thesis**

Gender specific influences of weight around conception on glucocorticoid sensitivity in different adipose tissue depots. Bos PM, Rattanatray L, MacLaughlin S, Muhlhausler B, Gardner DS, Symonds ME and McMillen IC. Nutrition Society Summer Meeting, Nottingham, UK (2008)

Differential expression of markers of insulin sensitivity in visceral adipose tissue depots in the neonatal sheep. Bos PM, Ekizoglou S, Ozanne SE, Symonds ME, McMillen IC, Gardner DS. 5th International Congress on Developmental Origins of Health & Disease, Perth, Australia (2007)

Influence of glucocorticoids on nutritional manipulation of regional adipose tissue distribution. Bos PM, Symonds ME, McMillen IC, Gardner DS. Perinatal Physiology: From Uterus to Brain meeting, Edinburgh, UK (2007) and Australian Society for Medical Research South Australia Scientific meeting, Adelaide, Australia (2007)

Influence of Obesity and Formula Feeding on Local Glucocorticoid Action in Regional Adipose Tissue (AT). Bos PM, Symonds ME, McMillen IC, Gardner DS. Joint meeting of The Neonatal Society and The Nutrition Society, Cambridge, UK (2006)

Local Glucocorticoid Action on Regional Adipose Tissue (AT) Development. Bos PM, Symonds ME, Gardner DS. Fetal and Neonatal Physiological Society meeting, Cambridge, UK (2006)

## Abstract

Adipose tissue development is regulated by a complex interaction between the local actions of insulin, glucocorticoids and insulin like growth factors (IGFs). A series of experiments was undertaken in which the normal development of individual adipose tissue depots and their development following periconceptional under- and overnutrition, formula feeding and juvenile obesity was investigated in sheep. Expression and abundance of glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenases (11β-HSDs), insulin receptor, p85 subunit of phosphatidylinositol 3-kinase (p85), glucose transporter 4 (Glut4), insulin like growth factor (IGF) 1 and 2 and their receptors (IGF-R) were measured as markers of sensitivity to glucocorticoids, insulin and IGFs in individual adipose tissue depots.

It was found that during early postnatal life omental adipose tissue grows faster than other depots. In all investigated groups there were marked differences in the expression of all investigated genes between adipose tissue depots.

No effect was found of periconceptional nutrition on expression of the investigated genes. Weight of the mother prior to conception was negatively associated with omental GR and 11β-HSD1. Free fatty acid levels at 4 months of age were related to omental and subcutaneous 11β-HSD1 expression. Perirenal expression of IGF1R at 4 months was negatively

correlated with perirenal and subcutaneous adipose tissue mass. IGF1R expression correlated with IR and GR expression.

Formula feeding resulted in reduced expression of Glut4 and increased 11β-HSD1 expression.

A combination of formula feeding and juvenile obesity resulted in a redistribution of adipose tissue in favour of the perirenal depot. Obesity *per se* resulted in a reduction of the expression of all genes and proteins examined.

We have shown significant differences in markers of tissue sensitivity to the actions of insulin, glucocorticoids and insulin like growth factors between different adipose tissue depots in the body, highlighting the importance of examining those depots individually in future studies.

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## Commonly Used Abbreviations

| | | |
|---|---|---|
| **A** | ACTH | adrenocorticotropic hormone |
| | AGA | average for gestational age |
| | ANOVA | analysis of variance |
| | ATP | adenosine triphosphate |
| | AVP | arginine vasopressin |
| **B** | 11β-HSD | 11β – hydroxysteroid dehydrogenase |
| | BMI | body mass index |
| | bp | base pairs |
| **C** | cAMP | cyclic adenosine monophosphate |
| | CC | control-control animal (fed 100% ME at all times) |
| | cDNA | complementary deoxyribonucleic acid |
| | CHD | coronary heart disease |
| | CR | control-restricted animal (fed 100% ME followed by 70% ME in the periconceptional period) |
| | CRH | corticotropin releasing hormone |
| | cT | comparative threshold |
| | CV | coefficient of variation |
| | CVD | cardiovascular disease |
| **D** | d | days |
| | DNA | deoxyribonucleic acid |
| **E** | ewe | ewe-reared animal |
| **F** | FF | formula fed lamb |

| | | |
|---|---|---|
| | FFA | free fatty acids |
| **G** | G3PDH | glycerol-3-phosphate dehydrogenase |
| | GAPDH | glyceraldehyde-3-phosphate dehydrogenase |
| | GA | gestational age |
| | GC | glucocorticoid |
| | GH | growth hormone |
| | GHRH | growth hormone releasing hormone |
| | GLUT | glucose transporter |
| | GR | glucocorticoid receptor |
| **H** | h | hours |
| | HBW | high birth weight |
| | HDL | high density lipoprotein |
| | HH | high-high fed animal (*ad libitum* during periconceptional period) |
| | HPA | hypothalamic pituitary adrenal |
| | HR | high-restriction feed animal (*ad libitum* followed by 70% ME during periconceptional period) |
| | HSL | hormone sensitive lipase |
| **I** | IGF | insulin-like growth factor |
| | IGFBP | insulin-like growth factor binding protein |
| | IGFR | insulin-like growth factor receptor |
| | IL | interleukin |
| | IR | insulin receptor |
| | IRS | insulin receptor substrate |
| | i.m. | intramuscular |
| | i.v. | intravenous |
| | IUGR | intra-uterine growth retardation |

| | | |
|---|---|---|
| **J** | | |
| **K** | KO | knock out |
| **L** | L | lean animal |
| | LBW | low birth weight |
| | LDL | low density lipoprotein |
| | LGA | large for gestational age |
| | LPL | lipoprotein lipase |
| **M** | ME | metabolisable energy |
| | MR | mineralcorticoid receptor |
| | mRNA | messenger ribonucleic acid |
| **N** | NADH | nicotinamide adenine dinucleotide |
| | NEFA | non-esterified fatty acid |
| **O** | O | obese animal |
| | OFF | obese formula fed animal |
| **P** | P85 | p85 subunit of phophatidylinositol 3 kinase |
| | PAT | perirenal adipose tissue |
| | PCON | periconceptional overnutrition |
| | PCR | polymerase chain reaction |
| | PCUN | periconceptional undernutrition |
| | PI3-kinase | phophatidylinositol 3 kinase |
| | PPAR$\gamma$ | proliferator-activated receptor-$\gamma$ |
| | PR | placentally restricted |

| | | |
|---|---|---|
| **Q** | qRTPCR | quantitative real-time polymerase chain reaction |
| **R** | RNA | ribonucleic acid |
| | RpP0 | ribosomal protein P0 |
| | RT-PCR | real-time polymerase chain reaction |
| | RXR | retinoid-x-receptor |
| **S** | SD | standard deviation |
| | SEM | standard error of the mean |
| | SGA | small for gestational age |
| | SREBP1c | sterol regulating element binding protein 1C |
| **T** | T2DM | type 2 diabetes mellitus |
| | TG | triglyceride |
| **U** | UK | United Kingdom |
| **V** | vLDL | very low density lipoprotein |
| **W** | WHO | World Health Organisation |
| | WHR | waist hip ratio |

**XYZ**

# 1. Literature Review

## 1.1 Obesity in our present society

In our present day society obesity is a massive health problem. Obesity is defined as a body mass index (BMI) of 30 kg/m$^2$ or higher and overweight is defined as a body mass index of 25 kg/m$^2$ or higher. The WHO estimated that globally 1.6 billion adults were classified as overweight, while 400 million were classified as obese in 2005 (WHO, 2006), furthermore the WHO predicts that by 2015 these numbers will have risen to 2.3 billion adults being classified as overweight and 700 million being classified as obese (WHO, 2006).

In England 43% of men and 33% of women are considered overweight, while 22% of men and 24% of women are classified obese (BHF, 2006). The number of obese adults has increased by more than 50% during the last decade (BHF, 2006).

According to the World Health Organization, 8-15% of all disease burden in developed countries is caused by overweight and obesity and 21% of all cases of coronary heart disease (CHD) are caused by overweight (Murray & Lopez, 2002).

Of particular worry is the prevalence of obesity amongst children. In the United States 16% of children aged 2-19 years were considered obese in

2001-2002 (Hedley *et al.*, 2004), while in the same period in Australia 5% of primary school children were obese. It has been shown that approximately 25-50% of obese children will remain obese in adulthood (Hedley *et al.*, 2004).

Obesity is an important risk factor for the metabolic syndrome, which is a co-occurrence of four metabolic risk factors and diseases; insulin resistance, type 2 diabetes mellitus (T2DM), dyslipidaemia and hypertension (Reaven, 1993). The metabolic syndrome is, like obesity, an important risk factor for the development of cardiovascular disease (CVD). CVD and T2DM are the major health problems in westernised countries accounting for 40% of all deaths in the United Kingdom (UK) in 2003 (BHF, 2005). Coronary heart disease and stroke are the two major clinical outcomes of CVD. Coronary heart disease on its own is estimated to cost the UK economy about £7900 million a year (BHF, 2005).

The metabolic risk factors associated with obesity are dependent on the regional organisation (distribution) of adipose tissue: increased central i.e. around the viscera, as opposed to peripheral e.g. subcutaneous adipose tissue is associated with higher cardiovascular risk (Krotkiewski *et al.*, 1983; Fujioka *et al.*, 1987; Despres *et al.*, 1989; Pouliot *et al.*, 1992; Wajchenberg, 2000; Busetto, 2001; Miyazaki *et al.*, 2002; Misra *et al.*, 2004; Despres & Lemieux, 2006). According to the INTERHEART case-control study approximately 63% of all heart attacks in Western Europe are secondary to abdominal obesity (Yusuf *et al.*, 2004). The risk of a heart attack doubles in people with an increased waist-hip ratio (WHR) compared to people with a

normal WHR (Yusuf *et al.*, 2004). The surgical removal of visceral adipose tissue leads to an improvement in metabolic status both in rodents and in humans (Gabriely *et al.*, 2002; Thorne *et al.*, 2002; Gabriely & Barzilai, 2003). A study by Butler and colleagues indicated that in adults of 20 years and older truncal skinfold thickness could independently predict the development of type 2 diabetes (Butler *et al.*, 1982). The prevalence of central obesity (defined as a WHR ≥ 0.95 in men and WHR ≥ 0.85 in women) in England was 33% in men and 30% in women in 2003 (BHF, 2006).

Because of the enormous impact that visceral obesity has on our society, particularly on our health and economy, it is important to understand the underlying aetiology of the development of visceral obesity.

## 1.2 Programming of adipose tissue depots in early life

A large number of studies have investigated factors in early life that can program the onset of obesity and cardiovascular diseases in childhood and adulthood. In 1986 Barker and his colleagues described the “early” origins of adult disease hypothesis which stated that ‘*environmental factors, particularly nutrition, act in early life to program the risks for the early onset of cardiovascular and metabolic disease in adult life*’ (Barker & Osmond, 1986). They based this hypothesis on the observation that the geographical distribution of mortality rates of ischaemic heart disease in England and

Wales during 1968 - 1978 was strongly related to the geographical distribution of neonatal mortality rates during 1921 - 1925 (Barker & Osmond, 1986).

Studies that investigated the effects of the Dutch hunger winter of 1944 – 1945 have further confirmed this hypothesis. Women that were pregnant during the Dutch hunger winter of 1944 - 1945 produced offspring that were more likely to become obese and suffer from other metabolic diseases such as diabetes and hypertension in later life (Ravelli *et al.*, 2000; Roseboom *et al.*, 2001, 2003).

Numerous other studies have further investigated the early origins of adult health and disease hypothesis and a strong relationship between birth weight and the development of obesity in later life has been demonstrated (McMillen & Robinson, 2005; Taylor & Poston, 2007; Vickers *et al.*, 2007; Yajnik & Deshmukh, 2008).

Some of the studies that confirmed this relationship were large studies that followed the 1958 British birth cohort. Studies based on the British birth cohort demonstrated that a combination of low birth weight and a high postnatal growth rate amplified the risk of developing obesity (Ravelli *et al.*, 1976; Parsons *et al.*, 2001). Another study examined the relationship between early growth patterns and the development of obesity in adulthood in a French cohort of 21-27 year olds with intra-uterine growth restriction or normal intra-uterine growth. It found that early growth patterns were not associated with body weight or BMI, but that a pattern of rapid growth in

early life was associated with an increase in the percentage body fat mass (Jaquet *et al.*, 2000).

Several other studies confirmed that small for gestational age (SGA) infants are more likely to have accelerated growth in early postnatal life, so-called 'catch-up growth' (Fitzhardinge & Steven, 1972; Albertsson-Wikland *et al.*, 1993) and that SGA infants have a higher relative body fat mass from 2-12 months of age (Hediger *et al.*, 1998; Ong *et al.*, 2000; Brown *et al.*, 2002; Gray *et al.*, 2002; Cole, 2004). These relationships between birth weight and catch up growth and adipose tissue mass in later life are also found in other large mammals such as sheep and pigs (Greenwood *et al.*, 1998; Poore & Fowden, 2004; Louey *et al.*, 2005; De Blasio *et al.*, 2007).

A large body of evidence has indicated that birth weight is not only related to the development of obesity in later life, but also to a distribution of adipose tissue which favours the central areas. The 1979 Amsterdam Growth and Health Longitudinal Study found associations between low birth weight (LBW) and a high waist circumference (in females) or a high waist-hip ratio (in males) in adult life (Te Velde *et al.*, 2003). Another study which looked at 7 to 12 year old American school children found that LBW was associated with relatively more central subcutaneous adipose tissue and an increase in truncal fat (Malina *et al.*, 1996). It estimated that 2-8% of the variation in relative subcutaneous adipose tissue distribution at school age is explained by variation in birth weight (Malina *et al.*, 1996). While Barker and colleagues estimated that for every kilogram decrease in birth weight resulted in a 7%

increase of subscapular skinfold thickness (Barker *et al.*, 1997). Loos and colleagues compared male twins at the age of 18-34 years. They found that the twin who was lighter at birth was also shorter and lighter as an adult, had a higher waist-hip ratio and less lean body mass compared to his heavier sibling (Loos *et al.*, 2001). Comparisons between Indian and Caucasian babies also indicated that poor intra-uterine growth was a predictor of a higher central adipose tissue mass at the age of 8 years (Bavdekar *et al.*, 1999; Yajnik, 2000).

Other studies found that growth restricted infants with catch up growth had an increased body mass index during the first year of life (Jaquet *et al.*, 1999) and a higher body mass index, higher adipose tissue mass and an increase in truncal fat distribution during childhood (Ong *et al.*, 2000) and in adult life (Law *et al.*, 1992; Leger *et al.*, 1997; Jaquet *et al.*, 2001; Parsons *et al.*, 2001).

Numerous experimental animal models have been developed that mimicked the relationship between intra-uterine growth restriction, catch up growth and central adiposity in later life. Maternal nutrient restriction in sheep and other animals did not lead to offspring with low birth weight, but did lead to offspring with increased postnatal growth rate, increased prevalence of obesity during childhood and an increase in visceral adipose tissue distribution (Heasman *et al.*, 1999; Poore & Fowden, 2004; Ford *et al.*, 2007; Symonds *et al.*, 2009). Vickers and colleagues showed that rat pups that were low birth weight as a result of maternal nutrient restriction, had an increased feed intake from weaning to adulthood, which was associated with

a greater retroperitoneal and gonadal adipose tissue mass. They also showed that these pups had higher levels of circulating leptin and insulin, but not glucose (Vickers *et al.*, 2000; Vickers *et al.*, 2001).

Other models that are often used to study the effects of a poor early nutritional environment on the development of adipose tissue are the models of placental restriction (PR). PR can be experimentally induced by uterine carunclectomy, maternal hyperthermia, single umbilical artery ligation, bilateral uterine artery ligation, placental embolisation and overfeeding of the adolescent ewe (Simmons *et al.*, 2001; Morrison, 2008). Placental restriction in sheep has been shown to result in asymmetrical foetal growth restriction with a higher reduction in body weight compared to crown-rump length or girth (Owens *et al.*, 1994; Robinson *et al.*, 1994; Kind *et al.*, 1995). Offspring of rats with placental insufficiency have increased postnatal growth rates and increased perirenal, gonadal and omental adipose tissue masses (Simmons *et al.*, 2001). Lambs that have been placentally restricted in utero have an increased postnatal growth rate, an increase in relative perirenal fat mass and an increase in whole body insulin sensitivity compared to controls at 6 and 8 weeks of age, as indicated by the capacity of insulin to suppress circulating FFA levels (Louey *et al.*, 2005; De Blasio *et al.*, 2007).

The ‘thrifty phenotype hypothesis’ provides a mechanism that explains the relation between low birth weight or a sub-optimal intra-uterine environment and the development of obesity in later life. The hypothesis states that poor nutrition in early life leads to permanent programmed changes in glucose-

insulin metabolism in the offspring leading to an increase in insulin resistance in later life (Hales & Barker, 2001).

It is clear that a restriction in nutrient supply during pregnancy can have detrimental effects on the later health of offspring, but it appears that these effects can even find their origin earlier in life during the periconceptional period. Exposure of the oocyte and/or embryo to maternal undernutrition (periconceptional undernutrition; PCUN) resulted in a decrease in foetal weight and an increase in relative perirenal adipose tissue in twin pregnancies in sheep (Edwards *et al.*, 2005). Furthermore, a relationship was seen in twin pregnancies between weight loss of the ewe and placenta and offspring size, such that an increase in weight loss resulted in relatively heavier placentae and larger foetuses at 55 days of gestation (MacLaughlin *et al.*, 2005). Furthermore, it has been shown that PCUN in sheep results in an increase in arterial blood pressure in the foetus and an increased and precocious activation of the HPA axis during late gestation (Edwards & McMillen, 2002a, b; Edwards *et al.*, 2005).

When maternal undernutrition extends from the periconceptional period into early pregnancy (i.e. from 60 days prior to conception and up to 30 days of pregnancy) a reduced foetal growth rate occurs with hyperactivation of the HPA axis and an increase in premature deliveries in sheep (Bloomfield *et al.*, 2003; Oliver *et al.*, 2005)

As opposed to challenges that restrict the nutritional supply to the foetus an increasing number of researchers are investigating the effects of an increased supply of nutrients to the foetus on the development of obesity. Rats that were fed a diet that consisted of 24% fat produced offspring with an increased body weight and and increased visceral adipose tissue mass (Khan *et al.*, 2003; Khan *et al.*, 2004; Taylor *et al.*, 2005).

Similar observations are seen in women who are obese during pregnancy. Obese women are more likely to develop gestational diabetes or reduced insulin sensitivity (Sebire *et al.*, 2001; LaCoursiere *et al.*, 2005). The increased nutrient supply, associated with the reduction in insulin sensitivity, can result in increased growth of the foetus and macrosomia (Sebire *et al.*, 2001; Yajnik *et al.*, 2002; Bergmann *et al.*, 2003; Catalano *et al.*, 2003; Gillman *et al.*, 2003; Jensen *et al.*, 2003; Ehrenberg *et al.*, 2004). Catalano and colleagues showed that the strongest predictor of foetal fat accretion was pregravid maternal insulin sensitivity. They hypothesised that obese women, having lower insulin sensitivities, are therefore more at risk of having a large baby (Catalano *et al.*, 1995). The Hyperglycaemia and Adverse Pregnancy Outcome Study Cooperative Research Group found that even in normal maternal glucose ranges there is an association between maternal glucose levels and birth weight of the offspring (Metzger *et al.*, 2008), while Jensen and colleagues showed that even in women with a normal glucose tolerance, obesity or being overweight was still associated with an increased risk of macrosomia (Jensen *et al.*, 2003).

In addition, babies that are born with a high birth weight are more likely to stay heavy throughout childhood and adulthood and thus have an increased risk of obesity in adulthood (Curhan *et al.*, 1996a; Curhan *et al.*, 1996b; Sorensen *et al.*, 1997; Rasmussen & Johansson, 1998; Parsons *et al.*, 2001; Pietilainen *et al.*, 2001; Gillman *et al.*, 2003; Oken & Gillman, 2003). Whether obesity during the periconceptional period and during pregnancy affects the distribution of adipose tissue in the offspring has yet to be established.

A possible mechanism that explains the relation between high birth weight and obesity in later life could be a genetic predisposition. Furthermore an increase in adipose tissue at birth, as is likely to be present in the high birth weight infant, leads to an increase of FFA exposure to the liver which ultimately leads to reduced skeletal muscle insulin sensitivity and therefore increased lipogenesis which leads to further adipose tissue growth and a perputeous cycle. This cycle and the underlying mechanism of this cycle are described in more detail in section 1.3.4.2.

Another nutritional challenge that could affect adipose tissue development and nutrition occurs during a critical period in early postnatal life, lactation. The effect of breastfeeding on the incidence of obesity and cardiovascular risk factors in adulthood has been a subject of debate in many studies over the last decades. Numerous studies in developed and developing countries have shown that breastfeeding leads to a reduced prevalence of obesity or overweight at school age and later on in life and that this effect is still present when confounding factors such as social class are taken into account (Pettitt

*et al.*, 1997; von Kries *et al.*, 1999; Toschke *et al.*, 2002). Some studies also showed a dose response relation between the duration of breastfeeding and the risks of developing obesity or overweight later in life (von Kries *et al.*, 1999; Gillman *et al.*, 2001). Interestingly, a study of the Dutch famine cohort showed no difference in BMI, waist-circumference and waist-hip ratio between breast and formula fed individuals (Ravelli *et al.*, 2000). Several large meta-analyses have been performed in order to elucidate the real relationship between breastfeeding and obesity. The general conclusion was a small but significant protective effect of breastfeeding on the incidence of obesity later in life (Dietz, 2001; Dewey, 2003; Arenz *et al.*, 2004; Harder *et al.*, 2005; Owen *et al.*, 2005). Both Harder and colleagues and Owen and colleagues managed to confirm a dose response relationship in their meta-analyses (Harder *et al.*, 2005; Owen *et al.*, 2005); whilst Dewey and colleagues concluded that the protective effect of breast feeding was more prominent in adolescence compared to childhood, suggesting a programmed effect (Dewey, 2003).

It seems to be clear that challenges of over- or undernutrition during the periconceptional, gestational and immediate postnatal period can have long lasting effects on health in later life, including significant changes in the prevalence of obesity and in particular visceral adipose tissue. The mechanisms that are responsible for these programmed effects, and particularly for the effect of increased visceral adipose tissue, are not completely elucidated yet and are subject of this study.

Cross ref show s a summary of animal models used to investigate challenges in early life on obesity (**Error! Reference source not found.**).

| Animal model | Intervention | Main outcome | Reference |
|---|---|---|---|
| Sheep | Maternal nutrient restriction | No effect on birth weight<br>↑ postnatal growth rate<br>↑obesity prevalence in childhood<br>↑ visceral adipose tissue distribution | Heasman *et al,* 1999<br>Ford *et al,* 2007<br>Symonds *et al,* 2009 |
| Rat | Maternal nutrient restriction | ↑feed intake from weaning in LBW<br>↑retroperitoneal & gonadal adipose tissue in LBW<br>↑circulating leptin and insulin | Vickers *et al*, 2000<br>Vickers *et al*, 2001 |
| Pig | LBW vs HBW comparison | ↑ fat depth in adult LBW animals<br>↓plasma leptin in LBW animals | Poore & Fowden, 2004 |
| Sheep | Placental restriction | Assymetrical fetal growth restriction<br>↑postnatal growth rate<br>↑relative perirenal adipose tissue<br>↑insulin sensitivity at 6 & 8 weeks postnatal age | Owens *et al,* 1994<br>Robinson *et al,* 1994<br>Kind *et al,* 1995<br>Louey *et al,* 2005<br>De Blasio *et al,* 2007 |
| Rats | Placental insufficiency | ↑postnatal growth rate<br>↑perirenal, gonadal & omental adipose tissue | Simmons *et al,* 2001 |
| Sheep | Periconceptional undernutrition | ↓foetal weight<br>↑relative perirenal adipose tissue in twins<br>↑arterial blood pressure in foetus<br>Precocious activation HPA axis late gestation | Edwards *et al,* 2005<br>Edwards & McMillen, 2002a |
| Sheep | Periconceptional undernutrition + maternal nutrient restriction up to day 30 gestation | ↓foetal growth rate<br>↑hyperactivation HPA axis<br>↑premature deliveries | Bloomfield *et al,* 2003<br>Oliver *et al,* 2005 |
| Rats | Maternal high fat diet | ↑birth weight<br>↑visceral adipose tissue mass | Khan *et al,* 2003<br>Khan *et al,* 2004<br>Taylor *et al,* 2005 |

**Table 1.1; Summary of animal models used to investigate challenges in early life on obesity**

## 1.3 Adipose tissue growth and development

Adipose tissue is an abundant tissue in the adult human body. Average body composition of an adult man is 9-18% adipose tissue and of an adult woman is 14-28% adipose tissue (DiGirolamo & Fine, 2000). Adipose tissue distinguishes itself from other tissues by its almost unlimited ability to expand in size. In obese people total fat mass may comprise 60 to 70% of body weight (Hausman *et al.*, 2001).

### 1.3.1 ADIPOSE TISSUE DEVELOPMENT IN EARLY LIFE

Human foetuses begin to store adipose tissue, in the form of multilocular or brown adipose tissue, from the 20$^{th}$ week of gestation onwards (Merklin, 1974; Himms-Hagen & Ricquier, 1998; Cannon & Nedergaard, 2004). In sheep, adipose tissue, in the form of multilocular or brown adipocytes, can be detected from as early as 70 days gestation. From about 70 – 110 days gestation brown adipose tissue undergoes a relatively accelerated growth as opposed to total body mass (Alexander, 1978). During late gestation adipose tissue grows in proportion to whole body mass in sheep (Alexander, 1978). The tissue starts to develop as individual clusters of lipid droplets which later confluence to form adipose tissue depots. The size of the clusters increases while the number of clusters remains relatively constant during growth (Ailhaud *et al.*, 1992). Just like in humans, in pig and sheep, brown adipose

tissue depots are detectable before birth (Merklin, 1974; Alexander, 1978; Hausman & Richardson, 1982). However the total contribution of adipose tissue to body mass is only 1.5% in newborn sheep, whilst in the human, adipose tissue contributes approximately 16% of body mass (Fowden, 1995).

During the first 7-30 days of postnatal life in a sheep adipose tissue undergoes rapid growth. Perirenal adipose tissue mass remains stable for the first two days of life but proliferates rapidly thereafter (Clarke *et al.*, 1997b). During early postnatal life white or unilocular adipose tissue in the perirenal depot grows relatively faster than the liver, lung, heart, kidneys and adrenal glands (Clarke *et al.*, 1997b). In foetal life most adipocytes contain multiple lipid locules. Tissue with predominantly multilocular adipocytes is called brown adipose tissue. During the first 2-3 weeks of postnatal life most of those multilocular adipocytes undergo a transformation and become unilocular cells. Tissue with predominantly unilocular adipocytes is called white adipose tissue (Gemmell *et al.*, 1972). These cells have one large lipid droplet, which can contribute to 80-95% of adipocyte cell volume. The nucleus and cytoplasm of the cells are eccentrically located and form a rim at the side of the cell. In sheep, as well as in humans, nearly all multilocular adipocytes have transformed and display the morphometric and molecular characteristics of mature unilocular adipocytes by 30 days postnatal age (Gemmell *et al.*, 1972; Clarke *et al.*, 1997b; Mostyn *et al.*, 2003).

### 1.3.2 ADIPOSE TISSUE GROWTH

In a state of positive energy balance then adipose tissue growth occurs. This growth can be either due to an increase in cell size (hypertrophy) or an increase in adipocyte number (hyperplastic growth). Many authors nowadays believe that adipocytes are unable to expand beyond a given maximum volume. After these cells have reached their maximum volume it is believed that the increased stretch will trigger local preadipocytes to differentiate into mature adipocytes. Thus according to this theory the adipocytes induce hyperplastic growth when hypertrophy is no longer possible (Bjorntorp, 1974; Faust *et al.*, 1978; Bjorntorp, 1991; Cinti, 2005). Other authors believe that maximum cell number is determined during early life, which limits the ability of hyperplastic growth (Spalding *et al.*, 2008).

#### *1.3.2.1 Lipogenesis*

After a meal the plasma free fatty acid (FFA) concentration rises. FFAs enter adipocytes where they are transformed into and stored as triglycerides. An essential factor in this process of lipogenesis is lipoprotein lipase (LPL).

LPL is an enzyme that is present on the luminal endothelial surface of tissues such as adipose tissue and skeletal muscle. It is essential in the hydrolysis of triacylglycerol from circulating chylomicrons and very low density lipoprotein (vLDL) into fatty acids that can enter the cell (Braun & Severson, 1992). LPL is produced mainly in adipocytes, myocytes and cardiomyocytes (Semenkovich *et al.*, 1989a). LPL mRNA levels in adipocytes are increased by the effects of insulin. Insulin however, does not

alter the rate of LPL gene transcription which suggests that insulin increases LPL mRNA stability (Semenkovich *et al.*, 1989b).

LPL mRNA expression in adipose tissue is influenced by peroxisome proliferator-activated receptor-γ (PPARγ). PPARγ, when combined with retinoid-x-receptor-alpha (RXRα), binds to peroxisome proliferator response elements on the DNA and acts as a general transcription factor. It stimulates LPL mRNA expression but also influences genes involved in adipocyte proliferation (Schoonjans *et al.*, 1996; Gregoire *et al.*, 1998; Auwerx, 1999; Kersten *et al.*, 2000; Combs *et al.*, 2002; Picard & Auwerx, 2002).

Once free fatty acids have entered the cell they bind to acetyl coenzyme A (acetyl CoA). Once bound to acetyl CoA, three FFA molecules can bind with one glycerol molecule to form a triglyceride. More details about this process can be found in Figure 1.1.

Glucocorticoids are also likely to have a lipogenic effect by stimulation of PPARγ and by stimulation of sterol regulating element binding protein 1C (SREBP1C), which also functions as a transcriptor factor in the regulation of LPL (Samra *et al.*, 1998). The possible role of glucocorticoids in lipogenesis will be discussed in more detail in paragraph 1.5.

Another factor that influences lipogenesis is growth hormone (GH). GH interferes with insulin signalling at the post-receptor level and therefore reduces insulin sensitivity and ultimately adipogenesis (Yin *et al.*, 1998).

An overview of the processes involved in lipogenesis can be found in **Error! Reference source not found.**.

#### *1.3.2.2 Lipolysis*

In periods of negative energy balance triglycerides in adipocytes are broken down into free fatty acids and released into the blood stream. This process of lipolysis is catalysed by the enzyme hormone sensitive lipase (HSL) and initiated in response to increased glucagon levels (Braun & Severson, 1992). The regulation of lipolysis is a very complicated process and involves numerous factors. Glucocorticoids have been shown to increase HSL in *in vitro* studies and therefore stimulate lipolysis (Slavin *et al.*, 1994). Insulin, on the other hand, has a direct inhibitory effect on lipolysis. The effects of insulin and glucocorticoids on adipose tissue growth will be discussed in more detail in paragraph 1.4 and 1.5 respectively.

Other factors that influence lipolysis include catecholamines, growth hormone and leptin. Adipose tissue is innervated by sympathetic nerve fibres with noradrenaline as the neurotransmitter. The nerve endings innervate the vessels up to the smallest capillaries and the adipose cells itself via neuro-adipocytic junctions which contain $\alpha_2$, $\beta_{1,}$ $\beta_2$ and $\beta_3$ receptors. Activation of beta-receptors leads to increased levels of cyclic AMP in the cell, which leads to an increase in the activation of protein kinase A, resulting in activation of hormone sensitive lipase and therefore stimulating lipolysis. Circulating adrenaline can also stimulate lipolysis through this pathway (Belfrage *et al.*, 1981; Bjorgell *et al.*, 1981; Stralfors & Belfrage, 1983; Frayn, 2003; Cinti, 2005).

Growth hormone has been shown to stimulate lipolysis by inhibiting the effects of insulin (Beck *et al.*, 1957; Zierler & Rabinowitz, 1963). Several studies have indicated that circulating FFA levels rise following continuous or pulsatile GH administration (Moller *et al.*, 1990a; Moller *et al.*, 1990b; Moller *et al.*, 1992).

Leptin is a 16kDA protein that is mainly produced by adipose tissue (Zhang *et al.*, 1994). The expression of leptin is directly related to lipid content and size of adipocytes (Lonnqvist *et al.*, 1997; Chilliard *et al.*, 2001; Muhlhausler *et al.*, 2002). Leptin is an important factor in energy homeostasis; it decreases appetite and increases energy expenditure. Leptin has been shown to stimulate fatty acid oxidation and release in adipocytes and skeletal muscle of rats both *in vitro* and *in vivo* (Shimabukuro *et al.*, 1997; William *et al.*, 2002).

An overview of the processes involved in lipolysis can be found in **Error! Reference source not found.**.

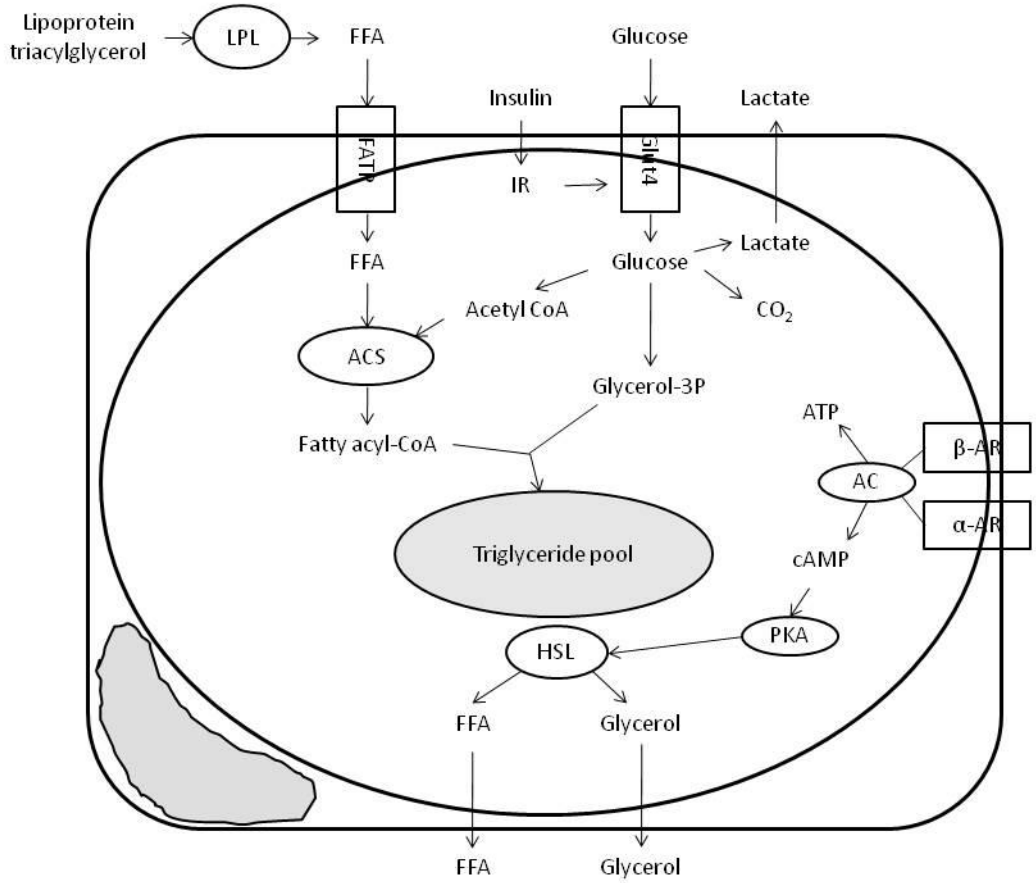


**Figure 1.1; Overview of lipogenesis and lipolysis.**

*AC,* adenylate cyclise; *ACS,* acyl-coenzyme A synthetise; *α-AR,* α-adrenoreceptor; *ATP,* adenosine triphosphate; *β-AR,* β-adrenoreceptor; *$CO_2$,* carbon dioxide; *FATP,* fatty acid transporter protein; *FFA,* free fatty acid; *Glut4,* insulin-sensitive glucose transporter 4; *Glycerol-3,* glycerol 3-phosphate; *IR,* insulin receptor; *HSL,* hormone sensitive lipase; *LPL,* lipoprotein lipase; *PKA,* protein kinase C. Modified from Avram and colleagues (Avram *et al.*, 2005)

#### *1.3.2.3 Adipogenesis*

The balance between increasing fat mass (lipogenesis, preadipocyte reproduction and differentiation) and decreasing fat mass (lipolysis, apoptosis, necrosis and dedifferentiation) depends on the balance between energy intake (i.e. as food) and energy expenditure (i.e. as physical activity).

Adipocytes develop from preadipocyte precursor cells. This process of adipogenesis is triggered by peroxisome proliferator-activated receptor γ (PPARγ) (Spiegelman & Flier, 1996). Numerous other factors influence the proliferation and differentiation of adipocytes and preadipocytes, including insulin, insulin like growth factors (IGFs), glucocorticoids and FFAs. It is now believed that preadipocytes can differentiate into new adipocytes at any period in life (Ailhaud *et al.*, 1990; Ailhaud *et al.*, 1991).

PPARγ when bound to retinoid-x-receptor-alpha acts as a transcription factor and has a key role in the induction of early growth arrest of adipocytes and in their terminal differentiation (Gregoire *et al.*, 1998; Auwerx, 1999; Kersten *et al.*, 2000; Combs *et al.*, 2002; Picard & Auwerx, 2002). Insulin has an important role in adipogenesis by promoting the clonal expansion of adipocytes (Gregoire *et al.*, 1998).

IGF1 is another important and essential factor in adipose tissue growth. It promotes clonal expansion of growth arrested preadipocytes *in vivo* and *in vitro* in rats (Smith *et al.*, 1988; Ramsay *et al.*, 1989b; Wright & Hausman, 1995; Gregoire *et al.*, 1998; Rajkumar *et al.*, 1999; Holzenberger *et al.*, 2001; Avram *et al.*, 2007). In cell lines, IGFs have stimulatory effects on DNA

synthesis and cell replication in embryo cultures and cell lines (Zapf *et al.*, 1978; Soret *et al.*, 1999). The effects of IGF1 on adipose tissue proliferation appear to be due to autocrine / paracrine mechanisms as opposed to endocrine mechanisms (Faust *et al.*, 1978; Lau *et al.*, 1990; Peter *et al.*, 1993; Considine *et al.*, 1996; Marques *et al.*, 1998; Marques *et al.*, 2000; Butler *et al.*, 2002).

Glucocorticoids have a role in adipogenesis by stimulation of PPARγ and stimulation of C/EBPa, factors that stimulate adipocyte differentiation. Cortisol has been shown to be essential for adipocyte differentiation (Hauner *et al.*, 1987; Samra *et al.*, 1998).

Besides the discussed external factors that promote adipogenesis, it appears that an increase in fatty acid levels also plays an important role in the induction of adipocyte differentiation (Ailhaud *et al.*, 1995; Sampath & Ntambi, 2004; Madsen *et al.*, 2005). This relationship is reflected in a correlation between plasma non-esterified fatty acids (NEFAs) during the first 24 hours after birth and relative perirenal adipose tissue mass (Duffield, 2007).

### 1.3.3 EXCESSIVE ADIPOSE TISSUE GROWTH

By definition, in most overweight and obese people adipose tissue growth has been excessive. As discussed previously, this process can be due to both hypertrophy and/or hyperplasia of adipose tissue; currently prevailing wisdom favours predominatly hypertrophic growth up to a ‘maximum cell

size' followed by hyperplastic growth thereafter. (Bjorntorp, 1974; Faust *et al.*, 1978; Bjorntorp, 1991; Cinti, 2005).

The maximum size an adipocyte can reach is genetically variable and dependent on the particular adipose tissue depot. This volume is called the 'critical cell size' (Bjorntorp, 1974; Faust *et al.*, 1978; Bjorntorp, 1991; Cinti, 2005). The critical cell size can is also dependent on the source of energy in the diet (Schoonmaker *et al.*, 2004). In mild obesity, cell size normally remains within its critical size and therefore mild obesity is described as being hypertrophic with regard to cell size and numbers. Whilst in severe obesity the critical cell size is exceeded and hyperplasia is induced (Spiegelman & Flier, 1996). Obesity with an onset in early life is also believed to be hyperplastic (Lemonnier, 1972; Spiegelman & Flier, 1996). Spalding and colleagues showed that in most people who would become obese the adipocyte number is already increased by the age of 10 years (Spalding *et al.*, 2008).

In obesity, the rise of the free fatty acid concentration in the blood will lead to a reduction in insulin stimulated glucose uptake in skeletal muscle. The pancreas will partially compensate this effect by secreting more insulin. However, the extra secreted insulin together with decreased clearance leads to peripheral hyperinsulinemia (Wajchenberg, 2000). The process of the development of insulin resistance secondary to obesity will be described in more detail in paragraph 1.3.4.2.

### 1.3.4 REGIONAL DIFFERENCES IN ADIPOSE DEPOTS

Obesity is a very important health problem in our present day society. The metabolic risk factors associated with obesity are dependent on the distribution of adipose tissue. Increased central as opposed to peripheral adipose tissue is associated with higher cardiovascular risks (Krotkiewski *et al.*, 1983; Fujioka *et al.*, 1987; Despres *et al.*, 1989; Pouliot *et al.*, 1992; Wajchenberg, 2000; Busetto, 2001; Miyazaki *et al.*, 2002; Misra *et al.*, 2004; Despres & Lemieux, 2006). According to the INTERHEART case-control study approximately 63% of all heart attacks in Western Europe are secondary to abdominal obesity (Yusuf *et al.*, 2004). The risk of a heart attack doubles in people with an increased waist-hip ratio (WHR) compared to people with a normal WHR (Yusuf *et al.*, 2004). The surgical removal of visceral adipose tissue leads to an improvement in metabolic status both in rodents and in humans (Gabriely *et al.*, 2002; Thorne *et al.*, 2002; Gabriely & Barzilai, 2003).

#### *1.3.4.1 Adipose tissue depots in the body*

Adipose tissue can be divided anatomically into subcutaneous fat, which has a superficial and a deep layer, and visceral fat. About 80 percent of total fat mass is located subcutaneously and about 20 percent intra-abdominal (Marin *et al.*, 1992a). Visceral adipose tissue is an amalgamation of adipose tissue that can be divided in intraperitoneal and retroperitoneal fat. Intraperitoneal fat can be found in the omentum and in the mesenterium, this depot is often called omental fat. Retroperitoneal fat is mainly found around

the kidneys and is called perirenal adipose tissue. Visceral adipose tissue can also be found pericardial and around the genitalia (Himms-Hagen & Ricquier, 1998; Fukuchi *et al.*, 2003). A study by Marin and colleagues investigated the relations between different adipose tissue depots and metabolic variables such as plasma insulin, blood glucose levels and glucose disposal rate during a euglycemic glucose clamp. Fasting insulin and fasting glucose were positively related to visceral and retroperitoneal adipose tissue mass. Glucose disposal rate was negatively related with total body fat, abdominal, visceral and retroperitoneal adipose tissue mass. They found that all relations were strongest for intra-peritoneal adipose tissue mass. Furthermore they found that adipose tissue lipid uptake was higher in omental than subcutaneous adipose tissue and that adipocytes in the omental adipose tissue depot were less sensitive to the antilipolytic effects of insulin. They suggested that the omental adipose tissue depot has a higher turnover rate of lipids compared to the other depots (Marin *et al.*, 1992a).

Special interest has been given to deep subcutaneous abdominal adipose tissue. Some authors believe that this adipose tissue depot should be considered a ‘central’ adipose tissue depot, with increased metabolic risks; others believe this depot is part of the subcutaneous or peripheral adipose tissue depot. Miyazakie and colleagues found that in adults with type 2 diabetes between the ages of 30-60 years, the amount of deep subcutaneous fat at L4-L5 level as determined by MRI was associated with both peripheral and hepatic insulin resistance (Miyazaki *et al.*, 2002). While both Abate and Goodpaster and colleagues found that an increase in

abdominal subcutaneous adipose tissue led to a similar or even greater increase in the risk of insulin insensitivity compared to an increase in visceral adipose tissue (Abate *et al.*, 1995; Goodpaster *et al.*, 1997) suggesting that the abdominal subcutaneous depot should be considered, at least with regards to risks of insulin resistance, as a visceral adipose tissue depot.

There is a wide variation of the distribution of fat within humans. Females tend to deposit more subcutaneous compared to visceral adipose tissue (Kotani *et al.*, 1994; Dua *et al.*, 1996; Legato, 1997). Men have for every given amount of total body fat on average twice as much abdominal fat as premenopausal women with the same amount of total body fat (Han *et al.*, 1997), which could be part of the explanation why men are more susceptible to developing cardiovascular diseases.

Different adipose tissue depots develop at different stages in life. Previous studies have shown that in large mammals, like sheep, at birth the most important visceral adipose tissue depot is the perirenal adipose tissue depot, while the subcutaneous depot is also present at birth (Clarke *et al.*, 1997b; Symonds & Stephenson, 1999). After birth during early postnatal life, the omental and the subcutaneous adipose tissue become more abundant. Little is known about the growth rate of those different depots and the factors that influence the distribution of adipose tissue between visceral fat depots. Furthermore it is not know whether the development of a specific depot at a certain stage in life of the individual is related to risks associated with that particular depot in later life.

#### *1.3.4.2 Molecular difference between adipose tissue depots and mechanisms that could explain increased risks associated with visceral obesity*

Several mechanisms have been put forward to explain the difference in risk patterns associated with a primarily centripedal distribution of adipose tissue. The most important of those mechanisms is the production of cytokines by visceral adipose tissue and the direct release of free fatty acids from the visceral depot to the liver via the hepatic portal vein. Visceral adipose tissue in comparison to subcutaneous adipose tissue is more sensitive to lipolytic stimulation by, for example, the sympathetic nervous system. On the other hand, it is less sensitive to antilipolytic stimuli by, for example insulin (Rebuffe-Scrive *et al.*, 1989; Arner *et al.*, 1990; Marin *et al.*, 1992a; Vikman *et al.*, 1996). Therefore visceral adipose tissue seems to have more lipolytic activity resulting in an increased flow of non esterified fatty acids (NEFA) into the portal vein (Svedberg *et al.*, 1990; Boden *et al.*, 1998; Smith & Zachwieja, 1999). These NEFAs are directly transported to the liver where they have several effects associated with an increased metabolic risk pattern. For example, an increase in NEFAs in the liver will lead to a decrease in hepatic insulin clearance and an increase in insulin secretion. Both factors lead to a decrease in glucose tolerance and an increase in insulin resistance. Increased NEFAs in the portal vein circulation also lead to an increase in the production of very low density lipoproteins by the liver, leading to dyslipideamia (Svedberg et al., 1990; Boden et al., 1998).

Therefore an increased lipolytic activity in the portal vein drainage area, caused by an increase of visceral adipose tissue, contributes to all symptoms of the metabolic syndrome. Figure 1.2 summarises the effects of visceral adiposity on the development of the metabolic syndrome (Figure 1.2).

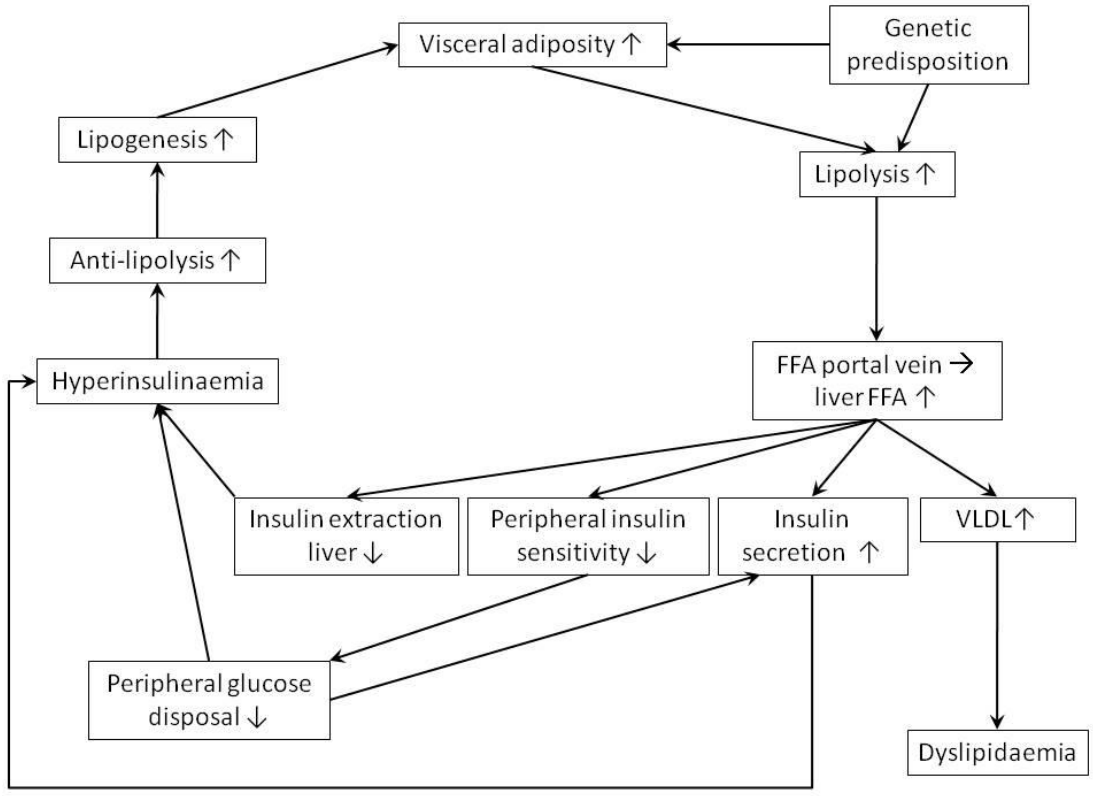


**Figure 1.2; Effects of visceral adiposity on the development of the metabolic syndrome**

Adapted from Wajchenberg and colleagues (Wajchenberg, 2000).

Numerous studies have further confirmed the differences in fatty acid turnover between visceral and subcutaneous adipose tissue. For example the uptake of triglycerides has been shown to be higher in omental compared to subcutaneous adipose tissue, but this was not correlated with LPL activity (Marin *et al.*, 1992a). Martin and Jensen found that triglyceride turnover higher was in abdominal versus gluteo-femoral adipose tissue in men *in vivo* (Martin & Jensen, 1991; Jensen, 1997). Differences in LPL activity between men and women have also been proposed as an explanation in the observed gender differences in fat distribution. LPL activity in women is higher in subcutaneous fat cells compared to omental fat cells and consequently adipocyte size is greater in subcutaneous tissue than in omental tissue, while the opposite was found of men (Arner *et al.*, 1991; Bouchard *et al.*, 1993).

Van Harmelen and colleagues investigated the proliferation and differentiation capacity of human adipocyte precursor cells from the omental and subcutaneous adipose tissue of obese subjects. They found that stromal cells from the subcutaneous region proliferated significantly faster than those from the omental region (Van Harmelen *et al.*, 2004). In a similar study Tchkonia and colleagues confirmed these findings (Tchkonia *et al.*, 2005). The increased proliferative capacity of the subcutaneous depot could be related to a difference in PPARγ mRNA expression between depots. PPARγ mRNA expression levels were significantly lower in visceral adipose tissue compared with subcutaneous fat tissue, although this was not found in obese

people (Lefebvre et al., 1998). Furthermore, subcutaneous adipose tissue was also found to be more sensitive to the PPARγ agonist troglitazone compared to visceral adipose tissue (Adams et al., 1997).

Besides differences in fatty acid turnover another theory attempts to explain differences in risk profile of different adipose tissue depots by differences in their production of cytokines. Cytokines such as Interleukin 6 (IL-6) increase hepatic triglyceride secretion (Nonogaki *et al.*, 1995). IL-6 production in omental tissue of obese subjects was higher than in subcutaneous abdominal adipose tissue of the same subject (Fried *et al.*, 1998; Wajchenberg *et al.*, 2002). Therefore visceral obesity could induce hypertriglyceridemia through elevated IL-6 levels which cause extra triglyceride synthesis in the liver. IL-6 stimulates corticotropin releasing hormone (CRH), adrenocorticotropic hormone (ACTH) and cortisol secretion while cortisol suppresses adipose IL-6 production (Path *et al.*, 1997).

Other molecular differences that have been found between adipose tissue depots include differences in androgen receptor levels and leptin mRNA expression. Finally, some genetic polymorphisms have been associated with adipose tissue distribution. Androgen receptor levels have been found to be higher in visceral fat than in subcutaneous fat. This difference has been used in an attempt to explain gender differences in adipose tissue distribution. Exposure to testosterone increases the number of androgen receptors while estrogen exposure decreases the number of androgen receptors which could

potentially be a mechanism that protects females from central adiposity (Haarbo *et al.*, 1991; Polderman *et al.*, 1994).

Leptin mRNA levels have been found to be greater in subcutaneous than in omental fat tissue. Leptin levels are also positively correlated to adipocyte size and adipose tissue triglyceride storage (Hamilton *et al.*, 1995; Montague *et al.*, 1997; Tritos & Mantzoros, 1997; Van Harmelen *et al.*, 1998; Wajchenberg *et al.*, 2002). Halleux and colleagues found that leptin secretion was stimulated by glucocorticoids in visceral adipose tissue. They therefore proposed that the regional distribution of GR and leptin might be linked (Halleux *et al.*, 1998).

Several polymorphisms are associated with fat distribution. Trp64Arg, a mutation of the β3-adrenergic receptor gene, is associated with visceral obesity and insulin resistance (Widen *et al.*, 1995; Kim-Motoyama *et al.*, 1997). Other polymorphisms that possibly influence regional fat distribution are the LPL HindIII polymorphism, the apo-B-100 gene EcoR-1 polymorphism, polymorphisms in the Mob-1 region of human chromosome 16p12-p11.2 and a polymorphic marker (LIPE) in the hormone-sensitive lipase gene on chromosome 19q13.1-13.2 (Pouliot *et al.*, 1994; Vohl *et al.*, 1995; Klannemark *et al.*, 1998). Also, the Bc/I restriction enzyme at the GR gene is associated with a more visceral fat distribution (Buemann *et al.*, 1997).

There is little doubt that an abundance of visceral adipose tissue increases metabolic risk. This paragraph has demonstrated that numerous molecular differences can be responsible for differences in adipose tissue distribution or differences in risks associated with different adipose tissue depots. It is however not known whether a differential sensitivity to the actions of regulators of adipose tissue growth during early life can explain adipose tissue distribution later in life. This thesis will focus on the differential sensitivity of adipose tissue depots in early life to the actions of such regulators and in particular the sensitivity of adipose tissue depots to the actions of insulin, glucocorticoids and insulin like growth factors. In the next section of this thesis we will explain the action of those regulators of adipose tissue growth in more detail.

## 1.4 The effects of insulin on adipose tissue

Insulin is a very important factor in adipocyte growth and development. Insulin is responsible for glucose uptake into the cell but also exhibits an inhibitory effect on lipolysis and a stimulatory effect on adipogenesis by promoting the clonal expansion of adipocytes and proliferation and differentiation of preadipocytes (Belfrage *et al.*, 1981; Gregoire *et al.*, 1998; Soret *et al.*, 1999). The effects of insulin on cells are dependent on a complex cascade of insulin signalling in the cell. If errors occur in this signalling cascade the cells can become resistant to the effects of insulin.

Insulin resistance is an important risk factor in the development of diabetes and cardiovascular disease. Insulin resistance is also one of the characteristics of the metabolic syndrome.

A study in sheep from our research group showed that plasma insulin during the first 24 hours of life is directly related to perirenal adipose tissue mass and the size of perirenal adipocytes at three weeks of age (Duffield, 2007).

Landin and colleagues showed that the anti-lipolytic effect of insulin was lower in obese compared to lean individuals and this was related to insulin resistance (Landin *et al.*, 1990). These data suggest that increased exposure of adipose tissue to insulin, as is the case in these obese, insulin resistant individuals, results in a reduced sensitivity of adipose tissue to the effects of insulin.

### 1.4.1 INSULIN SIGNALLING IN THE CELL

Adipose tissue deposition is influenced by several factors amongst which insulin is one of the most important ones. Insulin inhibits lipolysis and therefore promotes adipose tissue growth. The local tissue-specific effects of insulin are dependent on the level of circulating insulin, the density of membrane bound insulin receptors and the intracellular efficiency of insulin-stimulated second messenger signalling. The intracellular insulin signalling cascade involves binding of insulin to the α-subunit of insulin receptor (IR) which is a tyrosine-kinase transmembrane receptor (Cheatham & Kahn, 1995). Activation of the α-subunit leads to auto-phosphorylation of the β-

subunit of the receptor. Phosphorylation of the β-subunit of the receptor leads to tyrosine phosphorylation of insulin receptor substrate (IRS) 1 and 2 which leads to activation of phophatidylinositol 3 kinase (PI3-Kinase) which in turn activates Akt (also known as protein kinase B) (Alessi et al., 1997; Peterson & Schreiber, 1999). Activation of Akt leads to a translocation of glucose transporter 4 (Glut4) from the plasma to the cell membrane, leading to a passive influx of glucose into the cell (Rea & James, 1997) (Figure 1.3). Akt also leads to phosphorylation of glycogen synthase kinase-3, which results in modulation of glucose metabolism, cell growth / survival and differentiation (Whiteman *et al.*, 2002).

Other effects of insulin in the cell include the modulation of gene expression of SREBP1c and PPARγ. SREBP1C regulates lipid synthetic enzymes like pyruvate dehydrogenase, fatty acid synthase and acetyl CoA carboxylase in adipocytes, which results in modification of adipocyte growth and differentiation (Kim & Spiegelman, 1996; Vidal-Puig *et al.*, 1997; Foretz *et al.*, 1999; Shimomura *et al.*, 1999; Le Lay *et al.*, 2002). Insulin inhibits lipolysis by decreasing cAMP levels through phosphorylation and subsequent activation of cAMP specific phosphodiestenase-3 (Belfrage *et al.*, 1981; Smith & Manganiello, 1989; Degerman *et al.*, 1990).

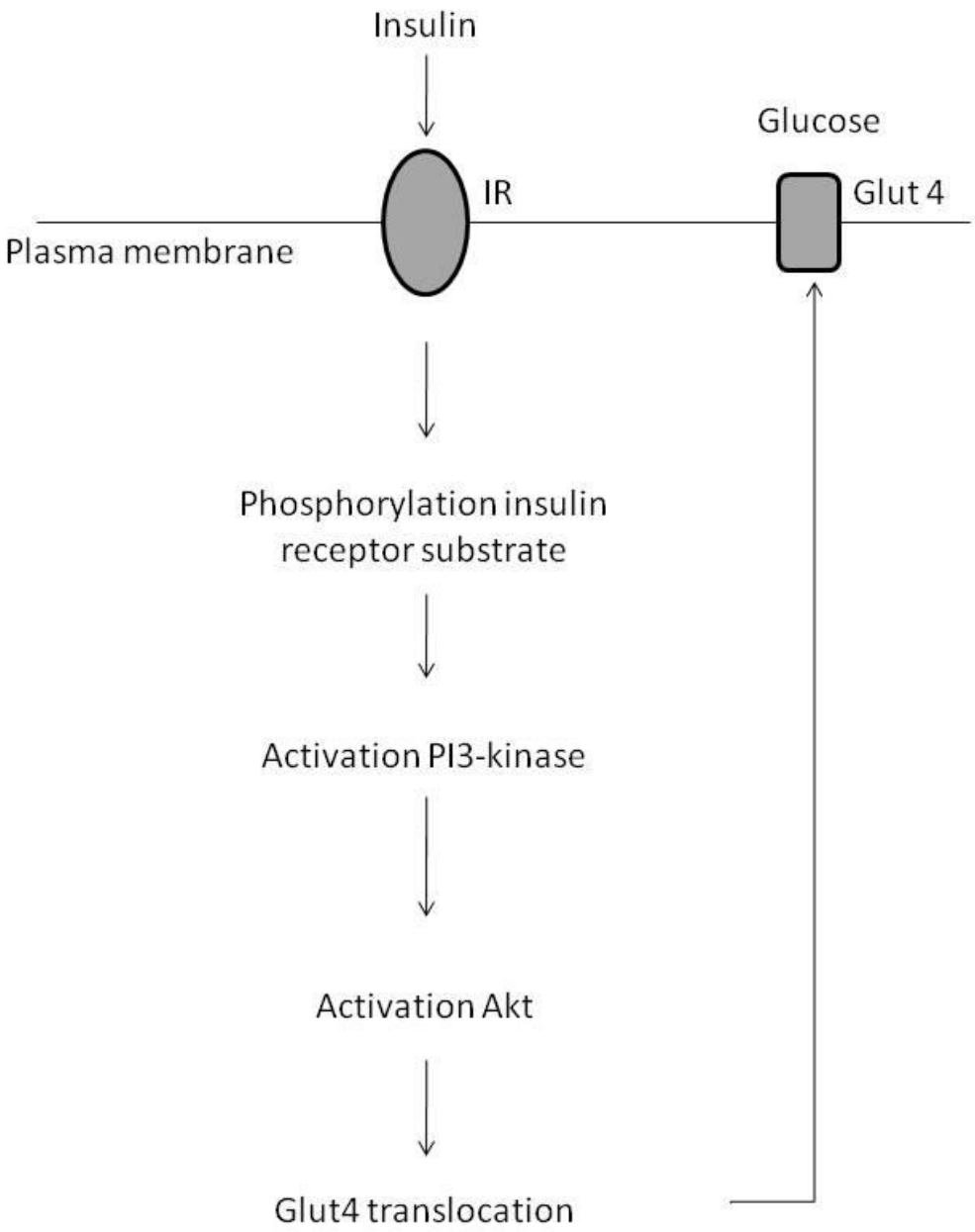


**Figure 1.3; The intra-cellular insulin signalling cascade**

Insulin binds to the insulin receptor (IR) which is present on the plasma membrane, this results in a phosphorylation of insulin receptor substrate, which leads to activation of PI3-kinase, which leads to activation of Akt, which leads to translocation of Glut4 to the cell membrane and influx of glucose into the cell.

### 1.4.2 MECHANISMS FOR RESISTANCE TO THE ACTIONS OF INSULIN AT THE TISSUE LEVEL

If errors occur in the insulin signalling cascade, cells become resistant to the effects of insulin. Different explanations for this phenomenon have been described in the literature.

It has been shown that PI3-kinase is essential for insulin stimulated glucose transport (Cheatham *et al.*, 1994). The most prominent subunit of PI3-kinase is the p85 subunit which catalyses the phosphorylation of several other phophatidylinositols. Blocking of PI3-kinase, using dominant-negative mutants or pharmacological agents, abolishes most metabolic effects of insulin on insulin sensitive cells inclucing Glut4 translocation, glucose uptake, anti-lipolysis and activation of fatty acid and DNA synthesis (Cheatham *et al.*, 1994; Hara *et al.*, 1994; Okada *et al.*, 1994; Alessi & Downes, 1998). If IRS undergoes a phosphorylation of serine instead of a tyrosine phosphorylation the p85 subunit of PI3-kinase disassociates and the insulin signal gets blunted (Le Marchand-Brustel et al., 2003; Tanti et al., 2004).

The inhibition of Akt either by adenoviral expression of a dominant-negative Akt or by the introduction of synthetic interfering RNA against Akt resulted in a reduction of insulin-stimulated Glut4 translocation and glucose uptake (Katome *et al.*, 2003).

Studies that investigated the effect of drugs as colchicines, vinblastine and nocodazole have highlighted the importance of the cytoskeleton in regulating Glut4 translocation. These drugs disrupt the microtubule structure, which has

been shown to block Glut4 vesicle trafficking (Guilherme *et al.*, 2000; Olson *et al.*, 2001).

### 1.4.3 INSULIN SIGNALLING IN DIFFERENT ADIPOSE TISSUE DEPOTS

Insulin seems to have different strengths of effect in different adipose tissue depots. The antilipolytic effects of insulin were observed to be weaker in visceral adipocytes compared to subcutaneous adipocytes in humans, indicating a relative insulin resistance of the visceral adipose tissue depot (Zierath *et al.*, 1998). *In vitro* studies of human adipocytes have found that the IR binding capacity is lower in visceral compared to subcutaneous adipocytes, which results in a reduction of the activation of IR signalling including a reduction in tyrosine phosphorylation of IR and IRS and a reduction in PI3-kinase activation (Zierath *et al.*, 1998). However, an *in vivo* study found the insulin stimulated glucose uptake per surface area to be higher in visceral compared to subcutaneous adipose tissue (Virtanen *et al.*, 2003). This difference in insulin-stimulated glucose uptake was also found in an *in vitro* study by Lundgren and colleagues. They found that the insulin-stimulated glucose uptake was twice as high in human omental compared to subcutaneous adipocytes. They also found an increase in Glut4 expression in omental compared to subcutaneous adipose tissue, whilst IRS-1 and PI3-kinase levels were similar between depots (Lundgren *et al.*, 2004). Furthermore they found that dexamethasone treatment resulted in a decrease in the cellular content of IRS-1 and Akt in omental adipose tissue, but resulted in no change in IRS-1 and Akt content in subcutaneous adipose

tissue, suggesting that omental adipose tissue is more sensitive to glucocorticoids compared to subcutaneous adipose tissue (Lundgren *et al.*, 2004).

The relatively increased glucose uptake in omental adipose tissue compared to subcutaneous adipose tissue could be partly explained by differences in protein abundance of molecules in the insulin signalling cascade. Protein levels of IR, IRS-2, p85, GSK-3α and GSK-3β were all higher in omental compared to abdominal subcutaneous adipose tissue. Furthermore, the tyrosine phosphorylation of IRS was also higher in the omental compared to abdominal subcutaneous adipose tissue, which would result in a greater phosphorylation of Akt (Giorgino *et al.*, 2005; Laviola *et al.*, 2006).

Although the above mentioned studies clearly indicate a relatively increased glucose uptake in the omental depot compared to the subcutaneous adipose tissue depot, some controversy remains regarding the exact depot specific differences in insulin action, as is indicated by a decreased anti-lipolytic effect of insulin in the omental depot.

### 1.4.4 EVIDENCE FOR THE DEVELOPMENTAL PROGRAMMING OF GENES INVOLVED IN INSULIN SIGNALLING

Numerous studies show the plasticity of the insulin signalling cascade and the subsequent development of whole body insulin sensitivity or resistance during its development in early life. Birth weight has been shown to influence glucose metabolism in early postnatal life and in adulthood in humans, rats,

guinea-pigs, pigs and sheep (Clarke *et al.*, 2000; Poore & Fowden, 2002; Kind *et al.*, 2003; Wolf, 2003; De Blasio *et al.*, 2007). Strong associations have been found between a low birth weight (LBW) and the development of insulin resistance and type 2 diabetes mellitus in adult life (Bavdekar *et al.*, 1999; Veening *et al.*, 2002). Bazaes and colleagues have shown that small for gestational age (SGA) infants have a modified glucose metabolism from as early as 48 hours in postnatal life. They had higher levels of plasma FFA, lower levels of plasma leptin and were more insulin sensitive compared to average for gestational age (AGA) infants (Bazaes *et al.*, 2003). In lambs of 45 days of age an inverse relationship was found between birth weight and insulin sensitivity (De Blasio *et al.*, 2007). Another study by Clarke and colleagues indicated that both at one and at three months of age lighter lambs had higher (i.e. quantitatively 'better') glucose tolerance compared to their heavier twin siblings (Clarke *et al.*, 2000). In guinea pigs of 90 days old (young adulthood) it was found that low birth weight was associated with hyperinsulinaemia and an increased insulin-glucose ratio (Kind *et al.*, 2003). In pigs of 1 year of age a decreased glucose tolerance was found in the pigs that had a LBW (Poore & Fowden, 2002). Taken together these studies suggest that low birth weight is associated with increased insulin sensitivity in early postnatal life and decreased insulin sensitivity in adulthood. The effects of low birth weight on reduced insulin sensitivity in adulthood are amplified when the individual becomes obese (Hales *et al.*, 1991; Valdez *et al.*, 1994; Lithell *et al.*, 1996; Jaquet *et al.*, 2000). In children with a high BMI an increased insulin resistance is already found from the age of 8 years in

children who were LBW compared to normal birth weight children with the same high BMI (Bavdekar *et al.*, 1999). Curhan and colleagues estimated that the adjusted odds ratio for whole body insulin resistance and T2DM was 1.75 in men with a LBW compared to men with an average or high birth weight (Curhan *et al.*, 1996b).

The effects of low birth weight are often mimicked in experimental studies that use maternal nutrient restriction or placental restriction to produce offspring with a low birth weight. Restriction of nutrients during pregnancy has been shown to decrease plasma levels of insulin and IGF1 in sheep (Osgerby *et al.*, 2002). The offspring of nutrient restricted rats had decreased levels of plasma insulin and increased levels of plasma glucose at both 3 and 11 weeks of postnatal life (Holemans *et al.*, 1996).

Rats that were protein restricted during pregnancy produced offspring that were insulin resistant and had a reduction in insulin stimulated glucose uptake in the muscle at 15 months of age, indicating the lasting effect (i.e. well into adulthood) of nutritional challenges that occurred during early life. The reduced insulin stimulated glucose uptake in the muscle was not attributed to changes in IR or Glut4 mRNA expression (Ozanne *et al.*, 2003). Offspring of protein restricted rats at the age of 3 months however, showed an increased basal glucose uptake in muscle cells, which was not associated with a change in plasma insulin. Increased levels of IR and Glut4 protein in muscle were found which may have contributed to the increased basal glucose intake in the muscle cells. The insulin stimulated glucose uptake in muscle however, was unchanged compared to control rats (Ozanne *et al.*,

1996). These different patterns of insulin stimulated glucose uptake at different stages in life suggests maternal protein restriction leads to an initial increase in insulin sensitivity in skeletal muscle in these offspring followed by a downregulation of insulin receptor levels and the development on insulin resistance in mature adulthood. In adipose tissue of offspring of protein restricted rats a similar observation is seen at 6 weeks of postnatal age. These rats show a decrease in IR number in the adipocyte, which may indicate a reduction in insulin sensitivity during adult life (Shepherd *et al.*, 1997). Numerous authors have also indicated that the development of obesity in later life in offspring that were growth restricted in foetal life in usually associated with the development of insulin resistance in adipose tissue (Jaquet *et al.*, 1998; Jaquet *et al.*, 1999; Phillips *et al.*, 1999; Jaquet *et al.*, 2000).

As opposed to a reduction in maternal nutrient intake some studies have investigated the effect of a nutritional challenge that consisted of an increased nutrient intake during pregnancy on insulin sensitivity in the offspring. An increase of maternal nutrient intake to 155% of maintenance energy intake during late gestation resulted in increased levels of foetal circulating glucose and insulin. This challenge however had no effect on foetal weight, foetal adipose tissue mass or plasma leptin (Muhlhausler *et al.*, 2002).

Other studies have investigated nutritional challenges in the periconceptional period, and have shown that insulin sensitivity can be programmed as early as the periconceptional period. Periconceptional undernutrition in sheep

resulted in a reduction in foetal sheep plasma glucose, insulin and IGF1, but not IGF2 during late gestation (Bauer *et al.*, 1995; Lee *et al.*, 1997; Gallaher *et al.*, 1998).

The programming effects of growth and nutrition during early life are certainly not limited to the ante-natal stage of life as is shown by the tremendous amount of studies that have investigated the effect of different growth patterns in early life on the development of metabolic diseases in childhood and adulthood (Barker *et al.*, 1993; Lithell *et al.*, 1996; Bavdekar *et al.*, 1999; McMillen & Robinson, 2005). An accelerated rate of growth during early postnatal life appears to be associated with early increased insulin sensitivity and a subsequent emergence of insulin resistance (Whincup *et al.*, 1997; Bavdekar *et al.*, 1999; Eriksson *et al.*, 2001). Numerous studies have also shown a particular increase in adipose tissue insulin resistance in adulthood in individuals that had an accelerated postnatal growth rate (Jaquet *et al.*, 1998; Jaquet *et al.*, 1999; Phillips *et al.*, 1999; Jaquet *et al.*, 2000). The increased risk of the development of insulin resistance in individuals that undergo an accelerated post-natal growth is further amplified if those individuals become obese during childhood or adulthood (Curhan *et al.*, 1996b; Whincup *et al.*, 1997; Bavdekar *et al.*, 1999; Eriksson *et al.*, 2001; Veening *et al.*, 2002).

The detrimental effects of low birth weight and an accelerated postnatal growth are often combined in individuals that undergo the so-called ‘catch-up growth’. It has been shown that individuals with the lowest birth weight often are the ones with the highest weight gain velocity in the first two months of

life and the highest insulin sensitivity during this early period of life (Gray *et al.*, 2002). Similarly piglets that were born low birth weight exhibited catch-up growth during early life compared to their higher birth weight siblings. These low birth weight piglets had a decreased glucose tolerance at 1 year of age (Poore & Fowden, 2002). In rats it has been observed that LBW pups (as a result of maternal nutrient restriction) had an increased feed intake from weaning to adulthood, leading to greater retroperitoneal and gonadal adipose tissue mass, and an accelerated growth rate when exposed to a hypercaloric post-weaning diet even though the weight in adulthood was not different between the low and average birth weight rats (Vickers *et al.*, 2000; Vickers *et al.*, 2001).

These studies strongly indicate that a combination of growth patterns during prenatal, early postnatal life and childhood leads to particular differences in whole body and tissue specific insulin sensitivity in later life. It is unknown to date whether changes in the early growth patterns can result in adipose tissue depot specific differences in the sensitivity of the particular depot to the actions of insulin.

## 1.5 The effects of glucocorticoids on adipose tissue

Glucocorticoids have an important influence in the pathogenesis of obesity. Patients with chronic elevated cortisol levels, like patients with Cushings Syndrome have a significant redistribution of adipose tissue towards the central depots (Lamberts & Birkenhager, 1976; Wajchenberg *et al.*, 1995).

### 1.5.1 GLUCOCORTICOID SIGNALLING IN THE CELL

Glucocorticoids exert their effects on cells through the glucocorticoid receptor (GR), which is a member of the steroid receptor family. GR is located in the cell nucleus and, after passive diffusion of glucocorticoids into the cell, binds to the free glucocorticoid. Binding of glucocorticoids to GR induces a conformational change in GR which leads to the dissociation of heat shock proteins that are attached to GR. Dissociation of heat shock proteins leads to formation of homodimers and exposure of the DNA and protein binding sites of GR. GR-homodimers then bind to hormone response elements in the DNA where they initiate or inhibit transcription (Lan *et al.*, 1984; Yamamoto, 1985; Scheidereit *et al.*, 1986; Strahle *et al.*, 1987) (Figure 1.4). GR can also function as a co-activator and bind to other proteins involved in transcription, such as glucocorticoid receptor interacting protein 1 or cAMP response element binding protein (Kwok *et al.*, 1994; Hong *et al.*, 1996).

It is believed that all the actions of glucocorticoids are initiated through inhibition or initiation of transcription by the glucocorticoid – GR complex (Duval *et al.*, 1983; Lan *et al.*, 1984; Fulton *et al.*, 1985).

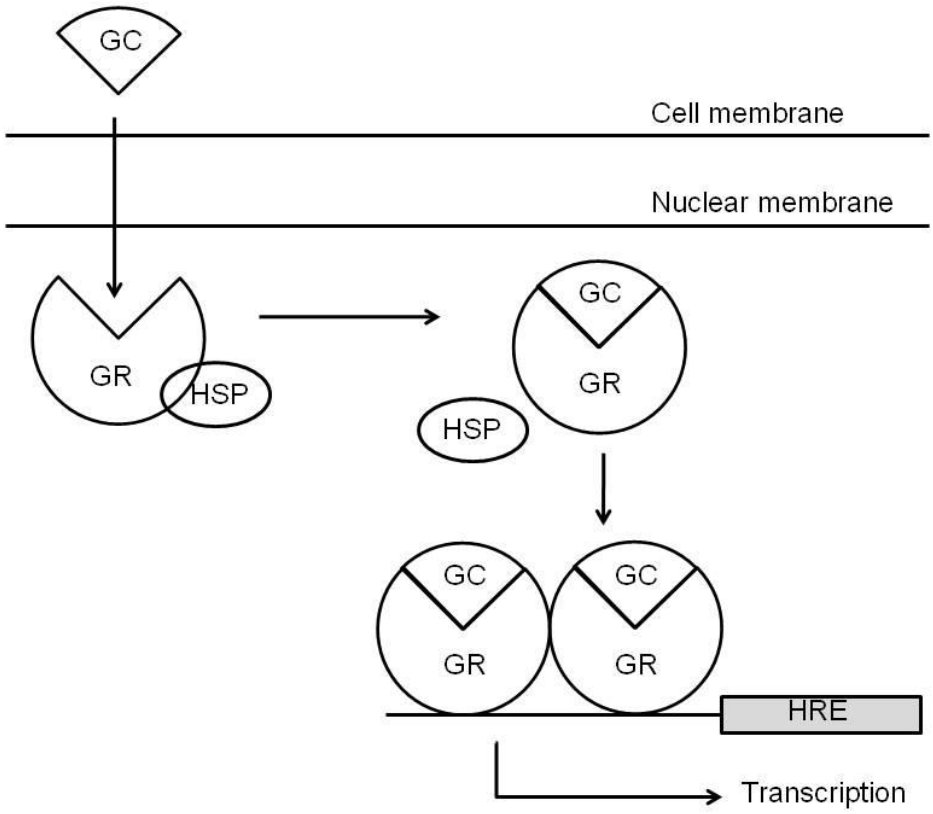


**Figure 1.4; Mechanism of glucocorticoid action in the cell**

Glucocorticoids (GC) enter the cell nucleus by passive diffusion where they bind to the glucocorticoid receptor (GR); this causes a conformational change of GR and leads to dissociation of the heat shock proteins (HSP). GR then forms homodimers and binds to hormone response elements (HRE) on the DNA where they initiate or inhibit transcription.

### 1.5.2 ACTIONS OF GLUCOCORTICOIDS IN ADIPOSE TISSUE

The effects of glucocorticoids on adipose tissue seem to be complex and diverse. *In vitro* studies in rats, have shown that glucocorticoids stimulate lipolysis by increasing the activity of hormone sensitive lipase (Fain *et al.*, 1963; Slavin *et al.*, 1994). These observations have been confirmed by human *in vivo* studies that showed an increase in fatty acid turnover with hypercortisolaemia (Divertie *et al.*, 1991; Dinneen *et al.*, 1995). A study by Samra and colleagues investigated the effects of a hydrocortisone infusion on plasma NEFA levels in human, and they found that plasma NEFA levels in the whole body increased, but that NEFA efflux from subcutaneous adipose tissue was reduced. This indicated a reduction in HSL in the subcutaneous depot as a response to glucocorticoids. The increased whole body lipolysis was considered to be most likely secondary to an increase in visceral lipolysis (Samra *et al.*, 1998). Furthermore, studies by Ottosson and colleagues in human, and Ramsay and colleagues in pigs, indicated that glucocorticoids can also stimulate lipoprotein lipase, at least *in vitro*, and therefore have stimulatory effects on lipogenesis (Ramsay *et al.*, 1989a; Ottosson *et al.*, 1994). Fried and colleagues investigated the effects of insulin and dexamethasone on LPL mRNA and activity in omental and abdominal subcutaneous adipose tissue in human. They found that insulin could increase LPL mRNA and activity only in abdominal subcutaneous adipose tissue, while dexamethasone increased LPL mRNA and activity in

both tissue, but the effects were more marked in the omental adipose tissue depot. When both insulin and dexamethasone were added together, a synergistic increase in LPL activity was seen (Fried *et al.*, 1993).

Besides effects on lipogenesis and lipolysis, glucocorticoids also have effects on adipogenesis. Glucocorticoids promote the differentiation of adipocyte precursor cells *in vitro* in human, sheep and pig (Hauner *et al.*, 1987; Ramsay *et al.*, 1989a; Casteilla *et al.*, 1991; Soret *et al.*, 1999). Glucocorticoids also augment IGF1 action *in vitro* in human and mice, (Conover *et al.*, 1985; Tronche *et al.*, 2004) and stimulate IGF1R expression in rats (Bennett *et al.*, 1984). In human and sheep, glucocorticoids are also known to stimulate PPAR-γ another important adipogenic transcription factors (Wu *et al.*, 1996; Vidal-Puig *et al.*, 1997; Soret *et al.*, 1999).

Furthermore glucocorticoids have been shown to have an inhibiting effect on glucose uptake in the human and rat adipocyte, while at the same time stimulating plasma insulin levels (Rizza *et al.*, 1982; Lenzen & Bailey, 1984; Carter-Su & Okamoto, 1985). Lundgren and colleagues found that the dexamethasone induced reduction in glucose uptake in the human adipocyte was only present in the omental and not in the subcutaneous adipose tissue depot (Lundgren *et al.*, 2004). Furthermore they found that dexamethasone treatment resulted in a decreased expression of insulin receptor substrate 1 in omental adipose tissue only (Lundgren *et al.*, 2004).

### 1.5.3 LOCAL AVAILABILITY OF GLUCOCORTICOIDS

People with elevated glucocorticoids, like patients with Cushing's Syndrome, display all characteristics of the metabolic syndrome; increased glucose intolerance, central obesity and hypertension. Most people with the metabolic syndrome however have normal plasma glucocorticoid levels. Nevertheless, the actual plasma glucocorticoid concentrations may not necessarily mirror circulating levels. This is because many tissues express the enzyme 11 beta hydroxysteroid dehydrogenase (11β-HSD), which was first discovered in 1953 (Amelung *et al.*, 1953). Nowadays at least two, only distinctly related, isoforms are known; 11β-HSD1 and 11β-HSD2.

11β-HSD1 and 11β-HSD2 have an important role in regulating glucocorticoid levels in local tissues. 11β-HSD1 is a bidirectional enzyme which can convert inactive cortisone into active cortisol and vice versa. *In vivo* 11β-HSD1 is thought to work mainly as a reductase to activate cortisone into cortisol. 11β-HSD2 works as a dehydrogenase and inactivates cortisol by converting it into cortisone (Figure 1.5).

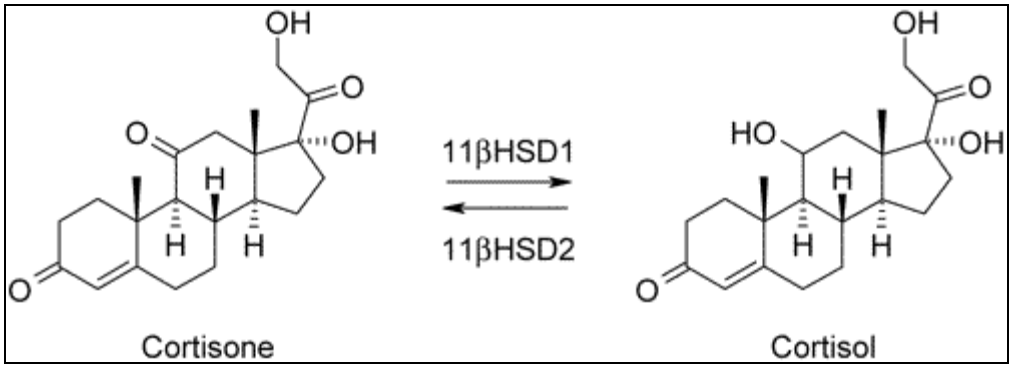


**Figure 1.5; Conversion of cortisone into cortisol and vice versa**

Function of 11-beta hydroxysteroid dehydrogenase enzymes type 1 and 2 in the conversion of cortisone into cortisol and vice versa.

In humans and large mammals cortisol is the main biologically active form of glucocorticoids. The equivalent of cortisol in rodents is corticosterone.

95% of the cortisol circulating in human blood is bound to proteins, mainly corticosteroid binding globulin (Dunn *et al.*, 1981), so the amount of freely available cortisol is low. At the diurnal peak cortisol levels are 400 – 600 nmol/l while nadir levels only reach 0.5 – 1 nmol/l (Dunn *et al.*, 1981). Cortisone shows no diurnal rhythm, is hardly bound to globulins and circulates at levels of 50 - 100 nmol/l (Walker *et al.*, 1992). On a local level 11β-HSD1 is capable of converting this cortisone into cortisol to ensure adequate cortisol availability as its affinity for substrate is much higher than 11β-HSD2.

11β-HSD1 is found abundantly in most mammalian tissues (Agarwal *et al.*, 1989; Moisan *et al.*, 1990; Ricketts *et al.*, 1998). 11β-HSD1 is NADP(H) dependent. It works *in vivo* mainly as a reductase to convert cortisone into its biological active form cortisol in humans and large mammals and it converts

11-dehydrocorticosterone into corticosterone in rodents (Low *et al.*, 1994; Hundertmark *et al.*, 1995; Jamieson *et al.*, 1995; Rajan *et al.*, 1996).

In humans and large mammals 11β-HSD2 converts the active form of cortisol into its inactive variant cortisone (Albiston *et al.*, 1994). In rodents 11β-HSD2 has a similar activity on corticosterone. The dehydrogenase conversion is nicotinamide adenine dinucleotide (NAD) dependent. The enzyme is mainly expressed in aldosterone target tissues such as the kidneys, colon, sweat and salivary glands, hippocampus, and the cardiovascular system (Smith *et al.*, 1996; Hirasawa *et al.*, 1997). Glucocorticoids have a higher affinity for the mineralocorticoid receptor (MR) than mineralocorticoids have. To ensure the availability of MR for aldosterone in order to regulate salt balance 11β-HSD2 deactivates cortisol in aldosterone target tissues (Arriza *et al.*, 1987; Edwards *et al.*, 1988; Funder *et al.*, 1988; Andrews & Walker, 1999).

### 1.5.4 GLUCOCORTICOIDS, THE METABOLIC SYNDROME AND OBESITY

Insulin resistance and glucose intolerance are, like most other components of the metabolic syndrome, associated with slightly higher cortisol levels (Phillips *et al.*, 1998; Reynolds *et al.*, 2001). High blood pressure is also associated with higher cortisol levels (Watt *et al.*, 1992; Filipovsky *et al.*, 1996). The tissue sensitivity for glucocorticoids is dependent on the density of glucocorticoid receptors. In hypertension and diabetes mellitus type 2 the expression of GR is increased (Walker *et al.*, 1996; Walker *et al.*, 1998;

Andrew *et al.*, 2002). In human skeletal muscle GR expression is related to insulin sensitivity and blood pressure (Reynolds *et al.*, 2002; Whorwood *et al.*, 2002). Panarelli showed that the metabolic syndrome is associated with a polymorphism of the GR gene (Panarelli *et al.*, 1998).

The C57B1/6 strand of mice is known for having the metabolic syndrome. These mice have higher levels of 11β-HSD1 in adipose tissue and less downregulation of 11β-HSD1 after a high fat diet (Morton *et al.*, 2004).

Obesity also shows several associations with glucocorticoid action. Visceral obesity is associated with lower morning peak cortisol levels (Ljung *et al.*, 1996; Phillips *et al.*, 1998; Rosmond *et al.*, 1998), but higher cortisol levels in the evening nadir (Ljung *et al.*, 1996; Rosmond *et al.*, 1998) and higher urinary cortisol excretions (Strain *et al.*, 1980; Marin *et al.*, 1992b; Pasquali *et al.*, 1993). Total cortisol production in obesity is increased (Andrew *et al.*, 1998; Fraser *et al.*, 1999; Stewart *et al.*, 1999). Hypothalamic-pituitary-adrenal (HPA) axis activity after stress is also increased with obesity (Marin *et al.*, 1992b; Hautanen & Adlercreutz, 1993; Pasquali *et al.*, 1999) and the negative feedback of glucocorticoid levels on HPA axis activity is decreased in obesity (Ljung *et al.*, 1996; Jessop *et al.*, 2001).

The effects of glucocorticoids on central and peripheral fat are different. Glucocorticoids appear to increase lipolysis and down regulate lipoprotein lipase in peripheral fat but stimulate preadipocyte differentiation and triglyceride synthesis in central fat (Samra *et al.*, 1998). In obesity in humans,

no upregulation of subcutaneous adipose tissue GR mRNA was found (Lindsay *et al.*, 2003; Wake *et al.*, 2003).

A study on leptin resistant Zucker rats showed that obesity is correlated with higher 11β-HSD1 activity in omental tissue and lower 11β-HSD1 activity in liver (Livingstone *et al.*, 2000). The reason for the lower 11β-HSD1 activity in the liver might be a compensation for the higher cortisol levels in the portal vein, which are due to the high 11β-HSD1 activity in omental tissue.

In humans the activity of 11β-HSD1 in omental fat is higher than in subcutaneous fat, suggesting a role for 11β-HSD1 in the development of visceral obesity (Bujalska *et al.*, 1999). The results from this activity assay however may be biased by the amount of GR, which has been shown to be higher in visceral than in peripheral fat (Masuzaki *et al.*, 2001).

Several studies found that the activity and mRNA expression of 11β-HSD1 in abdominal subcutaneous adipose tissue is increased in obese humans (Rask *et al.*, 2001; Rask *et al.*, 2002; Lindsay *et al.*, 2003; Wake *et al.*, 2003).

#### *1.5.4.1 11β-HSD1 overexpression*

Mice overexpressing 11β-HSD1 are obese and have more visceral fat than control mice (Masuzaki *et al.*, 2001). This relative increase in visceral fat may be explained by the levels of GRα which are much higher in visceral than in peripheral fat in mice (Masuzaki *et al.*, 2001). No downregulation of GRα is found in these animals. The obesity in these mice is caused by hypertrophy and not hyperplasia of adipose tissue (Masuzaki *et al.*, 2001).

The 11β-HSD1 overexpressing mice have more insulin resistance and glucose intolerance, especially after a high fat diet, and the levels of triglycerides and free fatty acids are higher in these mice (Masuzaki *et al.*, 2001). Corticosterone levels are higher in the portal veins of those mice but normal in the systemic circulation (Masuzaki *et al.*, 2001). This can be explained by a higher corticosterone production in omental tissue which leads to an overflow of corticosterone into the portal vein. The mice have increased plasma levels of angiotensinogen, angiotensin II, and aldosterone, suggesting activation of the circulating renin-angiotensin system (RAS). The mice are more likely to have salt-sensitive hypertension. Furthermore administration of a selective angiotensin II receptor AT-1 antagonist at a low dose abolishes the effect on blood pressure (Masuzaki et al., 2003). The mice also show dyslipidemia (Masuzaki et al., 2001). The 11β-HSD1 overexpressing mice all have symptoms of the metabolic syndrome suggesting a role for 11β-HSD1 in the pathogenesis of the metabolic syndrome. The 11β-HSD1 overexpressing mice also have elevated leptin levels and appear to be leptin resistant and exhibit hyperphagia (Masuzaki *et al.*, 2001).

#### *1.5.4.2 11β-HSD1 knock-out or deficiency*

11β-HSD1 knock out mice show no developmental defects, they are fertile and fully viable (Kotelevtsev *et al.*, 1997). In these mice lower activities of gluconeogenic enzymes have been found after fasting and lower glucose levels after stress or after a fatty meal (Kotelevtsev *et al.*, 1997; Morton *et al.*,

2004; Kerstens *et al.*, 2005). These mice also proved to be diabetes resistant and normotensive. As expected, they have lowered intra-adipose glucocorticoid levels but mildly elevated plasma glucocorticoid levels (Morton *et al.*, 2001). The mice also tend to distribute their adipose tissue more towards peripheral regions, indicating a role for 11β-HSD1 in the regulation of fat distribution. These mice have reduced triglyceride levels, raised high density lipoprotein levels, lowered hepatic fibrinogen synthesis and increased lipid beta oxidation (Morton *et al.*, 2001). The mice have reduced resistin, reduced TNFα, reduced leptin and increased adiponectin levels in fat tissue (Morton *et al.*, 2004). PPARγ receptor levels are higher in adipose tissue (Morton *et al.*, 2001). Altogether they seem to have a cardioprotective phenotype; with better glucose tolerance and increased insulin sensitivity, again, suggesting a role for 11β-HSD1 in the pathogenesis of the metabolic syndrome (Harris *et al.*, 2001).

The plasma corticosterone levels are modestly elevated, but the adipose tissue corticosterone levels are lowered. The HPA axis response to stress is appropriate in these mice (Harris *et al.*, 2001). These mice gain less weight after feeding a high fat diet, although they have hyperphagia. It is possible that these mice have an enhanced metabolic rate. In summary it seems that lower 11β-HSD1 levels lead to lower levels of glucocorticoid action and increased insulin sensitivity (Walker *et al.*, 1995; Andrews *et al.*, 2002; Andrews *et al.*, 2003).

11β-HSD1 deficiency has also been described in humans. These people have features of adrenal androgen excess. They have enhanced metabolic clearance of cortisol and compensatory hypothalamic-pituitary-adrenal gland axis activation. No coding mutations have been found of the 11β-HSD1 gene in this syndrome. The syndrome is called apparent cortisone reductase deficiency (Phillipov *et al.*, 1996; Draper *et al.*, 2003). Patients with this syndrome had mutations in the gene for hexose-6-phosphate besides the mutation in the 11β-HSD1 gene (Draper *et al.*, 2003). Hexose-6-phosphate is important for the production of the cofactor NADPH and therefore hexose-6-phospate may have an important role in the regional deposition of adipose tissue (Draper *et al.*, 2003; Bujalska *et al.*, 2005).

Carbonoxolone inhibits 11β-HSD1 activity and 11β-HSD2 activity in the kidney. Treatment with carbonoxolone enhances insulin sensitivity (Walker *et al.*, 1995) and inhibits hepatic glucose production in patients with diabetes mellitus type 2 (Andrews *et al.*, 2003).

#### *1.5.4.3 11β-HSD1 polymorphisms*

Besides the non-coding mutation which has a role in the aetiology of the syndrome of apparent cortisone reductase deficiency an intronic polymorphism in the 11β-HSD1 gene has been found. This polymorphism is associated with an increased body mass index, altered body composition and insulin resistance (Gelernter-Yaniv *et al.*, 2003). Caramelli et al investigated the relationship between obesity and common mutations in the

11β-HSD1 gene. They found no significant association, but their study was performed on only 8 obese patients (Caramelli *et al.*, 2001). In a larger study Draper et al looked at the association between genomic differences in the 11β-HSD1 gene and obesity. They also found only weak correlations (Draper *et al.*, 2002).

#### *1.5.4.4 11β-HSD2 knock out mice or mutations*

Patients with the syndrome of apparent mineralocorticoid excess (AME) have been shown to have more mutations in the 11β-HSD2 gene than normal controls (Mune *et al.*, 1995; Dave-Sharma *et al.*, 1998). In this syndrome glucocorticoids occupy mineralocorticoid receptors causing sodium retention, hypertension and hypokalemia. Carbonoxolone, which is found in liquorice, functions as an inhibitor of 11β-HSDs and excessive amounts of liquorice can cause symptoms similar to AME (Stewart *et al.*, 1987). 11β-HSD2 knock out mice have been produced and they also suffer from AME (Kotelevtsev *et al.*, 1999). It could be possible that due to a lack of 11β-HSD2 activity, an excess amount of cortisol is available. This excess amount of cortisol would then compete with mineralocorticoids for binding to the MR given the equal affinity each has for the MR receptor.

It is possible that 11β-HSD2 plays an important role in fat deposition by reducing the amount of available cortisol. It could therefore be protective for obesity and the metabolic syndrome. This role has not been investigated yet

and the differences of 11β-HSD2 levels between regional depots are still to be investigated.

### 1.5.5 GLUCOCORTICOID SENSITIVITY IN DIFFERENT ADIPOSE TISSUE DEPOTS

The effects of glucocorticoids on central and peripheral fat are different. Glucocorticoids appear to increase lipolysis and down regulate lipoprotein lipase in peripheral fat but stimulate preadipocyte differentiation and triglyceride synthesis in central fat in humans (Fried *et al.*, 1993; Samra *et al.*, 1998).

The influence of glucocorticoids on the regional fat depots is dependent on the concentration of glucocorticoid receptors (GR). GR concentrations in visceral adipose tissue were found to be higher than in subcutaneous adipose tissue (Rebuffe-Scrive *et al.*, 1985). In obesity in humans, no upregulation of GR mRNA was found (Lindsay *et al.*, 2003; Wake *et al.*, 2003).

Another essential component for local glucocorticoid action is 11β-HSD1 which converts inactive cortisone into active cortisol. In humans the activity of 11β-HSD1 in omental fat is higher than in subcutaneous fat, suggesting a role for 11β-HSD1 in the development of visceral obesity (Bujalska *et al.*, 1999; Walker, 2004). The results from this activity assay however may be biased by the amount of GR, which has been shown to be higher in visceral than in peripheral fat (Masuzaki *et al.*, 2001).

Several studies in obese humans, found that the activity and mRNA expression of 11β-HSD1 in abdominal subcutaneous adipose tissue was

increased compared to non-obese controls. This further emphasises the confusion around the specific position of abdominal subcutaneous adipose tissue as either visceral or peripheral (Rask *et al.*, 2001; Rask *et al.*, 2002; Lindsay *et al.*, 2003; Wake *et al.*, 2003).

A study on leptin resistant Zucker rats showed that obesity is correlated with higher 11β-HSD1 activity in omental tissue and lower 11β-HSD1 activity in liver (Livingstone *et al.*, 2000). The lower 11β-HSD1 activity which was found in the liver could be a compensation for the higher cortisol levels in the portal vein, which are due to the high 11β-HSD1 activity in omental tissue.

As discussed in the previous section, mice overexpressing 11β-HSD1 are obese and have more visceral fat than control mice (Masuzaki *et al.*, 2001), while 11β-HSD1 knock out mice tend to distribute their adipose tissue more towards peripheral regions, indicating a role for 11β-HSD1 in the regulation of fat distribution (Morton *et al.*, 2001).

### 1.5.6 EVIDENCE FOR THE DEVELOPMENTAL PROGRAMMING OF GENES INVOLVED IN GLUCOCORTICOID SIGNALLING

Glucocorticoids can have important roles in the programming of later health or disease of an individual. This is clearly demonstrated by the fact that glucocorticoid treatment during pregnancy alters birth weight in humans, sheep and mice (Reinisch *et al.*, 1978; Ikegami *et al.*, 1997; French *et al.*, 1999; Bloom *et al.*, 2001), increases blood pressure in the fetal, juvenile and adult sheep offspring and in the adult rat offspring (Benediktsson *et al.*, 1993; Dodic *et al.*, 1998; Dodic *et al.*, 1999; Dodic *et al.*, 2002; Jensen *et al.*,

2002) and leads to permanent hyperglycaemia in adult rats (Lindsay *et al.*, 1996; Nyirenda *et al.*, 1998; Sugden *et al.*, 2001). Prenatal glucocorticoid administration has been shown to accelerate maturation of particular organs in human, of which the lung is the most notable one (Ward, 1994).

Another clear demonstration of the essential role of glucocorticoids in the developmental origins of health and disease is provided by Langley-Evans and colleagues, who demonstrated that in the maternal protein restricted rat model, the development of hypertension by the age of 7 weeks in the offspring can be prevented by administration of glucocorticoid synthesis inhibitors to the pregnant dam (Langley-Evans, 1997).

Several animal models have indicated differences in glucocorticoid sensitivity or glucocorticoid signalling enzymes following pre-natal challenges such as maternal dietary restriction. Glucocorticoid sensitivity in the liver and visceral adipose tissue was significantly increased in rat offspring as a result of maternal nutrient restriction and placental 11β-HSD2 activity is selectively downregulated. These effects were present in fetal, neonatal, juvenile and adult offspring (Langley-Evans *et al.*, 1996; Nyirenda *et al.*, 1998; Bertram *et al.*, 2001; Cleasby *et al.*, 2003a). Hepatic GR levels are increased in the neonatal offspring of undernourished ewes and in the adult offspring of rats that were treated with dexamethasone during pregnancy (Nyirenda *et al.*, 1998; Whorwood *et al.*, 2001; Cleasby *et al.*, 2003b).

Chadio and colleagues showed that ewes that were nutrient restricted during early gestation had significantly higher cortisol levels. The offspring of those ewes had significantly higher areas under the curve for ACTH and cortisol in

response to a CRH challenge, indicating increased HPA axis activation (Chadio *et al.*, 2007).

A challenge of antenatal dexamethasone exposure demonstrates that programming of genes involved in glucocorticoid sensitivity also occurs in adipose tissue, by showing a significant increase in the expression of GR in visceral adipose tissue both in adult rats and in neonatal sheep (Whorwood *et al.*, 2001; Cleasby *et al.*, 2003a).

The programming of glucocorticoids and the HPA axis can occur very early in life as was shown by periconceptional undernutrition (PCUN) in a sheep model. Exposure of the oocyte and/or embryo to maternal undernutrition results in an increase in arterial blood pressure in the foetus and an increased and precocious activation of the HPA axis during late gestation (Edwards & McMillen, 2002a, b; Edwards *et al.*, 2005). When maternal undernutrition extends from the periconceptional period into early pregnancy (i.e. from 60 days prior to conception and up to 30 days of pregnancy) a reduced foetal growth rate occurs with hyperactivation of the HPA axis and an increase in premature deliveries in sheep (Bloomfield *et al.*, 2003; Oliver *et al.*, 2005)

It is clear that glucocorticoids play an important role in adipose tissue growth in the body. This role can include stimulation of lipolysis, lipogenesis and adipogenesis. The action of glucocorticoids on local tissues is dependent on the abundance of GR and the enzymes 11β-HSD1 and 11β-HSD2. It is also clear that the body sensitivity to glucocorticoids can be programmed from

very early on in life. In this thesis we aim to elucidate the exact role of glucocorticoids in the development of regional differences in adipose tissue in early life.

## 1.6 The effects of Insulin like growth factors on adipose tissue

IGF1 and IGF2 are both monomeric plasma proteins present in bound and free form in plasma and in most or all body tissues (Daughaday, 1989; Daughaday & Rotwein, 1989; Humbel, 1990). Both IGF1 and IGF2 are structurally similar to insulin (Rinderknecht & Humbel, 1978; Humbel, 1990; O'Dell & Day, 1998). IGF1 and IGF2 contribute to approximately 90% of the total extractable non-insulin insulin-like material in the serum (Froesch *et al.*, 1985). This similarity could well be responsible for the interactions that occur between IGFs and insulin at each other's receptors (Froesch *et al.*, 1985). The receptor for IGF1, IGF1R, is structurally very similar to IR. IGF1R can bind IGF1, IGF2 and insulin (Nielsen, 1992; O'Dell & Day, 1998). The receptor for IGF2, IGF2R, binds only IGF2 for internalisation and degradation (Nielsen, 1992; O'Dell & Day, 1998).

### 1.6.1 RELEVANCE OF IGFS IN GROWTH

IGFs are considered to be amongst the most important growth factors in the body. In humans IGF1 cord serum levels are directly related to body weight, length and head circumference (Gluckman *et al.*, 1983; Verhaeghe *et al.*,

1993; Geary *et al.*, 2003). IGF1 cord serum levels were found to be 40% lower in infants that were small for gestational age and 28% higher in infants that were large for gestational age compared to infants that were average for gestational age (Verhaeghe *et al.*, 1993). In sheep foetal body weight is directly related to circulating IGF1 levels (Owens *et al.*, 1994; Carr *et al.*, 1995; Kind *et al.*, 1995) and to liver IGF1 mRNA expression levels (Rhoads *et al.*, 2000). An infusion of IGF1 in rats that were hypophysectomised resulted in rats with increased body weight and bone width (Schoenle *et al.*, 1985).

Another example that illustrates the importance of IGF1 in growth is seen in the Pygmies that live in Central Africa; people who are known to have a short stature. They were found to have low basal IGF1 levels and no rise in IGF1 levels was found in response to exogenous growth hormone (GH) administration (Zapf *et al.*, 1978).

IGF1 homozygous knockout mice have been developed. These mice show foetal growth failure from day 13.5 of gestation. They also show growth retardation postnatally, which results in adult mice with a weight of 30-60% of normal adult weight (Baker *et al.*, 1993; Liu *et al.*, 1993).

In humans, one male has been reported to have a homozygous partial IGF1 deletion. He has reduced IGF1 concentrations and truncated protein. His basal and peak GH levels were elevated. At birth he showed symmetric growth retardation with a weight of 1.4 kg (3.9 SD below mean), a length of 37.8 cm (5.4 SD below mean) and a head circumference of 27 cm (4.9 SD below mean). In infancy and childhood he had severe growth failure.

Treatment with recombinant IGF resulted in an improvement of his linear growth and normalisation of his GH and insulin levels (Woods *et al.*, 1996; Woods *et al.*, 1997; Camacho-Hubner *et al.*, 1999).

Ashton and colleagues measured IGF1 levels in the foetus from 15 to 23 weeks of gestation. They found that the foetal levels were lower than cord blood levels at birth or adult levels. They also found that foetal IGF1 levels correlated with placental weight, foetal body weight and foetal body length (Ashton *et al.*, 1985). Langford and colleagues demonstrated that at 27 weeks of gestation IGF1 cord serum levels were lower in small for gestational age foetuses with placental insufficiency compared to average for gestational age babies or small for gestational age babies without placental insufficiency (Langford *et al.*, 1995).

After birth plasma IGF1 levels remain stable for the first 2 days in sheep. This period is followed by a rise in plasma IGF1 during postnatal day 3-7, during this period the relationship between plasma IGF1 levels and birth weight disappears. Plasma IGF2 levels remain stable from 12 hours after birth at a level comparable to the level in adult sheep (Gluckman *et al.*, 1983; Daughaday & Rotwein, 1989).

The relationship between IGF2 and body weight is not as clear as between IGF1 and body weight. Gluckman and colleagues reported no correlation between IGF2 cord serum levels at birth and size at birth (Gluckman *et al.*, 1983). While Geary and colleagues reported a correlation between cord plasma IGF2 levels and birth weight, length at birth and head circumference in a study of 987 singleton newborns (Geary *et al.*, 2003). Ashton measured

IGF2 levels in the foetus between 15 and 23 weeks of gestation and found no correlation between IGF2 levels and placental weight, foetal body weight or length (Ashton *et al.*, 1985). Verhaeghe and colleagues measured cord serum IGF2 levels during the 3rd trimester and at birth and found that IGF2 levels were 8-10% higher in large for gestational age newborns compared to average or small for gestational age newborns. He also reported that the levels of IGF2 were 6-10 times higher than IGF1 levels during this period (Verhaeghe *et al.*, 1993).

A knockout model for IGF2 has been developed. Mice are heterozygous null for IGF2. These mice have 10 times less IGF2 mRNA compared to homozygous wild type mice and they are 50% smaller at birth. There were however no differences in postnatal growth rate between wild type and knockout mice (DeChiara *et al.*, 1990).

#### *1.6.1.1 IGFs in adipose tissue growth*

IGF1 is one of the most important growth factors involved in adipose tissue growth. It has been shown to stimulate DNA synthesis and cell replication in embryo cultures and several cell lines (Zapf *et al.*, 1978). Most of the circulating IGF1 however is of hepatic rather than adipogenic orgin (Butler *et al.*, 2002). This together with evidence from different *in vitro* studies has led most researchers to believe that the effects of IGF1 on adipose tissue are delivered through autocrine and paracrine rather than endocrine

mechanisms (Faust *et al.*, 1978; Lau *et al.*, 1990; Peter *et al.*, 1993; Considine *et al.*, 1996; Marques *et al.*, 1998; Butler *et al.*, 2002).

IGF1 is an important factor in the proliferation and differentiation of preadipocytes. In cell culture it has proven, like glucocorticoids, to be an essential requirement for the induction of adipocyte differentiation (Gregoire *et al.*, 1998; Soret *et al.*, 1999). The role of IGF1 in adipocyte differentiation has also been shown to be present in other *in vitro* and *in vivo* studies (Smith *et al.*, 1988; Ramsay *et al.*, 1989b; Wright & Hausman, 1995; Rajkumar *et al.*, 1999; Holzenberger *et al.*, 2001).

Marques and colleagues performed an experiment in which IGF1 was stripped from the culture medium. Preadipocyte proliferation from inguinal and epididymal fat from rats was completely prevented in this study (Marques *et al.*, 2000). Holzenberger and colleagues showed with their experiment that a global IGF1R deficiency in transgenic mice led to growth restriction and a reduction in relative fat mass (Holzenberger *et al.*, 2001). Furthermore, Clarke and colleagues have demonstrated that IGF1 levels in plasma are correlated with lipid content in perirenal fat during the first month of postnatal life in sheep (Clarke *et al.*, 1997b).

#### *1.6.1.2 Factors that influence IGF function*

IGF levels are influenced by many factors which include growth hormone, insulin, NEFA and leptin levels. Insulin and growth hormone have been shown to have stimulatory effects of IGF1 secretion in porcine and human

preadipocyte and adipocyte cultures (Chen *et al.*, 1996; Wabitsch *et al.*, 2000). Work from a colleague in our laboratory has shown that there was no relationship between plasma insulin and IGF1 mRNA levels in perirenal adipose tissue of the sheep during the first three weeks of life. Furthermore it was shown that plasma leptin on postnatal day 1 was related to perirenal IGF1 mRNA expression at three weeks of age in both male and female lambs (Duffield, 2007).

The relevance of IGF1 in nutrition driven growth was demonstrated by a study by Rhoads and colleagues who showed that lambs that were given a balanced diet compared to lambs given a diet that lacked in energy and protein grew faster, but also had IGF1 plasma levels twice as high (Rhoads *et al.*, 2000). Several other studies showed the effects of nutrition on IGF expression and function. Hua and colleagues showed that sheep which were fasted for three days had a reduction in plasma IGF1 but not IGF2 levels both at the age of 4 months and at the age of 2 years (Hua *et al.*, 1995). They also showed a reduction in plasma IGF1, but not IGF2, levels and kidney IGF1 mRNA levels after fasting for 5 days in sheep at the age of six months (Hua *et al.*, 1993). Fasting did not, however, affect liver or skeletal muscle IGF1 mRNA levels in sheep at six months of age (Hua *et al.*, 1993; Rhoads *et al.*, 2000).

Another important factor for expression levels of IGF1 is gender. Sexual dimorphisms of IGF expression and function have been shown in numerous studies (Hindmarsh *et al.*, 1999; Fall *et al.*, 2000). At birth human males have higher levels of cord GH, IGF1 and IGF-binding protein-3 compared to

females (Geary *et al.*, 2003). In sheep, plasma IGF1 levels have been shown to be higher in males compared to females both in lambs and in adults (Van Vliet *et al.*, 1983; Gatford *et al.*, 1996). IGF1 mRNA levels in perirenal adipose tissue of 3 week old sheep were also higher in males compared to females and in subcutaneous adipose tissue a trend was shown for higher levels in males compared to females (Duffield, 2007). It could be that the increase in circulating IGF1 in males compared to females is secondary to an increased IGF1 transcription in adipose tissue (Nam & Marcus, 2000).

### 1.6.2 IGFS IN DIFFERENT ADIPOSE TISSUE DEPOTS

The expression levels of IGF1, IGF2 and their receptors have been shown to be adipose tissue depot-specific. A colleague from our laboratory has shown that at three weeks of age in sheep, IGF1 expression was higher in perirenal adipose tissue compared to subcutaneous adipose tissue, whilst IGF2, IGF1R and IGF2R were lower in the perirenal depot compared to the subcutaneous depot (Duffield, 2007). It has been suggested that depot specific expression levels of insulin like growth factor receptors could be an explanation for the variation in proliferative capacities of preadipocytes in different adipose tissue depots and the differences in metabolism between fat depots (Wang *et al.*, 1989; Bouchard *et al.*, 1993; Dieudonne *et al.*, 2000; Blaak, 2001). However expression levels of IGFs and their receptors in different visceral adipose tissue depots has not been investigated to date.

### 1.6.3 EVIDENCE FOR THE DEVELOPMENTAL PROGRAMMING OF GENES INVOLVED IN INSULIN LIKE GROWTH FACTOR SIGNALLING

Most of the developmental challenges that have shown to have a programming effect on insulin sensitivity and signalling have also been shown to affect insulin like growth factor signalling. IGF1 protein in foetal arterial blood has been shown to be related to placental as well as to foetal weight. IGF1 protein in foetal blood was also related to IGF1 mRNA levels in liver, kidney and skeletal muscle, but not lung or heart. IGF2 protein in foetal arterial blood however showed no relationship with placental or foetal weight or tissue levels of IGF mRNA (Kind *et al.*, 1995). Small for gestational age infants have lower levels of circulating IGF1, which is related to a reduced relative adipose tissue mass at birth (Enzi *et al.*, 1981). Intra-uterine growth restricted foetuses (IUGR), which were 50% smaller than control foetuses, had reduced levels of liver IGF1 mRNA (Rhoads *et al.*, 2000). Periconceptional maternal undernutrition has been shown to reduce foetal sheep plasma IGF1, but not IGF2 during late gestation (Lee *et al.*, 1997; Gallaher *et al.*, 1998). Nutritional challenges during pregnancy, such as maternal nutrient restriction or placental restriction have been shown to reduce plasma IGF1 and IGF mRNA in liver, kidney and skeletal muscle of the fetal sheep (Owens *et al.*, 1994; Kind *et al.*, 1995; Osgerby *et al.*, 2002). Placental restriction has also been shown to result in a reduction of IGF1 mRNA expression in perirenal adipose tissue during foetal life (Gemmell &

Alexander, 1978; Klaus, 1997). Other challenges such as hypoxaemia have also been shown to affect liver IGF1 mRNA in fetal sheep, but not IGF1 mRNA in kidney, muscle, lung or thymus. In addition, hypoxaemia had no effect on IGF2 mRNA levels (McLellan *et al.*, 1992).

It is clear that IGFs play an important role in adipose tissue development and that IGF expression and function can be programmed during early life. Whether this programming of IGF expression in early life remains present in the long term and whether this could influence the adipose tissue distribution in later life, however, remains uncertain.

## 1.7 Hypotheses

### 1.7.1 MAIN HYPOTHESIS

Adipose tissue distribution and development are regulated by a complex interaction between the local actions of insulin, glucocorticoids and insulin like growth factors on individual adipose tissue depots. Insulin promotes adipose tissue growth by promoting adipogenesis and lipogenesis and inhibiting lipolysis. Glucocorticoids decrease adipose tissue growth in the short term by promoting lipolysis but chronic exposure to high glucocorticoid concentrations stimulate adipose tissue growth through a stimulation of adipogenesis and lipogenesis. Insulin like growth factor 1 promotes adipogenesis.

*I propose that changes in adipose tissue distribution and development, as a consequence of exposure to different patterns of nutrition in the neonatal period, are secondary to changes in the balance between the actions of insulin, glucocorticoids and insulin like growth factors on regional adipose tissue depots.*

### 1.7.2 SPECIFIC HYPOTHESES

#### *1.7.2.1 Adipose tissue development and distribution in early postnatal life*

Individual adipose tissue depots develop at different stages in the perinatal period. The subcutaneous and perirenal adipose tissue depot start to develop during prenatal life, while the omental depot develops predominantly in postnatal life. During prenatal life the foetus is exposed to insulin, which drives growth. Adipose tissue which is exposed to high insulin levels can develop a relative resistance to the anti-lipolytic effects of insulin.

*I propose that the relatively prolonged exposure of perirenal and subcutaneous adipose tissue to the effects of insulin, compared to the omental adipose tissue depot, which develops mainly in postnatal life, make these tissues relatively more resistant to the local actions of insulin compared to the omental depot during the first few weeks of postnatal life. Therefore, after birth when enteral food intake commences, the tissue that is*

*most sensitive to the local actions of insulin will be the omental adipose tissue depot, which would result in an accelerated growth of this depot in immediate postnatal life.*

In chapter two we test this hypothesis by analysing growth of the individual adipose tissue depots and markers of the tissue sensitivity to the local actions of insulin in omental and perirenal adipose tissue during early life.

#### *1.7.2.2 Periconceptional undernutrition and its effects on glucocorticoid-insulin balance*

Exposure of the oocyte and/or embryo to maternal undernutrition (periconceptional undernutrition; PCUN) results in a premature activation of the HPA axis during late gestation. Furthermore it results in an enhanced cortisol response after a CRH challenge. This increased pituitary-adrenal activation occurs during late gestation, the period during which the subcutaneous and perirenal adipose tissue depot undergo rapid growth.
Offspring exposed to high cortisol levels in utero, such as in maternal nutrient restriction, have a greater response to HPA axis activation in later life. It is unknown to date whether this response is tissue specific.

*I hypothesise that PCUN will result in an enhanced glucocorticoid response in subcutaneous and perirenal adipose tissue depots which will persist after birth. When this period of periconceptional nutrient restriction occurs in*

*women that are already overweight, these effects are exaggerated, due to the increased insulin exposure of the perirenal and subcutaneous adipose tissue depot during prenatal life. Therefore these two depots become relatively even more insulin resistant and therefore more sensitive to the effects of glucocorticoids, which results in an even greater lipolytic activity in these depots and a greater nutrient supply for the omental depot.*

In chapter three we test this hypothesis by examining the effect on periconceptional undernutrition in ewes that were of a normal weight and ewes that were obese on adipose tissue growth and distribution in early life. We further test the described mechanism by investigating the expression of markers of the tissue sensitivity to the actions of insulin and glucocorticoids in the individual adipose tissue depots.

#### *1.7.2.3 Periconceptional undernutrition and the effects on insulin like growth factors*

Most tissues during development undergo a transition in the role of the insulin like growth factors and their receptors. This transition is, at least partly, mediated by glucocorticoids which stimulates IGF1R expression.

*I hypothesise that PCUN will result in a glucocorticoid mediated transition of IGF2R to IGF1R expression which happens mainly in the perirenal and subcutaneous adipose tissue depots which are present during foetal life.*

*Therefore the effects of IGF1 will be enhanced in the perirenal and subcutaneous adipose tissue depots, resulting in an increase in adipogenesis.*

In chapter four we test this hypothesis by examining the effects of periconceptional undernutrition in ewes that were of a normal weight and ewes that were obese on the expression of IGF1, IGF2, IGF1R and IGF2R in individual adipose tissue depots.

#### *1.7.2.4 Formula feeding and the effects on adipose tissue growth and distribution*

Formula feeding is associated with an increased food intake in early life and an increased plasma insulin concentration.

*I hypothesise that formula feeding results in a reduced sensitivity of adipose tissue to the actions of insulin. This reduction in sensitivity to insulin will switch the balance between insulin and glucocorticoids action on adipose tissue towards a stronger sensitivity of the adipose tissue to glucocorticoids. In the long term this increased glucocorticoid action will result in an increase in adipogenic growth in adipose tissue. The reduction in local sensitivity to the action of insulin will be greatest in the omental depot, which is developing rapidly during this period in life. The effects on increased adipose tissue*

*growth will therefore be greatest in this depot and lead to a redistribution of adipose tissue towards the omental depot.*

In chapter five we test this hypothesis by examining the effects of formula feeding or maternal milk feeding on the development and distribution of adipose tissue in lambs of three months of age. We further examine the expression of markers of the tissue sensitivity to the actions of insulin and glucocorticoids in individual adipose tissue depots.

#### *1.7.2.5 Adipose tissue distribution with adult-onset obesity*

Obesity is related to relative insulin resistance.

*I propose that the decreased response to the actions of insulin in adipose tissue will result in a reduction of the insulin mediated inhibition of 11β-HSD1 expression resulting in enhanced local production of cortisol resulting in an increase in adipogenesis and adipose tissue growth.*

*In obesity, however, the subcutaneous and perirenal depots are relatively less insulin resistant compared to the omental depot. In these depots insulin remains the main driver of growth. When the effects of obesity are combined with the effects of formula feeding an earlier shift towards glucocorticoid driven control of adipose tissue will occur in the omental depot, resulting in an exacerbation of the effects of an obesogenic lifestyle in people that were formula fed in early life.*

In chapter six we test this hypothesis by examining the effects of an obesogenic lifestyle in animals that were fed maternal milk and animals that were formula fed on adipose tissue distribution. We further examine the expression and abundance of markers of tissue sensitivity to the actions of insulin and glucocorticoids in individual adipose tissue depots.

# 2. Ontogeny of the distribution of adipose tissue and differential abundance of proteins in the insulin signalling cascade in adipose tissue in early life

## 2.1 Summary

It is well established that an increase in visceral adipose tissue mass relates to an increase in metabolic risks in an individual. In large mammals, like sheep, at birth the major visceral adipose tissue depot is the perirenal depot. Later in life the omental depot develops but little is known about the differential growth patterns of these individual adipose tissue depots. Insulin is considered to be a major driving factor regulating adipose tissue growth. The actions of insulin are dependent on the efficiency of a complex cascade of several factors that involve insulin signalling in the cell. It has not been investigated whether the tissue specific abundance of factors involved in the insulin signalling cascade is different between adipose tissue depots. We hypothesised that omental adipose tissue has a relative accelerated growth pattern compared to other visceral adipose tissue depots in early life. We further hypothesised that this accelerated growth pattern is secondary to a relatively enhanced efficiency of the insulin signalling cascade in this depot in early postnatal life.

Visceral adipose tissue was collected from sheep at different ages in early life and the growth of the individual visceral adipose tissue depots was

determined. The abundance of key factors in the insulin signalling cascade: IR, p85, Glut4 and IGF1R was determined in omental and perirenal adipose tissue from 7 and 14 day old lambs using Western Blotting.

We found that during the first three months of postnatal life omental adipose tissue has a relative accelerated growth compared to the other visceral adipose tissue depots. IR abundance was significantly higher in perirenal compared to omental adipose tissue while p85 abundance was significantly lower in perirenal compared to omental adipose tissue. Glut4 and IGF1R abundance were not different between adipose tissue depots. All investigated proteins had a higher abundance at 7 days of age compared to 14 days of age.

This study has clearly shown that visceral adipose tissue development in early life is depot specific and that markers of insulin signalling are different between depots in early life. A lower abundance of IR in omental compared to perirenal adipose tissue, combined with a comparable abundance of Glut4 between the depots, could potentially mean that the omental depot has a relatively more efficient insulin signalling cascade compared to the perirenal depot. However, this effect seems to be compensated by an upregulation of IR in the perirenal depot, leading to similar levels of Glut4 between tissues. Therefore, no conclusive evidence was found that the relative accelerated growth of omental adipose tissue in early postnatal life is driven by greater abundance of proteins involved in the insulin signalling cascade.

## 2.2 Introduction

Obesity is a very important health problem in our present day society. The metabolic risk factors associated with obesity are dependent on the distribution of adipose tissue. Increased central as opposed to subcutaneous adipose tissue is associated with a higher cardiovascular risk (Fujioka *et al.*, 1987; Despres *et al.*, 1989; Pouliot *et al.*, 1992; Yusuf *et al.*, 2004).

Mechanisms that have been supposed to explain the difference in risk patterns associated with a more central than peripheral adipose tissue distribution include the production of cytokines by visceral adipose tissue and the direct release of free fatty acids from the visceral depot into the portal vein. The increased release of free fatty acids into the portal vein could be secondary to a decreased sensitivity to antilipolytic stimuli, by for example insulin (Arner *et al.*, 1990; Hellmer *et al.*, 1992; Marin *et al.*, 1992a; Smith & Zachwieja, 1999). An increase in FFA in the portal drained viscera leads to a reduced insulin clearance and increased insulin secretion by the liver and ultimately insulin resistance. An increase in FFA in the portal vein also contributes to dyslipidaemia (Svedberg *et al.*, 1990; Boden *et al.*, 1998; Smith & Zachwieja, 1999).

Visceral adipose tissue does not consist of one major adipose tissue depot but rather an amalgamation of pericardial (around the heart), perirenal (around the kidneys), omental (around the mesentery and intestines)

adipose tissue, and, arguably, also the subcutaneous adipose tissue that surrounds the abdomen (Himms-Hagen & Ricquier, 1998). In contrast to all the research that has been focusing on the differences between central and peripheral adipose tissue there has hardly been any research focusing on the differences between the depots that are all considered central.

Previous studies have shown that in large mammals, like sheep, at birth the most important visceral adipose tissue depot is the perirenal adipose tissue depot (Clarke *et al.*, 1997a; Symonds & Stephenson, 1999). After birth, however, the omental adipose tissue depot starts to develop. Little is known about the growth rate of those different depots and the factors that influence the distribution of adipose tissue between visceral fat depots.

Adipose tissue deposition is influenced by several factors amongst which insulin is one of the most important ones. Insulin inhibits lipolysis and therefore promotes adipose tissue growth. The local tissue-specific effects of insulin are dependent on the level of circulating insulin, the density of membrane bound insulin receptors and the intracellular efficiency of insulin-stimulated second messenger signalling.

It has been shown that the anti-lipoloytic effect of insulin is lower in obese compared to lean individuals and that this is related to insulin resistance (Landin *et al.*, 1990). This suggests that increased exposure of adipose tissue to insulin results in a reduced sensitivity of adipose tissue to the effects of insulin.

We propose that the relatively prolonged exposure of perirenal and subcutaneous adipose tissue to the effects of insulin, compared to the omental adipose tissue depot, which develops mainly in postnatal life, make these tissues relatively more insulin resistant compared to the omental depot during the first few weeks of postnatal life. Therefore, after birth when enteral food intake commences, the tissue that is most insulin sensitive will be the omental adipose tissue depot, which would result in an accelerated growth of this depot in immediate postnatal life.

*In this chapter we test this hypothesis by analysing growth of the individual adipose tissue depots and markers of the tissue sensitivity to the local actions of insulin in omental and perirenal adipose tissue during early life.*

## 2.3 Methods

### 2.3.1 ONTOGENY OF THE DISTRIBUTION OF ADIPOSE TISSUE IN THE SHEEP

In this study, cross sectional data from multiple groups of nutritional control, twin sheep were used to delineate the postnatal ontogeny of regional adipose tissue depots in sheep. Data are: day 140 foetal samples (n=16, female [10] and male [6]); term, n=15 lambs (female [9] male [6]); day 7, n=7 lambs (female [1], male [6]); day 14, n=6 lambs (female [1] male [5]); one month of age, n=9 lambs (female [3] male [6]); three months of age, n=6 lambs (female [0] male [6]); six months of age, n=7 lambs (female [5] male [2]); 12 months of age, n=12 lambs (female [5] male [7]) and 36 months of age, n=8 lambs (female [0] male [8]). Details of the different animal cohorts can be found in Table 2.1. Animals were humanely killed using a pentobarbitone overdose and omental, perirenal and pericardial adipose tissue was collected, weighed and snap frozen in liquid nitrogen and stored at -80ºC for further laboratory analysis.

| Age | Breed | Number of females | Number of males | Total number |
|---|---|---|---|---|
| 140 day gestation | Welsh mountain | 10 | 6 | 16 |
| term | Welsh mountain | 9 | 6 | 15 |
| 7 days | Welsh mountain | 1 | 6 | 7 |
| 14 days | Scottish Blackface | 1 | 5 | 6 |
| 1 month | Welsh mountain | 3 | 6 | 9 |
| 3 months | Cross-bred Mule | 0 | 6 | 6 |
| 6 months | Welsh mountain | 5 | 2 | 7 |
| 12 months | Cross-bred Mule | 5 | 7 | 12 |
| 36 months | Scottish Blackface | 0 | 8 | 8 |

**Table 2.1; Overview of animal cohorts studied**

Different animal cohorts studied at specific ages. Information regarding breed and animal numbers in each cohort.

### 2.3.2 SAMPLE PREPARATION FOR REGIONAL ADIPOSE INSULIN SENSITIVITY ANALYSIS DURING THE FIRST TWO WEEKS OF POSTNATAL LIFE

Protein was extracted from perirenal and omental adipose tissues of the 7 and 14 day old lambs. Lambs of 7 and 14 days of age were chosen because it appeared that the difference in growth rate between perirenal and omental adipose tissue was greatest at these ages. Unfortunately no subcutaneous adipose tissue was available from these animals for further analyses.

#### *2.3.2.1 Protein extraction*

To extract protein a lysis buffer was made by combining 75 ml RIPA, 750 µl PMSF, 2250 µl aprotinin and 750 µl sodium orthovanadate. 2.5 g of adipose tissue was homogenised in 7.5 ml of the lysis buffer. 50 µl PMSF was added and the mixture was incubated for 30 minutes on ice. The mixture was then centrifuged at 10000 rpm for 10 minutes at 4ºC. The supernatant was collected and recentrifuged for another 10 minutes at 10000 rpm at 4ºC. The supernatant was again collected, aliquoted into 2 tubes and stored at -20ºC.

#### *2.3.2.2 Measurement of protein concentration*

The protein content was measured using a bicinchoninic acid kit. Reagent A was prepared using 0.5 g 1% bicinchoninic acid (BCA), 1 g 2% sodium carbonate, 0.08 g 0.16% sodium tartrate and 0.2 g 0.4% sodium hydroxide in water to a total volume of 50 ml. 10% sodium bicarbonate was used to achieve a pH of 11.25. Reagent B was prepared with 2 g of 4% copper sulphate in water to a total volume of 50 ml. Reagent C was prepared by combining reagent A and B in a ratio of 100:2. A serial dilution of a 1 mg/ml stock solution of BSA in 0.9% saline was used as a standard.

Samples were diluted 1:20 with distilled water. 50 µl of standard solution or sample was added to 1 ml of reagent C and incubated while shaking at 37ºC for 30 minutes. 200 µl from each tube was analysed in triplicate with a photospectrometer at 570 nm. All protein samples were diluted to the concentration of 1 mg/ml. SDS loading buffer (5 ml 1M Tris-HCL pH6.8, 1.54

grams dithiotreitol, 2 g SDS, 10 ml glycerol and bromophenol blue, mixed and diluted 1 in 5) was used to dilute the samples.

#### *2.3.2.3 Protein separation using electrophoresis*

A resolving buffer was prepared from 30 ml 40% acrylamide, 7.8 ml 2% bisacrylamide, 64.8 ml distilled water, 15 ml 3M Tris HCl (pH 8.8), 1200 µl 20% (w/v) SDS, 162 µl TEMED and 1620 µl 10% (w/v) APS. The resolving buffer was loaded in a prepared gelholder (2 glassplates rinsed with 70% ethanol and distilled water, fixed with sticky tape and clips), layered with a butanol-water mixture and left to set for 35 minutes. The butanol-water mixture was removed, a comb was placed and a stacking gel was added (7.5 ml 40% acrylamid, 3.9 ml 2% bisacrylamide, 39.9 ml distilled water, 7.5 ml 1M Tris HCl (pH 6.8), 600 µl 20% (w/v) SDS, 81 µl TEMED, 810 µl 10% (w/v) APS). The stacking gel was left to set for 15-20 minutes. The tape was removed and the gelholder was placed in a geltank. Running buffer (10x running buffer = 60 g Tris base, 288 g glycine, 100 ml 20% SDS, 2 L distilled water) was added and the comb was removed. Protein samples were heated to 100ºC for 5 minutes. 10 µl of each sample was loaded on the gel. A negative control was used which contained 100% SDS. One sample of omental and one sample of perirenal tissue were loaded twice, once with 10 µl and once with 20 µl in order to assess the adequate exposure to measure protein amounts. A marker was loaded to assess protein size. The gel tank was closed and an electrical power of 25V was applied overnight.

#### *2.3.2.4 Protein transfer onto Western Blotting membrane*

The following day protein was transferred onto a membrane. A transfer buffer was prepared as follows: 50x transfer buffer = 9.3 g SDS, 145.25 g Trisbase, 73 g Glycine, 500 ml distilled water. Filter paper and membranes were soaked for 1 minute in methanol and for 1 minute in distilled water prior to be soaked in 1x transfer buffer. A horizontal transfer chamber was used for sample transfer. 8 layers of filter paper were placed underneath the membrane; the gel was placed on top and covered with another 8 layers of filter paper. All layers were soaked in transfer buffer. The protein samples were transferred at 400mA, 10 W to a maximum of 21 V for 1 hour and 35 minutes. The membrane was then removed and placed in blocking buffer (20 ml 10x TBS, 0.5 ml Tween 20, 2g Marvel powdered skimmed milk in a total of 200 ml distilled water) overnight at 4ºC while continuously shaking.

#### *2.3.2.5 Blotting the membranes with primary and secondary antibodies*

The following morning the blocking buffer was drained and a dilution of primary antibody was added. Ovine antibodies used were to insulin receptor β-subunit (IR, 1:200) (Santa Cruz, Autogen Bioclear, UK), glucose transporter 4 (Glut4, 1:5000) (Abcam, Cambridgeshire, UK), p85 α-subunit of phophatidylinositol 3-kinase (p85, 1:1000) (Upstate Biotech) and insulin like growth factor 1 receptor β-subunit (IGF1R, 1:200) (Santa Cruz, Autogen Bioclear, UK). The dilutions were made using PBS buffer (10 tablets PBS, 20

g Marvel powdered skimmed milk, 2 ml Tween 20, distilled water up to a total of 2 L). The membranes were incubated with the primary antibodies for 1 hour at room temperature. The membranes were then washed in TBSE buffer (100 ml 10x TBS, 5ml 20% SDS, 10ml 0.5M EDTA, 10ml Triton X 100, 10g deoxycholic acid, distilled water up to a total of 1L) for 35 minutes. The membranes were then washed twice for 5 minutes each in fresh PBS buffer. The secondary antibodies anti-rabbit (Amersham, UK) were diluted 1:5000 in PBS. The membranes were incubated 1 hour at room temperature with secondary antibody, before being washed for 35 minutes with TBSE and twice for 5 minutes with PBS buffer.

#### *2.3.2.6 Chemiluminence and densitometric analysis of protein concentration*

Chemiluminence was performed by mixing reagent A and B from Millipore chemiluminence substrate (Millipore, USA). The membranes were incubated for 1 minute in substrate and then wrapped in clingfilm. The membranes were placed in a hypercasette and exposed in a dark room at different exposure times. Protein concentrations were analysed using densitometry. The exposure which produced a ratio of 1:2 of protein concentration for the sample that was loaded twice with a double amount was used for further analyses of the other samples. An example of a Western Blot image is shown in Figure 2.1.

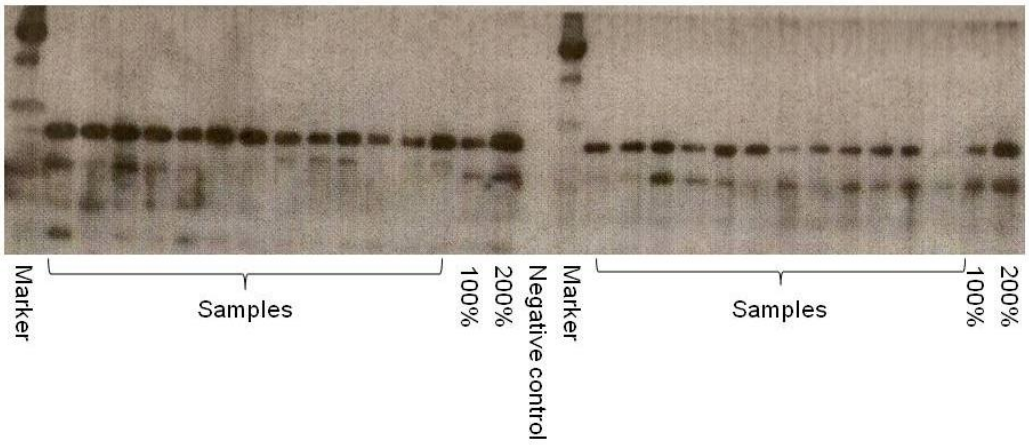


**Figure 2.1; Example of Western Blot gel image**

Gel image of P85 western blot. Marker: protein markers, Samples: unknown samples loaded with 10 µg protein, 100%: sample loaded with 10 µg protein, 200%: same sample as 100% loaded with 20 µg protein, negative control: loaded with SDS buffer.

### 2.3.3 STATISTICAL ANALYSIS

Differences in gene expression between depots and groups of lambs of different ages were determined using a multifactorial ANOVA with repeated measures using STATA 10.0 (StataCorp, College Station, Texas, USA) in which the outcomes for the different depots were considered to be repeated within each animal. Extensive consideration has been given to the choice of using analyses for repeated or independent measures. Data have been analysed using both methods obtaining similar results. It was felt that data from different depots could not be considered as indepent and therefore analyses using repeated measures were chosen to be presented here. Given the unequal gender distribution in our cohorts, the effect of gender could not

be analysed in this chapter. Results are presented as means ± standard error of the mean.

## 2.4 Results

### 2.4.1 General ontogeny of visceral adipose tissue distribution in lean sheep

Figure 2.2 illustrates the development of regional adiposity in female sheep. At birth approximately 90% visceral adipose tissue in the term lamb is located around the kidneys. Within 6 months, the relative distribution changes considerably with a rapid expansion of the omental depot and relative loss of the perirenal depot. After this time, the relative mass of each depot remains relatively stable (Figure 2.2). The absolute weight of each adipose tissue depot at different ages can be found in Table 2.2.

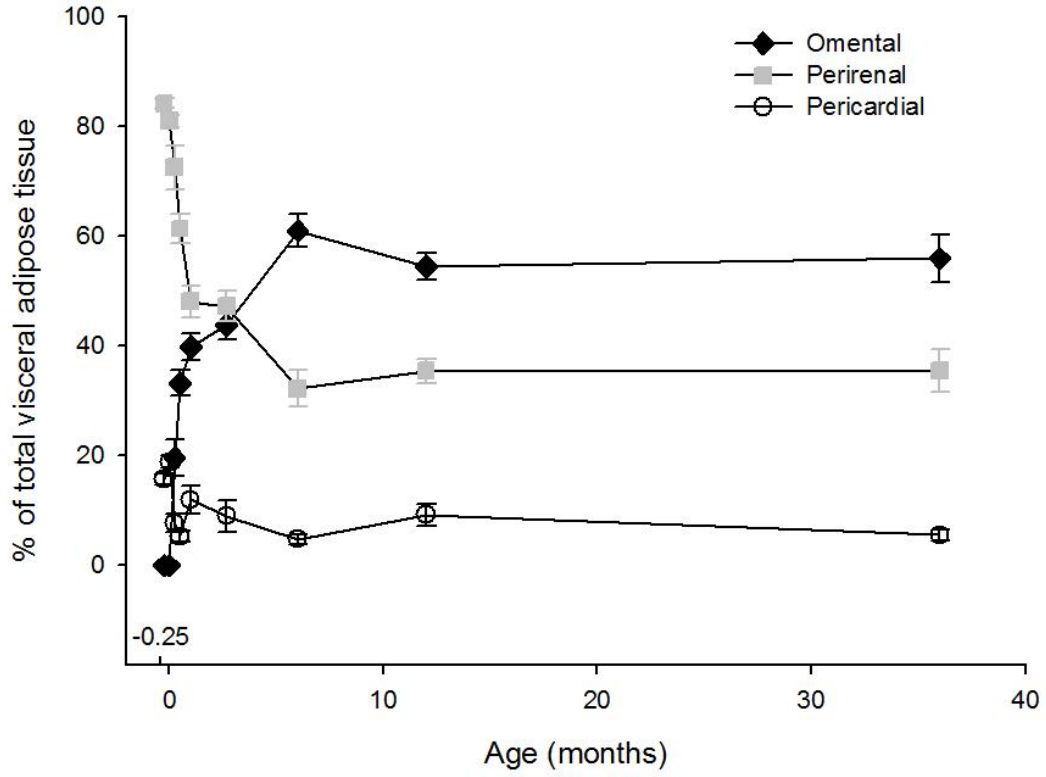


**Figure 2.2; Relative contribution of specific visceral adipose tissue depots to total visceral adipose tissue in control sheep over time**

Contribution of total omental, perirenal and pericardial adipose tissue to total visceral adipose tissue during prenatal (140 days gestation) and postnatal (up to 36 months) development in control sheep. Data are: 140 days gestation: n=16, term: n=15, 7 days postnatal: n=7, 14 days postnatal: n=6, 1 month: n=9, 3 months: n=6, 6 months: n=7, 12 months: n=12, 36 months: n=8.

| Age | Omental weight (g) | Perirenal weight (g) | Pericardial weight (g) |
|---|---|---|---|
| 140 days gestation | - | 20.1 ± 0.9 | 3.7 ± 0.3 |
| Term | - | 19.8 ± 1.1 | 4.5 ± 0.4 |
| 7 days | 11.7 ± 2.7 | 43.3 ± 6.8 | 4.5 ± 0.3 |
| 14 days | 46.7 ± 6.8 | 87.5 ± 14.9 | 8.2 ± 2.2 |
| 1 month | 99.4 ± 1.4 | 119.2 ± 12.6 | 29.6 ± 7.2 |
| 3 months | 340.0 ± 77.1 | 372.5 ± 98.1 | 49.2 ± 6.3 |
| 6 months | 523.6 ± 82.6 | 269.7 ± 39.7 | 36.2 ± 3.8 |
| 12 months | 714.7 ± 92.3 | 472.0 ± 80.9 | 92.7 ± 11.3 |
| 36 months | 712.9 ± 128.3 | 474.5 ± 112.0 | 63.5 ± 10.7 |

**Table 2.2; Absolute adipose tissue weight per depot at different ages**

Absolute weight in grams (mean ± SEM) of the different adipose tissue depots at different ages. Data are: 140 days gestation: n=16, term: n=15, 7 days postnatal: n=7, 14 days postnatal: n=6, 1 month: n=9, 3 months: n=6, 6 months: n=7, 12 months: n=12, 36 months: n=8.

### 2.4.2 VISCERAL ADIPOSE TISSUE DISTRIBUTION AT 7 AND 14 DAYS

The rapid expansion of the omental depot during the first six months of life starts within the first two weeks. The relative contribution of omental adipose tissue to total visceral fat increases from 19.6 ± 3.3% to 33.2 ± 2.3% during the period from 7 to 14 days (P = 0.008, Figure 2.3). During the same period in life there is a tendency towards a lower relative contribution of perirenal adipose tissue to visceral fat from 72.6 ± 4.0% to 61.4 ± 2.6% (P = 0.08, Figure 2.3).

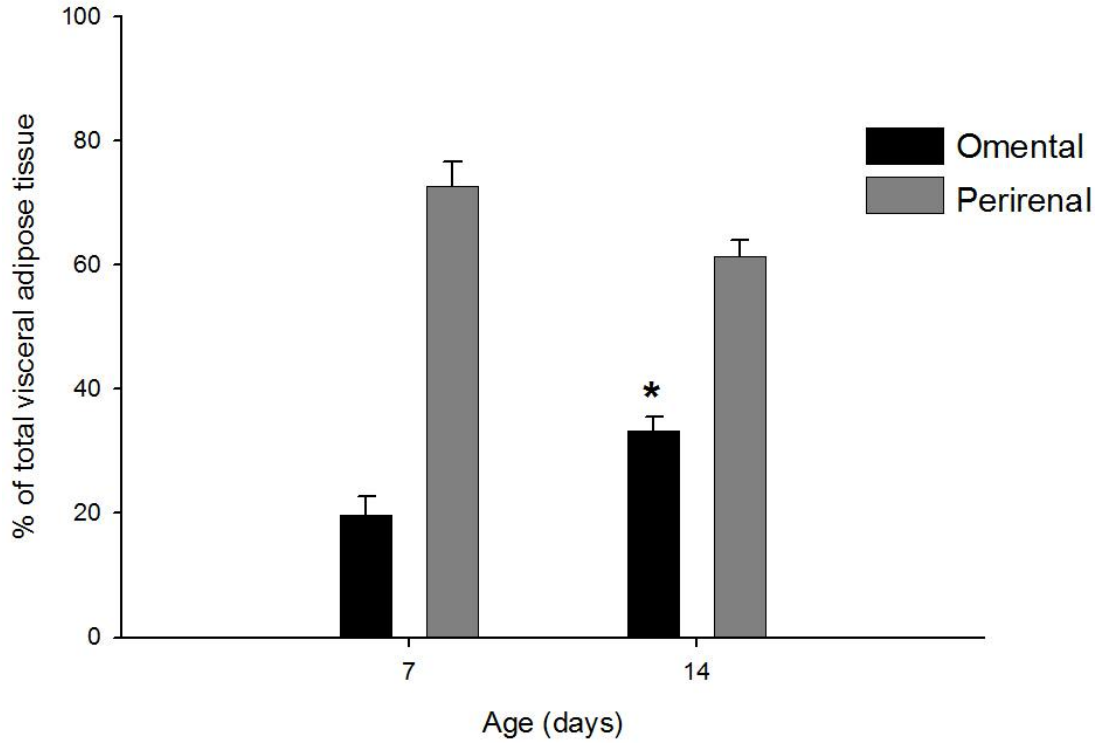


**Figure 2.3; Relative contribution of omental and perirenal adipose tissue to total visceral fat in control sheep at 7 and 14 days**

Omental and perirenal adipose tissue mass as a percentage of total visceral adipose tissue mass at 7 and 14 days of age. * indicate differences between 7 and 14 days. Data are: 7days: n=7, 14 days: n=6.

### 2.4.3 INSULIN RECEPTOR ABUNDANCE IN 7 AND 14 DAY OLD LAMBS

The abundance of insulin receptor was higher in 7 day old lambs compared to 14 day old lambs. This effect was significant in the perirenal as well as in the omental adipose tissue depot (P<0.001). At both 7 and 14 days of age IR abundance was higher in perirenal compared to omental adipose tissue (P=0.005, Figure 2.4). No interaction was found between age and adipose tissue depot.

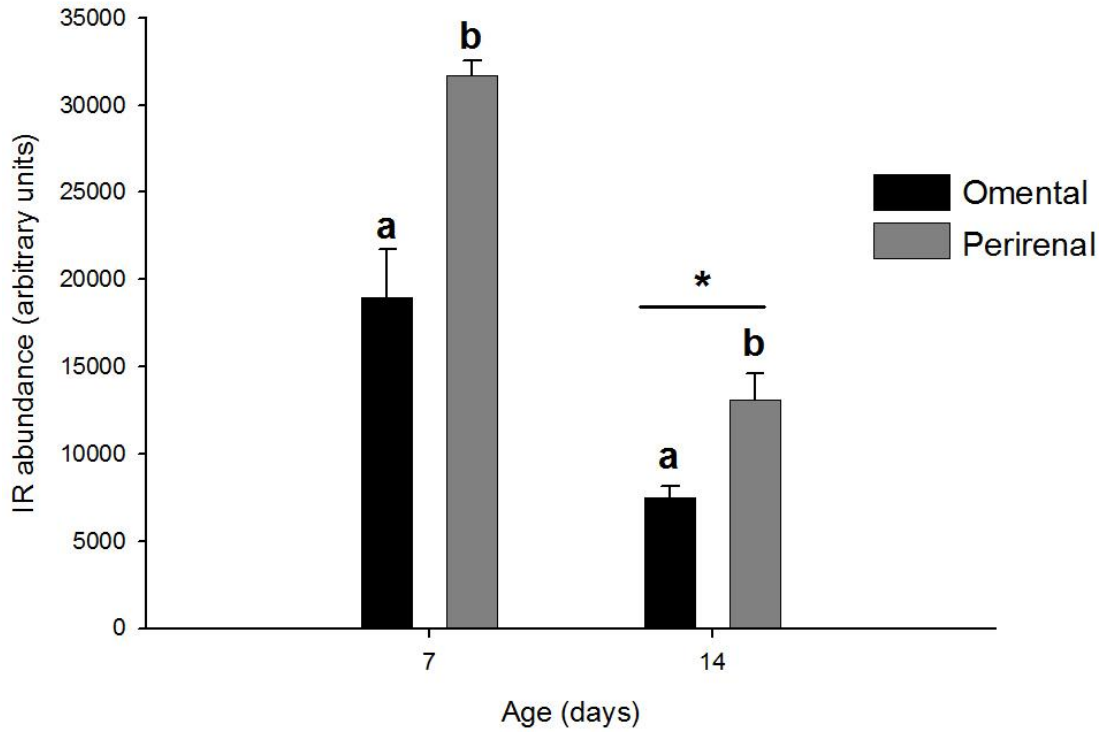


**Figure 2.4; IR abundance in omental and perirenal adipose tissue in control sheep at 7 and 14 days of age**

* indicate differences between 7 and 14 days, different letters indicate differences between depots. Data are: 7days: n=7, 14 days: n=6.

### 2.4.4 P85 ABUNDANCE IN 7 AND 14 DAY OLD LAMBS

The abundance of p85 was higher in 7 day old lambs compared to 14 day old lambs in perirenal and omental adipose tissue (P=0.007). p85 abundance was higher in the omental compared to the perirenal depot (P=0.011). No interaction was found between age and adipose tissue depot (Figure 2.5).

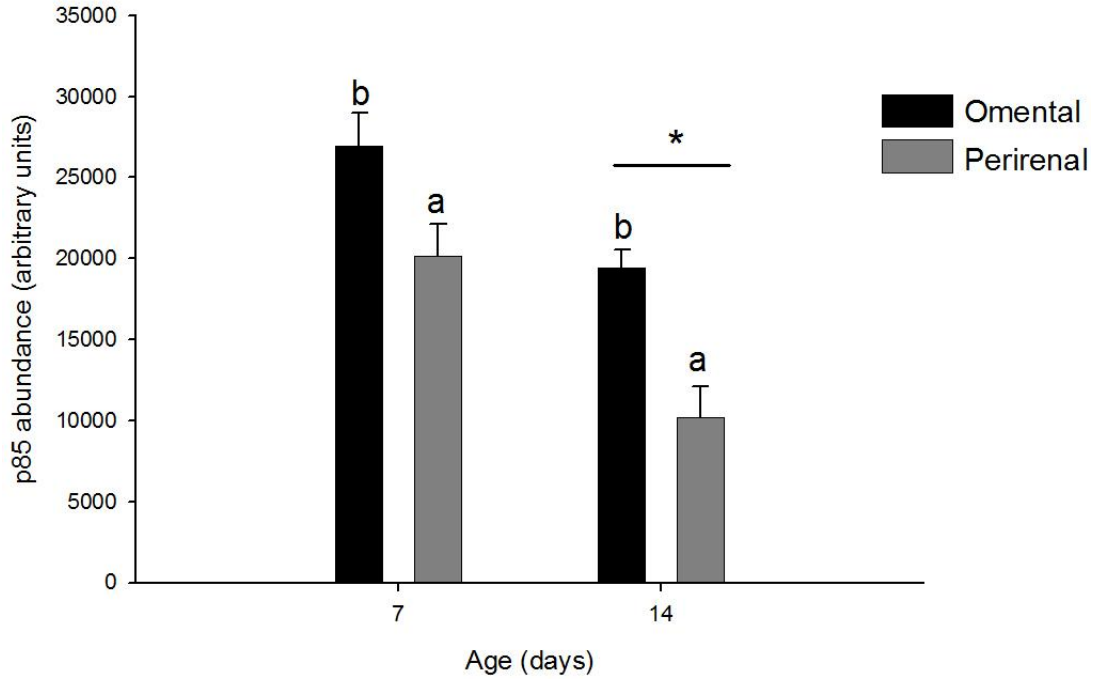


**Figure 2.5; p85 abundance in omental and perirenal adipose tissue in control sheep at 7 and 14 days of age**

* indicate differences between 7 and 14 days, different letters indicate differences between depots. Data are: 7days: n=7, 14 days: n=6.

### 2.4.5 GLUT4 ABUNDANCE IN 7 AND 14 DAY OLD LAMBS

Glut4 abundance tended to be higher at 7 days than at 14 days (p=0.06). The abundance was not significantly different between the perirenal and the omental adipose tissue depot (Figure 2.6).

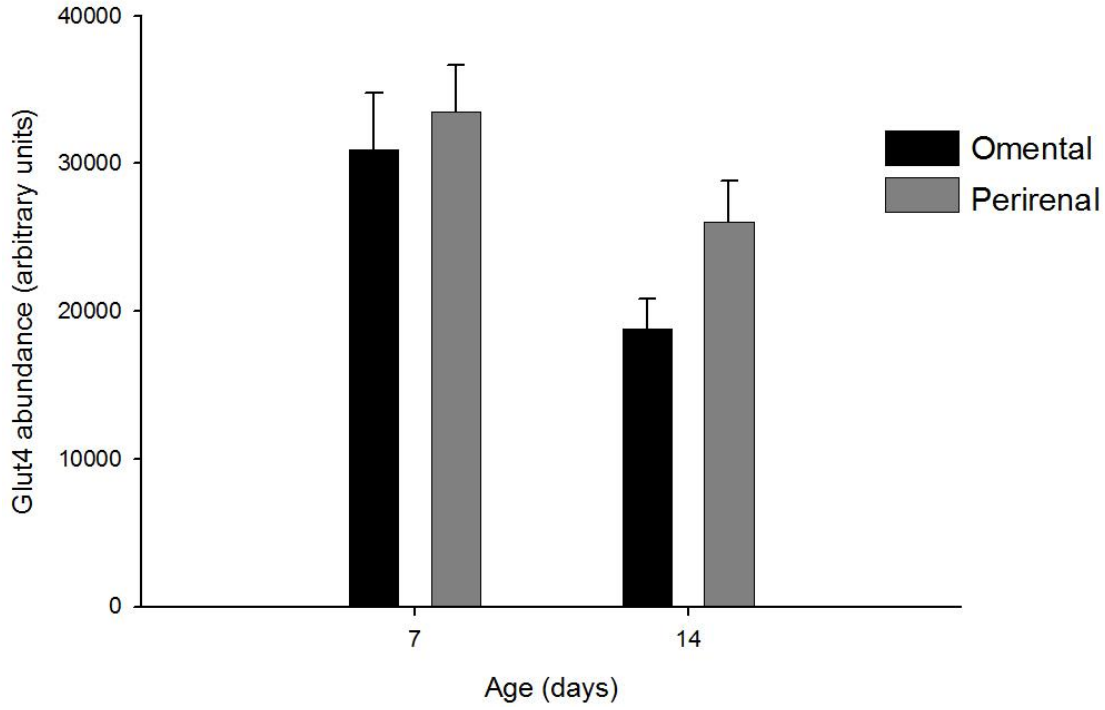


**Figure 2.6; Glut4 abundance in omental and perirenal adipose tissue in control sheep at 7 and 14 days of age**

Data are: 7days: n=7, 14 days: n=6.

### 2.4.6 IGF1R ABUNDANCE IN 7 AND 14 DAY OLD LAMBS

Protein abundance of IGF1R was higher in 7 day old lambs when compared to 14 day old lambs (P=0.034). There was no difference in the abundance of IGF1R between the two investigated depots, neither was there an interaction between the effect of age and depot (Figure 2.7).

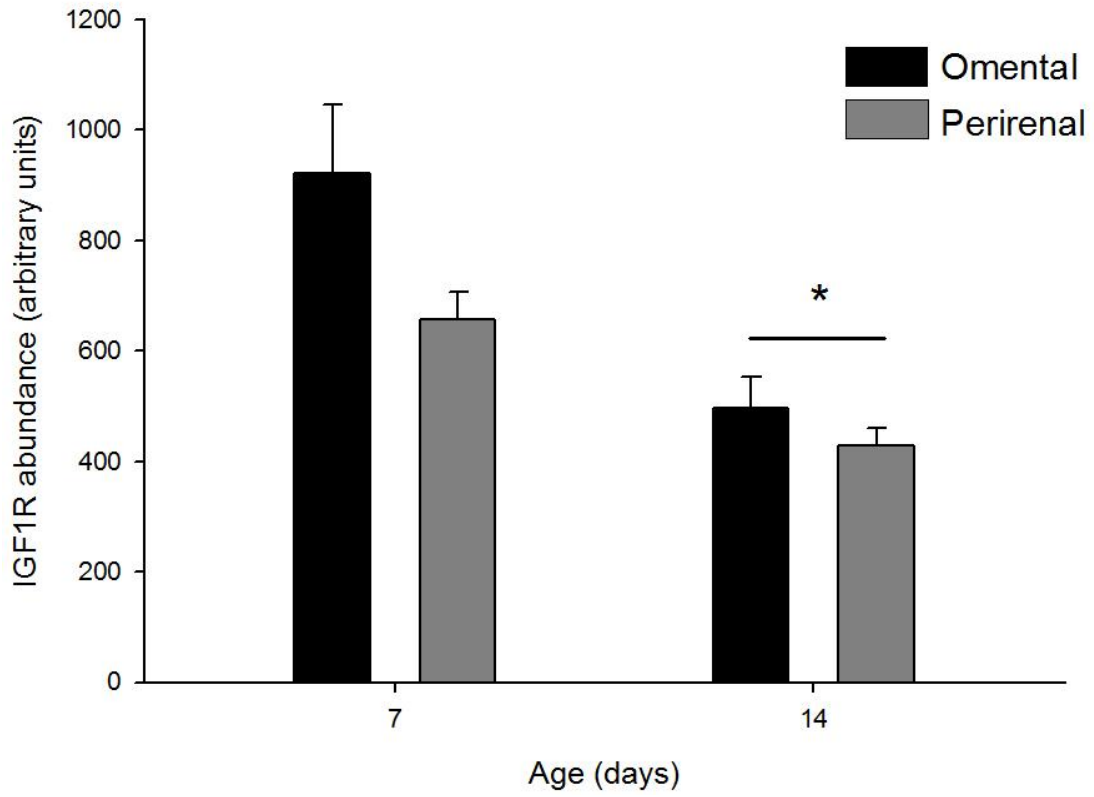


**Figure 2.7; IGF1R abundance in omental and perirenal adipose tissue in control sheep at 7 and 14 days of age**

* indicate differences between 7 and 14 days. Data are: 7days: n=7, 14 days: n=6.

The protein abundance of IR, P85, Glut4 and IGF1R in all animals is summarised in Table 2.3.

| | IR | | | P85 | | | Glut4 | | | IGF1R | | |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| | 7 d | 14 d | P-value | 7 d | 14 d | P-value | 7 d | 14 d | P-value | 7 d | 14 d | P-value |
| Omental | 18986 ± 2726 | 7515 ± 648 | <0.001 | 26939 ± 2077 | 19451 ± 1099 | 0.007 | 30932 ± 3861 | 18826 ± 2008 | 0.06 | 921 ± 125 | 496 ± 56 | 0.033 |
| Perirenal | 31678 ± 885 | 13076 ± 1513 | | 20135 ± 1991 | 10222 ± 1884 | | 33532 ± 3105 | 26056 ± 2794 | | 658 ± 49 | 429 ± 32 | |
| P-value | 0.005 | | | 0.011 | | | NS | | | NS | | |

**Table 2.3; Protein abundance of IR, p85, Glut4 and IGF1R in arbitrary units in perirenal and omental fat of 7 and 14 day old lambs**

Data are: 7days: n=7, 14 days: n=6.

## 2.5 Discussion

This study, using cross-sectional data, is the first to show relatively accelerated growth of the omental adipose tissue depot compared to the perirenal and pericardial depots during early neonatal life.

It has been shown in non-human primates, that the postnatal increase in adipose tissue mass is predominantly through tissue hypertrophy rather than hyperplasia (Lewis *et al.*, 1986, 1989), a finding recently corroborated in humans (Spalding *et al.*, 2008). Furthermore it is clear that the structure and function of adipose tissue is not homogenous throughout the body; and that increased visceral adipose i.e. omental and mesenteric, is implicated as key to the deleterious consequences of obesity (Despres & Lemieux, 2006; Hayashi *et al.*, 2008). Indeed, increased visceral adiposity (marked by an increased waist circumference) even in normal weight people, has been found to be a strong independent risk factor for death (Pischon *et al.*, 2008). Therefore, factors that influence the development, growth and metabolism of visceral adipose, specifically, are of particular interest.

Other experimental studies using animal models have described the neonatal period as important with respect to adipose tissue development, structure and function (McCance, 1962; Dietz, 1994). Here we show quite

clearly that the regional distribution of visceral adipose tissue in the sheep occurs during the first six months of life and changes little thereafter.

We hypothesised that the increased growth of the omental relative to the other visceral adipose tissue depots was secondary to a relative increased sensitivity to the actions of insulin in this depot.

This study has clearly shown that adipose tissue *per se* is metabolically different between regional depots; abundance of IR was much lower in the omental compared to the perirenal depot at both 7 and 14 days of age. This implies significant differences in insulin signalling and possibly local sensitivity to the actions of insulin between perirenal and omental adipose tissue. The depot specific differences between insulin receptor abundance are however not reflected by depot specific differences of other markers of the insulin signalling cascade. P85 abundance was higher in omental compared to perirenal adipose tissue while Glut4 abundance was not different between the two depots. This shows that individual markers of the insulin signalling cascade have fundamentally different abundance patterns between perirenal and omental adipose tissue. This could potentially mean a difference in the efficiency of the insulin signalling cascade in the two depots and therefore a potentially different sensitivity to the local actions of insulin. Several mechanisms could be responsible for these protein specific differences in the insulin signalling cascade. Sympathetic nervous system activation or cytokine stimulation, for example, in the perirenal adipose tissue

could result in serine phosphorylation of insulin receptor substrate (IRS), which causes the signal in the insulin signalling cascade to be blunted (Klein *et al.*, 1999; Le Marchand-Brustel *et al.*, 2003; Tanti *et al.*, 2004; Gual *et al.*, 2005).

We could speculate that the relatively prolonged exposure to insulin in the perirenal adipose tissue depot has resulted in a decreased efficiency of the insulin signalling cascade in this depot and that IR abundance has increased in an attempt to compensate for the decreased insulin signalling efficiency. Kasuga and colleagues showed that adipocytes of streptozotocin treated diabetic rats had increased expression of insulin receptor and increased insulin binding capacity, indicating that the mechanism of insulin resistance is at post-receptor level and receptor expression can be upregulated in a compensatory response (Kasuga *et al.*, 1978)

Unfortunately we were unable to investigate the expression of markers of local tissue sensitivity to the effects of glucocorticoids at this age due to degradation of the RNA samples that were collected from these cohorts. Therefore we do not know whether the differences in abundance of proteins of the insulin signalling cascade in the perirenal compared to the omental depot are associated with differences in the expression of markers of the local sensitivity to the actions of glucocorticoids. Future studies will have to investigate the local tissue sensitivity to the actions of glucocorticoids in both

depots to further elucidate the exact role of glucocorticoids in growth patterns of different adipose tissue depots during this critical period in early life.

Insulin like growth factor 1 receptor abundance was not significantly different between depots. However, at 7 days of age there was a strong trend towards a higher expression in the omental adipose tissue depot. This implies a possible difference in insulin like growth factor sensitivity between the two adipose tissue depots during this important period in early life. IGF1 sensitivity could have a role in the accelerated growth of the omental depot at this stage in life. However at 14 days of age this trend is not present anymore while omental adipose tissue remains to grow at accelerated levels, indicating that IGF1 sensitivity is, at least, not the only factor contributing to this increased growth rate.

All proteins that were investigated in this study showed higher abundance levels at 7 compared to 14 days of age independent of adipose tissue depot. Numerous studies have indicated increased levels of protein abundance or gene expression of several genes and proteins around birth (Liggins, 1976; Smith *et al.*, 2009). The increased levels of the investigated proteins at 7 days of age could be secondary to this rise in protein abundance around birth.

In conclusion, the increased growth of the omental depot confirms the hypothesis that this depot undergoes accelerated growth in early life.

However, Glut4 abundance was similar in the omental compared to ther perirenal adipose tissue depot at 7 and 14 days of age. Other proteins involved in the insulin signalling cascade had differential abundance patterns between the two depots, indicating that there are fundamental metabolic differences in the insulin signalling cascade between the two depots. No definite conclusion can be made on the effects of those differential abundance patterns on the specific tissue sensitivity to the local actions of insulin.

Given the differential growth rate of omental and perirenal adipose tissue during the first six months of life, the differences in local tissue sensitivity to the actions of insulin and glucocorticoids are likely to be most prominent at the age of three months. In chapter 3 and 5 we therefore examined local tissue sensitivity to the actions of insulin and glucocorticoids in perirenal and omental adipose tissue of lambs at the age of 3-4 months. We also investigated whether the differential growth of adipose tissue and the expression of key genes in the insulin signalling cascade could be influenced by nutritional challenges in the periconceptional period (Chapter 3) or the early postnatal period (Chapter 5).

# 3. Periconceptional nutrition influences adipose tissue development in sheep.

## 3.1 SUMMARY

With the increase in the incidence of obesity, the number of women that are entering pregnancy obese rises dramatically as well. Women that are obese when they enter pregnancy are relatively more insulin resistant than normal weight women during pregnancy. Associated with this increase in insulin resistance is an increase in the number of heavy babies born to mothers that were overweight or obese when they entered pregnancy. Heavier babies are more likely to become obese in adulthood. These obese adults then enter pregnancy themselves -an intergenerational cycle of obesity is established. It is to date not known whether this cycle relies on the weight of the women in the periconceptional period or later on in pregnancy. It is also not known whether the offspring of overnourished mothers have increased amounts of specific adipose tissue depots in their body.

This study investigated the effects of periconceptional overnutrition, followed or not followed by a period of dietary restriction on the development of adipose tissue in the offspring. The study used embryo transfer and donor mothers in a sheep model to isolate the period of periconceptional nutrition from changes in maternal nutrition later in pregnancy.

Female offspring from ewes that were overnourished during the periconceptional period had higher total fat mass compared to offspring of control-fed ewes. This increase in fat mass was attenuated if the period of overnutrition was followed by a period of nutrient restriction. This indicates that the periconceptional period is an important programming period for the development of adipose tissue in the female offspring.

The effect of periconceptional nutrition on adipose tissue development did not seem to be driven by changes in RNA expression of factors involved in the local sensitivity to the actions of insulin or glucocorticoids, since transcript expression of markers of each hormone were similar between treatment groups. However in offspring at the age of 4 months, independent of treatment group, the expression of markers of local sensitivity to the actions of insulin and glucocorticoids was highly depot specific. Furthermore the expression of markers of glucocorticoid sensitivity was negatively associated with weight of the donor ewe in the periconceptional period, but only in the omental adipose tissue depot of females. This study therefore shows that the omental and perirenal adipose tissue depot are significantly metabolically different at the age of 4 months and can be programmed by periconceptional nutritional status.

## 3.2 Introduction

It is clear that alterations in nutrient supply during pregnancy have significant programmed effects on the health of the offspring in adulthood. These effects include increased risks for diseases like type 2 diabetes mellitus, and cardiovascular disease.

It appears that these detrimental effects can find their origin as early in life as during the periconceptional period. Exposure of the oocyte and/or embryo to maternal undernutrition (periconceptional undernutrition; PCUN) resulted in a decrease in foetal weight and an increase in relative perirenal adipose tissue in twin pregnancies (Edwards *et al.*, 2005). Furthermore, a relationship was seen in twin pregnancies between weight loss of the ewe and placenta and offspring size, such that an increase in weight loss resulted in relatively heavier placentae and larger foetuses at 55 days of gestation (MacLaughlin *et al.*, 2005). Furthermore, it has been shown that PCUN results in an increase in arterial blood pressure in the foetus and a greater and precocious activation of the HPA axis during late gestation (Edwards & McMillen, 2002a, b; Edwards *et al.*, 2005).

When maternal undernutrition extends from the periconceptional period into early pregnancy (i.e. from 60 days prior to conception and up to 30 days of pregnancy) a reduced foetal growth rate occurs with hyperactivation of the

HPA axis and an increase in premature deliveries (Bloomfield *et al.*, 2003; Oliver *et al.*, 2005).

It has also been shown that antenatal exposure to glucocorticoids results in programming of the expression of genes involved in the local sensitivity to the actions of glucocorticoids in adipose tissue. Cleasby and colleagues demonstrated that antenatal exposure to dexamethasone resulted in a significant increase in the expression of GR in visceral adipose tissue in rats (Cleasby *et al.*, 2003a). Whorwood and colleagues showed a similar response to antenatal dexamethasone in sheep visceral adipose tissue (Whorwood *et al.*, 2001).

Since periconceptional undernutrition increases the exposure of the offspring prenatally to glucocorticoids, it is likely that PCUN will have programmed effects on the local sensitivity to glucocorticoids in adipose tissue that will last into later life.

In our present day society obesity is an ever growing problem. With the increase in the obesity prevalence over the last few decades there has also been an increase in the number of women that are entering pregnancy obese (Bergmann *et al.*, 2003; LaCoursiere *et al.*, 2005). LaCoursiere and colleagues reported that in 1991 25.1% of women entered pregnancy overweight or obese. In 2001 this number had increased by 40.2 % to 35.2% (LaCoursiere *et al.*, 2005). However, periconceptional overnutrition i.e. increased nutrient supply at this time, has been little explored in experimental studies but is becoming more prevalent in modern society.

Women who are overweight or obese at this time are more likely to have a reduced insulin sensitivity compared to lean women. During normal pregnancies the insulin sensitivity of the mother decreases in order to provide sufficient glucose supply to the foetus. Obese women, however, are more likely to have an insulin sensitivity that is already decreased even before pregnancy. The insulin sensitivity of obese women remains lower compared to that of lean women throughout pregnancy (Catalano & Ehrenberg, 2006).

An excessive reduction in insulin sensitivity during pregnancy can lead to an excessive nutrient supply to the foetus, as is the case in gestational diabetes. Obese women are more likely to develop gestational diabetes or reduced insulin sensitivity (Sebire *et al.*, 2001; LaCoursiere *et al.*, 2005). The increased nutrient supply, associated with the reduction in insulin sensitivity, can result in increased growth of the foetus and macrosomia (Sebire *et al.*, 2001; Yajnik *et al.*, 2002; Bergmann *et al.*, 2003; Catalano *et al.*, 2003; Gillman *et al.*, 2003; Jensen *et al.*, 2003; Ehrenberg *et al.*, 2004). Catalano and colleagues showed that the strongest predictor of foetal fat accretion was pregravid maternal insulin sensitivity. They hypothesised that obese women, having lower insulin sensitivities, are therefore more at risk of having a large baby (Catalano *et al.*, 1995). The Hyperglycaemia and Adverse Pregnancy Outcome Study Cooperative Research Group found that even in normal maternal glucose ranges there is an association between maternal glucose levels and birth weight of the offspring (Metzger *et al.*, 2008), while Jensen and colleagues showed that even in women with a normal glucose

tolerance, obesity or being overweight was still associated with an increased risk of macrosomia (Jensen *et al.*, 2003).

A combination of periconceptional undernutrition, which affects the HPA axis, and maternal obesity in the periconceptional period, which affects the insulin supply to the foetus, is likely to have complicated effects on adipose tissue growth and development in the offspring.

In this chapter we investigate these effects by using a model which combines periconceptional overnutrition, which functions as a model for maternal obesity, followed by periconceptional undernutrition, which functions as a model for dietary restriction. Embryo transfer and donor ewes were used after the periconceptional period to be able to look at the programmed effects of nutrition during the periconceptional period alone on adipose tissue development in the offspring. This enables us to distinguish the potentially beneficial effects of dietary restriction in the periconceptional period from dietary restriction during pregnancy.

I hypothesise that the premature activation of the HPA axis during late gestation in PCUN will result in increased expression of genes involved in the local sensitivity of the tissue to the actions of glucocorticoids in the subcutaneous and perirenal adipose tissue, which are undergoing rapid growth during this period of life. I further hypothesise that this increased expression in of genes involved in the local sensitivity of tissue to the actions

of glucocorticoids in these depots will persist after birth. The omental depot, however, develops after birth and I therefore hypothesise that after PCUN this depot is not exposed to the increased actions of local glucocorticoids. Thus the growth of this depot is primarily mediated by insulin in the postnatal period.

When this period of periconceptional nutrient restriction occurs in women that are already overweight, these effects are exaggerated, due to the increased insulin exposure of the perirenal and subcutaneous adipose tissue depot during prenatal life. Therefore these two depots become relatively more resistant to the local effects of insulin and therefore more sensitive to the local effects of glucocorticoids, which results in an even greater lipolytic activity in these depots and a greater nutrient supply for the omental depot.

## 3.3 Methods

### 3.3.1 ANIMALS AND EXPERIMENTAL DESIGN

All procedures were approved by The University of Adelaide Animal Ethics Committee and by the Institute of Medical and Veterinary Science Animal Ethics Committee.

23 Non-pregnant Merino ewes were included in this study. All ewes were weighed and body condition score was assessed by an experienced assessor using a scale of 1-5 with intervals of 0.5 (Russel *et al.*, 1969; Greenwood *et al.*, 2000). A body condition score of 1 represents an extremely emaciated animal and a body condition score of 5 represents an extremely obese animal.

During an acclimatisation period of 2 weeks the ewes were fed 100% metabolisable energy requirements (ME) as defined by the Agricultural and Food Research Council in 1993 (AFRC, 1993). The food consisted of pellets containing cereal hay, lucerne hay, barley, oats, almond shells, lupins, oat bran, lime and molasses (Johnsons & Sons Pty Ltd, Kapunda, South Australia, Australia).

After the acclimatisation period, the ewes were randomly allocated to one of four treatment groups. The control-control group (CC, n=6) received a diet of 100% metabolisable energy requirements (ME) for 4 months prior to conception. The control-restricted group (CR, n=6) received a diet of 100%

ME for 3 months followed by 70% ME for 1 month. The high-high group (HH, n=6) was fed *ad libitum* (170-190% ME) for 4 months. The high-restricted group (HR, n=5) was fed *ad libitum* (170-190% ME) for 3 months followed by a diet of 70% ME for 1 month prior to conception.

The reproductive cycle of all ewes was synchronised by the administration of a progesterone pessary (45 mg flugestone acetate, Intervet, Paris France) for two weeks. Superovulation was induced by the administration of FSH (Folltropin, equivalent to 180 mg NIH-HSH-P1 standard), administered by 6 intramuscular injections given over 3 days, twice daily, starting 48 hours before pessary removal and the final injection given 12 hours after pessary removal. Pessaries were removed on day 14 in the afternoon for donor ewes and 12 hours later in recipient ewes. Fresh semen was collected from a ram of proven fertility using the protocol specified by Kakar and colleagues (Kakar *et al.*, 2005).

Donor ewes were inseminated by laparoscopy with approximately $20 * 10^6$ spermatazoa being placed directly into the lumen of each uterine horn approximately 36 hours after pessary withdrawal.

Prior to the commencement of surgery a blood sample was collected by venipuncture from the jugular vein. 6 days after ovulation, the donor ewes were anaesthetised and embryos were collected by mid-ventral laparotomy. Each uterine horn was flushed with 20 ml PBS containing 5% HISS (v:v).

At embryonic day 6-7, donor embryos of good quality (≥ 8 cells and acceptable morphology) were transferred to adult recipient ewes by

laparotomy. Recipients ewes (n=41) were maintained on a control diet for the maintenance of singleton bearing pregnant ewes (100% ME) at all times. Pregnancies were monitored by ultrasonography at day 45 of gestation and then once a month until term. The pregnant ewes were allowed to give birth naturally (term = 147±3 days).

Lambs (n=41) remained with their mothers until weaning at three months of age. Ewes and lambs were housed in individual pens in an indoor housing facility. The ambient temperature in the housing facility was kept constant between 20 and 22°C. Animals were exposed to a 12 hour light, 12 hour dark cycle.

Ewes were weighed and their body condition was assessed and scored approximately every two weeks after commencing the feeding regime until delivery. Body condition score of the recipient ewes ranged from 3.0 ± 0.02 at the beginning of pregnancy to 3.2 ± 0.04 at the end of pregnancy, with no differences between treatment groups. Lambs were weighed at birth and approximately every two weeks until post mortem. Figure 3.1 shows a flow diagram of the experimental design used in this study (Figure 3.1).

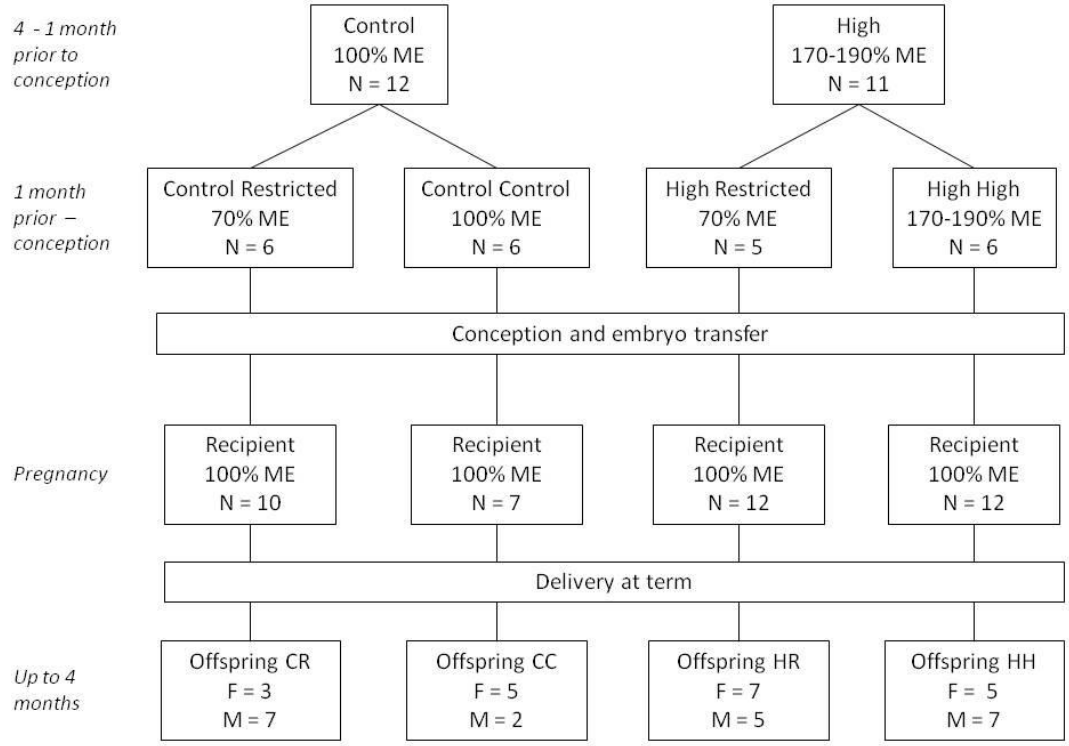


**Figure 3.1; Overview of the experimental design to investigate the effects of periconceptional nutrition on adipose tissue development in sheep**

ME: metabolisable energy requirements, F: female, M: male

Lambs (n=41) were humanely killed at 4 months of age with an overdose sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, New South Wales, Australia); all adipose tissue (perirenal, pericardial, subcutaneous, omental, axillary and gonadal) was collected and weighed at post mortem, samples were rapidly snap frozen in liquid nitrogen and stored at -80°C for further analysis.

### 3.3.2 PLASMA METABOLITES

Blood samples were collected from the jugular vein by venipuncture from all lambs at post mortem. Blood samples were collected into chilled heparinised tubes. All samples were centrifuged at 1500 x *g* for 10 minutes and plasma was isolated and stored into aliquots at -20°C for the measurement of non-esterified free fatty acids (FFA), glucose, insulin and cortisol.

#### *3.3.2.1 Glucose*

Plasma glucose concentrations were measured by enzymatic analysis using hexokinase and glucose-6-phosphate dehydrogenase to measure the formation of NADH photometrically at 340 nm (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland). The sensitivity of the assay was 0.5 mmol/l and the intra- and inter assay coefficients of variation were both < 5%.

#### *3.3.2.2 Insulin*

Plasma insulin concentrations were measured using a radioimmunoassay (Rat insulin kit, Linco Research, Inc., Missouri, USA), which was validated for use with sheep plasma. This assay has previously been shown to have a cross-reactivity of 100% with sheep insulin and no detectable cross-reactivity with related proteins (C-peptide, glucagon, somatostatin, pancreatic polypeptide or IGF-1) (Linco Research, Inc., Missouri, USA). The recovery of insulin from lamb plasma was 96.5 ± 3.7 %. When insulin was

measured in increasing volumes of lamb plasma, the displacement curve was parallel to the assay standard curve. Samples (10 µl) were assayed in duplicate and added to borosilicate glass tubes with 100 µl of hydrated $^{125}$I-Insulin and guinea-pig anti-rat insulin antibody and incubated overnight at 4°C. Precipitating reagent (1ml) was added and tubes were centrifuged for 25 min at 2000g, the contents were then aspirated and total counts measured by gamma counter. The sensitivity of the assay was 0.01 ng/ml and the intra and inter assay coefficients of variance were both <10%.

#### *3.3.2.3 Non-esterified fatty acids*

Plasma FFAs were measured by an *in vitro* enzymatic colorimetric method (Wako Pure Chemicals Industries Ltd, Osaka, Japan). The method relies upon the acylation of coenzyme A (CoA) by the fatty acids in the presence of added acyl-CoA synthetase (ACS). The acyl-CoA thus produced is oxidised by added acyl-CoA oxidase (ACOD) with generation of hydrogen peroxide. Hydrogen peroxide, in the presence of peroxidase (POD) permits the oxidative condensation of 3-methyl-N-ethyl-N-(β-hydroxyethyl)-aniline (MEHA) with 4-aminoantipyrine to form a purple coloured adduct which can be measured colorimetrically at 550nm (COBAS MIRA automated analysis system, Roche Diagnostica, Basel, Switzerland). The sensitivity of the assay was 0.25 ng/ml and the intra- and inter assay coefficients of variation were both < 10 %.

#### *3.3.2.4 Cortisol RIA*

Cortisol was extracted from foetal plasma samples in duplicate using dichloromethane (Bocking *et al.*, 1986) and measured with a radioimmunoassay (PerkinElmer Pty Ltd, Waltham, Massachusetts, USA) previously validated for use in foetal sheep plasma (MacLaughlin *et al.*, 2007). The efficiency of recovery of $^{125}$I-cortisol from foetal plasma using this extraction procedure was always greater than 90%. Samples were then reconstituted in assay buffer (Tris hydrochloride, BSA, and sodium azide). Rabbit anti-cortisol (1:450 dilution, MP Biomedicals, Seven Hills, NSW, Australia) was added followed by $^{125}$I-labeled cortisol (Amersham Pharmacia Biotech, Little Chalfont, UK). Tubes were vortexed and incubated at 37°C for 1 h before the addition of goat anti-rabbit serum (1:30 dilution, Chemicon, Billerica, MA, USA) and 20% polyethylene glycol (BDH Laboratory Supplies, Poole, UK). After centrifugation at 3700 × g and 4°C for 30 min, the supernatant was aspirated and the precipitate counted on a Gamma-counter (Packard, Downers Grove, IL, USA). The sensitivity of the assay was 0.2 nmol/L, and the intraassay and interassay coefficients of variation were <5% and 6.3%, respectively.

### 3.3.3 RNA EXTRACTION AND cDNA SYNTHESIS

RNA was isolated from frozen omental, perirenal and subcutaneous adipose tissue samples using Trizol reagent (Invitrogen, Groningen, The Netherlands) and purified using the RNeasy Mini Kit (Qiagen, Basel, Switzerland). In detail; 120-130 mg of adipose tissue was homogenised in 1

ml of Trizol reagent. Samples were left to stand at room temperature for 5 minutes. 200 µl chloroform was added to each sample. Samples were shaken vigorously for 15 seconds and allowed to stand at room temperature for 2-12 minutes prior to be centrifuged at 12000 *g* for 15 minutes at 4ºC. After centrifugation the upper aqueous, RNA containing, layer was transferred into a new eppendorf tube. Equal volume of cold 70% ethanol were added to the aqueous phase and the samples were mixed by inverting a few times. The solution was then transferred into a Qiagen column (in two steps). The liquid was eluted by centrifugation at 10000 rpm for 15 seconds at room temperature. Any flow through was discarded and these steps were repeated with the remainder of the sample. 700 µl of buffer RW1 from the RNeasy Mini Kit was added to each column and column were centrifuged for 15 seconds at 10000 rpm. Flow through was discarded and 500 µl of RPE buffer (RNeasy Mini Kit) with added ethanol (as per manufacturers' instructions) was added. Columns were centrifuged at 10000 rpm for 2 minutes and the flow through was discarded. Columns were then placed in a fresh collection tube and centrifuged at maximum speed for 1 minute to remove any left over buffer on the column membrane. Columns were again placed in a fresh collection tube. 30 µl of molecular grade water was added directly onto the membrane and columns were centrifuged for 1 minute at 10000 rpm to elute the RNA in water.

RNA was quantified by spectrophotometric measurements at 260 and 280 nm. cDNA was synthesised from 2 µg RNA using Superscript III (Invitrogen,

Groningen, The Netherlands) by reverse transcription. Controls containing no RNA transcript or no superscript were used to test for DNA contamination.

### 3.3.4 QUANTITATIVE REAL-TIME RT-PCR

Normalised mRNA expression for glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), insulin receptor β (IR), and glucose transporter 4 (Glut4) was determined in omental, perirenal and subcutaneous adipose tissue by reverse transcription real time PCR using the SYBR Green system in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, California). Each qRT-PCR well contained 5 µl SYBR Green Master Mix (Applied Biosystems), 1 µl each of forward and reverse primer (GeneWorks, Adelaide, South Australia, Australia) for the appropriate gene (Table 3.1), 2 µl water and 50 ng/µl cDNA (1µl) to give a total volume of 10 µl. Controls for each primer set containing no cDNA were included on each plate. Three replicates of cDNA for each adipose tissue sample were performed to determine intra-plate variation. Two quality control samples were run on each plate to determine inter-plate variation. Amplification efficiencies were determined from the slope of a plot of Ct (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (ranging from 1-100 ng/µl). The abundance of each transcript relative to the abundance of the reference gene ribosomal protein P0 (RpP0) was

calculated using Q-Gene analysis software (Muller *et al.*, 2002). RpP0 expression was not influenced by treatment, gender or adipose tissue depot. The expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) was examined as described above. The expression was however too low to be able to obtain reproducible results.

| **Primer** | **Sequence** | **Accession number** |
|---|---|---|
| GR | **F:** 5'-ACT GCC CCA AGT GAA AAC AGA-3'<br>**R:** 5'-ATG AAC AGA AAT GGC AGA CAT TTT ATT-3' | NM_001114186 |
| 11β-HSD1 | **F:** 5'-GCG CCA GAT CCC TGT CTG AT-3'<br>**R:** 5'-AGC GGG ATA CCA CCT TCT TT-3' | NM_001009395 |
| 11β-HSD2 | **F:** 5'-AGC AGG AGA CAT GCC GTT TC-3'<br>**R:** 5'-GCA ATG CCA AGG CTG CTT-3' | NM_001009460 |
| IRβ | **F:** 5'-CAT CCC CAG AAA ATC ATC TTC AG-3'<br>**R:** 5'-CAA GGG CTC TGC GTT TCC T-3' | Y16092 |
| Glut4 | **F:** 5'-GTG GCC ATC TTT GGC TTC GTG-3'<br>**R:** 5'-CGG CTG AGA TCT GGT CAA AC-3' | AY949177 |
| RpP0 | **F:** 5'-CAA CCC TGA AGT GCT TGA CAT-3'<br>**R:** 5'-AGG CAG ATG GAT CAG CCA -3' | NG_009485 |

**Table 3.1; Primer sequences for PCR analysis of GR, 11β-HSD1, 11β-HSD2, IRβ, Glut4 and RpP0**

F: forward primer sequence, R: reverse primer sequence

### 3.3.5 STATISTICAL ANALYSIS

Data are presented as the mean ± SEM. The effects of periconceptional overnutrition and dietary intervention on depot specific adipose tissue expression of GR, 11β-HSD1, IR and Glut4 were determined using a multifactorial ANOVA using SPSS for Windows version 16.0 (SPSS Inc, Chicago, Illinois). Differences in gene expression between depots were determined using a multifactorial ANOVA with repeated measures using STATA 10.0 (StataCorp, College Station, Texas, USA) in which the outcomes for the different depots were considered to be repeated within each animal. Extensive consideration has been given to the choice of using analyses for repeated or independent measures. Data have been analysed using both methods obtaining similar results. It was felt that data from different depots could not be considered as indepent and therefore analyses using repeated measures were chosen to be presented here. Bonferroni post hoc tests were used where more than two groups were present. Relations between variables were assessed by linear regression using SigmaPlot 9.0 (SPSS). A probability level of 5% ($P<0.05$) was considered to be significant.

## 3.4 Results

### 3.4.1 WEIGHT OF DONOR EWE

Body weight of the donor ewes in the HH and HR group was significantly higher compared to the CC and CR group at the time of embryo transfer. Dietary restriction did not result in a significant decrease in weight of the CR and HR group (Table 3.2). However the change in weight of the non-pregnant donor ewes between 25 weeks before conception and the day of embryo transfer (day 6-7 pregnancy) was significantly different in each of the groups ($P<0.001$, Figure 3.2).

| Weight of the ewe | Treatment group | | | |
|---|---|---|---|---|
| | CC | CR | HH | HR |
| 25 weeks prior to conception (kg) | 54.8 ± 1.2 | 54.5 ± 0.7 | 54.9 ± 1.1 | 55.9 ± 0.3 |
| 1 month prior to conception (kg) | 59.9 ± 1.2 | 58.1 ± 1.2 | 72.8 ± 3.6* | 70.9 ± 1.4* |
| Conception (kg) | 55.7 ± 0.8 | 53.6 ± 1.5 | 69.4 ± 2.9* | 65.1 ± 1.0* |
| Embryo transfer (kg) | 62.6 ± 1.4 | 58.6 ± 1.2 | 73.6 ± 3.1* | 68.7 ± 0.8* |

**Table 3.2; Weight of the donor ewes 25 weeks prior to conception, 1 month prior to conception, at conception and at the time of embryo transfer (day 6-7 pregnancy)**

Data are mean ± SEM. Asterisk (*) indicates a significant difference between the HH and HR group compared to the CC and CR group ($P < 0.01$). Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

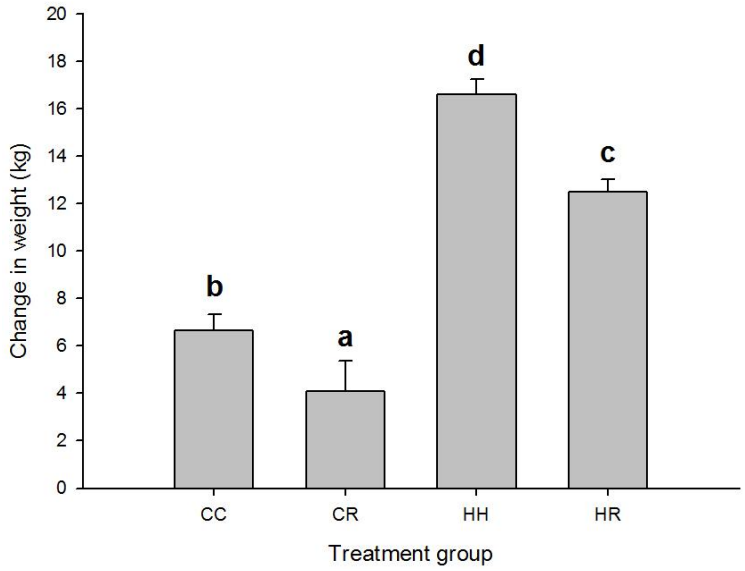


**Figure 3.2; Changes in the weight of the non-pregnant donor ewes between 25 weeks before conception to the day of embryo transfer**

Different letters indicate significant differences between treatment groups. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

### 3.4.2 LAMB BODY WEIGHT AND FAT MASS

There was no difference in lamb birth weight or weight at post mortem between the four treatment groups. Male lambs (n=21) were heavier than females (n=20) at birth and at four months of age (P<0.001 both time points, Figure 3.3). At post mortem female lambs had higher total fat mass compared to males despite a lower body weight (P=0.001, Figure 3.4). Female lambs in the high-high group had higher total fat mass compared to females in the control and the control-restricted group. This effect was attenuated when overnutrition was followed by a period of nutrient restriction (Figure 3.4). No differences were found between the weights of the individual adipose tissue depots and between the relative distribution of adipose tissue in the body between the different treatment groups. Table 3.3 gives an overview of the individual adipose tissue mass of the separate depots in males and females of the different treatment groups (Table 3.3).

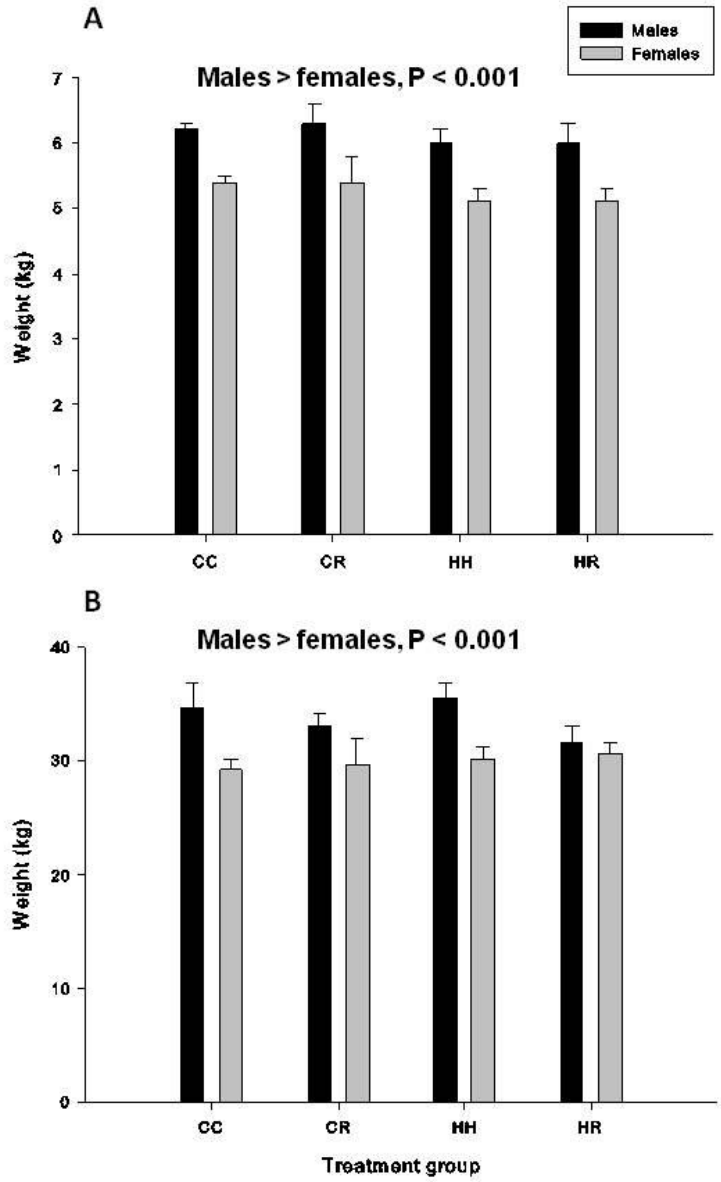


**Figure 3.3; Weight of the lambs at birth (A) and at 4 months of age (B)**

Males were significantly heavier than females at both timepoints. Males: n=21, Females: n=20. By group: CC: n=7, CR: n=10, HR: n=12, HH: n=12

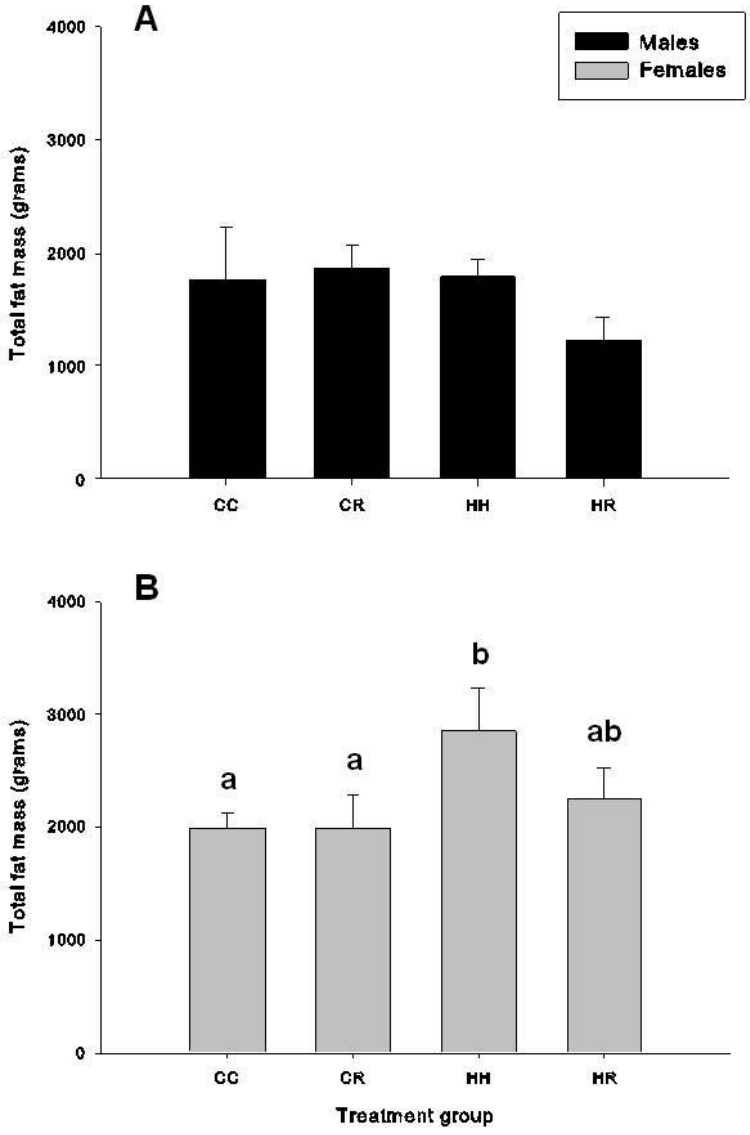


**Figure 3.4; Total adipose tissue mass at 4 months of age in male (A) and female (B) lambs.**

Males have significantly less total adipose tissue mass (P = 0.001). Different letters indicate differences between different treatment groups. Males: n=21, Females: n=20. By group: CC: n=7, CR: n=10, HR: n=12, HH: n=12

| Group | CC | | CR | | HH | | HR | |
|---|---|---|---|---|---|---|---|---|
| Gender | M (n=2) | F (n=5) | M (n=7) | F (n=3) | M (n=7) | F (n=5) | M (n=5) | F (n=7) |
| Perirenal (g) | 305.0 ± 123.5 | 425.5 ± 36.5 | 471.3 ± 146.5 | 403.7 ± 67.8 | 304.1 ± 35.5 | 741.8 ± 208.6 | 235.9 ± 36.3 | 587.3 ± 140.6 |
| Omental (g) | 230.5 ± 77.8 | 432.2 ± 41.9 | 322.4 ± 39.0 | 549.2 ± 176.5 | 425.9 ± 80.3 | 760.2 ± 183.8 | 223.7 ± 50.4 | 476.9 ± 47.5 |
| Subcutaneous (g) | 846.1 ± 321.7 | 751.0 ± 95.3 | 718.2 ± 99.5 | 736.1 ± 47.1 | 760.9 ± 79.3 | 989.5 ± 89.3 | 514.4 ± 90.0 | 948.8 ± 100.3 |
| Pericardial (g) | 81.2 ± 6.2 | 59.4 ± 5.1 | 60.3 ± 2.8 | 60.6 ± 3.9 | 62.2 ± 6.9 | 71.5 ± 8.4 | 52.4 ± 11.5 | 57.5 ± 5.7 |
| Axillary (g) | 109.5 ± 11.4 | 130.6 ± 12.2 | 119.6 ± 19.2 | 116.8 ± 23.1 | 120.0 ± 8.7 | 123.7 ± 8.9 | 82.7 ± 11.1 | 114.4 ± 5.0 |
| Gonadal (g) | 187.3 ± 77.7 | 181.3 ± 19.2 | 170.7 ± 21.6 | 125.8 ± 1.2 | 130.5 ± 22.8 | 182.6 ± 26.2 | 116.7 ± 11.3 | 144.2 ± 18.8 |
| Total (g) | 1572.3 ± 385.0 | 1798.6 ± 134.7 | 1691.8 ± 200.3 | 1866.4 ± 286.0 | 1656.0 ± 162.9 | 2661.9 ± 373.4* | 1109.0 ± 195.2 | 2116.8 ± 251.2 |

**Table 3.3; Absolute adipose tissue mass of the individual depots in male and female lambs at post mortem**

M: male, F: female. Female lambs had higher total fat mass compared to males. * signifanctly different from total fat mass in females in the CC and CR groups.

### 3.4.3 PLASMA METABOLITES

Periconceptional overnutrition and a period of dietary restriction did not lead to significant alterations in plasma glucose, insulin, free fatty acid and cortisol concentrations in the offspring at four months of age. There was no effect of gender on these plasma metabolite levels (Table 3.4).

| | Treatment group | | | | | | | |
|---|---|---|---|---|---|---|---|---|
| | CC | | CR | | HH | | HR | |
| | Male (n=2) | Female (n=5) | Male (n=7) | Female (n=3) | Male (n=7) | Female (n=5) | Male (n=5) | Female (n=7) |
| Glucose (mmol/L) | 3.8 ± 0.04 | 3.6 ± 0.1 | 3.6 ± 0.2 | 4.0 ± 0.2 | 3.8 ± 0.2 | 4.0 ± 0.1 | 3.6 ± 0.2 | 3.7 ± 0.1 |
| Insulin (ng/ml) | 0.3 ± 0.02 | 0.2 ± 0.01 | 0.3 ± 0.04 | 0.5 ± 0.2 | 0.4 ± 0.1 | 0.3 ± 0.1 | 0.4 ± 0.2 | 0.4 ± 0.1 |
| FFA (ng/ml) | 0.4 ± 0.1 | 0.6 ± 0.2 | 0.8 ± 0.1 | 0.9 ± 0.1 | 0.7 ± 0.2 | 0.5 ± 0.1 | 0.7 ± 0.1 | 0.7 ± 0.1 |
| Cortisol (nmol/l) | 17.9 ± 14.2 | 25.7 ± 5.3 | 23.3 ± 4.8 | 49.3 ± 22.9 | 25.0 ± 3.1 | 28.0 ± 7.3 | 19.7 ± 5.7 | 33.0 ± 7.2 |

**Table 3.4; Plasma glucose, insulin, free fatty acid and cortisol concentrations in the lambs at 4 months**

No significant differences were found.

### 3.4.4 PERIRENAL GENE EXPRESSION

There was no effect of periconceptional overnutrition and dietary restriction on the expression of GR, 11β-HSD1, IR and Glut4 in the perirenal adipose tissue depot. Neither was the expression of these genes influenced by gender in perirenal adipose tissue (Figure 3.5 and Figure 3.6).

### 3.4.5 OMENTAL GENE EXPRESSION

There was no effect of periconceptional overnutrition and dietary restriction on the expression of GR, 11β-HSD1, IR and Glut4 in the omental adipose tissue depot. Neither was the expression of these genes influenced by gender in omental adipose tissue (Figure 3.7 and Figure 3.8).

### 3.4.6 SUBCUTANEOUS GENE EXPRESSION

There was no effect of periconceptional overnutrition and dietary restriction on the expression of GR, 11β-HSD1, IR and Glut4 in subcutaneous adipose tissue. Neither was the expression of these genes influenced by gender in subcutaneous adipose tissue (Figure 3.9 and Figure 3.10).

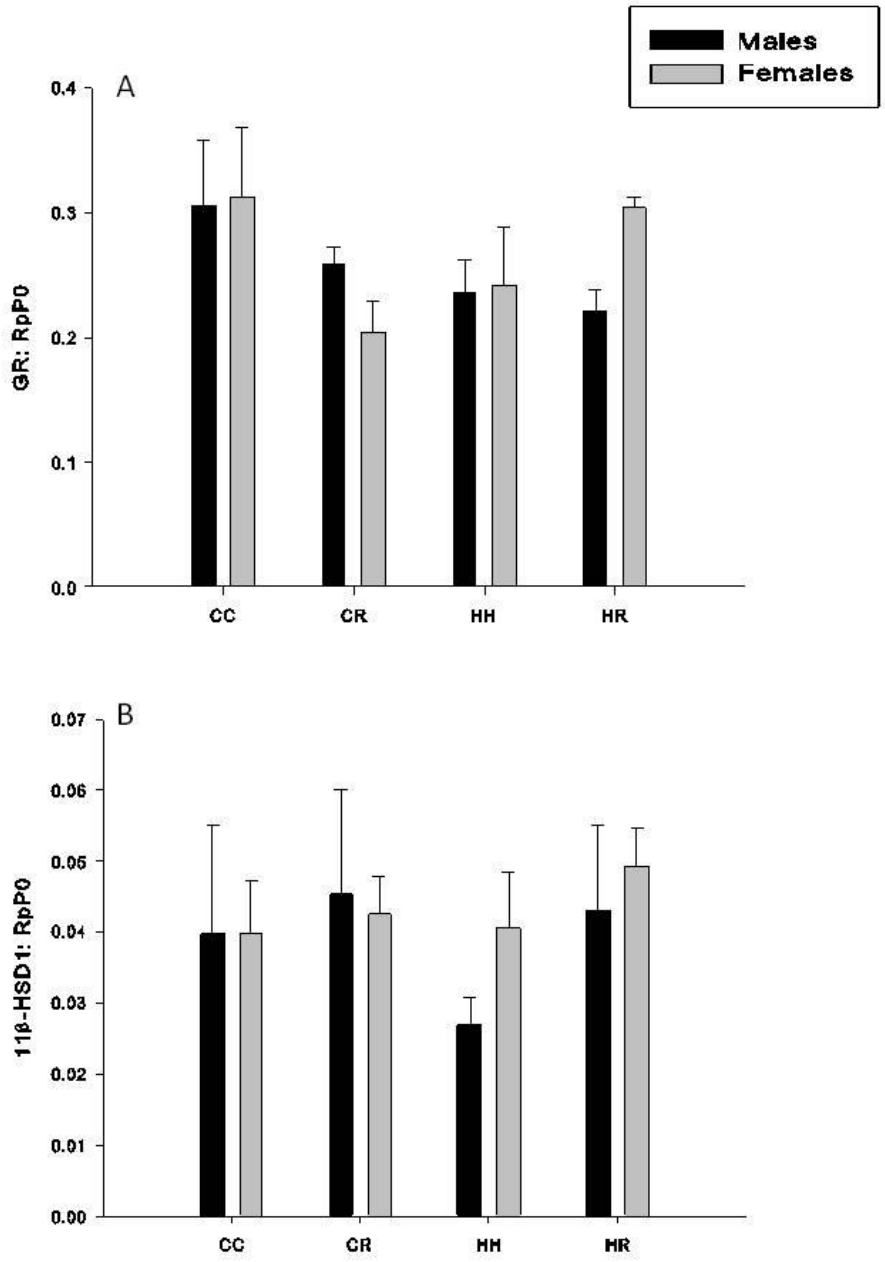


**Figure 3.5; Expression of GR (A) and 11β-HSD1 (B) in perirenal adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

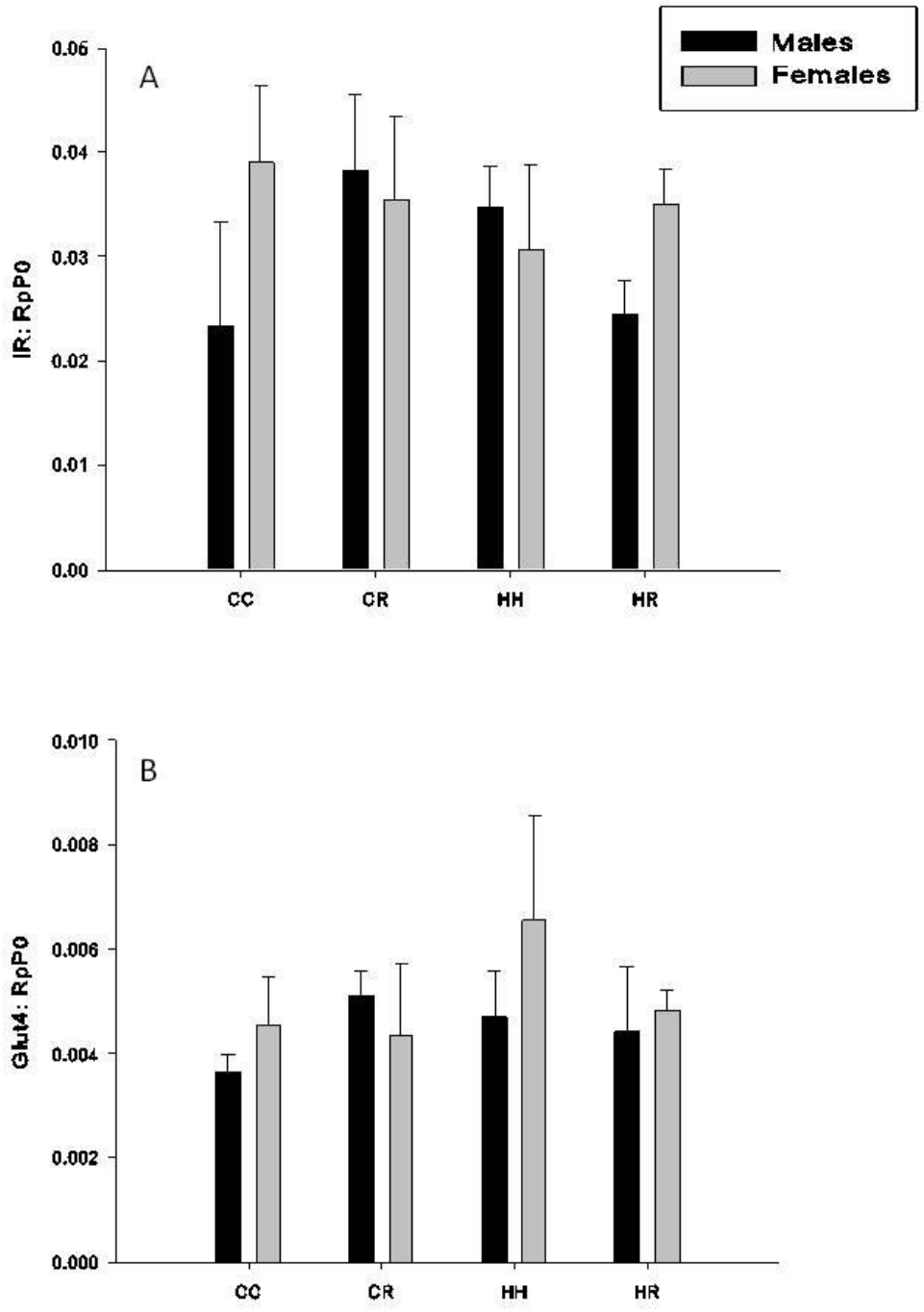


**Figure 3.6; Expression of IR (A) and Glut4 (B) in perirenal adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

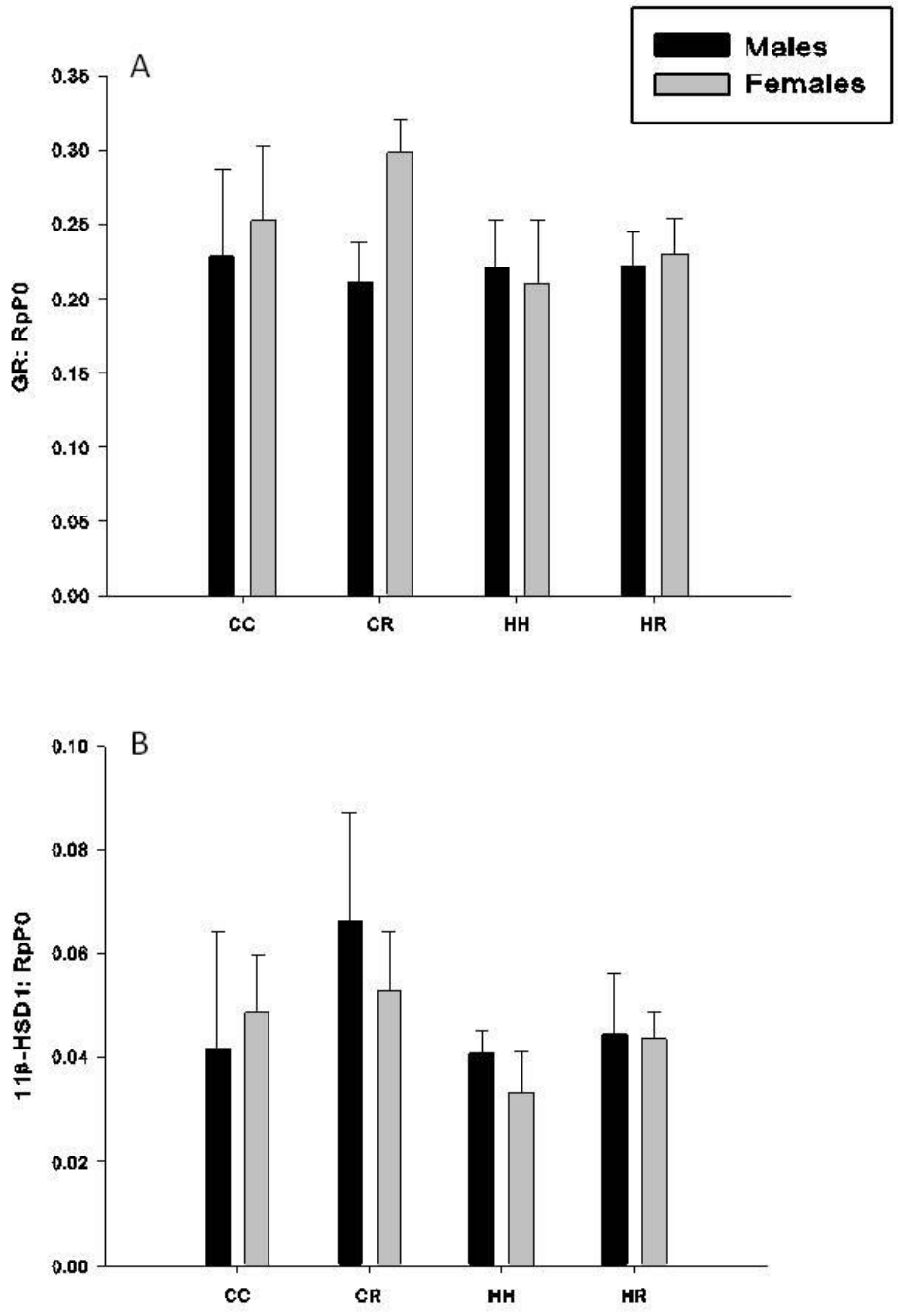


**Figure 3.7; Expression of GR (A) and 11β-HSD1 (B) in omental adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

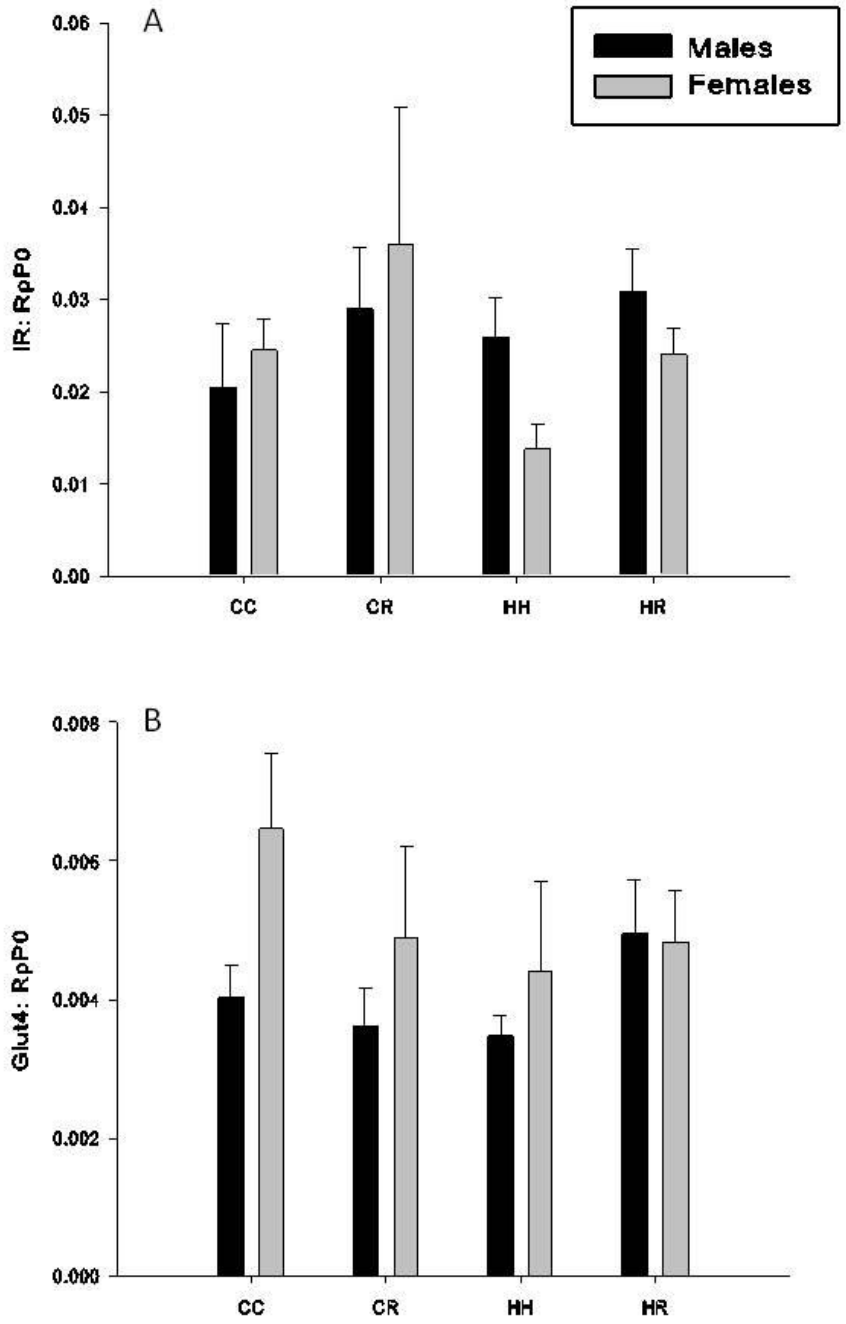


**Figure 3.8; Expression of IR (A) and Glut4 (B) in omental adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

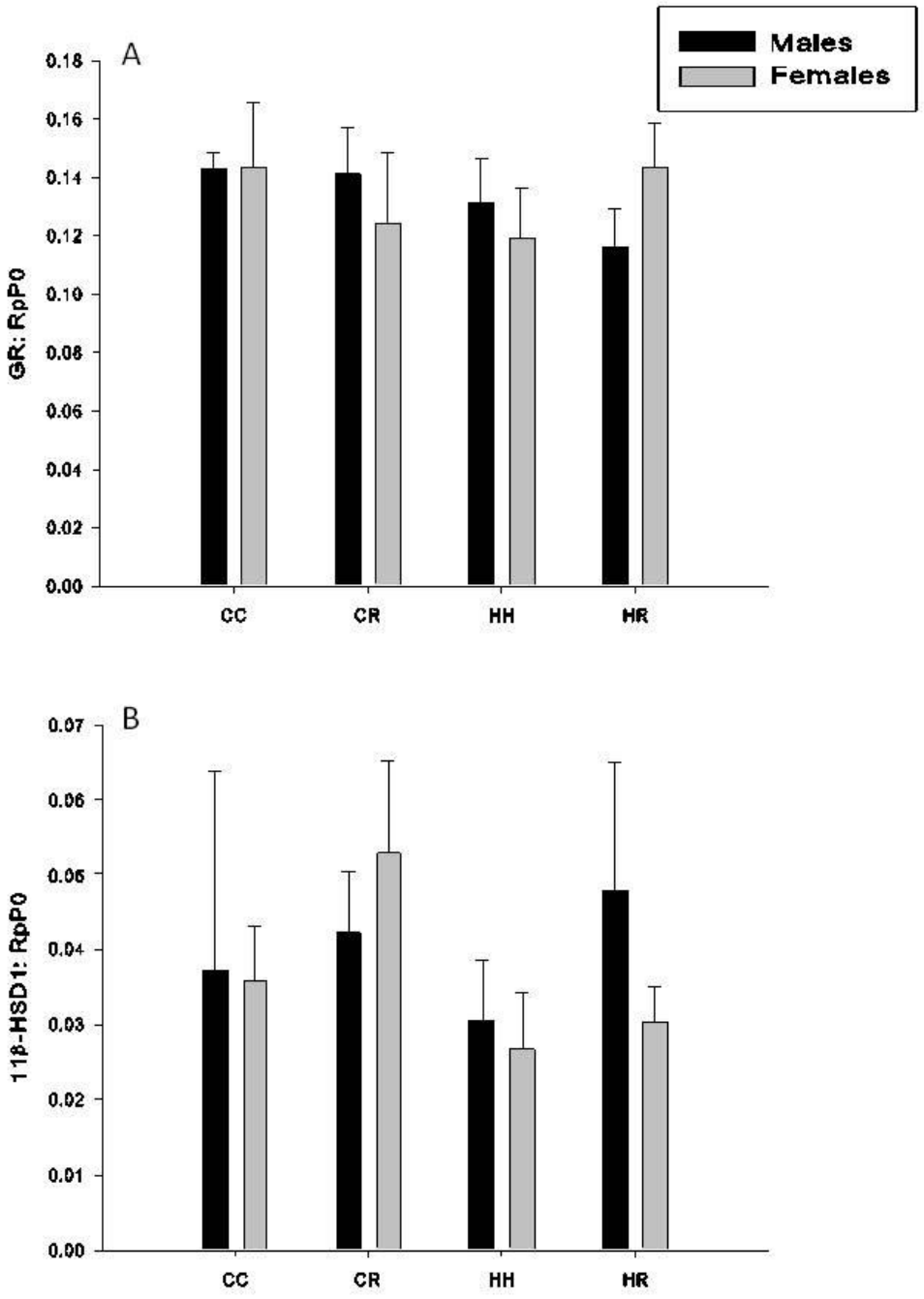


**Figure 3.9; Expression of GR (A) and 11β-HSD1 (B) in subcutaneous adipose tissue**
Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

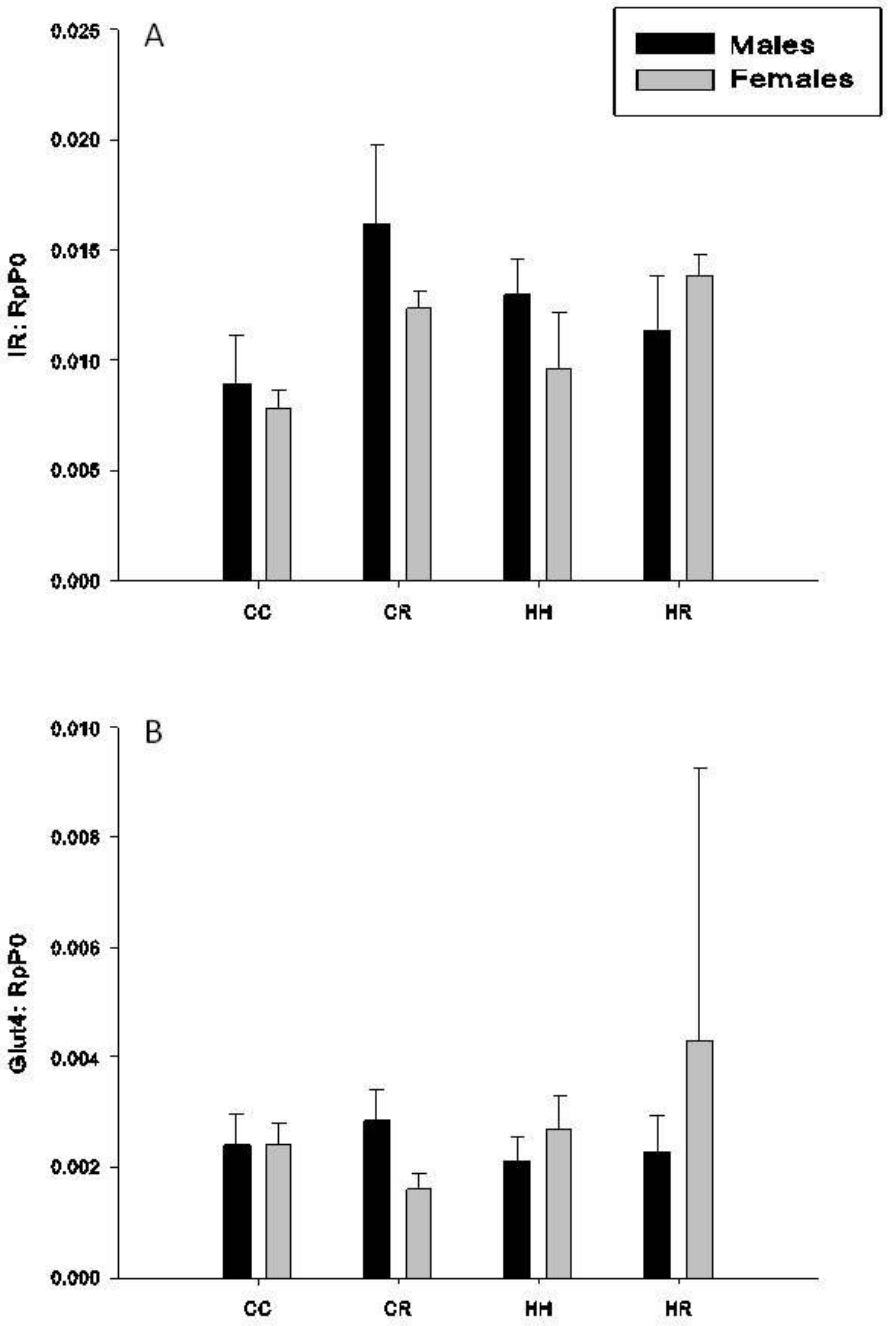


**Figure 3.10; Expression of IR (A) and Glut4 (B) in subcutaneous adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

### 3.4.7 INTERACTIONS BETWEEN WEIGHT OF THE DONOR EWE, PLASMA METABOLITES AND GENE EXPRESSION

Weight of the donor ewe at 14 days before conception only, was negatively associated with omental GR and 11β-HSD1 mRNA expression in female offspring at 4 months of age (GR: $P = 0.035$, $R^2 = 0.223$, n=20, 11β-HSD1: $P = 0.043$, $R^2 = 0.208$, n=20, Figure 3.11). FFA plasma levels at post mortem were associated with omental and subcutaneous 11β-HSD1 mRNA expression, but not with perirenal 11β-HSD1 mRNA expression (omental: $P = 0.003$, $R^2 = 0.209$, n=39, subcutaneous: $P = 0.030$, $R^2 = 0.121$, n=39, Figure 3.12).

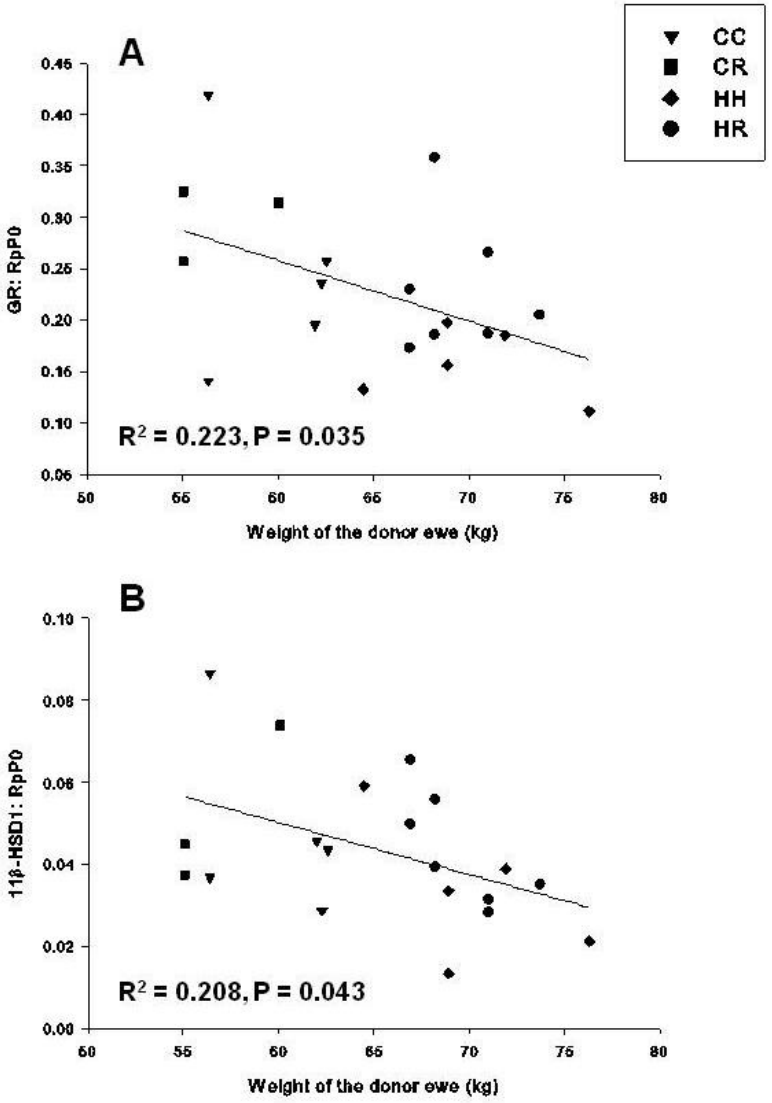


**Figure 3.11; Correlations between weight of the donor ewe 14 days before conception and omental GR (A) and 11β-HSD1 (B) expression in female lambs at 4 months of age**
N=20 in both graphs

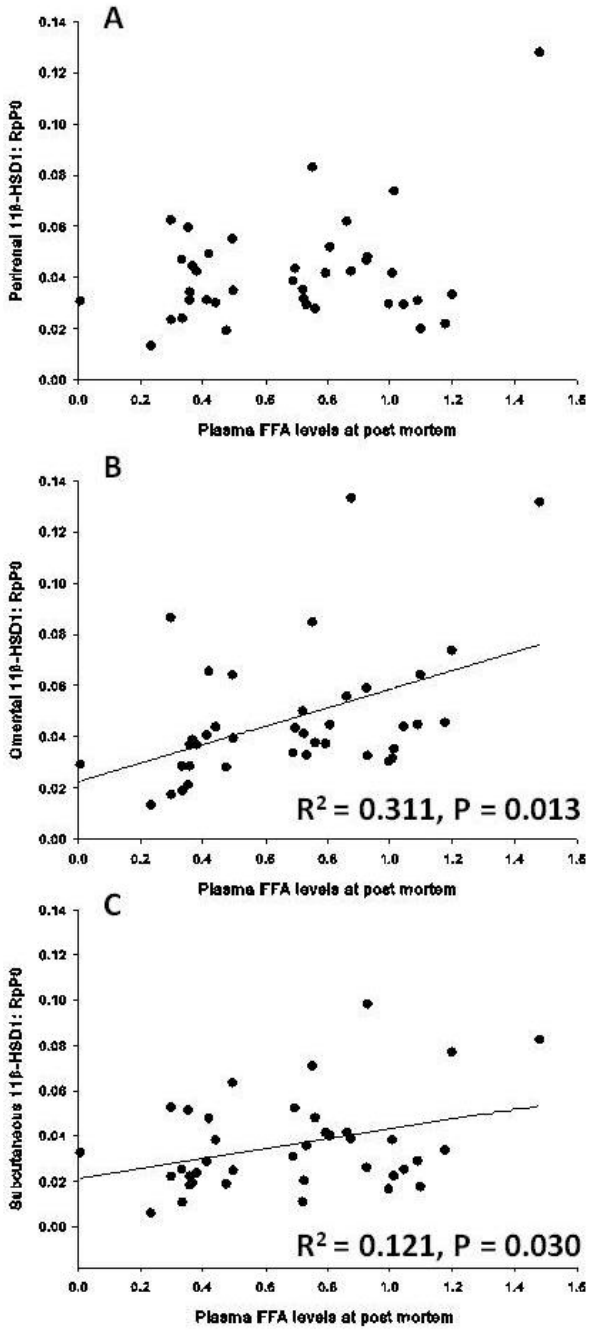


**Figure 3.12; Correlations between plasma FFA levels at post mortem and expression of 11β-HSD1 in perirenal (A), omental (B) and subcutaneous (C) adipose tissue**

N=39 for each depot

### 3.4.8 DEPOT SPECIFIC GENE EXPRESSION

Since there were no significant differences between groups or gender in the expression of the investigated genes, data were pooled to allow a comparison of gene expression between different adipose tissue depots. The expression of all investigated genes was highly depot specific, with the expression being lowest in the subcutaneous depot compared to both visceral adipose tissue depots (11β-HSD1: omental vs. subcutaneous: $P = 0.040$, perirenal vs. subcutaneous: not significant, all other genes: omental vs. subcutaneous $P < 0.001$, perirenal vs. subcutaneous $P < 0.001$). Expression of GR and IR was higher in perirenal adipose tissue than omental adipose tissue (GR: $P = 0.024$, IR: $P = 0.0011$). No differences between omental and perirenal expression levels were found for 11β-HSD1 and Glut4 (Figure 3.13 and Figure 3.14).

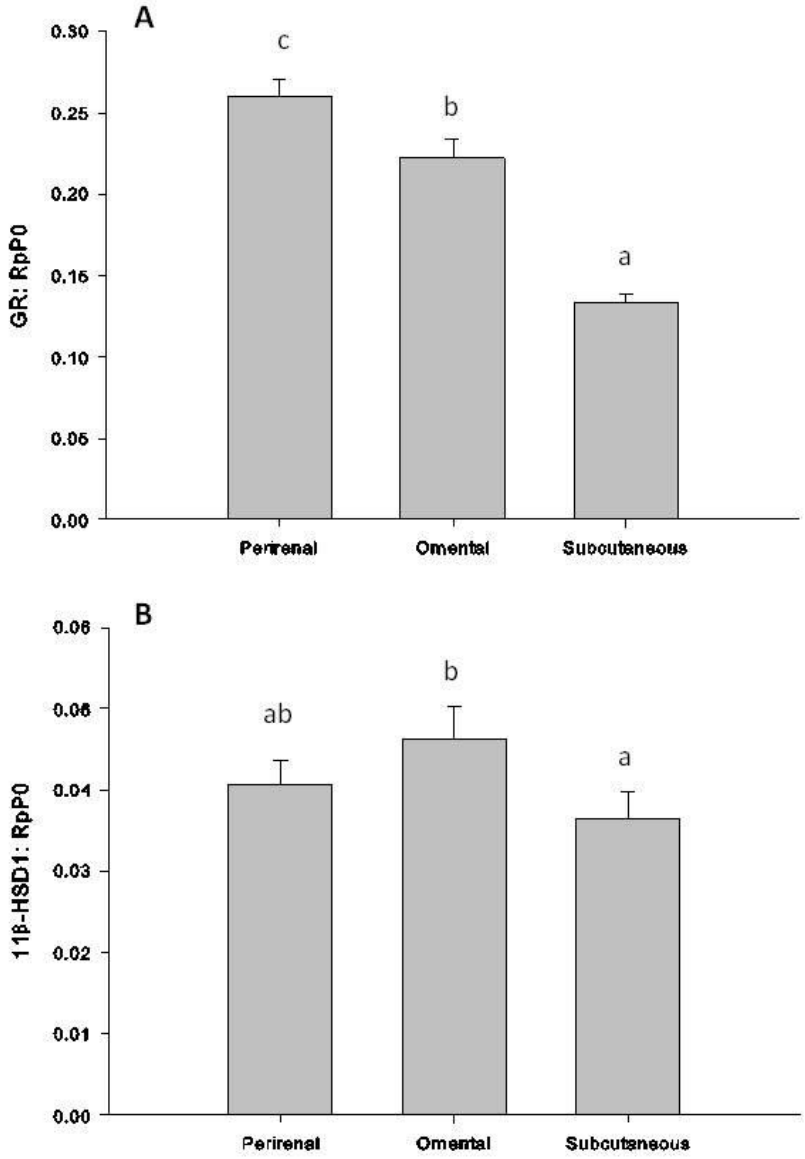


**Figure 3.13; Expression of GR (A) and 11β-HSD1 (B) across adipose tissue depots**

Lambs of 4 months of age. Different letters indicate significant differences between adipose tissue depots. Both graphs: n=41

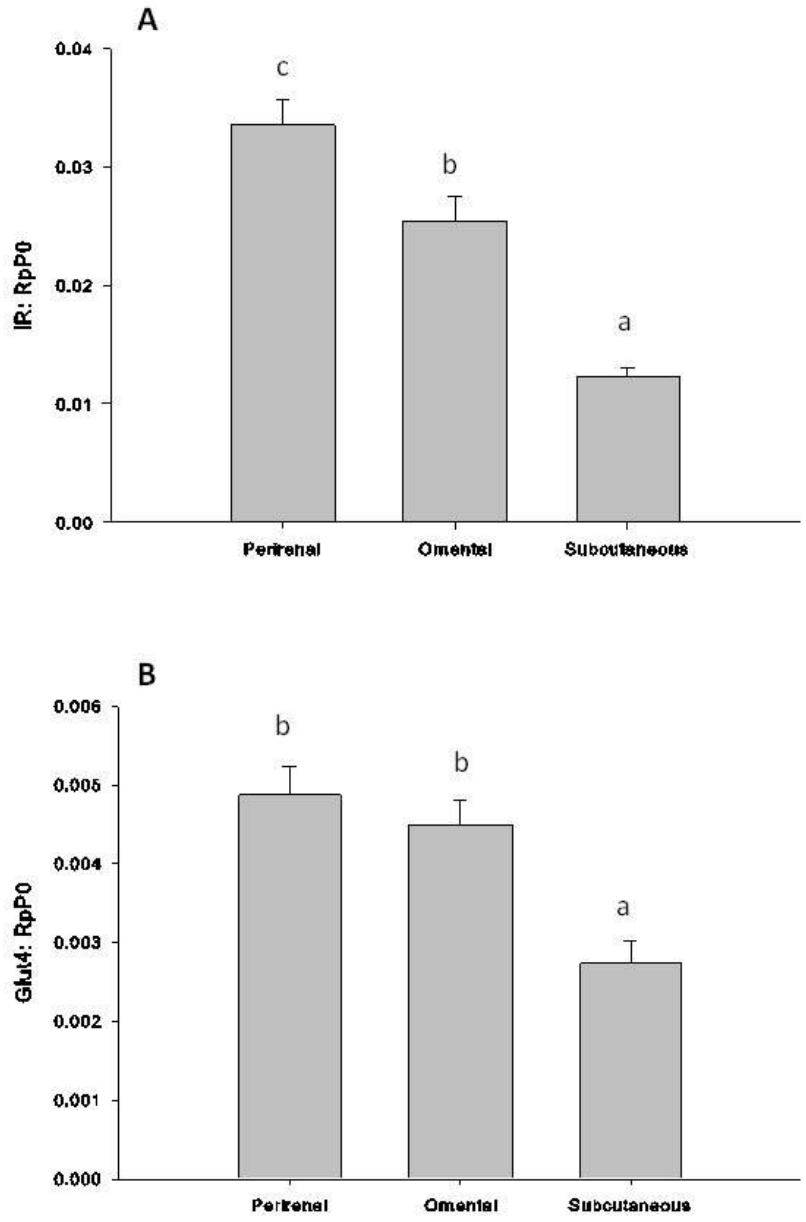


**Figure 3.14; Expression of IR (A) and Glut4 (B) across adipose tissue depots**

Lambs of 4 months of age. Different letters indicate significant differences between adipose tissue depots. Both graphs: n=41

## 3.5 Discussion

Periconceptional undernutrition did not result in a redistribution of growth towards the omental depot, as hypothesised. Neither did PCUN result in increased markers of local sensitivity to the actions of glucocorticoids in the perirenal or subcutaneous depot. However a reduction of weight of the ewe around conception did correlate with an increase in markers of sensitivity of the tissue to the actions of glucocorticoids, which could be a predictive adaptive response to the reduction in nutrient supply in the periconceptional period. The predictive adaptive response hypothesis suggests that the early life environment prepares an individual for a similar environment in later life and protects it from adverse effects of this type of environment (Gluckman & Hanson, 2004a, b). We could speculate that a low nutrient supply leads to mechanisms which protect the infant from decreased adipose tissue growth later in life, such as an up regulation of genes involved in glucocorticoid signalling. This response was only present in the omental depot, which is surprising since this depot is the last one to develop. However, given the anatomic location of the omental adipose tissue depot, it is widely recognised as the more metabolically active than other adipose depots and therefore more susceptible to the effects of changes in nutrition (Alvarez et al., 2002; Wilding, 2007; Hayashi et al., 2008; Lafontan & Girard, 2008). The relevance of the anatomic location of the omental adipose tissue depot

during this embryonic period of life is however uncertain, given that a significant amount of the nutrient supply from the umbilical cord bypasses the portal circulation and drains into the inferior vena cava.

Periconceptional overnutrition did not result in the hypothesised reduction in markers of tissue sensitivity to the action of insulin in any of the investigated depots. Periconceptional overnutrition did result in an increase in total adipose tissue mass in females, which could indicate a response to the increased substrate supply in this offspring. However, no changes were found between the expression of GR and 11β-HSD1 between dietary groups. It is therefore unlikely that the increased growth of adipose tissue mass in the female offspring of periconceptionally overnourished ewes is secondary to changes in the expression of markers of the local tissue sensitivity to the actions of glucocorticoids. Furthermore, no changes were found in the expression of IR and Glut4 between treatment groups. It is therefore unlikely that changes in the expression of key molecules in the insulin signalling cascade are underlying this difference in growth. However, we did not investigate other molecules involved in the insulin signalling cascade, such as IRS and PI3-kinase. Furthermore due to time limitations, we were unable to investigate protein abundance of proteins involved in the insulin signalling cascade in these samples. Future studies to investigate the expression and abundance of those and other relevant factors in the insulin signalling cascade could further elucidate whether increased tissue sensitivity to the actions of insulin in the female offspring of periconceptionally overnourished

ewes could underly the increased adipose tissue mass that is found in this group.

An interesting finding in this study was that a restriction of energy intake during the periconceptional period in previously overnourished ewes leads to a normalisation of adipose tissue mass in the offspring. This is an important finding since it highlights a potential beneficial effect of a period of dietary restriction in obese individuals before and around conception. Other studies have shown that especially in people with fertility problems a period of dietary restriction has shown to have positive effects on conception rate (Mitchell & Rogers, 1953; Kiddy *et al.*, 1992; Guzick *et al.*, 1994). This study indicates that the effects could be much longer lasting than the immediate period of conception. However, it is not possible to make definite conclusions regarding the effect of periconceptional undernutrition in overnourished individuals based on our study. Key differences between a real life human situation and our study include the use of an animal model and the use of embryo transfer and recipient ewes. The use of embryo transfer and recipient ewes does allow us to solely look at the periconceptional period and investigate the effects of dietary restriction in isolation from the further pregnancy environment on later pregnancy outcome.

We found that with an increase of the expression of 11β-HSD1 in the omental or subcutaneous adipose tissue depot the plasma FFA concentration rises. This could support our theory that glucocorticoids have a

stronger lipolytic than lipogenic effect on adipose tissue in the short term. Similar relations have been found between the effects of hypercortisolaemia and fatty acid turnover (Divertie *et al.*, 1991; Dinneen *et al.*, 1995). The absence of this correlation between perirenal 11β-HSD1 and plasma FFA could be explained by the fact that at the age of 4 months, the perirenal depot is relatively smaller compared to the other depots and would therefore contribute less to total plasma FFA levels.

The expression of markers of tissue sensitivity to the actions of insulin is lower in the subcutaneous adipose tissue compared to both visceral adipose tissue depots. This would support our hypothesis that this tissue which develops prenatally has a reduced tissue specific sensitivity to the actions of insulin in early postnatal life. However the perirenal adipose tissue also develops prenatally and does not show this reduction in the markers of tissue sensitivity to the actions of insulin. In order to make definite conclusion regarding tissue sensitivity to insulin more markers of the insulin signalling cascade need to be investigated. Furthermore we hypothesised that the actions of insulin are balanced against the actions of glucocorticoids and depot specific changes in this balance are responsible for differential growth of adipose tissue. In our study we also found a decrease in markers of sensitivity of the tissue to the actions of glucocorticoids in subcutaneous adipose tissue. This does not support our hypothesis that depot-specific differential growth patterns are secondary to changes in the balance of local

expression of markers of sensitivity to the local actions of insulin and glucocorticoid.

The programming of adipose tissue mass in females during the periconceptional period could not be explained by changes in markers of insulin or glucocorticoid signalling. Therefore we can not conclude that this increased growth of adipose tissue mass in females is driven by changes in local sensitivity to the actions of insulin or glucocorticoids. In the next chapter we examine the effect of periconceptional overnutrition on insulin like growth factors and their receptors in order to further investigate other mechanisms that could explain the increased adipose tissue mass that was found in female offspring of ewes that were overnourished in the periconceptional period.

# 4. Periconceptional nutrition and the expression of insulin like growth factor 1, 2 and their receptors in individual adipose tissue depots in sheep.

## 4.1 SUMMARY

Most tissues during development undergo a transition in the role of the insulin like growth factors and their receptors. This transition is, at least partly, mediated by glucocorticoids which stimulate IGF1R expression. We hypothesised that PCUN will result in a glucocorticoid mediated transition of IGF2R to IGF1R expression which happens mainly in the perirenal and subcutaneous adipose tissue depots as these are present during foetal life. Therefore the effects of IGF1 will be enhanced in the perirenal and subcutaneous adipose tissue depots, resulting in an increase in adipogenesis. Omental adipose tissue on the other hand, will undergo transition from IGF2R to IGF1R at a later stage in life. Therefore IGF2R remains abundant in early life, resulting in an enhanced clearance of IGFs and no enhanced effects of IGFs on adipogenesis in this tissue. We hypothesised that the growth of this depot therefore remains, during early postnatal life, insulin driven.

This study found no effects of PCUN on the expression of IGF1, IGF2,

IGF1R and IGF2R in omental, perirenal and subcutaneous adipose tissue of 4 month old offspring. However, the lack of an effect of PCUN on markers of tissue sensitivity to glucocorticoids could provide a potential explanation for the absence of an effect. The increased adipose tissue mass in female offspring of PCON ewes, which was found in Chapter 3, could not be explained by changes in expression of the IGFs and their receptors.

This study did find evidence for positive relations between GR and IGF1R and also between IR and IGF1R, indicating possible synergistic effects of glucocorticoids and IGFs and of insulin and IGFs. Interestingly a negative relationship was found between perirenal IGF1 expression and perirenal and subcutaneous adipose tissue mass, suggesting a regulatory role for the perirenal adipose tissue in the development of both adipose tissue depots.

Finally an increased expression of IGF2 and IGF2R in the omental depot, relative to the other depots was found, supporting our hypothesis that delayed development of this depot is associated with a delayed transition from IGF1 to IGF2 dominance.

## 4.2 Introduction

In the previous chapter we demonstrated that adipose tissue mass in females can be programmed as early as the periconceptional period. A high nutrient intake in the ewe resulted in an increase in total fat mass in the female offspring at four months of age. We investigated the effect of factors influencing sensitivity to glucocorticoids and insulin on depot specific adipose tissue growth. The increase in total omental adipose tissue mass in female offspring of ewes that were overnourished during the periconceptional period could not be explained by differences in the expression of the investigated genes.

In this chapter we will investigate other growth promoting factors that could be responsible for differential growth patterns of adipose tissue depots, namely the insulin like growth factors. Insulin like growth factor 1 (IGF1) and insulin like growth factor 2 (IGF2) are well known to promote DNA synthesis and cell replication in human serum (Zapf *et al.*, 1978). Actions of IGF1 include stimulations of proliferation and differentiation of preadipocytes both in vivo and in cell culture in a number of species (Smith *et al.*, 1988; Gregoire *et al.*, 1998; Rajkumar *et al.*, 1999; Soret *et al.*, 1999; Holzenberger *et al.*, 2001). Furthermore, human umbilical cord serum levels of IGF1 positively

correlate with body weight and length (Gluckman *et al.*, 1983; Verhaeghe *et al.*, 1993; Geary *et al.*, 2003).

The role of IGF2, although less clear than the role of IGF1, involves mainly the stimulation of antenatal growth as suggested by knockout mice studies and the relationship between size for gestational age and serum IGF2 concentrations that was found in human newborns (DeChiara *et al.*, 1990; Verhaeghe *et al.*, 1993). IGF2 is expressed in a range of tissues at high levels during early gestation in rats and sheep (van Dijk *et al.*, 1988; Han *et al.*, 1992), making it potentially vulnerable to dietary interventions in early gestation or the periconceptional period. Periconceptional maternal undernutrition has been shown to reduce foetal sheep plasma IGF1, but not IGF2 during late gestation (Lee *et al.*, 1997; Gallaher *et al.*, 1998). A study investigating the effects of periconceptional undernutrition on expression of IGF1, IGF2, IGF1R and IGF2R in sheep adrenal glands showed no effect of undernutrition on IGF gene expression (MacLaughlin *et al.*, 2007). However, a study that investigated the effect of a low protein diet during the preimplantation period in rats did show a significant reduction in IGF2 expression in the liver of male offspring only. The effect was only present if the diet was restricted to the preimplantation period and was abolished when the low protein diet continued throughout pregnancy (Kwong *et al.*, 2006).

Verhaeghe and colleagues reported that human serum levels of IGF2 were 6-10 times higher than IGF1 levels during late gestation (Verhaeghe *et al.*,

1993). Several studies have indicated that during development a transition occurs from predominantly IGF2R expression to IGF1R expression in a variety of species such as sheep, pigs and rats, and in a variety of tissues such as liver, adrenal glands, bones, kidneys and pancreas (Sara & Hall, 1990; Delhanty & Han, 1993; Li *et al.*, 1993; Peng *et al.*, 1998; Hyatt *et al.*, 2004). This transition is, at least partly, mediated by glucocorticoids which stimulate IGF1R expression (Bennett *et al.*, 1984; Li *et al.*, 1993; Lu *et al.*, 1994).

We hypothesise that the premature activation of the HPA axis in PCUN will result in a glucocorticoid mediated transition of IGF2R to IGF1R mRNA expression which happens mainly in the perirenal and subcutaneous adipose tissue depots because these are present during foetal life. Therefore the effects of IGF1 will be enhanced in the perirenal and subcutaneous adipose tissue depots, resulting in an increase in adipogenesis. Omental adipose tissue on the other hand, will undergo transition from IGF2R to IGF1R at a later stage in life. Therefore IGF2R remains abundant in early life, resulting in an enhanced clearance of IGFs and no enhanced effects of IGFs on adipogenesis in this tissue. The growth of this depot therefore remains, during early postnatal life, insulin driven.

## 4.3 Methods

### 4.3.1 ANIMALS AND EXPERIMENTAL DESIGN

The animal protocol and experimental design used in this study is identical to that described in detail in the previous chapter.

In brief: 23 Non-pregnant Merino ewes were included in this study. During an acclimatisation period of 2 weeks the ewes were fed 100% metabolisable energy requirements (ME) as defined by the Agricultural and Food Research Council in 1993 (AFRC, 1993).

After the acclimatisation period, the ewes were randomly allocated to one of four treatment groups. The control-control group (CC, n=6) received a diet of 100% metabolisable energy requirements (ME) for 4 months prior to conception. The control-restricted group (CR, n=6) received a diet of 100% ME for 3 months followed by 70% ME for 1 month. The high-high group (HH, n=6) was fed *ad libitum* (170-190% ME) for 4 months. The high-restricted group (HR, n=5) was fed *ad libitum* (170-190% ME) for 3 months followed by a diet of 70% ME for 1 month prior to conception.

The reproductive cycle of all ewes was synchronised and superovulation was induced. Fresh semen was collected from a ram of proven fertility and donor ewes were inseminated by laparoscopy with approximately $20 * 10^6$ spermatazoa being placed directly into the lumen of each uterine horn.

6 days after ovulation, the donor ewes were anaesthetised and embryos were collected by mid-ventral laparotomy. At embryonic day 6-7, donor embryos of good quality (≥ 8 cells and acceptable morphology) were transferred to adult recipient ewes by laparotomy. Recipients ewes were maintained on a control diet for the maintenance of singleton bearing pregnant ewes (100% ME) at all times. The pregnant ewes were allowed to give birth naturally (term = 147±3 days).

Lambs remained with their mothers until weaning at three months of age. Lambs (n=41) were humanely killed at 4 months of age with an overdose sodium pentobarbitone (Virbac Pty Ltd, Peakhurst, New South Wales, Australia); all adipose tissue was collected and weighed at post mortem, samples were rapidly snap frozen in liquid nitrogen and stored at -80°C for further analysis.

### 4.3.2 LABORATORY ANALYSIS

RNA was extracted and cDNA was synthesised from frozen omental, perirenal and subcutaneous adipose tissue as described in Chapter 3. Normalised mRNA expression for insulin-like growth factor 1 and 2 (IGF1 and IGF2) and their receptors (IGF1R and IGF2R) was determined by reverse transcription real time PCR as described in Chapter 3. The sequences of the individual primers can be found in Table 4.1.

| Primer | Sequence | Accession number |
|---|---|---|
| IGF1 | **F:** 5'-TTG GTG GAT GCT CTC CAG TTC-3'<br>**R:** 5'-AGC AGC ACT CAT CCA CGA TTC-3' | NM_001009774 |
| IGF2 | **F:** 5'- GCT TCT TGC CTT CTT GGC CTT-3'<br>**R:** 5'- TCG GTT TAT GCG GCT GGA T-3' | M89789 |
| IGF1R | **F:** 5'-AAG AAC CAT GCC TGC AGA AGG-3'<br>**R:** 5'-GGA TTC TCA GGT TCT GGC CAT T-3' | AY162434 |
| IGF2R | **F:** 5'- GAT GAA GGA GGC TGC AAG GAT-3'<br>**R:** 5'- CCT GAT GCC TGT AGT CCA GCT T-3' | AF327649 |
| RpP0 | **F:** 5'-CAA CCC TGA AGT GCT TGA CAT-3'<br>**R:** 5'-AGG CAG ATG GAT CAG CCA -3' | NG_009485 |

**Table 4.1; Primer sequences for PCR analysis of IGF1, IGF2, IGF1R, IGF2R and RpP0**

F: forward primer sequence, R: reverse primer sequence

### 4.3.3 STATISTICAL ANALYSIS

Data are presented as the mean ± SEM. The effects of periconceptional overnutrition and dietary intervention on depot specific adipose tissue expression of IGF1, IGF2, IGF1R and IGF2R were determined with an ANOVA using SPSS for Windows version 16.0 (SPSS Inc, Chicago, Illinois). Differences in gene expression between depots were determined using a multifactorial ANOVA with repeated measures using STATA 10.0 (StataCorp, College Station, Texas, USA) in which the outcomes for the different depots were considered to be repeated within each animal. Extensive consideration has been given to the choice of using analyses for repeated or independent measures. Data have been analysed using both methods obtaining similar results. It was felt that data from different depots

could not be considered as indepent and therefore analyses using repeated measures were chosen to be presented here. Bonferroni posthoc tests were performed when more than two groups were present. Relationships between variables were assessed by linear regression using SigmaPlot 9.0 (SPSS). A probability level of 5% ($p<0.05$) was considered to be significant.

## 4.4 Results

### 4.4.1 PERIRENAL GENE EXPRESSION

There was no effect of periconceptional overnutrition and dietary restriction on the expression of IGF1, IGF2, IGF1R and IGF2R in the perirenal adipose tissue depot. Neither was the expression of these genes influenced by gender in perirenal adipose tissue (Figure 4.1 and Figure 4.2).

### 4.4.2 OMENTAL GENE EXPRESSION

There was no effect of periconceptional overnutrition and dietary restriction on the expression of IGF1, IGF2, IGF1R and IGF2R in the omental adipose tissue depot. Neither was the expression of these genes influenced by gender in omental adipose tissue (Figure 4.3 and Figure 4.4).

### 4.4.3 SUBCUTANEOUS GENE EXPRESSION

There was no effect of periconceptional overnutrition and dietary restriction on the expression of IGF1, IGF2, IGF1R and IGF2R in subcutaneous adipose tissue. Neither was the expression of these genes influenced by gender in subcutaneous adipose tissue (Figure 4.5 and Figure 4.6).

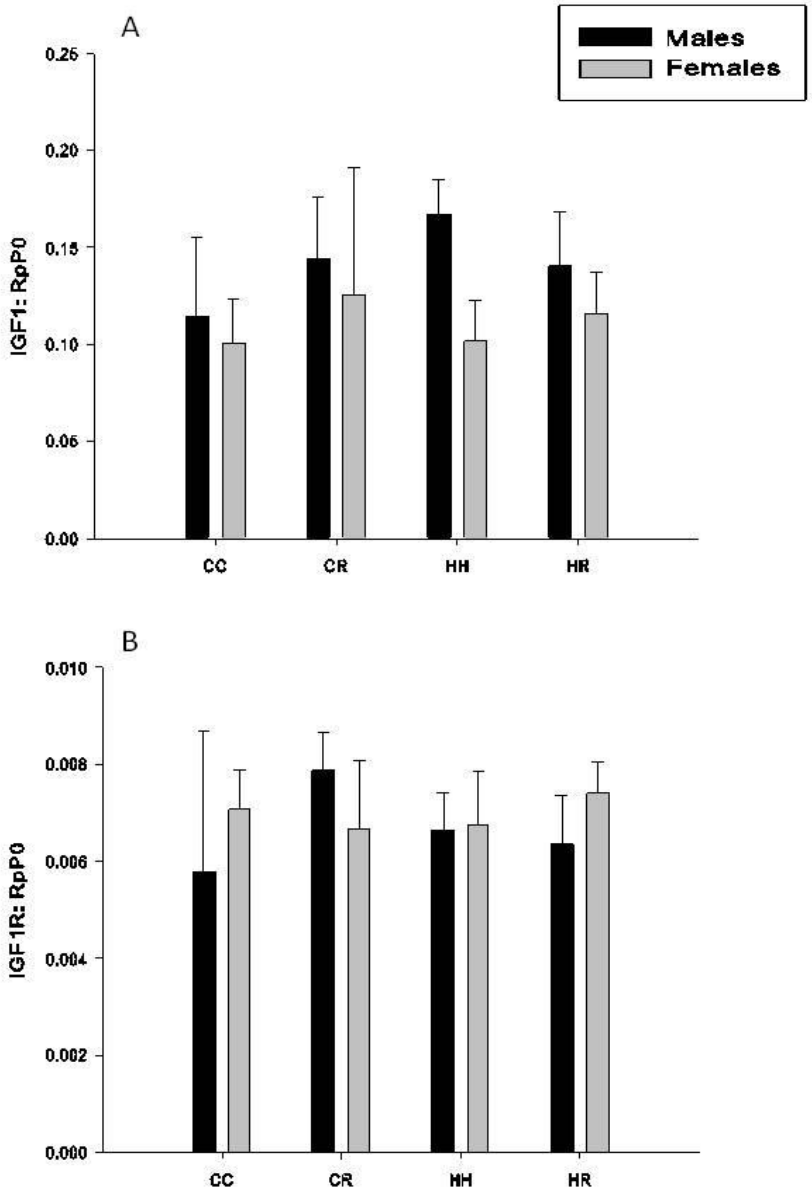


**Figure 4.1; Expression of IGF1 (A) and IGF1R (B) in perirenal adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

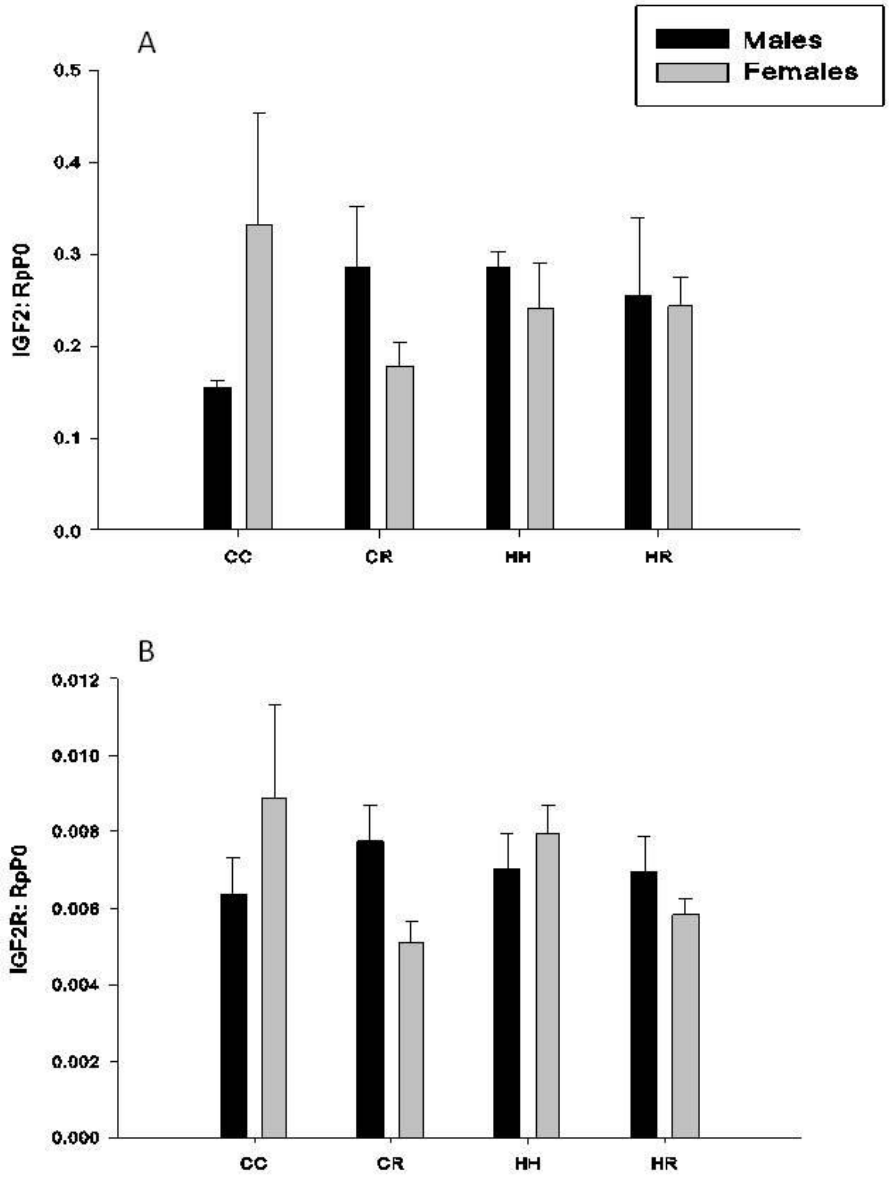


**Figure 4.2; Expression of IGF2 (A) and IGF2R (B) in perirenal adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

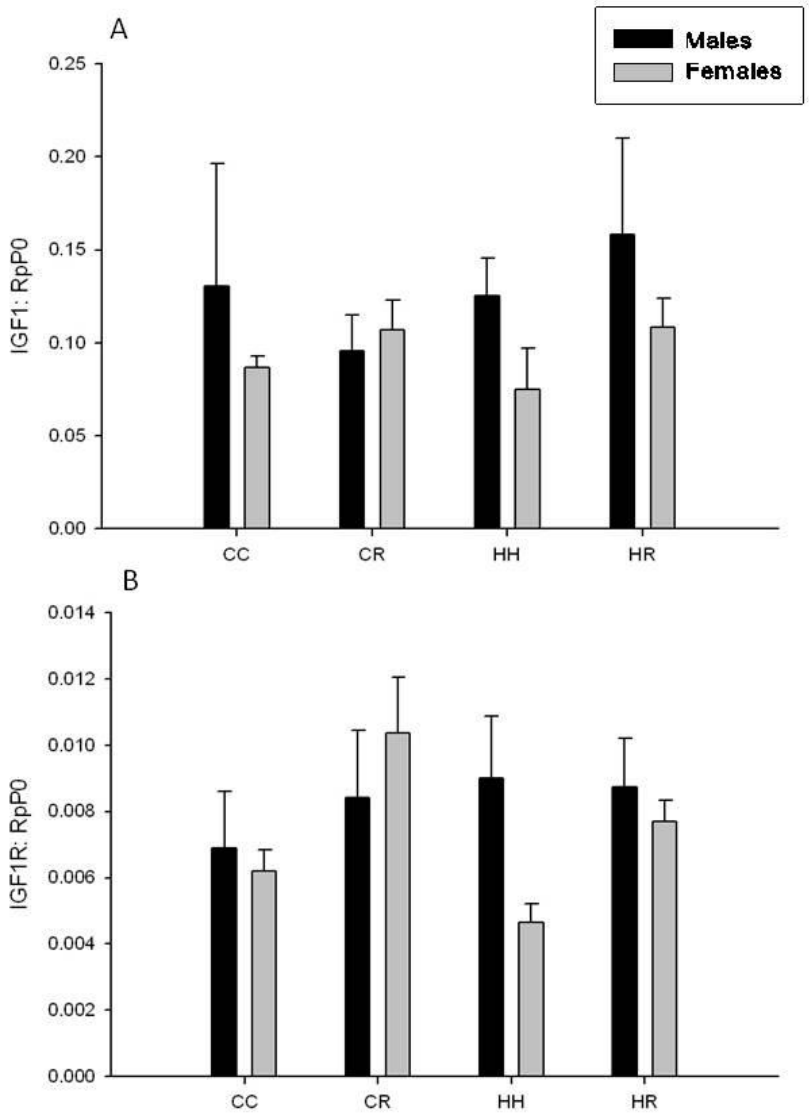


**Figure 4.3; Expression of IGF1 (A) and IGF1R (B) in omental adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

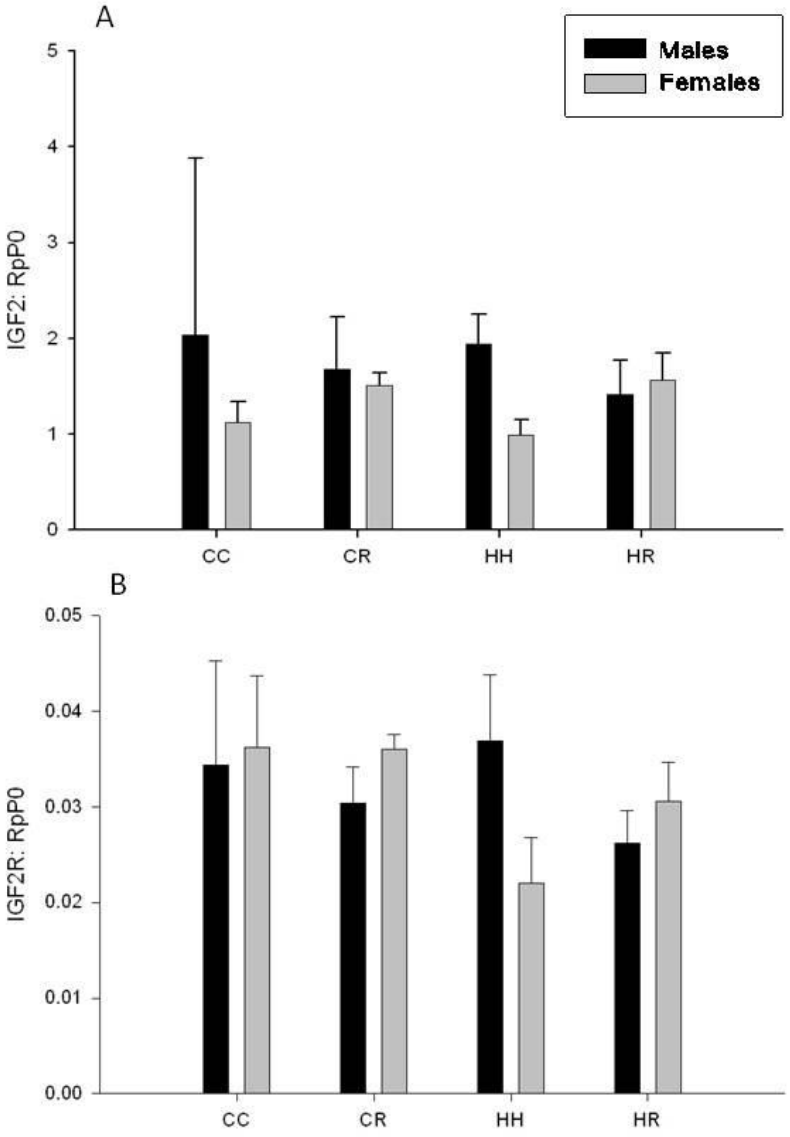


**Figure 4.4; Expression of IGF2 (A) and IGF2R (B) in omental adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

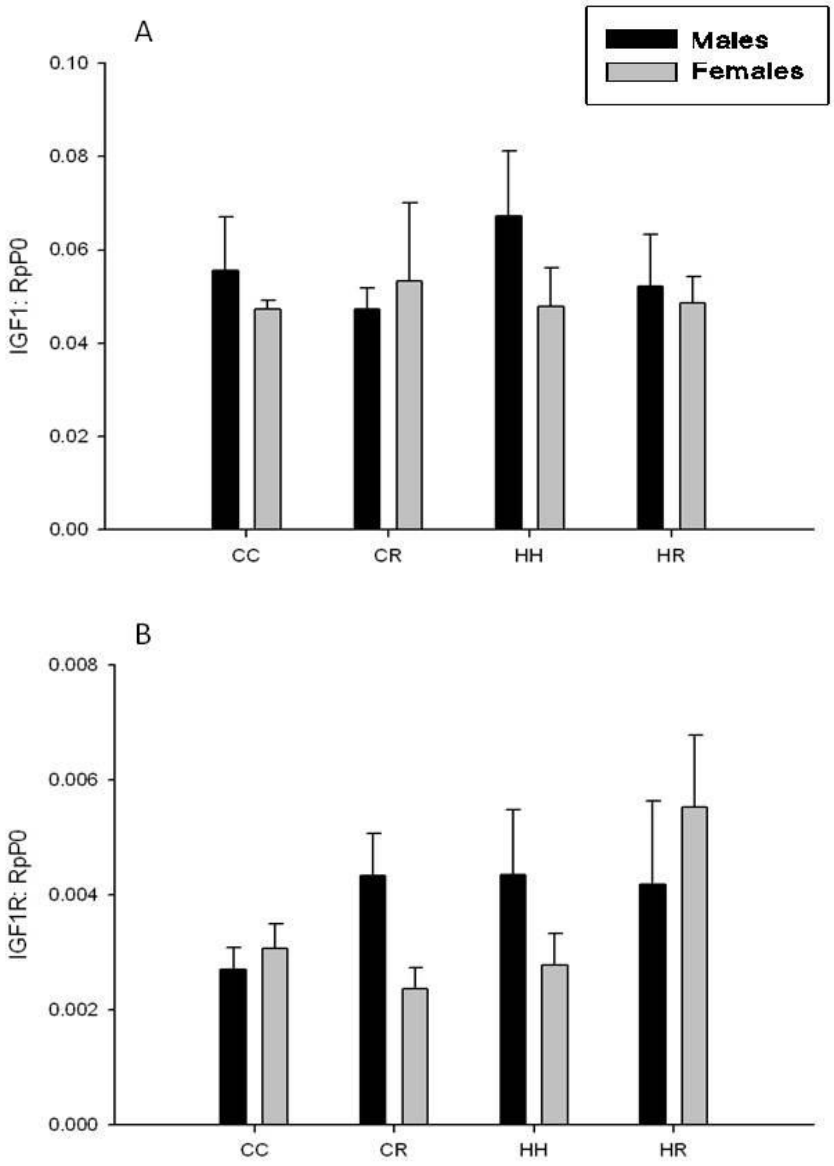


**Figure 4.5; Expression of IGF1 (A) and IGF1R (B) in subcutaneous adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

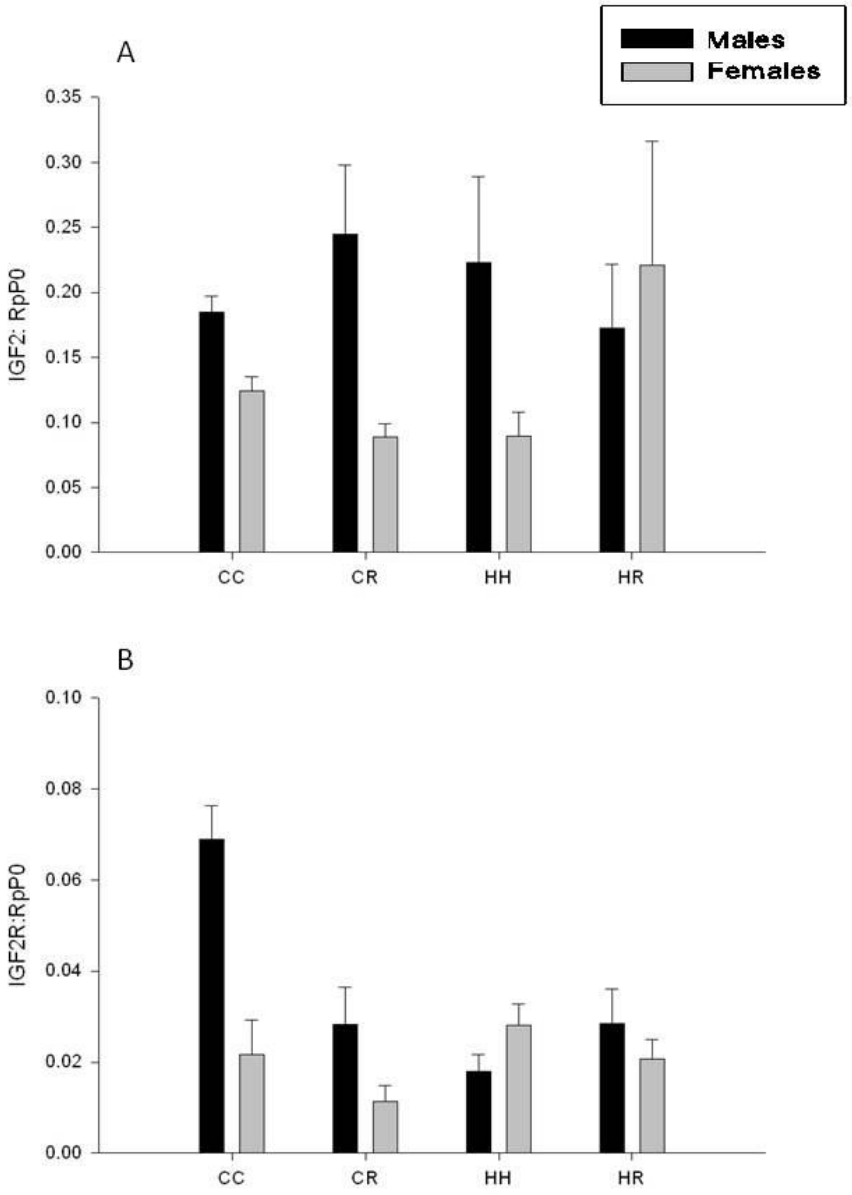


**Figure 4.6; Expression of IGF2 (A) and IGF2R (B) in subcutaneous adipose tissue**

Male and female lambs at 4 months of age. No significant differences were found. Data are: CC: n=7, CR: n=10, HR: n=12, HH: n=12

### 4.4.4 INTERACTIONS BETWEEN GENES, PLASMA METABOLITES AND DEPOTS

No interactions were found between the expression of IGF1, IGF1R, IGF2 or IGF2R expression and plasma metabolites.

In male lambs perirenal expression of IGF1 was strongly negatively associated with relative perirenal and subcutaneous adipose tissue mass (perirenal adipose tissue: P = 0.002, $R^2$ = 0.4191, n=21, subcutaneous adipose tissue: P = 0.005, $R^2$ = 0.3466, n=21, Figure 4.7). There was also a significant association between perirenal IGF1 and total fat mass, but this correlation was no longer significant when controlled for subcutaneous adipose tissue mass.

In both males and females the expression of GR was positively correlated with the expression of IGF1R. This correlation was significant in omental and perirenal adipose tissue although the slope of the regression line was significantly lower in omental compared to perirenal adipose tissue (omental: P = 0.003, $R^2$= 0.40, slope: 12.79, n=41, perirenal: P=0.019, $R^2$ = 0.27, slope: 17.26, n=41, Figure 4.8). The expression of IR was also positively correlated with the expression of IGF1R. This correlation was significant in all investigated depots and the slope was significantly higher in perirenal compared to omental adipose tissue (omental: P <0.0001, $R^2$ = 0.50, slope: 2.51, n=41, perirenal: P = 0.002, $R^2$ = 0.23, slope: 3.17, n=41, subcutaneous: P = 0.0254, $R^2$ = 0.12, slope: 0.81, n=41, Figure 4.9). A correlation between expression levels of GR and IR was no longer significant when controlled for IGF1R expression levels.

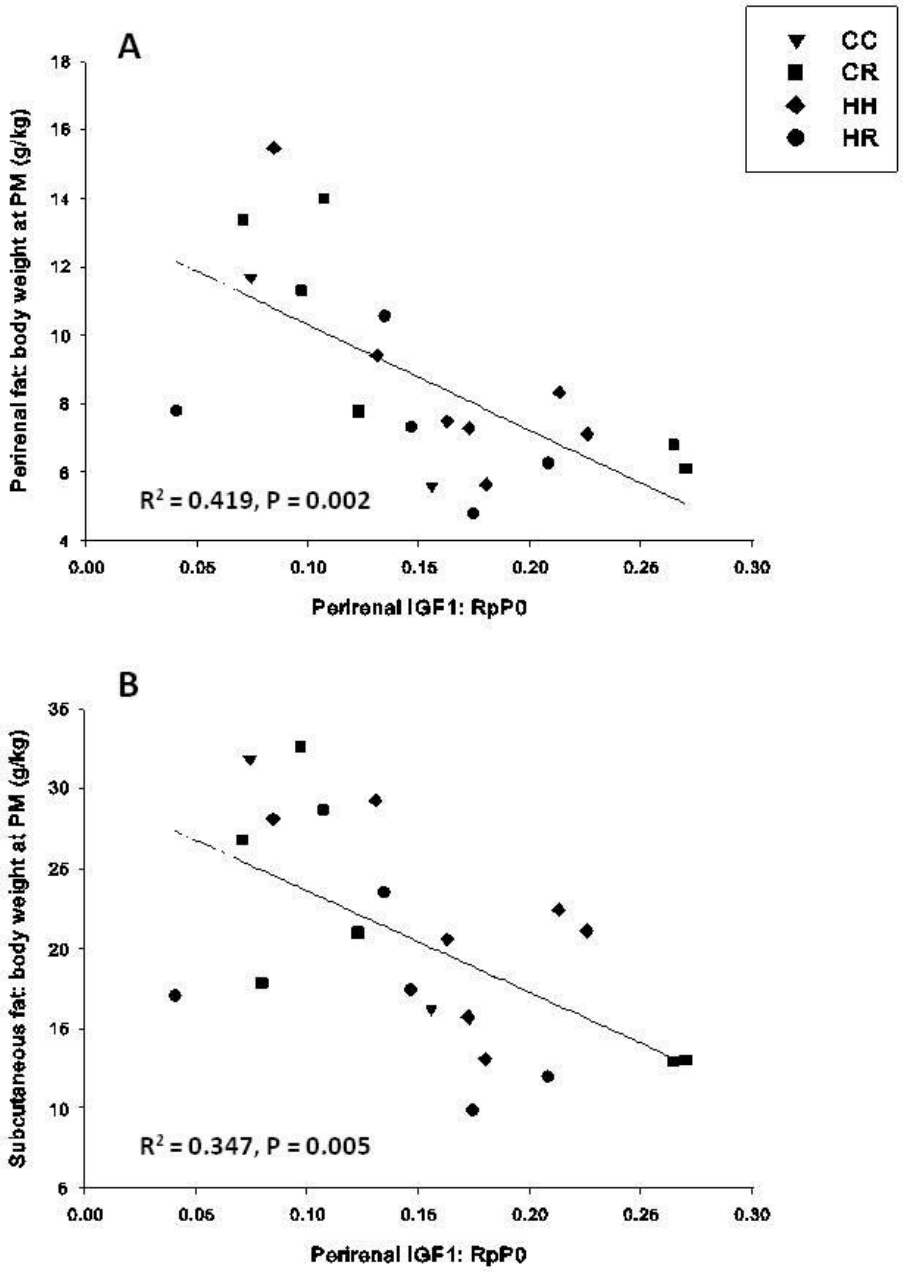


**Figure 4.7; Correlation between perirenal expression levels of IGF1 and relative perirenal (A) and subcutaneous (B) adipose tissue mass in males**

Male lambs at four months of age, n=21

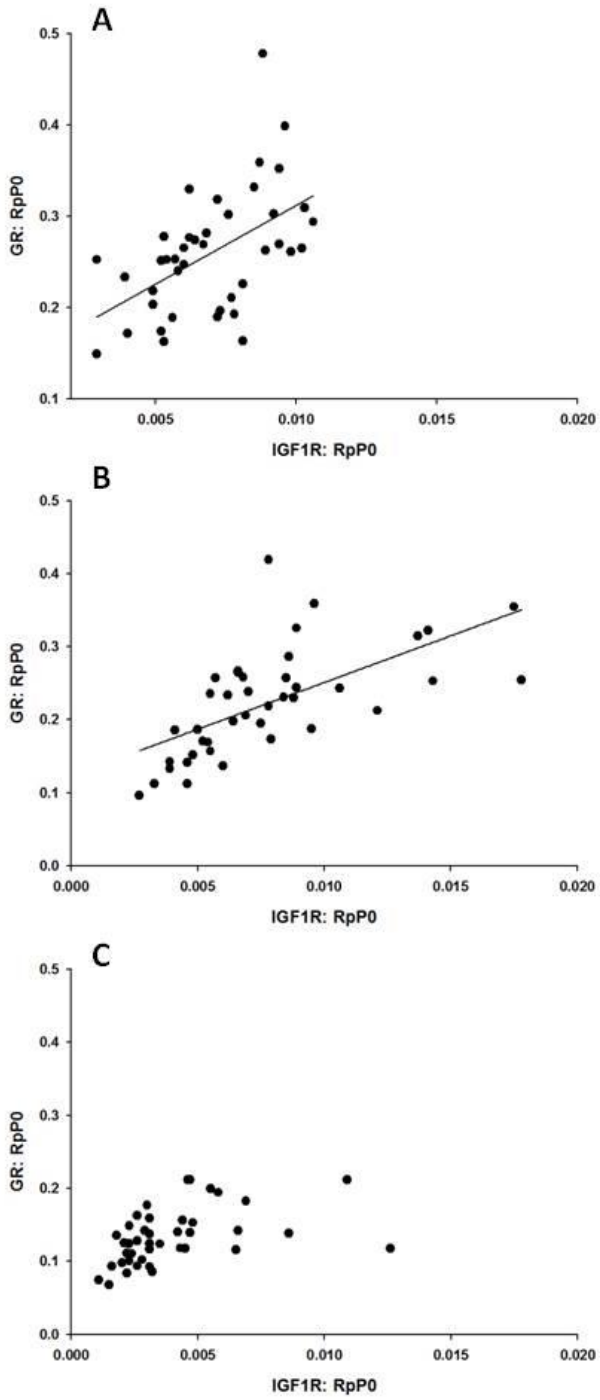


**Figure 4.8; Correlations between GR and IGF1R in perirenal (A), omental (B) and subcutaneous (C) adipose tissue**

Lambs at 4 months of age. A; perirenal (GR = 17.26 IGF1R + 0.1394, P = 0.019, $R^2$ = 0.27, n=41). B; omental (GR = 12.79 IGF1R + 0.1232, P = 0.003, $R^2$ = 0.40, n=41). C; subcutaneous (no significant correlation).

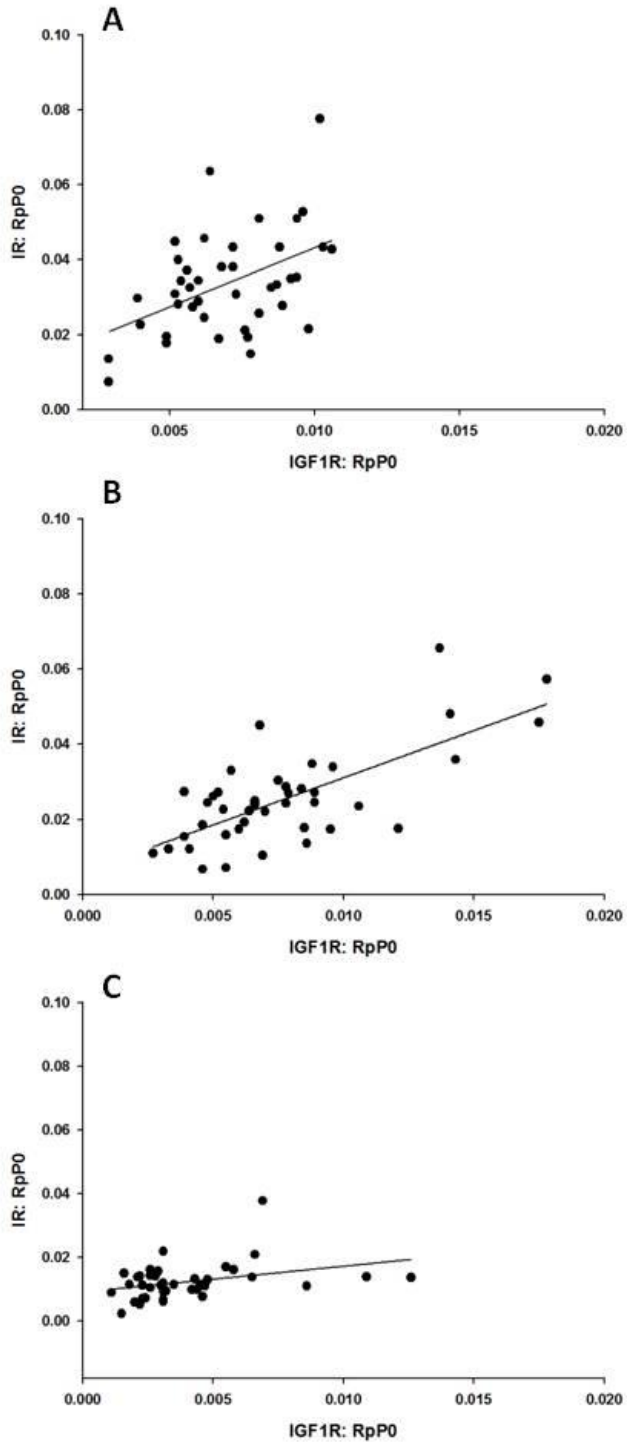


**Figure 4.9; Correlations between IR and IGF1R in perirenal (A), omental (B) and subcutaneous (C) adipose tissue**

Lambs at 4 months of age. A; perirenal (IR = 3.1684 IGF1R + 0.0116, P = 0.002, $R^2$ = 0.22, n=41). B; omental (IR = 2.5143 IGF1R + 0.0059, P < 0.001, $R^2$ = 0.50, n=41). C; subcutaneous (IR = 0.8146 IGF1R + 0.009, P = 0.025, $R^2$ = 0.12, n=41)

### 4.4.5 DEPOT SPECIFIC GENE EXPRESSION

Since there were no significant differences between groups or gender in the expression of the investigated genes, data were pooled to allow a comparison in gene expression between different adipose tissue depots. The expression of all investigated genes was highly depot specific. Expression of IGF1 and IGF1R were significantly lower in subcutaneous compared to omental and perirenal adipose tissue ($P < 0.001$, all cases, Figure 4.10). Expression of IGF2 was significantly higher in omental compared to perirenal adipose tissue ($P < 0.001$). The expression of IGF2 in subcutaneous adipose tissue was lower compared to both visceral adipose tissue depots ($P < 0.001$, both cases). Expression of IGF2R was also significantly higher in omental compared to both other depots ($P < 0.001$, omental *vs.* perirenal and $P = 0.006$, omental *vs.* subcutaneous). The subcutaneous expression of IGF2R was lower than the omental IGF2R expression but higher than the perirenal IGF2R expression ($P < 0.001$, Figure 4.11).

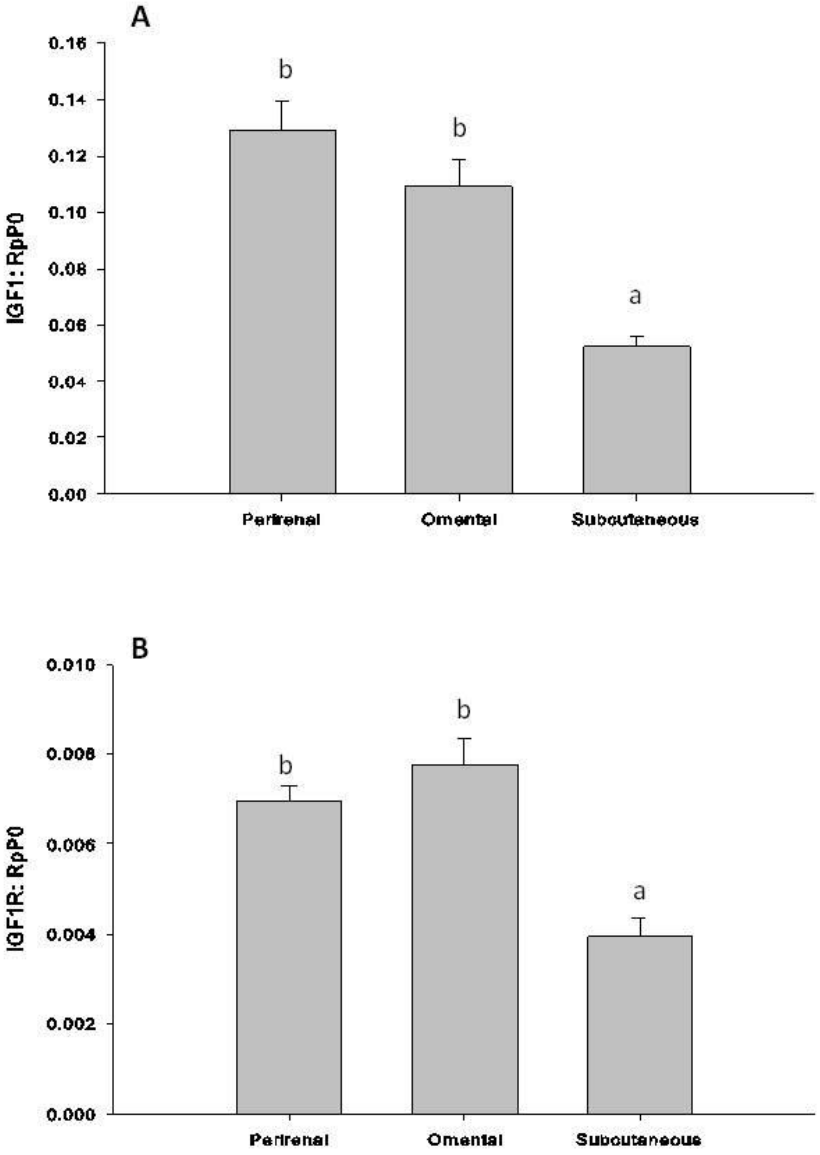


**Figure 4.10; Expression of IGF1 (A) and IGF1R (B) across adipose tissue depots**

Lambs of 4 months of age. Different letters indicate significant differences between adipose tissue depots. Both graphs: n=41.

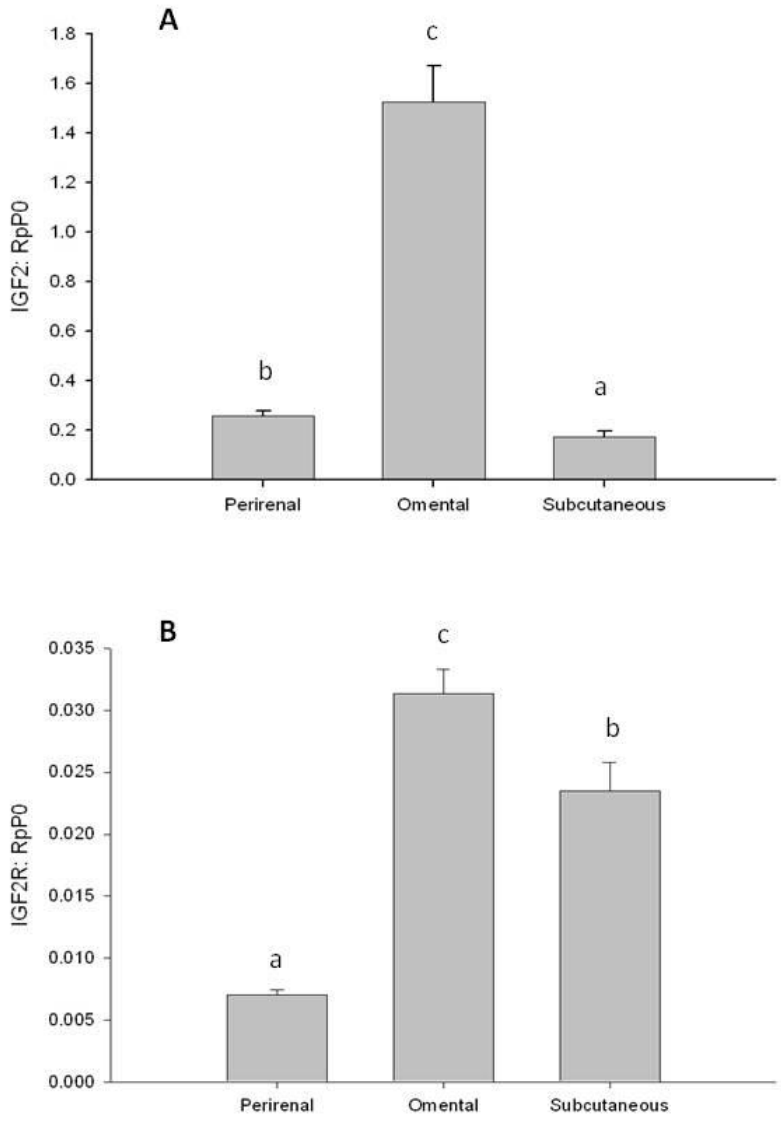


**Figure 4.11; Expression of IGF2 (A) and IGF2R (B) across adipose tissue depots**

Lambs of 4 months of age. Different letters indicate significant differences between adipose tissue depots. Both graphs: n=41.

## 4.5 Discussion

Periconceptional undernutrition did not result in the expected increased expression of IGF1 or IGF1R in any of the investigated depots. Furthermore, no effects of periconceptional overnutrition or nutrient restriction on the expression of IGF1, IGF2, IGF1R or IGF2R in perirenal, omental or subcutaneous adipose tissue were found. The increased amount of total adipose tissue which was found in periconceptionally overnourished (female) sheep cannot, therefore, be explained by differences in the expression of insulin like growth factors and their receptors.

However we did find an association between GR and IGF1R which supports our hypothesis that glucocorticoids stimulate IGF1R expression and therefore work synergistically with IGF1. Bennett and colleagues found similar relations in their study (Bennett *et al.*, 1984), while Conover and colleagues and Tronche and colleagues found that glucocorticoids stimulated IGF1 action *in vivo* (Conover *et al.*, 1985; Tronche *et al.*, 2004)

Since our hypothesis was initially based on an increase in markers of tissue sensitivity to the actions of glucocorticoids in perirenal and subcutaneous adipose tissue resulting in an increase in IGF1R, the absence of an effect of PCUN on IGF1 and IGF1R expression in those depots could be secondary to the absence in the effect of markers of the tissue sensitivity to glucocorticoids in PCUN. Given the relationship between markers of tissue

sensitivity to the actions of glucocorticoids and IGF1R it is therefore possible that the effects of PCUN on IGF1 expression are absent because PCUN does not alter tissue sensitivity to the actions of glucocorticoids as seen in the previous chapter.

Unfortunately, due to time limitations, we were not able to measure protein abundance of insulin like growth factors and their receptors in different adipose tissue depots in this study. Future data will further elucidate the effects of periconceptional nutrition on insulin like growth factors protein abundance.

The relationship between IGF1R and IR suggests that not only do glucocorticoids work synergistically with IGF1 but that insulin has the same effect. It has been shown in the past that insulin can directly stimulate plasma IGF1 concentrations (Gluckman *et al.*, 1987; Fowden, 1989). Furthermore, IGF1 and IGF2 plasma concentrations were related to foetal glucose concentrations, a relationship that was considered to be secondary to the effects of insulin (Gluckman, 1986; Oliver *et al.*, 1993). Our data suggest that the relationship between insulin and IGF1 not only exists at a plasma metabolite level, but also on a local tissue sensitivity level.

The relationship between IR and GR was no longer present when controlled for IGF1R which suggests that there is no direct synergistic effect between the actions of insulin and glucocorticoids. The lack of evidence for a synergistic effect between insulin and glucocorticoids could provide support for our hypothesis of a counter-acting balance between the two.

An interesting negative relationship was found between perirenal IGF1 expression and perirenal and subcutaneous adipose tissue mass. We expected IGF1 to have autocrine and paracrine adipogenic effects, so it was surprising to see a negative relationship. Even more surprising is the relationship between perirenal IGF1 expression and subcutaneous adipose tissue mass. This suggests some cross-talk between perirenal and subcutaneous adipose tissue, although the precise nature has yet to be defined. Since most of the plasma IGF1 is hepatic in origin, it is unlikely that this cross-talk is mediated by IGF1 itself. It could be that perirenal adipose tissue growth results in the production of a signalling factor, such as leptin or adiponectin, which subsequently results in endocrine effects on the growth of the subcutaneous depot and at the same time down-regulates IGF1 in the perirenal depot in an attempt to prevent it from further growth. Such a mechanism of cross-talk has been proposed before by Duffield and colleagues. They reported similar relationships between perirenal fat and the development of subcutaneous adipose tissue. They also found relationships between both perirenal adipose tissue mass and perirenal adipocyte size with the expression of leptin mRNA in subcutaneous adipose tissue (Duffield, 2007).

Finally we found increased levels of IGF2 and IGF2R in the omental depot compared to the other depots which supports our hypothesis that the delayed effects of omental growth coincide with a delayed switch from IGF2

to IGF1 dominance. Previous studies have indicated that this transition was driven, at least partly, by glucocorticoids in the liver and adrenal gland (Li *et al.*, 1993; Lu *et al.*, 1994). The omental depot which develops post-natally is not exposed to the cortisol surge which occurs before parturition (Liggins, 1976; Smith *et al.*, 2009) which could potentially explain a delayed switch from IGF2 to IGF1 dominance. A recent study in near-term baboons also found a higher abundance of IGF2R in omental compared to subcutaneous adipose tissue in the abdominal or femoral regions (Tchoukalova *et al.*, 2009). They suggested that a relative increase in the abundance of the clearance receptor IGF2R results in a lower bioavailability and activity of IGF2, which could result in a restraint differentiation of the omental adipose tissue. They did however not examine the abundance of IGF2. Our study has shown that both IGF2 and IGF2R are, at the age of 4 months, elevated in omental adipose tissue in sheep. The exact effects on the bioavailability remain to be investigated.

# 5. The effects of formula feeding on differential expression of key genes involved in adipose tissue growth.

## 5.1 SUMMARY

Formula feeding is associated with an increased food intake in early life and increased plasma insulin concentrations. We hypothesise that formula feeding results in a reduced sensitivity of adipose tissue to the actions of insulin. This reduction in sensitivity to insulin will switch the balance between insulin and glucocorticoid action in adipose tissue towards a stronger sensitivity of the adipose tissue to glucocorticoids, resulting in an increase in long term adipogenic growth. We hypothesise that the reduction in local sensitivity to the action of insulin will be greatest in the omental depot, which is developing rapidly during this period in life. The effects on increased adipose tissue growth will therefore lead to a redistribution of adipose tissue towards the omental depot.

We tested this hypothesis by examining the effects of formula feeding or maternal milk feeding on the development and distribution of adipose tissue in lambs at three months of age. We further examined the transcript expression of key markers of the tissue sensitivity to the actions of insulin and glucocorticoids in individual adipose tissue depots.

Formula feeding in this study did not lead to a redistribution of adipose tissue at the age of 3 months. Formula feeding did lead to a significant reduction in Glut4 expression in all depots. A reduction in Glut4 expression combined with an absence of an effect on insulin receptor expression could potentially mean that the tissue is more resistant to the actions of insulin as a result of formula feeding, suggesting that the reduction in insulin sensitivity could be secondary to a decrease in the efficiency of the insulin signalling cascade which occurs as early as at the age of 3 months.

Furthermore formula feeding resulted in an increase in 11β-HSD1 expression in all adipose tissue depots. The increased expression of 11β-HSD1 could in the long term have an adipogenic effect and be a mechanism that explains the increased growth of adipose tissue in formula fed infants. GR expression was higher in perirenal and subcutaneous adipose tissue which would support our hypothesis that these depots are relatively more sensitive to the actions of glucocorticoids compared to the omental depot during this period of life.

Furthermore an increase in IGF1 in the perirenal adipose tissue compared to omental and subcutaneous adipose tissue supports our hypothesis that there is a relationship between the actions of glucocorticoids and IGF1 in the tissue.

## 5.2 Introduction

It has long been known that breastfeeding as opposed to formula feeding has beneficial effects on short term health outcomes such as infections and long term health outcomes such as the development of asthma and allergies in later life (Saarinen *et al.*, 1979; Lucas *et al.*, 1990; Saarinen & Kajosaari, 1995). The effects of breastfeeding on the incidence of obesity and cardiovascular risk factors in adulthood, however, have been a subject of debate in many studies over the last decades. The general conclusion that was obtained from several large meta-analyses was that breastfeeding has a small but significant protective effect on the incidence of obesity later in life and this effect could have a dose-response element (Dietz, 2001; Dewey, 2003; Arenz *et al.*, 2004; Harder *et al.*, 2005; Owen *et al.*, 2005). The protective effect of breast feeding appears more prominent in adolescence compared to childhood, which suggests a possible programmed effect (Dewey, 2003). Given the present obesity epidemic, any possible prevention method of obesity is worth investigating; even very small effects can still have major impacts on society as a whole.

Several mechanisms have been proposed to explain the relationship between formula feeding and obesity. Formula fed infants have a higher food, energy and protein intake compared to breast fed infants, which leads

to an increased weight gain and fat mass (Heinig *et al.*, 1993; Dewey, 1998). Insulin like growth factor 1 (IGF1), an important growth factor in postnatal life, has been shown to be dependent on amino acid availability (Wheelhouse *et al.*, 1999). An increased protein intake in formula fed infants could therefore lead to increased levels of amino acids and increased IGF1 levels subsequently promoting increased growth and BMI in later life.

Another important driver of adipose tissue growth is insulin. Insulin acts through a series of second messenger molecules ultimately resulting in translocation of the glucose transporter 4 (Glut4) from storage vesicles in the cytosol to the cell membrane, leading to an increase in glucose uptake by the adipocyte and fat cell growth (Wallberg-Henriksson & Zierath, 2001). Children who were formula fed as opposed to being breast fed showed increased plasma insulin levels and prolonged insulin responses at the age of 6 years (Lucas *et al.*, 1980; Lucas *et al.*, 1981). Several studies have also shown a relationship between formula feeding and a decreased glucose tolerance (Pettitt *et al.*, 1997; Ravelli *et al.*, 2000; Singhal *et al.*, 2003). It is to date not known whether these effects of postnatal diet on later life obesity and glucose tolerance are mediated by programmed changes in adipose tissue insulin signalling proteins such as IR and Glut4.

Equally important for adipose tissue growth are the effects of glucocorticoids. Immediate effects of glucocorticoids include a counteracting of the effects of insulin and an increase in lipolysis (Dallman *et al.*, 2004). Long term effects

of glucocorticoids, however, involve adipose tissue growth. This effect seems to be adipose tissue depot specific as is apparent in patients with Cushing's syndrome who have increased central adiposity (Pasquali & Vicennati, 2000; Despres & Lemieux, 2006). It is not know to date whether formula feeding has an effect on systemic or tissue specific glucocorticoid action.

We hypothesise that formula feeding results in a reduced local sensitivity of adipose tissue to the actions of insulin. This reduction in sensitivity to insulin will switch the balance between insulin and glucocorticoid action on adipose tissue towards a stronger sensitivity of adipose tissue to glucocorticoids. We hypothesise that in the long term this increased glucocorticoid action will result in an increase in adipogenic growth potential. We expect that the reduction in local sensitivity to the action of insulin will be greatest in the omental depot, which is developing rapidly during this early postnatal period of life. We hypothesise therefore that the effects on increased adipose tissue growth will be greatest in the omental adipose tissue depot and lead to a redistribution of adipose tissue towards the omental depot.

In this chapter we test this hypothesis by examining the effects of formula feeding or maternal milk feeding on the development and distribution of adipose tissue in lambs at three months of age. We further examine the expression of markers of the tissue sensitivity to the actions of insulin and glucocorticoids in individual adipose tissue depots.

## 5.3 Materials and methods

### 5.3.1 ANIMALS AND EXPERIMENTAL DESIGN

All procedures were performed in accordance with the UK animals (Scientific Procedures) Act, 1986. 7 twin (all males) bearing Mule ewes were included in this study. They were fed a fixed amount of hay and concentrate pellets sufficient to fully meet their metabolisable energy requirements throughout gestation according to AFRC guidelines (AFRC, 1993). The diet contained adequate vitamins and minerals. Offspring were delivered spontaneously (term 147 ± 3 days).

After birth one of the lambs remained with the ewe and was maternally reared. They were group housed indoors with *ad libitum* access to hay and a fixed amount of concentrate pellets sufficient to fully meet the maternal nutrient requirements plus that needed to maintain lactation according to AFRC guidelines (AFRC, 1993).

Remaining lambs in the formula fed group were group housed indoors and had free access to formula milk which was fed according to the manufacturers' specifications (Volac International Ltd., Volac House, Orell, Royston, UK). Details of the macronutrient composition and availability of the formula milk are given in Table 5.1.

Unfortunately, due to practical issues, it was not possible to measure milk intake in either group.

| | Ewe milk* | Formula milk † |
|---|---|---|
| Protein (%) | ~5 | 4.8 |
| Fat (%) | ~6 | 4.8 |
| Carbohydrates (lactose) (%) | ~48 | 41.5 |
| Energetic value (MJ/kg) | 3-4 | 4.1 |

**Table 5.1; Macronutrient content of ewe and formula milk.**

*, values as quoted in standard text (Freer & Dove, 2002). †, as quoted by company analysis (Volac Ltd). The formula milk is comprised of whey protein powder (as protein source), vegetable oil (as fat source), wheat gluten and lactose (as carbohydrate source) with added vitamin and minerals (Vitamin A, 50,000 iu/kg; Vitamin D3, 6,000 iu/kg; and Vitamin E, 100 iu/kg), DL-methionine and calcium carbonate. Formula milk was fed 4x daily for the first 7 days (maximum intake 1L/day/lamb) and then 2x daily thereafter in a cold *ad libitum* feeding system (calculated at a maximum intake 1L/day/lamb) with concentrate feed available.

The weight of each lamb was recorded at weekly intervals. After approximately eleven weeks, just before weaning, all lambs were humanely killed by electrocortical stunning and exsanguination. All major organs were rapidly excised, weighed, and a representative sample snap-frozen in liquid nitrogen before being stored at -80°C until further molecular analysis was performed.

### 5.3.2 RNA EXTRACTION AND cDNA SYNTHESIS

RNA was isolated from frozen omental, perirenal and subcutaneous adipose tissue samples using Trizol reagent (Invitrogen, Groningen, The Netherlands) and purified using the RNeasy Mini Kit (Qiagen, Basel, Switzerland) as described in detail in chapter 3. RNA was quantified by spectrophotometric measurements at 260 and 280 nm. cDNA was synthesised from 2 µg RNA using Superscript III (Invitrogen, Groningen, The Netherlands) by reverse transcription. Controls containing no RNA transcript or no superscript were used to test for DNA contamination.

### 5.3.3 QUANTITATIVE REAL-TIME RT-PCR

Normalised mRNA expression for glucocorticoid receptor (GR), 11β-hydroxysteroid dehydrogenase type 1 (11β-HSD1), insulin like growth factor 1 (IGF1), insulin like growth factor 1 receptor (IGF1R), insulin receptor (IR), and glucose transporter 4 (Glut4) was determined in omental, perirenal and

subcutaneous adipose tissue by reverse transcription real time PCT using the SYBR Green system in an ABI Prism 7000 Sequence Detection System (Applied Biosystems, Foster City, California). Each qRT-PCR well contained 5 µl SYBR Green Master Mix (Applied Biosystems), 1 µl each of forward and reverser primer (GeneWorks, Adelaide, South Australia, Australia) for the appropriate gene (Table 5.2), 2 µl water and 50 ng/µl cDNA (1µl) to give a total volume of 10 µl. Controls for each primer set containing no cDNA were included on each plate. Three replicates of cDNA for each adipose tissue sample were performed to determine intra-plate variation. Two quality control samples were run on each plate to determine inter-plate variation. Amplification efficiencies were determined from the slope of a plot of Ct (defined as the threshold cycle with the lowest significant increase in fluorescence) against the log of the cDNA template concentration (ranging from 1-100 ng/µl). The abundance of each transcript relative to the abundance of the reference gene ribosomal protein P0 (RpP0) was calculated using Q-Gene analysis software (Muller *et al.*, 2002).

The expression of 11β-hydroxysteroid dehydrogenase type 2 (11β-HSD2) was examined as described above. The expression was however too low to be able to obtain reproducible results.

Due to time limitations the expression of IGF2 and IGF2R was not measured in these samples.

| Primer | Sequence | Accession number |
|---|---|---|
| GR | **F:** 5'-ACT GCC CCA AGT GAA AAC AGA-3'<br>**R:** 5'-ATG AAC AGA AAT GGC AGA CAT TTT ATT-3' | NM_001114186 |
| 11β-HSD1 | **F:** 5'-GCG CCA GAT CCC TGT CTG AT-3'<br>**R:** 5'-AGC GGG ATA CCA CCT TCT TT-3' | NM_001009395 |
| 11β-HSD2 | **F:** 5'-AGC AGG AGA CAT GCC GTT TC-3'<br>**R;** 5'-GCA ATG CCA AGG CTG CTT-3' | NM_001009460 |
| IR | **F:** 5'-CAT CCC CAG AAA ATC ATC TTC AG-3'<br>**R:** 5'-CAA GGG CTC TGC GTT TCC T-3' | Y16092 |
| Glut4 | **F:** 5'-GTG GCC ATC TTT GGC TTC GTG-3'<br>**R:** 5'-CGG CTG AGA TCT GGT CAA AC-3' | AY949177 |
| IGF1 | **F:** 5'-TTG GTG GAT GCT CTC CAG TTC-3'<br>**R:** 5'-AGC AGC ACT CAT CCA CGA TTC-3' | NM_001009774 |
| IGF1R | **F:** 5'-AAG AAC CAT GCC TGC AGA AGG-3'<br>**R:** 5'-GGA TTC TCA GGT TCT GGC CAT T-3' | AY162434 |
| RpP0 | **F:** 5'-CAA CCC TGA AGT GCT TGA CAT-3'<br>**R:** 5'-AGG CAG ATG GAT CAG CCA -3' | NG_009485 |

**Table 5.2; Primer sequences for PCR analysis of GR, 11β-HSD1, 11β-HSD2, IR, Glut4, IGF1, IGF1R and RpP0.**

F: forward primer sequence, R: reverse primer sequence.

### 5.3.4 STATISTICAL ANALYSIS

Data are presented as Mean ± SEM. The effects of formula feeding on gene expression within each tissue were determined using a paired T-test using SPSS for Windows (SPSS Inc, Chicago, Illinois). The differences between adipose tissue depots were determined using a multi-factorial ANOVA with repeated measures using STATA 10.0 (StataCorp, College Station, Texas, USA). In order to take into account that siblings across treatment groups had similar genetic backgrounds, each pair was treated as a repeated outcome

measure within the mother. Similarly *in vivo* data of lamb growth were analysed using a multi factorial ANOVA with repeated measures for lamb siblings within the maternal ewe and different time-points of growth within each lamb. Extensive consideration has been given to the choice of using analyses for repeated or independent measures. Data have been analysed using both methods obtaining similar results. It was felt that data from different depots within each lamb and data from siblings could not be considered as indepent and therefore analyses using repeated measures were chosen to be presented here. Different adipose tissue depots were treated as repeated outcomes within each lamb. Data from siblings were treated as repeated outcomes within each ewe. A probability level of 5% ($p<0.05$) was considered to be significant.

No significant correlations were found between adipose tissue mass and gene expression.

## 5.4 Results

### 5.4.1 LAMB BIRTH WEIGHT, GROWTH AND DISSECTION DATA

There was no difference in birth weight of the lambs in either nutritional group (Table 5.3). Lambs in the formula fed group were overall smaller compared to ewe reared lambs ($P< 0.001$) but there was no interaction between dietary treatment and time (Figure 5.1).

At post mortem there was no difference in lamb body weight, total visceral fat mass, omental, perirenal and pericardial adipose tissue mass between treatment groups (Table 5.3). The distribution of visceral adipose tissue was not significantly different between formula fed and ewe reared lambs (Figure 5.2). Absolute pancreas weight was lower in the formula fed animals compared to the ewe fed animals ($P=0.05$). Relative heart weight was higher in the formula fed compared to the ewe fed animals ($P=0.01$). No other differences in organ weight were found between the two treatment groups (Table 5.3).

| | **Ewe-fed Lambs (n=7)** | **Formula-fed lambs (n=7)** | **P value** |
|---|---|---|---|
| Birth weight (kg) | 5.1 ± 0.3 | 4.8 ± 0.2 | NS |
| Weight at post mortem (kg) | 39.2 ± 2.5 | 36.4 ± 1.8 | NS |
| **Adipose tissue (g)** | | | |
| Omental | 340 ± 77 | 347 ± 47 | NS |
| Perirenal | 373 ± 98 | 363 ± 51 | NS |
| Pericardial | 49 ± 6 | 54 ± 5 | NS |
| Total visceral | 762 ± 174 | 764 ± 97 | NS |
| Relative adiposity ($g.kg^{-1}$) | 18.5 ± 3.6 | 20.6 ± 2.0 | NS |
| **Organ weights (g)** | | | |
| Liver | 659 ± 63 | 612 ± 42 | NS |
| Lung | 529 ± 62 | 517 ± 39 | NS |
| Heart | 183 ± 12 | 191 ± 9 | NS |
| Kidney | 141 ± 14 | 126 ± 5 | NS |
| Spleen | 78 ± 7 | 72 ± 6 | NS |
| Pancreas | 47 ±5 | 34 ± 4 | 0.05 |
| Adrenal | 2.6 ± 0.3 | 2.4 ± 0.2 | NS |
| **Relative organ weights ($g.kg^{-1}$)** | | | |
| Liver | 16.7 ± 0.8 | 16.8 ± 0.5 | NS |
| Lung | 13.3 ± 0.9 | 14.5 ± 1.6 | NS |
| Heart | 4.7 ± 0.1 | 5.3 ± 0.2 | 0.01 |
| Kidney | 3.6 ± 0.2 | 3.5 ± 0.1 | NS |
| Spleen | 2.0 ± 0.1 | 2.0 ± 0.1 | NS |
| Pancreas | 1.2 ± 0.1 | 0.9 ± 0.1 | NS |
| Adrenal | 0.07 ± 0.0 | 0.07 ± 0.0 | NS |

**Table 5.3; Weight of lambs at birth and post mortem and organ weight at post mortem.**

Data are presented as means ± standard errors of the mean. NS: no significant difference

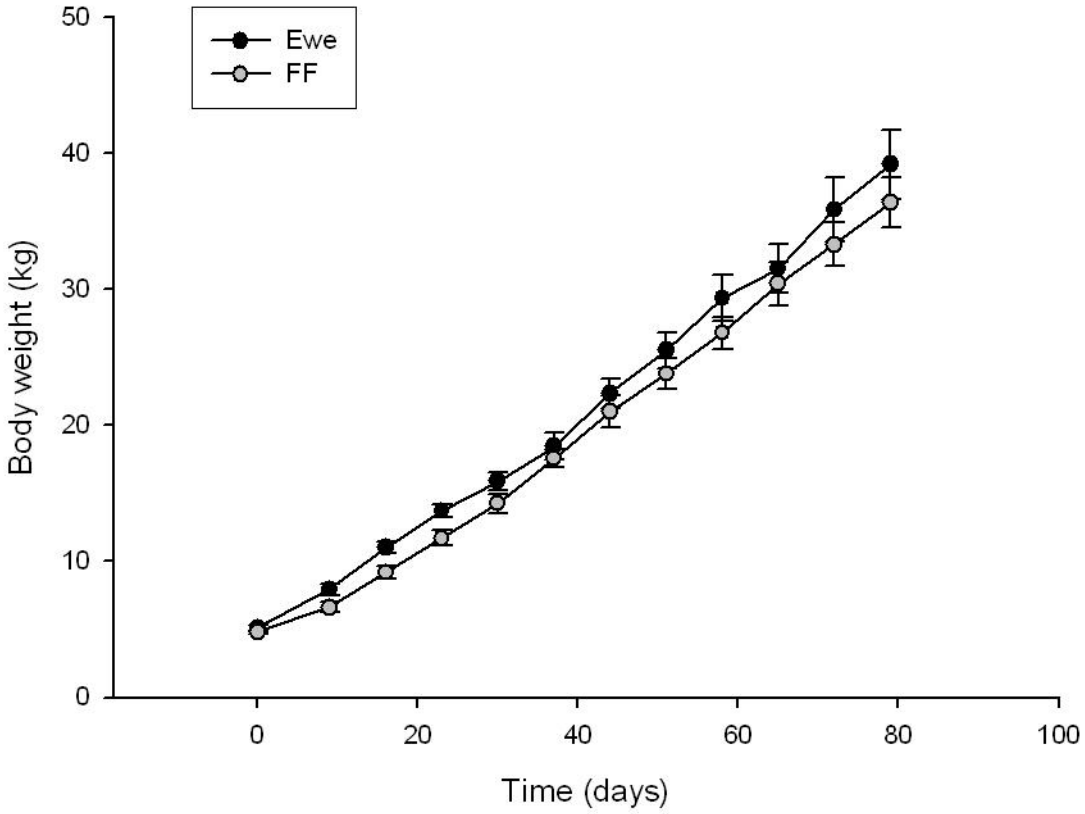


**Figure 5.1; Growth from birth to post mortem in ewe reared and formula fed lambs.** Lambs in the formula fed group were smaller compared to ewe reared lambs (P=0.001), there was no interaction between dietary treatment and time. Ewe means ewe reared lambs (black circles) n=7, FF means formula fed lambs (light circles) n=7.

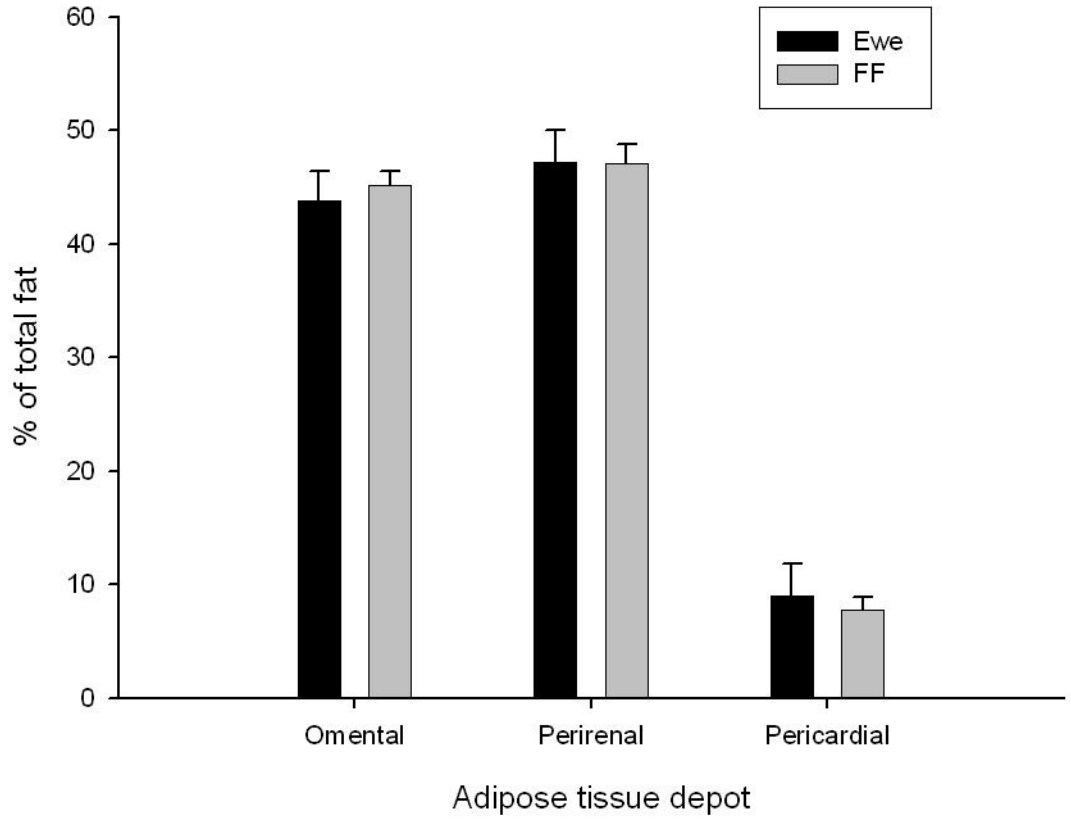


**Figure 5.2; Visceral adipose tissue distribution in formula fed and ewe reared lambs.** Omental, perirenal and pericardial adipose tissue mass expressed as a percentage of total visceral adipose tissue mass. Ewe means ewe reared lambs (black bars) n=7, FF means formula fed lambs (light bars) n=7.

### 5.4.2 ADIPOSE TISSUE EXPRESSION PROFILE

#### *5.4.2.1 Insulin like growth factor 1 and its receptor*

IGF1 expression was not altered by postnatal diet but was significantly higher in perirenal compared to omental and subcutaneous adipose tissue (perirenal vs. omental: P=0.004, perirenal vs. subcutaneous P=0.004) (Figure 5.3). IGF1R expression was down-regulated in the omental depot with formula feeding (P=0.016) (Figure 5.3).

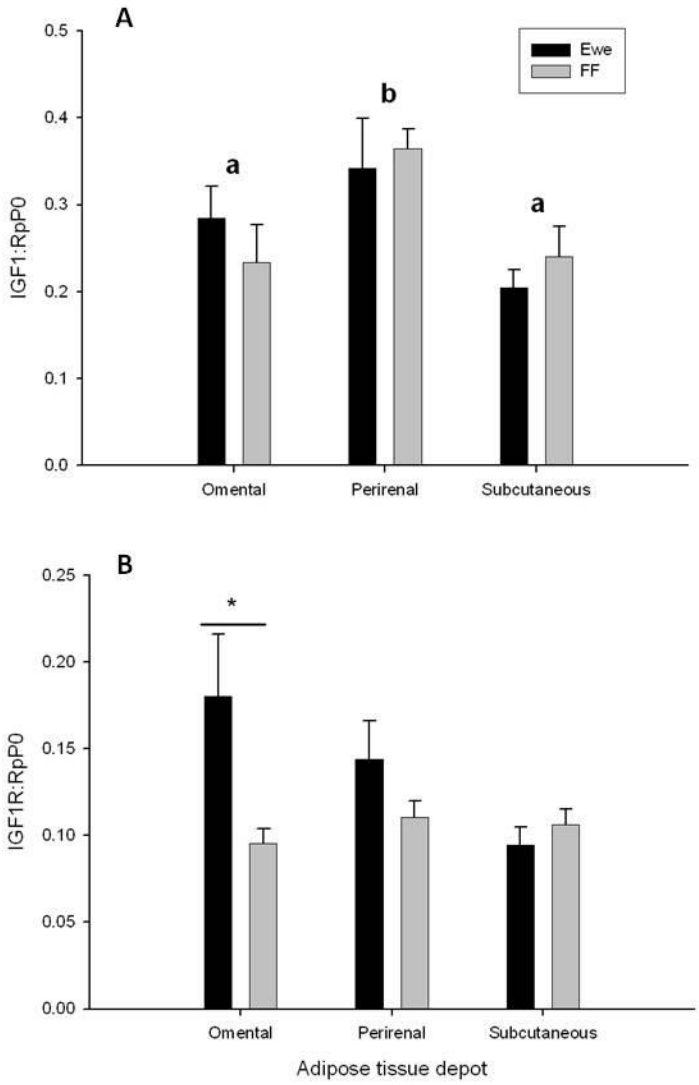


**Figure 5.3; Expression of insulin like growth factor 1 (A) and its receptor (B) relative to housekeeper ribosomal protein P0.**

Ewe means ewe reared lambs (black bars), FF means formula fed lambs (light bars). * indicates differences between ewe and formula fed lambs, different letters indicate differences between adipose tissue depots, $P<0.05$, $n=7$ in both groups.

#### *5.4.2.2 Expression of genes involved in insulin signalling*

Insulin receptor expression levels were not influenced by either diet or adipose tissue depot (Figure 5.4). Overall expression levels of Glut4 were decreased in the formula fed lambs (P=0.005) while being similar across adipose tissue depots (Figure 5.4).

#### *5.4.2.3 Expression of genes involved in glucocorticoid sensitivity*

There was no effect of formula-feeding on the expression of GR, but GR expression was significantly lower in omental *vs.* perirenal and/or subcutaneous adipose tissue (P<0.001 for effect of depot) (Figure 5.5). 11β-HSD1 expression was markedly upregulated by formula feeding relative to ewe reared animals in all investigated depots (P<0.001) with no effect of adipose region on this expression (Figure 5.5). 11β-HSD2 expression was too low to be adequately measured in all depots and diet groups.

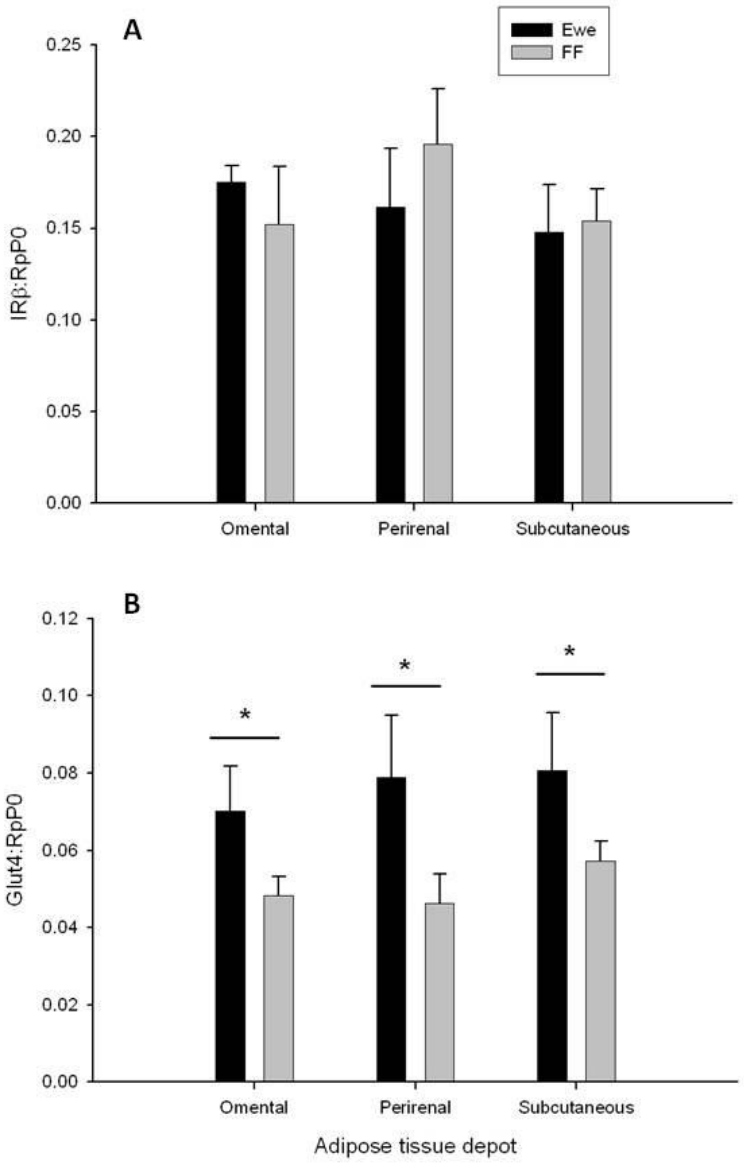


**Figure 5.4; Expression of insulin receptor (A) and glucose transporter 4 (B) relative to housekeeper ribosomal protein P0.**

Ewe means ewe reared lambs (black bars), FF means formula fed lambs (light bars). * indicates differences between ewe and formula fed lambs, different letters indicate differences between adipose tissue depots, $P<0.05$, $n=7$ in both groups.

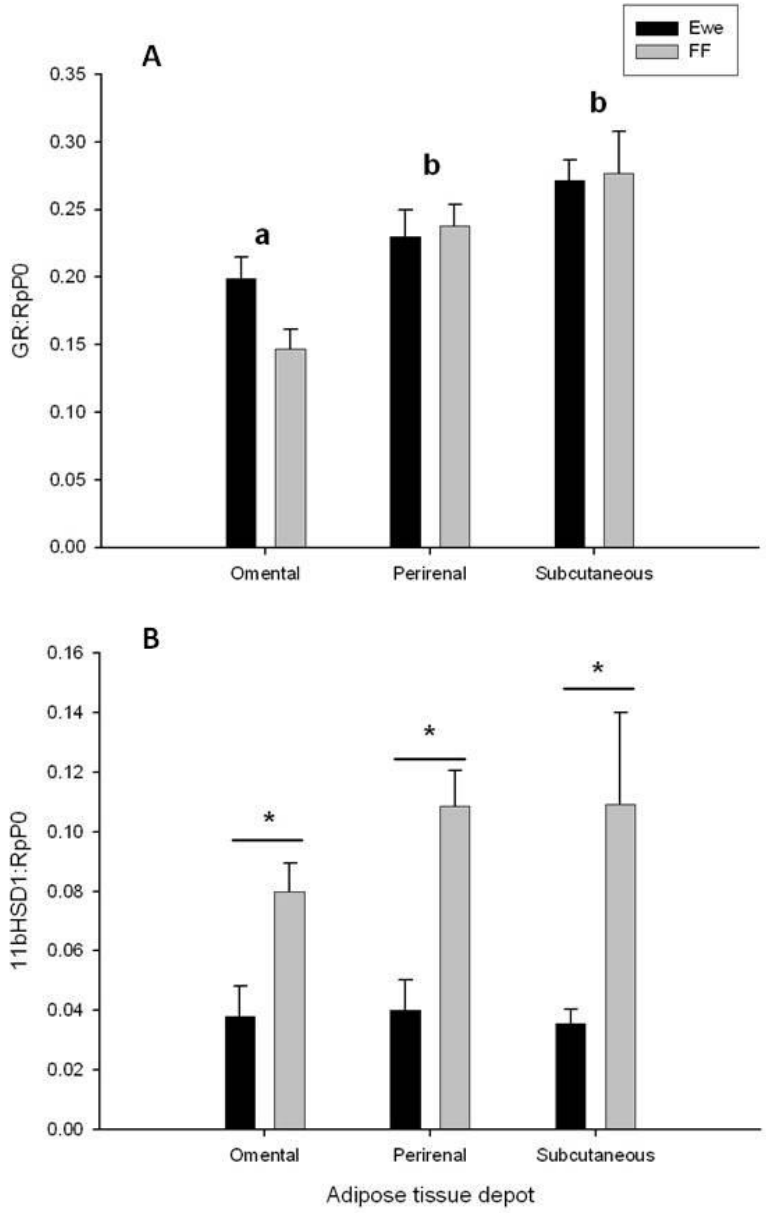


**Figure 5.5; Expression of glucocorticoid receptor (A) and 11β- hydroxysteroid dehydrogenase type 1 (B) relative to housekeeper ribosomal protein P0.**

Expression of 11β-HSD2 was too low to be adequately measured. Ewe means ewe reared lambs (black bars), FF means formula fed lambs (light bars). * indicates differences between ewe and formula fed lambs, different letters indicate differences between adipose tissue depots, $P<0.05$. FF (light bars): formula fed lambs, n=7 in both groups

## 5.5 Discussion

Formula feeding in this study did not lead to a redistribution of adipose tissue at the age of 3 months. Furthermore no increased growth, as seen in formula fed infants (Dewey *et al.*, 1993; Dewey *et al.*, 1995; Ong *et al.*, 2002; Stettler *et al.*, 2005), was found in the formula fed lambs. The mechanism that is often suggested as underlying the accelerated growth in formula fed infants, involves a higher protein and fat content of human formula as opposed to human breast milk, which leads to a 60-70% higher protein intake in formula fed infants (Heinig *et al.*, 1993). In sheep however, maternal milk has slightly higher protein and fat contents when compared to human breast milk (Freer & Dove, 2002), making it therefore more comparable to formula milk and possibly thereby abolishing these effects on growth rate in sheep.

Other theories regarding mechanisms that explain the effects of formula feeding however do not involve macro-nutrient content of the milk, but are dependent on hormonal factors and growth factors present in maternal milk. Despite ewe milk being more similar to formula milk in macro-nutrient content, micro-nutrients and factors such as hormones like leptin, growth factors and cytokines will still be different between ewe and formula milk. Differences in those factors could possibly explain some of the effects seen in this study.

Formula feeding did lead to a significant reduction in Glut4 expression in all depots. We could speculate that a reduction in Glut4 expression combined with an absence of an effect on insulin receptor expression could indicate a reduced efficiency of the insulin signalling cascade. This would mean that the tissue is more resistant to the local actions of insulin as a result of formula feeding. These observations would support our hypothesis that formula feeding results in reduced tissue sensitivity to the actions of insulin in adipose tissue. A reduced expression of Glut4 combined with similar expressions of IR could potentially indicate a malfunctioning insulin signalling cascade in formula fed animals, ultimately leading to a decreased translocation of Glut4 to the cell membrane when stimulated with similar levels of insulin. A decreased translocation of Glut4 leads to a decreased glucose uptake by the cell and increased plasma glucose levels, ultimately leading to an increased insulin secretion by the pancreas (Smith, 2002). The observations of increased insulin resistance and an increased prevalence of type 2 diabetes mellitus in formula fed individuals (Pettitt *et al.*, 1997; Ravelli *et al.*, 2000; Singhal *et al.*, 2003) could potentially be explained by a malfunctioning insulin signalling pathway, but in order to make any definite conclusions on this point more research into the individual intracellular effectors of the insulin signalling cascade in both dietary groups is necessary.

Unfortunately no plasma was available from these animals in order to relate any of our findings with regards to markers of local sensitivity to the actions

of insulin with plasma metabolite levels. Furthermore, due to time restrictions, we have not been able to investigate protein abundance of key markers in the insulin signalling cascade and have therefore not been able to confirm that altered RNA levels result in altered protein abundance in these animals. Furthermore our study was limited to male animals only and we have therefore not been able to examine whether the effect of formula feeding on adipose tissue is gender specific. Future studies will hopefully provide more elucidation on the exact insulin sensitivity of the adipose tissue in formula fed and maternally fed animals.

Furthermore formula feeding resulted in a marked increase in 11β-HSD1 expression in all adipose tissue depots, indicating that formula feeding does indeed result, at the local level, in an increased sensitivity of adipose tissue to the actions of glucocorticoids. The increased expression of 11β-HSD1 could potentially in the long term have an adipogenic effect and be a mechanism that explains the increased growth of adipose tissue in formula fed infants. Positive correlations between 11β-HSD1 expression and adipose tissue mass were found by Gnanalingham and colleagues (Gnanalingham *et al.*, 2005) indicating that 11β-HSD1 could well be an important factor in adipose tissue deposition. However this effect appears to be present across all depots and cannot be responsible for a redistribution of adipose tissue growth. Some studies have indicated an inhibitory effect of insulin on the expression of 11β-HSD1 (Hammami & Siiteri, 1991; Jamieson *et al.*, 1995; Liu *et al.*, 1996). It is possible that the increase in 11β-HSD1 in our formula

fed animals is secondary to a reduction of the inhibitory effects of insulin in adipose tissue in these animals which would further support our main hypothesis that adipose tissue growth is regulated by a balance between the counteracting effects of insulin and glucocorticoids, however this remains a mere speculation at this stage and future studies will need to further investigate this possible interaction.

GR expression was higher in perirenal and subcutaneous adipose tissue which would be in line with our hypothesis that these depots are relatively more sensitive to the actions of glucocorticoids compared to the omental depot during this period of life. In this study however the relative increased expression of GR in perirenal and subcutaneous adipose tissue did not result in a redistribution of adipose tissue growth and therefore this does not provide any evidence to support our hypothesis that the redistribution is secondary to changes in sensitivity of the tissue to the actions of glucocorticoids.

It was interesting to find that the depot specific expression of GR in this study was different compared to the depot specific expression of GR found in Chapter 3. In chapter 3 GR expression was higher in perirenal and omental adipose tissue compared to subcutaneous adipose tissue, while in this chapter the expression was higher in perirenal and subcutaneous adipose tissue compared to omental adipose tissue. We can only speculate regarding mechanisms that might be responsible for these altered expression patterns.

Possible factors that could be involved in those mechanisms include animal breed, housing and ambient temperature.

Insulin like growth factor 1 is often suggested as a potential candidate for the driver of increased growth in formula fed infants. The proposed mechanism of higher IGF1 activity is however, dependent on amino acid availability (Wheelhouse *et al.*, 1999). Given the fact that the formula used in this study had comparable protein contents to sheep maternal milk, it is unlikely that the amino acid availability was different between ewe reared and formula fed animals and it is therefore not surprising that IGF1 expression levels in adipose tissue were not different between dietary treatment groups. IGF1 expression was however, different between adipose tissue depots. An increase in IGF1 in the perirenal adipose tissue compared to omental and subcutaneous adipose tissue further supports our hypothesis that there is a relationship between the actions of glucocorticoids and IGF1 in these tissues.

# 6. The effect of postnatal nutrition and juvenile-onset obesity on regional adiposity in sheep: depot-specific adipose tissue sensitivity to glucocorticoid and insulin signalling.

## 6.1 SUMMARY

Obesity is related to relative insulin resistance. We hypothesised that the decreased response to the actions of insulin in adipose tissue will result in a reduction in the insulin mediated inhibition of 11β-HSD1 expression resulting in enhanced local production of cortisol and an increase in adipogenesis and adipose tissue growth. We further hypothesised that the subcutaneous and perirenal depots are relatively less insulin resistant compared to the omental depot. In these depots insulin remains the main driver of growth. Finally we hypothesised that if the effects of obesity are combined with the effects of formula feeding an earlier shift towards glucocorticoid driven control of adipose tissue will occur in the omental depot. This results in an exacerbation of the effects of an obesogenic lifestyle in people that were formula fed in early life.

In this chapter we tested this hypothesis by examining the effects of an obesogenic lifestyle in animals that, as infants, were fed either by their mothers or were formula fed throughout the lactation period on their adipose

tissue distribution and glucocorticoid and insulin sensitivity later in life. The latter were assessed through transcript expression profiles in regional adipose tissue for key regulatory elements of glucocorticoid and insulin signalling.

Juvenile-onset obesity *per se* did not result in a redistribution of visceral adipose tissue in this study. Interestingly, a combination of obesity and formula feeding produced adipose redistribution in favour of the perirenal depot. However, no differences in the expression of markers of tissue sensitivity to the actions of glucocorticoids or insulin were found that could potentially underlieunderlie this redistribution in adipose tissue.

Markers of the tissue sensitivity to glucocorticoids and to insulin were significantly reduced in response to obesity in both adipose tissue depots. No differences were found in markers of tissue sensitivity to glucocorticoids and to insulin between obese ewe fed and obese formula fed animals. This opposes our initial hypothesis that differences in the regional distribution of adipose tissue are secondary to regional differences in the balance between tissue sensitivity to the actions of insulin and glucocorticoids. It further opposes our hypothesis that high sensitivity to the actions of glucocorticoids or insulin in the long term results in adipogenesis.

A reduction in markers of tissue sensitivity to the actions of insulin was found in obese animals. This could reflect an increased resistance of the tissues to the actions of insulin, but the mechanism behind this tissue specific resistance to insulin action appears different in omental and perirenal

adipose tissue, given differential abundance patterns of Glut4 and IR in those depots.

Finally, we tested the obese offspring's HPA axis response to a combined CRH and AVP bolus but found the response was unaffected by juvenile-onset obesity, although, as expected the ACTH response was greater in females.

## 6.2 Introduction

Obesity is currently one of the biggest health challenges in the developed world (Kelly *et al.*, 2008). The World Health Organisation estimated that approximately 400 million adults were obese in 2005 (WHO, 2006). The simple cause of obesity is an imbalance between energy intake and energy expenditure favouring an excessive intake relative to expenditure, leading to increased energy deposition as lipid in adipose tissue. The metabolic risk factors associated with obesity are dependent on the distribution of adipose tissue. Increased central as opposed to peripheral adipose tissue is associated with higher cardiovascular risks (Krotkiewski *et al.*, 1983; Fujioka *et al.*, 1987; Despres *et al.*, 1989; Pouliot *et al.*, 1992; Wajchenberg, 2000; Busetto, 2001; Miyazaki *et al.*, 2002; Misra *et al.*, 2004; Despres & Lemieux, 2006).

Visceral adipose tissue in comparison to subcutaneous adipose tissue is more sensitive to lipolytic stimulation by, for example, the sympathetic nervous system. On the other hand, it is less sensitive to antilipolytic stimuli by, for example insulin (Rebuffe-Scrive et al., 1989; Arner et al., 1990; Marin et al., 1992a; Vikman et al., 1996). Since obesity is associated with a general reduction in insulin sensitivity, it is likely that these effects are most pronounced in omental adipose tissue.

Among the many influences that affect the occurrence of obesity, being formula fed as an infant, as opposed to being breast fed, has been shown to increase an individual's susceptibility to greater weight gain and obesity (Dewey *et al.*, 1993; Harder *et al.*, 2005) although more recent reports have questioned the strength of the effect (Michels *et al.*, 2007; Stettler, 2007). A number of studies have attempted to explain the increased risk of adult obesity after formula feeding. For example, the total energy and protein intake of formula fed infants is generally higher when compared to breast fed infants (Heinig *et al.*, 1993; Whitehead, 1995) potentially explaining the higher plasma insulin concentrations in formula fed infants (Lucas *et al.*, 1980).

In chapter five we hypothesised that formula feeding results in a decreased efficiency of the insulin signalling cascade and therefore a reduction in the tissue sensitivity to the actions of insulin. We further hypothesised that this reduced local insulin action is accompanied by an increase in local glucocorticoid action in adipose tissue which ultimately leads to an increase in adipose tissue growth. Our results from chapter five supported this hypothesis although an increase in adipose tissue growth was not present at the age of 3 months. We further hypothesised that the changes in tissue sensitivity to the actions of insulin and glucocorticoids would be greatest in the omental depot which develops entirely during the period of formula feeding (i.e. during lactation). However our data did not show an increase in omental adipose tissue at the age of three months. In this chapter we investigate the effects of exclusive formula feeding on adipose tissue in the

longer term (i.e. in 1 year old young adults) in juvenile-onset obesity.

We hypothesised that with obesity the decreased response of omental adipose tissue to insulin action will result in a reduction of insulin-mediated inhibition of 11β-HSD1 expression (Hammami & Siiteri, 1991; Jamieson *et al.*, 1995; Liu *et al.*, 1996) resulting in enhanced local production of cortisol; ultimately increasing adipogenesis and adipose tissue growth. We hypothesised that, in obesity, the subcutaneous and perirenal depots are relatively less insulin resistant compared to the omental depot. In these depots insulin remains the main driver of growth. We further hypothesised that when the effects of juvenile-onset obesity are combined with the effects of formula feeding an earlier shift towards glucocorticoid driven control of adipose tissue will occur in the omental depot. This results in an exacerbation of the effects of an obesogenic lifestyle in people that were formula fed in early life.

In this chapter we test this hypothesis by examining the effects of an obesogenic lifestyle in animals that were fed maternal milk and animals that were formula fed on regional adipose tissue distribution. We further examined the expression and abundance of markers of tissue sensitivity to the actions of insulin and glucocorticoids in individual adipose tissue depots. Finally we examined the plasma metabolite response to a combined bolus of corticotrophin and arginine vasopressin to further elucidate the effect of formula feeding on the HPA axis.

## 6.3 Materials and methods

### 6.3.1 EXPERIMENTAL PROTOCOLS

#### *6.3.1.1 Effects of Formula Feeding vs. Ewe Rearing on Adipose Tissue Development*

All procedures were performed in accordance with the UK animals (Scientific Procedures) Act, 1986. 22 pregnant Cross bred Mule sheep were included in this study. Throughout gestation all sheep were fed to requirement (i.e. 100% metabolizable energy requirements (7-13 MJ/day) according to AFRC guidelines (AFRC, 1993) allowing for increased metabolic demand with gestation and foetal number. All offspring were delivered spontaneously as twins and were either reared by their mothers (n = 14) or formula fed (n = 8) until weaning (at ~10 weeks). One twin lamb from each ewe was euthanased at 7 days post-partum for a separate study. Remaining lambs in the formula fed group had free access to formula milk which was fed according to the manufacturers' specifications (Volac International Ltd., Volac House, Orell, Royston, UK). Details of the composition and availability of the formula milk are given in Table 6.1.

| | Ewe milk* | Formula milk † |
|---|---|---|
| Protein (%) | ~5 | 4.8 |
| Fat (%) | ~6 | 4.8 |
| Carbohydrates (lactose) (%) | ~48 | 41.5 |
| Energetic value (MJ/kg) | 3-4 | 4.1 |

**Table 6.1; Macronutrient content of ewe and formula milk.**

Values as quoted in standard text (Freer & Dove, 2002). †, as quoted by company analysis (Volac Ltd). The formula milk is comprised of whey protein powder (as protein source), vegetable oil (as fat source), wheat gluten and lactose (as carbohydrate source) with added vitamin and minerals (Vitamin A, 50,000 iu/kg; Vitamin D3, 6,000 iu/kg; and Vitamin E, 100 iu/kg), DL-methionine and calcium carbonate. Formula milk was fed 4x daily for the first 7 days (maximum intake 1L/day/lamb) and then 2x daily thereafter in a cold *ad libitum* feeding system (calculated at a maximum intake 1L/day/lamb) with concentrate feed available.

#### *6.3.1.2 Interaction between Formula Feeding and an Obesogenic Postnatal Environment*

After birth, all mothers were fed a diet of hay *ad libitum* together with a fixed amount of concentrate pellets sufficient to fully meet their own metabolizable requirements, plus that needed to maintain lactation. All diets contained adequate minerals and vitamins. From weaning to 12 months of age, offspring were either group-housed in a barn (restricted activity), at a stocking rate of 17 animals per $50m^2$ with *ad libitum* access to hay and concentrate pellets to promote increased fat deposition (obese formula fed; OFF [n=8; 4 male, 4 female] and obese ewe-reared sheep; O [n=7; 5 male, 2 female]) or pasture grazed at a stocking rate of 17 animals per $3000m^2$ (unrestricted activity lean sheep ; L, n=7; 0 male, 7 female) with *ad libitum* access to grass and concentrate pellets. An overview of the animal methods used in this study can be found in Figure 6.1.

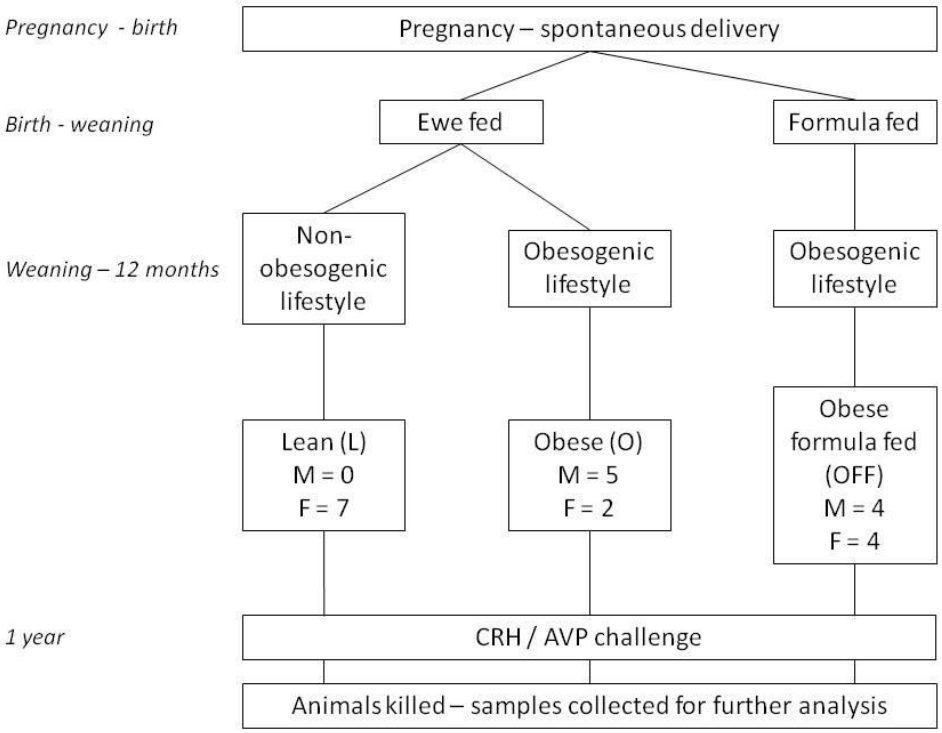


**Figure 6.1; Overview of animal methods used to investigate the effect of obesity and the combination of obesity and formula feeding on adipose tissue distribution in adulthood**

M: male, F: female. CRH: corticotrophin, AVP: arginine vasopressin

### 6.3.2 ASSESSMENT OF THE WHOLE BODY ADIPOSE SENSITIVITY TO EXCESS GLUCOCORTICOID

At 1 year of age (young, sexually mature adults) all sheep were instrumented with a venous (jugular) catheter and, after withdrawal of all food, but not water, for one day, were subjected to an I.V. combined bolus of corticotrophin (CRH, 0.5 $\mu g.kg^{-1}$) and arginine vasopressin (AVP; 0.1 µg/kg). Baseline samples were taken at -30 and -15 min prior the bolus at time zero. Thereafter regular blood samples were withdrawn at +5, 15, 30, 45, 60, 90, 120, 150 & 180 minutes. All blood samples were collected into chilled heparinised tubes, centrifuged (800*g*) for 5 min at 4 ºC and, after decanting of the supernatant plasma, were stored at -20 ºC for further analysis of plasma ACTH, cortisol, non-esterified fatty acids (NEFA), triglyceride (TG) and glucose concentrations. Plasma metabolites were analysed by auto-analyser (RX-Imola, Randox, Co Antrim, NI). Thereafter the sheep were humanely killed by electrocortical stunning and exsanguination and all major organs and adipose depots weighed and a representative sample snap-frozen in liquid nitrogen before being stored at -80°C until further molecular analysis was performed.

### 6.3.3 ESTIMATION OF TOTAL PLASMA ACTH AND CORTISOL

Radioimmunoassay kits were used for both ACTH (Diasorin, Slough, UK) and cortisol (Coat-a-Count, DPC, USA), validated for ovine plasma. The assays were conducted as per the manufacturer's instructions and as previously described (Gardner *et al.*, 2006). Plasma concentrations for both cortisol and ACTH were calculated from interpolation of a semi-log standard curve with $B/B_0$ (%) on the y-axis and either ACTH or cortisol on the x-axis. Inter and intra-assay coefficients of variation in our hands are between 5.0-8.0% and 10.0-12.0%, respectively for cortisol and ACTH (Gardner *et al.*, 2006).

### 6.3.4 SAMPLE PREPARATION FOR REGIONAL ADIPOSE INSULIN SENSITIVITY ANALYSIS

Protein was extracted from perirenal and omental adipose tissues using lysis buffer and protein content measured using a bicinchoninic acid kit as previously described (Gardner *et al.*, 2005). Western blot analysis was performed as previously described with the following modifications for sheep: Protein lysates from each animal were standardized to a final concentration of 0.5 mg/ml in Laemmli's sample buffer. Equal amounts of protein for each animal (10µg) were loaded onto 10% SDS polyacrylamide gels for separation by electrophoresis (Gardner *et al.*, 2005). The ovine antibodies used in this study were to insulin receptor β-subunit (IR) (Santa Cruz, Autogen Bioclear, UK), glucose transporter 4 (Glut4) (Abcam, Cambridgeshire, UK), p85 α-

subunit of phophatidylinositol 3-kinase (p85) (Upstate Biotech) and insulin like growth factor 1 receptor β-subunit (IGF1R) (Santa Cruz, Autogen Bioclear, UK). Secondary antibodies and an antibody-binding (enhanced chemiluminescence) kit were both obtained from Amersham, UK.

### 6.3.5 SAMPLE PREPARATION FOR REGIONAL ADIPOSE GR, 11B-HSD1, 11B-HSD2 MRNA EXPRESSION

Quantitative PCR was used to examine the expression of GR, 11β-HSD1, 11β-HSD2 and 18S in omental and perirenal adipose tissue (Table 6.2). PCR reactions were carried out in 20 ml volumes consisting of 1-SYBRs PCR mastermix (with ROX passive reference dye) (Qiagen Ltd, Crawley, UK), 500 nM forward primer, 500 nM reverse primer, and 7 ml nuclease-free water. Real time PCR was performed in a Techne QuanticaTM 14 real-time thermocycler (Techne, Barloworld Scientific Ltd) on all samples at 95°C (15 min) followed by 45 cycles of 95°C (15 s), annealing temperature (25 s), 72°C (25 s). Two negative control reactions were carried out with each set of samples analyzed: (1) no RNA template but RT and polymerase provided and (2) RNA and polymerase provided but no RT.

| Primer | Sequence | Annealing temp (°C) | Accession number |
|---|---|---|---|
| GR | **F:** 5' - ACT GCC CCA AGT GAA AAC AGA – 3'<br>**R:** 5' - ATG AAC AGA AAT GGC AGA CAT T – 3' | 59 | NM_001114186 |
| 11β-HSD1 | **F:** 5' – GCG CCA GAT CCC TGT CTG AT – 3'<br>**R:** 5' – AGC GGG ATA CCA CCT TCT TT – 3' | 58.5 | NM_001009395 |
| 11β-HSD2 | **F:** 5' – AGC AGG AGA CAT GCC GTT TC – 3'<br>**R:** 5' – GCA ATG CCA AGG CTG CTT – 3' | 60 | NM_001009460 |
| 18s | **F:** 5' – GAT GCG GCG GCG TTA TTC C – 3'<br>**R:** 5' – CTC CTG GTG CTG CCC TCC– 3' | 60 | NR_002170.3 |

**Table 6.2; Primer sequences for PCR analysis of GR, 11β-HSD1, 11β-HSD2 and 18s**

F: forward primer sequence, R: reverse primer sequence

### 6.3.6 STANDARD CURVE GENERATION FOR QPCR TRANSCRIPT ANALYSIS

Standards for GR, 11β-HSD1 and 11β-HSD2, and for the housekeeping gene 18s ribosomal RNA were made from cDNA obtained from a randomly selected sample of an L animal using semi-quantitative polymerase chain reaction (PCR). The method used oligonucleotide primers to GR, 11β-HSD1, 11β-HSD2 and 18s genes generating specific intron-spanning products. The PCR program comprised an initial denaturation stage (95°C, 15 min), amplification (stage I, 94°C (30 s); stage II, annealing temperature (30 s); stage III, 72°C (1 min), and final extension (72°C, 7 min; 8°C 'hold'). The PCR mixture (final volume 20 ml) contained 7 ml nuclease-free water (Ambion), 10 ml thermo-start PCR master mix (50 ml contains 1.25U Thermo-Start DNA polymerase, 1U thermo-start reaction buffer, 1.5mM $MgCl_2$ and 0.2mM each of denatured adenotriphosphate, denatured 20-

deoxycytidine 50-triphosphate, denatured 20- deoxyguanosine 50-triphosphate, and denatured 20-deoxythymidine 50-triphosphate (ABgene, Epsom, UK)), 1 ml forward primer, 1 ml reverse primer, and 1 ml RT (cDNA) product. The annealing temperature and cycle number of all primers were optimized and used in their linear range. Agarose gel electrophoresis (2.0–2.5%) and ethidium bromide staining confirmed the presence of both the product and 18s at the expected sizes. All procedures were performed with appropriate negative and positive controls as well as a range of molecular weight markers (MBI Fermentas, York, UK). The resultant PCR product was extracted (QIAquick gel extraction kit, Qiagens, cat no. 28704), sequenced, and results cross-referenced against the Genbank website to determine specificity of the target gene. After confirmation that the product was specific to the target gene, extracted PCR products were resuspended in nuclease-free water and a 10-fold serial dilution performed. Standards were stored at -20°C until use in quantitative PCR.

### 6.3.7 STATISTICAL ANALYSIS

The specific contrasts selected *a priori* for analysis in this study were; 1) an effect of region (e.g. omental or perirenal) on adipose tissue biology within female individuals (i.e. L group alone), 2) an effect of juvenile-onset obesity on similar adipose measurements and HPA axis function (i.e. L *vs.* O) and 3) an effect of being naturally reared or formula-fed on the previously described endpoints (i.e. O *vs.* OFF). Where data were nested within individuals (e.g.

different adipose regions within the same individual) the data were considered to be repeated measurements within each individual and were therefore analyzed by multifactorial ANOVA with the repeated measures of dietary group and adipose depot as the fixed effects and each individual as a random effect using STATA 10.0 (StataCorp, College Station, Texas, USA). Extensive consideration has been given to the choice of using analyses for repeated or independent measures. Data have been analysed using both methods obtaining similar results. It was felt that data from different depots could not be considered as indepent and therefore analyses using repeated measures were chosen to be presented here. Different adipose tissue depots were treated as repeated outcomes within each lamb. *In vivo* hormone challenge data were similarly analyzed by multivariate repeated measures ANOVA with calculated areas-under-the-curve on baseline corrected data using Graphpad Prism 5. Where fixed effects (e.g. adipose region, postnatal obesity, formula-feeding) were significant appropriate *post hoc* tests (Bonferroni) with correction for multiple comparisons were conducted. Gender could only be considered as a fixed effect in the analyses when considering the effect of formula-feeding on postnatal endpoints. Results are presented as estimated marginal means ± standard error of the mean.

## 6.4 Results

### 6.4.1 DISTRIBUTION OF ADIPOSE TISSUE IN LEAN AND OBESE EWE REARED SHEEP AND OBESE FORMULA-FED SHEEP.

The postnatal obesogenic environment, as expected, led to significantly heavier sheep at 1 year of age (L, 57.7±3.2; O, 89.2±2.2; OFF, 98.0±3.6 kg; P<0.001 L vs. O) (Figure 6.2). Obesity *per se* had no effect on the distribution of visceral adipose tissue distribution (Figure 6.3), but in conjunction with neonatal formula feeding a significant shift in visceral adipose tissue distribution was observed. In the OFF group, the perirenal depot, expressed as a percentage of total visceral adipose tissue, was relatively increased where that of the omental depot was decreased (P=0.013 for perirenal and P=0.030 for omental) (Figure 6.3). There was no difference in the proportion of pericardial fat mass between groups and therefore no further analyses were performed in this depot.

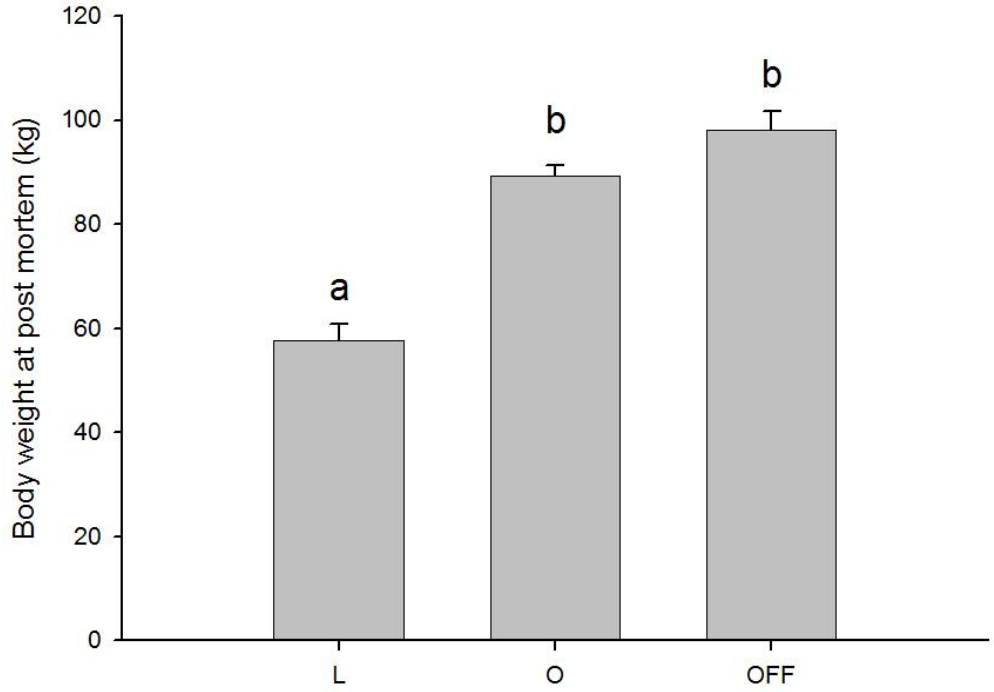


**Figure 6.2; Sheep weight at post mortem at the age of 1 year**

Different letters indicate significant differences between groups. L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8)

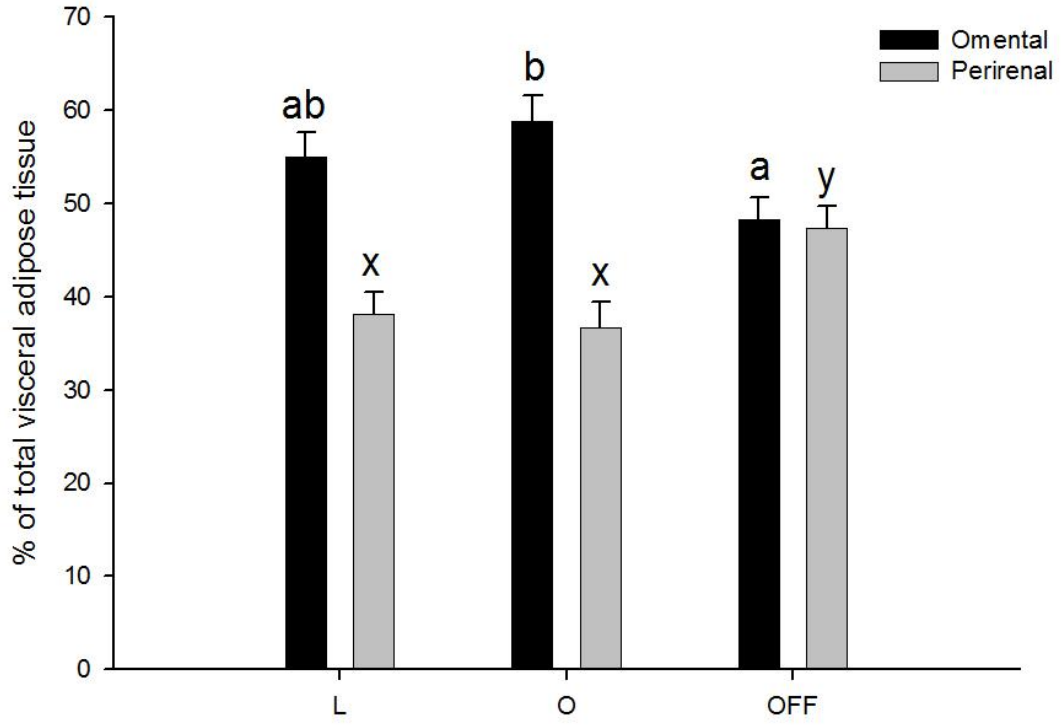


**Figure 6.3; Omental and perirenal adipose tissue weight relative to total visceral adipose tissue in lean sheep, obese ewe reared sheep and obese formula fed sheep.** Statistics are; bars with differing superscripts are significantly different at P<0.05 (*abc* for omental and *xyz* for perirenal adipose tissue). L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8).

### 6.4.2 ACTH AND CORTISOL RESPONSES TO THE COMBINED CRH AND AVP CHALLENGE

#### *6.4.2.1 ACTH*

Baseline ACTH was not different between groups at 12 months of age (L, 52.6±7.8; O, 59.7±8.6; OFF, 64.9±7.9 pg.ml-1). Upon bolus administration of corticotrophin and arginine vasopressin there were significant (P<0.001) similar increments in ACTH in all groups (Figure 6.4). Whilst the response was not modified by dietary group, gender did have a significant effect on the response (P=0.043) with no interactions between dietary group and gender (Figure 6.4). Consequently, the ACTH area-under-the-curve was significantly (P=0.039) influenced by gender (female, 77.86±8.98; male, 46.96±11.90; $pg.ml^{-1}.min^{-1}$). The areas under the curve for ACTH in response to a combined bolus of CRH and AVP can be found in Table 6.3.

#### *6.4.2.2 Cortisol*

Baseline cortisol was not different between groups at 12 months of age (L, 63.9±14.1; O, 81.2±14.1; OFF, 56.8±13.2 nmol.L-1). Upon bolus administration of CRH and AVP there were significant (P<0.001) increments in cortisol in all groups, with no main effects of dietary group, gender or any interaction between the two (Figure 6.5). Similarly, there was no impact of dietary group on the cortisol data when expressed as area-under-the-curve (L, 73.68±14.37; O, 80.34±15.91; OFF, 87.14.± 14.52 nmol.L-1.min-1). The

areas under the curve for cortisol in response to a combined bolus of CRH and AVP can be found in Table 6.3.

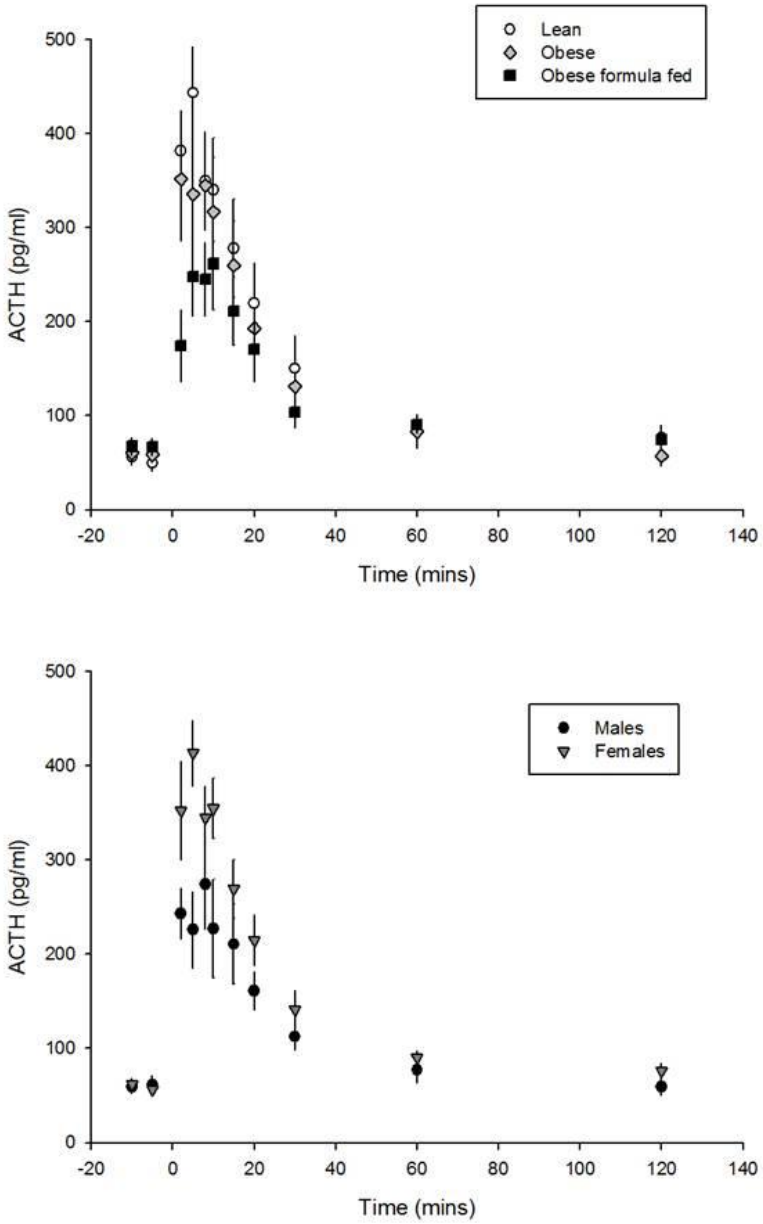


**Figure 6.4; Plasma ACTH responses to a combined I.V. bolus of CRH and AVP**

CRH (0.5 µg/kg) and AVP (0.1 µg/kg) were administered I.V. as a combined bolus and plasma collected for analysis of ACTH by RIA (see Methods). Statistics are P<0.05 female *vs.* male. L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8).

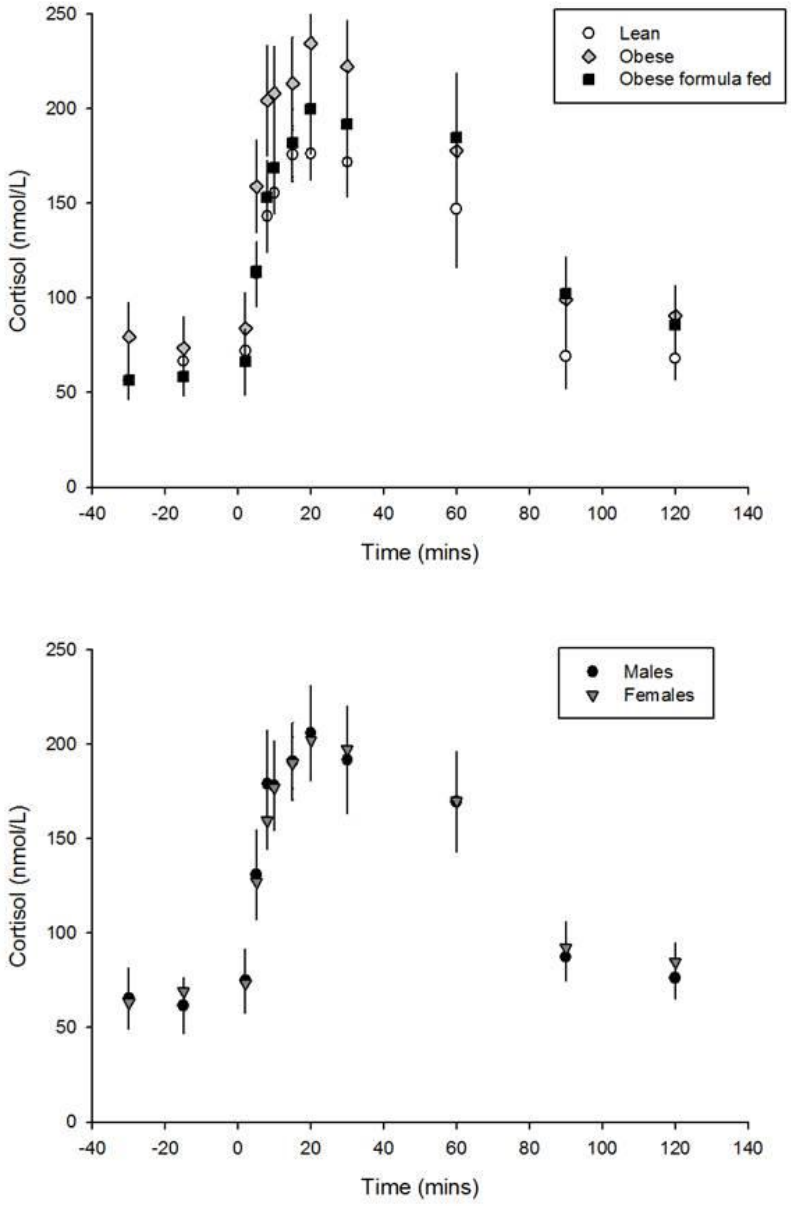


**Figure 6.5; Plasma cortisol responses to a combined I.V. bolus of CRH and AVP**

Data are given as means ± SEM at each timepoint. CRH (0.5 µg/kg) and AVP (0.1 µg/kg) were administered I.V. as a combined bolus and plasma collected for analysis of cortisol by RIA (see Methods). No significant differences were found. L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8).

### 6.4.3 ADIPOSE TISSUE SENSITIVITY TO HIGH CIRCULATING GLUCOCORTICOID

At 12 months of age there were no significant differences in resting plasma glucose, non-esterified fatty acids (NEFA) or triglycerides (TG) between dietary groups, but obese animals (O and OFF) tended (P=0.06, all cases) to have higher glucose, NEFA and TG concentrations (Glucose: L, 4.81±0.47; O, 5.01±0.84; OFF, 5.62±0.60 mmol.$L^{-1}$; NEFA: L, 0.29±0.09; O, 0.61±0.13; OFF, 0.45±0.08 mmol.$L^{-1}$; triglycerides L, 0.11±0.01; O, 0.18±0.02; OFF, 0.17±0.01 mmol.$L^{-1}$).

In response to the CRH and AVP bolus there was a significant, similar increase in plasma glucose concentrations in all groups (P<0.01) with no interaction between the effects of time and dietary group (Figure 6.6). For plasma NEFA, there was a significant decrease with time (P<0.001) but the response with time was different between lean (L) and obese animals (O; P<0.001 for interaction). Plasma NEFA in obese animals returned toward baseline concentrations earlier than in lean sheep (Figure 6.6). Plasma TG significantly increased in all groups following the CRH and AVP bolus and this increase tended to be greater in the obese groups (P=0.055) (Figure 6.6). None of the above changes in metabolites were influenced by gender.

The areas under the curve for plasma metabolites in response to a combined bolus of CRH and AVP can be found in Table 6.3.

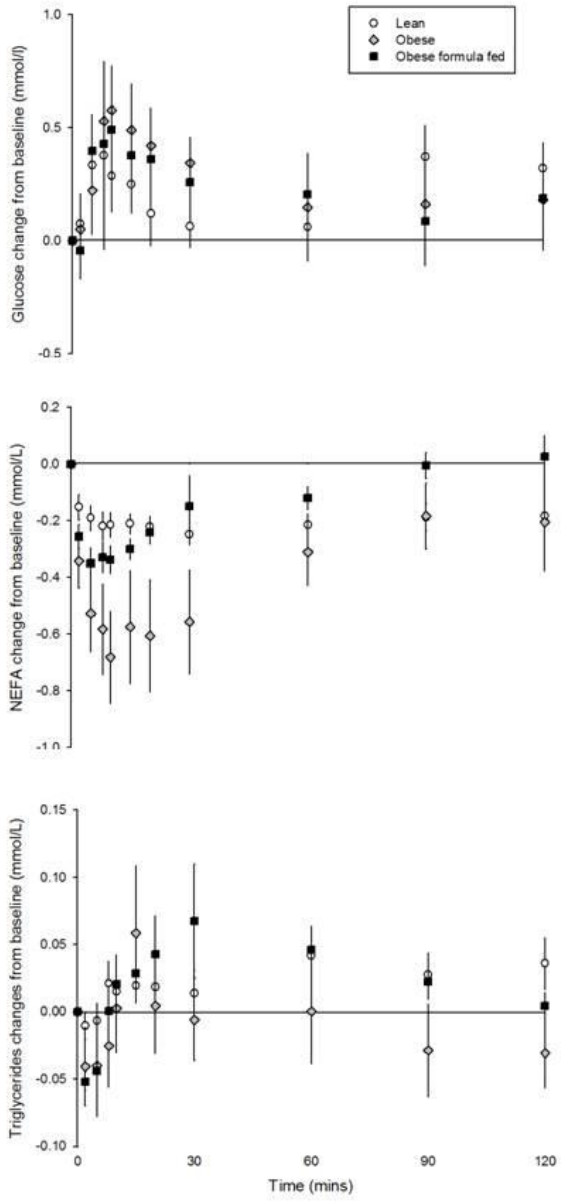


**Figure 6.6; Plasma glucose, non-esterified fatty acid and triglyceride response to a combined bolus of CRH and AVP**

Data are given as means ± SEM at each timepoint. CRH and AVP were administered as a combined bolus as described in the methods section. Plasma glucose, non-esterified fatty acid (NEFA) and triglyceride (TG) were measured on an autoanalyser (RX-Imola, Randox, Co Antrim). Statistics are *, P<0.05 L *vs.* O. For triglyceride the P-value was 0.055. L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8).

| | Lean | | Obese | | Obese formula fed | |
|---|---|---|---|---|---|---|
| | Male (n=0) | Female (n=7) | Male (n=5) | Female (n=2) | Male (n=4) | Female (n=4) |
| **ACTH** | - | 11077 ± 1600 | 12213 ± 2676 | 5748 ± 2153 | 6143 ± 1387 | 4636 ± 2479 |
| **Cortisol** | - | 8403 ± 1337 | 10567 ± 1651 | 9831 ± 1861 | 13514 ± 3126 | 7355 ± 4229 |
| **NEFA** | - | 24.7 ± 4.6 | 20.1 ± 10.0 | 53.2 ± 20.3 | 14.1 ± 5.3 | 20.6 ± 2.6 |
| **Triglyceride** | - | 3.87 ± 1.1 | 5.10 ± 4.18 | 2.73 ± 1.88 | 6.43 ± 2.32 | 1.64 ± 0.12 |
| **Glucose** | - | 29.9 ± 8.2 | 22.9 ± 8.2 | 51.0 ± 16.6 | 27.1 ± 12.5 | 35.8 ± 9.4 |

**Table 6.3; HPA axis and plasma metabolite areas under the curve after a combined bolus of CRH and AVP.**

Data are given as means ± SEM at each timepoint. CRH and AVP were administered as a combined bolus as described in the methods section. Statistics are ACTH: $P<0.05$ male *vs.* female, cortisol: ns, NEFA: $P<0.05$ L *vs.* O, triglyceride: $P=0.055$ for L *vs.* O, glucose: ns.

### 6.4.4 GR, 11B-HSD1 AND 11B-HSD2 EXPRESSION IN ADIPOSE TISSUE

GR mRNA expression was not different between omental and perirenal adipose tissue, but was lower in the omental depot in obese animals only (P=0.006 L vs. O; Figure 6.7). 11β-HSD1 expression was lower in the perirenal and omental depots in obese animals relative to expression in L (P<0.001, both cases; Figure 6.7). 11β-HSD2 mRNA expression was significantly lower in the omental region in obese compared to lean animals (P<0.001, Figure 6.7).

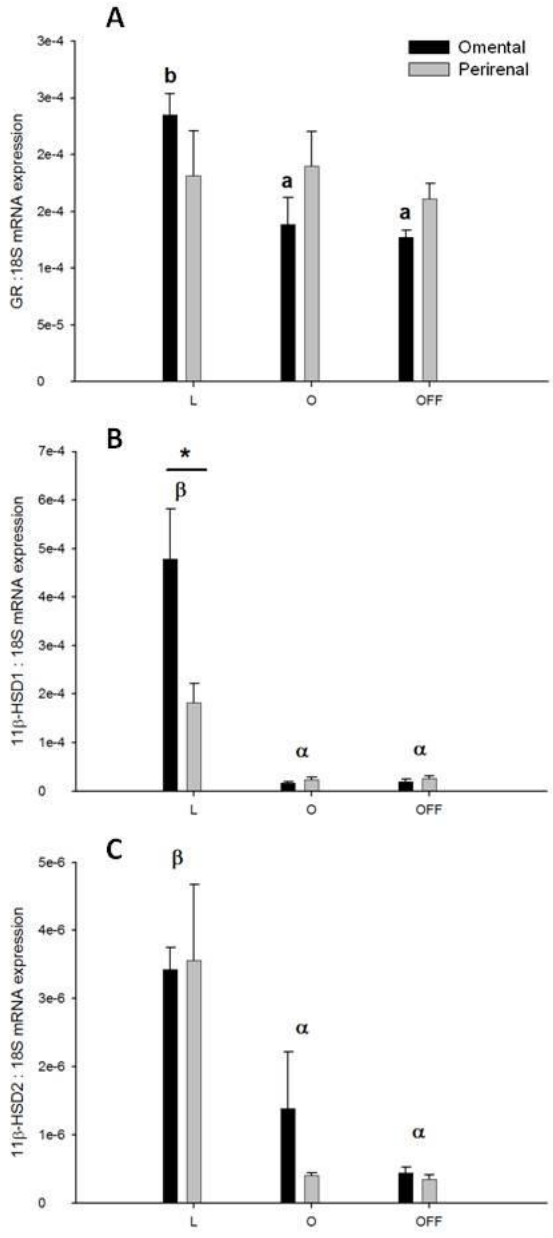


**Figure 6.7; Transcript expression of GR (A), 11β-HSD1 (B) and 11β-HSD2 (C) in omental and perirenal adipose tissue of lean and obese animals.**

Statistics are; * indicate significant difference between adipose tissue depots, bars with differing superscripts are significantly different (*abc* for omental and *xyz* for perirenal adipose tissue or *Greek letters* when no interaction is present and the superscripts relate to both depots are). L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8).

### 6.4.5 INSULIN SIGNALLING PROTEIN ABUNDANCE IN ADIPOSE TISSUE

Abundance of IR was consistently lower in perirenal relative to omental adipose tissue in all dietary groups (P<0.001). In the omental depot, IR abundance was lower in obese relative to lean animals (P<0.001 L vs. O). In the perirenal depot there were no differences in IR abundance between treatment groups (Figure 6.8). The abundance of Glut4 was markedly higher in omental relative to perirenal adipose tissue in all treatment groups (P<0.001). In addition, in omental, but not perirenal adipose tissue, GLUT4 abundance was lower in obese ewe-reared animals (O) relative to L (P=0.023; Figure 6.8). There was no effect of diet or obesity on P85 abundance (data not shown). IGF1R abundance was higher in the omental compared to the perirenal depot (P=0.001). IGF1R abundance in omental and perirenal depots was also lower in obese vs. lean animals (P=0.037, L vs. O, Figure 6.8).

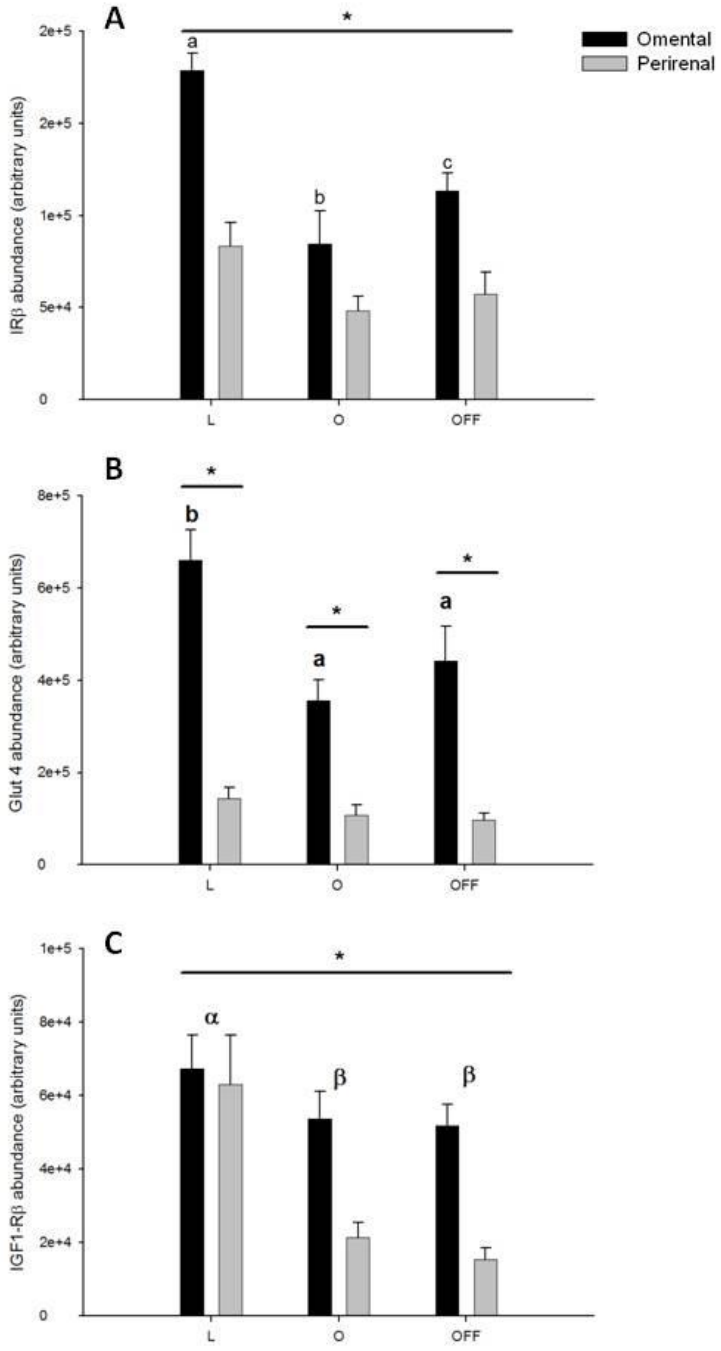


**Figure 6.8; Protein abundance of IR (A), GLUT4 (B) and IGF1-R (C) in omental and perirenal adipose tissue of lean and obese animals.**

* indicated differences between adipose tissue depots; bars with differing superscripts are significantly different (*abc* for omental and *xyz* for perirenal adipose tissue or *Greek letters* when no interaction is present and the superscripts relate to both depots). L, Lean (n=7); O, obese (n=7); OFF, Obese formula-fed (n=8).

## 6.5 Discussion

Obesity *per se* did not result in a redistribution of visceral adipose tissue in this study. Interestingly a combination of obesity and formula feeding resulted in a redistribution but in favour of the perirenal depot and not, as hypothesised, in favour of the omental depot. Furthermore no differences in the expression of markers of tissue sensitivity to glucocorticoids or insulin were found between obese maternally reared and obese formula fed animals. No conclusion can therefore be made which explains the redistribution of adipose tissue towards the perirenal depot in the formula fed animals. Other factors, such as leptin or adiponectin, could be responsible for the shift in adipose tissue distribution with obesity.

The increase in 11β-HSD1 and decrease in Glut4 expression which were found in formula fed animals at 3 months of age (Chapter 5) appears to be no longer present at the age of one year. However this study examined formula fed animals that subsequently became obese and obesity as such has dramatic effects on the expression of those genes which could overshadow any programmed effects secondary to formula feeding. A future study of formula fed animals that remain lean will further elucidate this possibility. Furthermore differences in the markers of adipose tissue sensitivity to the effects of insulin and glucocorticoids, which could explain

the redistribution of adipose tissue towards the perirenal depot, could also be overshadowed by the effects of obesity *per se*.

This study focussed on potential programmed effects of our treatments on the HPA axis and glucocorticoid and insulin sensitivity of visceral adipose tissue. In terms of overall HPA axis activity we observed no effect of obesity or formula feeding *per se*; simply that the overall response curve for ACTH was exaggerated in female animals relative to males – a common finding in adult animals (Roelfsema *et al.*, 1993; Andrew *et al.*, 1998; Canny *et al.*, 1999; Silva *et al.*, 2002; van Lier *et al.*, 2003). Unfortunately our study had a very unequal distribution of gender across treatment groups, with no males in the lean group. Our analyses did always take gender into account, it could however still be possible that differences exist between lean and obese males that are study has been unable to identify given the lack of lean males.

The excursions of plasma lipid metabolites, in particular NEFA, in response to high cortisol levels produced by an AVP and CRH challenge were, however, significantly affected by obesity; the return to baseline occurred much more rapidly in obese animals independently of whether these animals had been ewe reared or formula fed in early life. Such a response suggests altered adipose tissue sensitivity to cortisol. We therefore characterised transcript expression of key genes mediating adipose tissue sensitivity to cortisol in omental and perirenal tissue. In omental tissue of obese animals only, we found lower GR expression and, in both visceral depots, markedly

reduced 11β-HSD 1/2 expression in obese relative to lean animals. Given that the visceral depot (i.e. omental/mesenteric) is widely recognised as being more metabolically active than other adipose depots e.g. subcutaneous (Alvarez *et al.*, 2002; Wilding, 2007; Hayashi *et al.*, 2008; Lafontan & Girard, 2008) then the shortened NEFA excursion during the HPA challenge may well reflect the lower level of GR expression in this depot in obese animals. Furthermore, the marked reduction in adipose 11β-HSD1 would further compound this effect.

Markers of the tissue sensitivity to glucocorticoids and to insulin were significantly reduced in response to obesity in both adipose tissue depots. This opposes our initial hypothesis that differences in the regional adipose tissue distibution are secondary to regional differences in the balance between tissue sensitivity to the actions of insulin and glucocorticoids. It further opposes our hypothesis that high sensitivity to the actions of glucocorticoids or insulin in the long term results in adipogenesis. We can speculate that the reduction in markers of tissue sensitivity to glucocorticoids could however be a normal physiological response to an excess in nutrient supply, resulting in marked obesity. Such a response would suggest some feedback control over whole body adipose tissue expression of markers for glucocorticoid and insulin sensitivity mediated by adipose tissue itself. Unfortunately we have not been able to measure plasma insulin in those animals and are therefore unable to relate our findings to plasma insulin levels.

A reduction in markers of tissue sensitivity to the actions of insulin was seen in the omental depot of obese animals compared to lean animals. This reduction in IR and Glut4 abundance could potentially mean reduced tissue sensitivity to insulin. A reduction in the efficiency of the insulin signalling cascade could be secondary to serine phosphorylation of IRS instead of tyrosine phosphorylation. However serine phosphorylation of IRS would be expected to lead to a dissociation of the p85 subunit of PI3-kinase (Le Marchand-Brustel et al., 2003; Tanti et al., 2004). No differences in p85 abundance were found suggesting that the reduction in efficiency of the insulin signalling cascade occurs at another level than at PI3-kinase. Furthermore, a reduction in the abundance of IR could potentially mean reduced sensitivity to the actions of insulin starts at a receptor level, rather than downstream in the insulin signalling cascade.

While obese animals had significantly lower omental expression of IR and Glut4, they maintained IGF1R suggesting a potential mechanism for regional insulin resistance but maintenance of the tissue mass. For all the investigated proteins and genes, the effects of postnatally acquired obesity were more pronounced in the omental than in the perirenal adipose tissue depot, further implicating this depot as key to some of the deleterious consequences of central obesity (Despres & Lemieux, 2006; Pischon *et al.*, 2008). Unfortunately no subcutaneous adipose tissue was available to further compare visceral and peripheral depots and this could be a point of

investigation in future studies.

# Summary, conclusions and future directions

The risk enhancing effects of an increase in visceral adipose tissue in the body have been known for a long time. It has also been known for a long time that the development of obesity or visceral obesity in particular, can be altered by programming processes in early life. The mechanisms behind the processes that program the development of adipose tissue in early life are not fully understood at this stage. Furthermore, little is known about the differences between individual adipose tissue depots that are considered to be visceral adipose tissue.

Adipose tissue distribution and development are regulated by a complex interaction between the local actions of insulin and glucocorticoids on individual adipose tissue depots. Insulin promotes adipose tissue growth by promoting adipogenesis and lipogenesis and inhibiting lipolysis (Belfrage *et al.*, 1981; Gregoire *et al.*, 1998; Soret *et al.*, 1999). Glucocorticoids decrease adipose tissue growth in the short term by promoting lipolysis but chronic exposure to high glucocorticoid concentrations stimulate adipose tissue growth through a stimulation of adipogenesis and lipogenesis (Fain *et al.*, 1963; Hauner *et al.*, 1987; Ramsay *et al.*, 1989a; Divertie *et al.*, 1991; Dinneen *et al.*, 1995).

I proposed that changes in adipose tissue distribution and development, as a consequence of exposure to different patterns of nutrition from the periconceptional up to the neonatal period, are secondary to changes in the balance between the actions of insulin and glucocorticoids on regional adipose tissue depots.

No strong supportive evidence was found that adipose tissue growth and distribution is entirely dependent on the balance between the local sensitivity of individual depots to the actions of insulin and glucocorticoids.

Several different cohorts of animals were used in this thesis to investigate the effects of diet during the perinatal period on the expression of markers of tissue sensitivity to the actions of insulin, glucocorticoids and insulin like growth factors. Using different cohorts allowed us to look at a fast number of data. However, the use of different cohorts is also associated with limitations to the interpretation of data. Different breeds of sheep were used, animal experiments were run in different countries with different climates and animal facilities and experimental methods were slightly different between individual studies. All those factors limit the interpretation of data obtained from the different studies. Furthermore, we were faced with differences in the availability of tissues and samples between studies, making it impossible to perform certain analyses, such as RNA expression in our 7 and 14 day animals and analysis of subcutaneous tissue in a number of cohorts. Future studies could attempt to fill in those gaps and further elucidate the physiology

with regards to adipose tissue specific sensitivity to the actions of glucocorticoids, insulin and insulin like growth factors. Another limitation in this PhD project was time and this resulted in a lack of protein data from the periconceptional nutrition and formula feeding studies. Studies are currently being conducted to add those data of protein abundance to the data presented in this thesis.

The omental depot did undergo accelerated growth in early postnatal life as expected, with a significant lessening of the dominance of perirenal adipose toward overall fat mass. These changes occurred almost entirely during the first 12 weeks of life in sheep (i.e. through lactation) and were not influenced thereafter by a high caloric environment in juvenile-to-adult life, nor altered nutrition during lactation. We originally hypothesised that this differential growth of regional adipose was secondary to increased sensitivity to insulin in the omental depot, and decreased sensitivity in the perirenal depot. We therefore measured molecular markers of tissue sensitivity to the actions of insulin in both the omental and perirenal adipose tissue depot. The abundance of IR was much lower in the omental compared to the perirenal depot, but in the absence of changes to Glut4 abundance, we could not make any conclusions regarding the definite tissue sensitivity to the actions of insulin in either depot. Intracellular insulin signalling is effected through a highly complicated insulin signalling cascade (Cheatham & Kahn, 1995; Alessi *et al.*, 1997; Rea & James, 1997; Peterson & Schreiber, 1999; Whiteman *et al.*, 2002) and changes in IR abundance which are not related

to changes in Glut4 abundance could potentially indicate an altered efficiency of this signalling pathway. However, more measurements of other specific markers in the insulin signalling cascade and measures of actual tissue sensitivity to insulin are necessary to make any definite conclusions with regards to insulin sensitivity in the omental and perirenal adipose tissue depot during this period of life.

Unfortunately due to degradation of RNA of our 7 and 14 day old animals we were unable to measure markers of the local tissue sensitivity to glucocorticoids in those animals. We have therefore not been able to investigate the role of glucocorticoids on the different growth rate of individual adipose tissue depots during early postnatal life. Furthermore, other mechanisms such as decreased expression of leptin, adiponectin and growth hormone could potentially explain the differential growth rates that were found. This early postnatal period of life is the period in which brown (multilocular) adipose tissue transforms into white (unilocular) adipose tissue. It is possible that factors that influence this histological change in adipocytes are related to growth rate of the individual adipose tissue depots. Future studies will hopefully elucidate the exact mechanism that is responsible for this differential growth pattern of individual adipose tissue depots.

Exposure of the oocyte and/or embryo to maternal undernutrition (periconceptional undernutrition; PCUN) results in a premature activation of the HPA axis during late gestation (Edwards & McMillen, 2002a; Bloomfield *et al.*, 2003; Edwards *et al.*, 2005). Furthermore it results in an enhanced

cortisol response after a CRH challenge. This increased pituitary-adrenal activation occurs during late gestation, the period during which the subcutaneous and perirenal adipose tissue depot undergo rapid growth (Alexander, 1978). I hypothesised that PCUN will result in an enhanced glucocorticoid response in these depots which will persist after birth.

Women that are obese or overweight during pregnancy have a reduced insulin sensitivity and higher circulating plasma glucose resulting in a greater exposure of the foetus to insulin (Catalano & Ehrenberg, 2006). I hypothesised that when a period of periconceptional nutrient restriction occurs in sheep that are already overweight; the effects of PCUN would be exaggerated, due to the increased insulin exposure of the perirenal and subcutaneous adipose tissue depot during prenatal life. Therefore these two depots would become relatively more insulin resistant and more sensitive to the effects of glucocorticoids, resulting in an even greater lipolytic activity in these depots and a greater nutrient flux to the omental depot.

Our data showed that weight of the donor ewe during the periconceptional period was indeed associated with programming of markers of the local sensitivity to the actions of glucocorticoids, but only in the omental depot. The increased expression of markers of tissue sensitivity to the actions of glucocorticoids in omental adipose tissue in offspring of ewes that were lighter around pregnancy was not associated with a redistribution of adipose tissue. The increase in markers of tissue sensitivity to the effects of

glucocorticoids appeared to be a programmed response to a decrease in nutrient supply during the periconceptional period. However it is not clear why this response was only present in the omental depot. Given the anatomic location of the omental depot it is likely to be more responsive to the effects of nutrition compared to the other adipose tissue depots (Alvarez et al., 2002; Wilding, 2007; Hayashi et al., 2008; Lafontan & Girard, 2008), which could explain a programmed effect in this depot only. However it is unclear up to what extent the anatomical location of adipose tissue is relevant in this case where nutrition is umbilical and partly bypasses the portal circulation.

Furthermore periconceptional overnutrition did not result in changes in the investigated molecular markers of tissue sensitivity to the actions of insulin. Periconceptional overnutrition did result in an increased adipose tissue mass in females. This is a relevant finding considering the current epidemic of obesity and the increase of women that enter pregnancy obese (LaCoursiere et al., 2005). It appears that overnutrition or obesity in the periconceptional period not only has effects on fertility, diseases in pregnancy such as gestational diabetes and hypertension, macrosomia but also in the adipose tissue distribution of the offspring in later life, at least in females (Ryan, 2007). The effect of periconceptional overnutrition on adipose tissue mass was not associated with changes in markers of the tissue sensitivity to the actions of insulin or glucocorticoids and appeared to be merely an effect secondary to increased substrate supply. However, future studies to

investigate further key elements of the intracellular insulin signalling cascade, such as IRS and Akt, could further elucidate whether an increased tissue sensitivity to the actions of insulin in the female offspring of periconceptionally overnourished ewes could underly the increased adipose tissue mass that was found in this group. Future studies could also investigate other factors that could influence the increased adipose tissue growth in females such as growth hormone, leptin and adiponectin.

Our study into the effects of PCUN demonstrated a direct relationship between the expression of 11β-HSD1 in the omental or subcutaneous adipose tissue depot and plasma FFA concentration. This relationship supports our theory that glucocorticoids have a stronger lipolytic than lipogenic effect on adipose tissue in the short term. Similar relations have been found between the effects of hypercortisolaemia and fatty acid turnover (Divertie *et al.*, 1991; Dinneen *et al.*, 1995).

Most tissues during development undergo a transition in the role of the insulin like growth factors and their receptors (Sara & Hall, 1990; Delhanty & Han, 1993; Li *et al.*, 1993; Peng *et al.*, 1998). This transition is, at least partly, mediated by glucocorticoids which stimulate IGF1R expression (Bennett *et al.*, 1984; Li *et al.*, 1993; Lu *et al.*, 1994). We hypothesised that PCUN would result in a glucocorticoid mediated transition of IGF2R to IGF1R expression which would happen mainly in the perirenal and subcutaneous adipose tissue depots which are present during foetal life. Therefore the

effects of IGF1 would be enhanced in the perirenal and subcutaneous adipose tissue depots, resulting in an increase in adipogenesis.

Periconceptional undernutrition did not result in the expected increased expression of IGF1 or IGF1R in any of the investigated depots. However an association was found between GR and IGF1R which supports our hypothesis that glucocorticoids stimulate IGF1R expression and therefore work synergistically with IGF1. Similar associations have been found before by different *in vitro* and *in vivo* studies (Bennett *et al.*, 1984; Conover *et al.*, 1985; Tronche *et al.*, 2004). The absence of an effect of PCUN on markers of tissue sensitivity to the action of glucocorticoids could potentially provide and explanation for the lack of an effect of PCUN on the expression of insulin like growth factors and their receptors; as such an effect would be driven by glucocorticoids. The role of growth hormone and the GH-IGF axis in adipose tissue remain largely unclear at this stage and further investigation of growth hormone and growth hormone receptor expression in different adipose tissue depots could potentially bring new insides into depot specific adipose tissue development.

In lambs at 4 months of age we found increased levels of IGF2 and IGF2R in the omental depot compared to the perirenal and subcutaneous depots which was consistent with our hypothesis that the delayed effects of omental growth coincide with a delayed switch from IGF2 to IGF1 dominance.

Our cohort of periconceptional under and overnutrition used donor ewes, embryo transfer and recipient ewes in order to be able to examine the effects of nutrition during the periconceptional period alone on long term adipose tissue development in the offspring. This study has shown that overnutrition during the periconceptional period alone leads to an increase in adipose tissue mass in the female offspring. In real life however, an embryo is rarely exposed to an environment of overnutrition during the periconceptional period alone, followed by an environment of normal nutrition during the rest of pregnancy. Future studies could combine the effect of periconceptional overnutrition with overnutrition later in pregnancy to see whether the effects of the offspring remain the same or might even be exacerbated.

Formula feeding is associated with an increased food intake in early life and an increased plasma insulin concentration (Lucas *et al.*, 1980; Lucas *et al.*, 1981; Heinig *et al.*, 1993; Dewey, 1998). We hypothesised that formula feeding would result in a reduced sensitivity of adipose tissue to the actions of insulin. This reduction in sensitivity to insulin would switch the balance between insulin and glucocorticoid action on adipose tissue towards a stronger sensitivity of the adipose tissue to glucocorticoids. In the long term this increased glucocorticoid action would result in an increase in adipogenic growth in adipose tissue. The reduction in local sensitivity to the action of insulin would be greatest in the omental depot, which is developing rapidly during this period in life. The effects on increased adipose tissue growth

would therefore be greatest in this depot and lead to a redistribution of adipose tissue towards the omental depot.

Formula feeding resulted in a decrease of Glut4 and an increase in 11β-HSD1 expression which could potentially mean a reduction of the tissue sensitivity to the effects of insulin and an increase of tissue sensitivity to the effects of glucocorticoids. A reduction in tissue sensitivity to the actions of insulin could be a mechanism that explains the observations of increased insulin resistance and an increased prevalence of type 2 diabetes mellitus in formula fed individuals (Pettitt *et al.*, 1997; Ravelli *et al.*, 2000; Singhal *et al.*, 2003). Unfortunately we were not able to relate our findings to plasma levels of glucose, insulin and cortisol and neither have glucose tolerance tests been performed in our animals. We can therefore not make any definite conclusions regarding the relevance of the findings of decreased Glut4 and increased 11β-HSD1 expression. Furthermore these effects were not depot specific and did not result in the expected redistribution of adipose tissue. An increase in 11β-HSD1 could mean an increase in the sensitivity of the tissue to the effects of glucocorticoids in formula fed animals. This effect could potentially result in long term adipogenic effects of glucocorticoids and an increase of adipose tissue mass in formula fed infants. Furthermore the increase in adipose tissue 11β-HSD1 secondary to formula feeding, combined with a possible reduction in tissue specific sensitivity to insulin, favours our hypothesis of a balance between the effects of glucocorticoids and the effects on insulin in the tissue. These effects were however not

depot specific and were not significant enough to result in a redistribution of adipose tissue depots as we initially hypothesised.

In all the animal cohorts that we examined we found marked differences of expression of markers of tissue sensitivity to the actions of insulin, glucocorticoids and insulin like growth factors between adipose tissue depots. Tissue specific expression patterns however appear not consistent in our different cohorts. Insulin receptor abundance for example is higher in perirenal compared to omental adipose tissue at 7 and 14 days of age, while IR expression at 3 months of age is not significantly different between the depots, at 4 months of age is higher in perirenal compared to omental adipose tissue and at 1 year of age is higher in omental compared to perirenal adipose tissue. This inconsistency in expression patterns makes it difficult to interpret our data. Obviously a number of key elements are different between our cohorts and could provide potential explanations for these differences. These explanations include the use of protein abundance *vs.* RNA expression, different breeds and different environmental circumstances. It is therefore difficult to make any definite conclusion regarding expression of our markers of local tissue sensitivity to insulin, glucocorticoid and insulin like growth factor over time. Our data do however highlight, for the first time, that there are significant differences in the expression of those key markers between omental and perirenal adipose tissue. Our data further highlight that those expression patterns are likely to change significantly over time. It is therefore important for future studies to

recognise the important in examining depot specific effects rather than investigating adipose tissue as one organ with similar responses throughout the body or throughout the abdomen.

Obesity is related to relative insulin resistance (Wajchenberg, 2000). I hypothesised that the decreased response to the actions of insulin in adipose tissue results in a reduction of the insulin mediated inhibition of 11β-HSD1 expression (Hammami & Siiteri, 1991; Jamieson *et al.*, 1995; Liu *et al.*, 1996) resulting in enhanced local production of cortisol resulting in an increase in adipogenesis and adipose tissue growth.

In obesity, however, the subcutaneous and perirenal depots are relatively less insulin resistant compared to the omental depot. I hypothesised that in these depots insulin would remain the main driver of growth. When the effects of obesity are combined with the effects of formula feeding an earlier shift towards glucocorticoid driven control of adipose tissue would occur in the omental depot. I hypothesised that this would result in an exacerbation of the effects of an obesogenic lifestyle in people that were formula fed in early life.

Obesity did not result in tissue specific changes in the expression of markers of tissue sensitivity to insulin and glucocorticoids, nor did it result in the expected redistribution of adipose tissue growth. A combination of formula feeding and obesity did result in a redistribution of adipose tissue growth but was opposite from what was expected - namely in the direction of the

perirenal depot. This redistribution could not be explained by differential expression of markers of the tissue sensitivity to the effects of insulin or glucocorticoids. Possible other mechanisms that could explain this redistribution could be differential expression of leptin and adiponectin.

The increase in 11β-HSD1 and decrease in Glut4 expression which were found in formula fed animals at 3 months of age appeared to be no longer present at the age of one year. However, the investigated animals at one year of age which were formula fed were all significantly obese and obesity *per se* had significant effects on the expression of markers of tissue sensitivity to the effects of insulin and glucocorticoids. These effects were of a considerable magnitude which could potentially mask any effects of formula feeding *per se*. Future studies will therefore focus on the effects of formula feeding in lean animals.

The reduction in markers of tissue sensitivity to glucocorticoids and to insulin in response to obesity was present in both adipose tissue depots. This opposes our initial hypothesis that differences in the regional adipose tissue distibution are secondary to regional differences in the balance between tissue sensitivity to the actions of insulin and glucocorticoids. It further opposes our hypothesis that high sensitivity to the actions of glucocorticoids or insulin in the long term results in adipogenesis.

Given this large body of evidence against our hypothesis, we feel that we can conclude that adipose tissue growth and distribution in sheep are not

directly explained by changes in the tissue specific expression of markers of tissue sensitivity to the actions of insulin, glucocorticoids and insulin like growth factors.

The formula feeding study was the only study that supported evidence that had opposing effects on adipose tissue sensitivity to the actions of glucocorticoids and the actions of insulin. However, this effect was not depot specific and did not result in a redistribution of adipose tissue growth.

Our hypothesis was based on glucocorticoids promoting lipolysis in the short term, however no single relationship was found between tissue sensitivity to the effects of glucocorticoids and a reduced short term adipose tissue growth.

Some relationships did favour the expected long term adipogenic effects of glucocorticoids, such as the increased expression of 11β-HSD1 in formula fed animals. Furthermore relationships were found that supported our hypothesis of a synergistic relationship between glucocorticoids and IGF1.

Our studies have clearly shown, for the first time, the differential growth patterns of adipose tissue depots during postnatal life. Furthermore our studies have clearly shown, in a large number of cohorts and animals, that expression of markers of tissue sensitivity to the actions of insulin, glucocorticoids and insulin like growth factors is significantly different

between different adipose tissue depots in the body, highlighting the importance of examining those depots individually in future studies.

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