Alterations induced by juvenile obesity on the renal tissue of nutrient restricted offspring

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Abstract

Human epidemiological studies have indentified obesity as an independent risk factor for renal disease. In addition, maternal nutrient restriction (MNR) during gestation results in a series of adaptations that may predispose those offspring to obesity and hypertension. Recent reports demonstrated that obese sheep exposed to MNR during early to mid gestation have a predisposition to ectopic lipid deposition in the heart and a rise in necrotic adipocytes, which are markers of severe metabolic dysfunction. Surprisingly, in this model of MNR, the renal tissue of those offspring showed an apparent reduction in cell apoptosis. However, renal diseases associated with obesity have a slow progression and their mechanisms are not completely understood. In the light of these results, the main hypothesis of my thesis is that the renal amelioration observed in those nutrient restricted (NR) obese offspring is a product of post-injury responses, inducing scarring and others adaptations to obesity. Therefore, some of the main regulatory factors in renal and perirenal adipose tissue (PAT) development were analysed in seven-day-old and one-year-old obese sheep offspring exposed to MNR (3.5 KJ/days) from 30 to 80 gestational days (term ≈ 145 days).

At one week of age, the renal composition and gene expression showed small changes between NR offspring and those born to control mothers. However, in PAT of NR offspring, an increased in expression of the methyltransferase DNMT-1 and a decrease in mRNA abundance of IGF-2 were observed.

At six months of age, obesity onset was accompanied by raised plasma cortisol and leptin concentration in NR offspring compared to control. By one year of age, whilst plasma leptin concentration was similar between the obese groups, in the PAT of the NR offspring there was an increase in gene expression of pro-inflammatory factors and DNMT-1, suggesting advanced adipose tissue remodelling. In kidney, regardless of in utero diet, obesity induced similar amounts of oxidative stress, activation of cellular proliferative factors and collagen deposition. Although, both obese groups had equal activation of pro-apoptotic factors (e.g p-53 and Bax), renal iron and mRNA abundance of the death receptor, Fas only increased in obese offspring born to control fed mothers. A major finding in the NR kidney was increased ectopic triglyceride deposition, indicating early onset post-injury in response to sympathetic activation and lipotoxity.

The main conclusion of my thesis is that functional changes observed in the adipose tissue lead the kidney to an initial cycle of cell proliferation, apoptosis and arrest, followed by tissue remodelling, characterised by the presence of collagen. However, this adaptation to obesity is accompanied by an increase in lipid deposition in the kidney of the NR group that may be a sign of an advanced state of metabolic dysfunction.

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This thesis I dedicate to my uncle, Sami Perla, who passed away during the course of my studies.

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Declaration

The work of this thesis was carried out in the Division of Human development, Academic Child Health, School of Clinical Sciences, University of Nottingham between September 2006 and January 2010.

The thesis and publications represent my own work, undertaken under the supervision of Professor Michael Symonds and Dr. Helen Budge. This report is an accurate representation of the work performed. No other study representing this work has been carried out within this School.

Hernan P. Fainberg

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Abbreviations

ACE	Angiotensin Converting Enzyme
ACTH	Adrenocorticotropic hormone
ANG I/II	Angiotensin I/II
ATP	Adenosine triphosphate
ATR	Angiotensin receptor I/II
Bax	Bcl-2-associeted X protein
Bcl-2	B-cell lymphoma 2
BH_4	Bihydrobiopterin
BMI	Body mass index
BSA	Bovine serum albumin
C/EBP	CCAAT/enhancer-binding proteins
CAT	Catalase
CHOP	Cyclophosphamide hydroxydaunorubicin oncovin prednisone
CLS	Crown-like structures
CRH	Corticotropin-releasing hormone
CRP	C-reactive protein
CVD	Cardiovascular diseases
db/db	Mouse leptin receptor deficient
DNA	Deoxyribonucleic acid
DNMT	DNA methyltransferase
eNOS	Endothelial Nitric oxide synthase
ER	Endoplasmatic reticulum
ESRD	End-stage renal disease
FAD	Flavin adenine dinucleotide
Fas	Fas receptor (APO-1)
FFA	Free-fatty-acids
FSG	Focal segmental glomerusclerosis
GFR	Glomerular filtration rate
GH	Growth hormone
GLUT	Glucose transporter
GpX	Glutathione peroxidise
H_2O_2	Hydrogen peroxide
HIF	Hypoxia inducible factors
HK	Hexokinase
HSL	Hormone-sensitive lipase
Ig	Immunoglobulin
IGF	Insulin-like growth factor
IL	Interleukin

iNOS	Inducible Nitric oxide synthesise
IR	Insulin receptor
IRS	Insulin receptor substrate family
JNK	c-Jun N-terminal Kinase
L	Lean Group
LDL	Low-density lipoprotein
MCP-1	Monocyte-chemotactic protein-1
MDA	Malonyldaildehyde
MNR	Maternal nutrient restriction
NaCl	Sodium Chloride
NADPH	Nicotinamide adenine dinucleotide phosphate
NF-κB kinase	Nuclear factor kappa-light-chain-enhancer of activated B-cell
NHANES	National Health and Nutrition Examination Surveys
nNOS	Neural nitric oxide synthesise
NO	Nitric oxide
NOS	Nitric oxide synthase
Nox	Non-phagocyte NADPH oxidase
NPY	Neuropeptide Y
NR	Nutrient restricted group
NR-O	Nutrient restricted Obese group
O	Oxigen
$O2^{\cdot -}$	Superoxide
$ONOO^-$	Peroxinitrite
OR	Odd ratio
ORG	Obesity related glomerulopathy
OxS	Oxidative stress
PAI-1	Plasminogen activator inhibitor-1
PCK	Phosphoenolpyruvate carboxykinase
PCR	Polymerase chain reaction
PPAR-α	Peroxisome proliferator-activated receptor α
pRb	Retinoblastoma Protein
RAS	Renin-angiotensin system activity
RNA	Ribonucleic acid
ROS	Reactive oxygen species
RPF	Renal plasma flow
SHR	Spontaneously hypertensive rats
SOD	Superoxide dismutases
SREBP	Sterol regulatory element binding protein
T_4	Thyroxine

TBARS	Thiobarbituric acid substances
TG	Triglycerides
TGF	Transforming growth factor
TNF	Tumor necrosis factor
UCP	Uncoupling protein
UPR	Unfolded protein response
VEGF	Vascular endothelial growth factor
WHO	World Health Organisation
VLDL	Very low-density lipoprotein
ZDF-fa/fa	Zucker diabetic rats
8-OHdG	8-hydro-deoxyguanosine
11-βHDS	11-β hydroxysteroid dehydrogenase

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Chapter 1 – Introduction

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1.1 Maternal nutritional status during pregnancy and its influence on offspring in later life

1.1.1 Early life nutrition and its influence on adult development

In humans, as with other animals, health and life span are sensitive to external environmental conditions. Variation in nutrition at different stages of development might play an important part in overcoming health problems. Numerous human epidemiological and animal studies have suggested that early nutrition modulates the physiological response to an environmental challenge by reducing susceptibility to chronic diseases in adulthood (Stein *et al.*, 1996).

A landmark publication by Kermack *et al.*, analysing the rise in life expectancy in Sweden and the United Kingdom between 1751 and 1930, concluded that the improved welfare of infants and adolescents was the strongest determining factor of non-transmissible diseases in later life (Kermack *et al.*, 2001). Other research conducted by Forsdahl also supported this finding and added that children and adolescents exposed to significant poverty, followed by a period of prosperity, are also at high risk of developing chronic heart disease in later life (Forsdahl, 1977). In the early 20th century, the impact of improved infant dietary patterns was recognised as one factor in the gradual reduction in cardiovascular mortality in adulthood in the industrialised world (Barker *et al.*, 1992).

Studies on the effects of early nutrition also include the maternal environment during the different stages of pregnancy in humans (Painter *et al.*, 2005). For instance, the effects of poor nutrition during pregnancy, which may affect fetal and infant development, were associated with an increased risk of cardiovascular disease in offspring in adulthood. In 1986, Baker *et al*. noted a similarity between the geographical distribution of stroke and cardiovascular diseases (1968-78) and neonatal mortality records (1921-5) in England and Wales (Barker & Osmond, 1986). This study was the first to demonstrate the potential importance of fetal development as a risk factor for heart disease in later life. Epidemiological studies of offspring exposed to the Dutch Famine (1944-5) confirmed these findings, adding that perinatal, and later infant, nutrition both influence adult body mass index (BMI), cardiovascular disease, cognitive disorders and other chronic diseases (Roseboom *et al.*, 2000; Painter *et al.*, 2005). In 2000, Law *et al.* reported that blood pressure was inversely proportionate to body size at birth among children born in several developing countries commonly affected by maternal undernutrition (Law *et al.*, 2001). Furthermore, past studies conducted in Western countries have shown that this pattern in blood pressure persists into adulthood, even without an increase in body weight (Barker *et al.*, 1989; Osmond *et al.*, 1993). Additional epidemiological human observations and animal models have strongly suggested that perinatal and early life growth affects the predisposition to adult obesity, cardiovascular diseases and type 2 diabetes in later life (Hales *et al.*, 1991) .

1.1.2 The thrifty phenotype and other hypotheses

In an attempt to unify several human epidemiological and animal studies with long term chronic diseases associated with exposure to suboptimal fetal and neonatal

environments, a number of hypotheses have been proposed. In 1991, Lucas proposed the principle of '*fetal programming'* (Lucas, 1998). This term describes the link between adaptive responses in the offspring to an insult generated during pregnancy, including malnutrition, resulting in long term physiological or somatic consequences. Later, in 1992, Barker & Hales *et al*. produced a theory termed the '*thrifty phenotype hypothesis'* (Hales & Barker, 2001). The central proposal of this hypothesis is that it is possible to trace the sources of the metabolic syndrome back from adulthood to a maternal suboptimal environment (including diet) during pregnancy or early infancy. As a consequence, early adaptations to malnutrition might, for example, reduce the secretion of insulin, affecting the activity of the insulin dependent organs and changing patterns of body development (Fig.1.1). Such observations may explain the effects of exposure to suboptimal diet during fetal growth, resulting in deficient development of pancreatic-β cell mass and function, which increases the risk of type 2 diabetes in later life (Hales *et al.*, 1991; Ravelli *et al.*, 1998; Petrik *et al.*, 1999; Jaquet *et al.*, 2000). It was proposed that such adaptations would become detrimental when, in later life, an individual is exposed to a richer diet than experienced in the prenatal environment. Thus, enhanced calorie intake in later life would induce glucose intolerance, leading to obesity.

A large body of animal data and human studies support the '*thrifty phenotype hypothesis'*. The epidemiological data collected by Hale *et al*. showed that Hertfordshire men (1920-30) who were underweight at birth and in early infancy displayed an increased predisposition to impaired glucose tolerance and non-insulin dependent diabetes (Hales *et al.*, 1991). Extensive studies, conducted on small and large mammals, show that malnutrition during pregnancy may have a permanent effect on pancreatic cell

insulin secretion, cognitive development and changes in blood pressure, leading to adverse effects on the longevity of the offspring (Petrik *et al.,* 1999; Oliver *et al.,* 2002; Erhard *et al.*, 2004; Ozanne *et al*., 2005) In addition, the concepts of '*fetal programming'* and '*thrifty phenotype hypothesis*' were extended to include the existence of critical or sensitive periods during pregnancy, during which the effects of these prenatal adaptations to a suboptimal intrauterine environment generate adverse outcomes for specific body tissues in later life (Hoek *et al.,* 1998; Ravelli *et al.,* 1998; Roseboom *et al.,* 2000). Evidence regarding the existence of such critical periods came from retrospective studies examining men and women who were *in utero* during the Dutch Famine. By dividing this entire period of gestational malnutrition into trimesters, it was possible to determine that different outcomes occurred, depending on the period of pregnancy in which the fetus was exposed to famine (Painter *et al.*, 2005). Famine exposure during the first three months of gestation contributed to an increased risk in coronary heart disease and neurodevelopmental disorders in adulthood (Hoek *et al.*, 1998; Roseboom *et al.,* 2000). Offspring who were exposed to the famine during the second trimester showed an increase in renal-vascular diseases (Painter *et al.,* 2005). Finally, prenatal exposure to famine during late gestation resulted in low weight at birth and was associated with permanent changes in insulin-glucose metabolism and obesity in adults (Ravelli *et al.*, 1998).

In spite of the significant body of evidence from human and animal studies that validates this hypothesis, alternative explanations exist. In 2001, Frayling and Hattersley proposed the *'fetal insulin hypothesis'*, suggesting that mutation of a single gene has two different independent outcomes: low birth weight and type 2-diabetes (Hattersley *et al.*,

1998). One possible cause of insulin deficiency is the presence of a glucokinase autosomal recessive mutation in the fetus, which is associated with low weight at birth and reduction of insulin secretion. However, permanent glucokinase deficiency caused by this type of mutation is unusual. Studies conducted with low weight identical twins have demonstrated that their weight at birth is regulated by different independent genes (Njolstad *et al.*, 2003).

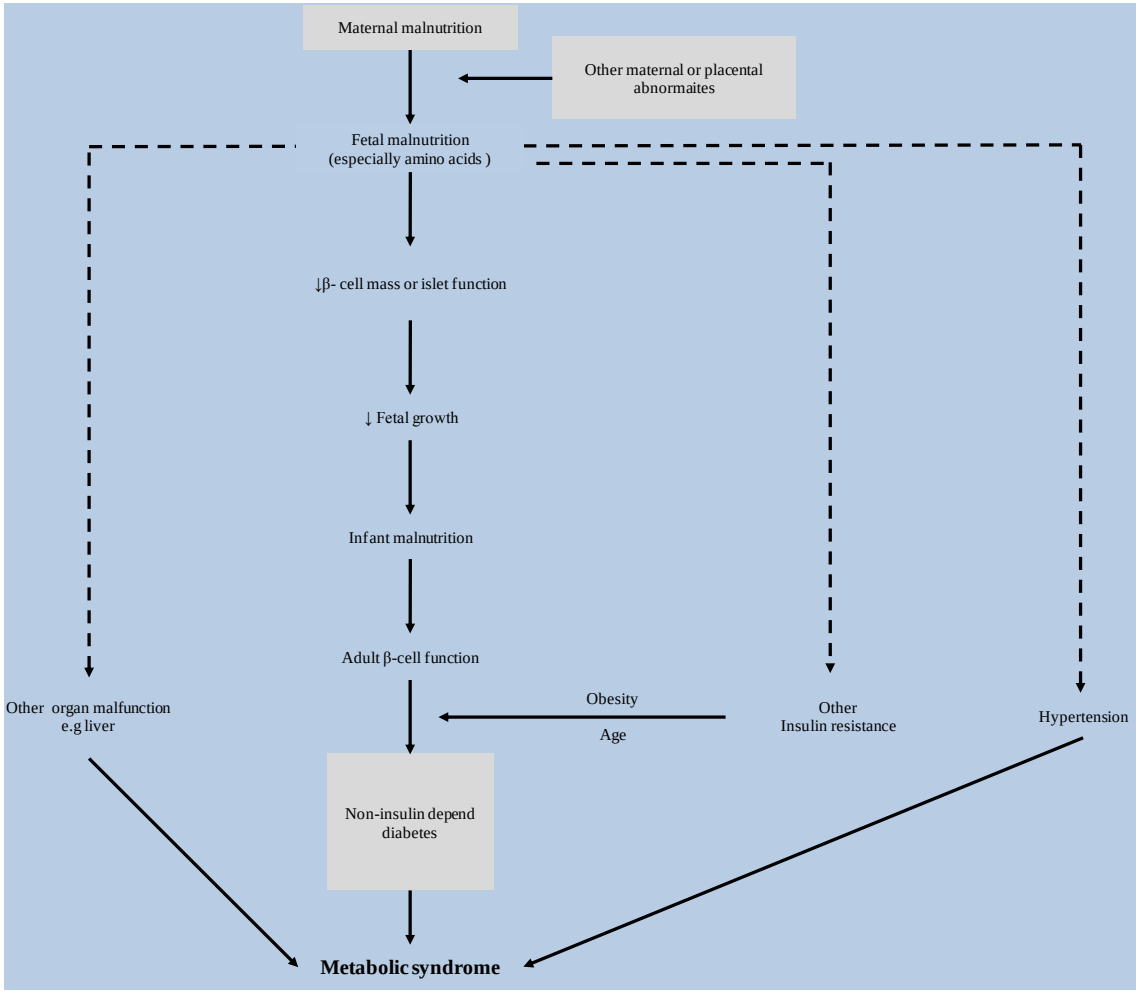

Figure 1.1 The original diagrammatic interpretation of the thrifty phenotype hypothesis by Hales and Barker

1.1.2.1 Fetal suboptimal nutritional environment as a future health factor

As the Dutch Famine illustrated, there are critical windows in fetal development during which the maternal environment may cause irreversible alterations in growth and metabolism. These adaptations occur in response to specific nutritional conditions in early life which are limited to susceptible critical periods during early development and have a persistent life effect. In addition, their outcome can be specific and measurable, may differ among individuals and is a response to a specific maternal or postnatal environment (Waterland & Garza, 1999). For instance, Painter *et al*., observed that, following exposure to the Dutch Famine during early-mid gestation, all newborns had a normal weight at birth. However, during adulthood, these individuals developed a series of specific health complications, which included a rise in microalbuminuria and airways obstruction (Painter *et al.*, 2005).

Human epidemiological studies report that maternal body status (age, weight and life style) and diet composition (micro and macro-nutrients) at the time of conception and during pregnancy can have a large influence on the offspring outcome (Naeye, 1990; Christian *et al.*, 2003; Olson *et al.*, 2008). Godfrey *et al*. suggested that a high carbohydrate intake in early pregnancy suppresses placental growth, which limits the capacity of the mother to supply nutrients to the fetus and compromises its growth during late gestation (Godfrey *et al.*, 1996). Several studies have demonstrated that perinatal mortality and other abnormalities are associated with high or low maternal body weight in comparison to normal weight mothers before or during pregnancy (Lechtig *et al.*, 1975; Barker *et al.*, 1990; Nucci *et al.*, 2001; Olson *et al.*, 2008). For

instance, excessive pregnancy weight gain may increase the risks of obesity in children (Olson *et al.*, 2008).

Another important area of research in intrauterine growth is the role of micronutrients during pregnancy, which has not been fully elucidated. Individual micronutrients, such as folic acid, zinc, iron and vitamin A have received attention in several studies (Katz *et al.*, 2000; Osendarp *et al.*, 2000; Christian *et al.*, 2003). Trials in malnourished populations in Peru and Bangladesh did not confirm an improvement in birth weight or neonatal mortality with antenatal zinc supplementation (Caulfield *et al.*, 1999; Osendarp *et al.*, 2000). However, a large trial among malnourished rural communities in Nepal, which tested maternal supplementation with folic acid and iron, achieved a modest increase in birth weight (Christian *et al.*, 2003). Iron supplementation in mothers suffering from anaemia is known to reduce the synthesis of cortcotropin releasing hormone, causing a reduction in fetal cortisol and consequential increased fetal growth. In addition, iron deficiency increases oxidative stress of erythrocytes and the fetoplacental unit, which may result in intrauterine growth retardation and preterm delivery (Allen, 2001). Finally, iron supplementation may increase maternal appetite and energy consumption during pregnancy, inducing fetal growth (Lawless *et al.*, 1994; Allen, 2001).

To many authors, it has become apparent that the nutritional status of the mother alters the metabolic features of the offspring, changing the functions of different key organs. One suggestion is that these adaptations prepare the newborn for survival under unfavourable nutritional conditions, such as famine. An alternative option is that these organs are simply immature and their final physiological readiness depends on the right

nutritional postnatal environment (Zidar *et al.*, 1998; Bateson *et al.*, 2004). However, it is important to analyse the real consequences of these adaptations of offspring in adulthood in such a nutritional environment.

1.1.2.2 Adaptation to new nutritional environments

The success of an adaptation to any environmental challenge, including changes in diet, can be achieved by a period of phenotypic modifications. Bateson *et al*. suggested that maternal physical and nutritional status is able to modify the chances of adaptation. In adverse circumstances, small size or underdeveloped organs and acute metabolic responses may be seen as advantageous for survival by reaching reproductive age faster, even if the consequence of this would be a reduction in lifespan (Bateson *et al.*, 2004). For example, male offspring of maternal protein-restricted rats show normal glucose tolerance at an early age, but they mature faster than their controls, achieving adulthood more quickly. However, at 15 months of age, they show signs of advanced ageing by an increase in insulin secretion, poor glucose tolerance and a reduction in renal telomeres (Tarry-Adkins *et al.*, 2006; Tarry-Adkins *et al.*, 2009). Such responses could be seen as having short term advantages, but long term disadvantages, by accelerating the rate of the ageing process to environmental changes, such as an increase in food consumption. Following the '*thrifty phenotype hypothesis'*, a poor prenatal environment and modifications in organ function, along with changes in nutrition, might generate an adverse adaptation in later life (Barker *et al.*, 2002). Migrant or historically low income populations exposed to an excess of nutrition in adult life best illustrate these inappropriate adaptive responses. Additional associations between intrauterine deprivation and other physiological adaptations are found to increase the risk of

development of renal and cardiovascular diseases (Eriksson *et al.*, 2001; Barker *et al.*, 2002; Jimenez-Cruz *et al.*, 2003; Hoehner *et al.*, 2006). High blood pressure was found to be prevalent in children and adults exposed to maternal and infant under-nutrition. Eriksson *et al*. reported that there is an association between early infant obesity and coronary heart disease, suggesting that its pathogenesis was influenced by events *in utero* (Eriksson *et al.*, 2001). A study of Pima Indians, who were exposed to poor nutrition in early life in their communities, confirmed a positive association between increased access to food and the increased risk of type 2 diabetes and renal dysfunction in adulthood (Nelson *et al.*, 1998). Moreover, poor intrauterine growth and the development of decreased glucose tolerance are linked with the rapid progression of kidney diseases and injuries, such as IgA nephropathy, membranous nephropathy and glomerulosclerosis. This suggests that these renal adaptations to unfavourable fetal-maternal environment are not beneficial in the long term (Barker *et al.*, 1989; Nelson *et al.*, 1998; Zidar *et al.*, 1998).

1.1.3 The sheep as animal model for studies of maternal nutritional manipulation

Epidemiological studies, as with other retrospective studies, have weaknesses that may limit their ability to provide definitive answers. The choice of methodology used to quantify and analyse data, the time between exposure to variables and measurable outcomes, as well as the researcher's perspective, may lead to variable interpretations (Wadsworth *et al.*, 1985; Barker & Osmond, 1986; de Onis *et al.*, 1998). For further investigation and to verify the biological relevance of such observations, it may be necessary to create and use animal models.

Thus, in an attempt to validate the epidemiological data and understand the biological mechanisms behind the association between maternal nutrition and long term metabolic changes in offspring, several animal models have been developed. These models employ a large number of techniques, including maternal nutrient restriction and hormonal insults in order to simulate similar conditions, as seen during the Dutch Famine (Ravelli *et al*. 1976).

1.1.3.1 Prenatal nutrition manipulation in sheep and its consequences

Due to its ability to generate an offspring of similar weight to human newborns (≈ 4.5 kg), with fully developed organs at birth, the sheep is one of the most reliable animal models for the study of the effects of prenatal undernutrition at specific stages of pregnancy (Symonds *et al*., 2007).

Studies using the offspring of undernourished pregnant sheep mothers throughout all or late (around the final one-third of gestation; term ≈145 days) gestation showed a reduction in weight at birth (Oliver *et al.*, 2002). A global reduction in maternal nutrition in the period of late gestation (0.3 to 0.5 MJ per day ≈ 0.03-0.05% of metabolic needs; 105 gestational days to term 145 days) affects the concentrations of fetal plasma glucose, decreasing the lean mass tissue as the main factor of weight loss in the newborn (Oliver *et al.*, 2002). However, several groups demonstrated that maternal undernutrition (3.8 MJ/d ≈ 50% of metabolic needs) around early to mid gestation generates placental growth and morphological changes, without affecting weight at birth (Heasman *et al.*, 1998). Such alterations in placental development may reduce its capacity to transport supplies, compromising fetal organ development mainly in the later stages of gestation (Heasman *et al.*, 1998; Bispham *et al.*, 2003; Jaquiery *et al.*, 2006).

Maternal nutrient restriction impacts on metabolic programming in insulin-sensitive tissues. Livers of 3-year-old offspring exposed to early to mid gestation nutrient restriction were disproportionately smaller to body weight and showed an increase in mRNA abundance of glucocorticoid receptor, which is possible evidence of reduced gluconeogenic activity. In addition, this adaptation includes an imbalance between mitogenic and pro-apoptotic mechanisms, such as a reduction in IGF hepatic sensitivity (Hyatt *et al.*, 2007). A reduction of maternal nutrition during late gestation also showed a decrease in GLUT 4 abundance in adipose tissue, possibly one of the factors triggering an increase in fat mass (Gardner *et al.*, 2005). This was followed by glucose intolerance and insulin resistance, which may increase glucose uptake by adipose tissue in an insulin-independent manner. Moreover, insulin secretion to a glucose challenge was increased, suggesting a possible reason for the reduction of GLUT 4 abundance in nutrient restricted offspring. Both nutritional interventions may induce permanent changes in mitochondrial function in the liver and adipose tissue (Gardner *et al.*, 2005; Hyatt *et al.*, 2007).

As the epidemiological observations illustrate, animal studies also suggest a strong correlation between maternal nutrition and the risk of cardiovascular diseases in later life (Barker *et al.*, 1990). Although, at 1 year of age, ovine offspring born of nutrient restricted mothers between 0 to 30 days of gestation have similar systolic and diastolic resting blood pressure, *Gardner et al*. showed evidence of altered baroreflex function and renin-angiotensin system activity (RAS) (Gardner *et al.*, 2004). This data suggests that those offspring have an increased risk of developing cardiac dysfunction in later life due to a leftward shift of the baroreceptor , which is a symptom preceding hypertension

in humans (Zidar *et al.*, 1998). The similar relationship between maternal nutrition and alterations in blood pressure is also seen in nutritional intervention at mid gestation. Whilst blood pressure in offspring exposed to maternal nutrient restriction in early to mid gestation is reduced before puberty, in late juvenile life there is a leftward shift of the baroreceptor reflex and this is followed by an increase in blood pressure relative to control offspring by adulthood (Gopalakrishnan *et al.*, 2005). In addition, at 6 months of age, these offspring have a clear reduction in nephron number, which may be an indication of early renal deficit function and later cardiovascular health decline (Gopalakrishnan *et al.*, 2004). The human epidemiological data showed similar patterns in renal and cardiovascular development to that seen in sheep studies (Zidar *et al.*, 1998). Finally, it is important to note that, in the end, these interventions had no observable detrimental effects on viability of these nutrient restricted offspring.

1.1.3.2 Fetal programming and glucocorticoids

The long term effects of intrauterine treatment to corticosteroids, including dexamethasone, have been studied extensively in the sheep model. Dodic *et al*. demonstrated an acute effect on cardiovascular activity after 48 hours' exposure to cortisol (5 mg/h) in pregnant ewes between 26 and 28 days of gestation. This overexposure resulted in high blood pressure in the offspring at 2 months postnatal age (Dodic *et al.*, 2002). Similar studies with dexamethasone (0.5 mg/h) exacerbated the induction of programmed hypertension by increasing cardiac output with age, although the same dose of dexamethasone given at 64-66 days gestation had no effect (Dodic *et al.*, 1999).

A possible explanation for this outcome of acute treatment is that the steroid intervention given by Dodic *et al*. coincided with the first stages of nephrogenesis, altering future renal functional development. Nephrogenesis in sheep starts at approximately 26 days of gestation and the process is complete at around 130 days' gestation, *i.e* before term (≈ 145 days) (Moritz *et al.*, 2005). Furthermore, it has been shown previously that the mesonephrons at 26-30 days of gestation express functional glucocorticoid receptor (Peers *et al.*, 2001b). It was suggested that cortisol or dexamethasone infusions produce increased blood pressure via glucorticoid receptors (Dodic *et al.*, 1999; Dodic *et al.*, 2002). Therefore, following exposure to dexamethasone during early nephrogenesis (0.48 mg/h, for 48 hours), the sheep not only became hypertensive, but renal morphology was also altered by a significant reduction in the number of nephrons, followed by an increase in glomerular volume at 7 years of age. This reduction in nephron numbers was associated with enlarged and dilated proximal tubes and greater accumulation of collagen type I and II in the tubular periadventitia of the renal cortical and cardiac vessels (Wintour *et al.*, 2003). In addition, studies in a similar sheep model indicated a rise of mRNA abundance for elements of the RAS in the fetal kidney and vascular vessels at 130 days of gestation (Moritz *et al.*, 2002). The hypertension was followed by alterations in cardiac output, associated with left ventricular hypertrophy (Dodic *et al.*, 1999; Dodic *et al.*, 2001).

At 127 days of gestation, the physiological results obtained from angiotensin II infusions more resembled a neonatal or adult response than a fetal response, showing an acceleration in renal maturity. Moreover, there was also a rapid increase in mRNA renal expression of the same RAS elements, including angiotensinogen and angiotensin

receptor (ATR1 and ATR2), which is commonly found in postnatal, but not fetal kidneys (Peers *et al.*, 2001a). Alterations in RAS responses persisted well after birth: at 10-14 days of age, newborn lambs showed alterations in baroreflex and vascular responses after an angiotensin challenge. The changes in cardiac activity were accompanied by an increase in sympathetic activity and a reduction in nitric oxide vasodilatory function (Segar *et al.*, 2006).

Taken together, these results suggest that early prenatal steroid over-exposure caused a premature maturation of the fetal kidney, normally associated with the end of the nephrogenesis process. In addition, exposure to glucocorticoids induces premature development of coronary artery dysfunction, triggered by alterations in RAS and other vascular regulatory hormones.

Different results were obtained when the fetus was exposed to dexamethasone or cortisol at late gestation (around 100-40 gestational days). Prenatal cortisol infusion (0.1 mg/h) caused significant increases in basal systolic and diastolic blood pressure respectively, without inducing renal morphological changes (Molnar *et al.*, 2002). Furthermore, an enhanced vascular response to angiotensin II was registered, but not to noradrenaline (Tangalakis *et al.*, 1992). Glucocorticoid infusion decreased the concentrations of fetal noradrenaline and adrenaline, which may explain, in part, the enhanced vascular sensitivity to angiotensin II (Forhead & Fowden, 2004).

As seen following early prenatatal exposure to dexamethasone, late infusions also produced microvascular dysfunction in adulthood at 5 months of age. This dysfunction was characterised by a combination of enhanced endothelin-1 vasoconstriction and abnormal independent vasodilatation, as characterised by a reduction in nitric oxide

release in the endothelium. An imbalance between these two functions may contribute to microvascular dysfunction as one of the factors leading to hypertension in these animals (Molnar *et al.*, 2002; Molnar *et al.*, 2003).

Such studies of glucocorticoid administration during fetal development provide examples of changes induced in renal development and key tissues of the cardiovascular system. However, subsequent findings in maternal nutrient restriction at early to mid gestation indicate that overexposure to glucocorticoids is unlikely within up to a 50% reduction in maternal nutrition during this period of gestation (Bispham *et al.*, 2003; Jaquiery *et al.*, 2006).

1.1.3.3 Glucocorticoid exposure and relationship to maternal nutrient restriction

The natural placental barrier to maternal glucocorticoids is regulated by placental 11-β hydroxysteroid dehydrogenase type 2 (11-βHDS 2), which converts cortisol to its inert form, cortisone (in humans and sheep). During the final stages of pregnancy until birth, in sheep and other animal species, there is a rise in circulating cortisol concentration, which accelerates the maturation of the organs and metabolic pathways, in preparation for life outside the maternal womb (Moritz *et al.*, 2005).

Previous studies of undernutrition in pregnant rats (*i.e* 50% reduction in energy intake) show an increase in maternal and neonatal glucocorticoids (Lesage *et al.*, 2001). In sheep, acute restriction in nutrient intake throughout pregnancy results in the activity reduction of 11-βHDS 2, associated with placental remodelling (Whorwood *et al.*, 2001; McMullen *et al.*, 2004). A reduction of 50% of the metabolic needs in maternal nutrition during the period of rapid placental growth (28-77 days) in sheep resulted in a decrease in 11-βHDS 2 expression in the placenta and some fetal tissues, including kidneys and

adrenals (Whorwood *et al.*, 2001). Furthermore, due the increase in abundance of glucocorticoid receptor and angiotensin 1 receptor mRNA, these tissues showed the same pattern of renal development as seen in glucocorticoid over-exposure (Whorwood *et al.*, 2001; Dodic *et al*.,1999). However, pregnant sheep NR from 28 to 80 days of gestation (50% of metabolic need during gestation), followed by a control diet after this period, showed plasma cortisol concentrations lower than their controls (Bispham *et al.*, 2003). Similar results were obtained in pregnant ewes that had been undernourished from 60 days before mating to 30 days after mating (Bloomfield *et al.*, 2004). Jaquiery *et al*. showed that, immediately after an acute decrease in nutrition from 30 days prior to mating to the first 50 days of pregnancy, only a minor increase in maternal of glucocorticoids was produced. However, the mothers quickly adapted to prolonged undernutrition and cortisol concentrations returned to basal levels (Jaquiery *et al.*, 2006). In a different study in which a moderate maternal NR was induced at early mid gestation, similar adaptation was followed by an increase in free fatty acids and a decrease in leptin, IGF-I and T_4. Bispham *et al*. suggested that this is a mechanism to reduce maternal carbohydrate oxidation and promote lipolysis, thereby maintaining glucose supply to support the normal development of placenta and fetus. Furthermore, fetuses of sheep exposed to pre-conceptional undernutrition also showed accelerated maturation of the HPA axis (Bloomfield *et al.*, 2004). Bloomfield *et al*. showed that fetal lambs, whose mothers were undernourished in the periconceptional period, had accelerated maturation of their HPA axis, resulting in a shortened gestation length, which may have an impact on health in adult life (Bloomfield *et al.*, 2004).

1.2 Obesity

Obesity has been one of the leading public health concerns in industrialised societies for the last 40 years. There is an increasing prevalence in the USA as well as in Europe and this is steadily progressing to the rest of the world. Obesity among adults has been defined by the WHO as a body mass index or BMI above 30 kg/m^2 (WHO, 2006). Children and adolescents regarded as overweight are recorded as ≥ "95^{th} percentile of the sex-specific BMI for their age growth charts" (WHO, 2003). A close analysis of the data from the National Health and Nutrition Examination Surveys (NHANES) indicates that, between 1980 and 2002, obesity prevalence doubled in adults (aged 20-74 years) and the prevalence of overweight children and adolescents (aged 6–19 years) tripled in the USA (Ogden *et al.*, 2006). In Europe, several countries also showed an increase in the prevalence of obesity, including a major increase in the United Kingdom over the last three decades. In 1980, among men (aged 16-60 years), the obesity rate was approximately 6%, rising to 15% by 1995 and 22% by 2003. More recent statistics from the WHO indicate that obesity affects 400 million adults worldwide today (WHO, 2006).

Obesity is associated with a large range of chronic diseases, including hypertension, ischemic heart disease, type 2 diabetes and a combination of these chronic diseases, now called metabolic syndrome (Hotamisligil *et al.*, 1993; McCance *et al.*, 1994; Festa *et al.*, 2000). It has been recognised that adipose tissue is an active participant in numerous physiological and pathophysiological processes. Under increasing exposure to glucose and insulin, as seen in obesity, the adipocytes are able to express and secrete several inflammatory molecules, including TNF α and IL-6

(Hotamisligil *et al.*, 1993; Fried *et al.*, 1998; Bastard *et al.*, 2002). These may result in an activation of the innate immune system, exceeding local effects in adipose tissue but also having systemic effects, such as macrophage infiltration into other tissues, including kidneys, pancreas and others. Therefore, obesity is described as low grade inflammation which may subsequently lead to the metabolic syndrome (Plomgaard *et al.*, 2007).

Previous epidemiological observations and animal models show a good correlation between suboptimal maternal environments during fetal or neonatal development, which may alter the carbohydrate metabolism of the offspring in later life, leading to obesity (Gale *et al.*, 2001; Budge *et al.*, 2004). Furthermore, there is evidence in rodents that a maternal low protein diet during pregnancy and lactation not only alters postnatal growth and appetite behaviour but also programmes the expression of lipogenic genes in the offspring (Zambrano *et al.*, 2006). In sheep, maternal nutrient restriction in late gestation enhances fetal fat deposition and reduces lean mass (Budge *et al.*, 2004). Similar results in humans exposed to a suboptimal maternal environment show a decrease in muscle growth and an increase in adipogenesis (Ravelli *et al*., 1998). In this context, it is important to first analyse, in depth, the epidemiological relationship between the prenatal environment, postnatal growth and its association with adult adiposity.

1.2.1 Epidemiological associations between maternal nutrient restriction and obesity

Widespread reports indicate a complex relationship between maternal nutritional status and offspring adult fat mass (Ravelli *et al*.,1998; Roseboom *et al*., 2000). A large

number of studies have reported that there is a correlation between birth weight and adult BMI or body composition. These reports suggest that a higher prevalence of adult obesity were found at both extremes of the birth weight distribution in human population (low birth weight ≤ 2.500 g; high weight birth ≥ 4.500 g) (Bavdekar *et al.*, 1999; Boney *et al.*, 2005). In addition, the analyses of the Dutch Famine and additional studies of human cohorts showed evidence of metabolic alterations associated with '*fetal programming'* in individuals born in the normal range of human birth weight (Ravelli *et al.*, 1999). These differences suggest that there is a much more complex association between growth in utero and obesity. For instance, there is a small prenatal inverse effect of fetal growth and the risk of developing abdominal obesity in men who had experienced a rapid period of infant growth (Kuh *et al.*, 2002; Singhal *et al.*, 2003). Others studies suggests that there is an increased risk of adult obesity among people with a low weight at birth, whose weight increases rapidly in middle and late childhood. An Indian study demonstrated that lower weight at birth is associated with obesity and other characteristics, such as higher fasting plasma insulin, higher systolic blood pressure and an increased fat mass in early childhood (Bavdekar *et al.*, 1999). Body composition, including percentage of body fat and fat distribution, may also be programmed during early development and contribute later to the risk of obesity and associated disease. For example, in a study of children aged between 6 and 9 years, low-birth-weight children have a higher percentage of fat than children with a higher birth weight, after adjustments for current weight and height. This relationship may explain the prevalence of obesity associated with cardiovascular diseases, found in these individuals in later life (Kuh *et al.*, 2002; Elia *et al.*, 2007).

Additional studies have reported the opposite: that high birth weight associated with maternal health status is the main factor for child or adult obesity. A large number of observations found a borderline correlation between heavier birth weight and maternal obesity or gestational diabetes. Bone *et al*. found that more than 50% of 11 year old children exposed to high levels of maternal insulin were considered overweight for their age group and this relationship continues in later life (Boney *et al.*, 2005). Another cohort found a small positive effect of neonatal increased weight on the adult BMI at age 33; birth weight was directly associated with maternal weight during pregnancy (Parsons *et al.*, 2001). Even with the bulk of the evidence proving the influence of maternal health, nutritional status, and placental function on fetal and birth characteristics, the influence of parental height and other genetic factors on BMI fetal programming is still unclear (Singhal *et al.*, 2003; Painter *et al.*, 2008). Data extracted from the Dutch Famine demonstrates that exposure to reduced nutrient supply (1680 to 2360 KJ/day) during early gestation only, resulted in an increase in adiposity in later life, despite birth weight being in the normal range ($\approx$ 3.3 kg). Only in the female population exposed to this famine during the first trimester of pregnancy was there an associated rise in body weight and BMI at 50 years of age (Ravelli *et al.*, 1999). Interestingly, Roseboom *et al*. found that those women who were exposed to the famine in early gestation transmitted their phenotype to the next generation (Painter *et al.*, 2008). These transgenerational effects are characterised by increased neonatal adiposity and a decline in health in later life.

1.2.2 Inflammation during obesity

Several epidemiologic studies described obesity as a low chronic inflammatory response, associated with an abnormal secretion by the adipose tissue of cytokines or adipokines, such as tumour necrosis factor-α (TNF-α) and interleukin-6 (IL-6) (Hotamisligil *et al.*, 1993; Bastard *et al.*, 2002). The over-expression of adipokines by adipose tissue contributes to the elevation of some pro-inflammatory elements, inducing an activation of the immune system (Lord *et al.*, 1998; Farooqi *et al.*, 2002; Barzilay *et al.*, 2006). This increase in adipokines has a predominant role in the development of a large number of pathologies and co-morbidities associated with obesity, such as type 2 diabetes, cardiovascular diseases (CVD) and renal dysfunction (Engstrom *et al.*, 2003).

Several observations noted large differences in the metabolic activity and cardiovascular risk associated with the location of excessive adipose tissue in the body. Depot differences between visceral (including omental and peririnal fat) and subcutaneous fat cells differ metabolically (Fried *et al.*, 1998; Tchernof *et al.*, 2006; Lee *et al.*, 2009). For example, visceral fat cells are more hyperlipolytic in response to catecholamine stimulation and less sensitive to inhibition of lipolysis by insulin than subcutaneous adipocytes (Greenberg *et al.*, 1992; Fried *et al.*, 1998; Bastard *et al.*, 2002). This particular characteristic of visceral adipocytes increases their propensity to release high levels of free-fatty-acids (FFA). In addition, their anatomical proximity to the portal vein and the generation of FFA would be critical to the function of other organs, such as the liver, by stimulating hepatic gluconeogenesis and reducing insulin clearance, leading to ectopic fat accumulation (Castell *et al.*, 1988; Luc *et al.*, 2003). Morphologically, adipocytes from the omental rather than subcutaneous fat are smaller,

which may reflect a low storage capacity for fat, leading to much quicker lipid saturation and cell dysfunction (Tchernof *et al.*, 2006; Tchoukalova *et al.*, 2007). Furthermore, visceral adipose tissue from obese subjects has also been found to secrete higher levels of leptin, TNF-α and IL-6 and lower levels of adiponectin, compared to that of lean individuals (Belanger *et al.*, 2006; Tchernof *et al.*, 2006). Results from organ tissue cultures showed that adipose tissue production of IL-6 may be inhibited by glucocorticoids and not by insulin (Fried *et al.*, 1998; Bastard *et al.*, 2002). IL-6 also stimulates adrenal cortisol release by stimulating hypothalamic CRH and ACTH. The release of cortisol may act as a feedback inhibitor of IL-6 expression from adipose tissue and this might cause an abnormal increase in cortisol secretion, a common feature of visceral obesity (Fried *et al.*, 1998). Maachi *et al*. found a significant correlation between the secretion of C-reactive protein (CRP) from the liver and circulating levels of visceral fat adipokines, TNF-α, leptin and IL-6 in obese women. Of the inflammatory markers available, CRP is the most independent predictor of cardiovascular and inflammatory events in humans and its concentration in plasma is mostly linked with obesity (Maachi *et al.*, 2004). Finally, the accumulation of visceral adiposity also increases additional biological inflammatory markers, including monocyte-chemotactic protein-1 (MCP-1) and plasminogen activator inhibitor-1 (PAI-1), which are precursors of macrophage infiltration in adipose tissue (Samad *et al.*, 1996; Sartipy & Loskutoff, 2003; Pandey *et al.*, 2005).

1.2.2.1 Adipose maturation and macrophage infiltration

Several findings indicate that the adipocytes and immune cells share a number of genes and properties. Rosen *et al*., and later Hotamisligil *et al*., showed that fat cells produce a number of components of the alternative complement activation, including adipsin-factor D and pro-inflammatory cytokines, such as TNF-α (Rosen *et al.*, 1989; Hotamisligil *et al.*, 1993). Furthermore, proliferating pre-adipocytes develop a phagocytic phenotype in response to several stimuli. *In vitro* experiments show that a possible stimulus is cell-to-cell contact between pre-adipocytes and macrophages (Cousin *et al.*, 1999; Charriere *et al.*, 2003). Following phagocytosis, the microbicide activity of pre-adipocytes, as seen in other immune cells, occurs via an oxygen dependent mechanism (Cousin *et al.*, 1999). Thus, the possibility that T-cells simulate pre-adipocytes to become active phagocytes may influence the biology of both cell types. This has important immunological implications as well as metabolic pathogenic states (Tontonoz *et al.*, 1998).

Numerous genes that code for transcription factors, such as cytokines, inflammation signalling molecules and fatty acid transporters, are essential for adipocyte biology and are also expressed in macrophages (Makowski *et al.*, 2001). Another cross-talk between the energy metabolism control and immune responses is angiotensin II that increases macrophage activity and leucocytes migration. In lean individuals, 85% to 95% of the total plasma angiotensinogen is synthesised by the liver and secreted into the bloodstream as a precursor of the vasoreactive angiotensin II protein (Van Harmelen *et al.*, 2000; Massiera *et al.*, 2001a). Angiotensinogen is also known to be expressed by adipose tissue and to play an important role in adipocyte development. Aubert *et al.*

reported that angiotensinogen expression in adipose cells is responsive only to glucocorticoids, and not insulin, as in liver cells (Aubert *et al.*, 1997). Therefore, in obese individuals, the angiotensinogen contribution from adipocytes increases by up to 60% (Van Harmelen *et al.*, 2000). The mRNA expression of angiotensinogen is enhanced multiple-fold during adipocyte differentiation in human and rodents (Massiera *et al.*, 2001; Tchoukalova *et al.*, 2007). Following exposure to angiotensin II, culture pre-adipocytes from mice and humans increased lipogenesis and triglyceride accumulation, leading to cell hypertrophy (Tchoukalova *et al.*, 2004). When the pre-adipocyes stopped proliferating and differentiated into adipocytes, the immune phagocyte-like cell phenotype disappeared. During this stage, the adipocytes secrete protaglandin I_2, a potent inducer of pre-adipocyte differentiation, suggesting that angiotensin II is implicated in the formation of new adipocytes (Tchoukalova *et al.*, 2004). In addition, it has been demonstrated that increased concentration of angiotensin II stimulates the release of TNF-α, PAI-1 and MCP-1 by pre-adipocytes, possibly triggering the macrophage infiltration of the adipocyte hypertrophy commonly associated with obesity (Kim *et al.*, 2006; Tsuchiya *et al.*, 2006; Takahashi *et al.*, 2008).

Another adipose-specific molecule that changes during adipocyte-cell differentiation and regulates the immune system is leptin. After adipocyte differentiation, the abundance of leptin mRNA increases remarkably. Lord *et al.* observed that administration of leptin to starved mice had a specific effect on T-lymphocytes, by increasing cytokine release and proliferation. In addition, leptin is able to reverse the process of adipocyte maturation induced by angiotensin II, by increasing

lipolysis. Leptin administration to ob/ob obese mice (leptin-deficient) activates gene products involved in the inflammatory process (Lord *et al.,* 1998; Farooqi *et al.,* 2002).

Immunochemical and microarray analysis of perirenal and additional depots of adipose tissue revealed that the source of these altered pro-inflammatory molecules is macrophages. These locally present macrophages are responsible for almost all adipose tissue TNF-α expression and significant amounts of iNOS (inducible nitric oxide synthesise) and IL-6 expression (Soukas *et al.*, 2000). This increase in macrophage infiltration could either represent the cause, or be a consequence, of the low-grade inflammatory state associated with obesity. The cellular and molecular mechanisms involved in this process are unknown, but it has been suggested that the activation of unfolded protein response (UPR) by hypertrophied adipocytes is involved (Ozcan *et al.*, 2004). Triglyceride accumulation enhanced adipose tissue dysfunction was followed by UPR pathway activation, which in turn perturbed inflammation signalling present in pre-adipocytes and in endothelial cells, leading to macrophage infiltration (Fig 1.2) (Ozawa *et al.*, 2005).

1.2.2.2 Insulin resistance

Following adipocyte hypertrophy, there is an elevation in fatty acids, glucose and plasma concentrations of several cytokines, characteristic of obesity and other inflammatory states. Hyperglycemia and hyperlididemia in obesity eventually contribute, in part, to the development of insulin resistance in human and several animal models of obesity (Dobrian *et al.*, 2001; Lee *et al.*, 2009) .

At a cellular level, insulin resistance starts with the inhibition by autophosphorylation of the β subunits on the thyrosine residues of the insulin receptor,

after binding to insulin. This event is followed by the subsequent inhibition of the autophosphorylation of the insulin receptor substrate family (IRS) and other additional substrates (Hotamisligil *et al.*, 1996; Paz *et al.*, 1997; Aguirre *et al.*, 2002). Thus, a chain reaction is induced by the rising concentrations of elements involved in inflammatory adipocyte responses commonly associated with obesity (Furukawa *et al.*, 2004; Maachi *et al.*, 2004). For instance, the exposure of insulin response cells to several cytokines, such as TNF and IL-6 or high concentrations of FFA, produces a strong stimulus for the inhibition of the serine residues of intracellular Insulin receptor substrate 1 (IRS-1). The phosphorylation inhibition of IRS-1 and insulin receptors reduces the ability of insulin intracellular action (Dresner *et al.*, 1999). Insulin resistance activates the mechanisms of stress by overloading the capacity of several cell compartments (Hotamisligil *et al.*, 1996; Yin *et al.*, 1998; Aguirre *et al.*, 2000). One organelle affected by insulin resistance is the endoplasmatic reticulum (ER), which has the function of synthesising and secreting several proteins. Inside the ER, a number chaperones constantly assemble and correctly fold these proteins. Exposure to cytokines or high concentrations of glucose can interfere with the ER function (Ozcan *et al.*, 2004). Obesity overloads the functional capacity of the ER, initiating the UPR mechanism. Depending on the severity of the ER stress, the cell can activate inflammatory elements, such as CHOP and GADD 153, therefore contributing to insulin resistance (Oyadomari *et al.*, 2001; Nakatani *et al.*, 2005). Another cell organelle that causes enhanced activation of the inflammation pathway is the mitochondria (Rossetti *et al.*, 1997; Furukawa *et al.*, 2004; Lin *et al.*, 2005b). Increased glucose and FFA metabolism induce mitochondria dysfunction, which leads to a rise in oxidative stress

(OxS). In obesity, there is chronic elevation of OxS which, in turn, enhances the activation of inflammatory pathways (Furukawa *et al.*, 2004).

The inhibition of the insulin pathway and the dysfunction of the mitochondria and ER induce the activation the NF-κB kinase, PCK C family, and JNK. Activation of these serine/threonine kinases demonstrates the importance of the cross-walk between the metabolic and immune pathways (Brown *et al.*, 1995; Chen *et al.*, 1995).

1.2.2.3 Lipotoxicity

The term lipotoxity is associated with the damaging effects of cellular lipid saturation on organ dysfunction, including renal disease (Carroll & Kyser, 2002). These include the involvment the of acute toxicity effects produced from cellular accumulation of long chain nonesterified fatty acids and their metabolites (fatty acyl CoA, diacylglycerol and ceramide) (Dresner *et al.*, 1999; Avramoglu *et al.*, 2003). Excess FFA availability promotes a decrease in normal oxidative metabolism, which is often followed by triglyceride accumulation in non-adipose cell organs of the body, including skeletal and cardiac myocytes, hepatocytes, pancreatic β-cells and mesangial cells (van der Vusse *et al.*, 1992; Cowley *et al.*, 2001; Wang *et al.*, 2003; Abrass, 2004; Johnson *et al.*, 2004). This process results in a chronic cellular dysfunction linked to obesity (Lowell & Shulman, 2005). The lipid deposition in non-adipose tissue is followed by low adiponectin secretion, leptin resistance or deficiency and cytokines release from the adipose tissue, inducing dysfunctional effects in skeletal muscle, liver, pancreas, heart and kidney (Yamauchi *et al.*, 2001). Lipotoxity is followed by an accumulation of neutral lipids in cells triglycerides (TG). Several observations have shown that TG themselves are used as storage. However, as toxicity comes mainly from FFA and their

by-products that accumulate as a metabolic failure of breakdown or esterification of the triglycerides, the effects of FFA metabolism vary from cell to cell and also differ between different body tissues (El-Assaad *et al.*, 2003).

1.2.2.3.1 Skeletal muscle

In skeletal muscle, the excess of FFA deposition plays a vital contribution to insulin resistance, through an elevation in intracellular fatty acyl CoA that activates PKC θ and IκB, causing inhibition of the insulin receptor substrate-1 serine phosphorylation (Dresner *et al.*, 1999). Subsequently, activation of phosphatidylinsitol-3 kinase and AKT is limited, resulting in the translocation of GLUT 4 from the cell membrane to the cytosol with a consequent reduction in glucose-insulin regulation uptake (Dresner *et al.*, 1999). This process is characterised by a shift in the source of oxidative mitochondrial metabolism from glucose to FFA by the skeletal muscle (Yamauchi *et al.*, 2001). An increase of intracellular FFA in skeletal myocytes can result in a decrease in mitochondrial density or a reduction in respiratory enzyme activity (Yamauchi *et al*., 2001). In addition, FFA deposition in non-adipose tissues produces a decrease in circulating cytokines, such as adiponectin that regulate rates of mitochondrial oxidation of FFA, reducing further the insulin action. Petersen *et al*. have shown a genetic predisposition in offspring of patients with type 2 diabetes to a reduction in mitochondrial oxidative phosphorylation. Using magnetic resonance spectroscopy, they demonstrated a reduction of 60% in muscle glucose uptake, associated with 80% of intramyocellular lipid content. It is possible to link this increase in lipid intracellular content to mitochondrial dysfunction, reflected in a 30% reduction in mitochondrial phosphorylation (Petersen *et al.*, 2004). However, a recent study

demonstrated that lipid overload *in vivo* or *in vitro* did not induce apoptosis in skeletal muscle cells, even after mitochondrial functional changes (Turpin *et al.*, 2009).

1.2.2.3.2 Pancreas

An accumulation of lipids in the pancreas elevates the expression of elements that promote endogenous fatty acid synthesis, such as sterol regulatory element binding protein-1c (SREBP-1c) in several animal models (Wang *et al*., 2003; Jiang *et al*., 2006; Wu *et al*., 2006). In cultured pancreatic β-cells, the over-expression of SREBP-1c induced an increase of lipid droplets and an inhibition in insulin secretion stimulated by glucose, followed by cell dysfunction (Wang *et al.*, 2003). Analysis of gene expression revealed that β-cell dysfunction is characterised by an abnormal expression of genes dedicated to carbohydrate metabolism, lipid biosynthesis, cell growth and apoptosis (Johnson *et al.*, 2004). A response to glucose or lipid uptake in the pancreas is an increase in the secretion of hormone-sensitive lipase (HSL), specifically in β-cell. This is reflected in an increase in lipid deposition from triglycerides (El-Assaad *et al.*, 2003). Winzell *et al*. proposed that HLS is involved in mediating β-cell lipotoxicity by providing ligands for peroxisome proliferator-activated receptor α (PPAR-α) and other lipid activated transcription factors, which in turn alter the expression of critical genes involved in free radical production, such as the uncoupling protein (UCP). Furthermore, a rise in UCP-2 abundance promoted proton leaking in pancreatic β-cell mitochondria. Thereby, the proton loss during respiration reduces the production of adenosine triphosphate (ATP) by the mitochondria, which in turn impairs glucose-stimulated insulin secretion (Winzell *et al.,* 2003). High glucose and FFA induced activation and expression of UCP-2 in the mitochondria of pancreatic cell, suppressing ATP production

normally induced by glucose. This limits the closing of plasma membrane of ATP-potassium channels, leading to mitochondrial depolarisation and increasing the calcium influx required for insulin release (Joseph *et al*., 2004). In addition, UCP-2 over-expression reduces the cytosolic ratio between ATP and superoxide production in response to glucose, which later increases mitochondrial free-radical production, and lipid overload-induced apoptosis, with a progressive loss of β-cell mass (Joseph *et al.*, 2004).

1.2.2.3.3 Liver

The increase in visceral adiposity is associated with larger amounts of lipid released in to the portal vein, directly impacting the liver. The triglycerides excess deposition leads to hepatocyte insulin resistance in a similar fashion to skeletal muscle. Therefore, the cellular pathway involved includes increased production of reactive oxygen species from lipid oxidation via mitochondrial electron transport chain, peroxisomal β-oxidation and microsomal cytochrome P450 and gene expression of TNF-α (Browning & Horton, 2004). The increase in FFA intake promotes an abnormal retention of lipids within the hepatocyte cells, further aggravated by excess FFA delivery from lipolysis in visceral adipocytes, and resulting in non-alcoholic fatty liver disease. FFA treatment of liver cells results in mitochondrial dysfunction via the translocation of Bax (a Bcl-2 protein that induces cell apoptosis usually through mitochondria membrane permeabilization) to the mitochondria and lysosomes. Destabilisation of the lysosomes releases cathepsin B, a lysosomal cysteine protease into the cytosol and this process culminates in the expression of TNF-α, involving the innate immune system activation (Feldstein *et al.*, 2004).

1.2.2.3.4 Heart

Intracellular accumulation of FFA has been proposed to play an important role in the pathogenesis of heart disease (Schwartz *et al.*, 1994). For instance, lipid deposition in the hearts of Zucker diabetic rats (ZDF) (fa/fa) induced a reduction of myocardial contractility and was associated with unresponsiveness to leptin (Zhou *et al.*, 2000). At cellular level, these animals showed an increase of lipids which was higher than that of controls and was associated with an increase in with oxidative stress (Schwartz *et al.*, 1994). *In vitro* observation demonstrated that saturated FFA and high glucose induce apoptosis of cardiac myocytes through the generation of reactive oxygen species independent of FFA synthesis (Listenberger *et al.*, 2001). Similarly, as reviewed by Rodrigues *et al.*, human diabetic cardiomypathy is associated with increase myocardial triglyceride content, increasing the risk for arrhythmia and reduction in contractile function (Rodrigues *et al.*, 1995).

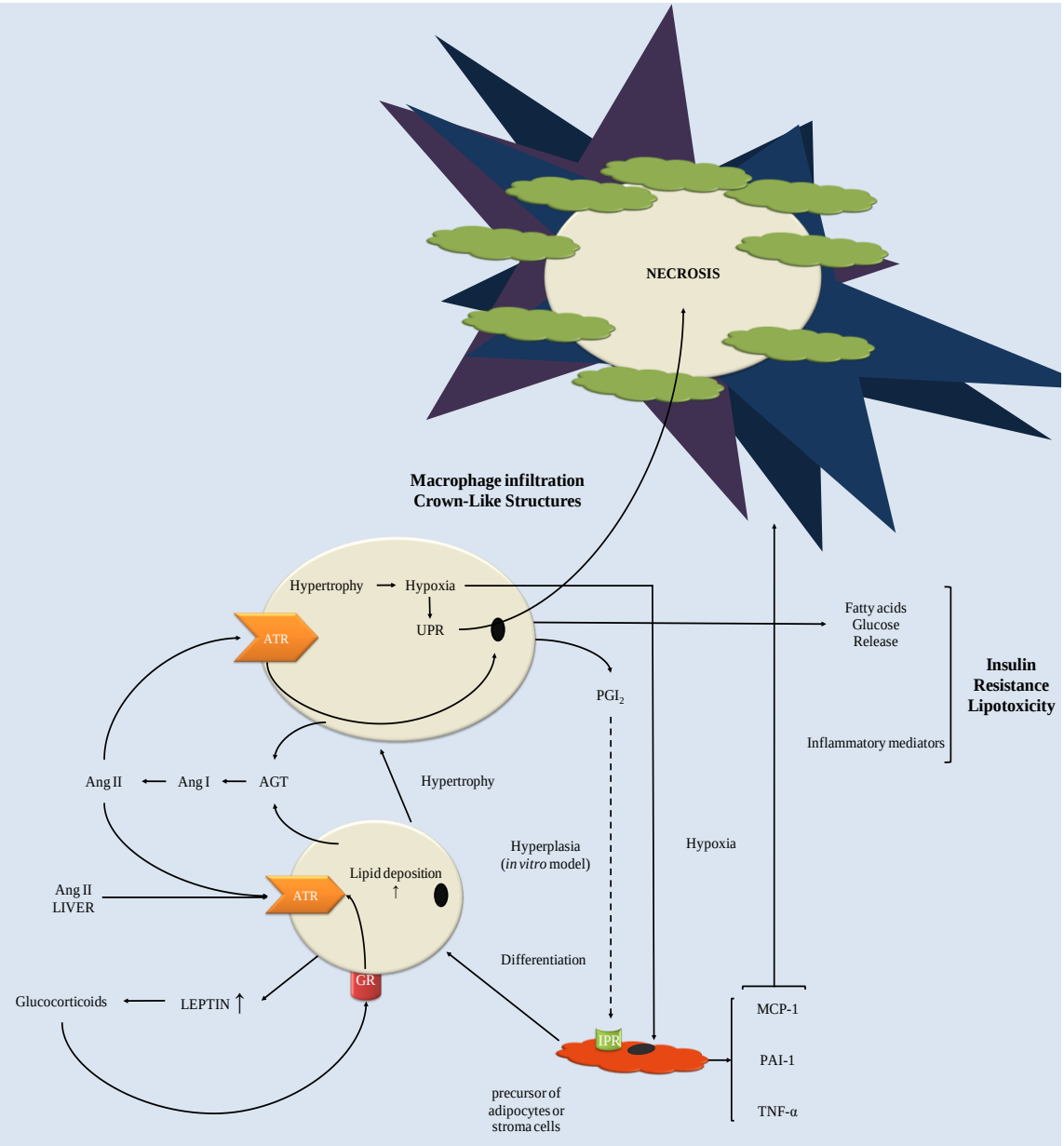

Figure 1.2 Proposed model of adipose tissue development adapted from Aihaud *et al.* 2002. AGT, angiotensinogen; Ang I, angiotensin I; Ang II, angiotensin II; IP, prostacyclin; PGI_2, prostacyclin; UPR, unfolding protein response; TNF-*α*, tumour necrosis factor; PAI-1, plasminogen activator inhibitor-1; MCP-1, monocytes chemotactic protein-1

1.3 Obesity and kidney disease

1.3.1 Epidemiology of obesity and kidney disease

The association between obesity and the development of renal diseases is well documented. In a large Japanese cohort (n > 100000), followed up after 17 years, reported that obesity increased the risk of end-stage renal disease (ESRD) among men. This relationship persisted even after adjustments for hypertension and proteinuria, suggesting that obesity *per se* is an independent risk factor for ESRD (Iseki *et al.*, 2004). Further research, based on a large population study (n > 300000 adults from both sexes) conducted by Hsu *et al.* in the USA, supported this finding and showed that excess weight remained an independent risk factor, even after adjustment for other risk factors, such as age, race, sex, education, smoking, diabetes and proteinuria. Compared with individuals of normal weight (BMI 18.5 to 24.9 Kg/m^2), the adjusted odds ratio (OR) for ESRD was 1.87 (95% CI, 1.64 to 2.14) which increased with BMI to 7.07 (CI, 5.37 to 9.31) for those with morbid obesity (BMI $\geq$ 40 kg/m^2) (Hsu *et al.*, 2006). A large cross-sectional population study demonstrated that being overweight, from the age of 20 to any time later in life, was linked with an increased risk of chronic kidney disease (CKD) for both men and women. In addition, in this cohort, the risk for CKD in obese people considerably increased the risks of hypertension and type 2 diabetes (Ejerblad *et al.*, 2006). Taken together, the data in these population-based studies shows that obesity is an independent risk factor for the development of renal diseases. Nevertheless, because of the association between obesity, diabetes and hypertension, the final impact of obesity on CKD is still unclear. For instance, as reviewed by Kincaid-Smith, hypertension and morbid obesity coexist in 50 to 70% of cases (Kincaid-Smith, 2004).

Although, metabolic and vascular dysfunction are prevalent in obese individuals, several studies have shown that renal disease is at least temporally related to obesity, independent of the development of hypertension or diabetes (Chen *et al.*, 2003b). For instance, excess weight plays a fundamental role in the development of proteinuria and renal damage in patients with severe renal mass reduction (Gonzalez *et al.*, 2005). Furthermore, it was demonstrated that obesity accelerates the increase of proteinuria in IgA nephropathy through remodelling of the glomerular membrane (Tanaka *et al.*, 2007). Therefore, these previous publications demonstrate mediate statistical and renal physiological analysis of the role of obesity as a precursor or contributor to renal diseases.

1.3.1.1 Epidemiology of metabolic syndrome and kidney disease

Epidemiological studies have demonstrated a strong association between the metabolic syndrome and several markers of kidney disease, such as an increase in microalbuminuria. A retrospective study conducted by Kurella *et al*. analysed an adult population of more than 10000 non-diabetic participants suffering with arteriosclerosis and studied their risk for the development of CKD over 9 years. They found that the metabolic syndrome reduced kidney function over time compared with healthy participants, even after adjustment for hypertension and diabetes (Kurella *et al.*, 2005). Chen *et al*. studied a cohort of more than 6000 adult participants followed over 21 years, which also demonstrated a higher risk for renal dysfunction in comparison to non-diabetic men and women with metabolic syndrome. Importantly, in this study, the relationship persisted even after the exclusion of diabetes as a trait of metabolic syndrome (Chen *et al.*, 2004a). Several clinical studies have also found that the risk for

microalbuminuria was independent of hypertension and associated with different components of metabolic syndrome, such as abdominal obesity and BMI (Chen *et al.*, 2004a; Lin *et al.*, 2007). The combination of insulin resistance, hyperinsulinemia and abdominal obesity before the development of clinical diabetes might be a critical risk factor in the development of CKD (Chen *et al.*, 2003b). Thus, metabolic syndrome and renal function may have a common starting point long before the appearance of hypertension and diabetes.

1.3.2 Renal structure and function in obesity

1.3.2.1 Kidney morphology

Fetal kidney development is a very complex process, controlled by a large number of genes in constant modification (Bard, 2002). The fetal mammalian kidney develops from the intermediate mesoderm. Kidney development, nephrogenesis, proceeds through a series of three successive phases during gestation: the pronesphros, mesonephros and metanephros (Bards, 2002). Only metanephros is a permanent structure of the kidney; the early two structures degrade during fetal growth. In the sheep, the formation of the nephrons starts at day 30 of gestation, increasing the number of functional nephrons until birth (Moritz *et al*., 2005). In humans, around the 36th week of pregnancy, approximately 1 million nephrons are formed in each kidney (Brenner *et al*., 1996). This arrest of nephron development at birth is a common feature of renal development in several mammals, including sheep, although in rats and other rodents this process extends for up to two weeks after birth *i.e.* lactation period (Moritz *et al*., 2005). As, in humans, approximately two thirds of the nephrons are developed during

the last trimester of gestation, different challenges during renal development may have a major impact on subsequent nephrogenesis.

The fully developed mammalian kidney is divided morphologically into two separate sections: the cortex and the medulla. The main function of the kidney is to filter blood and excrete foreign substances. These functions are performed by its basic unit, the nephron. These are S-shaped structures, which expand from the kidney cortex to the medulla and are composed of different cell types, such as mesangial, podocytes and endothelial cells (Brenner et al., 1996).

1.3.2.2 Changes in renal function and activity in obesity

In humans, the progression of renal changes associated with obesity is better characterised than other mammals and is usually linked with an increase in microalbuminuria and variable degrees of proteinuria (Adelman *et al.*, 2001; Kambham *et al.*, 2001; Chen *et al.*, 2004a). However, some observations have demonstrated that the first possible morphological alteration that precedes the appearance of proteinuria, in obesity without apparent renal dysfunction, is tubulointerstitial nephropathy. Rea *et al*. studied the biopsies of 49 obese and 41 non obese renal donors, who met acceptance criteria such as microalbumin excretion (< 30mg/day), fasting blood glucose (< 110 mg/dl) and normal blood pressure. They found no difference in glomerulopathy, tubular atrophy or internal fibrosis. However, the obese donors had a larger glomerular planar surface area and more tubular dilatation, than the non-obese controls (Fig. 1.3/A-E). These parameters correlated with patient weight and urinary microalbumin secretion (Rea *et al.*, 2006). In obese individuals with advanced renal insufficiency, the majority of the renal biopsy observations were negative for nephrotic syndrome but the common

feature was slow and progressive proteinuria (Praga *et al.*, 2000; Kambham *et al.*, 2001). Over a 7 year period, Praga *et al*. followed 15 obese patients who had been diagnosed with focal segmental glomerulosclerosis (FSG), but they found an absence of nephrotic syndrome, despite nephritic range proteinuria. At the end of this study, 33% of patients were on chronic dialysis and another 13% showed advanced chronic insufficiency. An estimation analysis (Kaplan-Meier) of this study reveals that, under these parameters such as BMI > 30 kg/m^2, the renal survival after 5 years was 77% and 5 years later declined to 49% (Praga *et al.*, 2001) (Fig. 1.3/H). Thereby, changes in glomerular function and structure have been termed obesity related glomerulopathy (ORG). Furthermore, Kambham *et al*. found that ORG shows an increase in patients with a BMI > 30 kg/m^2. Morphologic features include the presence of glomerulomegaly, a predominance of sclerosis lesions and less podocyte injury than non-obese FSG patients (Kambham *et al.*, 2001) (Fig. 1.3/G). In addition to the structural changes, non-diabetic obese patients showed an alteration in several haemodynamic parameters, such as an elevation in renal plasma flow (RPF) and glomerular filtration rate (GFR) (Chagnac *et al.*, 2000), possibly induced by the vasodilatory action of insulin in renal microvessels (Hayashi *et al.*, 1997). The significance of these results is unclear, but demonstrates that chronic obesity produces alterations or adaptations in renal tissues, affecting functionality and morphology.

1.3.3 Mechanisms of renal disease in obesity

The exact mechanisms by which obesity initiates the process of renal dysfunction are unknown. However, it is known that the obesogenic environment alters several inflammatory and metabolic effects and haemodynamic factors, which potentially lead to a decrease in renal functionality. A number of epidemiological studies demonstrated that inflammation is linked to metabolic syndrome in patients with CKD. A cross-sectional study of 94 CDK patients found an association between a high concentration of the inflammation marker CRP (> 3.0 mg/l) and BMI ≥ 30kg/m^2 compared to patients with normal BMI (< 25 kg/m^2) (Ramkumar *et al.*, 2004). Another larger cohort demonstrated a correlation between microabumiruria, obesity and serum CRP and that the association existed even after the exclusion of diabetes as a contributory factor (Nakamura *et al.*, 2004). The gene and immunhistochemistry analysis of glomeruli dissected from patients with obesity, proteinuria and biopsy-proven ORG demonstrated the involvement of several key genes in lipid metabolism (SREBP-1), inflammation (TNF-α and IL-6) and insulin resistance (VEGF and GLUT-1) in renal dysfunction (Wu *et al.*, 2006) (Fig. 1.3/G,F). These results emphasise the role of elements associated with metabolic syndrome in the formation of obesity-related glomerular injuries.

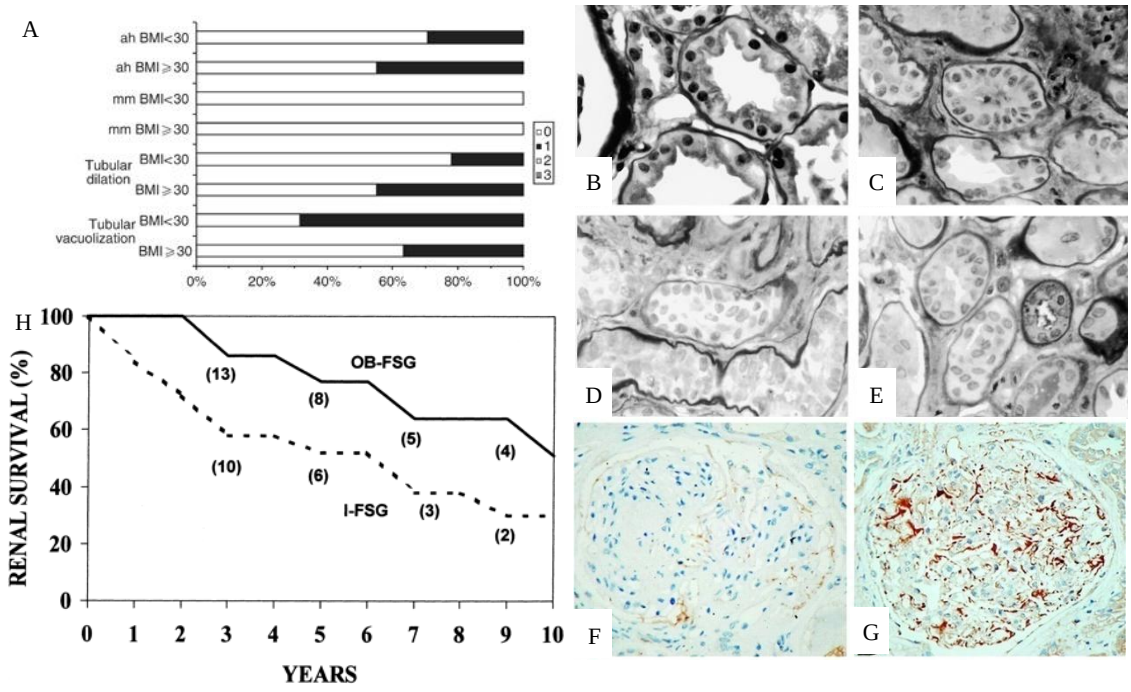

Figure 1.3 Statistics and morphology of early renal injuries in obese or over-weight subjects without diabetes. A. Distribution of histology scores of renal biopsies in obese non-diabetic donors (Rea *et al*. 2006). Renal cross sections observed in obese patients: Biopsy (B) - evidence of tubular injury. Pictures (C-E) - evidence of obesity associated tubular dilatation or cellular hyperplasia (Herbert *et al*. 2000) (original magnification x 100; PAS staining). By contrast, the second biopsy (F:control; G: obese patient) was immuno-stained with VEGF-A antibodies and demonstrates a typical obesity-related glomerulopathy (original magnification x400). H (Kaplan-Meier analysis) - the increased statistical risk of renal failure in years among obese-associated focal glomerulosclerosis (OB-FSG) in relation to non-obese patients suffering from the same disease (I-FSG). Number in brackets represents the patients at risk at every period (Paga *et al*., 2001).

1.3.3.1 Role of cytokines

1.3.3.1.1 Leptin

Five leptin receptors are known to be active in the kidney, only two of which are relatively abundant (ob-Ra and ob-Rf) and located in the inner medullar region (Lee *et al.*, 1996; Vaisse *et al.*, 1996; Wang *et al.*, 1996). However, fewer leptin receptors detected are in other areas of the kidney, including the glomeruli, proximal and distal tubules and terminal collecting ducts (Serradeil-Le Gal *et al.*, 1997). Cell culture studies demonstrate that leptin stimulates the growth of glomerular, but not mesangial, cells, indicating a specific effect on cell proliferation. In rats, infusion of leptin also stimulates mRNA expression and production of the profibrogenic cytokine TGF-β1, an increase in glomerular collagen type IV renal deposition, glomerulosclerosis and proteinuria (Wolf *et al.*, 1999). In addition, leptin increases glucose uptake and type I collagen in obese, leptin-deficient mice. Hans *et al*., also postulated that leptin activates the interglomerular TGF-β system through its receptors, thereby contributing to glomerulosclerosis in obesity (Han *et al.*, 2001). Therefore, the distribution and activity of leptin appears to have a direct effect on renal structure and function, possibly playing a role in glomerulosclerosis.

After its secretion by the adipose tissue, leptin is removed from the plasma by the kidneys, primarily by glomerular filtration (Sharma *et al.*, 1997). Cumin *et al*. infused lean rats with leptin and demonstrated that leptin is extracted intact and digested by the kidney. Furthermore, leptin was only present in small quantities in urine, indicating its metabolic degradation in the renal tubules. After binephrectomy, plasma leptin concentrations did not increase and its degradation rate was unchanged, suggesting that

the capacity for leptin extraction by the kidneys was not overwhelmed (Cumin *et al.*, 1997).

Leptin may also affect the kidneys directly, by increasing sympathetic nerve trafficking and inducing renal sodium retention, which can cause hypertension (Hall *et al.*, 2004). Furthermore, hyperleptinemia induces abnormal sodium handing and decreases nitric oxide production by an increase in systemic and intrarenal oxidative stress, an effect that may promote hypertension (Beltowski *et al.*, 2004). Controversially, Stenvinkel *et al*. have shown, in severe chronic kidney failure patients, that serum leptin increases at the initiation of peritoneal dialysis treatment and is inversely related to inflammation and followed by changes in lean body weight. These may possibly explain the survival advantage for obese patients with ESRD (Stenvinkel *et al.*, 2000). In addition, leptin appears to be an independent predictor for cardiovascular events (even after adjustment for CRP concentration and other metabolic risk factors) (Wallace *et al.*, 2001). Thus, the increase in secretion of leptin by adipose tissue would appear to play an important role in endothelial dysfunction, leading to deterioration in renal structure and function.

1.3.3.1.2 TNF-α

Cunningham *et al*. demonstrated that TNF-α is a key mediator in the development of acute renal failure by the induction of an inflammation response to bacterial endotoxines. The effects of TNF-α associated with renal fibrosis are mediated through both receptors, TNFR1 and TNFR2, with TNFR1 playing a predominant role. After binding to TNFR1, macrophage infiltration results, leading to renal apoptosis (Cunningham *et al.*, 2002). One of the possible mechanisms involving TNF-α/TNFR1 is

activation of the innate immune system by the increase in reactive oxygen species (ROS), such as oxygen superoxide. This, in turn, induces the recruitment of additional cytokines such as MCP-1 (Chen *et al*., 2004). Furthermore, the neutralisation of endogenous TNF-α reduces glomerular inflammation and injuries in glomerulonephritis (Khan *et al.*, 2005).

1.3.3.2 Insulin and IGF-1

The early progression of renal disease in obese pre-diabetic Zucker rats (fa/fa rats) started at 6 weeks with the appearance of podocytes but no mesangial cell injury. This process was followed by a glomerular infiltration of monocytes and macrophages associated with hypertrophy, as well as secondary tubuleinterstitial damage. Therefore, in obese fa/fa rats, the first signs of glomerular hypertrophy appeared in conjunction with hyperinsulemia, without hypertension or renal matrix deposition and before additional indicators of renal dysfunction (Coimbra *et al.*, 2000). Therefore, these results demonstrate the importance of insulin as an early factor in renal dysfunction. As a weak vasodilatator, a chronic high concentration of insulin does not cause hypertension. However, insulin increases endothelial dependent vasodilatation, which could contribute to glomerular hypertension, leading to a slow process of renal dysfunction. High plasma insulin concentrations significantly increases sodium retention, without producing changes in blood pressure (Brands *et al.*, 2009). *In vitro* observations indicate that insulin and insulin-like growth factor-1 (IGF-1) share common pathways involving proliferation of mesangial cells, altering the glomerular structure (Abrass *et al.*, 1988; Hiromura *et al.*, 2002). In addition, high doses of IGF-1 and insulin induce an increase in secretion of extracellular matrix proteins by mesangial and tubular cells affecting

glomerular architecture and functionality (Abrass *et al.*, 1994; Hirschberg, 1996). After exposure to insulin *in vitro*, mesangial cells chronically maintained the production of these pro-inflammatory collagen proteins and also changed cell morphology, suggesting permanent modifications in gene expression (Abrass *et al.*, 1994; Abrass *et al.*, 1995). In part, the secretion of collagen type I and IV can by reduced *in vitro* by exposure of the renal tubules to IGF-1 receptor antibodies (Hirschberg, 1996). The histological analysis of transgenic knockout glomerular IGF-1 receptors in mice has shown the formation of abnormal and small glomeruli. This study suggests that IGF signalling is essential in maintaining the function of the podocytes as keepers of the integrity of the renal structure, an alteration that may play an important role in the development of glomerulosclerosis (Bridgewater *et al.*, 2008).

Insulin seems to interact with ANG II, increasing the contractile response in cultured mesangial cells. Kreisberg *et al*. suggested that this loss of contractile response by mesangial cells, induced by a reduction in insulin production, could lead to an increase in glomerular flow which may result in glomerulosclerosis (Kreisberg, 1982). In addition, treatment with IGF-1 (100nmol/L) for 1 to 4 weeks resulted in mesangial cells which failed to contract normally in response to ANG II. These effects are due to an increase in lipid accumulation, as lipid removal restored the contractile response (Berfield *et al.*, 2002; Berfield *et al.*, 2006). Furthermore, lipid saturated mesangial cells were unable to migrate in response to IGF binding protein 5, an essential response to glomerular injury and they lost phagocyte phenotype, an indicator of an increase in the severity of the injury (Berfield *et al.*, 2002).

1.3.3.3 The renin angiotensin system

The RAS is an enzymatic cascade in which angiotensinogen is transformed first to angiotensin I (ANG I) and later to ANG II by the actions of renin and angiotensin converting enzyme (ACE). In obese individuals and animal models, these proteins are produced in excess by adipose tissue and the liver despite sodium retention and an apparent increase in extracellular fluid volume (Fig.1.1) (Hall, 1994; Engeli *et al.*, 2005).

One of the components of this system is ANG II, known to be associated with the progression of renal injury. In rats, after renal mass reduction, it has been possible to detect an increase in hypertension parallel to a rise in oxidative stress (Hasdan *et al.*, 2002). A specific *in vitro* study demonstrated that ANG II dependent cell contraction in rat micro-tissue strips from the medulla is mediated by the generation of oxygen superoxide anions ($O_2^{\cdot -}$)(Mori & Cowley, 2003). Zafari *et al*. have shown that ANG II induced a rise in reactive oxygen species in vascular cell cultures through an increase in NADPH oxidase activity linked to cell hypertrophy (Zafari *et al.*, 1998). ANG II also induces IL-6 synthesis which is mediated by lipoxygenase, a peptide associated with the inflammatory response (Luchtefeld *et al.*, 2003). The $O_2^{\cdot -}$ has been found to be the main cause of the reduction in nitric oxide vasodilatory activity. In patients with renovascular hypertension, there is impairment in the endothelium-dependent vasodilatation, mediated in part by an increase in oxidative stress (Higashi *et al.*, 2002). Previous studies in rats have shown that infusions of ANG II induce different effects on the kidneys, depending on dose and duration of the treatment (Lombardi *et al.*, 1999; Welch *et al.*, 2005). In renal cortical tissue, low doses (200ng/kg x min/sc) of ANG II for two

weeks cause hypoxia, which is followed by inefficient use of oxygen. At these concentrations, ANG II may induce a mild hypoxia through reduced renal oxygen use as well as enhanced NO production and reduced superoxide generation, at least in the renal cortex. However, in the renal cortex at the end of this treatment, ANG II exposure causes severe hypoxia and increases the generation of $O_2^{\cdot -}$, inducing apoptosis by increased NADPH oxidase in the renal cortex (Welch *et al.*, 2005). In the medulla, chronic infusion of ANG II increases the synthesis of NO without generating oxidative stress and is accompanied by a reduction in contraction (Zhang *et al.*, 2005). In addition, rats exposed to ANG II (435ng/kg min) over two weeks experience structural and functional changes, leading to salt sensitive hypertension. The infusion is associated with acute hypertension, renal dysfunction and vascular damage. At sites of renal injury, there was a reduction in nitric oxide synthetase abundance both in the medulla and cortical (Lombardi *et al.*, 1999). Therefore, these specific regional effects of chronic ANG II on the kidney may increase the expression of markers of oxidative stress. Brenner *et al*. demonstrated that blocking of the Ang II type I receptor with losartan significantly reduced the development of proteinuria, in patients with type 2 diabetes and nephropathy (Brenner *et al.*, 2001). Similar treatment reduced the mRNA abundance TNF-α, PAI-1, MCP-1 and additional markers of oxidative stress in adipose tissue of obese rats, thereby ameliorating the effects of obesity (Kurata *et al.*, 2006). Some human studies support these findings: Giacchetti *et al*. observing regional adipose differences in gene expression of components of the RAS. In visceral adipose tissue, there was raised expression of angiotensinogen and the ANG II type 1 receptor compared with subcutaneous fat of both obese and lean individuals. Thereby, the

authors suggested that the presence of these RAS components in visceral adipose tissue may play an important role in the development of renal dysfunction in obesity (Giacchetti *et al.*, 2002). Moreover, higher plasma concentrations of angiotensinogen, rennin and aldosterone and ACE activity levels were found in obese compared to slim menopausal women and these were reduced by later weight loss (Engeli *et al.*, 2005). However, the gene expression of RAS components in visceral fat was not influenced by weight loss (Engeli *et al.*, 2005). Finally, the increase in RAS components and, in particular, ANG II by obesity may affect renal function and structure through an increase in inflammation, hypertension and an increase in lipid metabolism.

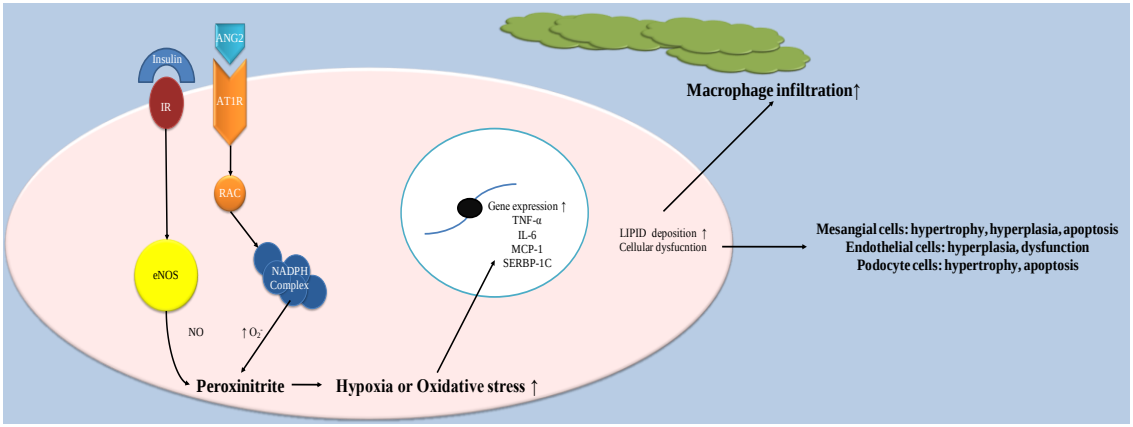

Figure 1.4 Graphical representation of cellular dysfunction in renal tissues proposed in this section and the different fate for same of different cells type residing in the renal tissues.

1.3.4 Haemodynamic role in renal disease

1.3.4.1 Hypertension

As reviewed by Montani *et al*., obesity predisposes to hypertension, which leads to significant structural and functional renal alterations, such as increased glomerular filtration rate (GFR), renal plasma flow (RPF) and urinary albumin excretion (Montani *et al.*, 2002). During the course of obesity, there is an increase in systemic levels of FFA, leptin and insulin, which in turn may increase activity of the sympathetic nervous system (Kassab *et al.*, 1995). The conjunction of these factors promotes vasonconstriction, sodium and water retention (Montani *et al.*, 2002). As obesity progresses, there is an induction of insulin resistance, which is followed by cytokine production, exacerbated endothelial dysfunction and a rise in vascular tone (Hotamisligil *et al.*, 1993). In addition, the increase in lipid deposition in adipose tissue and the sympathetic activation increases the synthesis of RAS components, resulting in further sodium and water retention. The combination of these factors is the main promoter of hypertension associated with obesity (Giacchetti *et al.*, 2002).

1.3.4.2 Hyperfiltration

In the first stages of chronic renal disease, there is usually an elevation in GFR along with an increase in RPF, which has been observed in animal models and non-diabetic obese patients (Chagnac *et al.*, 2000; Williams *et al.*, 2007). These haemodynamic alterations probably occur as a result of a change in arteriolar/efferent dilatation balance and an increase in glomerular pressure. These effects are likely to be mediated by a rise in RAS component activity and abnormal sodium sensitivity

(Henegar *et al.*, 2001; Deji *et al.*, 2009). In addition, these changes in haemodynamic features are followed by morphological alterations, including renal sclerosis and the consistent presence of glomerulomegaly (Deji *et al.*, 2009). In dogs, obesity is followed by a rise in GFR and RPF and renal structural changes, such as matrix expansion and glomerulosclerosis (Henegar *et al.*, 2001). Moreover, Kassab *et al*. observed an increase in cumulative sodium retention as a possible response to a high fat diet, in part, due to enhanced tubular reabsorption by sympathetic activation. Hall *et al*. proposed that this may result in reduced salt delivery to the macula densa, increasing the activity of RAS and in turn leading to hypertension (Hall, 1994). Moreover, weight loss in severely obese subjects without overt renal disease was followed by improved glomerular haemodynamics. These improvements were associated with a decrease in blood pressure and GFR (Chagnac *et al.*, 2003). Therefore, the elevated obesity-related hyperfiltration induces slow, progressive renal dysfunction, particularly when obesity and hypertension are simultaneously present.

1.3.5 Renal lipotoxicity

There is an strong association between inflammatory renal diseases and the accumulation of lipids the renal on the epithelium (Kimmelstiel, 1936). Recently, several animal models supported the possibility that diet-induced obesity increased renal lipid accumulation and can cause glomerulosclerosis (Jiang *et al.*, 2005; Proctor *et al.*, 2006). Generally, FFAs are attached to albumin which, in the kidneys, is filtered through glomeruli and reabsorbed into the proximal tubes. Kamijo *et al*. demonstrated that FFA binds to albumin and the interaction of these two is the main contributor for nephrotic syndrome through reabsorption into the proximal tubes (Kamijo *et al.*, 2002).

The mechanisms of incorporation of non-polar lipids and promotion of lipotoxicity involve proximal tubular cells, which in turn, activates the innate immune system inducing an inflammatory response leading to cell apoptosis (Arici *et al.*, 2002). An additional mechanism of lipotoxicity involves the exposure of mesangial cells to TG-rich lipoproteins, such as LDL. As a consequence, the mesangial cells undergo cell proliferation, whereas oxidised LDL has a cytotoxic effect (Nishida *et al.*, 1999). Furthermore, oxidised LDL stimulates the secretions of extracellular matrix and cytokines such as IL-6 and TNF-α from mesangial cells (Nishida *et al.*, 1999). In addition, as previously described, the over-exposure to IGF-1 accelerates the uptake of lipids in rat glomerular mesangial cells, which become lipid-laden foam cells. Therefore, lipid uptake is mediated through increased endocytosis and its accumulation affects cellular organisation, which causes impairment in normal cell function such as phagocytosis and migration (Berfield *et al.*, 2002). Over-accumulation of lipids by mesangial cells reduces the contractile response to ANG II. Following removal of the excess in lipid from these mesangial cells, the contractile response is restored (Berfield *et al.*, 2002). Administration of ANG II to rats induces the expression of SREPB-1 and results in renal lipid accumulation of TG and cholesterol. There was also a significant increase in the expression of factors associated with renal dysfunction and inflammation, such as VEGF and collagen IV, leading to glomerulosclerosis and proteinuria (Jiang *et al.*, 2005). Changes in ANG II metabolism are also associated with an abnormal accumulation of renal iron and increased amounts of superoxide. Several studies demonstrated that increase FFA induces renal dysfunction through an enhanced generation of ROS. In obese ZDF prediabetic rats, there is an increase in circulating

FFA, which promotes the activation of NADPH oxidase, a major producer of vascular ROS. In this model of obesity, there is a reduction in aceltylcholine as a vasodilator, involving a possible reduction in NO as the main cause of endothelial dysfunction (Chinen *et al.*, 2007). Short term elevation in FFA (2 hours) in healthy insulin-sensitive subjects induces endothelial dysfunction via a reduction in NO production and action (Steinberg *et al.*, 1997). The increase in oxidation may have a modulatory effect on the expression of genes related to aging and fibrogenesis (Saito *et al.*, 2005). Jiang *et al*. have found that the inhibition of lipogenic genes in mesangial cells also reduced the expression of genes involved in inflammation and modulation of fibrosis (Jiang *et al.*, 2007).

1.4 The kidneys and oxidative stress

1.4.1 Concept of oxidative stress

ROS are derivates of oxygen metabolism produced by all living organisms (Gardner & Fridovich, 1992). These oxygen species are short lived and, because of their high reactivity, are able to interact with all surrounding macromolecules, including lipids, carbohydrates and nucleotides. When the production of ROS exceeds the antioxidant capacity of the cell, it starts a degenerative process called denominated oxidative stress (OxS) (Chen *et al.*, 2004b). Thereby, due to its cytotoxicity and reactivity, an excess of ROS can induce several processes associated with cell damage, including lipid peroxidation, DNA and protein modification (Bae *et al.*, 1997). Production of ROS also has important functions in the cell cycle and is involved in several biochemical processes, including cellular differentiation, growth arrest,

apoptosis, immunity and inflammation (Ding *et al.*, 2000; Levy *et al.*, 2000; Chen *et al.*, 2004b). The classification of ROS also includes oxygen metabolic by-products of oxygen metabolism, such as superoxide ($O_2^{\cdot-}$) and hydrogen peroxidate (H_2O_2), along with reactive nitrogen species, such as nitric oxide (NO) and peroxynitrite radical ($ONOO^{\cdot}$) (Beckman *et al.*, 1990). Although ROS are short-lived molecules, their detection is generally based on the trace of modified end products. Thus, during lipid peroxidation, unstable products of the reaction between FFA and $O_2^{\cdot-}$ break down into smaller and more stable products, including malonyldaildehyde (MDA), acrolein, 4-hydroxynonenal (HNE) or better denominated thiobarbituric acid substances (TBARS). Additional examples of ROS-damaged products include oxidised nucleic acid bases 8-hydro-deoxyguanosine (8-OHdG) and oxidised proteins (nitrated tyrosines). Moreover, the reaction between NO and $O_2^{\cdot-}$ decreases its vascular function as a vasodilator, leading to metabolic dysfunction, which has been implicated in inflammation and early renal damage (Ece *et al.*, 2006). The reaction between $ONOO^-$ and proteins results in the nitration of tyrosine residues and the formation of 3-nitrotyrosine, which could potentially lead to vascular damage (Amirmansour *et al.*, 1999). At the molecular level, the nitration of tyrosine may alter protein function, initiating protein damage. Thus, the presence of 3-nitrotyrosien has been used as a marker for $ONOO^-$ (van der Vliet *et al.*, 1995).

1.4.2 The antioxidant System

All aerobic organisms possess a complex antioxidant defence system, which can be divided into two main components: a group of antioxidant enzymes and organic compounds. The first group consists of a coordinate enzymatic cascade, which includes

the superoxide dismutases (SOD), catalase (CAT) and glutathione peroxidise (GpX) (Fig. 1.5). The second group is a non-enzymatic group, which includes the vitamins C, E, β, reduced glutathione and other compounds.

The first enzyme to be activated in the enzymatic cascade is SOD, which is able to convert $O_2^{\cdot-}$ to a the less reactive form H_2O_2 ($O_2^{\cdot-} + O_2^{\cdot-} + 2H \rightarrow H_2O_2 + O_2$) (Gardner & Fridovich, 1992). There are three SOD isoforms in mammals: cytosolic Cu/Zn SOD, mitochondrial Mn-SOD and extracellular EC-SOD (Mates *et al.*, 1999) and all these enzymes are able to neutralise $O_2^{\cdot-}$ at very high reaction rate (Meier *et al.*, 1998). At a cellular level, there are two main sources of $O_2^{\cdot-}$: one is the respiratory chain in the mitochondria and the other is the activation of NADPH by cytokines or RAS (Chen *et al.*, 2004b). SOD in endothelial cells has a protective role, attenuating the effects of induced superoxide anion by cytokines, such as TNF-α. These results suggest that SOD has anti-inflammatory properties and may play an important role in the reduction of the inflammatory response (Lin *et al.*, 2005a). Moreover, EC-SOD has an important role in maintaining the extracellular oxidant/antioxidant equilibrium, thus regulating the adaptation of several tissues, including the kidneys, to a low oxygen environment (Suliman *et al.*, 2004). The by-product of the neutralisation of $O_2^{\cdot-}$ by SOD is H_2O_2. This molecule, unlike $O_2^{\cdot-}$, can diffuse across membranes (Antunes & Cadenas, 2000) and is also a highly reactive free radical. Two enzymes, CAT and GpX, are involved in the neutralisation of H_2O_2. CAT transforms H_2O_2 to water and molecular oxygen ($2\ H_2O_2 \rightarrow CAT \rightarrow\ 2\ H_2O + O_2$) very efficiently. There are five GpX isoforms expressed in mammals, and the abundance of each type varies according to the tissue. All of them are found in the kidneys (de Haan *et al.*, 1998). Although GpX shares the substrate with

CAT, these enzymes also have the capacity to reduce lipoproteins and other hydroperoxides to their corresponding hydroxylated compounds, using glutathione as a hydrogen donor (ROOH + 2GSH →GpX→ROH + GSSG + H_2O). Furthermore, the GpX enzymes are able to neutralise 95% of the total H_2O_2, being the major source of protection against low levels of OxS (Sies *et al.*, 1997).

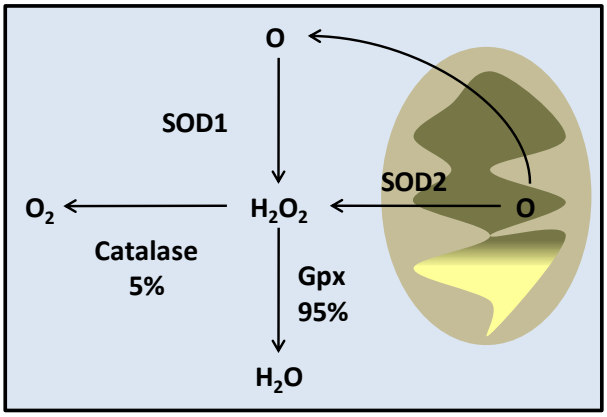

Figure 1.5 Superoxide neutralisation by superoxide dismutase family (SOD)

1.4.3 Vascular endothelial cells and nitric oxide production

Vascular endothelial cells have a number of important functions. They provide a natural barrier between the vessel wall and the lumen and maintain vascular tone by secreting a number of mediators. Thus, the dysfunction in the endothelium induces several processes, including platelet aggregation and coagulation, involved in cardiovascular health. Endothelial cells produce a large number of compounds, which mainly induce vasoconstriction, including endotheilin-1 or vasodilatation, such as nitric oxide (NO).

A number of observations demonstrate that endothelial NO production is induced by insulin-receptor pathways and the uptake of glucose (Montagnani *et al.*, 2002; Chen

et al., 2003a). By this action, insulin stimulates the endothelial cells to produce NO, which is a potent vasodilator and anti-thrombotic mediator. Additional metabolic components, such as adiponectin, also have vascular actions by stimulating the production of NO in endothelial cells (Chen *et al.*, 2003a). Furthermore, insulin NO mediated stimulation can be suppressed by IL-6. In vitro exposure to IL-6 results in impaired IRS-1 phosphorylation and in a reduction of NO production in endothelial cells (Andreozzi *et al.*, 2007). Additional evidence indicates that ANG II is also able to interfere in NO generation through the insulin pathway (Andreozzi *et al.*, 2004). Finally, the anti-diabetes drug, metformin, restores the NO insulin mediated synthesis (Davis *et al.*, 2006). These observations suggest a link between obesity-insulin resistance and NO secretion.

1.4.3.1 Nitric oxide synthase (NOS)

Nitric oxide (NO) is a key biological messenger and one of the few known gaseous signalling molecules. This molecule plays a major role in several biological processes, such as vasodilatation and the inhibition of platelet aggregation. NO, also known as the endothelium-relaxing factor (EDRF), is biosynthesised endogenously by a family of enzymes called nitric oxide synthase (NOS).

There are three major isoforms of NOS: inducible (iNOS), neural (nNOS) and endothelial (eNOS). In addition, the activation of each of these enzymes can be induced by four other cofactors to produce NO, including flavinmononucleotide (FMN), calmodulin, bihydrobiopterin (BH_4) and flavin adenine dinucleotide (FAD).

The most common reaction used in the production of NO involves the oxidation of the nitrogen atom of L-arginine, which is catalysed by all three isoforms of NOS. ROS

can consume NO in a series of reactions that leads to a reduction in its vasodilatation properties (Nathan, 1992). Exposure to ROS can transform NO to other forms of reactive nitrogen species such as nitorsonium cation (NO^+), nitroxyl anion (NO^-), or peroxynitirtite ($ONOO^-$). Oxygen reacts with NO to produce $ONOO^-$, which may dissociate into NO and $OH^\cdot$ ONOO oxidises the zinc-thiolate centre of NOS, producing a decrease in NO generation. Furthermore, H_2O_2 reacts with several renal proteins containing iron, producing hydroxyl radical (HO.), which in turn produces biologically inactive forms of NO, such as nitrite NO_2 and nitrate NO_3 (Haber-Weiss reaction) (Beckman *et al.*, 1990). Thus, a decrease in NO activity is associated with OxS and hypertension.

In rats with induced chronic myocardial infarction, an increased production of NO by eNOS was observed. However, the increased formation of $O_2^{\cdot -}$ inactivated most of the NO and this process was only reversed by treatment with exogenous superoxide dismutase (SOD). This observation demonstrated that the affinity between NO and $O_2^{\cdot -}$ is higher than the affinity of $O_2^{\cdot -}$ by SOD and this accelerated its degradation (Bauersachs *et al.*, 1999).

An additional modulation in the production of NO is a decrease in substrate, cofactors or toxins (Shimokawa *et al.*, 1991; Pou *et al.*, 1992). For instance, pertussis, a toxin associated with porcine artery injuries, is able to alter the cell signal of eNOS leading to a reduction in NO production (Shimokawa *et al.*, 1991). Diet can also regulate NO production. Ren *et al*. have shown, during low NaCl intake in rabbits, that nNOS function alters renal afferent arteriole diameter in the macula densa. This data indicates that macula densa nNOS is activated by low NaCl, suggesting that NO brings

the glomerular filtration rate to an appropriate level during renin secretion, a result of low sodium intake (Ren *et al.*, 2001). Increased NO production by nNOs can also reverse OxS and hypertension through improved parasympathetic activity in spontaneously hypertensive rats (SHR) (Heaton *et al.*, 2007). The oxidation of cofactor BH_4 reduces its capacity to be used in the production of NO. The absence of BH_4 leads eNOS to produce $O_2^{\cdot -}$ instead of NO, starting a process called NO uncoupling, which leads to an increase in OxS (Kerr *et al.*, 1999). Thus, NO has important roles in the kidney, including the regulation of tubular NaCl transportation, modulation of renal sympathetic nerves and cell antioxidants.

1.4.3.1.1 Production of NO

As reviewed by Higashi *et al*., mechanical forces, such as laminar and oscillatory shear stress, lead to alterations in endothelial function mediated through changes in NO bioavailability. Several studies observed that laminar shear stress, through an increase in physical activity, increased NO production and had an important role in the regulation of vascular tone (Higashi *et al*., 2002). In vitro data indicates that shear stress in endothelial culture cells stimulates the gene expression of eNOS and the production of NO (Uematsu *et al.*, 1995). In dogs, shear stress induced by 10 days of exercise increased the expression of eNOS mRNA and protein abundance in epicardial coronary arteries, leading to acetylcholine induced release (Sessa *et al.*, 1994). Oscillatory shear stress results in increased production of ROS via NADPH (Vaziri *et al.*, 2003). Although there is a large body of evidence about the effects of these forces in vascular tissues, less is known about the kidney. *In vitro* studies in podocytes demonstrate that mechanical stress can lead to glomerulosclerosis through a re-organisation of the actin

cytoskeleton, elevating the probability of podocyte detachment (Friedrich *et al.*, 2006). Furthermore, exposure to continuous shear stress leads to an activation of ATR1 mRNA expression in cultured podocytes and in turn to cell apoptosis (Durvasula *et al.*, 2004).

An additional pathway that contributes to the production of NO is the insulin/IGF1. The activation of this pathway promotes vasodilatation by activating the insulin receptor through PI3-Kinase/AKT that leads to the activation of eNOS, starting the secretion of NO (Montagnani & Quon, 2000). Thereby, IGF-1 and insulin decrease vascular contraction. The local mechanisms by which these hormones may decrease vascular reactivity include the partial release of nitric oxide (NO) (Hayashi *et al.*, 1997). An increase in NO generation is observed in early stages during streptozotocin treatment in diabetic rats, which show signs of glomerular hyperfiltration, due to greater expression of glomerular endothelial nitric oxide synthase (eNOS) (Veelken *et al.*, 2000). In addition, insulin also promotes vascular contraction by inducing an increase in cytosolic calcium. Thereby, the contractive actions of ANG II in mesangial cells might be attenuated by an increase in insulin resistance. Thus, insulin and IGF-1 are active in promoting vascular relaxation and contraction, in part via increases in NO bioavailability and ANG II activity (Kreisberg, 1982; Standley *et al.*, 1991).

1.4.3.2 NADPH oxidase family and HIF system

Several studies demonstrate that the different isoforms of NADPH are the main source of ROS, thereby having an important role in the development of renal and vascular dysfunction (Zafari *et al.*, 1998). The first member of the NADPH family to be discovered was the phagocyte $Gp91^{phox}$ (phagocyte oxidase), presumed to be activated as part of the vessels' response to injury and inflammation. Additional studies have

shown the existence of different $Gp91^{phox}$ isoforms, which are expressed in other cell types, including: fibroblast, endothelial cells and smooth muscle cells. To avoid confusion, the enzymes in these cells are called Nox (non-phagocyte NADPH oxidase) (Touyz *et al.*, 2002; Chamseddine & Miller, 2003).

NADPH oxidase is a multiple protein electron transport system that includes the membrane-bound flavocytochrome b_{559} (formed by $gp91^{phox}$ and $p22^{phox}$) and three cytosolic proteins ($p47^{phox}$, $p67^{phox}$ and Rac 1 or 2). Flavocytochrome b_{559} bears the NADPH binding site and a redox centre (FAD and heme). Electrons flow through the redox centres, where NADPH transforms oxygen to superoxide (Koshkin *et al.*, 1997). Shiose *et al*. have reported a novel NADPH homologue, Nox 4 that is abundant in adult and fetal kidneys (Shiose *et al.*, 2001). Later tissue and cell analysis revealed the expression of all additional members of the Nox family, including Nox 1, Nox 2, Nox 3 and Nox 5, in renal tissues (Suh *et al.*, 1999; Cheng *et al.*, 2001; Shiose *et al.*, 2001).

ANG II regulates these enzymes by phosphorylating the $p47^{phox}$ unit, inducing the subsequent activation of NADPH oxidase and the generation of ROS (Touyz *et al.*, 2002). It is well established that the ROS produced from Nox by over-exposure to ANG II has hypertrophic and proliferative effects on mesangial cells and fibroblasts (Chamseddine & Miller, 2003; Gorin *et al.*, 2003). Infusion of ANG II for 7 days in rats (1mg/Kg/d) causes endothelial dysfunction by raising vascular superoxide production and increasing the expression of NADPH oxidase subunit $p22^{phox}$, Nox1 and $gp91^{phox}$. The infusion also led to eNOS uncoupling, which may further enhance oxidative stress in vascular tissue, thereby decreasing NO bioavaibility (Mollnau *et al.*, 2002).

In acquired forms of hypertension in rodents, such as surgical nephrectomy, there was a rise in oxidative stress markers (lipid and protein oxidation), followed by a reduction in cellular SOD isoforms. These alterations were possibly induced by the significant elevation of Nox activity and expression in renal tissues (Vaziri *et al.*, 2003). The abundant expression of Nox 4 protein and mRNA in the proximal convoluted tubule may suggest that it has a function as oxygen sensor (Geiszt *et al.*, 2000). It is appropriate to presume that function exists because these cells are the major regulators of renal oxygen consumption and are sensitive to hypoxia (Bernhardt *et al.*, 2007).

The key mediators of cellular adaptation to low oxygen levels are the hypoxia inducible factors (HIFs), HIF-1 and HIF-2. They consist of two subunits: an oxygen-sensitive unit-α (or HIF-α) and a constitutively expressed β-unit, also known as HIF-β. During hypoxia, the activation of HIF is dependent upon stabilisation of the HIF-α subunit and its translocation to the nucleus, where it forms a functional complex with additional co-activators (CBP/p300). Under normal levels of oxygen, HIF-α is degradated after binding to the von Hippel-Lindau tumour suppressor protein (Jaakkola *et al.*, 2001). This interaction requires iron, oxygen and 2-oxogutarate for the hydroxylation of the proline residues within the HIF-α subunit (Jaakkola *et al.*, 2001; Elkins *et al.*, 2003). However, in vascular smooth muscle cells, ANG II is able to activate HIF-1 by over expressing HIF-1α protein.

The activation of HIF-1α by ANG II is different from hypoxic induction. Under aerobic conditions, the protein induction of HIF-1α by ANG II is through the activation of two different pathways. One activates the IGF-1 receptor for HIF-1α through the PI3K pathway for protein translation. The second mechanism involves the tyrosine

kinase receptor activation by ANG II, essential in both the induction and activation of HIF-1 (Lauzier *et al.*, 2007). Thereby, the activation of HIF 1 and 2 influences genes that play important roles in cell metabolism in adaptive responses to hypoxia, including the angiogenic gene VEGF and the glucose transporter GLUT-1 (Warnecke *et al.*, 2003).

In several *in vivo* animal models of kidney disease, these reno-protective effects of the HIF system were identified by cobalt chloride preconditioning, producing an inhibition of prolyl-hydroxylation (Matsumoto *et al.*, 2003). These observations emphasise the importance of the stabilisation of HIF-α before the onset of renal injury. On the other hand, deletion of the pVHL gene from glomerular cells in rats is sufficient to induce a rapid proliferation of podocytes, resembling glomerular disease (Ding *et al.*, 2006). Moreover, Higgings *et al*. demonstrated that the activation of renal epithelial HIF-1α, without chemical preconditioning, promoted excessive fibrosis, whereas the inhibition of HIF-1α inhibited the development of tubulointersititial fibrosis. This protective effect was associated with a decrease in collagen deposition and cell infiltration (Higgins *et al.*, 2007). These results demonstrate the fragile balance between the activation of oxygen sensing and oxidative stress.

1.5 Conclusions and future perspectives

Overall, the data collected from human and animal studies has expanded our understanding of how the early nutritional environment affects growth. The inclusion of additional studies that focus on the effects of obesity adds to our knowledge of the basic pathways and morphological changes that contribute to the development of renal

dysfunction. However, our understanding of the interaction between the kidneys, the obesogenic environment and early nutritional manipulation is incomplete.

In recent years, our laboratory has made considerable progress in the characterisation of the metabolic outcomes resulting from a compromised intrauterine environment during organogenesis. Using sheep as an animal model, the predominant responses to a reduction in fetal nutrient supply, between early to mid-gestation, were changes in insulin sensitive tissues (*i.e*. muscle, liver and adipocyte) (Sharkey *et al*., 2009; Chan *et al*., 2009; unpublished observations). It is important to note that these sheep, at adolescence, showed considerable weight gain. Furthermore, it was also observed that, at one year of age, regardless of maternal diet during gestation, exposure to an obesogenic environment, produced an increase in visceral adipose tissue deposition and dysfunction characterised by perturbed insulin signalling, an abundance of crown-like structures and activation of pro-inflammatory genes (Sharkey *et al.*, 2009). In parallel, obesity in this animal model led to hyperinsulemia, hyperleptemia and a considerable increase in plasma FFAs concentration (Sebert *et al.*, 2009). Of further note, there was evidence of renal structural changes, such as glomerular hypertrophy, which appeared before the onset of hyperglycemia (Williams *et al*., 2007). In sheep, at one year of age, exposure to an obesogenic environment produced renal structural changes, which included glomerulomegaly with Bowman's capsule expansion and glomerulosclerosis (Williams *et al.*, 2007; Sharkey *et al.*, 2009). These markers of renal dysfunction were particularly exacerbated in obese sheep not exposed to maternal nutrient restriction.

Stimuli from the surrounding environment, in addition to alterations induced by *in utero* nutrition, also contribute to the renal adaptation to the obesogenic environment. The induction of oxidative stress by certain metabolic pathways, such as RAS and insulin axis, and their influence in renal development and function provide good tools to understand the interaction between the kidneys and different tissues. Finally, discrimination between, and identification of, early changes induced *in utero* and obesogenic adaptation may lead to new reno-protective interventions and improved maternal and offspring health outcomes.

1.6 Main hypothesis

The main hypothesis of my study was that the relative structural renal amelioration observed in those obese offspring previously exposed to maternal nutrient restriction is a product of post-injury responses, inducing scarring and other adaptations to obesity. In addition, the severity of the renal dysfunction is at a similar stage to that observed in other tissues of this animal model, including the adipose tissue of both obese groups. The following chapters were designed for these aims:

1. Description of the effects induced by maternal nutrient restriction in renal and adipose tissue in early postnatal life prior to exposure to an obesogenic environment.
2. Identification of structural renal alternations and their molecular markers and whether these exhibit different progression in the renal disease induced by obesity when offspring have previously been exposed to maternal nutrient restriction compared to those who were born to control fed mothers.

3. Due to the interaction between the adipose tissue maturation and renal health, the last aim was to identify a potential alteration induced by maternal nutrient restriction in adipose tissue, which may affect differentially the progression of renal disease between both obese groups.

Chapter 2 - Materials and Method

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2.1 Study Protocols

The laboratory procedures were all conducted at the School of Clinical Sciences, Division of Human Development, Academic Child Health, and University of Nottingham. The animal research was conducted with the approval of the Home Office and performed according to the terms of the Animals Act. In addition, all experimental protocols were followed according to national legislation, with the ethical approval of the University of Nottingham.

All the laboratory protocols were followed, under the United Kingdom Control Substances Hazardous to Health (COSHH: SI No 1657, 1988) code of laboratory practice, and administered by the University of Nottingham. Unless otherwise stated, all the chemicals and reagents were obtained from Sigma-Aldrich Company (Gillingham, UK) and equipment was obtained from Laboratory Supplies. Details of all suppliers and the standard solutions used can be found in the Appendix.

2.2 Animal Study

Welsh Mountain ewes of similar age and body composition were selected for this study. Throughout the experiment, animals were kept at the University of Nottingham's Joint Animal Breeding Unit at the Sutton Bonington Campus. The ewes were mated with Texel rams. At 30 days of pregnancy, the ewes were scanned by real time ultrasound to determine fetal number. Only mothers pregnant with twins (n=26) were selected for this study.

Ewes were randomly allocated to either a nutrient restricted group (NR or NR-O, n=12), fed at 50% of metabolic energy requirements (3,5MJ/day) from day 30 to 80 of gestation, a control fed group euthanased at seven days of age (C, n=7), an obese group (O, n=7) or lean group (L, n=8). Both the latter groups were fed to 100% of the requirements (7MJ/day). After day 80 of gestation until term, the diet of all the groups was restored to 100% of the calculated metabolic need (12-13MJ/day)(Clarke *et al.*, 1998).

Throughout the period of experimentation, the animals were fed daily between 8.00 am and 9.00 am and all feed intake was recorded. The metabolic requirements during the period of pregnancy were based on the Agricultural and Food Research Council recommendations (1980)(Council, 1980). The diet for the mothers consisted of 1kg of chopped hay per day and an increasing proportion of barley (200-500g per day), depending on nutritional group and period of pregnancy. In addition, each group had free access to water and was given adequate minerals and vitamins.

Twenty-two newborn (12 female and 10 male) sheep, which were born from control fed mothers, were randomly assigned to the Lean or Obese control groups. This was undertaken irrespective of gender, because previous observations in 1 year old sheep demonstrated no difference in metabolic responses (Gardner *et al*., 2005). The maternal nutrient restricted group was composed of twenty-two newborns, in which 6 were born male and 18 were females.

At 7 days after birth, one offspring from each mother from the groups C and NR were humanly euthanased and their tissues were immediately taken for molecular measurements and preservation at -80^{o}C until further analysis (Fig. 2.1) (Council, 1992).

The remaining offspring from all the groups were kept with their mother during lactation for 10 weeks. During this period, all mothers were fed a diet to fully meet the metabolic needs during lactation. Then, between the 4^{th} and the 12^{th} month of postnatal life, the physical activity of the offspring of O and NR-O groups was restricted (by housing at a stocking rate of 17 animals per $50m^2$) and they were fed hay and concentrated pellets to promote obesity (Williams et al., 2007). Lean offspring remained at pasture, and exhibited a four-fold increase in physical activity compared to the other groups (Williams et al., 2007). This increase was confirmed by accelerometry ('Actiwatch', Linton Instrumention, UK) every 30 seconds over a 24 hour period. All the groups had free access to water during the experimentation.

At one year of age, the sheep were humanely euthanased, using an electric shock. The tissue samples, including the kidney and perirenal adipose tissue (PAT), were collected and snap frozen in liquid nitrogen and stored at -80^oC until further analysis. Prior to preservation, all vital organs, including the kidneys, were weighed and dissected. From this tissue, some samples were extracted for immunochemistry analysis

and were preserved in 10% formol saline (10% (v/v) formaldehyde in 0.9% (v/v) sodium chloride) (Fig. 2.1).

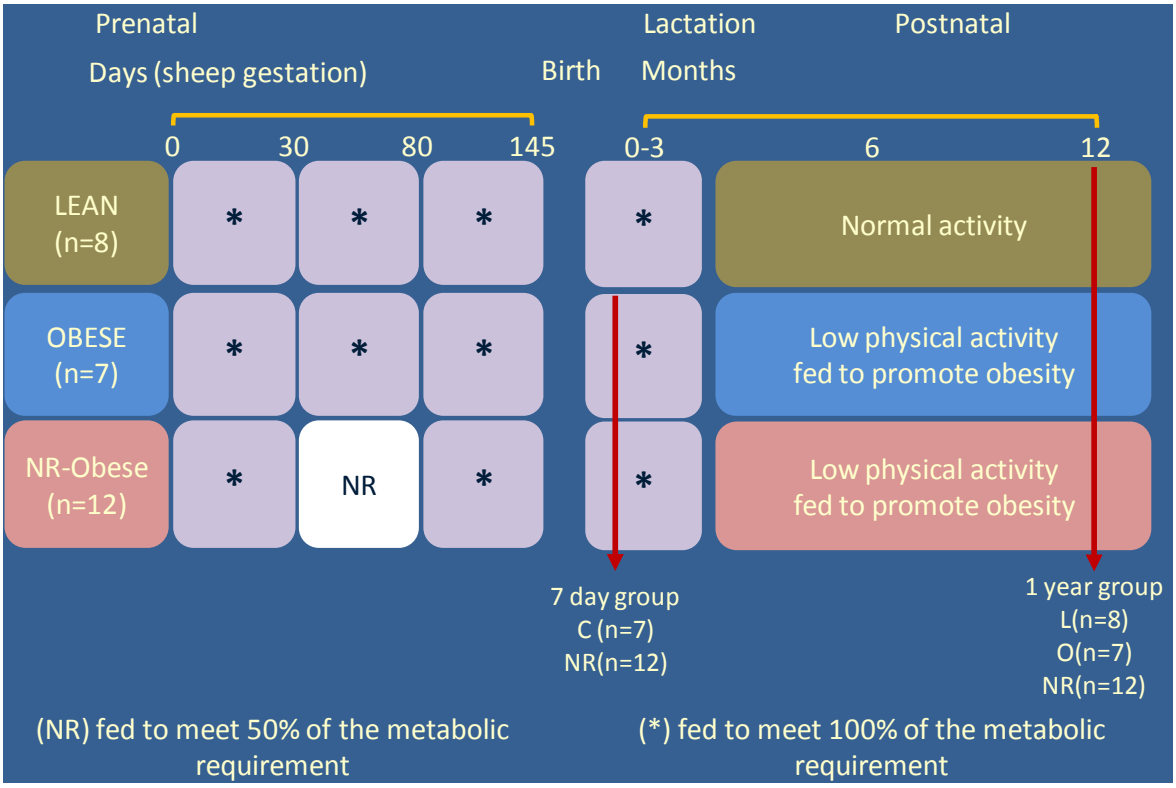


Figure 2.1 Schematic diagram describing the animal model used in this study

In this study, in addition to Welsh mountain flock, Border Leicester cross Swaledale sheep were also used for the perirenal fat ontogeny of the DNA methyltransferase (DNMT)-1. Previous studies had established that there were no distinguishable differences in molecular development between these breeds (Gnanalingham *et al*., 2005). All mothers included in this study were fed to nutritional levels stipulated by the Agricultural and Food Research Council throughout gestation (7 MJ/day). Randomly selected animals were euthanased either at birth (n=7) or at 30 (n=9) days after birth, and their organs dissected following similar procedures as described above.

2.2.1 Collection of plasma samples from lambs

In order to collect venous blood at one year of age, all offspring were kept in a barn for one week prior to surgical implantation of arterial and venous catheters. During a period of 24 hours before surgery, food, with exception of water, was withdrawn from all animals. For the insertion of the catheters, an anaesthetic was applied (propofol, Rapinovet; 6mg.kg^{-1}; National Veterinary Supplies Ltd.) and animals were maintained with 3-4% l.min^{-1} O_2. In the area to be catheterised, the neck was shaved and cleaned. The incision was closed after the operation. In addition, the sheep received a dose of antibiotic, to prevent any infection. This included 10mg.kg^{-1} I.M. propcaine penicillin (Duphapen, Fort Dodge Animal Health Ltd, Southampton, UK) and a dose of analgesia 2mg.kg^{-1} I.M. flunixin meglumine (Finadyne, Schering-Plough, Kenilworth, UK) for 3 days after surgery. Catheter patency was maintained by a flushing 50 I.U/ml^{-1} of saline heparin per day. The normal pattern of feeding was restored 1 hour after surgery. No animal exhibited any signs of physical discomfort during, or after, this procedure.

After blood sampling, and immediately after surgery, the 10 ml blood samples were placed immediately into heparinised tubes and kept at 4^{o}C. Aliquots of the blood previously collected were used to analyse: sodium concentration [Na^{2+}], calcium [Ca^{2+}], potassium [K^{+}], and chloride [Cl^{-}]. In addition, haematocrit and of blood glucose and lactate concentrations were recorded.

2.3 Tissue analysis

After their removal from the ultrafreezer (-80^{o}C), tissues selected for analysis were preserved in a closed polystyrene box containing dry ice. For each analysis,

selected amounts of kidney cortex or PAT were dissected from each sample, depending on the assay to be performed. Each sample received a code number (in order to blind the analysis) and their dissected weights were recorded on an Excel data sheet. The remaining tissue was returned to the ultrafreezer immediately. In addition, the selected samples were kept in dry ice or returned to the ultrafreezer for later use.

2.3.1 Protein extraction from frozen tissues

Protein extraction consists of a series of procedures to free the proteins from the matrix and stabilise them in a suitable working buffer of the assay of interest. The first step is to homogenise the tissue and release the protein content from the cell. The average mammalian cell, which builds the renal tissue, is approximately 10μm in diameter (Mathews *et al.*, 1999). It possesses a cytoskeleton, which maintains the cell shape, and it is enclosed by a membrane built of a thin bilayer of amphipathic phospholipids. For this reason, mammalian cells lack rigidity and are easy to disrupt by sheer force, such as that produced by a simple homogeniser. The second consideration is the type of buffer to use, which depends on the analysis to be performed and the type of proteins to be analysed. For the study of whole cell proteins, RIPA buffer (a phosphate-base buffer) is commonly used because it has similar properties, such as acidity and density, to the cell cytosol. Another important characteristic is its compatibility with a wide range of protease inhibitors, thereby preventing proteolysis (Wilson & Walker, 2001) (Nevalainen *et al.*, 1996). For that reason, RIPA buffer is the optimum medium for studying total protein concentrations. It is also an ionic buffer, able to denature some proteins and disrupt protein-protein interactions. Therefore, it should not be used in enzyme activity tests. Another important limitation of RIPA buffer is that it is an ionic

buffer, increasing the background of some assays, such as in the detection of lipid peroxidation (Mathews *et al.*, 1999).

To study mitochondrial proteins, a dense buffer is needed to separate the mitochondrial fraction from the cytosol. The basic principle of this procedure is that the homogenisation of the tissue is followed by high speed centrifugations, producing a pellet, the mitochondrial fraction. The components that help in this process are: sucrose, tween-20 (ionic detergent), and different derivates of glycerol. The buffer has two important ingredients: EDTA, which helps to prevent mitochondrial swelling by chelating calcium ions, and albumin, which removes free fatty acids, reducing respiration and preserving the mitochondria structure (Symonds *et al*., 1992).

2.3.1.1 Whole cell lysate procedure

The procedure used for the extraction of whole cell protein fractions from the renal cortex was an adaptation from the method published by Haimovich et al (Haimovich *et al.*, 1991). To each 100 mg dissected of renal cortex were added 300 µl RIPA buffer containing the protease inhibitors, phenylmethylsulphonyfluore (PMSF) (10µg/ml), aprotinin (30 µl per ml of RIPA) and sodium orthovanadate (100 mM). Each sample was homogenised using a Dispomix® (GC biotech B.V., Alphen aan den Rijn, Holland) homogeniser at 9000 rpm for 45 seconds, until the tissue was completely homogenised and no clumps of tissue were present. To each lysate was added 30 µl of PMSF (10 mg/ml) before incubation at 4^{o}C for 30 minutes. Following this, the homogenates were centrifuged at 10000 rpm for 10 minutes at 4^{o}C and from each sample only the supernatants were placed in a sterile 500 µl microcentrifuge tubes in

aliquiots of 100 µl. These protein samples were stored at -20^{o}C until required for further analysis.

2.3.1.2 Mitochondrial cell fraction procedure

The procedure used for the mitochondrial fraction from the renal cortex was an adaptation from the method published by Symonds et al. (Symonds *et al*., 1992).

To each 1 g of renal cortex dissected was added 10 ml homogenisation buffer. Each sample was homogenised using a Dispomix® homogeniser at 9000 rpm for 45 seconds, until the tissue was completely homogenised and no clumps of tissue were present. Homogenisation buffer was added to achieve a final volume of 2 ml buffer per 100 mg of tissue. Following this, the crude homogenate was centrifuged (T-1045, Kontron AG, Germany) at 900 x g at 4^{o}C for 10 minutes. The protein concentration of each crude homogenate was determined using a Bradford Assay (Sigma), 200 µl aliquot was separated from each crude sample and stored at -20^{o}C, until further analysis.

The remaining supernatant was filtered through two layers of surgical gauze to remove any lipids and centrifuged at 13000 x g at 4^{o}C for 30 minutes. Finally, the mitochondrial fractions were placed in a sterile 500 µl eppendorff tubes in aliquiots of 100 µl. These protein samples were stored at -20^{o}C, until required for further analysis.

2.3.1.3 Bradford protein assay

The protein concentrations were determined by the Bradford protein assay (Sigma, Steinheim, Germany). This assay is based on changes in absorbance produced by the reaction between Coomassie dye and its binding to proteins, which shifts its colour from red to blue, depending on protein concentration. This change in colour occurs during the

formation of the complex dye and protein. The Coomassie reagent passes a free proton to the ionic groups found in proteins, which opens their tertiary structure. This positions the positive amine groups of the proteins in proximity to the negative charge of the dye. These changes in protein charge increase the ionic interaction, which stabilises the blue form of Coomassie, so that the intensity of the colour can be used to measure protein concentration in a linear fashion by reading absorbance (Bradford, 1976).

The benefit of using this assay is that it is less susceptible to interference from chemicals that may be present in protein samples, with the exception of high or low concentrations of detergents such as SDS. At low concentrations (from 0.0033% to 0.067% v/v), SDS inhibits the protein binding sites, resulting in an underestimation of the concentration of the protein in solution. High concentrations of SDS will produce the opposite effect. Therefore, thus, a period of optimisation is needed, with known concentrations of protein and SDS, to calculate the critical concentration (Zor & Selinger, 1996). Another disadvantage is the short linear range of detection of this assay (from 2µg/ml to 120µg/ml), necessitating dilution of samples (1:20) before analysis.

2.3.1.4 Bradford assay procedure

The tissue homogenates (mitochondrial or whole cell) were diluted 1:20 with RIPA buffer. Bovine serum albumin (BSA) was diluted as described by the manufacturer (Sigma) in six dilutions (0, 0.25, 0.5, 1, 1.25, and 2 mg/ml), in order to establish a standard curve. The standards and the samples were placed in a 96 well plate in duplicate. In each well were placed 5 µl of sample and then 250 µl of Bradford reagent was added and briefly mixed on a shaker platform before incubation at room temperature for 5 minutes. Then, the absorbance of each sample was measured at 595

mm by a spectrophotometer (Anthos Labtec Instruments, Cambridge, UK). The protein concentration was calculated and corrected for the original dilution factor with a coefficient of variance of less than 5% between duplicates deemed acceptable reproducibility.

2.3.2 Immunoblotting (Western blotting)

Immunoblotting is an analytical technique designed for the detection and quantification of specific proteins expressed by the cell. This technique involves two steps for the detection of protein abundance: the first step being based on the use of gel electrophoresis to separate proteins by their molecular weight (Burnette, 1981). After the separation, the proteins are transferred to a membrane (usually nitrocellulose or PVDF) in which the proteins are detected by specific antibodies (during this project only polyclonal antibodies were used) (Renart *et al.*, 1979). The second antibody is conjugated with an enzyme and visualised using enhanced chemiluminescence detection system. To prove the reproducibility of the assay, each Western blot was performed in duplicate for these studies.

Sodium dodecylsulphate (SDS) is an ionic detergent which gives a negative charge and denatures proteins by removing their secondary and tertiary structures (disulphite bonds to sulphhydryl bonds), thus allowing the complete separation of proteins by their molecular weight. In addition, SDS binds specifically in a mass ratio of 1.4g SDS: 1g protein confers a negative charge equal to the molecular length. It is usually necessary to reduce further the disulphite bonds to avoid random disulphite bridges. This is done by adding 2-mercaptoethanol or dithiothreitol (DDT). To ensure the complete denaturation, it is also necessary to boil this mix at 100^{o}C for 10 minutes.

After the proteins become negatively charged, they are loaded in the wells of a polyacrylamide gel. Together with the samples, a reference sample and protein marker or ladder (Bio-Rad) are also loaded, usually at the first and second well of each gel. The reference sample used is a common protein sample and enables the calculation of an intra-assay coefficient of variance. The marker is a commercial mix of proteins, having a known molecular weight and is used to measure the target protein size.

The SDS-protein complexes are then electrophoresed, as they move through the positive electrode at a rate inverse to their molecular weight (usually measured in kilo daltons, KDa). The resolution of the gel depends on the concentration of the acrylamide and the cross-linking bisacrylamide used in its preparation. This determines the pore size, which can increase the separation between lower molecular weight proteins.

In order to facilitate the immune-detection, the separated proteins are transferred into a membrane made of nitrocellulose. The gel is placed on top of the nitrocellulose membrane and both are covered by filter paper to facilitate capillarity, which is involved in the protein transfer during transmission of the electric current. All of this is introduced into a transfer cassette, that is placed in an electroblotting tank together with a buffer solution (Towbin *et al.*, 1979). By applying an electric current, the proteins are pulled from the gel into the nitrocellulose membrane in the same pattern as separated by the SDS-PAGE gel. The effectiveness of the transfer is tested by staining the membrane with Ponceau S dye. After this test, it is possible to distain the dye by applying water alone or a saline solution with a detergent.

Since the membrane has the capacity of binding proteins non-specifically, it is necessary to block it with a known non antigenic protein like casein so that antibodies will not

react to them, preventing any false positives and noise. The detection process starts by incubating the primary antibody at an appropriate concentration, incubation time and temperature, with the membrane in constant agitation. The antibodies are diluted in a buffer composed of 3% milk powder and 0.2% detergent (Tween-20) in a saline solution (TBS). The concentration, time and temperature of incubation of the primary antibody depend on an optimisation process for each target protein. To reduce non-specific binding at the end of the incubation, the membrane is rinsed in order to remove unbonded or unspecific antibodies. In order to detect the complex antibody target protein, the membrane is exposed to secondary antibody. These are produced from a different animal species and are able to bind to the heavy chains of the primary antibody. In addition, the secondary antibody is commonly attached to a reporter enzyme, such as horseradish peroxidase (HPR). When exposed to a chemoluminescent agent, the reaction results in the emission of light or luminescence proportional to the protein abundance in the initial sample.

The light emitted by this reaction is detected by an image detection system or CCD camera (Fujifilm LAS-100 cooled CCD camera, Fuji Photo Film Co. Ltd, Tokyo Japan) which produces a digital image of the Western blot. The image created by this process is analysed by densitometry (Aida version 2.0, Raytek Scientific Ltd., Sheffield), which evaluates the density of the bands found in the image. The intensity values of each band reflect abundance of the polypeptide within the initial sample.

2.3.2.1 Western blot procedure

Polyacrylamide gel preparation

Polyacylamide gels were cast, using a dual gel caster. This casting system consists of two glass plates: a small one sits on the top of a larger one. On the last plate, two silica 1 mm spacers were attached. Both plates were vertically assembled within the casting apparatus, according to the manufacturer's instructions (mini-PROTEAN® 3 cell, Bio-Rad). Each 10 ml 12% of polycylamide resolving gel consisted of: 3.3 ml of deionised water, 4 ml of 30% acrylamide-bisacrylmide solution, 2.5 ml of ph 8.8; 1.5 M Tris ([hydroxymethyl] amino-methane, 0.2% (w/v) SDS), 100 µl of 10% SDS (w/v), 100 µl 10% Ammonium Persulphate (w/v) and 4 µl N,N,N',N' Tetramethylethylenediamine (Temed). The mix was poured on the top and between the plates to a level of 3 cm below the top. To avoid gel-oxidation 2 ml of isopropanol was added to top of the gel solution. The polymerisation of the resolving gel was conducted at room temperature for 45 minutes. After this incubation, the isopropanol was rinsed with deionised water. Another gel is added on to top of the polycylamide resolving gel (called: stacking gel). The mix of this last gel consisting of: 6.8 ml deionised water, 1.7 ml of 30% acrylamide-bisacrylmide solution, 1.25 ml of ph 6.8; 1 M Tris ([hydroxymethyl] amino-methane, 0.2% (w/v) SDS), 100 µl of 10% SDS (w/v), 100 µl 10% Ammonium Persulphate (w/v) and 10 µl N,N,N',N' Tetramethylethylenediamine (Temed). The mix was poured on the top of the resolving gel and a 10 well comb inserted in order to produce the loading wells and left to polymerise for 15 minutes at room temperature.

Sample preparation

The protein concentration of mitochondrial or whole cell samples was determined, using the Bradford assay. Aliquots of each sample were diluted to an initial concentration of 4.2 mg protein/ml with mitochondrial homogenisation buffer (10 mM Tris ([hydroxymethyl] amino-methane ph 7.4, 250 mM Sucrose and 1 mM EDTA) or protein homogenisation buffer (10 % (v/v) Glycerol, 2 % w/v SDS, 5 % 2-mercaptoethanol made up to volume with 50 mM Tris (ph 6.8) to the final volume of 20 µl. To this, 50 µl protein or mitochondrial buffer was added to each sample, followed by 14 µl loading dye (0.00 1% Bromophenol blue, 16 % (v/v) Glycerol in deionised water). In order to denaturate the proteins within the diluted fractions and to expose the epitopes to the antibodies, 20 µl aliquot of each sample were incubated at 100^{o}C for 10 minutes. This procedure was followed by incubation in ice, until the stacking gel was ready for use.

Sample loading and electrophoresis

The polymerised gels were transferred to a vertical electrophoresis unit (Bio-Rad Ltd., Hercules, CA, USA). The reservoir and sample wells were filled with 1 x running buffer (25 mM Tris [hydroxymethyl] amino-methane, 250 mM glycine, 0.1 % (w/v) SDS).

Before loading the protein samples, the wells were cleansed of unpolymerised acrylamide, using a Pasteur pipette and the running buffer. 15 µl of protein sample was loaded from the second to ninth well. The first well was reserved for the protein ladder (Precision Plus pre-stained standards, Bio-Rad) and the last well was excluded from use because of its proximity to the silica spacer, which alters polymerisation. The second

well was reserved for the reference sample and 5 µl of loading buffer was loaded on the last well.

The samples from the different animals were blinded and randomised, using a research randomised web site http://www.randomizer.org/ . Finally, the gels were electrophoresed at 200V for 45 minutes per gel.

Electrophoresis transfer onto nitrocellulose membrane

Proteins are transferred from the polyacrylmide gel to a nitrocellulose membrane (Hybond-C Super; Amersham Ltd, UK) using a Mighty Small II electrophoretic chamber (SE 250; Hoefer Pharmacia Biotech Inc.). The gel and the membrane are opposed between two layers of blotting paper soaked in transfer buffer (48mM Tris [hydroxymethyl] amino-methane, 39 mM Glycine 20% (v/v) Methanol, 0.037% (v/w) SDS). Gel and membrane were firmly placed together and air bubbles removed by light transverse pressure. Following this, both gel and membrane were sandwiched between two Scotch-Brite scouring pads (3M: Bracknell, UK) and held in position vertically in the cassettes within the blotting tank in the order cathode – gel – membrane – anode (Fig 2.1), to allow transfer of the negatively charged polypeptides onto the membrane. The tank was filled with transfer buffer and a potential difference of 80 mA applied across for 1 hour. A cool water stream passes throughout the high voltage transfer, so that proteins were not denatured by the transfer process.

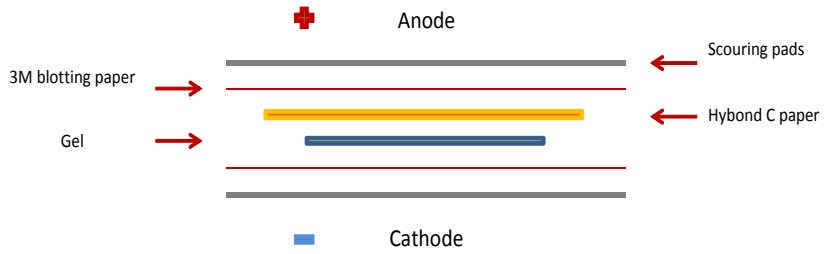


Figure 2.2 Western blotting- graphical description of the transfer unit

Protein staining

Following electroblotting, the consistency of electrophoresis and blotting was examined by irreversible staining of the nitrocellulose membrane. The membrane was then incubated at room temperature with 10% diluted Ponseau S stain (2% (w/v) Ponseau S, 30% (w/v) Trichloroacetic acid, 30% (w/v) Sulphosalicylic acid in deionised water; further diluted 1:10 in deionised water) for 10 minutes and excess dye eluted with deionised water. Ponseau S stain reversibility binds to protein and reveals a series of multiple bands, arising vertically in lanes from each well (Fig 2.2). Following the visualisation, it was possible to distain the Ponseau S dye by applying TTBS (Tween-Tris buffered saline (0.2% (v/v) Tween-20 in 20 mM Tris [hydroxymethyl] amino-methane, 500mM Sodium Chloride, ph 7.5)) for 10 minutes at room temperature in constant agitation. The final confirmation of the efficiency of the transfer was assessed by staining the gel in Coomassie Brilliant Blue (Simply Blue™, Invitrogen, CA, USA) solution for 1 hour. Excess stain is removed by deionised water after 5 minutes in constant agitation. This procedure stains any proteins remaining on the gel after electroblotting, with the absence of protein staining after this procedure confirms the complete transfer of polypeptides.

Immunodetection

In order to prevent potential non-specific binding of antibodies to the areas of membrane not occupied by polypeptides from the sample originally loaded, the nitrocellulose membranes are incubated with a protein containing solution (10% Marvel dried milk, Primier Brands in TTBS) to 'block' these sites. Each membrane was incubated with blocking buffer overnight at 4^{o}C. The membrane was rinsed with TTBS, before incubation for 1 hour at room temperature with 10 ml of primary antibody solution (TTBS, 3% (w/v) Marvel and antibody active against the target protein). The antibodies against SOD1 (Fig 2.2) and SOD2 were purchased from Abcam plc, UK; p85 was purchased from Milliopore Ltd, USA and VDAC1 was home produced (Mostyn *et al*., 2003).

Each antibody was validated by measuring the band that it generated against the protein ladder. In addition, the result of this assay was compared with the bands produced by non-specific immunoblots, one of which is tested with non-specific rabbit serum (all the antibodies used in this project were raised by rabbits) the other of which is exposed only to the secondary antibody. The antibody was validated only if the band to be tested was similar to the molecular weight of the protein of interest as published in the literature. Moreover, the band does not have the same molecular weight as the bands found in the other two non-specific immunoblots. To optimise the incubation conditions of the antibody, identical gels were exposed to different concentrations of primary antibodies, incubation times and temperatures (see table 2.1).

At the end of the incubation of the primary antibody, the membranes were rinsed and washed three times for 10 minutes in 100 ml of TTBS in constant agitation. Then, the membranes were incubated for an extra hour with the secondary antibody (1:1000

dilution, PO 217; Dako Ltd. UK). This antibody was also re-suspended in 10 ml of 3% dried milk in TTBS. The membranes were washed again and rinsed as previously described.

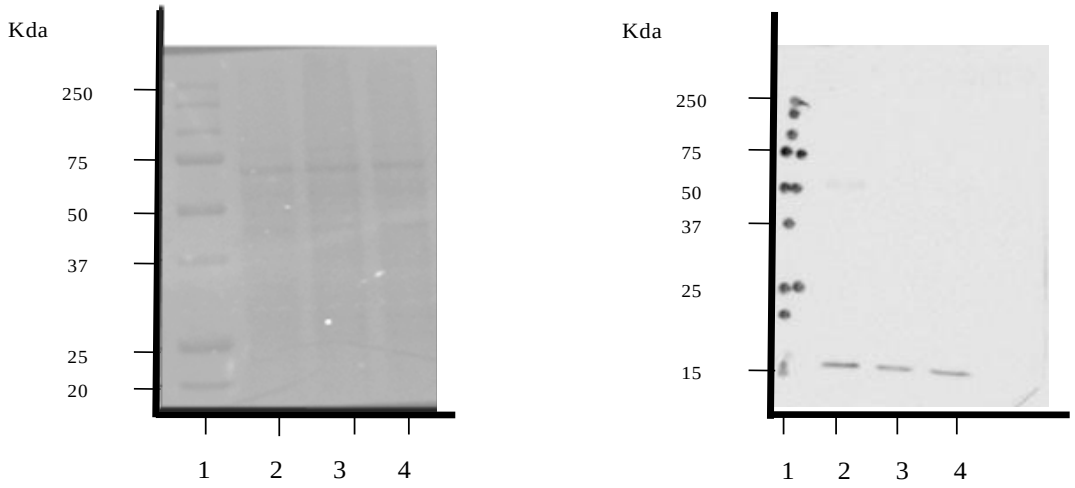


Figure 2.3 Immunoblot images. On the left Ponceau S stain test and on the right a western blot of SOD1. Line 1 protein standard, lines from 2 to 4 three are different samples representing each 1 year old group (2:L;3:O;4 NR-O).

Table 2.1: Technical details of the antibodies used during this project

Antibody	Supplier	Catalogue number	Protein type	Protein size (Kda)	Primary antibody dilution factor	Incubation time and temperature
p85	Upstate	06-195	Cytosol	85	1/1000	1 hour at room temperature
SOD1	Abcam	ab13498	Cytosol	15	1/500	Overnight at room temperature
SOD2	Abcam	ab13533	Mitochondria	25	1/1000	1 hour at room temperature
VDAC	In house	N/A	Mitochondria	34	1/1500	1 hour at room temperature

Enhanced chemiluminescence

The membranes were washed in constant agitation in TBS for 30 minutes and the excess fluid drained. To each membrane 6 ml of Immobilom western (Millipore Ltd. MA, USA) was applied and the membrane incubated for 5 minutes. The excess reagent was drained and the membrane placed in the CDD camera to generate a digital image. The optimum time of exposure for all immunoblots was 30 seconds. By visualisation of the digital image, it was possible to determine the intensity of the antibody-target peptide's signal and relative background. By comparing intensity of the pixels of bands against the background of the immunoblot, it was possible to determine the abundance of the protein of interest. To confirm the reproducibility of the assay, each gel was performed in duplicate on different occasions.

2.3.3 Nitric Oxide measurement

A large number of colorimetric methods exist to determine the presence of nitric oxide (NO). However, the most common technique used in clinical publications is based on the Griess reaction (Tsukahara *et al.*, 1997; Dobrian *et al.*, 2001; Beltowski *et al.*, 2004). This technique does not directly measure the concentration of NO for a number of reasons. Firstly, NO is produced in trace quantities by a large number of cells, including neurons, endothelial cells and platelets under normal physiological conditions. Secondly, NO is a highly reactive free radical, able to interact with ozone, reducing its half life to few seconds. For these reasons, the Greiss reaction is based on the measurement of the final products in vivo of NO, which is Nitrite (NO_2^-) and Nitrate (NO_3^-) (Granger *et al.*, 1996). It is possible to measure the relative concentration of both products. The remaining NO in the samples is transformed by NADPH to both final

products. Then, NO_3^- can be converted to NO_2^- with nitrate reductase, an enzyme extracted from bacteria (*e.g. Escherichia Coli*). The total NO_2^- is exposed to sulphanilamide and N-(1-naphthly) ethylenediamine to produce an azo dye (yellow colour) with the colour product being proportional to the concentration of NO_2^- present in each sample. Thus, the colour intensity is used for the measurement of NO concentration by absorbance reading. The absorbance spectrum of this assay is at 540 mm, with an increase in absorbance is equal to the quantity of dye that is proportional to the amount of NO present in the sample (Granger *et al.*, 1996).

However, there are a large number of factors that may interfere with the assay, including reagents present in biological fluids and components from the Griess reaction. For that reason, there are a number of variants to this reaction, designed to reduce interference from such sources. Plasma or tissue homogenate assays require protein extraction by precipitation or by ultrafiltration. In addition, NADPH is an essential cofactor in the activity between of L-arginine and enzymes NO synthase (NOS), which interferes with the chemistry of the Griess reaction (Greenberg *et al.*, 1995). This limitation is overcome by adding small quantities of sample or removing the excess of NADPH by the metabolic action of lactate dehydrogenase (LDH) (Tracey *et al.*, 1995). The determination of nitrite and nitrate (NO_x) described above is performed using a standard commercial kit (Nitric Oxide Synthase Assay Kit, Calbiochem Inc., Darmstadt Germany).

2.3.3.1 Nitric oxide measurement procedure

Sample preparation

For this procedure, one sample from renal cortex was dissected of each animal euthanased at 1 year of age from this study. 100 mg of tissue were added to 300 µl of phosphate buffered saline (PBS; 137 mM NaCl, 10 mM Phosphate, 2.7 mM KCl, ph 7.4) and homogenised using a Dispomix® 9000 rpm for 45 seconds until complete homogenisation. The homogenates were then centrifuged (5810R, Eppendorf, SLS, UK) at 10000 rpm for 10 minutes at 4^{o}C and the supernatant placed in sterile 1 ml eppendroff tubes. The samples were analysed for protein concentration by the Bradford method (described earlier at 2.3.1.4). The protein concentration was expressed in mg/ml. The tissue supernatants were ultracentrifuged (T-1045, Kontron AG, Germany) at 10000 x g for 15 minutes at 4^{o}C. Later, the resulting supernatants were ultrafiltrated through two filters a 0.45 µm (Ministart, Sartorius Ltd., Gottingen, Germany) and 10 KDa (Vavispin 2, Sartorius Ltd., Stonehouse, UK) to separate complete all proteins from each sample.

Performing the assay

The NO_2^- solution was diluted 5, 10, 15, 20, 25µM and a blank, to establish standard curve. These and the samples were placed in a 96 well plate in duplicate using 60 µl of standard or sample. The samples were blinded and randomised using a research randomised web site http://www.randomizer.org/. 10 µl of freshly prepared NADPH solution (1mM), 10µl of nitrate reductase was then added and the sample incubated at room temperature for 60 minutes. Then 10 µl of cofactors solution plus 10 µl LDH solution, and samples incubated for another 20 minutes at room temperature. Finally, 50 µl of Griess reagent added to each well. The plate was left for 10 minutes at room

temperature for the colour reaction to occur. The intensity of the reaction was measured at 540 mm by a spectrophotometer. The standard data was expressed as a function of absorbance at 540 mm and concentration of nitrate (µM) calculated. The final NO_2^- concentration was normalised by the protein content of each sample found by the Bradford assay ([NO_2^- µM]/ [protein mg/mg]).

In order to optimise the linear detection range, different serial renal NO dilutions were tested using the same protocol as described earlier.

2.3.4 Triglyceride extraction from renal tissue

The Folch, Less and Stanley method is a simple method for the preparation of total pure lipid extracts from various tissues including the kidney (Folch *et al*., 1957; Saito *et al*., 2005). This procedure exploits on the interaction between the buffer chloroform-methanol-saline (8:4:3) and cellular lipids for separation of the homogenised tissue into two phases in which the lower phase is the total pure lipid extract. After homogenisation and gravity filtration, due the polarity and density of the suspension solution and the lipids, the cellular and tissue debris can be separated simply by adding and mixing one fifths of water or a salt buffer (*e.g.* 0.9% NaCl) of the total volume. The chloroform-methanol solution can be removed by applying a nitrogen stream and the pure lipids faction can be easily measure by any commercial triglyceride assay (Randox Ltd, Antrim, UK).

2.3.4.1 Triglyceride tissue deposition procedure

Tissue preparation

The tissue samples (500 mg of renal cortex) were minced in 10 ml (w/v) cold chloroform/methanol (2:1) on ice, using a dispomix® homogeniser as described previously (2.3.1.1), then the samples were agitated at room temperature for 15-20 minutes. The extracts were filtered through filter (24 cm) paper (Whatman Ltd, Banbury, UK) into centrifuge tubes, and filtration was completed by adding 1 ml of chloroform. The filtrates washed by mix them with 2 ml of 0.9% (w/v) NaCl solution and centrifuged at low speed (2000 rpm) for 10 minutes for the separation the lipid and aqueous phases. The aqueous layers (upper phase) were removed with a Pasteur pipette and the organic phase was dried by applying a nitrogen stream and then the pellets were dissolve in 200 µl of (v/v) tert-butyl alcohol/Triton X-100.

Performing the assay

Triglycerides were measured enzymatically by using a commercial kit (Randox Ltd, Antrim, UK) and the absorbance read using a plate reader at 500 mm. Triglyceride concentration was expressed as a ratio of triglyceride found in micrograms and weight in grams of the sample previously dissected (mg/g).

2.3.5 Superoxide dismutase activity assay

The total activity of superoxide dismutase (SOD) enzymes can be measured using a modified version of the method of Beauchamp and Fridovich (Fridovich, 1970). Measurement of the activity of total SOD utilised a tetrazolium salt for the detection of superoxide radicals generated by xanthine oxidase and hypoxanthine. One unit of SOD is defined as the amount of enzyme needed to exhibit 50% dismutation of the superoxide

radical. The activity test was performed using a commercial kit (Superoxide Dismutase Kit II, Calbiochem Inc., Darmstadt Germany).

2.3.5.1 Superoxide dismutase activity procedure

Sample preparation

As described earlier, 100 mg of dissected renal cortex was added to 1 ml of suspension buffer (20mM HEPES buffer ph 7.2, 1mM EGTA, 210 mM Mannitol, 70 mM Sucrose), homogenised and centrifuged at 1500 x g for 5 minutes at 4^{o}C and the supernatant removed for the assay and stored at -80^{o}C. The samples were analysed for protein concentration by the Bradford method (described at 2.3.1.4).

Performing the assay

Standards for the assay at 0, 0.025, 0.05, 0.1, 0.15, 0.2 and 0.25 U/ml were prepared and placed together with the samples in a 96 well plate in duplicate, blinded and randomised, as previously described. In each well, 200 µl of the diluted radical detector was added plus 20 µl of diluted xanthine oxidate was added and incubated at room temperature in constant agitation for 20 minutes. The absorbance was read using a plate reader at 450 mm. The activity of SOD was expressed as a function of total SOD activity U/ml.

To fully validate and optimise this methodology, different, serially diluted homogenate samples were measured to find the protein concentration detection range of the assay. The detection range of this assay varied from a protein concentration of 0.02 to 0.1 mg/ml. All the samples were diluted to the mid detection range equal to 0.05 mg/ml.

2.3.6 Thiobarbituric acid reactive substances (TBARS)

The thiobarbituric acid reactive substances (or TBARS) method has been used extensively in food manufacturing for the last 50 years and is the gold standard method to measure global oxidative stress a well-established mechanism of cellular injury. Originally, this technique was based on the ability of thiobarbituric acid (TB) to react with malondialdehyde (MDA) and used as an index of lipidperoxidation (Armstrong & Browne, 1994; Yagi, 1994). MDA is the stable byproduct of the decomposition by free radical of polyunsaturated fatty acids (Tappel, 1973).

The assay starts by heating the sample with TBARS at low pH. Then, the reaction with TB and MDA produces a pink chromogen, which is possible to measure at 532 mm (Kosugi *et al.*, 1987). However, previous findings have demonstrated that TB might react with other components. This acid is also able to react with aldehydes and other components associated with oxidative stress induced by obesity, producing similar colour dye (Fogelman *et al.,* 1980). Moreover, MDA is a reactive compound able to bind to different cell components such as lysine, albumin and DNA. For that reason, it can be overestimated four to five fold when TB is used just to determine MDA (Gutteridge, 1986). Therefore, many authors have suggested that the results need to be termed TBARS and not MDA concentration (Csallany *et al.*, 1984; Draper *et al.*, 2001). This procedure is still the most wildly employed assay to determine lipid peroxidation (Armstrong & Browne, 1994; Draper *et al.*, 2001; Kim *et al.*, 2001; Ece *et al.*, 2006). The TBARS assay was performed using a commercial kit (OxiSelect™ TBARS assay Kit, Cell Biolabs Inc., CA, USA).

2.3.6.1 Thiobarbituric acid reactive substances (TBARS) procedure

Sample preparation

100 mg of dissected renal cortex were resuspended in 1 ml of lysis buffer (Cell Lytic^{TM} MT mammalian tissue lysis, Sigma, Steinheim, Germany) and 10 µl of protease inhibitor cocktail was added (Sigma). Each sample was homogenised as described previously (2.3.1.1) and centrifuged at 1600 x g for 10 minutes at 4^{o}C before the supernatant was removed for the assay and stored at -80^{o}C. The samples were analysed for protein concentration by the Bradford method (Section 2.3.1.4).

Performing the assay

Each standard and sample was prepared in duplicate and randomised as described previously (see 2.3.3.1). The standard curve solution was diluted from 0 to 250 µM of MDA and 100 µl of sample or MDA standard was added. 100 µl of SDS lysis solution was then added and incubated for 5 minutes at room temperature. 250 µl of TB reagent was added, immediately incubated at 95^{o}C for 1 hour and then cooled in ice for 5 minutes, followed by centrifugation for 15 minutes at 3000 rpm. All the samples were read spectrophotometerically at 540 mm excitation and 590 nm emission. The concentration of MDA calculated and was expressed as: [MDA µM]/[Protein mg/ml].

To validate this methodology, firstly the lysis buffers and the protein inhibitors were tested for any interference with the assay. Optimal results were obtained by using the Sigma lysis buffer and its inhibitor cocktail. The detection range was between 0.3 to 7.5 mg/ml of protein with all the samples diluted to a range of 3 mg/ml. To test a possible inference of lipids with a higher degree of unsaturated bonds and lipoprotein,

homogenate samples from of both one year old obese groups were tested at different protein concentrations against the one year old lean group.

2.3.7 Histology

2.3.7.1 Immunohistochemistry

Immunohistochemistry (IHC) is a technique used to locate specific proteins in a cell and exploits the interaction between antibody-antigen. The visualisation of these interactions can be accomplished by the use of a dye, enzyme, radioactive element or conjugated antibody, as are used in other techniques, such as Western bloting (Mason & Sammons, 1978; Ramos-Vara, 2005).

Tissues were preserved as described in Section 2.3 and were then paraffin wax embedded. These techniques permit the preservation of the morphological structures of the tissues with a high visual resolution. Formaldehyde preserves the majority of peptides and the general structure of cellular organelles because it is able to bind to and preserve amino acids (Lysine, Tyrosine and other) stopping the process of protein degradation. Five µm serial sections, optimal for antibody binding and light microscopy, were prepared from the paraffin wax tissue blocks using a sledge microtome.(Eltoum *et al.*, 2001).

Following slicing and mounting onto microscopy slices, the sections were deparaffinised with xylene and brought to water and then peroxidase blocked to prevent exogenous peroxidases being detected by the horseradish peroxidase (HRP) conjugated antibody. Epitope retrieval was performed by citrate buffer at 95^{o}C for 15 minutes (Shi *et al.*, 2000). Sections were then incubated with the primary antibody, followed by the

secondary antibody, which binds specifically to the primary antibody and is conjugated with HRP enzyme and, after applying a DAB substrate, allows the localisation of the protein of interest (Merz *et al.*, 1995 ; Kiernan, 2000).

2.3.7.2 Immunohistochemistry procedure

Tissue processing

Samples of 1 year old sheep kidneys were fixed in 10% formol saline (10% (v/v) formaldehyde in 0.9% (v/v) sodium chloride) and embedded in paraffin wax and sectioned using a microtome (Sledge microtome, Anglia Scientific, UK) into 5 µm sections, floated out onto warm water (45^{o}C), these were then placed onto Superfrost plus glass microscope slides (Mezel-Glazer Inc., Braunchweig, Germany) and baked on a hot plate for 15 minutes. The slices were dried overnight at 37^{o}C.

In total, 130 slides from kidney sections (NR-O (n=10 x 5); O (n=8 x 5); L (n= 8 x 5)) and 26 from PAT (NR-O (n=10); O (n=8); L (n=8)) were prepared for this assay and for subsequent procedures conducted in these studies. Identification of samples was double blinded as previously described (2.3.2.1) before any further experimentation was carry out.

Performing the assay

The Bond-Max automated slide processor (Bond software version 3.4A) and Bond Polymer Refine Detection System (Vision Biosystems, Mount Waverley, Australia) was used for the immunhistochemical processing with proliferative cell nuclear antibody (PCNA). The rabbit PCNA antibody was purchased from Abcam PLC (ab18197).

Twenty six sections, including a positive control (sheep placentome) and two negative controls (a section of sheep kidney and another placentome section) were

placed in random order in a Bond-Max tray. The slides were dewaxed with toluene, incubated in citrate buffer at 95^{o}C for 15 minutes and then peroxide blocked for 5 minutes at room temperature. Each slide was incubated with 150µl of rabbit anti PCNA primary antibody (1:2500 dilutions) for 15 minutes. The sections were washed twice with Bond Wash solution and with deionised water after which they were incubated with 100 µl of secondary antibody for 8 minutes and rewashed. The section were then incubated with 3, 3' Diaminobenzindine (DAB) for 10 minutes followed by 5 minutes incubation with haematoxylin and again washed with water.

Slides were imaged using a Leica DM RB microscope and images captured using the OpenLab software (Improvision OpenLab 4.0.2) and analysed blinded using Volocity 4 (v4.2.1, Improvision Ltd, Coventry, UK) quantification software.

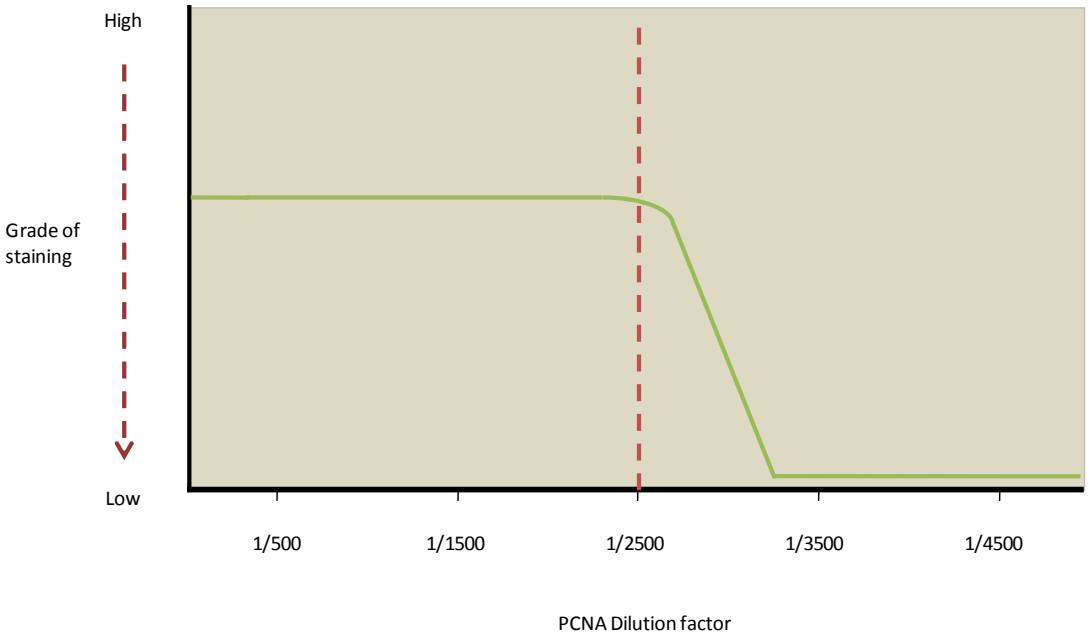
In order to ensure that the optimum PCNA antibody concentration was used, serial antibody dilutions were performed as described in table 2.2, prior to the assay:

Table 2.2 PCNA antibody dilution series used to optimise to immunohistochemical staining

Optimization number	Antibody	Dilution 1	Dilution 2	Dilution 3	Dilution 4	Dilution 5
1st	PCNA	1 /250	1 /1000	1 /2000	1 /4000	1 /8000
2nd	PCNA	1 /2500	1 /3000	1 /3500	1 /3500	1 /4500

The optimum dilution is that which produces good staining but with the majority of positive staining loss after the next more dilute antibody is used (see Fig. 2.3) (Stevens & Lowe, 2005). The loss should be dramatic and not gradual. Gradual loss will indicate non-specific bind of the antibody (Table 2.3).

Table 2.3 Graph illustrating the optimum PCNA antibody dilution factor. Note that the red line symbolises the optimum dilution found during the optimisation



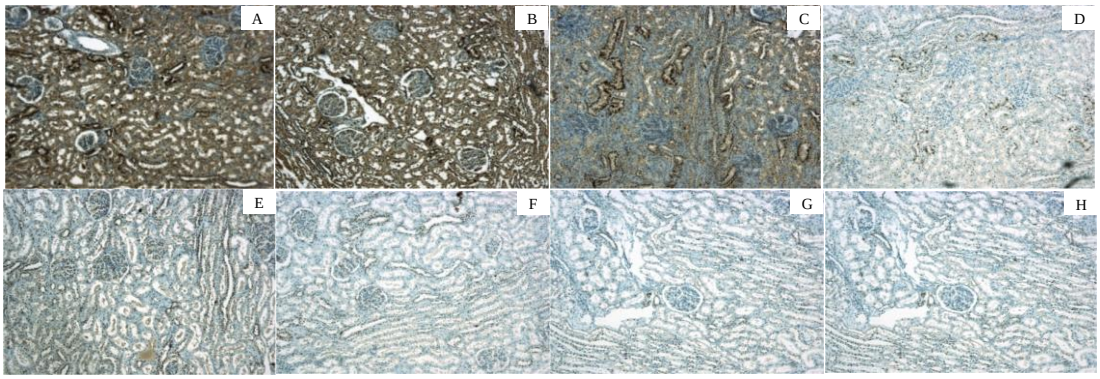


Figure 2.4 First and second renal section stained with PCNA assigned for the optimisation of the immunostaining (20X magnification). Antibody dilution: A 1/250; B 1/1000; C 1/2000; D 1/4000; E 1/2500; F 1/3000; G 1/3500; H 1/4500. Optimal dilution: 1/2500.

Semi-quantification of PCNA stained sections

From each section, three pictures were taken, which represented random fields of renal cortex using a magnification of x 1:20. Prior to any analysis, each section was viewed microscopically to recognise and identify the main structures of the renal cortex and specific cells that were positive stained, after which the numbers of positive stained cells were counted.

The mean number of positive cells in each individual random field was estimated by using quantification software (Volocity 4, Improvision, Coventry, UK).

2.3.7.3 Histochemistry

Histochemistry refers to a series of techniques that exploit specific reactions between chemical dyes and components within the tissue. Some commonly performed histochemical techniques are Perls' Prussian blue, used to demonstrate iron deposition and Masson trichrome, which is a three-colour staining protocol for the observation of collagen, keratin and cells within the tissue (Ishizaka *et al.*, 2002; Bharwan *et al.*, 2009).

Prussian blue staining

Perls' Prussian blue allows estimation of the deposition of non-haemoglobin iron in formalin fixed, wax embedded sections by staining erythrocytes, normoblasts, macrophages and other cells containing particulate iron. Biologically active iron is normally attached to protein as haemosiderin and ferritin, which are impossible to detect. However, treatment with dilute mineral acid releases the ferric (Fe^{+3}) form in the presence of cyanide ions and iron is seen as small blue or blue-green granules. This technique has recently been used to demonstrate iron deposition in mice renal tissue (Ishazaka *et al*. 2005; Saito *et al*., 2005).

Performing the assay

For the assessment of iron deposition, 26 paraffin-embedded renal sections and their controls (positive, liver; negative, placenta and a renal section without exposure to potassium ferric ferrocyanide) were prepared as described in Section 2.3.6.1. The sections were deparaffinised and hydrated. The slices were immersed in freshly prepared Perls' Prussian blue solution (2% hydrochloric acid mixed with 2% potassium ferrocyanide) for 20 minutes. The samples were washed three times in distilled water for 2 minutes and then counterstained with nuclear fast red stain (0.1 g nuclear fast red, 5% V/V aluminium sulphate, 100 ml distilled water; Sigma (N3020)) for 5 minutes. After staining, the sections were dehydrated in 95% alcohol and two changes of absolute alcohol the slices were cleared in xylene and mounted in a resinous medium (DPX). All analysis was performed independently by two assessors using reference slides to access consistency of grading assessment. For assessment of iron deposition, the slides were analysed in a blinded fashion using a semi-quantitative index (1-4) as described by Wintour *et al*.. Grade 1= no staining, 2= 1-10 granules 3= 10-20, 4= 20 and more. Twelve random fields were chosen per kidney section and were evaluated at x 20 magnification.

Masson trichrome

Masson's trichrome is frequently used to differentiate collagen from other fibres, particularly keratin and to identify increased collagenous tissue in disease as seen in renal dysfunction (Bharwan *et al.*, 2009). The tissue sections were stained with Harris' haematoxylin, a nuclear stain. The sections then were treated with acid fuchsin solution (0.5 g of acid fuchsin; 0.5 ml glacial acetic acid; 100 ml distilled water). As result of the exposure to acid dye, all the acidophilic components including the cytoplasm and

collagen will stain after this stage. The sections then were treated with phospmolybdic acid (25g phosphomolybdic acid; 20 ml water) to facilitate the diffusion of the acid stains into the cytoplasm, which is less permeable than collagen. Chemical compounds such as phosphomolybdic acid has numerous acidic groups that most likely act as mediator between collagen and light green, which is the collagen dye. Finally, the pH of the phosphomolybdic acid also increases the colour intensity of the collagen green light dye. A quality control is not required for this technique as every tissue has an internal control due the presence of collagen and other components. Both this technique and Prussian blue are suitable for formalin fixed tissue sections. The expected results are: collagen green-blue, nuclei black, cytoplasm and keratin red.

Performing the assay

Twenty-six slides from renal tissue and PAT were dewaxed by immersion in two changes of xylene each for three minutes, followed by two changes of absolute alcohol each for two minutes and other three minutes in 70% alcohol before washing in distilled water for three minutes. The trichrome was applied by immersion of the fixated sample into Harris' haematoxylin and then the slices were incubated in three different solutions: A (acid fuchsin; 5 minutes), B (phosphomybdic acid; 5 minutes), and C (light green SF yellowish; 5 minutes). In between each solution the samples were rinsed in water. Finally, the sections were immersed in 1% acetic acid for two minutes, two changes of absolute alcohol each for two minutes, followed by two changes of xylene each for 3 minutes. The sections were then mounted in a resinous medium (DPX). All analysis was independently performed blind by two assessors using reference slides to access consistency of grading assessment.

For assessment of fibrosis, the slides were analysed using a Nikon Eclipse 90i microscope with CCD high speed camera and analysed in a blinded fashion using a semi-quantitative index (0-4) as described by Wintour *et al*. Grade 0= no positive green-blue (collagen) staining, 1= 25%, 2=26-49%, 3= 50-74% and 4=75-100%. Twelve random fields were chosen per kidney section and were evaluated at x 20 magnification.

2.3.8 RNA extraction

The total RNA was isolated from renal tissues and perirenal adipose tissue sampled at 7 days and 1 year of age using Tri-reagent (Sigma Chemical Co. Poole, UK)(Chomczynski & Sacchi, 1987) and a RNA extraction kit (RNeasy, Qiagen Ltd., Crawley, UK), which included a DNAse digestion step. Tri-reagent contains guanidine thicyanate and phenol, which dissolves RNA during tissue homogenisation. The addition of chloroform separates the aqueous phase containing the RNA, which is passed to an RNeasy mini column for further purification.

The RNeasy columns are designed for a rapid and effective isolation of up 100 µg of total RNA for frozen tissues. These columns contained a RNA nucleotide selective silica membrane to enable the combination of reagents to enhance the quantity and quality of the purification.

Once extracted, the quantity and preliminary integrity evaluation of the RNA was determined spectophotomerically (Nanodrop® Technologies, Wilminton, USA), calculating the concentration of RNA at 260 mm (1 OD unit= 40 µg RNA/µl) (Wilson & Walker, 2001). By also calculating the absorption at 280nm, it is possible to estimate the presence of protein contaminants. The optimum 260/280 absorbance ratio for RNA is 1.8 to 2.0 (Wilkinson, 1995; Wilson & Walker, 2001).

To further ensure the quality of the total RNA, samples were checked using the Agilent 2100 Bioanalyser (Agilent Technologies Inc., Santa Clara, USA). This spectrophotometer uses 1ng of RNA and is able to give an extremely accurate readout showing the 18S and 28S peaks, along with the degradation of any kind (Bustin & Nolan, 2004; Fleige & Pfaffl, 2006).

In order to avoid any contamination and degradation of RNA, all the materials were autoclaved and the working areas were cleansed of ribonucleases using RNaseZAP® (Sigma Chemical Co., Poole, UK). In addition, all the reagents, including the water, were RNAse and ribonuclease free. All the procedures were conducted with filter tips while wearing gloves and a laminar flow cabinet was used to set up the reactions. Moreover, all the stages of the reaction were performed on ice, to minimise RNA degradation. After preparation, the samples were stored at -80^{o}C until further use.

2.3.8.1 RNA extraction procedure

RNA was extracted from 100 mg renal cortex or 1 g PAT 7 days and 1 year old sheep in random order as described previously. Samples were fully homogenised at 9000 rpm for 45 seconds and left to stand at room temperature for 5 minutes to ensure complete dissociation of nucleoprotein complexes. Following this, 200µl of chloroform per 1 ml of tri-reagent was added, briefly mixed by vortex and then incubated for 15 minutes at room temperature, followed by a centrifugation (Microcentaur, Sanyo, SLS LTD; UK) at 13000 rpm for 10 minutes at 4^{o}C. The objective of this centrifugation was to separate the top aqueous phase (from the phenol phase), which was transferred to a sterile 1.5 ml microcentrifuge tube. Then, to each sample, an equal volume (to the separate aqueous phase) of 70% ethanol was added and mixed well by pipetting. All of

the mixture was transferred to an RNAeasy spin mini column and centrifuged (Microcentaur, Sanyo, SLS LTD; UK) at 10000 rpm for 15 seconds at room temperature.

To each sample, 350 µl of RW1 buffer (contains Guanidine Isothiocyanate and 70% ethanol) was added and centrifuged for 15 seconds at 10000 rpm (Microcentaur, Sanyo, SLS LTD; UK). After this step, 80µl of DNAase I solution it was added directly to the RNAeasy column membrane and incubated for 15 minutes at room temperature. Another 350 µl of RW1 was added, centrifugated. 500µl of RPE buffer (contains Guanidine Isothiocyanate) was then added and centrifuged. The empty columns were spun at 13000 rpm for 1 minute. All the columns were placed in a new 1.5 µl collection tube and the RNA was eluted by adding 30 µl of RNAse free water and centrifuged at 10000 rpm.

The RNA concentrations from these extractions were determined by spectrophotometer analysis. One µl was pipetted from each RNA preperation and placed on the Nanodrop® spectrophometer.

2.3.9 Reverse Transcriptase polymerase chain reaction (RT-PCR)

RT-PCR is a molecular biology technique that allows the amplification of a defined sequence of RNA and is used for the detection of a low copy number messenger RNA (mRNA). It is widely used in the diagnosis of genetic diseases and as a first step in the cellular determination of gene expression (Baumforth *et al.,* 1999).

Firstly, the RNA strand is treated with the reverse transcriptase enzyme that transcribes its complementary DNA (cDNA). The most common sources of reverse transcriptases are extracted from avian myeloblastosis virus (AM) or Moloney murine

leukaemia virus. Throughout my thesis, the reverse transcriptase used was extracted from the AM virus, which is able to synthesis cDNA at 42^{o}C preserving the RNA secondary structure (Sambrook & Russel, 2001). The product of this reaction is that the cDNA can be used as a platform using other techniques such as, amplification by polymerase chain reaction (PCR) or quantification of mRNA expression by real-time polymerase chain reaction (QPCR).

2.3.9.1 RT-PCR procedure

Total RNA isolated previously was diluted to 1µg/µl in a final volume of 5µl in a 0.5 ml microcentrifuge tube and labelled with an individual sample ID. Following, 8µl RT mix was added to all the tubes. This master mix consisted of 4 µl of reverse transcriptase buffer (100mmol/l dithrithreitol), 0.5µl RNAse inhibitor (final concentration 1.0IU/µl) and 2µl deoxyribonucleotide triphosphate (dNTP; final concentration 10nmol/l), 1µl of random hexamers primers (Roche Co. Basel, Switzerland) and RNAse free water (Ambion, Applied biosystems, Warrington, UK) to the final volume of 17µl. The reaction mixtures were heated to 25^{o}C for 5 minutes and then 1µl of reverse transcriptase was added (Roche Co. Basel, Switzerland). Reverse transcription was allowed to proceed for 10 minutes at 25^{o}C, then for 1 hour at 42^{o}C. The reaction was stopped and then cooled to 8^{o}C.

2.3.10 Primer design

Oligonucleotide primers are sequences of nucleic acids essential for the first step of DNA replication by any type of polymerase reaction. They are required because their binding to the genome marks the point where the DNA polymerase starts or finishes the

imprinting of the new DNA sequence. The DNA polymerase starts replication at the 3' end of the primer, and then copies the opposite strand. The oligonuclotides primers replace the *novo* RNA strand which starts the DNA replication or synthesis in nature (Sambrook & Russel, 2001; Gibson & Muse, 2004).

One of the parameters for the correct design of the primers is the similar melting temperature (T_m) of both primers. This is importance as the reaction, annealing to the DNA in PCR occurs for both primers at the same time. A significant differance between temperatures may result in an incorrect sequence of DNA or failure to extend at all. In addition, a correct T_m avoids that the primers annealing with other primers during the reaction, producing primer dimmers.

A common method in the design of primers is the use of software such as Beacon Designer, which is able to produce specific primers. This software can avoid the formation of primers loops or mishybridisation, by reducing the addition of mononucleotide repeats. This effect occurs when the primers anneal with themselves as internal hairpins or loops, hindering the annealing with the template DNA. For efficient results in real-time PCR (QPCR), the amplificon sizes should not exceed 250 bp.

In addition, to ensure the effectiveness of the DNA amplification, the primer sequences are designed from a unique DNA sequence. An online BLAST search engine was used that searches and compares all known DNA sequences that have similarity to the primers and identifies the boundaries between exon/introns on the DNA sequence, avoiding any amplification of possible genomic DNA. Therefore, it is possible to eliminate primers that may yield non-specific DNA amplifications (Wilson & Walker, 2001; Bustin & Nolan, 2004; Bustin *et al.*, 2005; Fleige & Pfaffl, 2006).

2.3.10.1 Primer design for QPCR procedure

The primers for QPCR used during this research were designed using the Beacon Designer 4.0 (Premier Biosoft, Palo Alto, USA). All the primers were designed specifically for QPCR reactions. To minimise nonspecific primer annealing, the melting temperature for the primers was set at 60^{o}C. The lengths of the primer were 15-30 bases each. The cytosine (C) and guanine (G) content (the number of Gs and Cs in the primer as a percentage of the total bases) was around 30-80% to avoid the formation of loops or mishybridisation of the primers.

The design of the primers was based on known mRNA sheep sequences published on the National Center for Biotechnology Information (NCBI) online database (http://www.ncbi.nih.gov). To ensure that the primer sequences would not cross hybridise with other sequences within the genome, the target sequence was tested using the BLAST search engine from the NCBI (http://www.ncbi.nlm.nih.gov/BLAST). To avoid amplification of contamination genomic DNA, primers were designed to include cross exon/intron boundaries wherever possible.

The oligonucleotides primers were purchased form Sigma-Genosys (Sigma Chemicals Co. St. Lous, USA) and were resuspended in RNA free water to achieve a stock concentration of 100µmol/l. Table 2.4 describes the sequence and annealing temperature of each primer use during this project:

Table 2.4 QPCR sequences use in this thesis

Gene	Chapter	Symbol	Specie	Publication or PubMed sequence	Primer sequence (5'-3')	Annealing temp °C
18S ribosomal RNA	2	18s	*Sus scrofa*	AK2372246.1	Foward GAT GCG GCG GCG TTA TTC C Reverse CTC CTG GTG GTG CCC TCC	58°C
Insulin-like growth factor-1	3	IGF-1	*Sus scrofa*	DQ784687.1	Foward CTT CAG TTC GTG TGC GGA GAC Reverse CTC CAG CCT CCT CAG ATC ACA G	60°C
Insulin-like growth factor-2	3,5	IGF-2	*Ovis aries*	MacLaughlin *et al*., 2007	Foward GCT TCT TGC CTT CTT GGC CTT Reverse TCG GTT TAT GCG GCT GGA T	60°C
Insulin-like growth factor-2 Receptor	3	IGF-2R	*Ovis aries*	MacLaughlin *et al*., 2007	Foward GAT GAA GGA GGC TGC AAG GAT Reverse CCT GAT GCC TGT AGT CCA CTT	60°C
Growth hormone recpetor	3	GHR	*Ovis aries*	NM001009323.1	Foward AAG CCT GGA GGA AAC CAT ACG Reverse TGC CAC TGC CAA GGT CAA C	60°C
Insulin-like growth factor-1 Receptor	3,4	IGF-1R	*Ovis aries*	AY162434.1	Foward TCT AAC TTT GTC TTT GCA AGA ACC A Reverse TCA CTG GCC CAG GAA ATG TC	60°C
TNF receptor superfamily	4	Fas	*Ovis aries*	Moorjani *et al*., 2003	Foward CGG GAT CTG GGT TCA CTT GTC Reverse AAC AGG TGC TCA CGA TAT AGG C	57°C
Tumor protein p-53	4	p-53	*Ovis aries*	X81705	Foward CGG GTG GAA GGG AAT TTA CGC Reverse ATG AAG TTG TAG TGG ATG GTG GTG	60°C
Bcl-2 associeted X protein	4	Bax	*Ovis aries*	DQ323116.1	Foward TGA AGC GCA TTG GAG ATG AA Reverse CTT GAG CAC CAG TTT GCT GG	60°C
B-cell CLL/lymphoma 2	4	Bcl-2	*Ovis aries*	AF515848	Foward TTC GCC GAG ATG TCC AGT CAG C Reverse TTG ACG CTC TCC ACA CAC ATG ACC	59°C
Glucose Transporter 1	4	Glut-1	*Ovis aries*	U89029.1	Foward GCA GGA GAT GAA GGA GGA GAG C Reverse GCA GCA CCA CGG AAA TGA G	60°C
Hypoxia-induceble factor 1 α	4	HIF-1α	*Ovis aries*	AY485676	Foward CGT GTT ATC TGT CGC TTT GAG TC Reverse ATC CAC TTT CAT CCA TTG ATT GCC	58°C
Hexokinase 1	4	HK-1	*Ovis aries*	AM492192	Foward TAA TCC GAC AGC CAC AGT CA Reverse AGG GCG ATG AAA TCT CCT TT	60°C
Insulin receptor	4	IR	*Ovis aries*	AY157728.1	Foward CTG CAC CAT CAT CAA CGG AA Reverse CGT AAC TTC CGG AAG AAG GA	60°C
Vascular endothelial growth factor A	4,5	VEGF-A	*Ovis aries*	MN001025110	Foward GCC TTG CCT TGC TGC TCT AC Reverse GGT TTC TGC CCT CCT TCT GC	60°C
DNA(cytosine-5)-methyltransferase 1	3,4,5	DNMT-1	*Ovis aries*	AY228548	Foward GGG AAG CGA ATG GAT GTC TA Reverse GTT CTT CCT CGG GTT TTC GT	60°C
endothelial Nitric Oxide synthase	4,5	eNOS	*Ovis aries*	MN00603	Foward AAC CAT CCT GTA CGC CTC TG Reverse ATT CCC AAA GGT GCT GGT C	60°C
inducible Nitric Oxide synthase	4,5	iNOS	*Ovis aries*	MN00625	Foward CCC CAA GCT CTA CAC TTC CA Reverse ACA GGA GAG TTC CAC CAG GA	60°C

2.3.11 Polymerase chain reaction (PCR)

Polymerase chain reaction or PCR is widely used for the amplification of a chosen region of the DNA molecule. PCR as RT-PCR involved the use of two primers, which are complementary to opposite strands of the double stranded DNA sequence to be amplified (Zheng *et al.*, 2006). The key to this reaction is the use of the Tag polymerase enzyme (extracted from the bacteria *Thermus aquaticus*), which maintains its activity for long periods of time at high temperatures (>105^{o}C) (Saiki *et al.*, 1988). The high temperatures are essential for the reaction allowing the separation of the double strand

DNA and the binding of the primers to it. During this reaction, the polymerase enzyme synthesises the new DNA strand from the DNA basic components, the nucleotides, and by adding them after the binding of the primers on the template DNA.

The basic PCR reaction is a sequence of several cycles (approx 40 cycles) of denaturation, hybridisation and synthesis resulting in the exponential amplification of the DNA target sequence (Wilkinson, 1995; Sambrook & Russel, 2001; Wilson & Walker, 2001). The product of each PCR reaction was tested using an Aragose gel for DNA genomic contamination, optimisation of the amplification process (T_m), and each PCR product was sent for DNA sequencing for the validation of the primers using the QMC sequence service. The resulting chromatogram were then analysed by using Chromas Lite computer software (http://www.technelyium.com.au) and checked for the correct genes by BLAST.

A sample from each PCR reaction was used to measure the concentration using the Nanodrop® spectrophotometer. The results of this measurement were used to set the standard of the QPCR reaction.

2.3.11.1 PCR procedure

As previously described, the PCR reaction was performed by keeping the cDNA in an ice box, working with gloves and filter tips. The reaction was performed in a final volume of 20µl using 1µl of cDNA, 10µl of PCR master mix (ABgene Ltd. Epson, UK), 1µl forward and 1µl reverse primers at a final concentration of 100µmol/l in 7µl of RNA free water. PCR conditions are described in Table 2.5:

Table 2.5 PCR standard programme

Time	Temperature	Step
4 Minutes	105°C	Initaition
15 Minutes	96°C	Enzyme activation
	Number of cycles 45	
30 Seconds	94°C	Denaturation
30 Seconds	60°C	Annealling
60 Seconds	72°C	Extension
7 Minutes	72°C	Final extension
	One single step	
Indefinitely	8°C	Hold

The cDNA was run in an electrophoresis agarose gel (3%) and gel extracted using QIAquick gel extraction kit (Qiagen).

2.3.12 Aragose gel electrophoresis

DNA molecules carry a negatively charge, which enable their separation in an electric field, with the shorter molecules moving faster and migrating farther than the longer ones in the direction of the cathode. The matrix in which the proteins move is built up of a material called agarose (3% (w/v) aragose pH7), which is dissolved in TAE buffer (2M Tris ([hydroxymethyl] amino-methane, 0.2% (w/v) SDS, 1 M Glacial acetic acid , 0.5M Na_2 EDTA ph8, deionsate water up to 1000ml) by heating. After a short period cooling 0.01% of ethidium bromide is added, a dye which fluoresces under ultra violet (UV) light when intercalated into DNA. 20µl of PCR product was mixed with 5µl of 5x gel loading buffer (0.0016% (v/v saturated bromophenol blue, 0.15% (w/v)

EDTA, 20% (v/v) Glycerol) and 5 µl of 1Kb sizes marker (ABgene) were loaded in different lines. The electrophoresis was then performed at a constant 120 V for 1 hour. For the visualisation of the DNA, the gel was exposed to a UV transilluminator CCD camera (Fuji film luminescent image analyser LAS-1000 V1.0).

2.3.13 DNA extraction from agarose gel

The gel extraction was performed using a gel extraction kit (QIAquick®, Qiagen, West Sussex, UK) that combines spin-column technology with the selective binding properties of a silica membrane which absorbs the DNA in the presence of a high concentration of salt, while allowing contaminants to pass through. After further purification steps, the purified DNA can be eluted.

The PCR products were run on a 3% aragose gel for 1 hour at 120 V. After visualisation, the bands were cut and transferred to a 1.5 ml microcentrifuge tube. For each 100 mg of aragose gel, 300 ml of QG was added, and incubated at 50^{o}C for 10 minutes with vortexing at intervals of 3 minutes to facilitate the dissolution of the gel. Once the gel was completely dissolved, 100 µl of Isopropanol was added per 100 mg of gel, for precipitation of the DNA. The mix was transferred to a sterile spin column and centrifuged at 13000 rpm for 1 minute. The resulting flow was discarded and another 500 µl of QG buffer was added, to eliminate any traces of agarose. Again, the column was centrifuged as previously described and the flow was discarded.

Next, 750 µl of PE buffer was added and centrifuged for 1 minute at 13000 rpm. The empty column was centrifuged again at full speed to eliminate any traces of PE (contain ethanol). Finally, the column was transferred to a clean microcentrifuge tube to

which 30µl of RNA free water was added and incubated for 1 minute at room temperature. The DNA was eluted by centrifugation at 13000 rpm during 1 minute.

2.3.14 Real-Time polymerase chain reaction (QPCR)

Real time PCR (QPCR) is a kinetic reaction that monitors the increases of a signal, normally fluorescent, as an indicator of amplicon production during each PCR cycle (Lee *et al.*, 1993). This signal increases in direct proportion to the amount of PCR product in a reaction and is detected when it rises above a set background and detection correlated with an initial amount of target template (Higuchi *et al.*, 1992; Higuchi *et al.*, 1993; Bustin, 2000).

The signal is normally measured at a fixed threshold level by the operator and is called the cycle point (Cp) (Wittwer *et al.*, 1997). This parameter is defined as the cycle number at which the signal exceeds the fixed threshold. During the reaction, the monitoring of the signal creates a sigmoid line until it reaches a plateau. During the linear log face, this signal can be compared with other samples in the reaction (Higuchi *et al.*, 1993). Normally, the QPCR reactions are performed in a 96 well plate, in which the samples can be compared with the standard curve of known concentrations of DNA and curve is also used to calculate the efficiency of the reaction (Bustin *et al.*, 2005). These known concentrations of DNA are a series of dilutions usually extending from 10^{-1} ng/µl to 10^{-9} ng/µl. The plotting of the Cp values versus the log of the cDNA concentration of the standard curve creates a slope, which reflects the amplification efficiency (E). E can be calculated by the formula (Lekanne Deprez *et al.*, 2002; Fleige & Pfaffl, 2006):

$$E = 10^{[-1/slope]}$$

Equation 2.1 Mathematical model proposed by Pfaffl for the equation of QPCR efficiency.

The efficiency of the QPCR reaction should be 90-100% (-3.6> slope >-3.1 or 1.9≥ E ≥2). However, a number of variables can affect the efficiency of the QPCR including length of the amplification, secondary structure of the product and primer quality (Bustin & Nolan, 2004; Yuan *et al.*, 2006).

The fluorescent signal emitted by a DNA binding agent like SYBR green, which attaches specifically to double stranded DNA, increases proportionally with the amount of double stranded DNA produced in each cycle during the length of reaction. The disadvantage of SYBR is the requirement for extensive optimisation because it is unable to discriminate its binding to any double stranded DNA (Ririe *et al.*, 1997; Morrison *et al.*, 1998).

As mentioned in previously (Section 2.3.9), the specificity of the reaction is determined by the quality of the primers. The specificity of reaction can be measured by creation of a melting curve that allows the visual comparison between different melting temperatures of specific products. This allows a search for any suspected nonspecific products. The melting temperature is a function of the length and sequence of the final product and this will be observed as different peaks on the graph. The specificity of the reaction will be marked as a single peak or similar melting temperature (Ririe *et al.*, 1997; Morrison *et al.*, 1998).

The quantity of gene expression between different samples needs to be normalised against housekeeping gene expression of the same sample, thereby normalising any possible variation between different samples. A housekeeping gene is one with a

constant mRNA abundance during the cell life cycle, *i.e* 18S gene which was tested under a range of physiological conditions in comparison to other housekeeping genes, such as GAPDH (Sabek *et al.*, 2002; Schmid *et al.*, 2003; Bustin & Nolan, 2004).

The most efficient way to analyse QPCR results is by using the mathematical model for relative quantification developed by Pfaffl (Pfaffl, 2001) (equations 2.1 and 2.2).

$$Ratio = \frac{(E_{target})^{\Delta Cp_{Target}\ (control-sample)}}{(E_{ref})^{0}}$$

$$Ratio = (E_{target})^{\Delta Cp_{Target}\ (control-Sample)}$$

Equation 2.2 Mathematical model also proposed by Plaff for the relative gene expression by QPCR procedures under constant reference gene expression. CP values in the sample and the control are equal and represent ideal housekeeping expression and condition.

2.3.14.1 QPCR procedure

In all the runs, all the unknown and standard curve samples were placed in a heat sealled 96 well plate (ABgene) in duplicate. For each gene, DNA that made up the standard curve was obtained from DNA extracted from Aragose gels. The internal controls used in each reaction were: no primer control (NPC, containing all reaction reagent minus primers) amd no reverse transcriptase control (NRT), which is a mock reverse transcription containing all the RT-PCR reagents except the reverse transcriptase. The latterallows testing the presence of genomic DNA (Bustin *et al.*, 2005).

As mentioned in the previously (Section 2.3.13), a period of optimisation is needed for the correct use of SYBR green (Thermo, ABgene Ltd. Epson, UK). This

consisted of the monitoring of nonspecific products, such as primer dimmer (melting curve graph) and efficiency of the reaction. For improving reaction conditions, at least three different primer conditions (100, 300 and 500 nmol/l) and three different annealing temperatures (58, 60 and 62^{o}C) were tested.

The QPCR reactions were carried out in a final volume of 20 µl, which included 1µl of both forward and reverse primers, 10 µl of SYBR green master mix with ROX passive reference, and 7 µl of free RNA water. Once the plates were loaded with all the reactions they were heat sealed and placed in a PCR machine (Techne Quantica®, Staffordshire, UK). Table 2.6 describes the temperature and conditions used for the QPCR of the housekeeping gene 18s:

Table 2.6 QPRC standard programme

Time	Temperature	Step
4 Minutes	94^{o}C	Denaturation
Number of cycles 45		
10 Seconds	94^{o}C	Denaturation
25 Seconds	60^{o}C	Annealling
15 Seconds	72^{o}C	Extension
15 Minutes	Ramp from 75 to 72^{o}C	Melt Curve
One single step		
Indefinitely	8^{o}C	Hold

The data collected from the QPCR reaction was exported to an Excel datasheet. Each sample Cp was tested for coefficient of variance and its mean was normalised against the 18S housekeeping gene of the same sample.

2.3.15 8-Hydroxy 2-deoxy Guanosine elisa assay

8-hydroxy 2 deoxy Guanosine (8-OHdG) is a byproduct of the reaction between reactive oxygen and nitrogen species. Excessive concentrations of 8-OHdG is a well established marker of oxidative stress (Spencer *et al.*, 1995) . The generation of 8-OHdG is a response to both normal metabolic processes and of other environmental factors. Rising levels of 8-OHdG are associated with tissue injury due to oxidative stress as well as with a number of pathological conditions including cancer, diabetes and hypertension. In tissues, plasma and cell lysates 8-OHdG may be found as a free nucleoside or attached to DNA (Hirai *et al.*, 2006; Shen *et al.*, 2007; Tomohiro *et al.*, 2007).

This assay is based on a competitive ELISA where 8-OHdG, conjugate binding 8-OHdG protein and an anti conjugate 8-OHdG goat antibody compete for a limited and fixed amount of anti-goat monoclonal HRP conjugated antibody. The amount of 8-OHdG monoclonal antibody is inversely proportional to the concentration of 8-OHdG attached to the well. The plate is washed to remove any unbound reagents and then Ellmna's Reagent (which contains the substrate to HRP) is added to the well. The product of this enzymatic reaction produces a yellow colour and can be detected at absorption of 450 nm. The intensity of the colour, is inversely proportional to the amount of free 8-OHdG present in each sample (Hempelmann *et al.*, 1987; Chou *et al.*, 2007). For a schematic explanation of this assay see Figure 1.4.

The determination of 8-OHdG described above was performed using a commercial kit (OxiSelect™ 8-OHdG assay kit, Cell Biolabs Inc., CA, USA).

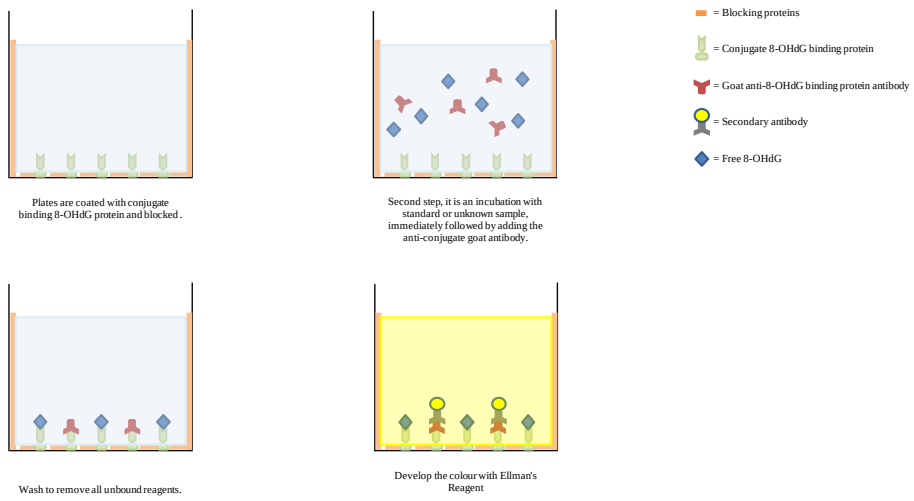


Figure 2.5 graphical explanation of the 8-OHdG competitive elisa

2.3.15.1 8-Hydroxy 2 deoxy Guanosine elisa procedure

Sample preparation

1g of renal cortex was homogenised using a Dispomix® homogeniser at 9000 rpm for 45 seconds in 5 ml buffer (0.1 M phosphate buffer, pH 7.4, 1 mM EDTA) and centrifuged at 1000 x g for 10 minutes. From the resulting supernatant, the DNA was extracted using a commercially available extraction kit (DNeasy®, Qiagen, West Sussex, UK). The concentration of each sample was verified using Nanodrop® spectrophotomer Then the DNA was digested using nuclease P1 (Sigma) for 2 hours at 37°C in 20 mM Sodium acetate, followed by incubation with 1 unit alkaline phosphatase at 37°C for 30 minutes per 100µl of DNA. After this incubation, the samples were heated at 100°C for 10 minutes. The reaction mixture was centrifuged for 5 minutes at 6000 x g. Finally, the

supernatant DNA samples were determined spectrophometrically and stored at 4^{o}C until use.

Performing the assay

Each standard and sample to be analysed was prepared in duplicate and randomised as described in previously (Section 2.4.4.1). The first step was the coating of the plate: 1mg/ml of 8-OHdG conjugate was diluted to 10µl/ ml with 1 time PBS. To each well 100 µl of 8-OHdG conjugate solution was added and incubated overnight at 4^{o}C. Next day, the unbound 8-OHdG conjugate was removed by washing each well with 300µl of dionased water. The plate was blotted in paper towels to remove excess fluid. Then, 200 µl of assay diluent buffer was added to each well and blocked for 1 hour at room temperature.

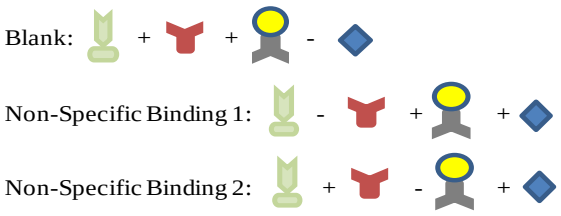
The standard curve solution was diluted with the assay diluent in a concentration range of 0 ng/µl to 20 ng/µl 8-OHdG. Then, 50 µl of unknown sample or 8-OHdG standard was added to the wells of the conjugated plate. The plate was incubated for 10 minutes on an orbital shaker. Next, 50 µl of diluted (1:1000) anti-8-OHdG antibody was added to each well and the plate was incubated was 1 hour on an orbital shaker at room temperature. Following, each well was washed by applying three times 250 µl of wash buffer thorough aspiration. After the last wash, the plate was emptied by taping on a paper towel to remove the excess of wash buffer. Then, 100 µl of the diluted secondary antibody was added to all the wells, followed by 1 hour of incubation at room temperature in constant agitation. The plate was washed three times. Immediately, 100µl of Ellmna's Reagent was added to each well including the blank and control wells. The plate was incubated for 5 minute on an orbital shaker at room temperature. To stop the reaction, 100µl of Stop Solution was added into each well. Finally, the absorbance was

read by the spectrophotometer using 450 nm as the primary wave length. The concentration of 8-OHdG was expressed as: [8-OHdG ng/µl].

To validate this methodology, firstly the standard curve was tested, and each dilution was repeated in duplicates to guarantee the reproducibility of the assay. The parameters tested were coefficient of variance between same concentration, absorption value of the blank and R^2 value. In addition, different concentrations of DNA sample were tested by serial dilution and finding the detection range of this assay as described in previous sections (Section 2.4.4.1). The detection range of this assay was between 25-100 ng/ml of DNA. All the samples were diluted to 50 ng/ml in deionised water.

The controls used for this assay were:

List of control used



2.4 Statistical analysis

To assist in the study design and to limit the sample sizes that would be required in each group and not erroneously accept (type II error) or reject (type I error) the null hypothesis, a power calculation was performed. Sample size was calculated to assess the effect of measured variables (such as weight) obtained from an intervention (NR or obesity) or group at 80% power. The sensitivity of the power calculation is a function of the group sizes, statistical controls and reliability of the measures. Therefore, by increasing sample sizes it is possible to increase the statistical power. In the research literature, it is generally accepted that a power of 80% is needed as a standard for adequacy. Power calculations were performed using Samplepower ™ (SPSS Inc., Chicago, Illinois, USA).

Previous to any statistical analysis, all the values collected in each assay were transferred up to an Excel datasheet. Then, the coefficient of variance (*CV*) of all the duplicate values were analysed and for which a $CV \leq 0.95\%$ confirmed low variation between duplicates and the reproducibility of the assay. The data points that fulfil this requirement were used for the analyses.

The distribution of the data collected in each assay was the main factor for the chosen statistical test to perform. For testing normal distribution, the data collected of each group were subjected to the Kolmogorov-Smirnov test for which a $P \leq 0.05$ confirmed that the data was normally distributed. Depending on the significance of this analysis, parametric or nonparametric tests were utilised for the analysis of the data. For consistency, the data in these studies are presented as mean and standard of error (*SEM*).

During the length of this study, the NR-O group and L group are considered to be exposure to more than one intervention (*e.i* maternal nutrient restriction and exposure to an obesogenic environment). For that reason, to obtain a biological meaningful analysis, the L group was compared only with the O group, who was exposed only to one intervention (obesogenic environment). Subsequently, the means of the two obese groups (NR-O and O) were compared independently of the L group. Thus, depending on their type of distribution based on the significance of the Kolmogorov-Smirnov test, either an independent parametric (T-test) or non-parametric test (Mann Whitney U) was assigned to compare these two groups. When an independent T-test was applied ,it was followed by a Levene's test to evaluate equal variances. If test result was $p > 0.05$, equal variances between the groups could be assumed. More specific tests are detailed throughout the results chapter and further explanation is given when relevant. The statistical tests were performed using SSPS software (version 14.0).

Chapter 3 - The effect of maternal nutrient restriction between early to mid gestation on renal and perirenal adipose tissue at seven days of age

Chapter 3 - The effect of maternal nutrient restriction between early to mid gestation on renal and perirenal adipose tissue at seven days of age

3.1 Introduction

3.1.1 Early effects of maternal nutrient restriction

The thrifty phenotype hypothesis suggests that low birth weight compromises adult health and well being by increasing the risk of the development of metabolic, endocrine and cardiovascular diseases (Hales & Barker, 2001). However, the causes of low birth weight are complex and many retrospective studies have shown that maternal health and nutritional status play a part in the programming of future health outcomes, irrespective of birth weight (Hales & Barker, 2001, Olson et al., 2008). These findings are supported by evidence collected from offspring exposed to the Dutch Famine (Roseboom et al., 2001). Moreover, another important conclusion from this cohort is that outcomes in later life depended on the trimester of gestation in which there was exposure to famine. For instance, offspring exposed during the second trimester were not smaller or lighter than non-exposed newborn but they had an increased prevalence of renal disease and other metabolic alterations in later life (Painter et al., 2005). Furthermore, evidence obtained from large animal models of maternal nutrient manipulation shows that the only examples that lead to a reduction in birth weight are those offspring exposed to severe undernutrition throughout pregnancy, or during, the last third of gestation (Whorwood *et al*., 2001; Oliver *et al*., 2002; Budge *et al*., 2004).

It is important to note that the majority of studies describing the development of renal tissue influenced by the maternal nutritional environment were conducted in

singleton offspring, which may differ from the present study that utilised twin bearing mothers. Previous studies have shown that twin and singleton ovine offspring have different insulin metabolic responses, which may affect the patterns of renal and adipose tissue development (Bloomfield *et al*., 2004; Rumball *et al*., 2008).

As in humans, a reduction in maternal nutrition during early to mid gestation in sheep (30 to 80 days of 145 day pregnancy) produced offspring of similar weight to those born from standard fed mother but with significant alteration in fat mass, blood pressure and renal structure (Whorwood *et al*., 2001; Bispham *et al*., 2005; Gopalakrishnan *et al*., 2005). Near term singleton fetuses (≈140 days of 145) exhibited a rise in fat deposition in conjunction with an increase in mRNA abundance for IGF-1 and -2 receptors (Bispham *et al*., 2003). This effect was accompanied by an increase in mRNA abundance of other key regulatory components of fat metabolism, including UCP-2 and PPAR-α (Bispham *et al*., 2005). Additional alterations in mRNA gene expression of critical pathways in renal and adipose tissue development included the glucocorticoid and angiotensin 1 receptors (Whorwood *et al*., 2001). In another similar cohort, at six months of age, the nutrient restricted offspring showed a reduction in blood pressure in conjunction with a decrease in nephron number (Gopalakrishnan *et al*., 2005). These results suggest that maternal nutrient restriction during early-to-mid gestation potentially alters the gene expression of key physiological and endocrine pathways, affecting the normal patterns of renal and adipose tissue development and subsequently altering the risk of cardiovascular disease in later life (Tchoukalova *et al*., 2004; Gopalakrishnan *et al*., 2005).

3.1.2 Changes in the growth hormone (GH)-IGF axis in renal and adipose tissue

Linear growth is unique to childhood and growth hormone (GH) is a key endocrine regulator of postnatal growth (Low *et al*., 2001). However, during early childhood, the normal GH production and release is mainly nutritional dependent (Ogilvy-Stuart *et al*., 1998). One of the mechanisms by which maternal nutrient restriction modulates postnatal growth may be through alterations in the GH-IGF axis, that has a major role in kidney and adipose tissue development (Brennan *et al.*, 2006). During the neonatal period, the receptors of this axis are detectable at very high levels in developing renal and adipose tissue (Ymer & Herington, 1992; Holzenberger *et al.*, 2001). IGF-1 and its homolog IGF-2 are both involved in embryonic development, whereas only IGF-1 maintains a role in growth regulation after birth. In small animals, IGFs play an essential role in renal development by inducing metanephric differentiation, thereby modulating nephron number (DeChiara *et al.*, 1990; Liu *et al.*, 1993). In addition, IGF-1 production is GH dependent (Tannenbaum *et al.*, 1983). IGFs, by binding to IGF-1 receptors (IGF-1R), play a role in the negative feedback regulation of GH in juvenile animals and, to a lesser degree, in adults (Tannenbaum *et al.*, 1983).

In vitro, GH appears to regulate the number of IGF receptors in adipocytes by modulating the stimulatory effects of IGF (Lonnroth *et al.*, 1987). Genetically induced IGF-1R deficient mice are seen to have disproportionate postnatal growth, particularly in adipose tissue, characterised by a reduction in adipocyte number per fat pat and a raised lipid content (Holzenberger *et al.*, 2001). A possible explanation for these effects has come from cell culture studies, which indentified IGFs as important regulators of adipocyte development (Deslex *et al.*, 1987).

As well as having a role in adipose and renal tissue development, several observations indicate an additional role for the IGF-GH axis in mature kidney function. Infusion of GH into healthy adults causes an increase in glomerular filtration rate (GFR) and renal plasma flow (RPF). Hirshberg *et al.* have demonstrated that renal IGF-1 is the main component for GH regulation of kidney function (Parving *et al.*, 1978; Hirschberg & Kopple, 1989). Thus, alterations in the IGF-GH axis by reducing prenatal nutrition could compromise the early development of renal and adipose tissues, thereby increasing the risk of metabolic and renal dysfunction in adulthood.

3.1.3 Common factors in cell maturation in renal and adipose tissue

In newborn sheep, brown adipose tissue represents the main adipose tissue depot, particularly around the kidneys. Over the first two weeks of postnatal life, the majority of brown adipocytes in this depot completely disappear and are replaced by white adipose tissue (Finn *et al.*, 1998). Comparative microarray analysis of pre-adipocytes and white adipocytes revealed the involvement of DNA methyltransferases (DNMT) as one of the key regulators of the differentiation process (van Beek *et al.*, 2008). One of these proteins, DNMT-1, is associated with the maintenance of methylation patterns during replication and *de novo* DNA methylation.

It is known that, during the cell differentiation process, DNMT-1 interacts with the retinoblastoma protein, an important developmental protein with a role in the regulation of energy expenditure (Robertson *et al.*, 2000; Hansen *et al.*, 2004). DNA methylation has the potential to affect the expression of other essential genes that are active during cell differentiation, such as IGF-2 and its receptor (Kaneda *et al*., 2004). A decrease in the expression of IGF-2 has been linked with a reduction in muscle mass and an increase

in fat deposition in pigs (Nezer *et al.*, 1999). Recent findings have demonstrated that lactogens are able to control the differentiation and growth of brown adipose tissue in neonatal mice through the up-regulation of IGF-2 (Viengchareun *et al.*, 2008).

In addition, DNMT-1 activity and alterations in DNA methylation patterns have been implicated in gene expression and morphologic changes in renal tissues, induced by a suboptimal maternal environment (Pham *et al.*, 2003). For instance, exposure to uteroplacental insufficiency in rats decreased the methylation in specific areas of the p-53 promotor. This triggered excessive apoptosis, altering the process of organogenesis and potentially reducing nephron numbers. Interestingly, this effect was associated with a reduction in DNMT-1 mRNA expression in the renal cortex, followed by a reduction in IGF-1 gene expression (Pham *et al.*, 2003). Thus, the enzymatic activity and expression of DNA methyltransferases, such as DNMT-1, may be critical factors for postnatal growth of the kidney.

3.1.4 Scientific rationale, aim and hypothesis

Previous human and animals studies have indicated an association between the maternal environment *in utero* and an increased risk of later renal disease. It has also been postulated that the development of adipose tissue during embryogenesis and early fetal life may play a role in the modulation of kidney function in adulthood. Furthermore, as a key endocrine organ, alterations of adipose tissue function, caused by later exposure to an obesogenic environment, may increase susceptibility to kidney disease. The hypothesis to be examined in this chapter was whether a targeted reduction in maternal nutrition over the period of early organogenesis in sheep affected early postnatal maturation of adipose and renal tissues. In addition, it was postulated that

exposure to maternal nutrient restriction will result in an altered pattern of expression of key developmental genes involved in postnatal development, such as cell cycling, glucose utilisation and DNA methylation. Therefore, the effects of maternal nutrient restriction during this period of postnatal development on the gene expression of components of the IGF-GH axis, IR, HK-1, GLUT-1, DNMT-1, eNOS, iNOS and p-53 in renal tissue and IGF-1, IGF-1R, IGF-2, IGF-2R and DNMT-1 in perirenal adipose tissue of seven-day-old sheep, were investigated.

3.2 Methods and materials

A full description and details of all methods, including animal experimentation, gene expression analysis used in this chapter can be found in Chapter 2, with the exception of the estimation of nephron number assay that was kindly carried out and supervised by Dr. David S. Gardner with the collaboration of Prof. Michael E. Symonds. In addition, all statistical tests utilised are also explained in Chapter 2 or in the results section of this chapter.

3.3 Results

3.3.1 The effects of maternal nutrient restriction between early to mid gestation on birth weight, parameters of renal development and fat mass

As expected, there was no difference in body weight at birth between groups and this relationship persisted until the time of dissection at seven days of postnatal age. In addition, there were no effects of maternal nutrient restriction on kidney or perirenal adipose tissue weights. However, the ratio between kidney and body weight was lower in offspring exposed to maternal nutrient restriction ($p<0.01$; Fig. 3.1.B). Nephron numbers per group were similar. Importantly, the mean increase in total body weight was directly related with the increase in fat in each group during this first week of neonatal life ($p<0.01$; Fig. 3.2).

In addition, NR offspring exhibited a trend towards greater pancreas weight compared to the controls ($p= 0.053$; Fig 3.1.A). Finally, no difference was recorded between the groups in the relative mass of the remaining tissues dissected or body dimensions measured at seven days of age (Table 3.1).

Table 3.1 Physiological characteristics at birth and at dissection time (adapted from Williams *et al*., 2007)

Growth Parameter	NR Group (n=12)	Control Group (n=7)	P
Weight at birth (kg)	3.19 ± 0.25	3.18 ± 0.15	NS
Weight at 7 day (g)	4.58 ± 0.25	4.24 ± 0.29	NS
Crown-rump length (mm)	43.11 ± 0.72	42.43 ± 1.00	NS
Total kidney weight (g)	27.28 ± 1.49	29.42 ± 1.10	NS
Kidney length (mm)	38.86 ± 0.86	39.25 ± 0.85	NS
Kidney breadth (mm)	25.57 ± 2.49	27.28 ± 1.49	NS
Kidney Depth (mm)	24.29 ± 1.90	26.50 ± 1.94	NS
Nephon numbers per kidney	$5.25 \times 10^5 \pm 2.34 \times 10^4$	$5.36 \times 10^5 \pm 1.04 \times 10^5$	NS
Perirenal adipose tissue weight (g)	49.50 ± 5.45	40.19 ± 7.18	NS
Total fat (g)	65.75 ± 7.24	53.25 ± 7.47	NS
Ratio total fat body weight at 7 day (g/g)	13.74 ± 0.97	12.44 ± 1.25	NS
Ratio perirenal adipose tissue kidney weight (g/g)	1.81 ± 0.16	1.35 ± 0.35	NS
Mean increase weight per day (g/d)	193.29 ± 31.29	159.14 ± 31.29	NS

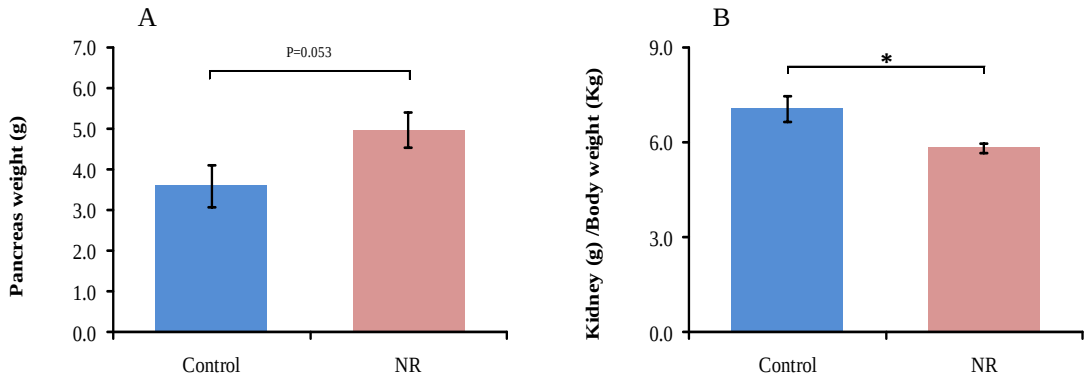


Figure 3.1 (A) Effect of maternal nutrient restriction on pancreatic weight (p=0.053) and (B) ratio of kidney weight to body weight at seven days of age (*p<0.05). Values are mean ± SEM.

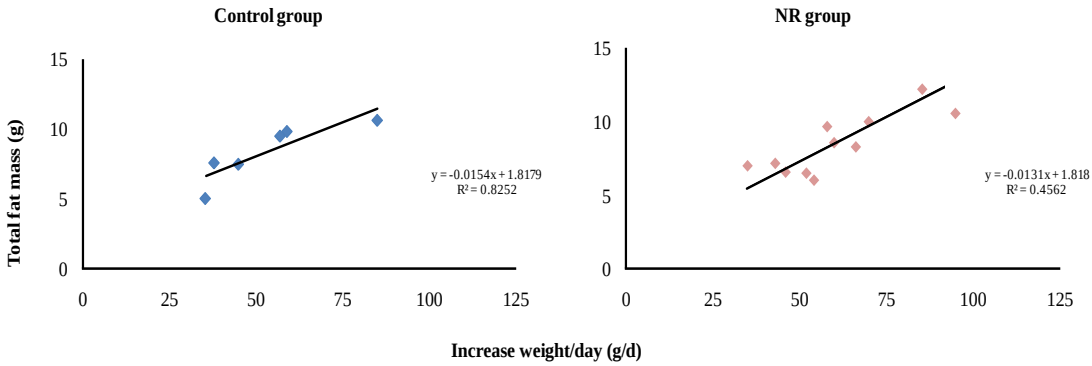


Figure 3.2 Linear association between the mean of weight increase and the total fat mass in both nutrition groups at seven days.

3.3.2 Effects of maternal nutrient restriction between early to mid gestation on the renal IGF-GH axis

3.3.2.1 Renal IGF-1, IGF-2, IGF-1R and IGF-2R gene expression in seven-day-old offspring

There was no difference in IGF-1 mRNA abundance in the kidneys between groups but, interestingly, it was negatively correlated to total fat mass ($p<0.01$: Fig. 3.3) and to body weight at seven days of age ($p<0.001$; Fig. 3.4). There was no difference between the groups in renal IGF-1R, IGF-2 and IGF-2R mRNA expression (Table 3.2).

Table 3.2 Renal mRNA expression of IGF-1, IGF-2 and their respective receptors at seven days of age

Gene	NR Group	Control Group	P
IGF-1 ($2^{-\Delta CT}$)	0.95 ± 0.14	1.00 ± 0.13	NS
IGF-2 ($2^{-\Delta CT}$)	2.50 ± 0.71	1.00 ± 0.16	NS
IGF-1R ($2^{-\Delta CT}$)	0.81 ± 0.12	1.00 ± 0.24	NS
IGF-2R ($2^{-\Delta CT}$)	0.86 ± 0.11	1.00 ± 0.11	NS

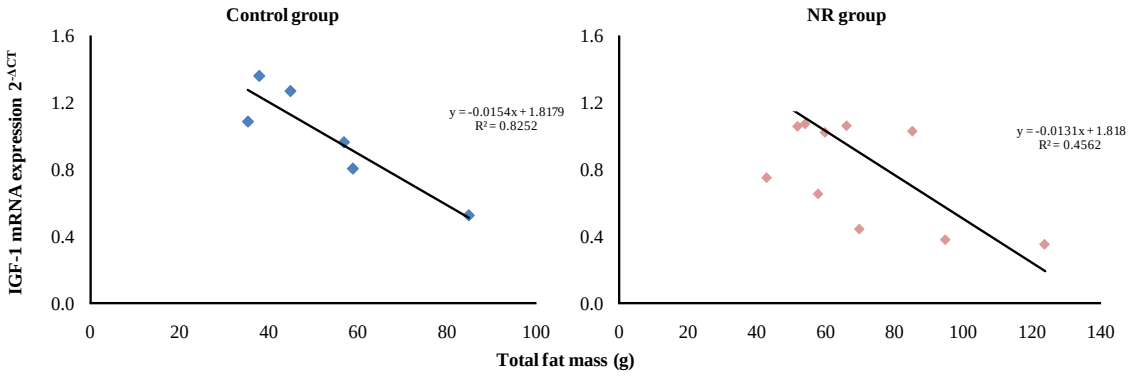


Figure 3.3 Scatter plots illustrating the negative relationship between IGF-1 renal gene expression and total fat mass at 7 days of age

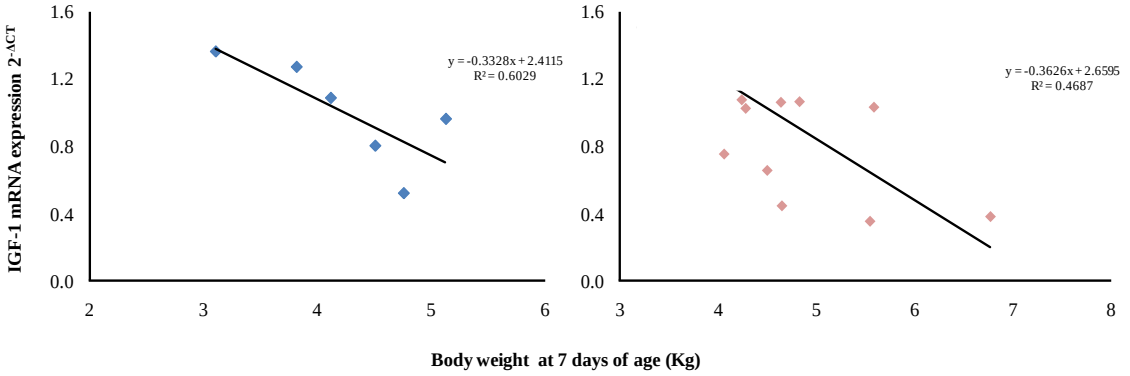


Figure 3.4 Scatter plots illustrating the negative relationship between renal IGF-1 gene expression and body weight at 7 days of age

3.3.2.2 Renal gene expression of growth hormone receptor (GHR) and measurement of renal oxidative stress

Seven days after birth, GHR was the only renal gene tested in this study to show a decrease as a result of maternal nutrient restriction ($p<0.05$; Fig. 3.5). To evaluate the effect of reduced GHR mRNA expression, concentrations of thiobarbituric acid reactive substances (TBARS) were measured in the kidney cortex and were found to be significantly decreased ($p<0.01$; Fig. 3.5.B) in the offspring of nutrient restricted mothers.

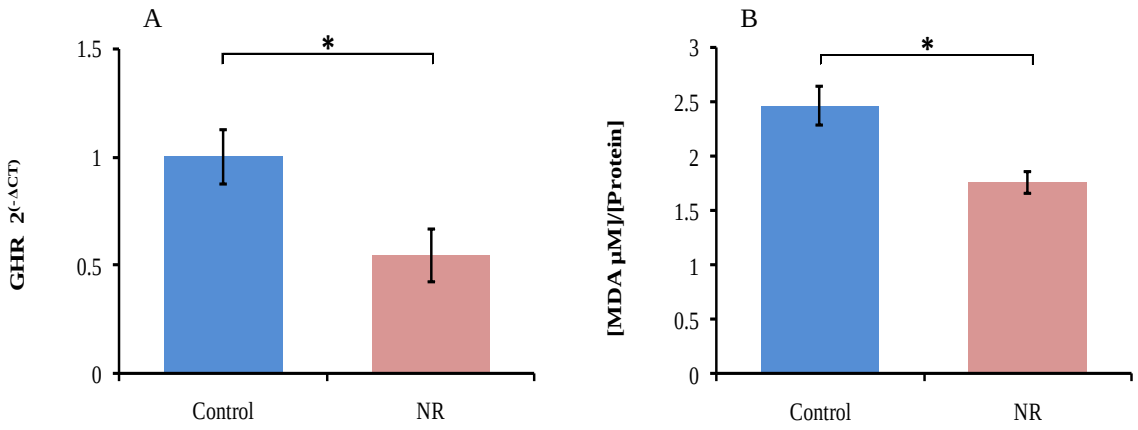


Figure 3.5 (A) Renal GHR mRNA abundance and (B) TBARS concentration observed in NR and control offspring at one week of age. Values are expressed as means ± SEM. Significant differences between NR and control offspring *($p<0.05$).

3.3.3 Renal gene expression for glucose sensing factors

Maternal nutrient restriction in early to mid gestation did not influence the mRNA abundance in the renal cortex of glucose sensing factors, such as glucose transporter 1 (GLUT-1) or hexokinase 1 (HK-1). Furthermore, there was no difference in the renal mRNA abundance of the renal insulin receptor between nutrition groups (Table 3.3).

3.3.4 Renal mRNA gene expression of DNMT-1, the pro-apoptotic gene p-53, iNOS and eNOS

Maternal nutrient restriction did not influence the mRNA expression of renal DNMT-1, p-53, iNOS and eNOS (Table 3.3).

Table 3.3 Renal gene expression of glucose sensing factors, p-53, DNMT-1, iNOS and eNOS at 7 days after birth

Gene	NR Group	Control Group	P
GLUT-1 ($2^{-\Delta CT}$)	0.75 ± 0.23	1.00 ± 0.39	NS
HK-1 ($2^{-\Delta CT}$)	1.21 ± 0.20	1.00 ± 0.21	NS
IR ($2^{-\Delta CT}$)	0.89 ± 0.45	1.00 ± 0.13	NS
DNMT-1 ($2^{-\Delta CT}$)	1.10 ± 0.19	1.00 ± 0.18	NS
p-53 ($2^{-\Delta CT}$)	1.44 ± 0.39	1.00 ± 0.16	NS
eNOS ($2^{-\Delta CT}$)	1.07 ± 0.18	1.00 ± 0.23	NS
iNOS ($2^{-\Delta CT}$)	1.70 ± 0.39	1.00 ± 0.18	NS

3.3.5 IGF-1, IGF-2, IGF-1R and IGF-2R and DNMT-1 mRNA expression in perirenal fat of seven-day-old offspring

There was no difference in the expression of IGF-1, IGF-1R mRNA between groups (Table 3.4), but, IGF-2 gene expression was significantly lower ($p<0.05$; Fig. 3.6.A) in perirenal adipose tissue from maternal nutrient restricted offspring. This was associated with a similar trend in IGF-2R mRNA abundance ($p= 0.075$; Fig. 3.6.B). In the perirenal adipose tissue of early to mid gestation maternal nutrient restricted offspring, at seven days of age, there was a two-fold greater abundance of DNMT-1 mRNA ($p<0.05$; Fig. 3.7) compared to controls.

Table 3.4 IGF-1 and IGF-1R mRNA expression in perirenal adipose tissue in 7-day-old offspring

Gene	NR Group	Control Group	P
IGF-1 ($2^{-\Delta CT}$)	1.08 ± 0.13	1.00 ± 0.13	NS
IGF-1R ($2^{-\Delta CT}$)	0.88 ± 0.10	1.00 ± 0.21	NS

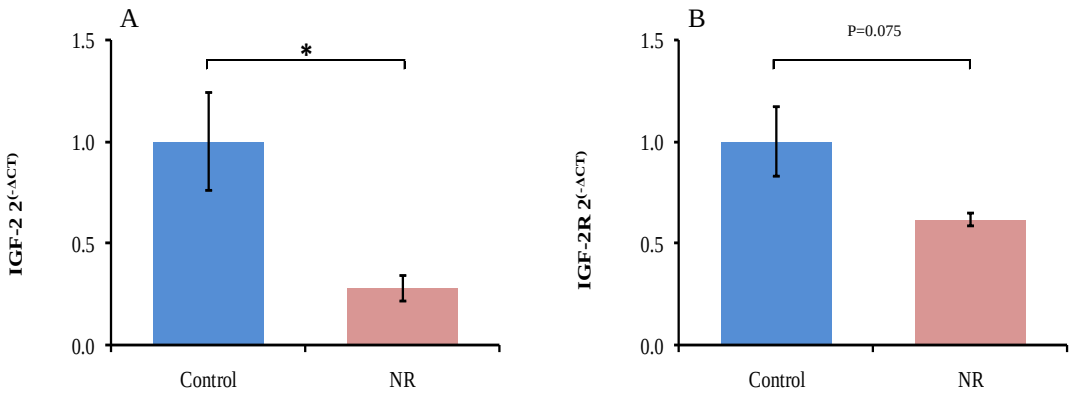


Figure 3.6 (A) Gene expression of IGF-2 (significant control vs. NR offspring *p<0.05) and (B) abundance of IGF-2R mRNA in neonatal perirenal adipose tissue in NR and control offspring at seven days of age (p< 0.075). Values are expressed as means ± SEM.

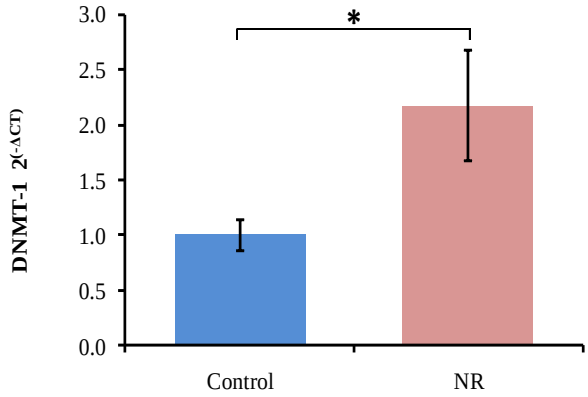


Figure 3.7 Gene expression of perirenal adipose tissue DNMT-1 in seven-day-old offspring born from maternal nutrient and control fed mothers. Values are expressed as means ± SEM. Significant differences between NR and control offspring* (p<0.05)

3.4 Discussion

In the present study, exposure to maternal nutrient restriction between early and mid gestation had little effect on the kidney in terms of weight and size, as measured at seven days after birth, although a reduction in the ratio of kidney-body weight was observed. The few significant changes noted in these renal tissues included a decrease in GHR mRNA expression and a reduction in oxidative stress. In contrast, perirenal adipose tissue of these offspring exhibited significant changes in gene expression, associated with the process of adipocyte differentiation (van Beek *et al.*, 2008; Viengchareun *et al.*, 2008). Thereby, following early exposure to maternal nutrient restriction, adipose tissue is characterised by a decrease in gene expression of IGF-2, IGF-2R and an increase in the methyltransferase DNMT-1.

3.4.1 Effects of maternal nutrient restriction on body weight, renal and adipocyte development

Consistent with previous studies, weight at birth was not affected by exposure to maternal nutrient restriction during early to mid gestation (Whorwood *et al.*, 2001). However, epidemiological studies of the Dutch Famine offspring survivors have shown that, despite there being no significant effect on birth weight, exposure to famine during early gestation predisposed offspring to metabolic disorders in adult life (Ravelli *et al.*, 1976). In addition, throughout their first week of postnatal life and regardless of maternal nutrition during gestation, both groups of offspring showed similar increases in total body weight and in other parameters of physical development. These results

support the view that endocrine regulation of postnatal growth, which is nutrient dependent, may act in a similar manner in both groups.

As demonstrated in previous studies, maternal nutrient restriction during early to mid gestation did not alter additional parameters of renal composition, such as nephron number or total weight (Whorwood *et al.*, 2001; Brennan *et al.*, 2005). However, in my study, the only change in renal composition influenced by reduced nutrition *in utero* was a decrease in the ratio of kidney to body weight, whilst previous studies in newborn ovine singletons had shown an increase (Whorwood *et al.*, 2001; Brennan *et al.*, 2005). These findings suggest that maternal nutrition has different consequences for renal development depending on whether the offspring are singleton or twins (Whorwood *et al.*, 2001; Brennan *et al.*, 2005).

It has previously been proposed that exposure to maternal glucocorticoids during early nephrogenesis can alter renal physiology. As demonstrated by Dodic *et al*., an infusion of dexamethasone (a cortisol analogue), administered to sheep mothers from 26 to 28 days of gestation, decreased nephron numbers in offspring, followed by renal dysfunction and hypertension in later life (Dodic *et al.*, 2002). However, exposure to moderate undernutrition in mothers bearing singleton or twins resulted in a rapid decrease in cortisol secretion. These results may explain the different effect on renal composition in response to a suboptimal maternal nutritional environment compared with those offspring exposed to dexamethasone during gestation (Dodic *et al.*, 2002;Bispham *et al.*, 2003; Jaquiery *et al.*, 2006; Rumball *et al*., 2008). Interestingly, in twin pregnancies, maternal cortisol remained low for at least 20 days after returning to a standard maternal diet. On the other hand, in the plasma of mothers bearing singletons,

the concentration of cortisol is normalised in parallel to the increase in food intake (Bispham *et al.*, 2003; Jaquiery *et al.*, 2006). Possibly due to this slow increase in maternal cortisol levels, the twin fetal environment may produce a different maturation pattern of the kidneys, affecting renal composition and also generating different endocrine responses, as compared with singleton offspring exposed to maternal nutrient restriction (Whorwood *et al.*, 2001; Bloomfield *et al.*, 2004; Brennan *et al.*, 2005). Another important observation in pregnant sheep bearing twins is that undernutrition led to a constant decrease in maternal glucose and insulin plasma concentrations until the end of gestation (Rumball *et al*., 2008). These parameters were unaffected by the increase in food intake during the re-feeding period, an effect not seen in singleton pregnancies exposed to nutritional manipulation (Bispham *et al.*, 2003). Thereby, an absence of change in total fat mass or renal composition observed in both groups of offspring at seven days after birth may, in part, reflect the maternal compensatory response to a reduction in food intake during twin pregnancy.

3.4.2 The effect of maternal nutrient restriction on renal endothelial function

Nephrogenesis is affected not only by the actions of glucocorticoids, but also depend on additional endocrine components, including elements of the renin-angiotensin system (RAS) (Hall, 2003; Moritz *et al*., 2004). For example, in rats, inhibition of the angiotensin 1 receptor (AT1R) during postnatal nephrogenesis is associated with a reduction in nephron number and renal mass (Woods & Rasch, 1998). The hypertensive effects of angiotensin are mediated through AT1R by the up-regulation of the NADPH protein complex, which contributes to the generation of oxidative stress and subsequent endothelial dysfunction (Zafari *et al.*, 1998).

In previous studies, it has been found that exposure to maternal nutrient restriction in singleton sheep offspring between early to mid gestation resulted in a significant increase in the expression of renal AT1R, suggesting an activation of the RAS in renal tissue leading to possible early endothelial dysfunction (Whorwood *et al.*, 2001).

The oxidative stress analysis (*i.e.*TBARS) of renal tissues at seven days of age, in the present study showed a reduction in lipid peroxidation in the maternal nutrient restricted group. In addition, renal gene expression of nitric oxide synthase enzymes, eNOS and iNOS, were not affected by maternal NR, suggesting similar endothelial function regulation between nutrient groups, an observation reinforced by similar mRNA expression of p-53 and DNMT-1. Overall, the reduction in oxidative stress and the gene expression of markers associated with endothelial function may suggest that the RAS in twin offspring exposed to maternal NR is down regulated, another possible difference in renal development compared with singletons.

3.4.3 Gene expression for renal cellular glucose uptake and utilisation

Previous findings have suggested that important long term changes in metabolism and the endocrine system may be induced by exposure to maternal nutrient restriction, independent of fetal number or birth weight (Oliver *et al.*, 2002). Nutrient restriction, confined to early gestation, negatively impacts on offspring glucose-insulin homeostasis. Oliver *et al*. reported increased pancreatic insulin release in offspring, at 5 and 30 months of age, exposed to severe undernutrition from 60 days before conception to 30 days of gestation (Oliver *et al.*, 2002). Raised insulin can induce endothelial vasodilatation, which could contribute to glomerular hypertension, leading to a slow process of renal dysfunction (Brands *et al.*, 2009). Numerous enzymatic systems in the

kidney are affected by plasma insulin concentration , including the hexokinase family (HK1-3), which plays a central role in cellular glucose uptake and utilisation (Robey *et al.*, 2002; Henningsen *et al.*, 2003). Previous observations have demonstrated that exposure to high concentrations of insulin can induce mRNA expression of HK-1 in the glomeruli of obese rodents (Henningsen *et al.*, 2003). In addition, insulin itself is also able to regulate IGF production with high IGF-1 concentrations suppressing insulin action in mesangial cells (Abrass *et al*., 1988). Unfortunately, we were unable to collect plasma to measure insulin concentration during the neonatal period.

However, offspring exposed to maternal NR had a heavier pancreas, which may be possible sign of a different pancreatic development influenced by the *in utero* environment (Fowden *et al*., 2005). Further exploration of pancreatic function and structure is needed to understand the biological relevance of these differences in weight.

3.4.4 The renal IGF-GH axis

The IGF-GH axis has a key role in the maintenance of kidney structure and function (Doi *et al.*, 1988). Although most actions of GH are mediated by IGF-1, both share common functions; but over-exposure to IGF-1 or GH has different consequences for the kidney. Infusion of GH or IGF-1 in mice causes renal hypertrophy, glomerulosclerosis and lipid accumulation, leading to an increase in kidney weight (Doi *et al.*, 1988; Quaife *et al.*, 1989; Berfield *et al*., 2006).

Increases in the gene expression of IGFs and their receptors in several tissues, including the kidney, have also been observed in singleton fetuses exposed to similar *in utero* nutritional manipulation during different periods of gestation (Bispham *et al.*, 2003; Brennan *et al.*, 2005; Hyatt *et al.*, 2007). Furthermore, changes in activation of

this axis may be the main mechanism by which maternal nutrient restriction is associated with impaired glucose tolerance and future alterations in renal functioning in offspring (Hyatt *et al.*, 2007; Bridgewater *et al*., 2008). However, evidence collected in this study indicates that a reduction in maternal diet between early to mid gestation in twin offspring had no influence with respect to renal gene expression of the IGFs. In addition, as stated previously, no changes in kidney composition and endothelial function between the groups were observed. Furthermore, the identical gene response of renal IGF-1 to increased body weight or fat mass, regardless of maternal nutrition, may indicate a similar regulation of this hormone in both offspring groups. My results might therefore suggest that the factors governing normal body growth during this period of life, such as neonatal nutrition, also regulate the gene expression of renal IGF-1, thus emphasising the similarities in renal endocrine responses in both groups.

Another important element in the regulation of this axis is the hormone ghrelin. Predominantly produced by the stomach, ghrelin is a potent inducer of GH secretion, glucose oxidation and food intake (Arvat *et al.*, 2000; Wren *et al.*, 2001). As previously shown, a period of nutrient restriction in early to mid gestation resulted in an appreciable decrease in NPY mRNA abundance in the hypothalamus at 7 days of age (Sebert *et al.*, 2009). This component of appetite regulation is able to reduce the activity of ghrelin, inducing a decrease in cellular metabolic activity and indirectly reducing oxidative stress in the kidney (Kalra & Kalra, 2004). Consequently, the functional alteration associated with the regulation of appetite through the actions of NPY, observed in the hypothalamus, may be the most likely factor influencing the reduction in oxidative stress and GHR seen in the kidneys of the maternal nutrient restricted

offspring. Further studies are needed to explore the influence of appetite regulation by the hypothalamus in neonatal renal function.

3.4.5 Possible factors altering the perirenal fat maturation in maternal nutrient restricted seven-day-old offspring

It has been shown previously that the maturation of brown fat cells in mice, induced by lactogens, occurs through the prolactin receptor and is characterised by an increase in IGF-2 and UCP-1 gene expression in adipose tissue at birth (Viengchareun *et al.*, 2008). Cell culture experimentation using prolactin receptor deficient pre-adipocytes demonstrated that the process of differentiation can be triggered by introducing exogenous IGF-2, which stimulated glucose uptake (King *et al.*, 1980; Sinha *et al.*, 1990; Viengchareun *et al.*, 2008). Importantly, GH, at least *in vitro*, regulates IGF-2 receptor abundance (Lonnroth *et al.*, 1987). The results of the present study show that my model of maternal nutrient restriction decreases the gene expression of IGF-2 and its receptor during the neonatal period. It is possible that this reduction in mRNA abundance may have consequences for the stimulation of energy use and the differentiation of perirenal adipose tissue. This hypothesis is reinforced by other observations, including reduced NPY mRNA expression in the hypothalamus, which is linked to adipocyte hyperplasia that might subsequently increase gene expression of DNMT-1 on perirenal fat, as observed in NR neonatal offspring (Kalra & Kalra, 2004; van Beek *et al.*, 2008; Sebert *et al.*, 2009). At the molecular level, DNMT-1 is associated with cell differentiation and proliferation in pre-adipocytes through interaction with the retinoblastoma protein (pRb) (Hansen *et al.*, 2004; van Beek *et al.*, 2008). For instance, the inactivation of this complex leads to an inhibition of the

adipocyte differentiation process, whilst obese pRb deficient mice show an increase in energy expenditure, resulting in weight loss (Hansen *et al.*, 2004).

It has been suggested that an irregular pattern of adipocyte differentiation may lead to insufficient capacity to store and assimilate increased nutrition intake (Miyoshi *et al*., 2009). Ultimately this failure might lead to adipocyte dysfunction and necrosis, associated with insulin resistance in later life (Salans *et al*., 1973; Cinti *et al*., 2005; Spalding *et al*., 2008). Sharkey *et al*. have recently reported that adipose tissue dysfunction and inflammation in one-year-old offspring born to maternal nutrient restricted (28-80 gestation days) mothers, following exposure to a post-weaning obesogenic environment (Sharkey *et al*., 2009). There was an increase in markers associated with necrosis in adipocytes, such as crown-like structures. In addition, the perirenal adipose tissue of these obese offspring showed clear signs of inflammation and macrophage infiltration (Sharkey *et al*., 2009). They also displayed increased ectopic lipid storage in cardiac tissue and raised plasma insulin, in comparison with obese controls (Chan *et al.*, 2009; Sebert *et al.*, 2009). In maternal nutrient restricted neonatal sheep, IGF-2 and its receptor mRNA expression was decreased in perirenal fat, whilst there was no effect on IGF-1 and its receptor. Given that gene regulation of IGF-1 and its receptor in adipose and other peripheral tissues is related to nutrition intake, these similarities in gene expression are not surprising in this animal model (Wren *et al.*, 2001; Giovannuci *et al*., 2003). However, I further propose that changes in gene expression of IGF-2, IGF-2R and DNMT-1 may indicate a cellular phenotypic alteration, which may ultimately limit the lipid storage capacity of the adipocyte, becoming critical only after exposure to an obesogenic environment.

3.5 Summary

Offspring exposed to nutrient restriction during the period of early organogenesis, compared with those born to mothers fed to appetite throughout gestation, showed a reduction in the expression of GHR in conjunction with a decrease in oxidative stress within the kidney. However, the greatest differences between gestational nutrition groups were found in the adipose tissue surrounding the kidneys. The gene expression of IGF-2 and its receptor were decreased, accompanied by an increase in DNMT-1 mRNA abundance. All these effects may be a consequence of the alterations on gene expression of the appetite control elements in the hypothalamus, such as NPY, possibly programmed by their *in utero* nutritional experience. Owing to the role of these genes in adipose tissue development and energy metabolism, there may be long term implications for the storage capacity of this tissue and its response to increased nutrition consumption. These mechanisms could explain, in part, the process of adipocyte hypertrophy and other effects associated with extreme obesity seen when the siblings of these offspring are exposed to an obesogenic environment (Sharkey *et al*., 2009).

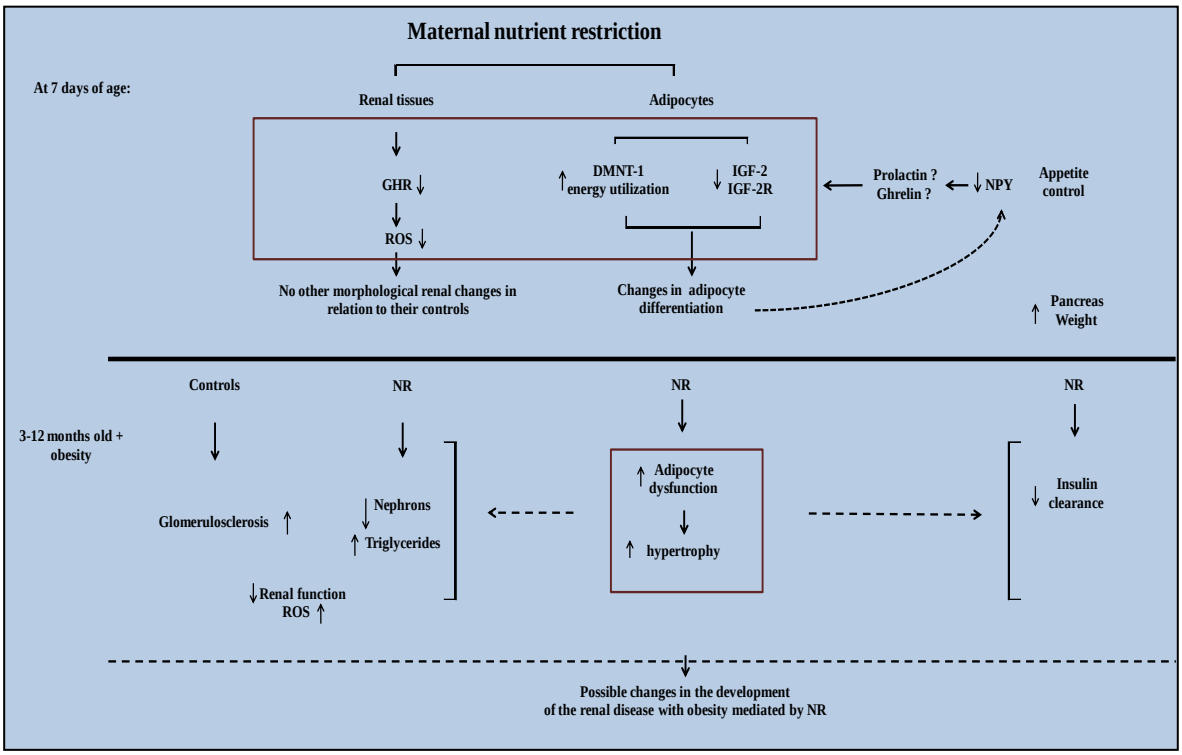


Figure 3.8 Summary of findings

Chapter 4 - Effects of obesity on renal tissue of young sheep exposed to maternal nutrient restriction between early to mid gestation

Chapter 4 - Effects of obesity on renal tissue of young sheep exposed to maternal nutrient restriction between early to mid gestation

4.1 Introduction

4.1.1 The effects of leptin on renal function

It was observed in several human cohorts that an adverse uterine environment may induce permanent functional changes within the kidneys, leading to hypertension and subsequent cardiovascular and renal dysfunction in later life (Barker *et al.*, 1990; Law *et al.*, 2002; Hughson *et al.*, 2003). Analysis of renal autopsies have demonstrated a strong relationship between birth weight and nephron number in both the infant and adult kidney (Hughson *et al.*, 2003). However, the authors of this report noted a substantial variation in adult nephron number in the lower range of birth weights, suggesting an innate ability of the kidney to develop correctly, even in a suboptimal maternal environment (Hughson *et al.*, 2003). As described in chapter 3, birth weight and kidney development, including nephron number, was not influenced by maternal nutrient restriction in early to mid gestation. However, this manipulation in maternal nutrient supply affected the expression of genes associated with adipose tissue development. Thus, the influence of maternal nutrient restriction on renal tissue is possibly limited to alterations in metabolic and hormonal responses associated with lipid storage and appetite control in these offspring (Chapter 3; Sebert *et al.*, 2009). As previously observed, the model of maternal nutrient restriction I have adopted has produced different renal responses when these offspring experienced an excessive increase in weight gain after weaning, such as a reduction in renal apoptosis and gene expression of

elements of the RAS (Williams *et al.*, 2007; Sharkey *et al*., 2009; Chan *et al.*, 2009; Sebert *et al.*, 2009).

In recent years, it has been suggested that leptin, a hormone secreted by adipose tissue, is partly associated with obesity-induced hypertension (Rahmouni *et al.*, 2005). Importantly, a short isoform of the leptin receptor is highly expressed in the kidney, potentially influencing renal function and structure (Serradeil-Le Gal *et al.*, 1997). Previously it was observed that acute or chronic exposure to leptin influenced blood pressure and renal function by altering the synthesis of nitric oxide (NO) (Beltowski *et al.*, 2002; Beltowski *et al.*, 2004). Acute infusion of human leptin (1 mg/Kg) into rats resulted in increased NO synthesis, through the stimulation of NO synthase enzymes, but did not induce activation of the vascular-sympathetic nerve activity system or produce renal sodium retention (Beltowski *et al.*, 2002). However, long term leptin administration (7days-0.25mg/Kg), as observed during hyperleptinemia, decreased NO synthesis, followed by abnormal sodium retention and vasoconstriction (Beltowski *et al.*, 2004). As expected, in obese mice obesity, a peripheral or cerebroventricular administration of leptin produced increased renal sympathetic nerve activity of similar intensity to that found in controls (Rahmouni *et al.*, 2005). However, the increase in arterial blood pressure following an infusion of leptin was more than 10 mmHg higher in obese compared with lean mice (Rahmouni *et al.*, 2005). Furthermore, the strong correlation between body weight and leptin serum abundance suggests the development of a leptin-resistant mechanism in obesity (Considine *et al.*, 1996). This adverse response can be set *in utero* following severe undernutrition in rats (a 30% reduction compared with *ad libitum* energy intake) throughout gestation, resulted in elevated

leptin concentrations in adult life, which was also associated with an increase in adiposity and food intake (Vickers *et al.*, 2000). In addition, human adipocytes exposed *in vitro* to angiotensin II secreted leptin in a dose dependent fashion (Skurk *et al.*, 2005). These observations may explain, in part, the abnormalities in renal function induced by obesity (Skurk *et al.*, 2005).

4.1.2 Renal dysfunction and angiotensin II

The activation of the RAS has been associated with renal injury, mediated in part by an increase in reactive oxygen species and pro-inflammatory cytokines, through impairment to endothelium-dependent vasodilatation (Hasdan *et al.*, 2002; Higashi *et al.*, 2002; Luchtefeld *et al.*, 2003). Angiotensinogen is the precursor of ANG II and is mainly synthesised by two tissues: the liver and the adipose tissue (Lynch & Peach, 1991). However, unlike the liver, adipose tissue not only expresses angiotensinogen, but also expresses all of the enzymes involved in its conversion to ANG II. Observations *in vitro* and *ex vivo* demonstrated that ANG II is an important component in adipose tissue development (Massiera *et al.*, 2001a). Comparative studies of angiotensinogen adipose deficient mice showed that the lack of expression of this gene generates a phenotype unable to increase weight in response to a high fat diet (Massiera *et al.*, 2001b). The opposite genetic manipulation in mice results in increased adiposity, plasma leptin and insulin concentrations, together with increased abundance of renal ATR1 protein (Kim *et al.*, 2006). Long term infusion of ANG II in rats promotes abnormal accumulation of lipids and iron in renal tissue, which may play a crucial role in the development of renal damage, causing free radical injuries, such as lipid peroxidation in the proximal tubules (Kawabata *et al.*, 1997 ; Ishizaka *et al.*, 2002; Saito *et al.*, 2005). Finally, exposure to

maternal nutrient restriction (i.e 50% of total metabolic requirements) during early to mid gestation (28-77 days) is also associated with an increase in ATR1 gene expression in several tissues, including kidney, liver, adrenal and lungs in newborn sheep (Whorwood *et al.*, 2001).

4.1.3 Mechanism of apoptosis and proliferation in renal tissues

It has been suggested that both apoptosis and cell proliferation play a major role in the development of renal disease. In the kidney, RAS assumes an important role by inducing both processes (Cao *et al.*, 2000; Bhaskaran *et al.*, 2003). After binding to one of its receptors, ANG II induces cell apoptosis in renal tubular cells through the activation of Fas, a member of TNF cytokine receptor (Muller *et al.*, 1998; Bhaskaran *et al.*, 2003). Interestingly, gene expression of Fas is under the control of p-53, which is the major regulator of the cell cycle and whose activation is associated with DNA damage (Bouvard *et al.*, 2000). For the subsequent development of the intracellular apoptotic pathway, p-53 targets the activation of another gene called Bax, a pro-apoptotic member of Bcl2. In response to stress, Bax forms a homodimer and binds to a mitochondrial pore called the voltage-dependent anion channel (VDAC). These actions allow the release of cytochrome c from the mitochondria, which results in cell death (Shimizu *et al.*, 1999). Aizawa *et al*. found that the protein abundance of Bax in renal tissues of rats rises after an infusion of ANG II, in a dose and time dependent manner and was followed by apoptosis (Aizawa *et al.*, 2001). ANG II also stimulates cell proliferation or hypertrophy in the rat kidney and this effect is possibly mediated by several angiogenic factors, such as vascular endothelial factor-A (VEGF-A) (Kitayama *et al.*, 2006). Although its role in renal disease is controversial (Flyvbjerg *et al.*, 2002; Advani *et al.*, 2007). Recently, in

transgenic (mRNA-2)27 rats that over-express renin, VEGF mRNA expression increased two fold and the administration of different VEGF blockers resulted in severe glomerulosclerosis (Advani *et al.*, 2007). In contrast, treatment with VEGF antibodies in db/db mice was able to reduce the renal injury associated with diabetes, such as increased kidney weight, glomerular volume, mesangial matrix expansion and creatinine clearance (Flyvbjerg *et al.*, 2002). It is therefore possible that the different effects of VEGF-A reflect the complexity of the factors involved in the adaptation of renal tissue to obesity.

4.1.4 Scientific rationale, aim and hypothesis

Previous epidemiological observations reported a link between maternal nutrition during gestation and subsequent changes in metabolic responses in offspring later in life (Ravelli *et al*., 1976; Painter *et al*., 2005). In addition, it was postulated that obesity *per se* can modulate the kidney, leading to a slow process of structural and functional deterioration (Rea *et al*., 2001; Henegar *et al*., 2001). Williams *et al*. observed a reduction in the number of apoptotic renal cells linked to obesity as a unique feature of the development of glomerulosclerosis in offspring exposed to maternal nutrient restriction during early to mid gestation (Williams *et al.*, 2007). Later work conducted by Sharkey *et al.*, who applied the same model of maternal manipulation, observed a rise in the unfolded protein response in the kidney as a possible adaptation to obesity (Sharkey *et al*., 2009). However, the extent to which further molecular changes in the kidney may have been influenced by the endocrine status of these offspring has not been fully investigated. Furthermore, none of the previous studies has established a possible mechanism that would explain the reduction in cell apoptosis seen in these offspring.

My study was designed to identify the possible reasons for the reduction in renal apoptosis observed in this model of nutrient restriction. It is hypothesised that the reduction in apoptosis observed in the kidneys of the juvenile sheep exposed to maternal nutrient restriction, compared with those offspring born to mothers fed to appetite, is associated with the post-injury responses such as cell proliferation, which may be an additional degenerative adaptation to obesity in renal tissue (Nangaku & Eckardt, 2007). Therefore, several cellular responses induced by obesity and associated with renal dysfunction in this tissue were investigated.

4.2 Methods and materials

A full description and details of all methods, including animal models and gene expression analysis used in this chapter, can be found in Chapter 2, with exception of angiotensin II challenge assay and the analysis of cysteine concentration in plasma. These were kindly carried out and supervised by Drs. David S. Gardner with the collaboration of Prof. Michael E. Symonds. In addition, all statistical tests are also explained either in Chapter 2 or in the appropriate section of this chapter.

4.3 Results

4.3.1 Body composition, renal and metabolic status

The results of this section (4.3.1) have been reported previously by Williams *et al.* and are summarised in table 4.1 (Williams *et al.*, 2007; Sharkey *et al.*, 2009).

4.3.1.1 Body composition

After nine months of exposure to an obesogenic environment (65% reduction in physical activity and 30% increase in food intake), there was no difference in body weight between both *in utero* nutrition groups. However, the combined postnatal nutritional intervention and relative physical inactivity influenced the body weight and composition of these sheep in relation to the lean group, who remained in an unrestricted field environment. Finally, the weight of the kidneys and the adipose tissue was influenced only by obesity and not by the previous maternal nutritional environment (Williams *et al.*, 2007).

Table 4.1 Morphometry in one-year-old lean and obese sheep (Williams *et al.*, 2007).

Parameter	Lean	Obese	NR-Obese group	P
Body weight (kg)	58.58±2.46	88.71±2.67*	85.83±4.57*	P<0.01
Total kidney weight (g)	117.27±9.20	155.70±6.68*	164.86±7.16*	P<0.01
Kidney lenght (mm)	73.38±5.26	75.71±2.45	75.71±2.45	NS
Kidney depth (mm)	28.57±1.49	26.57±1.99	29.56±2.79	NS
Perirenal fat (g)	553.88±93.48	2692.14±294.33*	2783.23±197.92*	P<0.05
Omental (g)	82.50±8.64	328.43±29.36*	232.60±105.80*	P<0.05
Viseral fat (g)	1422.00±513.80	7242.71±349.32*	6614.68±333.30*	P<0.01

*** Statistical significance in lean group vs. both obese groups, regardless of maternal diet**

4.3.1.2 Metabolic status

At one year of age, there was no difference in plasma leptin between those obese groups of offspring, although exposure to the obesogenic environment had elevated the concentration of this hormone, relative to the lean group. Similar trends were recorded in other plasma hormones, such as IGF-1, cysteine and free fatty acids (Table 4.2) (Williams *et al.*, 2007). The concentration of glucose was similar between the three groups, but plasma insulin concentration increased with obesity, a response that was amplified in offspring born to nutrient restricted mothers (Fig. 4.1) (Sebert *et al.*, 2009).

Table 4.2 Mean plasma concentrations of hormones and metabolites at one year of age in lean and obese sheep, either exposed to maternal nutrient restriction or fed to appetite throughout pregnancy (Sebert *et al.*, 2009)

Parameter	Lean	Obese	NR-Obese group	P
Leptin (ng/mg)	3.0±0.5	18.9±1.4*	22.3±1.5*	P<0.01
IGF-1(ng/ml)	6.31±0.48	16.92±3.55*	13.09±1.06*	P<0.05
Cysteine (μM)	127.9±11.7	183±10.9*	180.1±16.3*	P<0.05
Free fatty acids (mmol/liter)	0.31±0.05	0.64±0.16*	0.56±0.06*	P<0.05
Glucose (mmol/liter)	4.47±0.58	5.77±0.62	5.17±0.51	NS

***Statistical significance in lean group vs. both obese groups, regardless of maternal diet**

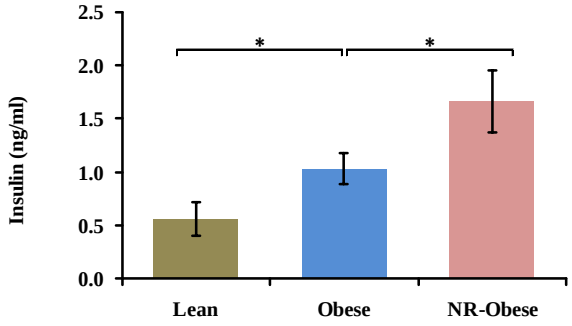


Figure 4.1 Insulin concentration in 1 year old lean and obese sheep born to mothers exposed to nutrient restriction or fed to appetite (*p<0.05) (Sebert *et al.*, 2009)

4.3.1.3 Glomerular filtration rate (GFR), blood pressure and angiotensin II challenge

Glomerular filtration rates were similar in both obese groups but the values obtained shown a tendency be to higher than the lean group (Table 4.3). In addition, there were clear differences in baseline systolic and diastolic blood pressure between the obese group and those obese offspring previously exposed to maternal nutrient restriction. Following a similar trend, the infusion of angiotensin II resulted in a similar rise in arterial blood pressure in both obese groups, as compared to the lean group (Table 4.3) (Williams *et al.*, 2007; Sharkey *et al*., 2009).

Table 4.3 Glomerular filtration rate, basal blood pressure and systolic blood pressure response to angiotensin II infusion in lean and obese offspring, either born from mothers exposed to nutrient restriction during early to mid gestation or those fed to appetite throughout pregnancy (Williams *et al*., 2007; Sharkey *et al*., 2009)

Parameter	Lean	Obese	NR-Obese group	P
Blood pressure-systolic (mmHg)	105±2	116±3*	119±3*	P<0.01
Blood pressure-diastolic (mmHg)	78.1±1.5	88.6±2.3*	86±2.2*	P<0.01
Angiotensin II challenge- systolic blood pressure (mmHg/Sec)	849.3±55.07	1174±94.72*	1019±84.98*	P<0.05
Glomerular filtration rate (ml/min)	93.5±9.9	138±15*	114±17*	P<0.08

***Statistical significance in lean group vs. both obese groups, regardless of maternal diet**

4.3.2 Iron staining, Masson's trichrome stain, lipid, TBARS and 8-OHdG concentrations in renal tissues

Iron deposits in renal tissues were observed using Prussian blue staining. A significant reduction in iron deposition was seen in both obese maternal nutrient restricted (NR-O) and lean (L) groups, compared with the obese (O) group. The different levels of iron deposition were estimated semi-quantitatively (for more details see chapter 2, page 93) ($p<0.05$; Fig. 4.2.B). More distinct deposits of iron were observed in both obese groups in the cortical regions and, to a lesser degree, in the medulla (Fig. 4.3). Further analysis revealed that iron deposition occurred occasionally in glomerular cells, but was predominantly seen in the tubular structures. In contrast, exposure to an obesogenic environment significantly increased the lipid abundance in those sheep exposed to maternal nutrient restriction ($p<0.05$; Fig. 4.2.A). Analysis of Masson's trichrome stained kidney sections revealed that the renal cortex and medulla in

lean sheep exhibited a low abundance of collagen compared with both obese groups. The most striking characteristic of this histological measurement from sheep exposed to an obesogenic environment, independent of maternal nutrition during pregnancy, was a marked presence of collagen in the renal cortex (Fig. 4.3). The data was analysed in a semi-quantitative manner (for more details see chapter 2, page 95). Finally, exposure to an obesogenic environment raised the abundance of renal TBARS / 8-OHdG associated metabolites to similar concentrations in both obese groups, without modulation by *in utero* nutrition supply (Table 4.4).

Table 4.4 Collagen abundance observed using Masson's trichrome in kidney sections and TBARS/8-OHdG concentration in renal tissues of lean and obese sheep, born either to mothers exposed to nutrient restriction during early to mid gestation or fed to appetite throughout pregnancy

Parameter	Lean	Obese	NR-Obese group	P
Collagen (arbitrary units)	2.51±0.21	3.32±0.26*	3.57±0.15*	P<0.05
8-OHdG ([8-OHdG ng/µl]/[Protein µg/µl])	0.74±0.05	1.02±0.08*	0.99±0.05*	P<0.05
TBARS ([MDA µM]/[Protein µg/µl])	3.91±0.12	4.50±0.14*	4.30±0.25*	P<0.05

*** Statistical significance in lean group vs. obese group, regardless of maternal diet**

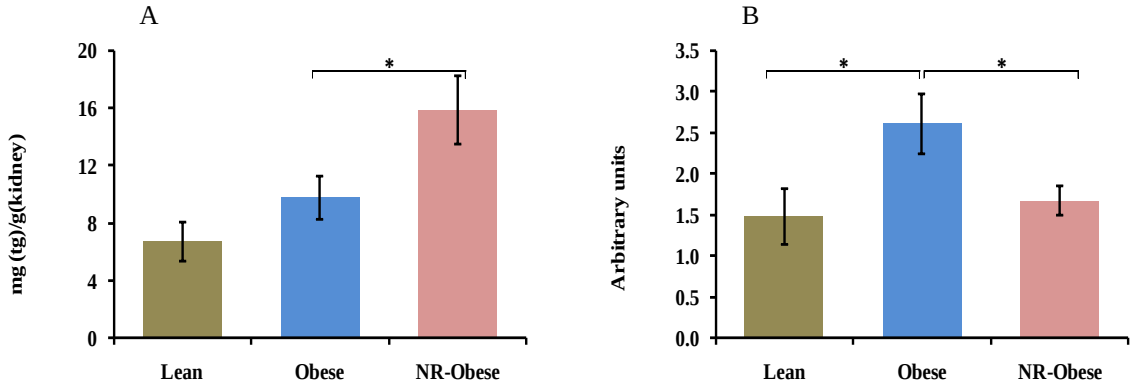


Figure 4.2 (A) The effect of prenatal nutrient restriction and postnatal obesity on triglyceride content in renal and (B) on renal iron deposition as assessed by Prussian blue staining tissues of lean, obese or prenatally nutrient restricted obese offspring. (*P<0.05) Data is expressed as mean± SEM.

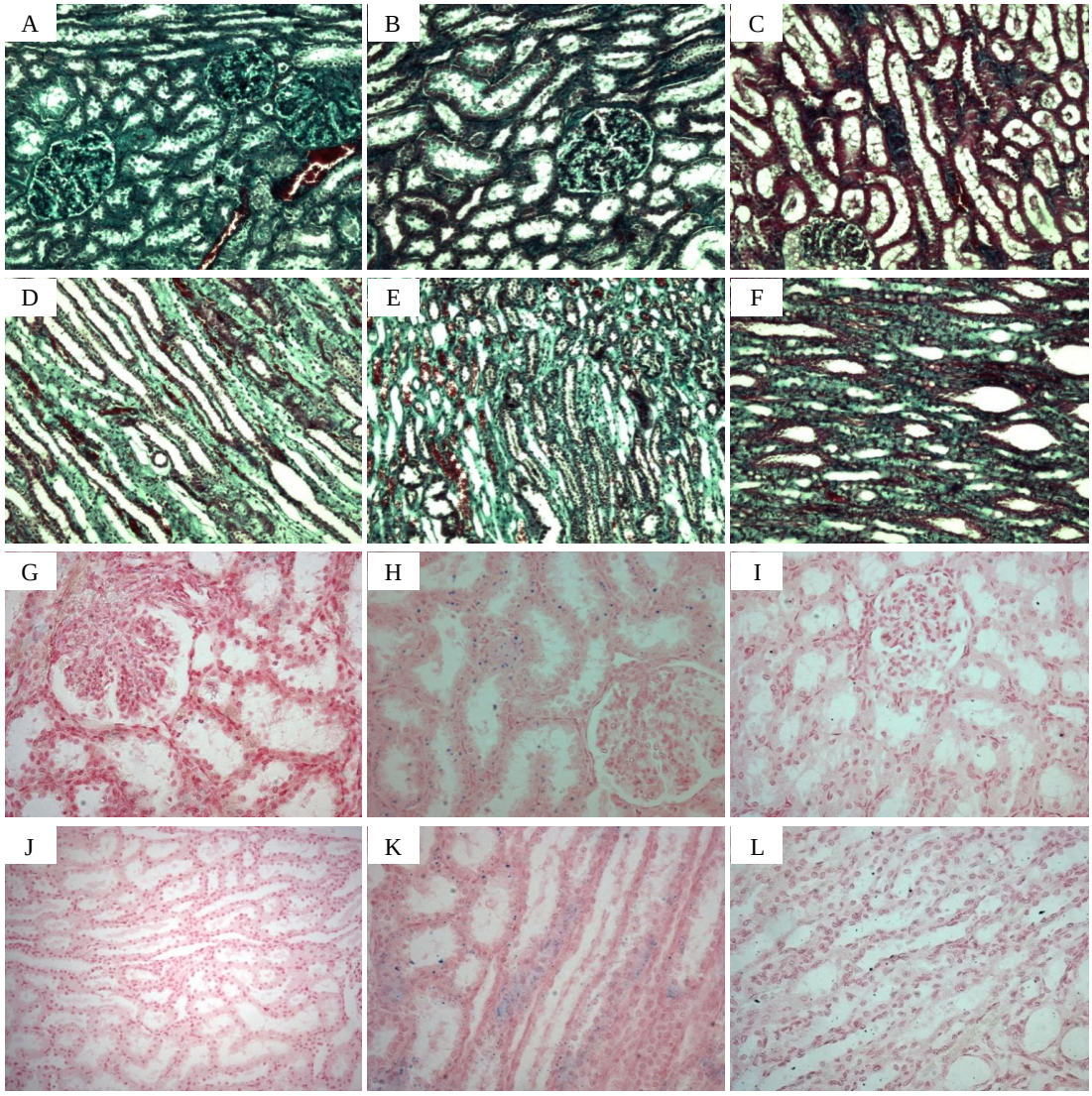


Figure 4.3 Representative renal Masson's trichrome stain and iron staining in renal cortex and medulla of lean, obese or prenatally nutrient restricted obese offspring. The sections A to F were stained with Masson's trichrome staining for the detection of collagen and G to L were stained with Prussian blue for the observation of iron deposition in the kidneys. Renal sections C, F, I and L were obtained from lean sheep, B, E, H and K belong to the obese group and finally A, D, G and J were generated from prenatally nutrient restricted obese sheep. The colours red, dark brown or black, observed on the renal sections stained with Masson's trichrome, are cell nuclei and keratin, whereas blue to green are collagen peptides. A predominant green-blue staining was observed in the renal cortex and with less intensity in medulla of both obese groups independent of maternal nutrition during pregnancy (A, B, D, and E). In contrast, the cortex and medulla sections obtained from the lean group were usually stained dark brown or red and green (C and F). Although no visible iron depositions were observed in the renal cortex and medulla of the lean (I and L) or obese nutrient restricted sheep (G and J), iron deposits, indicated by blue dots, were seen in the medullary ray (K) and cortical proximal tubules (H) in sheep belonging to the obese group (Magnification X 10).

4.3.3 Renal nitric oxide (NO)

The analysis of Nitrite (NO_2^-) and Nitrate (NO_3^-) showed that the production of NO was higher in those obese offspring born from standard fed mothers compared to the nutrient restricted obese group ($p<0.05$; Fig. 4.4). Finally, the gene expression of renal iNOS and eNOS was similar among the three groups (Table 4.5).

Table 4.5 Renal mRNA abundance of iNOS and eNOS

Parameter	Lean	Obese	NR-Obese group	P
iNOS ($2^{-\Delta CT}$)	1.00±0.26	1.35±0.39	1.23±0.25	NS
eNOS ($2^{-\Delta CT}$)	1.00±0.28	1.56±0.43	1.70±0.26	NS

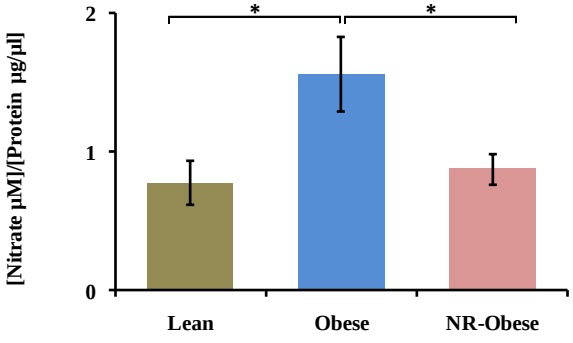


Figure 4.4 NO_3^- abundance in renal tissue. Values are expressed as means ± SEM. Significant differences between lean and obese groups (*p<0.05) and between obese and maternal nutrient restricted obese group (*p<0.05).

4.3.4 Regulation of genes associated with apoptosis

Real time PCR analysis showed that renal expression of the pro-apoptotic gene Fas was increased with obesity, although this response was not observed in those obese offspring born to nutrient restricted mothers ($p<0.05$; Fig. 4.5). In addition, gene expression of Bax and p-53 increased in both obese groups compared with lean animals (Table 4.6).

Table 4.6 The effect of prenatal nutrient restriction and postnatal obesity in renal tissues on the gene expression of Bax and p-53

Parameter	Lean	Obese	NR-Obese group	P
Bax ($2^{-\Delta CT}$)	1.00±0.15	2.68±0.21*	2.67±0.46*	P<0.05
p-53 ($2^{-\Delta CT}$)	1.00±0.34	3.61±1.05*	1.94±0.48*	P<0.05

*** Statistical significance in lean group vs. obese groups, regardless of maternal diet**

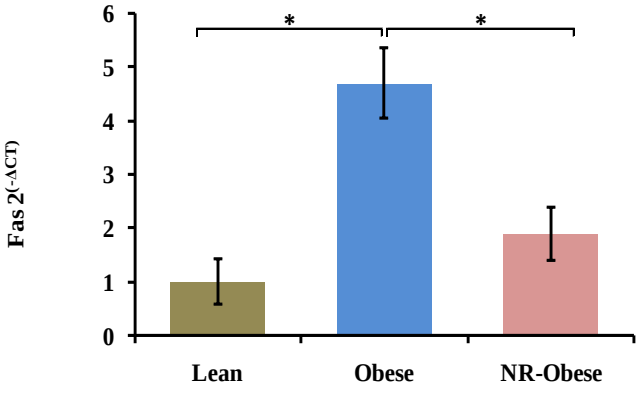


Figure 4.5 mRNA abundance of the pro-apoptotic receptor Fas. Values are expressed as means ± SEM. Significant differences between lean and obese groups* ($p<0.05$) and between obese and maternal nutrient restricted obese group*($p<0.05$).

4.3.5 Renal markers of cell survival

4.3.5.1 Immunohistochemical analysis of proliferating cell nuclear antigen (PCNA)

PCNA positive immune-stained cells are used as a marker for cell proliferation as this protein is mainly expressed during the DNA synthesis phase of the cell cycle (Leonardi *et al*., 1992). In the renal cortex, the PCNA staining was, in general, detected in tubular cells and with less intensity around the Bowman capsule and glomerulus. Exposure to an obesogenic manipulation in the offspring of mothers fed to requirements throughout gestation resulted in an increase of PCNA both in the tubular cells and around the Bowman capsule to a moderate degree, as well as inside the glomerulus. Similar results were recorded in the obese and in the obese nutrient restricted groups (Fig. 4.6).

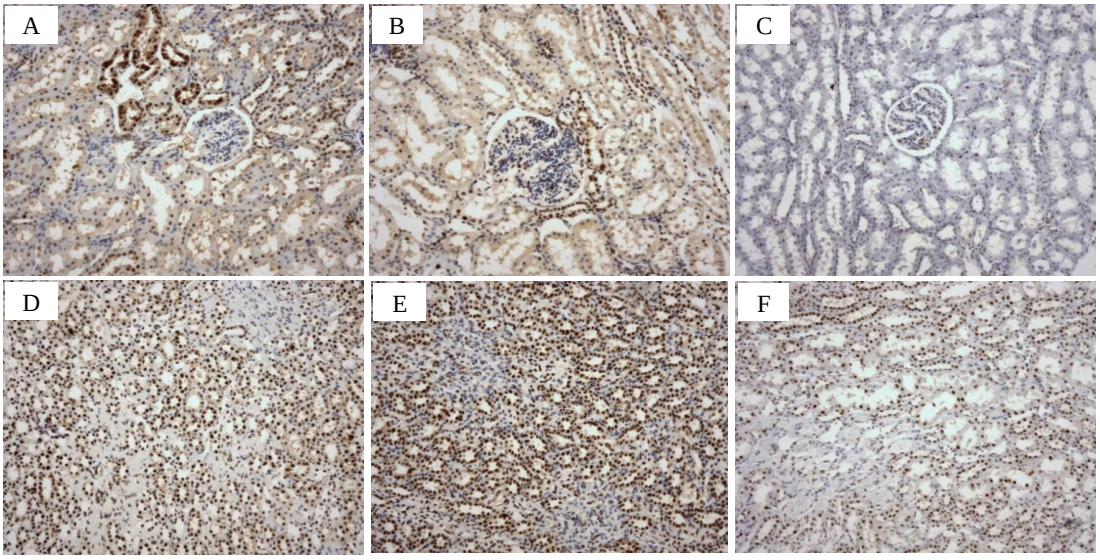


Figure 4.6 Immuno-stained paraffin embedded 5µm sections of sheep kidney for proliferating cell nuclear antigen (PCNA). The renal section A (cortex) and D (Medulla) were generated from maternal nutrient restricted obese sheep. Figures B and E represent the renal cortex and medulla of obese group and in the same order C and F sections were produced from the lean group (magnification x 10).

4.3.5.2 Regulation of genes associated with cell proliferation, angiogenesis and hypoxia

All genes linked with the cell replication machinery, such as VEGF-A, DNMT-1 Bcl-2, Glut-1 and IGF-1R, showed a statistically significant elevation in mRNA abundance by exposure to an obesogenic environment, compared with the lean group. However, maternal nutrient restriction did not influence the mRNA abundance of this group of genes (Table 4.7).

Although gene expression of HIF-1α increased with obesity, this response was amplified in offspring born to NR mothers ($p<0.05$; Fig 4.7).

Table 4.7 Abundance of proteins and genes associated with cell proliferation and angiogenesis at 1 year of age in lean and obese sheep born to mothers that were fed either a control diet throughout pregnancy or nutrient restricted at early and mid-gestation

Parameter	Lean	Obese	NR-Obese group	P
PCNA (arbitrary units) (fig.4.7)	1.17±0.44	3.23±1.01*	2.54±0.70*	P<0.05
VEGF-A ($2^{-\Delta CT}$)	1.00±0.29	3.01±0.88*	3.44±0.544*	P<0.05
Glut-1 ($2^{-\Delta CT}$)	1.00±0.21	2.59±0.57*	2.04±0.22*	P<0.05
Bcl2 ($2^{-\Delta CT}$)	1.00±0.36	3.67±0.57*	2.77±0.52*	P<0.05
DMNT-1 ($2^{-\Delta CT}$)	1.00±0.24	3.42±0.40*	2.44±0.44*	P<0.05
IGF-IR ($2^{-\Delta CT}$)	1.00±0.35	4.31±0.79*	2.74±0.47*	P<0.05

*** Statistical significance in lean group vs. obese groups, regardless of maternal diet**

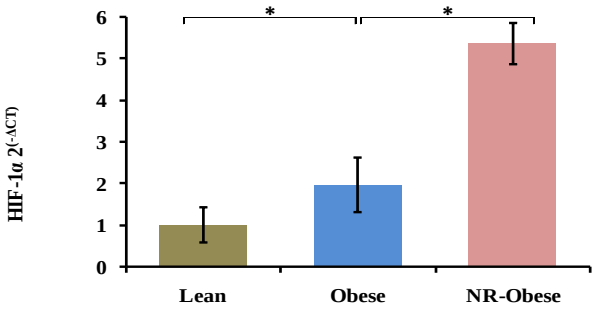


Figure 4.7 mRNA abundance of HIF-1α at 1 year of age in lean and obese sheep, regardless of maternal diet. Values are mean ± SEM. Significant differences between lean and obese groups (p<0.05) and between obese and NR-obese groups

4.3.6 Expression of proteins associated with mitochondrial function

Changes in expression of proteins associated with the release of mitochondrial superoxide or detoxification can be use as reliable markers of cell function (Wilcox, 2005). The antioxidant proteins measured were the mitochondrial SOD-2 and its cytosolic isoform SOD-1. In addition, the abundance VDAC-1 was analysed, which has been shown to control the flow of metabolites and ions from the mitochondria (Tan & Colombini, 2007). After exposure to an obesogenic environment, the only renal protein analysed in this thesis that increased in expression as a result of maternal nutrient restriction, is the mitochondrial pore VDAC-1 ($p<0.05$; Fig. 4.8.A). There was no difference in SOD-2 protein expression between obese groups, although its abundance was significantly higher than in the lean group. Finally, the SOD-1 renal content was similar in the three groups (Table 4.8). However, the total SOD enzymatic activity was higher in those obese sheep exposed to maternal nutrient restriction in relation to the obese group born from control fed mothers ($p<0.05$; Fig. 4.8.B).

Table 4.8 Protein abundance of SOD-1 and SOD-2 in renal tissue at 1 year of age in lean and obese sheep born from mothers that were fed either to appetite during gestation or nutrient restricted during the period of nephrogenesis

Parameter	Lean	Obese	NR-Obese group	P
SOD-1 (% reference)	23.69±5.76	32.57±6.95	29.99±2.01	NS
SOD-2 (% reference)	10.46±0.65	12.79±0.85*	11.97±0.65*	P<0.05

*** Statistical significance in lean group vs. obese groups, regardless of maternal diet**

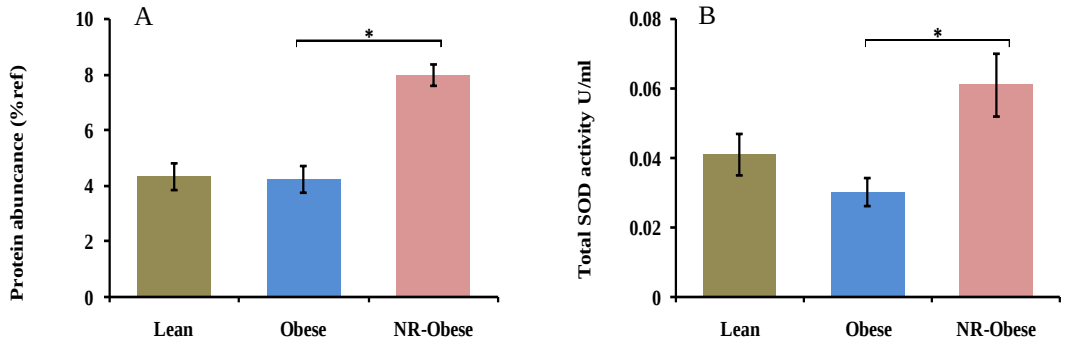


Figure 4.8 (A) The effect of prenatal nutrient restriction and postnatal obesity on renal abundance of the mitochondrial pore VDAC and on renal total SOD enzymatic activity (B). Statistics are expressed as Mean ± SEM (*p<0.05).

4.3.6.1 Renal gene expression of glucose sensing factors

Maternal nutrient restriction followed by exposure to an obesogenic environment had no influence on the mRNA abundance of glucose sensing factors such as, HK-1 and IR. However, the obese group showed a significant increase in gene expression of glucose sensing factors, compared with the lean group (Table 4.9).

Table 4.9 mRNA abundance of genes associated with glucose sensing

Parameter	Lean	Obese	NR-Obese group	P
HK1 ($2^{-\Delta CT}$)	1.00±0.20	2.26±0.43*	1.70±0.23*	P<0.05
IR ($2^{-\Delta CT}$)	1.00±0.47	2.68±0.72*	2.80±0.59*	P<0.05

*** Statistical significance in lean group vs. obese groups, regardless of maternal diet**

4.4 Discussion

The main findings of this study are that *in utero* nutrient restriction, followed by post-weaning exposure to an obesogenic environment in juvenile sheep, results in a differential regulation of the proliferative-apoptotic pathways in renal tissue. The potential cell survival effect seen in the NR-O group was characterised by an increase in abundance of the mitochondrial pore VDAC-1. However, unexpectedly, similar levels of oxidative stress and the expression of additional pro-apoptotic and proliferative factors were found in renal tissue of both obese groups. Further analysis of the renal cortex of those sheep exposed to maternal nutrient restriction indicated an increase in lipid deposition, which may be the main source of oxidative stress. Activation of the apoptotic pathway with obesity, but not seen in NR-O animals was characterised by an increase in mRNA abundance of the cell death receptor, Fas. This is associated with a rise in oxidative stress and cell damage, possibly catalysed by the presence of iron (Bouvard *et al.*, 2000; Ishizaka *et al.*, 2002). Thus, maternal nutrient restriction during early to mid gestation, followed by a period of accelerated weight gain, may affect the sequence of cell proliferation, hypertrophy and apoptosis, which are linked to a progressive decline in renal function.

4.4.1 Evidence of cell proliferation on renal tissue induced by obesity

Results obtained in this study show that a common characteristic of both obese groups was an increase in mRNA abundance from genes such as VEGF-A and GLUT-1, that are associated with angiogenesis, in conjunction with a rise in cell proliferation factors, including IGF-1R and IR mRNA in renal tissue (Nangaku & Eckardt, 2007).

These changes in gene expression are possibly influenced by the increased plasma insulin concentration, a response amplified in offspring born to NR mothers (Hiromura *et al*., 2002; Sebert *et al.*, 2009). On the basis of other *ex-vivo* observations in the early pathogenesis of renal damage in obese *fa/fa* rats, the onset of hyperinsulemia was associated with glomerular and tubular cell hyperplasia, followed by other forms of renal dysfunction, such as cell hypertrophy and lipid accumulation (Coimbra *et al.*, 2000). The changes in plasma insulin and IGF-1, in addition to the changes in gene expression of their renal receptor, possibly indicate similar responses in my sheep model (Sebert *et al.*, 2009; Williams *et al.*, 2007). A combination of these factors may induce the expression of several proteins involved in cell survival, proliferation and intracellular energy consumption homeostasis (Warnecke *et al.*, 2003; Advani *et al.*, 2007). Furthermore, several *in vitro* observations have indicated the ability of insulin and IGF-1 to induce cell proliferation through increased expression of HIF-1α, an action mediated by the activation of IGF-1R and by an increase in the generation of ROS, leading to hypoxia (Page *et al.*, 2002; Mori & Cowley, 2003; Lauzier *et al.*, 2007). However, HIF-1α plays an important role in the regulation of fibrosis *in vivo*, increasing cell motility and invasiveness, indicating a link between the activity of this transcriptional factor and the development of renal injury (Higgins *et al.*, 2007).

Due to differences in the oxygen tension between the renal cortex (30 to 50 mmHg) and medulla (10 to 25 mmHg), certain renal structures, such as the proximal tubule or the thick ascending limbs, are more vulnerable to hypoxia insults (Lubbers & Baumgartl, 1997; Chen *et al.*, 2009). Consequently, an obesogenic environment, due to a rise in cellular metabolic activity, may increase the kidneys sensitivity to changes in

oxygen delivery and thus its susceptibility to hypoxic insults (Mollnau *et al.*, 2002; Page *et al.*, 2002). Further evidence of mitochondrial dysfunction, associated with hypoxia and the reduction in oxygen delivery, is indicated by the rise in protein abundance of SOD-2, detected in both obese groups (Hussain *et al.*, 2004).

A reduction in oxygen delivery may explain the presence of proliferative factor PCNA in areas susceptible to hypoxic insults, such as the tubules themselves. In addition, PCNA immunostained cells were also found in the Bowman capsule and golmerulus on the kidney cortex of both obese groups of sheep, irrespective of the maternal *in-utero* environment. One explanation for the increase in PCNA abundance may be that it represents the first stage of a compensative cell-division response to a non-lethal hypoxic insult, commonly associated with exposure to an obesogenic environment (Flyvbjerg *et al.*, 1989; Johnson *et al.*, 1992; Raz *et al.*, 2003). Thus, increased exposure to anabolic hormones, such as IGF-1, insulin and leptin that are stimulated by the obesogenic environment, might be responsible for accelerated abnormal abundance of PCNA, as observed in the kidney of this model of juvenile obesity.

My study suggests a significant increase in proliferative response, or at least a rise in the abundance of factors involved in the first stage of a mitotic response in both obese groups. An additional sign of the activation of the cell division mechanism was the increase in mRNA abundance of DNMT-1, detected in both obese groups. DNMT-1 acts in conjunction with PCNA during specific phases of the cell division cycle (Leonhardt *et al.*, 1992). Importantly, a previous publication observed a reduction in molecular markers of apoptosis in the renal cortex, which might result in hyperplasia or

hypertrophy, which may possibly indicate a different development of vascular dysfunction between those obese offspring exposed to maternal nutrient restriction, compared with the obese group (Williams *et al.*, 2007). Finally, in the present animal model, Williams *et al*. found that juvenile-onset obesity *per se* led to an increase in kidney size, which may be a result of cell hyperplasia. However, this was accompanied by similar increases in GFR and blood pressure, which are important markers of early kidney disease (Henegar et al., 2001; Williams *et al.*, 2007).

4.4.1.1 The VDAC role in the mitochondria with obesity in the maternal nutrient restricted group

The success of cellular adaptation to hypoxia by HIF-1α depends on the coordinated expression of gene products that facilitate glucose and oxygen uptake for the completion of the cell cycle in order to increase oxygen delivery by an increase in vascularisation (angiogenesis) (Jaakkola *et al.*, 2001; Warnecke *et al.*, 2003; Advani *et al.*, 2007).

A family of enzymes known as hexokinases are key mediators of glucose uptake and utilisation at the cellular level (HK1-3). Robey *et al.* have suggested that increases in glucose metabolism and exposure to fibrosis are capable of simulating HK mRNA abundance in mesangial cells (Robey *et al.*, 1999; Robey *et al.*, 2000). An important property of these enzymes is their ability to interact with the mitochondrial pore, VDAC-1, that facilitates kinase access to ATP, thereby increasing glucose uptake (Viitanen *et al.*, 1984; Chang *et al.*, 1996). Furthermore, this interaction can improve mitochondrial activity and it reduces the induction of apoptotic effects by growth factor withdrawal (Gottlob *et al.*, 2001). *In vitro*, cells expressing low abundance of VDAC-1

showed a reduction in ATP synthesis capacity and a substantial reduction in cell proliferation (Abu-Hamad *et al.*, 2006). *In vivo*, the absence of VDAC-1 leads to impaired glucose tolerance (Anflous-Pharayra *et al.*, 2007). An increased abundance in this ionic channel, through its binding to HK-1, may be a temporary adaptation in cells undergoing mitosis to overcome the energetic mitochondrial deficits induced by hypoxia (Tanaka *et al.*, 2003). A comparable adaptation may be occurring in the renal cortex of obese maternal nutrient restricted offspring.

An increased concentration of intracellular glucose-6-phosphatase, which is associated with normal cellular oxygen diffusion, interrupts the mitochondrial-hexokinase by inducing structural changes in both proteins, allowing other factors to bind to this channel (Mulichak *et al.*, 1998). Bcl2 is one of a number of proteins that associate with VDAC-1 at normal cellular oxygen tension. Essential to this interaction is ATP availability and low cytosolic abundance of pro-apoptotic proteins, such as Bax (Shimizu *et al.*, 1999; Gottlob *et al.*, 2001). Abu-Hamad *et al.* showed that over-expression of VDAC-1 *in vitro*, without a rise in protein abundance of HK-1 or Bcl2, induces cell apoptosis (Abu-Hamad *et al.*, 2006). For that reason, an increase in glucose-6 phosphate, in conjunction with a rise in abundance of the Bax protein, leads to the formation of the complex Bax-VDAC-1 and subsequent cytochrome C release, initialising the apoptotic cascade (Vyssokikh *et al.*, 2004). Therefore, I propose that the decrease in the presence of molecular markers of renal apoptosis, in conjunction to an increase in VDAC-1 abundance and total SOD enzymatic activity observed in NR obese sheep, might indicate an improvement in cellular oxygen metabolism (Williams *et al.*,

2007). These molecular changes possibly lead to cell division, a sign of an advanced adaptation to a hypoxic environment.

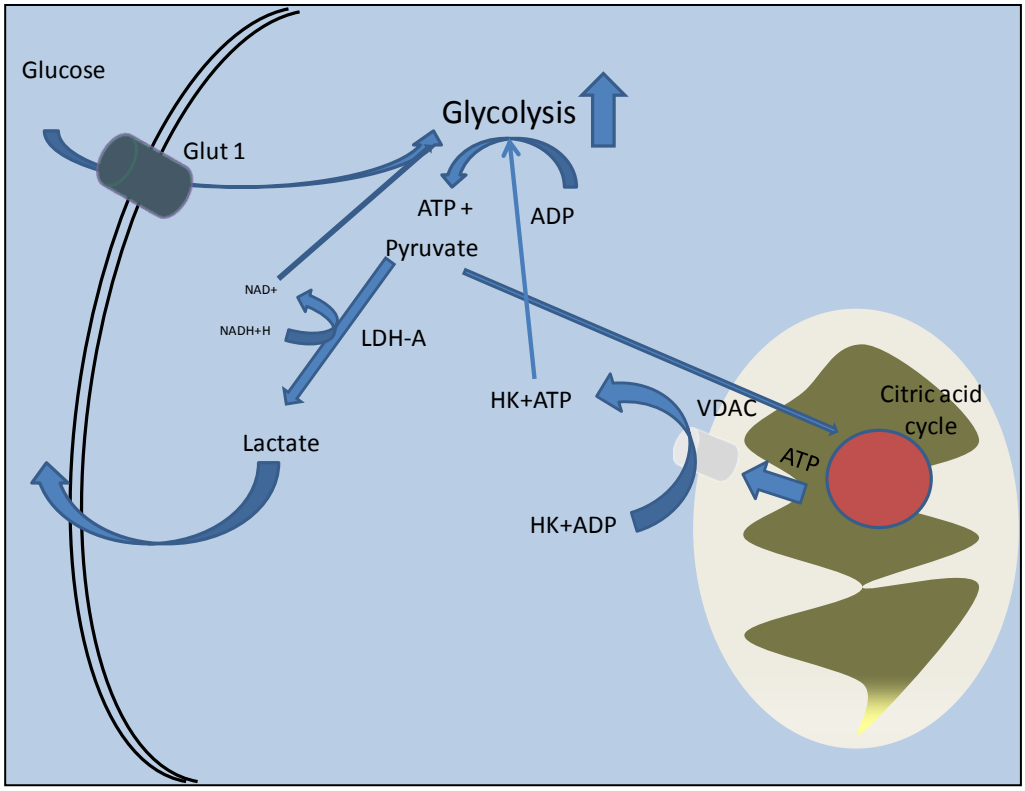


Figure 4.9 The proposed interaction between VDAC-1 and HK1 in the transportation of ATP, which potentially might improve the mitochondrial function under hypoxic conditions (Adapted from Vander Heiden *et al*., 2009).

4.4.2 Evidence for cell-arrest and cell-hypertrophy in the renal tissue in both obese groups

Histological examination of renal biopsies from non-proteinuric overweight patients has indicated an increase in the glomerular surface and tubular dilatation, compared to lean controls. These results suggest that inflammation may be one of the early events in renal dysfunction, possibly preceding the appearance of other symptoms, such as increased serum creatinine or changes in blood pressure (Rea *et al.*, 2006). In an attempt to examine renal functional and morphological changes linked to early onset obesity, Henegar *et al*. exposed dogs to a high fat diet intake. After 7 to 9 weeks, their body weight, GFR and mean arterial pressure was higher compared with lean controls. In addition, the high fat diet caused hyperinsulinemia, hyperliptemia and activation of RAS. These hemodynamic alterations were accompanied by further renal structural changes, which included expansion of the Bowman's capsule, followed by an increase in glomerular cell proliferation and mesangial matrix expansion (Henegar *et al.*, 2001). In my study, using juvenile sheep, exposure to an obesogenic environment also induced similar alterations in plasma concentration of several hormones, such as leptin, and an increased sensitivity to ANG II, which may influence renal functional and structural changes, as observed in this model (Williams *et al*., 2007; Sharkey *et al*., 2009).

In obese patients, high serum concentration of leptin is associated with obesity and reflects the increased amount of adipose tissue (Considine *et al.*, 1996). This hormone is able to affect renal structure and function directly, mainly through the short form of its receptor (Ob-Ra), which is highly abundant in the kidneys (Gal *et al*., 1997; Wolf *et al*., 1999; Rahmouni *et al*., 2005). Furthermore, short term exposure to leptin may affect

kidney structure and functionality through increased production of NO. However, chronic hyperliptinemia leads to an increase in intrarenal oxidative stress, producing NO deficiency (Beltowski *et al*., 2004). Thereby, the changes in the amount of NO detected in renal tissue of both obese groups suggests the existence of different degrees of leptin resistance. Release of NO by leptin can modify the activities of angiogenic factors, such as VEGF-A, inducing endothelial cell proliferation. For that reason, when this hormone is infused into naive rats, it induces cell division of glomerular endothelial cells, followed by fibrosis (Wolf *et al*., 1999; Nakagawa *et al*., 2006).

The action of leptin in cell proliferation may be mediated by an increase in glucose uptake, increasing the activity of other factors, including elements of the renal RAS (Han *et al*., 2001; Vidotti *et al.*, 2004). Subsequent activation of the RAS induces different acute effects on renal tissue, including hyperplasia and hypertrophy. In first instance, exposure to ANG II induced a pronounced proliferation of vascular smooth cells, which are followed by tubulointerstitial fibrosis injury (Johnson *et al.*, 1992). However, long term exposure to ANG II in renal tissues leads to the activation of the cell arrest mechanism (Hannken *et al*., 1998). This is a direct consequence of the vasoconstricting action of ANG II, which takes place through the generation of reactive oxygen species, inducing DNA damage and triggering the activation of pro-apoptotic proteins, such as the tumour suppressor p-53 and Bax (Hannken *et al.*, 1998; Mori & Cowley, 2003). Furthermore, cell arrest induces a rise in RNA and protein content, leading to an increase in cell size or cell-hypertrophy, possibly reflected in our obesity model by the increase in kidney mass (Fine & Norman, 1989; Williams *et al*., 2007). Cell hypertrophy is associated with a slow, degenerative process, commonly linked with

podocyte loss of function and glomerulosclerosis (Wiggins *et al.*, 2005). The increased sensitivity to ANG II, seen in both groups of obese juvenile sheep, may be an indicator of a similar proliferative-hypertrophy process. Therefore it is likely that obesity *per se*, independent of *in utero* nutrition, appears to induce those genes involved in processes such as proliferation and apoptosis, to activate cell arrest or hypertrophy in renal tissue (Fig. 4.10).

4.4.3 Characterisation of the apoptotic process observed in the obese groups

After the initial compensatory proliferative-hypertrophic stage, the progression of renal insufficiency associated with obesity involves inflammation, leading to glomerulosclerosis and tubuleinterstitial fibrosis, both part of the renal scarring process (Cao *et al.*, 2000; Coimbra *et al.*, 2000; Henegar *et al.*, 2001). As metabolic disease develops, the ensuing hyperinsulemia and renal injury leads to a slow but progressive reduction in glomerular and tubular cell numbers, inducing deterioration in renal structure and function (Chagnac *et al.*, 2000; Praga *et al.*, 2001; Wiggins *et al.*, 2005).

Various models of prenatal diet have shown the potential and ability to modulate renal structure and function (Whorwood *et al.*, 2001; Gopalakrishnan *et al.*, 2005; Williams *et al.*, 2007). In the absence of obesity, maternal nutrient restriction between early to mid gestation in young sheep, reduced the nephron number associated with increased cytochrome C abundance in the kidney, at 6 months of age (Gopalakrishnan *et al.*, 2005). A previous study, which analysed renal dysfunction in year old obese sheep exposed to maternal nutrient restriction, showed an apparent reduction in certain markers associated with inflammation and apoptosis, compared with the obese group (Williams *et al.*, 2007). The main difference that I observed from the previous findings

of Williams *et al*., was an equal increase in expression of the gene, Bax, which is regulated directly by p-53, suggesting perhaps that the pathways associated with renal injury are in progress in both obese groups, although the developmental pattern of renal dysfunction differs.

Cell death by apoptosis, unlike necrosis, is an ATP dependent cellular pathway that is genetically controlled by a transcriptional factor p-53, which in turn, depending on the cellular stimuli, activates a series of other genes, including Bax and Fas. This process includes rapid changes in cellular morphology, such as contraction of the cytoplasm, nucleus and DNA degradation (Yonish-Rouach *et al.*, 1991; Bouvard *et al.*, 2000). Exposure to ROS activates the main regulator of cell apoptosis, p-53 due to increased cellular and DNA damage (Muller *et al.*, 1998). Data obtain from the TBARS and 8-OHdG tests indicate similar levels of oxidative stress, possibly explaining the equally high gene expression of p-53 and Bax in both obese groups.

Possible differences in the intensity of apoptosis between obese groups may be based on the stage of development of the metabolic disease, which may be altered by exposure to metabolites, hormones and cytokines, particularly those secreted by the surrounding adipose tissue (Hotamisligil *et al.*, 1995; Massiera *et al.*, 2001a; Sharkey *et al*., 2009). Sharkey *et al*. have demonstrated, using this animal model, much more advanced adipocyte dysfunction with increased formation of crown-like structures in those obese offspring exposed to maternal nutrient restriction compared with the obese group (Sharkey *et al*., 2009). Several studies have demonstrated that adipose tissue, especially in obese individuals, is able to secrete hormones and cytokines, including ANG II and TNF-α, potentially influencing inflammation and healing in renal tissue

(Hotamisligil *et al.*, 1995; Giacchetti *et al.*, 2002; Kim *et al.*, 2006). Thereby, the evolution of the fibrotic process depends heavily on apoptosis (Verrecchia *et al.*, 2002). Interestingly, it has been shown that exposure to TNF-α influences the secretion of collagen, affecting the fibroblast contractile activity, thus demonstrating that the balance of cytokines influences the outcome of the wound healing process (Goldberg *et al.*, 2007). After the inflammatory-fibrotic stage, the myofibroblasts undergo apoptosis or differentiation to vascular cells, leading to complete tissue re-epithelialisation, as characterised by a total inhibition of cell death (Desmouliere *et al.*, 1995).

By staining kidney sections with Masson's trichrome and by analysis of mRNA expression of the pro-inflammatory receptor Fas, I indirectly assessed the advance of the fibrotic process. Therefore, on the basis of this data and information published previously, I speculate that the kidneys of the NR-O animals were in a more advanced stage of tissue re-epithelialisation or tissue scarring compared with the (control) obese group. This hypothesis is further supported by evidence of a decrease in mRNA abundance of inflammatory or pro-apoptotic genes, the reduction in cell death and the excess of collagen observed in the kidneys of the prenatally nutrient restricted and obese animals (Williams *et al.*, 2007).

4.4.3.1 The role of iron and lipid accumulation in renal tissue

As previously noted in other tissues, including the heart and liver, the effects of postnatal obesity in the model studied here are influenced by *in utero* nutrient restriction, which also increases ectopic triglyceride accumulation in the renal cortex, compared with the obese group (Chan *et al.*, 2009; unpublished observation). Jiang *et al*. showed that obesity in mice (C57BL/6J) induced by high fat feeding caused a robust

lipid accumulation in glomerular and tubuleinterstitial cells. In addition, it was observed that this effect was accompanied by glomerulosclerosis, which resulted in a significant renal mRNA over-expression of collagen and fibronectin (Jiang *et al.*, 2005). A possible inducer of this type of renal injury associated with obesity is the RAS. For instance, chronic ANG II administration is able to induce a short term increase in the accumulation of iron in the renal tubular and vascular cells, which is followed by lipid deposition (Saito *et al.*, 2005). Importantly, when iron is exposed to a reducing environment, such as the cysteine-glutathione cycle or the activation of RAS, it is able to promote injuries in renal tissue through the production of free radicals (Mori & Cowley., 2003 ;Okada *et al.*, 1993).

Importantly, over activation of RAS, as observed in cases of severe obesity, may contribute to alterations in iron transport by promoting liver dysfunction through an increase in lipid accumulation (Bataller *et al.*, 2005; Engeli *et al.*, 2005; Kolak *et al.*, 2007). In this model, it has previously been demonstrated that chronic obesity influenced by maternal nutrient restriction causes an acceleration of metabolic dysfunction in the liver, characterised in particular by ectopic lipid accumulation. These changes in liver function possibly affect iron delivery, which in turn, may influence the lipid accumulation in other tissues, such as the heart and kidneys (Chan *et al.*, 2009; unpublished observation).

Free fatty acid accumulation *per se* may also result in endothelial dysfunction, mediated by increased production of ROS (Chen *et al.*, 2004). However, it was suggested that damaged tubular cells can incorporate exogenous triglycerides, as part of a physiological response to injury in addition to the synthesis of fatty acids induced by

exposure to an obesogenic environment (Jiang *et al.*, 2005; Zager *et al.*, 2007). This combination of circumstances may rapidly increase the cellular triglyceride reserves, whereas the chemistry of fatty acid metabolism may have differential effects on the survival of cells. Listenberger *et al*. have suggested that triglyceride accumulation, particularly of unsaturated fatty acids, inhibits apoptosis by the ability to neutralise the toxic effects of oleic and palmitate acid in non-adipose tissues (Listenberger *et al.*, 2003). This may represent an initial cellular response against lipotoxicity, which may explain the slow progress of renal diseases associated with obesity (Listenberger *et al.*, 2003). In this model of juvenile obesity, renal triglyceride accumulation suggests an association with a reduction in cell death. Accumulation of triglycerides may be a temporary protection against some of the negative effects of rapid weight gain, such as hyperliptemia and an increase in ANG II sensitivity. However, in the long term, the incorporation of fatty acids raises the susceptibility to cell injuries induced by ROS and ATP depletion, leading to apoptosis (Chinen *et al.*, 2007). Thus, the presence of iron and the accumulation of triglycerides may represent two different sources of oxidative stress of equal intensity, triggering different outcomes that might be different stages of the same overall disease process (Okada *et al.*, 1993; Chinen *et al.*, 2007).

4.4.4 Summary

As described in the literature, exposure to an obesogenic environment induced in sheep renal tissue results in a series of cellular events: proliferation, apoptosis and hypertrophy The results of my study have determined that maternal nutrient restriction during early to mid gestation resulted in a marked acceleration of this degenerative process in renal tissue, in response to juvenile obesity.

Activation of pro-survival and fibrogenic genes in both obese groups may be the biological consequences of hypoxic HIF-1α activation, suggested by up-regulation angiogenic genes, such as GLUT-1 and VEGF-A. Hypoxia in the kidneys could have resulted from a rise in the activity of the RAS. Due the heterogenic renal cellular population and the length of exposure to an obesogenic environment, my results indicate that the three processes of proliferation, apoptosis and hypertrophy may have occurred simultaneously in the kidneys of both obese groups by one year of age. Renal dysfunction in the obese group was characterised mainly by the prevalence of apoptosis, associated with an outgoing inflammation catalysed by iron deposited in the renal tubule. Conversely, in the kidneys of the maternal nutrient restricted obese group, my findings suggest that, whilst there is an active pro-apoptotic response, this occurs on a smaller scale compared with the obese group. This reduction in cell death is in conjunction with a lack of expression of genes associated with the inflammatory process. Furthermore, the increase in abundance of the mitochondrial pore VDAC, combined with an increase in activity of the SOD system and the reduction in apoptosis may indicate that a large number of cells are dividing or becoming hypertrophic. Another important feature is the equal presence of collagen in all obese animals. However, with the reduction in apoptosis along with differences in inflammatory responses and the increase in lipid deposition, I propose that the NR-O group are at a more advanced stage of renal scarring than the obese group. This advanced stage of disease is in accord with additional findings from other tissues of the same animals (Chan *et al*., 2009). Maternal nutrient restriction in early to mid gestation, followed by a period of postnatal obesity, has a much greater impact on the liver, heart and adipose

tissue than on the obese offspring born from mothers fed *ad libitum*. Finally, these adaptations seen in renal tissue caused by obesity may be strongly influenced by the appreciable dysfunction observed in other tissues. In particular, the influence of adipose tissue, which is able to modify the abundance of metabolites, cytokines and hormones, has a significant impact on kidney health (Fig. 4.10).

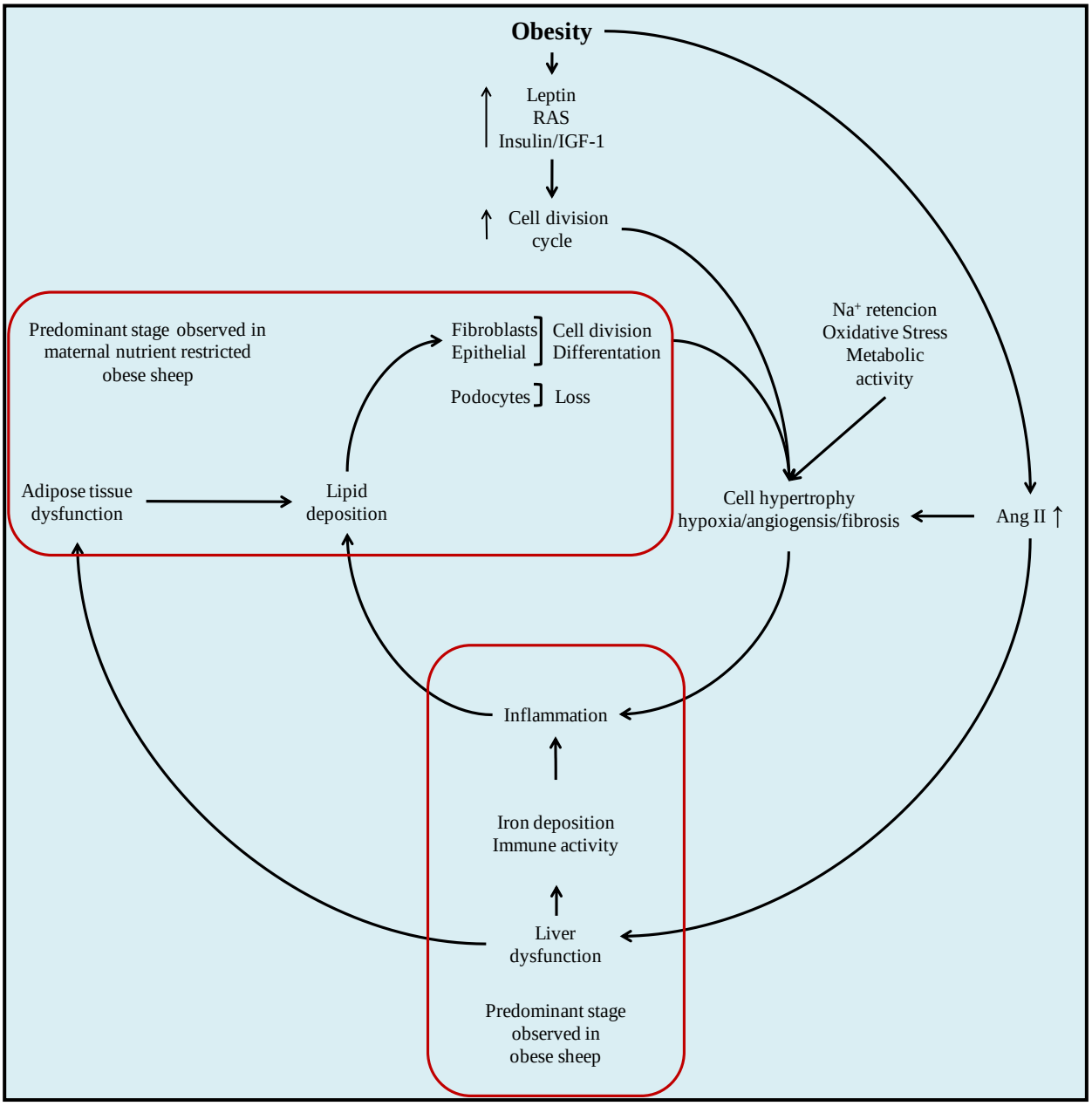


Figure 4.10 Schematic summary of findings of this chapter. Exposure to an obesogenic environment induced in renal tissue results in a series of cellular events: proliferation, apoptosis and hypertrophy and all these processes are followed by collagen and lipid deposition. The red areas represent the main outgoing stage in each obese group in this study.

Chapter 5 - Influence of maternal nutrient restriction from early to mid gestation on the development of the adipose tissue in response to juvenile obesity

Chapter 5 - Influence of maternal nutrient restriction from early to mid gestation on the development of the adipose tissue in response to juvenile obesity

5.1 Introduction

5.1.1 Sensitive periods for the development of the adipose tissue

Epidemiological and clinical studies have demonstrated that there is an association between early life diet, commencing *in-utero* and then extending over the first years of postnatal life, with respect to the onset of later obesity. To elucidate the potential influence of maternal nutrition, Ravelli *et al*. conducted a retrospective study of young males (<19 years old) prenatally exposed to the Dutch famine of 1944-5. This investigation indicated that the prevalence of obesity was higher in individuals who had suffered a reduction in nutrient supply *in utero* during the first trimester of gestation. Furthermore, exposure to the famine during the second half of pregnancy produced a leaner phenotype, leading the authors to conclude that early fetal nutritional deprivation may interfere with the normal development of the adipose tissue (Ravelli *et al.*, 1976). Importantly, findings from animal models exposed to maternal nutrient restriction during early to mid gestation also suggested substantial metabolic alterations in the adipose tissue, both at birth and in early adulthood (Whorwood *et al*., 2001; Sharkey *et al*., 2009).

Adipose tissue enlargement is thought to be the result of three cellular mechanisms: hyperplasia (increase in cell number), hypertrophy (increase in cell size) and or a combination of both (Jo *et al.*, 2009). Several studies have followed the development of adipose cellularity in relation to fat cell morphology, in obese and lean

individuals throughout life and revealed the existence of critical stages on adipose tissue development (Knittle *et al.*, 1979; Spalding *et al.*, 2008). It has been suggested that one particularly sensitive stage for adipose tissue growth in human beings lasts from early childhood to puberty, during which time there is a high level of cell division (Baum *et al.*, 1986). A number of studies have demonstrated that adipose tissue of infants is characterised by the extensive generation of new adipocytes, especially during the first year of life (Baum *et al.*, 1986). In subsequent years, there is a small and temporary decline in the rate of cell division but a second increase in adipocyte numbers may then follow between 9 and 13 years of age (Salans *et al.*, 1973; Knittle *et al.*, 1979). After this second peak in cell division, there is a strong decline in new cell production, stabilising the number of adipocytes. The mature cell population of adipocytes them remains constant during adulthood, although human beings do possess a constant reserve of pre-adipocytes located on the connective tissue or stroma (Salans *et al.*, 1973; Gimble *et al.*, 1989; Spalding *et al.*, 2008). Thereby, individuals who become obese during early childhood and remain obese after puberty, have adipose tissue with a tendency to be hyperplastic, but with relatively normal sized cells. On the other hand, in obese subjects exposed to an obesogenic environment during adulthood, the adipose tissue develops larger cells, though cell number remains on a par with that of lean adults (Knittle *et al.*, 1979). A plausible reason for these differences in cell composition is that the ratio between adipocyte death and differentiation during adulthood does not exceed more than 10% annually. This rate is unaffected by the early onset of obesity, indicating the existence of a strong mechanism that regulates the generation of new fat cells (Spalding *et al.*, 2008). However, this modest increase in new adipocytes can be

compromised by an increase in adiposity, reducing further the capacity of stroma cells to differentiate and leading to cell hypertrophy (van Harmelen *et al.*, 2003). Thus, the size of adipocytes, particularly those belonging to upper-body depots, are the strongest determinants of metabolic abnormalities, due to the expansion of abdominal fat (Tchoukalova *et al.*, 2008).

5.1.1.1 Mechanisms of adipocyte differentiation

Adipocyte differentiation involves the coordinated activation of a large number of genes and transcriptional factors that are heavily influenced by hormones, cytokines, nutrient and signalling molecules. These factors in turn regulate the characteristics of the mature adipose tissue depot including cell size and number (Massiera *et al.*, 2001a; Farooqi *et al.*, 2002; van Beek *et al.*, 2008).

Recent observations have begun to elucidate the molecular mechanisms involved in general pre-adipocyte differentiation, beginning with their activation in early postnatal life (Fu *et al.*, 2005; van Beek *et al.*, 2008; Viengchareun *et al.*, 2008). One of the first regulators of adipogenesis is the hormone, prolactin, which stimulates IGF-2 gene expression in brown adipocytes through its receptor, inducing early stages of cell differentiation, growth and the absorption of nutrients (Sinha *et al.*, 1990; Viengchareun *et al.*, 2008). Other important transcriptional factors involved in the regulation of adipogenesis are CCAAT/enhancer-binding proteins (C/EBP)-β and δ. These in turn induce the expression of C/EBP-α and peroxisome proliferator-activated receptor γ (PPAR-γ) that plays a key role in the expression of other transcriptional factors associated with adipogenesis. In contrast to other members of this group, C/EBP-α promotes specific aspects of the adipocyte phenotype, including insulin sensitivity and

lipid accumulation (Cao *et al.*, 1991; Wu *et al.*, 1999). Other important factors in the regulation of early adipogenesis are hormones such as glucocorticoids. By binding to their receptor, which expressed in human pre-adipocytes, glucocorticoids induce the expression of C/EBP-α leading to mature cell phenotype (Joyner *et al.*, 2000; Hausman *et al*., 2000). Notably, local activation of C/EBP-α in adipose tissue is dependent on the enzyme 11β-HSD-1, which transforms inert cortisone to active cortisol in humans and sheep (Livingstone *et al.*, 2000; Rask *et al.*, 2001). In addition, C/EBP-α is able to mediate the activity of other components involved in cell differentiation. One such example is the retinoblastoma protein (pRB) that also induces the expression of UCP-1 in brown fat cells and affects the rate of lipid accumulation in mature adipocytes (Cram *et al.*, 1998; Hansen *et al.*, 2004). Interestingly, during cell replication, pRB interacts with DNMT-1, a DNA methyltransferase involved in terminal differentiation in adipocytes and other cells types (Robertson *et al.*, 2000; Rai *et al.*, 2006; van Beek *et al.*, 2008). As stated previously in Chapter 3, DNMT-1 is also essential for the maintenance of DNA methylation motifs and its deletion inhibits the process of cell differentiation (Li *et al.*, 1992).

5.1.1.2 Factors that inhibit adipocyte differentiation

This is a heterogenic group of molecular signals that control or repress the process of adipogenesis, and this includes inflammatory cytokines and hormones. Cytokines are important regulators of adipogenesis, the most well known of which are TNF-α and IL-6. In obese individuals, the plasma serum concentrations of both these cytokines are highly elevated and is usually linked to severity of their metabolic disease (Hotamisligil *et al.*, 1993; Vgontzas *et al.*, 1997; Fried *et al.*, 1998). For example, in obese mice

(ob/ob) with null mutations on both genes encoding the TNF-α receptor there is a significant improvement in insulin sensitivity (Uysal *et al.*, 1997; Nisoli *et al.*, 2000). Conversely, when TNF-α gene expression is limited to only adipose tissue, in null mutated ob/ob mice, insulin action is normalised but does not prevent cell hypertrophy, which is a common feature in this animal model of obesity (Xu *et al.*, 2002). Furthermore, in *in vitro* studies of fully differentiated 3T3-L1 adipocytes, exposure to TNF-α can reduce the mRNA expression of genes associated with cell differentiation, such as C/EBP and PPAR-γ, thus reversing the adipocytic phenotype (Ron *et al.*, 1992; Zhang *et al.*, 1996). Treatment of adipocytes with TNF-α and IL-6 promotes lipolysis by increasing the activity of hormone-sensitive lipase (HSL) (Fried *et al.*, 1998; Laurencikiene *et al.*, 2007). In addition, hormones such as growth hormone (GH), also inhibit adipogenesis and induce lipolysis through the stimulation of HSL and the inhibition of adipogenic DNA transcriptional factors (Dietz & Schwartz, 1991; Huppertz *et al.*, 1996; Asada *et al.*, 2000; Chen *et al.*, 2001). GH can promote cell differentiation. Its effects are mediated by IGF-1, which inhibits lipolysis through a reduction in the abundance of growth hormone receptor (GHR) (Bengtsson *et al.*, 1993; Asada *et al.*, 2000). Therefore, hormone and cytokine activity are considered to be potential regulators of adipose tissue metabolism, influencing the outcome of obesity related complications (Clement *et al.*, 2004; Ukropec *et al.*, 2008).

5.1.2 Adipocyte death, macrophage infiltration and tissue remodelling

Recently, Sharkey *et al*. observed that after a period of fast weight gain, NR offspring exhibit signs of adipose tissue dysfunction characterised by an increase in crown-like structures (CLS), with similar inflammatory features, as seen in other models

of severe obesity (Cinti *et al*., 2005; Sharkey *et al*., 2009). The consumption of a high fat diet in rodents, particularly one composed mainly of saturated fatty acids, accelerates the process of adipocyte hypertrophy or hyperplasia. This commonly coincides with an increase in the expression of immune related genes, such as TNF-α and iNOS (inducible nitric oxide synthase) within the adipose tissue (Soukas *et al.*, 2000; Strissel *et al.*, 2007). When adipose tissue begins to secrete these pro-inflammatory mediators, an infiltration of macrophages occurs in parallel, incrementing further the level of inflammation and also affecting insulin sensitivity (Hotamisligil *et al.*, 1993; Clement *et al.*, 2004). Cinti *et al.* attributed the outgoing phagocyte infiltration to an increase in necrosis-like cell death. This might be associated with the negative effects of adipocyte hypertrophy observed in humans and obese (db/db) mice models (Cinti *et al.*, 2005). Dead adipocytes become surrounded by scavenging macrophages, which are postulated to absorb cell debris and toxic lipid droplets and form CLS (Cinti *et al.*, 2005; Strissel *et al.*, 2007). Although CLS are commonly detected in extreme obese subjects, they are normally observed at a very low frequency (Cinti *et al.*, 2005). It has been suggested that the macrophage infiltration is a vital mediator in adipose tissue remodelling. This is due to the fact that adipocyte necrosis is associated with matrix deposition, angiogenesis and differentiation of new adipocytes (Nishimura *et al.*, 2007; Strissel *et al.*, 2007). For instance, in the case of hypertrophic adipocytes there is a reduction in oxygen diffusion, leading to hypoxia in the adipose tissue. This is a potent inducer of angiogenesis, an essential initiator of adipogenesis. Therefore, hypertrophic adipocytes and their subsequent removal may create an angiogenic microenvironment in which pre-adipocytes can potentially differentiate to mature adipocytes (Fukumura *et al.*, 2003;

Nishimura *et al.*, 2007). This is possible because new adipose cells can arise from progenitor cells within the adult white fat depots or the bone marrow, a process that may be stimulated by a high fat diet in rodents (Crossno *et al.*, 2006; Rodeheffer *et al.*, 2008).

5.1.3 Scientific rationale, aim and hypothesis

As observed in Chapter 3, at seven days of age, offspring born from nutrient restricted mothers showed a different gene expression pattern within adipose tissue, compared to those control offspring born from mothers fed to requirements throughout gestation. These changes in gene expression include genes associated with adipose tissue growth, such as IGF-2, IGF-2 receptor and DNMT-1, linked to terminal cell differentiation, suggesting that there might be different mechanism involving cell maturation (Chapter 3). However, the extent to which those alterations persist during adulthood and the effect on other tissues in response to an obesogenic environment remains to be further established. Furthermore, whether an alteration in the nutritional status *in utero* alters susceptibility to obesity in adulthood and promotes adipocyte hypertrophy and cell death is still unclear. However, Sharkey *et al.* demonstrated that maternal nutrient restriction during early to mid gestation in sheep is implicated in increased markers of cellular stress, such as increased abundance of CLS in the adipose tissue, in response to juvenile obesity (Sharkey *et al.*, 2009). Importantly, the appearance of these formations is linked to multiple cytotoxic stressors, including endoplasmatic reticulum stress, hypoxia, reactive oxygen species and the release of free fatty acids from the adipose tissue (Clement *et al.*, 2004; Cinti *et al.*, 2005; Nishimura *et al.*, 2007). These cytotoxic stresses activate inflammatory pathways, thus influencing the

rate of ectopic lipid deposition in tissues such as the kidneys, inducing tissue remodelling (Kolak *et al.,* 2007; Strissel *et al.,* 2007). Furthermore, changes in adipogenesis may also potentially affect kidney function and health (Chapter 4). For that reason, my next study was designed to elucidate whether maternal nutrient restriction during the period of maximal organogenesis, in addition to an obesogenic postnatal environment, still affects the mechanisms of adipocyte maturation. My current hypothesis is that maternal nutrient restriction, during this period of gestation (*e.g.* early to mid gestation), when compared to *ad libitum* nutrition, would modify perirenal adipose tissue development. Alteration of adipocyte maturation when followed by a period of fast weight gain may produce additional changes in the secretion of hormones and metabolites. This would lead to subsequent changes in gene expression associated with lipolysis, angiogenesis, inflammation and terminal cell differentiation- specifically GHR, VEGF-A, iNOS and DNMT-1.

5.2 Methods and materials

A full description and details of all methods, including animal models, gene expression analysis in this chapter can be found in chapter 2. It is important to mention that the ontology of DNMT-1, the PAT of histological section using Masson trichrome technique and cortisol and leptin plasma quantifications were kindly carried out by Mr Mark Pope, Mrs Victoria Wilson and Dr David S. Gardner. Finally, all statistical tests are also explained in chapter 2.

5.3 Results

5.3.1 Perirenal adipose tissue development over time and the influence of maternal nutrient restriction and obesity

As expected, between birth and both the first week and month of postnatal life, the adipose tissue-body weight ratio increased equally in all animals ($p<0.05$; Fig. 5.1). This process of adipose tissue growth continued up to one year of age and was amplified by the exposure to an obesogenic environment irrespective of the prenatal nutritional environment ($p<0.05$; Fig. 5.1) (Williams *et al*., 2007).

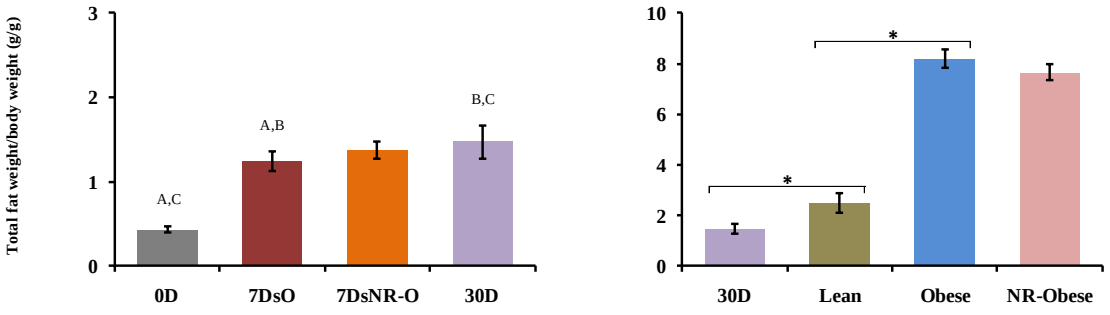


Figure 5.1 Increase in the ratio total fat-body weight at different time points from birth to one year old in sheep. The data are expressed as mean ± SEM. Description of statistical analysis (A) offspring born to control diet mothers at birth (0D) versus 7 day old siblings from the obese group (7DsO) ($p<0.05$) and (C) 0D versus offspring born to control diet mothers from birth to 30 days of age (30D) ($p< 0.05$). (B) $p< 0.05$ 30D versus 7DsO. * $p<0.05$ 30D versus one year old lean group; Lean group versus Obese group.

5.3.2 Metabolic, endocrinological and physical adaptation in response to an obesogenic environment at 6 months of age

At 6 months of age, the body weight of those sheep exposed to an obesogenic environment, regardless of maternal diet during gestation, were for first time statistically heavier than the lean group (Table 5.1) and the weight observed was similar for both

obese groups (Sharkey *et al*., 2009). An adaptation that was accompanied by raised plasma IGF-1 (Table 5.1). At this age offspring born to NR mothers exhibit both raised leptin ($p<0.05$; Fig. 5.2.A) and cortisol ($p<0.05$; Fig 5.2.B).

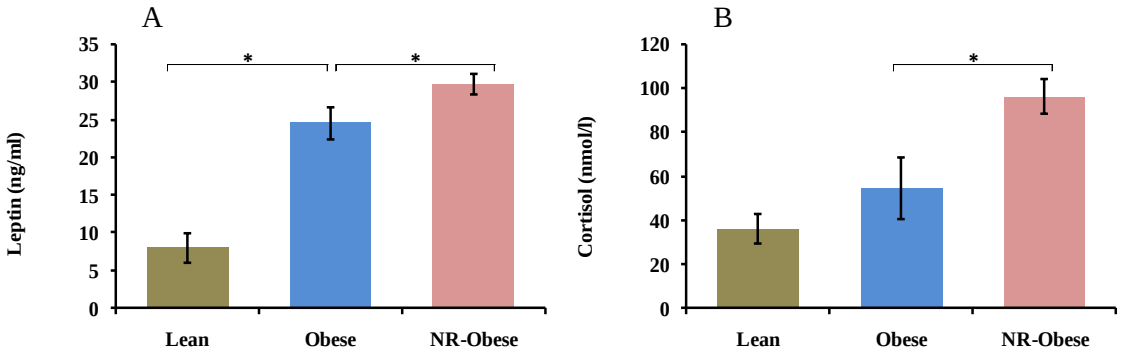


Figure 5.2 (A) Leptin and (B) Cortisol concentration in plasma of 6 month old lean and obese offspring born to mothers exposed to nutrient restriction or fed to appetite (*p<0.05)

5.3.3 Adipose tissue gene expression

5.3.3.1 Ontogeny of DNMT-1 in the adipose tissue

Gene expression of DNMT-1within the perirenal adipose tissue increased from birth until the age of 30 days and was clearly affected by maternal nutrient restriction at 7 days of age, demonstrated by a significant rise in mRNA abundance compared with the one week old offspring born to control fed mothers ($p<0.05$; Fig 5.3). Then, between 30 days and 1 year of age, the gene expression of DNMT-1 in the perirenal adipose tissue strongly declined but obese offspring born to NR mothers still exhibited raised DNMT-1 mRNA abundance. Whereas obesity *per se* had no effect on DNMT-1 gene expression ($p<0.05$; Fig. 5.3).

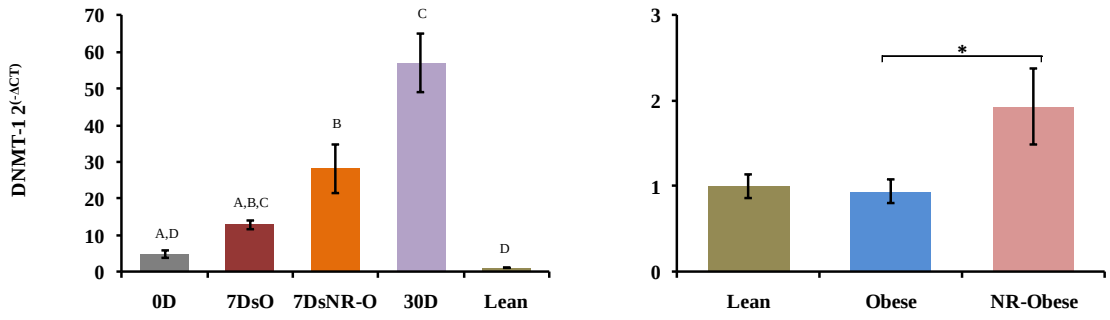


Figure 5.3 Increase in mRNA expression of the gene DNMT-1 in the perirenal adipose tissue depot at different time points from birth to one year old in sheep. The data are expressed as mean ± SEM. (A) p<0.05 offspring born to control diet mothers at birth (0D) versus 7 day old siblings from the obese group (7DsO). (B) p<0.05 statistical analysis of 7 day old offspring born to control fed mothers (7DsO) versus 7 day old NR sheep (7DsNR-O). (C) p<0.05 Statistical analysis of 30 day of offspring born from control mothers versus 7DsO. (D) Newborn offspring born to control fed mothers (0D) versus one year old lean sheep. * p<0.05 Statistical significance between the offspring born to mothers exposed to nutrient restriction and those born to mothers fed to appetite at 1 year of age.

5.3.3.2 Regulation of genes associated with adipose tissue remodelling and collagen deposition

Obesity resulted in a down regulation of VEGF-A mRNA expression at one year of age (Table 5.1), irrespective of prenatal diet. Gene expression of iNOS was significantly up-regulated by obesity but this effect was amplified in offspring born to NR mothers ($p<0.05$; Fig. 5.4). Histological staining by the Masson trichrome technique showed clear evidence of the deposition of collagen peptides on the areas of crown-like structures, together with infiltration of immune cells or division of adipose stroma indigenous cells (Fig. 5.5).

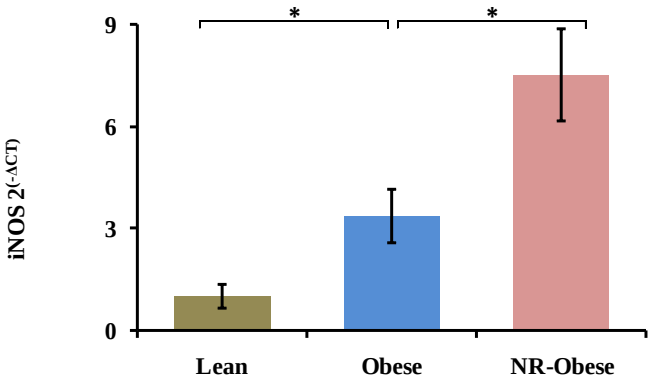


Figure 5.4 Data expressed as mean ± SEM. It shows the influence of pre-natal nutrient restriction and postnatal obesity on the gene expression of iNOS in the perirenal adipose tissue. Statistics are * p< 0.05 Obese versus Lean and NR-Obese group.

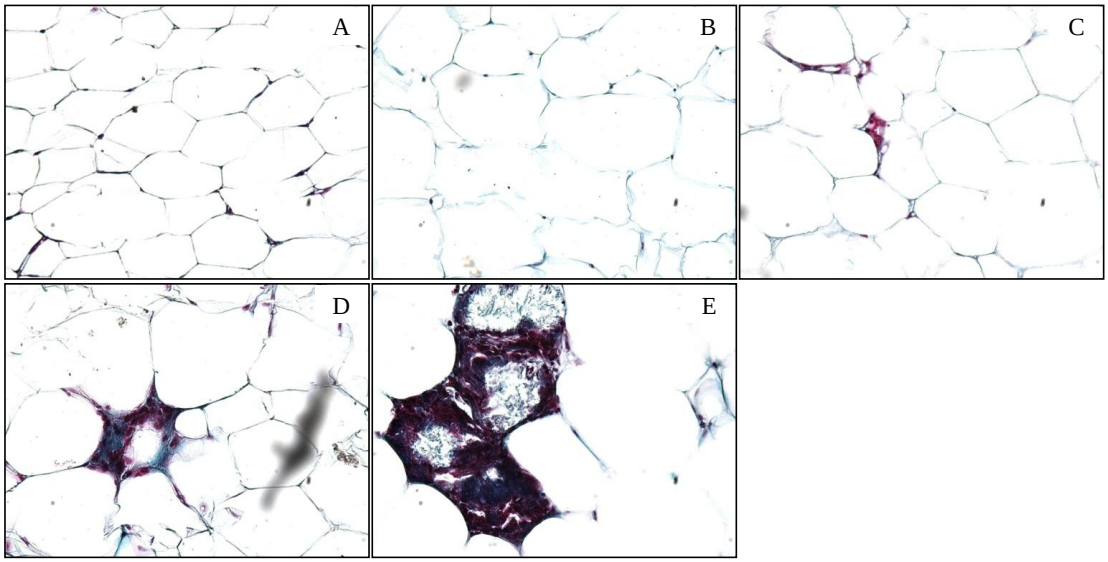


Figure 5.5 Representative histological sections obtained from perirenal adipose tissue (PAT) stained by Masson trichrome technique. A) Perirenal adipose tissue of a lean subject. B) PAT of obese offspring born to mothers fed standard diet during gestation without crown-like structure. C) PAT from obese offspring exposed to maternal nutrient restriction showing an early adipocyte dysfunction. D) PAT from an obese offspring born to appetite fed mother at the site of the death adipocyte apparent showing collagen deposition (green) surrounded by infiltrating or dividing stroma cells (red). E) Advanced crown-like structure observed on obese nutrient restricted sheep. Note the extensive collagen deposition (green-blue) and large number of infiltrating or dividing cells (red), which is evidence of adipose tissue remodelling (Magnification X 20).

5.3.3.3 Regulation at gene expression of receptors belonging to the IGF-GH axis

Obesity, in addition to maternal nutrient restriction, resulted in a trend towards low mRNA expression of growth hormone receptor (p=0.079; Fig. 5.6)). However, at one year of age, after 9 months exposure to an obesogenic environment, the gene expression of IGF-1 receptor was similar in obese and lean animals (Table 5.1).

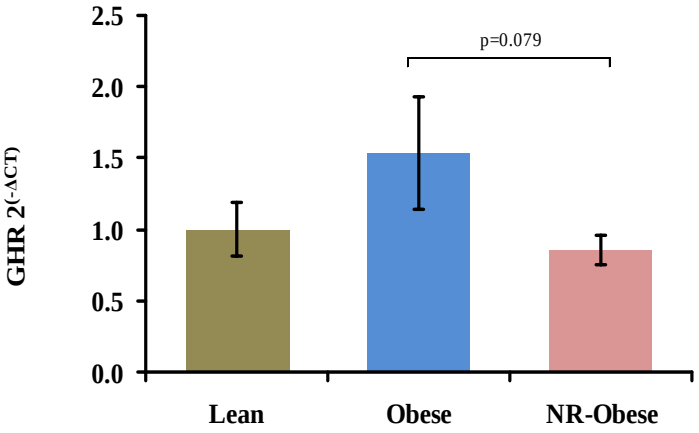


Figure 5.6 Data expressed as mean ± SEM. It shows the influence of pre-natal nutrient restriction and postnatal obesity on the gene expression of GHR in the perirenal adipose tissue. Statistics are p= 0.079 Obese versus NR-Obese group.

Table 5.1 IGF-1 plasma concentration at six months of age, mRNA abundance of IGF-1R and VEGF-A at one year of age and the total rate of weight increase in lean and obese juvenile offspring, born to mothers either exposed to nutrient restriction during early to mid gestation or fed to appetite throughout pregnancy

Parameter	Lean	Obese	NR-Obese group	P
IGF-1(ng/ml)	6.31±0.48	16.92±3.55*	13.09±1.06*	P<0.05
IGF-1R ($2^{-\Delta CT}$)	1.00±0.19	1.28±0.17	1.43±0.18	NS
VEGF-A ($2^{-\Delta CT}$)	1.00± 0.26	0.45±0.11*	0.33±0.09*	P<0.05
Increase weight increase (AUC- kg/month)	416± 34.52	617±13.36*	610±13.36*	P<0.05
Weight at six months (kg)	41. 00±2.08	61.74±1.68*	60.08±1.80*	P<0.05

*** Statistical significance in lean group vs. both obese groups, regardless of maternal diet**

5.4 Discussion

The main finding of this part of my thesis is that the gene expression of DNMT-1, an enzyme involved in cellular differentiation increases in parallel to adiposity within the perirenal adipose tissue during the first 30 days of postnatal life. Importantly, the mRNA abundance of this gene strongly declines in juvenile offspring and it is not influenced by obesity *per se*. Therefore, it follows a similar pattern of adipogenesis, as described in humans (Spalding *et al.*, 2008). Furthermore, I also observed that, in offspring exposed to maternal nutrient restriction, the gene expression of DNMT-1 was further raised at one week of age compared to those offspring born from mothers fed *ad libitum* throughout gestation, without any change in fat mass. Interestingly, this adaptation persisted up to in one year of age, after nine months' exposure to an obesogenic environment. In addition, in those offspring born to NR mothers, it was accompanied by several hormonal and histological changes in the adipose tissue in response to exposure to an obesogenic environment (Sharkey *et al*., 2009). These include an elevation in plasma leptin and cortisol at six months of age whilst at one year of age, the appearance of cytotoxic stressors within adipose tissue, such as CLS, which might be linked to increases in lipid ectopic deposition observed in the kidneys, heart and liver of these animals (Chan *et al*., 2009; unpublished observation). Overall, these results suggest the strong possibility that maternal nutrient restriction produced a different adipose tissue phenotype, which accelerated the process of adipocyte maturation when these NR offspring are exposed to an obesogenic environment (Sharkey *et al*., 2009; Chapter 4).

5.4.1 DNMT-1 gene expression and its possible role in adipocyte development

Although the function of DNMT-1 in the adipose tissue remains to be elucidated, one putative role is in adipogenesis (Takebayashi *et al.*, 2007; van Beek *et al.*, 2008). It is essential for cell replication, as it is primarily responsible for copying methylation patterns after cell mitosis (Leonhardt *et al.*, 1992; Takebayashi *et al.*, 2007). Previous analysis has revealed an increase in gene expression of DNMT-1 during the process of adipocyte differentiation (van Beek *et al.*, 2008). It was demonstrated recently that the role of DNMT-1 in the process of adipocyte differentiation is mediated through the formation of a complex with pRB (Robertson *et al.*, 2000; Hansen *et al.*, 2004; Dali-Youcef *et al.*, 2007). Thereby, DNMT-1 may be involved in one of the first molecular events occurring in adipocyte differentiation, the activation of C/EBP-β and δ, and initiation of the transcriptional cascade that results in the deposition of lipids, and which drives these cells to maturity (Cao *et al.*, 1991; Chen *et al.*, 1996; Richon *et al.*, 1997; Wu *et al.*, 1999). Gene expression of DNMT-1 appears to increase gradually during the first month of postnatal life in ovine adipose tissue. It may therefore follow the characteristics of adipocyte differentiation, including lipid accumulation and, in turn, the subsequent rise in the weight of the perirenal fat depot. Importantly, gene expression of DNMT-1 then declines up to one year of age and the expression of this gene is not affected by obesity *per se*. This may be possible because pBR has opposing roles during the adipocyte life cycle, which are independent of DNMT-1; pRB can interact with other proteins including PPAR-γ, repressing adipose differentiation and inducing cell arrest (Cram *et al.*, 1998; Fajas *et al.*, 2002). Thus, due to this role in cell differentiation, the

mRNA abundance of DNMT-1 may reflects certain similarities with the process of adipogenesis (Spalding *et al.*, 2008).

5.4.1.1 Evidence for changes in adipocyte maturation rate induced by maternal nutrient restriction

As seen as in Chapter 3, at birth and at one week of age, there were no major morphometric differences observed between offspring born to control fed or nutrient restricted mothers. During this first week of postnatal life, the majority of changes observed in adipose tissue with maternal nutrient restriction were primarily at the level of gene expression. This extensive analysis included several other tissues, such as hypothalamus, kidney, liver skeletal muscle and heart (Sebert *et al.*, 2009).

The only hypothalamic gene of those tested previously by Serbert *et al*. that was shown to be affected by maternal nutrition at 7 days of age was the neuropeptide Y (NPY), leading to a reduction in mRNA abundance (Sebert *et al.*, 2009). In the hypothalamus, NPY controls the appetite regulating network by stimulating food intake and its activity is enhanced by glucocorticoids and inhibited by leptin, thereby suppressing appetite (Schwartz *et al.*, 1996; Pinto *et al.*, 2004; Kuo *et al.*, 2007). Importantly, immunohistological analysis demonstrated that leptin secretion increases in parallel to maturation of adipocytes (Bornstein *et al.*, 2000). Unfortunately, Sebert *et al*. was unable measure the concentration of leptin in plasma at seven days of age (Sebert *et al.*, 2009). However, the reduction in mRNA abundance of NPY in the hypothalamus in neonatal animals born to maternal nutrient restricted mothers could be mediated be an increase in plasma leptin (Sebert *et al.*, 2009). This is in agreement with the current hypothesis that increased DNMT-1 mRNA abundance is associated with enhanced

adipocyte development. Further evidence of an alteration in the adipose tissue function induced by maternal nutrient restriction during early neonatal life has been demonstrated by the reduction in mRNA abundance for both IGF-2 and its receptor (Chapter 3).

Additionally, differing plasma concentrations of leptin and cortisol also point towards metabolic alterations in the adipose tissue induced by maternal NR. At six months of age, plasma leptin was increased in all obese animals but this adaptation to increased fat mass was enhanced in those offspring exposed to *in utero* maternal nutrient restriction. Another significant outcome in the obese offspring exposed to maternal nutrient restriction after three month exposure to an obesogenic environment was an increase in plasma cortisol concentration. This effect may be mediated as inhibitory feedback, resulting from a potential rise in sympathetic activity induced by leptin (Malcher-Lopes *et al.*, 2006). Furthermore, 6 months later, no differences in body mass were found in either obese group and they also showed similar increases in weight gain (Sharkey *et al*., 2009). Finally, these changes in adipocyte development may have profound implications in later life, as extreme obesity and the subsequent hyperleptinemia are associated with the appearance of cytotoxic stress markers on adipose tissue, such as CLS, influencing lipid deposition and dysfunction in other tissues, including the kidney (Cinti *et al.*, 2005; Kolak *et al.*, 2007; Deji *et al.*, 2009).

5.4.2 The effects of maternal nutrient restriction in adipose tissue remodelling in juvenile obese sheep

After nine months' exposure to an obesogenic environment, all offspring, irrespective of the prenatal nutritional environment exhibited a similar increase in plasma concentrations of leptin, FFA and triglycerides compared with the lean animals although plasma insulin concentrations were further raised in offspring born to nutrient restricted mothers (Williams *et al.*, 2007; Sebert *et al.*, 2009). In contrast, differences between the two obese groups within their adipose tissue were apparent both morphologically and at the level of gene expression. Recently, Sharkey *et al.*, using the same animal model, showed that maternal nutrient restriction in addition to exposure to an obesogenic environment induced an activation of the unfolded protein response (UPR). This adaptation is characterised by the induction of endoplasmatic reticulum stress, which might promote infiltration of macrophages and ultimately induced adipose tissue remodelling (Roll *et al*., 1991; Cinti *et al*., 2005; Sharkey *et al*., 2009). Thereby, the increased presence of CLS may explain the rise in gene expression of iNOS and other factors associated with the inflammatory response also observed by Sharkey *et al*. such as CCR-2 (CC chemokine receptor-2), particularly within the adipose tissue of the NR-O group (Sharkey *et al*., 2009).

In addition to their role in immunity, macrophages are able to promote vascular remodelling and hypoxia plays a pivotal role in this adaptive response (Hosogai *et al.*, 2007; Duffield *et al.*, 2008). As observed in the present animal model, the adipocytes of both obese groups became equally hypertrophic, which might limit their capacity for oxygen diffusion, leading to cell death (Cinti *et al.*, 2005; Hosogai *et al.*, 2007; Sharkey

et al., 2009). Importantly, hypoxia has been reported to induce endoplasmatic reticulum stress as an initial pathway for cellular adaptation, triggering the process of angiogenesis (Fukumura *et al.*, 2003; Hosogai *et al.*, 2007). Adipocyte necrosis, as demonstrated by Strissel *et al.*, is associated with matrix deposition and the cell differentiation. This may explain the increase in abundance of DNMT-1 mRNA as observed in the NR-O group compared with the O group (Strissel *et al.*, 2007). A recent *in vivo* study, based on histological examination of adipose tissue, demonstrated that angiogenesis in obesity requires a coordinated action between macrophages, differentiating adipocytes, stroma and blood cells (Nishimura *et al.*, 2007). This close interplay forms angiogenic microenvironments within the adipose tissue, which are found to occur in parallel to CLS at the early stages of obesity (Nishimura *et al.*, 2007). Importantly, inside these clusters is an increase of VEGF-A protein abundance and its inhibition with anti-VEGF antibodies not only reduces angiogenesis, but also inhibits adipogenesis in obesity (Nishimura *et al.*, 2007). Tumour cells are also exposed to hypoxia and reticulum endoplasmatic stress with their survival also depending on angiogenesis, of which a critical mediator in tumour vascular development is also VEGF-A (Abcouwer *et al.*, 2002). However, it was observed in wild-type endothelial cells, that VEGF-A is inhibited at gene expression level by RNAse enzymes, when UPR is activated by hypoxia or glucose deprivation. In addition, tumour cells undergo a series of DNA mutations and epigenetic changes before starting the process of vascularisation (Drogat *et al*., 2007); however to date, such a mechanism has not been demonstrated to occur in adipocytes (Drogat *et al.*, 2007; Nishimura *et al.*, 2007). Under those circumstances, the adipose tissue cells may depend on macrophage activation for the induction of

angiogenesis, however, due to the low level of mRNA abundance of VEGF-A this process may have been inhibited, which explains the possible decline in adipogenesis observed in obese adults (van Harmelen *et al.*, 2003; Strissel *et al.*, 2007).

One particularly interesting outcome from my study was that the obese offspring born to nutrient restricted mothers showed a tendency to exhibit low GHR mRNA abundance, when compared to obese offspring born from control-fed mothers at one year of age. This may be indicative of differences in adipocyte metabolism (Asada *et al.*, 2000). Further illustration was the fact that the adipocyte size was similar in both obese groups, regardless of maternal diet during gestation, whilst there were substantial increases in cytotoxic stressors on the adipose tissue of the NR-O compared with that of the O group (Sharkey *et al*., 2009). Importantly, several authors have demonstrated that the number of CLS is positively correlated with increased adipocyte size in obese humans and rodents (Clement *et al.*, 2004; Cinti *et al.*, 2005). Thus, a reduction in gene expression of GHR might predispose the adipose tissue to abnormal lipid metabolism, leading to cell hypoxia and cell necrosis (Asada *et al.*, 2000; Hosogai *et al.*, 2007; Ukropec *et al.*, 2008).

In addition, adipose tissue dysfunction is associated with an increase in triglyceride content in non-adipose organs, such as the liver of extremely obese humans (Kolak *et al.*, 2007). Free lipid droplets released from necrotic adipocytes are a source of toxic fatty acids that could not only damage other white adipocytes but also peripheral organs, including the kidneys (Kolak *et al.*, 2007). Zager *et al*. recently reported that renal cortical accumulation of non-saturated free fatty acids, such as triglycerides, is a normal cell mechanism that may confer a certain degree of cellular resistance in

response to a toxic challenge (Zager *et al.*, 2007). In the present model of obesity, a substantial increase in triglyceride incorporation was observed in several tissues, including the kidneys, liver and heart of obese offspring born to NR mothers (Chan *et al.*, 2009). Compromised renal function was observed in all juvenile obese sheep, however in obese maternal nutrient restricted offspring, enhanced ectopic lipid deposition and tissue remodelling in the kidneys coincided with a substantial decrease in renal apoptosis compared with the obese group (Williams *et al.*, 2007; Chapter 4). Thus, the increase in crown-like structures and inflammation of the adipose tissue may induce a series of local events, such as adipocyte necrosis, proliferation of inflammatory cells and remodelling of the adipose tissue. Systemic events may also be initiated, including increased lipid accumulation in non-adipose tissues. These might be indicative of different stages in the adaptation to the toxic effects induced by obesity (Kolak *et al.*, 2007; Strissel *et al.*, 2007; Chan *et al.*, 2009).

5.5 Summary

In this study it was observed that maternal nutrient restriction targeted between early to mid gestation altered the expression of central genes related to white and brown adipocyte differentiation during early neonatal life, such as DNMT-1 and IGF-2 (see also Chapter 3). Interestingly, the over-expression of DNMT-1 persisted in juvenile maternal nutrient restricted offspring, even after nine months' exposure to an obesogenic environment. This was not the case in obese offspring born from mothers fed *ad libitum*. The importance of DNMT-1 is that it has a major role in terminal differentiation, replicating the DNA methylation motif after cell division (Rai *et al.*, 2006). In addition, changes in the epigenome have been recently associated with susceptibility to metabolic

complications linked with obesity in adulthood (Dolinoy *et al.*, 2006). In my study, during the first month of neonatal life there was an increase in DNMT-1 mRNA abundance in offspring born to control fed mothers, followed by a substantial elevation in the weight of perirenal fat. At one year of age, the expression of this gene had strongly declined and was not affected by obesity *per se*. These results suggest a potential role for DNMT-1 in adipocyte differentiation. At 6 months of age, which included 3 months' exposure to an obesogenic environment, all the obese animals were equally overweight compared with the lean group. As seen in humans, an increase in plasma leptin was observed but this adaptation was amplified in offspring born to nutrient restricted mothers, indicating a difference in adipocyte adaptation to nutrition intake (Bornstein *et al.*, 2000; Farooqi *et al.*, 2002). Indeed, at one year of age, when the adipose tissue of both obese groups was compared, different developmental adipocyte characteristics were found in those offspring born from nutrient restricted mothers. This may be related to a limited storage capacity for excess lipid. The perirenal adipose tissue of the NR-O group exhibited the formation of crown-like structures and signs of an inflammatory response, which might be associated with a reduction in oxygen cellular diffusion (Hosogai *et al.*, 2007; Sharkey *et al.*, 2009). These events are of significant importance for evaluating the phenotype of mature adipocytes, whereas the cells of both obese groups, regardless of maternal nutrition during gestation, were similar in size and number (Sharkey *et al.*, 2009). Therefore, these results indicate that the change in gene expression of DNMT-1 induced by the *in utero* nutritional environment may have a significant effect in the final adult adipocyte phenotype (Fig. 5.7).

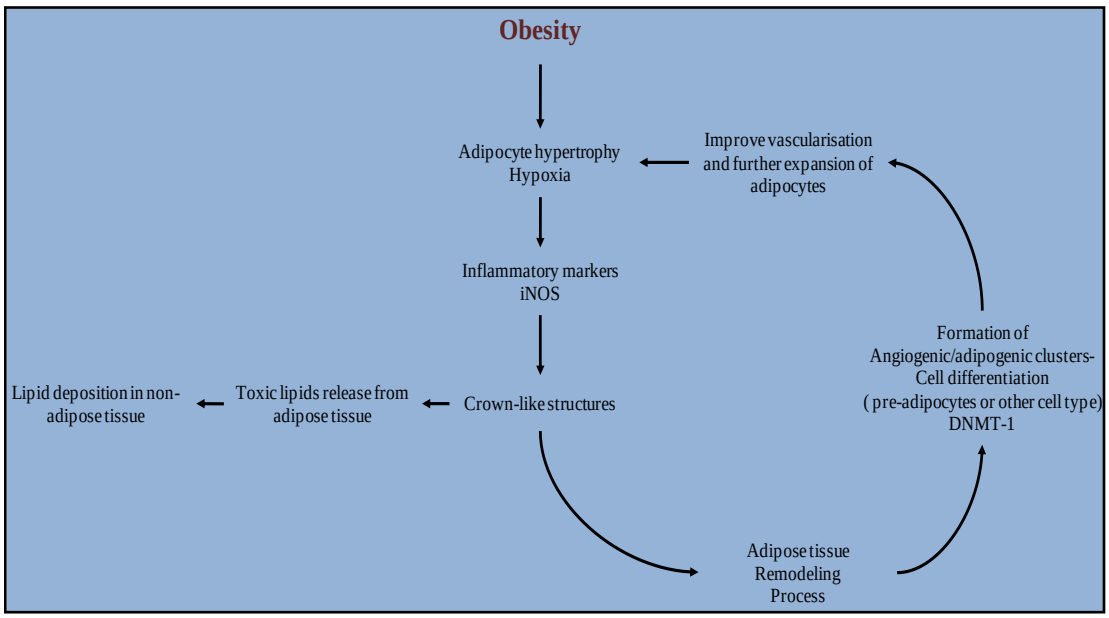


Figure 5.7 Schematic summary of findings of this chapter.

Chapter 6 - Conclusion

Chapter 6 – Conclusion

6.1 Previous studies of the effects of maternal nutrient restriction during gestation and their influence on this investigation

Many publications, from the pioneering 1934 publication by Kermack *et al.* until today, including later epidemiological research of the studies of non-transmittable diseases, support the concept that maternal welfare and early life nutrition influence adulthood health outcome (Kermack *et al.*, 2001; Stettler *et al.*, 2005; Olson *et al.*, 2008). In addition, different animal models have validated the role of the gestational environment as an important factor in the development of metabolic associated diseases in later life (Gardner *et al.*, 2004; Bispham *et al.*, 2005). Over the past two decades, research in this area has determined some of the maternal mechanisms by which an alteration in nutrition during gestation leads to long term life consequences for offspring (Jaquiery *et al.*, 2006; Hyatt *et al.*, 2007; Rumball *et al.*, 2008). Important conclusions from these detailed investigations include an understanding of the molecular and physiological mechanisms, which alter the metabolism of these offspring, leading to a decline in renal-cardiovascular function (Gardner *et al.*, 2004; Gopalakrishnan *et al.*, 2004). The present thesis incorporates another factor in this field: the induction of obesity and patterns of adaptation in renal tissues, including the interaction of the kidneys with the surrounding adipose tissue in a population exposed to maternal nutrient restriction during the period of maximal organogenesis. This is an emerging area of interest owing to the increasing prevalence of obesity worldwide in recent years and its

association with the development of renal disease (Iseki *et al.*, 2004). Thus, the modulation induced by the changes in maternal-fetal nutrient supply during early to mid gestation (30 to 80 days, term ≈145 days; sheep) may alter the metabolic phenotype of those offspring, influencing a different pattern of adaptation to obesity in comparison to obese sheep born to mothers fed to appetite (Williams *et al.*, 2007; Sharkey *et al*., 2009).

6.2 Effects of maternal nutrient restriction in early to mid gestation on renal and perirenal adipose tissue

Previously, Williams *et al.*, using the same obesogenic animal model as my study, observed that juvenile obesity in sheep, regardless of maternal diet during gestation, follows a similar pattern to that seen in humans: chronic hyperleptinemia, hypertension and a reduction in vascular-renal functions (Chagnac *et al.*, 2000; Williams *et al.*, 2007). However, the Williams *et al*. investigation found that the common molecular pathologic markers associated with renal dysfunction, such as responses to pro-inflammatory cytokines, angiotensin II and the induction of apoptosis, were apparently reduced in obese offspring born to NR mothers (Henegar *et al.*, 2001; Rea *et al.*, 2006; Williams *et al.*, 2007). Subsequent examinations under similar experimental conditions have shown different patterns of adaptation in the adipose tissue surrounding the kidneys of both obese groups (Sharkey *et al*., 2009). Therefore, exposure to maternal nutrient restriction in one year old obese sheep had influenced an increase in pro-inflammatory markers within the adipose tissue in relation to juvenile obese offspring born to control fed mothers (Sharkey *et al*., 2009).

Therefore, my experimental studies were undertaken to elucidate possible early developmental differences, induced by maternal nutrient restriction in offspring kidneys and surrounding adipose tissue, by seven days postnatal age.

At the end of the first week of postnatal life, measured physiological parameters in these NR offspring had not significantly altered, compared with those offspring born to mothers who were fed to appetite. Possibly for that reason, only small changes were induced by maternal nutrient restriction in the renal tissues. These included a decrease in the mRNA abundance of the growth hormone receptor and a significant reduction in oxidative stress (Chapter 3). However, this alteration in prenatal nutrition induced changes in genes associated primarily with adipocyte differentiation in the perirenal adipose tissue. At seven days of age, a decrease in mRNA expression of IGF-2 and its receptor was observed in perirenal adipose tissue of offspring exposed to maternal nutrient restriction. The activity of these genes is linked to the development of brown adipocytes throughout the interaction with prolactin receptors (Viengchareun *et al.*, 2008). An additional gene affected by maternal nutrient restriction in this fat depot was DNA methyltransferase-1 (DNMT-1), whose mRNA abundance increased in the perirenal adipose tissue of those offspring exposed to prenatal undernutrition. DNMT-1 is involved in cell differentiation and lipid accumulation in white fat cells (Hansen *et al.*, 2004; Dali-Youcef *et al.*, 2007; van Beek *et al.*, 2008a).

The changes in activity of DNMT-1 and IGF-2 may have both short and long term metabolic consequences. For instance, alterations in the expression of these genes could affect the production of leptin by adipose tissue and its action in appetite regulation, may explain the different hypothalamic NPY mRNA abundance observed during at this

age between both prenatal nutritional groups. (Schwartz *et al.*, 1996; Sebert *et al.*, 2009). In the long term, such changes in gene expression within the adipose tissue may underlie differences in metabolic adipocyte function and the subsequent ectopic lipid deposition in non-adipose tissues, seen at one year of age in both obese groups (Sharkey *et al*., 2009; Chan *et al.*, 2009).

6.3 Effects of obesity in renal tissue of young sheep exposed to maternal nutrient restriction between early to mid gestation

The present study describes some of the different renal adaptations induced by exposure to an obesogenic environment in both prenatal nutritional groups of offspring. *In utero* nutrient restriction, followed by nine months exposure to an obesogenic environment in young sheep, results in a different regulation of the proliferative - apoptotic pathways in renal tissues, - a common characteristic of processes associated with tissue dysfunction, scarring and healing (Abrass *et al.*, 1995; Verrecchia *et al.*, 2002; Zager *et al.*, 2007). The kidneys of both obese groups showed major similarities that are associated with a reduction in renal function: an increase in collagen deposition and oxidative stress compared with the renal tissues of the lean group (Han *et al.*, 2001; Chen *et al.*, 2004). However, both obese groups differed in the predominant adaptive process observed in the kidneys. In my study, as in the findings of Williams *et al*., an increase in cell renal apoptosis was also observed, possibly catalysed by the deposition of iron in offspring born to appetite fed mothers (Ishizaka *et al.*, 2002; Williams *et al.*, 2007). On the other hand, in those maternal nutrient restricted obese offspring, cell proliferation or cell hypertrophy appears to be the predominant process, which may be a consequence of past renal injuries or dysfunction induced by the fast weight gain (Praga

et al., 2001). Whereas detailed analysis of the renal cortex of NR obese sheep indicated that there was an increase in triglyceride ectopic deposition, this may be a post-injury response to toxic exposure and a mechanism possibly linked to cell survival (Listenberger *et al.*, 2003; Zager *et al.*, 2007). Whilst this cellular mechanism serves as an adaptation that could reduce temporary cell apoptosis, it may also lead to other forms of cell dysfunction commonly seen in renal tissue, including mesangial hyperplasia and podocyte hypertrophy (Berfield *et al.*, 2002; Wiggins *et al.*, 2005; Berfield *et al.*, 2006). Given these considerations, I speculate that obese subjects exposed to maternal nutrient restriction may pass through a similar pro-apoptotic stage, as seen as in the kidneys of those obese offspring born to control fed mothers. This interpretation of the present findings is also compatible with the grade of dysfunction observed in other tissues in this animal model, which may be a consequence of the level of dysfunction observed in the surrounding adipose tissue (Sharkey *et al*., 2009; Chan *et al.*, 2009).

6.4 The influence of the adipose tissue development on renal adaptation in maternal nutrient restricted offspring after exposure to juvenile obesity

As previously seen by Williams *et al*., the kidneys of both obese groups showed important morphological and functional similarities, associated mainly with the adverse conditions induced by exposure to an obesogenic juvenile environment. However, indices of renal pathology, such as expression of factors associated with inflammation, elements of the RAS and cells apoptosis were reduced in obese NR offspring. Interestingly, this renal adaptation was accompanied by an accumulation in triglycerides, Listenberger *et al*. have suggested that a physiological response by non-adipose cells represents a temporary protection against the toxic effects of certain lipid

derivates (Listenberger *et al.*, 2003). These cytotoxic metabolites may be released by necrotic adipocytes, forming a pathological hallmark of severe obesity (Cinti *et al.*, 2005; Kolak *et al.*, 2007). Sharkey *et al*. observed that exposure to an obesogenic environment in juvenile maternal nutrient restricted offspring increases the markers of cellular stress within the adipose tissue compared with those offspring born to mothers fed to appetite (Sharkey *et al*., 2009). Therefore, the hypothesis of my study was to elucidate the means by which maternal nutrient restriction, under the experimental conditions applied in this animal model, affects the mechanisms associated with adipocyte maturation. These alterations may induce several changes, including alteration in the secretion of hormones and also in the expression of genes associated with the cell cycle and the function of adipocytes over a period of time.

The main finding of my study is that, in the adipose tissue of those offspring exposed to maternal nutrient restriction, there may be an alteration in the process of cell differentiation, the most important feature of which is the increase in mRNA abundance of the methyltransferase DNMT-1. The ontogeny of this gene reveals that, during the first month of neonatal life, there is a time dependent rise in the mRNA abundance of DNMT-1 in the adipose tissue surrounding the kidneys. At juvenile age, the mRNA abundance of this gene strongly declines and is not influenced by obesity *per se*, thus following a similar pattern seen in adipogenesis in humans (Spalding *et al.*, 2008). In addition, exposure to an obesogenic environment in those offspring born to nutrient restricted mothers induced more acute hormonal, histological and inflammatory responses compared with the obese group. These include an elevation in leptin and cortisol at six months of age and the appearance of markers of cellular stress within the

adipose tissue, such as an increase in the mRNA abundance of iNOS (an inflammatory associated gene) and an increase in the frequency of necrotic adipocytes at one year of age (Soukas *et al.*, 2000; Cinti *et al.*, 2005; Sharkey *et al*. 2009). Furthermore, in perirenal adipose tissue, the gene expression of DNMT-1 is enhanced in offspring exposed to an obesogenic environment following maternal nutrient restriction, suggesting evidence of some adipose tissue remodelling or cell differentiation (Rai *et al.*, 2006; Strissel *et al.*, 2007; van Beek *et al.*, 2008).

Several authors have described the link between the inflammation and infiltration of macrophages in adipose tissue of older obese animals but little is known about the role that both processes play during obesity (Cinti *et al.*, 2005; Strissel *et al.*, 2007). In adipose tissue, macrophages may interact with stroma cells, promoting angiogenesis and tissue remodelling under a specific angiogenic microenvironment in the area surrounding the necrotic adipocyte (Nishimura *et al.*, 2007). The interaction of macrophages and stroma cells under this angiogenic environment allows the formation of blood vessels, improving cellular oxygen diffusion for the induction of adipogenesis or further enlargement of fully differentiated adipocytes (Hosogai *et al.*, 2007; Nishimura *et al.*, 2007; Strissel *et al.*, 2007). However, a reduction in expression of the angiogenic gene, VEGF-A mRNA, may indicate that this process is inhibited in my obesogenic animal model. Therefore, the presence of matrix proteins, such as collagen peptides in addition to increase in expression of DNMT-1, may suggest that other cell types associated with tissue remodelling, other than adipocytes, are undergoing terminal differentiation. It is important to note that the rate of increase in weight, adipocyte numbers and sizes and the majority of metabolites analysed, including cortisol and

leptin, during this study were similar in both obese groups at one year of age (Sharkey *et al*., 2009; Sebert *et al.*, 2009).

Taken together, these observations suggest that maternal nutrient restriction influences of the development of adipose tissue by inducing acceleration in the rate of adipocyte maturation, possibly due a reduction in vascularisation. Finally, the chronic cytotoxic process, induced by the release of lipids and insoluble proteins from necrotic adipocytes, generates a unique adaptive-degenerative path for the kidneys and other surrounding organs in response to an obesogenic environment (Yang *et al.*, 2006; Kolak *et al.*, 2007; Chan *et al.*, 2009).

6.5 Final conclusions

Despite the many confounding factors induced by exposure to obesity, the current results indicate that a reduction in maternal nutrition supply during early to mid gestation may reset the overall development of offspring perirenal adipose tissue. In addition, this prenatal nutritional intervention may affect other physiological responses linked to the development of adiposity, such as early life appetite regulation and hormone *milieu*, including the secretion of leptin and cortisol. Moreover, substantial changes were observed in gene expression within the adipose tissue at seven days and one year of age in those offspring exposed to prenatal undernutrition. Some of these genes, such as IGF-2 and DNMT-1, may bring to light evidence of a developmental alteration involving an epigenetic mechanism. Although there are clear differences between species, the ovine animal model is able to mimic many molecular aspects commonly observed in obese human dysfunction. Therefore, from the results of my study, it would appear that adipose tissue dysfunction influences the inflammatory stage

in neighbouring renal tissue. Finally, these responses in perirenal adipose tissue possibly lead from an initial cycle of cell apoptosis to temporary cell resistance to lipotoxity, characterised by an increase in lipid deposition in renal tissue, which might after all, be part of the same long term degenerative process.

6.6 Future perspectives

6.6.1 Future work in the adipose tissue

As clear differences in gene expression of DNMT-1 have been shown here, future investigations of the epigenetic modifications in the perirenal adipose tissue need to include offspring exposed to postnatal obesity and prior maternal nutrient restriction. In addition, this would enable us to estimate the importance of the inheritance of genomic DNA methylation and the influence of maternal nutrient restriction, as well as the later phenotypic changes induced by exposure to an obesogenic environment. Moreover, for completion of an epigenetic analysis, further methylation tests on the promoters of specific adipogenic genes would add to our understanding of the cell mechanisms involved in this model of prenatal nutrition and juvenile obesity. Finally, a combination of basic histology and *in situ* hybridisation for genes such as DNMT-1 and VEGF-A on perirenal adipose tissue, might help identify which types of cells are undergoing differentiation. This analysis would have the potential to answer important questions about the function of this methyltransferase on adipocyte maturation, in what is thought to be a highly heterogenic cell population within adipose tissue.

6.6.2 Future work in renal tissue

In order to increase our understanding regarding the fundamental cellular mechanisms through which the accumulation of triglycerides exerts effects on renal tissues, further investigations are required. These should include a series of analyses to determine whether the lipids accumulated originate from sources exogenous or endogenous to the kidney. Therefore a specific comparison of the lipids' content from the perirenal adipose tissue and kidneys would help to reveal the cellular process behind this interesting effect.

As discussed in Chapter 2, as similar oxidative stress values were obtained in both obese groups, TBARS may not be the most suitable assay to perform on renal tissues. A more accurate marker of lipid peroxidation and oxidative stress, such as specific markers for MDA and protein oxidation, could provide more information. This might enable differentiation of the origin of oxidation and its relationship to the cellular mechanism involved in lipid cellular accumulation. Finally, lipid staining of renal histological sections and their comparison with the immuno-staining of PCNA would be useful to identify cell type and renal structures susceptible to lipid accumulation.

6.7 Limitations of this study

6.7.1 The effects of maternal nutrient restriction in lean animals

A limitation of this study is the lack of a lean one-year-old maternal nutrient restricted group. However, previous studies by our research group characterised extensively the phenotype differences between lean animals with and without maternal nutrient restriction (Gopalakrishnan *et al.*, 2004). Thus, the current hypothesis of this

project was limited to an examination of the effects of obesity on maternal nutrient restricted offspring and their patterns of renal adaptation. Therefore, a future investigation using tissues from lean one-year-old animals, who had experienced maternal nutrient restriction during the same period of gestation, would strengthen the outcome of the present study and, importantly, would allow detailed comparisons to be conducted using this control group.

6.7.2 The effects of gender on maternal nutrient restriction and obesity

The role of gender has not been addressed in the present study. Some authors have noted that exposure to the prenatal *in utero* environment can induce specific gender effects, particularly in hypertension and insulin sensitivity (Ozaki *et al.*, 2001; Poore *et al.*, 2007). For instance, male rats exposed to maternal nutrient restriction are more susceptible to a reduction in nephron number, changes in RAS function and hypertension than female dams (Woods *et al.*, 2004). Nevertheless, obesity *per se* in humans is a factor independent of gender, but there are minor regional differences in adipose tissue metabolism in females (Chen *et al.*, 2003; Tchernof *et al.*, 2006). Unfortunately, in my study it was not possible to measure specific gender effects, as the study was not powered to address this question. The gender imbalance occurred by chance, as the initial nutritional intervention at early gestation (at 30 gestational days; term ≈ 145 days) required that the mothers were randomised and it was impossible to know the gender of the fetus at this stage of pregnancy. As described in Chapter 3, similar renal characteristics were observed, including nephron number, in both groups of offspring at seven days, regardless of maternal nutrition. Several parameters influenced by gender, such as blood pressure, renin-angiotensin responses and renal function, as

assessed by Williams *et al*., deteriorated in a similar manner in both obese groups after exposure to an obesogenic environment (Williams *et al*., 2007). Furthermore, the rate of increase in body weight over time and the amounts of oxidative stress at systemic or renal level were also similar between both obese groups. I am aware that some parameters analysed during this investigation were possibly affected by gender. Nonetheless, it seems likely that gender has not played a significant role in this particular obesogenic animal model of maternal nutrient restriction.

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Appendices

Appendices

A. Abstracts, original presentation and conferences attended

Fainberg HP, Gardner DS, Sinclair KD, Budge H, Symonds M.E (2009) - *In-utero nutritional programming of kidney development and its long term impact on renal oxidative stress following juvenile obesity*
Oral presentation- Main Meeting of the Physiological Society, Dublin, RoI, July 2009

Maternal nutrient restriction, coincident with early fetal kidney development, can increase glucocorticoid action in the newborn kidney. Fetal adaptations in the kidney may also determine the long term consequences following juvenile obesity and the onset of hypertension. One important mechanism implicated in both obesity and renal disease is enhanced oxidative stress, leading to glomerulosclerosis. Two key regulators of this process are the angiogenic hypoxia inducible factor-1α and the pro-apoptotic receptor Fas. The present study, therefore, examined how the early in-utero diet results in renal adaptations to oxidative stress induced by juvenile obesity.

Pregnant sheep (n=26) were randomly assigned to a normal (7 MJ/day) or nutrient restricted diet (NR, 3.5 MJ/day), from 30-80 days of gestation (term = 147 days) and fed to requirements at all other times. After weaning, the NR (NR-O, n=11) and obese (O, n=7) offspring were reared in an obesogenic environment to promote fat deposition. The lean group (L, n=8) remained out to pasture. At one year of age, all sheep underwent measurements of plasma cysteine concentration and were then humanely euthanased with kidneys sampled for renal malondialdehyde (MDA) concentrations and real-time gene expression of the Fas and HIF-1α genes.

Obesity raised the total plasma cysteine (L 12.8±1.1; O 18.3±1; NR-O 18±1.6 ($p<0.05$)) and intra-renal (*i.e.* MDA) oxidative stress in both obese groups. In addition, although gene expression of HIF-1α also increased with obesity, this response was amplified in offspring born to NR mothers (L 1±0.285; O 1.964±0.552; NR-O 5.353 ±1.025 $2^{-(\Delta CT)}$ ($p<0.05$)), whereas mRNA abundance for Fas was reduced (NR-O 1.89±1.56; O 4.68 ±1.72 $2^{-(\Delta CT)}$ ($p<0.05$)).

Our study suggests that abnormal patterns of renal adaptation are induced by juvenile obesity in NR offspring, with persistent changes in markers of oxidation. We have, therefore, shown, for the first time in this model of fetal programming, that increased systemic and renal oxidative stress alters the gene regulation of angiogenic and pro-apoptotic genes such as HIF-1α and Fas. These differential adaptations may delay the onset of glomerulosclerosis without necessarily improving vascular function.

Fainberg HP, Saeed A, Gardner DS, Budge H, Symonds ME (2008) - *Differences in renal adaptation to oxidative stress in juvenile offspring, following maternal nutrient restriction*
Poster presentation- Beijing Joint Conference of Physiological Sciences, Beijing China, October 2008

Introduction

The gestational diet of offspring modulates environmental adaptation, altering renal oxidative stress (OxS). We demonstrated that maternal nutrient restriction (NR) from early to mid-gestation decreases apoptosis in renal cells, common in obesity associated with glomerulosclerosis[1]. This study follows the effects of renal growth hormone receptor (GHR) on OxS in newborn sheep, following early obesity.

Methods

Pregnant twin-bearing sheep (n=18) were randomly assigned to normal (C, 7 MJ/day) or nutrient restricted diets (NR, 3.5 MJ/day), from days 30 to 80 gestation and fed to requirements to term (147 days). One twin from each mother was euthanased after seven days. All kidneys were collected and analysed for expression of the pro-apoptotic p53 and GHR genes and malondialdehyde (MDA) concentrations. Procedures conformed to U.K. legislation.

Results

MDA concentrations were higher in the C group (C 2.465 SE 0.180; NR 1.764 SE 0.102, [MDA µM]/ [protein µg/µl] ($p<0.05$)). NR reduced the expression of GHR (C 1.00 SE 0.12; NR 0.54 SE 0.12, $2^{-(\Delta CT)}$ ($p<0.05$)) whilst p53 expression was similar.

Conclusions

Low concentrations of MDA found in NR animals may result from GHR down regulation, which controls the expression of several antioxidant enzymes. Decreased OxS in the NR group was unconnected to reduced apoptotic markers, like p53. This may reduce glomerulosclerosis, protecting NR offspring from renal damage induced by early obesity, as previously demonstrated.

Fainberg HP, Saeed A, Gardner DS, Budge H, Symonds ME (2008) - *Evidences in the reduction of renal oxidative stress in offspring of nutrient restricted sheep, following juvenile obesity*
Poster Presentation - Nutrition Society Annual Summer Meeting, Nottingham UK, July 2008

Obesity is associated with an increase in reactive oxygen species (ROS), and the development of renal damage. We have previously demonstrated that exposure to a period of maternal nutrient restriction (NR), between early-to-mid gestation, prevents the occurrence of glomerulosclerosis that follows juvenile obesity. The aim of this study was to elucidate whether this adaptation is accompanied by a reduction in renal ROS production.

Pregnant sheep (n=26) were randomly assigned to a normal (7 MJ/day) or nutrient restricted diet (NR, 3.5 MJ/day), from days 30 to 80 gestation (term = 147 days) and fed to requirements at all other times. Nutrient restricted (NR-O, n=11) and obese (O, n=7) offspring groups were reared in an environment of restricted activity and increased energy dense food to promote fat deposition and, thus, obesity following weaning at 10 weeks postnatal age. The lean group (L, n=8) remained out to pasture. All sheep were humanely euthanased at one year of age and kidneys sampled for nitric oxide (NO) determination, superoxide dismutase (SOD) activity measurement and immunoblotting for SOD1 and SOD2. All animal procedures had local Animal Ethics Committee Approval and were performed in accordance with U.K. legislation.

SOD2 abundance showed an increase in obese offspring compared to lean animals (O 127.85 SE 6.46; L 104.59 SE 6.46 arbitrary units ($p<0.01$)): whereas the SOD1 abundance was similar. However, the SOD activity was higher in NR-O compared with O (O 0.03 SE 0.004; NR-O 0.059 SE 0.009 U/ml ($p<0.05$)). In addition, NO oxidation was lower in the NR-O, compared with O, offspring (O 1.562 SE 0.3; NR-O 0.866 SE 0.1 Nitrate µM/ [protein µg/µl] ($p<0.05$)).

Exposure to nutrient restriction over the period of early kidney development when followed by juvenile obesity is associated with an increase in renal SOD activity and reduction in NO concentrations.

These adaptations may reduce the production of ROS and contribute to avoidance of the early renal damage induced by obesity. The factors that may protect offspring born to nutrient restricted mothers from early-onset of glomerulosclerosis induced by obesity are currently being investigated.

Fainberg HP, Saeed A, Sébert SP, Rashid A, Gardner DS, Budge H, Symonds ME (2008) - *Evidence for a role for the voltage-dependent anion channel protein (VDAC) in the reduction of renal oxidative stress following juvenile obesity in offspring of nutrient restricted sheep*

Poster presentation - International Symposium Demonstrating early programming in human and animal models, Granada Spain, April 2008

Renal injuries are linked to obesity through a rise in oxidative stress (OxS) and mitochondrial dysfunction. The voltage-dependent anion channel 1 protein (VDAC) is a critical gatekeeper regulating mitochondrial function able to control the cell cycle. Exposure to maternal nutrient restriction (NR) from early to mid-gestation reduced the number of apoptotic renal cells link to obesity, a feature of the development of glomerulosclerosis. This study evaluates the status of VDAC expression in regard to OxS in this NR ovine model.

Pregnant sheep (n=26) were randomly assigned to a normal (7 MJ/day) or nutrient restricted diet (NR, 3.5 MJ/day), from days 30 to 80 gestation (term = 147 days) and fed to requirements at other times. After weaning, the nutrient restricted (NR-O, n=11) and obese (O, n=7) offspring were reared in an obesogenic environment to promote fat deposition, and thus obesity. The lean group (L, n=8) remained out to pasture. At one year of age, all sheep were humanely euthanased and kidneys sampled for the following analysis: 1) immunoblotting for VDAC, 2) real time gene expression of pro-apoptotic BAX and anti-apoptotic Bcl-2 as well as the endothelial (eNOS) and inducible (iNOS) nitric oxide synthase and 3) nitric oxide (NO) concentration. All procedures were in accordance with U.K. legislation, with local Animal Ethics Committee approval.

The analysis of VDAC abundance showed a doubling ($P<0.05$) in NR-O compared to the O group whereas VDAC was similar in the lean and obese groups. The expression of BAX and Bcl-2 were up regulated with obesity ($P<0.05$) and the gene expression of iNOS and eNOS was similar. NO production was higher in O, compared to the NR-O (O 1.562 SE 0.3; NR-O 0.866 SE 0.1 Nitrate µM/ [protein µg/µl] ($p<0.05$)).

The rise in VDAC abundance in the NR-O may reduce the level of mitochondrial dysfunction induced by BAX/Bcl-2 in obesity, as suggested by reduction in NO concentrations. This adaptation may reduce the production of OxS and protect NR offspring from renal injuries induced by early onset of obesity. However, this is not related to up regulation of the NOS isoforms. Further analyses are needed to characterize the sources of the increase in renal NO in NR animals.

Fainberg HP, Gardner DS, Budge H, Symonds M.E (2007) - *Protective Renal adaptations in obese young sheep exposed to maternal nutrient restriction between early to mid gestation.*

Poster presentation - 5th International Congress on Developmental Origins of Health & Disease, Perth Australia, November 2007

AIMS

Maternal nutrient restriction (NR) between early to mid gestation protects the kidney against later glomerulosclerosis following obesity. This study aimed to elucidate whether a defined period of NR prevents renal apoptosis before the onset of obesity associated kidney disease.

STUDY DESIGN

Twenty six sheep were randomly allocated into either NR, (NR, n=11, 50% metabolic energy requirements) or control nutrition (100 % requirements) from gestation days 30 to 80 (term ~148 days) and to an obesogenic (O, n=7) or lean (L, n=8) postnatal environment induced by restricted physical activity with ad libitum feeding after weaning, or pasture, respectively. At 1 year of age, renal gene expression of Fas, Bax and Bcl-2 were measured using real time PCR.

RESULTS

Offspring exposed to the obesogenic environment were fatter (relative fat mass g/kg: L - 24 ± 3;

O - 80 ± 3; NR-O - 74 ± 4 ($p<0.01$ compared to L).

	L	**O**	**NR – O**
Fas mRNA	1.1 ± 0.4	$3.3 \pm 0.5^{**}$	$1.4 \pm 0.3^{\#}$
Bax mRNA	1.1 ± 0.3	$2.5 \pm 0.2^{*}$	$2.4 \pm 0.3^{*}$
Bcl-2 mRNA	0.3 ± 0.1	$0.8 \pm 0.1^{*}$	0.7 ± 0.1

Values are means ± SEM; *=P<0.05, **=P<0.01 compared to L; #=p<0.05 compared to O

CONCLUSIONS

Obesity caused upregulation of the apoptotic pathway. Exposure to NR during fetal development prevented the usual increase in renal Fas expression with obesity and may act to prevent glomerulosclerosis.

B. Title of publications and awards

H.P Fainberg, (D.S Gardner[1], H. Budge and M.E Symonds) (2010) *Alterations induced by juvenile obesity on the renal tissue of nutrient restricted offspring*
Nutrition Society Postgraduate Symposium, Dublin, RoI, February 2010 - Winning paper of the Nutrition Society postgraduate competition 2010.

Sébert SP, Hyatt MA, Chan M, Yiallourides M, Fainberg HP, Patel N, Sharkey D, Stephenson T, Rhind SM, Bell C, Budge H, Gardner DS, Symonds ME *Influence of prenatal nutrition and obesity on tissue specific fat mass and obesity-associated (FTO) gene expression*. Reproduction 2009; [Epub ahead of print]

Sharkey D, Fainberg HP, Wilson V, Gardner DS, Symonds ME, Budge H *The impact of early-onset obesity and hypertension on the unfolded protein response in renal tissues of juvenile sheep.* Hypertension 2009; 53:925-31

Sharkey D, Gardner DS, Fainberg HP, Sébert SP, Bos P, Wilson V, Bell R, Symonds ME, Budge H *Maternal nutrient restriction during pregnancy differentially alters the unfolded protein response in adipose and renal tissue of obese juvenile offspring.* Faseb J 2009; 23:1314-24

C. Details of Suppliers

Abcam plc, 332 Cambridge Science Park, Milton Road, Cambridge, CB4 6FW; www.abcam.com

ABgene Limited UK, ABgene House, Blenhem Road, Epsom, KT19 9AP; www.abgene.com

Amersham Biosciences, GE Healthcare UK Limited, Pollards Wood, Nightingales Lane, Chalfont St. Giles, Buckinghamshire, HP8 4SP; www.gelifesciences.com.

BioRad Laboratories Ltd, Bio-Rad House, Maxted Road, Hemel Hempstead, Herfordshire, HP2 7DX; www.bio-rad.com.

Cambridge Bioscience Ltd, 24-25 Signet Court, Cambridge, CB5 8LA; www.bioscience.co.uk.

Dako UK Ltd, Denmark House, Angel Drove. Ely, Cambridgeshire, CB7 4ET; www.daco.co.uk.

IDS.Ltd, Boldon, Business Park, Boldon, NE35 9DP; http://www.idstld.com.

Invitrogen Ltd, 3 Fountain Drive, Inchinnan Business Park, Paisley, PA4 9RF; http://www.invitrogen.com.

Merk Chemicals Ltd, Padge Road, Nottingham, NG9 2JR; www.merckboisciences.co.uk.

Qiagen, Qiagen House, Fleming Way, Crawley, West Sussex, RH10 9NQ; www.qiagen.com.

Severn Biotech Ltd, Unit 2, Park Lane, Kidderminster, DY11 6TJ; www.severnbiotech.com.

Sigma-Aldrich Company Ltd, The Old Brickyard, New Road, Gillingham, Dorset, SP8 4XT; www.sigma-aldrich.com.

Sigma-Genosys Ltd, Sigma-Aldrich House, Homefield Business Park, Homefield Road, Haverhill, Suffolk, CB9 8QP; www.sigma-genosys.com

D. Standard buffers and stock solutions

Reagents for RNA work

Tri Reagent: Pre-made solution (Sigma-Aldrich)

Chloroform: Used for phase separation of RNA (Fisher Scientific, UK)

Isopropanol: Used to precipitate RNA (Fisher Scientific, UK)

75% Ethanol: 75ml ethanol and 25 ml deionised water (RNAse free).

Reagents for protein work

Whole cell lysate solution:

RIPA buffer: 1 x PBS (pH 7.4), 0.1% SDS, 1 % Igepal CA-630 (Sigma-Aldrich) and 0.5 % Sodium Deoxycholate

Protease inhibitors: 10 mg/ml PMSF, 30 µl/ml of buffer Aprotinin (Sigma-Aldrich) and 100 mM Sodium Orthovanadate

Immunoblotting (western Blotting) solutions:

Transfer buffer: 14 g Tris-base, 7.25g glycine, 500 ml methanol, 9.25 ml 10% SDS and deionised H_2O to 2.5 L.

Ponceaux S dye: 2 g Ponceaux S, 30 g trichloroacetic acid (TCA), 30 g salpasalcyclic acid and deionised water to 100 ml (used 1 part dye to 9 parts deionised water)

TBS: 20 mM Tris (pH 7.4), 500 mM NaCl, pH to 7.5 and deionised water to 2.5 L.

TTBS buffer: 0.2 % Tween in TBS

Blocking buffer: 1 % milk fat (Marvel®) in 70 ml TTBS

Primary/Secondary antibody buffer: TTBS containing 3 % (w/v; Marvel®) and antibody of interest.

Electrophoresis buffers

DNA/RNA electrophoresis

5 x TBE: 44 mM Tris-borate (pH 8) and 2.5 mM EDTA

50 x TAE: 242 g Tris Base, 57.1 ml glacial acetic acid, 100 ml 0.5 M EDTA (pH 8) and made up to 1 L using deionised water

Ethidium Bromide: A 5 mg/ml solution was prepared in 1 x TBE buffer

100 Bp DNA ladder (0.5 mg/ml): Purchased from ABgene. 2 µl DNA marker was added to 3 µl of 1 x loading dye and 7 µl RNAse free water. 12 µl was loaded on the agarose gel.

1 x DNA loading dye: 30% (w/v) glycerol and (w/v) bromophenol blue.

Protein electrophoresis (SDS-PAGE)

30% bis- acrylamide: pre-made solution purchased from Severn Biotech

12% SDS-PAGE resolving gel: 6.6 ml deionised water, 8 ml bis-acrylamide, 5 ml 1.5 M Tris-HCl (pH 8.8) , 200 µl 10 % SDS, 200 µl 10% Ammonium presulphate (APS) and 8 µl N,N,N',N'-Tetramethlylethylenediamine (TEMED).

5% SDS-PAGE stacking gel: 6.8 ml deionised water, 1.7 ml 30 % bis-acrylamide, 1.25 ml 1 M Tris-HCl (pH 6.8), 100 µl 10 % SDS, 100µl 10% APS and 4 µl TEMED

Protein dissociation buffer: 50 M Tris (pH 6.8), containing: 10% glycerol, 10% SDS, made up to 10 L with deionised water

Gel loading dye: stock: 0.1 g bromophenol blue, 14.9 ml 0.01 M NaOH and 23.5 ml deionised water; working solution: 400 µl stock solution, 800 µl glycerol and 3.8 ml deionised water

SDS-Precision Plus Protein^{TM} standard: Pre-stained standard marker purchased from Bio-Rad

Coomassie blue staining solution: purchased from Bio-Rad

Reagents for immunohistochemistry

10% Formalin sock: 100 ml (37/40%) formaldehyde (Fisher Scientific, UK) in 900 ml saline

0.05 M TBS (pH 7.2-7.6) stock solution: 175.2 g NaCl, 12.12 g Tris, approx. 40 ml M HCl to pH and 2 L deionised water. This was diluted 1: 10 for use

0.25% solution TBS/Bovine Serum Albumin (BSA): TBS-0.25% BSA 0.25 g BSA and 100 ml TBS. Once made the solution was filtered through 0.225 µm filters, aliquoted out into 10 ml portions and stored at -20^{o}C until use

0.1 M Citrate buffer (pH 6) stock solution: 29.41 g Tris-sodium citrate, 900 ml deionised water, pH using 1 M HCl and make up to 1 L with deionised water. This was diluted 1:10 for use

6% Hydrogen Peroxide (H_2O_2) in Methanol: 5 ml 30% H_2O_2 (sigma-Aldrich), 295 ml methanol. Once this solution was made up, it was stored at 4^oC and use within a week

Normal Swine Serum: This was commercially bought serum (DAKO, cat. 0901) and use in 1:10 dilution was made using TBS/BSA as the diluents

Scott's Tap Water: 2 g potassium bicarbonate, 20 g magnesium sulphate and 1000 ml deionised H_2O

Acid Alcohol: 99 ml 70% alcohol and 1 ml conc. HCl