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EFEITOS DA NUTRIÇÃO NA INFÂNCIA PRECOCE NO DESENVOLVIMENTO DO TECIDO ADIPOSO

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RESUMO

Introdução: A obesidade e o excesso de peso têm uma etiologia multifactorial que resulta da interacção de factores genéticos e ambientais. Estas condições apresentam uma elevada prevalência nos países ocidentais e actualmente nos países em desenvolvimento. O tecido adiposo tem uma função importante na regulação da resposta inflamatória associada à obesidade. As alterações nutricionais no período pré e pós natal podem ter consequências futuras na função do tecido adiposo. A nutrição influencia a reprogramação de respostas inflamatórias através de alterações da expressão genética.

Objectivo: O objectivo deste estudo foi determinar a relação entre a restrição alimentar durante a gestação, associada ou não a crescimento pós-natal acelerado, e a expressão genética de marcadores inflamatórios no tecido adiposo subcutâneo de animais jovens.

Material e métodos: Um grupo de ovelhas com gestação gemelar foi alimentado de acordo com as suas necessidades energéticas (R; n=10) e outro grupo com restrição nutricional de 60% deste valor (N; n=10) desde os 110 dias de gestação até ao termo (147 dias). Em dez crias de cada grupo foi promovido o crescimento pós-natal acelerado (A) e nas restantes o crescimento foi limitado (S). As crias foram eutanasiadas aos 17 meses de vida. Amostras de tecido adiposo subcutâneo foram colhidas e conservadas a -80ºC até à análise de mRNA para os genes que codificam a adiponectina, leptina, interleucina (IL) -6 e -18, *monocyte chemoattractant protein-1* (MCP-1), factor de necrose tumoral (TNF)-α, *toll-like receptor* (TLR)-4, *fat mass and obesity-associated* (FTO) gene e *glucose-responsive protein* (GRP) 78 utilizando a técnica de *polymerase chain reaction* em tempo real.

Resultados: O peso de nascimento encontrava-se diminuído após restrição calórica materna. Aos 17 meses de idade o peso corporal e quantidade de massa gorda eram semelhantes entre os grupos. A expressão genética de adiponectina encontrava-se diminuída nas fêmeas que sofreram crescimento pós-natal acelerado (NS: 0.671±0.21, NA: 0.22±0.04 ($p < 0.05$)). A quantidade de mRNA para FTO e IL-6 estava diminuída, no grupo do sexo feminino que sofreu restrição nutritiva *in utero* (ex. FTO - RA: 1.3±0.3, NA: 0.56±0.04 $p < 0.05$)). Não houve alterações significativas na determinação de leptina, TNF-α, MCP-1, TLR-4, IL-18 ou GRP-78 em nenhuma intervenção.

Conclusões: O tecido adiposo subcutâneo apresenta alterações limitadas na sua expressão genética após a manipulação do meio nutricional pré e pós-natal. As principais alterações estão relacionadas com o aumento ponderal após o nascimento, período durante o qual há maior deposição de tecido adiposo. Apesar da quantidade de tecido adiposo ser semelhante há diferenças na expressão genética entre os grupos que podem ter consequências na idade adulta.

Palavras-chave: restrição nutricional, crescimento pós-natal, expressão genética, inflamação

ABSTRACT

Introduction: Obesity and overweight have a multifactorial aetiology. Genetics and environment interact and have a different impact in establishing a condition with high prevalence in industrialized countries and also in developing countries. Adipose tissue plays an important role in regulating inflammatory responses related to obesity. Changes in both the pre and postnatal nutritional environment can have a long term impact on adipose tissue function in the offspring. This includes a resetting of inflammatory and related responses as a consequence of changes in gene expression.

Objectives: The aim of my thesis was, therefore, to determine the extent to which exposure to a nutrient restricted diet in late gestation, with or without accelerated postnatal growth, determines gene expression of inflammatory markers in subcutaneous adipose tissue of young adult offspring.

Material and methods: Pregnant twin-bearing sheep were either fed to requirements (R; n=10) or nutrient restricted to 60% of this amount (N; n=10) from 110 days up to term (147 days). Ten offspring in each group were then reared by their mother as singletons in order to promote postnatal growth (accelerated weight gain – A). Ten twin offspring from N group were reared by their mother together in order to restrict postnatal growth (standard weight gain – S). Offspring were humanely euthanized at 17 months of age, adipose tissue was sampled and stored at -80^{o}C until analysis of mRNA abundance for the genes encoding adiponectin, leptin, interleukin-6 (IL-6), monocyte chemoattractant protein (MCP) 1, tumor necrosis factor (TNF) α, toll-like receptor (TLR) 4, IL-18 fat mass and obesity-associated (FTO) gene and glucose-responsive

protein (GRP)78 using real-time PCR. Appropriate institutional animal ethics committee approval was obtained.

Results: Following maternal caloric restriction, birth weight was reduced. At 17 months of age mean body weight was similar between groups as was total fat mass. Gene expression for adiponectin was decreased in females with accelerated postnatal growth (NS: 0.671±0.21, NA: 0.22±0.04 ($p<0.05$)) whereas mRNA abundance for the FTO and IL-6 genes were both reduced by maternal nutrient restriction (e.g. FTO - RA: 1.3±0.3, NA: 0.56±0.04 $p<0.05$)). There were no changes in mRNA abundance for leptin, TNF-α, MCP-1, TLR-4, IL-18 or GRP-78 with any of the interventions.

Conclusion: Subcutaneous adipose tissue exhibits limited changes in gene expression following manipulation of either the pre and postnatal nutritional environments. Postnatal growth coincides with the period in which significant amounts of subcutaneous adipose tissue are being deposited and is a critical period to determine adipocyte function. Even though fat mass quantity is similar between the different groups there are significant changes in gene expression programming that may influence adult life health.

Key-words: nutrient restriction, catch-up growth, gene expression, inflammation

ABREVIATIONS:

AGA- adequate for gestational age

BAT - brown adipose tissue

cDNA – complementary Deoxyribonucleic Acid

CVD – cardiovascular diseases

Ct – cycle threshold

DM – Diabetes mellitus

ER – endoplasmic reticulum

FTO – fat mass and obesity gene

GRP-78 – glucose-regulated protein 78

HAP - hypothalamic-pituitary-adrenal

IL-6 – interleukin 6

IL-18 – interleukin 18

IUGR – intra-uterine growth restriction

Kcal – kilocalories

MCP-1 – monocyte chemoattractive protein 1

NA – **N**utrient restricted group with **A**ccelerated postnatal growth

NS – **N**utrient restricted group with **S**tandard postnatal growth

PCR – polymerase chain reaction

RA – fed to **R**equirements group with **A**ccelerated postnatal growth

RT – reverse transcription

SAT - subcutaneous adipose tissue

SGA – small for gestational age

TNF-α – tumor necrosis factor – α

TLR-4 – toll-like receptor 4

UCP-1 - uncoupling protein 1

UPR – unfolded protein response

VAD – visceral adipose tissue

WAT - white adipose tissue

INTRODUCTION

Obesity

Obesity and overweight are defined as abnormal or excessive fat accumulation that may impair health (WHO, 2010). Worldwide, according to the most recent WHO projection in 2005 approximately 1,6 billion adults were overweight and at least 400 million adults obese. Future predictions indicate further increases, not only in western societies but also in developing countries.

Obesity is a disease with a multifactorial aetiology. Genetics and environment interact and have a different impact, in establishing a disease with high prevalence in industrialized countries and also in developing countries where a process of westernization is occurring (Tabacchi, 2007). A complete study of the influence of each of the different involved factors is very complicated and difficult. The most common form of obesity is polygenic and involves a combination of individual genetic variants that can increase the risk of obesity (Bray, 1998 and Hill, 1998).

A more recent serious problem is the increase in obesity among children and adolescents (Tabacchi *et al*, 2007). In Europe, the International Obesity Task Force estimates the prevalence of overweight children is 24% (Lobstein *et al*, 2004). The increasing incidence of childhood obesity is related to a combination of genetic, environmental, psychosocial and socioeconomic factors. Ultimately, obesity results from a dysregulation of caloric intake and energy expenditure. A complex interplay between each individual's genetic predisposition and the environment affects an intricate system that controls appetite and energy expenditure (Skelton and Rudolph, 2007). The excessive consumption of energy-dense, sweetened and pre-prepared food,

absence of family meals, large portion sizes, excessive television viewing and sedentary lifestyle are all associated with a greater prevalence of obesity (Symonds, 2007). Overweight status in the pediatric population is associated with significant comorbidities, which if untreated are likely to persist into adulthood. The probability of obesity persisting into adulthood increases from 20% at 4 years old to 80% by adolescence (Krebs *et al*, 2007).

Adipose tissue

Considering obesity as a major public health issue, many studies and information have been gathered in order to understand the primary factors. Adipose tissue has been one of the primary target organs investigated. There are two types of adipose tissue depending on its cell structure, location, colour, vascularisation and function: white adipose tissue (WAT) and brown adipose tissue (BAT). WAT is the primary site of energy storage in the form of triglycerides lipid droplets, whereas BAT contains multilocular adipocytes and is characterised as possessing a unique uncoupling protein (UCP 1) (Vázquez-Vela et al, 2008). The latest has a large number of mitochondria and is specialized in heat production and, therefore, energy expenditure. Nevertheless, in humans and in sheep, BAT is most abundant in the newborn for regulating thermogenic processes, while adults have mainly WAT (Vázquez-Vela *et al*, 2008).

Adipose tissue contains different cell types. Only one third of the tissue is constituted by adipocytes and the rest is represented by fibroblasts, macrophages, stromal cells, monocytes and preadipocytes (Vázquez-Vela *et al*, 2008). The process by which adipose cells are derived from a mesenchymal preadipocyte involves an

orchestrated series of differentiation steps mediated by a cascade of specific transcription factors (Flier and Maratos-Flier, 2005). In humans, preadipocytes are converted into adipocytes in the late embryonic stage (Cornelius *et al*, 1994). Nevertheless, the ability of differentiation of preadipocytes is always present and depends on the body energy status and the storage needs (Vázquez-Vela *et al*, 2008).

Adipose mass increases by enlargement of adipose tissue which can undergo hyperplasia and hypertrophy. In growth stages, adiposity is increased mainly through hyperplasia (Vázquez-Vela et al, 2008). In the adult life, the capacity of the preadipocytes to become fully functional mature adipocytes declines (Vázquez-Vela *et al*, 2008). However, adipogenesis in adults can still occur, and the inability of an individual to increase cell numbers by this process contributes to the development of metabolic diseases (Dubois *et al*, 2006).

Over the last two decades, studies have reemphasized the point that obesity is not a homogenous condition and that the regional distribution of adipose tissue is important to understand the relation of obesity to disturbances in glucose and lipid metabolism (Flier and Maratos-Flier, 2005).

Location and distribution of adipose tissue explains its different functions. Subcutaneous adipose tissue (SAT) is located underneath the skin and is responsible, in part, for the distinct and different body compositions between males and females. Visceral adipose tissue fills in the space between organs and helps maintains their normal position (Guyton and Hall, 2000).

Many early studies have shown that excess fat in the upper part of the body, considered “android or male-type obesity”, more often correlates with increased mortality and risk for disorders such as type 2 diabetes mellitus (DM), dislipidemia, hypertension and atherosclerosis. It is considered that the detrimental influence of

abdominal obesity on metabolic processes is mediated by the intra-abdominal fat depot. Generally, visceral or omental adipose tissue (VAD) is the most active, which means, it is more sensitive to lipolysis, through catecholamines and β-adrenergic receptors, and more resistant to insulin, releasing free fatty acids into the porta vein (Hermsdorff and Monteiro, 2004). However, studies show that the presence of obesity and insulin resistance is associated with changes in the specific proinflammatory, lipogenic, adipogenic and insulin signalling resistance pathway genes with no preferable localization in SAT or VAT depots (Dolinková *et al*, 2008).

In total, considering that the SAT has a higher total mass, when compared to VAT, the former might have an equally important function in the relationship between obesity and its associated diseases (Hermsdorff and Monteiro, 2004).

Until recently, WAT was considered just a fat depot primarily involved in the storage and release of fatty acids, thermal insulation and a mechanical role. Now, it is regarded as a dynamic major secretory organ with multiple functions (Wajchenberg, 2000). This concept rapidly developed following the discovery of leptin in 1994; a 16 kDa protein secreted by adipocytes that was found to be the product of *obese* (ob) gene. Since then, WAT has been found to produce more than 50 cytokines and other adipokynes. These act through endocrine, paracrine and autocrine mechanisms, in a wide variety of physiological or physiopathologic processes, including food intake, insulin sensitivity, vascular sclerotic processes, immunity and inflammation (Lago *et al*, 2007). They are currently considered to play a crucial role in crosstalk among adrenal, immune and central and peripheral nervous systems, among others (Lago *et al*, 2007).

Adipose tissue development

In humans, the primary fat formation takes place relatively early in the prenatal period around the 14th to 16th week, with gland-like aggregations of epitheloid precursor cells, called lipoblasts, or preadipocytes being formed in specific locations. Subsequently, a secondary fat formation takes place after the 23rd week of gestation as well as in the early postnatal period. In this case, the differentiation of fusiform precursor cells, which accumulate lipid to ultimately coalesce into a single large fat droplet in each cell, leads to the dissemination of fat depots formed by unilocular white adipocytes in many areas of connective tissue (Yang, 2005).

BAT has a similar development but it is preferably located in the interscapular and cervical regions, axillae and along lymphatic ganglia (Saldanha, 1999 and Banister, 1995).

Therefore, there is no doubt that maternal nutritional factors in the last months of gestation and during the first months of life influence the adipocytes physiology (Saldanha, 1999).

The third trimester of gestation represents a critical period for the development of obesity in later life. Although relatively small, adipocytes are already present in the main fat depots at the beginning of third trimester. At birth, WAT reportedly accounts for approximately 16% of total body weight (with BAT constituting 2-5%). During the first year of life, an increase in total body fat from approximately 0.7 kg to 2.8 kg normally occurs (Yang, 2005).

Obesity and inflammation

It is thought that excessive or disproportionate gain of adipose tissue may be causal to its dysfunction at many levels. One important concept is that it acts as a central mediator of the inflammatory response. Obesity is now regarded as a pro-inflammatory state with several markers of inflammation elevated in obese subjects (Lau *et al*, 2005). Type 2 DM is also an inflammatory condition in which inflammatory mechanisms contribute to accentuate insulin resistance (Dandona *et al*, 2004). Excess WAT can contribute to the maintenance of this state in three ways: through inflammation-inducing lipotoxicity; by secreting factors that stimulates the synthesis of inflammatory agents in other organs; and by secreting inflammatory agents itself (Hotamisligil *et al*, 1993). Inflammatory pathways are upregulated in obese adipose tissue leading to increased expression of down-stream cytokines such as tumor necrosis factor α (TNF- α), interleukin 6 (IL-6), interleukin 18 (IL-18) and monocyte chemoattractant protein (MCP-1), among others (Gregor and Hotamisligil, 2007).

Although adipose tissue is a central site of inflammation in obesity, the resultant metabolic sequelae are systemic. These proinflammatory adipokynes appear to contribute significantly to the "low-grade inflammatory state" of obese subjects with metabolic syndrome (Trayhurn *et al*, 2006). The metabolic syndrome (syndrome X, insulin resistance syndrome) consists of a constellation of metabolic abnormalities that confer increased risk of cardiovascular disease (CVD) and DM. The criteria for the metabolic syndrome have evolved since the original definition by the World Health Organization in 1998, reflecting growing clinical evidence and analysis by a variety of consensus conferences and professional organizations. The major features of the

metabolic syndrome include central obesity, hypertriglyceridemia, low HDL cholesterol, hyperglycemia, and hypertension (Flier and Maratos-Flier, 2005).

Adipose tissue in obesity is characterised with chronic inflammation and other possible insults such as hypoxia, oxidative stress, and mechanical stress attributable to hypertrophy. These cumulatively result in organelle dysfunction, particularly in mitochondria and endoplasmic reticulum (ER). It is considered that a saturated or dysfunctional adipose tissue must send its lipid load elsewhere for storage, leading to ectopic accumulation of fat in other organs (Gregor and Hotamisligil, 2007). Ectopic lipid deposition has a range of adverse physiological consequences. Lipids are cytotoxic to most cells including those in the pancreas where it has been implicated in the pathogenesis of diabetes. Excess lipid deposition in endothelial cells contributes to hypertension, in part by inducing resistance to the vasodilatory effect of insulin (O´Rourke, 2009). Lipid accumulation in the liver defines steatosis with progression to steatohepatitis whereas in skeletal muscle it has been associated with peripheral insulin resistance (O´Rourke, 2009). (Figure 1)

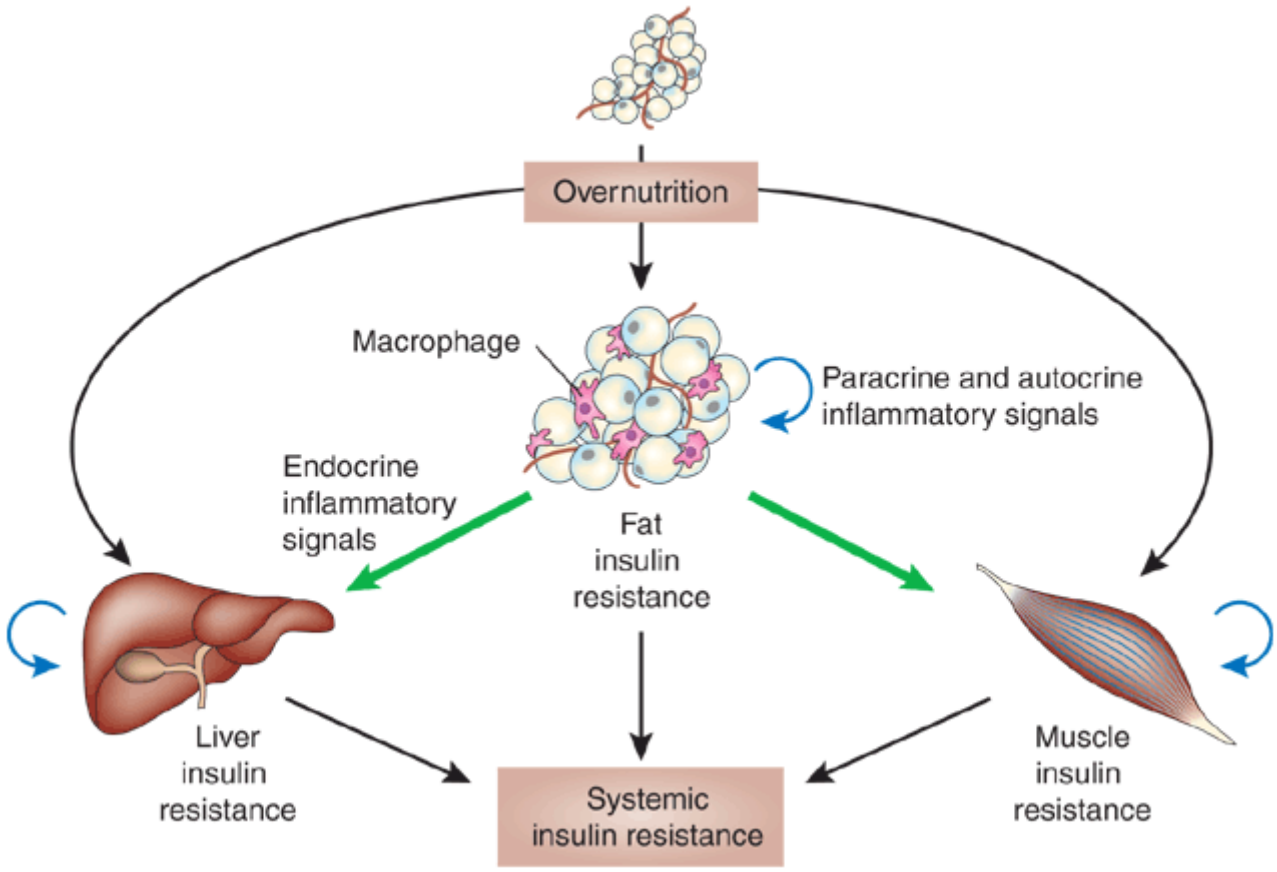


Figure 1. The development of systemic insulin resistance in obesity-induced inflammation and stress.

In obesity, adipose tissue is under a constant state of metabolic stress, resulting in the activation of the stress and inflammatory response, which leads to the accumulation of macrophages. In this state, adipocytes release cytokines, adipokines and free fatty acids, which can act in a paracrine or autocrine fashion to amplify the proinflammatory state within adipose tissue and cause localized insulin resistance. Adipose tissue also serves as an endocrine organ whereby these cytokines, adipokines and free fatty acids travel to liver and muscle and may decrease insulin sensitivity. In addition to the adipose tissue–derived factors, stress and inflammatory signals can arise independently within liver and muscle, and result in local insulin resistance within these organs (De Luca and Olefsky, 2006).

Adipokines

Adipose tissue is a rich source of adipokines whose secretion can increase with obesity that promotes inflammation (O´Rourke, 2009). Expression of positive regulators of inflammation is increased in SAT from obese humans and mice, whereas calorie restriction causes a reversion of the adipose tissue transcriptome toward that similar to lean subjects, with down-regulating of pro-inflammatory cytokines and upregulation of anti-inflammatory cytokines (Sharkey *et al*, 2009).

Many proteins are expressed by both lymphocytes and adipocytes, including adiponectin, IL-6 and MCP-1, which reflects the close relationship between adipocytes and immunological cells. Collectively, these adipokines demonstrate a remarkable multiplicity of functions that regulate immune, endocrine and development processes. Virtually all adipokines share important immunoregulatory activities (O´Rourke, 2009).

Leptin, from the greek *leptos* means lean and it regulates energy metabolism, increasing energy expenditure and decreasing energy consumption. It is now considered that leptin controls adipose tissue growth through its action in the central nervous system. Leptin concentration and expression is directly associated with adiposity and body weight changes (Vázquez-Vela *et al*, 2008). Immunohistochemical assays show that leptin is present in mature adipocytes and not preadipocytes (Vázquez-Vela *et al*, 2008). Adipose tissue also expresses leptin receptors, suggesting an autocrine and paracrine role of the hormone. Leptin also regulates immune responses. Hyperleptinemia during obesity is often associated with leptin resistance, a condition in which fat storage capacity is augmented and the rate of oxidation diminished. Leptin resistance is proposed as an important cause of adipocyte dysfunction and lipid overload

in non-adipose tissue leading to ectopic accumulation of lipids in skeletal muscle, heart and pancreas (Unger, 2003). It also regulates immune function through a stimulation of responses to inflammatory challenge, inducing oxidative stress and inflammation in endothelial cells (Dandona, 2004).

Known direct actions of leptin on immune responses include the following:

- It promotes phagocyte function (Zarkesh-Esfahani *et a*l, 2001) and induces the synthesis of nitric oxide and several pro-inflammatory cytokines in macrophages and monocytes (Raso *et a*l, 2002).
- It induces chemotaxis and the release of reactive oxygen species by neutrophils (Caldefie-Chezet *et al*, 2001).
- It influences the proliferation, differentiation, activation and cytotoxicity of natural killer cells (Tian *et al*, 2002).
- It may protect dendritic cells from apoptosis (Lam *et al*, 2006).
- It modifies T-cell balance, induces T-cell activation, and alters the pattern of T-cell cytokine production (Farooqi *et al*, 2002).

Adiponectin is a protein expressed primarily in mature adipocytes. In contrast, to the effects of other factors secreted by the WAT, it has a protective role against the onset of CVD, type 2 DM, metabolic syndrome and other immune-related diseases. Although adiponectin was discovered soon after leptin, its role in protection was only recognized several years later. Its anti-inflammatory and anti-atherogenic functions are because it reduces the expression of TNF-α and resistin, macrophage chemotaxy and inhibition of the inflammatory pathway within the endothelial tissue. Adiponectin improves insulin sensitivity through an increase in fatty acid oxidation and glucose uptake by skeletal

muscle and WAT; reduces liver production of glucose, improving glycaemia and the control of circulating free fatty acids and triglycerides (Lago *et al*, 2007).

The plasma concentration of adiponectin is negatively related to the quantity of visceral and subcutaneous adipose tissue. Circulating concentrations are lower in obese patients and increase with weight loss (Hermsdorff and Monteiro, 2004).

TNF-α is a multi-functional cytokine that can regulate many cellular and biological processes including immune function, cell differentiation, proliferation, apoptosis and energy metabolism (Cawthorn and Sethi, 2008). The concept of inflammation in relation to the metabolic syndrome was first proposed in 1993 (Hotamisligil *et al*, 1993), when it was demonstrated that adipocytes constitutively express the proinflammatory cytokine TNF-α and that its expression in obese rodents is greatly increased (Dandona *et al*, 2004), where it is a mediator of obesity-related insulin resistance and type 2 DM (Cawthorn and Sethi, 2008). This proposal was supported by studies in which gene knock out of TNF-α action restored insulin sensitivity (Cawthorn and Sethi, 2008). The evidence for a similar role in humans is less convincing (Cawthorn and Sethi, 2008). Within adipose tissue, TNF-α is primarily produced by macrophages. Increased adipocyte death in expanding adipose tissue induces chemoattractant signals that recruit monocytes and release more TNF-α (Cawthorn and Sethi, 2008).

IL-6 is an interleukin that acts as both a pro-inflammatory and anti-inflammatory cytokine. It is secreted by T cells and macrophages to stimulate the immune response, specifically, in the induction of acute phase reactions. It is implicated in a wide variety of inflammation-associated disease states, including obesity and susceptibility to DM.

IL-6 also plays a role in controlling the extent of inflammatory responses, by suppressing the level of proinflammatory cytokines and inducing anti-inflammatory molecules. These paradoxical activities may be viewed as an attempt to bring the host back to normal homeostasis (Xing *et al*, 1998).

MCP-1 belongs to the family of chemotactic cytokines known as chemokines. It plays a role in the recruitment of monocytes, memory T cells and dendritic cells to sites of tissue injury and infection. MCP-1 is produced as a protein precursor containing signal peptide of 23 amino acids and a mature peptide of 76 amino acids, and is a monomeric polypeptide, with a molecular weight of ~13 kDa. As with many other chemokines, MCP-1 is located on chromosome 17 in humans. The cell surface receptors that bind MCP-1 are CCR2 and CCR4 (Jiang *et al*, 1992).

Toll-like receptor (TLR) is a key regulator of both innate and adaptive immune responses. Lipotoxicity plays an important role in direct activation of the innate immune system via TLR signalling. It provides a direct molecular link between hyperlipidemia, a central clinical feature of obesity, and activation of the innate immune system. Free fatty acids are important ligands for TLR-4 which is expressed in a wide range of cells, including macrophages and adipocytes. When activated it upregulates inflammatory cytokine expression, and induces insulin resistance. TLRs plays a role in other co-morbidities of obesity including atherosclerosis and hepatic steatosis (O´Rourke, 2009). TLR-4 is associated with macrophage infiltration and their levels are positively correlated with the magnitude of adipocyte hypertrophy and hyperplasia, suggesting that macrophage–related inflammation may regulate the development of obesity (Zhang *et al*, 2009).

Interleukin-18 is a proinflammatory cytokine produced by macrophages and other cells that belong to the IL-1 superfamily. In conjunction with IL-12 it induces cell-mediated immunity following infection and is stimulated by microbial products such as lipopolysaccharide. After IL-18 stimulation, natural killer cells and some T cells release another important cytokine interferon-γ (or type II interferon) that also plays an important role in macrophage activation. A proinflammatory state of a cell can be induced by both TLR-4 and IL-18 as they share similar intracellular signalling pathways (Zhang *et al*, 2009).

Fat mass and obesity associated gene, also known as FTO is a gene on human chromosome 16 in which certain variants appear to be correlated with obesity in humans. This gene is widely expressed in a range of human tissues, with its expression being highest in the brain including the hypothalamus, one of the main appetite regulating centres (Sébert *et al*, 2009). In mice, hipotalamic FTO mRNA abundance is modulated by nutritional status, with starvation down-regulating FTO mRNA transcription (Gerken *et al*, 2007). These results show an association between energy intake and FTO polymorphisms, suggesting that the product of the FTO gene could be involved in energy balance regulation (Gerken *et al*, 2007). In addition, the FTO gene is ubiquitously expressed in all peripheral tissues tested to date. This includes some of the key tissues or organs involved in the control of energy metabolism and cardiovascular function (Gerken *et al*, 2007). The extent to which alterations of FTO regulation in these organs can be involved in obesity and obesity-related disorders remain unknown.

A study of 38,759 Europeans for variants of FTO identified an obesity risk allele (SNPrs9939609). In particular, carriers of one copy of the allele weighed on average 1.2

kg more than people with no copies, whilst carriers of two copies (16% of the subjects) weighed 3kg more and had a 1.67-fold higher rate of obesity. This gene is also associated with increased risk of type 2 DM (Frayling *et al*, 2007). FTO gene expression is upregulated in the hypothalamus following a combination of *in utero* exposure to nutrient restriction and juvenile obesity (Sébert *et al*, 2009).

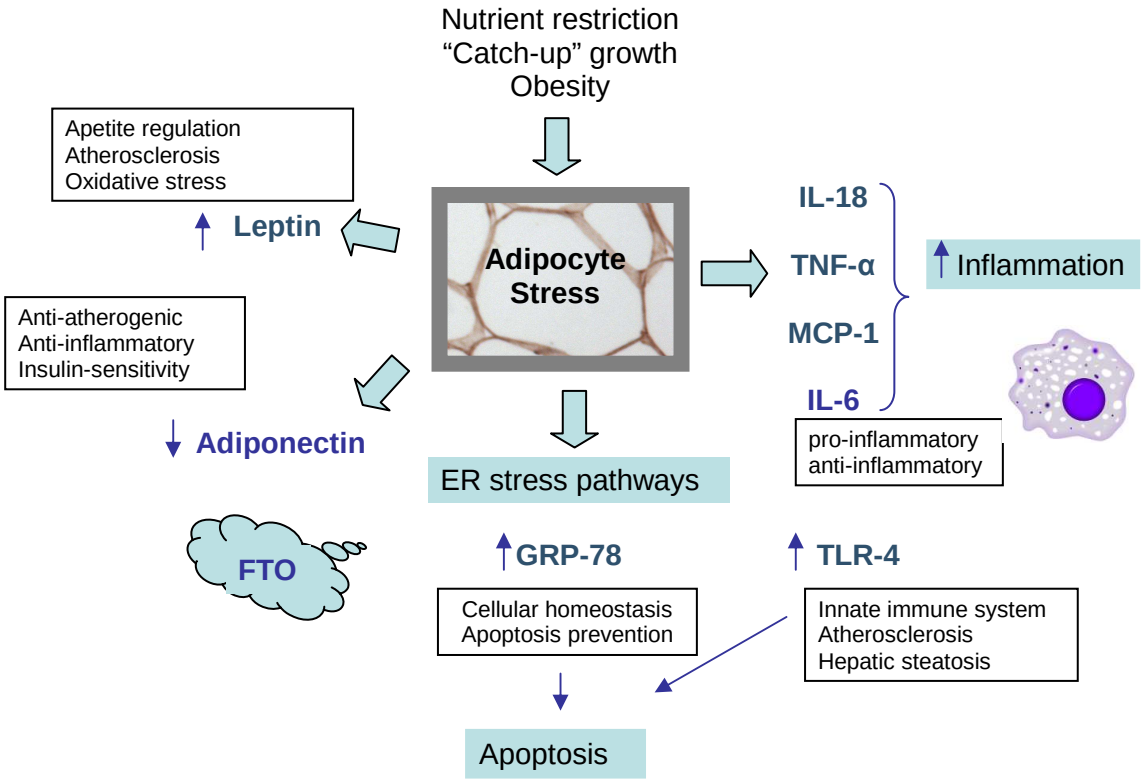


Figure 2 – Disadvantageous nutritional environment induces an adipocyte stress response with expression of different adipokines. IL-18, TNF-α and MCP-1 are associated to an inflammatory response. IL-6 regulates inflammation either by induction or suppression in order to maintain homeostasis. TLR-4 is activated by free fatty acids and is responsible for metabolic effects. GRP-78 is expressed in the ER to prevent apoptosis. Leptin is a saciety hormone and is overexpressed in obese. Adiponectin is a "protective" adipokine and its expression is higher in lean individuals.

Endoplasmic reticulum stress pathways

An emerging concept to explain the vast array of maladaptive responses with obesity is organelle dysfunction, such as ER stress with subsequent activation of the unfolded protein response (UPR). Within stressed adipocytes, activation of UPR initially aims to promote cell survival by increasing crucial ER chaperones, such as glucose-regulated protein 78 (GRP78), to restore cellular homeostasis (Gregor and Hotamisligil, 2007). If this process fails, the UPR will programme cell death via apoptotic ways, through the activation of a homologous protein - CHOP (Sharkey *et al*, 2009).

Obesity is a well defined pathologic state; however, its main causes remain an area of intense debate. Many epidemiological and prospective clinical studies in humans and animals, have demonstrated that modifications in the nutritional environment during fetal and early life can have a substantial impact on later health and disease, including the onset of obesity (Symonds *et al*, 2007).

Fetal nutritional programming of adult health and disease

The intrauterine environment plays a critical role in the growth and development of the offspring. Infants who have suboptimal growth *in utero* have an increased incidence of adult disease (Barker, 1992).

In the context of the effects of early life nutrition on growth and development, “programming” was defined as the lasting consequences of a stimulus or insult during a

sensitive period in early life (Lucas, 1999). Events during gestation dictate permanent influences on fetal physiology. The majority of morphological features are present before birth and this represents the time of the greatest ability for plasticity or adaptation. However, adaptations to insults may have long term adverse consequences in later life. The "fetal origins of adult disease hypothesis" (Barker, 1992) proposes the theory that fetal programming occurs when inadequate nutrition of the mother impairs fetal nutrient supply that ultimately causes permanent physiological changes which can predispose to later cardiovascular disease, hypertension, impaired glucose tolerance and type 2 DM (Barker, 1994).

In various populations and different countries, decreased glucose tolerance in adults has been associated with low birth weight. Associations between decreased rates of fetal growth and impaired insulin-glucose metabolism have also been found in children and adolescents (Ravelli *et al*, 1998).

The Dutch famine, which occurred in the western part of the Netherlands at the end of World War II (late November, 1944, to early May, 1945) provided a unique opportunity to study the fetal effects of severe maternal undernutrition during pregnancy in humans (Ravelli *et al*, 1998). At its peak, the official daily rations for the general population varied between 400 and 800 Kcal (1680 and 3360 KJ). In this study, 702 people who were born between Nov 1, 1943 and February 28, 1947 in Amsterdam, for whom prenatal and birth records could be assessed were include. Glucose and insulin responses to a standard oral glucose test were compared in participants exposed to famine in different stages of gestation and compared with those born after this period (i.e. Controls). These results suggest that prenatal exposure to famine, especially during late gestation, is linked to decreased glucose tolerance in adults. It also assessed that poor nutrition may lead to permanent changes in insulin-glucose metabolism, even if the

effect on fetal growth is small (Ravelli *et al*, 1998, Ravelli *et al* 1999, Roseboom *et al*, 2006).

It is now well established that changes in the macronutrient or micronutrient composition of the maternal diet, either throughout or at specific stages of pregnancy, can have pronounced effects on the fetus (Roseboom *et al*, 2006).

Low birth weight and "Catch-up growth"

In developed countries, where fetal growth restriction is generally the result of compromised placental nutrient transfer rather than a decreased intake of nutrients by the mother, the infant undergoes a period of accelerated ("catch-up growth") growth during early postnatal life when its nutrient supply is no longer constrained (Muhlhausler and Smith, 2008). Emergence into a postnatal environment of plenty is associated with increased accumulation of subcutaneous and visceral adipose tissue in a short period of time (Muhlhausler and Smith, 2008).

A number if studies have shown that fast post-natal "catch-up" growth may have adverse effects on late metabolic health, particularly in small for gestational age (SGA) infants. However, it is not known at which period after birth the accelerated growth may be disadvantageous, with some studies pointing to the immediate early post-natal period (Ong *et al*, 2000; Soto *et al*, 2003; Singhal *et al*, 2003; Singhal *et al*, 2007). Several findings indicate that accelerated growth in childhood further increases the risk (Forsen *et al*, 2000; Eriksson *et al*, 2001; Eriksson *et al*, 2003). Nevertheless, a potential confounder in many previous studies may be lack of correction of catch-up growth for low birth weight *per se*. Given the fact that SGA infants at the time of birth are shifting from an adverse and sparse nutritional intra-uterine environment into one of potentially

excess nutrition, this may be important. The potential state of cellular nutrient restriction in SGA infants at the time of birth may itself facilitate a more efficient nutritional uptake and energy metabolism and thereby increased post-natal growth rate (Vaag, 2009).

Follow-up at age 13-16 years of preterm infants from a randomized intervention trial of neonatal nutrition showed a positive association between weight gain during the first two weeks of life and insulin resistance in adolescence irrespective of birth-weight (Singhal *et al*, 2003). Another study reported that weight gain of term newborn infants from birth to six months was positively associated with metabolic risk at 17 years of age (Ekelund *et* al, 2007).

Effect of gender in nutrient restriction consequences

Gender-specific differences in fetal growth and development following nutritional manipulation *in utero* have been demonstrated in different aspects. Namely, nutrient restriction has more effect in impairing nephrogenesis in males than in females (Jeffrey *et al*, 2007). In addition, sex-specific regulation of the fetal and adult hypothalamic-pituitary-adrenal (HPA) axis per se has been noted previously (Gardner *et al*, 2006). Furthermore, it is clear that responses to challenges that have been shown to incur developmental consequences exhibit a distinct sex-specific bias (Gardner *et al*, 2006). For example, after maternal restraint stress in rats during late gestation (resulting in maternal HPA axis activation), only the adult female offspring are affected; males appear largely unaffected (Gardner *et al*, 2006). Prenatal alcohol exposure again leads to an increase in adult female, but not male, HPA axis activity (Gardner *et al*, 2006).

Animal models

Sheep are one preferred animal model to study adipose tissue manipulation. They are comparable to humans in several fundamental aspects of fetal development. Both are precocial thermoregulators, with BAT being most abundant around the time of the birth, a period that triggers the non-shivering thermogenesis, which disappears during the post-natal period (Symonds and Budge, 2009).

Specific organ growth rates, fetal metabolic rate and protein turnover are similar in sheep and humans and very different to rodents. Because a greater proportion of energy intake is required for maintenance of the individual, postnatal growth is much slower in sheep and humans compared with small mammals (Symonds and Budge, 2009).

Sheep and humans, unlike rodents, are born with a mature hypothalamic-pituitary - thyroidal/adrenal axis. The central neural network for the regulation of appetite develops before birth in the sheep as in humans. Rodents develop this network in the early postnatal period when it is leptin dependent (Symonds and Budge, 2009).

Aim

The aim of my project is to demonstrate initial alterations in the metabolic functions of adipose tissue which may form potential mechanisms implicated in the link between catch-up growth and later obesity. Furthermore, as maternal nutrient restriction during fetal development is also associated with later obesity and adverse metabolic outcomes, it will elucidate the effects of the fetal as well as early postnatal nutritional environments on adipokine function.

Hypothesis

It is hypothesised that deleterious metabolic outcomes in young adulthood are associated with impaired adipokine function following a period of rapid early postnatal growth and that these effects are amplified when individuals were exposed to maternal nutrient restriction during late gestation.

MATERIAL AND METHODS

Laboratory work was conducted at the University of Nottingham in collaboration with Universidade de Coimbra. All experimental protocols were carried out with local Ethics committee approval under United Kingdom's legislation. All procedures carried out in the UK were performed according to the Animals (Scientific Procedures) Act, 1986 under Home Office Project License No. 30/1157 (chapters 3,5,6,8).

Laboratory protocols were carried out at the University of Nottingham in accordance to the UK Control of Substances Hazardous to Health (COSHH 2002) regulations on laboratory practice issued by this university. Unless other wise stated, all chemicals and reagents were obtained from Sigma-Aldrich Company Ltd and all laboratory equipments from Scientific Laboratory Supplies.

1. Animals

Twenty five twin bearing Bluefaced Leicester cross Swaledale sheep of similar body weights and age were used. Sheep were confirmed as being pregnant with twins by ultra-sound scanning.

They were randomly allocated into two different nutritional groups that were then maintained until giving birth normally at term (145±2 days).

Dietary manipulation was performed at three critical stages:

1. Prenatal: during the last trimester of pregnancy (110 days to term)
2. Lactation: 0 to 3 months of age
3. Post-weaning: 3 to 17 months of age

1. **Gestation**: A control group of 10 mothers were therefore fed to completely meet their metabolisable energy requirements (R) and the remaining 15 were all nutrient restricted (N) and thus fed to a diet that was 60% of this amount (Table 1).

Nutrient restricted (**N**): (n=15)	mothers received 60% of normal energy requirement
Fed to Requirements (**R**): (n=10)	mothers received 100% of normal energy requirements

Table 1. Prenatal manipulation: Two different prenatal groups were made according to mother's energy intake during late gestation stage.

The diet compromised a mix of 40% concentrated pellets and 60% straw nuts. In addition all mother had *ad libitum* access to a mineral block to ensure adequate micronutrient supply. Mothers were weighted on a weekly basis prior to feeding after which their total food requirements were adjusted as necessary.

2. **Lactation**: Mothers were fed to fully meet their metabolisable requirements throughout lactation. Offspring groups were designed to examine the effect of being reared as a twin (i.e. with competition from a sibling for available food) or as a singleton. Those raised as a singleton experienced accelerated growth (**A**) and those raised as twins experienced standard growth (**S**) (Table 2)

Offspring were weighted twice a week during the first month of postnatal life and once a week thereafter until weaning.

3. **Post-weaning**: All offspring were weaned at three months of age after which time they were all raised in the same restricted indoor environment so as to promote obesity and weighed monthly (Table 2).

Taking into account a small number of animal losses between birth and the end of weaning, final groups and compositions with respect to gender were:

	Groups	Females	Males	Total
RA	**R**equirement **A**ccelerated growth Obesogenic environment	4	5	9
NA	**N**utrient restricted **A**ccelerated growth Obesogenic environment	7	2	9
NS	**N**utrient restricted **S**tandard growth Obesogenic environment	6	2	8

Table 2. Number of participants by group and gender.

Normal energy requirement (NER) for each animal was calculated according to animal body weight and fetal number, as determined by ultrasound, in order to account for the requirements for the maintenance of the sheep and the growth of the fetus to produce a 4,5 Kg lamb (Agricultural Research Council, 1980). Since NER increase during gestation, associated with fetal growth, feed was adjusted fortnightly on the basis of maternal weight and gestation throughout the experimental period.

Food consumption and daily energy intake were measured by weighing of refusals on a daily basis.

At 17 months of age, which corresponds to young adulthood, all offspring were humanely euthanized by electrical stunning and exsanguination after overnight fast.

Representative samples of subcutaneous fat were then isolated, weighted and snap frozen in liquid nitrogen and stored at -80ºC.

2. Fat mass quantification: Dual-Energy X-ray Absorptiometry (DEXA) measurements

DEXA is a scanning technique that measures the differential attenuation of two x-rays as they pass through the tissues. It differentiates bone mineral from soft tissue and subsequently divides the latter into fat and lean mass. The method provides information on total body composition, and also on the composition of individual body segments.

Fat mass quantification was made to all offspring at 7 months and at 17 months of age.

3. Tissue preparation

Initial preparation of samples for tissues analysis was carried out on dry ice. Samples of approximately 2g were taken from the stock sample and weighed.

- **RNA extraction**

RNA extraction has 3 main steps:

a) Discard the fat

Disruption of cell walls and plasma membranes is required to release the RNA contained in the sample. For this step, Trizol reagent was used, this is a single homogenous solution for the isolation of total RNA. It is phenol/guanidine based and disrupts cell walls, denatures the proteins (including RNAse) and selectively extracts RNA when combined with chloroform.

The sample with Trizol was homogenised (Ultra-Turrax T25, Janke and Kunkel, IKA-Labortechnik). After this, they were mixed (Whirlimixer TM, Fissons Scientific Equipment) centrifuged at 2500 rpm (Coolspin 2, Thermo Life Sciences). This ensured that the fat and the RNA had been thoroughly separated. The lower aqueous phase contained the RNA. This was then collected on a sterile RNAse free tube. The fat that remained in the initial tube was disposed.

b) RNA extraction

For every 1ml of RNA sample, 0.2 ml of chloroform was added. The samples were incubated at room temperature and then centrifuged (Dupont instruments, Sorvall, RC-5B Refrigerated Superspeed Centrifuge, Thermolife Sciences). The components in the test had three clear layers, an upper clear phase that contained the RNA, an interphase containing genomic DNA and a lower red phase that contained the cell components.

c) RNA precipitation

The upper aqueous phase containing the RNA was collected and placed onto a new sterile RNAse free tube. The next step used RNeasy (QIAGEN Gmbh, Qiagen Str. 1, 40724 Hilden, Germany) spin columns. This was carried out in three steps:

I. Binding RNA to the column

II. Washing

III. Elution

The sample was then transferred into a sterile, RNAse free tube, labelled and stored at -80ºC. Approximately, 30 µl of RNA was extracted from each sample.

- **RNA concentration measurement**

RNA concentrations were determined using a spectrophotometer (Nano drop Spectrophotometer ND-1000). As the sample had been eluted from the RNeasy kit using RNAse free water, this same water was used to set the background of the spectrophotometer. After this, 1,5ul of each sample were placed in the spectrophotometer and their concentrations and purity measured.

RNA has specific wavelength absorption of 260 nm, therefore, measurements were taken with this wavelength and RNA concentration and purity were assessed. A ratio of absorption between 1.6 and 2,9 was considered acceptable.

- **DNAse treatment**

The principle of this step is to remove any remaining traces of genomic DNA from our samples. DNAse, the enzyme that specifically digests double stranded DNA, is used. The enzyme and the DNA particles are then removed, so that after reverse transcription is carried out the newly produce cDNA cannot be damaged.

4. Reverse transcription

Polymerase chain reaction (PCR) requires double stranded DNA, which is then able to be amplified. Therefore reverse transcription (RT) is needed to convert the RNA into cDNA.

The appropriate programme was selected on the semi-quantitative PCR machine (Techne Touchgene gradient, Scientific Laboratory supplies ref. 2650, Cambridge, UK).

The samples were placed in the PCR machine for elongation to occur.

5. Primer design

The primers used had already been designed before this experiment.

6. Semi- quantitative PCR

In order to establish the efficiency of RT and prepare standards for the different primers, semi-quantitative PCR was used.

To ensure that RT had been successful, 18s gene was tested, as this would be expressed in all samples.

The 18s gene, is a housekeeping gene and is the structural RNA for the small component of eukaryotic cytoplasmic ribosomes, and thus one of the basic components of all eukaryotic cells. A positive result is indicative of the presence of cDNA.

6.1. Semi-quantitative PCR

Thermocycler was programmed to run 45 cycles, each one consisting of the distinctive PCR phases (Figure 3):

1. *Denaturation step*: This step is the first regular cycling event and consists of heating the reaction. It causes melting of DNA template and primers by disrupting the hydrogen bonds between complementary bases of the DNA strands, yielding single strands of DNA.

2. *Annealing step*: The reaction temperature is lowered to 60°C allowing annealing of the primers to the single-stranded DNA template. Stable DNA-DNA hydrogen bonds are only formed when the primer sequence very closely matches the template sequence. The polymerase binds to the primer-template hybrid and begins DNA synthesis.

3. *Extension/elongation step*: the reaction temperature is increase to 72ºC for 1 minute. At this step the DNA polymerase synthesizes a new DNA strand complementary to the DNA template strand by adding nucleotides. The extension time depends both on the DNA polymerase used and on the length of the DNA fragment to be amplified. Under optimum conditions, i.e., if there are no limitations due to limiting substrates or reagents, at each extension step, the amount of DNA target is doubled, leading to exponential amplification of the specific DNA fragment.

4. *Final elongation*: This single step is occasionally performed at a temperature of 70-74°C for 5-15 minutes after the last PCR cycle to ensure that any remaining single-stranded DNA is fully extended.

5. *Final hold*: This step at 4-15°C for an indefinite time may be employed for short-term storage of the reaction.

The cycle number and annealing temperature are primer dependent.

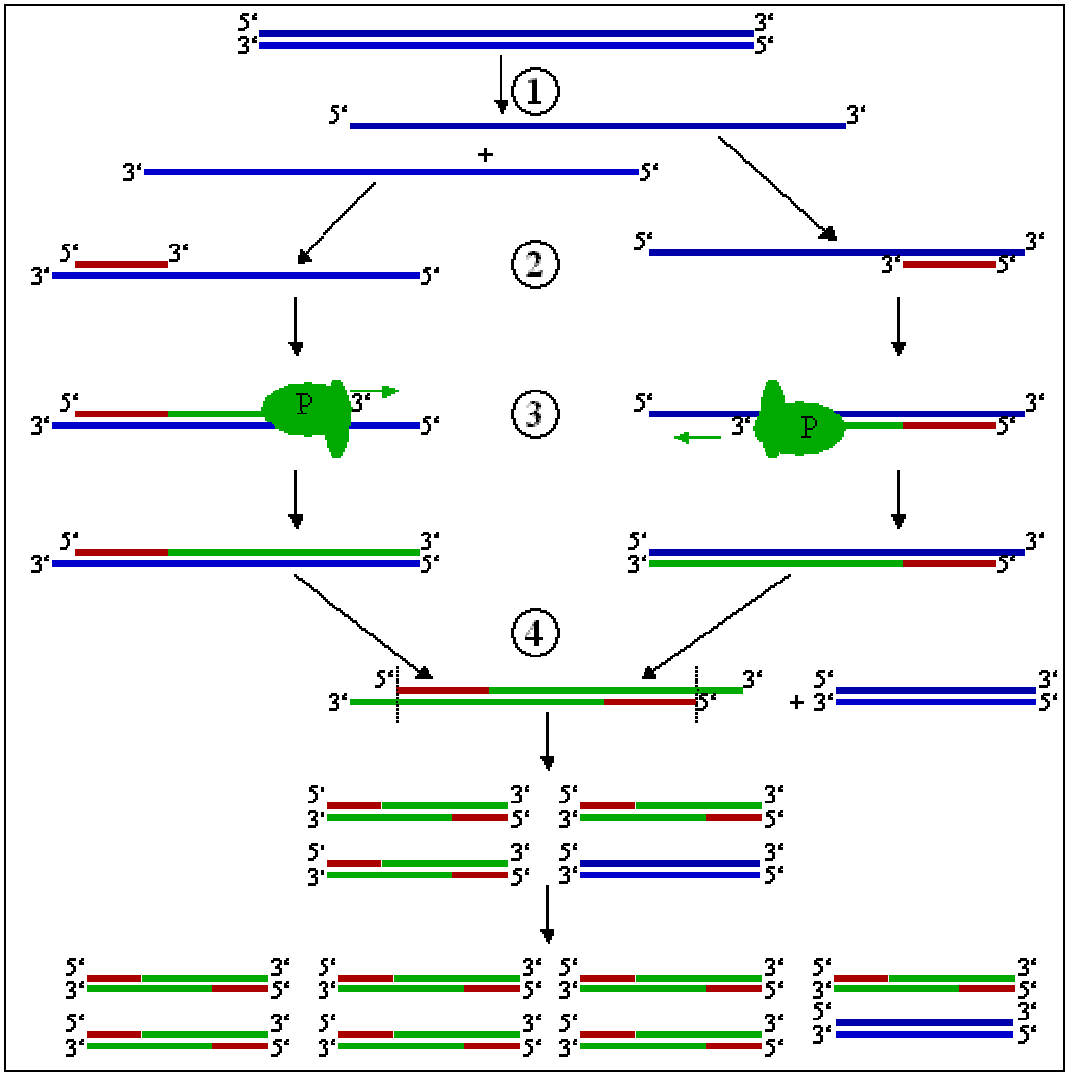


Figure 3. Principles of polymerase chain reaction

1) Denaturation stage: the doublestranded template DNA is split into two single strands. 2) Annealing stage: the primers bind to their complimentary sections on the template DNA. 3) Amplification stage: the enzyme polymerase (P) extends the primers with nucleotides to form new DNA strands. 4) The amount of DNA is doubled during each cycle. The newly formed DNA strands act as templates in the consecutive cycle

Adapted from Wikipedia (Wikipedia & contributors, 2006)

6.2 Gel electrophoresis

To analyse the PCR products obtained by conventional PCR, they can be injected in wells in an agarose gel. An electrical current is then applied to the gel, which causes the DNA molecules to move towards the anode. Smaller molecules move quicker through the gel, so the different products are separated by size. The DNA can be visualised with ultraviolet light after staining with ethidium bromide.

6. 3. DNA extraction and sequencing

The DNA bands of the gels were cut out and DNA was extracted from the gel using a QIAquick Gel Extraction Kit (Qiagen$^{(R)}$). A sample of the extracted DNA was sent to the Queen´s Medical Center sequencing service for sequencing to confirm the right product.

Another sample of the DNA was used to prepare standards for the standard curve in real time PCR.

7. Real time polymerase chain reaction

Real time PCR uses a signal, normally fluorescent, which increases with the increased amount of DNA formed by the PCR reaction. When the signal rises above a set background, it is detected. The signal normally increases in an S-formed curve until it reaches a plateau. During the linear increscent, the signal can be compared with the signal from other samples. A standard curve with known DNA concentrations is usually run alongside, to compare the signals of the samples and to calculate the DNA concentrations of the unknowns. The signal is normally measured at a threshold level:

Cycle threshold (Ct). The cycle number at which the signal crosses the Ct is correlated to the initial concentration of DNA in the template.

DNA binding agents, like SYBR green, bind specifically to double stranded DNA. They emit a fluorescent signal when they bind to double stranded DNA. During each cycle of the PCR the amount of double stranded DNA increases with the fluorescent signal.

For real time PCR, a standard curve was used to enable to calculate the efficiency of the reaction. For the standard curve, DNA was used that was extracted from an agarose gel as described before in this section. The first standard used was diluted to a concentration of 1 ng/µl. This standard was diluted 1:10 and the product was diluted 1:10 again. This process was repeated 10 times, so the final standard had a concentration of 1×10^{-9} ng/µl.

Complementary DNA was amplified using a real-time thermocycler (Quantica, Techne incorporated, Barloword Scientific Ltd, Stone, UK), using SYBR green based Taq polymerase reaction mix (Absolute® blue QPCR SYBR® green, Thermo scientific, Epsom, UK). Two negative controls with RNAse free water were also run. All samples were tested in duplicate.

8. **Immunohistochemistry**

In order to further characterize the tissue samples available, paraffin-embedded subcutaneous adipose tissue sections were cut at 5 µm and mounted on Super Frost Microscope Slides (Menzel-Glaser, Braunschweig, Germany). Five randomly taken samples were fixed, and subsequent histological analysis performed (Sharkey et al, 2009).

Immunohistochemical techniques can be used to demonstrate the presence of antigens in tissues and cells. Staining was carried out on the Bond-max histology system using Bond Polymer Refine Detection (Vision biosystems, Mount Waverley, Australia, DS 9800) which utilizes a controlled polymerization technology to prepare polymeric HRP-linker antibody conjugates. Slides are heated and stained as follows:

- The specimen is incubated with hydrogen peroxide to quench endogenous peroxidase activity
- A user-supplied specific primary antibody is applied
- A post primary antibody solution enhances penetration of the subsequent polymer reagent
- A poly-HRP anti-mouse/rabbit IgG reagent localizes the primary antibody
- The substrate chromogen visualizes the complex via a brown precipitate
- Hematoxylin (blue) counterstaining allows the visualization of cell nuclei

Using Bond Polymer Refine Detection reduces the possibility of human error and inherent variability resulting from individual reagent dilution, manual pipetting and reagent application.

The antibody used was GRP-78. This immunostaining procedure, even though it is not its main goal, allowed the visualization of the cell size and shape.

Slides were imaged using Nikon Eclipse 90i microscope with high speed colour camera (micropublisher 3.3RTV; Qimaging, Surrey, BC, Canada) and analysed using Volocity 4 (v.4.2.1; Improvision, Coventry, UK) quantification software.

9. Statistical analysis

The real time PCR results were used for statistical analysis. The point where the fluorescent signal crosses the threshold level is called the crossing points (Cp or Ct). This point can be used to calculate differences in the amount of RNA of the gene of interest between samples. All Ct values of genes of interest are normalised against Ct values of the same sample of 18S to take into account differences in RNA concentrations between samples. All Ct values are also normalised against the mean Ct value of all subcutaneous samples. Results are expressed as fold changes compared to the subcutaneous tissue (Yuan *et al*, 2006).

$$\Delta Ct = Ct_{\text{target gene}} - Ct_{\text{housekeeping gene}}$$

Equation 1.

$Ct_{\text{target gene}}$ is the mean crossing point for the gene

$Ct_{\text{housekeeping gene}}$ is the mean crossing for 18s gene

ΔCt is the mean of the difference between the crossing point of the target gene of the individual sample and the mean crossing point of the housekeeping gene

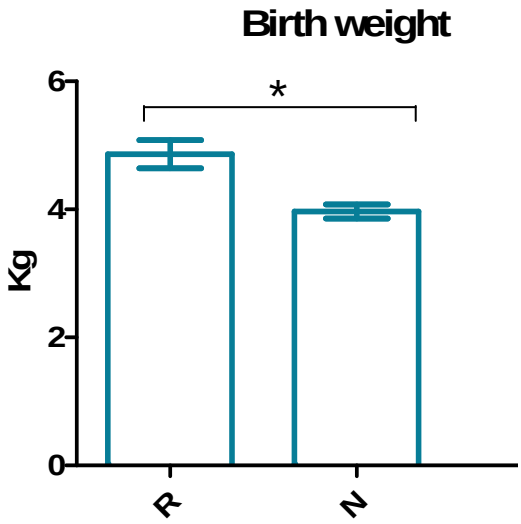
$$\text{Gene expression} = 2^{-\Delta Ct}$$

Equation 2. Gene expression

All data were analyzed using SPSS software 1(v.17.0; SPSS, Chicago, IL, USA). The fold change data were assessed for normality using Kolmogorov-Smirnoff. The influence of maternal nutrition (NA *vs* RA) or early postnatal growth (NS *vs* NA) was determined, according to parametric distribution, using impaired *t* Student or Mann-Whitney U tests. Data is expressed as mean values with their standard errors. For all comparisons, statistical significance was accepted when a probability of 5% was observed ($P<0.05$).

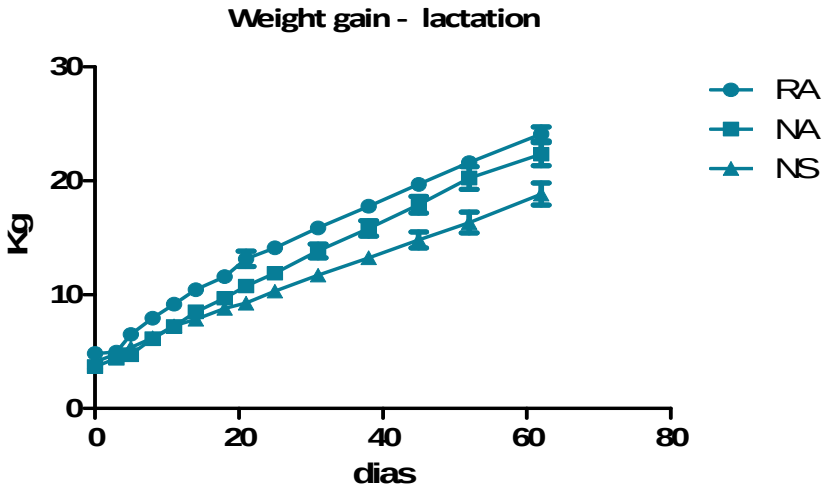
RESULTS

Following maternal caloric restriction, birth weight was reduced (graph 1) without any change in length of gestation.



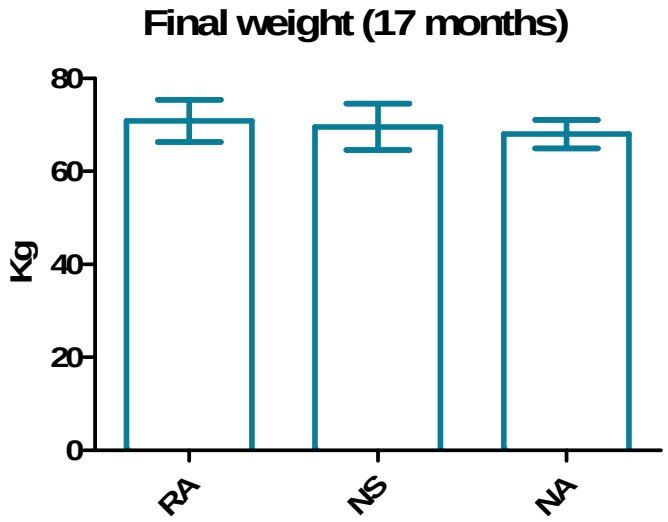
Graph 1 – The impact on birth weight of the offspring born to R mothers (fed to requirements; n=9) and N mothers (nutrient restricted) (n=17). (*P<0,05)

Throughout lactation, the low birth weight offspring remained lighter than the group that was fed to requirements (graph 2). The groups that were allowed to accelerate their growth (RA, NA) grew faster and thus weighed more by end of lactation (i.e. 90 days of age) (graph 2).

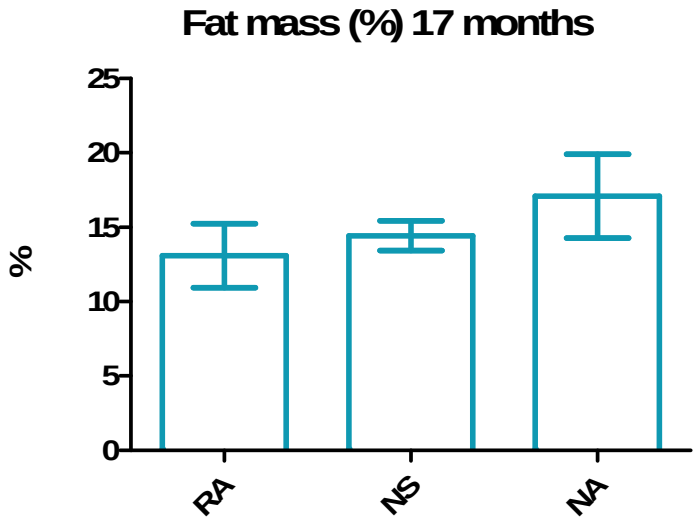


Graph 2 - Fetal restriction and the timing of postnatal growth. Weight gain of RA (n=9), NA (n=9) and NS (n=8) offspring during lactation.

After weaning all offspring born to nutrient restricted mothers required a further 4 months before their weight matched the other group. Possibly, as a consequence of showing a slower weight gain prior to weaning, the NS offspring exhibited the fastest rate of post-weaning growth. Nevertheless, no offspring born to nutrient restricted mothers grew more than controls and at 17 months of age mean body weight (graph3) was similar between groups as was total fat mass (graph 4).



Graph 3 – No difference in adult (17 months old) offspring body weight. RA (n=9), NA (n=9) and NS (n=8).

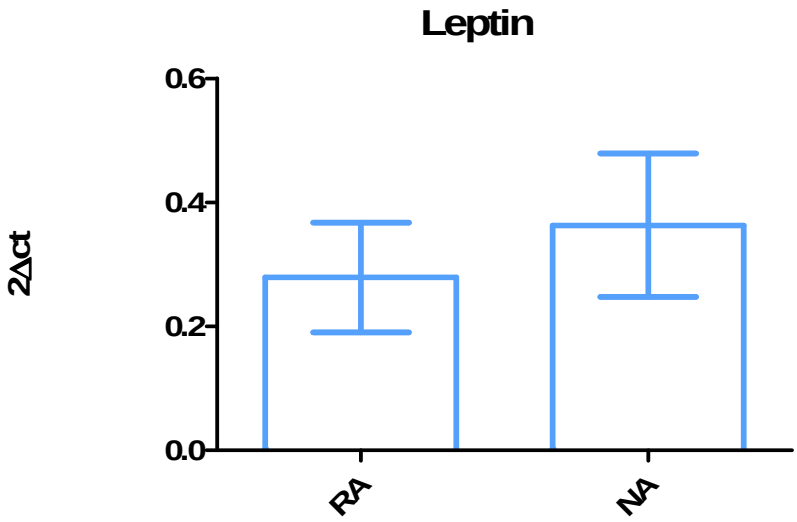


Graph 4 – No difference in adult (17 months old) offspring fat mass percentage. RA (n=9), NA (n=9) and NS (n=8).

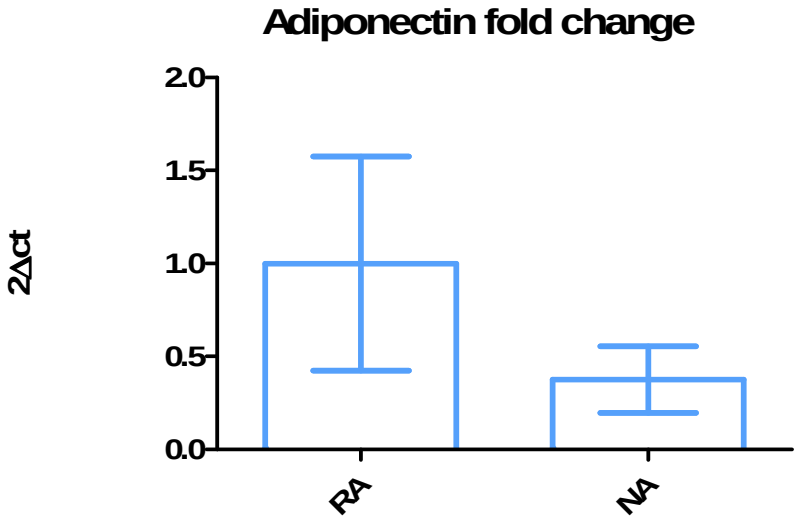
Gestational nutrient restriction and the gene expression of inflammatory markers of subcutaneous adipose tissue

Comparing gene expression for adipokines between offspring born to nutrient restricted (NA) or control fed mothers (RA) a range of responses were found.

There was no difference in the gene expression of leptin (graph 5) or adiponectin (graph 6).

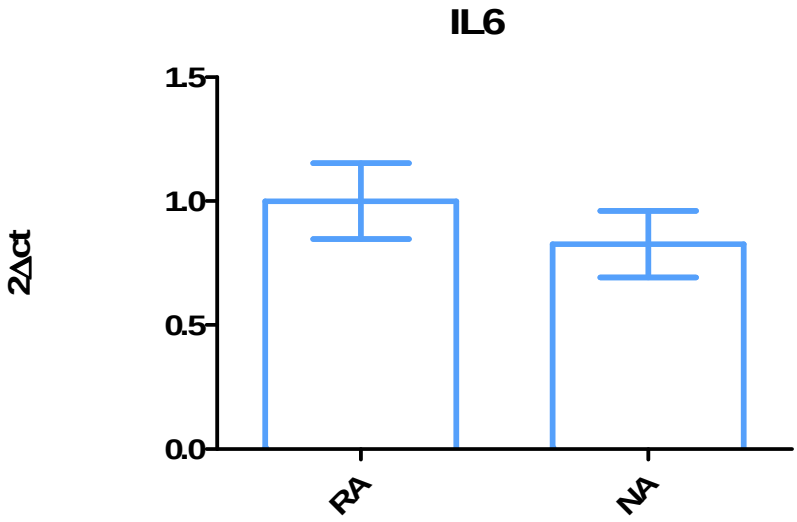


Graph 5 – No difference in leptin gene expression fold change in RA (n=9) and NA (n=9).

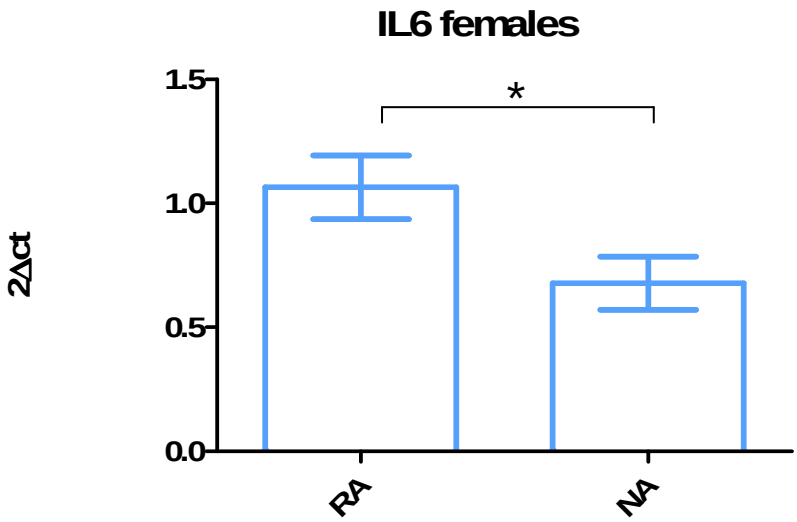


Graph 6 - No difference in adiponectin gene expression fold change in RA (n=9) and NA (n=9).

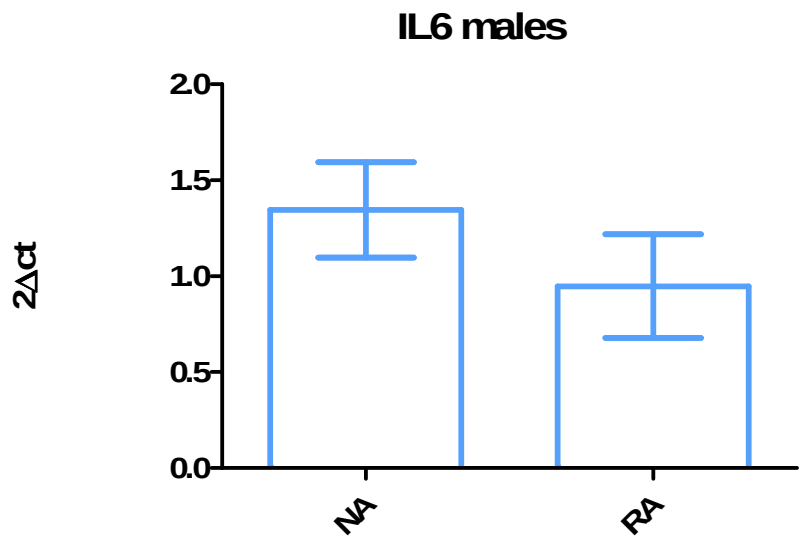
The gene expression of IL6 in the mix gender groups did not show any differences (graph 7). However when the group was split by gender, females showed a more active inflammatory response. IL6 gene expression was higher in females that were fed to requirement during gestation (graph 8). In males no difference was found.



Graph 7 - No difference in Interleukin 6 (IL-6) gene expression fold change in RA (n=9) and NA (n=9).

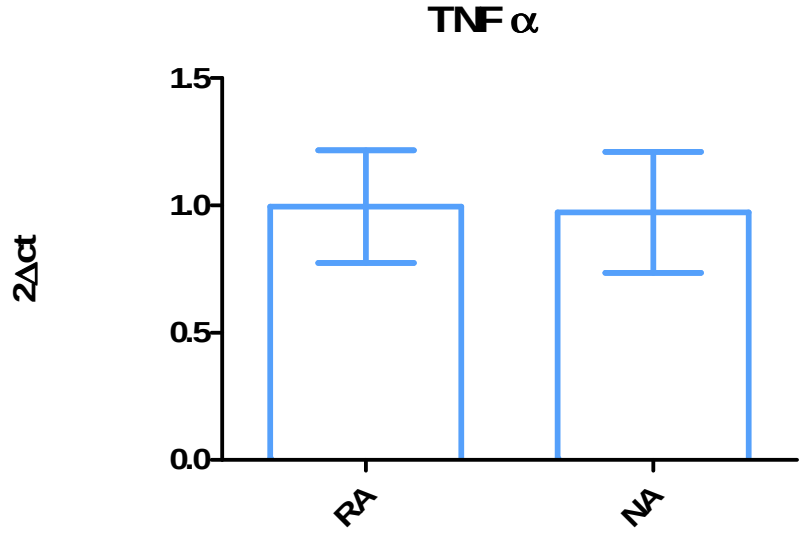


Graph 8 - Interleukin 6 (IL-6) gene expression fold change for females. Statistically higher gene expression in females from RA group (n=4) than in females from NA group (n=7) (*P<0,05).

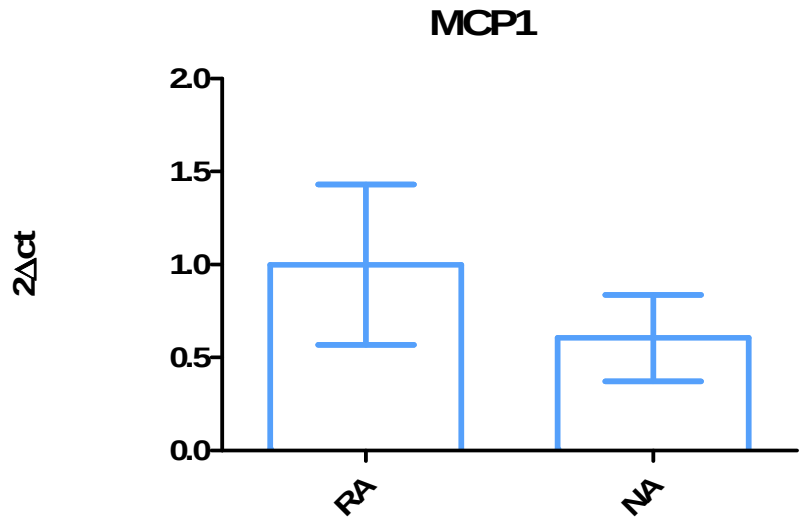


Graph 9 - Interleukin 6 (IL-6) gene expression fold change for males. Statistically higher gene expression in males from RA group (n=5) than in males from NA group (n=2)

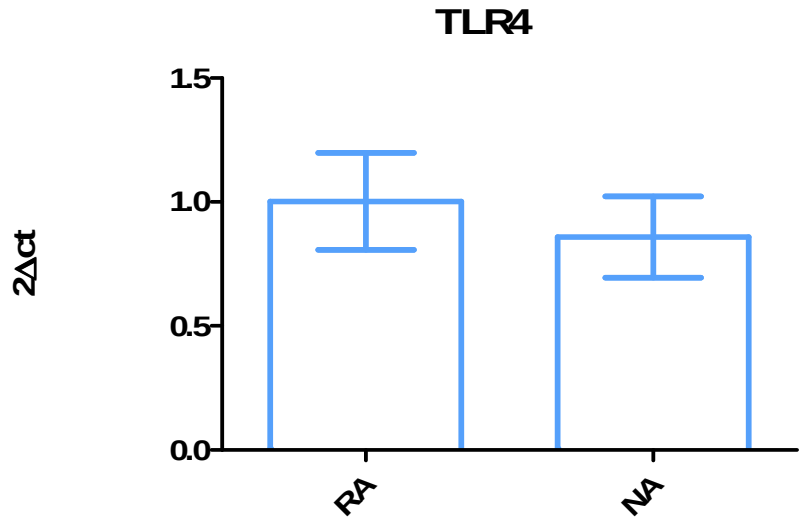
There was no difference in the gene expression of TNF-α, MCP-1, TLR-4, IL18 and GRP-78 (graphs 10, 11, 12, 13 and 14), either in mixed gender groups or males and females.



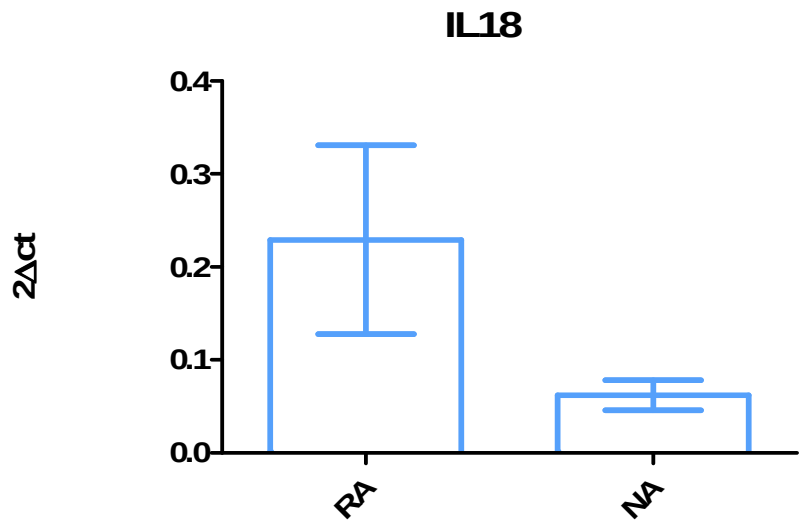
Graph 10 – No difference in Tumor necrosis factor (TNF) α gene expression fold change in RA (n=9) and NA (n=9).



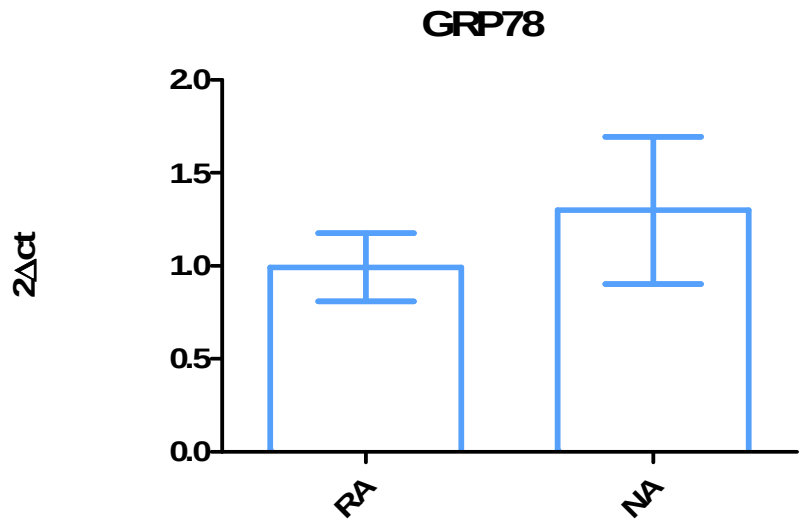
Graph 11 - No difference in Monocyte chemotactic protein-1 (MCP-1) gene expression fold change in RA (n=9) and NA (n=9).



Graph 12 – No difference in Toll-like receptor 4 (TLR-4) gene expression fold change in RA (n=9) and NA (n=9).

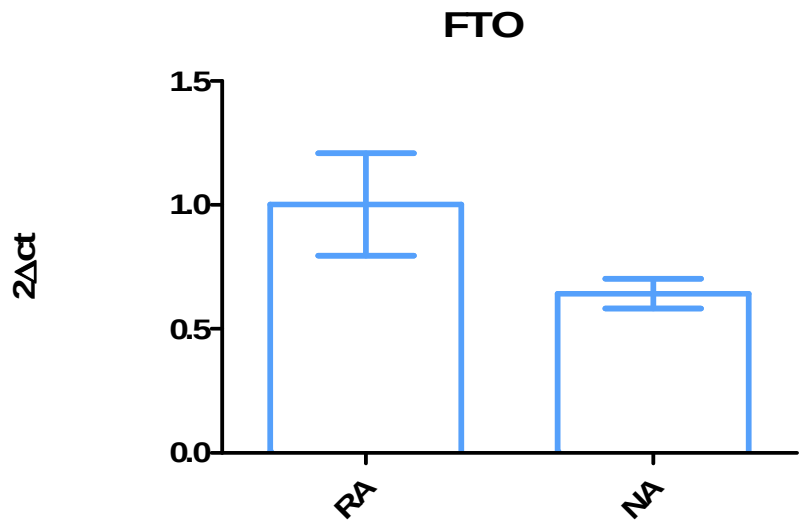


Graph 13 – No difference in Interleukin 18 (IL-18) gene expression fold change in RA (n=9) and NA (n=9).

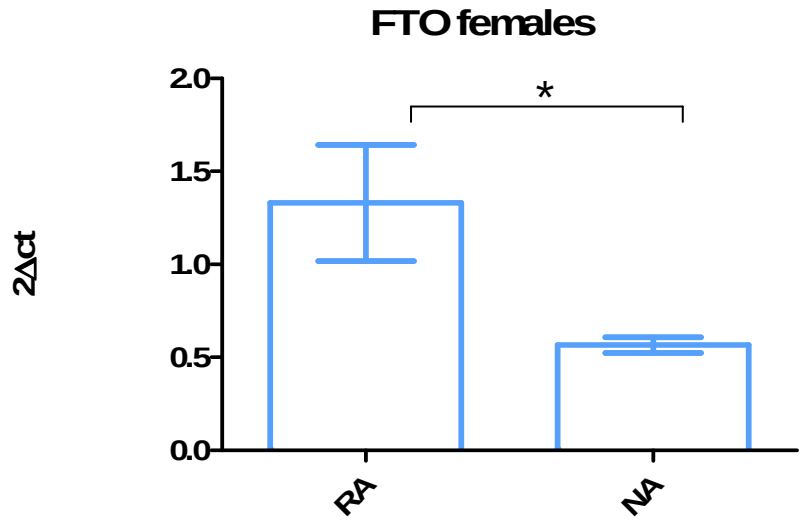


Graph 14 - No difference in Glucose regulated protein 78 (GRP-78) gene expression fold change in RA (n=9) and NA (n=9).

The gene expression of FTO gene in the mix gender groups was not significant different, however when the group was split by gender there was a higher expression of this gene in the females that were fed to requirement during gestation (graphs 15 and 16).



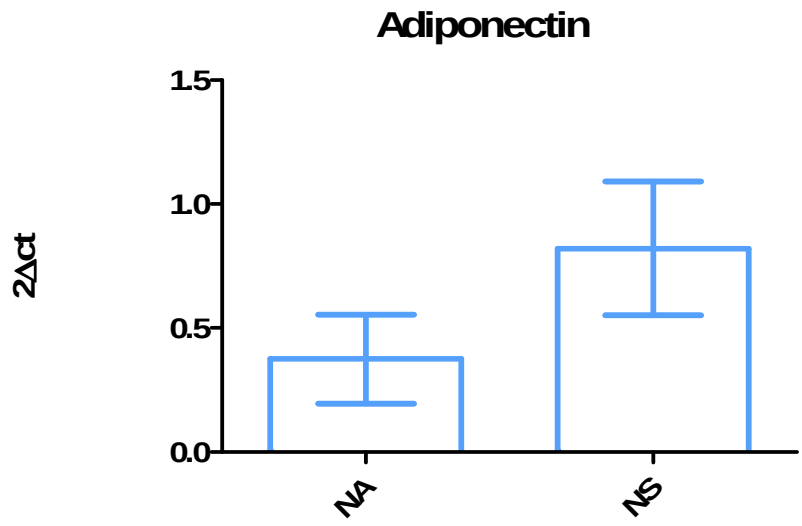
Graph 15 - No difference in Fat mass and obesity (FTO) gene expression fold change in RA (n=9) and NA (n=9).



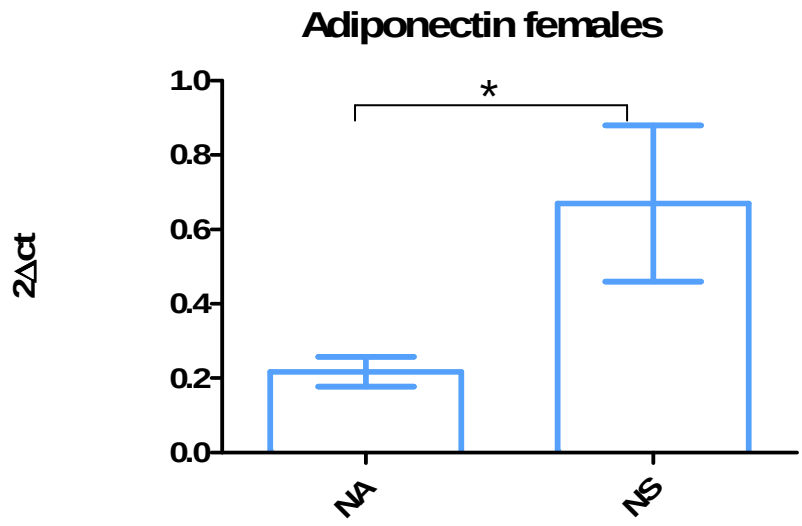
Graph 16 - Fat mass and obesity (FTO) gene expression fold change for females. Statistically higher gene expression in females from RA group (N=4) in comparison to females from NA group (n=7) (*P<0,05).

Postnatal accelerated growth and the gene expression of inflammatory markers of subcutaneous adipose tissue

The most significant result found was related to adiponectin gene expression. In the mix gender group no significant difference was found (graph 17). However when the group was split by gender, there was a difference amongst the females. The female group that showed a standard growth had a significantly higher gene expression of adiponectin (Graph 18). No difference was found in the males.

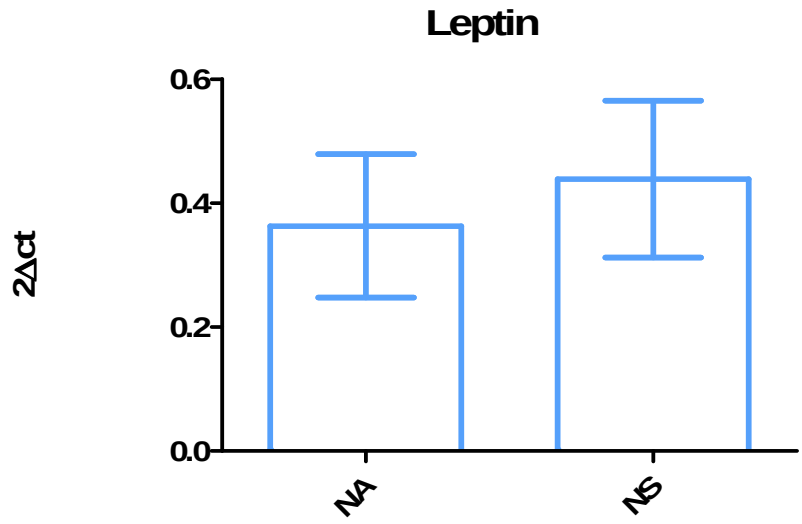


Graph 17 – No difference in leptin gene expression fold change in NA (n=9) and NS (n=8) groups.

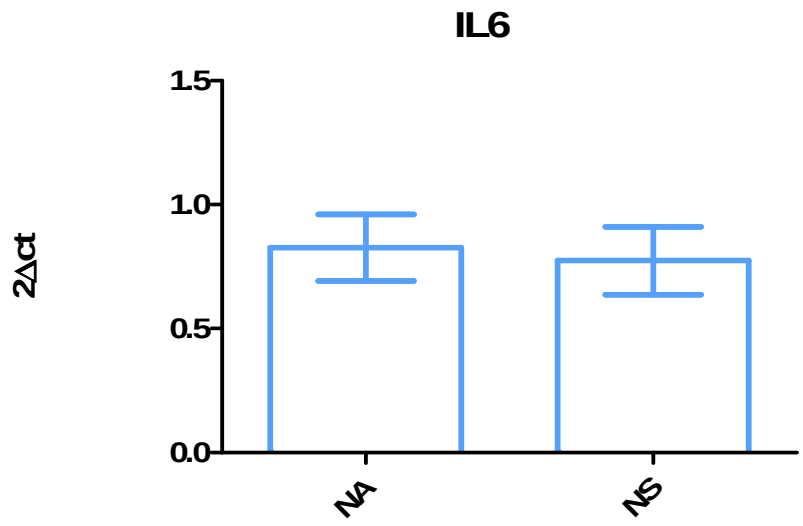


Graph 18 –Adiponectin gene expression fold change for females. Females from the group that had an accelerated growth (NA; n=7) was associated to a significant lower adiponectin gene expression in comparison to females from NS group (n=6). (*p<0,05).

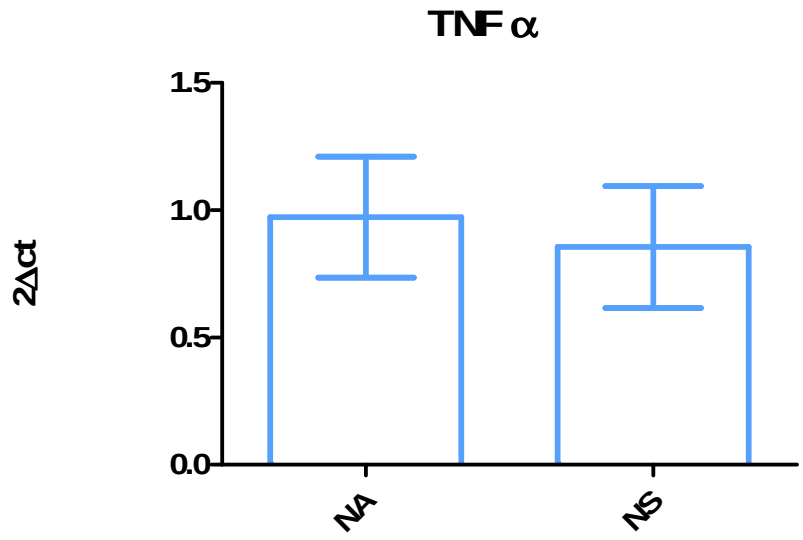
The other genes analysed showed no significant difference among these two groups as illustrated in the following graphs. The results were also analysed as mix gender groups and split by gender. In either female or male no significant differences were found.



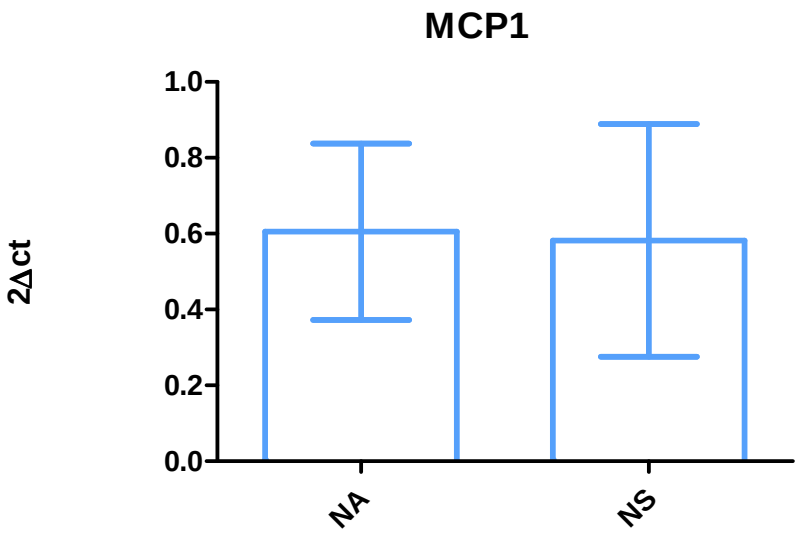
Graph 19 – No difference in leptin gene expression fold change in NA (n=9) and NS (n=8) groups.



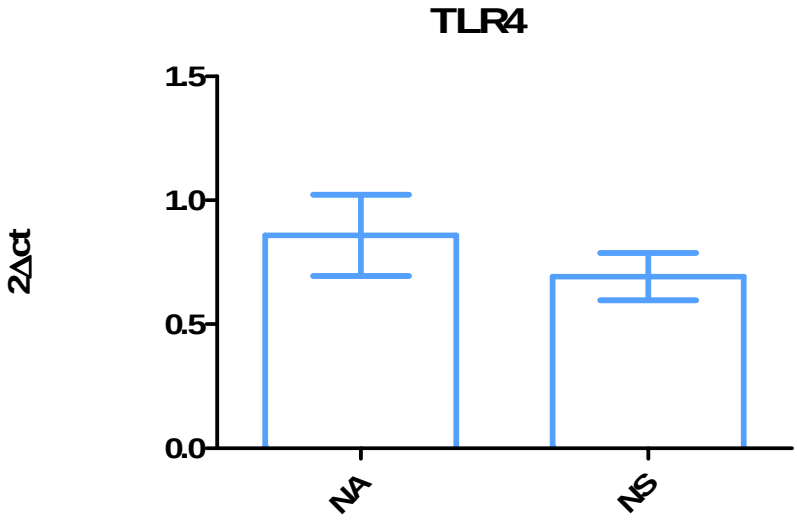
Graph 20 – No difference in Interleukin 6 (IL-6 gene expression fold change in NA (n=9) and NS (n=8) groups.



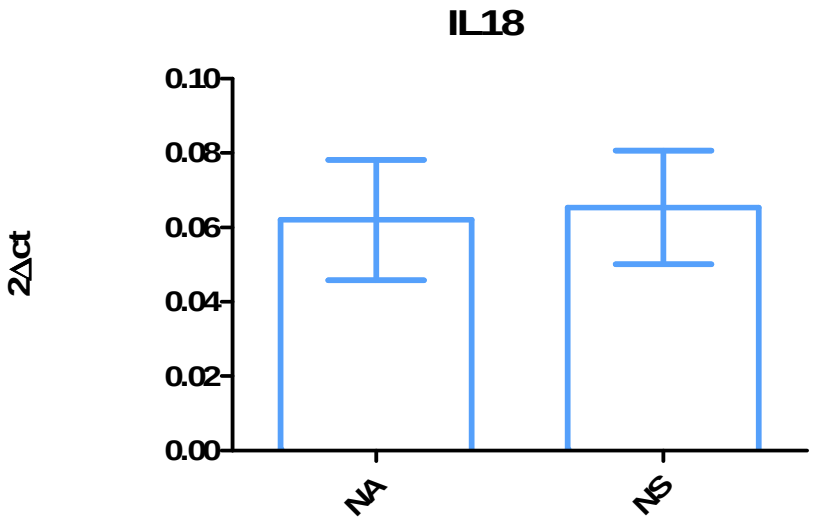
Graph 21 – No difference in Tumor necrosis factor alpha (TNF-α) gene expression fold change in NA (n=9) and NS (n=8) groups.



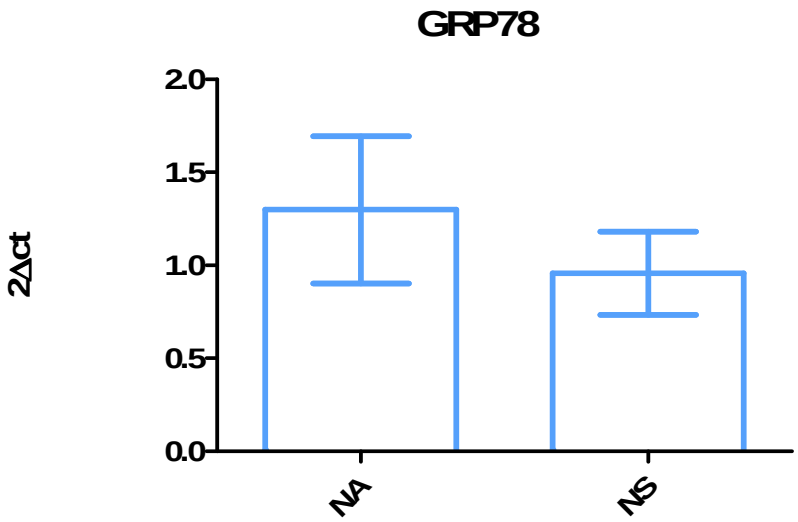
Graph 22 – No difference in Monocyte chemoattractive protein-1 (MCP-1) gene expression fold change in NA (n=9) and NS (n=8) groups.



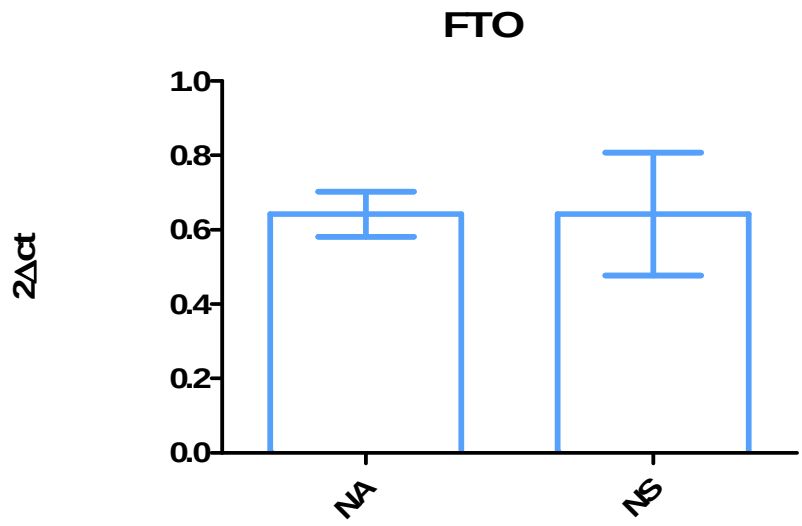
Graph 23 – No difference in Toll-like receptor 4 (TLR-4) gene expression fold change in NA (n=9) and NS (n=8) groups.



Graph 24 – No difference in Interleukin 18 (IL-18) gene expression fold change in NA (n=9) and NS (n=8) groups.



Graph 25 - No difference in Glucose regulated protein 78 (GRP-78) gene expression fold change in NA (n=9) and NS (n=8) groups.



Graph 26 - No difference in Fat mass and obesity (FTO) gene expression fold change in NA (n=9) and NS (n=8) groups.

Immunohistochemistry

The adipose tissue slices were analysed microscopically and no significant differences were found between groups. One representative image for each group is shown.

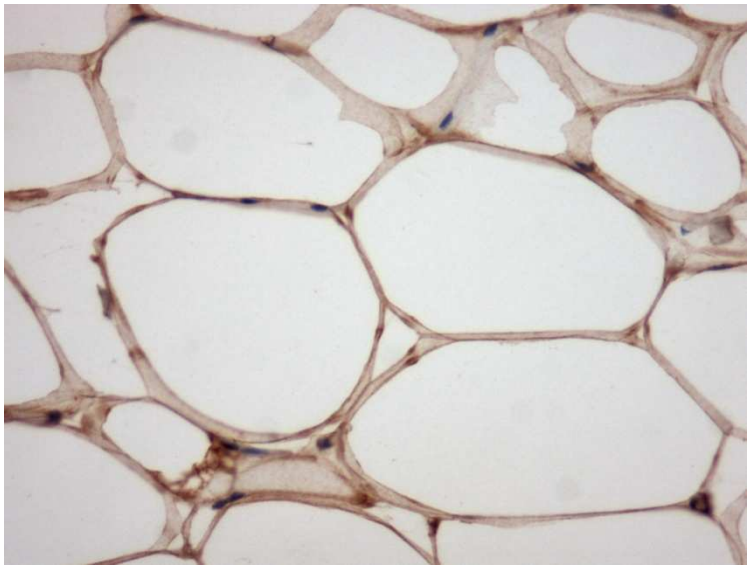


Image 1 – Adipose tissue of fed to requirement group with accelerated growth (RA). Representative micrograph (x 40).

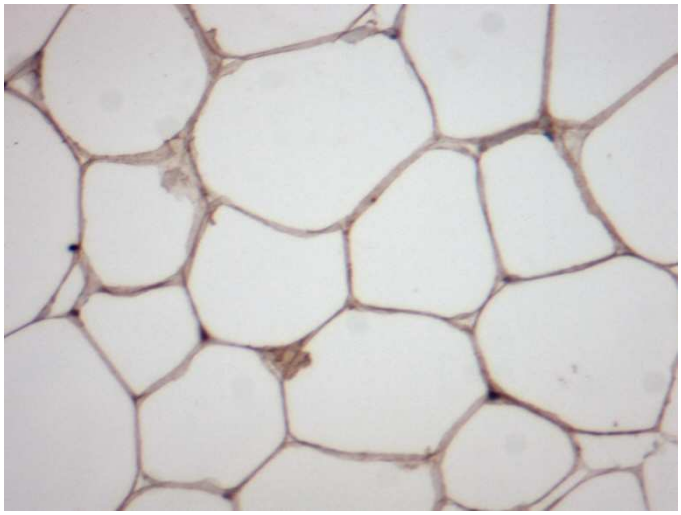


Image 2 – Adipose tissue of nutrient restricted group with accelerated growth (NA). Representative micrograph (x 40).

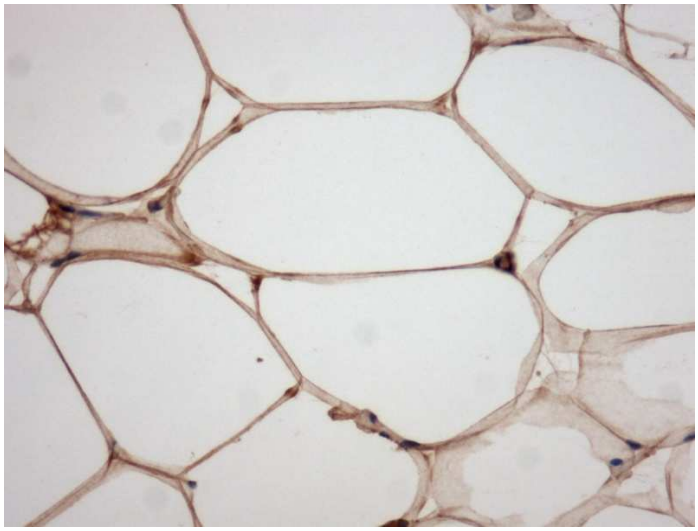
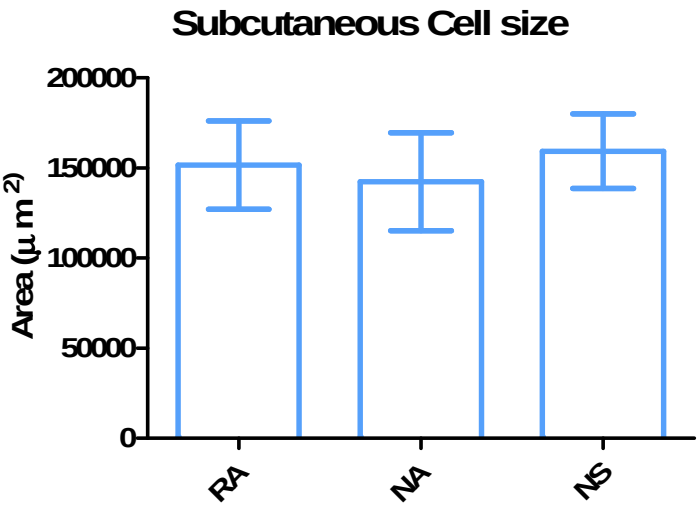
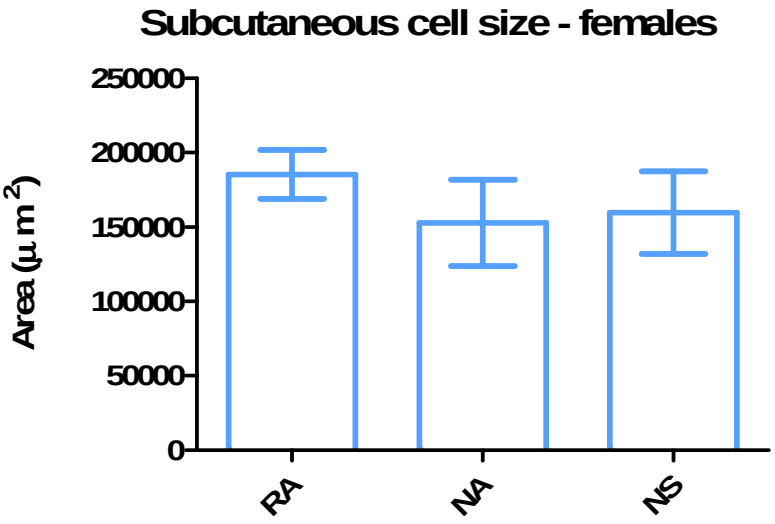


Image 3 – Adipose tissue of nutrient restricted group with standard growth (SA). Representative micrograph (x 40).

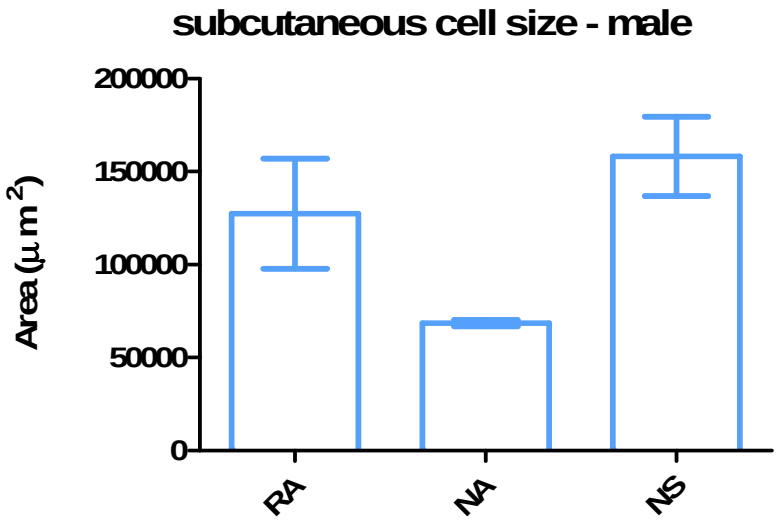
The images were further analysed in order to achieve a cell size average for each group. The average cell size was similar to the offspring of all groups (Graph 27).



Graph 27 – Similar cell area (μm^2) in the three groups (RA n=9; NA n=9; NS n=8).



Graph 28 – Similar cell area (μm^2) for females in all groups (RA n=4; NA n=7; NS n=6).



Graph 29 – Similar cell area (μm^2) for males in all groups (RA n=5; NA n=2; NS n=2).

DISCUSSION

The rapid growth of infants during the first year of life and continued growth, albeit at lower rates, from twelve months of age through to adolescence imposes unique nutritional needs. The needs for growth are superimposed on relatively high maintenance needs coincident to the higher metabolic and nutrient turnover rates of infants and children compared with adults. Because the high rates of growth are accompanied by marked developmental changes in organ function and composition, failure to provide sufficient nutrients during this time is likely to have adverse effects on development as well as growth. Provision of these special nutrient needs, particularly during early life, is therefore essential (Skelton and Rudolph, 2007).

Early life development proceeds as a complex set of interdependent yet distinct and precisely integrated biological programmes. Following fertilization, development is initiated by selective reading of the genetic code leading to the generation of the various cell types, organs and organ systems that constitute mammals. Early development is dependent on the expression of the maternal and fetal genomes and is regulated, in part, by maternally derived supply of nutrients. Nutrients are required as building blocks for the synthesis of macromolecules, energy production and as biological cofactors for enzymatic reactions. Nutrients also serve as information molecules that interact with the genome and trigger or facilitate developmental programmes (Ong *et al*, 2000).

Mammalian development is dependent critically upon the function of the maternal and fetal genomes and the availability of maternally or placentally synthesized small molecules including hormones, cytokines and growth factors.

Maternal health also is essential for the successful initiation and progression of mammalian development. The fetal environment is maintained by the placenta, which facilitates and regulates the flow of maternally derived vitamins, minerals and other essential nutrients to the fetus while simultaneously protecting it from deleterious compounds (Ong *et al*, 2000).

Mammalian development proceeds as a precisely orchestrated temporal programme that requires coordinated changes in fetal and maternal gene expression as well as a complete and regulated maternally derived nutrient supply. Maternal nutritional status influences all stages of fetal development and birth outcomes including birthweight and disease susceptibility. In many cases, deficiency or excess of particular nutrients at a critical time will impair specific genetically programmed development processes. Therefore the risk of these impairments is associated with a specific development period or "critical window". During each "critical window", nutrients and gene-nutrient interactions can influence DNA synthesis rates, cell proliferation rates, cell signalling, cell differentiation, cell migration and can actually "imprint" the expression levels of specific genes in a way that can impact on the organism throughout life.

The present study demonstrates initial alterations in the metabolic functions of adipose tissue which may impact upon potential mechanisms implicated in the link between nutrient restriction and rapid catch-up growth and later obesity.

My study analyzed for the first time inflammatory markers following intra-uterine growth restriction and accelerated post-natal growth.

Intra-uterine growth restriction

The differences found in gene expression were not dependent or proportional to the weight or fat mass quantity they had at the time they were euthanized, but instead they are the result of gene expression programming that occurred earlier in adipose tissue development.

Each developmental stage from conception, birth and trough lactation contributes to the setting of energy homeostasis of an individual (Symonds *et al*, 2009). In this respect, the final period of gestation in both humans and sheep is critical in determining both birth weight and postnatal growth. My study emphasizes different profiles in young adult offspring following a 40% reduction in maternal feed availability from 110 days gestation up to term. These adaptations are likely to be detrimental for the metabolic outcomes at birth and through the life cycle that will ultimately predispose upon the onset of metabolic disease. These different developmental windows then have a substantial influence on the long term tissue specific outcomes.

IL-6 gene expression

Cytokines are soluble signals of paramount importance mediating cell-to-cell communication during inflammatory and immune responses. Among cytokines the first discovered was IL-6, which is still a subject of intensive investigations today because of its ubiquity and functional diversity. IL-6 is a cytokine that is commonly produced at local tissue sites and released into circulation in almost all situations of homeostatic perturbation. It has remained unclear whether IL-6 has got proinflammatory or anti-inflammatory activities in immune reactions (Xing *et al*, 1998). Indeed, the lack of

understanding of other functional aspects of this cytokine in both local and systemic inflammatory responses has led to a confounding description about the nature of IL-6 as summarised below (Xing *et al*, 1998).

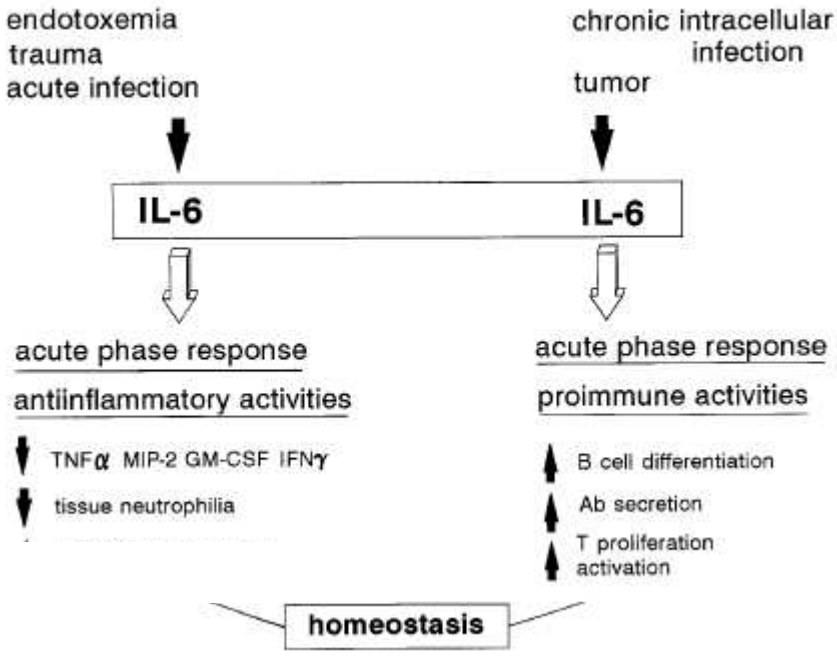


Figure 6 - A model of biologic functions of IL-6 in acute and chronic diseases. During local or systemic acute inflammatory responses, IL-6 is involved in the induction of acute phase reactions and controlling the level of acute inflammatory responses by downregulating the expression of proinflammatory cytokines: TNF-α (Tumor necrosis factor α), MIP-2 (macrophage-inflammatory protein-2); GM-CSF (Granulocyte and monocyte colony stimulating factor), IFN-ɣ (Interferon ɣ) and upregulating anti-inflammatory molecules

In my study I would expect that IL-6 would be more expressed in the group submitted to nutrient restriction which can be considered a homeostatic challenge. However, nutrient restricted offspring, and only females, had a lower gene expression of IL-6.

No difference was found when comparing the accelerated and standard early postnatal growth in the nutrient restricted groups.

FTO gene expression

Until very recently there were no consistent reports of gene variants that influence the risk obesity. Genome-wide association studies have, however, shown strong relationships between a common variant of the fat mass and obesity gene (FTO) and body mass index that is prevalent from as early as 7 years of age (Frayling *et al*, 2007). Indeed, this variant predisposes individuals to diabetes through an effect on body mass, a relationship that has been confirmed in a separate study based on a European cohort (Frayling *et al*, 2007).

In my study I found higher FTO gene expression in the female "fed to requirement" group. The potential divergence in response to variations in the FTO gene is further indicated by its widespread tissue distribution, with gene expression being greatest in the hypothalamus. Clearly, more information is required in relation to the regulation of the FTO gene from a development point of view and how it responds to an environment of excess or insufficient energy supply.

In resume, females from RA group had an expected nutritional gestational environment and birthweight but an accelerated post-natal growth. This means that early post-natal growth by itself contributes to a higher expression of FTO and IL-6. However, this effect disappears in those individuals that were nutrient restricted during gestation.

In conclusion, adverse outcomes of accelerated postnatal growth may be accentuated in AGA infants.

. **Accelerated post-natal growth**

In large animals, the post-natal period represents the main critical window of fat growth. Fat distribution changes substantially over the first few weeks of life, as fat is the most rapidly growing organ through this period and is soon found throughout the body. On the other hand, in term infants of normal body weight fat is present around the internal organs as well as subcutaneously, and it is the latter depot that grows substantially after birth. An appreciable amount of this fat can then be mobilised during the weaning period, although this process has nor been well documented. There is, however, increasing evidence that excess fat deposition can occur at this time and is very likely to have adverse long-term consequences (Symonds, 2009).

There is still controversy about the ideal weight gain for low birth weight newborns or premature babies. Catch-up growth is a more complex process than simply an accentuation of normal weight gain. During catch-up growth, energy intakes may be as high as 4.5 times basal expenditure. At high rates of weight gain, there is a limitation in the deposition of lean tissue, with excessive deposition of adipose tissue. As the rate of lean tissue deposition increases, there is a disproportionate increase in the demand for nutrients relative to energy. Associations between low birth weight, particularly thinness at birth, and increased risks for disease in adulthood, such as type 2 DM and CVD are now well established. In many population studies these associations are enhanced by and even depend on the development of obesity in adulthood (Eklund *et al*, 2007). Therefore any relationship between small size at birth and obesity in later life may contribute to the pathogenesis underlying the fetal origins hypothesis.

The greatest variation in rates of weight gain is seen in the first 1-2 years of life when infants may show significant "catch-up" or "catch-down" growth. These variable

growth rates often compensate for intrauterine restraint or enhancement of fetal growth and by two years of age growth usually follows the genetic trajectory. In recent birth cohort studies, subjects who were small or thin at birth and then showed rapid childhood growth had the greatest risk for disease in adulthood (Ong *at al*, 2000).

A normal response in growing animals following a period of food restriction is compensatory growth (Metcalfe and Monaghan, 2001).

This is dependent on sufficient food subsequently being available to support the accompanying increased metabolic demands. My study demonstrates that low birth weight offspring following fetal intra-uterine restriction are predisposed to early accelerated postnatal growth. A primary factor determining this response appears to be food availability. It is only seen when offspring were raised as singleton and thus there is no competition for available milk from an age matched sibling. Both the time course and magnitude of potentially adverse outcomes were influenced by the time after birth in which growth was promoted in the growth restricted offspring. Such a transitory period of rapid postnatal growth is representative of that observed in the term human newborn small for gestational age (Metcalfe and Monaghan, 2001).

With my study it can be seen that in the nutrient restricted females that had a standard growth, in comparison to those who suffered an accelerated growth, adiponectin had a higher gene expression. This hormone exists in lower quantity in obese individuals, and it is considered a protective mediator against cardiovascular pathologies, glucose intolerance and inflammation. Here we can see that if “catch-up” growth is avoided, higher adiponectin will be expressed and these individuals will benefit from its protection, irrespectively of the nutrient restriction *in utero*.

Other genes

In my study, there were no statistically significant differences in the other genes tested. This limited effect of nutrient restriction during gestation and early growth on SAT gene expression might be related to the fact that this tissue is less metabolically active in comparison to, for example, VAT. Deleterious metabolic effects of obesity are associated to the accumulation of abdominal fat resulting in an increase in waist size. This central obesity is an indicator used in the diagnosis of the metabolic syndrome. Thus, an increase in abdominal fat mass appears to be important in the pathogenesis of insulin resistance, dyslipidemia, hypertension, inflammation, protrombotic state and ultimately, cardiovascular risk (Vázquez-Vela *et al*, 2008, Wajchenber, 2000). VAT is composed of several fat depots, such as perirenal fat which has been found to have significant inflammatory response in nutrient restricted juvenile obese offspring (Sharkey *et al*, 2009; Williams *et al*, 2007).

In addition to the detrimental effects of VAT, human and animal studies have suggested a possible protective role for subcutaneous fat. In humans, increased subcutaneous leg fat is associated with decreased risk of disturbed glucose metabolism and dyslipidemia, independent of abdominal fat (Porter *et al*, 2009). Thiazolidinedione treatment, which increases total fat mass, mostly in subcutaneous fat stores, improves insulin sensitivity (Miyazaki *et al*, 2002). Removal of VAT by omentectomy results in decreased glucose and insulin levels in humans, (Thorne *et al*, 2002), whereas removal of SAT by liposuction does not always result in improvements in glucose metabolism or lipid levels (Klein *et al*, 2004; Giugliano *et al*, 2004). Transplantation of subcutaneous fat into visceral compartments in mice produces decreases in body weight and total fat

mass and improved glucose metabolism, suggesting that subcutaneous fat may be intrinsically different from visceral fat in ways that are beneficial (Tran *et al*, 2008). Therefore, it is hypothesis that abdominal subcutaneous fat might be a protective fat depot in terms of cardiometabolic risk factor prevalence (Porter et al, 2009).

VAT and SAT differ not only in anatomic location but also in cytokine secretion profile. Although the relationships between VAT and SAT secretion profiles and cardiometabolic pathogenesis are, at present, unclear, it may be that paracrine and perhaps endocrine factors contribute to the differential effects of VAT and SAT (Porter et al, 2009).

Considering SAT is a protective fat depot, this might explain the limited changes in inflammatory markers gene expression found in my study.

Further investigation is needed in to characterize SAT function and association to inflammatory responses in obesity.

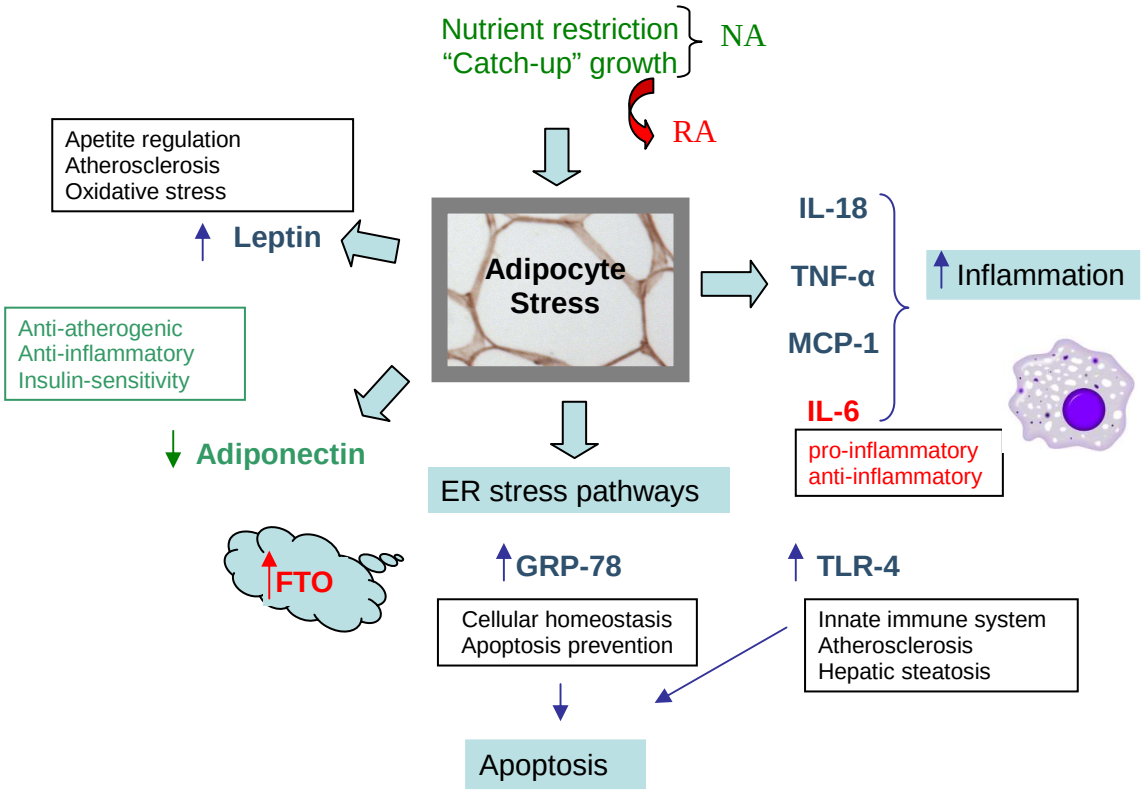


Figure 7 – Accelerated post-natal growth in maternal fed to requirement offspring (RA) was associated to higher expression of IL-6 and FTO (in red).

Nutrient restricted female offspring with accelerated post-natal growth (NA) had lower expression of adiponectin (in green).

Conclusions

Subcutaneous adipose tissue suffers limited alterations of its gene expression after nutritional manipulation.

However, differences were found in three of the studied genes: IL-6, FTO and adiponectin, which shows that subcutaneous adipose tissue is also important for the metabolic homeostasis of the individual. Even though fat mass quantity is similar there are significant changes between groups, indicating gene expression programming with later consequences.

Adverse outcomes of early accelerated postnatal growth may be accentuated in AGA infants, with higher expression of FTO and IL-6. When comparing nutrient restricted offspring, “catch-up” growth is associated with lower protection provided by adiponectin.

The main changes are related to increased postnatal growth and coincide with the period in which significant amounts of subcutaneous adipose tissue is being deposited.

This study attempts to add one more step to avoid forced accelerated post-natal growth in all newborns in order to prevent adult obesity and obesity-related disorders.

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